Nitrite and Nitrotyrosine Concentrations in Articular Cartilage, Subchondral Bone, and Trabecular Bone of Normal Juvenile, Normal Adult, and Osteoarthritic Adult Equine Metacarpophalangeal Joints

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ABSTRACT. ***Objective.*** Osteoarthritis (OA) is a chronic debilitating joint disorder in which the importance of inflammation is increasingly recognized. In advanced cases, both the articular cartilage and the underlying bony layers are affected, but the exact sequence of events and their localization in the initial phase of pathogenesis remain uncertain. We measured nitric oxide (NO) end products in tissue layers that constitute the bearing surface of the joint, as possible indicators of physiological and pathological processes.

Methods. Nitrite as a measure for NO and nitrotyrosine was measured in articular cartilage, subchondral bone, and the underlying trabecular bone of the proximal articular surface of the first phalanx of healthy mature horses (n = 15; age range 5–18 yrs), mature horses affected by OA (n = 15; age range 8–22 yrs), and unaffected juvenile horses (n = 13; age range 6 months–4 yrs). Data were correlated with cartilage damage, as quantified by the Cartilage Degeneration Index.

Results. In all 3 layers the nitrite concentration was higher in OA joints (cartilage, $p < 0.001$; subchondral and trabecular bone, $p < 0.05$). The concentration of nitrite was significantly higher in cartilage and subchondral bone of juvenile horses compared with mature horses ($p < 0.001$). Nitrotyrosine concentrations were significantly higher in subchondral bone of OA horses compared with healthy controls ($p < 0.001$), but significantly lower in trabecular bone of juvenile horses ($p < 0.01$).

Conclusion. The similarities observed over the 3 tissue layers support the concept of the bearing surface of the joint as a functional entity. Nitrite concentration seems to be a good indicator of tissue metabolic activity, but cannot discriminate between physiological (juvenile animals) and pathological (OA cases) processes. The increased nitrotyrosine levels in subchondral bone of OA-affected animals suggest that this layer is important in early or moderate OA, and implies a role of oxidative stress in the development of the disease. (J Rheumatol 2006;33:1662–7)

Key Indexing Terms:
OSTEOARTHRITIS BONE-CARTILAGE RELATIONSHIP NITRIC OXIDE
NITROTYROSINE OXIDATIVE STRESS

Osteoarthritis (OA) is a debilitating condition that may affect a large number of joints in both humans and horses[1,2]. It has a multifactorial etiology and there is large individual variation in the speed with which the disease progresses. Age, genetics, trauma, and more general biomechanical influences are among the factors that contribute to the osteoarthritic process[2]. Classically, OA has been viewed as a mainly degenerative disorder of mobile joints, characterized by deterioration of articular cartilage and the formation of new bone at the joint surfaces and margins[3]. However, recent debate strongly questions the noninflammatory character of the disease[4]. Another contentious issue is the site where the disease initiates. Although it is well known that synovial membrane and subchondral bone and ligaments as well as cartilage may be implicated, the major focus for OA research has remained primarily on the articular cartilage. Degradation of articular cartilage typical of OA with no signs of pathological changes in the underlying subchondral bone has been reported[5,6], but other studies claim that sclerosis of the subchondral bone plate precedes articular cartilage damage[7-14].

Nitric oxide (NO) is a substance that fulfils a physiological role in tissue homeostasis, where it has a regulatory function, but is also upregulated under inflammatory conditions, exerting cytotoxic effects through the creation of oxidative stress. NO is formed from the basic amino acid l-arginine by the enzyme NO synthase (NOS), of which 3 major forms have been identified, 2 constitutive calcium-dependent isoforms

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(eNOS and nNOS) and the inducible calcium-independent isoform (iNOS). The latter is thought to be largely regulated by inflammatory cytokines, such as interleukin 1, tumor necrosis factor-α, and interferon-γ, and can induce apoptosis or programmed cell death following various insults[15-18]. In the case of tissue damage, it plays a dual role. On the one hand NO and its byproducts, reactive oxygen species (ROS), play a part in the inflammatory component of OA. On the other hand it may act in a protective way by suppressing cell proliferation and synthesis, thus attenuating the inflammatory reaction but also impairing the formation of repair tissue and new extracellular matrix components[16,19,20]. NO is a highly volatile substance that is rapidly degraded into nitrite. Consequently, nitrite is commonly used as a marker for NO synthesis[18]. As nitrite is continually cleared from the body by renal excretion, nitrite concentrations measured in tissue can be considered representative for ongoing NO production[21].

Nitrotyrosine is formed following the reaction of NO with superoxide-producing peroxinitrite, which (among other processes) nitrosylates tyrosine rings in proteins, thereby producing nitrotyrosine[22]. It has been identified as an additional marker of inflammation and enhanced NO production, and the presence of nitrotyrosine has been suggested to represent oxidative damage of cellular proteins. Further, it correlates with diseases such as OA and neurological disorders[23,24]. Nitrotyrosine is not cleared continually, and may thus accumulate.

The concept that the bearing surface of the joint should be comprehensively visualized as a functional entity, rather than a separate tissue layer, is becoming more widely accepted. In the horse, this concept has been applied to the metacarpophalangeal (MCP) joint in mature and juvenile horses to monitor changes related to development[25,26] and to joints with early OA, in which it was shown that signs of early OA were most evident in the subchondral bone[28]. We decided to follow a similar whole-joint approach to evaluate the potentially important role of NO and its end products in chronic joint disorders. Nitrite and nitrotyrosine concentrations were determined in the 3 layers that constitute the bearing surface of the joint, i.e., cartilage, subchondral bone, and trabecular bone, in specimens from normal, osteoarthritic, and juvenile equine MCP joints to test the hypothesis that nitrite levels may be influenced by both physiological (growth and development) and pathological (cartilage degeneration) processes. For nitrotyrosine it was hypothesized that this would be a more specific indicator for damaged tissue that would be increased only in pathological conditions. It was further hypothesized that changes in nitrite and nitrotyrosine levels would show considerable similarities through the composing layers of the bearing surface of the joint.

MATERIALS AND METHODS

Joints. Forty-three right MCP joints from slaughter horses were harvested immediately after death and stored at –20°C until processing. All horses were Warmblood horses, but no clinical data were available. Articular cartilage degeneration was quantified with help of the Cartilage Degeneration Index (CDI)[27]. In this method, the amount of India ink uptake as a measure for cartilage degeneration is quantified across the entire cartilage surface by digital imaging of the native and stained articular cartilage surfaces. The increase in mean grey level is used as a basis for calculation of the CDI (range from 0 to 100%). This procedure was performed for the entire articular surface of the proximal first phalanx, resulting in a general CDI, and for a specific area of interest (CDI_1) located halfway between the medial edge of the sulcus articularis and the medial border of the articular surface, adjacent to the dorsal articular margin. This site is where cartilage degeneration is known to start in the equine MCP joint and from where it gradually spreads over the joint[28].

The study cohort was divided into 3 groups. The first group (n = 15; mean age 10.2 yrs, range 5–18 yrs), classified as normal, had a $CDI_1 < 25\%$, indicative of none to minor degenerative changes[29]. The second (n = 15; mean age 14.7 yrs, range 8–22) had CDI_1 values > 25%, indicative of mild to severe cartilage changes; and the third group (n = 13; mean age 2.4 yrs, range 6 months–4 yrs) consisted of juvenile horses. These horses all had $CDI_1 < 25\%$.

Sampling procedure. After establishing the CDI, a 6 mm slice of phalanx I containing cartilage and bone was cut in dorsopalmar direction, perpendicular to the articular surface and through the center of the medial fovea, using a bandsaw. Cartilage of the area of interest was removed with a scalpel and the subchondral and trabecular bone layers were separated with a milling cutter at a standardized distance from the cartilage-subchondral bone interface. Each bone and cartilage sample was homogenized in a microdismembrator (Braun, Melsungen, Germany) with a stainless steel ball-mill under liquid nitrogen. After suspension in distilled water, samples were stored at –80°C until further analysis.

Protein measurement. Samples were dried under vacuum (Savant, Hollbrook, NY, USA), and proteins were extracted by boiling in 200 μl 2% sodium dodecyl sulfate (SDS) in 50 mM Tris (pH 6.8) for 10 min. The protein concentration was measured using a modified method of Lowry, *et al*[30]. Briefly, samples were boiled 10 min in 30 mM SDS, 160 mM Na_2CO_3, and 12 mM KNa-tartrate in 80 mM NaOH. Samples were incubated 10 min in 1.5 mM $CuSO_4$, and the color was enhanced with 0.1 M Folin-Ciocalteu's phenol reagent for 30 min at room temperature. Samples were measured spectrophotometrically (Biorad 3550, Biorad, Veenendaal, The Netherlands) at 750 nm. Bovine serum albumin (BSA) was used as a standard. All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Nitrite assay. Nitrite, a stable end product of NO, was measured in cartilage and subchondral bone samples using the spectrophotometric Griess reaction[18,31-33]. Twenty microliters of sulfanilamide (1% w/v) in phosphoric acid (5%) were added to 200 μl of the sample and incubated 10 min at room temperature before adding 20 μl naphthylethylenediamine (1.4%). Samples were incubated 2 min and centrifuged for 5 min at 13,000 rpm. Seventy-five microliters of supernatant was transferred to a 96 well plate and the optical density was measured at 540 nm (Biorad). Nitrite concentrations were calculated by comparison with the optical density of standard solutions of sodium nitrite (Merck, Darmstadt, Germany). The nitrite concentration was expressed as μmol/mg protein. Samples were analyzed in triplicate.

Nitrotyrosine ELIFA. An enzyme-linked immunoflow assay (ELIFA) was used in order to (semi-) quantify the amount of adsorbed nitrotyrosine by microplate filtration, in which diluted SDS-extracted protein samples are drawn past a nitrocellulose membrane with a constant flow rate of 40 μl/min/well [Easy-Titer ELIFA microplate filtration device (Pierce, Rockford, IL, USA) with Microperpex S peristaltic pump (LKB, Bromma, Sweden)]. Samples were diluted with Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris, pH 7.5). Washing buffer (200 μl/well) TBS-T (TBS with 0.05% Tween-20) was subsequently drawn through 3 times at the same flow rate. Nitrotyrosine was detected by drawing 50 μl mouse anti-nitrotyrosine antibody (TBS-T, 0.1% BSA, 0.5 μg/ml antibody) through the membrane. After washing 3 times, 50 μl secondary rabbit anti-mouse (Ig) antibody, conjugated with horseradish peroxidase diluted 5000× in 0.1% BSA in TBS-T, was drawn through. The membrane was washed another 3 times. Bound horseradish peroxidase was detected by the substrate 3,3,5,5-tetramethylbenzidine,

which was drawn past the membrane and collected in a 96 well polystyrene plate containing 50 μl 2 M H_2SO_4 per well. The optical density of the wells was measured at 450 nm with a microplate reader, and the nitrotyrosine contents were calculated and expressed relative to a standardized sample (arbitrary units/mg protein). Samples of each layer were analyzed simultaneously, and in triplicate.

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM). Differences between the OA, non-OA, and the juvenile horses were tested using the nonparametric Kruskal-Wallis test, completed with post-hoc Dunn's multiple comparison tests. Correlations between different parameters were tested using the nonparametric Spearman correlation coefficient test. Data were considered statistically significant when $p < 0.05$. Statistical analysis was performed with GraphPad Prism software (GraphPad, San Diego, CA, USA).

RESULTS

Nitrite in cartilage and bone. NO concentration in cartilage, as indicated by nitrite level, was significantly higher in the group with cartilage damage (OA) compared to those with healthy cartilage (Figure 1A). Similarly, nitrite concentrations in subchondral and trabecular bone were significantly higher in horses with cartilage lesions compared with controls (Figure 1B, 1C).

In the juvenile horses, a significantly higher nitrite concentration was measured in cartilage and subchondral bone compared with the mature horses classified as normal joints. In trabecular bone, the levels were comparable with those in normal, mature horses (Figure 1A, 1B, 1C).

Nitrotyrosine in cartilage and bone. There was a significantly higher nitrotyrosine level in the subchondral bone of the animals classified as having cartilage damage compared with the normal animals (Figure 2B). However, this was not the case in either cartilage or trabecular bone (Figure 2A, 2C).

In the juvenile age class, nitrotyrosine levels in both cartilage and subchondral bone were similar to those found in mature horses. In contrast, in trabecular bone, the nitrotyrosine level was significantly lower than in normal, mature animals (Figure 2A, 2B, 2C).

Relationship of nitrite and nitrotyrosine levels with CDI. For mature animals, in all 3 layers a positive correlation existed between the CDI and the specific tissue nitrite concentration. The correlation (r) was strongest in the cartilage layer ($r = 0.7$; $p < 0.001$) and slightly lower in both subchondral and trabecular bone ($r = 0.5$; $p < 0.01$). For nitrotyrosine in the adult animal group, there was no significant correlation with CDI in articular cartilage ($r = 0.1$; nonsignificant), a strong correlation in subchondral bone ($r = 0.8$; $p < 0.001$; Figure 3), and a considerably less strong correlation in the trabecular bone layer ($r = 0.4$; $p < 0.05$). The relation between nitrite and nitrotyrosine in subchondral bone revealed a strong and significant correlation ($r = 0.7$; $p < 0.0001$; Figure 4).

DISCUSSION

The main findings of our study were that the patterns of nitrite in the 3 layers examined showed similarities, and that there appeared to be a considerable and significant increase in nitrotyrosine in the subchondral layer of OA specimens that was strongly correlated to the CDI, which was used as a measure for cartilage degeneration.

NO acts as a mediator that is upregulated in various physiological and pathophysiological processes in the body in which tissue activity is increased. Growth is such a process; NO content has been shown to be increased during development and maturation[34]. NO is also upregulated in fracture repair, which can be seen as a physiological response to a pathologic event[35,36].

In our study, nitrite levels in normal, mature horses may be considered as representative of a steady-state condition. Levels were comparable in the subchondral bone plate and the underlying trabecular bone. In the cartilage layer, the level was lower in terms of μmol per mg protein, but will still be of about the same absolute level as in the bony layers, because the protein content of cartilage is much higher than that of bone. The lower levels of NO in relation to protein content are in agreement with the substantially lower cellular activity of articular cartilage compared to bone in mature individuals, resulting in extremely long turnover times for collagen[37,38].

In the juvenile animals, nitrite levels were higher in the

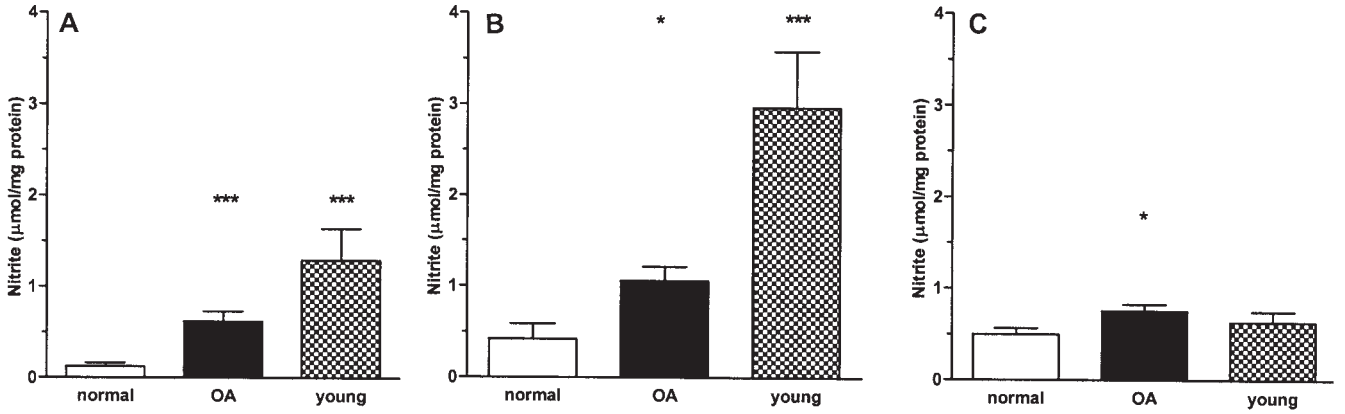

Figure 1. Nitrite content (μmol/mg protein; mean ± SEM) in cartilage (A), subchondral bone (B), and trabecular bone (C) in samples from the proximal first phalanx of normal mature joints (n = 15), OA joints (n = 15), and normal immature joints (n = 13). Significant differences with normal mature joints: *$p < 0.05$, ***$p < 0.001$.

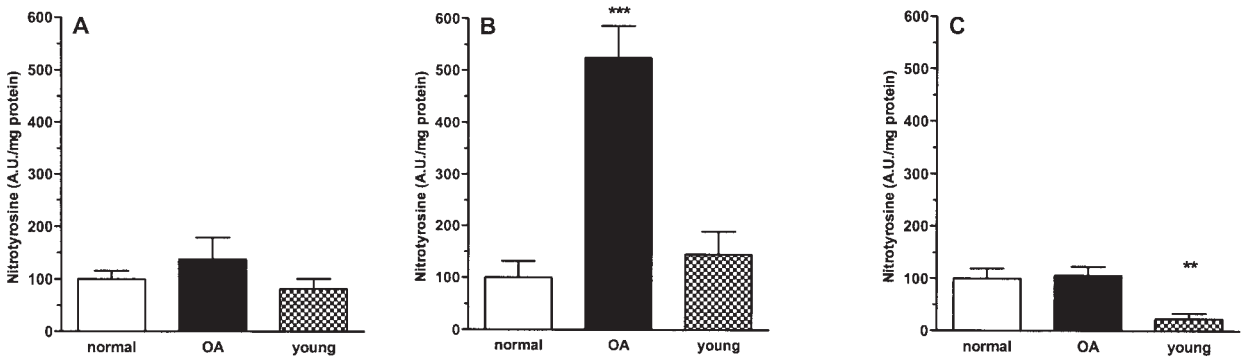

Figure 2. Nitrotyrosine content (arbitrary units/mg protein; mean ± SEM) in cartilage (A), subchondral bone (B), and trabecular bone (C) in samples from the proximal first phalanx of normal mature joints (n = 15), OA joints (n = 15), and normal immature joints (n = 13). Significant differences with normal mature joints: **p < 0.01, ***p < 0.001.

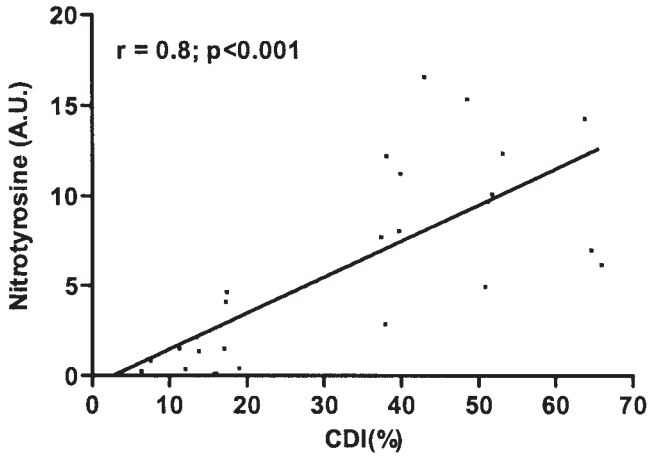

Figure 3. Correlation between Cartilage Degeneration Index (CDI; %) and nitrotyrosine content (arbitrary units) of subchondral bone in samples from the proximal first phalanx of mature horses.

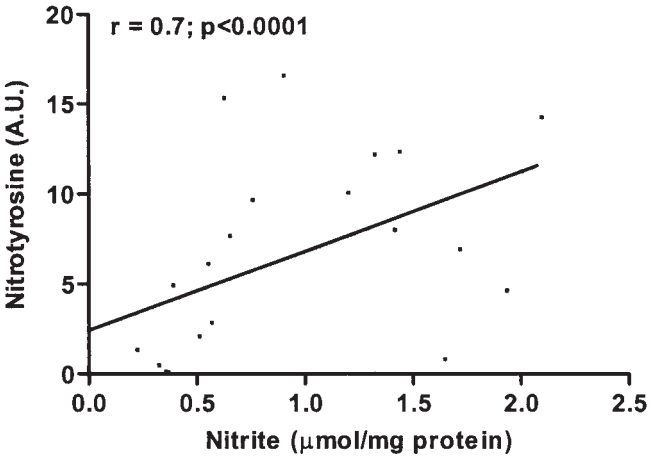

Figure 4. Correlation between nitrite (μmol/mg protein) and nitrotyrosine content (arbitrary units) of subchondral bone in samples from the proximal first phalanx of mature horses.

cartilage and subchondral bone layers compared to the mature horses. In many of the animals from this age class, the process of endochondral ossification may not have ceased completely, yet there is extensive metabolic activity in both the cartilage and the underlying subchondral bone plate, which would account for the higher levels in these 2 layers but not in the layer of trabecular bone underneath.

Nitrite levels were higher in specimens from OA-affected animals compared to normal specimens. We did not measure NOS isoforms in this study and thus cannot tell with certainty how the increased amounts of nitrite in these animals were formed. Taking the unaffected animals as normal controls in which nitrite will be principally generated by eNOS and nNOS, it follows that the higher nitrite levels in the OA-affected animals may be due to upregulation of iNOS. This is known to negatively affect chondrocyte proliferation and synthetic activity, which may result in functional consequences. In an *in vivo* study, topical administration of the NO donor glyceryltrinitrate affected the biomechanical characteristics of articular cartilage in the femorotibial joints of sheep, presumably via a disturbance of chondrocyte metabolism[39]. The decrease in proteoglycan content of articular cartilage in the same sample set as used in our study, as reported[40], is in agreement with this observation.

Nitrite levels in OA-affected specimens were higher in all 3 layers that constitute the bearing surface of the joint. The increase in nitrite over all layers, and the positive correlation of nitrite level with CDI in all these layers, suggest that in (early or moderate) OA, increased cellular activity is present throughout the entire bearing surface of the joint, and not only in the articular cartilage layer. These results support findings that extracellular matrix composition in equine MCP joint specimens affected by (early) OA showed changes in all 3 layers[40]. This observation agrees with earlier work[13,41,42], and supports the concept of the bearing surface of the joint as an integrated and interrelated structure, rather than as a compilation of largely independent tissue layers. The observation further stresses the importance of the bone layers underlying the articular cartilage. The mechanism by which mutual influence

of the layers beneath the joint surface or crosstalk between them occurs is not clear. The subchondral bone plate and the underlying trabecular bone are different in structure, but in fact form a continuum with common vascularization. This does not apply to the articular cartilage layer. It has been suggested, however, that cytokines, growth factors, and other mediators may seep through the bone-cartilage interface and provoke cartilage breakdown[43]. Structural pathways for this process may be formed by the micro-cracks that have been demonstrated to exist in the calcified layer of articular cartilage[44].

Whereas nitrite is an indicator only for NO production, protein-associated nitrotyrosine is a more suitable marker for damage induced by reactive nitrogen intermediates derived from NO. It is metabolized in the process of protein turnover, but, as many extracellular matrix proteins of connective tissues have a long half-life, nitrotyrosine can to a certain extent be seen as a measure for accumulated damage. In our study nitrotyrosine levels in mature animals are thought to represent normal steady-state values. Levels in the juvenile group were at a similar level as in the mature individuals in the cartilage and subchondral bone layers. This means that although NO levels were significantly higher in those layers in the juvenile animals, resulting in nitrosylation occurring at a higher rate, the higher protein turnover rate compensates for this. In the trabecular bone of such juvenile horses, nitrotyrosine was significantly lower. This may be explained by the fact that in this layer the NO concentration was similar to the concentration in mature horses; the higher protein turnover rate will then result in lower nitrotyrosine levels.

In joints with osteoarthritic changes, nitrotyrosine levels were significantly and substantially increased in the subchondral bone layer. This may be interpreted as a significant increase in oxidative stress in this layer. It is thought that elevated NO levels affect principally osteoclast activity in bone. A reduced osteoclast activity might result in sclerosis. In the specimens used for our study no significant changes in bone mineral density were observed; however, ash content was significantly higher in the subchondral bone plate of OA-affected specimens than in nonaffected ones[40]. In addition, the correlation of nitrotyrosine with the CDI of the cartilage layer is highest in the subchondral bone layer. These findings suggest that there may be a relationship between the cartilage degeneration and the production of nitrotyrosine in the underlying subchondral bone plate. It should be emphasized in this context that a substantial amount of the stress generated by joint loading is absorbed by subchondral bone rather than cartilage, which may account for more oxidative stress in the subchondral bone layer compared to cartilage, and hence may result in more accumulation of nitrotyrosine. An alternative explanation might be differences in the presence of antioxidants between the layers. In both cases the substantial increase in nitrotyrosine levels in the subchondral bone, but not in cartilage, might serve as an element in the continuing discussion of where OA is initiated[45].

We conclude that the nitrite concentrations in the 3 layers that constitute the bearing surface of the joint suggest a close and functional interrelation between these tissue layers. The high nitrotyrosine levels in the subchondral plate of OA-affected specimens and the relatively strong correlation with the quality of the overlying cartilage further strengthen this concept, and give additional evidence for a substantial role of this structure in (early) OA.

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