Study of the Effect of Boron Supplementation in the Feed of Broiler Chickens on the Histological Structure of the Tibia

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Abstract
Previous research has demonstrated the role played by boron in the synthesis of estrogens, vitamin D and other steroid hormones and, therefore indirectly in bone matrix mineralisation. The study presented concerns the effects of different source and levels of boron on the mineralisation of bony tissue. Experimental work involved the preparation of fixed histological samples of tibial tissue from sampled broiler chickens in five experimental treatment groups: the feed concentrate (FC) of the control group (1) was not boron supplemented; group 2 were received 20 mg B/kg FC in the form of boric acid; group 3–20 mg B/kg FC as phosphate glass; group 4–10 mg B/kg FC in the form of calcium fructoborate; group 5–10 mg B/kg FC as phosphate glass. Histomorphometric parameters evaluated were the volume of bone trabeculae (BV/TV, %) and mean bone trabecular thickness. Histomorphometric analysis of transverse sections of the proximal extremity of the tibia showed that bone trabeculae volume (BV/TV, %) was greater for groups 4 (52.87%) and 2 (50.19%). The ratio was 43.22% in the control group, 45.70% for group 3 and 45.57% or group 5.

Keywords: boron, broiler chickens, histomorphometric parameters, tibia

1. Introduction
Bone tissue is continuously produced by osteoblasts, modified with the help of osteocytes and destroyed by osteoclasts. The process of mineralization occurs in two tightly linked steps. The first step consists of the laminar secretion of the bony matrix, by osteoblasts. The second stage involves the actual osteoid mineralisation. Bone hardness and rigidity are conferred by the presence of mineral salts in the osteoid matrix, and especially of calcium salts and of phosphate hydroxide precipitated as thermodynamically stable hydroxyapatite (HAP) crystals. These fix themselves between and on the collagen fibers, thus ensuring osteoid mineralisation. The 3D dendritic HAP crystal morphology provides an enormous surface for exchange (2m²/gram of crystal) between the HAP crystals and the interstitial liquid [1]. However, the formation of HAP crystals is only possible if the solubility product of Ca²⁺ and PO₄³⁻ ion concentrations is exceeded. The osteoblasts generate matrix vesicles rich in alkaline phosphatase, which causes accumulations of Ca²⁺ and PO₄³⁻, and in pyrophosphatases which cleave PO₄³⁻ ions from larger molecules. The matrix vesicles bud from the osteoblast apical pole and are stored in the matrix. They provide the control element for mineral deposition in osteoid. After initial precipitation of HAP crystals, they grow rapidly through accretion and intermesh to generate larger crystalline entities. In this way, mineralisation is propagated in the newly formed osteoid. Alkaline phosphatase (ALP) facilitates osteoid matrix mineralisation, and can be used as a phenotypically marker indicator for osteoblasts. On the matrix

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produced mineral deposits (calcium phosphates and carbonates) then form, creating crystals of hydroxyapatite – \( \text{Ca}_10(\text{PO}_4)_6(\text{OH})_2 \) – and thus forming a core mineral deposit, surrounding the osteoblasts, that remain included in the gaps of the newly formed tissue. From this point, the osteoblast reduces its synthetic activity and is transformed into an osteocyte adult cell, which persists in the lacunae of the formed tissue. Calcium plays an important role in bone physiology and in homeostasis. It is stored in bone during bone formation and is released during bone resorption. Intestinal calcium absorption is a complex process requiring the presence of sex hormones [2], especially estrogens. Previous research has established the involvement of boron in the synthesis of estrogens, vitamin D and other steroid hormones, it being essential in the process of -OH group addition in steroid biosynthesis. The presence of -OH groups can lead to large differences of hormonal activity, the difference between testosterone and estrogen, for example, being due to the presence or absence of a single hydroxyl group [3-7].

2. Materials and methods

The research undertaken involved a study of the effect of boron supplements from various sources and at different dosages on the mineralisation of bone tissue in broiler chicken. ROSS 308 hybrid animals were used divided into five experimental treatment groups each of 30 birds. The control group (1) was not boron supplemented; group 2 were administered 20 mg B/kg FC in the form of boric acid; group 3–20 mg B/kg FC as phosphate glass; group 4–10 mg B/kg FC in the form of calcium fructoborate; group 5–10 mg B/kg FC as phosphate glass. Histological examination was made on tibial bone specimens taken from five sampled animals in each treatment group. Following fixation in 10% neutral formol, decalcification with 15% trichloroacetic acid, serial dehydration, clearing and paraffin wax embedding, trichromic Mallory stained sections were examined for histomorphometric analysis using an Olympus CX41 microscope provided with a digital camera and Quick Photo 2.2 software. Among the parameters recomended by the American Society for Bone and Mineral Research [8-11], we assessed the volume of bone trabeculae (BV/TV, %) or the percentage of bone tissue in a given volume and the mean width of bone trabeculae.

3. Results and discussion

Of the serial transversal sections made through diaphysial-epiphysial cartilage in the control group showed proliferating chondroblasts separated by extracellular matrix material and contained high levels of collagen II. Among the proliferating cells there were epiphysial blood capillaries (Figure 1). The cells showed intense secretory activity (Figure 2) with large quantities of matrix material present and a tendency towards hypertrophy. They were rounded, have large nuclei and markedly granular cytoplasm suggesting the accumulation of proteinaceous secretory material, such as collagen, glycoproteins and a series of growth factors. Ramifying blood vessels were found among these cells. In the lower zone of the hypertrophic region chondroblasts showed marked hypertrophy and were beginning to degenerate through apoptosis, their place being taken by osteoblasts which actively secrete around them the bony matrix which subsequently becomes mineralised. Hypertrophic chondroblasts, osteoblasts responsible for the secretion of the bone matrix and osteoclasts, positioned at the periphery of the bone matrix, were all to be observed in the newly formed bone lamellae. Results of histomorphometric study of transverse sections the proximal extremity of the tibia show that the volume of bone trabeculae (BV/TV, %) is 43.22%, with the median thickenss of bony trabecules being 36.9 μ (Figure 3). The periphery of the body of the tibia was formed of cortical bone consisting of osteones, of circular cross section, in the process of formation (Figure 4). Median cortical bone thickness was 1081.3 μ and the overall diameter of the tibia was 0.75 cm.
Transverse sections from the proximal extremities of individuals from treatment group 2 (which received 20 mg B/kg FC as boric acid) showed a structure formed from spongy bone tissue (Figure 5). Median trabecular thickness, as determined by histomorphometric measurement of the bone, was 53.55 μ while trabecular bone volume (BV/TV, %) was 50.19%.

Although trabecular ossification was not complete, on a morphological view, the trabecules presented a more compact appearance than those of the control group. The substance of the trabecules is largely made up of deposited fibres of collagen I and of osteina impregnated with crystals of hydroxyapatite (Figure 6). Within the structure of the osteina numerous osteoplasts are evident, cavities occupied by osteocytes, differentiated bony cells which have lost their capacity for secretion. Both on the outer surface of the trabecules, and within the body of them, osteoblasts, either isolated or in clusters, were present, as were hypertrophic chondroblasts and also osteoclasts (Figure 6); they have a role in the resorption of the matrix and in bone remodelling.

The presence of osteoblasts and hypertrophic chondroblasts indicates that ossification had already started. The occurrence of this process is also suggested by the presence of small cavities in the structure of the bony trabecules.

The cortical bone in the periphery of the tibial shaft was formed of circular osteones. Median thickness of the cortical bone was 1365.3 μ while the external diameter to the tibia was 0.90 cm.
For treatment group 3 (given 20 mg B/kg FC as phosphate glass) microscopic analysis of histological sections of epiphysial plate cartilage showed, in the zone below the hypertrophic region of the cartilage, an intense process of bone formation marked by the presence of active osteoblasts and hypertrophic chondroblasts in the axillary part of the bony lamellae (Figure 7). The periphery of the bony trabecules was formed of complete bony lamellae in which the secretory activity of the osteoblasts had ceased and, following bone mineralization, they had differentiated into osteocytes. Microscopic study of histological sections of the proximal extremity of the tibia showed both the presence of bony trabecules and cavities bounded by them (Figure 8). Bony trabecules had a mean thickness of 61.0 µ. In this case too it could be observed that although the trabecules had been formed through deposition of collagen I fibres followed by bony mineralisation there could already be observed on the restricted surfaces osteoblastic activity and chondroblastic hypertrophy. Bony trabecular volume (BV/TV, %) determined by histomorphometry of bony tissue was 45.70%.

Transverse sections of tibial shaft showed an intense process of ossification, with involvement of the periosteum, in particular the osteoprogenitor cells in cellular layers of the periosteum. These cells establish themselves in the previously established cavities and, following their transformation into osteoblasts, begin to produce the young connective tissue. The
osteoblasts in the cavities had the strongly basophilic cytoplasm associated with active protein synthesis. Cortical bone thickness was 1514 µ, with median bone diameter being 0.70 cm.

Individuals from treatment group 4 (which received 10 mg B/kg FC in the form of calcium fructoborate showed a dense bone trabecules at the proximal extremity of the tibia, that bounding of cavities, generally, small in size (Figure 9).

The bony trabecules has developed fairly uniformly and has a median thickness of 53.7 µ. The trabecular bony volume (BV/TV, %), measured by histomorphometry, is 52.87%.

A noteworthy aspect in individuals from this treatment group is the strong stimulus to the process of osteogenesis. The process occurs intensely both in the cartilage of the epiphysial plate, being evidenced by the proliferation, hypertrophy and secretory activity of chondroblasts, and aslo at the level of the trabecules of the spongy bone (Figure 10). They are composed predominantly of condensation fibres of collagen I and osteina impregnated with mineral salts. Axially the presence of hypertrophic chondroblasts and osteoblast secretory activity was noted. On the endosteal surface of the trabecule are to be found osteoblasts arranged in a single layer while on restricted areas, active osteoclasts (Figure 11). Microscopically observable features indicate an active process of morphogenesis shown by the development of angiogenesis and activity of the osteoblasts, these being present on the areolar surface of the bony trabecules. While the bony matrix is being syntheised and mineralised the process of bone remodelling, involving osteoclasts, also take place. These are giant multinulate cells which arise from blood monocytes. Thus trabecular structures are formed by a process in which both osteoblasts (which synthetise the bony matrix) and osteoclasts (which resorb surplus matrix material) are involved. There is a close cooperation between the secreting osteoblasts and the resorbing osteoclasts with the differentiation and activation of the osteoclasts being dependent on the formation of a RANK – RANK – L type coupling [12 – 14]. Thus the RANK-L factor, synthetised by the osteoblasts, bind to the RANK receptor on the membrane of osteoclast cell lines and thus both induce the formation and differentiation of the osteoclasts and stimulate the activity of mature osteoclasts.

Transverse sections of the tibial diaphisis showed the process of ossification by aposition, with the involvement of cells of the periosteum, the process being accompanied by the formation of osteones and an intensification of angiogenesis. The thickness of the cortical bone in this treatment group was approximately 1245.5 µ while the mean diameter was approximately 0.85 cm.

![Figure 9](image.png)

**Figure 9.** Group 4. Tibia – proximal extremity: trabecules and cancellous cavities (200x, trichromic Mallory stain).
Figure 10. Group 4. Tibia – inferior zone of diaphysial – epiphysial cartilage: bony trabecules in formation (1000x; trichromic Mallory stain).

Figure 11. Group 4. Tibia – proximal extremity. Bony trabecules (1000x; trichromic Mallory stain).

Individuals in treatment group 5 (which received 10mg B/kg FC in the form of phosphatic glass) the epiphysial cartilage showed intense activity, the hypertrophic layer being very thick. Immediately underlying this layer the chondroblasts showed intense secretory activity, the cells were characterized, morphologically, by oval shape, large and eccentric nucleus, and granular cytoplasm. Newly formed bony trabecules showed conjunctive lamella and mineralised osseina on at the periphery of those, the axial portion being occupied by a mixed population of cells ranging from chondroblasts in apoptosis to chondroblast and osteoblasts which were active (Figure 12).

In the proximal extremity of the tibia (Figure 13) the trabecules has a median thickness of 55.6 μ while the volume of bony trabecules (BV/TV, %) is 45.57%. Histomorphometric analysis of transverse sections from the shaft of the tibia showed median cortical bone thickness of 1096.4 μ, and median diameter of approximately 0.75 cm.

Figure 12. Group 5.- Tibia – newly formed bony trabecule (1000x, trichromic Mallory stain).

Figure 13. Group 5- Tibia – proximal extremity (100x; trichromic Mallory stain).

From our analysis results, it appears that in all experimental variants, boron stimulates proliferative activity and secretion of osteoblasts. In all experimental variants, trabeculae bone thickness is greater than control, while the highest values of bone volume were reported at groups 2 and 4, in which boron was administered by boric acid and calcium fructoborate.
Our results are consistent with those obtained by Rico et al., [15], who studied influence of boron supplementation on vertebral and femoral bone mass in rats on strenuous treadmill exercise. The femur weight, bone mineral content and density, trabecular bone volume and trabecular thickness, were significantly higher in the exercise plus boron group.

Boron affects the composition and physical characteristics of bone, by stimulating the action of hormones involved in bone growth and bone and/or a mechanism to stimulate the formation and maturation of organic matrix on which calcification occurs [16].

Recently, it has been demonstrated that B in the form of boric acid potently activates the mitogen-activated protein kinase signaling pathway to markedly increase cell proliferation and growth at low concentrations and inhibits these activities at high concentrations [17]. These results are relevant to bone biology given that it has been demonstrated that many of these signaling cascades are required for mesenchymal cell commitment, osteoblast differentiation, and proliferation [18–20].

It has been reported that boron may be beneficial for optimal calcium metabolism and, as a consequence, optimal bone metabolism [21]. In pigs, boron supplementation has shown beneficial effects on bone characteristics [22, 23]. In chickens, studies have found that a supplement of boron will alleviate some of the symptoms associated with vitamin D deficiency [24]. It is generally accepted that moderate dietary supplementation of the chicken diet with boron increases the strength of bone and augments the bone ash content without detrimental effects [25, 26]. In these male rat studies, dietary boron administration (>200 ppm) was associated with a 10% greater vertebral resistance to a crushing force by biomechanical testing.

4. Conclusions

For individuals from the control group the median trabecular thickness was 36.9 µ, smaller than that found for individuals in other treatment groups. The median thicknesses for respective treatment groups being 53.5 µ (treatment 2), 61.0 µ (treatment 3), 53.7 µ (treatment 4), and 55.6 µ (treatment 5). In all the treatment groups apart from the control the process of ossification was continuing, as shown by the appearance of the trabecules and evidence of osteoblast activity. Serial transverse sections of the proximal extremity and the body of the tibia show the continuation of the process of morphogenesis in which the cells which arise in the periosteum play an important role as do both the osteoblasts to be found on the surface of the cancellous cavities of the trabecule, through the synthesis of bone matrix and also osteoclasts which act by resorption of excess matrix material.

A noteworthy feature of individuals in group 4 was the powerful stimulation of osteogenesis. The process occurs intensively both in the diaphysial-epiphyseal cartilage, being signalled by the proliferation, hypertrophy and secretory activity of the chondroblasts, and in the trabecules of the spongy bone. These are largely formed through the deposition of fibres of collagen I and oseina impregnated with mineral salts, and axially by the presence of hypertrophic chondroblasts and as a result of osteoblast secretory activity. The cancellous cavities of the trabecules are lined with a single layer of osteoblasts, and, one restricted surfaces, active osteoclasts.

Histomorphometric analysis of transverse sections from the proximal extremity of the tibia showed that bony trabecule volume (BV/TV, %) was greater for individuals from groups 4 (52.87%) and 2 (50.19%). The ratio was 43.22% for individuals from the control group, 45.70% for individuals from group 3 and 45.57% for individuals from group 5.

References

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