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Prenatal nicotine exposure-induced intrauterine programming alteration increases the susceptibility of high-fat diet-induced non-alcoholic simple fatty liver in female adult offspring rats

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The novelty of the work: "Two intrauterine programming" involve in the intrauterine origin of high-fat diet-induced NAFL in female offspring rats by prenatal nicotine exposure

Abstract

Previous studies have indicated that intrauterine growth retardation (IUGR) fetus was faced with high susceptibility of adult metabolic syndrome (MS). Non-alcoholic simple fatty liver (NAFL) is considered to be the hepatic manifestation of MS. In the present study, we evaluated the susceptibility of high-fat diet-induced NAFL in female adult IUGR offspring rats induced by prenatal nicotine exposure, and we further explored the underlying intrauterine programming mechanism for this phenomenon. The IUGR rat model was established by prenatal exposure to nicotine (2 mg/kg.d), the liver tissues from the female fetuses and the female adult offspring fed with normal or high-fat diet were collected. The female adult offspring in the nicotine group showed low birth weight and postnatal catch-up growth, as well as severe NAFL under high-fat diet. Moreover, increased gene expression involved in hepatic insulin-like growth factor 1 (IGF1) pathway, gluconeogenesis and lipid synthesis, decreased gene expression of lipid output accompanied with elevated serum triglyceride level, were observed. The female fetuses in the nicotine group showed down-regulated hepatic IGF1 pathway, and also exhibited a similar pattern of increased gluconeogenesis and lipid synthesis and decreased lipid output to those in the adults. The present study demonstrated the intrauterine origin of increased susceptibility to high-fat diet-induced NAFL in female offspring rats by prenatal nicotine exposure, which is most likely mediated by "two intrauterine programming". That is, the first glucocorticoid-IGF1 axis programming induced postnatal catch-up growth, aggravated glucose and lipid metabolic disorder, lead to an increased susceptibility to adult NAFL, while the second hepatic glucose and lipid metabolic programming enhanced hepatic lipogenesis and reduced lipid oxidation and output, and promoted NAFL.

Key words: Non-alcoholic simple fatty liver; Prenatal nicotine exposure; Intrauterine programming; Insulin-like growth factor 1; Glucose and lipid metabolic function.

Introduction

Metabolic syndrome (MS) is a combination of several disorders, which include hypertension, hyperglycemia, dyslipidemia, and obesity. It often leads directly to a series of diseases, such as fatty liver, diabetes, and cardiovascular diseases. Fatty liver refers to a clinical syndrome characterized as liver steatosis and excessive fat (mainly relevant to triglyceride, TG) storage. Fatty liver can be clinically divided into alcoholic fatty liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). NAFLD can be further divided into non-alcoholic simple fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), and NASH related hepatic cirrhosis and hepatocellular carcinoma.[1,2] The incidence of NAFLD presented an increased trend over the years. Epidemiological evidence has shown that the prevalence of NAFLD is estimated to be 20%-30% in Europe and the United States, and 12%-24% in the Asia-Pacific region.[3,4]

The developmental origin of NAFLD has recently become the topic of global interest in metabolic studies. Shown mainly as low birth weight, intrauterine growth retardation (IUGR) has been reported to be one of the key risk factors for developing NAFLD.[5] A recent report by Cao et al. demonstrated that the susceptibility of NAFLD is increased in IUGR adult sheep induced by prenatal hypoxia.[6] Prenatal food restriction induced IUGR adult rats has been shown to display NAFLD and a high risk state of inflammation.[7] Moreover, female IUGR offspring rats by prenatal dexamethasone exposure presented much more accumulation of liver lipid with a high-fat diet.[8] Furthermore, clinical evidence also indicated the correlation/relevance between IUGR and NAFLD in children. A population-based study showed that among 90 Italian children with biopsy-proven NAFLD, the prevalence of IUGR with NAFLD was approximately 4 times higher than that in all children.[9] All these findings indicate that IUGR fetus after birth is faced with high susceptibility to NAFLD, and this susceptibility will stay into adulthood and present high prevalence of NAFLD induced by several acquired factors such as high-fat diet, inflammation and so on.

Cigarette smoke contains a variety of compounds that may be harmful to the developing fetus.

Among all smoking products, nicotine is considered one of the aversive components that perturb fetal development.[10] Epidemiological investigations and animal experiments demonstrated that the risk of adult MS is increased in fetuses with IUGR by prenatal smoke or nicotine exposure.[11-13] As one type of evolution of liver lesions, NAFL is the most common NAFLD, often associated with obesity, glucose or lipid metabolic disorders, and considered as the clinical characteristics or hepatic manifestation of MS.[14] However, an association between prenatal nicotine exposure and increased susceptibility to NAFL, as well as the underlying mechanism remains unclear.

Studies have suggested that hepatic de novo lipogenesis may mediate the generation of original NAFL, mainly referring to lipogenic transcriptional factor-sterol regulatory element binding protein-1c (SREBP1c).[15] Insulin-like growth factor 1 (IGF1) plays essential roles in IUGR caused by an unfavorable intrauterine environment and postnatal catch-up growth.[16] In the liver, IGF1 could induce the expression of SREBP1 and mediate hepatic lipid synthesis.[17] In our previous study, we confirmed that prenatal nicotine exposure might result in IUGR and maternal glucocorticoid (GC) over-exposure.[18,19] Furthermore, we proposed an underlying mechanism of "hypothalamic-pituitary-adrenocortical (HPA) axis-associated neuroendocrine metabolic programmed alteration" for susceptibility to MS in these IUGR offspring with and without high-fat diet.[20,21] In brief, with an over-exposure to maternal GC, the development of the fetal HPA axis of the IUGR fetus was inhibited; meanwhile, the glucose and lipid metabolism were affected, which could most likely be attributed to the increased fetal circulatory GC level;[19] after birth, the IUGR offspring showed low basal activity but enhanced sensitivity of HPA axis to chronic stress, as well as the GC-dependent alterations of glucose and lipid metabolic function, especially catch-up growth and steatosis of multiple organs under high-fat diet.[20,21] Therefore, we speculate that the correlations between GC and hepatic IGF1 pathway might be associated with IUGR formation, and further contribute to the catch-up growth and development of NAFL in adult IUGR offspring rats.

Since it has been reported that female offspring with IUGR acquire more susceptibility to

hepatic steatosis,[8] in this study, we evaluated the susceptibility of high-fat diet-induced NAFL in female adult offspring rats by prenatal nicotine exposure. We further explored its intrauterine programming mechanism by observing the metabolic phenotypes, gene expression of hepatic glucose and lipid metabolic function, as well as hepatic IGF1 pathway before and after birth. This work may help to elucidate the developmental toxicity of nicotine and explain the susceptibility to adult NAFL for IUGR offspring.

Results

Adult offspring rats

Body weight and food intake

As shown in Table 1, the female offspring rats in nicotine group showed significantly lower body weight than the control at postnatal week (PW) 1 (P<0.01). When fed a normal diet, the body weight of female rats was lower than the control at PW24 (P<0.01), while the corresponding body weight growth rate was significantly higher than that of the control at PW24 (P<0.01). However, when fed a high-fat diet, the body weight in nicotine group was close to that of the control group and the corresponding body weight growth rate was significantly higher than that of the control at PW24 (P<0.01). Food intake of the nicotine group exhibited no significant difference compared with control either under normal diet or high-fat diet (data not shown).

Serum glucose and TG levels

When fed with a normal diet, the serum glucose and TG levels were close to those of their respective controls for the female adult offspring rats in nicotine group (Fig. 1A, 1B). However, both the serum glucose and TG levels in nicotine group were much higher than their respective controls when fed with a high-fat diet (P<0.05, P<0.01, Fig. 1A, 1B).

Liver histological analysis

Hema-toxylin and eosin (H&E) staining showed that with normal diet, the hepatocytes of female

adult rats in the nicotine group (Fig. 2B) had no obvious pathological changes when compared to those in the control group (Fig. 2A), accompanied with a similar Kleiner score (< 1) in these two groups (Fig. 2E). However, when fed with the high-fat diet, the control group displayed scattered hepatocyte micro-vesicular steatosis (Fig. 2C), while the nicotine group showed prevalent macrovesicular steatosis (Fig. 3D), with a significantly increased Kleiner score (> 3) compared to the control (P<0.01, Fig. 2E), indicating the formation of NAFL in female adult offspring in the nicotine group.

Gene expression of hepatic IGF1 signal pathway, metabolic nuclear factor and key enzymes

With normal diet, only the expression of glucose transporter 2 (GLUT2) in nicotine group were significantly increased, as compared with the control (P<0.05, Fig. 3). However, under the high-fat diet, the expression of all key genes involved in hepatic IGF1 signal pathway, including IGF1, insulin-like growth factor 1 receptor (IGF1R), insulin receptor substrate 2 (IRS2) and GLUT2, were significantly increased in the nicotine group (P<0.05, P<0.01, Fig. 3).

We further detected the expression of hepatic metabolic nuclear factors, SREBP1c and fork-head box transcription factor O1 (FoxO1) and key enzymes related to glucose metabolism and lipid metabolism, including glucose-6-phosphatase (G6Pase), fatty acid synthase (FASN), acetyl-CoA carboxylase α (ACCα), AMP-activated protein kinase α (AMPKα), carnitine palmitoyltransferase 1 α (CPT1α) and microsomal triglyceride transfer protein (MTTP). The results indicated that the expression of G6Pase and FASN (involved in gluconeogenesis and lipogenesis, respectively), as well as CPT1α and MTTP (involved in lipid oxidation and output, respectively), were all significantly increased in the nicotine group, as compared with the control (P<0.05, P<0.01, Fig. 3). While for the nicotine group with high-fat diet, the expression of G6Pase, SREBP1c, FoxO1, FASN and ACCα (involved in lipogenesis) were all significantly increased, accompanied with significant decrease in AMPKα and CPT1α expression (involved in lipid oxidation) as well as decreased MTTP expression (involved in lipid output) (P<0.05, P<0.01, Fig. 3).

Fetal rats

Body weight, serum glucose and TG levels

The female fetal rats in the nicotine group showed much lower body weight and higher IUGR rate than those in the control group (P<0.01, Fig. 4A, 4B). Furthermore, serum glucose level were significantly increased while the TG level were significantly decreased in the nicotine group (P<0.01, Fig. 4C, 4D), compared to the control.

Histological and hepatocellular ultrastructure analysis

H&E staining revealed that female fetal rats in the nicotine group exhibited a reduced cellularity of parenchyma cells and an increase in both multinucleated giant cells and vacuolar parenchyma cells (Fig. 5B), as compared with the control (Fig. 5A). Further ultrastructural observation demonstrated that, in the parenchyma cells from the nicotine group (Fig. 5D), not only the number of mitochondria was decreased, but also the mitochondrial structure was damaged due to swelling deformation, and cristae mitochondria were destroyed, as compared with the control (Fig. 5C). In addition, some degranulation of rough endoplasmic reticula had occurred and a mass of large glycogen granules had accumulated in the cytoplasm of hepatic parenchyma cells in nicotine group (Fig. 5D).

Gene expression of hepatic IGF1 signal pathway and glucose and lipid metabolic pathways

As compared with the control, the expression of key genes involved in hepatic IGF1 signal pathway, including IGF1, IGF1R and insulin receptor (INSR), were significantly decreased in the female fetal rats (P<0.05, Fig. 6). On the other hand, the expression of genes involved in gluconeogenesis or lipogenesis (G6pase, SREBP1c, FoxO1, FASN and ACCα), were significantly increased, while the expression of AMPKα, CPT1α and MTTP, which are involved in lipid oxidation or output, were significantly decreased in the nicotine group (P<0.05, Fig. 6), compared with the control. The genes involved in adiponectin/leptin pathways were unchanged in the nicotine group.

Discussion

An increased susceptibility to NAFL in female adult offspring rats with IUGR induced by prenatal nicotine exposure

A large number of studies have pointed out an increased risk of adult MS in prenatal nicotine exposure-induced IUGR offspring, however, the potential role of prenatal nicotine exposure in susceptibility to adult NAFL has not been reported. Histological examination and Kleiner score are the "gold standard" for NAFLD diagnosis.[22] In the present study, we found that when fed with a normal diet, the female adult offspring in the nicotine group exhibited light steatosis, but large-areas of macrovesicular steatosis was found under the high-fat diet, accompanied with significantly higher Kleiner score (> 3) than that of the control. These results indicated that prenatal nicotine exposure had an effect on stimulating the hepatic lipid accumulation and increasing the susceptibility to high-fat diet-induced NAFL in female offspring. This was consistent with the finding that liver lipid accumulated much more easily in female IUGR offspring rats with the high-fat diet.[8]

Increased susceptibility to NAFL is mediated by intrauterine programming of hepatic glucose and lipid metabolism

Hepatic lipid dysregulation and lipid accumulation are the major mechanisms of NAFL, including increased de novo lipid synthesis and lipid input, as well as reduced fatty acid oxidation and lipid output.[17] In this study, severe NAFL was observed in prenatal nicotine exposure-induced female offspring rats with the high-fat diet. These female offspring showed enhanced hepatic lipid synthesis (SREBP1c, FoxO1, FASN and ACCα), as well as decreased lipid oxidation (AMPKα and CPT1α) and decreased output (MTTP), all of which may contribute to the formation of NAFL. We also observed the up-regulated gluconeogenesis (G6Pase), which may contribute to the increased blood glucose level, and the latter may further prompt hepatic lipid synthesis by inducing SREBP1c and FASN expression.[23]

Hepatic ultrastructure changes and lipid accumulations have been reported in a variety of IUGR fetal models.[6,24] In this study, the hepatic ultrastructure analysis of fetal rats in the nicotine

group also indicated the increased vacuolar parenchyma cells, decreased number of mitochondria accompanied with their destroyed structure, and the accumulated glycogen granules. Hepatic multiplex gene expression analysis further detected the increased gluconeogenesis and lipogenesis (G6pase, SREBP1c, FoxO1, FASN and ACC), as well as decreased lipid oxidation and output (AMPKα, CPT1 and MTTP). Decreased TG level in fetal blood was also detected, which may presumably be due to impaired hepatic lipid output, while MTTP as a key molecule for very low density lipoprotein output is significantly decreased.[25] All these results implied that prenatal nicotine exposure could impair the structure and function of fetal liver, resulting in enhanced hepatic gluconeogenesis and lipid synthesis, and reduced lipid oxidation and output. This was consistent with the alterations of adult offspring rats under the high-fat diet.

Intrauterine programming is the process by which the structure and function of tissues are altered permanently by insults acting during intrauterine period.[26] The consistent alteration of hepatic glucose and lipid metabolism before and after birth intrigued the possibility that this alteration is originated from intrauterine programming. The programmed glucose and lipid metabolic function alteration induced by prenatal nicotine exposure could promote the formation of high-fat diet-induced NAFL in adult offspring.

Intrauterine GC-IGF1 axis programming contributes to catch-up growth and aggravates the metabolic disorders

GC is an important regulatory hormone that regulates fetal growth, development and maturity *in utero*, and plays a vital role in multiple metabolic processes. IGF1 plays an insulin-like growth-promoting role and is crucial for pre- and post-natal growth. *In utero*, blood IGF1 level in IUGR fetuses may be modified by GC alterations, but not growth hormone.[27-29] Our previous studies showed that IUGR fetus induced by prenatal nicotine exposure suffered maternal GC overexposure but had decreased blood GC levels when fed with a normal diet or a high-fat diet after birth.[19-21] The present study further indicated the significantly reduced body weight accompanied with

down-regulated hepatic IGF1 signal pathway *in utero*. Moreover, the catch-up growth appeared and was more obvious under the high-fat diet after birth, accompanied with significantly enhanced hepatic IGF1 signal pathway in the female adult offspring. The negative correlation between serum GC-IGF1 and body weight before and after birth suggested an adaptive alteration of "intrauterine GC-IGF1 axis programming" in IUGR offspring by prenatal nicotine exposure.

IGF1 is considered as the main factor for adverse intrauterine environment-induced IUGR and postnatal catch-up growth.[16,30] Catch-up growth induced by IGF1 has been observed in IUGR offspring in many studies, which is closely related to the high risk of developing adult MS.[31] We speculate that the IUGR and postnatal catch-up growth of offspring with prenatal nicotine exposure might be induced by the adaptive alteration of "intrauterine GC-IGF1 axis programming". Moreover, elevated serum glucose and TG levels were also observed in adult offspring of the nicotine group, suggesting the up-regulated hepatic IGF1 pathway could aggravate the metabolic disorders under high-fat diet, which could promote the occurrence of NAFL.

Possible epigenetic mechanism for intrauterine programming alteration

Numerous studies suggested that epigenetics provides a molecular link between the prenatal environment effects on genes and subsequent susceptibility to adult diseases.[32,33] In Lane and Fu's studies, the reduced expression of CPT1 in IUGR rat persisted from fetal to adult period, which was induced by site-specific changes in histone H3 acetylation and predisposed to develop diabetes.[34,35] The expression of histone deacetylase silence signal regulating factor 1 (SIRT1) in IUGR rats consistently decreased after birth, which may cause liver steatosis by affecting the expression of several transcription factors.[36] Hepatic G6Pase expression was increased in newborn offspring with prenatal protein restriction, which was also mediated by epigenetic modification.[23] In addition, a growing number of studies suggested that abnormal epigenetic modifications play an important role in intrauterine programming.[37,38] For example, an epigenetic modification was considered to have participated in perinatal stress response programming of HPA axis.[39,40] Our previous studies also

indicated possible roles of epigenetic modifications of several fetal genes, such as hippocampal 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2), adrenal steroid acute regulatory protein (StAR) and liver IGF1, in HPA axis-associated neuroendocrine metabolic programming induced by prenatal caffeine or nicotine exposure.[41-43] Therefore, in the present study, the possible epigenetic mechanism such as epigenetic modifications of liver IGF1, G6Pase and SIRT1, may also involve in intrauterine programming of GC-IGF1 axis as well as hepatic glucose and lipid metabolism, which still needs further investigation.

Dosage range of nicotine exposure and its relevance with human's daily life

Smoking can cause many health problems; however, more than one third of people in the world smoke, and approximately 20%–50% of women smoke during pregnancy, while 25%–29% keep smoking until the end of their pregnancy.[44,45] Additionally, 50% of non-smoking mothers are exposed to passive smoking during pregnancy.[45] In the present study, 2.0 mg/kg.d nicotine were used to establish a stable IUGR rat model, based on our previous studies. This dosage is much less thanother lab's research, where 6.0 mg/kg.d nicotine was infused continuously *via* osmotic mini pumps to pregnant rats, and this procedure mimicked maternal cigarette smoke exposure.[46,47] Therefore, by using the dose conversion between humans and rats (human: rats = 1: 6.17),[48] 2.0 mg/kg.d nicotine exposure in pregnant rats is equivalent to nicotine exposure in a pregnant women of about 70 kg who smokes 2.3 cigarettes daily (a regular cigarette contains about 10 mg of nicotine).[49] Slightly more than two cigarettes a day is a very conservative estimation of a dosage for a regular smoker. Under this dosage, the alterations of intrauterine programming of GC-IGF1 axis as well as glucose and lipid metabolic function are obvious in female offspring rats. Therefore, the effects exerted by this dosage of nicotine can provide reference, at least partly, to estimating risks in population-based studies.

Materials and methods

Chemicals and reagents

Nicotine (CAS No 54-11-5, with a purity of 98%) was provided by Sanqiang Co, Ltd (Weifang, China). Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). Glucose oxidase assay kit was provided by Mind Bioengineering Co., Ltd. (Shanghai, China). TG 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 RT-PCR genes (PAGE purification) and GeXP multiplex gene expression analysis (HPLC purification) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals and agents were of analytical grade.

Animals and treatments

Specific pathogen-free Wistar rats (weighing 200–240 g for females and 260–300 g for males) were provided by the Experimental Center of Hubei Medical Scientific Academy (No. 2009-0004, Hubei, China). Animal experiments were performed at the Center for Animal Experiment of Wuhan University (Wuhan, Hubei, P.R. China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All experiment procedures involving animals were approved by and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Animals were held under temperature-controlled conditions on a 12-h light-dark cycle and had ad libitum access to standard chow and tap water. The present study comprised three independent experiments: fetal rats, adult offspring rats fed with the normal or high-fat diet. Pregnancies and nicotine treatments were the same for these three experiments, i.e., after one week of acclimation, two female rats were mated with one male rat for one night. Then the day was tagged as gestational day 0 (GD0) when sperms were observed in the vaginal smear of the two female rats and mating was

thus confirmed successful. Pregnant females were then transferred to individual cages. The rat IUGR model was induced by prenatal nicotine exposure as described in previous studies.[19,20] Pregnant rats were randomly divided into two groups: control and nicotine group. Starting from GD11 until term delivery (GD21-22), the nicotine group was subcutaneously administered with 1.0 mg/kg nicotine twice per day. The control group was given the same volume of saline.

In the experiment of fetal rats, 8 pregnant rats from each group were euthanized on GD20 under isoflurane anesthesia. Pregnant rats with litter size between 8 and 14 were considered qualified. The female fetuses were quickly removed and proceeded for body weight, and IUGR was diagnosed when the body weight of a fetus was two standard deviations lower than that of the control group.[50] Fetal blood samples were collected and serum was isolated. Fetal livers were separated and collected. Samples collected from each littermate were pooled together and immediately frozen in liquid nitrogen, followed by storage at -80°C for subsequent analyses. One fetal liver from each group was randomly selected and routinely fixed in 4% paraformaldehyde for light microscopic and transmission electron microscopic (TEM) analysis.

In the experiment of adult offspring rats fed with normal diet, 8 pregnant rats from each group were kept till normal delivery. On postnatal day 1 (PD1), the numbers of pups were randomly normalized to 8 per litter to ensure adequate and standardized nutrition. After weaning (at PW4), one female pup per litter was randomly selected from each group and fed with normal diet (providing 22% of its calorie intake as protein, 63% as carbohydrate, and only 5% as fat). The offspring rats were weighed weekly. The rate of body weight growth was calculated as follows: Gain rate of body weight (%) = [(body weight at PW24–body weight at PW1)]/body weight at PW1×100%. At PW24, the offspring rats were anesthetized with isoflurane and decapitated in a room separate from that where the other animals were kept. Serum was prepared from the subjects' blood and stored at -80°C until used for measurement. The livers were dissected, and a section of 5 randomly selected livers from each group were fixed in 4% paraformaldehyde solution for light microscopic analysis, while

all the 8 livers from each group were immediately frozen and stored at -80°C for gene expression analyses.

In the experiment of adult offspring rats fed with high-fat diet (containing 88.0% of corn flour, 11.5% of lard, and 0.5% of cholesterol, which provided 18.9% kcal from protein, 61.7% kcal from carbohydrate and 19.4% kcal from fat),[51] the animal treatment was the same as the adult rats fed with normal diet except the diet intake.

Analysis for blood samples

Serum glucose and TG levels were detected by biochemical assay kits following the manufacturer's protocol.

Light microscopic analysis and Kleiner score

Liver tissues stained with H&E was processed by standard procedures in gradient alcohols and xylene, paraffin embedded. Sections were observed and photographed with an Olympus AH-2 light microscope (Olympus, Tokyo, Japan). Five H&E sections of each group were selected and five random fields of each section were scored under the microscope.

The grading and staging of NAFLD were scored according to the system in the previous study by Kleiner et al.[22] The scoring system comprises 3 histological features that are evaluated semi-quantitatively: steatosis (0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2). A Kleiner score is calculated by the sum of the steatosis, inflammation and ballooning scores. Sections with a score of no less than 5 were diagnosed as NASH, while sections with a score of greater than 3 were diagnosed as NAFL. Steatosis was graded on a 4-point scale: grade 0 for no or negligible, grade 1 for mild, grade 2 for moderate and grade 3 for severe.

TEM analysis

1-mm^3 tissue blocks of liver samples were placed in 3% glutar-aldehyde/1.5% paraformaldehyde solution with 0.1 mol/L PBS. Samples were postfixed for 1.5 h in 1% osmium tetroxide/1.5% potassium ferrocyanide solution and washed in 0.1 mol/L PBS, dehydrated in gradient concentrations

of ethanol, and embedded in Epon 618. Epoxy blocks were sliced on a ultratome (LKB-V, LKB, Stockholm, Sweden, 70 nm), stained with uranyl acetate and lead citrate, and examined with a Hitachi Hu-12A transmission electron microscope (Hitachi, Co., Tokyo, Japan). Digital images were acquired directly by a computer.

Real-time quantitative RT-PCR

Total RNA extraction, reverse transcription as well as real-time quantitative PCR analysis were performed following the procedures in our previous report.[19] The target genes include: IGF1, IGF1R, IRS2, GLUT2, G6Pase, SREBP1c, FoxOl, FASN, ACCα, AMPKα, CPT1α, MTTP and housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). Primer sequences and annealing conditions for each gene are listed in Table S1 (Supplementary information Table S1). RT-PCR products were routinely verified by gel-resolved to exclude the formation of splicing variants.

Multiply gene expression analysis

Multiplex analysis containing 23 target genes and 3 housekeeping genes were completed using a multiplex primer designed by using the GenomeLabTM eXpress Profiler software (Beckman-Coulter, Fullerton, CA). Multiplex optimization (e.g., primer validation and attenuation) was performed according to the manufacturer's instructions. Briefly, a primer pair was considered valid if only one PCR product of less than one nucleotide differed from its predicted size after being run on the GenomeLab GeXP Genetic Analysis System (Beckman-Coulter, Fullerton, CA). The 23 target genes are: insulin/IGF1pathways (IGF1, IGFBP3, IGF1R, INSR, IRS1, IRS2, GSK3β and G6Pase), metabolic nuclear factors (SREBP1, FoxO1, PPARα and HNF4), key enzymes related to lipid metabolism (FASN, ACCα, CPT1α, AMPKα, MTTP, APOB and HMGCR) and adiponectin/leptin pathways (AdipoR2, LepR, JAK2 and mTOR2). Three housekeeping genes in this study are: GAPDH, ACTB and HPRT. The primer pairs of these genes are given in Table S2 (Supplementary information Table S2).

To account for the different scale of expression of each gene, the proportion of each reverse primer in the multiplex reverse transcription reaction was adjusted to obtain similar peak signals for each gene. Reverse transcription was performed on 100 ng of RNA as a template according to the GenomeLab GeXP Genetic Analysis System protocol. Real-time PCR amplification was performed by using a mixture of the forward primers, and the resulting reactions were analyzed by capillary electrophoresis on the GenomeLab GeXP with the GeXP Start kit reagents. Relative RNA expression levels were calculated against a pooled RNA standard using the GeXP Quant Tool software and normalized to GAPDH, β-actin, and HPRT expression levels. Typical spectra of fetal liver multiplex gene expression of the control and nicotine groups are shown in Fig. S1A and S1B (Supplementary information Figure S1), respectively.

Statistical analysis

SPSS 17 (SPSS Science Inc., Chicago, Illinois) and Prism (GraphPad Software, La Jolla, CA, USA) were used for data analysis. Quantitative data were expressed as the mean ± S.E.M. and evaluated with Independent Samples t-test. The mean weights for each litter were calculated and used for statistical analysis. For enumeration data, the body weight growth rate as well as IUGR rate was arcsine square-root transformed before t-test evaluations. The Kleiner score was evaluated with Mann-Whitney U test. Statistical significance was defined as $P<0.05$.

Conclusions

The present study systematically demonstrated the intrauterine origin of increased susceptibility to high-fat diet-induced NAFL in female offspring rats with prenatal nicotine exposure, which is most likely mediated by intrauterine programming of hepatic glucose and lipid metabolic function and GC-IGF1 axis (Fig. 7). The first GC-IGF1 axis programming may induce postnatal catch-up growth and aggravate glucose and lipid metabolism disorder, leading to an increased susceptibility to adult NAFL, while the second hepatic glucose and lipid metabolic programming may enhance hepatic

lipogenesis and reduce lipid oxidation and output, promoting the occurrence of NAFL.

The authors declare that there are no conflicts of interests.

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Table 1 Effects of prenatal nicotine exposure on body weights in female adult offspring rats with normal diet or high-fat diet.

Parameters	Normal diet		High-fat diet	
	Control	Nicotine	Control	Nicotine
Weight (g, W1)	12.7±1.2	9.2±0.5**	12.9±0.5	9.7±0.4**
Weight (g, W24)	248.8±18.1	226.7±10.2**	451.1±23.2	482.3±12.8
Growth rate (%)	1883.1±285.3	2529.3±242.4**	3415.1±280.3	2918.4±431.7**

Mean ± S.E.M., n=8 offspring from 8 pregnant rats. **P<0.01 vs. respective controls.

Figure legends

Fig. 1 Effects of prenatal nicotine exposure on serum glucose (A) and triglyceride (B) levels in female adult offspring rats with normal or high-fat diet. Mean ± S.E.M., n=8 offspring from 8 pregnant rats. $^{*}P<0.05$, $^{**}P<0.01$ vs. respective controls.

Fig. 2 Effects of prenatal nicotine exposure on liver histology and Klenier score in female adult offspring rats with the normal or high-fat diet (HE, ×400). A: liver histology of female adult rats in the control group with the normal diet; B: liver histology in the nicotine group with the normal diet; C: liver histology in control group with the high-fat diet; D: liver histology in female nicotine group with the high-fat diet; E: Klenier score comparison between the control and nicotine groups with the normal or high-fat diet. Mean ± S.E.M., n=5 offspring from 8 pregnant rats. $^{**}P<0.01$ vs. respective control.

Fig. 3 Effects of prenatal nicotine exposure on the expression of hepatic insulin-like growth factor 1 (IGF1) pathway, metabolic nuclear factor and key enzymes in female adult offspring rats with the normal or high-fat diet. IGF1R: IGF1 receptor; IRS2: insulin receptor substrate 2; GLUT2: glucose transporter 2; G6Pase: glucose-6-phosphatase; SREBP1c: sterol regulatory element binding protein 1c; FoxOl: fork-head box transcription factor O1; FASN: fatty acid synthase; ACCα: acetyl-CoA carboxylase α; AMPKα, AMP-activated protein kinaseα; CPT1α: carnitine palmitoyl acyl-CoA transferase 1α; MTTP: microsomal triglyceride transfer protein. Mean ± S.E.M., n=8 offspring from 8 pregnant rats. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* respective controls.

Fig. 4 Effects of prenatal nicotine exposure on body weight (A), intrauterine growth retardation (IUGR) rate (B), serum glucose (C) and triglyceride (D) levels in female fetal rats on gestational day 20. Mean ± S.E.M., n=8 litters from 8 pregnant rats. $^{**}P<0.01$ vs. control.

Fig. 5 Effects of prenatal nicotine exposure on liver histology and ultrastructure in female fetal rats on gestational day 20. A: HE staining of liver tissue from the control group (HE, ×400); B: HE staining of liver tissue from the nicotine group (HE, ×400); C: ultrastructural observation of parenchyma cells from the control group (TEM, ×10000); D: ultrastructural observation of parenchyma cells from the nicotine group (TEM, ×10000). SM: swollen mitochondria; AGG: accumulated glycogen granule.

Fig. 6 Effects of prenatal nicotine exposure on the expression of genes in hepatic insulin-like growth factor 1 (IGF1) pathway, metabolic nuclear factor and key enzymes in fetal rats. IGFBP3: insulin-like growth factor binding protein 3; IGF1R: IGF1 receptor; INSR: insulin receptor; IRS1/2: insulin receptor substrate 1/2; GSK3β: glycogen synthase kinase 3β; G6Pase: glucose-6-phosphatase; SREBP1: sterol regulatory element binding protein 1; FoxOl: fork-head box transcription factor O1; PPARα: peroxisome proliferator activated receptor α; HNF4: hepatocyte nuclear factor 4; FASN: fatty acid synthase; ACCα: acetyl-CoA carboxylase α; CPT1α: carnitine palmitoyl acyl-CoA transferase 1; AMPKα, AMP-activated protein kinaseα; MTTP: microsomal triglyceride transfer protein; APOB: apolipoprotein B; HMGCR: HMG-CoA reductase; AdipoR2: adiponectin receptor 2; LepR: leptin receptor; JAK2: Janus kinase 2; mTOR2: mammalian target of rapamycin complex 2. Mean ± S.E.M., n=8 litters from 8 pregnant rats. $^{*}P<0.05$, $^{**}P<0.01$ vs. control.

Fig. 7 The intrauterine programming alteration increases the susceptibility of high-fat diet-induced non-alcoholic simple fatty liver (NAFL) in female adult offspring rats with prenatal nicotine exposure. GC: glucocorticoid; IGF1: insulin-like growth factor 1.

Abstract

Previous studies have indicated that intrauterine growth retardation (IUGR) fetus was faced with high susceptibility of adult metabolic syndrome (MS). Non-alcoholic simple fatty liver (NAFL) is considered to be the hepatic manifestation of MS. In the present study, we evaluated the susceptibility of high-fat diet-induced NAFL in female adult IUGR offspring rats induced by prenatal nicotine exposure, and we further explored the underlying intrauterine programming mechanism for this phenomenon. The IUGR rat model was established by prenatal exposure to nicotine (2 mg/kg.d), the liver tissues from the female fetuses and the female adult offspring fed with normal or high-fat diet were collected. The female adult offspring in the nicotine group showed low birth weight and postnatal catch-up growth, as well as severe NAFL under high-fat diet. Moreover, increased gene expression involved in hepatic insulin-like growth factor 1 (IGF1) pathway, gluconeogenesis and lipid synthesis, decreased gene expression of lipid output accompanied with elevated serum triglyceride level, were observed. The female fetuses in the nicotine group showed down-regulated hepatic IGF1 pathway, and also exhibited a similar pattern of increased gluconeogenesis and lipid synthesis and decreased lipid output to those in the adults. The present study demonstrated the intrauterine origin of increased susceptibility to high-fat diet-induced NAFL in female offspring rats by prenatal nicotine exposure, which is most likely mediated by “two intrauterine programming”. That is, the first glucocorticoid-IGF1 axis programming induced postnatal catch-up growth, aggravated glucose and lipid metabolic disorder, lead to an increased susceptibility to adult NAFL, while the second hepatic glucose and lipid metabolic programming enhanced hepatic lipogenesis and reduced lipid oxidation and output, and promoted NAFL.

Key words: Non-alcoholic simple fatty liver; Prenatal nicotine exposure; Intrauterine programming; Insulin-like growth factor 1; Glucose and lipid metabolic function.

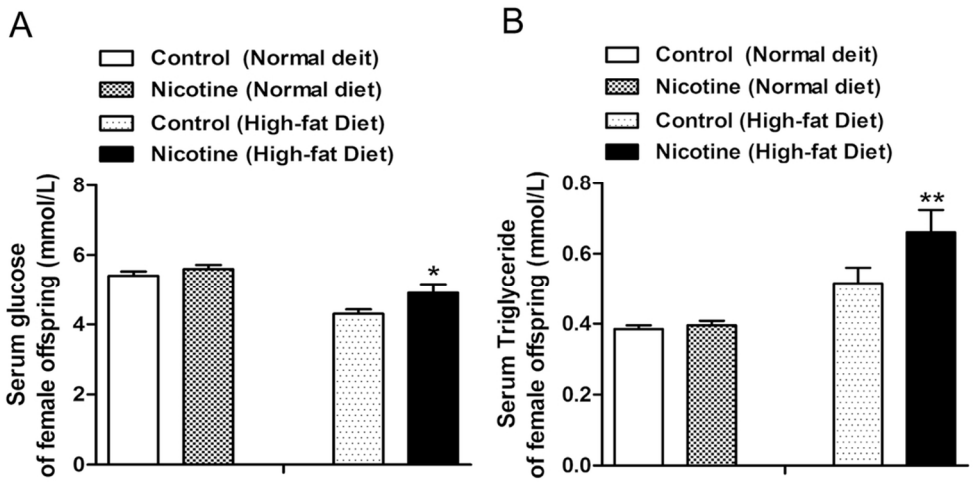

Fig. 1 Effects of prenatal nicotine exposure on serum glucose (A) and triglyceride (B) levels in female adult offspring rats with normal or high-fat diet. Mean ± S.E.M., n=8 offspring from 8 pregnant rats. *P<0.05, **P<0.01 vs. respective controls.
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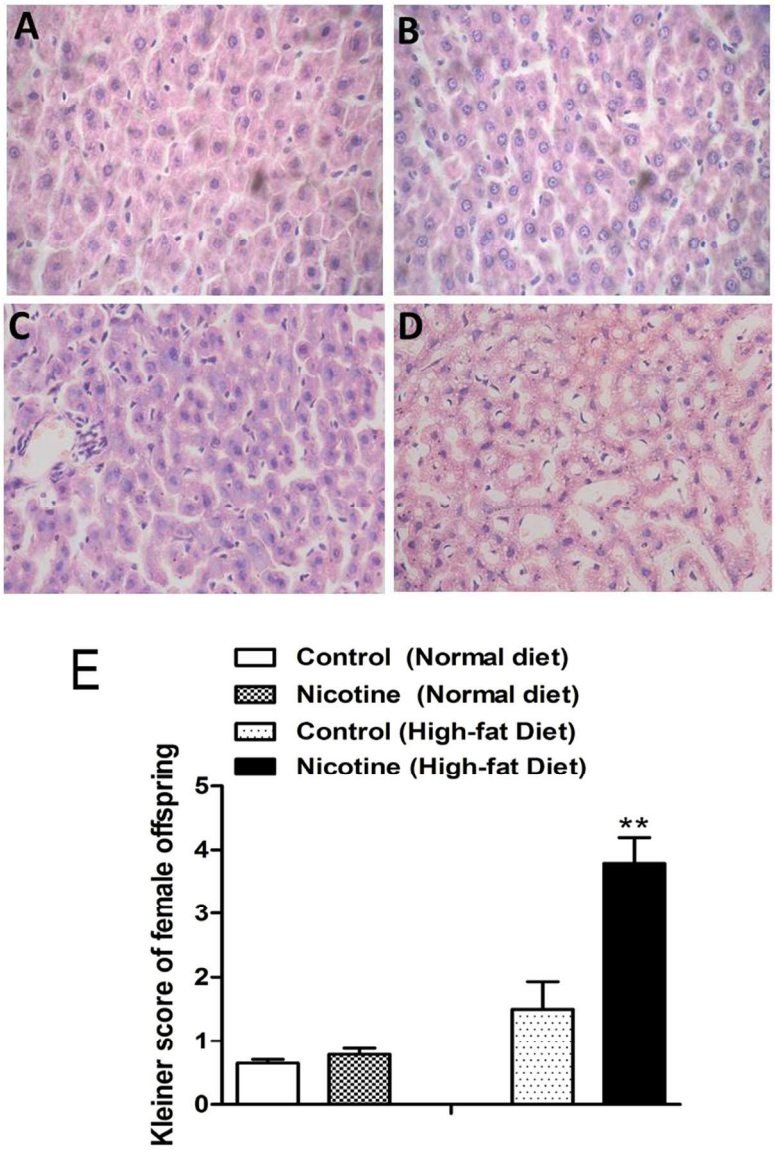

Fig. 2 Effects of prenatal nicotine exposure on liver histology and Klenier score in female adult offspring rats with the normal or high-fat diet (HE, ×400). A: liver histology of female adult rats in the control group with the normal diet; B: liver histology in the nicotine group with the normal diet; C: liver histology in control group with the high-fat diet; D: liver histology in female nicotine group with the high-fat diet; E: Klenier score comparison between the control and nicotine groups with the normal or high-fat diet. Mean ± S.E.M., n=5 offspring from 8 pregnant rats. **P<0.01 vs. respective control.
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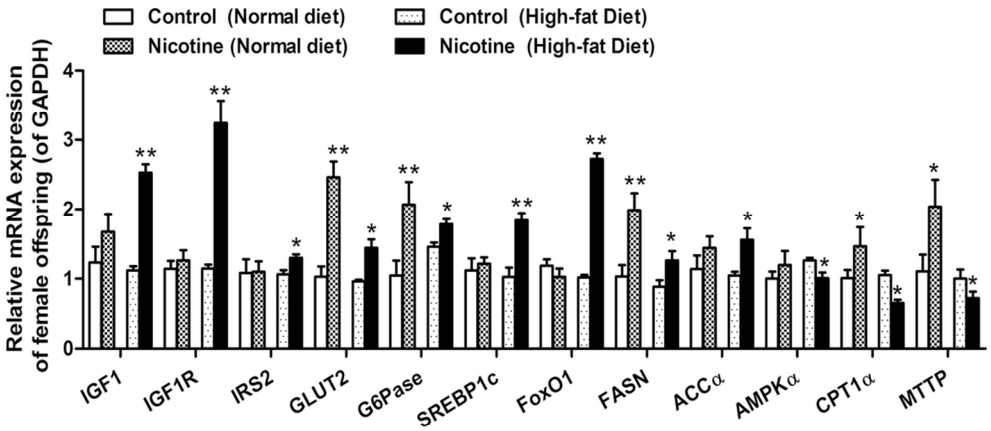

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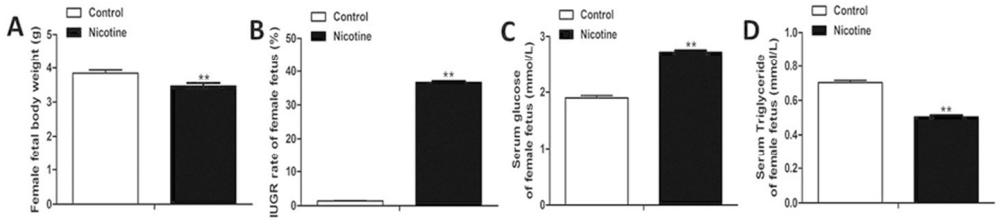

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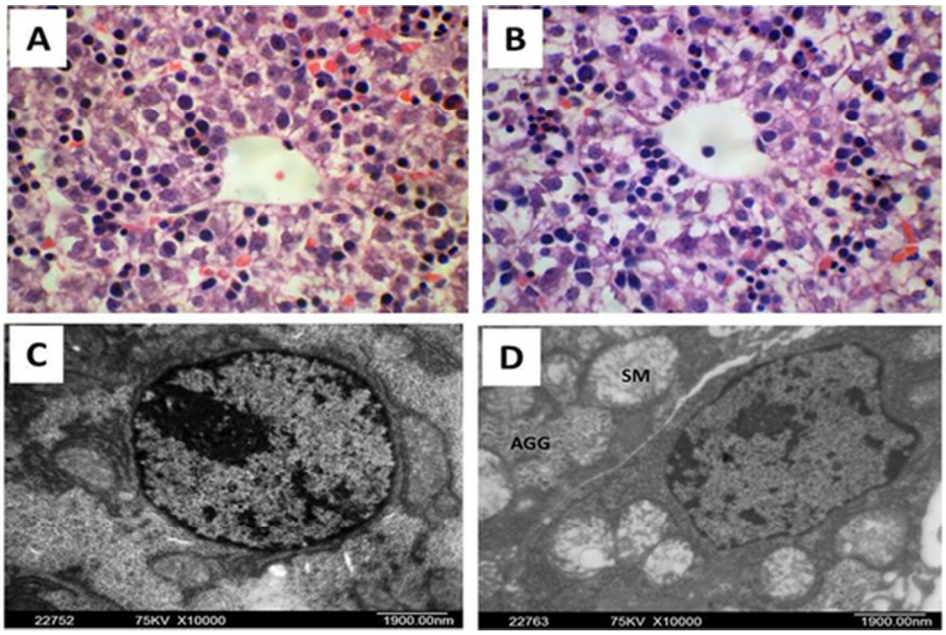

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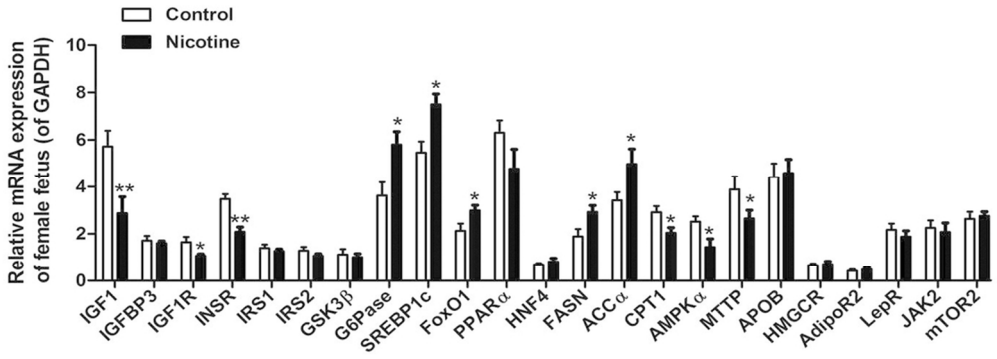

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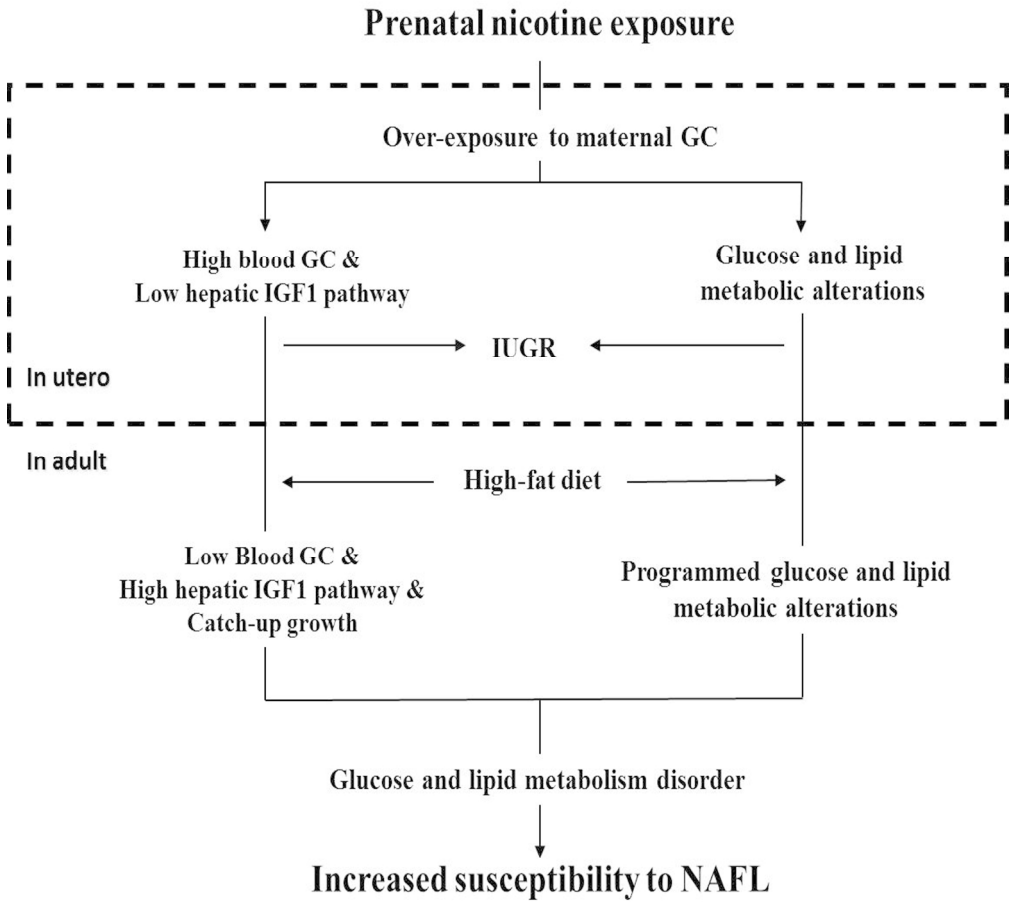

Fig. 7 The intrauterine programming alteration increases the susceptibility of high-fat diet-induced non-alcoholic simple fatty liver (NAFL) in female adult offspring rats with prenatal nicotine exposure. GC: glucocorticoid; IGF1: insulin-like growth factor 1.
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