Repair of Metadiaphyseal Fracture of the Humerus in Treating Animals with Calcium Preparation in Combination with Vitamin D3

The study of bone repair after fracture in the treatment of animals with calcium in combination with vitamin D3 was conducted in 36 two-month white lab male rats weighting 150±15 g, complying with the rules of humane treatment of animals. It was revealed that at modeling metadiaphyseal humeral fractures in control and experimental animals, which are additionally given calcium and vitamin D3, bone adhesion appears by desmal type (intramembranous ossification): granulation tissue - fibroreticular tissue - osteoid - bone tissue. Based on the qualitative morphological and quantitative characteristics of callus formation in animals, we can recommend the use of calcium and vitamin D in the comprehensive medical treatment for fractures due to their pronounced stimulatory effect on the course of reparative osteogenesis.

Keywords: Humerus, fractures, animals, bone repair, treatment.

UDC: 616.717-001.5-011.891.5-003.93-08

Introduction

Fractures lead to disturbance of mineral density and osteopenia not only in the injured bone, but also in other skeletal bones that adversely affects the formation of peak of bone mass in children and may be a risk factor for subsequent fractures (Korz et al., 2006; Wiel et al., 1994). Mineral density and the quality of bones forming in a child depend on many factors as follows: adequate intake of calcium and vitamin D3, levels of physical activity, hormonal regulation, features of nutrition, state of gastrointestinal tract, etc. (Cattermole et al., 1997; Povoroznyuk et al., 2001). In this regard, preparations of calcium and vitamin D3 which have various biological values are recommended to prevent osteopenic state (Jackson et al., 2006). As Hamilton (2010) noted, calcium plays the important role in mineralization process and skeleton formation, whereas vitamin D regulates calcium metabolism and influences on the structural and functional states of bone and muscle tissues.

Study of the role of calcium and vitamin D3 in optimization of bone repair in children is a relevant scientific field, but this issue is not well studied.

The purpose of research is to study repair of metadiaphyseal fractures of the humerus in treating young rats with calcium in combination with vitamin D3.

Materials and methods

The study of bone repair after fracture in the treatment of animals with calcium in combination with vitamin D3 was conducted in 36 two-month white lab male rats (body weight 150±15 g), at subject to the rules of humane treatment to animals (European convention, 1986).

Modeling of metadiaphyseal humeral fracture was carried out under general intramuscular anaesthesia (aminazine at 10 mg/kg, ketamine at 50 mg/kg) under aseptic conditions. To reproduce the fracture, incision was made by lateral access in 1/3 the length of the humerus (closer to distal part). The muscles were moved by blunt raspatory and an oblique saw cut in metadiaphyseal zone was carried out by the special circular saw.
Proximal and distal parts of bone were compared together and treated with dry antibiotic (penicillin), and then the skin was sutured (Prolene). The limb plaster bandage immobilization was performed. The rats were withdrawn from the experiment by an overdose of ether for anesthesia at the 3, 5, 7, 14, 21 and 28 days.

Two series of experiments were carried out:

Series 1 - modeling of metadiaphyseal humeral fracture with plaster bandage fixation - control group (18 animals);

Series 2 - modeling of metadiaphyseal humeral fracture with plaster bandage fixation and medicamentous treatment - experimental group (18 animals). The rats were administered calcium with vitamin D3 (Calcium D3 Nycomed at a dose of 1.8 g per 1 kg body weight) per so in the morning in a suspension of distilled water, from the 3rd day after the operation for whole experiment duration.

For histological studies, we isolated fragments of bones of rats with the fracture site and fixed them in 10% solution of neutral formalin, decalcified in 4% solution of nitric acid, dehydrated in alcohols of increasing strength and embedded in celloidin, according to the technique of Sarkisov et al. (1996). Coloration of histological sections was carried out by hematoxylin eosin, picrofuchsin by Van Gieson, and toluidine blue at pH 2.5. Colored sections were analyzed under a microscope MICROS, as well as in polarized light (Polmy-A). Preparations were pictured by a digital camera Canon EOS-300D.

Morphometric studies were performed for comparative analysis of cellular composition and dynamics of bone callus formation at different periods of research. The number of osteoblasts as bone forming cells in callus was determined in interfragmental fissure territory. The relative square (on the section) of new tissue formation (fibroreticular and bone) in interfragmental fissure during observation periods was investigated using the square-mesh ocular inserts with 289 points by Avtandilov’s method (1990). The number of blood vessels was counted in the field of microscope view (at magnification of 200), as well as on territory of whole interfragmental region (at the 14th day). Morphometric studies were conducted on two longitudinal sections of bone for each animal.

Statistical method. The obtained digital data were processed by methods of variational statistics using Student’s t-criterion (application package STATISTICA 5.11 for Windows). The level of significance was accepted as 95%.

Results of research

Macroscopically, the operated limb at the fracture site was thickened at the 3, 5 and 7 days. In subsequent periods of observation, the fracture zone was not revealed.

At the 3rd day, a microscopic analysis has revealed a hematoma with foci of degenerative changes in the bone marrow and small bone fragments between the ends of fragments both in compact and cancellous bones of distal part of the humerus in experimental and control animals. Blood elements as neutrophils and lymphocytes were marked among the filaments of fibrin. Single macrophages, poorly differentiated connective tissue cells, and fibroblasts were located mainly in marginal parts of the hematoma. Areas with destructive alterations - cracks, crevices, territories without osteocytes were determined in the bone trabeculae of cancellous maternal bone nearby the fracture region. Compact bone on the border of traumatic injury also had no osteocytes, the fragmental margins were usurated. Over defect area, the periosteum was absent, and over bone areas adjacent to the fracture site it was edematous with swollen collagen fibers and lysed cells.

No distinctive features with control group animals have been found.

At the 5th day, the hematoma in callus of experimental rats was not observed, compared with the control. In interfragmental area, there was fibroreticular tissue of osteoblastic type interspersed in areas with osteoid and single woven trabeculae nearby the maternal bone (Figure 1A). Only in central part of interfragmental zone were revealed small
territories of granulation tissue which consisted mainly of fibroblasts. Poorly differentiated connective tissue cells were determined in a small number.

**FIGURE 1. A) NEW BONE TRABECULAE FORMATIONS ADHESIVE TO MATERNAL BONE. OSTEOID. FIBRORETICULAR TISSUE IN INTERFRAGMENTAL AREA (EXPERIMENTAL GROUP). COLORING BY HEMATOXYLIN AND EOSIN. ENLARGEMENT 200. B) AREA OF INTERFRAGMENTAL FISSURE IN THE FRACTURE SITE, FILLED WITH GRANULATION TISSUE. THIN-WALL VESSELS (CONTROL GROUP). COLORING BY HEMATOXYLIN AND EOSIN. ENLARGEMENT 100.**

We paid attention to the presence of a large number of blood vessels with large lumens in granulation tissue. Signs of hemorrhage associated with disturbance of permeability of vessel walls were not established. Vessels in fibroreticular tissue had narrow lumens; their density was lower than in granulation tissue. The number of vessels of different caliber in the field of microscope view ranged from 12 to 20. In fibroreticular tissue, islands of osteoblasts forming the “prototype” of osteoid were revealed. Osteoblasts had large nuclei with loose chromatin. The nuclei were surrounded by the basophilic cytoplasm, indicating activebiosynthesis in cells.

In interfragmental zone of control animals granulation tissue was prevailed (Figure 1B). Areas of the tuned hematoma with the filaments of fibrin, mononuclear leukocytes, macrophages, stellate cells of fibroblastic type were still determined. The number of polymorphonuclear leukocytes was marked to decrease. Areas of fibroreticular tissue and osteoid were minor; woven bone trabeculae were not identified. The number of vessels in the field of microscope view ranged from 9 to 15.

The maternal bone tissue of animals of both groups, as in the cortex, as also in the areas of cancellous bone had signs of destructive changes similar to those described at the 3rd day. The fragmental margins in control animals were usurated, destructive changes in the maternal bone were more significant and on extensive territories. Formation of the periosteum was noted, but it covered the defect not completely. The periosteum was presented by loosely packed bundles of collagen fibers with high density of fibroblasts. In control animals the periosteum which structure was not different from those in experimental animals was detected only in the maternal bone nearby the fracture.

For the 7th day, rats of experimental group had fibroreticular tissue of osteoblastic type, the osteoid and woven bone trabeculae between the fragments of bone tissue. Small foci of granulation tissue were detected only in central parts of interfragmental fissure. Numerous blood vessels of different sizes (from 12 to 24 in the field of microscope view) were determined in fibroreticular tissue, as in granulation one. New bone trabeculae formations with high density of osteoblasts and single osteocytes on the surface were determined throughout territory of interfragmental fissure.
In contrast to that, in interfragmental fissure of compact bone in control animals were marked extensive territories of fibroreticular tissue which was characterized by high density of fibroblastic and osteoblastic cells, as well as small areas of granulation tissue. Capillaries were normal with wide lumen and venules with expressed external membrane. The number of vessels in the field of microscope view amounted from 8 to 17. In the region of trabecular bone were determined areas of osteoid with signs of ossification, as well as few new bone trabeculae formations with high density of osteocytes.

The periosteum near the interfragmental fissure of animals of both groups was thickened due to fibroblastic proliferates and dilation of osteoblastic layer. In a certain distance from the injury, the periosteum had a structure similar to the norm. Compact bone at the border of saw cut in experimental group of animals had signs of posttraumatic adjustment, whereas in control animals it had destructive changes.

For the 14th day, composition and architectonics of callus formed in interfragmental fissure of the humerus in experimental rats were significantly different from the control. In callus between the fragments of compact bone in experimental rats were determined young bone trabeculae forming a network (Figure 2A).

**Figure 2.** A) **New bone trabeculae formations with high density of osteoblasts in marginal parts.** Osteocytes on the surface of trabeculae. The periosteum over the area of traumatic injury (experimental group). Coloring by hematoxylin and eosin. Enlargement 100. B) **New bone trabeculae formations and fields of fibroreticular tissue in the fracture area.** Posttraumatic changes in maternal bone (control group). Coloring by hematoxylin and eosin. Enlargement 125.

New bone trabeculae formations contained osteoblasts and osteocytes with high density on the surfaces. Osteoblasts were located on the marginal surface of bone trabeculae by dense layer, indicating active bone formation leading to an increase in their width. Woven bone tissue was detected in deep parts of cancellous bone, forming a dense plexus with the maternal bone trabeculae adjacent to the area of traumatic injury.

The control animals had extensive areas of fibroreticular tissue and foci of granulation tissue in callus. New bone trabeculae formations were found only in the region of interfragmental fissure of maternal trabecular bone (Figure 2B). For an objective comparison of the activity of bone formation in experimental and control animals, we counted the number of osteoblasts through the territory of forming callus (Table 1).

Fibroreticular tissue with small foci of new red bone marrow formation, capillaries, venules, and arterioles was located in intertrabecular spaces of maternal bone nearby the
interfragmental fissure. Blood vessels were detected on the territory of formed callus too, but their number was lower compared to experimental animals (Table 1).

**Table 1. The number of blood vessels in osteoblasts (M±M) in callus of interfragmental fissure in the humerus of rats at the 14th day**

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Number of blood vessels</th>
<th>Number of osteoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (animals without</td>
<td>21.51 ± 2.21</td>
<td>237.83 ± 21.68</td>
</tr>
<tr>
<td>additional treatment) (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental (animals with</td>
<td>34.74 ± 2.15 P &lt; 0.01</td>
<td>381.81 ± 20.91 P &lt; 0.01</td>
</tr>
<tr>
<td>additional treatment) (n=6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: P - significant differences to control.

The data in Table 1 are evidence of that the density of blood vessels on the territory of callus formation was significantly (P<0.01) higher in 1.75 times in experimental animals than in control group. The number of osteoblasts in callus of experimental rats was higher in 1.61 times (P<0.01), in comparison with the number of osteoblasts in callus of control animals.

The maternal compact and cancellous bones adjacent to the defect area had signs of reparative adjustment. Fibroreticular tissue evolved in the cracks and crevices, bedding of new bone tissue formations were appeared in cell-free areas. The periosteum over the defect and at a distance from the area of traumatic injury was thickened due to formation of the dense fibrous and osteogenic layers.

At the 21st day, bone callus with different organization of trabecular network was located in interfragmental fissure in the site of compact and cancellous bones in experimental animals. The callus was presented by lamellar bone tissue with small-loop network of bone trabeculae in the cortex area, and by bone trabeculae forming large-loop network in cancellous bone. The periosteum over the defect was narrow and presented by osteoblastic layer which had thin layer of fibrous tissue above.

In interfragmental fissure in control group of animals, fields of fibroreticular tissue were still remained in the cortex, and foci of collagen-fiber tissue in the periosteum area. The periosteum over callus zone was presented by dilated fibrous layer. An insignificant activation of osteoblastic layer of the periosteum was observed. The interfragmental fissure in cancellous bone was made by new bone trabeculae formations forming small-loop network.

For the 28th day in experimental animals, bone callus which was presented by lamellar bone tissue with high density of osteocytes was revealed in interfragmental fissure of the cortex zone (Figure 3A). Bony canals were made by fibroreticular tissue (fibrous tissue), and contained blood vessels. In the area of cancellous bone, new bone trabeculae formations developed large-loop network. Fibrous tissue was marked only in small areas, mainly in intertrabecular spaces.

In control animals, the ends of fragments of maternal cortex were connected by new bone trabeculae formations of both woven and lamellar bones structure, as well as by areas of fibrous tissue which was presented mainly in intertrabecular spaces of new bone trabeculae formations of the callus, interspersing with islands of the bone marrow. A narrow strip of fibroreticular tissue was identified over bone trabeculae in the periosteum region as well (Figure 3B).

The adjacent maternal bone of animals of both groups had mild destructive changes manifested in the absence of cells at the ends of fragments.

For objectification of the data, we performed morphometric analysis of callus tissues in animals of experimental and control groups (Table 2).
**Figure 3.** A) Lamellar bone in interfragmental fissure of the cortex. High density of osteocytes. Intertrabecular spaces of callus in the area of cancellous bone, filled with the red bone marrow (experimental group). Coloring by hematoxylin and eosin. Enlargement 200. B) New bone trabeculae formations with high density of osteocytes and areas of fibroreticular tissue in interfragmental fissure. Osteocyte-free site in maternal bone (control group). Coloring by hematoxylin and eosin. Enlargement 125.

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Control (Untreated animals)</th>
<th>Experimental (Treated animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroreticular</td>
<td>34.81 ± 1.97</td>
<td>14.5 ± 1.62 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Bone</td>
<td>64.3 ± 2.23</td>
<td>85.1 ± 2.56 ( P &lt; 0.01 )</td>
</tr>
</tbody>
</table>

**Notes:**
- \( P \) - significant differences between the indexes of animals of experimental and control groups.
- \( P_1 \) - significant differences between the indexes of squares of bone and fibroreticular tissues.

Table 2 shows that by the 28th day in the field of interfragmental fissure in experimental animals is formed callus, in which the squares of bone tissue are significantly \( (P<0.01) \) differ from control, exceeding them in 1.32 times, while the square of fibroreticular tissue was 2.4 times less \( (P<0.01) \).

**Discussion**

Based on the research conducted, we have revealed that at modeling metadiaphyseal humeral fractures both in control and experimental animals which were additionally given calcium and vitamin D3, bone adhesion appears by desmal type (intramembranous ossification): granulation tissue - fibroreticular tissue - osteoid - bone tissue. This is a typical variant of secondary type of adhesion described by several researchers (Einhorn, 1995; Frost, 1989; Thompson et al., 2002; Webb et al., 2000). In callus, the density of fibroblasts is increased, macrophages, lymphocytes, neutrophils, poorly differentiated connective tissue cells are marked for the 3rd day. Single osteoblasts with bone forming function are found (Frost, 1989). The main functions of neutrophils are the secretion of numerous cytokines regulating cell proliferation and differentiation in the early stages of fracture healing, as well as phagocytosis (Serov et al., 1995). Macrophages are detected in
the focus of inflammation. Macrophage presents itself as not only a phagocytic cell, but also as a secretory one, which excretes about 60 soluble substances - monokines capable to regulate various processes not only in the region of traumatic injury, but also in the organism, i.e. macrophage, being a cell of the local inflammatory focus, promotes the inclusion of other body systems into process, in particular, enhances the formation and differentiation of precursors of granulocytes in the bone marrow (Serov et al., 1995).

Macrophage is a regulator of inflammatory process, exerting chemotactic action, providing cell co-operation, producing colony stimulating factor which stimulates the functions of endothelial cells.

The foci of new bone formation are detected for the 7th day, and at the 14th day of the study callus is presented by extensive territories of cancellous bone. Fibroreticular tissue of osteoblastic type with high density of osteoblasts is appeared between bone trabeculae in central part of the defect. The use of Calcium D3 Nycomed preparation activates angiogenesis that was confirmed by morphometric studies. This leads to an increase in the number of osteoblasts in callus of experimental animals. By the 28th day, the square of bone tissue of callus in animals receiving the drug was 1.32 times higher than in the control. Thus, lamellar bone tissue was prevailed in callus of experimental animals.

Based on the qualitative morphological and quantitative characteristics of callus formation in animals, we can conclude that the use of calcium and vitamin D has pronounced stimulatory effect on the course of reparative osteogenesis and their use can and should be recommended in the comprehensive medical treatment for fractures.

References

Avtandilov, G., 1990. Medical morphometry, Moscow: Medicine
European convention, 1986. European convention for the protection of vertebrate animals used for experimental and other scientific purpose, Council of Europe 18.03.1986 - Strasbourg