Maternal high-fat diet programs Wnt genes through histone modification in the liver of neonatal rats

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Abstract

Maternal high-fat (HF) diets during gestation and lactation have been shown to contribute to metabolic disorders in offspring. Molecular and epigenetic mechanisms underlying this connection may be essential for the prevention and treatment of the fetal origins of metabolic diseases. The current study examined the impact of maternal HF diets on Wnt signaling and histone modification in offspring. Time-pregnant Sprague–Dawley rats were fed either control diet or HF diet during gestation and lactation and then the neonatal offspring of both groups were investigated. The neonatal offspring born to dams fed on HF diets exhibited increases in serum glucose and liver triglyceride levels. Maternal exposure to the HF diet also repressed the mRNA expression of Wnt1 and nuclear β-catenin protein in the liver of offspring. The altered Wnt1 gene expression may be due to the changes of acetylation of H4 at its promoter as well as acetylation of H4 and methylation of H3K9 at coding region. Maternal exposure to the HF diet induced suppression of the Wnt/β-catenin signaling pathway through histone modification, potentially increasing the risk of metabolic syndrome.

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Introduction

The prevalence of type II diabetes, obesity, and other metabolic syndromes has remarkably increased not only in developed countries but also in developing countries, which has been considered to be in large part due to an imbalance nutritional status, such as excessive intake of dietary fat (Lichtenstein et al. 1998, Suh et al. 2001, Donahoo et al. 2008). The fetal period is a key developmental window to nutritional exposure, and excessive nutritional intake during this period has been shown to contribute to some features of metabolic disorders, such impacts being independent of postnatal nutritional environment (Chechi & Cheema 2006, Martin-Gronert & Ozanne 2010). The link between maternal nutritional status and health of offspring has been explained by the developmental origins of the health and disease paradigm, which indicates that maternal nutritional factors lead to fetal adaptations that can cause long-term structure, physiology, and metabolic changes (Hales & Barker 1992, 2001, Godfrey & Barker 2001).

Recently, quite a few animal studies have indicated that a maternal high-fat (HF) diet increases offspring susceptibility to the later development of a metabolic syndrome-like phenotype (Singh et al. 2003, Elahi et al. 2009, Odaka et al. 2010). In animal models of maternal HF feeding, offspring born to mothers fed a HF diet during gestation only or during gestation and lactation exhibited a markedly increased adiposity, metabolic disorders, poor glycemic control, insulin resistance, hepatic metabolic dysfunction, and development of nonalcoholic fatty liver disease (NAFLD; Bayol et al. 2005, Ashino et al. 2011). Though the physiological outcome of a maternal HF diet on offspring has been well investigated, further studies are needed to unravel the molecular and epigenetic mechanisms, which may be essential for the prevention and treatment of the fetal origins of metabolic disorders.

The liver plays an important role in whole body energy balance and metabolism, and the development of fetal liver is a key target for altered maternal nutritional conditions (Hyatt et al. 2008, Ghouri et al. 2010, Savage & Semple 2010). Many studies have shown an effect of nutritional programming on liver metabolism and gene expression (Brameld et al. 2000, Buckley et al. 2005). As a critical regulator of liver development, the Wnt signaling pathway is essential for embryonic liver development, postnatal liver growth, and regeneration (Negishi et al. 2010). The Wnt signaling pathway is involved in almost every aspect of embryonic development and influences homeostatic self-renewal in many mature tissues (Peifer & Polakis 2000). In addition to its original attribution to liver developmental biology,
Wnt signaling has recently been identified as a regulator of various endocrine functions and a participant in the development of metabolic syndrome (Jin 2008, Schinner 2009).

As such, we propose that maternal HF diets influence Wnt gene expression through histone modification in offspring and subsequently trigger Wnt signaling cascades, which could affect fetal and postnatal liver development and downstream target genes, and predispose the offspring to metabolic diseases. To investigate this hypothesis, time-pregnant rats were fed a HF diet during gestation and lactation and then physiological outcomes, mRNA expression of Wnt genes, and nuclear protein levels of the neonatal offspring were detected. Histone modification, including acetylated histone 3 (H3Ac), acetylated histone 4 (H4Ac), dimethylated histone 3 at lysine 4 residues (H3K4Me2), and trimethylated histone 3 at lysine 9 residues (H3K9Me3) for promoter and coding region of Wnt genes were examined by chromatin immunoprecipitation (ChIP) assay.

Materials and methods

Animals and treatment

Twelve virgin female Sprague–Dawley rats (80 days old) were taken from Shanghai Slac Laboratory Animal, Inc. (Shangai, China) and fed with standard laboratory chow for 1 week for adaptation. Mating was performed by housing females with adult male rats overnight, and pregnant rats were randomly assigned to control (C, n=6) or HF diet (HF, n=6), which were modified according to the AIN-93 diet formula (Table 1) throughout gestation and lactation periods. Animals were individually housed in standard polycarbonate cages and maintained in a humidity- and temperature-controlled room on a 12 h light:12 h darkness cycle with access to water and chow ad libitum. Twenty-four hours after birth, litter sizes were standardized to eight pups to minimize variation in offspring’s nutrition status during suckling. Twelve rat offspring (two offspring were randomly chosen per litter) for each group were killed at postnatal day 7. The left lobe of liver was dissected out, frozen in liquid nitrogen, and stored at −80 °C for future analysis. The animal protocol for this study was approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University, School of Medicine.

Blood sample collection and analysis

Dam blood samples were collected from the heart after overnight fasting and offspring blood samples were collected after 2 h fasting. Serum was collected after centrifugation at 3000 g for 15 min and stored at −80 °C until subsequent analysis. Serum aliquots were used to measure serum glucose by glucose oxidase method (Rongsheng Biotechnology, Inc., Shanghai, China). Serum insulin was analyzed with a RIA kit (RI-13K, Linco Research, St Charles, MO, USA). Serum triglyceride (TG) and cholesterol contents were measured enzymatically by automatic biochemistry analyzer (Hitachi 7600).

Liver histology and TG and glycogen analysis

Liver TG content was assayed using a method described previously (Kim et al. 2003). Briefly, ∼0.1 g frozen tissue was homogenized in chloroform/methanol (1 ml, 2:1 vol/vol) over ice. Homogenates were shaken overnight before adding 0.6% NaCl and then the samples were centrifuged at 370 g for 10 min to separate phases. The TG-containing organic layer was separated and air-dried. The isolated lipids were resuspended in 250 μl ethanol and then determined spectrophotometrically. Liver glycogen content was measured spectrophotometrically using a commercially available assay kit (Nanjing Jiancheng Bioengineering, Inc., Nanjing, China). Frozen liver samples were processed for sectioning by cryostat (Leica CM1900, Heidelberg, Nussloch, Germany) after being embedded in O.C.T. compound (Sakura Finetek USA, Torrance, CA, USA). Liver sections were then stained with hematoxylin and eosin (H&E) and photographed using fluorescence microscope (Nikon Eclipse80i, Tokyo, Japan).

Real-time quantitative RT-PCR (qPCR)

Frozen liver samples were ground with a mortar and pestle in liquid nitrogen and total RNA was isolated with TRI reagent (Sigma). All purified RNA samples

<table>
<thead>
<tr>
<th>Table 1 Diet composition</th>
<th>Control (C)</th>
<th>High-fat (HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of calorie (kcal%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>64</td>
<td>35</td>
</tr>
<tr>
<td>Fat</td>
<td>16</td>
<td>45</td>
</tr>
<tr>
<td>Ingredient (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corn starch</td>
<td>437.2</td>
<td>72.8</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>102</td>
<td>172</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Lard</td>
<td>47</td>
<td>177.5</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
exhibited A260/A280 ratios $\geq 1.6$. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription of 2 μg total RNA in a 20 μl reaction volume. The entire procedure was performed in a DNA 2720 Thermal Cycler (Applied Biosystems). The samples were heated at 37 °C for 2 h for reverse transcription followed by 85 °C for 5 s to inactivate reverse transcriptase and terminate the reaction. To measure the relative amount of mRNA, cDNA samples were analyzed in a 96-well plate using a 7300 Real-Time PCR System (Applied Biosystems) and iTag SYBR Green Supermix with ROX (Bio-Rad). The reactions were activated at 95 °C for 1 min. Ribosomal protein L7a mRNA level was also measured to use for normalization of data. After PCR, a dissociation curve was generated by stepwise increase in the temperature from 55 to 95 °C to ensure that a unique product was amplified in the reaction. Primers for qPCR were designed using Vector NTI software (InforMax, Inc., Frederick, MD, USA; Table 2).

**Nuclear extraction and western blotting**

Frozen muscle samples (150 mg) were ground in liquid nitrogen and then lysed with lysis buffer (10 mmol/l HEPES, pH 7.9, 10 mmol/l sodium chloride, 1.5 mmol/l magnesium chloride, 1 mmol/l EGTA, 0.2 mol/l sucrose, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulphonyl fluoride, 1× protease inhibitor cocktail, 4× phosphatase inhibitor cocktail 1, 4× phosphatase inhibitor cocktail 2, and 0.5% NP-40) on ice for 5 min and centrifuged. Crude nuclei were collected from lysis of the resuspended pellet and sonicated on ice. After centrifugation at 10,000 g, the supernatants were nuclear protein extracts. Lowry assay was used to determine protein content, and samples containing 40 μg protein were resolved by 10% SDS–PAGE. Western analysis was performed using a wet transfer protocol, using 1:1000 rabbit polyclonal antibody against β-catenin (#9587, Cell Signaling Technology, Danvers, MA, USA) to detect β-catenin protein, goat anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Western blot images were captured and analyzed by Chemi Doc system (Bio-Rad). An anti-CREB antibody (RB-17000P0, NeoMarkers, Fremont, CA, USA) was used as an internal control for each sample, and the result of β-catenin protein expression levels was normalized to the expression of CREB.

**Chromatin immunoprecipitation**

ChIP analysis was performed according to a modified protocol (Chen *et al.* 2004). Briefly, 200 mg frozen liver samples were ground in liquid nitrogen, resuspended in PBS, and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was resuspended in nuclei swelling buffer containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer containing protease inhibitors. The resulting chromatin was sonicated (Fisher Scientific model D100 Sonic Dismembrator, Pittsburgh, PA, USA) on ice with eight bursts for 40 s at power setting 5 with 2 min cooling interval between each burst. The sample was then centrifuged at 16 100 g for 10 min at 4 °C to remove cell debris from the crude chromatin lysate. One milliliter sheared chromatin was diluted in total 10 ml ChIP dilution buffer. Ten percent of the diluted lysate was subsequently incubated overnight on a hematology mixer with 2 μg primary antibodies at 4 °C (Table 3). Preblocked salmon sperm DNA/protein A agarose beads (60 μl, 50% slurry; Upstate Biotechnology, USA) were used for reverse transcription of 2 μg total RNA in a 20 μl reaction volume. The entire procedure was performed in a DNA 2720 Thermal Cycler (Applied Biosystems). The samples were heated at 37 °C for 2 h for reverse transcription followed by 85 °C for 5 s to inactivate reverse transcriptase and terminate the reaction. To measure the relative amount of mRNA, cDNA samples were analyzed in a 96-well plate using a 7300 Real-Time PCR System (Applied Biosystems) and iTag SYBR Green Supermix with ROX (Bio-Rad). The reactions were activated at 95 °C for 1 min. Ribosomal protein L7a mRNA level was also measured to use for normalization of data. After PCR, a dissociation curve was generated by stepwise increase in the temperature from 55 to 95 °C to ensure that a unique product was amplified in the reaction. Primers for qPCR were designed using Vector NTI software (InforMax, Inc., Frederick, MD, USA; Table 2).

**Table 2** Primer sequence used in qPCR and ChIP assay

<table>
<thead>
<tr>
<th>Gene (Ensembl ID)</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt1 (ENSRNO-G0000014627)</td>
<td>(+ 1663)CCCCTGACCTCCTGTGTATCAC</td>
<td>(+ 1742)TGAAGGCCAGGTGTTGGGTT</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>Wnt3a (ENSRNO-G0000003039)</td>
<td>(+ 210)CAAGGCGGGCATCCAAGAGT</td>
<td>(+ 281)CTGTTGCTGACAGTGGTGAGTC</td>
<td>Histone modification at promoter</td>
</tr>
<tr>
<td>Wnt5a (ENSRNO-G0000015618)</td>
<td>(+ 1256)GCCTGCTGACTGACCAGTTTAAGA</td>
<td>(+ 1357)TCCCAAATCTCCCGTCGACTTT</td>
<td>Histone modification at coding region</td>
</tr>
</tbody>
</table>

**Table 3** Antibodies used in ChIP assay

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3Ac</td>
<td>Upstate, Temecula, CA, USA</td>
<td>06-599</td>
</tr>
<tr>
<td>H4Ac</td>
<td>Upstate</td>
<td>06-866</td>
</tr>
<tr>
<td>H3K4Me2</td>
<td>Upstate</td>
<td>07-030</td>
</tr>
<tr>
<td>H3K9Me3</td>
<td>Upstate</td>
<td>cs200604</td>
</tr>
<tr>
<td>IgG</td>
<td>Santa Cruz</td>
<td>sc-2027</td>
</tr>
</tbody>
</table>

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Lake Placid, NY, USA) were then added to the chromatin for 2 h followed by centrifugation at 370 g for 1 min at 4 °C. Supernatant of normal rabbit IgG was saved as the input control for PCR after clean up. The pellets containing immunoprecipitated complexes were washed sequentially with 1 ml low-salt solution, high-salt solution, and LiCl solution and twice with TE (pH 8-0). Antibody/protein/DNA complexes were eluted from protein A agarose beads by adding twice 250 µl of the elution buffer followed by shaking at 37 °C at 300 r.p.m. for 15 min and flash spin down at room temperature. The combined supernatants were incubated at 65 °C for 4–5 h after addition of 20 µl 5 M NaCl and 1 µg RNase A to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K at 37 °C for 1 h to remove protein and DNA was purified with a Wizard SV Gel and PCR Clean up System (Promega). Five percent of immunoprecipitated DNA was used for each real-time PCR. The standards and the samples were simultaneously amplified using the same reaction master mixture in a 25 µl reaction volume, and primers were used to amplify genomic sequences at the promoter and coding region of Wnt1.  

**Statistical analysis**

Values were expressed as mean ± S.E.M., and differences were evaluated using one-way ANOVA test. The level of significance was set at P<0.05. All analyses were conducted using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).  

**Results**

**Body weight and serum analysis in dams**

There was no difference in dam body weight between the C and HF groups throughout gestation (Fig. 1A). At the time of being killed (7 days after giving birth to offspring), serum glucose, insulin, TG, and cholesterol levels of dams fed the HF diet exhibited no significant difference from dams fed the C diet (Table 4). The litter size of HF dams was 10·67 ± 2·08, which did not differ from the litter size of C dams (11·33 ± 2·42; P>0·05).  

**Physiological observations in offspring**

The birth weight of HF offspring was higher than that of C offspring (7·11 ± 0·45 vs 6·56 ± 0·18 g), but the difference was not statistically significant (P>0·05). At postnatal age of day 7, the body weight of HF offspring was significantly higher than that of C offspring (15·12 ± 0·82 vs 13·03 ± 0·47 g; P<0·05; Fig. 1B). Serum glucose levels were higher in HF offspring than in C offspring (P<0·05), while serum insulin, TG, and cholesterol levels of both groups showed no significant difference (Table 5). There was no significant difference in liver glycogen content between HF offspring and C offspring (Fig. 2A). Quantitative determination revealed that the liver TG level was about 1·4-fold higher in HF offspring than in C offspring (P<0·05, Fig. 2B). Liver structure tested by H&E staining was normal and did not differ qualitatively between the C and HF offspring (Fig. 2C).

**Wnt gene mRNA expression in liver of offspring**

The mRNA expressions of the Wnt gene family, including Wnt1, Wnt3a, and Wnt5a, were examined. The mRNA expression of Wnt1 decreased 2·5-fold.
Nuclear β-catenin protein levels in the liver of offspring

Because Wnt1 participates in the canonical Wnt signaling pathway and β-catenin is the major effector of this pathway, we detected the nuclear β-catenin protein level in offspring liver. Nuclear β-catenin protein was significantly lower in the liver of HF female offspring when compared with C (Fig. 4; $P<0.05$), a result that is consistent with the trend observed in the mRNA expression of Wnt1.

Histone modifications at the Wnt1 promoter and coding region in offspring

ChIP assay was performed to determine whether the altered expression of Wnt1 was regulated by the changes in chromatin structure. Figure 5A shows that there was a significant decrease in H4Ac in the HF offspring when compared with C offspring ($P<0.05$) at the Wnt1 promoter region, but there was no statistical difference in H3Ac, H3K4Me2, and H3K9Me3 between both groups. At the Wnt1 coding region, H3Ac was significantly decreased ($P<0.05$) by 3.0-fold while H3K9Me3 was increased by 2.2-fold in the HF offspring when compared with C (Fig. 5B). There was no difference in the IgG binding within promoter and coding regions (Fig. 5A and B), which was used as a negative control to verify the validity of the results at the promoter and coding regions of the gene. The ChIP results indicate that the altered Wnt1 gene expression in response to a maternal HF diet may occur due to the changes in acetylation of H4 at its promoter as well as acetylation of H4 and methylation of H3K9 at coding region.

Discussion

In the current study, there were no significant differences in body weight and serum profile between the rat dams fed a C and HF diet during gestation, whereas the neonatal offspring born to dams fed a HF diet exhibited early metabolic defects, including increased serum glucose and liver TG levels compared with those born to control dams. This result indicates that the impact of a maternal HF diet on offspring physiological outcomes is independent of maternal obesity as the body weight of HF-fed rat dams was not heavier than that of C dams during gestation.

In a recent systematic review, Ainge et al. (2011) identified that the risk of diabetes and obesity development in offspring born to HF-fed dams appears to be independent of maternal obesity, birth weight, and postweaning macronutrient intake. However, there are contradictions among the animal models of maternal HF feeding. An animal study designed to separate the effects of maternal diet and maternal adiposity suggested that maternal obesity, not maternal HF diet per se, leads to insulin resistance and increased body weight that persists into adulthood in offspring (White et al. 2009). Possible reasons for the controversial results could be the different type of dietary fat.
and upregulated hepatic lipogenesis in groups of offspring from HF-fed mothers. In this study, the metabolic disorders observed in neonatal offspring of HF-fed dams may be due to the impairment of fetal liver development, which was programmed by the suppressed Wnt/β-catenin signaling pathway. In future studies, the downstream target genes of Wnt/β-catenin signaling in response to maternal HF as well as how it regulates hepatocellular events, such as differentiation, proliferation, oxidative stress, and morphogenesis, need to be identified.

Lately, there is increasing interest in the role of epigenetic mechanisms mediating the impact of maternal nutritional status on the health of offspring (Burdge et al. 2007). In a maternal protein restriction animal model, gene expression was programmed through histone modification but not DNA methylation in the offspring mammary gland and liver (Zheng & Pan 2011). Histone modifications, mainly including methylation and acetylation, regulate gene expression without changing the DNA sequence. Acetylation of histone H3 and H4 is associated with actively transcribed genes, while methylation of histone has either positive or negative effects on gene transcription, depending on methylation location. Methylation of histone H3 at lysine residues 9 (H3K9) leads to an inhibition of gene transcription, whereas methylation of H3 at lysine residues 4 (H3K4) correlates with active transcription (Alvarez-Venegas & Avramova 2005,

used and the proportion of energy contributed by dietary fat, which are quite distinct from each other in these studies. Fat content of the experimental HF diet ranged from 34 to 60% of total energy, and most studies used saturated fat as the main source of fat in the diet (Giraudo et al. 2010, Ainge et al. 2011). In our study, the fat in the HF group provided 45% of the total calories and was mainly composed of lard, which is rich in saturated fatty acids fat.

The Wnt family, including Wnt1, Wnt3a, and Wnt5a, transmits information from the cell surface to the nucleus by binding to frizzled proteins on target cells, which in turn induces the Wnt signaling cascade and then influences embryonic development as well as cell fate determination (Willert & Jones 2006). In the current study, we report for the first time that maternal exposure to a HF diet programmed the canonical Wnt signaling pathway in the liver of offspring by decreasing the expression of Wnt1 and the protein level of β-catenin. Although the role of specific Wnt genes remains obscure during liver development, the function of canonical Wnt signaling pathway and β-catenin has been confirmed in multiple physiological and pathological aspects of liver, including development, regeneration, growth, and carcinogenesis (Clevers 2006, Behari 2010). Ober et al. (2006) identified an important role of Wnt signaling as a positive regulator of liver specification and induction in zebrafish. As the liver plays a pivotal role in glucose and fatty acid metabolism over both short and long periods, any alteration in the fetal development of the liver could exert long-term influences on metabolic capacity. Bruce et al. (2009) reported that maternal HF intake contributes to the development of NAFLD progression and hepatic steatosis.
Different from C, P ratio to the input DNA, the bar values are mean trimethylated histone 3 at lysine 9 residues. Data are shown as a

Histone modification at Wnt1 promoter and coding regions in offspring. Histone modifications within the promoter region (A) and coding region (B) of Wnt1 in the liver of offspring born to dams fed the C or HF diet. Normal rabbit antibody, IgG, was used as a negative control to show nonspecific binding. H4Ac, acetylated histone 4; H3Ac, acetylated histone 3; H3K4Me2, dimethylated histone 3 at lysine 4 residues; H3K9Me3, trimethylated histone 3 at lysine 9 residues. Data are shown as a ratio to the input DNA, the bar values are mean ± S.E.M. (n = 12). * Different from C, P < 0.05.

Pal-Bhadra et al. 2007). Our ChIP experiment showed maternal HF diet-induced histone modifications in the liver of offspring that had previously been associated with transcriptional regulation, including acetylation of H3 and H4 and methylation of H3K9. These histone coding alterations provide experimental support for the hypothesis that maternal nutritional conditions can cause epigenetic changes that persist long after the exposure is done in the offspring.

In summary, the current study showed that maternal exposure to a HF diet induced suppression of Wnt1 mRNA expression and nuclear β-catenin protein as well as physiological outcomes including elevated blood sugar and increased fat accumulation in the liver of neonatal offspring. With the ChIP result, we suggest that the histone coding alterations might contribute to a mechanism that stably links the transient exposure conditions in early life to changes in the gene expression programming prevailing long after the exposure is done.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

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Author contribution statement

W Cai and Kf Yang designed the experiment. Kf Yang conducted the animal experiments and analyzed the data; Jl Xu and W Shi assisted with laboratory analysis and data collection; Kf Yang drafted the manuscript and W Cai gave important advice to paper writing.

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