ANALÝZA NAPĚTÍ A STEREOLOGIE HLADKÉ SVALOVÉ A POJIVOVÉ TKÁNĚ

STRESS AND STEREOLOGICAL ANALYSIS OF SMOOTH MUSCLE AND CONNECTIVE TISSUE

Petra KOCHOVÁ, Miroslav HOLEČEK[1]

Abstrakt

Počítačové modelování tkání ve spolupráci s lékařskou komunitou pomáhá odhalit příčiny vzniku a šíření nemocí v živé tkáni. Pro verifikaci těchto modelů je nutné provádět odpovídající experimenty. Cílem této práce je popis měření mechanických vlasností pojivové a hladké svalové tkáně nožního integumentu plicnatého plže. Tato tkáň byla vybráno pro své velmi dobré vlastnosti. Tkáň byla během měření zatížena jednoosým napětím ve směru kolmém a rovnoběžném k hlavní ose těla plže a při různých rychlostech zátěžové síly. Z výsledných závislostí byl určen Youngův modul elasticity a mezní napětí tkáně. Výsledná měření byla srovnána se stereologickou kvantifikací hlavních složek měřené tkáně a analýzou porušení tkáně.

Klíčová slova: hladká svalová tkáň, pojivová tkáň, stereologie, mechanické vlastnosti, porušení tkáně.

Abstract

In cooperation with medical community, the computer modeling of living tissue helps to find the causes of emergence and spread of the diseases of living tissue. It is necessary to arrange suitable experiments in order to verify the models. The aim of this work is to describe the measurement of mechanical properties of smooth muscle and connective tissue of the integument of the pulmonate gastropod's foot. This kind of tissue has been chosen for its suitable properties. During performed experiment, the tissue was uniaxially loaded at various velocities and in various directions (longitudinal and perpendicular to the main axis of gastropod's body). The Young's moduli and values of ultimate strength have been found. We present also results of the stereological quantification of volume fractions of the main components of the tissue and the analysis of tissue rupture.

Keywords: smooth muscle tissue, connective tissue, stereology, mechanical properties, tissue rupture

INTRODUCTION

The mechanical properties of soft tissue are of increasing interest for medical diagnosis and surgical simulation. In the former case, mechanical testing may aid in deciding whether or not to remove tissue when other tests are inconclusive or inconvenient. The object of this study was to investigate the mechanical properties and microstructure of gastropod smooth muscle and connective tissue by using the uniaxial loading. Specifically, to identify if mechanical properties vary using tensile tests by various load velocity and in various directions of loading, to find

[1] Ing. Petra KOCHOVÁ, doc. RNDr. Miroslav HOLEČEK, Department of Mechanics, Faculty of Applied sciences, University of West Bohemia, Plzeň, kochovap@kme.zcu.cz, holecek@ums.zcu.cz
Lektoroval: RNDr. Matej DANIEL, PhD., KAMaM, SjF TU v Košiciach, matej.daniel@tuke.sk

percentage area of main tissue components, and to find the propagation directions of tissue rupture.

MATERIAL AND METHOD

In this study we analyzed the samples (10x5x5mm) taken away from the sole of pulmonate gastropod's foot. These samples were measured by two measurement devices to find the modulus of elasticity and the ultimate strength. The pieces of tissue taken away from the place close to the measured specimens were fixed in formaldehyde and prepared for stereological study.

THE SMOOTH MUSCLE AND CONNECTIVE TISSUE OF GASTROPODS

Biological tissue is any substance made up of cells that perform a similar function within an organism. The cell is the structural and functional unit of all living organisms, and is sometimes called the "building block of life." Some organisms, such as bacteria, are unicellular, consisting of a single cell. Other organisms, such as humans, are multicellular, (humans have an estimated 100,000 billion or 10^{14} cells; a typical cell size is 10 μm, a typical cell mass 1 nanogram).

There are four basic types of tissue in the body of all animals, including the human body and lower multicellular organisms such as insects. These compose all the organs, structures and other contents. Epithelium - Tissues composed of layers of cells that cover organ surfaces such as surface of the skin and inner lining of digestive tract. The tissues serve for protection, secretion, and absorption. Connective tissue - As the name suggests, connective tissue holds everything together. Blood is considered a connective tissue. These tissues contain extensive extracellular matrix. Muscle tissue - Muscle cells contain contractile filaments that move past each other and change the size of the cell. Muscle tissue also is separated into three distinct categories: visceral or smooth muscle, which is found in the inner linings of organs; skeletal muscle, which is found attached to bone in order for mobility to take place; and cardiac muscle which is found in the heart. Nervous tissue - Cells which form the brain, spinal cord and peripheral nervous system.

The smooth muscle and connective tissue of gastropod chosen for mechanical experiments contain smooth muscle cells, cholinergic neural fibrils, fibroblasts, immune and fat cells and connective tissue (collagen fibrils), see Fig. *1*.

To understand the mechanical behavior of whole tissue we have to learn the behavior of individual isolated cells. Although different cells show different structure arrangement, the main constructive elements are similar: the liquid membrane envelops the cell and its components and the net of fibrils (filaments) holds the cell shape and helps organize the cell inside. The structural elements of the cell are soft, in contrast to the hard concrete and steel of buildings and bridges. The mechanical properties of soft materials may be quite different from those of the hard structures, e.g. the soft rubber becomes more resistant to stretching when heated. The origin of rubber elasticity is in the variety of molecular configurations of polymers.

Individual smooth muscle cell is spindle-shaped, 2-5 μm in diameter and 20-50 μm in length, with in cytoplasm peripheral embedded nucleus, see Fig. *2*. High dynamic actin filaments, stitch of fibrous intermediary filaments and microtubules form the structural grid of cell cytoskeleton (net of proteins). The intermediary filaments and actin filaments of cell cytoskeleton are arranged in 3-D net and anchored into the cell membrane by dense bodies and by dense plaques, in cell inside and in cell membrane, respectively. The thin actin filaments (6 nm in diameter) anchored in dense bodies and dense plaques together with thick myosin filaments (15-20 nm in diameter) built the contractive apparatus. The connection between the cells is mediated by

desmosomes which serve like mechanical junctions and gap junctions which transmit the information between cells.

Smooth muscle cells occur partly alone partly in form of fascicle systems longitudinal, transversal or radial to main axis of gastropod body orientation. Smooth muscle cells are covered by thin layer of basal lamina around which lie the different thick and disordered network of collagen fibrils joining the cells and increasing the stiffness of the cell, see Fig. *2*.

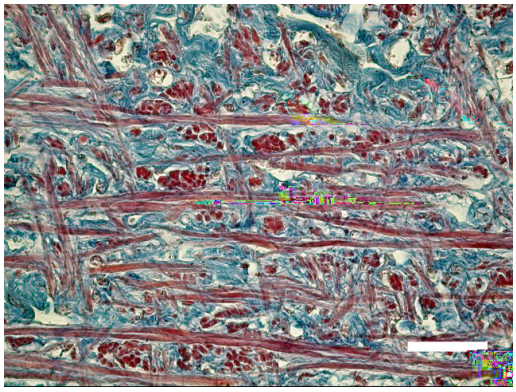

Fig.1 A deeper layer of sole integument with collagen ligament. The 3D net of muscle cells is visible, scale 100 μm. According to [11]

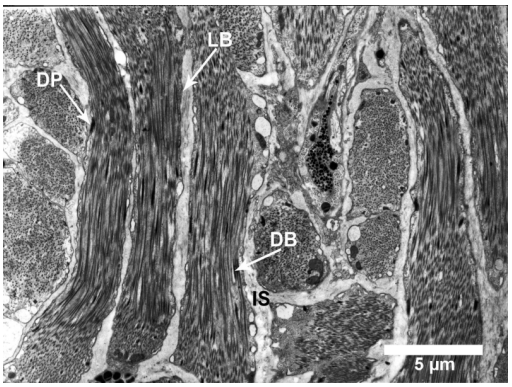

Fig.2 The sections through smooth muscle cells (longitudinal, transversal), DB – dense bodies, DP – dense plaques, LS – basal lamina, IS – intercellular spaces filled by collagen fibrils, scale 5 μm. According to [11]

MEASUREMENT OF TISSUE STIFFNESS

The modulus of elasticity of investigated tissue was measured at the Department of Macromolecular Physics of the Mathematical-Physical Faculty of the Charles University in Prague in the experimental laboratory. The measurement device was DMA-7e (dynamical mechanical analyzer, Perkin Elmer company).

The living tissue and the tissue fixed by formaldehyde were measured. All samples were clamped into the jaw of the measuring device. The exact width and thickness were measured by a digital caliper, the height of sample was found by the device. Strapped sample was embedded into a beaker with physiological solution to protect the sample before dehydration.

For measurement of mechanical properties of gastropod tissue, uniaxial loading in two directions (longitudinal and transversal direction to the main axis of gastropod body) and with various velocity rates of mechanical loading namely 2, 10, 20 and 50 mN/min were used. All samples were pre-stressed by the force of 10 mN during 3 minutes by transversal and 4 minutes by longitudinal loading. After pre-conditioning the load grew linear from 10 to 100-300 mN.

The elongation of smooth muscle tissue on template load was checked. The least squares method was used on the resultant stress-strain relations and the Young's moduli of material were obtained.

MEASUREMENT OF ULTIMATE STRENGTH

The ultimate strength of the smooth muscle tissue was measured at the Department of Mechanics of the Faculty of Applied Sciences of the University of West Bohemia in Pilsen on materials testing machine SC-FR050TH, company Zwick GmbH & Co.

The samples were clamped into the jaw of the measuring device. The width and thickness were measured by a digital caliper.

The failure of the gastropods connective and smooth muscle tissue was observed. The uniaxial loading was applied in the direction longitudinal to the gastropod's main body axis. The load velocities were 2 and 3 mm/min and 3 mm/min for living and fixed tissue, respectively.

The values of ultimate strength were obtained from stress-strain relations.

THE STEREOLOGY OF TISSUE

The stereology measurement was used to find the percentage area fractions of the main tissue components.

The microscope Olympus BX51, with objectives UPlanSApo 4x/0.40 and 20x/0.750, the digital camera Camedia C5060WZ directed by the software QuickPhoto Micro 2.0 (Olympus C&S, Prague, Czech Republic) were used for observation and recording.

An additional piece of the tissue adjacent to the sample used for the mechanical test was taken for histological processing. The tissue was placed in the 10% buffered formalin and processed by common paraffin technique. Each sample was cut into 72 serial sections (thickness of 5 μm) with transversally oriented cutting plane, and stained with Mallory trichrome and hematoxylin-eosin.

We assessed the relative proportions of the smooth muscle cells, hemocoelic spaces and other tissue components in the area of 12 histological sections through the integument of each specimen. We calculated a mean value of individual values of percentage fractions of the main components.

The software Ellipse3D created by company ViDiTo, Košice, Slovak Republic was used for stereological study of the tissue.

This software covering the Buffon needle problem was used to estimate the length density measurement as follows. The expected number of intersections between smooth muscle cells and the rupture was calculated:

$$P_L = l\frac{2}{\pi}L_A,$$

where L_A is the value of estimated length density of smooth muscle cells, P_L is the expected number of intersections between smooth muscle cells and the rupture, and l is the length of the rupture, see [10].

RESULTS

The samples started to elongate immediately after applying the load. Pre-stress and pre-conditioning of samples were not reflected during evaluation.

The values of Young's moduli of the living tissue loaded in the transversal direction by 2, 10 and 20 mN/min loading velocity rates were 24.221 ± 2.449, 29.509 ± 3.162 and 28.471 ± 2.778 kPa, respectively, see Fig. 3. The values of Young's moduli of the living tissue loaded in the longitudinal direction by velocity rates 2, 10 and 20 mN/min were 27.967 ± 2.928, 36.354 ± 4.259 and 42.192 ± 7.521kPa, respectively, see Fig. 4.

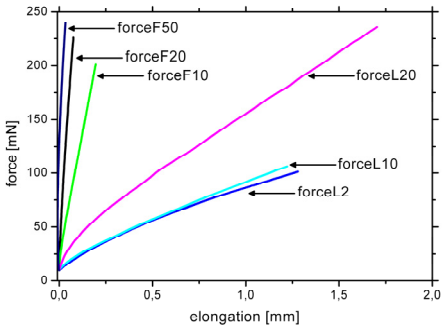

Fig.3 The force vs elongation for smooth muscle tissue of gastropods during loading in the transversal direction to the main axis of the body. The living tissue at velocity rates 20 (forceL20 - pink), 10 (forceL10 - turquoise) and 2 mN/min (forceL2 - blue). The fixed tissue at velocity rates 50 (forceF50 – dark blue), 20 (forceF20 - black) and 10 mN/min (forceF10 -green)

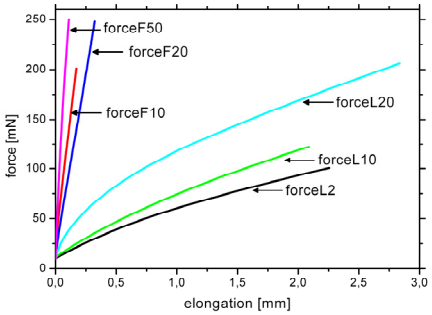

Fig.4 The force vs elongation for smooth muscle tissue of gastropods during loading in the longitudinal direction to the main axis of the body. The living tissue at velocity rates 20 (forceL20 - turquoise), 10 (forceL10 - green) and 2 mN/min (forceL2 - black). The fixed tissue at velocity rates 50 (forceF50 - pink), 20 (forceF20 - blue) and 10 mN/min (forceF10 - red)

The stiffness values of fixed tissue loaded in the longitudinal direction to main body axis for rates of loading 10, 20 and 50 mN/min were 565.788 ± 75.779, 434.200 ± 68.688 and 327.240 kPa respectively, see Fig. 3. The stiffness values of fixed tissue loaded in the transversal direction to main body axis for rates of loading 10, 20 and 50 mN/min were 770.238, 504.087 ± 18.238 and 410.166 ± 28.160 kPa, respectively, see Fig. 4.

The values of ultimate strength for living tissue for loading velocities 2 and 3 mm/min were 1.59 ± 1.39 N (883.33 ± 216.67 kPa) and 2.16 ± 0.10 N (1201.85 ± 56.79 kPa), respectively,

see *Fig. 5*. The values of ultimate strength for tissue fixed by formaldehyde for loading velocities 3 mm/min were higher then for the living tissue, the values were 8.31 ± 0.82 N (4618.52 ± 453.09 kPa), see Fig. 5.

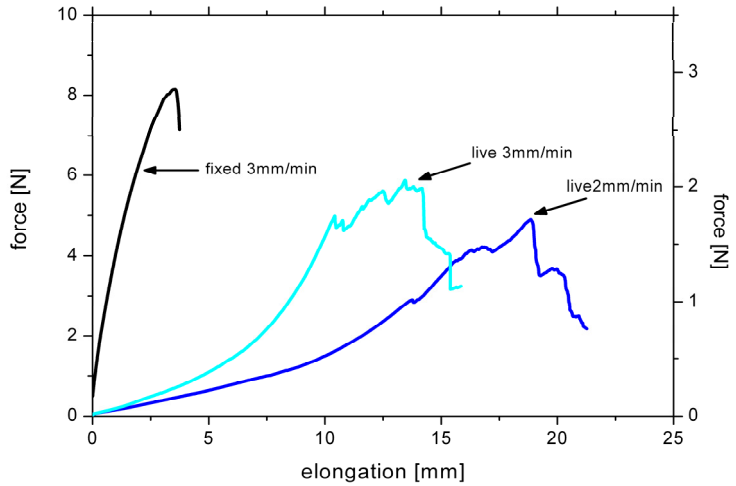

Fig.5 The force vs elongation with ultimate strength for smooth muscle tissue of gastropods during uniaxial loading in the longitudinal direction to the main axis of body. The velocities for living tissue 2 (live 2mm/min - blue), 3 (live 3 mm/min - turquoise) and for fixed tissue 3 mm/min (fixed 3mm/min - black)

It was obvious that the rupture line ran through the connective tissue, see Fig. 6. The results of the stereological study concerning the measurement of preferred directions of the tissue rupture are summarized in Tab. 1. The significant difference between the theoretical number P_L and the real number P_L' of points of intersection of smooth muscle cells and rupture convincingly contradicted the hypothesis that the direction of the rupture was running though the connective tissue surrounding the smooth muscle cells.

The stereological study showed mean percentage area fractions of the smooth muscle cells: 44.45 ± 7.67%, hemocoel 36.01 ± 5.32%, others cells (neural fibrils, fibroblasts, immune and fat cells) and collagen fibrils 19.54 ± 4.55%. These components are responsible for mechanical properties of the tissue.

L_A - the values of estimated length density, P_L - expected number of intersections between smooth muscle cells and the rupture, P_L'- real number of points of intersection of smooth muscle cells and the rupture, l - length of the rupture and p - p-value of Wilcoxon matched pairs test **Table 1**

sample	L_A	P_L	P_L'	*l [μm]*	*P*
106/05	1.36E-01	141.76	32.50	1.63E+03	2.22E-03
105/05	1.27E-01	68.85	19.92	8.53E+02	2.22E-03
	1.64E-01	148.87	22.00	1.43E+03	2.22E-03
107/05	9.45E-02	67.59	9.17	1.12E+03	1.17E-02

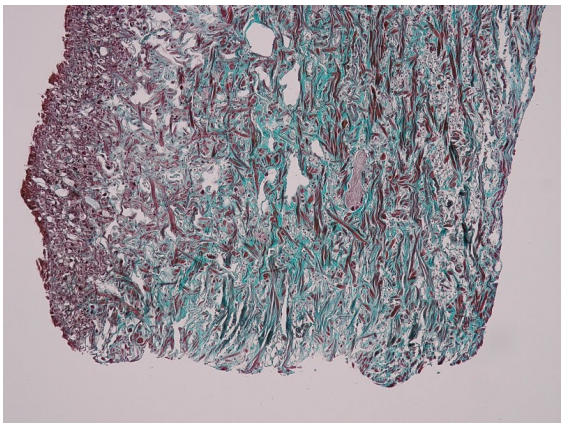

Fig.6 The rupture of the tissue significantly going through the connective tissue

DISCUSSION

The results show that the value of elongation depends on the value and velocity of loading. Smooth muscle tissue embodies non-linear behavior, see [7, 9, 12]. The high dynamics of the tissue is the reason of the change of stiffness for various load velocity rates, see [1]. This dynamics is caused by reordering of interior cytoskeleton. Sharp increase of loading induces the cell membrane depolarization and it results in contraction, the cell becomes stiffer. Slow loading leads to elongation of tissue, "tissue plasticity". By virtue of this are smooth muscle tissues like that in urinary bladder able to hold the liquid inside of the tissue without significant increase of pressure in the liquid inside. This property of smooth muscle tissue is called "stress relaxation" and leads to the fact that the slow loaded tissue appears to be less tough than the fast loaded tissue for which appears the muscle contraction.

The stiffness increase is due to the structural and morphological changes which are produced by fixation. Formaldehyde forms the cross-net between the proteins, builds gel which holds the cell constituents in state "in vivo". Soluble proteins are fixed into structural proteins and become insoluble. Formaldehyde causes protein denaturation and irreversibly makes covalent binding between molecules of neighboring proteins and changes so the original tertiary structure. The result is a 3-D net of intra- and extracellular proteins which increases the stiffness of cells.

The reasons for higher value of ultimate strength in fixed tissue are the morphological changes, which are produced by fixation agent formaldehyde, discussed up.

The direction of the rupture doesn't run though the connective tissue surrounding the smooth muscle cells shows that the least rigidity is in these tissue components.

CONCLUSION

The results of measurement of smooth muscle tissue proved that the stiffness of this tissue depended on the level and velocity of loading. The slow loading causes exteriorization of tissue plasticity known as "stress relaxation". The mechanical properties of the tissue corresponded to morphological composition of the tissue: smooth muscle cells 44.45%, hemocoel 36.01%, other cells and collagen and elastin fibrils 19.54%. This work determined a range for ultimate strength of gastropod connective and smooth muscle tissue and quantified a trend in failure behavior of this type of tissue. It was found that the rupture preferred the connective tissue which didn't surround the smooth muscle cells.

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