## MORPHOLOGY, PHYSIOLOGY AND PATHOPHYSIOLOGY

# AUTOMATED MORPHOMETRIC STUDIES OF COLLAGEN FIBERS AS AN AUXILIARY METHOD FOR DIAGNOSING COLD INJURY

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## ABSTRACT

***Background.*** *Cold injury is one of the most significant problems for the northern regions of the Russian Federation. Frostbite is defined as a complex of changes caused by the action of low temperatures, which lead to morphological changes in damaged tissue structures. As a result, the skin with underlying tissues and the intercellular matrix, the components of which are collagen fibers, are damaged, which eventually leads to remodeling and a protracted course of the wound process. Morphometric studies in combination with quantitative analysis of microphotographs (histological specimens) using GIS technologies make it possible to distinguish altered collagen fibers under the influence of low temperatures from relatively healthy tissues.*

***The aim of the study.*** *To assess the possibility of using computer analysis of microphotographs in a complex of morphometric studies of collagen fibers in local cold injury.*

***Materials and methods.*** *The study included 84 patients with III and IV degree frostbite of the lower extremities. Morphological study of tissues and microphotography were performed using Leica DM2500 microscope (Leica, Germany). The thickness of collagen fibers was measured based on visual measurements of characteristic areas of the microphotograph. Computer analysis of tissue microphotographs of the zone of cryoinjury was performed using the ArcINFO software (Esri, USA). Statistical processing of the study results was carried out using the SPSS Statistics software package (IBM Corp., USA). Graphs and diagrams were constructed using MS Office Excel (Microsoft Corp., USA).*

***Results.*** *With frostbite, severe tissue damage occurs, accompanied by the destructive processes of the extracellular matrix components. Low temperatures contribute to changes in the width and orientation of collagen fibers in the damaged area. In this regard, a change in the texture of the histological specimen image leads to a change in the numerical characteristics of the standard deviation of the curvature coefficient in the studied area of the microphotograph. Thus, in the late reactive period, the described complex of morphometric studies makes it possible to classify particular microphotograph as having pathological signs or as a sample of healthy tissue.*

***Key words:*** *morphometry, collagen fibers, cryoinjury, frostbite, standard deviation, curvature coefficient, raster image*

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# АВТОМАТИЗИРОВАННЫЕ МОРФОМЕТРИЧЕСКИЕ ИССЛЕДОВАНИЯ КОЛЛАГЕНОВЫХ ВОЛОКОН КАК ВСПОМОГАТЕЛЬНЫЙ СПОСОБ ДИАГНОСТИКИ ХОЛОДОВОЙ ТРАВМЫ

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## РЕЗЮМЕ

***Актуальность.*** *Холодовая травма является одной из наиболее значимых проблем для северных регионов нашей страны. Отморожения определяются как комплекс изменений, возникающих вследствие действия низких температур, которые приводят к морфологическим сдвигам в повреждённых тканевых структурах. В результате повреждаются кожа с подлежащими тканями и межклеточный матрикс, компонентами которого являются коллагеновые волокна, что в конечном итоге приводит к ремоделированию и затяжному течению раневого процесса. Морфометрические исследования в комплексе с количественным анализом микрофотографий (гистологических препаратов) с помощью ГИС-технологий позволяют отличить изменённые коллагеновые волокна под действием низких температур от относительно здоровых тканей.*

***Цель исследования.*** *Оценить возможность применения компьютерного анализа микрофотографий в комплексе морфометрических исследований коллагеновых волокон при местной холодовой травме.*

***Материалы и методы.*** *В исследование включены 84 больных с отморожениями нижних конечностей III и IV степени тяжести. Морфологическое изучение тканей и микрофотосъёмка выполнялись на микроскопе Leica DM2500 (Leica, Германия). Измерение толщины коллагеновых волокон выполнено на основе визуальных измерений характерных участков микрофотографии. Компьютерный анализ микрофотографий тканей зоны криоповреждения выполнен в программе ArcINFO (Esri, США). Статистическая обработка результатов исследования осуществлялась с помощью пакета программ SPSS Statistics (IBM Corp., США). Построение графиков и диаграмм выполнено с помощью пакета MS Office Excel (Microsoft Corp., США).*

***Результаты.*** *При отморожениях происходит выраженное повреждение тканей, сопровождающееся деструктивными процессами компонентов внеклеточного матрикса. Низкие температуры способствуют изменению ширины и ориентации коллагеновых волокон в зоне повреждения. В этой связи изменение текстуры изображения гистологического препарата приводит к изменению численных характеристик стандартного отклонения коэффициента кривизны в исследуемой области микрофотографии. Таким образом, в позднем реактивном периоде описанный комплекс морфометрических исследований позволяет отнести ту или иную микрофотографию к числу имеющих патологические признаки либо к образцу здоровых тканей.*

***Ключевые слова:*** *морфометрия, коллагеновые волокна, криотравма, отморожения, стандартное отклонение, коэффициент кривизны, растровое изображение*

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## INTRODUCTION

Frostbite is one of the most severe types of cold injury and often leads to a high level of disability and death of victims [1]. During cold alteration, severe dystrophic, inflammatory, and necrotic changes develop after tissue warming, which contribute to damage to cells, intercellular matter, and connective tissue [2, 3].

One of the most characteristic symptoms of the development of pathological processes in tissues is a change in the structure of collagen fibers. In general, the changes include a change in the thickness of the fibers and a transition from a highly dispersed fine-fiber structure to a coarse-dispersed one with thickened linear structures. The listed changes in collagen fibers are visually recorded during morphometric studies.

In order to reduce the subjective component in the evaluation of tissue samples, it seems advisable to apply some numerical estimates of microphotographs, in particular, the study of the texture of the sample. This approach would reduce the time for mass evaluation of samples and eliminate the features introduced by the level of training of the specialist performing the diagnosis. The general purpose of this approach is to develop a set of numerical characteristics that would allow us to classify a sample as having pathological features or as a healthy tissue sample. Similar studies are known in the work on the assessment of the structure and condition of collagen by the second harmonic generation signal, which provides the results of numerical characterization of collagen fibers in a bundle and the mutual organization of bundles of collagen fibers [4]. It should also be noted that there is no universal method to quantify microphotographs suitable for any collagen structure [4].

Any raster image, in particular microphotography of collagen fibers, can be represented as a surface, the height of which corresponds to the numerical characteristic of brightness [5]. In this case, the black pixel (brightness 0) will correspond to a height of 0 m, and the white pixel (brightness 255) will correspond to a height of 255 m. Taking the pixel size as a fixed spatial value (for example, also for 1 m), we get a three-dimensional surface. It should be noted that the direct assessment of the spectral characteristics of the samples (color) is not a reliable source of classifying features. The color may vary depending on the reagents used and the characteristics of lighting. Therefore, texture assessment is the most reliable and promising approach.

For surface analysis, there is a fairly set of geoinformation technology tools [6-8], traditionally used for spatial analysis (regardless of spatial coverage). It is justified to use such a surface characteristic as curvature coefficient at a given point.

The change in the organization of collagen fiber bundles, their width and length is accompanied by a large number of brightness changes, i. e. (in the representation described above) local surface bends within a certain studied area. The more bends such a surface has, the higher the total and average values of the curvature coefficient (CC) will be. It is expected that the CC value will be significantly higher for an image of a histological specimen (transformed into a surface) with a large number of thin contrast fibers than for an image with a smaller number of thicker fibers. In this regard, the authors of the article proposed an algorithm that allows using the CC value as a reliable numerical criterion for classifying a sample as having pathologies or no pathologies.

## THE AIM OF THE STUDY

To assess the possibility of using computer analysis of microphotographs in a complex of morphometric studies of collagen fibers in cold injury.

## MATERIALS AND METHODS

The study included 84 patients with frostbite of the lower extremities of the III and IV degrees of severity in the period of granulation and epithelialization (day 30). The age of the victims ranged from 25 to 45 years. Men made up the majority of the group of patients – 78 %, women – 22 %. All the victims with local cold injury of the distal segments of the feet underwent surgical treatment at the regional center for thermal trauma at the Chita City Clinical Hospital No. 1 in the period from 2016 to 2020. All the patients gave written voluntary informed consent. Tissue fragments measuring 1 × 1 × 0.5 cm were taken with a scalpel under local anesthesia during surgical treatment in the affected area at the border of damaged and healthy tissues (in the demarcation zone). The study was performed in accordance with the Regulations of Declaration of Helsinki of the World Medical Association (rev. 2013).

Autopsy material was used as a control. Tissue sampling from the lower extremities was performed within 24 hours after the biological death of the patients. The control group consisted of 50 patients who died from acute trauma, without severe concomitant diseases, of a similar age category.

Exclusion criteria: age – under 18 y. o. and over 50 y. o.; tuberculosis; cachexia of various etiologies; sepsis; chronic obstructive pulmonary disease; diabetes mellitus; vascular and nerve diseases of extremities; rheumatism; premorbid heart rhythm disorders; acute inflammatory diseases; atherosclerosis; acute cerebral circulation disorder.

Histological specimens were prepared according to a generally accepted method. Sections of 5–10 μm thickness were stained with hematoxylin, eosin and picrofuxin according to Van Gieson. Histological microslides of tissues of patients with local cold injury and tissues of patients of the control group were examined using Olympus CX21 microscope (Olympus, Japan), microphotography was performed using a Leica DM2500 microscope (Leica, Germany), at the Department of Pathological Anatomy of the Chita State Medical Academy.

Morphometric studies to determine the thickness of collagen fibers were performed for the control group and patients with frostbite on day 30 with local cold injury. The measurement of the thickness of collagen fibers was performed on the basis of visual measurements of characteristic areas of microphotograph, the areas for measurements were selected based on diagnostic experience. For each microphotograph, at least 10 measurements of the thickness of collagen fibers were performed at the same magnification of the microscope (× 5). The complex of morphometric studies includes computer analysis of images of collagen fibers, which suggests the author's method of evaluating microphotographs in the field of view. The following sequence of actions was adopted for processing microphotographs:

Step 1. Creating surfaces. To perform this task, the RGB image in JPG format was converted to grayscale by channel-by-channel averaging (the numerical values of the brightness of each pixel in R, G, B channels are added and divided by 3). Contrast, gamma and histogram correction, other digital brightness correction methods were not applied in order to ensure the most homogeneous, content-independent spectral brightnesses of the samples. The resulting gray image is transformed into an ArcINFO GRID type surface (matrix with height values), where each cell is assigned a height numerically equal to the brightness of the gray image on a scale from 0 to 255.

Step 2. Surface filtration. The resulting image may contain digitization (technical features of the microscope) and compression (defects in the JPG algorithm) defects. Samples from different researchers may have different types of artifacts. For this purpose, the final surface was filtered. The sliding window method with a radius of 2 pixels is used for filtering, window shape – circle. The idea of the filtering algorithm is that a circle with a radius of 2 pixels is built around the current cell of the surface, the brightness of all pixels inside the circle is added and divided by the number of cells. The resulting average value is assigned to the current cell. Next, the analysis window moves one pixel to the side.

Step 3. Calculation of the curvature surface. The surface is calculated using the ArcGIS package using the Curvature function. The calculation of the numerical characteristic of the curvature goes along a 3 × 3 cell section, the final results are assigned to the central cell. The calculated curvature coefficient is multiplied by 100 for ease of use – it allows us to work with whole numbers, not fractional numbers. The result is a surface of CC values, the range of values is from 0 to 1800.

Step 4. Calculation of Curvature statistics. For each image, a block structure was formed in the form of squares measuring 100 × 100 pixels (which corresponds to a 64 × 64 μm matrix cell). The size of the block is determined by the size of the digital image, the field of view and the size of the collagen fibers. It is necessary to follow the principle when the block size is at least an order of magnitude larger than the average fiber thickness and when the field of view is at least an order of magnitude larger than the block. The size of 100 × 100 pixels satisfies these conditions.

Statistics of CC values were calculated for each image and for each block. The «Average» value is not representative, as it is close to 0 for all blocks and images – this is a consequence of peculiarities of the algorithm itself. However, the value of the standard deviation for CC values shows the presence of two well-defined classes correlating with the presence or absence of pathologies in the images. Calculating the values of standard deviations not for the entire images, but for blocks (with their subsequent filtering) allows us to exclude from consideration tissue samples and structures present on tissue samples, but not the subject of analysis (background, extraneous inclusions, accompanying tissues, etc.).

Step 5. Displaying the results. The results of the computer analysis of microphotographs are presented in the form of classified images by class: Control → Transitional class → Patients.

Statistical processing of the study results was carried out using the SPSS Statistics software package v. 25.0 (IBM Corp., USA). The results of the study are presented in the form of median, quartiles 1 and 3 – Me (Q1; Q3). Taking into account the number of the studied groups, the assessment of the normality of the distribution of quantitative characteristics was carried out using the Kolmogorov-Smirnov test. The statistical significance of the differences in the indicators was assessed by comparing the calculated and critical values of the Kruskal-Wallis test, followed by determining the significance level of $p$.

The results are presented in the form of boxplots of collagen fiber thickness and values of CC standard deviations in the studied area of the microphotograph for the groups (Control, Patients) in the figures. The image of the results of the computer analysis of microphotographs is presented in the form of classified images by class: Control → Transitional class → Patients in the pictures.

## THE RESULTS OBTAINED AND DISCUSSION

As a result of the study, a significant increase in the thickness of collagen fibers was revealed in a group of patients with cold injury in the late stages of cryoinjury relative to the control group.

Figure 1 shows an example of the results of determination the thickness of collagen fibers for microphotographs from the control group and the group of patients.

In the field of view of the microphotograph, significant differences are observed in the form of changes in the thickness and orientation of the collagen fibers of the cryoinjury zone. This fact confirms the significant damage to the tissues of the frostbite zone at a late date (on day 30). Destructive processes in collagen fibers manifest themselves in the form of changes in their thickness and orientation. The so-called tissue disorganization of the cryoinjury zone occurs.

In the late reactive period, in patients with cryoinjury, the fiber thickness is 2.8 times higher relative to the control group ($p < 0.001$).

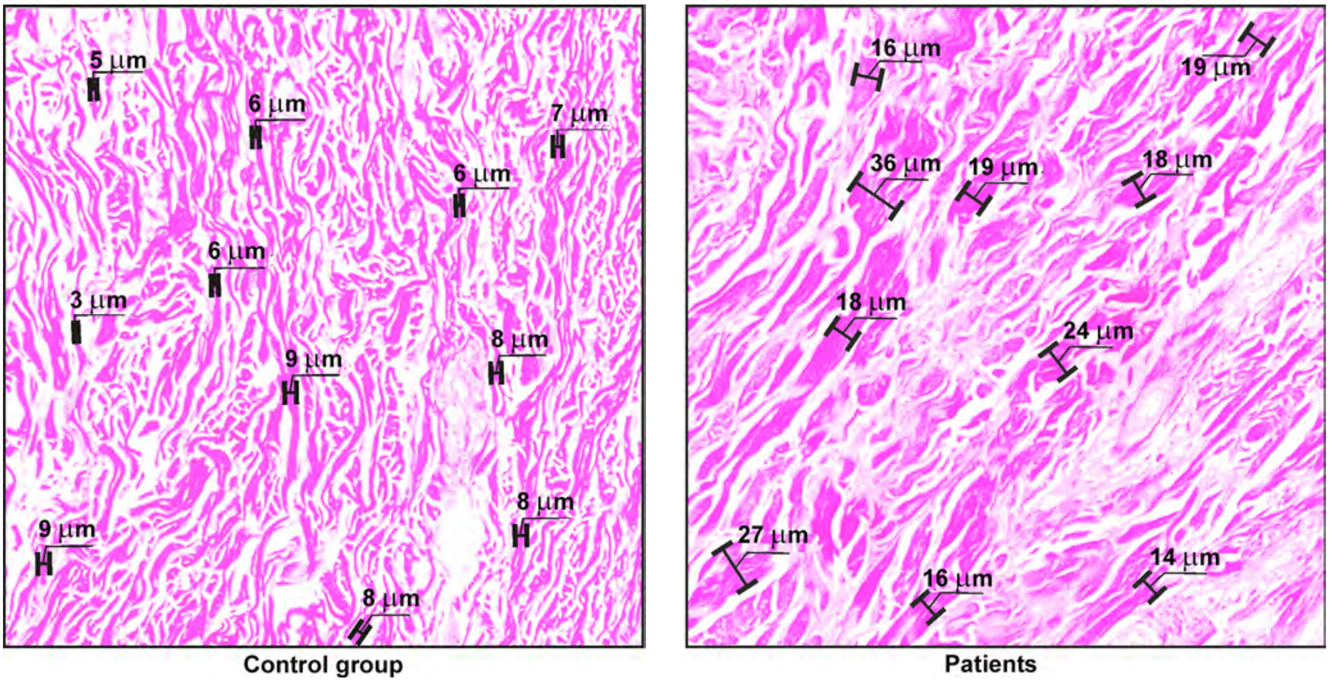


***FIG. 1.***

*Thickness of collagen fibers in the control group and in the group of patients with frostbite. Van Gieson picrofuxin staining. Magnification during microphotography ×5. Photo made by the author*

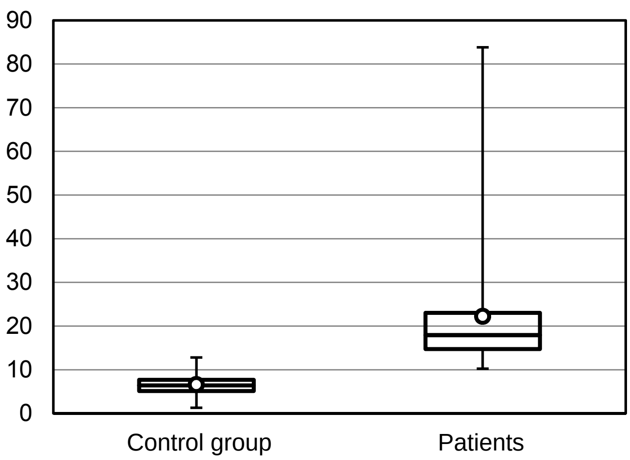


***FIG. 2.***

*Fiber thickness (microns) in the control group and in the group of patients with local cold injury*

Collagen disorganization has enzymatic and non-enzymatic mechanisms. Enzymatic mechanisms are carried out by the interaction of collagenases and their inhibitors, which regulate the intensity of catabolic processes. Non-enzymatic mechanisms are associated with the formation of additional intermolecular links under the influence of end-products of glycation of UV irradiation or mechanical pressure (edema), as well as a result of carcinogenesis. Different regulatory pathways and degradation mechanisms intersect with each other at different stages, rather than working independently of each other [9–11].

Thus, secondary dystrophic and inflammatory changes in tissues under the influence of low temperatures lead to damage to collagen and its disorganization. This phenomenon pathogenetically explains the large number of late complications in patients with frostbite and the characteristic prolonged course of reparative processes.

As a result of computer analysis of images of collagen fibers for the control group and the group of patients, the standard deviation of the curvature coefficient in the studied area of the microphotograph was determined.

In the late reactive period, in patients with cryoinjury, the standard deviation of CC in the studied area of the microphotograph is 1.8 times lower relative to the control group ($p < 0.001$).

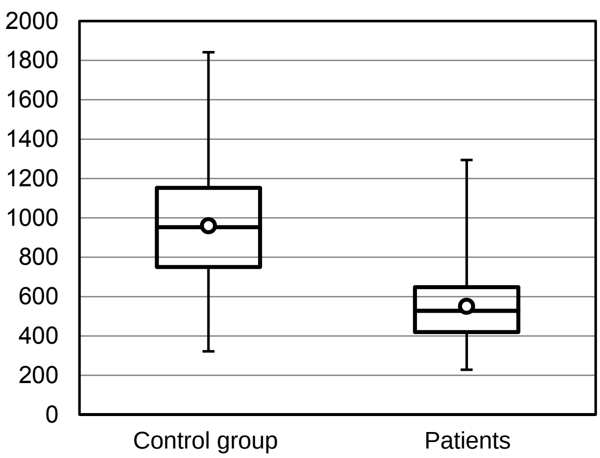


***FIG. 3.***

*Standard deviation of the curvature coefficient (standard units) in the studied area of microphotograph*

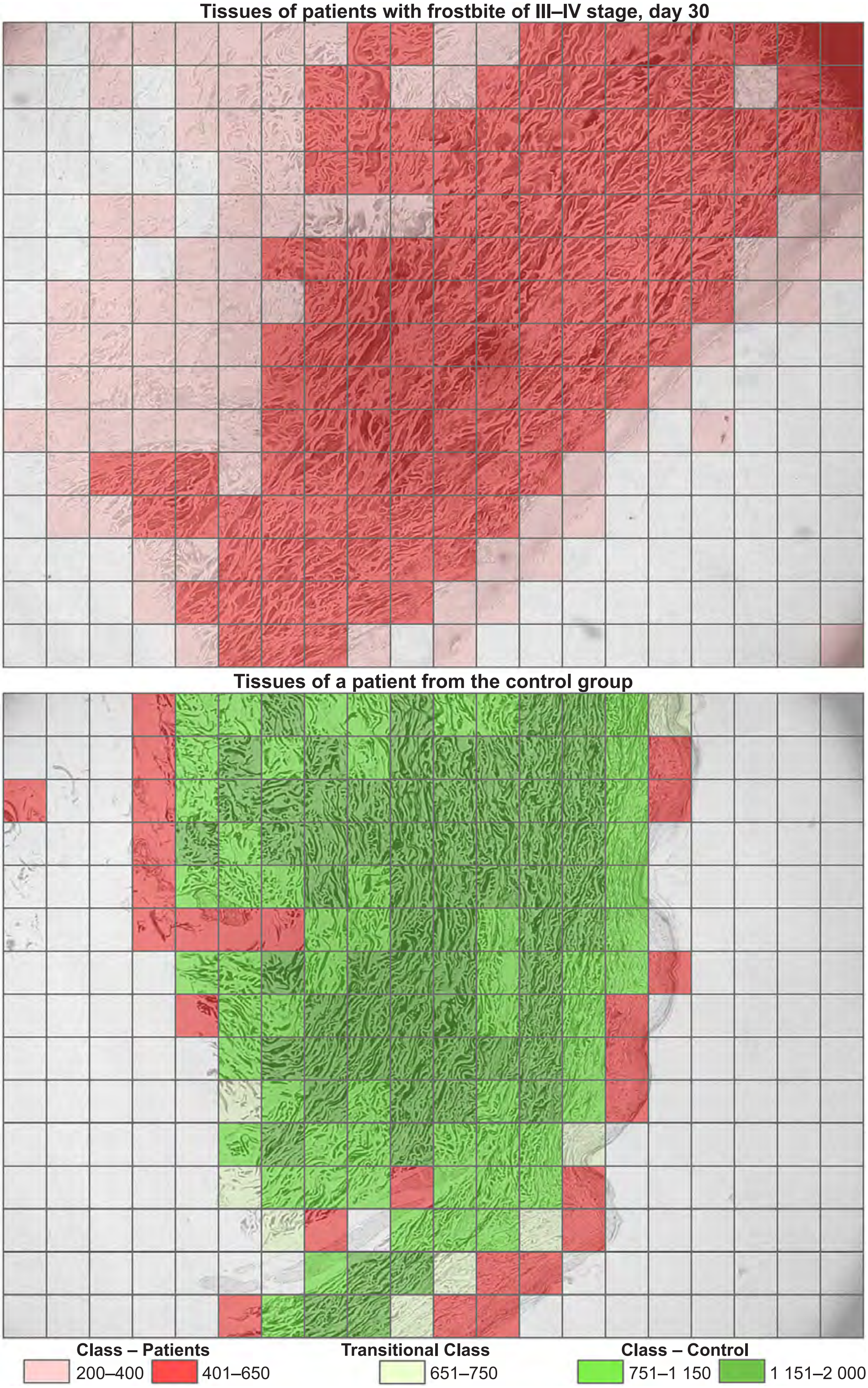


***FIG. 4.***

*Classification of microphotographs according to the values of the standard deviation of the curvature coefficient. Photo made by the author*

With significant cryoinjury of tissues, changes in the thickness and orientation of collagen fibers occur, which is well matched in the field of view of micrography. In this regard, a change in the texture of the microphotograph leads to a change in the numerical characteristics of the standard deviation of CC in the studied area. This is confirmed by a strong inverse correlation between the measured thickness of collagen fibers and the standard deviation of CC in the studied area of the microphotograph ($r_S = -0.75$; $p < 0.001$).

Thus, the characteristic of the standard deviation of the CC allows us to attribute one or another microphotograph to the number of pathological signs or to a sample of healthy tissues. This fact makes it possible, based on the obtained statistical indicators of the standard deviation of the CC, to classify the field of study of microphotograph by classes: Control → Transitional class → Patients (Fig. 4).

Based on the classification obtained, the difference between the microphotograph of tissues of patients with frostbite of III–IV stage on day 30 and the control group is clearly demonstrated (Fig. 4).

The number of colored blocks in each class characterizes the area of damage to collagen fibers in the field of view of the microphotograph.

## CONCLUSIONS

Low temperatures lead to qualitative changes in collagen fibers and contribute to a change in their width and orientation in the area of damage. With frostbite, severe tissue structure damage occurs, accompanied by the destructive processes of the extracellular matrix components. Changes in the tissue structure and their organization are important for the morphological characteristics of tissues in various physiological conditions and pathologies, including cold injury.

Computer analysis of microphotographs allows classifying the image of a histological preparation by the value of CC and classifying one or another microphotograph as having pathological signs or as a sample of healthy tissues. Thus, the characteristic changes in collagen fibers during local cold injury in the late reactive period are a factor that makes it possible to perform automated computer diagnostics of microphotographs using GIS technologies.

### Conflict of interest

The authors of this article declare the absence of a conflict of interest.

## REFERENCES


1. Alekseev RZ, Tomsky MI, Golderova AS, Potapov AF, Alekseev YuR, Semenova SV, et al. Prevention of necrosis in frostbite with tissue glaciation. *International Journal of Applied and Fundamental Research*. 2015; (8-1): 35-41. (In Russ.). [Алексеев Р.З., Томский М.И., Гольдерова А.С., Потапов А.Ф., Алексеев Ю.Р., Семенова С.В., и др. Предупреждение развития некроза при отморожениях с оледенением тканей. *Международный журнал прикладных и фундаментальных исследований*. 2015; (8-1): 35-41].

2. Gruzdeva OS, Mikhailichenko MI, Shapovalov KG. Morphological features of tissue changes during limb frostbite. *The Transbaikalian Medical Bulletin*. 2022; (1): 104-113. (In Russ.). [Груздева О.С., Михайличенко М.И., Шаповалов К.Г. Морфологические особенности изменения тканей при отморожениях конечностей. *Забайкальский медицинский вестник*. 2022; (1): 104-113]. doi: 10.52485/19986173_2022_1_104

3. Gruzdeva OS. Pathological anatomy of frostbite. *The Transbaikalian Medical Bulletin*. 2020; (4): 137-144. (In Russ.). [Груздева О.С. Патологическая анатомия отморожений. *Забайкальский медицинский вестник*. 2020; (4): 137-144]. doi: 10.52485/19986173_2020_4_137

4. Dudenkova VV, Shirmanova MV, Lukina MM, Fedelstein FI, Vitkin A, Zagainova EV. Evaluation of the structure and condition of collagen by the second harmonic generation signal. *Biological Chemistry Reviews*. 2019; (59): 181-218. (In Russ.). [Дуденкова В.В., Ширманова М.В., Лукина М.М., Фельдштейн Ф.И., Виткин А., Загайнова Е.В. Оценка структуры и состояния коллагена по сигналу генерации второй гармоники. Успехи биологической химии. 2019; (59): 181-218].

5. Kapralov EG, Koshkarev AV, Tikunov VS. *Fundamentals of geoinformatics:* textbook for university students; in 2 books. Moscow: Akademia; 2004. (In Russ.). [Капралов Е.Г., Кошкарёв А.В., Тикунов В.С. *Основы геоинформатики:* учебное пособие для студентов вузов; в 2 кн. М.: Академия; 2004 ].

6. Kurlovich DM. *GIS analysis and modeling*. Minsk; 2016. (In Russ.). [Курлович Д.М. ГИС-*анализ и моделирование*. Минск; 2016].

7. Savelyev AA, Mukharamova SS, Chizhikova NA. *Fundamentals of spatial analysis in raster GIS:* study guide. Kazan; 2015. (In Russ.). [Савельев А.А., Мухарамова С.С., Чижикова Н.А. *Основы пространственного анализа в растровых ГИС:* учебно-методическое пособие. Казань; 2015].

8. Shikhov AN, Cherepanova ES, Pyankov SV. *Geoinformation systems: Methods of spatial analysis*. Perm: Izdatelstvo PGNI; 2017. (In Russ.). [Шихов А.Н., Черепанова Е.С., Пьянков С.В. *Геоинформационные системы: методы пространственного* анализа. Пермь, Издательство ПГНИ; 2017].

9. Kistenev YuV, Vrazhnov DA, Nikolaev VV, Sandykova EA, Krivova NA. Investigation of the spatial structure of collagen using methods of multiphoton microscopy and machine learning. *Biological Chemistry Reviews*. 2019; (59): 219-252. (In Russ.). [Кистенев Ю.В., Вражнов Д.А., Николаев В.В., Сандыкова Е.А., Кривова Н.А. Исследование пространственной структуры коллагена с применением методов многофотонной микроскопии и машинного обучения. *Успехи биологической химии*. 2019; (59): 219-252 ].

10. Potekhina YuP. Structure and functions of collagen. *Russian Osteopathic Journal*. 2016; 1-2(32-33): 87-99. (In Russ.). [Потехина Ю.П. Структура и функции коллагена. *Российский остеопатический журнал*. 2016; 1-2(32-33): 87-99].

11. Aziz J, Shezali HA, Radzi Z, Yahya NA, Abu Kassim NH, Czernuszka J, et al. Molecular mechanisms of stressresponsive changes in collagen and elastin networks in skin. *Skin Pharmacol. Physiol*. 2016; (29): 190-203. doi: 10.1159/000447017

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