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Research Paper

Microhardness of human cancellous bone tissue in progressive hip osteoarthritis

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ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form
20 June 2016
Accepted 18 July 2016
Available online 22 July 2016

Keywords:
Microhardness
Osteoarthritis
Hip
Cancellous bone
Mineralization

ABSTRACT

Bone tissue is a biological system in which the dynamic processes of, among others, bone formation or internal reconstruction will determine the spatial structure of the tissue and its mechanical properties. The appearance of a factor disturbing the balance between biological processes, e.g. a disease, will cause changes in the spatial structure of bones, thus affecting its mechanical properties.

One of the bone diseases most common in an increasingly ageing population is osteoarthritis, also referred to as degenerative joint disease. It is estimated that in 2050 about 1300 million people will show symptoms of OA. The appearance of a pathological stimulus disturbs the balance of the processes of degradation and synthesis of articular cartilage, chondrocytes and the extracellular matrix, and the subchondral bone layer. As osteoarthritis progresses, study of the epiphysis reveals increasingly widespread changes of the articular surface and the internal structure of bone tissue.

In this paper, the authors point out the differences in the mechanical properties of cancellous bone tissue forming the proximal epiphysis of the femoral bone during the progressive stages of OA. In order to determine microproperties of bone trabeculae, specimens from different stages of the disease ($N=9$) were subjected to microindentation testing, which made it possible to determine the material properties of bone tissue, such as microhardness HV and Young's modulus E. In addition, mechanical tests were supplemented with Raman spectroscopy, which determine the degree of bone mineralization, and measurements of structural properties based on analysis using microCT. The conducted tests were used to establish both quantitative and quantitative description of changes in the structural and mechanical properties connected with reorganization of trabeculae making up the bone in the various stages of osteoarthritis. The proposed description will supplement existing knowledge in the literature about identification of the processes occurring during the development of this disease.

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http://dx.doi.org/10.1016/j.jmbbm.2016.07.022
1751-6161/

1. Introduction

Osteoarthritis is one of the most frequent diseases of the osteoarticular system in the ageing population and is associated with the most stressed joints, such as the hip and the spine (Murphy et al., 2010). In year 2010, Global Burden of Disease Study, stated that the increase of musculoskeletal disorders cases was greater than previously predicted. At present, WHO estimates that 10–15% of adults over the age of 60 have signs and symptoms associated with progressive OA. According to the projections of the United Nations, by 2050 over 20% of the world population over the age of 60 will have symptomatic OA. In one-third of this group, i.e. 40 million, OA will cause disability and immobilization.

The hip joint is particularly vulnerable to degenerative changes due to its design and the complexity of loads, which are the resultant force of upright stabilization of the body and the range of movements performed in the joint. Continuous overloading may cause chronic inflammation within the joint and contribute to the formation of osteoarthritic changes. Patients with diagnosed coxarthrosis, constituting approximately 30% of all patients with OA, complain of limited joint mobility, impeded locomotion, and pain when walking. Patients in the advanced stages of the disease undergo total hip arthroplasty in order to recover lost mobility in the joint. The procedure involves resection of the damaged proximal femoral epiphysis and implantation of an endoprosthesis.

Osteoarthritis is the result of biological and mechanical changes disturbing the balance of the processes of degradation and synthesis of articular cartilage, chondrocytes and the extracellular matrix, and the subchondral bone layer. So far, osteoarthritis has been considered a disease of elderly (Felson et al., 2000), which results in a significant reduction in the quality of bone, making it more susceptible to damage. However, recent reports indicates that the occurrence of OA should not be associated exclusively with old age. It is generally suggested that risk factors contributing to disease development include obesity and the sex of the patient. Women, due to the rapid change in their hormonal balance, have a higher risk of occurrence of osteoarthritis. Other factors that increase the likelihood of the disease include the lifestyle and genetic predispositions (Perilli et al., 2007).

As a result of degenerative changes, the joint is loaded non-physiologically. The imbalance between the interacting external and internal forces that stabilize the epiphysis in the acetabulum will initiate a series of changes within the bone. The increasing pressure on the bone tissue stimulates the processes of intensive reconstruction of the trabecular structure within the epiphysis, there is a noticeable prevalence of resorption processes over osteogenic processes, and the spatial structure of bone tissue adjusts to new biomechanical conditions. Due to overgrowth of bone tissue, the epiphysis loses its normal, sphere-like shape and becomes asymmetrical. The growing friction between joint elements leads to a gradual attrition of the articular surface. Initially, local loss of cartilage is observed, which expands until the bone is completely exposed. The gradual degradation of the articular surface leads to an overgrowth of bone tissue in the subchondral region and directly impacts on the reorganization of the structure. It is why the analysis of the cartilage structure has been found to be a good parameter in determining the stage of OA (Outerbridge, 1961). The change of biomechanical conditions in the subchondral region leads to micro injuries in the bone structure, which are one of the factors causing of cysts formation. Intensive metabolic and biomechanical processes taking place in the bone tissue disrupt the mineral balance within the joint, inducing a change in the degree of mineralization of the pathologically altered tissues. Recognized classifications of osteoarthritis are based mainly on the description of changes taking place only in the cartilage tissue area. Changes within the cartilage tissue occurring in the first stage of OA manifest themselves by softening and swelling and by slow gradation of tissue in stressed areas leads to the complete exposure of the subchondral bone. However, those classifications do not take into account changes in the properties of bone tissue in progressive stages of the disease and, in our opinion, in order to characterize the complexity of the processes occurring in the proximal femoral epiphysis in the various stages of the disease (Kozun et al., 2014a, 2014b), it is necessary to quantify the lesions associated with other tissues of the joint, i.e. entire cross-section of bone tissue. The main causes of these changes are not only changes of the mechanical properties of bone tissue, largely related to changes in the joint loading conditions, but also a change in the quality of the tissue itself, associated with disturbances of its metabolic and mineralization processes.

The aim of this study was to determine the micromechanical properties of bone trabeculae obtained from human proximal femoral epiphysis, representing progressive stages in the development of osteoarthritis. Therefore, we conducted microindentation testing, which allowed us to determine the material properties of bone tissue, such as *HV* microhardness and Young's modulus *E*. In addition, mechanical tests were supplemented with Raman spectroscopy, which makes it possible to determine the degree of mineralization of bone tissue, and measurements of structural parameters of the tissue using microCT. The tests were conducted in three regions of interest within the femoral head, which allowed us to obtain distributions of the examined parameters.

2. Material

The research material consisted of proximal femoral epiphyses ($N=9$) obtained from patients with diagnosed osteoarthritis who had undergone total hip arthroplasty. Biological material was obtained form 7 male and 2 female, age 55 ± 23 relatively great dispersion of the subject age results form fact that early stages of OA are more likely to occur in younger patients while advanced degeneration in elderly patients. The scope of research program has been approved by the local bioethics committee (KB-86/2010) as well all the patients gave consent. Until the performance of the tests, the biological material was stored in polyethylene containers at the temperature of $-20\,^{\circ}$C.

In order to include specimen for the study authors relied on the medical diagnosis, patient X-ray documentation

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complemented with examination of the geometry of the proximal epiphysis, and the degree of damage to cartilage tissue. Based on that material was divided into three classes according to the recognized classification of osteoarthritis proposed by Outerbridge. For the purpose of this study form each classes three epiphyses being most representative for the group were chosen and grouped grades I, II, and III.

Each epiphyses was used to prepare two 1-mm thick slices from the center of the proximal femoral epiphysis (Fig. 1A). Obtained specimens were mounted in acrylic resin, which filled in the pores of the cancellous tissue, thus providing them with support during the microhardness test and eliminating the adverse effect of bending of trabeculae during testing. Whereas preparation of the sample surface is crucial for microhardness testing the samples were ground under constant flow of water with sandpaper of varying grit (from 240 to 2000) and polished, thereby obtaining plain section. The microhardness testing was carried out for three regions of interest (ROI) (Fig. 1B). The first measurement group comprised the subchondral region ROI-S located 5–15 mm beneath the articular surface, the second group – the medial region ROI-M up to 25 mm, and the third group – the distal region ROI-D up to 35 mm under the articular surface. The measurement regions were located along the axis of symmetry of the femoral head. These ROI regions were specified in order to determine changes in the structural and mechanical properties within the proximal femoral epiphysis in the various stages of the osteoarthritis of the hip.

3. Methods

In order to determine the hardness of bone trabeculae, we used the microindentation method, in which the hardness measurement specifies the resistance of a solid body to elastic deformations caused by local pressure of the indenter (force in mN). It is assumed that the indentation does not undergo elastic recovery after force removal. In the case of microhardness measurements both the method (Johnson and Rapoff, 2007), storage and preparation (Dall'Ara et al., 2007) of samples may affect the results, thus it is very important to appropriately prepare the specimen surface that will be subjected to impression. The surface should have the lowest possible roughness and should be plane-parallel, so that the indenter travels perpendicularly to the sample plane. What is more, in the case of microindentation test of bone trabeculae, special care should be taken when choosing the measurement site. Where possible, testing should be performed in the middle section of the trabecula in order to avoid the adverse effect of the mounting method on the obtained results.

Microhardness testing was carried out using a MicroCombi Tester® (CSM Instruments) with a Vickers indenter (*HV*). Parameters were calculated using the Oliver and Pharr (2004) method, allowing for the determination of the hardness of the test material without the need of measuring the diagonals of impression under the microscope. The values of the determined parameters are calculated based directly on the normal force values and the displacement of the indenter. The measurement method used in this way provides high accuracy, thanks to which it can be used for measurements both at the micro- and nanoscales (Sahar et al., 2005). In addition, the Oliver and Pharr method allows to determine the actual depth along which contact is made between the indenter and the specimen, which is used to calculate the indenter/sample contact surface and the *HV* hardness. The microhardness test can also be used to determine the Young's *E* modulus, which measures the stiffness of the test material.

3.1. *The degree of bone tissue mineralization*

From the point of view of its composition, bone tissue is a composite material consisting of inorganic and organic compounds, whose share may vary depending on the type of bone, age, diet, physical activity, or the ongoing disease processes. One of the parameters determining the quality of the bone and providing valuable information about the properties of tissue at the micro and nano scales is the degree of mineralization related to hydroxyapatite content (HAp). It has been theorized that osteoarthritis is accompanied by bone demineralization. The

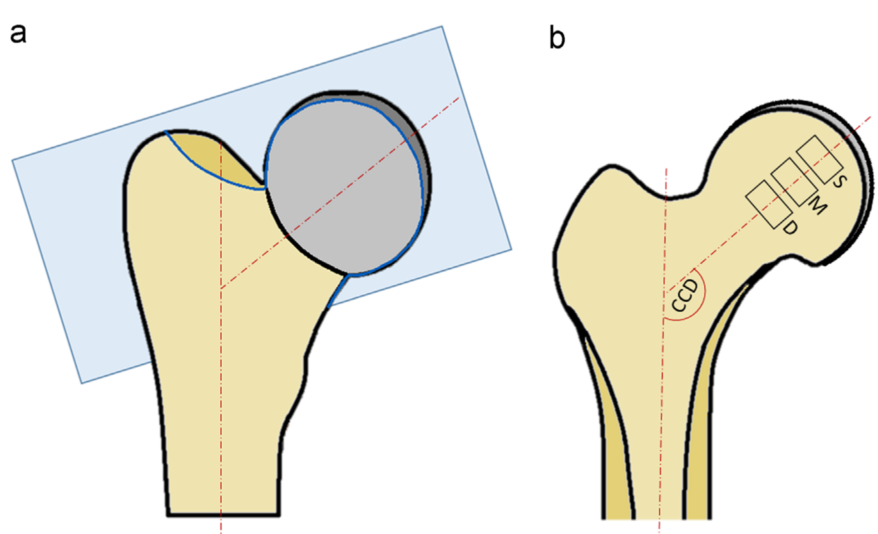

Fig. 1 – Schematic illustration of the cutting plane of the epiphyses (A) and the defined regions of interest – ROI (B).

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degree of bone mineralization can be determined from the Raman spectrum as a ratio of intensity of integral vibration bands $\nu^1(PO_4)$ (~960 cm^{-1}) derived from hydroxyapatite to the amid I band (~1 656 cm^{-1}), amid III band (~1 270 cm^{-1}), or collagen. The analysis of the ratio of carbonates to phosphates makes it possible to obtain information on the composition of the bones, which changes depending on the bone architecture, age, and mineral crystallization (Yerramshetty et al., 2006; Akkus et al., 2004).

The degree of mineralization of tissue was determined for trabeculae of the subchondral region coming from the second slice of the central part of the epiphysis. Raman 2D maps (FT-Raman Spectrometer Nicolet® NXR9650-Thermo Scientific) were made using an excitation radiation source (500 mW) laser Nd:YVO4 (λ=1064 nm). The signal was recorded using an air-cooled InGaAs detector in the spectral range of 4000–50 cm^{-1} with resolution of 8 cm^{-1} and the measurement accuracy of the wave number equal to 0.8 cm^{-1}.

3.2. *Trabeculae bone thickness*

Cancellous bone tissue forming the proximal epiphysis of the femoral bone is a spatial arrangement of bone trabeculae, which are classified as parallel plate-like elements with negligible space between them (Stauber and Muller, 2006). An important phase in the course of microhardness testing is choice of the appropriate measurement site, so that the results are reliable. Furthermore, form the point of the application the thickness of the analysed trabeculae should be large enough that the indenter does not perforate it during the test simultaneously force can not cause bending of the trabecula. In that case we can be sure that the measured results are only effect of the penetration process on the specimen by the indenter. Therefore, before the indentation test, the samples were registered using a SkyScan1172 microCT scanner (Bruker®, Belgium). The obtained images were used for analysis of 3D parameters, which made it possible to determine the structural properties of trabeculae forming the cancellous structure of the analysed samples. The average thickness of bone tissue ($Tb \cdot Th$), which determined the choice of the site of microhardness testing was analysed. On the basis of the conducted quantitative analysis it was determined that the mean trabecular thickness $Tb \cdot Th$ ranged from 0.24 mm to 0.31 mm. The above results were used to select the measurement site based on the width of the central part of the trabeculae and establish indentation depth, which should not exceed ~1% of the trabecular thickness.

3.3. *Statistics*

The obtained results were statistically analysed using the OriginPro8.0 software. The normal distribution was confirmed with Kolmogorov–Smirnov test, analysis of variance (ANOVA) and the significance levels were tested in compare to results obtained for the various stages of osteoarthritis.

4. Results

The relationship between the measured hardness value and the indentation depth [even for homogeneous materials] may influence microhardness testing. It is why the choice of proper measurement parameters is important. The force and depth of indentation, shall depend on microstructural properties of bone tissue, in this case lamellae, which are the basic building blocks of osteon and are organized into layers with a thickness of 1–3 μm and 2–4 μm. The main aim of this study is microhardness analysis of bone trabeculae; therefore, for the purposes of this study it was assumed that measurements would be made at the indentation depth of 2–3 μm (Hengsberger et al., 2002). Microhardness measurements use force-controlled indentation, with the penetration depth being the resultant value. In order to verify the measurement assumptions, preliminary studies were carried out to determine the indentation depth for various force values between 25 mN and 100 mN. From the analysis of the results it was determined that the obtained depth values for the force of 50 mN and the load time of 15 s corresponded best to the assumed indentation depth. On the basis of the results it was observed that the value of the applied normal force had no significant effect on the obtained hardness values. Microindentation testing was conducted for designated ROI by performing from 18 to 22 indentation tests in the central axis of the trabecula, with the distance between individual measuring points of no less than 2.5 times the diagonal of the impression (Fig. 2). Independent analysis of the results obtained from each proximal femoral epiphysis for particular regions of interest revealed that the data set is homogeneous, that motivated authors to perform further analyses on average of the 55–60 indentation per ROI. The obtained results are summarized in Table 1 are comparable with the results obtained by other researchers (Dall'Ara et al., 2011). Indentation tests conducted on a micro scale allow to specify hardness at the level of individual elements of the structure of cancellous bone tissue (trabeculae and osteons).

4.1. *The degree of bone mineralization*

The next stage of the research was the measurement of the degree of bone mineralization, in which Raman 2D maps were made for the various stages of osteoarthritis. The obtained maps were used to determine intensity of the phosphate band and the collagen band, whose ratio determinates the degree of bone mineralization (DBM). On the basis of the obtained results it can be concluded that the greatest DBM value in the subchondral region is demonstrated by the bone tissue obtained from patients classified as the first stage of OA. As the disease progresses, the degree of mineralization decreases. Because of this decrease, the trabeculae become weaker and weaker and the reduction of collagen content in the structure makes the bone less elastic and, consequently, more susceptible to damage. Table 2 shows the obtained values of the degree of bone mineralization for the various stages of OA.

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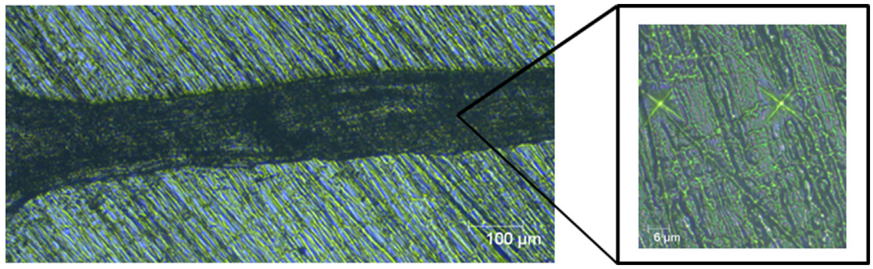

Fig. 2 – The cross-section of the femoral head trabecula, (magnification 200 ×) and impressions left by the Vickers indenter on trabecula surface (magnification 500 ×).

Table 1 – Mean values of the microhardness (*HV*) and Young's modulus (*E*) for bone trabeculae of the various regions of the proximal femoral epiphysis in progressive stages of osteoarthritis.

	I stage OA		*II stage OA*		*III stage OA*	
	HV *M* (SD)	E [GPa] *M* (SD)	HV *M* (SD)	E [GPa] *M* (SD)	HV *M* (SD)	E [GPa] *M* (SD)
ROI-S	44 (4)	9,0 (1,2)	41* (4)	7,0* (1,7)	39* (3)	8,6* (1,4)
ROI-M	53 (5)	11,8 (1,7)	43*(4)	7,2* (2.3)	45* (4)	10,2* (2,0)
ROI-D	45 (4)	8,7* (2,2)	43 (5)	8,3* (2,2)	44 (4)	10,7* (2,3)

M – Mean value, SD – Standard deviation.
* Statistically significant differences at the level $p<0{,}05$ in comparison to stage I OA.

Table 2 – Mean values of the degree of bone mineralization for ROI-S in the progressive stages of OA.

DBM [%]	*I stage OA*	*II stage OA*	*III stage OA*
M	71,3	68,5*	63,1*
SD	3,4	3,4	3,7

* Statistically significant differences at the level $p<0{,}05$ in comparison to stage I OA.

5. Discussion

Bone is a material with strong hierarchical structure, in which several levels can be distinguish: macro level (long bone), meso level (tissue), micro level (trabecula or osteon), and ultra level, where one can distinguish the lamella level or even individual HAp crystals (Currey, 2012). By studying bone tissue with the use of new measurement methods, we can compile a more detailed description of bone tissue, which is especially important in the case of a pathologically altered tissue. Studies of the mechanical properties of cancellous bone tissue are typically conducted on the tissue scale (meso level) on cubic or cylindrical samples of a specified volume (Kozun et al., 2014b; Nikodem, 2012; Tassani et al., 2010; Turner and Burr, 1993). The processes associated with reconstruction of bone tissue and its adaptation to the changing conditions often take place at the cellular level. Therefore, we should ask ourselves whether the determination of the properties of the entire structure at the meso level is sufficient. What is the impact of the spatial organization, compared to the properties of the bone tissue itself, on the obtained properties of individual elements forming the structure? Therefore, it is vital to determine if the observed changes stem directly from differentiation of bone structure as a result of disturbed balance between biological processes, which are observed as an increase in density (Li and Aspden, 1997) or degree of bone mineralization (Hart et al., 1994; Schneider et al., 2002).

In order to determine the properties of cancellous bone tissue at the microscopic level, Boivin et al. conducted a study in which, using microindentation techniques, they determined the averaged value of trabecular hardness from the entire cross-section of the proximal femoral epiphyses (Dall'Ara et al., 2011; Ziv et al., 1996). The aim of the conducted tests was to compare trabecular hardness of osteoporotic bone tissue in relation to normal bone tissue (Boivin et al., 2008). Similar studies were carried out by Dall'Ara et al. (2011), who analysed microhardness of the osteoarthritic tissue. In the presented studies, quantitative analysis was applied to bone trabeculae originating from the entire cross-section of the epiphyses, treating them as equivalent. Moreover, lack of information on the stage of the disease (OP or OA) and its correlation with the micromechanical properties of bone tissue precludes detail and complete description necessary to determine the pathomechanism of the disease. Therefore, it was, documented in an analysis on a macro scale that, as a result of osteoporosis, the strength parameters are reduced by 20–25% whereas osteoarthritis causes an increase in the value of mechanical parameters in the range of 30–50% (Li and Aspden, 1997). Analysis of the hardness values in the literature on a micro scale shows that in the case of normal bone the differences in hardness between compact and cancellous bone tissues are insignificant and, what is more, they can be regarded as the same kind of tissue with different spatial organization. As osteoarthritis progresses, bone hardness decreases and compact bone tissue becomes weakened in relation to cancellous tissue. It follows that on a micro scale osteoarthritis leads to almost twice the decrease in bone quality, making it more susceptible to injury (Dall'Ara et al., 2011; Boivin et al., 2008).

Taking into account the fact that in the case of osteoarthritis we have to consider both structural changes within bone tissue and metabolic changes, tissue differences must be assumed in accordance with advancement of the disease. In the literature there are OA classifications based on the amount of damage and the progressive erosion of cartilage as

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well as changes in the shape of the pathologically altered epiphysis. In our opinion, presentation of the results averaged for the entire cross-section of the epiphysis (Dall'Ara et al., 2011; Coats et al., 2003) does not fully reflect the entire scope of changes taking place during OA and only shows that such tissue is different from normal tissue. It should also be noted that the trabecular structure within the femoral head forms a distinctive arc (Van Rietbergen et al., 2003); therefore, we should consider whether bone tissue of the individual regions, especially along the main load direction, will not become structurally more isotropic.

Osteoarthritis results in a disturbance of the biomechanical conditions throughout the joint due to changes in the geometry of, among others, femoral head. This changes the load distribution in the joint; also, the surface area on which the forces are transferred from the acetabulum is reduced. A reduction of the above area increases the value of the compressive forces acting on the articular surface, which directly affects the subchondral region and constitutes a mechanical stimulus that triggers "negative" reconstruction processes in bone tissue. Intensive metabolic processes accompanying bone tissue reconstruction disturb the mineral balance within the tissue. The rapid leaching of minerals from bones into the bloodstream and the joint will cause demineralization of bones and a reduction in their strength and hardness, resulting in their increased fragility (Hengsberger et al., 2002; Kazanci et al., 2006; Yerramshetty and Akkus, 2008). The minerals accumulating in the articular capsule, synovial fluid, and articular cartilage start to crystallize and the resulting crystals initiate and deepen the processes of degradation of articular cartilage. The problem of cartilage degradation was often discussed in many works on changes in the metabolism of articular cartilage under the influence of mechanical factors (Glaser and Putz, 2002; Radin et al., 1984; Hellmich et al., 2004). When physicochemical changes occur that disturb the processes of mineralization, the collagen and mineral structure cannot be rebuilt and there is a reduction in the degree of bone mineralization.

Subject chosen for this study were representatives form largest set of femoral heads and aimed to represent the characteristic features of a given stage of OA regardless of the patients age. On the basis of the conducted studies we can conclude that, as osteoarthritis progresses, statistically significant changes occur in the values of microhardness *HV* of bone trabeculae (Fig. 3) and the degree of mineralization between the various stages of the disease and the regions of bones. What is more, the results indicate a heterogeneous nature of the structure within the proximal epiphysis. Assuming that in the first stage of the disease the occurring changes are the smallest and the bone tissue does not differ significantly from normal tissue, we can say that bone trabeculae of the subchondral region have hardness with the value of 44 HV. It was observed that, as the distance from the articular surface increases, the trabeculae are characterized by greater hardness. The properties of the distal region of the proximal femoral epiphysis are similar to the subchondral tissue.

As the disease progresses in the subchondral region, which is located in the immediate impact zone of the forces, the microhardness becomes reduced by 11%, which is associated with a reduction of bone tissue mineralization by 11.5%, Fig. 4 shows consistent between parameters. It can, therefore, be concluded that, as the disease advances, significant histological changes occur in the subchondral region that affect the mechanical properties of bone trabeculae. As the subchondral bone tissue degrades, the acting forces propagate into the proximal femoral epiphysis, which will be loaded to a greater extent. In the second stage of OA there is a visible reduction in hardness by 19%, which may be the result of intensive restructuring due to changes in the biomechanical conditions resulting from a change in the direction of the acting forces. When the structure becomes adjusted to new conditions, loading begins to strengthen, which is reflected in an increase of trabecula microhardness. Despite the fact that the study was conducted on relatively small number of subject obtained data of 60 results for each group gave consistent mean values as indicated by SD lower than 10% which contributed to the decision not to enlarge measuring groups.

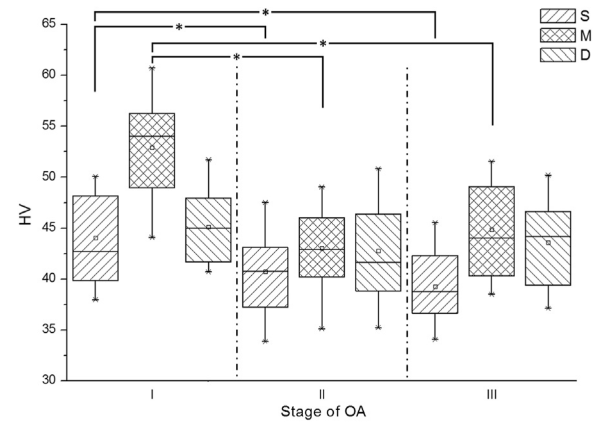

Fig. 3 – Graph of microhardness *HV* values in relation to ROI in the progressive stages of OA, *statistically significant differences at the level $p<0{,}05$.

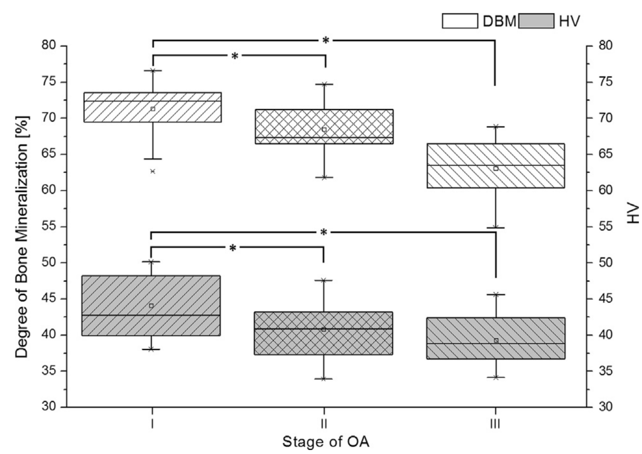

Fig. 4 – Graph showing relationship between changes in microhardness *HV* and DBM in relation to subchondral region ROI-S in the progressive stages of OA, *statistically significant differences at the level $p<0{,}05$.

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The conducted studies showed that in the distal part of the epiphysis shows no statistically significant changes in bone hardness during the OA progression. However, it can be assumed that in later stages of the disease, with the increasing weakening and destruction of the tissue in the subchondral region, the structure of the distal part of the epiphysis will gradually take over its function, which will manifest as changes in the micromechanical properties.

6. Conclusion

Our research shows that the progress of osteoarthritis is accompanied by significant and irreversible changes in the bone tissue. Between the various stages of the disease, we can observe statistically significant changes in the degree of bone mineralization, microhardness, and Young's modulus. In addition, our research indicated that bone tissue within the proximal epiphysis is not homogeneous in its mechanical properties; therefore, the measurement site must be taken into account. According to the authors, description of a disease entity such as osteoarthritis in a generalized way, which does not consider the stage of the disease and treats the tissue from the entire epiphysis in a uniform manner, is not sufficient for a complete description of the pathomechanism of the disease. Certainly hardness is not a suitable parameter for clinical diagnosis of the OA stage, however by showing differences in the hardness value authors wants to raise an important issue of the changes in the trabecular bone tissue. Presented study aimed to concentrate on changes in the trabecular bone material properties and provide micromechanical properties specially valid for numerical simulation and giving the scope of bone quality due to progression of the OA. Moreover by distinguishing regions of interests within the cross-section of the femoral hades we wanted to determine if the bone tissue is constant in the properties in regard to the specificity of disease. Therefore, the presented research is part of the study aiming to provides an extended, quantitative view of the changes in the quality of bone tissue in OA and expand the current stage of knowledge.

Acknowledgment

We would like to thank Professor Szymon Dragan, Clinical Department of Orthopedic and Traumatologic Surgery, Wrocław Medical University, for providing us with the study material. This work has been supported by the National Science Centre under the project No. N N518 505139.

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Please cite this article as: Tomanik, M., et al., Microhardness of human cancellous bone tissue in progressive hip osteoarthritis. Journal of the Mechanical Behavior of Biomedical Materials (2016), http://dx.doi.org/10.1016/j.jmbbm.2016.07.022

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