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ORIGINAL PAPER

MICROSCOPIC AND ULTRASTRUCTURAL CHARACTERISATION OF THE DIAPHRAGMATIC CENTRAL TENDON UNDER STANDARD-PRESSURE PNEUMOPERITONEUM OF VARYING DURATION

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Abstract

Introduction. In-depth study of the ultrastructure and cellular composition of the central tendon opens new perspectives in understanding the biomechanics of respiration under conditions of laparoscopic pneumoperitoneum.
The objective of the study was to determine the dynamics of microscopic and ultrastructural changes in the central tendon of the diaphragm under the influence of standard-pressure pneumoperitoneum.
Materials and methods. The experiment was performed on 55 male rats, divided into a control group and three experimental groups. Pneumoperitoneum was simulated by inserting a Veress needle into the abdominal cavity and creating an intra-abdominal pressure of 10 mmHg. After that, the central tendon was harvested for morphological study.

Résumé

Caractérisation microscopique et ultrastructurale du centre tendineux du diaphragme sous pneumopéritoine à pression standard de durée variable

Introduction. L'étude approfondie de l'ultrastructure et de la composition cellulaire du centre tendineux ouvre de nouvelles perspectives pour la compréhension de la biomécanique respiratoire dans les conditions du pneumopéritoine laparoscopique.
L'objectif de l'étude a était de déterminer la dynamique des changements microscopiques et ultrastructuraux du centre tendineux du diaphragme sous l'influence d'un pneumopéritoine à pression standard.
Matériel et méthodes. L'expérience a été réalisée sur 55 rats mâles, répartis en un groupe témoin et trois groupes expérimentaux. Le pneumopéritoine a été

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Results. After 5 hours of pneumoperitoneum, the most pronounced changes were detected, the area of damage to the central tendon expanded, and the foci of destruction with lymphocytic-macrophage infiltration were combined with edema of the ground substance. The absence of mucoid swelling when stained with Alcian blue excluded the autoimmune factor of collagen disorganization. Submicroscopically, disorganization of bundles and expansion of interfibrillar spaces were detected. Multiple fragmentation and lysis of collagen fibrils were detected, with preservation of their transverse periodicity in intact areas. Macrophages with numerous lysosomes and phagosomes, as well as lymphocytes forming an inflammatory infiltration, were present in the destruction zones.
Conclusions. Prolonged exposure to standard-pressure pneumoperitoneum leads to progressive destructive changes in the structure of the central tendon of the diaphragm.

Keywords: tendons, intra-abdominal pressure, artificial pneumoperitoneum, ultrastructure, light microscopy, electron microscopy.

List of abbreviations:
CO_2 – carbon dioxide
CT – central tendon
PP – pneumoperitoneum
CG – control group
EG – experimental groups

Résultats. Après 5 heures de pneumopéritoine, les modifications les plus prononcées ont été détectées: la zone de lésion du centre tendineux s'est étendue, et les foyers de destruction présentant une infiltration lympho-macrophagique se sont associés à un œdème de la substance fondamentale. L'absence de gonflement mucoïde lors de la coloration au bleu Alcian a permis d'exclure un facteur auto-immun dans la désorganisation du collagène. À l'échelle submicroscopique, une désorganisation des faisceaux et un élargissement des espaces interfibrillaires ont été observés. Une fragmentation multiple et une lyse des fibrilles de collagène ont été détectées, avec une préservation de leur périodicité transversale dans les zones intactes. Des macrophages contenant de nombreux lysosomes et phagosomes, ainsi que des lymphocytes formant une infiltration inflammatoire, étaient présents dans les zones de destruction.
Conclusions. L'exposition prolongée au pneumopéritoine à pression standard entraîne des modifications destructrices progressives de la structure du centre tendineux du diaphragme.

Mots-clés: tendons, pression intra-abdominale, pneumopéritoine artificiel, ultrastructure, microscopie optique, microscopie électronique.

Introduction

The widespread adoption of laparoscopic technologies in clinical practice has become the gold standard for treating a diverse range of abdominal pathologies. However, despite the minimally invasive nature of this approach, it imposes a specific physiological burden on the body due to the requirement for induced tension pneumoperitoneum[1-3]. The insufflation of carbon dioxide (CO_2) at a pressure of 12-15 mmHg leads to a complex array of pathophysiological reactions, dominated by mechanical compression and ischaemia-reperfusion injuries[4-6].

Within the abdominal wall structure, the diaphragm represents the most dynamic and functionally burdened element. During laparoscopy, it is subjected to direct mechanical stress, being forced to counteract artificially elevated intra-abdominal pressure[7,8]. While the muscular portion of the diaphragm possesses a degree of elasticity and an adaptive capacity through alterations in myosymplast tone, the central tendon (CT) remains a relatively rigid structure. This central tendon ensures the stability of the diaphragmatic dome and serves as the primary fixation point for muscle bundles, making its integrity critically essential for maintaining respiratory excursion[9].

The clinical significance of investigating the CT stems from its unique histological architecture. Composed of dense regular connective tissue, it is physiologically adapted to accommodate pressure fluctuations. However, prolonged static pneumoperitoneum creates conditions for the mechanical 'overstrain' of the collagenous framework[10]. At the microscopic level, this may manifest as alterations in the spatial orientation of fibres, while at the ultrastructural level, it potentially leads to the degradation of the ground substance and disruption of fibroblast metabolic activity. A critical question remains unresolved, how does the duration of surgical intervention influence the threshold of tissue decompensation?

Elevated intra-abdominal pressure inevitably results in the compression of the microvasculature traversing the central tendon. The development of localised ischaemia, compounded by mechanical stretching, establishes a hypoxic environment for fibroblasts – the primary synthesisers of collagen and

elastin[11,12]. Ultrastructural analysis enables the visualisation of early markers of cellular energy depletion – such as mitochondrial vacuolisation and the dilation of endoplasmic reticulum cisternae – well before the onset of structural degradation detectable via light microscopy.

The duration of modern laparoscopic operations can vary from 20–30 minutes to several hours[13,14]. The effect of pneumoperitoneum (PP) exposure on the structural integrity of the diaphragm remains controversial. Most existing studies focus on changes in the lungs or heart, ignoring the diaphragm itself. Investigation of the microscopic and ultrastructural architecture of the CT at standard pressure, but of different duration, will allow determining the time during which the central tendon of the diaphragm is able to maintain its morphofunctional stability.

Understanding the morphological basis of changes in the CT is necessary for the development of myoprotection and optimization of intraoperative pressure parameters. This will reduce the risk of postoperative respiratory complications associated with diaphragmatic dysfunction, especially in patients with concomitant lung pathology. In-depth study of the ultrastructure and cellular composition of the CT opens new perspectives in understanding the biomechanics of breathing under conditions of laparoscopic pneumoperitoneum.

The objective of the study was to determine the dynamics of microscopic and ultrastructural changes in the CT of the diaphragm under the influence of standard-pressure PP, depending on its exposure duration, to establish adaptive and destructive processes within it.

Materials and methods

Experimental research on laboratory animals was conducted in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (18.03.1986), Council Directive 2010/63/EU, and the Law of Ukraine "On the Protection of Animals from Cruelty". The experiment was performed on sexually mature six-month-old rats of the reproductive period, weighing 215.0 ± 20.0 grams. The animals were kept in an accredited vivarium at I.Ya. Horbachevsky Ternopil National Medical University, Ukraine. Manipulations were carried out in the morning at an indoor temperature of 18-20°C, relative humidity of 60-80 %, and illumination of 200 lx. Euthanasia was performed by intraperitoneal administration of thiopental sodium at a dose of 75 mg/kg of body weight.

Male rats were divided into a control group (CG) and three experimental groups (EG). The control group included 10 animals in which no pathology was simulated, these results were considered the baseline for the CT structure, against which the EG data were compared. All animals in the experimental groups were subjected to simulated standard-pressure PP for different durations: the first EG – 1 hour; the second EG – 3 hours and the third EG – 5 hours. Each EG consisted of 15 rats.

Rats were fasted for 6 hours prior to anaesthesia but had ad libitum access to water. Two hours before anaesthesia, water was removed from the cages. The animals were anaesthetized by intramuscular injections of xylazine (10 mg/kg) and ketamine (90 mg/kg) using separate syringes. They were then fixed in a supine position on the operating table. Prior to creating the PP via CO_2 insufflation into the abdominal cavity, the anterior abdominal wall was shaved, and the surgical field was treated three times with 10 % povidone-iodine solution and covered with sterile drapes. A 3-mm skin and subcutaneous tissue incision was made. A Veress needle was inserted into the abdominal cavity through the surgical wound and connected to a KARL STORZ Electronic Endoflator 264300 20. The device parameters were set to an intra-abdominal pressure of 10 mmHg and a CO_2 flow rate of 0.1 L/min.

Immediately following the completion of the designated period for each EG, the diaphragm was harvested, and the CT was isolated (Fig. 1).

The harvested CT specimens were fixed in a 10% neutral buffered formalin solution. Following fixation, the histological material was dehydrated in a graded series of ethanol and embedded in paraffin. Sections of 5-7 μm thickness were cut from each paraffin block using a microtome. After deparaffinization, the sections were stained with haematoxylin and eosin and Alcian blue. For electron microscopy, small fragments of the

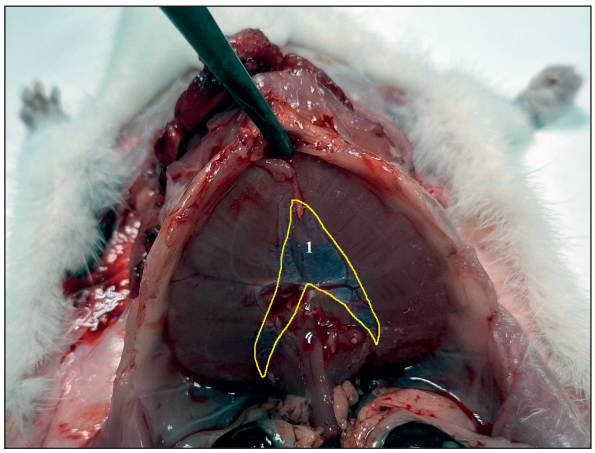


Figure 1. Isolation of the rat diaphragmatic CT: 1 – CT.

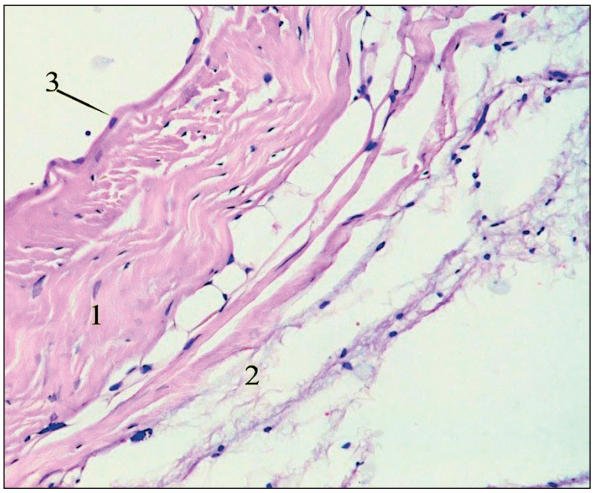


Figure 2. Structure of the CT of the diaphragm (control group): 1 – dense bundles of collagen fibers; 2 – loose connective tissue; 3 – peritoneum. Hematoxylin and eosin staining. × 200.

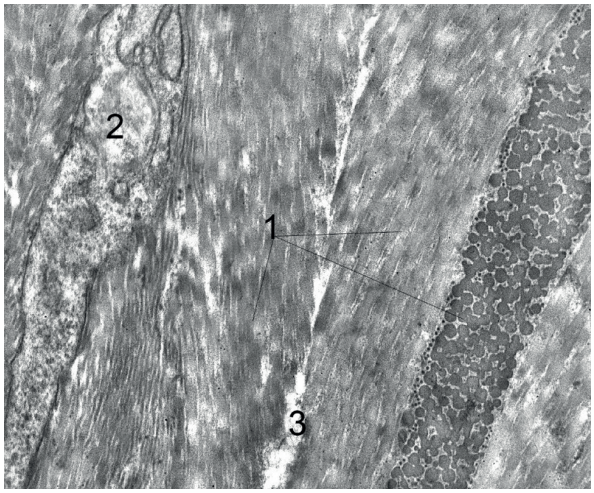


Figure 3. Submicroscopic structure of the CT of the diaphragm (control group): 1 – collagen fibril bundles; 2 – fibroblast cytoplasm fragment; 3 – thin layer of amorphous substance. Electron micrograph. Magnification: × 25 000.

CT were collected and fixed in a glutaraldehyde solution buffered with 0.1 M phosphate buffer (pH 7.36) containing sucrose.

Results

When evaluating the results obtained in the control group of animals, it was found that the CT of the diaphragm fully corresponds to the properties of dense regular connective tissue. It is represented by dense bundles of collagen fibers that form an aponeurotic sheet. Collagen fibers are homogeneously eosinophilic with minor changes in the intensity of staining. Its thickness varies slightly in different sections. From the side of the thoracic cavity, it is covered with diaphragmatic fascia, and from the side of the abdominal cavity, with parietal peritoneum. Microscopically, the latter has a loose consistency and contains fatty tissue. The cellular component is sparse and consists of fibroblasts, fibrocytes, and histiocytes (Fig. 2). Submicroscopically, the cytoplasm of the cells contains branched tubules of the granular endoplasmic reticulum, cisterns of the Golgi complex, and polysomes, which indicates active synthetic processes, in particular, the production of procollagen. There is a small amount of amorphous substance in the intercellular matrix (Fig. 3).

When reproducing PP at standard pressure for 1 hour, degenerative changes occurred, collagen fibers were fragmented, and macrophage-lymphocyte infiltration occurred in response. Loosening and edema created the impression of tendon thickening (Fig. 4). At the electron microscopic level, disorganization of the collagen matrix was determined. Interfibrillar spaces were expanded due to edema and the accumulation of amorphous ground substance. Fibroblasts were edematous, with vacuolization of the cytoplasm, expansion of endoplasmic reticulum cisternae, and uneven contours of the nuclei, signs of chromatin condensation were possible. Single macrophages and lymphocytes were detected in the interfascicular spaces, which indicates reactive inflammatory changes (Fig. 5).

Three hours after the creation of PP at standard pressure, the tissue of the CT loses its compactness, the longitudinal parallel arrangement of collagen fibers is disturbed, which is due to their deformation, fragmentation, and partial lysis. In the areas of fiber disintegration, an intense macrophage-lymphocyte infiltration with an admixture of neutrophils is determined (Fig. 6). Submicroscopic changes were manifested by the loss of dense ordering, bundles of collagen fibrils are disconnected, and their parallel orientation is disturbed. Fibrils are deformed, fragmented, and in places show signs of lysis, interfibrillar spaces are expanded due to edema and the accumulation of amorphous ground substance. In the areas of collagen disintegration, macrophages with numerous lysosomes and phagosomes, lymphocytes with a dense nucleus, as well as single neutrophils with granules are detected (Fig. 7).

After five hours of standard pressure PP, the greatest changes were detected, since the duration of the pathological factor was the longest. In the CT, the lesion zone expanded, multiple foci of destruction infiltrated by lymphocytes and macrophages were adjacent to edema of the ground substance. At the same time, Alcian blue staining did not reveal signs of systemic disorganization of collagen fibers, in particular mucoid swelling, which excluded the presence of an autoimmune factor in this case (Fig. 8). Submicroscopically,

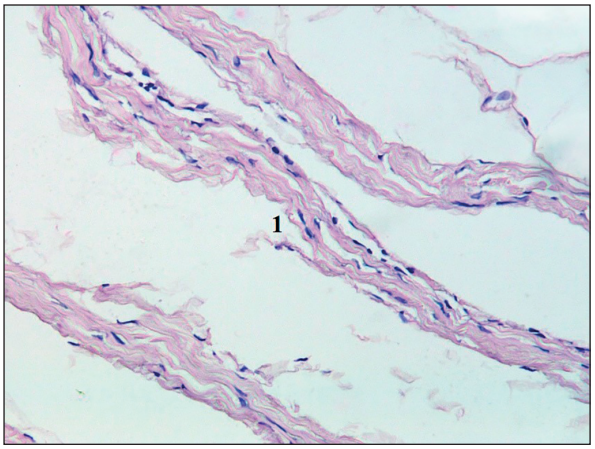


Figure 4. The structure of the CT after 1 hour of PP: 1 – loosening of the structure with fragmentation of collagen fibers. Hematoxylin and eosin staining. × 200.

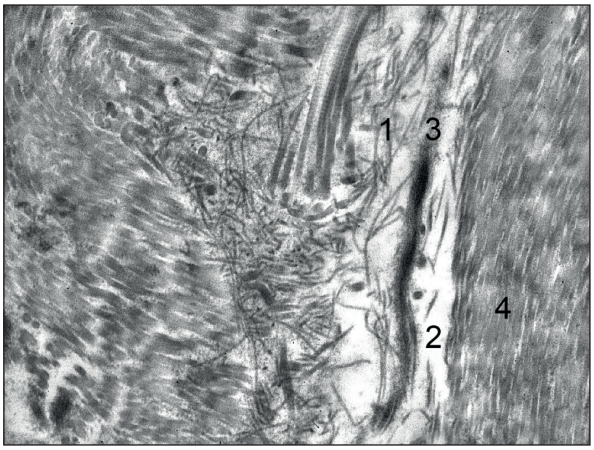


Figure 5. Submicroscopic structure of the CT of the diaphragm after 1 hour of PP: 1 – frayed and fragmented collagen fibrils; 2 – amorphous component; 3 – homogeneous, osmiophilic fibrils; 4 – fibrils with unaltered ultrastructure. Electron micrograph. Magnification: × 21 000.

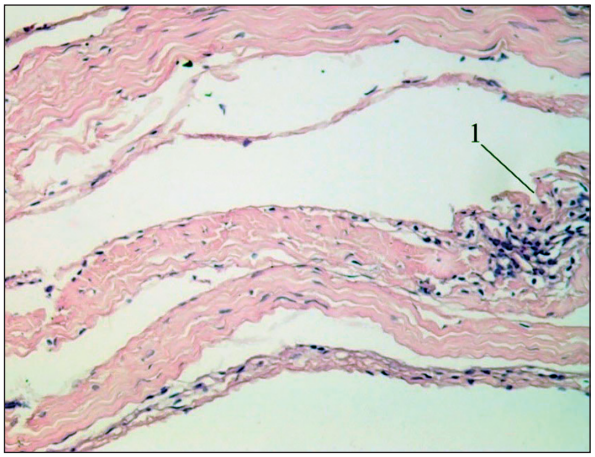


Figure 6. Structure of the CT after 3 hours of pneumoperitoneum: 1 – cellular infiltration. Hematoxylin and eosin staining. × 200.

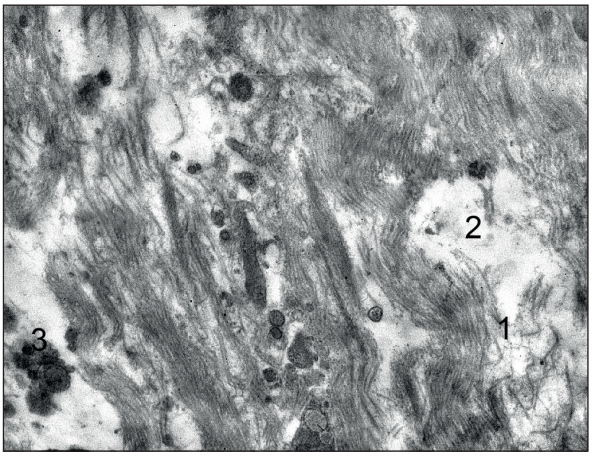


Figure 7. Submicroscopic structure of the CT of the diaphragm after 3 hours of PP: 1 – frayed and fragmented collagen fibrils; 2 – amorphous component; 3 – cellular debris. Electron micrograph. Magnification: × 17 000.

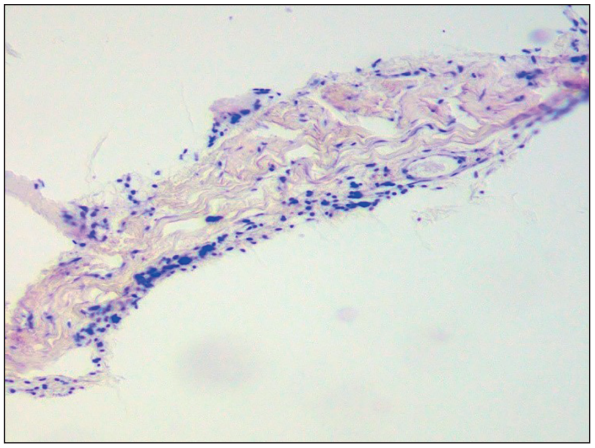


Figure 8. Structure of the CT after 5 hours of PP. Stained with Alcian blue. × 100.

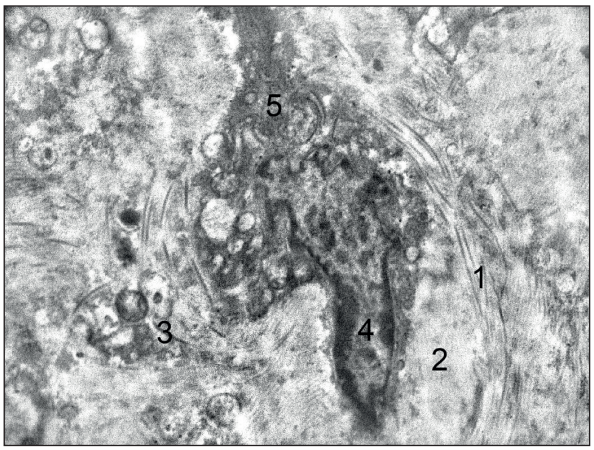


Figure 9. Submicroscopic structure of the CT after 5 hours of PP: 1 – frayed and fragmented collagen fibrils; 2 – amorphous component; 3 – cellular debris; 4 – deformed, osmiophilic nucleus; 5 – fibroblast cytoplasm. Electron micrograph. Magnification: × 9 000.

after 5 hours of standard pressure PP, partially disorganized bundles of collagen fibrils are determined in the central tendon, interfibrillar spaces are expanded due to edema of the amorphous ground substance. Multiple foci of fragmentation and partial lysis of collagen fibrils are detected, with preservation of their transverse periodicity in relatively intact areas. In the destruction zones, macrophages with numerous lysosomes and phagosomes, as well as lymphocytes forming inflammatory infiltration, are present (Fig. 9).

Discussion

Analysis of the obtained results indicates that prolonged exposure to standard pressure PP (12-15 mm Hg) is an aggressive factor that causes a staged disorganization of the dense regular connective tissue of the CT of the diaphragm. The data we obtained for the intact group of animals are fully consistent with the classical ideas about the microscopic architectonics of the CT, where collagen fibers provide high mechanical strength and stability of the diaphragm dome[15,16]. However, when reproducing the conditions of PP, we observed the transformation of the CT from an inert structure into a zone of active reactive inflammation and destruction.

The morphological disorganization of the CT is closely linked to the profound alterations in the topography and functional state of the diaphragm. Under the influence of increased intra-abdominal pressure, we observe a significant cranial displacement (elevation) of the diaphragm dome, which leads to a reduction in the volume of the thoracic cavity. This displacement limits the excursion of the diaphragm during the respiratory cycle, creating a restrictive pattern of ventilation. Furthermore, the constant mechanical stress on the CT in its elevated position causes a redistribution of tension, where the loss of collagen bundle integrity directly compromises the passive elastic recoil of the diaphragm. Consequently, the reduction in thoracic space combined with the structural weakening of the tendinous core forms a pathological basis for postoperative respiratory dysfunction and impaired gas exchange.

Initial degenerative changes and collagen bundle loosening were observed as early as 1 hour after PP exposure. This is consistent with previous studies that have shown that even short-term increases in intra-abdominal pressure can mechanically stretch the mesothelial lining and underlying connective tissue[17]. According to the concept of mechanotransduction, fibroblast stretch activates the release of pro-inflammatory mediators, which is confirmed in our study by the appearance of early macrophage-lymphocyte infiltration. The swelling of the amorphous substance that we observed at the submicroscopic level is likely a consequence of impaired microcirculation and increased capillary permeability under the influence of direct compression.

The results of 3-hour exposure to PP deserve special attention. The loss of tissue compactness and fragmentation of fibrils indicate that the adaptive reserves of the connective tissue are exhausted. Our data are consistent with works describing the effects of ischemia-reperfusion during laparoscopy[18–20]. We suggest that collagen fragmentation is a consequence not only of the mechanical factor but also of the activation of matrix metalloproteinases, which are produced by infiltrating macrophages and neutrophils. The lysosomes and phagosomes we detected in the cytoplasm of macrophages are direct evidence of active phagocytosis of collagen degradation products.

The most pronounced changes after 5 hours of PP demonstrate critical matrix disorganization. Multiple foci of collagen lysis in combination with edema create a morphological basis for a decrease in the elastic properties of the diaphragm. It is interesting that when stained with Alcian blue, we did not detect mucoid swelling. This is of fundamental importance, since it allows us to differentiate the acute traumatic-ischemic effect of PP from systemic autoimmune diseases of the connective tissue, where the accumulation of glycosaminoglycans is a key marker. The absence of an autoimmune component indicates that the pathogenesis of damage in PP is predominantly local mechanical and hypoxic in nature. The preservation of the transverse periodicity of collagen in intact areas even after 5 hours indicates the focal nature of the damage, which leaves a potential opportunity for regeneration when the pressure is removed.

From the point of view of clinical morphology, the identified changes in the structure of the CT are important for understanding postoperative diaphragm dysfunction. Disorganization of the collagenous core may be one of the causes of impaired respiratory biomechanics in patients after prolonged laparoscopic interventions. The obtained data confirm the need for monitoring intra-abdominal pressure and limiting the duration of pneumoperitoneum to prevent irreversible destruction of the diaphragm.

Conclusions

Prolonged exposure to standard pressure PP leads to progressive destructive changes in the structure of the CT of the diaphragm. After 3 hours, a violation of the compactness and parallel orientation of collagen fibers, with signs of their deformation and fragmentation against the background of macrophage-lymphocyte infiltration, was detected. Maximum destruction reaches a peak after 5 hours, which is manifested by the expansion of fragmentation zones and partial lysis

of collagen fibrils while maintaining their transverse periodicity in intact areas. Swelling of the amorphous ground substance and the presence of macrophages with numerous lysosomes and phagosomes confirm the active phase of resorption of damaged collagen. At the same time, the absence of mucoid swelling (according to Alcian blue staining) indicates the absence of systemic autoimmune disorganization of connective tissue under these conditions.

Authors' Contributions:

Conceptualization, methodology, design of the work I.D., M.K., wrote the manuscript, analysis M.K., Z.N., project administration, draft preparation O.Ya., O.D., interpretation of data for the work, editing, validation S.P., Z.N. All authors have read and agreed to the published version of the manuscript.

Compliance with Ethics Requirements:

"The authors declare no conflict of interest regarding this article"

"The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law."

"All institutional and national guidelines for the care and use of laboratory animals were followed"

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