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A temporary decrease in mineral density in perinatal mouse long bones

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ABSTRACT

Fetal and postnatal bone development in humans is traditionally viewed as a process characterized by progressively increasing mineral density. Yet, a temporary decrease in mineral density has been described in the long bones of infants in the immediate postnatal period. The mechanism that underlies this phenomenon, as well as its causes and consequences, remain unclear. Using daily μCT scans of murine femora and tibiae during perinatal development, we show that a temporary decrease in tissue mineral density (TMD) is evident in mice. By monitoring spatial and temporal structural changes during normal growth and in a mouse strain in which osteoclasts are non-functional (*Src*-null), we show that endosteal bone resorption is the main cause for the perinatal decrease in TMD. Mechanical testing revealed that this temporary decrease is correlated with reduced stiffness of the bones. We also show, by administration of a progestational agent to pregnant mice, that the decrease in TMD is not the result of parturition itself. This study provides a comprehensive view of perinatal long bone development in mice, and describes the process as well as the consequences of density fluctuation during this period.

Introduction

The ability of long bones to fulfill their mechanical function is influenced by their material properties, which are primarily determined by their mineral density and architecture [1]. During perinatal development, the requirement for mechanical competence of bones increases significantly [2,3]; thus, a corresponding consistent increase in mineral density can be expected [4–6]. Surprisingly, however, it was noted that during normal human development a brief decrease in mineral density occurs in the immediate postnatal period [7–9]. This phenomenon is more prominent in premature infants, and is termed metabolic bone disease of prematurity [10]. This syndrome might lead to osteopenia, rickets and multiple fractures [10,11]. Premature births occur in 10% of pregnancies [12], and preterm infants have recently been shown to have decreased bone mineral density in adulthood [13,14]. Thus, the improved survival rate of increasingly younger preterm infants could pose a significant long term public health issue in terms of osteoporosis and fracture risk [15]. A better understanding of the normal course of bone development is an important prerequisite for the design of efficient preventive and therapeutic approaches for the management of such congenital bone diseases.

The mechanism responsible for the perinatal decrease in mineral density in humans is a subject of much speculation. It has been suggested to be the result of a "dilution effect," due to the increase in the relative contribution of new, poorly mineralized bone tissue [16], or to the relatively greater rate of enlargement of the marrow cavity compared to periosteal expansion [17], or to the decreased availability of minerals due to the separation of the embryo from the umbilical cord after delivery [18]. To date, the exact mechanism remains unresolved.

The few studies that have assessed peripartal mineral density in humans have made use of a variety of non-invasive modalities. The accuracy of modalities such as peripheral computed tomography (pQCT) and dual-energy X-Ray absorptiometry (DXA) [19] is debatable [20]. Bone mineral density data acquired using speed of sound (SOS) are influenced by additional features of the bone such as its elasticity and structure [21–23]. Moreover, the assessment of perinatal bone density in humans is usually based on a relatively small number of individuals with long intervals between data points, especially before birth. These constraints are the main reasons for the limited information regarding the precise onset of density reduction in humans, as well as the limited success in elucidating its mechanism.

While the mouse is the main animal model for studying skeletogenesis, the question of whether or not the aforementioned perinatal decrease in bone density occurs in mice has, so far, not been addressed. The relatively few studies which describe, quantitatively, early bone growth in mice examined the skeleton only every few days, and thus overlooked critical perinatal time points in bone development [6,16,24]. Nevertheless, these published data do indicate a

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perinatal decrease in cortical thickness [6,24] or bone density [16]; however, the authors of these reports did not investigate this phenomenon in depth.

In this study, we examined developing long bones of mice using a temporal sequence of μCT scans, in order to elucidate perinatal mineral density fluctuations. We show a temporal reduction in tissue mineral density (TMD), which is initiated before parturition and is caused primarily by increased bone resorption by osteoclasts. We then demonstrate a corresponding reduction in bone stiffness. Finally, we show indications suggesting hormonal regulation of perinatal endosteal bone resorption, possibly in preparation for parturition.

Methods

Animals

ICR mice were purchased from Harlan Laboratories (Jerusalem, Israel). In all timed pregnancies plug date was defined as post-coitus (PC) 0.5. Days were counted consecutively from this time point. At each time point, eight mice from at least two litters were evaluated, except for PC16.5 and PC23.5, when six mice were evaluated. The interval between time points was 24 h, except for the interval between PC21.5 and PC23.5, which was 48 h. To examine whether the same phenomenon can also be observed in other mouse strains, hind-limbs of C57BL/6 mice were also examined during the same perinatal period. The effect of osteoclastic activity was evaluated in a mouse strain that carries a null mutation in the *Rous sarcoma oncogene* (*Src*) gene. In this mutant, a deficiency of osteoclast function occurs, leading to osteopetrosis [25]. *Src*-null mice were purchased from Jackson Laboratories. The genetic background of these mice was C57BL/6. To create src mutants, mice heterozygous for the mutation were crossed; wild-type littermates were used as controls. Embryos were harvested from timed-pregnant females, which were sacrificed by CO_2 intoxication. The gravid uterus was removed and suspended in a bath of cold phosphate-buffered saline (PBS), and the embryos were harvested after amnionectomy and removal of the placenta. Tail genomic DNA was used for genotyping. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Weizmann Institute of Science.

Micro-computed tomography (μCT)

Micro-computed tomographic analysis was performed according to recently published guidelines [26]. Samples were scanned by an eXplore Locus SP scanner (GE Healthcare, London, Ontario, Canada) at 45 kV and 120 μA. Nine hundred projections at a total integration time of 3850 ms were acquired for each individual scan over 360° of rotation. These projections were corrected using low-end and high-end outlier replacement in conjunction with a sinogram-based long-term trend correction. The projections were then reconstructed using a filtered cone-beam back-projection algorithm with a Ram-Lak filter, to generate images with an isotropic voxel size of 6.7 μm [27]. Hydroxyapatite (HA) calibration phantoms (GE Medical) were used to facilitate conversion of the linear attenuation of a given voxel to mg HA/cm^3. A global threshold was used to separate bone tissue from background [28]. Morphologic traits were measured by the software provided by the μCT manufacturer (Advanced Bone Analysis, Microview 2.2), averaged over each bone type and time point, and standard deviations for each parameter were calculated. These traits included bone length (mm), defined as the distance between the two growth plates, bone volume (BV, cm^3), tissue mineral content (TMC, mg HA) and tissue mineral density (TMD, mg HA/cm^3). Tissue mineral measurements of content and density, which are calculated from the attenuation value of the scanned bone tissue only and do not include attenuation values from non-bone voxels, were chosen over bone mineral measurement due to the porous nature of mice bones during the investigated period [26]. A series of consecutive transverse slices from the mid-diaphysis of tibiae at PC18.5 and PC19.5, covering 5% of the bone length, were used to measure cortical TMD and cross-sectional moment of inertia (CSMI) for interpretation of mechanical testing results.

Mechanical testing

Mechanical testing was carried out on 16 tibiae (8 each from PC18.5 and PC19.5 mice). Bones were tested by three-point bending [29] using a custom-made miniature mechanical testing device, using a load cell with force resolution of 0.015 N (AL311BN-6I, Sensotec, Honeywell, USA) and a linear motor with 1/61 μm step (PI M-235.5DG, Physik Instrumente (PI) GmbH, Germany). Tibiae were chosen owing to their relatively high aspect ratio, which reduces shear effects [30]. Right tibiae were removed and cleaned of adhering soft tissue under microscopic control, wrapped in saline-soaked gauze and stored at −20 °C until testing. Prior to testing, the frozen tibiae were slowly thawed at room temperature. Bones were placed on two stationary supports with rounded profiles (0.2 mm diameter), such that the supports were located equidistant from the ends of the bone. The distance between the stationary supports was 1.15 mm. The medial aspect of each tibia was loaded by a moving anvil with a rounded profile (0.2 mm diameter) at the middle of the distance between the stationary supports. All tibiae were first loaded with 0.01 N of preload, followed by eight load cycles between 0.01 and 0.02 N to allow the tibia to accommodate to the load and position. The tibiae were then loaded at a constant rate (60 μm/min) until fracture or to a maximum displacement of 180 μm. The tibiae were irrigated continuously with PBS during loading. Force-displacement data were collected at 20 Hz. The resulting load–displacement curves were used to calculate whole-bone stiffness (i.e. the slope of the linear portion of the load–displacement curve), yield load, ultimate load and area under the curve at 120 μm displacement (AUC). The yield point was defined as the load at which the load–deformation relationship ceased to be linear, and was determined by adapting the 0.03% offset criterion [31].

Evaluation of the accuracy of mineral density determination by μCT scanning

μCT scanning has become an accepted method for analysis of geometry, morphology and mineral density distribution of small bones in basic bone research and pre-clinical studies [32,33]. Specifically, it has recently been established as an accurate tool for murine bone densitometry [27]. While absolute values are not entirely reliable due to beam hardening artifacts, comparisons between different samples scanned by the same parameters are valid [26]. In contrast to bones of mature mice, perinatal murine bones are small, ranging in length from 1 to 4 mm, and contain many voids. In addition, they exhibit low mineral density since they are only partially mineralized (Supplemental Fig. S1A). We therefore wanted to evaluate the accuracy and precision of mineral density determination by μCT scanning and analysis protocols used in this study.

To this end we first compared images of transverse sections of mouse embryo bones obtained by backscatter electron microscopy to images of the same sections obtained by μCT, and showed that the mineral distribution determined by both methods was very similar. We then performed thermogravimetric analysis on whole femora that were scanned by μCT and found excellent correlation between mineral content values obtained by both methods. We also tested the calibration of mineral density by scanning phantoms prepared by another μCT manufacturer, and the density values determined by these scans were very close to those reported for these phantoms. Finally we tested the precision of our methodology by scanning bones repeatedly on different days, and demonstrated very small differences between the different scans of the same bone. These evaluations are described in detail in the Supplemental methods part.