Callus area and glycosaminoglycans content of the traumatized tibia in rats

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Key words: *post-traumatic, bone repair, callus, osteotomy, perforation.*

Summary. *The aim of the present work is the quantification of the post-traumatic bone healing histology (total callus morphometry) and histochemistry of the glycosaminoglycans (GAG) content (microspectrometry) in rats tibia after segment osteotomy and bicortical perforation of trained and immobilized animals.*

Material and methods. A quantified investigation of post-traumatic bone repair histology and GAG content after osteotomy and perforation of tibia in 105 young adult male Wistar rats has been performed. The repair was studied in normal and affected (training and immobilization) animals at 1–42 days after operation.

Results. The posttraumatic bone repair is an ordinary process of osteohisto- and organogenesis, and dependent on the environment factors (mode and degree of trauma, training of animals, etc.). The repair is trauma-dependent; after osteotomy the total callus area is significantly larger respectively to perforation. Otherwise, the training did not significantly influence the repair callus area and GAG content and therefore did not accelerate the bone repair, whereas the immobilization of animals depressed these processes and the bone repair was inhibited.

Conclusions. Quantified study of histology and histochemistry of bone repair after perforation gave important new, more detailed results on the reparative histogenesis of the bone tissues: repair dynamics of callus areas, dynamics of GAG concentration, effects of mode and degree of trauma, training and immobilization on the repair process.

Introduction

Posttraumatic bone repair is a specific and complex phenomenon, which in favorable conditions leads to the complete restoration of the bone. Traumatology and orthopaedics is based on the use of knowledge about repair morphology of the skeletal hard (osseus, chondral) tissues. The problems of posttraumatic bone repair morphology after trauma (opposite to other mesenchymal tissues) are: formation of the external and internal callus as a special material for repair process, two different bone formation pathways – dismal (intramembranous) and (endo)chondral ossification, role of fracture fixation and blood supply in this process, interactions between callus tissues.

Different experimental models were made (external and internal bone fracture, scrap, incision-slit-cutting-fissura, standard defect of osteotomy, etc.) for qualitative and quantitative study of the bone repair after surgical injuries of different degrees (1).

A lot of local internal (blood supply, state of osteogenic zones etc.) and external factors (physical activity etc.) influence the bone healing. In trained rodents bone repair is a bit stimulated (2–6), whereas in immobilized or hypokinetic ones after tenotomy, it is inhibited and complicated by osteoporosis, pseudoarthroses, etc. (2, 3, 5, 7, 8).

Posttraumatic bone repair process and its study with histology, histochemistry and immunohistology is complicated. A new suitable histological model of bone posttraumatic reparation for qualitative and quantitative complex investigation in the same morphological material in situ during norm as well as various external factors was elaborated previously (9, 10).

The aim of the present work is the quantitation of the post-traumatic bone healing histology (total callus morphometry) and histochemistry (microspectrometry of GAGs) in rats' tibia after resection (segment) osteotomy and bicortical perforation of trained and immobilized animals.

Material and methods

In the present experimental work 105 male young

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Table 1. **General organization of experiment**

Content of experiments	No. of rats
1. Resection osteotomy of tibia	
– control	22
– physical training	5
– immobilization	6
	33
2. Bicortical perforation of tibia	
– control	44
– physical training	14
– immobilization	14
	72

adult (growing) Wistar-rats with the body weight 200–220 g were used. The animals were investigated from the 1st to the 42nd day (Table 1).

The guidelines for the care and use of the animals were approved by the Ethical Committee of the University of Tartu, and complied with laws that regulate handling of laboratory animals and their utilization.

The animals were divided into 2 groups: 1) osteotomy (33 rats); 2) perforation of cortex tibiae (72 rats). The groups were subdivided into following groups: 1) controls; 2) trained and 3) immobilized animals. The training was performed in special swimming pool with size of 40 cm × 40 cm × 70 cm at the water temperature 22±2°C. In the immobilized subgroups rats were separated into narrow boxes (cages), one animal in each box; so their ability to move was significantly reduced. In experiments both right and left limb were employed. Bilateral limb has been used as described by other authors (11–13).

Anesthesia was induced with intramuscular injection of ketamine 50 mg/kg b. w. and diazepam 5 mg/kg. Prophylaxis of infection was carried out with ampicillin of 7.5 mg/kg i. m. It was started 2 hours before the operation and continued during 3 days. The operations were performed under strictly aseptic conditions.

Macroscopic and microscopic investigation

The scarification was performed by decapitation of animals anaesthetized with the ketamine and diazepam. The average size of the material collected for histological evaluation was 0.5–1.0 cm. Histology was carried out in the Department of Anatomy, Tartu University and histochemistry of glycosaminoglycans in the Department of Anatomy, Kuopio University. The material was fixed with formalin and Zenker formol by Maximov and demineralized with the solution of Shampy (Group 1), or by EDTA (Group 2). Paraffin embedded slices with a thickness of 7 μm were stained with hematoxylin and eosin, azure 2-eosin, Heidenhain iron hematoxylin, by van Gieson, alcian blue and safranin-O (for histochemistry of glycosaminoglycans).

Computerized histomorphometry

The microanatomical pictures of callus were photographed by light-microscope "Olympus" BX-50 and saved electronically. Further the process was performed with computer program Adobe Photoshop 5.0 under simultaneous visual control of light-microscopy. The above pictures were analyzed with Adobe Photoshop observing the total area of callus as well as the areas of hard (osseus and chondral tissues), and soft callus (connective tissue, degenerative inflammatory tissues). The painted areas of different colors were summarized in pixels and calculated in percents.

Glycosaminoglycan quantitation

Tissue sections were deparaffinized with xylene (3×5 min.), hydrated in ethanol solutions and brought to water. Glycosaminoglycans were stained with 0.5% safranin-O in 0.1 M sodium acetate buffer, pH 4.6, for 10 min. The stain has been shown to bind stoichiometrically to glycosaminoglycans. Stained sections were mounted with DPX (Difco, East Molesey, U.K.) and covered with a coverslip. Quantitation of glycosaminoglycans was performed with a light microscope connected to a thermoelectrically cooled Photometrics CH250 camera (Photometrics Inc., Tucson, AZ, USA). A monochromatic plate (l=492 nm) and PL Fluotar x4 objective were used for calibration and measurement. Light intensity transmitted through two randomly selected sections was measured. Staining intensity was converted into optical density (OD) by the logarithmic function: OD=log (I/I°) where I is the measured and I° oncoming light intensity. The measured light intensities were converted to OD values using a calibration curve (r=0.999). OD average from both sections was used to give an estimate of the glycosaminoglycan content of a section (14, 15).

Statistics

The statistical analysis was performed using the one sample t test (Graph Pad Quick Calcs: Analyze continuous data) at the level of significance p less 0.05 ($p<0.05$).

Results

Morphology of the post-traumatic bone repair callus was measured quantitatively by the means of histomorphometry (Table 2) and microspectrometry (Fig. 1, 2).

There exists a clear difference between the Group 1 and Group 2. Total callus area after the osteotomy is equal to 31.6±3.1; after the perforation – 20.1±1.1 ($p<0.05$). Hard callus area is 2 times (Group 1) or even 4 times (Group 2) higher than the soft callus area (21.3±2.8 and 10.3±1.7, or 15.8±0.8 and 4.3±0.5 respectively). In training groups similar results are given. Hard callus areas are in both groups about 3 times higher as the soft callus areas, i. e. in Group 1 is a bit increased and in Group 2 decreased compared to control. Training is more effective in the osteotomy group.

In immobilized animals the total callus area in

Table 2. **Areas of callus tissues 28 days after the internal fracture, osteotomy and perforation in control and under the conditions of training and immobilization (percentage of callus area ±SD). Computer field in use: 16.7%; 120000 pixels (100%)**

Group and number of animals	Total callus	Hard callus+	Soft callus+
Osteotomy			
Control 8	31.6±3.1	21.3±2.8	10.3±1.7
Training 5	35.8±3.3	25.9±3.5	9.9±2.6
Immobilization 4	20.4±3.4*	8.1±1.0*	12.3±2.5
Perforation			
Control 5	20.1±1.1**	15.8±0.8	4.3±0.5
Training 3	25.7±3.2	19.5±2.1	6.2±0.9
Immobilization 3	21.2±2.8	6.6±0.7*	14.6±1.7*

+ hard callus is bone and chondral callus, soft callus is the inflammatory area with adjacent cells and mixed connective tissue.

* differences between the values of this group are significant ($p<0.05$).

** differences between the values of this group and control of Group 1 are significant ($p<0.05$).

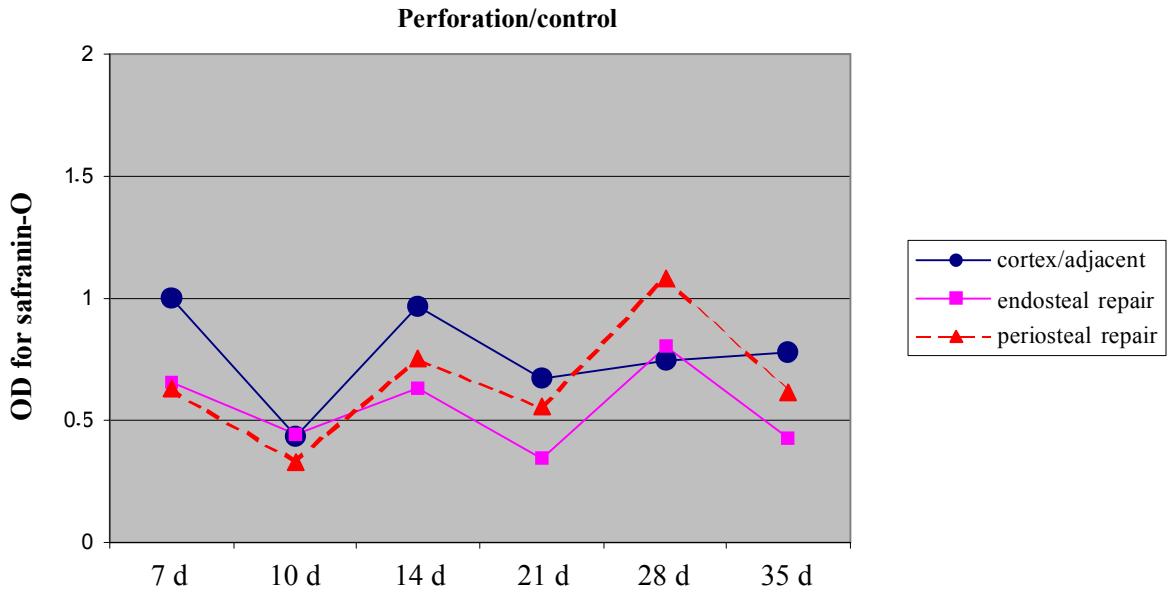

Fig. 1. **The posttraumatic bone repair in rat tibia, 4–42 days after resection osteotomy**
Glycosaminoglycans (GAG) concentration of the cortex/adjacent, periosteal and endosteal callus. Safranin-O staining. Microspectrometry.

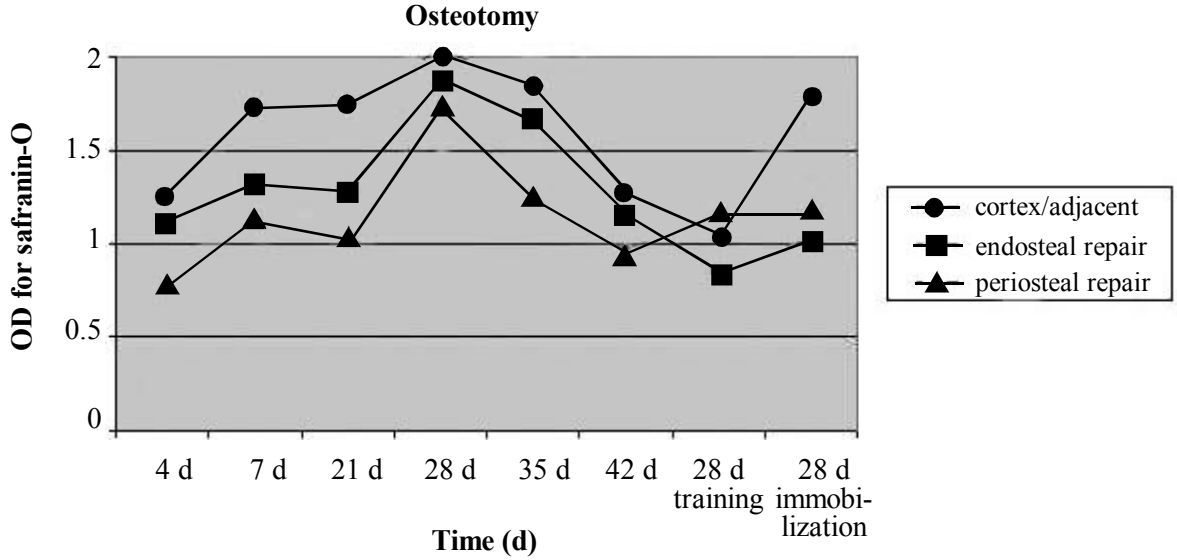

Fig. 2. **The posttraumatic bone repair in rat tibia, 7–35 days after perforation, control group GAG concentration of the cortex/adjacent, periosteal and endosteal callus**
Safranin-O staining. Microspectrometry.

Group 1 fell significantly compared to control (31.6±3.1 and 20.4±3.4 respectively; $p<0.05$). In Group 2 the total callus area is similar to control (20.1±1.1 and 21.2±2.8 respectively). In both immobilized groups the hard callus area is 1.5–2 times lower than the soft callus area. In both groups after immobilization the hard callus formation was strongly affected, while the total callus forming was affected only after the osteotomy.

New results of morphometry are in accordance with results of histology (10).

Microspectrometry also gives new results:

In osteotomy group GAG concentration in repairing bone callus 7 to 35 days is much higher than in perforation group (Fig. 1, 2).

GAG content 28 days after resection osteotomy in training group both in periosteal and endosteal callus as well as in cortex/adjacent area is lower compared to control group. In the immobilization group also a decrease in endosteal and periosteal callus areas is observed.

Discussion

Two main factors of post-traumatic bone repair are observed – degree and mode of the injury and external physical loadings. It is known that callus formation is dependent on the mode and degree of the injury (1, 16–18). It is known that absolutely stable environment leads to the primary bone repair (17) and flexible fixation causes formation of more extensive callus and the dominant ossification pathway is secondary, endochondral. The primary bone repair is also seen in bone defect healing. By these criterions our resection osteotomy model and perforation model correspond to the flexible and absolute fixation, respectively. In the Group 2 (perforation), where the movements at the bone defect site were absent, bone repair occured rapidly and with minimal amount of callus, whereas in the Group 1 (osteotomy), where the stabilization was not absolute, some delayed repair of hard callus was observed with high amount of callus (Table 2). Absence of motion at the bone defect site (Group 2) diminishes soft tissue reaction, stimulates the vascularization of endosteal callus and activates bone marrow reaction in bone repair. Although the start of ossification is delayed (measured with temporo-spatial parameters) the complete ossification of the defect is achieved more rapidly compared to osteotomy (Group 1) (9).

The total bone callus after osteotomy on the 28th day was equal to 31.6±3.1; after the perforation 20.1±1.1; $p<0.05$. Our data suggests, that the total areas of bone tissue callus in Groups 1 and 2 were opposite to the degree of the trauma: a more serious trauma in Group 1 caused smaller values in the bone tissue callus area development and a slight trauma in Group 2 caused a very large development of bone tissue callus (45.8±3.4 and 85.7±6.6) (9). In the groups, hard callus (bone, cartilage) formed 65–75% of the total callus. Our results suggest a dependence of the callus formation on the functional requirements to the repairing bone (necessity of consolidation, etc.).

The bone repair is influenced by numerous mechanical, physical, chemical, neural, endocrine, the general environment and local factors (17, 19, 20). In our

experiments the bone repair total callus area as well as GAG content in extracellular matrix of bone were not stimulated with moderate physical load (swimming), but was inhibited by limited possibility to move (immobilization). The physical activity did not additionally enhance the bone total callus areas, while immobilization caused its fall in ostotomy group, and hard callus fall in perforation group. Physical activity (swimming) may accelerate the bone repair in osteotomized tibia of rats (4), but it is also known that sufficient physical activity for a successful bone tissue repair may be achieved even by normal weight bearing, whereas the tenotomized rat tibia repair is inhibited (3).

We used our new experimental model (bicortical perforation), comparable to osteotomy, for quantitation of different tissue factors and repair dynamics after injury in mechanically stable and unstable environment (osteotomy, perforation) after training, or immobilization of animals. New comparable results were given (great variety of tissue reactions, enhancement or inhibition of bone repair after different injuries and external loadings, etc.).

Concentration of glycosaminoglycans (GAG) was measured by microspectrometer after staining histological sections with safranin-O (21). It was noticed that 11-week immobilization of canine knee causes a significant softening (fall of the GAG concentration) of lateral femoral and tibial cartilages. After remobilization period of 50 weeks the alterations of cartilage biomechanical properties were disappeared. Immobilization causes the reversible changes in cartilage markers and synovium metabolism including HA in young beagles. Increased weight bearing did not change the concentration of markers (22).

In our experiments the GAG concentration was higher at 28th day after osteotomy and low after perforation of tibia. In training group the GAG content after osteotomy fell compare to control.

Conclusions

Total callus area in post-traumatic bone repair of rat tibia after osteotomy is in control and training group 1.5 time higher as in other groups (osteotomy of immobilized animals, perforation – all groups).

In the osteotomy group the hard callus area is 2 times and in the perforation group even 4 times higher than the soft callus area. In training groups similar results are given. Hard callus areas are in both groups about 3 times higher as the soft callus areas, i.e. in Group 1 is a bit increased and in Group 2 decreased compared to control.

In immobilized animals the total callus area in Group 1 (osteotomy) fell significantly compared to control, in Group 2 (perforation) the total callus area is similar to control. In both groups after immobilization the hard callus area is 1.5–2 times lower than the soft callus area. In both group after immobilization the hard callus formation is strongly affected, while the total callus forming was affected only after the osteotomy.

At 28th day of osteotomy callus total area is 1.5 and GAG concentration is 2 times higher compared to perforation. After physical loading (training) total callus area is the same, compared to the control group, but GAG concentration fell without any correlation between them. After immobilization the callus area as well as GAG content are decreased.

Acknowledgements

We wish to thank prof. H. J. Helminen for kind support to the present work, senior research assistant M. Lammi and senior lecturer R. Tammi for good advices in the study of histochemistry and immunohistochemistry, research assistant M. Hyttinen for teaching of GAG concentration measurements and the whole Department of Anatomy of the University of Kuopio for helpful affirmation.

This work was supported in part by Estonian Scientific Foundation Grant 3007.

Kaulinio rumbo (kaliuso) ir glikozaminoglikanų kitimas po žiurkių blauzdikaulio traumos

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Raktažodžiai: potraumatinis kaulo gijimas, kaulinis rumbas (kaliusas), osteotomija, perforacija.

Santrauka. *Darbo tikslas.* Kiekybiškai histologiškai (atlikta bendra kaliuso morfometrija) ir histochemiškai (atlikta mikrospektrometrija tirti glikozaminoglikanų kiekiui) ištirti potrauminį kaulo gijimą žiurkių blauzdikaulyje treniruotiems ir imobilizuotiems gyvūnams po segmentinės osteotomijos ir bikortikalinės perforacijos.

Medžiaga ir metodai. Kiekybiški tyrimai po trauminio kaulo gijimo histologiškai ir glikozaminoglikanų kiekio po osteotomijos ir blauzdikaulio perforacijos atlikti 105 Wistar veislės jauniems suaugusiems žiurkių

patinams. Buvo stebimas kontrolinių ir paveiktų (treniruotų, imobilizuotų) gyvūnų gijimas 1–42 parą po operacijos.

Rezultatas. Potrauminio kaulo gijimas vyksta kaip osteohistogenezės ir organogenezės įprastinis procesas, kuris priklauso nuo aplinkos faktorių (traumos pobūdžio ir gylio, gyvūnų treniruotumo). Gijimas priklauso nuo traumos: po osteotomijos susidaręs kaliusas yra žymiai didesnis negu perforacijos metu. Tačiau treniruotumas neturi įtakos kaliuso srities gijimui ir glikozaminoglikanų kiekiui ir dėl to nepagreitina kaulo gijimo, o imobilizuotiems gyvūnams šie procesai slopinami, todėl kaulo gijimas sulėtėja.

Išvados. Atlikus kiekybinius histologinius ir histocheminius kaulo gijimo po perforacijos tyrimus, gauta daug informacijos apie kaulinio audinio reparatyvinę histogenezę: kaliuso srities gijimo dinamiką, glikozaminoglikanų koncentracijos kitimą, traumos pobūdžio ir gylio efektą, treniravimą ir imobilizaciją gijimo proceso metu.

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Received 13 June 2003, accepted 29 September 2003
Straipsnis gautas 2003 06 13, priimtas 2003 09 29