

Structural Changes in Intervertebral Discs at Chronic Staphylococcus Aureus Osteomyelitis of the Tibia

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Abstract Inflammatory process is not limited only local effects but could affect other organs and tissues. In the present study, we investigated the influence of tibial osteomyelitis caused by Staphylococcus aureus on extracellular matrix components of Wistar rat's intervertebral discs. Histochemical assay was carried out on sulfated glycosaminoglycans, neutral glycoproteins and collagen fibers of nucleus pulposus. Immunochemical method was applied to I and II type collagen, fibronectin and fibulin-2. The role of persisting staphylococcal infection in the initiation and development of degenerative changes of the fibrous cartilage of intervertebral discs was demonstrated. Progressive disorders in sulfated glycosaminoglycans metabolism accompanied by changes of a fibrous component and collagen type predominance replacement suggest fibrous transformation in intervertebral discs. Increase of neutral glycoproteins due to separate fractions, in particular, fibulin-2 could be considered as compensatory reaction on progressing overpatchings of fibrocartilage extracellular matrix components. Reorganizations mentioned are supposed to promote further dystrophic-degenerative changes in intervertebral discs.

Keywords: *intervertebral disks, nucleus pulposus, bacterial infection, staphylococcus aureus, collagen, glycosaminoglycans, neutral glycoproteins, fibronectin, fibulin-2*

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1. Introduction

Progress in antimicrobial therapy methods developing is undoubted however staphylococcal infections are still supposed to be at the bottom of morbidity and mortality. Staphylococcus aureus in the United States each year causes more cases of infectious diseases than tuberculosis, viral hepatitis and AIDS combined [7] and methicillin-resistant strains are especially deleterious [11]. Evident symptoms of infection are highly focused, while minor manifestations of macro- and microorganisms interaction are not less important. In particular, it was shown a direct relationship between the focal persisting bacterial infection and the development of degenerative-dystrophic changes in rabbit intervertebral discs [12]. Considerably that pathological changes in the intervertebral discs could manifest the syndrome of degenerative-dystrophic changes in mesenchymal derivatives during local chronic inflammatory process [13]. In the present study, we investigated the influence of tibial osteomyelitis caused by Staphylococcus aureus on the ratio of extracellular matrix components in rats.

2. Materials and Methods

2.1. Study Design

The experiment was performed in thirty male Wistar rats (180-220 g, 2,5 months old). All animals were treated according to protocols approved by the animal care institutional review board. Eighteen rats were subjected to the experimental tibial myelitis. Using sterile surgical conditions shin-bone trepanation was carried out under halothane anesthesia, hole was plugged with cotton thread containing Staphylococcus aureus, strain 209 (10^7 cfu). Animals were decapitated 1, 2 and 3-months after surgery. Six intact rats were used as a control. Six animals with the trephined tibia followed by the introduction of sterile cotton thread were used as an additional control.

2.2. Immunohistochemistry

Specimens (tail intervertebral disks) were fixed in 12% formalin followed by dehydration in ethanol and embedding in paraffin. One section was used for the conventional hematoxylin and eosin method. Collagen fibers were stained by Van Gieson's picrofuchsin, sulfated glycosaminoglycans (SGAGs) – by alcian blue (pH 1,0) and neutral glycoproteins – by McManus PAS reaction [16]. The extracellular matrix parameters were estimated by immunocytochemistry based on indirect streptavidin biotin peroxidase method as described previously [3,4] according to the manufacturer's instructions. Triton X-100 (0,1%) was used for antigen demasking procedure for 5 min. Deparaffinized sections were incubated with primary antibodies: Anti-Collagen Type I (COL-1, Mouse IgG,

Santa Cruz Inc.), Anti-COL2A1 (M2139, Mouse IgG2, Santa Cruz Inc.), Anti-Fibronectin (Isotype: Mouse IgG1, Clone: IST-9, Santa Cruz Inc.) and Anti-Fibulin-2 (H-250, rabbit polyclonal, Santa Cruz Inc.). All incubations were performed for 60 min at room temperature. Immunostaining was performed using Novocastra Peroxidase Detection System (Ready-to-Use) kit (Code No. RE7110-K), which employed the streptavidin-biotin technique and DAB Substrate/Chromogen System for visualization.

2.3. Image Analysis

Sections were viewed by light microscopy (area: 64500 mkm² [15], magnification: × 400 per each experimental group). Staining intensity was analyzed quantitatively using Image J 1.42g software (National institutes of Health, USA). RGB channels were applied to reveal tinctorial characteristics of collagen fibers and neutral glycoproteins (Red) and SGAGs (Blue).

2.4. Data Analysis

The results were performed as a percentage obtained by the following relationship: % structure = S_s/S_t , S_s is the stained fibroblasts area and S_t is the total investigated area. Results were expressed as the mean (± SEM). Statistical analyses were performed using Kruskal-Wallis H test and Mann-Whitney U test with Bonferroni correction. Statistical significant was accepted at $p \leq 0.05$.

3. Results

The localized osteomyelitis model we analysed led to changes in rat health status (fever, appetite loss). After 1 month from the date of inoculation *Staphylococcus aureus* (Figure 1, A) necrosis of bone marrow, productive inflammatory process in endosteum and periosteum, osteoclast-mediated bone resorption. Later on, in 2 and 3 months after inoculation of *Staphylococcus aureus* bone marrow offered granulation tissue fields bounded the necrosis foci (Figure 1, B).

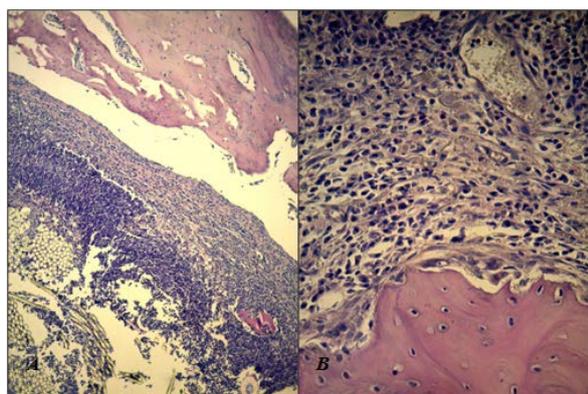


Figure 1. (A) Rat tibia 1 month after *S. aureus* inoculation. Bone marrow necrosis, osteoclastic bone resorption. (B) Rat tibia 2 month after *S. aureus* inoculation. Focal necrotic zones are bounded by granulation tissue, osteoclastic bone resorption (Hematoxylin and eosin staining. × 400)

Bone trabeculae in spongy bone tissue were fragmented, few osteocytes were located irregularly. Focal dispersions could be visualized in bone matrix (Figure 2).

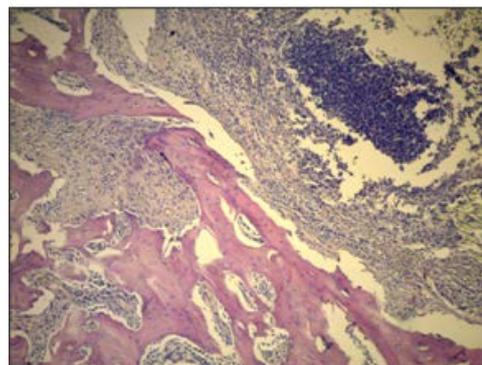


Figure 2. Rat tibia 3 month after *S. aureus* inoculation. Bone trabeculae fragmentation, focal dispersions, irregularly location of osteocytes. Hematoxylin and eosin staining. × 400

1 month after staphylococcal infection nidus has been produced statistically significant changes of tinctorial properties and staining intensity of sulfated glycosaminoglycans (Table 1).

Table 1. Analysis of extracellular matrix of intervertebral discs (nucleus pulposus), M±m

	Inflammation			
	Control	1 month	2 month	3 month
Neutral glycoproteins				
AR	24,6±0,8	24,7±0,9	28,7±1,2*	32,9±1,6*
SI	61,0±2,6	64,4±3,1	69, 5±2,2*	79,87±1,7*
Red	162,9±1,2	161,6±1,8	152,2±1,8*	145,4±1,3*
Sulfated glycosaminoglycans				
AR	32,3±0,9	30,4±1,1	25,8±0,90*	24,3±0,9*
SI	106,3±0,8	88,1±1,2*	87,6±1,2*	83,6±2,1*
Blue	192,0±0,9	179,4±1,5*	158,2±1,3*	158,7±0,9*

* - $p < 0,05$ compared to control

It suggests early involvement of nucleus pulposus proteoglycans in pathological process. Despite relative area of SGAGs was not changed; staining intensity was significantly decreased. Moreover, changes in tinctorial properties of SGAGs observed in the early stages of staphylococcal infection (Figure 3) may indicates changes in the ratio of different sulfated glycosaminoglycans forming a viscoelastic frame of nucleus pulposus tissue that conforms to data about dystrophically-degenerative alterations in of intervertebral discs [6,8].

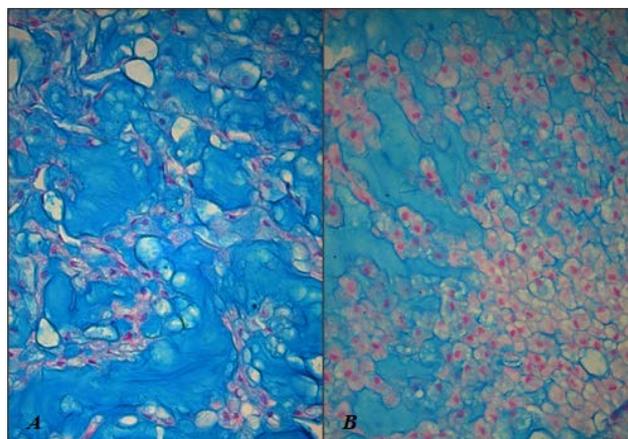


Figure 3. Fragment of rat intervertebral disk. (A) Moderate content of sulphurated glycosaminoglycans in nucleus pulposus 1 month after *S. aureus* inoculation. (B) Decrease of sulphurated glycosaminoglycans in nucleus pulposus 3 month after *S. aureus* inoculation. Alcian blue, pH 1,0 and carmalum staining. × 400

1 month after infection model reproducing structure of neutral glycoproteins was changed due to some of their fractions: despite invariable fibronectin distribution of nucleus pulposus relative area of fibulin-2 increased more than 10 times (Table 2).

2 months after inoculation of *Staphylococcus aureus* in the tibia we observed changes in tinctorial properties, distribution and staining intensity of collagen fibers and neutral glycoproteins of extracellular matrix (Table 1) as a result of alterations in constituents and predominance of collagen type compared to the control staining intensity of SGAGs reduced by 1,3 times. Immunohistochemical analysis of neutral glycoproteins revealed significant increase of fibronectin area and permanent increase of fibulin-2 area accompanied by 2-times staining intensity increase compared to preceding period. Area and staining intensity of SGAGs was below than a control ($p < 0,05$) (Table 2).

Table 2. Analysis of extracellular matrix of intervertebral discs (nucleus pulposus), M±m

	Control	Inflammation		
		1 month	2 month	3 month
Collagen fibers I type				
AR	23,2±1,3	23,2±1,4	23,9±1,0	39,9±1,3*
SI	87,8±1,6	48,7±1,8*	40,5±2,1*	65,8±1,7*
Collagen fibers II type				
AR	5,4±0,4	20,9±1,4*	39,34±0,5*	45,5±2,1*
SI	32,6±1,1	36,8±1,7*	47,5±1,5*	65,8±1,1*
Fibronectin				
AR	3,4±0,1	3,5±0,1	3,9±0,1*	4,3±0,0*
SI	56,9±1,2	58,4±1,1	35,7±1,6*	33,8±1,4*
Fibulin-2				
AR	3,4±0,1	36,5±0,9*	31,4±0,9*	31,4±0,8*
SI	34,3±0,9	27,2±1,0*	73,3±1,7*	94,9±1,3*

* - $p < 0,05$ compared to control

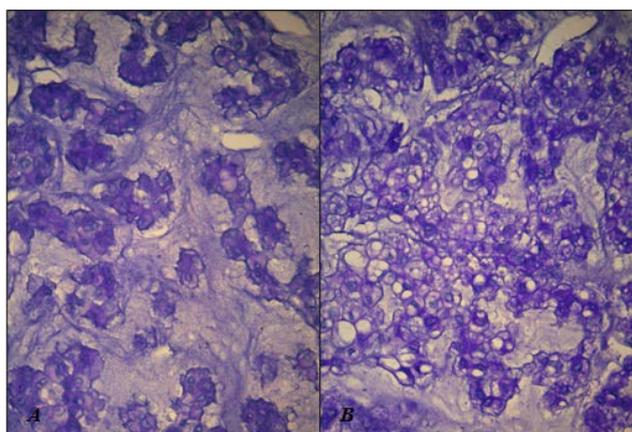


Figure 4. Fragment of rat intervertebral disk. (A) Moderate content of neutral glycoproteins in nucleus pulposus 1 month after *S. aureus* inoculation. (B) Increase of neutral glycoproteins in nucleus pulposus 3 month after *S. aureus* inoculation. PAS-reaction. $\times 400$

Immunohistochemical analysis of collagen type I in nucleus pulposus revealed a decline of DAB-positive staining intensity along with increased area (by 7 times compared to control) and staining intensity of collagen type II. It could be in support of fibrous transformation in nucleus pulposus progressing within 2 months after *S. aureus* inoculation that is taken into account when investigating of degenerative changes of intervertebral disks [18].

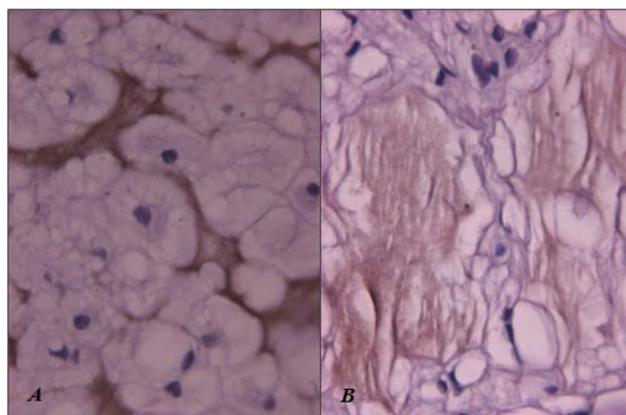


Figure 5. Fragment of rat intervertebral disk. (A) Moderate content of II type collagen in nucleus pulposus 1 month after *S. aureus* inoculation. (B) Vast gaps lacking of chondroblasts and filled with II type collagen in nucleus pulposus 3 month after *S. aureus* inoculation. Immunostaining for II type collagen. $\times 400$

3 months after bacterial infection nidus creation in the tibia progressing most evident decrease of SGAGs area and staining intensity were revealed. Violations of the SGAGs metabolism and consequently proteoglycans at persisting staphylococcal infection are likely reflected on viscoelastic characteristics of intervertebral discs [17]. Moreover, it was noted further growth neutral glycoproteins area together with persistent shift in the fractions ratio (Table 1).

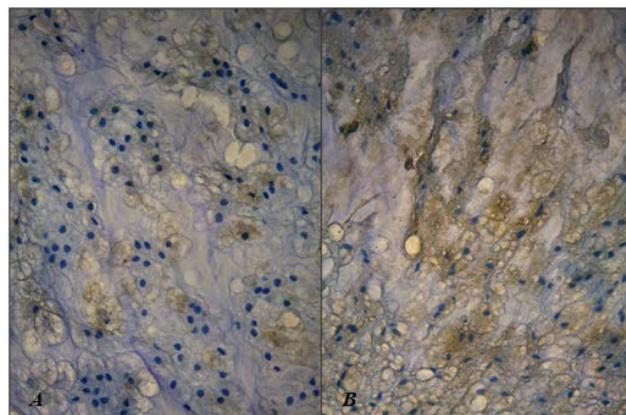


Figure 6. Fragment of rat intervertebral disk. (A) Moderate content of fibulin-2 in nucleus pulposus 1 month after *S. aureus* inoculation. (B) Increase of fibulin-2 in nucleus pulposus 3 month after *S. aureus* inoculation. Immunostaining for fibulin-2. $\times 400$

4. Discussion

Last years more attention is paid to cell therapy and tissue engineering aimed at tissue recovery at the degenerative processes in the intervertebral discs [10]. The knowledge concerning pathophysiological processes in target tissues, in particular, at chronic staphylococcal infection, is important for development of tissue engineering strategies.

In experimental tibial osteomyelitis model any relationship between plugged cotton thread without containing *Staphylococcus aureus* and intervertebral disc degeneration was not demonstrated. Unlike experimental groups with infectious agent inoculation which revealed advancing osteomyelitis of the tibia, there was no evidence of local and generalized inflammation in rats

already after 1 week after sterile thread plugging. A month after tibial trephination any violations in biomechanics of operated limb and movement pattern were not observed. However, it should be noted changes of microcirculation in parenchymatous organs persisted until 2 months after traumatic injury of the tibia. Subsequently compensatory hypertrophy and polyploidization of hepatocytes reactions were revealed [19]. Changes fibrous cartilage intervertebral disks, typical for groups with inoculation of *Staphylococcus aureus* was not observed.

Several studies have shown that the cells of the annulus fibrosus exhibit a chondrocyte phenotype with the predominant expression of type II collagen [1,2,5]. The increase of vascularization in external departments of the annulus fibrosus in the presence of bacterial infection nidus [20] could possibly affect the metabolic processes rate in the intervertebral disc nucleus pulposus. This may explain the significant increase in the area of collagen type II during the whole experiment, compared with intact animals, as well as significant increase of the relative area of type I collagen fibers in a long-term period that conforms to lesser role of type I collagen in the nucleus pulposus architectonics [5]. The experimental data demonstrate an increase of the integrated density of collagen type II. Polarization of collagen type ratio in favor of type II collagen could suppose fibrotic changes in the nucleus pulposus in the presence of bacterial infection [18]. Significant increase of collagen type I and II relative areas and also fibronectin expression increase were observed. It is noteworthy because fibronectin along with other extracellular matrix components, such as integrins, plays an important role in collagen fibrils formation [9], [14]. The above changes of the nucleus pulposus extracellular matrix combined with a progressive decline in relative area and staining intensity of sulfated glycosaminoglycans. Violations of sulfated glycosaminoglycans and, therefore, proteoglycans are likely reflected on the elastic and viscous properties of the intervertebral discs [17]. Increase of neutral glycoproteins due to separate fractions, in particular, fibulin-2 could be considered as compensatory reaction on progressing overpachings of fibrocartilage extracellular matrix components. Thus, reorganizations in extracellular matrix of the nucleus pulposus at prolonged staphylococcal infection are supposed to promote further dystrophic-degenerative changes in the intervertebral discs.

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