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Research article

TIBIA GROWTH AND DEVELOPMENT IN BROILER CHICKS REARED UNDER CONTINUOUS LIGHT AND MELATONIN DIETARY SUPPLEMENTATION DURING THE FIRST TWO WEEKS OF LIFE

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The first few weeks after broilers hatch are the period of most intense bone growth and development, and the time when they are most susceptible to the influence of various external and internal factors. Research in the recent decades has focused on the involvement of melatonin in bone development during early life in chickens. Melatonin release from the pineal gland has a circadian rhythm, with the highest levels circulating during the night and decreasing during the light phase of the day. Various types of lighting are used in intensive broiler production. In this study, the effects of melatonin on the tibial structure and growth of broilers were investigated. During the first two weeks of life, two groups of chickens were kept under continuous light and fed the same diet, with the experimental group receiving melatonin in the amount of 0.03 g/kg of feed. The results obtained showed that the addition of melatonin in the diet had positive effects on the development and growth of the tibia, which was expressed in a significantly greater thickness of the diaphysis and cortical bone of the diaphysis, higher breaking force and higher values of alkaline phosphatase activity. The cortical bone mineral density of the tibia did not differ significantly between the groups of chicken.

Keywords: broilers, growth, development, light, melatonin, tibia

INTRODUCTION

Intensive broiler production is often associated with leg weakness, resulting in economic losses. Numerous studies have been conducted to explore the characteristics of the dynamics of bone metabolism and the factors that influence it. Particular attention has

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been paid to the growth, development and structure of the tibia in the first weeks after hatching, when these processes are most intense and crucial for the development of the tibia in broilers [1,2].

After hatching, the long bones grow in length and width. They elongate by proliferation, hypertrophy, and differentiation of chondrocytes in the epiphyseal growth plate of the long bones. The most intense growth in length of the tibia occurs in the first two weeks of life of broiler chickens [3].

The long bones grow in width due to the activity of osteoblast cells from the periosteum. In the process of bone apposition, the osteoblasts secrete a matrix towards the outer surface of the bone. The matrix has an organic composition and consists of type I collagen fibers, proteoglycans, and glycoproteins. After some time, calcium and phosphorus salts depose on the surface of the deposited organic matrix. Bones are becoming mineralized, and the cortical bone is formed (the outer layer of long bones that covers the epiphyses and builds the wall of the diaphysis, and surrounds the bone marrow cavity). Calcification is supported by the enzyme alkaline phosphatase produced by osteoblasts. On the endosteal surface, osteoclasts' activity leads to bone tissue resorption and increases the width of the medullary canal. These two processes are known as bone modeling [4]. Inside the bone, at the osteon level, bone remodeling takes place: osteoclasts resorb and remove mineralized bone, and then osteoblasts form a new bone matrix, followed by mineralization [5,6]. The amount of mineral material per unit of bone volume is expressed as bone mineral density (BMD) [7,8]. The results of a study by Williams et al [9] indicate that a high growth rate significantly impacts BMD; fast-growing commercial broiler chickens show a decrease in BMD of the tibia diaphysis compared to slow-growing chickens.

The growth and development of bones at a young age are under the complex influence of several hormones that act on the cells of bone tissue: Growth hormone (GH), which stimulates osteoblasts directly or indirectly through the synthesis of insulin-like growth factors (IGF I and IGF II); parathyroid hormone (PTH), which enhances the resorptive function of osteoclasts to increase the Ca concentration in the blood; calcitonin, which has an inhibitory effect on osteoclast function [10-14]. In the last forty years, much attention has been paid to the influence of melatonin on metabolism, growth and bone development. Melatonin synthesis in the pineal gland exhibits circadian rhythmicity, so that the level of melatonin in the blood is highest during the night, i.e. in darkness, while it decreases under conditions of the light phase [15-18]. The basic and primary function of melatonin is to act as a neutralizer of free radicals and stimulates antioxidant processes in the body [19-21]. In addition, melatonin is involved in a variety of physiological functions and plays an important role in bone modeling [22-24] and bone remodeling [25-27] through its effects on osteoblasts and osteoclasts. Amstrup et al. [28] found that bone mineral density (BMD) significantly decreased in pinealectomised animals compared to control animals, highlighting the importance of adequate melatonin levels. Dietary supplementation with melatonin increases the strength of the femur and tibia bones of laying hens, as measured by breaking force

[29]. Our study investigated the effects of melatonin on the tibia of broiler chickens raised under continuous light and fed dietary melatonin supplementation.

MATERIAL AND METHOD

The experiment, approved by the Veterinary Directorate in MAFW of the Republic of Serbia (Approval number: 323-07-00069/2017-05), was conducted on a total of 320 one-day ROSS 308 broiler chickens of both sexes and lasted from day 0 to day 14 after hatching. Broilers were divided into two groups (M - experimental group and C - control group), with four replicates per group and 40 broilers in each replicate. They were kept in a floor housing system with continuous lighting (CL) in a 24L:0D (light: dark) ratio and with feed and water *ad libitum*. The complete feed mixture was the same for both groups and was prepared according to the recommended feeding program and nutrient requirements prescribed by the hybrid manufacturer (ROSS 308 Broilers: Nutrition Specifications, Aviagen, 2014). In contrast to the control group (C), the experimental group (M) received feed with melatonin addition (0.03 g/kg synthetic melatonin, N-acetyl-5-methoxytryptamine EINECS number: 200-797-7; purity 99%; Elephant Co., Serbia).

At the end of the experimental period of 14 days, blood was taken from five chickens (n=5) from each group (C, M) by cardiac puncture to determine the activity of the enzyme alkaline phosphatase. The enzyme was determined by spectrophotometry (A15 Random Access Analyzer, BioSystem, Spain) using the corresponding kit AFA-250 (Bioanalytica, Serbia). Simultaneously, ten chickens from each group (C, M) were sacrificed by neck dislocation. The left tibias (n=10) of the sacrificed chickens were used to test the bone strength (breaking force, kg), which was determined by the IPNIS instrument in the three-point bending test [30,31]. The bones were placed on two supports with a distance of 40 mm. A force was applied in the anterior-posterior plane of the bone at the mid-diaphysis with a uniform loading rate of 5k per minute set on the instrument. The right tibias (n=10) were used to study bone structure and cortical bone mineral density. Computed tomography of the tibial bones was performed at the Department of Radiology and Radiation Hygiene of the Faculty of Veterinary Medicine in Belgrade using a single-slice device CT Somatom Ar Star (Siemens Medical Systems, Germany) with 110 kV, 63 mAs and contiguous 5 mm thick slices in the bone reconstruction algorithm. Radiographs were obtained using the ZooMax Gold veterinary radiography system and were acquired in both lateral views at 55 kV and 16 mAs. Radiographs of the cortical bone were acquired in lateral and anterior-posterior positions at 48 kV, 10 mAs. Images were processed using the AGFA 10-X CR system.

Bone mineral density of cortical bone were measured in the following cross-sectional areas (n=10): cortical BMD in the proximal epiphysis, cortical BMD in the middle diaphysis, and cortical BMD in the distal epiphysis. Bone density was measured at these sections at four locations: anterior, posterior, lateral, and medial. Using these

four values, the average bone density (HU - Hounsfield unit) was calculated. On the bone scans, the length of the tibia from proximal to distal end (cm) and the outer and inner diameters were measured in the cross-section of the middle part of the diaphysis (n=10). Cross-sectional area as an indicator of macroscopic bone structure was calculated using the following formulas:

$$TA\ (mm^2) = \pi/4\ (AP \times LM)$$

$$MA\ (mm^2) = \pi/4\ (ap \times lm)$$

$$CA\ (mm^2) = TA - MA = \pi/4\ (AP \times LM - ap \times lm)$$

Where:

TA – Total diaphyseal cross sectional area, MA – Medullar diaphyseal cross sectional area, CA – Cortical diaphyseal cross sectional area, AP – Outer (*outside*) diameter in antero-posterior direction; LM – Outer (*outside*) diameter in latero-medial direction; ap - Internal diameter in antero-posterior direction; lm – Internal diameter in latero-medial direction.

Statistical analysis

Results were analyzed using the STATISTICA statistical software package, version 8, StatSoft. Inc. (www.statsoft.com), and t-test was performed to determine the degree of statistical significance of differences between groups, independently by group.

RESULTS

The influence of melatonin supplementation on the mean values of the parameters of bone metabolism of the tibiae of broiler chickens is shown in Table 1.

Table 1. Parameters of bone metabolism of the tibia of 14-day-old broiler chickens

Parameter	Group		P - value
	Control	Experimental	
Length (cm)	5.58 ± 0.10	5.78 ± 0.13	0.003**
TA (mm^2)	12.93 ± 0.98	14.78 ± 1.41	0.008**
MA (mm^2)	0.94 ± 0.18	1.05 ± 0.25	0.357
CA (mm^2)	11.98 ± 0.94	13.73 ± 1.23	0.006**
BMD proximal epiphysis (HU)	471.90 ± 16.64	474.00 ± 24.81	0.845
BMD distal epiphysis (HU)	488.09 ± 33.54	507.87 ± 34.89	0.266
BMD diaphysis (HU)	712.79 ± 79.47	693.59 ± 59.39	0.592
Breaking force (kg)	6.99 ±0.717	7.81 ± 0.998	0.049*
Alkaline phosphatase activity (IU/L)	8,811.41 ± 1752.30	10,041.43 ± 3931.35	0.054

TA – Total diaphyseal cross sectional area; MA – Medullar diaphyseal cross sectional area; CA – Cortical diaphyseal cross sectional area; BMD – Bone Mineral Density; **– Statistically highly significant difference ($P < 0.01$); * – Statistically significant difference ($P < 0.05$).

The tibia length of the chickens that received melatonin in the diet was significantly higher ($P < 0.01$) compared to the control group that did not receive melatonin. The effect of melatonin on appositional growth of the tibia is evidenced by the results of measuring the diameter and calculating the cross-sectional areas in the middle part of the diaphysis. The total cross-sectional area (TA) and the cortical cross-sectional area (CA) were significantly higher in the experimental group ($P < 0.01$). The differences in the value of medullary cross-sectional area (MA) were not significant. Bone density was not significantly different between groups at 14 days of age. In both groups, the bone density of the cortical part of the diaphysis was higher than the cortex density of the proximal and distal epiphysis. In chickens fed melatonin, tibial breaking force and alkaline phosphatase enzyme activity value (ALP) were significantly higher ($P < 0.05$) and showed a tendency to increase ($P = 0.054$), respectively, in the experimental group compared with chickens in the control group.

DISCUSSION

The circadian rhythm of vertebrates is maintained by mechanisms called circadian pacemakers. Birds have multiple pacemakers in the retina, pineal gland, and suprachiasmatic nucleus (SCN), a bilateral structure in the anterior part of the hypothalamus [18]. Through output signals, the pacemakers coordinate the circadian rhythm of peripheral organs and tissues. One of the most important output signals is melatonin, whose plasma concentration changes rhythmically during the day and reaches the highest levels during the night (darkness). When chickens [15] and turkeys [16,17] are exposed to constant light during the night, the rhythm of melatonin synthesis and secretion is not disturbed, but there is a decrease in melatonin concentration compared to the normal night time concentration. This can have numerous negative consequences, especially in young organisms at a young age, because melatonin is involved in a number of physiological functions. One of these functions is to affect metabolism, growth, and bone development, especially during the first weeks after hatching when these processes are most intense and are critical for tibia development in broiler chickens [1-3]. Our results show that the length of the tibia of chickens that received melatonin in the diet was significantly higher than that of chickens that did not receive melatonin. This is consistent with [32], who claimed that melatonin plays an important role in the proliferation, hypertrophy and differentiation of chondrocytes in the epiphyseal growth plate during endochondral ossification and postnatal long bone length growth.

The appositional growth of long bones in width occurs through the activity of osteoblast cells originating from the periosteum. The results of our experiment show that the tibia of chickens that received melatonin had a significantly higher diaphysis thickness (TA), cortical cross-sectional area, and breaking force compared to the tibia of chickens that did not receive melatonin. Cortical bone occurs by periosteal bone apposition, a process of matrix deposition at the outer surface of the bone, that results

in increased bone width and skeletal strength [14]. Taylor et al. [29] found that litter with a high level of melatonin in their diet had the highest breaking forces of the femur. The stimulatory effect of melatonin on the function of osteoblasts in our experiment was also shown through the values of alkaline phosphatase (ALP) enzyme activity which showed a tendency towards increase in chickens of the treated group (10041.4 IU/L) compared to the control group (8811.4 IU/L).

The results of numerous studies indicate that melatonin has a direct effect on bone growth and development through a stimulatory effect on osteoblast proliferation and differentiation, while at the same time inhibiting osteoclast function and reducing bone tissue breakdown [22,23,25,26].

Cortical bone mineral density of the tibia-diaphysis and proximal and distal epiphysis did not differ significantly between the two groups of chickens. This can be explained by the results reported by [5] and [9]. The high growth rate has a significant effect on bone mineralization. Fast growing broilers have lower tibial mineralization (density) and increased porosity compared to slower growing chickens. The high growth rate of the chicks results in heavy bone deposition on the periosteal surface, which in turn leads to an increase in the diameter of the diaphysis of the tibia. Mineralization of the matrix is slower.

The direct effects of melatonin on osteoblasts are achieved via the MT2 receptors for melatonin on osteoblasts [27,33]. Moreover, Nakano et al. [24] demonstrated the expression of melatonin receptors and calcitonin in osteocytes and proposed a new mechanism underlying the suppressive effect of melatonin on osteoclasts through the upregulation of calcitonin secretion by osteocytes. The indirect, receptor-independent effect of melatonin on bone is due to its action as an antioxidant and neutralizer (scavenger) of free radicals released during bone resorption by osteoclast activity.

CONCLUSION

The results of this study show that melatonin has a significant effect on bone growth and development during the first two weeks of life of chickens after hatching. Exposure of young chickens to constant light throughout the day may result in decreased melatonin synthesis and consequent negative effects on the physiological functions and development of the whole organism, especially the bones. On the other hand, the addition of melatonin to the diet of chickens raised under constant light, as shown by the results of this study, improves tibial length, diaphyseal cross-sectional area, bone strength, and alkaline phosphatase activity.

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Authors' contributions

DV designed and coordinated the study; ML, ZŠ and VP designed and performed the experiment on the farm, and contributed in determination of bone strength; IB collected the samples, performed and interpreted statistical analyses; RR contributed in blood analyses; MLM and KN performed radiological analyses of the chicken bones; DV, IB and RR contributed in the writing, translation and preparation of the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The authors do not have any financial or personal conflicts of interest that could bias the study.

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RAST I RAZVOJ TIBIJE KOD BROJLERA GAJENIH POD STALNIM SVETLOM I SA DODATKOM MELATONINA U HRANI TOKOM PRVE DVE NEDELJE ŽIVOTA

Duško VITOROVIĆ, Ivana BOŽIČKOVIĆ, Miloš LUKIĆ, Renata RELIĆ, Zdenka ŠKRBIĆ, Veselin PETRIČEVIĆ, Mirjana LAZAREVIĆ MACANOVIĆ, Nikola KRSTIĆ

Prva nedelja nakon izleganja brojlera je period najintenzivnijeg rasta i razvoja kostiju i njihove najveće podložnosti uticaju različitih spoljašnjih i unutrašnjih faktora. Istraživanja u poslednjim decenijama skrenula su pažnju na učešće melatonina u razvoju kostiju tokom ranog uzrasta pilića. Sekrecija melatonina iz epifize ima cirkadijalni ritam sa najvećim nivoom cirkulacije tokom noći, dok se smanjuje tokom svetle faze dana. U intenzivnoj proizvodnji brojlera koriste se različiti režimi osvetljenja. Ova studija je ispitivala uticaj melatonina na strukturu tibije i rast brojlera. Tokom prve dve nedelje života, dve grupe pilića su držane na stalnom svetlu i hranjene istom ishranom, uz dodatak melatonina u količini 0,03 g/kg hrane u oglednoj grupi. Dobijeni rezultati su pokazali da suplementacija melatonina u ishrani ima pozitivne efekte na razvoj i rast tibije, izražene kroz značajno veću debljinu dijafize i korteksa dijafize, veću silu loma i veće vrednosti aktivnosti alkalne fosfataze. Mineralna gustina kortikalne kosti tibije nije se značajno razlikovala između grupa pilića.