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# Prenatal caffeine exposure induced a lower level of fetal blood leptin mainly via placental mechanism

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ABSTRACT

It's known that blood leptin level is reduced in intrauterine growth retardation (IUGR) fetus, and placental leptin is the major source of fetal blood leptin. This study aimed to investigate the decreased fetal blood leptin level by prenatal caffeine exposure (PCE) and its underlying placental mechanisms. Pregnant Wistar rats were intragastrically administered caffeine (30–120 mg/kg day) from gestational day 9 to 20. The level of fetal serum leptin and the expression of placental leptin-related genes were analyzed. Furthermore, we investigated the molecular mechanism of the reduced placental leptin's expression by treatment with caffeine (0.8–20 μM) in the BeWo cells. In vivo, PCE significantly decreased fetal serum leptin level in caffeine dose-dependent manner. Meanwhile, placental mRNA expression of adenosine A2a receptor (Adora2a), cAMP-response element binding protein (CREB), a short-type leptin receptor (Ob-Ra) and leptin was reduced in the PCE groups. In vitro, caffeine significantly decreased the mRNA expression of leptin, CREB and ADORA2A in concentration and time-dependent manners. The addition of ADORA2A agonist or adenylyl cyclase (AC) agonist reversed the inhibition of leptin expression induced by caffeine. PCE induced a lower level of fetal blood leptin, which the primary mechanism is that caffeine inhibited antagonized Adora2a and AC activities to decreased cAMP synthesis, thus inhibited the expression of the transcription factor CREB and target gene leptin in the placenta. Meantime, the reduced transportation of maternal leptin by placental Ob-Ra also contributed to the reduced fetal blood leptin. Together, PCE decreased fetal blood leptin mainly via reducing the expression and transportation of leptin in the placenta.



## 1. Introduction

Leptin is a satiety hormone and also can inhibit the apoptosis of trophoblastic cells (Attig et al., 2013; Toro et al., 2014). Several studies have documented that the lower blood leptin is closely associated with intrauterine growth retardation (IUGR) (Bouret and Simerly, 2007; Attig et al., 2013). Epidemiological and animal studies have reported that fetal blood leptin level is lower in offspring with IUGR (Shekhawat et al., 1998; Pighetti et al., 2003; Franco-Sena and Goldani, 2010; Struwe et al., 2010; Wang et al., 2010; Fahlbusch et al., 2012). Increasing specific studies have reported that fetal blood leptin is correlated to the development of hypothalamic–pituitary–adipose axis, adipose tissue, liver, bone and muscle (Bouret and Simerly, 2007; Attig et al., 2013). Moreover, leptin supplementation to piglet neonate enhanced their growth rate and was associated with an apparent improvement in the growth of several organs, including their digestive, reproductive and immune systems (Attig et al., 2013). Therefore, some researchers suggest that blood leptin can become biomarkers for IUGR (Lepercq et al., 2001; Struwe et al., 2010; Fahlbusch et al., 2012).

Placenta, white adipose tissue and maternal blood are the main sources of fetal blood leptin, and placental leptin is the major source during pregnancy (Mise et al., 2007; Kyriakakou et al., 2008). A study has suggested that the decrease of leptin level in IUGR fetus is possibly related to an inhibited expression of placental leptin (Lorenzo et al., 2010), but its molecular mechanism has not been clarified. The expression of leptin is regulated by the cyclic adenosine monophosphate (cAMP) in normal placenta (Maymo et al., 2011). The intracellular cAMP successively activates protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) (Ge et al., 2011; Maymo et al., 2011), then the activated ERK promotes the phosphorylation of cAMP-response element binding protein (CREB) to up-regulate placental leptin expression (Coya et al., 2001; Szkudelski et al., 2005). However, in IUGR placenta, the pathway of leptin production has not been clarified.

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Caffeine is a xanthine alkaloid that widely existed in coffee, tea, soft beverages, foods and some analgesic drugs and exerts its effects via antagonizing adenosine receptors (ADORs). Epidemiological and clinical studies have indicated that caffeine has embryo toxicity and maternal caffeine ingestion is one of the risk factors attributable to IUGR (Guilbert, 2003; Momoi et al., 2008; Peck et al., 2010). Many animal studies also have demonstrated that caffeine induces the adverse pregnancy outcomes (IUGR, preterm birth etc.) by impairing the development of fetal rats (Golding, 1995; Jacombs et al., 1999; Burdan, 2004). Our previous study has shown that prenatal caffeine exposure (PCE) could result in developmental toxicity: e.g. increased numbers of absorbed and stillborn fetuses and decreased fetal bodyweight (Huang et al., 2012). We also found that PCE could over-expose the fetus to maternal glucocorticoids and induce a hypothalamic–pituitary–adrenal (HPA) axis-associated neuroendocrine metabolic programming alteration, resulting in IUGR and an enhanced susceptibility to metabolic syndrome in adult offspring rats (Liu et al., 2012; Xu et al., 2012a; Xu et al., 2012b; Wang et al., 2014; Ao et al., 2015). The aim of this study is to confirm a lower level of blood leptin and clarify its placental underlying mechanisms on rat IUGR model by PCE and human BeWo cells treated by caffeine. This study will provide a valuable experimental basis for illustrating placental mechanisms of IUGR caused by PCE.

## 2. Materials and methods

### 2.1. Materials

Caffeine was obtained from Sigma-Aldrich Co., Ltd. (St Louis, MO, USA). TRIzol was purchased from Invitrogen Co. (Carlsbad, CA, USA). Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). The SYBR Green dye was purchased from ABI (Foster City, CA, USA). CGS-21,680 and forskolin were obtained from Selleck (St Louis, MO, USA). All other chemicals and reagents used were of analytical grade. All of the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The rat leptin enzyme-linked immuno sorbent assay (ELISA) kit and adenosine A2a receptor (Adora2a) were obtained from Abcam (Cambridge, MA, USA). Reverse transcription and real-time reverse-transcription PCR (RT-PCR) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The total protein detection kit was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The MTS assay kit was purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). The human BeWo cells were purchased from the China Type Culture Collection (CTCC, Wuhan, China).

### 2.2. Animals and treatment

Specific pathogen-free Wistar rats weighing 180–220 g (female) and 260–300 g (male) were obtained from the Experimental Center of the Hubei Medical Scientific Academy (no. 2006-0005, Hubei, China). The animal work described in this study was performed in the Center for Animal Experimentation of Wuhan University (Wuhan, Hubei, PR China), which has been accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine (permit number: 14,016) and performed in accordance with the committee's Guidelines for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their sufferings.

The animals were allowed to acclimate for at least one week before being subjected to the experimental conditions. Each male rat was mated with two female rats overnight. The day in which the evidence of mating was observed (vaginal smear with sperm cells) was designated gestational day (GD) 0. The animals were housed under standard conditions and allowed free access to standard chow and water. Pregnant rats were randomly divided into 4 groups: one control group and three caffeine groups with different doses. From GD9 to GD20, the pregnant rats were intragastrically treated with caffeine (30, 60 and 120 mg/kg day) and the control rats were sham-treated with the same volume (10 ml/kg) of saline. On GD20, the pregnant rats were anesthetized with isoflurane. The number of pregnant rats in each group was set to 12, and the litter size of each pregnant rat was set to 8–14 live fetuses. The placentas were quickly removed to weigh, and IUGR was diagnosed when the body weight of a fetus was two standard deviations less than the mean body weight of fetuses in the control group (Engelbregt et al., 2001). Fetal blood and placenta were divided into four groups: female control, female different-doses caffeine, male control and male different-doses caffeine. Fetal blood samplings were performed as cutting the bilateral carotid arteries and carefully catch drops of the blood into EP tubes. Serum samples were prepared from blood by centrifugation at 12,000 × *g*, 4 °C for 15 min. Fetal serum samples collected from littermates were pooled together by sex, then 4 litter serum samples from each group were randomly merged to count as one independent sample for subsequent detection (n = 3) because of meager fetal blood. The principle of pooling placental tissues was that 6 placentas from 2 littermates (3 placentas from each littermate) in the same group were pooled for homogenization into one sample. All samples were immediately frozen in liquid nitrogen, followed by storage at −80 °C for subsequent analyses.

### 2.3. Maternal serum caffeine and fetal serum leptin measurement

An Agilent 7890A GC coupled to an Agilent 5975C quadrupole mass selective detector (Santa Clara, CA, USA) was employed to measure the concentration of maternal serum caffeine concentration (n = 4) as described previously (Wang et al., 2014). Fetal serum sample was measured for leptin level using ELISA kit according to the manufacturer's protocol. The intra- and inter-assay coefficients of variation for leptin were 10% and 12% respectively.

### 2.4. Total RNA extract and quantitative real-time PCR

Placental tissues (10 mg) were taken from the same position in each placenta. Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol. Detailed protocols for total RNA extraction and RT have been described by Tan et al.(2012).

To determine the mRNA expression of ADORs, adenylyl cyclase (AC), leptin, and CREB, a quantitative real-time PCR was performed with SYBR Green dye. Primers were designed using Primer Premier 5.0, and their sequences are shown in Table 1. PCR was performed in 96-well optical reaction plates using the ABI Step One Plus™ real-time PCR System (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl containing: 1 μl of cDNA template, 0.4 μl of each primer, 10 μl of 2× SYBR Green, 0.4 μl 50× ROX, and 7.8 μl of DEPC-$H_2O$. The PCR cycling conditions used were as follows: pre-denaturation, 95 °C for 30 s and denaturation, 95 °C for 5 s; the annealing conditions for each gene are listed in Table 1. To precisely quantify the gene transcripts, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was measured and used as quantitative control. Relative target amplicon expression was assayed using the linear regression method with $R^2$ greater than 0.99 for each gene and expressed as arbitrary units.

### 2.5. Western blotting

Western blotting was performed as previously described (Wen et al., 2014). Briefly, the cells were rinsed with ice-cold PBS, and then lysed for 30 min at 4 °C in RIPA lysis buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 1% NP40, 2.5 mM EDTA, 10% glycerol, 1% Triton X-100) containing protease inhibitors (2 mM NaF, 2 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 1 mM protein inhibitor), and quantified using a BCA kit following the protocol. Then, total 40 μg of proteins was loaded to each lane, separated by SDS-PAGE (10% gels) and blotted onto