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Basic Study

Abnormal dietary calcium intake during the pregnancy and lactation aggravates the development of obesity in their male offspring by affecting the differentiation potential of bone mesenchymal stem cells

Calcium intake affects BMSCs differentiation potential

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Abstract

BACKGROUND

Currently, the mechanisms of abnormal dietary calcium intake in early life on the later obesity had not clearly explained, which still requires more assumptions.

AIM

We present the efficient possibility that abnormal dietary calcium intake during the pregnancy and lactation could derive the differentiation potential of bone mesenchymal stem cells (BMSCs) by regulating the related gene expression profiles through to adulthood.

METHODS

Four-week female C57BL/6J mice were fed by deficient, low, normal and excessive calcium reproductive diets throughout the pregnancy and lactation. Their BMSCs were obtained from the male offspring at 7-days to measure the differentiation potential by Wnt/β-Catenin signaling pathway. Then the weaning male pups were fed by the high-fat-diet (HFD) for 16 wk along with normal-fat-diet as the control. Meanwhile, their serum was collected for biochemical analyse, the adipose tissues were excised for histological examination, immunohistochemistry, proportions of immune cells and related gene expressions on the adipogenic differentiation and Wnt/β-Catenin signaling pathway.

RESULTS

Notably, comparing with the control group, deficient, low and excessive dietary calcium intake during the pregnancy and lactation could aggravate the development of obesity with larger adipocytes, more serious inflammatory infiltration and higher serum metabolism-related indicators by affecting the related gene expressions on the adipogenic differentiation (*PPARγ, C/EBPa, Fabp4, LPL, Adiponectin, Resistin* and *Leptin*) in the adipose tissues under high fat induction ($P<0.05$). What is more, it showed the

similar specific gene expressions in the BMSCs under maternal abnormal dietary calcium intake to successfully polarize the adipogenic differentiation and suppress osteogenic differentiation *in vivo* respectively(P<0.05). Furthermore, the related mechanistic insights were gained to worsen this adipogenic differentiation potential of BMSCs and adulthood adipose tissues through the Wnt/β-Catenin signaling pathway.

CONCLUSION

These above results suggested that abnormal dietary calcium intake in early life might program the adipogenic differentiation potential of BMSCs, which was related with the abnormal expression of many genes in the Wnt/β-Catenin signaling pathway to reserve more preadipocytes to aggravates the development of obesity in the adulthood with HFD induction. This deeper understanding of early-life calcium intake could play a significant role on preventing the later obesity.

Key Words: Calcium, obesity, bone mesenchymal stem cells, Wnt/β-Catenin signaling pathway, adipogenic differentiation, male offspring.

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Core Tip: Abnormal dietary calcium intake during the pregnancy and lactation could aggravate the development of obesity with larger adipocytes and more serious inflammatory infiltration by affecting the related genes expressions on the adipogenic differentiation in the adipose tissues with high fat induction. Meanwhile, it showed the differently specific gene expressions in the BMSCs to successfully polarize the adipogenic differentiation and suppress osteogenic differentiation *in vivo* respectively.

Furthermore, the related mechanistic insights were gained to worsen this adipogenic differentiation potential of BMSCs and adulthood adipose tissues through the Wnt/β-Catenin signaling pathway. This deeper understanding of early-life appropriate calcium intake could play a significant role on preventing the later obesity.

INTRODUCTION

Obesity has represented as a worldwide crucial noncommunicable health crisis with rising prevalence in the past decades due to excess calorie intake, fat accumulation and further adiposity[1-2], it can pose a severe metabolic disorders such as non-alcoholic steatohepatitis, type 2 diabetes, cardiovascular diseases and cancers[1-3]. These above pathological complications are characterized both by the hypertrophy and hyperplasia of adipocytes to cause the dynamic expansion in the subcutaneous and visceral white adipose tissues, in which the hyperplasia is a complicated process including the disorder commitment of mesenchymal stem cells (MSCs) to preadipocytes and terminal differentiation from the preadipocytes to mature adipocytes[4-6]. As we all known, MSCs (CD29+, CD90+, Sca-1+, CD31+, CD34+, CD45− and CD49d−), as a group of cells with multi-lineage differentiated potential and self-renewal capacity, are the major sources of adipocytes under the stimuli condition, in which the pattern on the key coordinated cascade of transcription factors are included peroxisome proliferators-activated receptor gamma (*PPARγ*) and CCAAT/enhancer -binding protein α (*C/EBPa*), lipoprotein lipase (*LPL*) and fatty acid binding protein 4 (*FABP4*), with the significant secretory molecules from the mature adipocytes such as Leptin, Adiponectin and Resistin[7,8]. In this process, the mechanisms governing the adipogenic differentiation of MSCs can be regulated by the coordination of complex networks in many signaling pathways including JAK2/ STAT3, SIRT1/SIRT2, ERK1/ERK2, TGF-β/BMP, Wnt/β-Catenin and RHO-family GTPase signalling cascades[9,10], in which the activation of canonical Wnt/β-Catenin signaling can inhibit the adipogenic differentiation and promote the osteogenic differentiation through the endogenous regulator *CTNNB1, Wnt1, Wnt10a, Wnt10b, Wnt5a, Gsk3β, Axin2* and *TGF7L2* [11,12]. What is more, it has been

demonstrated that the differentiated potential of MSCs mainly occurs in the early life. Their numbers and differentiated potential are significantly declined along with the age[12], so the adequate and appropriate nutrition exposure in early life, especially the pregnancy and lactation, is important for the differentiated potential of MSCs to affect the later metabolic disturbances in the adulthood[13-16].

Calcium, as a functional nutrient, plays an important role on the regulation of energy balance and glucose uptake in the battle against obesity to enter an exciting phase from epidemiology and clinical investigations, as well as cellular and molecular researches[17-19]. However, it is reported that the daily calcium intake is still lower than the recommended nutrient intake in the pregnant women and possible more calcium intake in the infants after birth[20], so the imbalance of calcium intake in the early life may have detrimental effects on the later health. Our previous study also had proved that dietary insufficient or excessive calcium intake during the pregnancy and lactation could increase the body weight gain by affecting the structures of gut microbiota, and related gene expression on the lipolysis and liposynthesis in their offspring with a high fat diet induced obese mouse mode and epidemiological cohort[21-24]. However, the specific mechanisms by which maternal calcium intake modulates the body weight, fat and glucose homeostasis of their infants are still not fully understood. Recently, some researches also have stated that the Ca^{2+} formed in the culture media at many different sources has osteo-induction properties to promote the osteogenic proliferation and differentiation of MSCs in vitro[25,26], which also has been proved that neonatal calcium deficiency appeared to reduce the osteogenic priming of MSCs by enlarging a sub-population of potentially adipogenic cells using the piglets and mice in vivo. Meanwhile, it has been reported that the decision process of MSCs into the adipo-osteogenic cells is competing and reciprocal[27-28], so whether maternal inappropriate dietary calcium intake can increase the adipogenic differentiation potential of MSCs is still unclear among their male offspring.

Thus, this study was designed to investigate the effects of abnormal dietary calcium intake during the gestation and lactation on the adipogenic differentiation

potential of Bone MSCs (BMSCs) to aggravate the development of obesity among their male offspring and explore the possible signaling pathways. This deeper understanding of early-life calcium intake will play a significant role on preventing the later obesity.

MATERIALS AND METHODS

Animal Procedures

Forty four-week-old C57BL/6N female mice were obtained from Beijing Vital River Laboratory Animal Technology (License SCXK (Beijing)) and housed at the Animal Center in the Academy of Military Medical Sciences under a 12-h light/dark cycle (lights-on 08:00) with adequate food and water intake at 22°C and 50% humidity. All the mice were randomly divided into four groups (n=15/group) and fed with the deficient (DC, 0.05%), low (LC, 0.25%), normal (NC, 0.70%) and high-calcium (HC, 1.20%) reproductive diets respectively for 6 weeks. The five female mice in each group were executed to determine the contents of calcium and other metabolic indicators before mating. Then the remaining mice (n=10/group) were mated with 10-week-old C57BL/6N male mice (Beijing Vital River Laboratory Animal Technology) (2:1/cage) to create their male offspring, and continued on their own diets throughout the whole gestation and lactation. According to the previous study [21-23]. the male offspring were used as the subjects to discuss the development of obesity by the different calcium intervention during the pregnancy and lactation in our mouse model, in which the 7-day-old male offspring (n = 9/group from more than three cages) in each group were executed to obtain the BMSCs, while at 21-day-old, the male offspring (n = 10/group) were weaned onto the high fat diet (HFD, 34.9% fat by weight, 60% kcal, No. H10060) to 16-week-old (NC-HFD, DC-HFD, LC-HFD and HC-HFD groups), with the normal diet (4.3% fat by weight, 10% kcal, No. H10010) as the control (NC-C). All above diets were prepared by Beijing HFK Bioscience Co., LTD (http://www.hfkbio.com/) and shown in **Table 1**.

At the whole procedure, the body weights, food intake and energy intake of the male offspring in the NC-C, NC-HFD, DC-HFD, LC-HFD and HC-HFD groups were recorded weekly. Then they were anesthetized by the carbon dioxide inhalation after their blood samples were collected through eye-drop. Immediately, their adipose tissues including the epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), kidney adipose tissue (KAT) and brown adipose tissue (BAT) were freely dissected from the surrounding tissues, in which some were fixed by the 10% phosphate buffered formalin, some were stored in the phosphate buffered saline (PBS) to analyze the percents of immune cells and the remaining others were frozen in liquid N_2. Then the serum samples were separated and obtained at 3000 r/min for 15 minutes after stewing 30 minutes at the room temperature. After the experiment finished, all tissues were transferred and stored in a -80°C refrigerator until use. All animal studies were approved and conducted in accordance with the Beijing Academy of Military Medical Sciences Guide for the Care and Usage Committee of Laboratory Animals. Meanwhile, the animal protocol used in this study was approved by the Ethics of Animal Experiments in the Academy of Military Medical Sciences in China (No. IACUC-DWZX-2019-704).

Measurement of the biochemical indicators

The concentrations of serum calcium, glucose, triglyceride (TG) and total cholesterol (TC) were respectively measured by the coloristic methods using the enzymatic assay kits (Maccura Biotechnology Co., Ltd, Sichuan, China). Meanwhile, all male mice were orally garaged with 20% (weight/volume) glucose (2.0g/kg) after a 10-hour overnight fasting and collected the blood samples from the tail vein at the 15min, 30min, 60min, 90min and 120 min to determine the contents of glucose (OGTT) at the 13th week. Then the insulin tolerance test (ITT) was chosen one week after the OGTT, in which the blood samples were collected from the tail vein for serial blood glucose determinations after 2-hour fasting.

Analysis of the immune cells in the adipose tissues

According to the reference[29], the stromal vascular cells (SVCs) were extracted from the eWAT and iWAT in the PBS. Then their infiltration and percentages of immune cells as M1 macrophages ($CD45^+CD64^+CD11C^+$), M2 macrophages ($CD45^+CD64^+$ $CD11C^-$) and ATDC cells ($CD45^+CD64^-/CD11C^+$) were determined using the BD FACSCanto II Flow Cytometer (BD Biosciences, USA) and analyzed by the FlowJo flow cytometry software (Treestar Inc., Ashland, USA).

Histological Analysis of the adipose tissues

The adipose tissues (eWAT, iWAT, KAT and BAT) in the NC-C, DC-HFD, LC-HFD, NC-HFD and HC-HFD groups were randomly embedded in the paraffin to cut into 6μm sections, then they were stained with hematoxylin and eosin (HE) to measure the adipocyte sizes and inflammatory infiltration under the light microscope at magnification 200×, which were analyzed by the Image-pro Plus. All histological analysis of the above adipose tissues were determined by the Servicebio company in Beijing, China.

Gene expressions on the adipogenic differentiation and Wnt/β-Catenin signaling pathway in the adipose tissues by RT-PCR

Total RNA in the adipose tissues (eWAT, iWAT, KAT and BAT) was extracted using the TRIzol Reagent (No.15596-206, Invitrogen, Carlsbad, USA), and the cDNA was reversely transcribed by Transcript® One-Step gDNA Removal and cDNA Synthesis SuperMix (No.AT311-02, TransGen Biotech, China), which were measured for the gene expressions by RT-PCR following the manufacturer (No.AQ101-03, TransStart® Green qPCR SuperMix, TransGen Biotech, China).

The related gene expressions on the adipogenic differentiation (*PPARγ*, *C/EBPa*, *LPL*, *Fabp4*, *Adiponectin*, *Resistin* and *Leptin*) and Wnt/β-Catenin signaling pathway (*CTNNB1, Wnt1, Wnt10a, Wnt10b, Wnt5a, Gsk3β, Axin2* and *TGF7L2*) were determined by RT-PCR (CFX-96, Bio-Rad, USA) with 36B4 as the invariant internal gene (**Supplementary Table 1**). Then the above gene expressions were normalized using the $2^{-\blacktriangle CT}$ method.

BMSCs derivation and maintenance of the male offspring

The BMSCs from the 7-day-old male offspring were isolated and cultured as follows: The tibia and fibula from three pups with different mothers were isolated after washing with PBS to remove the residual muscle and blood under the sterile conditions. They were shredded into the small pieces of 2mm^3 and digested in the 0.1% type II collagenase (No.17101015, Gibco™) at 37°Cfor 40 min, then they were transferred into the α-MEM medium (No.11900024, Gibco™) containing 10% fetal bovine serum (FBS, No.10091, Gibco™), 100U/mL penicillin, and 100mg/mL streptomycin. The medium with FBS should be changed every three days. When the adherent BMSCs were reached a confluence of approximately 80-90%, they were collected by the 0.25% trypsin (Gibco™, #25200056) and subcultured at a ratio of 1:3 for the further expansion and identification until the P3 generation, which were used for the subsequent experiments.

Detection of the Cell Cycles and surface antibody of BMSCs by the Flow cytometry

The P3 generation BMSCs (1×10^6) from the DC, LC, NC and HC groups were phenotypically fixed, permeabilized, stained and characterized by the following antibody permeabilization process according to the manufacturer's instructions. Exactly, the mouse phycoerythrin (PE)-conjugated monoclonal antibodies as Sca-1 (AB_2539218, MA5-17834), CD90 (AB_469640, 25-0900-82) and CD31 (AB_657735, 17-0311-82) (eBioscience, Waltham, MA, USA), and fluorescein isothiocyanate (FITC)-conjugated antibodies including CD29 (AB_2572449, 11-0291-82), CD34 (AB_465021, 11-0341-82) and CD45 (AB_465050, 11-0451-82) and CD49d (AB_465083, 11-0492-82) (eBioscience, Waltham, MA, USA) were performed, while the Cell Cycle and Apoptosis Analysis kit (Beyotime, C1052) was obtained to measure the cell cycle of BMSCs. All above signals were recorded by the Flow Cytometry with a FACS calibur system (Becton Dickinson) and analyzed using the FlowJo software (**Supplementary Figure 2** and **Table 2**).

Differentiation potential capacity of BMSCs

To identify the adipogenic differentiation potential of BMSCs in different groups, P3 BMSCs (8×10^4) were cultured with α-MEM medium containing 10%FBS and the related adipogenic inducer (10^{-3}mM dexamethasone, 0.5 mmol/L isobutyl

methylxanthine, 0.2 mmol/L indomethacin, and 10 μg/mL insulin (Sigma, Germany)) for seven days, in which the medium was changed every three days. while their self-differentiated groups (3×10^4, without the above inducer) were as the controls. Then the above BMSCs were stained with Oil Red O (Sigma, Germany) and measured the related gene expressions of adipogenic differentiation (*PPARγ*, *C/EBPa*, *LPL*, *Fabp4*, *Adiponectin*, *Resistin* and *Leptin*)[29] .

The osteogenic differentiation capacity of BMSCs was assessed by incubating the cells (3×10^4) with α-MEM medium containing 10% FBS and osteogenic inducer (10^{-7} mM dexamethasone, 0.5 mmol/L ascorbic acid, and 10 mmol/L β-glycerol phosphate (Sigma, Germany)) for ten days, while their self-differentiated groups (3×10^4, without the above inducer) were as the controls. To demonstrate the osteogenic differentiation capacity of BMSCs, they were identified by the immunocytochemical stain with alkaline phosphatase(ALP)[29]. Meanwhile, the expressions of related genes on the osteogenic differentiation (*Runx2*, *ALP*, *COL1A1*, *Osteocalcin* and *Osteopontin*) were determined by the RT-PCR.

Quantitative real-time PCR of the BMSCs

Total RNA in the P3 BMSCs, adipogenic and osteogenic differentiated BMSCs and their related self-differentiated BMSCs was extracted using the TRIzol Reagent (No.15596-206, Invitrogen, Carlsbad, USA), and their cDNA samples were reversely transcribed by Transcript® One-Step gDNA Removal and cDNA Synthesis SuperMix (No.AT311-02, TransGen Biotech, China), which were measured for the gene expressions by RT-PCR following the manufacturer (No.AQ101-03, TransStart® Green qPCR SuperMix, TransGen Biotech, China).

The genes involving in the adipogenic differentiation (*PPARγ*, *C/EBPa*, *LPL*, *Fabp4*, *Adiponectin*, *Resistin* and *Leptin*), osteogenic differentiation (*Runx2*, *ALP*, *COL1A1*, *Osteocalcin* and *Osteopontin*) and Wnt/β-Catenin signaling pathway (*Wnt1*, *Wnt10a*, *Wnt10b*, *Wnt5a*, *CTNNB1*, *Gsk3β*, *Axin2* and *TGF7L2*) (**Supplementary Table 2**) were carried out using the RT-PCR (CFX-96, Bio-Rad, USA) with 36B4 as the

invariant internal control. Then the assays were performed in triplicates and normalized to the internal standard mRNA levels using the $2^{-\blacktriangle\blacktriangle CT}$ method.

Statistical analysis

All statistical analyses were conducted using the SPSS 21.0, with the α level of 0.05 and a effect coefficient of 0.90. All values were expressed as Mean±Standard deviation (Standard error), in which the Percent Percent plot was chosen to determine the normality of data. Then the differences among all groups were tested and analyzed the variances for the repeated measurement data based on whether the data was normally distributed (normal distribution: *t* test and ANOVA for the continuous variables and χ^2 test for the categorical variable. non-normal distribution: Mann-Whitney U test) The value of $P<0.05$ was considered to be statistically significant.

RESULTS

Abnormal dietary calcium intake during the pregnancy and lactation could aggravate the development of obesity among their male offspring

As shown in the **Supplementary Figure 1**, there were no significant differences of body weight, daily dietary intake, related indexes of glucose (OGTT and ITT) and lipid (TC and TG) among the maternal DC, LC, NC and HC groups, with lower bone calcium, phosphorus and Ca/P in the DC and LC groups and higher contents in the HC group, which all proved that the animal models were successful.

Among the male offspring, their body weight and mental state at weaning were not significantly different among the NC-C, DC-HFD, LC-HFD, NC-HFD and HC-HFD groups (Figure 1A). While at the adulthood, their body weights (**Figure 1A**), energy intake (**Figure 1B**) and concentrations of metabolism-related indicators in the serum (TG, TC and glucose, **Figure 1C to 1F**) were all higher in the HFD groups (DC-HFD, LC-HFD, NC-HFD and HC-HFD) than those in the NC-C group ($P<0.05$). Likewise, the circulating glucose response to the glucose load, as indicated by OGTT and ITT (**Figure 1G** and **1H**), showed that there were higher glucose levels in the HFD groups after the intraperitoneal glucose administration ($P<0.05$). Moreover,

comparing with those in the NC-HFD group, maternal low (LC-HFD) and excess (HC-HFD) dietary calcium intake could aggravate the development of obesity, with significant higher TC and TG (P<0.05). In contrast, the body weight in the DC-HFD group was lower and the content of TG was higher than those in the NC-HFD group (P<0.05).

Abnormal dietary calcium intake during the pregnancy and lactation could cause the infiltration disorders of immune cells in the adipose tissues among their male offspring

The percentages of M1 macrophages, M2 macrophages and ATDC cells were demonstrated in the eWAT (**Figure 1I**) and iWAT (**Figure 1J**). In the eWAT, the percentages of M1 macrophages and ATDCs were higher, and M2 macrophages were lower in the obese (DC-HFD, LC-HFD, NC-HFD and/or HC-HFD) groups than those in the NC-C group (P<0.05). Further comparison among the HFD groups showed that the percentages of M1 macrophages and ATDCs were increased in the DC-HFD, LC-HFD and HC-HFD than those in the NC-HFD group (P<0.05). While in the iWAT, the percentages of M1 macrophages and ATDCs were significant higher, and M2 macrophages were lower in the HFD groups than those in the NC-C group (P<0.05). Further comparison among the four HFD groups showed that comparing with the NC-HFD group, maternal low (LC-HFD) and high (HC-HFD) dietary calcium intake could aggravate the disorder of M1 and M2 macrophages (P<0.05), which were not found in the DC-HFD group (P>0.05).

Abnormal dietary calcium intake during the pregnancy and lactation could affect the weights and morphology of adipose tissues among their male offspring

The adipose tissue weights (eWAT, iWAT, KAT and BAT) (**Figure 2A**) and adipose tissue weight/body weight (**Figure 2B**) were all higher in the four HFD groups than those in the NC-C group (P<0.05). What is more, comparing with those in the NC-HFD group, maternal deficient (DC-HFD, eWAT and eWAT/body weight, P<0.05), low (LC-HFD, eWAT, KAT, BAT, eWAT/body weight, KAT/body weight and BAT/body weight, P<0.05) and excess (HC-HFD, BAT and BAT/body weight, P<0.05) dietary

calcium intake groups could increase the weights of partly eWAT, iWAT, KAT and BAT, and the related adipose tissue weight/body weight.

Comparing with the NC-C group, the proliferation and differentiation of adipocytes in the eWAT (**Figure 2C** and **2D**), iWAT (**Figure 2C** and **2E**), KAT (**Figure 2C** and **2F**) and BAT (**Figure 2C** and **2G**), shown by H&E, were more prominent in the HFD groups (P<0.05). Furthermore, among the HFD groups, maternal abnormal dietary calcium intake (DC-HFD, LC-HFD and HC-HFD groups) could aggravate the disorders of proliferation and differentiation of adipocytes (eWAT, iWAT and BAT) among their male offspring, which were shown by much bigger adipocytes in the eWAT, iWAT and BAT (P<0.05).

Abnormal dietary calcium intake during the pregnancy and lactation could regulate the target gene expressions in the adipose tissues among their male offspring

As shown in **Figure 3,** comparing with the NC-HFD group, the related gene expressions on the adipogenic differentiation (*PPARγ*, *C/EBPa*, *LPL*, *Fabp4*, *Adiponectin*, *Resistin* and *Leptin*) and Wnt/β-Catenin signaling pathway (*Wnt1, Wnt10a, Wnt10b, Wnt5a, CTNNB1, Gsk3β, Axin2* and *TGF7L2*) of the eWAT, iWAT, KAT and BAT in the DC-HFD, LC-HFD and HC-HFD were more disordered.

Exactly, in the eWAT (**Figure 3A** and **3B**), comparing with the NC-HFD group, higher expressions of *PPARγ*, *Adiponectin* and *Wnt5a,* and lower levels of *C/EBPa, CTNNB1* and *TCF7L2* were shown in the DC-HFD group (P<0.05), while higher expressions of *C/EBPa*, *Fabp4*, *LPL*, *Adiponectin* and *Leptin,* and lower levels of *Resist, CTNNB1* and *TCF7L2* in the LC-HFD group (P<0.05), meanwhile, higher expressions of *PPARγ*, *C/EBPa*, *LPL*, *Fabp4*, *Adiponectin*, *Leptin* and *Wnt5a,* and lower levels of *Wnt1, CTNNB1* and *TCF7L2* were demonstrated in the HC-HFD group (P<0.05). Among the four HFD groups in the iWAT (**Figure 3C** and **3D**), the expressions of *PPARγ*, *Adiponectin*, *Resistin* and *Leptin* were higher in the DC-HFD, LC-HFD, and HC-HFD groups (with higher *Fabp4* in the HC-HFD) than those in the NC-HFD group (P<0.05), with the significantly lower *C/EBPa, Wnt1,Wnt10a, Wnt10b, Wnt5a, CTNNB1* and *Gsk3β* in the DC-HFD group (P<0.05), lower *C/EBPa, Wnt1,Wnt10a, Axin2* and

TCF7L2 in the LC-HFD group ($P<0.05$), lower *CTNNB1* and *TCF7L2* in the HC-HFD group ($P<0.05$). As shown in the KAT among the HFD groups (**Figure 3E** and **3F**), the expressions of *PPARγ*, *LPL*, *Wnt10b* and *Gsk3β* were higher, with the significantly low levels of *Wnt5a, CTNNB1* and *Axin2* in the DC-HFD (Accompanied with higher contents of *Adiponectin* and *Resistin*), LC-HFD (higher contents of *Adiponectin,* with lower levels of *C/EBPa* and *Resistin*), and HC-HFD (lower contents of *Wnt10a* and *Resistin*) groups than those in the NC-HFD group ($P<0.05$). What is more, in the BAT (**Figure 3G** and **3H**), the expressions of *C/EBPa*, *LPL*, *Adiponectin*, *Resistin,* Leptin *and TGF7L2* were higher, with the significantly lower expressions of *Wnt10a,Wnt5a* and *CTNNB1* in the DC-HFD (lower expression of *Wnt1*), LC-HFD (higher *Wnt10b* and lower *Gsk3β*), and HC-HFD (lower expression of *Wnt1*and *Gsk3β*) than those in the NC-HFD group ($P<0.05$).

Effects of abnormal dietary calcium intake during the pregnancy and lactation on the adipogenic and osteogenic differentiation potential of BMSCs

The morphology of BMSCs at the P0 (**Figure 4A**) and P3 generations (**Figure 4B**) were similar in the DC, LC, NC and HC groups, with no significant differences of the pluripotent stem cells (G0G1 Phase) and purity of BMSCs (Sca-1**+,** CD90+, CD29+, CD34-, CD31-, CD45- and CD49d-) in the P3 generation ($P>0.05$, **Table 2** and **Supplementary Figure 2).**

In the P3 generation of BMSCs, maternal deficient (DC group) and low (LC group) dietary calcium intake could promote the adipogenic differentiation potential capacity of BMSCs, with higher levels of *PPARγ*, *C/EBPa*, *Fabp4*, *Adiponectin* and *Leptin* (**Figure 4C,** $P<0.05$). Meanwhile, maternal excess dietary calcium intake (HC group) could easily induced the osteogenic differentiation of BMSCs, with higher levels of Runx2, ALP, *COL1A1*, Osteocalcin and *Osteopontin* and lower levels of *PPARγ*, *LPL*, *Adiponectin* and *Leptin* (**Figure 4C** and **4D,** $P<0.05$). Under the adipogenic agent induction (**Figure 5),** maternal abnormal dietary calcium intake (DC, LC and HC groups) could promote the adipogenic differentiation of BMSCs, with more lipid drops (**Figure 5A**) and higher expression levels of related genes (*PPARγ*, *C/EBPa*, *Fabp4*, *LPL*,

Adiponectin, Resistin or *Leptin,* **Figure 5C** and **Fig.5D**). Like as the expression levels of genes on the adipogenic differentiation in the P3 generation (**Figure 4C),** comparing with these in the NC group, the expressions of *PPARγ*, *C/EBPa*, *Fabp4*, *LPL, Adiponectin, Resistin* or *Leptin* in the DC and LC groups were higher under the self-differentiation status (**Figure 5B**), with lower expressions in the HC group. Under the osteogenic agent induction (**Figure 6),** maternal excess dietary calcium intake (HC group) could promote the osteogenic differentiation of BMSCs, with more calcium nodule (**Figure 6A**) and higher expression levels of Runx2, ALP, *COL1A1* and *Osteocalcin* (**Figure 6C and 6D**), which was also similar as these in the condition at the self-differentiation status (**Figure 6B**). Furthermore, compared to the NC group, the potential osteogenic differentiation of BMSCs was weaker (**Figure 6A**), with lower expressions of Runx2, ALP, *COL1A1* and *Osteocalcin* in the DC and LC groups both under the osteogenic agent induction (**Figure 6C**) and self-differentiation status (**Figure 6D**).

Abnormal dietary calcium intake during the pregnancy and lactation could regulate the gene expressions of BMSCs on the Wnt/β-Catenin signaling pathway under the different interventions

The important genes of BMSCs on the Wnt/β-Catenin signaling pathway (*CTNNB1, Wnt1, Wnt10a, Wnt10b, Wnt5a, Gsk3β, Axin2* and *TGF7L2*) were measured among the male offspring from maternal different dietary calcium intake groups under different interventions including the P3 generation, adipogenic and osteogenic inductions (**Figure 7)**.

In the P3 generation without the induction, compared with the NC group, maternal deficient (DC group) and low (LC group) dietary calcium intake could inhibit the expressions of *Wnt1, Wnt10a, CTNNB1* and *Axin2* ($P<0.05$), while there were significantly higher levels of *Wnt10b, CTNNB1, Gsk3β* and *TGF7L2*, with lower expressions of *Wnt10a* and *Wnt5a* in the HC group ($P<0.05$) (**Figure 7A)**. Under the adipogenic agent induction, maternal abnormal dietary calcium intake (DC, LC and HC groups) could decrease the expression levels of *Wnt10a, Wnt10b, CTNNB1, Gsk3β* and

TGF7L2 to promote the adipogenic differentiation of BMSCs (**Figure 7D,** $P<0.05$**)**, while in the self-differentiation status, maternal deficient (DC group) and low (LC group) dietary calcium intake could inhibit the expressions of *Wnt1, CTNNB1* and *Axin2* ($P<0.05$), while there were significantly higher levels of *Wnt1, Wnt10a, Wnt10b, Wnt5a, CTNNB1, Axin2, Gsk3β* and *TGF7L2* in the HC group than those in the NC group (**Figure 7B,** $P<0.05$), which were consist with those in the P3 BMSCs. Furthermore, under the osteogenic induction, comparing with the NC group, the expressions of *Wnt1, Wnt5a* and *TGF7L2* were lower in the DC group ($P<0.05$), *Wnt1* was lower in the LC group ($P<0.05$), and there were significantly higher levels of *Wnt10a, Wnt10b, CTNNB1, Axin2, Gsk3β* and *TGF7L2* in the HC group (**Figure 7E**, $P<0.05$**)**. Meanwhile, in their self-differentiation status, maternal deficient (DC) and low (LC) dietary calcium intake could inhibit the expressions of *Wnt1, Wnt10a, Wnt5a, CTNNB1* and *TGF7L2* ($P<0.05$), while there were significantly higher levels of *Wnt1, Wnt10a, Wnt10b* and *Axin2* in the HC group than those in the NC group (**Figure 7C**, $P<0.05$).

DISCUSSION

The inappropriate consumption of nutrients and occurrence of obesity presents a greatest global public health problem, which needs more novel therapies[30]. A substantial body of researches have demonstrated that chronic deficient and excessive calcium exposure is as an important contributing factor for the development of obesity by controlling the de novo lipogenesis and lipolytic signals through regulating the related gene expressions[31-33]. Over the past several years, there were also compelling evidence implicating that maternal calcium dysfunction could directly affect the fat synthesis and metabolism of their offspring, which come from the animal models[34-37]. In agreement with our finding using the mouse model that abnormal dietary calcium intake during the pregnancy and lactation could aggravate the development of obesity by elevating the cytosolic calcium with much more/Larger adipocytes, and infiltration disorders of immune cells.

It is agreed that the obesity is driven both by the hypertrophy and hyperplasia of adipocytes in the process of adipogenic differentiation to cause the expansion of fat depots[38]. The lineage-tracing models had proved that the number of adipocytes in a given fat depot was primarily determined in the early life and mostly stable through the adulthood for the remarkable hypertrophic potential of differentiated adipocytes with the HFD induction[39-42]. Meanwhile, it has proved that the modulation of cytosolic calcium can regulate the early stages of murine adipocyte differentiation and thermogenic capacity of BAT [43]. And the propensity of adipogenesis to generate new adipocytes in different adipose tissues (eWAT, iWAT, KAT and BAT) highlights the unique characteristics of fat depots. Thus, we should discuss the roles of abnormal dietary calcium intake during the pregnancy and lactation on the potential adipogenesis in different adipose tissues among their male offspring, which was consistent with the existing findings that the imbalance of dietary calcium intake in early life could affect the proliferation and differentiation of eWAT, iWAT and BAT with more adipose tissue weights. However, it still remains to be elucidated the related mechanisms. Recently, the lineage-tracing studies in the animal models suggested that there were two-step phases in the adipogenic differentiation, including the specific preadipocyte formation (from MSCs to preadipocyte) and terminal adipocyte maturation (from preadipocyte to mature adipocytes), in which there were a number of critical transcription factors and related extracellular signals in the above process[44,45]. The committed preadipocytes from the pluripotent MSCs shoud be activated by the regulator of *PPARγ*, *C/EBPa*, *C/EBPβ* and *FABP4*, while at the differentiation of mature adipocytes (second stage), they express all the biomarkers of early adipocyte differentiation as well as the peptide hormones such as *Adiponectin*, *Resistin*, *Leptin*, adipose triglyceride lipase (*ATGL*), *LPL* and *Perilipin 1*[44,45]. All above transcription factors are involved in the specific Wnt signaling pathway to affect the adipogenic differentiation[46-48]. In our study, abnormal dietary calcium intake during the pregnancy and lactation (DC-HFD, LC-HFD and HC-HFD) could aggravate the disorder expressions of related genes on the proliferation and differentiation of

adipocytes (*PPARγ, C/EBPa, Fabp4, LPL, Adiponectin, Resistin* or *Leptin*) and Wnt/β-Catenin signaling pathway (*CTNNB1, Wnt1, Wnt10a, Wnt10b, Wnt5a, Gsk3β, Axin2* or *TGF7L2*) in the eWAT, iWAT, KAT or BAT in the adulthood of their male offspring, which could more clearly explain the possible causes for the development of obesity in vitro.

In the early stage, MSCs as the capable progenitor multipotential cells, are delicately balanced for their terminal adipo-osteogenic differentiation commitment[49-51]. It has been also reported that this decision process of MSCs is competing and reciprocal, which is precisely achieved by a variety of critical and external cues in fact including the phytocannabinoids, conjugated linoleic acid, calcium, chemical, physical, and biological factors[52-58], in which numerous investigations *in vitro* have demonstrated that deficient calcium exposure could inhibit the osteogenesis[59-61], and conversely very little is known about its effects of inappropriate dietary calcium intake during the pregnancy and lactation on the potentially adipogenic differentiation to reserve more preadipocytes and aggravate the development of obesity in adulthood with the HFD induction although the bone-fat induction are balanced[55, 62]. And it is the major novelty of our study, in which our results proved that maternal deficient and low dietary calcium intake could aggravate the potential adipogenic differentiation and suppress osteogenic differentiation of BMSCs, while maternal excess dietary calcium intake played a opposite differentiation role without the exogenous stimuli. In response to reagent induction, both maternal deficient, low and excessive dietary calcium intake all could successfully polarize the adipogenic differentiation and suppress osteogenic differentiation. All above results were consistent with the results in the adult offspring with the HFD induction. As we all known, the terminal differentiation of BMSCs is obtained through a coordinated and highly orchestrated program of triggering different signaling pathways and activate various transcription factors that guide the programming alterations of MSCs to commit the lineage to cause the pathophysiologic processes of obesity[63-65]. Thus, our research for screening out the roles of different calcium exposure in the early life on the expressions of related

transcription factors and signal pathways to regulate both osteoblast and adipocyte differentiation of BMSCs is necessary, which proved that the imbalance of terminal adipo-osteogenic differentiation by abnormal calcium exposure in the early life were due to the above genes expressions and Wnt/β-Catenin signaling pathway on the proliferation and differentiation of BMSCs among the male offspring. Furthermore, there were still some limitations in this study: Firstly, it requires a more complicated and explicit mechanism procedure including the Western blot. Secondly, this conclusion should be verified in the other MSCs and other animal models to ensure its feasibility and effectiveness.

CONCLUSION

In summary, these above results suggested that abnormal dietary calcium intake during the gestation and lactation might aggravate the development of obesity by affecting the adipogenic differentiation potential of BMSCs, which was related with the abnormal expression of many genes in the Wnt/β-Catenin signaling pathway among the male offspring. Taken together, our data provided an interesting understanding of the programs for multipotential differentiation of BMSCs that abnormal dietary calcium exposure in early life could polarize the adipogenic differentiation to reserve more preadipocytes, which was a high risk factor for the development of obesity in the adulthood with the HFD induction. Meanwhile, maternal deficient calcium exposure could inhibit the osteogenic differentiation to cause the low body weight. So the most interesting and worth mentioning of this study is that we have moved forward the preventive factors of obesity, which was not from the neonatal period, but from the period of maternal germ cells or fertilized egg formation.

ARTICLE HIGHLIGHTS

Research background

Obesity is characterized both by the hypertrophy and hyperplasia of adipocytes to cause the dynamic expansion in the adipose tissues, in which the commitment of MSCs

to preadipocytes is the important process for the hyperplasia. And our previous study had proved that dietary insufficient or excessive calcium intake during the pregnancy and lactation could increase the body weight gain in their offspring with a high fat diet induced obese mouse mode and epidemiological cohort. However, whether maternal inappropriate dietary calcium intake can increase the adipogenic differentiation potential of MSCs is still unclear.

Research motivation

This study was designed to investigate the effects of abnormal dietary calcium intake during the gestation and lactation on the adipogenesis differentiation potential of BMSCs from the male offspring and explore the possible signaling pathways, which might contribute to aggravate the development of obesity, with more excessive lipids accumulation in the adulthood.

Research objectives

We presented the efficient possibility to derive the hyperplasic adipogenesis from bone mesenchymal stem cells by regulating the gene expression profiles through to adulthood.

Research methods

Four-week-old female C57BL/6J mice were fed by deficient, low, normal and excessive calcium reproductive diets throughout the whole pregnancy and lactation. And their BMSCs were obtain from the offspring at 7-day-old to measure the adipogenic differentiation potential through the Wnt/β-Catenin signaling pathway. Then the weaning male pups were fed a high-fat diet (HFD) for 16 wk along with a normal-fat diet as the control. Their serum was collected for biochemical analyses. Adipose tissues were excised for histological examination, immunohistochemistry, gene expressions of adipogenic differentiation, and for determining the proportions of immune cells by flow cytometry.

Research results

Maternal deficient, low and excess dietary calcium intake could aggravate the development of obesity with much more/Larger adipocytes and higher serum metabolism-related indicators by affecting the related genes expressions on the adipogenic proliferation and differentiation (*PPARγ, C/EBPa, Fabp4, LPL, Adiponectin, Resistin* and *Leptin*) in the adipose tissues with high fat induction, which showed the similar specific gene expressions in the BMSCs to successfully polarize the adipogenic differentiation and suppress osteogenic differentiation *in vivo* and *in vitro* respectively. What is more, the related mechanistic insights were gained to worsen this different differentiation in the BMSCs and adulthood adipose tissues through Wnt/β-Catenin signaling pathway.

Research conclusions

Abnormal dietary calcium intake during the pregnancy and lactation might program the adipogenic differentiation potential of BMSCs, which was related with the abnormal expression of many genes in the Wnt/β-Catenin signaling pathway to reserve more preadipocytes to aggravates the development of obesity in the adulthood with HFD induction.

Research perspectives

The most worth mentioning of this study is that we have moved forward the preventive factors of obesity, which was not from the neonatal period, but from the period of maternal germ cells or fertilized egg formation.

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ORIGINALITY REPORT

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