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Reparative histogenesis of bone tissue in femoral fractures in rats using biocomposite material along with immunocorrection

V. V. Reshetnyak[1], V. V. Burdeyniy[2], V. V. Pronin[3], Ye. A. Iskaliev[4]

[1,2] FSBEI HE "Kostroma State Agricultural Academy" (FSBEI HE Kostroma SAA), Karavaevo, Kostroma Oblast, Russia

[3] FGBI "Federal Center for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

[4] Veterinary Clinic "AiBolit", Kostroma, Russia

[1] *e-mail: resh_vv76@mail.ru*

[2] *e-mail: van@ksaa.edu.ru*

[3] *ORCID 0000-0002-6240-3062, e-mail: pronin@arriah.ru*

SUMMARY

The paper studies the effect of the RVI biocomposite material belonging to the group of osteoplastic biocomposite materials, the RV-2 immunomodulator – a synthetic dipeptide inducing an immunocorrective effect, and combinations of these drugs on the reparative histogenesis of bone tissue in femoral fractures in rats. It was found that the remodeling of the primary bone callus into the secondary one in the fracture of the studied animals was of a diverse nature. This process was the most pronounced in the group where the components were used in complex, i.e. the bone defect was filled with RVI during the surgery, as well as RV-2 was injected intramuscularly to rats at a dose of 10 mcg per 1 kg of live weight for five days, starting immediately after the surgery. Well-formed coarse-fibrous connective tissue callus was recorded in animals of this group. The connective tissue was stained more intensely which indicates a denser arrangement of fibers in the callus. Focal cartilage tissue spanning bone fragments was observed within the callus. At the periphery of the site the cartilaginous callus was subjected to endochondral ossification with replacement by coarse-fibrous trabeculae with elements of lamellar bone tissue having haversian canals in the center. The inter-girdle spaces were filled with elements of the myeloid bone marrow in the forming bone tissue. Markedly proliferated osteoblasts were visible in the cambial layer of the periosteum. The bone tissue ratio increased up to (60.21 ± 2.62)%, which significantly exceeded the same indicator in the control group and in all experimental groups. The low content of connective tissue and the high ratio of bone tissue indicated more active osteogenesis processes and reparative regeneration in comparison with other groups.

Keywords: rats, immunomodulator, biocomposite material, bone callus, bone, cartilage and connective tissues, chondrocytes

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For correspondence: Vladimir V. Reshetnyak, Candidate of Science (Veterinary Medicine), Associate Professor, Head of the Department of Internal Non-Infectious Diseases, Surgery and Obstetrics, FSBEI HE Kostroma SAA, 156530, Russia, Kostroma Oblast, Kostroma Raion, Karavaevo, *e-mail: resh_vv76@mail.ru.*

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Репаративный гистогенез костной ткани при переломах бедренной кости у крыс при использовании биокомпозиционного материала на фоне иммунокоррекции

В. В. Решетняк[1], В. В. Бурдейный[2], В. В. Пронин[3], Е. А. Искалиев[4]

[1,2] ФГБОУ ВО «Костромская государственная сельскохозяйственная академия» (ФГБОУ ВО «Костромская ГСХА»), пос. Караваево, Костромская обл., Россия

[3] ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

[4] Ветеринарная клиника «АйБолит», г. Кострома, Россия

[1] *e-mail: resh_vv76@mail.ru*

[2] *e-mail: van@ksaa.edu.ru*

[3] *ORCID 0000-0002-6240-3062, e-mail: pronin@arriah.ru*

РЕЗЮМЕ

Проведено изучение влияния биокомпозиционного материала РВИ из группы остеопластических биокомпозиционных материалов, иммуномодулятора РВ-2 – синтетического дипептида, обладающего иммунокорригирующим действием, и комбинаций этих препаратов на репаративный гистогенез костной ткани при переломах бедренной кости у крыс. Установлено, что ремоделирование первичной костной мозоли во вторичную в области перелома у исследуемых животных носило разноплановый характер. Наиболее выраженно данный процесс протекал в группе с комплексным использованием компонентов, когда костный дефект во время операции заполняли препаратом РВИ и внутримышечно в течение пяти дней, начиная сразу после операции, инъецировали РВ-2 в дозе 10 мкг на 1 кг живой массы крыс. У животных этой группы регистрировали картину хорошо сформированной грубоволокнистой соединительнотканной мозоли. Соединительная ткань была окрашена более интенсивно, что свидетельствует о более плотном расположении волокон в костной мозоли. В ее толще отмечено наличие очагово расположенной хрящевой ткани, которая коммутировала между собой костные отломки. На периферии хрящевая мозоль подвергалась энхондральной оссификации с замещением грубоволокнистыми костными трабекулами с элементами появления пластинчатой костной ткани с гаверсовыми каналами в центре. В толще формирующейся костной ткани межбалочные пространства заполнены элементами миелоидного костного мозга. В камбиальном слое надкостницы видна выраженная пролиферация остеобластов. Доля костной ткани увеличена до (60,21 ± 2,62)%, что достоверно превышает аналогичный показатель как в контрольной, так и во всех опытных группах. Низкое содержание соединительной ткани и высокая доля костной ткани свидетельствуют о более активно протекающих процессах остеогенеза и репаративной регенерации в сравнении с другими группами.

Ключевые слова: крысы, иммуномодулятор, биокомпозиционный материал, костная мозоль, костная, хрящевая и соединительная ткани, хондроциты

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Для корреспонденции: Решетняк Владимир Вячеславович, кандидат ветеринарных наук, доцент, заведующий кафедрой внутренних незаразных болезней, хирургии и акушерства ФГБОУ ВО «Костромская ГСХА», 156530, Россия, Костромская область, Костромской район, пос. Караваево, *e-mail: resh_vv76@mail.ru.*

INTRODUCTION

The steady increase in the intensity of living process due to the rapid development of technological progress inevitably leads to an increase in the number and severity of skeletal traumatic injuries [1]. They account for up to 52.1% of all non-infectious disease cases. At the same time, mechanical injuries are most often recorded in animals (within the range of 32.7–44.5%) and are frequently complicated by fractures of tubular bones [2, 3].

Many morphological aspects of bone fracture healing are still poorly understood. Thus, questions remain regarding mechanisms of growth inhibition, maturation and remodeling of bone callus, as well as the link between inflammation, regeneration and fibrosis in case of slow healing and non-healing fractures. Traditional means and methods of treatment for this pathology do not always prevent the development of various complications [4]. In this regard, the need for further study of the mechanisms of bone fracture healing, the search for new materials and methods of treatment aimed at activating the reparative processes during healing can be justified [5].

At present a large number of drugs and biologically active substances have been developed and used to correct osteogenesis in both human and veterinary surgery and orthopedics. In this regard the use of biocomposite materials and drugs that have an immunomodulatory effect is very promising. Most publications reflect the results of medical studies, while scientific data in the veterinary field are limited to individual reports [2, 5–8]. In all cases, the authors provide data on effectiveness of separate use of drugs, while the possibility of their combined use presents both scientific and practical interest.

Based on the above, the aim of the paper was to study the effect of the RVI biocomposite material, the RV-2 immunomodulator and their combinations on the reparative histogenesis of bone tissue in femoral fractures in rats.

MATERIALS AND METHODS

The experiments were carried out at the Academic Department of Internal Non-Infectious Diseases, Surgery and Obstetrics and the Clinical and Diagnostic Center of the FSBEI HE **"**Kostroma State Agricultural Academy" (Kostroma), and the histological studies were conducted on the basis of the Preclinical Testing Center of the FGBI "ARRIAH' (Vladimir).

The study was performed using 30 mongrel white rats of 5–6 months of age weighing 200–250 g, which were kept in the animal facility under equal conditions and received a standard food diet in accordance with international regulatory documentation (Directive 2010/63/EU of the European Union on the protection of animals used for scientific purposes, and the interstate standard GOST 33215-2014 "Guidelines for accommodation and care of laboratory animals. Rules for equipment of premises and organization of procedures").

Two products were used for the study: RVI – preparation of the group of osteoplastic biocomposite materials

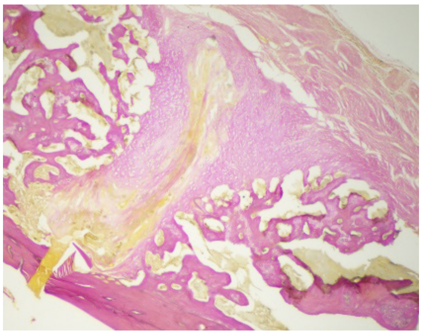

Fig. 1. Bone callus formation, control group (Van Gieson staining, magnification 40×)

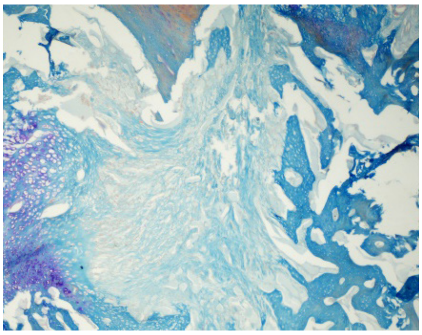

Fig. 2. Formation of cartilage islets, control group (Mallory staining, magnification 40×)

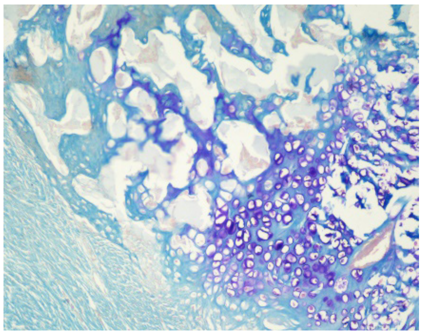

Fig. 3. Formation of bone trabeculae, control group (Mallory staining, magnification 100×)

in the form of granules, which is a synthetic hydroxyapatite with added collagen and an antibiotic (LLC company "Intermedapatit", Russia) and RV-2 – a synthetic dipeptide, which is an analogue of the active center of one of the native thymic hormones, having an immunomodulatory effect and stimulating the regeneration processes in case of their disturbance (DEKO Company Ltd., Russia).

All animals were divided into six groups (n = 5 in each group): one control group and five experimental groups. A common diaphyseal femoral fracture was modeled in animals in operating room conditions using adequate anesthesia, followed by intragrade osteosynthesis to fix the bone fragments.

No additional therapeutic measures were performed in the control group. During reposition between the ends of the fracture in rats of experimental group 1 the bone defect was loosely (at two-thirds) filled with RVI granules pre-moistened with isotonic sodium chloride solution; the rats of groups 2, 3 were intramuscularly injected with RV-2 at a dose of 10 mg per 1 kg of body weight once a day for five days immediately after surgery (group 2) or on day 5 (group 3) after surgery. In the other two groups both drugs were used: in group 4 – according to the schemes of groups 1 and 2, in group 5 – according to the schemes of experimental groups 1 and 3.

The animals were removed from the experiment and euthanized using carbon dioxide on day 45 after surgery. The whole femoral bones taken for the surgery were fixed in a 10%-neutral formalin solution; the callus (the fusion) was separated at the dorsal and ventral sides, and the pin was removed using a sharp scalpel (scissors, forceps).

Decalcifying fluid and histology equipment manufactured by Kreonika Ltd. (Russia) were used in the study.

After decalcification the biomaterial was placed in TLP720 tissue processor and was poured in paraffin using ESD-2800 filling station. Histological sections 5–8 microns thick were obtained using a semi-automatic rotary microtome RMD-3000. The sections were stained with hematoxylin and eosin in an ALS-96 automatic linear steiner, and Van Gieson and Mallory staining was additionally conducted to detect connective tissue.

The sections were studied using MICMED-6 microscope (LOMO, Russia). E31S PM video camera and ToupView Software (Hangzhou ToupTek Photonics Co., Ltd., China) were applied for measurements and photographic documentation. A measuring scale of the camera was calibrated using the object micrometre plate for transmitted light OMP (LOMO, Russia).

The ratio of tissue components (bone, cartilage, and connective tissues) in the area of bone fragment alignment was determined in the five fields of view for each of the five sections from all groups.

The measurement results were processed using the variation and statistical analysis and Statistica 7.0 Software as well as the calculation of the mean values (*M*) and errors (*m*). The differences were considered reliable if $P \leq 0.05$.

Table
Morphometric parameters of structures in the area of bone callus formation in rats of the control and experimental groups, % (n = 5)

Group number	Bone tissue	Cartilage tissue	Connective tissue
control	55.31 ± 2.80	14.43 ± 1.26	30.35 ± 2.46
Experiment 1	42.43 ± 3.62*	15.77 ± 1.41	41.91 ± 3.67*
Experiment 2	44.60 ± 2.96*	16.51 ± 1.54	38.94 ± 2.92*
Experiment 3	40.64 ± 3.38*	23.06 ± 1.87*	36.45 ± 2.87*
Experiment 4	60.21 ± 2.62*	23.58 ± 1.93*	16.37 ± 1.33*
Experiment 5	33.11 ± 2.14*	18.42 ± 1.76*	48.52 ± 3.28*

*P < 0.05 as compared to the control group.

RESULTS AND DISCUSSION

Histological examination revealed that a bone callus as an apparent coarse-fibrous connective tissue was formed in the contact zone between two bone fragments in animals of the control group (Fig. 1). Islands of cartilage tissue with well-distinguishable single chondrocytes, as well as isogenic cell groups were observed in the callus (Fig. 2). Randomly oriented bone plates with a large number of fibroblasts on their surface were found at the contact region of bone fragments. A large number of osteoblasts were found deep in the newly formed bone trabeculae, their periosteal surface was represented by fairly densely arranged connective tissue fibers, intensely colored according to Mallory (Fig. 3). Ratio of bone, cartilage and connective tissue was 55.31 ± 2.80; 14.43 ± 1.26; 30.35 ± 2.46%, respectively (Table).

At the same time, the bone callus in rats of experimental group 1, as compared with the control one, differed in a less pronounced morphological structure (Fig. 4). The bone intermediary zone was composed of bundles of large-cell spongy bone tissue. The bone marrow cavity in the fusion zone was filled with myeloid bone marrow with arteries of different calibers (Fig. 5). In addition, less intensive formation of young bone plates was found in this group, and the ratio of bone tissue at the site of the defect was significantly lower and amounted to 42.43 ± 3.62%, with no significant differences in the content of cartilage tissue between the groups. At the same time, the ratio of connective tissue increased to 41.91 ± 3.67% ($P \leq 0.05$, Table).

In experimental group 2 the callus was represented by a pronounced, dense, formed fibrous connective tissue. Single, randomly oriented bone plates were recorded in the area of bone fragment alignment (Fig. 6). Closer to the periosteum, the formed cartilage tissue with single

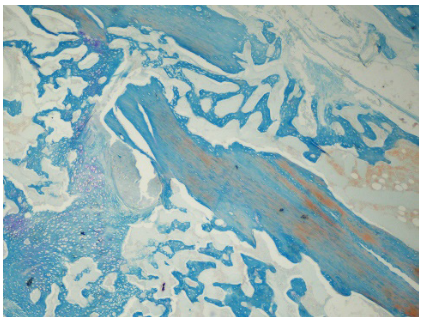

Fig. 4. Formation of bone callus, experimental group 1 (Mallory staining, magnification 40×)

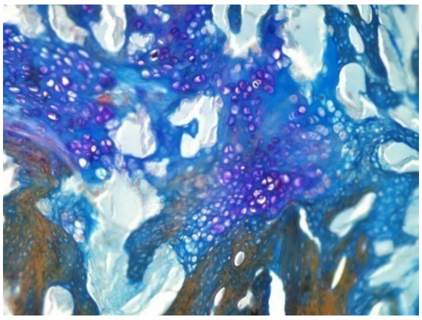

Fig. 5. Formation of bone trabeculae, experimental group 1 (Mallory staining, magnification 100×)

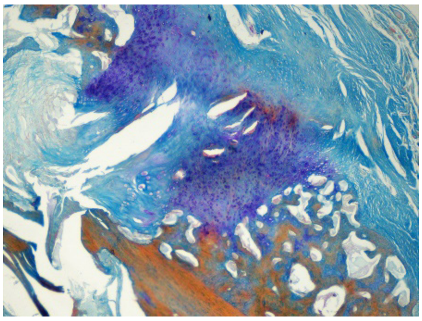

Fig. 6. Bone callus structures, experimental group 2 (Mallory staining, magnification 40×)

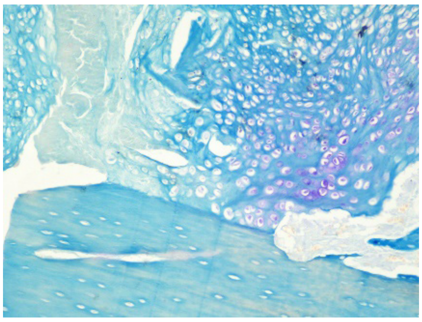

Fig. 7. Cartilaginous tissue of the bone callus, experimental group 2 (Mallory staining, magnification 100×)

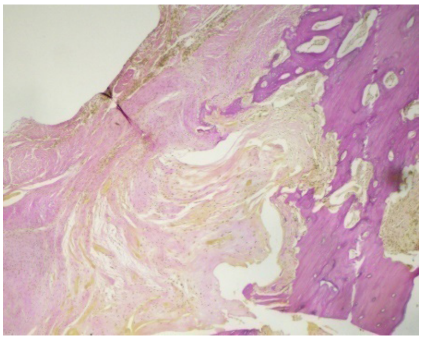

Fig. 8. Bone callus, experimental group 3 (Van Gieson staining, magnification 40×)

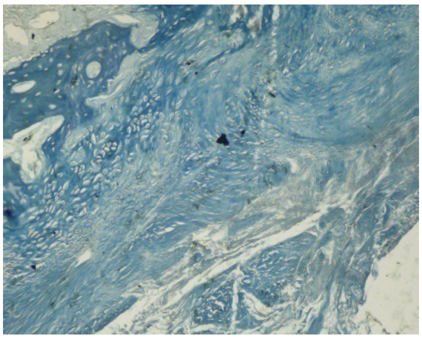

Fig. 9. Bone, experimental group 3 (Mallory staining, magnification 100×)

chondrocytes was visualized (Fig. 7). The specific parameters of bone and cartilage tissue did not have reliably significant differences as compared with experimental group 1. However, in comparison with the control group, a lower ratio of bone tissue along with a higher connective tissue content ($P < 0.05$, Table) and insignificantly increased cartilage tissue content was observed.

Morphometric parameters of bone callus structures in rats of experimental group 3 were not subjected to reliably significant changes as compared with experimental groups 1 and 2. It should be noted that there was a trend for decrease in the proportion of bone tissue and reliably significant differences in the content of cartilaginous tissue in comparison with experimental groups 1 and 2 (Table). The callus was well pronounced, with a predominance of coarse fibrous connective tissue, showing less intense fuchsinophilic properties when stained according to Van Gieson protocol, which indicates a looser arrangement of fibers (Fig. 8). Chondrocytes were widely spaced and basophilic intercellular matter was predominant in the cartilaginous callus. The formation of immature bone plates was noted on the periphery of the cartilaginous callus (Fig. 9).

A well-formed coarse-fibrous connective tissue callus was recorded in animals of experimental group 4. The connective tissue was stained more intensely, indicating a denser arrangement of fibers in the callus. Focally located cartilage tissue which commuted between bone fragments was found in it (Fig. 10). At the periphery of the site the cartilaginous callus was subjected to endochondral ossification with replacement by coarse-fibrous trabeculae with elements of lamellar bone tissue having haversian canals in the center. In the forming bone tissue, the inter-girdle spaces were filled with elements of the myeloid bone marrow (Fig. 11). In the cambial layer of the periosteum, a pronounced proliferation of osteoblasts was visible (Fig. 12). The bone tissue ratio increased up to 60.21 ± 2.62%, which reliably exceeds the same indicator in the control group, as well as in all experimental groups. The low content of connective tissue and the high ratio of bone tissue indicate more active processes of osteogenesis and reparative regeneration in comparison with other groups.

The bone callus was insignificantly pronounced in experimental group 5, on the periphery it was represented by loosely arranged bundles of coarse-fibrous connective tissue with deeply visible cartilagious islands (Fig. 13). The defect area was filled with myeloid substance and islands of weakly fuchsinophilic, coarse-fibrous, connective tissue (Fig. 14). In the periosteal zone, the rudiments of bone trabeculae formed by the replacement of reticulofibrous tissue were visualized (Fig. 15). The bone volume ratio was the lowest (33.11 ± 2.14%) in all the groups presented, along with the highest indicator (48.52 ± 3.28%) of the connective tissue ratio (Table).

CONCLUSION

The presented data indicate that the most stimulating effect on reparative osteogenesis was produced when using a combination of the RVI biocomposite material based on synthetic hydroxyapatite with added collagen and an antibiotic along with a five-day immunocorrection course with RV-2 preparation from the group of synthetic dipeptides, starting from day 1 after surgery. This

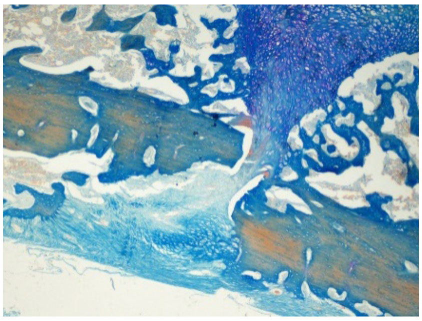

Fig. 10. Bone, experimental group 4 (Mallory staining, magnification 40×)

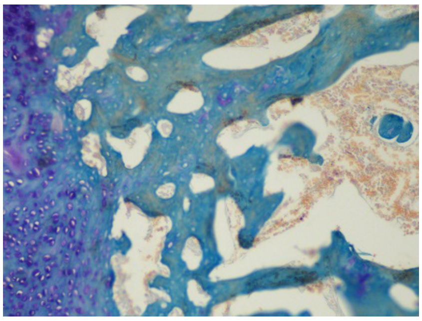

Fig. 11. Bone callus, experimental group 4 (Mallory staining, magnification 40×)

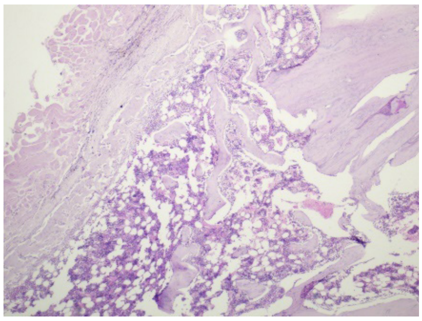

Fig. 12. Bone tissue, experimental group 4 (hematoxylin and eosin staining, magnification 40×)

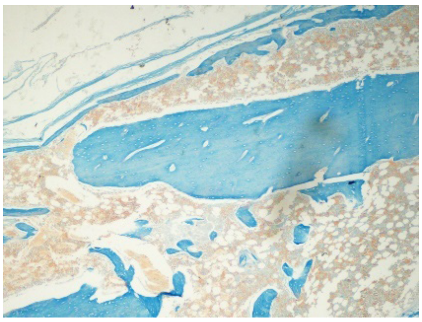

Fig. 13. Bone callus, experimental group 5 (Mallory staining, magnification 40×)

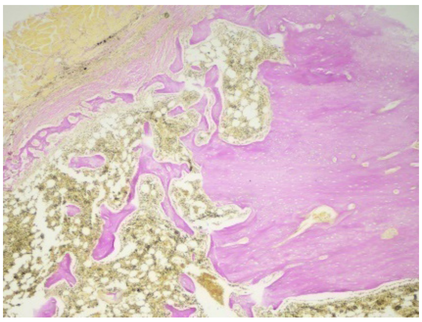

Fig. 14. Bone callus, experimental group 5 (Van Gieson staining, magnification 40×)

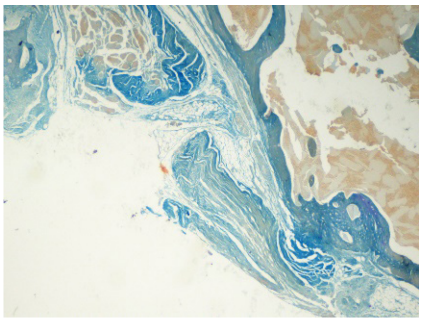

Fig. 15. Bone callus (formation of bone trabeculae), experimental group 5 (Mallory staining, magnification 40×)

is confirmed by a higher ratio of bone tissue and a low content of connective tissue at the sites where fragments fit together, as well as active ossification processes and appearance of elements of lamellar bone tissue.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Vladimir V. Reshetnyak, Candidate of Science (Veterinary Medicine), Associate Professor, Head of Department of Internal Non-Infectious Diseases, Surgery and Obstetrics, FSBEI HE Kostroma SAA, Karavaevo, Kostroma Oblast, Russia.

Vasiliy V. Burdeyniy, Doctor of Science (Veterinary Medicine), Professor, Department of Epizootology, Parasitology and Microbiology, FSBEI HE Kostroma SAA, Karavaevo, Kostroma Oblast, Russia.

Valery V. Pronin, Doctor of Science (Biology), Professor, Head of the Centre for Preclinical Tests FGBI "ARRIAH", Vladimir, Russia.

Yevgeniy A. Iskaliyev, Chief Veterinary Practitioner of "AiBolit" Veterinary Clinic, Kostroma, Russia.

Решетняк Владимир Вячеславович, кандидат ветеринарных наук, доцент, заведующий кафедрой внутренних незаразных болезней, хирургии и акушерства ФГБОУ ВО «Костромская ГСХА», г. Кострома, Россия.

Бурдейный Василий Владимирович, доктор ветеринарных наук, профессор кафедры эпизоотологии, паразитологии и микробиологии ФГБОУ ВО «Костромская ГСХА», г. Кострома, Россия.

Пронин Валерий Васильевич, доктор биологических наук, профессор, руководитель центра доклинических исследований ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Искалиев Евгений Айдарханович, главный врач ветеринарной клиники «АйБолит», г. Кострома, Россия.