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AGE-RELATED CHANGES IN HUMAN INTERVERTEBRAL DISCS. A COMPARATIVE LIGHT-MICROSCOPIC, ELECTRON-MICROSCOPIC AND HISTOCHEMICAL STUDY

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Abstract — **The aim of present study is to evaluate age-related changes in human intervertebral discs using light-microscopy, transmission electron microscopy and histochemical techniques. Intervertebral discs from 10 adult human cadavers and 10 patients undergoing spinal surgery due to degenerative diseases of the spinal column, from different age, were examined. Using aforementioned techniques we established diminished number of chondrocytes and apoptosis of cells. Clusters of cells, changes in the lamellar structure, thickness and calcification of intervertebral discs were also found, as well as positive reaction for nicotinamide adenine dinucleotide phosphate-diaphorase, which is a marker for nitric oxide synthase and an indirect marker for nitric oxide, linked with the degenerative changes in the intervertebral disc**

Keywords - **Intervertebral disc, Human, Age-related changes, Degenerative changes.**

I. INTRODUCTION

The main components of the intervertebral disc (IVD) are nucleus pulposus (NP), annulus fibrosus (AF) and cartilage end plates. The nucleus pulposus is composed of proteoglycans and diffuse networks of collagen fibrillae. The nucleus is surrounded by concentric collagen fibrillae which form AF. The complicated organization of collagen fibers in AF with NP takes part of the perfect biomechanical structure of IVD. With aging this strongly organized lamellar structure changes (Gruber and Hanley, 2002; Landzhov, 2009). Cartilage end plates are parts of IVD, which lie between the vertebral body and annulus fibrosus (Landzhov, 2006a; Landzhov, 2007). They are thin hyaline cartilage plates and form the anatomical and physiological boundary of the disc which facilitates the diffusion of nutrients from the vertebra to the disc. Nutrients and metabolites that supply disc cells pass through the cartilage end plate (Whalen et al., 1985; Urban et al., 2004; Landzhov, 2008). IVD are avascular structures and have a low capacity for regeneration and repair. Because of this the age related changes are particularly definitive and impair normal disc function. This could provoke disc herniation, commonly presented with pain and work disability.

In this article, we investigated the different changes that occurred in the IVD in different age groups using light-microscopic, ultrastuctural and histochemical studies.

II. MATERIALS AND METHODS

The current study includes IVD from humans: 10 from cadavers (between 15 and 85 years old) and 10 from patients with degenerative diseases of the spinal column (between 19 and 75 years old).

Light-microscopic technique

For the light-microscopic study routine was used the light-microscopic Masson technique. The material was fixed in 10% formaldehyde for 3 days. After that the specimens were washed, dehydrated and became brighter. Afterwards they were embedded in paraffin, and cut into 8 microns thick slices. This allowed for the cells and the intercellular matrix to be observed.

Ultrastructural study

The samples were fixed in 3% glutaraldehyde for 2 hours. After that they were rinsed several times with 0.1% phosphate buffer to remove the fixative solution. Afterwards a subsequent incubation was conducted in a 1% osmium tetraoxide solution for two hours. Then, the pieces were dehydrated in ethanol (50, 70, 95, 100%). Next, the samples were treated for 30 minutes with a 2:1 mixture of propylene oxide and epon. The pieces were embedded in Durcupan (Fluka, Buchs, Switzerland). Afterwards all of the slices were cut on an ultramicrotome (LKB, Stockholm-Bromma, Sweden). Different regions of the IVD were identified on semi-thin sections. The ultrathin sections (60 nm thick) were taken for transmission electron microscopy and were

contrasted with 2.5% uranyl acetate, lead nitrate, and sodium citrate. For observation, electron microscope Hitachi 500 was used.

Histochemical study

For the histochemical examination, the slices were incubated in solution containing 0.2 mg/ml NBT (nitroblautetrazoliumchloride), 1 mg/ml NADPH – tetranatriumsalt and 0.5 % Triton X – 100 diluted in 0.1 M Tris-HCl buffer with pH 7.4 at 37°C for 30-60 minutes. The reaction was finished with 0.1 M Tris HCl, pH 7.6. Afterwards, the sections were rinsed three times for 5 minutes in the same phosphate buffer, air-dried for 24 hours and cover-slipped using Entellan. The result was that NADPH-d stained cells were visible as a blue reaction product inside cells.

III. RESULTS

The morphological changes that we have found and described affect: the intercellular matrix; the distribution, the shapes and number of cells and position, shapes and thickness of collagen fibers.

Light-microscopy clearly presents the age-related changes in localization and number of the cells. Cell density decreased and apoptosis increased. Simultaneous to this increase, the degenerative change occurs in all parts of the disc. Clusters of cells were formed and concentric tears appeared. The quantity of apoptotic cells was greater in older than in younger specimens.

Light-microscopy clearly revealed the typical wavy way of the collagen fibers in young individuals (Fig. 1) and the changes that occur with aging (Fig. 2).

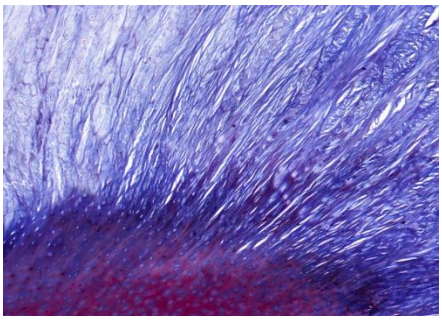

Fig. 1. Light-microscopic photomicrograph of IVD from 25 year old individual. Masson (x 100).

Well presented changes in the parallel structure in the lamellas and their disorganization (in degenerative samples) were detected. Numerous ruptures in the intercellular matrix were observed. In the specimens from young individuals, the wavy direction is better seen than in the older ones. The collagen fibers decreased in number and between them there were wider spaces which are optically empty.

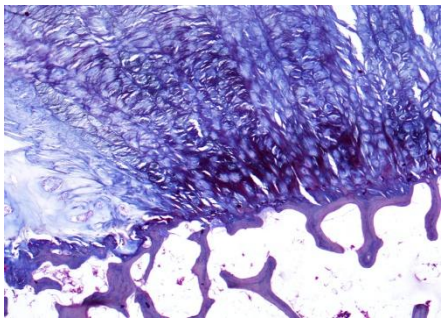

Fig. 2. Light-microscopic photomicrograph of IVD from 65 year old individual. Masson (x 100).

Degenerative changes were observed in the morphology of the cells, confirmed by transmission electron microscopy. Commonly the fibrocytes are flattened, while the chondrocytes are placed in lacunes with normal content and are inserted between and along the collagen fibers (Fig. 3).

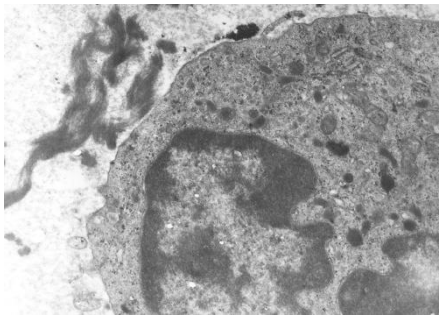

Fig. 3. Electron-microscopic photomicrograph of IVD from 25 year old individual. (x 2400).

In older people the number of lacunar volumes increased (Fig. 4). The changes in the cell population concerned mainly their shape and size.

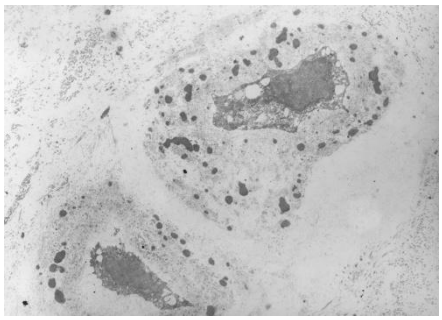

Fig. 4. Electron-microscopic photomicrograph of IVD from 65 year old individual. (x 8400).

The histochemical study revealed greater number of positive NADPH-d cells in degenerated discs (Fig. 5) than in control (Fig. 6) discs.

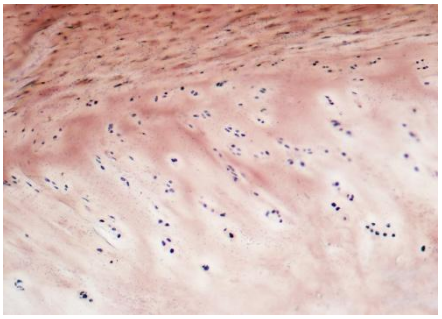

Fig. 5. NADPH-d stained cells in human IVD, specimen from 66 year old individual. (x100).

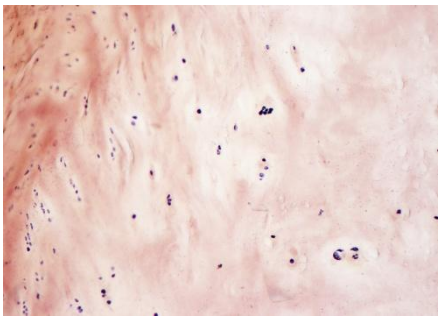

Fig. 6. NADPH-d stained cells in human IVD, specimen from 23 year old individual. (x100).

The quantity of the positive NADPH-d cells increased with the increase of degenerative changes. The lamellas in annulus fibrosus became more disorganized, and the connection between annulus fibrosus and cartilage and plates disrupted. Differences between expressions of NADPH-d positive cells in different parts of the disc were also established. The quantity of the chondrocytes in the outer AF is greater than those in the inner region. On the other hand, many NADPH-d positive cells were observed on the border of the vascular canals. This nitric oxide tends to come from endothelium.

IV. DISCUSSION

The biomechanics of the IVD is closely related with the density and the direction of the collagen fibers of the AF. They have a very typical direction which slowly changes in the different zones of the disc (Buckwalter, 1995; Antoniou et al., 1996; Duance et al., 1998; Eck et al., 1999; Gruber and Hanley, 2002; Landzhov et al., 2005; Landzhov et al., 2006a). The annular lamellas are arranged parallelly in several layers. The inner layers surround the periphery of NP. Their directions depend on the degree of the mechanical load of the IVD (Landzhov et al., 2007; Delcheva, 2008; Landzhov et al., 2012a). Outer lamellas' angle increases according to the longitudinal axis of the disc. The collagen fibers in each lamella have a parallel direction. The direction of the collagen fibers is different in the different zones of the AF (Gathercole and Keller, 1991). The amount of Type I collagen increases, especially in the inner annulus.

Along with this, changes also occur in the number of the cells (Errington et al., 1998; Gruber and Hanley, 2002; Johnson et al., 2001; Bruehlmann et al., 2002; Landzhov et al., 2005). The degenerative changes in AF are often related with the abnormal mechanical load of the spinal column and with changes of the amount of the proteoglycans (Wassilev and Dimova, 1970; Buckwalter, 1995; Antoniou et al., 1996; Duance et al., 1998; Eck et al., 1999; Johnson et al., 2001; Gruber and Hanley, 2002; Landzhov et al., 2006b). Usually these changes also affect the cartilage end plate. There are many studies on laboratory animals (bipedal mouse). After limb amputation are observed changes in the position of the fibers of AF (Wassilev and Dimova, 1970). Even though there are many experiments about this, the relation between the extreme load and the changes in the structure of IVD is still unknown.

As well as the macroscopic changes in the different zones of the disc, the age-related process is also presented with morphological changes (Gruber and Henly, 2002; Landzhov, 2006b; Bocheva et al., 2006; Ovtscharoff et al., 2006). The numbers of cells decrease during the years due to damage of the metabolite transport. Moreover, the cells lose their ability to replicate. It should be depicted that degeneration is difficult to be distinguished from age-related changes. In the literature, there are different theories concerning disc degeneration, but the most popular is that structural damage causes internal disc stresses, which impairs disc cell metabolism (Wassilev and Dimova, 1970; Buckwalter, 1995; Antoniou et al., 1996; Duance et al., 1998; Eck et al., 1999; Gruber and Hanley, 2002; Landzhov et al., 2005; Landzhov, 2007)

In the last years, numerous authors examined the cellular and molecular activity of IVD to understand the pathophysiology of the low back pain (Burke et al., 2002; Landzhov and Stokov, 2007; Landzhov et al., 2008; Hadjipavlou et al., 2008; Landzhov et al., 2012a). Different authors report that there is a correlation between proinflammatory mediators and the production of NO (Brisby et al., 2000; Kohyama et al.; 2000; Furusawa et al., 2001; Dzambazova et al., 2012). NO is a messenger molecule that is synthesized from L-arginine. It is a result of the metabolism of the cells and activates many pathways by diffusing across membranes. It is synthesized from three different enzymes: inducible, endothelial and neuronal (Dzambazova et al., 2012; Hinova-Palova et al., 2014).

The number of positive cells increases with aging and degeneration respectively, as presented by our results. The quantity of NO depends on the types of degenerative diseases

(Johnson et al., 2001; Landzhov et al., 2008; Landzhov et al., 2009; Landzhov et al., 2012b). The material from degenerative discs showed greater number of positive cells than normal control discs. With this we confirm that NO is a molecule that regulates cell metabolism and increases its quantity simultaneously with the increase of cyclic tensile stretch and disc degeneration (Liu et al., 2001; Rannou et al., 2003). According to Hashizume et al. (1997) and Landzhov et al. (2007) the main production of NO comes from newly formed blood capillaries and granulates tissue around them. The increased number of NOS-positive chondrocytes in young individuals is a result of chondrocyte maturation (Teixeira et al., 2005).

V. CONCLUSION

Age-related changes in IVD are one of the main factors for the appearance of low back pain. Often the degeneration starts as a result of abnormal mechanical load. This strength is related with changes in the cells, proteoglycans and collagen fibers, all intercellular and extracellular structures. The process that occurs within IVD with aging is not well-known.

Cluster formations and new blood vessels appear with cell proliferation and degeneration. These changes are very important in order to understand the problems which occur in the disc. The current study presents the morphological changes in the IVD in different ages and gives us a possibility for more precise definition for the complicated changes which happen in the cells and in the intercellular matrix during degenerative diseases.

REFERENCES

[1] Gruber HE, Hanley EN Jr (2002) Observations on morphologic changes in the aging and degenerating human disc: secondary collagen alterations. BMC Musculoskelet Disord 3:9.

[2] Landzhov B (2009) Age-related changes in human intervertebral discs and their relation with degenerative diseases. PhD thesis, Sofia, Bulgaria.

[3] Landzhov B (2006a) Ageing and changes in the cartilage end plates in humans. Compt Rend Acad Bulg Sci 59:987-990.

[4] Landzhov B (2007) Neovascularisation of Cartilage end plates in humans. Trakia J Sci 5 (Suppl. 2):35.

[5] Whalen JL, Parke WW, Mazur JM, Stauffer ES (1985) The intrinsic vasculature of developing vertebral end plates and its nutritive significance to the intervertebral discs. J Pediatr Orthop 5:403-410.

[6] Urban JP, Smith S, Fairbank JC (2004) Nutrition of the intervertebral disc. Spine, 29:2700-2709.

[7] Landzhov B (2008) Age related changes in the cells of intervertebral cartilage end plates. Acta Morphol Anthropol 13:327-330.

[8] Buckwalter JA (1995) Aging and degeneration of the human intervertebral disc. Spine (Phila Pa 1976) 20:1307-1314.

[9] Antoniou J, Steffen T, Nelson F, Winterbottom N, Hollander AP, Poole RA, Aebi M, Alini M (1996) The human lumbar intervertebral disc: evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing, and degeneration. J Clin Invest 98:996-1003.

[10] Duance VC, Crean JK, Sims TJ, Avery N, Smith S, Menage J, Eisenstein SM, Roberts S (1998) Changes in collagen cross-linking in degenerative disc disease and scoliosis. Spine (Phila Pa 1976) 23:2545-2551.

[11] Eck JC, Humphreys SC, Hodges SD (1999) Adjacent-segment degeneration after lumbar fusion: a review of clinical, biomechanical, and radiologic studies. Am J Orthop (Belle Mead NJ) 28:336-340.

[12] Landzhov B, Yotovski P, Yotovski V, Tzvetkova R, Ovtscharoff W (2005) Ultrastructural study of age-related changes of human intervertebral discs. Praemedicus since 1925 1-2:19-22.

[13] Landzhov B, Yotovski P, Yotovski V, Tzvetkova R, Stokov L, Ovtscharoff W (2006a) Changes in anulus fibrosus during degenerative diseases in human intervertebral discs. Light-microscopic study. Praemedicus since 1925 27:32-35.

[14] Landzhov B, Stokov L, Vladimirov B, Bozhilova-Pastirova A, Ovtscharoff W (2007) Degenerative changes in the herniated intervertebral disc. Trakia J Sci 5 (Suppl. 2):35.

[15] Delcheva D, Choneva I, Landzhov B, Vladimirov B (2008) Correlation between degenerative changes and herniation in the human intervertebral disc. Comparative morphological, MRI and clinical study. VII International congress of medical sciences, Sofia, 8-11 May, p. 263.

[16] Landzhov B, Malinova L, Dzambazova E, Bozhilova-Pastirova A, Ovtscharoff W (2012a) Comparative morphological and clinical study of intervertebral discs herniation in humans with low back pain in different age. Compt Rend Acad Bulg Sci 65:89-94.

[17] Gathercole LJ, Keller A (1991) Crimp morphology in the fibre-forming collagens. Matrix 11:214-234.

[18] Errington RJ, Puustjarvi K, White IR, Roberts S, Urban JP (1998) Characterisation of cytoplasm-filled processes in cells of the intervertebral disc. J Anat 192:369-738.

[19] Johnson WE, Eisenstein SM, Roberts S (2001) Cell cluster formation in degenerate lumbar intervertebral discs is associated with increased disc cell proliferation. Connect Tissue Res 42:197-207.

[20] Bruehlmann SB, Rattner JB, Matyas JR, Duncan NA (2002) Regional variations in the cellular matrix of the

annulus fibrosus of the intervertebral disc. J Anat 201:159-171.

[21] Wassilev M, Dimova R (1970) Influence of mechanical factors on the structure of intervertebral disks. Arch Orthop Unfallchir 68:273-281.

[22] Landzhov B, Vladimirov B, Bozhilova-Pastirova A, Ovtscharoff W (2006b) Degeneration in the structures of human intervertebral disc. Compt Rend Acad Bulg Sci 59:991-996.

[23] Landzhov B (2006b) The elastic network of fibers in human intervertebral disc changes with ageing and disc diseases. Praemedicus since 1925 27:36-38.

[24] Bocheva A, Landzhov B, Vladimirov B, Bozhilova-Pastirova A (2006) Age-related changes in human nucleus pulposus. 29th Balkan Medical Week, Varna, 28-30 September, Abstract book, p. 55.

[25] Ovtscharoff W, Landzhov B, Vladimirov B, Stokov L, Bozhilova-Pastirova A (2006) Degeneration of anulus fibrosus in human intervertebral disc. Scripta Scientif Med, 38 (Suppl. 2):74.

[26] Burke JG, Watson RW, McCormack D, Dowling FE, Walsh MG, Fitzpatrick JM (2002) Intervertebral discs which cause low back pain secrete high levels of proinflammatory mediators. J Bone Joint Surg Br 84:196-201.

[27] Landzhov B, Stokov L (2007) NADPH-d reactivity in the herniated and nonherniated human intervertebral disc. 10 th Congress of the BOTA, 206-209.

[28] Landzhov B, Stokov L, Vladimirov B, Bozhilova-Pastirova A, Ovtscharoff W (2008) Degenerative changes in the human intervertebral discs. Histochemical study. Acta Morphol Anthropol, 13:331-334.

[29] Hadjipavlou AG, Tzermiadianos MN, Bogduk N, Zindrick MR (2008) The pathophysiology of disc degeneration: a critical review. J Bone Joint Surg Br 90:1261-1270.

[30] Brisby H, Byröd G, Olmarker K, Miller VM, Aoki Y, Rydevik B (2000) Nitric oxide as a mediator of nucleus pulposus-induced effects on spinal nerve roots. J Orthop Res 18:815-820.

[31] Kohyama K, Saura R, Doita M, Mizuno K (2000) Intervertebral disc cell apoptosis by nitric oxide: biological understanding of intervertebral disc degeneration. Kobe J Med Sci 46:283-295.

[32] Furusawa N, Baba H, Miyoshi N, Maezawa Y, Uchida K, Kokubo Y, Fukuda M (2001) Herniation of cervical intervertebral disc: immunohistochemical examination and measurement of nitric oxide production. Spine (Phila Pa 1976) 26:1110-1116.

[33] Dzambazova EB, Landzhov BV, Bocheva AI, Bozhilova-Pastirova AA (2011) Effects of D-kyotorphin on nociception and NADPH-d neurons in rat's periaqueductal gray after immobilization stress. Amino Acids 41:937-944.

[34] Hinova-Palova DV, Edelstein L, Landzhov B, Minkov M, Malinova L, Hristov S, Denaro FJ, Alexandrov A, Kiriakova T, Brainova I, Paloff A, Ovtscharoff W (2014) Topographical distribution and morphology of NADPH-diaphorase-stained neurons in the human claustrum. Front Syst Neurosci 8:96.

[35] Landzhov B, Dzhambazova E, Bozhilova-Pastirova A, Bocheva A, Ovtscharoff W (2009) Degenerative cartilage changes in humans with gonarthrosis. Sci Res J S-W Uni 2:43-47.

[36] Landzhov B, Malinova L, Ovtscharoff W (2012b) Histochemical and magnetic resonance imaging study of degenerative diseases in human intervertebral discs. Compt Rend Acad Bulg Sci 65:389-392.

[37] Liu, GZ, Ishihara H, Osada R, Kimura T, Tsuji H (2001) Nitric oxide mediates the change of proteoglycan synthesis in the human lumbar intervertebral disc in response to hydrostatic pressure. Spine 26:134-141.

[38] Rannou F, Richette P, Benallaoua M, Francois M, Genries V, Korwin-Zmijowska C, Revel M, Corvol M, Poiraudeau S (2003) Cyclic tensile stretch modulates proteoglycan production by intervertebral disc annulus fibrosus cells through production of nitrite oxide. J Cell Biochem 90:148-157.

[39] Hashizume H, Kawakami M, Nishi H, Tamaki T (1997) Histochemical demonstration of nitric oxide in herniated lumbar discs. A clinical and animal model study. Spine 22:1080-1084.

[40] Teixeira CC, Ischiropoulos H, Leboy PS, Adams SL, Shapiro IM (2005) Nitric oxide-nitric oxide synthase regulates key maturational events during chondrocyte terminal differentiation. Bone, 37:37-45.