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The effect of weight loading and subsequent release from loading on the postnatal skeleton

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ABSTRACT

Introduction: The relationship between load and the structure and mechanical properties of mature bones has been thoroughly described. In contrast, this relationship has been studied much less in immature bones, which consist of bony tissue and cartilaginous growth plate, during the postnatal period. This paper describes the effect of an externally applied load on the bones of young fast-growing chicks; in particular, we examine the effect on the growth plate, which regulates longitudinal bone growth, and the consequences in terms of bone structural and mechanical properties.
Materials and methods: The tibial growth plates from chicks subjected to external load and control chicks, immediately after loading and following 5 days of load release, were studied by histological staining and quantitative PCR. The contralateral tibiae were mechanically tested by three-point bending and their structural features determined by micro-CT.
Results: At the end of the external loading period, the tibias of the experimental group were shorter and their growth plate narrower than in controls. However, at this time point, effects were not yet apparent in the bones' structural or mechanical parameters. After a further 5 days of no external load, bones and growth plates of the experimental group demonstrated the phenomenon of 'catch-up': the thickness of the growth plate exceeded that of the control; however the relative expression of genes controlling chondrocyte differentiation (collagen II and X) did not change, while the expression of factors related to growth-plate ossification (osteopontin, alkaline phosphatase) and cartilage and bone calcification (matrix and bone Gla proteins) was upregulated as a result of the catch-up process. At this time, however, the tibiae of the experimental group showed inferior mechanical and structural properties relative to the control group.
Conclusion: External loading during bone elongation negatively affects the mechanical and structural properties of the skeleton. The effect is first noticeable in the growth plate, which regulates bone growth, and is exhibited in the bone phenotype after a lag period.

Introduction

The morphological, structural and material features of the skeleton in vertebrates are genetically programmed; they can also be modified by epigenetic factors, the most important of these being local tissue stress and strain states [1,2]. These strains are created by intrinsic muscle forces and external loads. The ability of bone tissue to adapt its mass, shape and internal architecture to its mechanical loading environment is a fundamental concept of bone biology [3–6]. Indeed, the idea that bone structure is controlled locally to best fulfill its mechanical function has become a central tenet of orthopedics; furthermore, many paleontological and bioarcheological studies of skeletal material are based on this concept [6].

Many studies have investigated the effect of increased or decreased load on the structure of mature bones, both *in vivo* and *in vitro* [7–13]. Increased load has been shown to cause an increase in bone mass while decreased load (due to disuse by enforced rest, space flight or other unloading protocols) leads to a loss in bone mass. These effects occur through the processes of bone modeling and remodeling, and involve the cells of the bone tissue (osteocytes, osteoblasts and osteoclasts).

Mechanical loads are also known to play an important role during skeletal development in the postnatal period. It is well known, for instance, that various types of physical activity in children affect the geometry and composition of their bones [14–16]. However, studies describing the detailed interaction between loading during this time period and bones' structural and mechanical features are rare. In particular, the mechanism by which such influence is exerted is not

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well understood, and details regarding the relationship between load magnitude and duration and such quantitative properties as strength, stiffness and yield load are currently lacking. Our study sheds some light on this complex biological question.

During the postnatal period, long bones consist of osseous tissue and the cartilaginous growth plate which is responsible for their axial growth. The rate of bone elongation is regulated on one side of the growth plate by the rate of resting-cell division, chondrocyte proliferation and hypertrophy, and on the opposite side by penetration of blood vessels and cartilage resorption at the border of the growth plate and the metaphyseal bone [17–20]. These processes are tightly regulated by systemic (endocrine) and local (paracrine) factors, and determine overall body dimensions as a result of skeletal growth. It is reasonable to assume that mechanical load during this time will affect the chondrocytes in the growth plate and result in morphological and mechanical effects on the developing bone. This study examines these effects.

Animal studies suggest that growth-plate chondrocytes may have a finite proliferative capacity that is gradually exhausted, causing growth to slow and eventually cease altogether [21,22]. It has been shown that conditions that suppress growth-plate chondrocyte proliferation conserve the proliferative capacity of the chondrocytes, thus slowing senescence. Consequently, after transient growth inhibition, growth plates retain a greater proliferative capacity and are less senescent, enabling the phenomenon of catch-up growth [23]. In this way, above-normal growth rates occur when the cause for the growth retardation is removed [24].

The broiler chick was used as the animal model for this study because it is bipedal (compared to most other laboratory animals which are quadrupeds), rendering the results at least partially applicable to humans, and because broilers have been genetically selected for extremely high rates of growth (increasing their weight during the first 5 weeks after hatch by 5000%, from a hatching weight of 40 g to a weight of 2 kg at 5 weeks) [25]. Such 'spurt growth' was generally assumed to occur exclusively in humans during the first year of life [26]. Furthermore, several studies have suggested that the skeleton is more responsive to mechanical stimuli when growth is rapid [27–29], a fact which might accentuate the effects of loading in our broiler model.

In this paper, we describe the use of a previously reported protocol [30] to study the effects of external mechanical loading on the differentiation, mineralization, and ossification processes in the growth plate of fast-growing bones, and on the resulting phenotype of these bones in terms of architecture and mechanical properties. We hypothesize that such loading has deleterious effects on both the growth plate and the bone, and that load removal will be followed by recovery through the phenomenon of catch-up.

Materials and methods

Animals

The experiments were approved by the Ethics Committee for Animal Experimentation, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Israel.

Eighty 1-day-old broiler chicks (Cobb) were obtained from a commercial hatchery (Brown Hatcheries, Hod Hasharon, Israel), raised in constant-temperature battery brooders at 34 °C, and fed an age-appropriate diet, according to National Research Council recommendations, ad libitum. After a 24-h period of adaptation, the chicks were divided into two groups: 'load' (40 chicks) and 'control' (no load) (40 chicks).

Loading model

Two-day-old chicks in the 'load' group were harnessed for 4 days with small bags (2.5×4 cm) filled with sand weighing 10% of their BW, as previously described [30]. At the end of this loading period (when they were 6 days old), 20 chicks from the 'load' group were sacrificed (6-day load, 6dL) while the other 20 chicks were released from loading and allowed to grow for a further 5 days free of load (11 days release from load, 11dRL). Chicks belonging to the control group were raised under the same conditions, but without artificial loads (6-day control, 6dC and 11-day control, 11dC).

At the end of the experiment, chicks were anesthetized by inhalation of isoflurane, USP Terrell (Minrad Inc., USA), followed by cervical dislocation. Tibia lengths were measured with a ruler (±0.1 mm), then prepared for histological assessment or stored at −20 °C for mechanical and architectural evaluation.

Histological staining and in-situ hybridization

The effect of external load on the growth plate was examined by histological staining and *in-situ* hybridization. Growth plates and the adjacent cortical bone tissue were fixed overnight in 4% paraformaldehyde (Sigma Chemical, St Louis, MO, USA) in PBS at 4 °C. The samples were dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast. Thin (5 µm) sections were prepared. The sections were stained with Alcian blue and Von Kossa staining (0.6% Alcian blue 8GX in 70% ethanol and 2% silver nitrate exposed to sunlight). Tartrate-resistant acid phosphatase (TRACP) and alkaline phosphatase (ALP) [31] staining were performed as previously described [30].

The sections were hybridized with digoxigenin-labeled antisense probes for collagen (COL) type I, II or X, or with ^{35}S-labeled probes (10 ng) for bone Gla protein (BGP) and matrix Gla protein (MGP)

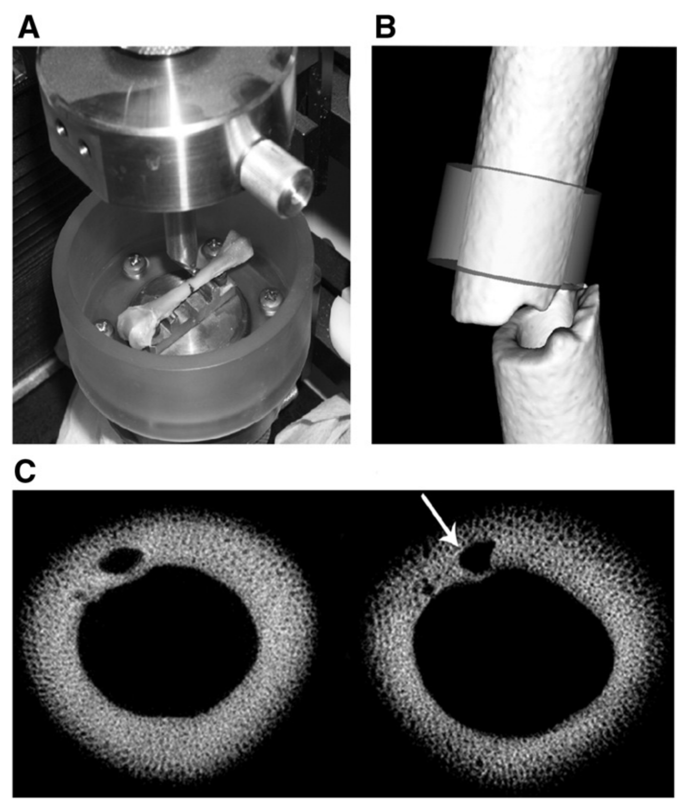

Fig. 1. (A) Three-point bending test set-up with custom-built saline-containing testing chamber. Bones were placed on the two testing supports such that their posterior surface faced the supports, which were equidistant from the ends of the bone. The distance between the supports was set so that both supports contacted the diaphysis. (B) A 1.0-mm transverse section (35 slices) of the diaphysis of the tibia, adjacent to the fracture line, was used to measure the cortical parameters. (C) A typical example of 2D transverse sections of the micro-CT scan to demonstrate the differences in architectural parameters between chicks with (right) or without (left) mechanical load at 11 days of age. Images were reconstructed from the same anatomical region of the tibia by identifying the nutrient foramen (white arrow).