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# Impact of prenatal hypoxia on fetal bone growth and osteoporosis in ovariectomized offspring rats

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ABSTRACT

Prenatal hypoxia causes intrauterine growth retardation. It is unclear whether/how hypoxia affects the bone in fetal and offspring life. This study showed that prenatal hypoxia retarded fetal skeletal growth in rats, inhibited extracellular matrix (ECM) synthesis and down-regulated of insulin-like growth factor 1 (IGF1) signaling in fetal growth plate chondrocytes *in vivo* and *in vitro*. In addition, ovariectomized (OVX) was used for study of postmenopausal osteoporosis. Compared with the control, OVX offspring in prenatal hypoxic group showed an enhanced osteoporosis in the femurs, associated with reduced proteoglycan and IGF1 signaling. The results indicated prenatal hypoxia not only delayed fetal skeletal growth, but also increased OVX-induced osteoporosis in the elder offspring probably through down-regulated IGF1 signaling and inhibition of ECM synthesis, providing important information of prenatal hypoxia on functional and molecular bone growth and metabolism in fetal and offspring.



## 1. Introduction

Intrauterine growth restriction (IUGR), also known as fetal growth restriction [1,2], affects approximately 30 million newborns per year [1,3]. About 52% of stillbirths and 10% of perinatal mortality per year are associated with IUGR [1,3]. IUGR happens with many causes, one common cause is placental insufficiency, in which the tissue that transfers oxygen and nutrients to the fetus is not working correctly [4]. Chronic fetal hypoxia is encountered in about 30% of IUGR [4,5]. In fact, many factors could induce *in utero* fetal hypoxia, including smoking, alcohol, drugs, high altitude, pulmonary diseases, and high blood pressure [6–9]. Accumulated evidence showed adverse intrauterine environment can lead to a retardation of fetal skeletal growth [10,11]. Our previous study showed that prenatal hypoxia not only increased the incidence of fetal absorption and stillbirth, but also increased IUGR in rats [12]. Those data suggested that hypoxia during pregnancy might affect fetal skeletal growth. However, the mechanisms underlying the retardation of fetal skeletal growth following prenatal hypoxia remain undefined.

Insulin-like growth factor 1 (IGF1) is an important regulator of bone growth [13,14]. During embryonic bone development, principal actions of IGF1 concern the control of bone growth through stimulating the secretion of extracellular matrix (ECM) in growth plate chondrocytes [14,15]. ECM includes collagen and proteoglycans, and plays an important role in intrauterine skeletal growth [16]. Previous studies have confirmed that prenatal adverse factors, such as smoking and caffeine, can cause IUGR and fetal growth retardation by inhibiting the expression of IGF1 signaling pathway in the growth plate chondrocytes [10,11]. For example, several components of IGF1 signaling pathway, such as IGF1 receptor (IGF1R), insulin receptor substrate (IRS)-1 and IRS2, and serine-threonine protein kinase (AKT), were down-regulated in femoral growth plate chondrocytes of fetal rats following prenatal exposure to nicotine [10]. The down-regulation of IGF1 signaling that was involved in inhibiting the synthesis of ECM by growth plate chondrocytes, eventually led to skeletal growth retardation in fetal rats [10,11]. An important question explored in the present study was whether and how prenatal hypoxia-produced poor bone

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development induce bone diseases in later life. In light of this, osteoporosis was considered since ECM gene and IGF1 signaling were involved in the processing of postmenopausal osteoporosis [17–20], which may provide interesting clues or ideas for further exploring early prevention or treatments of bone diseases.

As one of the most important and clinically relevant stresses during pregnancy, prenatal hypoxia can affect fetal development [5]. This study speculated that prenatal hypoxia may affect fetal skeletal growth probably through IGF1 signaling pathway that could inhibit the synthesis of ECM in the growth plate chondrocytes. The present study characterized skeletal growth changes in fetuses following prenatal hypoxia and clarified the relationship between the inhibition of IGF1 signaling and delayed ECM synthesis in fetal growth plate chondrocytes. Additionally, to study the effect of hypoxia on osteoporosis in the elder offspring, ovariectomized (OVX) was performed for postmenopausal osteoporosis in bone microstructure and molecular analysis. The data gained should provide important information on the impact of prenatal hypoxia on bone growth and metabolism in both fetuses and offspring.

## 2. Materials and methods

### 2.1. Experimental animal and tissue

Sprague-Dawley rats (4 months old, 300 g) were obtained from Soochow University Experimental Center (SYXK 2002-0037) and housed in a controlled environment of 22 °C with a 12-h light/dark cycle for at least one week to acclimate laboratory environment. One nulliparous female rat was placed together with two proven male breeders overnight for mating. The day upon which vaginal smear was observed, was designated as gestational day 0 (GD 0). Pregnant rats were randomly divided into the control (21% oxygen) and hypoxia group (10.5% oxygen) from GD 4 to GD20 (N = 25 each). Hypoxia was achieved and maintained by a mixture of nitrogen gas and air in an individual metal chamber. The control group was housed with only air flowing through the chambers [12]. All animals were fed with standard food and tap water. Some of the pregnant rats were sacrificed on GD20 for fetal studies. Others were allowed to give birth naturally for offspring experiments. Pups were kept with their mothers until weaning. The female offspring were tested at 9 months old. Pregnant rats were anesthetized at GD20 with sodium pentobarbital (100 mg/kg; Hengrui Medicine, Jiangsu, China) intraperitoneally. Fetal body length and weight were measured immediately, and IUGR rate was calculated according to the criteria [21]. Fetal left femurs (one per litter) were isolated under a dissection microscope. Some femurs were fixed in 10% paraformaldehyde solution for 24 h. Then the femurs were dehydrated and embedded in paraffin for histological investigation. All other femurs were harvested for subsequent experiments. Some female offspring received a bilateral ovariectomy by a minimally invasive surgical technique at 9 months old under isoflurane anesthesia [22]. All rats were sacrificed for harvesting tissue 1 month following the surgery [23]. The left femurs were for bone microstructure analysis using micro-computed tomographic (CT) (GE eXplore Locus SP, USA) for osteoporotic changes. All procedures were approved by the Institutional Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals.

### 2.2. Quantitative real-time PCR (qRT-PCR) and Western blot analysis

Total RNA was isolated from fetal femur growth plates or trabecular bone of OVX rats using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions, then reversed transcribed using the first-strand cDNA Synthesis Kit (Toyobo Corp., Shanghai, China). qRT-PCR was performed using the SYBR Green Supermix Taq Kit (Takara Biotechnology Co., Ltd., Dalian, China) and analyzed using the Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). $\Delta\Delta$Ct method was used to comparatively quantify the amount of mRNA levels [24]. The primer sequences were listed in Table 1. The protein abundance of IGF1, IGF2, IGF1R (IGF1 Receptor), AKT1/2, and IRS1 in femur growth plates were assessed by Western blot normalized to β-actin. Antibodies against IGF1, IGF2, IGF1R, AKT1/2, IRS1, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, California, USA). In each pregnant rat, the fetal femoral growth plates were dissected under an anatomy scope for RT-PCR and Western blot analysis. Fetal tissue from each pregnant rat were combined and analyzed as one sample. All experiments were repeated three times with independently prepared tissue lysates. qRT-PCR and western blot analyses were performed as previously described [24,25].

**Table 1**
The list of the primers used in this study.

| qRT-PCR primers | Sequence | |
|---|---|---|
| Gene Name | sense | anti-sense |
| Aggrecan | CAGGAATCC CTAGCTGCTT A | CTGAGATCACTGCAGCGATGA |
| Col2A1 | GAGTGGAAGAGCGGAGACTACT | CTCCATGTTG CAGAAGACTTTC |
| Col1A1 | GCCAAGAAGA CATCCCTGAA | ATAGCACGCC ATCGCACACA |
| IGF1 | CTGGTGGACG CTCTTCAGTT | TTCAGCGGAG CACAGTACATC |
| IGF2 | GTCGATGTTGGTG CTTCTCA | TTG AAGGCCTGC TGAA GTAG |
| IGF1R | GAATGAAGTCTGGCTCCGGA | TCAGCTGCTGATAGTGCTTG |
| AKT1 | GGATACCATG AACGACGTAG | GTGCCAC TGAGAAGTTG TTG |
| AKT2 | CTCTCTCAAG CTAGGTGACA | TAGATGAGAGG ATCTCCCGA |
| IRS1 | CACCTATGCCA GCATCAAC | GTGAGGTCCTGGTTGTGAAT |
| IRS2 | GCCGCCGTGGTGAAAGAGTAA | GACAGCGGAGAGGCAAGCCCT |

### 2.3. Histological and immunohistochemical assays

Fetal femurs were fixed in 10% paraformaldehyde, decalcified, and embedded in paraffin as previously described [26]. The fetal femur sections were stained with hematoxylin and eosin (HE) for measuring femur length (FL) and the calcifying zone length (CL). Safranin O-fast staining was performed to measure proteoglycan levels according to the manufacturer's instructions (Beijing Solarbio Science & Technology Co., Ltd., China). Immunohistochemistry was performed with a DAB staining kit (GeneTech Co., Ltd., Shanghai, China) for determining expressions of IGF1, IGF2, IGF1R, AKT1/2, IRS1, Aggrecan, Col2A1, and Col1A1 proteins in femur growth plates. The primary antibodies against IGF2, IGF1R, AKT1/2, IRS1, Aggrecan, Col2A1, Col1A1, and the appropriate secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). The slices were visualized under a Nikon microscope, and all images were captured and analyzed using Image Pro Plus software (version 6.1, Media Cybernetics, Silver Spring, USA). The staining was determined by counting the mean optical density (MOD) in 10 different fields for each sample.

### 2.4. Isolation and culture of fetal growth plate chondrocytes

Fetal growth plate chondrocytes were isolated from fetuses at GD20 as previously described [10,27]. Growth plate chondrocytes were cultured in DMEM (HyClone), containing 20% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO2 and 95% air humidified incubator. After two passages, the cells were incubated with 1.0% O2 for hypoxia or normoxia, respectively. After cultured for 48 h, cells were harvested. Total RNA was extracted from cells for qRT-PCR. All the experiments were repeated for three times.