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Histomorphometric characteristics of the metaepiphyseal plate of the distal femur of lambs during the period of their intensive growth

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Abstract

Introduction Growth zone injuries are quite common and account for 15–30 % of all skeletal bone injuries in children. Complications occur in 2–14 % of patients. An adequate experimental animal model is needed to develop new methods for treating growth zone injuries.

The purpose of the work is to identify patterns in the dynamics of histomorphometric characteristics of the metaepiphyseal cartilage of the distal femur of lambs during the period of their intensive growth.

Materials and methods The metaepiphyseal cartilage of the distal femur of 12 lambs (aged 3.5 and 5.5 months, 5 males and 7 females) previously participating in an experiment on the effect of osteosynthesis pins on the structural reorganization of the metaepiphyseal cartilage was studied. Histological, immunohistochemical, and histomorphometric studies were performed.

Results The zonal structure of the metaepiphyseal plate along with an increased proportion of PAS-positive structures in the outer layer of the border zone and in the calcified cartilage zone were determined. Masson staining revealed fuchsinophilic areas of the border zone matrix in the metaepiphyseal cartilage of animals aged 5.5 months, as well as an increase in the proportion of fuchsinophilic areas of the calcified cartilage zone compared to animals aged 3.5 months, which indicated increased mineralization. CD34 expression at 3.5 months was detected in the outer layer of the border zone, at 5.5 months the depth of vascular invasion increased, but did not reach the proliferating cartilage zone. A decrease in the thickness of the metaepiphyseal cartilage at the age of 5.5 months by an average of 18.2 % is due to a decrease in the thickness of the border zone by 1.9 times, while the thickness of the proliferating cartilage zone increased by 1.2 times.

Discussion The changes observed in the main substance of the metaepiphyseal cartilage indicated that the processes of matrix calcification are more intense in lambs by the age of 5.5 months. The depth of vascular penetration from the diaphysis is more pronounced than from the epiphysis. Fractures in the growth zone during the period of intensive growth can be caused by the predominance of the border zone and by the proliferating cartilage zone.

Conclusion Histomorphometric changes in the metaepiphyseal plate of the distal femur of lambs during the period of their intensive growth were characterized by a decrease in its thickness due to a marked decrease in the thickness of the reserve zone, while the thickness of the proliferating cartilage statistically significantly increased. The depth of vascular invasion in the border zone increased, but did not reach the proliferating cartilage zone, changes in the tinctorial characteristics of the ground substance indicated the activation of matrix calcification processes from the subchondral bone of the epiphysis and endomorphic ossification from the diaphysis.

Keywords: lambs, metaepiphyseal cartilage, histology, immunohistochemistry, histomorphometry

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INTRODUCTION

The growth zone (physis, metaepiphyseal plate, metaepiphyseal cartilage) is hyaline cartilage located between the epiphysis and metaphysis of tubular bones and is responsible for their longitudinal growth [1]. According to statistics, growth zone injuries are quite common and account for 15–30 % of all skeletal bone injuries in children; complications occur in 2–14 % of patients [2, 3]. Distal femur fractures are the most common in children, with Salter–Harris type II fractures dominating [4, 5].

Currently, there is an urgent need to develop effective methods of treating children with growth plate injuries that not only prevent the formation of "bone bridges" but also create conditions for complete regeneration of hyaline cartilage and normal bone growth [6, 7].

Experimental studies aimed at developing new methods for treating children with growth zone injuries and correcting pathological conditions associated with impaired function are relevant. An adequate animal model is needed to study the healing of metaepiphyseal plate damage. In preclinical studies, small mammals such as mice, rats, and rabbits are used as experimental models [8, 9, 10, 11, 12, 13]. Rodent models are not suitable for extrapolating experimental data to humans not only because rodent growth zones do not close during maturation, but also because of differences in their histological structure, blood supply, cell cycle, and growth time. Also, small animal models cannot simulate the biomechanical conditions of human skeletal loads [14, 15]. According to a number of authors, dogs and sheep are the most suitable experimental animals for studying the metaepiphyseal plate. Sheep are more similar to humans in terms of weight and bone size, and the long bones of their limbs are subject to similar loads. Moreover, their growth zone closes with age, and young sheep have plexiform bone structures similar to those of children during periods of rapid growth [15, 16, 17, 18]. Despite the availability of extensive information on the growth and development of animals, many phenomena of this biological process remain unexplored to date. In the available literature, we did not find data on structural changes in the metaepiphyseal plate of lambs during their growth. To determine the differences in the qualitative and quantitative characteristics of the metaepiphyseal cartilage from the norm, there is a need to compare them with similar characteristics of the metaepiphyseal cartilage of intact animals.

The **purpose** of the work was to identify patterns in the changes of histomorphometric characteristics of the metaepiphyseal cartilage of the distal femur of lambs during the period of their intensive growth.

MATERIALS AND METHODS

The object of study was the metaepiphyseal plate of the distal femur of the contralateral limb in 12 lambs (5 males, 7 females), which had previously participated in an experiment on the effect of osteosynthesis pins on the structural reorganization of the metaepiphyseal cartilage [19], at the age of 3.5 and 5.5 months (body weight — (21.92 ± 0.85) and (28.92 ± 2.4) kg, respectively). This is the period of rapid growth of the long bones in lambs. From 7 months of age, the growth rate decreases, while the muscle mass accumulates [20].

The animals were euthanized after premedication with a solution of 1 % diphenhydramine (0.02 mg/kg) and 2 % rometar (1 mg/kg), and a lethal dose of barbiturates. The animals were kept in the experimental conditions in accordance with the following regulatory documents: GOST R 33044-2014; PS SanPiN 3.3686-21; GOST 33215-2014; GOST 34088-2017.

For microscopic study, fragments of the distal articular end of the femur were cut out; next, the samples were fixed in a 10 % solution of neutral formalin for 48 hours. Decalcification was carried out in a mixture of hydrochloric and formic acid solutions (1:1), washed in water, dehydrated in alcohols, and embedded in paraffin using a standard method.

To obtain reliable information on the qualitative and quantitative characteristics of the metaepiphyseal cartilage, its zonal structure was considered, and paraffin sections of adequate orientation and thickness were used [21, 22]. Longitudinal serial sections along the femur axis (5.00 μm) were produced on an HM 450 microtome (Thermo Scientific, USA), which were stained with Alcian blue pH 2.5 and PAS-Reaction, using the three-color method according to Masson.

The immunohistochemical reaction was performed using the angiogenesis marker CD34 (CD34 [EP373Y]) (Abcam, UK) and the peroxidase detection system with diaminobenzidine with the micropolymer ab236469 (Rabbit specific HRP/DAB Detection IHC Detection Kit-Micropolymer, Abcam, UK). All stages of the immunohistochemical reaction were performed according to the protocol of the manufacturer for antibodies. Sections were counterstained with hematoxylin.

Histomorphometric examination and digitalization were performed on an AxioScope.A1 microscope with an AxioCam digital camera (Carl Zeiss MicroImaging GmbH, Germany) using the Zenblue image analysis program (Carl Zeiss MicroImaging GmbH, Germany).

The thickness of the metaepiphyseal cartilage (h, mm) was measured as the distance between its upper and lower borders with an interval of about 20 μm; 20 measurements were taken for each case. The thickness of the metaepiphyseal cartilage zones (h, μm) and their percentage ratio were determined. The boundary between the border zone and the proliferating cartilage zone was considered to be the point of appearance of the first flattened chondrocyte in the column; the boundary between the proliferating cartilage zone and the vesicular cartilage zone was considered to be the point of appearance of the first rounded chondrocyte; the boundary between the vesicular cartilage zone and the calcified cartilage zone was considered to be the point of appearance of areas of resorbable matrix [23]. The percentage of each zone was determined as a portion of the total thickness of the metaepiphyseal cartilage equal to 100 %.

Quantitative data were analyzed using descriptive statistics. The distribution pattern of data was assessed using the Kolmogorov test; the measure of central tendency was presented as a median and quartiles (Me (p25–p75)). Hypotheses about differences in the compared groups were tested using the Mann–Whitney test; differences were considered significant at $p < 0.05$ (AtteStat software, version 9.3.1).

RESULTS

On histotopograms, the metaepiphyseal plate of the distal part of the femur of lambs was visualized as a slightly sinous line. Light-optical study of paraffin sections showed that the metaepiphyseal cartilage had a zonal structure. Four zones were clearly distinguished: the border or reserve zone at the epiphyseal bone; the zone of proliferating or colum cartilage; the zone of vesicular (dying) cartilage; the zone of calcified (calcifying) cartilage (Fig. 1).

In the border zone, chondrocytes were located by one, rarely in the form of two-membered isogenic groups. In all other zones, a colum arrangement of cartilaginous cells was observed;

in the proliferative zone, chondrocytes were flattened, in the zone of vesicular cartilage they were enlarged in size, rounded in shape; and in the zone of calcified cartilage, the majority of cells showed signs of destruction (Fig. 1).

According to the tinctorial characteristics of the ground substance of the border zone, two layers were distinguished: outer and inner. The outer layer, when stained with alcian blue, was as follows: PAS is close to the bone, PAS is positive (the PAS reaction at the age of 5.5 months was more intense than at the age of 3.5 months), and the inner layer was stained with alcian blue pH 2.5 in blue (Fig. 1).

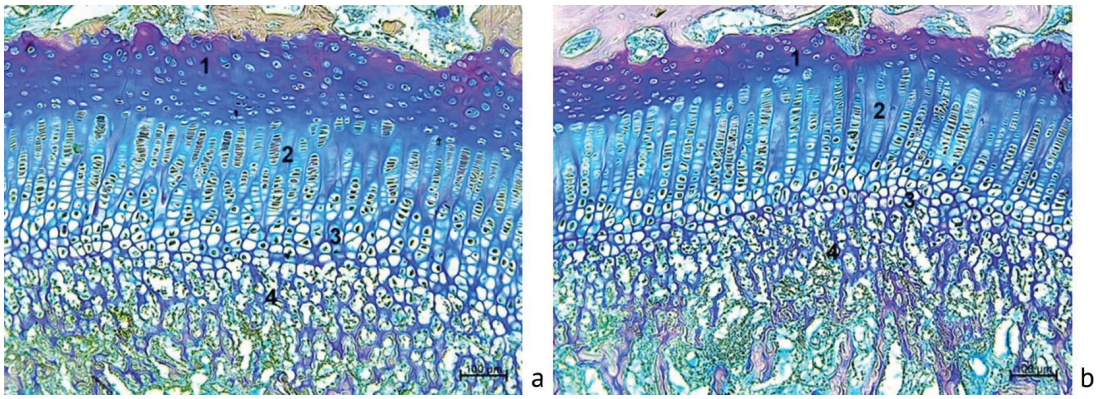

Fig. 1 Metaepiphyseal cartilage of the distal femur of a lamb: *a* age 3.5 months; *b* age 5.5 months. Border (reserve) zone (1), zone of proliferating cartilage (2); zone of vesicular cartilage (3); zone of calcified cartilage (4). Paraffin section, stained with Alcian blue pH 2.5 and PAS reaction. Magnification ×100

When stained using the three-color Masson method, the intercellular substance of the border zone in animals aged 3.5 months was stained blue, while at the age of 5.5 months there were extensive areas of the intercellular substance stained red, indicating calcification of the matrix (Fig. 2 a, c).

The intercellular substance of the zones of proliferating and vesicular (mature) cartilage with alcian blue: PAS was stained in an intense blue color, which indicates a high content of sulfated glycosaminoglycans. In the zone of calcifying cartilage, the matrix was partially resorbed, at the age of 5.5 months it was more PAS-positive (Fig. 1 b). When stained according to Masson, the red color prevailed (Fig. 2 d). Cartilaginous islands were located in the direction of the longitudinal axis of the bone and were the basis for appositional bone growth (Fig. 2 b, d).

Blood vessels feeding the metaepiphyseal cartilage were located both from the epiphysis and metaphysis sides (Fig. 3). In animals aged 3.5 months, the vessels from the epiphyseal subchondral bone side were in contact with the border zone and penetrated into it to a small depth (Fig. 2 a). Positive immunohistochemical staining for CD34 was recorded in the epiphyseal vessels at the border with the border zone (Fig. 3 a). During growth, the depth of vessel penetration increased; at the age of 5.5 months, vascular invasion was observed up to the border with the proliferating cartilage zone (Fig. 2 c; 3, c). CD34 expression from the metaphysis side was more pronounced than from the epiphysis side (Fig. 3 b, d).

The results of morphometric studies showed that the thickness of the metaepiphyseal cartilage of lambs during the period of their intensive growth from 3.5 to 5.5 months decreased by an average of 18.2 % and amounted to 0.66 (0.57–0.64) and 0.54 (0.46–0.59) mm, respectively. The decrease in the thickness of the metaepiphyseal cartilage at the age of 5.5 months is due to a significant decrease in the thickness of the border zone by 1.9 times, while the thickness of the proliferating cartilage zone increased by 1.2 times (Fig. 4, Table 1).

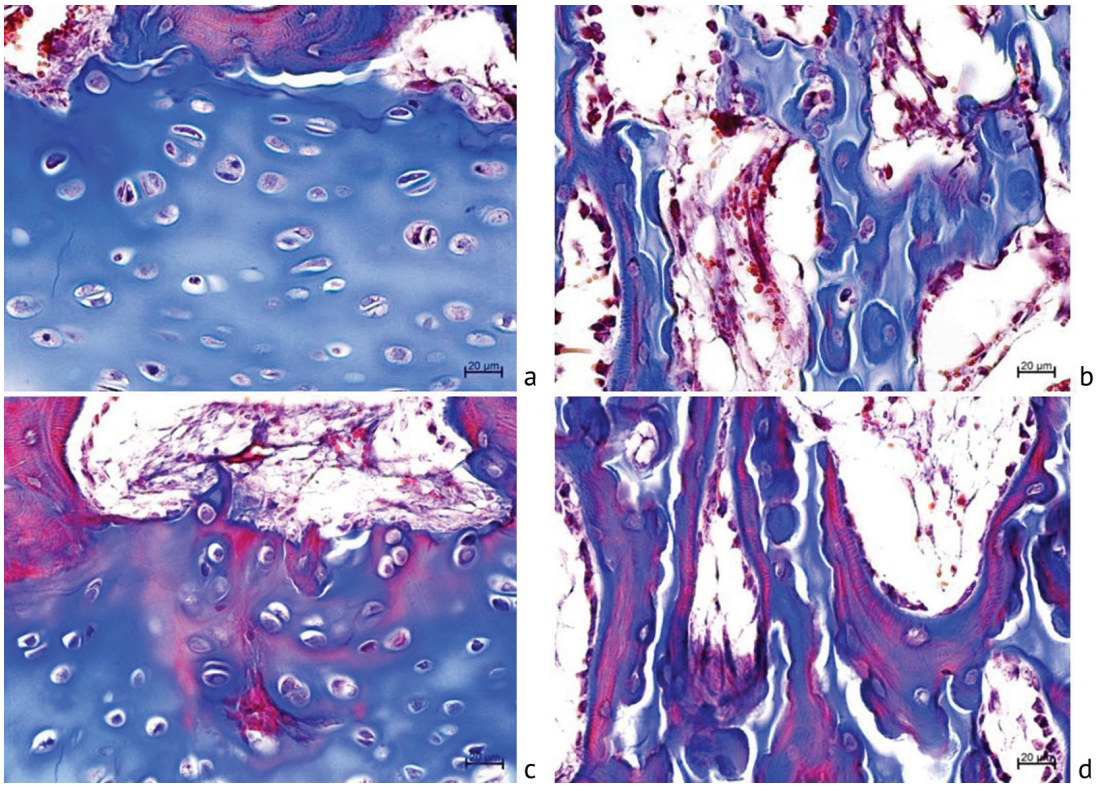

Fig. 2 Metaepiphyseal cartilage of the distal femur of a lamb: *a, b* age 3.5 months; *c, d* age 5.5 months. Border zone (*a, c*), deep penetration of vessels, presence of a mineralization front, formation of bone tissue (*c*). Zone of calcified cartilage (*b, d*). Pronounced mineralization of the matrix, osteoblasts on the surface of the calcified cartilage produce bone matrix (*d*). Paraffin section, stained with the three-color Masson method. Magnification ×400

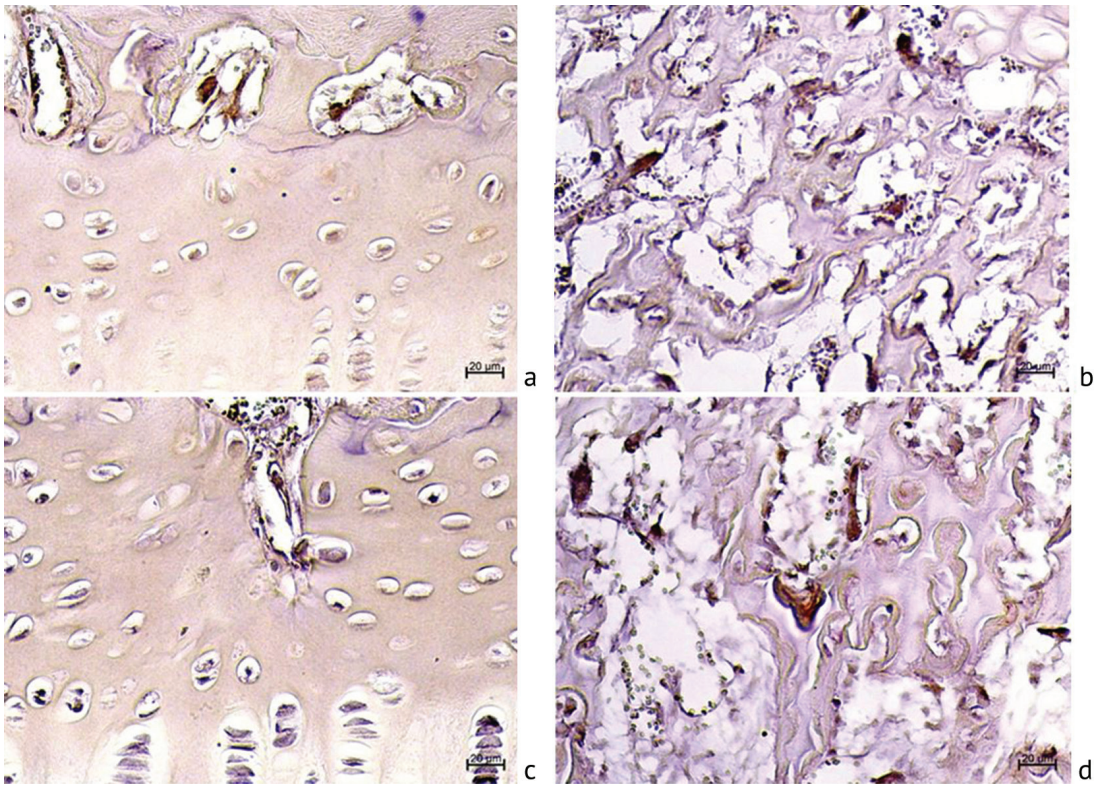

Fig. 3 Paraffin sections of metaepiphyseal cartilage of the distal femur of lambs: *a, b* age 3.5 months; *c, d* age 5.5 months. Positive immunohistochemical staining for CD34 in the epiphyseal vessels at the border with the border zone (*a*) and in the depth of the border zone (*c*). CD34 expression in the endothelium of the microcirculatory bed vessels from the diaphysis side (*b, d*). Magnification ×400

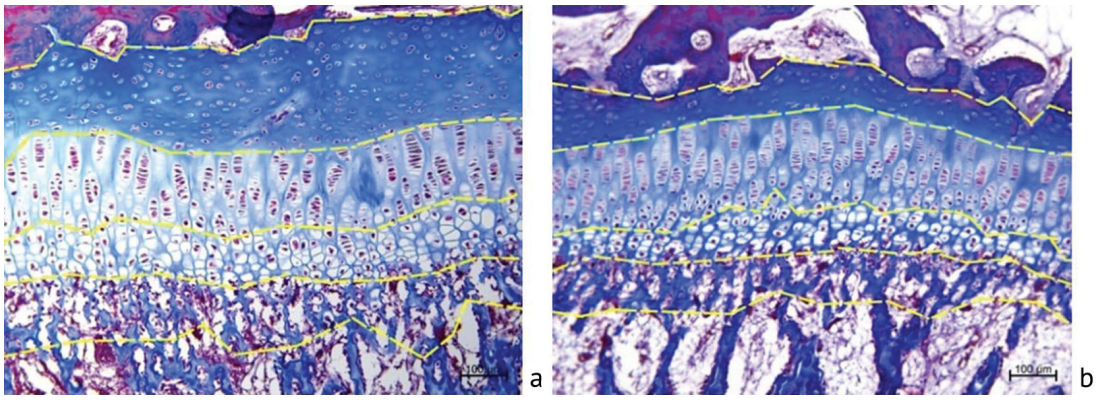

Fig. 4 Metaepiphyseal cartilage of the distal part of the femur of a lamb: *a* age 3.5 months; *b* age 5.5 months. The boundaries of the metaepiphyseal cartilage zones are indicated by yellow dotted lines. Paraffin section, stained with the three-color method according to Masson. Magnification ×100

Table 1

Thickness of metaepiphyseal cartilage zones of the distal femur in lambs

Zones of metaepiphyseal cartilage	Thickness (*h*, μm)		*p*
	3.5 months	5.5 months	
Border	238.45 (214.29–252.18)	124.76 (107.17–132.62)	0.0001
Proliferating cartilage	195.47 (185.18–198.64)	235.74 (217.37–249.27)	0.0001
Vesicular cartilage	125.49 (107.38–134.99)	128.57 (116.35–138.92)	0.4223
Calcified cartilage	106.16 (92.44–128.28)	116.31 (93.41–120.84)	0.5222

Note: Mann–Whitney test was used; the differences were statistically significant at $p < 0.05$.

The percentage ratio of zones of metaepiphyseal cartilage of the distal femur in lambs is presented in the diagram (Fig. 5). At the age of 5.5 months, a pronounced decrease in the proportion of the reserve zone and an increase in the proportion of the zone of proliferating chondrocytes were recorded.

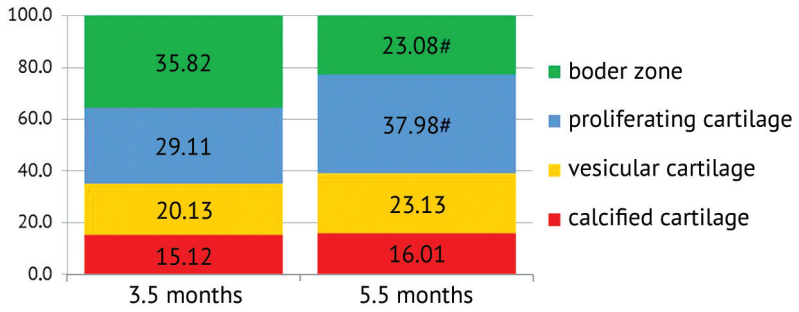

Fig. 5 Diagram of the percentage ratio of zones of metaepiphyseal cartilage of the distal femur in lambs: # statistically significant differences between the age of 3.5 and 5.5 months at p < 0.05.

DISCUSSION

The growth plate (physis) is formed by hyaline cartilage and is the weakest structure in the child's skeleton; it is often the site of injury or fracture. Treatment of children with fractures in the growth plate is a complex problem, since the consequences of such an injury can lead to growth impairment [24]. Experimental models of growing sheep are few in orthopaedic studies [19, 25], and data on sex differences in structural reorganization of the physis of lambs are absent.

It is known that growth rates are gender specific. According to a number of authors, a growth spurt in girls is registered at the age of 11–12 years while in boys 1.5–2 years later. Complete closure of growth zones occurs in girls by 16 years, in boys by 18 years [26, 27].

Metaepiphyseal cartilage is characterized by pronounced organ specificity, due to its topography and provisional function [23]. There is no unanimity among researchers in the classification of metaepiphyseal cartilage zones. Today, there are several classifications that distinguish from three to six zones, all of which are based on the functional activity of cartilage cells and the degree of their differentiation [23, 28, 29].

The results of our study demonstrate for the first time the features of structural reorganization of the metaepiphyseal cartilage of the distal femur during the period of intensive growth in lambs. At the age of 3.5 and 5.5 months, the zonal structure of the metaepiphyseal plate is clearly defined with the allocation of a border or reserve zone, a zone of proliferating chondrocytes, a zone of vesicular cartilage or hypertrophied chondrocytes and a zone of calcified cartilage.

The functional unity of bone and cartilage determines the normal development and functioning of the main units of the skeleton. At the present stage, the contact of the subchondral epiphyseal bone and the border zone of the metaepiphyseal cartilage, its formation and structure, are poorly studied, in contrast to the interface between articular cartilage and subchondral bone [29].

In all the cases, an increase in the PAS reaction was noted in the intercellular substance of the outer layer of the border zone when stained with alcian blue-PAS. It is the outer layer that is close in color to the subchondral epiphyseal bone; an increase in the proportion of PAS-positive structures was also seen in the calcified cartilage zone. A high content of glycosaminoglycans was noted by staining with alcian blue-PAS in the border zone, the zone of proliferating and hypertrophied chondrocytes.

The three-color Masson method differentiates mineralized and demineralized structures; mineralized structures exhibit affinity for acid fuchsin and are stained red [30]. Masson staining revealed fuchsinophilic areas of the matrix of the border zone in the metaepiphyseal cartilage of animals aged 5.5 months, as well as an increase in the proportion of fuchsinophilic areas of the calcified cartilage zone compared to the animals aged 3.5 months. It confirmed increased mineralization.

The changes observed in the tinctorial characteristics of the ground substance of the metaepiphyseal cartilage indicate that the processes of matrix calcification from the side of the subchondral bone of the epiphysis and the processes of endophysial ossification from the side of the diaphysis are more intensive in lambs by the age of 5.5 months. It is known that the nutrition of the metaepiphyseal cartilage is produced by diffusion from both sides, from the vessels of the epiphyseal and diaphyseal bones, but the invasion of vessels from the side of the diaphysis is more pronounced [23, 31].

The immunohistochemical study of paraffin sections of metaepiphyseal cartilage detected CD34 expression in 3.5-month-old lambs in the outer layer of the border zone; vessels penetrated from the subchondral bone of the epiphysis into the border zone to a small depth; at 5.5 months, the depth of vascular invasion in the border zone increased, but did not reach the zone of proliferating cartilage. The depth of vessel penetration from the diaphysis was more pronounced than from the epiphysis.

Metaphyseal plate fractures are the prevailing injury in young children (7–11 years), whereas in children over 12 years of age, ligament ruptures associated with low-energy trauma and muscle damage with high-energy trauma are observed more [2, 31].

Celarek et al. in an experimental model of fractures in the growth zone in sheep of different ages found that the fracture ran parallel to the growth zone at the age of 1.5 months, but at the age of 3.5–7 months, in the region of the metaepiphyseal plate through the zone of proliferating cartilage [25].

According to our data, during the period of intensive growth of lambs from 3.5 to 5.5 months, the thickness of the metaepiphyseal cartilage was reduced by an average of 18.2 %, due to a significant decrease (1.9 times) in the thickness of the reserve zone. At the age of 5.5 months, a statistically significant increase in the thickness of the proliferating chondrocyte zone (1.3 times) was revealed relative to the age of 3.5 months. In lambs aged 3.5–5.5 months, the proportion of the border zone and the proliferating cartilage zone was more than 50 % of the total thickness of the metaepiphyseal cartilage. These zones are characterized by the prevalence of the cell component and a low portion of intercellular substance [23, 32], which may indicate their vulnerability and inability to withstand high mechanical loads.

Limitation of this work was a small sample; a study with a larger number of growing animals of different ages would allow us to study gender patterns of structural reorganization of the metaepiphyseal cartilage, expand the statistical analysis, and thus to identify differences in the ratios of metaepiphyseal cartilage zones.

CONCLUSION

Histomorphometric changes in the metaepiphyseal plate of the distal femur of lambs during the period of their intensive growth were characterized by a decrease in its thickness due to a marked decrease in the thickness of the reserve zone, while a statistically significant increase in the thickness of the proliferating cartilage zone was revealed. In the animals aged 5.5 months, the depth of vascular invasion in the border zone increased, but did not reach the proliferating cartilage zone; changes in the tinctorial characteristics of the ground substance indicated activation of matrix calcification processes from the subchondral bone of the epiphysis and endophysis ossification processes from the diaphysis. The new data obtained on the dynamics of histomorphometric characteristics of the metaepiphyseal cartilage of the distal femur of lambs during the period of their intensive growth are the basis for experimental studies of pathological conditions of the growth zone and methods for their correction.

Conflict of interest Not declared

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The authors bear full responsibility for submitting the final version of the manuscript to be published.

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