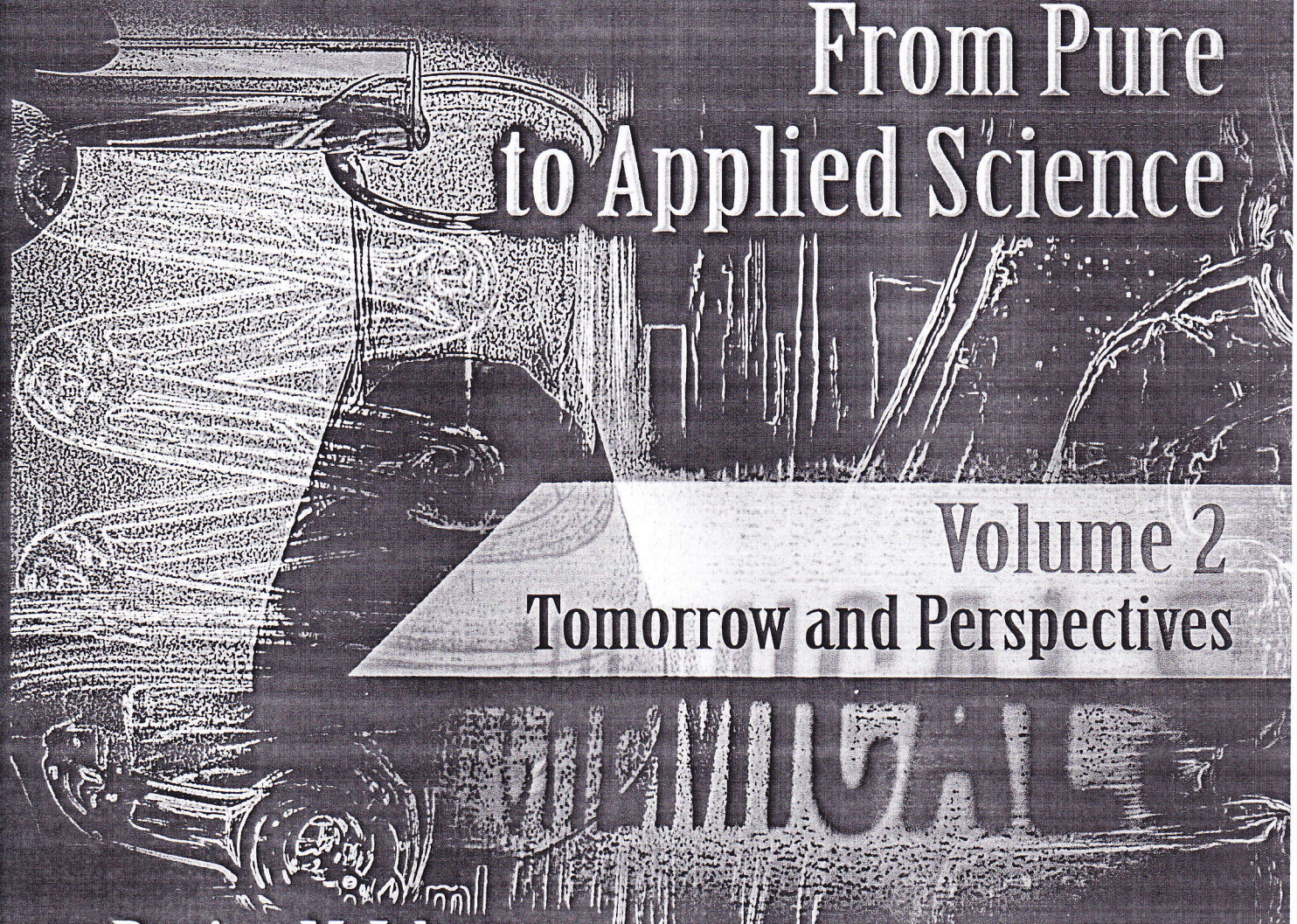
Kinetics, Catalysis and Mechanism of Chemical Reactions

From Pure to Applied Science



Volume 2
Tomorrow and Perspectives

Regina M. Islamova
Sergei V. Kolesov
Gennady E. Zaikov
Editors

Chemistry Research and Applications

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CHEMISTRY RESEARCH AND APPLICATIONS

KINETICS, CATALYSIS AND MECHANISM OF CHEMICAL REACTIONS

FROM PURE TO APPLIED SCIENCE
VOLUME 2 - TOMORROW AND PERSPECTIVES

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AND
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Chapter 1

REACTIVE OXYGEN SPECIES IN JOINT PHYSIOLOGY: POSSIBLE MECHANISM OF MAINTAINING HYPOXIA TO PROTECT CHONDROCYTES FROM OXYGEN EXCESS VIA SYNOVIAL FLUID HYALURONAN PEROXIDATION*

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ABSTRACT

The present paper proposes an original hypothesis on so far unrecognized function of synovial fluid hyaluronan in articular joint physiology. Since there are day-night periods of physical activity, the synovial joint experiences stages of hyperemia and ischemia, respectively. Hence, the synovial fluid may face the increased or decreased levels of oxygen, leading to periods of hyperoxia or hypoxia of the cartilage. Though, what chondrocytes most require and prefer is a relative hypoxia. In fact, it is an increased oxygen supply and resulting oxidative stress that has been reported to be responsible for chondrocyte dysfunction and senescence.

A large body of evidence suggests that hyaluronan catabolism is a non-enzymatic process, occurs *via* oxidative degradation and has typical features of peroxidative chain reaction. Apparently, as the concentration of oxygen in the synovial fluid is low during night, the free oxygen radical-mediated degradation of hyaluronan may be significantly inhibited, while in the morning, with starting physical activity and with joint reoxygenation, it may burst again. Initiation of hyaluronan peroxidation is suggested to be mediated by reactive oxygen species, particularly hydrogen peroxide, produced by chondrocyte mitochondria. Importantly, massive oxygen consumption occurs during propagation of the hyaluronan peroxidation; then a decreased availability of oxygen results in slowing down the chain reaction. This feedback mechanism may prevent a

*Dedicated to Professor Radomír Nosáľ, MD, DSc on the occasion of his 70th birth anniversary.
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decrease of oxygen concentration in the synovial fluid below the level that is optimal for normal functioning of articular chondrocytes.

Keywords: synovial joint hyperemia, ischemia; hypoxia-reoxygenation; hyperoxia; aggrecan.

INTRODUCTION

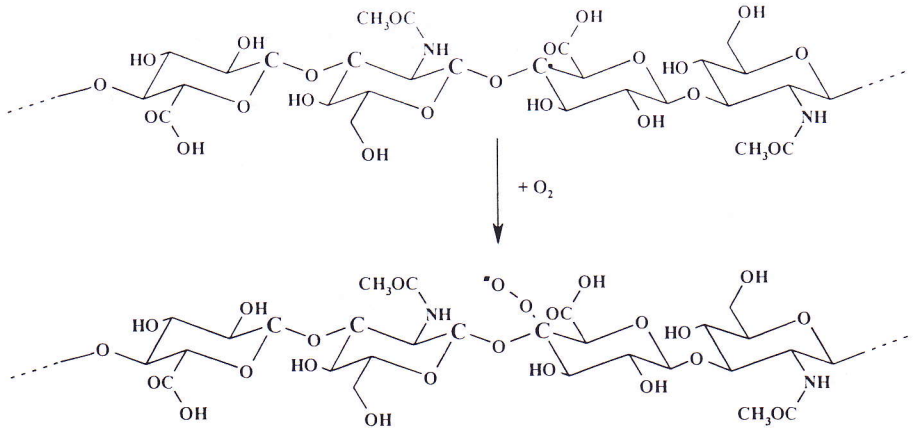
In this paper we present our hypothesis on a so far not recognized function of high-molar-mass hyaluronan (HA, hyaluronic acid) in normal physiology of synovial joints. It is generally accepted that one of the major functions of high-molar-mass HA is to maintain a proper viscoelasticity of the synovial fluid (SF) enabling it to work as a shock absorber, thus ensuring smooth joint movement, i.e. free articulation. MegaDalton-sized HA, synthesized by hyaluronan synthases, is extruded into the SF by type B synoviocytes embedded within the synovium – a few-cell-layer membrane [1]. The synovium also supplies articular cartilage, an avascular structure without innervation, with nutrients and oxygen, which diffuse through the SF into the cartilage.

In adulthood, the articular cartilage consists of amitotic yet metabolically very active chondrocytes, responsible for the production and maintenance of the extracellular matrix, representing a dense net of highly-organized macromolecules [2, 3].

It appears relevant to emphasize several important facts in the physiologic functioning of the synovial joint: (*i*) there is no direct blood supply of the inner structures of the joint, and thus delivery of O_2 and nutrients to the cartilage depends on their passive diffusion through the SF; (*ii*) chondrocytes require relatively hypoxic conditions for their normal functioning; (*iii*) as there are day-night periods of physical activity of man, the joint, specifically the synovium, experiences stages of hyperemia or ischemia [4]. By taking into account all the above mentioned facts, respective enhancement and attenuation of synovial filtrate production during day and night may be expected. Thus, as a result of the changing physical activity, the SF may encounter increased or decreased levels of oxygen, leading to periods of hyperoxia or hypoxia of the cartilage. However, what chondrocytes most require and prefer is relative hypoxia [5-8]. In fact, it is an increased oxygen supply and resulting oxidative stress that are responsible for chondrocyte dysfunction and/or senescence [9-14].

CIRCADIAN CYCLE VS. HYALURONAN HALF-LIFE IN HUMAN SF

Let us summarize some relevant consequences of the above-mentioned specific aspects of synovial joint physiology. As the joint is mostly supplied by oxygen during daytime, while hypoxia in the SF occurs overnight, the joint experiences an almost regular circadian cycle of hypoxia and reoxygenation. And, strikingly, the HA half-life ($t_{1/2}$) in the SF of healthy man is about 12 hours [15, 16]. This allows speculation on a possible relationship between the two processes. At this point a closer look at HA catabolism in the SF is required. Peculiarly, there is no enzyme to catabolize high molar mass HA, which nevertheless undergoes degradation, and the HA fragments are removed from the SF via drainage of the lymphatic system. Indeed, a large body of evidence has suggested that HA catabolism is non-enzymatic and occurs via



Scheme 1. Entrapment of oxygen by the hyaluronan *C*-macroradical, yielding a peroxyl (macro) radical.

its oxidative degradation, which has typical features of peroxidative chain reaction [17]. Initiation of the reaction involves hydrogen abstraction by a strong oxidant, such as hydroxyl radical, and results in the creation of hyaluronan *C*-macroradical. During propagation, molecular oxygen reacts with the *C*-macroradical (Scheme 1), and the oxygen addition is followed by immediate macromolecule cleavage yielding two fragments, one with a carbonyl, the other with a peroxyl group [18-20].

Eventually, the H radical is transferred from an adjacent HA macromolecule, thus forming the hyaluronan *C*-macroradical and keeping the oxidative HA degradation in process, which results in the consumption more and more oxygen molecules. The stage of termination usually occurs when substrates of the chain reaction are consumed. Apparently, as the oxygen delivery is rather limited during night and so the concentration of oxygen is low in the SF, the radical reaction may be terminated or significantly retarded by the end of the day and overnight. On the other hand, the chain reaction should burst again in the morning with starting physical activity and resulting reoxygenation of the joint.

CHONDROCYTE "NORMOXIA"

Logically, a question arises in this connection. Is the synthesis of megaDalton–sized HA, its oxidative degradation and subsequent removal of one half of the synthesized HA from the SF within 12 hours not too "luxurious" an event for the organism? We raised the hypothesis that high-molar-mass HA, besides acting as the main factor of high viscoelasticity of the SF, plays an important role in keeping a low oxygen concentration in the vicinity of the articular cartilage. Actually, close to the synovial membrane, the partial pressure of oxygen was found to be 13 %, decreasing in the SF towards the cartilage and reaching 6 % at the surface of the cartilage, while in the deep layers of the cartilage it is down to 1-2 % [4]. These conditions would be hypoxic for other tissues, yet not for the cartilage. It is important to note that articular chondrocytes appreciate this low oxygen level, which is in fact "normoxic" for them [4, 6, 7]. Conceivably, resulting from low oxygen availability, chondrocytes obtain most of their energy from glycolysis, as the activity of their oxidative phosphorylation is limited.

Compared to other tissues, mitochondria in chondrocytes are not abundant and they are often equipped with an incomplete set of respiratory chain complexes [4].

Thus, metabolically active chondrocytes operate at a relatively low oxygen tension of ~2 %. Supposing that this rather low oxygen concentration is optimal for normal chondrocyte functioning, an effective way of maintaining oxygen at a low level should be desirable. This is of particular importance during daytime when massive supply of oxygen into the SF may occur and so its excess has to be removed to attain the optimal level. In support of our original hypothesis, we suggest a possible mechanism of keeping the oxygen concentration low in the SF. The mechanism involves synovial fluid hyaluronan peroxidation, consumption of oxygen excess and regulated production of free oxygen radicals serving as signaling molecules.

As already mentioned, quite a good portion of oxygen is spent under physiological conditions during non-enzymatic HA catabolism, i.e. during its oxidative degradation. In fact, massive oxygen consumption occurs during propagation of the peroxidative chain reaction. It is noteworthy that for each cleavage of hyaluronan one molecule of oxygen is consumed and for thousands of cleavages readily occurring in the radical-chain-reaction propagation phase thousands of oxygen molecules should be removed. As native/parent HA is up to the size of several megaDaltons, many fragmentations do occur, and thus the consumption of oxygen is high. Importantly, decreased availability of oxygen slows down the chain reaction. We suppose that this feedback mechanism may prevent the decrease of oxygen concentration in the SF below the optimal ("normoxic") level for chondrocytes.

Initiation of Radical Chain Reaction – Reactants for Fenton Type Reaction

So far our novel concept covers a feasible mechanism readily removing oxygen excess from the SF and providing a conceivable explanation why the HA half-life is approximately 12 hours. Nevertheless, one more important question of the chain reaction has to be explained. How does the chain reaction begin, since for its initiation the presence of a strong oxidant is required. May the hydroxyl radical ($^{\bullet}OH$) be involved? If so, where and how is it produced? Our hypothesis comprises also these considerations. Based on principles of radical and macromolecular chemistry, we suggest a possible way of $^{\bullet}OH$ generation and its involvement in the process.

In general, hydroxyl radicals are usually formed in biological systems via Fenton reaction: hydrogen peroxide (H_2O_2) reacts with ions of transition metals, such as Fe^{II} or $Cu^{I/II}$, yielding hydroxyl radical and hydroxyl anion. In fact, the concentration of both metals in the SF and cartilage is high enough and they are kept in their reduced state by the presence of ascorbate to be able to react readily with H_2O_2 and thus to produce hydroxyl radicals [21, 22]. Hydrogen peroxide, on the other hand, is *in vivo* usually created by dismutation of two superoxide anion radicals, which are produced by activated phagocytes, e.g. neutrophils. However, a situation involving phagocytic cells is under physiological conditions rather questionable as their presence in the normal SF is unlikely. In addition, there is no detectable

superoxide dismutase activity in the SF to catalyze superoxide dismutation, and the spontaneous reaction is too slow to produce enough hydrogen peroxide. So, where do the molecules of H_2O_2 come from?

Based on the fact that joint cartilage and chondrocytes in particular prefer hypoxic conditions, chondrocytes themselves should conceivably possess some mechanism of sensing the O_2 tension. These cells are actually metabolically quite active as they produce and maintain the extracellular matrix, i.e. cartilage. Yet chondrocytes produce most of their energy by the glycolytic pathway, although they hold mitochondria. At this point we would speculate that chondrocyte mitochondria may serve as oxygen sensors via producing H_2O_2, a common by-product of oxidative phosphorylation. Thus under increased supply of oxygen to the cartilage, chondrocytes may actually be a significant H_2O_2 source. Since H_2O_2 is quite stable, its uncharged molecules can diffuse readily out of the cells, passing membranes and reaching the extracellular matrix. Notably, both oxygen and H_2O_2 move along their gradients, which have opposite directions. Molecules of H_2O_2 may meet and react with transition metal ions bound to aggrecans in the outer layer of the extracellular matrix, which is in close contact with high-molar mass SF-hyaluronan (Figure 1). However, the objection arises why H_2O_2 rather than oxygen does react with transition metal ions, since they both pass it on their respective way from and to the chondrocyte. The answer is that metal ions are complexly bound within aggrecan associates, and thus their reaction with oxygen molecules is limited, while they react readily with hydrogen peroxide.

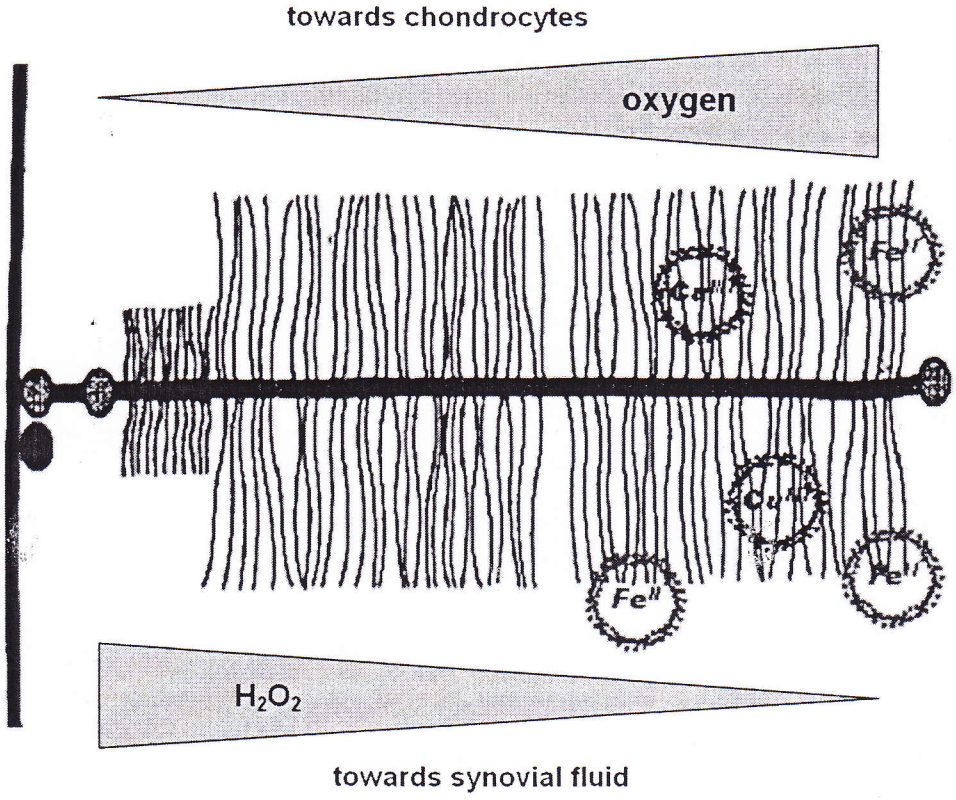


Figure 1. Depiction of transition metal ions (Fe^{II} and $Cu^{I/II}$) trapped by aggrecan in its outer shell and the opposite gradients of oxygen and hydrogen peroxide in the articular cartilage.

(This situation may somewhat resemble the condition of transition metal ions reactivity within active sites of metalloproteins.) Hence, Fenton reaction of hydrogen peroxide with Fe^{II} or $Cu^{I/II}$ may promptly occur at the surface of cartilage, producing enough hydroxyl radicals to initiate peroxidative degradation of SF-hyaluronan. This process consumes oxygen excess in the reaction propagation stage. In turn, a decrease of oxygen tension results in reduced chondrocyte mitochondrial respiration leading to decreased H_2O_2 formation and thus to retardation of hydroxyl radical production. Subsequently, the chain reaction may cease due to the absence of factors of initiation and propagation, i.e. hydroxyl radicals and oxygen. This is thus the mechanism responsible for keeping the oxygen tension at the desired level, a process which has an autoregulatory nature.

CLINICAL SIGNIFICANCE

It is important to appreciate that, compared to other tissues, articular cartilage, and chondrocytes in particular, prefer relatively hypoxic conditions [8, 11]. Conversely, high oxygen level has been demonstrated to be toxic for chondrocyte normal functioning [5, 23, 24]. Our original concept suggests a possible mechanism that may be involved in maintaining a low oxygen tension. During daytime physical activity, within the synovial membrane an increased blood circulation may result in increased concentration of oxygen and exert an adverse impact on chondrocyte function. Therefore, processes of sensing high oxygen tension and decreasing it are likely to be involved. It is well known that HA of the SF serves as a lubricant of the synovial joint. Previously, it has been reported that oxidative cleavage of HA is the main process of its catabolism in the SF [17]. During the propagation phase of HA oxidative degradation, a considerable amount of oxygen can be consumed, which may be a key process in maintaining the oxygen concentration at a desirably low level in the SF. The process of HA peroxidation may be understood as analogy to that of lipid peroxidation, which has been recently reported of having a dual function, i.e. playing not only deleterious role but also a physiological one [25]. We suggest that extracellularly released reactive oxygen species, particularly H_2O_2 produced in chondrocyte mitochondria, may serve as physiological messengers. In fact, the reactive oxygen or nitrogen species, which are capable of membrane-crossing and readily diffusion through extracellular space, such as hydrogen peroxide and nitric oxide, are known to serve as signaling molecules [26-28]. Generally, during the last decade, it appeared relevant that free oxygen radicals are involved not only in many pathological processes but also in physiological ones, particularly in cellular signaling [29-31]. And, that is why it may not be unequivocally distinguishable whether reactive oxygen species are cause or consequence of tissue injury [32]. Consequently, usage of antioxidants in treating pathologies involving oxidative stress requires thorough understanding of the involvement of reactive oxygen species in biological processes [33].

We are certainly aware that the described hypothesis has to be verified. To date, the literature provides only few indications and lack of evidence. We see several possible approaches to test our hypothesis. The first and logical one would be a circadian sampling of the SF from healthy volunteers and determination of HA fragments in samples of the active (daytime) and resting (nighttime) joint, which however presents serious ethical problems. Moreover, determination of HA fragments, particularly those with carbonyl and peroxyl

groups, is itself not an easy task since the SF contains a bunch of other high-molar-mass compounds as well as plenty low-molar-mass ones, which all may interfere with analysis. Probably the only feasible approach would be the use of magnetic resonance imaging (MRI) or spectroscopy (MRS), which are both non-invasive techniques, yet with certain limitations [34, 35]. Particularly MRI is generally suitable for imaging structural changes, usually due to pathological processes, while we need to follow subtle physiological changes in the molecule of HA. As for MRS, it can detect freely movable molecules, which may be again a problem for HA fragments determination in the highly viscous SF. Thus, to prove our novel hypothesis may be a real challenge for further investigations.

In conclusion, we are confident that the described processes are involved in the overall mechanism that keeps the oxygen concentration in the SF at optimal level for normal functioning of the articular cartilage. The suggested mechanism is initiated by chondrocytes whose mitochondria serve as oxygen sensors with H_2O_2 serving as a paracrine mediator. On balance then, the key process of the mechanism involves oxidative HA degradation in order to remove oxygen excess in the SF. If our suggestion is confirmed, its importance for clinical practice should become relevant since endogenous SF-hyaluronan may readily be destroyed by surplus oxygen in preparations generally injected in air-saturated, i.e. hyperoxic, solutions. Patients receiving intraarticular treatment would thus require the preparation to be applied into the joint in a hypoxic solution.

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