Nitroxide Synthase Activity as a Marker of Early Stages of Experimental Cartilage Dysmetabolism

A. V. Cherepovskii, A. I. Dubikov, S. V. Nikulin, L. A. Belogolovykh, and E. E. Medved'

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 137, No. 5, pp. 589-592, May, 2004
Original article submitted December 8, 2003

Time course of nitroxide synthase activity in the knee joint cartilage was studied in animals with experimental anterior instability of the knee joint. A significant increase in nitroxide synthase activity in chondrocytes was paralleled by a progressive decrease in glycosaminoglycan content in the cartilaginous matrix and subsequent destruction of the cartilage cytoarchitectonics.

Key Words: *nitrogen oxide; nitroxide synthase; cartilage; osteoarthrosis*

The search for biochemical "markers" reflecting the course of degenerative dystrophic process in the cartilaginous tissue, which can be used for monitoring of the process activity and prediction of its outcome is in progress [9]. However, despite numerous studies, no highly specific standard tests for wide practical use were proposed [1].

The study of the biological role of NO, an active tissue metabolite, in the development of cartilaginous degeneration, is a promising trend.

NO is a product of synthases (a group including 3 types or 3 isoenzymes) encoded for by different genes [3,11].

We studied the time course of nitroxide synthase (NOS) activity in the cartilages of laboratory animals with induced posttraumatic anterior instability of the knee joint (KJ).

MATERIALS AND METHODS

The study was carried out on 16 random-bred male rats (180-200 g) kept under standard vivarium conditions. Anterior KJ instability was created by crossing the patella ligament (extraarticular surgery). The animals were sacrificed by intraperitoneal injection of thiopental on days 30 (group 1), 60 (group 2), 90 (group 3), and 150 (group 4). The cartilages of the femoral condyles were studied. Control group consisted of 3 intact animals.

Histochemical location of NOS was determined by the method [6] based on the formation of insoluble diformazan precipitate in the presence of nitroblue tetrazolium (substratum) and NADPH (cosubstratum). This reaction makes possible to evaluate enzyme activity, because the density of diformazan precipitate is directly proportional to the molecular content of NOS [6]. Routine histological methods included staining with hematoxylin and eosin and with 1% toluidine blue. The thickness of the cartilage zones and their cytoarchitectonics were studied under a Karl Zeiss microscope. Computer imaging of all micropreparations was realized through a videotype system attached to Vickers M-85 microdensitometer. Digital processing of the images was carried out. Enzyme activity (optical density of granules) and matrix staining intensity were expressed in optical density units.

Differences between the means were significant at $p<0.05$.

RESULTS

Normally KJ cartilage in animals is clearly divided into zones: surface, intermediate, and deep. Each zone

Vladivostok State Medical Institute. ***Address for correspondence:*** aihavlad@online.vladivostok.ru. Dubikov A. I.

0007-4888/04/1375-0517

was characterized by specific cytoarchitectonics. The surface zone (69.9±3.97 μ) was presented by relatively small tangent-oriented chondrocytes (CC). The intermediate zone (131.5±7.69 μ) contained larger cells situated mainly in uni- and bicellular lacunae. The deep zone (183.8±3.60 μ) consisted of solitary CC.

Normally, CC of all cartilage layers positively react to NADPH-diaphorase, blue diformazan granules were seen in cell cytoplasm. Optical density of diformazan precipitate in CC differed in different zones of the cartilage (Fig. 1, *a*). The staining was most intensive in the surface zone and least intensive in the deep zone (Table 1).

The total content of glycosaminoglycans (GAG) in the cartilage matrix characterizing synthetic activity of CC is normally high. Evaluation of toluidine blue staining intensity gradient showed the highest concentration of GAG in the deep zone and the least in the surface zone (Table 1).

All studied parameters underwent most pronounced changes in group 1. Optical density of diformazan precipitate increased significantly in CC of all cartilage zones: the cell cytoplasm was bright blue. Activity of NADPH-diaphorase was maximum in cells of the deep zone. Toluidine blue staining showed a significant and virtually similar decrease in GAG content in the matrix of all cartilage zones. The thickness of the deep and intermediate zones of the cartilage increased significantly (Table 1). The cytoarchitectonics of the intermediate zone changed: 2- and 3-cell lacunae predominated and 4-cell lacunae appeared.

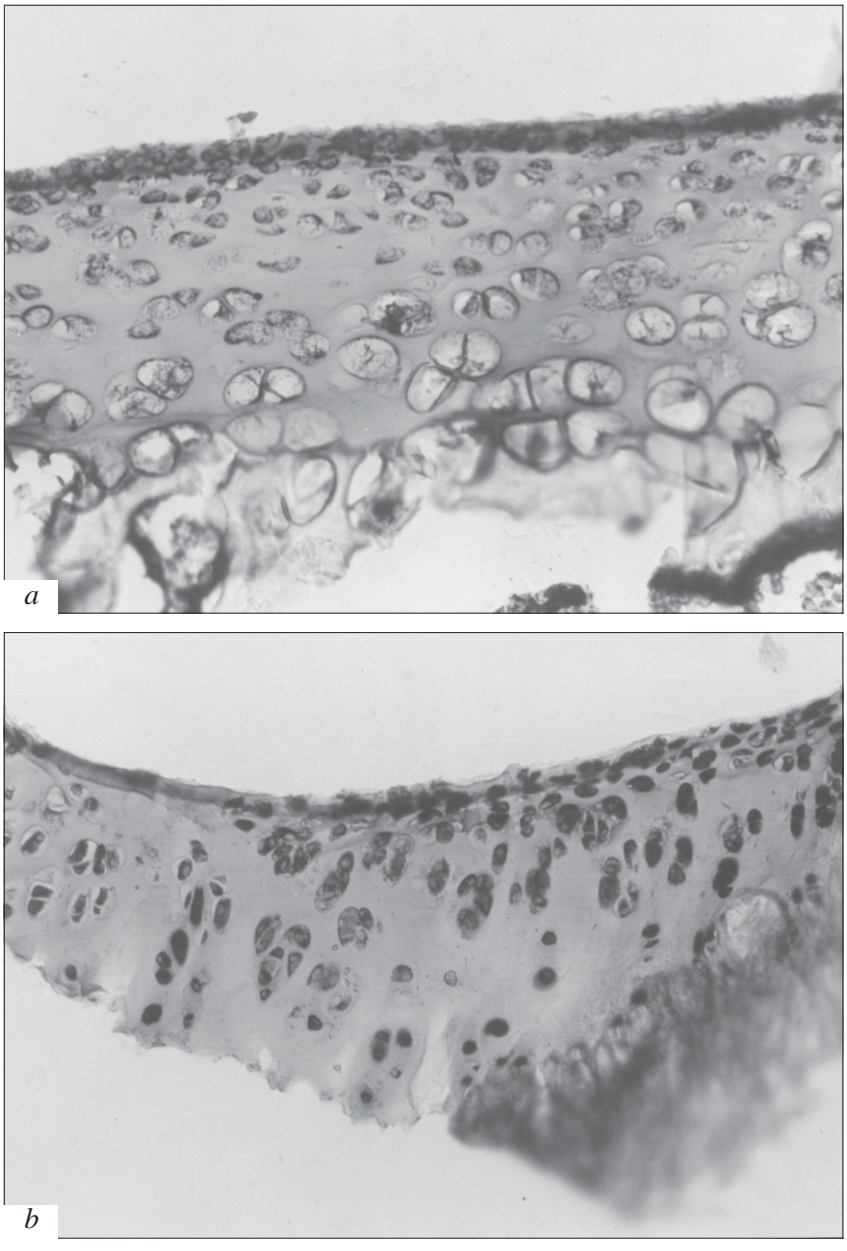

Fig. 1. Nitroxide synthase activity in rat articular cartilage chondrocytes in health (*a*) and on day 150 of the experiment (*b*). Hope, Vincent method, ×400.

TABLE 1. Histochemical and Morphometrical Characteristics of KJ Cartilage in Different Zones in Experimental Anterior Instability (*M*±*m*)

Day of experiment		Density of diformazan precipitate, opt. dens. units	Intensity of toluidine blue staining, opt. dens. units	Thickness of cartilage zones, μ
Normal	I	53.40±3.35	34.10±0.99	69.90±3.97
	II	31.90±2.85	56.90±1.24	131.50±7.69
	III	4.13±3.35	82.00±0.45	183.8±3.6
30	I	71.10±0.37**	21.00±1.24***	70.60±1.49
	II	49.10±0.5***	37.90±2.11***	163.80±2.48**
	III	46.30±1.98***	40.50±2.73***	202.10±2.98**
60	I	65.40±0.37**	17.90±1.49***	44.90±2.48**
	II	60.80±1.36***	30.00±1.74***	183.50±2.11***
	III	69.60±2.61***	45.00±1.74***	207.10±4.09***
90	I	63.40±2.48*	23.50±1.61***	31.80±0.99***
	II	70.50±1.49***	26.30±0.62***	77.80±6.82**
	III	69.10±1.61***	21.40±0.87***	146.60±5.33***
150	I	83.9±3.1***	17.10±0.74***	22.10±0.74***
	II	82.90±1.86***	15.00±0.74***	72.5±6.2***
	III	89.90±3.71	10.90±0.74***	137.8±4.8***

Note. Cartilage zones: I: surface; II: intermediate; III deep. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$ compared to normal.

In group 2 NADPH-diaphorase activity remained significantly increased in cells of all layers of the cartilage. Qualitative changes were detected in studies with toluidine blue staining: staining intensity of extraterritorial matrix sharply decreased, the content of GAG in the territorial matrix remained below the normal. The thickness of all cartilage zones decreased significantly in comparison with the normal; the surface and intermediate zones were thinner than in group 1 animals (Table 1). The cytoarchitectonics radically changed: the intermediate and deep zones were presented by 10-12-cell lacunae arranged in columns.

In group 3 animals activity of NADPH-diaphorase remained significantly above the normal. Toluidine blue staining revealed focal matrix metachromasia and low density of staining. The number of cartilage cells sharply decreased, multicellular lacunae disappeared. The thickness of all cartilage zones decreased significantly in comparison with group 2 (Table 1).

In group 4 optical density of diformazan precipitate in all zones of the cartilage was significantly higher than in group 3. Toluidine blue staining showed a significant decrease in the staining intensity in comparison with group 3; focal metachromasia persisted. The thickness of the cartilage zones also decreased significantly in comparison with group 3 (Table 1). Cartilage structure was still characterized by low number of cells and the absence of multicellular lacunae (Fig. 1, *b*).

According to published data, increased production of NO in CC is observed in excessive or aberrant exercise modulating the KJ surfaces [4,7,13,14] and in osteoarthrosis [2,10,12,15]. Structurally the degenerative effect of high NO concentration is realized mainly through inhibition of proteoglycan synthesis in the cartilage matrix [5,8].

Our findings indicate experimental anterior KJ instability leads to phasic changes in the cartilage ultrastructure. NOS activity in CC significantly increased during the earliest period of injured joint functioning. This increase was paralleled by early significant increase in GAG content. Since proteoglycan production is the main function of CC, we can hypothesize that increased NOS activity under these conditions leads to inhibition of synthetic activity in CC.

On the other hand, changes in the cartilage cell composition at this stage are characterized by CC proliferation in the intermediate (functionally most active) zone. Proliferation in combination with detected thickening of the intermediate and deep zones of the cartilage can be regarded as reactive adaptation changes (group 1).

Further exposure to the traumatic factor is associated with permanently high activity of NOS in the cartilage cells. This is paralleled by progressive decrease in GAG content primarily in the extraterritorial matrix (group 2). This progress attests to drastic inhibition of CC synthetic activity. Qualitative changes in the cytoarchitectonics are justified under these conditions. The formation of giant multinuclear lacunae under conditions of decreased functional activity of CC suggests that the cartilage reached the limit of structural compensation of functional disorders. The

significant thinning of all zones of the cartilage, first detected at this stage (group 2), is quite expected.

Further changes in the cartilage ultrastructure under conditions of traumatic exposure are characterized primarily by disorganization of the cytoarchitectonics: number of CC decreased, multicellular lacunae disappeared, and all zones of the cartilage became thinner (groups 3, 4). Synthetic activity of CC progressively decreased: GAG content in the matrix continues to decrease. These changes are observed against the background of increasing NOS activity in CC (group 4). This attests to failure of the compensatory potential of the cartilage and its destruction.

Hence, histochemical changes in the cartilage are detected at the earliest stages of KJ functioning under conditions of experimental posttraumatic instability. These changes are characterized by an appreciable and rapid (within 30 days) increase in NOS activity in cartilage cells and subsequent persistence of high activity of the enzyme. This process is initiated against the background of intact cytoarchitectonics and hence, has no structural manifestations. However, the increase in NOS activity forms fatal functional defect of CC manifesting by early suppression of the production of structural components of the cartilage matrix by these cells and thus creating prerequisites for progressive destruction of the cartilage. We believe that NO can be regarded as a specific marker of early stages of cartilage dysmetabolism.

REFERENCES

1. N. N. Kornilov, K. A. Novoselov, and N. V. Kornilov, *Travmatol. Ortoped.*, No. 2, 47-59 (2002).
2. A. R. Amin and S. B. Abramson, *Curr. Opin. Rheumatol.*, **10**, No. 3, 263-268 (1998).
3. D. S. Bredt**,** *Free Radic. Res.*, **31**, No. 6, 577-596 (1999).
4. C. Fink, B. Fermor, J. Weinberg, *et al.*, *Osteoarthritis Cartilage*, **9**, No. 5, 481-487 (2001).
5. J. Y. Jouzeau, C. Cipoletta, N. Presle, *et al.*, *Ibid.*, **7**, No. 4, 382-385 (1999).
6. V. T. Hope and S. R. Vincent**,** *Histochem. Cytochem.*, **37**, 653-661 (1989).
7. K. Kobayashi, H. Mishima, S. Hashimoto, *et al.*, *J. Orthop. Res.*, **19**, No. 5, 802-808 (2001).
8. P. M. Van der Kraan, E. L. Vitters, H. M. van Beuningen, *et al.*, *Osteoarthritis Cartilage*, **8**, No. 2, 82-86 (2000).
9. L. S. Lohmander, *Baillier's Clin. Rheum.*, **2**, No. 1, 37-61 (1988).
10. B. H. Min, H. J. Kim, H. Lim, *et al.*, *Exp. Mol. Med.*, **33**, No. 4, 299-302 (2001).
11. C. Nathan and Q.-W. Xie, *Cell*, **78**, 915-918 (1994).
12. J. Salvatierra, G. Escames, P. Hernandez, *et al.*, *J. Rheumatol.*, **26**, No. 9, 2015-2017 (1999).
13. D. Spreng, N. Sigrist, T. Jingi, *et al.*, *Am. J. Vet. Res.*, **61**, No. 5, 530-536 (2000).
14. K. Takahashi, S. Hashimoto, T. Kubo, *et al.*, *J. Orthop. Res.*, **19**, No. 3, 500-503 (2001).
15. K. Vuolteenamo, T. Moilanen, N. Al-Saffar, *et al.*, *Osteoarthritis Cartilage*, **9**, No. 7, 597-605 (2001).