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Full Length Research Paper

Effect of progressive decrease of oxygen tensions on rat articular chondrocytes: a Light and scanning electron microscopy study

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Oxygen is a critical parameter proposed to modulate the functions and structures of chondrocytes. Articular cartilage is comprised of chondrocytes surrounded by a dense extracellular matrix. Collagen forms the structural skeleton of the tissue. At the onset of cartilage degeneration, due to surface fibrillation, such gradients have been proposed to break down, thus contributing to the progression of the disease. This article investigates the effect of low oxygen percentage at different periods on the rat articular chondrocytes at the histological level. Forty healthy young male albino rats were used in this study and divided into four groups. The control group admitted in normal environment at sea level rates. The second, third and fourth groups admitted in high altitude environment above sea level for ten days, twenty days and thirty days respectively. At the end of the previous mentioned periods the rats were anesthetized and the knee joint dissected and the menisci were taken and prepared using hematoxylin and eosin (H and E), Masson's trichrome stains for light microscopy and prepared also for scanning electron microscopy (SEM) examination. On progression of low oxygen tension, by H and E stain highly destructed chondrocytes, pyknotic nuclei, no clear lacunae and shrunken cytoplasm were observed and by Masson's trichrome stain more concentrations of collagenous fibers that are destructed and on SEM examination chondrocytes showed disintegration of chondrocytes with disrupted microvilli on their surface with elevations containing aligned hydroxyapatite crystallites and destructed collagen fibrils were observed. Low oxygen tensions could modulate the chondrocytes and collagen fibrils and promote its extracellular matrix production.

Keywords: Varying Oxygen Tensions, Rat articular Chondrocytes, Light and Scanning Electron Microscopy

INTRODUCTION

Articular cartilage (AC) is located on the surfaces of

bones in all joints enabling smooth frictionless movement while also dissipating stresses in the joint and acting as a loadbearing surface. The structure of AC is described to consist of parallel fibres, orientated perpendicularly to the bone, which bend under pressure but completely recover

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once the load has been removed. Since then, the study and understanding of AC has undergone large transformations. Today, research on AC is even more important due to the rising numbers of people suffering from diseases such as osteoarthritis and rheumatoid arthritis, debilitating conditions caused by a breakdown in the structure and functionality of AC (Hughes et al., 2005). AC is comprised of chondrocytes surrounded by a dense extracellular matrix providing the tissue with its unique biomechanical properties. Collagen forms the structural skeleton of the tissue, enclosing a hydrated proteoglycan (PG) gel that exerts an internal pressure of approximately 2-3atm (Urban et al., 1979; Basser et al., 1998). The collagen skeleton resists both the pressure from the PGs and the shear stresses produced during joint movement (Basser et al., 1998; Bank et al., 2000). Type 11 collagen has been recognized as the major collagenous component of cartilagenous tissues for a number of years (Miller, 1976).

Most living cells and tissues are exposed to reduced levels of oxygen depending on the distance to the source of oxygen (Semenza, 2001; Distler et al., 2004). Sudden loss of physiological oxygen levels, as observed in coronary heart disease or brain infarction, leads to massive cell death within these organs. Certain tissues and cells, however, are able to survive in hypoxic or nearly anoxic environments. The most impressive cells in this regard are articular chondrocytes that are embedded in an extensive extracellular matrix with an extremely long diffusion distance from the nourishing arteries. To survive these hypoxic or anoxic environmental conditions, mammalian cells possess highly conserved adaptive mechanisms (Distler et al., 2004; Wenger and Gassmann, 1997).

Oxygen is a critical parameter proposed to modulate the functions of chondrocytes ex-vivo as well as in damaged joints (Ströbel et al., 2010). Oxygen is a critical parameter proposed to modulate chondrocyte metabolic activity (Malda et al., 2003). Indeed, articular cartilage is generally exposed to a finely regulated gradient of relatively low oxygen percentages (from about 10% at the surface to about 1% in the deepest layers) (Silver, 1975) which is essential for maintenance of specialized tissue function (Gonsalves et al., 2000).

During the onset of cartilage degeneration, possibly due to surface fibrillation and/or microfractures of the subchondral bone, such gradients have been proposed to break down (Grimshaw and Mason, 2000), thus contributing to the progression of the disease.

The meniscus is a specialized fibrocartilaginous tissue located in the knee joint where it functions to aid joint stability, protect articular cartilage, absorb shock and transmit load. Meniscal cartilage displays a poor repair capacity, especially when injury is located in the

avascular region of the tissue. In this study, we will explore the effect of oxygen at different grades oxygen tension on the chondrocytes and to determine whether low oxygen tension affects the matrix density, as well as quantity, of cartilage formed at histological and scanning electron microscope level.

MATERIALS AND METHODS

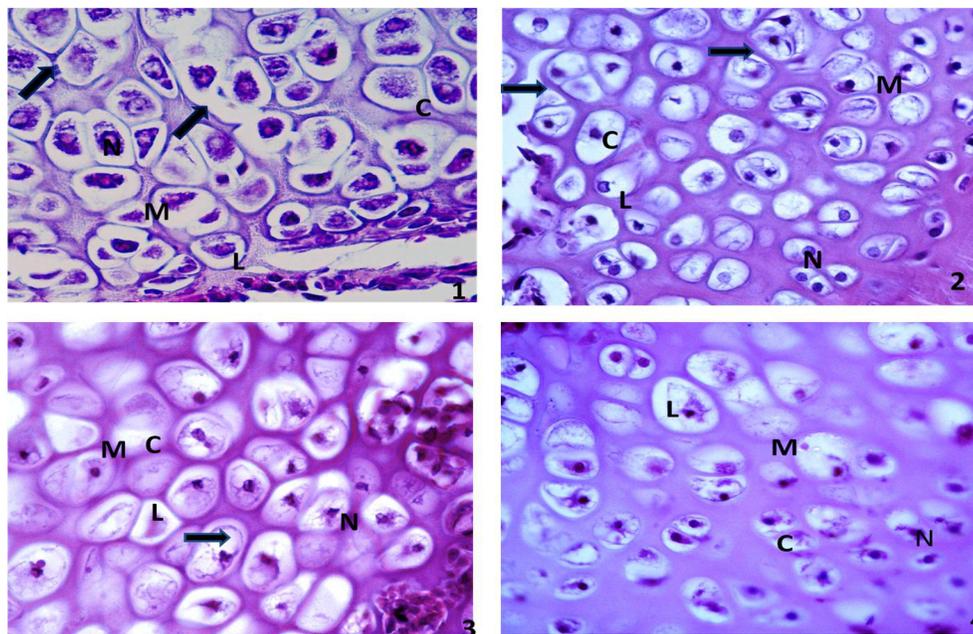
Forty healthy young male albino rats, having average weight of 200 grams for each, were used in this study. The animals obtained from the animal house of King Khalid University at sea level where they were fed with standard feed and allowed free water excess. The rats will divide into four groups (10 rats for each). The control group admitted in normal environment in Darb city at sea level for one month, where the animals housed in open mesh-wire cages in temperature-controlled room at 22-24°C and 50-60 % relative humidity with 12-hr light dark cycle (Soldani et al., 1997). The second, third and fourth groups admitted in high altitude environment (in the animal house of Collage of Medicine King Khaled University Abha city 2200 meters above sea level) for ten days, twenty days and thirty days respectively where they were fed with standard feed and allowed free water excess. At the end of the previous mentioned periods the rats anesthetized by inhalation of ether solution, the knee joint dissected and the menisci were taken and prepared for light and scanning electron microscopy examination.

For light microscopy

Formaline-fixed tissue was dehydrated in a graded series of ethanols, cleared in amyl acetate, embedded in paraffin and sectioned at 10 µm. Sections mounted on glass slides were stained with hematoxylin and eosin and Masson's trichrome stains Grogan et al. (2003).

For scanning electron microscopy (SEM)

Cartilage tissue fixed either in 95% alcohol or in glutaraldehyde was treated with sodium hypochlorite in commercial Clorox (Boyde, '721, used either full-strength or diluted to 50%, in order to remove organic material and expose mineralizing surfaces of tesserae. Specimens were washed in running water, dehydrated with a series of ethanols and mounted with Duco cement on 1/8-inch aluminum stubs. After drying in air they were coated with gold in a sputtering apparatus at the College of Medicine Electron Microscopy Laboratory, and viewed there in a JEOL Scanning Electron Microscope, model JSMU3,



[Plate A] Photomicrographs of the rat's knee joint cartilage stained with haematoxylin and eosin (Original magnification 400) from:

- Control group (Figure 1) showing chondrocytes (C) lying centrally within its lacunae (L) that are arranged in groups (arrows). The cells show finely granular cytoplasm that contains discrete vacuoles and are surrounded by a cartilage matrix (M) and centrally located nuclei (N).

- Second group (Figure 2) showing disrupted chondrocytes (C) lying centrally within its lacunae (L) that are arranged in groups (arrows). The cells show finely granular cytoplasm that contains discrete vacuoles and are surrounded by cartilage matrix (M) which is faint around most of the chondrocytes with pyknotic centrally located nuclei (N).

- Third group (Figure 3) showing disintegrated chondrocytes (C) lying within its lacunae (L) which are not clear. The cells show its cytoplasm with many vacuoles inside it and are surrounded by a cartilage matrix (M) which is devoid in most of the cells. Note more spaces inside the chondrocytes in most of them (arrow) with pyknotic centrally located nuclei (N) are clearly seen.

- Fourth group (Figure 4) showing highly destructed chondrocytes (C) with pyknotic nuclei with no clear lacunae (L), shrunken cytoplasm that contains many vacuoles and surrounded by a faint cartilage matrix (M). Note most of the cells have atrophic nuclei (N).

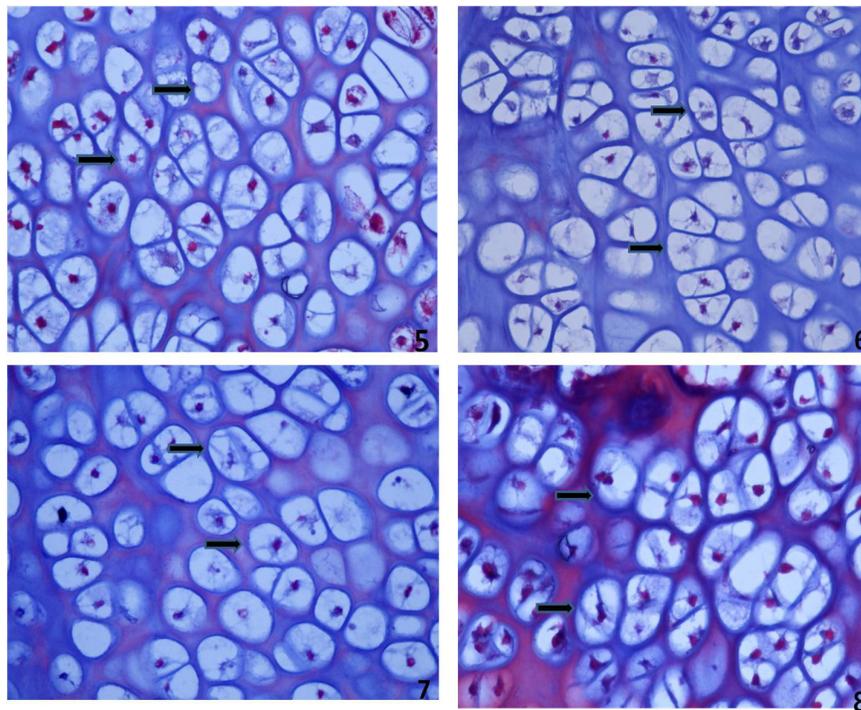
operating in the secondary electron mode at 15 Kv. Micrographs were taken on 4- X 5- inch Polaroid film, type 55 P/N. The samples were then prepared for SEM Stolz et al. (2009).

RESULTS

Hematoxylin and eosin stain

In the present study, sections from a rat' cartilage of the first group (control group) demonstrated that the chondrocytes were lied centrally within its lacunae that are arranged in groups. These cartilage cells showed finely granular cytoplasm that contains discrete vacuoles and were surrounded by a cartilage matrix and centrally located nuclei (Plate A, Figure 1). While the sections from a rat' cartilage of group two that admitted in high altitude

environment for ten days showed enlarged and swollen chondrocytes lied centrally within its lacunae that were arranged in groups. The cells appeared with finely granular cytoplasm that contains discrete vacuoles and were surrounded by a cartilage matrix which was faint around most of the chondrocytes (Plate A, Figure 2). In the third group that admitted in high altitude environment for twenty days showed disrupted chondrocytes lied within its disrupted lacunae. The cells appeared its cytoplasm with many vacuoles, were surrounded by a cartilage matrix which is devoid in most of the cells and more spaces inside the chondrocytes in most of them were clearly seen (Plate A, Figure 3). Lastly the fourth group that admitted in high altitude environment for thirty days showed highly destructed chondrocytes with pyknotic nuclei with no clear lacunae. The cells appeared with shrunken cytoplasm with many vacuoles and were surrounded by a faint cartilage matrix (Plate A, Figure 4).



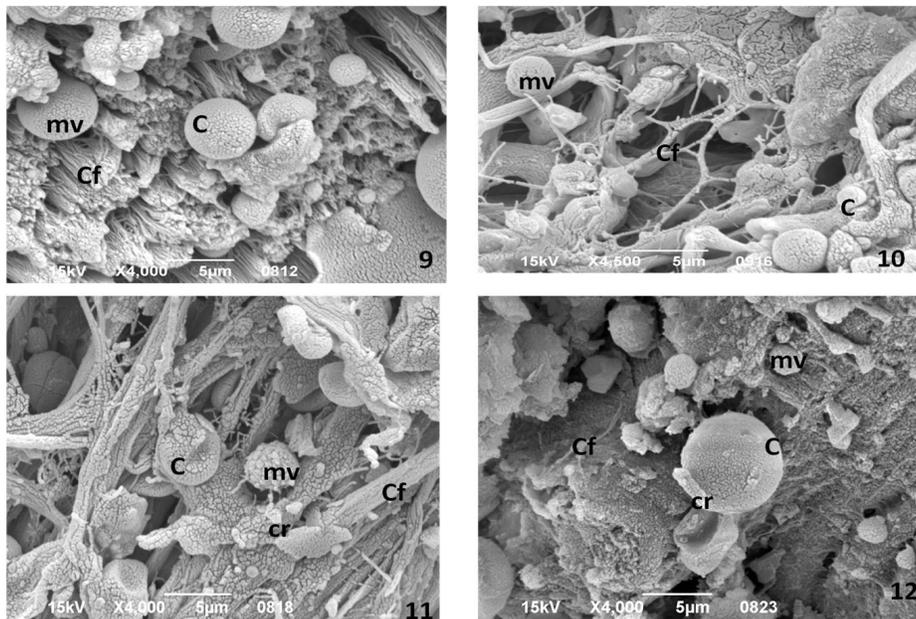
[Plate B] Photomicrographs of the rat's knee joint cartilage stained with Masson's trichrome stain (Original magnification 400) from:

- Control group (Figure 5) showing chondrocytes with its nuclei, a vacuolated cytoplasm and separated by little intercellular substance that are associated with concentrations of collagenous fibers (arrows).
- Second group (Figure 6) showing chondrocytes with disrupted nuclei and a vacuolated cytoplasm and separated by more or less little intercellular substance and are associated with concentrations of collagenous fibers especially around the disrupted chondrocytes (arrows).
- Third group (Figure 7) showing chondrocytes with disturbed nuclei and a clear cytoplasm and separated by more spaced with condensed collagen fibers (arrows) around most of the chondrocytes.
- Fourth group (Figure 8) showing chondrocytes with spherical nuclei and a vacuolated cytoplasm and separated by little intercellular substance and are associated with more concentrations of collagenous fibers that are destructed around some of the chondrocytes (arrows).

Masson's trichrome stain

In the present study, sections from a rat' cartilage of the first group (control group) demonstrated chondrocytes with its nuclei and a vacuolated cytoplasm and separated by little intercellular substance and are associated with concentrations of collagenous fibers (Plate B, Figure 5). While the sections from a rat' cartilage of the second group that admitted in high altitude environment for ten days showed chondrocytes with disrupted nuclei and a vacuolated cytoplasm and separated by little intercellular substance and were associated with concentrations of collagenous fibers especially around the disrupted chondrocytes (Plate B, Figure 6). The sections from a rat'

cartilage of the third group that admitted in high altitude environment for twenty days showed chondrocytes with disturbed nuclei and a clear cytoplasm and were separated by more spaces with destructed collagen fibers around most of the chondrocytes. The intercellular substance and were associated with different concentrations of collagenous fibers (Plate B, Figure 7). Lastly the sections from a rat' cartilage of the fourth group that admitted in high altitude environment for thirty days showed chondrocytes with spherical nuclei and a vacuolated cytoplasm and were separated by little intercellular substance and were associated with more concentrations of collagenous fibers that are destructed around most of the chondrocytes (Plate B, Figure 8).



[Plate C] Scanning Electron micrographs of the rat's knee joint cartilage (Bar 5µm) from:

- Control group (Figure 9) showing the chondrocytes (C) that are spherical in shape and has microvilli (mv) on their surface and wrap around the collagen fibrils (Cf) which are abundant and extending from all areas of the chondrocyte cell creating a dense network.
- Second group (Figure 10) showing round chondrocytes (C) with abnormal microvilli (mv) on their surface and wrap around the collagen fibrils which fibrils are abundant and disrupted extending from all areas of the chondrocyte cell creating a dense network. Chondrocytes were crimped and detached from the collagen fibrils (Cf).
- Third group chondrocytes (Figure 11) showing round chondrocytes (C) with abnormal and enlarged microvilli (mv) on their surface and wrap around the collagen fibrils (Cf) which abundant and destructed extending from all areas of the chondrocyte cell creating a dense network. Chondrocytes were crimped and detached from the collagen fibrils. Here the elevations containing aligned hydroxyapatite crystallites (cr) project prominently above the level of microvilli.
- Fourth group chondrocytes (Figure 12) showing round chondrocytes (C) with disrupted microvilli (mv) on their surface with elevations containing aligned hydroxyapatite crystallites (cr) project prominently above the level of microvilli. Atrophy of some chondrocytes is seen. Collagen fibrils (Cf) are destructed and extending from all areas of a lesser network.

Scanning electron microscopy

SEM micrographs of chondrocytes of group 1 one of the present study showed the chondrocytes that were spherical in shape and had microvilli on their surface and wrapped around the collagen fibrils which were abundant and extended from all areas of the chondrocytes created a dense network (Plate C, Figure 9). While the SEM micrographs from a rat' cartilage of group two that admitted in high altitude environment for ten days showed rounded chondrocytes with abnormal microvilli on their surface that wrapped around the collagen fibrils which were abundant and disrupted extending from all areas of the chondrocytes created a dense network. Chondrocytes were crimped and detached from the collagen fibrils (Plate C, Figure 10). The SEM micrographs from a rat' cartilage of group three that admitted in high altitude environment for twenty days

showed rounded chondrocytes with abnormal and enlarged microvilli on their surfaces and wrapped around the collagen fibrils that were abundant and destructed extending from all areas of the chondrocytes created a dense network. Chondrocytes were crimped and detached from the collagen fibrils. Also, there were elevations containing aligned hydroxyapatite crystallites projected prominently above the level of microvilli (Plate C, Figure 11). SEM micrographs of chondrocytes from a rat' cartilage of group four that admitted in high altitude environment for thirty days showed rounded chondrocytes with disrupted microvilli on their surface with elevations containing aligned hydroxyapatite crystallites projected prominently above the level of microvilli. Some disintegration of chondrocytes was seen. Collagen fibrils were destructed extending from all areas of a lesser network (Plate C, Figure 12).

DISCUSSION

Articular cartilage is an avascular tissue with the primary mode of nutrient delivery (e.g. oxygen, glucose) supplied to chondrocytes via diffusion from the synovial fluid. It has been estimated that in vivo oxygen tensions decrease with depth from the cartilage surface, with the gradient dependent on the rate of oxygen transport through cartilage and the rate of cellular consumption (Zhou et al., 2004). Mathematical models have predicted that the oxygen tension at the cartilage superficial zone decreases with depth from 5% at the superficial zone to approximately 1% in the subchondral bone region (Zhou et al., 2004) while experimental models have estimated that the oxygen tension varies between 7% and 1% (Silver, 1975).

The present study revealed a typical time- and dose-dependent degeneration of articular cartilaginous tissues, showing the progress of cartilage degradation. The cells appeared with shrunken cytoplasm with many vacuoles and were surrounded by a faint cartilage matrix and on SEM examination chondrocytes showed disrupted microvilli on their surface with elevations containing aligned hydroxyapatite crystallites projected prominently above the level of microvilli. Some disintegration of chondrocytes with destructed collagen fibrils extending from all areas of a lesser network was seen. In the study done by Ströbel et al. (2010), they found that at low, more physiological (5%) oxygen percentage has a dual role in HAC metabolism, namely to enhance the proteoglycan and collagen synthesis and at the same time to reduce the activity of two key catabolic enzymes involved in cartilage breakdown (that is, metalloproteinases). As a consequence, articular cartilage exposure to 19% oxygen reduced the de novo formation of cartilage tissue and induced degradation of pre-deposited collagen fibrils, leading to structural features similar to those found in osteoarthritic tissue. Interestingly, articular cartilage appeared to be highly sensitive to the oxygen percentage applied during differentiation culture in pellets, but not during expansion in monolayers.

However, low oxygen tensions and hypoxia-inducible factor-1a are important factors in articular chondrocyte behaviour during cartilage homeostasis and osteoarthritis. Hypoxia-inducible factor-1a is a highly conserved transcription factor that has key functions in controlling energy generation, cell survival and matrix synthesis by articular and growth-plate chondrocytes (Pfander and Gelse, 2007). Low oxygen tension (5%) was observed to promote extracellular matrix production by chondrocytes cultured in the absence of TGF- β 3, but was inhibitory in the presence of TGF- β 3. In contrast, a low oxygen tension enhanced chondrogenesis of infrapatellar fat pad constructs in the presence of TGF-

β 3, leading to superior mechanical functionality compared to chondrocytes cultured in identical conditions (Buckley et al., 2010).

In the current study revealed a typical time- and dose-dependent of oxygen tension by Masson's trichrome stain more concentrations of collagenous fibers that are destructed around most of the chondrocytes. Subsequent cartilaginous tissue formation in pellets was not affected as qualitatively assessed by safranin-O staining. At the oxygen concentrations evaluated, no effect of oxygen tension was observed on proliferation, oxygen consumption, and yield of lactate on glucose administration. For future investigations of chondrocytes and oxygen, the bioreactor system, which allows precise control and monitoring of oxygen tension, holds promise (Malda et al., 2004).

Moreover, in this article, the cells appeared with shrunken cytoplasm with many vacuoles and were surrounded by a faint cartilage matrix in some chondrocytes of the articular cartilage. Osteoarthritic chondrocytes are considered to be still metabolically active and may not only be characterized by an increased synthesis of matrix destructive enzymes, but also by an enhanced gene expression of type II collagen and several other matrix components depending on the stage of osteoarthritis (Aigner et al., 2001). This activity of osteoarthritis chondrocytes is generally appreciated as a response to restore the extracellular matrix. In different studies with biochemical as well as molecular biology approaches, the synthesis of type II collagen was found to be increased between four and seven-fold during osteoarthritis (Lippiello et al., 1977; Nelson et al., 1998).

In this work there was disruption of some chondrocytes on progression of low oxygen tension. Oxygen tension would appear to play a key role in regulating chondrogenesis during tissue development and regeneration. There is a strong relationship between oxygen concentrations and tissue differentiation during limb development (Bassett CA, Herrmann 1961) and during in vivo fracture repair with low oxygen tension favouring the formation of bone through endochondral ossification (Heppenstall et al., (1975) & Brighton and Krebs (1972) & Ham 1930). In cartilage explant models, low oxygen tension has been shown to promote cartilage specific extracellular matrix production (Pfander et al., 2003 & Ysart and Mason 1994), with oxygen tensions of 5% shown to significantly increase proteoglycan and collagen synthesis compared to culturing at 20% or at anoxic conditions (1%) only (Fermor et al., 2007). In addition, when combined with intermittent dynamic compression, oxygen tension has been shown to have a significant effect on the induction of inflammatory mediators in cartilage explants such as nitric oxide and prostaglandin E2 (Fermor et al., 2005). From a

regenerative medicine or tissue engineering perspective, oxygen tensions and oxygen gradients would also appear to play a key role in regulating the phenotype and biosynthetic activity of cells intended for therapeutic applications (Malda et al., 2003).

Within synovial joints, oxygen supply to articular chondrocytes is very limited and depends on the oxygenbinding capacity of synovial fluids and its flow during joint motion (Lund-Olesen, 1970; Schneider et al., 1996; Lee et al., 2007). It has been shown that oxygen tensions vary from around 6% at the joint surface to 1% in the deep layers of healthy articular cartilage (Silver, 1975; Ogata et al., 1976). Despite secondary inflammatory mechanisms during osteoarthritis, which are accompanied by an increased blood vessel formation in the synovial membrane and neoangiogenesis from the underlying bone into the deep zone of osteoarthritis cartilage, the hypoxic environment, in which articular chondrocytes have to exist, seems to be more pronounced in osteoarthritis.

CONCLUSION

This study demonstrates that low oxygen tensions could modulate the chondrocytes, collagen fibrils and promote its extracellular matrix production.

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