# COLLAGEN STRUCTURAL CHANGES IN EARLY RADIATION-INDUCED DAMAGE

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**The aim of the investigation** is complex study of the mechanisms of radiation-induced collagen damage and following recovery of its structure on various levels of multilayer organization.

**Materials and Methods.** Rat tail tendons were used as the model of collagen containing tissue. The animals were exposed to radiation at the dosage of 2, 4, 6, 8, and 10 Gy using gamma-apparatus "Luch-1" (Russia) *in vivo*. The collagen structure was studied 1 and 7 days after the radiation by trypsin-resistance test, differential scanning calorimetry, confocal microscopy with second-harmonic generation imaging, and cross polarization optical coherence tomography.

**Results.** There were determined certain regularities of collagen structural changes depending on radiation time. One day after the radiation of collagen containing tissue the leading processes were those of collagen structural damage at molecular level due to reactive oxygen species exposure. Within a week after the radiation, the collagen structure recovered partially, and therefore, there recovered its resistance to proteolytic activity. Ionizing radiation initiated the cascade of reactions beginning with direct and indirect protein damage, and resulting in its remodeling as a result of nonenzymic bridging between triple helix forming quaternary structure of collagen.

**Key words:** collagen structure; ionizing radiation; radiation-induced damage; collagen remodeling.

The problem of radiation-induced changes of normal tissues is still of current interest [1]. The degree of radiolesions at early or late stages after radiation exposure can vary from clinically insignificant changes to major complications that have a significant effect on patient's life quality [2]. For the development of effective preventive measures, diagnostic and treatment modalities, it is required to carry out a detailed study of the mechanisms of damage growth and following regeneration of biological tissues.

The important role in tissue changes induced by ionizing radiation belongs to progressing microcirculation abnormality, as well as collagen destruction that results in scar changes and mediated vascular obliteration [3–5]. Until now collagen molecule fragmentation and degradation of its structure due to the direct peptide chain breaking, as well as cross linkage formation in indirect impact of reactive oxygen species have been considered as the prominent mechanisms of radiation-induced damages of structural and functional status of collagen [6–9]. For the past 5 years, using modern techniques there have been carried out the investigations that have made it possible to acquire extensive information on the mechanisms of radiation-induced collagen damage [10–13]. However, these researches are aimed to study collagen remodeling processes occurring when skin allografts (alloderm) are sterilized by ionizing radiation. In *in vitro* experiments there were used radiation doses (2–30 kGy) much exceeding the doses used in radiation therapy. The dynamics of changes and following recovery of collagen structure *in vivo* in radiation doses used in radiation oncology is unresearched so far.

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**The aim of the investigation** was to study dose-time relations of collagen changes on different levels of its multilayer organization exposed to ionizing radiation at early stages of radiation-induced damage.

**Materials and Methods.** Rat tail tendons were used as the model of collagen containing tissue. This tissue due to quasi-crystalline uniaxial molecule folding into fibrils, fibrils into fibers, and fibers — into bundles, is an ideal model system, the changes of which can be definitely interpreted. The experiments were carried out on white outbred rats (28 animals) kept in standard conditions of animals quarters. When carrying out the experiments, ethical principles were kept inviolate according to European Convention for the protection of vertebrata used for experimental and other scientific purposes (the Convention was passed in Strasburg, 18.03.1986, and adopted in Strasburg, 15.06.2006). The animals were exposed to single radiation under general anesthesia (Zoletil, 50 mg/kg) on external-beam radiotherapy apparatus "Luch-1" (Russia) *in vivo* in the doses of 2, 4, 6, 8, and 10 Gy. The collagen structure was studied 1 and 7 days after the radiation by a complex of methods.

Trypsin-resistance test is a classical approach to reveal the degradation degree of collagen molecules since this proteolytic enzyme induces the depolymerization of damaged molecules only, while three-helix intact molecules appear to be tryprin-resistant [14]. The samples of prepared tendon were air-dried for 24 h at 37°C, then incubated in trypsin solution (concentration — 1 mg/ml, enzyme-substrate ratio — 1:10) with sodium azide (0.02%) added. The quantitative characteristic of trypsin-resistance is sample weight loss after incubation.

Water sorption ability of collagen matrix model depends not only on its composition but also on the interaction of matrix biopolymers determining the amount of polar groups available for water adsorption, and other characteristics of its molecular and supramolecular structure. Thus, the change in water balance is an integral characteristic of changes that have impact on mechanical properties of connective tissue as well. Water content in samples was measured gravimetrically, after swelling of dried preparations in 0.16 M NaCl solution for 6 h at 37°C. Water content was represented as weight of water per 1 g of dried tissue preparation.

Differential scanning calorimetry (DSC) is direct and most effective technique for recording thermal denaturation of proteins on thermograms. It enables to determine the proportion of intact macromolecules (by transition heat helix-coil — $\Delta H$) and perfection of fibrillar packing (by denaturation temperature — $T_d$); moreover, $T_d$ dynamics indicates the changes of the degree of intercross-linking of collagen fiber [15]. The tendon samples for DSC were kept at –20°C, and immediately before the analysis they were withdrawn and incubated in 0.15 M NaCl solution for 1 h at room temperature. The investigations were performed on a differential scanning calorimeter "Mettler TA 4000". The samples weighting 7–10 mg were put into a sealed aluminium box and heated in DSC30 cell from 5 to 100°C at scan rate 10°/min. The observed heat effect of collagen denaturation $\Delta H$ was counted on per sample dry basis.

To describe collagen structure at the level of fibers and bundles there was used multiphoton microscopy with second-harmonic generation (SHG). The researches were carried out on microscope "LSM Meta" (Karl Zeiss, Germany). Excitation was performed by pulse (100 fs) radiation of Ti:sapphire-laser (MaiTai HP, Spectra Physics, USA) at 800 nm length wave, pulse recurrence frequency being 80 MHz. Every image line was averaged by 8 scans to improve signal-noise ratio. The samples were fixed in 4% formaldehyde solution; and immediately before imaging collagen bundles ~400 μm in diameter were isolated from tissue preparations and placed between cover glasses to prevent dehydration.

Cross polarization optical coherence tomography (CP OCT) was used to monitor the changes at the level of tendon general architectonics [16]. This modality enables to image an object in direct and orthogonal polarization simultaneously with resolution of 10–15 μm, at a depth of 1–2 mm in real time mode. The presence of tissue anisotropic properties results in interferential pattern in a receiving signal, and it is manifested on tomograms as a regular repetition of dark and bright bands with $z_b$ period. The researches were carried out using CP OCT *in vivo* throughout the length of the prepared tendon under general anesthesia.

**Results.** There was determined a certain regularity of collagen structural changes depending on post-radiation period. Weight loss of intact tendon samples in trypsin solution was no more than 2% due to the proteolysis of noncollagen proteins of tissue matrix. 24 h after radiation there was observed a 5–10% increase in tendon weight loss compared to intact samples, the differences between the weight loss of intact and irradiated samples appeared to be statistically significant regardless of radiation dose. Sample weight loss was growing linearly with the increase of radiation dose. A week after radiation sample weight loss reduced, thought it did not reach the initial level (Fig. 1).

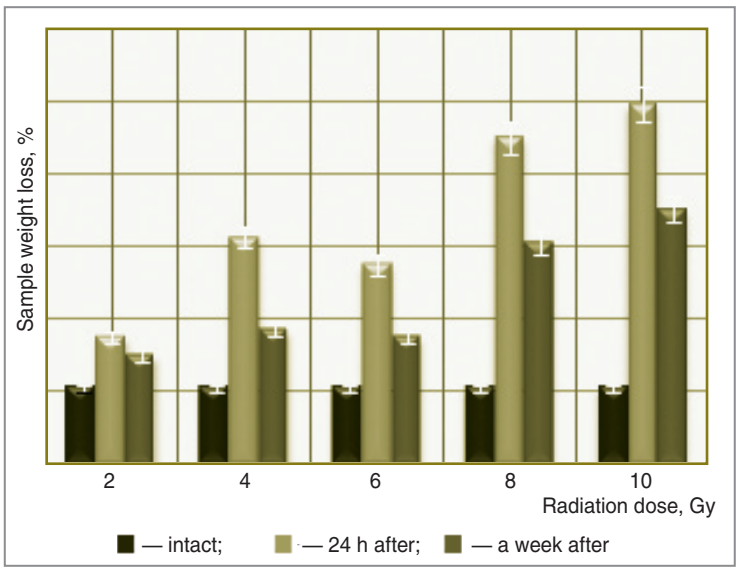


**Fig. 1.** Weight loss of tendon samples in trypsin solution depending on the dose and post-radiation period

On DSC thermograms of tendon samples (Fig. 2) when heated two processed are seen. The first process proceeds at 25°C, is exothermic and related to the formation of hydrogen bonds between polar groups of collagen and water molecules [15]. The second process is endothermic and reflects the ingress of heat in helix-coil transition of collagen macromolecules, i.e. denaturation process [15, 17–19]. For intact samples the exothermic transition heat was $\Delta H$=–(5±1) J/g. Denaturation was performed at 66.7±1.2°C and was characterized by heat $\Delta H_d$ = 42.1±0.5 J/g of dry tissue preparation. This value corresponds to the heat of collagen denaturation in tissues of tendons and ligaments [18, 19]. Ionizing effect had significant influence on the type and characteristics of denaturation endotherm, which was presented by several peaks, maximum ones being at 63.7±1.2°C, 67.0±1.5°C and 71.0±1.5°C, and $\Delta H$ value decreased up to 31.1±3.5 J/g 24 h after radiation. A week after radiation exposure denaturation temperature increased, its growth correlated positively with radiation dose (Fig. 2, *b*), and peaks with maximum values lower than collagen $T_d$ in intact tissue disappeared (Fig. 2, *a*, curve *4*).

24 h after the radiation, sorption capacity of dried tendon samples increased significantly, that indicated the molecular structural damage and proved the results of trypsin-resistance test (Fig. 3, *a*). A week after the radiation the tendon sorption capability decreased proportionally to radiation dose (Fig. 3, *b*).

The study of tendon structure using multiphoton microscopy on SHG-images of intact samples showed fibers to be closely packed in parallel primary and secondary bundles (Fig. 4, *a*), on which there was regular acute-angled folding (crimps) characteristic of tendon tissue [20]. SHG-images of irradiated samples 24 h after the radiation demonstrated fiber structure to be preserved, though there was significant stratification of secondary bundles, particularly typical of folding area (Fig. 4, *b*, *c*). A week after the radiation there was a marked increase in the number of these crimps compared to intact samples (Fig. 4, *d*).

Tissue anisotropy including birefringent effect is due to the ordered structure of rod-like collagen molecules in tendon [21]. The difference in refraction index for orthogonal polarization produces the repetition of dark and bright bands on CP OCT-images (Fig. 5), oscillation period of interference signal being 90 μm. The analysis of

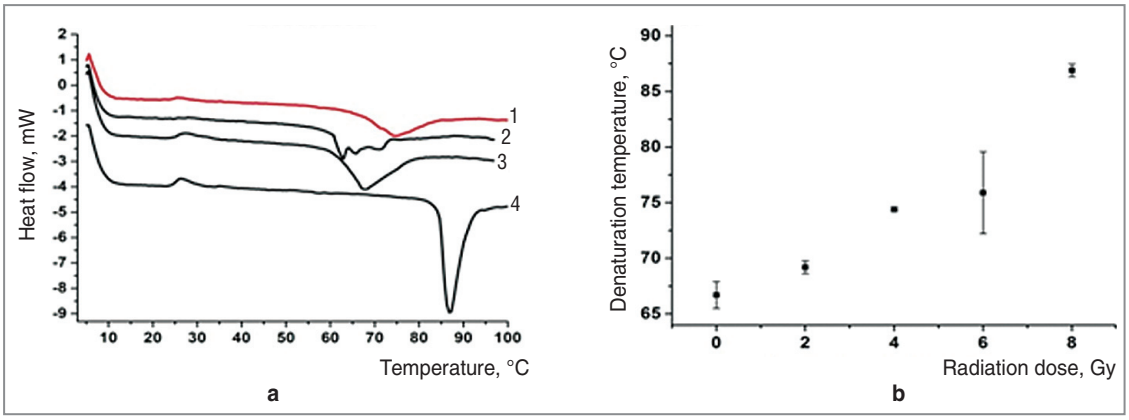


**Fig. 2.** Typical DSC-thermograms of tendon samples (*a*): *3* — intact; *2* — 2 Gy, 24 h; *1* — 4 Gy, week; *4* — 8 Gy, week and the dependence of collagen denaturation temperature in tendon tissue a week after ionizing radiation on radiation dose (*b*)

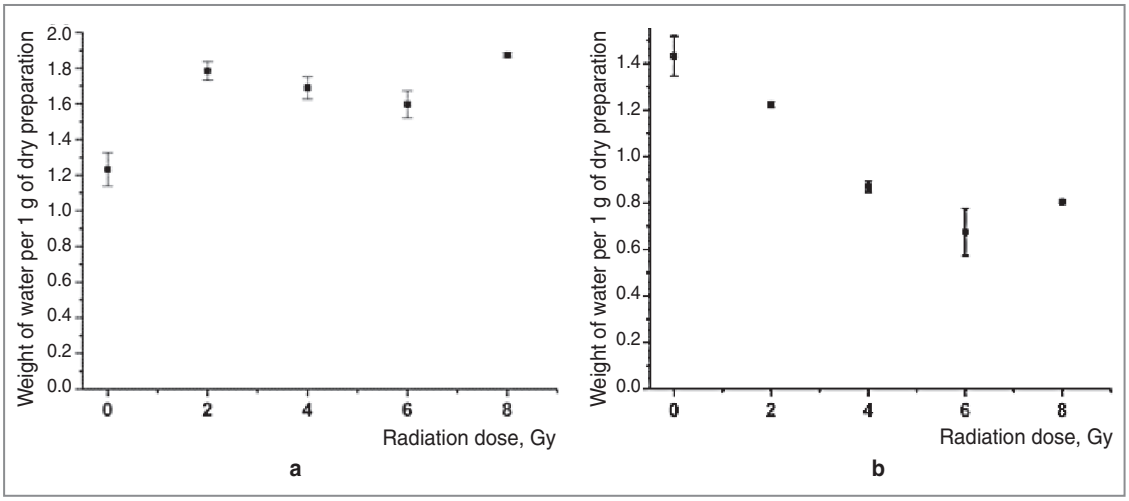


**Fig. 3.** The change of hydration level of dry samples of irradiated tendons of rat tails 24 h (*a*) and one week (*b*) after the radiation

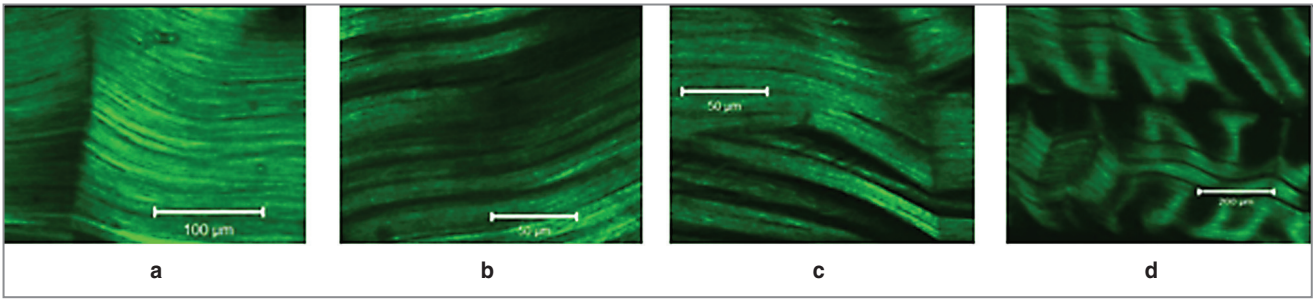


**Fig. 4.** SHG-images of rat tail tendons: *a* — intact sample; *b* — 24 h after radiation, 4 Gy; *c* — 24 h after radiation, 6 Gy; *d* — a week after radiation, 6 Gy

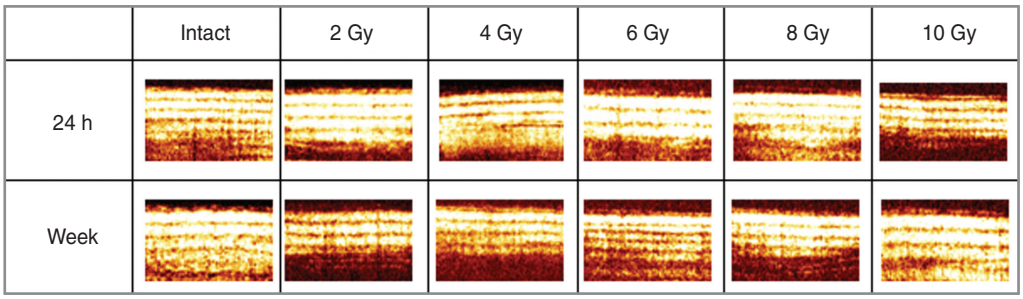


**Fig. 5.** Cross polarization optical coherence tomography images of tendon depending on radiation dose and post-radiation periods

CP OCT-images of tendon samples exposed to ionizing radiation in the doses from 2 to 10 Gy showed oscillation period to remain unchanged in these post-radiation periods, and the absence of serious damages of ordered anisotropic collagen packing.

**Discussion.** The fact of collagen structural damage caused by ionizing radiation has been known since the middle of XX c. and widely covered in literature [7–9]. The authors have pioneered in representing the dynamics of degenerative and regenerative processes of anionic protein of connective tissue *in vivo* at all levels of its multilayer organization depending on the dose and post-radiation period. Collagen changes have been found to develop mainly at molecular level, as well as fiber level, and have several stages. The first stage is characterized by partial bond damage of three-helix structure that is reflected in decreased resistance to proteolytic impact, denaturation temperature reduction and the increase of sorption capacity compared to intact samples. Low-temperature peak (shoulder) on DSC-thermograms 24 h after the radiation indicates the appearance of less stable collagen fracture in the samples (See Fig. 2). Thus, 24 h after the radiation there is partial matrix disorganization consisting in the degradation of some collagen macromolecules and the damage of their interaction with other chemical components of matrix.

A week after the radiation there is observed the rearrangement of collagen structure with the increase in the number of cross-links that is supported by denaturation temperature increase and the reduction of sorption capacity of samples compared to the control ones. These cross-links can form due to the condensation of amino groups of lateral chains of the residues of lysine and allysine, which in its turn forms due to oxidative deamination of $NH_2$-group by reactive oxygen species, the generation of which is observed under gamma radiation. Malonaldehyde may play a significant role in radiation-induced damage, its level in the body increases greatly under ionizing radiation [11]. A week after radiation $\Delta H$ value of helix-coil transition and exothermic heat effect of formation of hydrogen bonds of water-bridging structures return to the level of intact tendon. It means that collagen macromolecules in these samples are in conformation of triple helix. Significant increase of $T_d$ of basic collagen fraction indicates the difficulties in the formation of amorphous phase from quasi-crystalline [22]. These difficulties are related to the excess of intermolecular cross-links limiting polypeptide chain mobility and preventing the transition of triple helix into a random coil [22, 17].

There should not be ruled out that the stratification of tertiary bundles observed in SHG-images of tendon is related to damage fibroblasts located between secondary and tertiary bundles [22]. The observed increase of crimps in irradiated samples correlates with a well-known fact of reduction of Young's modulus of connective tissues exposed to gamma radiation. The growing number of crimps is the indicator of tension decrease in tissues (including internal stress), and their presence ensure tissue stretching in low stress levels [20].

When tissue architectonics is changed, there is the change of the degree of its anisotropy and refraction index; as well there changes oscillation period of interference signal and, therefore, the band width on CP OCT-tomograms. Thus, CP OCT is extremely sensitive diagnostic modality in collagen matrix degradation, especially in tissues with dense and organized packing, as it is in tendon. The absence of qualitative and quantitative changes on CP OCT-tomograms of irradiated tendons clearly indicates the architectonics of connective tissue in total doses from 2 to 10 Gy to be

generally preserved. Our findings agree the results reported in the work [13], in which by means of transmission electron microscopy there was shown the absence of collagen fibril changes and their packing in rat skin tissue exposed to ionizing radiation in the dose of 10 Gy.

**Conclusion.** 24 h after the radiation of collagen-containing tissue the leading processes are those of collagen structural damage at molecular level due to reactive oxygen species exposure. Within a week after the radiation, the collagen structure partially recovers and, therefore, there recovers its resistance to proteolytic activity. Ionizing radiation initiates the cascade of reactions beginning with direct and indirect protein damage, and resulting in its remodeling as a result of nonenzymic bridging between triple helixes forming quaternary structure of collagen.

### References


**1.** Coppes R. Evaluation of normal tissue response to particle irradiation from preclinical studies: radiobiological effectiveness and dose distribution. Prediction, recognition, evaluation and eradication of normal tissue effects (PREVENT). Abstracts. 20–21 March; Brussels, Belgium. *Radiother Oncol* 2011; 98: S10. DOI:10.1016/S0167-8140(11)71729.

**2.** *Cancer Therapy Evaluation Program, Common Terminology Criteria for Adverse Events, version 4.03, DCTC, NCI, NIH, DNNS*. June 14, 2010. http://ctep.cancer.gov.

**3.** Antonakopoulos G.N., Hicks R.M., Berry R.J. The subcellular basis of damage to the human urinary bladder induced by irradiation. *J Pathol* 1984; 143(2): 103–116.

**4.** O'Sullivan B., Levin W. Late radiation-related fibrosis: pathogenesis, manifestations, and current management. *Seminars in Radiation Oncology* 2003; 13(3): 274–289.

**5.** Bidhu K. Mohanti, Bansal M. Late sequelae of radiotherapy in adults. *Supportive Care in Cancer* 2005; 13(10): 775–780.

**6.** Braams R. The effect of electron radiation on the tensile strength of tendon. *Int Jour Appl Radiat Biol* 1961; 4: 27–31.

**7.** Bowes J.H., Moss J.A. The effect of gamma radiation on collagen. *Radiat Res* 1962; 16: 211–223.

**8.** Bailey A.J., Bendall J.R., Rhodes D.N. The effect of irradiation on the shrinkage temperature of collagen. *Int J Appl Radiat Isotopes* 1962; 13: 131–136.

**9.** Bailey A.J. Irradiation-induced changes in the denaturation temperature and intermolecular crosslinking of tropocollagen. *Radiat Res* 1967; 31(2): 206–214.

**10.** Gouk S.-S., Lim T.-M., Teoh S.-H., Sun W.Q. Alterations of human acellular tissue matrix by gamma irradiation: histology, biomechanical property, stability, in vitro cell repopulation, and remodeling. *J Biomed Mater Res Part B: Appl Biomater* 2008; 84B: 205–217.

**11.** Sun W.Q., Leung P. Calorimetric study of extracellular tissue matrix degradation and instability after gamma irradiation. *Acta Biomaterialia* 2008; 4: 817–826.

**12.** Shah N.B., Wolkers W.F., Morrissey M., et al. Fourier transform infrared spectroscopy investigation of native tissue matrix modifications using a gamma irradiation process. *Tissue engineering* 2009; A(15): 1–8.

**13.** Balli E., Comelekoglu U., Yalin E., et al. Exposure to gamma rays induces early alterations in skin in rodents: mechanical, biochemical and structural responses. *Ecotoxicology and Environmental Safety* 2009; 72: 889–894.

**14.** Bruckner P., Prockop D.J. Proteolytic enzymes as probes for the triple-helical conformation of procollagen. *Anal Biochem* 1981; 110(2): 360–368.

**15.** Miles Ch.A., Ghelashvili M. Polymer-in-a-box mechanism for the thermal stabilization of collagen molecules in fibers. *Biophysical Journal* 1999; 76: 3243–3252.

**16.** Kuranov R.V., Sapozhnikova V.V., Turchin I.V., Zagaynova E.V., Gelikonov V.M., Kamensky V.A., Snopova L.B., Prodanetz N.N. Complementary use of cross-polarization and standard OCT for differential diagnosis of pathological tissues. *Opt Express* 2002; 10: 707–713.

**17.** Ignatieva N.Yu., Zakharkina O.L., Andreeva I.V., et al. Effects of laser irradiation oncollagen organization in chemically induced degenerative annulus fibrosus of lumbar intervertebral disc. *Lasers Surg Med* 2008; 40: 422–432.

**18.** Bigi A., Cojazzi G., Roveri N., Koch M.H.J. Differential scanning calorimetry and X-ray diffraction study of tendon collagen thermal denaturation. *J Biol Macromol* 1987 December; 9: 363–367.

**19.** Willett T.L., Labow R.S., Lee J.M. Mechanical overload decreases the thermal stability of collagen in an in vitro tensile overload tendon model. *J Orthop Res* 2008; 26(12): 1605–1610.

**20.** Franchi M., Fini M., Quaranta M., et al. Crimp morphology in relaxed and stretched rat Achilles tendon. *J Anat* 2007 Jan; 210(1): 1–7.

**21.** Kastelic J., Galeski A., Baer E. The multicomposite structure of tendon. *Connect Tissue Res* 1978; 6(1): 11–23.

**22.** Kannus P. Structure of the tendon connective tissue. *Scand J Med Sci Sports* 2000; 10(6): 312–320.