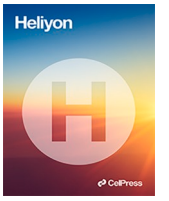
Heliyon 10 (2024) e23287

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



# Histoarchitecture of stromal collagen fibers in gastrointestinal hollow organs of mice after a 30-day space flight

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ARTICLE INFO

*Keywords:*
Orbital flight
Weightlessness
Stomach
Intestine
Connective tissue
Collagen fibers
C57BL/6 N mice

ABSTRACT

The digestive organs are highly sensitive to the influence of orbital flight factors and can limit the professional activities of crew members aboard the International Space Station. Connective tissue, as a system-forming matrix of the integrative-buffer metabolic environment, is of particular relevance in space biomedicine, ensuring the functioning of internal organs under an altered gravitational stimulus. However, the adaptive mechanisms of the fibrous extracellular matrix of the gastric and intestinal connective tissue have not been fully investigated under prolonged microgravity weightlessness. Using histochemical techniques, we experimentally studied the state of collagen fibers in the specific tissue microenvironment of the gastric and intestinal membranes in C57BL/6 N mice after a 30-day space flight, subsequent 7-day ground readaptation, and in animals of the relevant control groups. The 30-day stay of laboratory animals aboard the Bion-M 1 biosatellite resulted in a reduction in the fibrous extracellular matrix of connective tissue in the studied digestive organs, excepting the gastric lamina propria. Increased fibrillogenesis was revealed in the gastrointestinal mucous membranes of animals 7 days after biosatellite landing compared with the parameters of animals in the space flight group. During the experiment with ground simulated orbital flight conditions, changes in collagen fibers were not significant compared to the vivarium control group. Thus, the results obtained evidence gravisensitivity of the fibrous extracellular matrix of the intraorgan connective tissue. This fact also highlights the necessity to further improve gastrointestinal tract-related preventive measures for astronauts during orbital flight.

## 1. Introduction

Connective tissue performs a crucial biological mission in ensuring organ activity. The microenvironment conditions created to implement the functional activity of cells and their derivatives in all body tissues are adequate to meet the levels of external and internal challenges. The conditions of the gravitational stimulus on Earth are a factor to which both the cellular elements of the

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https://doi.org/10.1016/j.heliyon.2023.e23287
Received 21 September 2023; Received in revised form 29 November 2023; Accepted 30 November 2023
Available online 5 December 2023

connective tissue and the components of the extracellular matrix are adapted. The structures of the connective tissue of internal organs, acting as a soft skeleton, respond to changes in gravity during space flight. The launch of the Russian Bion-M 1 biosatellite provided new opportunities to identify structural and functional effects in the body of mammals after a long stay in the conditions of weightlessness, including the state of the connective tissue of the digestive system. On the one hand, the space flight duration was significantly increased, reaching 30 days for the first time in the history of biosatellite launches. On the other hand, the research program of the experiment was designed so that it allowed studying the gastrointestinal connective tissue readapted to the standard gravity after landing and the morphological and functional background of these readaptation mechanisms in the biological objects that had not previously been studied.

The digestive system is one of the critical systems of the body that determines the usefulness of astronauts' adaptation to the factors of long-term orbital flight. As demonstrated earlier in the experiment involving Mongolian gerbils after a 12-day flight on the Foton-M3 spacecraft, specific changes reflecting the result of gravitational unloading developed in the interstitium of the digestive organs [1–5]. In other model studies on cultures, a violation of the differentiation and functional activity of fibroblasts in relation to the remodeling of the extracellular matrix was shown [6–9]. However, the work performed is not enough to evaluate the fibrous components of the interstitium of the digestive system in vivo. Thus, current challenges of space gastroenterology imposing the need for further research on the digestive system under conditions of weightlessness determined the aim of the present study: to investigate the state of the connective tissue under the influence of orbital flight factors.

## 2. Results

### 2.1. The stomach

The fibrous component of the connective tissue was adequately detected in all structures of the stomach wall of mice from the vivarium control group (Fig. 1A and 2A). In the lamina propria of the gastric mucosa, mainly reticular fibers located between the

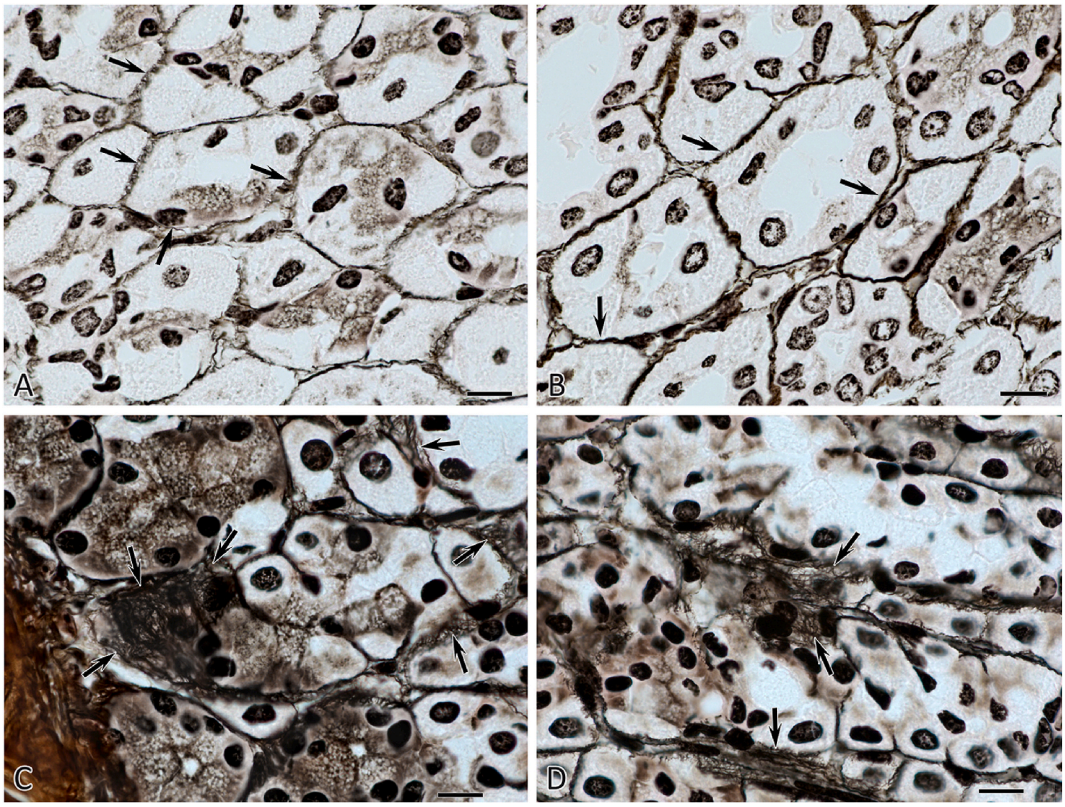


**Fig. 1.** Gastric mucosa of C57BL/6 N mice. Fixation: 10 % neutral formalin. Technique: Foot's silver nitrate impregnation. Groups of vivarium control animals for the 30-day space flight (A), biological control – ground-based experiment simulated 30-day space flight in the flight equipment model "BIOS-MLZH" (B), space flight group of animals that spent 30 days under conditions of weightlessness (C) and readaptation group including animals examined in 7 days after a 30-day space flight (D). Notes: (A) Reticular fibers are localized between the fundic glands of the stomach of the lamina propria (arrow). (B) The number of reticular fibers in the stroma did not change compared to that in the vivarium control group (arrow). (C) High content of reticular fibers in the lower sections of the proper glands of the stomach (arrow). (D). The stroma of the mucosa retains an increased content of impregnated fibrous structures (arrow). Scale bar: 10 μm.

fundic glands of the stomach were detected (Fig. 1A and B). Some of them repeated histotopography of the basement membrane of the proper gland, extending almost the entire thickness of the gastric mucosa. Within the muscularis mucosae, reticular fibers were accompanied by smooth myocytes, forming a network of fine intertwining fibers with mild argyrophilia. In the submucosa, there was a greater number of reticular fibers of various calibers located next to the bundles of fibrous structures, mainly represented by type I collagen. It should be noted that upon impregnation with silver, these fibers were stained in more brownish-yellow shades compared to almost black impregnated reticular fibers. A high content of reticular fibers was detected in the muscularis externa of the stomach

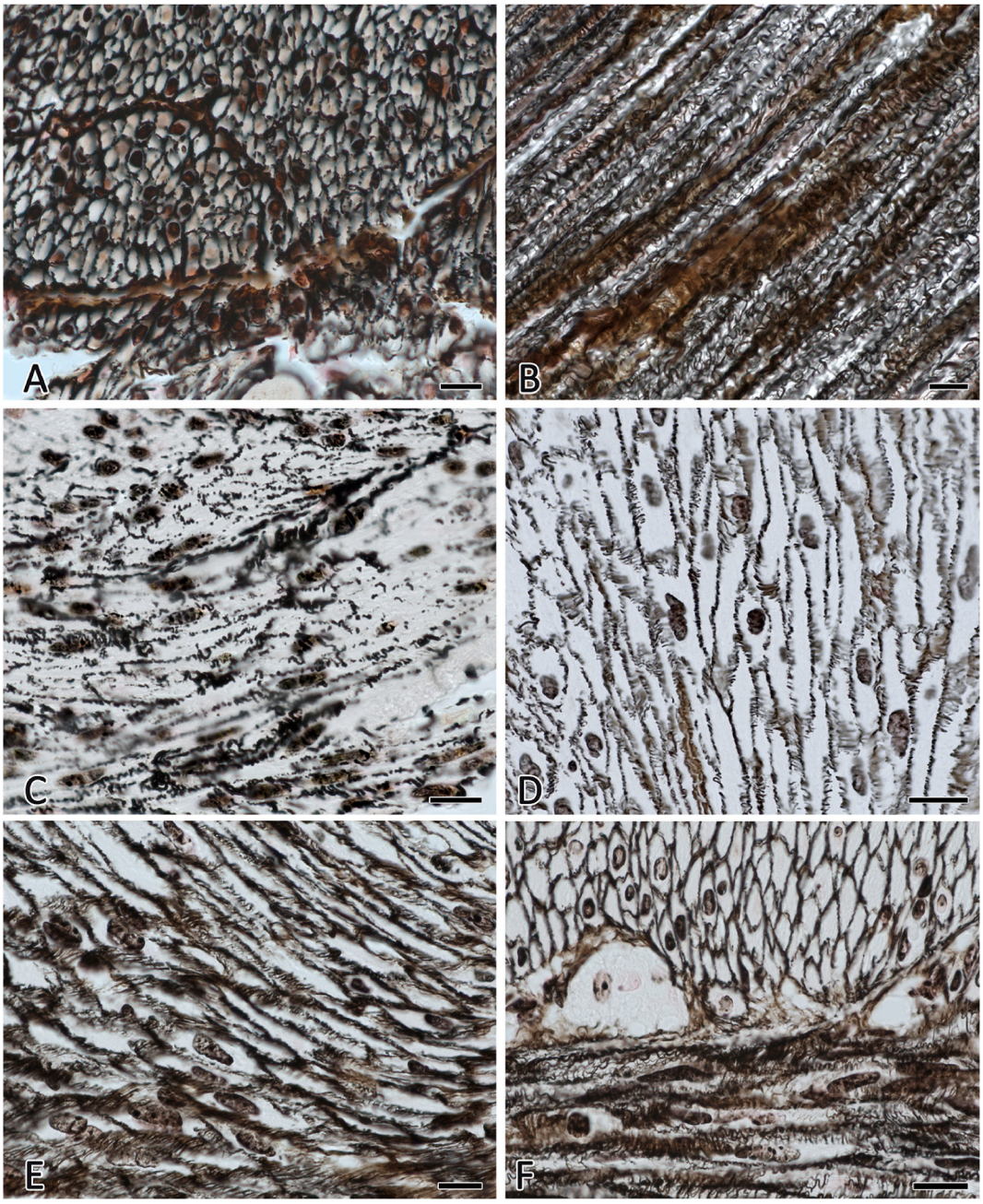


**Fig. 2.** Muscularis externa of the stomach of C57BL/6 N mice. Fixation: 10 % neutral formalin. Technique: Foot's silver nitrate impregnation. Notes: (A,B) - a developed network of reticular fibers in the endomysium of the gastric muscular membrane in animals from the vivarium control for the 30-day space flight group (A) and animals from the group of the ground-based experiment simulated a 30-day space flight in the BIOS-MLZH flight equipment Model (B). (C, D) Reduction of reticular fibers in the gastric muscular membrane in mice after space flight. (E, F) Increased representation of impregnated fibrous structures in the endomysium of the gastric muscular membrane in mice 7 days after BION-M 1 biosatellite landing. Areas with a reduced content of reticular fibers are preserved (E). Scale bar: 10 μm.

(Fig. 2A). Layers of smooth myocytes were separated by accumulations of loose fibrous connective tissue containing reticular fibers of various thicknesses.

Within the muscularis externa, reticular fibers contacting the myocyte plasmalemma were arranged in a specific sequence and formed a dense network consisting of contractile items. The reticular fibers in the muscularis externa of the organ were oriented both in the direction of the long axis of smooth myocytes and transverse (Fig. 2A). Sometimes unevenly distributed argyrophilic material in the form of pulverized grains or larger clumps contacted the myocyte plasmalemma. Collagen fibers, including reticular fibers, were constantly detected in the serosa.

After orbital flight, various transformations of collagen fibers related to the parameters of animals from the vivarium control group were found, and their severity depended on histotopography in the stomach wall. The processes of reticular fiber disorganization combined with changes in their quantitative parameters became evident. The increased representation of connective tissue in the gastric mucosa was due to the formation of microloci with a high content of reticular fibers (Fig. 1C). These areas often extended through the entire thickness of the gastric mucosa. However, the main trend was a locally increased number of impregnated fibrous structures within limited areas of the mucosal surface. Histotopographically, this was typical of the lower third of the gastric proper glands or the area of the fundus. The most likely reason for such changes seems to be the specific trophism of the gastric mucosa under space flight conditions; this specificity determines the extent of spread.

The microloci of the gastric proper glands with violated integrity of the argyrophilic fibrous structures composing the basement membrane were determined. For the tinctorial features of the reticular fibers, there was an increased degree of argyrophilia in the gastric interstitium of C57BL/6 N mice, whereas after staining according to van Gieson, an increased intensity of fuchsin staining of the fibers was more often detected, and in some cases, there was an increased picrinophilia in sites manifesting morphological signs of homogenization of fibers or their bundles. There was an evidentially decreased representation of reticular fibers in the muscularis mucosae of the gastric mucosa. Similar alterations were detected in the area of the submucosa. The number of large-caliber reticular fibers decreased, and granular-like accumulations of impregnated material and intensely stained individual fiber fragments were often observed. The reduction in bundles of collagen fibers was combined with increased fuchsinophilia and signs of edematous events.

There were detected areas of homogenization of collagen fibers, some of them acquired picrinophilia after staining using the van Gieson method. The reason for this, apparently, may be not only hemodynamic disturbances with the development of tissue hypoxia, but also the direct effect of the galactic component of radiation on the supramolecular structure of the fibrous phase of the intercellular matrix of connective tissue, leading to the formation of a large number of reactive groups interacting with the dye. This fact evidences dystrophic changes leading to partial disorganization of collagen fibers within microloci, or implementation of a directed mechanism of their lysis.

The most noticeable postflight changes in the fibrous structures of the extracellular matrix were found in the muscularis externa of the stomach. The number of reticular fibers significantly decreased (Table 1, Fig. 2C and D). These data coincided with a decrease in the expression of type III collagen in the pericellular matrix of smooth muscle cells (Table 2).

A similar trend in the representation of connective tissue was observed in the study of micropreparations after staining according to Masson-Goldner staining (Fig. 3A–D).

In the muscularis externa, rather large loci were sometimes formed, in which fibrous connective tissue structures, including argyrophilic structures, were almost completely absent (Fig. 1C). In the endomysium, the mutual arrangement of fibers relative to each other changed. The number of reticular fibers localized transversely to the long axis of smooth myocytes was significantly reduced (Fig. 2C and D). Reduced impregnated fibers were histotopographically characterized by a predominantly parallel orientation related to the long axis of smooth myocytes. Loci of endomysium with accumulation of granular argyrophilic material were often detected. Changes in the affinity for the dye were detected throughout the reticular fibers, which manifested as a pronounced variability from

**Table 1**
The index of the reticular fiber content in the muscular membrane of the digestive organs in C57BL/6 N mice (relative units, staining technique: silver impregnation method).

| Groups of animals | | Stomach | Jejunum | Colon |
|---|---|---|---|---|
| Experiment on board the "BION-M″ 1 biological satellite | VC-SF | 0.214 ± 0.019 | 0.156 ± 0.011 | 0.188 ± 0.012 |
| | SF | 0.154 ± 0.011*,** | 0.084 ± 0.002 *,** | 0.112 ± 0.007 *,** |
| | VC-RSF | 0.202 ± 0.012 | 0.144 ± 0.014 | 0.173 ± 0.013 |
| | RSF | 0.244 ± 0.017*,** | 0.179 ± 0.011* | 0.208 ± 0.018*,** |
| Ground-based experiment simulated conditions on board the "BION-M″ 1 biological satellite for animals staying in the flight equipment model "BIOS-MLZH" | VC-BC | 0.208 ± 0.022 | 0.165 ± 0.018 | 0.178 ± 0.020 |
| | BC | 0.214 ± 0.017 | 0.173 ± 0.021 | 0.171 ± 0.024 |
| | RBC | 0.197 ± 0.023 | 0.154 ± 0.018 | 0.170 ± 0.014 |
| | VC-RBC | 0.212 ± 0.018 | 0.152 ± 0.016 | 0.182 ± 0.022 |

Notes: statistical significance of differences: * –$p<0.05$ compared with the index of animals of the relevant vivarium control group; ** –$p<0.05$ compared to the relevant biological control. Abbreviations: (VC-SF) The vivarium control group animals for the 30-day space flight group; (SF) The space flight group of animals that spent 30 days under conditions of weightlessness; (VC-RSF) The vivarium control group for the readaptation group including animals examined in 7 days after a 30-day space flight; (RSF) The readaptation group including animals examined in 7 days after a 30-day space flight; (VC-BC) The vivarium control group animals for the biological control group – ground-based experiment simulated 30-day space flight in the flight equipment model "BIOS-MLZH"; BC The biological control group – ground-based experiment simulated 30-day space flight in the flight equipment model "BIOS-MLZH"; (RBC) The readaptation group including animals examined in 7 days after ground-based experiment simulated 30-day space flight in the flight equipment model "BIOS-MLZH"; (VC-RBC) The vivarium control group animals for the readaptation group including animals examined in 7 days after ground-based experiment simulated 30-day space flight in the flight equipment model "BIOS-MLZH".

**Table 2**
Expression of type III collagen in the muscular membrane of the digestive organs in C57BL/6 N mice (relative units).

| Groups of animals | | Pericellular matrix of smooth myocytes of the stomach | | | | Pericellular matrix of smooth myocytes of the jejunum | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Expression Intensity (%) | | | | Expression Intensity (%) | | | |
| | | Not identified | + (*M* ± *m*) | ++ (*M* ± *m*) | +++ (*M* ± *m*) | Not identified | + (*M* ± *m*) | ++ (*M* ± *m*) | +++ (*M* ± *m*) |
| Experiment on board the "BION-M″ 1 biological satellite | VC-SF | 2,2 ± 0,2 | 56,4 ± 3,2 | 34,2 ± 3,3 | 7,2 ± 0,7 | 3,4 ± 0,4 | 51,3 ± 4,2 | 40,1 ± 3,5 | 5,2 ± 0,4 |
| | SF | 5,4 ± 0,4*$^{\Delta}$ | 66,3 ± 4,7*$^{\Delta}$ | 24,9 ± 2,1*$^{\Delta}$ | 3,4 ± 0,2*$^{\Delta}$ | 7,6 ± 0,4*$^{\Delta}$ | 63,3 ± 5,3*$^{\Delta}$ | 26,3 ± 2,2*$^{\Delta}$ | 2,8 ± 0,3*$^{\Delta}$ |
| | VC-RSF | 1,7 ± 0,2 | 52,3 ± 4,4 | 37,6 ± 3,1 | 8,4 ± 0,7 | 3,3 ± 0,3 | 54,3 ± 3,7 | 36,2 ± 4,2 | 6,2 ± 0,3 |
| | RSF | 2,3 ± 0,3 | 44,1 ± 4,1*$^{\Delta}$ | 42,3 ± 3,8*$^{\Delta}$ | 11,3 ± 1,0*$^{\Delta}$ | 4,7 ± 0,4*$^{\Delta}$ | 43,9 ± 3,4*$^{\Delta}$ | 45,6 ± 4,3* | 5,8 ± 0,6$^{\Delta}$ |
| Ground-based experiment simulated conditions on board the "BION-M″ 1 biological satellite for animals staying in the flight equipment model "BIOS-MLZH" | VC-BC | 1,9 ± 0,2 | 57,4 ± 4,1 | 33,9 ± 2,9 | 6,8 ± 0,4 | 4,1 ± 0,5 | 46,3 ± 4,4 | 43,4 ± 3,8 | 6,2 ± 0,4 |
| | BC | 1,8 ± 0,3 | 58,3 ± 4,3 | 32,8 ± 3,3 | 7,1 ± 0,6 | 3,9 ± 0,2 | 44,2 ± 4,4 | 47,6 ± 4,7 | 4,3 ± 0,4 |
| | RBC | 2,3 ± 0,3 | 57,4 ± 5,1 | 34,7 ± 2,2 | 5,6 ± 0,5 | 2,8 ± 0,3 | 49,6 ± 5,1 | 40,2 ± 3,5 | 7,4 ± 0,4 |
| | VC-RBC | 2,8 ± 0,3 | 60,3 ± 5,4 | 31,4 ± 1,7 | 5,5 ± 0,7 | 3,3 ± 0,4 | 52,6 ± 6,2 | 39,5 ± 3,1 | 4,6 ± 0,2 |

Notes: statistical significance of differences: * –$p<0.05$ compared with the index of animals of the relevant vivarium control group; ** –$p<0.05$ compared to the relevant biological control. The abbreviated names of the experimental groups are shown in Table 1. Legend: + weak expression, ++ moderate expression, +++ pronounced expression.

areas with a low degree of staining to the formation of microloci with a high degree of impregnation. In addition, there were identified formations represented by conglomerates of impregnated material without an ordered structure, having an elongated shape and sometimes reaching rather large sizes.

The results obtained in C57BL/6 N mice from the 7-day recovery group after orbital flight demonstrated no return of the connective tissue state in the gastric mucosa to the level of the vivarium control group. First, it is related to the quantitative parameters of reticular fibers. In the places where cells of the gastric proper glands, characterized by the most pronounced signs of dystrophy under space flight conditions, were localized, an increased content of argyrophilic structures remained (Fig. 1D). An increased number of reticular fibers was also observed in the layers of connective tissue separating the proper glands of the stomach (Fig. 1D). There was an increase in argyrophilia in all micropreparations.

Notably, the number of reticular fibers increased in the muscularis mucosae and, especially, in the muscularis externa (Table 1, Fig. 2E and F). Increased content of type III collagen in the extracellular matrix around smooth muscle cells (Table 2). Argyrophilic fibers with a transverse direction toward the long axis of smooth myocytes were restored. To a lesser extent, the content of reticular fibers increased due to fibrous structures localized in the endomysium parallel to the long axis of smooth myocytes and those located in the perimysium. Concurrently, attention was drawn to the high content of impregnated granular formations in the muscularis externa, which could evidence incomplete processes of reticular fiber lysis combined with active biogenesis under conditions of adaptation to the usual level of Earth's gravity. In addition, there was a restoration of the content of endomysium argyrophilic structures contacting the basement membrane of smooth myocytes, which was especially clearly observed in cross sections (Fig. 1E and F). The frequency of detection of gastric smooth muscle loci with pronounced reduction of the network of reticular fibers in the endomysium was significantly reduced compared to that in animals from the space flight group, although such areas were being constantly detected.

In terms of the changes detected in the fibrous extracellular matrix of the stomach in C57BL/6 N mice from the space flight group, the study results of animals from the ground-based space-flight-simulated biological control group were crucial. The analysis of the biomaterial performed immediately after a 30-day stay in the BIOS-MLZH flight equipment model and 7 days after the space flight simulation demonstrated that the main changes in the fibrous component of the connective tissue occurred in the gastric mucosa and affected mainly the state of the reticular fibers. However, the trend toward a decreased representation of reticular fibers was not relevant compared to the parameters of animals from the vivarium control group (Fig. 1B). The changes mainly concerned the tinctorial features of the reticular fibers. Dystrophic changes in the integumentary epithelium and the proper glands of the stomach were rare and were observed within microareas. In the muscularis mucosae, the topography of fibrous elements practically did not differ from the patterns observed in mice from the vivarium control group. The study of the fibrous skeleton of the submucosa demonstrated a high content of reticular fibers in close contact with fibrous structures formed by type I collagen. In the muscularis externa, there was a tendency toward an increased index of the content of reticular fibers compared to the parameters of animals from the control group; however, no statistical significance was obtained. The fibrous structure histotopography did not undergo significant changes (Fig. 2B). The results obtained in animals examined 7 days after the simulated experiment demonstrated that microscopic pictures did not differ in most signs from the parameters of animals from the vivarium control group.

### 2.2. The intestine

In the wall of the jejunum in animals from the vivarium control group, reticular fibers were identified in all membranes, while collagen fibers were predominantly located in the submucosa and serosa (Figs. 3E and 4A). In the serosa, reticular fibers were localized in the subserous layer and had a large caliber and a high level of argyrophilia. In the muscularis externa, impregnated fibrous elements

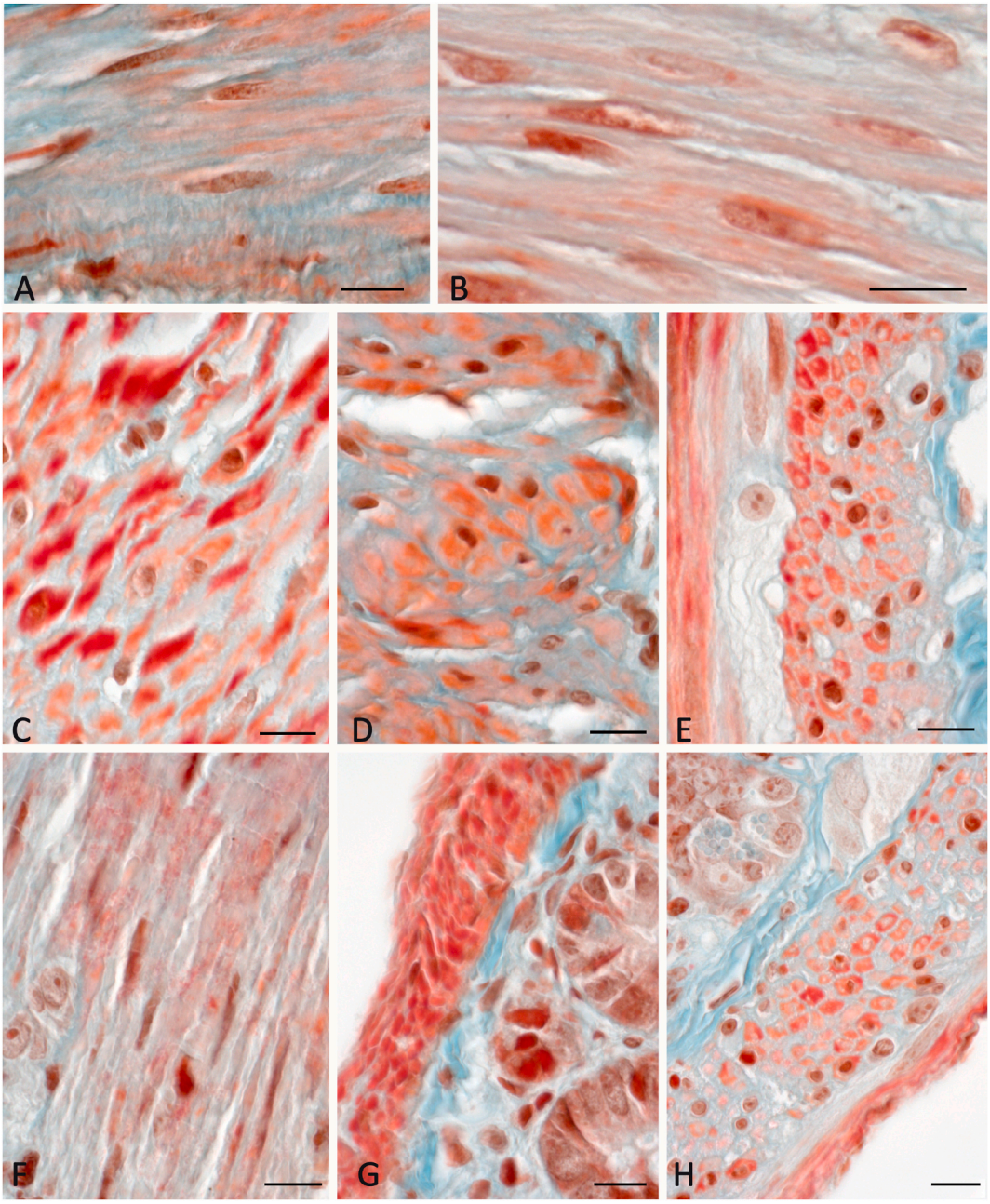


**Fig. 3.** Muscularis externa of the gastrointestinal hollow organs in C57BL/6 N mice. Fixation: 10 % neutral formalin. Technique: Masson's trichrome stain (Goldner variant). (A–D) stomach, (E–H) jejunum. (A,E) The vivarium control group. (B,F,G) A decreased content of the connective tissue in the stroma of the muscular layer after a 30-day space flight compared to the vivarium control group. (C,D,H) Seven-day post-flight rehabilitation is accompanied by an increased representation of the fibrous component in smooth muscle tissues both in the stomach (C,D) and in the jejunum (H) compared to the 30-day space flight group. Scale bar: 10 μm".

were detected both in the circular layer and in the longitudinal layer (Fig. 4A). Concurrently, reticular fibers created a network oriented mainly along the long axis of smooth myocytes (Fig. 4A). On cross sections, the basement membrane of smooth myocytes contacting the structures of the endomysium was well defined.

Within the submucosa, the largest caliber reticular fibers were detected. As in the stomach, they were located together with bundles of collagen fibers, which were well identified after staining by van Gieson and Masson-Goldner techniques.

In the lamina propria of the mucosa, reticular fibers, which formed the skeleton of the villi and were freely located in the intercryptal stroma, were predominantly detected. In the villi, reticular fibers could form a fine-mesh network with varying degrees of expressiveness (Fig. 5A).

After the space flight, quantitative and qualitative changes in the fibrous component of the connective tissue were detected in all membranes of the jejunum compared to the parameters of animals from the vivarium group (Fig. 3F and G). In the subserous layer, the reticular fibers acquired a different-sized appearance with a variable level of argyrophilia throughout. In the muscularis externa, a complete loss of reticular fibers was detected in some loci due to decreased argyrophilia (Fig. 4C and D). The caliber of reticular fibers simultaneously decreased along with their number (Table 1). The content of type III collagen around the smooth muscle membrane myocytes was significantly reduced (Table 2). However, sometimes there were impregnated fibrous fragments of considerable thickness, supporting signs of a specific disintegration of the stromal component. A large amount of impregnated material in the form of grains, as well as fragments of reticular fibers with high argyrophilia, was detected (Fig. 4C). Histotopographic features of the impregnated fibrous structure localization changed compared with the patterns typical for animals from the vivarium control group.

In particular, the ordered reticular fiber localization was lost in the muscularis externa, and the fibers became sparser and changed direction (Fig. 4C and D). Similar patterns were detected in the lamina propria of the mucous membrane of the jejunum. The reduction in reticular fibers was marked in the stroma of the villi (Fig. 5C, D, E).

The intercryptal stroma was also characterized by a decreased representation of argyrophilic fibers, which was combined with their decreased caliber and accumulation of a large number of fragments with a high degree of impregnation. In the submucosa, the content of collagen fibers decreased, and they acquired a higher level of fuchsinophilia.

The study of the biomaterial taken from the jejunum of C57BL/6 N mice 7 days after biological satellite landing demonstrated significant qualitative and quantitative changes in the system of the fibrous extracellular matrix of the connective tissue compared to the parameters of animals from the space flight group. This was primarily due to an increased expressiveness of the reticular skeleton in the lamina propria and the muscularis externa (Table 1; Fig. 3H; Fig. 4E and F and 5F). A network of thickened fibrous structures was formed, and its topography took the form typical of the animals from the vivarium control group. Concurrently, a large amount of granular impregnated material with high argyrophilicity and fragments of reticular fibers was found, which is obviously a reflection of

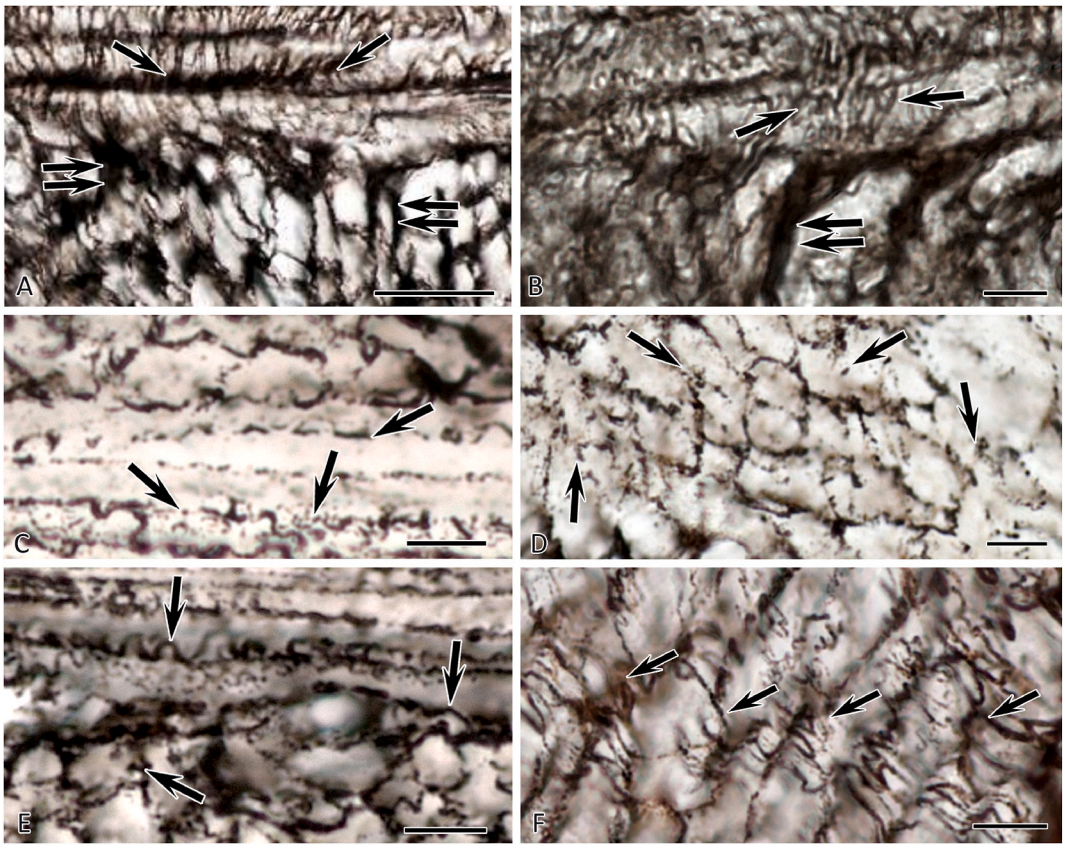


**Fig. 4.** Histoarchitectonics of reticular fibers in the muscularis externa of the jejunum of C57BL/6 N mice. Fixation: 10 % neutral formalin. Technique: Foot's silver nitrate impregnation. Notes: (A) The vivarium control group for the 30-day space flight group. Reticular fibers are clearly visible on longitudinal (arrow) and transverse (double arrow) sections of smooth muscle layers. (B) The group of the ground-based experiment simulated a 30-day space flight in the "BIOS-MLZH" flight equipment model (biological control). The state of histoarchitectonics of reticular fibers does not change compared to animals from the vivarium group. (C, D) The 30-day space flight group. The reduction of reticular fibers in the interstitium of the muscular layer with signs of fragmentation and formation of granular impregnated material is detected (arrow). (E,F) The group of readaptation including animals examined 7 days after the space flight. There is an increased number of reticular fibers in the muscular membrane (arrow). Scale bar: 5 μm.

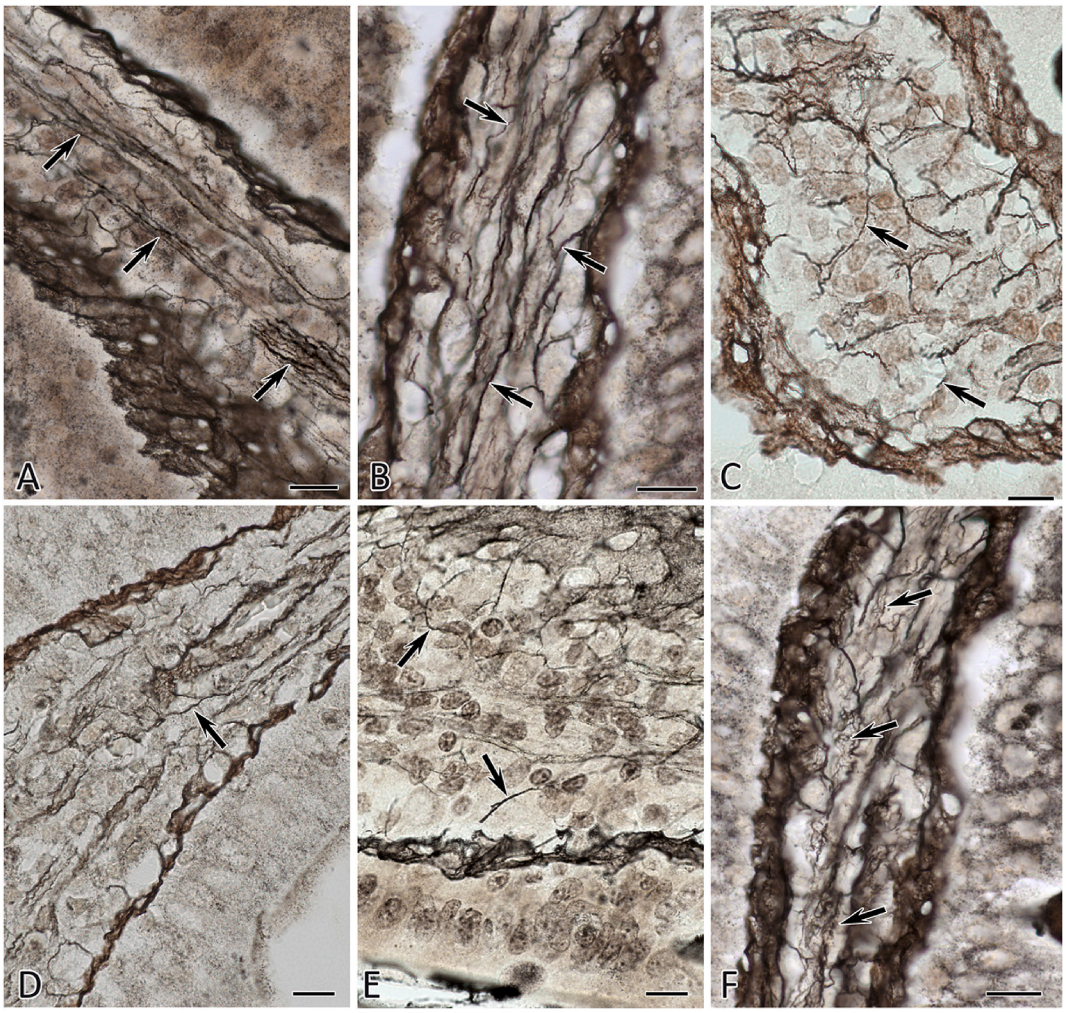


**Fig. 5.** Histoarchitectonics of reticular fibers in the villi stroma of the jejunum of C57BL/6 N mice. Fixation: 10 % neutral formalin. Technique: Foot's silver nitrate impregnation. (A) The vivarium control group for animals from the 30-day space flight group. (B) The group of the ground-based experiment simulated a 30-day space flight in the "BIOS-MLZH" flight equipment model (biological control). In the stroma of the villi, a network of reticular fibers is well defined (arrow). (C,D,E) The space flight group. The reduction in reticular fibers in the stroma of the villi (arrow). (F) The readaptation group including animals examined 7 days after a 30-day space flight. In the stroma of the villi, a high content of different-sized reticular fibers is detected (arrow).

the processes of incomplete readaptation of the jejunal stroma to the standard level of the Earth's gravity. The content of collagen fibers in the submucosa and serosa increased.

The study results demonstrated that the fibrous extracellular matrix of the connective tissue was present in all membranes of the large intestine in animals from the vivarium group. Reticular fibers were located mainly in the lamina propria and the muscularis mucosae. In the muscularis externa of the colon, a more pronouncedly developed network of reticular fibers was noted compared to the muscularis externa of the jejunum. These fibers had a reasonably large caliber and were characterized by uniform argyrophilia throughout. After a 30-day space flight, argyrophilic fragments and accumulations of impregnated granular material were found in the lamina propria of the colon mucosa. In general, the content of reticular fibers decreased in all membranes of the organ. In animals from the flight group, in the muscularis externa of the colon, loci without argyrophilic structures were detected, in some cases extending over significant areas of the colon wall. Notably, the detected reticular fibers were characterized by uneven staining throughout, and they were thickened or thinned. Concurrently, in 7 days of ground adaptation after space flight, a pronouncedly increased number of reticular fibers was observed.

Collagen fibers in the colon wall were predominantly distributed in the submucosa, although they were observed in small amounts in the subserous layer of the serosa. Within the submucosa, collagen fibers formed bundles of various sizes. In animals from the group after space flight, attention was drawn to an increased fuchsinophilia along with a trend toward a decreased content of collagen fibers in the submucosa compared to parameters of animals from the biological control group. There were signs of edema unevenly spreading in the area of the submucosa. Concurrently, areas of homogenized collagen fiber bundles and increased fuchsinophilic properties were

detected, and picrinophilic loci were detected in the submucosa. Seven days after space flight, the level of fuchsinophilia of collagen fiber bundles decreased, and the tinctorial properties of fibrous structures approached the level typical of animals from the vivarium control group. Notably, signs of edematous events within the submucosa were detected.

The analysis of the biomaterial taken from the small and large intestines in animals from the biological control groups after flight simulation demonstrated that the main changes in the state of the fibrous connective tissue occurred in the muscularis externa and affected the tinctorial properties of the reticular fibers. In particular, reticular fibers of different sizes that had unequal argyrophilia throughout were found in the intercryptal stroma of the jejunum of mice. In the stroma of villi, there was a tendency to increased representation of the network of reticular fibers along with signs of decreased argyrophilic properties, although their number did not differ from that detected in animals of the vivarium control group (Fig. 5B). In some animals, reticular fiber fragments were detected with an increased intensity. However, this was not as pronounced as in animals from the spaceflight group. It is noteworthy that the state of the reticular fibers in the muscularis externa did not undergo significant alterations (Fig. 4B).

The amount of the fibrous component in the large intestine practically did not change after the completed simulated experiment or after 7 days of stay in conditions of simulated readaptation (Table 1).

## 3. Discussion

Before discussing the results obtained, we would like to note that the role of altered gravity in the formation of morphological equivalents of the body's response has been the subject of numerous scientific discussions for a long time [1–4,10–21]. The data obtained in this study are consistent with the research results studying the digestive organs of Mongolian gerbils after a 12-day space flight on the Foton-M3 spacecraft [1–4]. Intraorganic connective tissue can be considered a gravity-dependent system that largely determines the specifics of the developing morphological and functional changes in other organ components in orbital flight [1–4,17,22].

As in the study investigating Mongolian gerbils, the data obtained in the experiment on C57BL/6 N mice evidence intensive adaptive remodeling of the extracellular matrix of the digestive connective tissue under space flight conditions, which can be mediated by a decreased efficiency of fibrillogenesis and violated processes of intercellular substance restoration. This assumption is also consistent with the results of an embryological experiment on the 'Mir' orbital station involving Japanese quail chicks, which demonstrated a weakly developing connective tissue of the gastrointestinal stroma under conditions of weightlessness, including a looser arrangement of fibers in embryos and chicks [23].

It can be assumed that one of the fundamental reasons for alterations in histoarchitectonics and tinctorial properties of the fibrous extracellular matrix of the studied connective tissue are the features of the amorphous component and acid–base balance that are formed during space flight. Notably, an altered biosynthesis of collagen proteins and fibrillogenesis can ultimately affect the properties of individual reticular fibers and the spatial structures arranged by these reticular fibers, loops, networks, etc. [22,24–26]. Perhaps this is the reason for the decrease in the content of type III collagen in the extracellular matrix of the muscularis externa of both the stomach and the small intestine. This is of particular importance for the endomysium of the gastric and intestinal muscularis externa. The results obtained demonstrate a high potential of fibrous elements of the connective tissue for readaptation processes in animals after space flight. However, based on the morphological features, they cannot be considered completed in 7 days of the postflight period.

Under conditions of weightlessness, the digestive connective tissue undergoes specific structural and functional rearrangements, reflecting both adaptive and alternative gravity-induced processes. Signs of increased interaction of selective dyes with collagen proteins may evidence depolymerization of fibrous structures, which results in the release of a significant number of reactive groups. This allows admitting the presence of either direct effects of cosmic radiation on the crosslinking of amino acids in collagen molecules or the phenomena of disorganization of fibrous structures resulting from the developing trophic disorders. In addition, it is impossible to exclude the influence of matrix metalloproteinases, which can actively function under conditions of gravitational unloading, including the influence of mast cell proteases [27–29]. In particular, the bioeffects of cosmic radiation were demonstrated in an experiment on cultured human fibroblasts exposed for 14 days aboard the ISS [30]. It should be taken into account that under orbital flight conditions, due to the development of hemodynamic changes, digestive connective tissue functions in an environment with different properties [31,32]. Obviously, under weightlessness, a decreased efficiency of extracellular fiber assembly in the connective tissue is due to a significant restructuring of the microenvironment in the amorphous component of the interstitium: a change in the pH level, the content of hyaluronan, other glycosaminoglycans, proteins, water, etc. Collagen fibrillogenesis in the extracellular matrix is accompanied by the aggregation of molecules into supramolecular structures: protofibrils, microfibrils, fibrils, and fibers [24]. In the initial stage of the developing interstitium fibrous phase, tropocollagen molecules form pericellular accumulations (mesophases); in these accumulations, the distance between the molecules is filled with liquid. This liquid crystal state of the initial stage of fiber formation is called a tactoid [24]. Tropocollagen molecules occupy an energetically favorable position in relation to each other in tactoids, and the shape of the molecules takes part in the stabilization of this state together with the action of electrostatic forces, the energy of molecular kinetic motion, and the influence of the surrounding liquid phase. To initiate the onset of developing supramolecular aggregates of collagen, it is necessary to bring tropocollagen molecules closer to a certain distance, which requires a decreased volume of the aqueous medium between them. This is achieved either as a result of an increased concentration of tropocolagen molecules by cellular secretion growth or by increased liberalization into the extracellular space of glycosaminoglycan molecules that can interact with water from tactoids by the osmotic mechanism. Therefore, this initiates the assembly of protofibrils from tropocollagen molecules. Microfibrils, fiber fibrils or collagen bundles are further formed. However, to successfully form a collagen fiber, well-defined conditions are needed, and they depend on water content, tropocollagen concentration, osmotic pressure, temperature, ionic strength, pH, and many other factors. Moreover, even when the necessary requirements are met, the effect of fiber

formation will depend on the amount of proteoglycans, complexing ions, ATP, ascorbic acid, enzymes, etc. [22,24,25]. There is no doubt that in weightlessness, the processes of fiber formation occur under conditions of a specific tissue microenvironment that are different from those on the ground [22,25]. That is why, apparently, in orbital flight, the processes of physiological regeneration of collagen fibers cannot be realized in full, since the consequences of venous stasis in the organs of the digestive system are accompanied by modified parameters of the integrative-buffer metabolic environment.

Moreover, under conditions of weightlessness, apparently, the process of remodeling or adaptation of the stroma of the organ is actively developed. In this case, the processes of disorganization of collagen fibers can be of great significance due to both the formation of specific trophic disorders and removal of the static load that occurs under the conditions of the Earth's gravity. Thus, the processes of accelerated reduction combined with decelerated formation of new fibers lead to a decreased volume of fibrous structures in the interstitium of the digestive system under conditions of weightlessness.

The dynamics of the content of the fibrous extracellular matrix in the wall of the digestive organs during space flight are expressed mainly by reduction processes decreasing the integrative role of the extracellular phase of the gastrointestinal connective tissue. The processes of accelerated lysis of fibrous structures, together with their decelerated formation, result in a decreased volume in the interstitium of the stomach and intestines, which is potentially considered one of the manifestations of connective tissue intercellular matrix remodeling in accordance with the achieved state of the adaptive, or "cosmic", norm [33]. In the stomach (excepting the lamina propria) and intestines, weightlessness caused a reduction in the fibrous phase of the connective tissue, which was accompanied by decreased morphometric parameters of their structures, including the muscularis externa. A decreased representation of reticular fibers in the wall of the stomach and intestines correlated with the results of morphometric analysis [1,2]. This suggests that the indicated morphological changes in the interstitium, primarily associated with the loss of fibrous collagens by the muscular layer, may have an impact on the weakening motor function of the gastrointestinal tract, as noted in a number of studies, including those conducted on board orbital stations [34–38].

The results obtained related to the state of connective tissue structures under conditions of weightlessness show that the cell and the extracellular matrix surrounding it are a single formation with a very high ability to measure the level of gravity in various environmental conditions. This emphasizes the existence of the cell in combination with other structural components of a specific tissue microenvironment as an integral mobile entity with powerful potential for implementing adaptive responses under the influence of microgravity. The findings on alterations of the collagen fibrous structure and metabolism in the extracellular matrix of the gastric and intestinal connective tissue obtained in mice and Mongolian gerbils as a result of a long flight of animals in the near-Earth orbit support potential similar changes in other organs, including vessels.

A number of experimental studies have assumed that the state of the intercellular substance of the connective tissue of various gastrointestinal organs after a space flight or under ground-based simulated physiological effects of weightlessness is closely related to the features of the mast cell population [3,4,39]. Notably, the active secretion of proteases, tryptase and chymase, which are capable of activating matrix metalloproteinases and, as a result, accelerating the degradation of collagen fibers, seems to be especially important [22,25,27–29,40]. The ability of mast cells to migrate in all layers of the walls of hollow organs and to actively secrete various factors determines their potential active participation in gastric and intestinal stroma remodeling during space flight.

We could not detect the identified changes in the extracellular matrix during space flight during the ground experiment. These facts are important evidence of the architectural role of microgravity in the formation of changes in the histological state of the extracellular matrix of the connective tissue of the studied organs of the digestive system. Local events occurring in the specific tissue microenvironment of the hollow organs of the digestive system of mice during space flight are caused by changes in hemodynamics, which is accompanied by a certain slowdown in the outflow of blood from both the stomach and intestines. This leads to the development of phenomena of overflow with blood during orbital flight and edema, which in turn can lead to corresponding changes in both the immune landscape of organ membranes and the structures of the extracellular matrix. Obviously, during the ground experiment, the state of the integrative-buffer metabolic environment In the gastrointestinal hollow organs of mice compared to the real space flight was significantly different, and could not lead to a change in histoarchitecture of stromal collagen fibers.

## 4. Materials and methods

### 4.1. Space flight opportunity

Biosatellite "Bion-M″ No. 1 was launched on a Soyuz 2-1a rocket from the Baikonur Cosmodrome on April 19, 2013. The descent module successfully landed on May 19, 2013, in the vicinity of Orenburg. During a 30-day orbital flight, the Bion-M No. 1 biosatellite made 477 orbits around the Earth at an altitude of 560–590 km. The microclimate parameters of the animals were kept at a temperature of 20–27 °C, relative humidity 20–75 %, and 12-h daylight hours (lights turned on at 09.00). Upon landing, laboratory rodent habitats were dismounted from the module. After a brief examination at the landing site, the mice were transported to Moscow to the Institute of Biomedical Problems, where 12 h after landing, preparation and taking of biosamples for research began. Housing and climate parameters were replicated in the subsequent ground-based experiment (July 26 to August 26, 2013) [41–44].

### 4.2. Experimental design

The experiment involved 58 male C57BL/6 N male mice (Table 3) with an initial body weight equal to 27.1 ± 0.74 g. Of these, 5 animals were included in the 30-day space flight group, and their biomaterial was sampled 9–11 h after biosatellite landing. Five animals were included in the group to study processes of digestive organ readaptation to the standard level of gravity; after returning

**Table 3**
Experimental groups of C57BL/6 N mice.

| Experiment | Groups of animals | Number of animals |
|---|---|---|
| Experiment on board the "BION-M″ 1 biological satellite | VC-SF | 8 |
| | SF | 5 |
| | VC-RSF | 8 |
| | RSF | 5 |
| Ground-based experiment simulated conditions on board the "BION-M″ 1 biological satellite for animals staying in the flight equipment model "BIOS-MLZH" | BC | 8 |
| | VC-BC | 8 |
| | RBC | 8 |
| | VC-RBC | 8 |
| *Total number of experimental animals* | | *58* |

Notes: For abbreviated names of the experimental groups, see Table 1.

from an orbital flight, they were kept for 7 days in standard conditions. The group of ground-simulated space flight conditions (biological control) included 16 animals; of these, half of the animals were kept in the BIOS-MLZH flight equipment model for 30 days, while the others were subjected to 7 days of readaptation, similar to that of animals from the postflight group. This experiment was carried out in a specially prepared mock-up of ground-based equipment for keeping mice, which was technically a complete copy of the equipment installed on the BION-M1 biosatellite. A ground simulation of a 30-day space flight was carried out in the period from 07/26/2013 to 08/26/2013 in this complex "BIOS-MLZH". The conditions for keeping the mice were maintained in a climate chamber, in which the temperature, humidity, gas composition and other climatic parameters that were recorded during a real space flight were fully reproduced. Thus, the ground-based experiment in the flight equipment model "BIOS-MLZH" for 30 days exactly repeated the conditions of detention that accompanied laboratory mice during their real space flight on the biological satellite "BION-M″ 1. There were four groups of vivarium control mice, 8 animals each, comparable to each of the four groups above (Table 3). Comprehensive information on the nutrition and other key details of animal care for C57BL/6 N mice included in the BION-M1 biosatellite flight program is presented in the relevant publications [41–43].

The animals of the flight and backup flight groups were transported to Baikonur 7 days prior to launch [41]. This transportation had no negative effect on the condition of the mice, as evidenced by a minor decrease in body weight (3 %, 0.9 g). For transportation, specialized containers made of opaque plastic with air filters (produced by the 'Pushchino' breeding station, Russia) were used [42,43]. During transportation and further experiments, the animals had access to a special "flight" food developed by T.S. Guryeva and E. I. Mednikova at the State Scientific Center of the Russian Federation - Institute for Biomedical Problems of the Russian Academy of Sciences (IBMP RAS); this was a paste of standard compound feed, casein as a thickener, with a 76–78 % water content [42,43]. Paste-like feedstuff consisted of base feed (extruded standard feed, 87.2 %), fat-free casein (11.6 %), melting salt (NaHPO4, 0.1 %), and sorbic acid (0.3 %) (per 100 g of dry product) [43]. The feed was balanced in terms of amino acid composition; macro- and micro-elements (premixes) and vitamins were additionally introduced. In particular, 100 g of dry food contained 44.5 % protein, 2.36 g lysine, 1.46 g methionine + cystine, 0.28 g tryptophan, 34.6 % carbohydrates, 9.4 % ash, 2.28 % calcium, 2783.4 mg magnesium, 3.66 mg zinc, 56.18 mg iron, 0.81 mg vitamin A, 0.06 mg vitamin D, 4.65 mg vitamin E, 1.10 mg vitamin B1, 3.15 mg vitamin B2, 25.2 mg vitamin B6, and 5.59 mg vitamin K3; the energy value was 361.4 kcal [42,43].

Mice in the flight and control groups were fed 5.52 ± 0.88 g of paste-like feed per 10 g of body weight on average. Taking into account the water content of the paste-like feed (76–78 %), the consumption of dry matter was 1.27 ± 0.2 g/10 g of body weight, which did not differ from the consumption of standard compound feed. After the flight, the body weight of the animals varied significantly; however, in general, these changes in the animals of the main experimental groups and animals from the vivarium control groups in the flight and ground experiments were inversely dependent: during the space flight (SF), the mice gained more body weight than the mice of the vivarium control group (VC), and in the ground control experiment (BC), they gained less weight than the corresponding vivarium control (VC-BC) [42]. Supposedly, these changes are associated with a decreased intensity of physical activity in mice under conditions of weightlessness.

### 4.3. Histoprocessing

After decapitation of the animals, fragments of the fundus of the stomach, jejunum and colon, at least 10 mm long, were fixed in 10 % neutral formalin at room temperature. According to the standard protocol for sample preparation, fragments of the stomach and intestines were immersed in a series of alcohol solutions and xylene solutions and embedded in paraffin.

### 4.4. Tissue Probe staining

Paraffin Sections 6μm thick, made along the long axis of the obtained fragments of the stomach and intestines, were stained with Mayer's hematoxylin and eosin to perform the survey microscopy [45]. Staining according to Masson-Goldner helped obtain a general view of the content of the extracellular matrix of connective tissue in the wall of the gastrointestinal organs and smooth muscles [46,47] (Table 4). To identify the collagen structures of the fibrous extracellular matrix of the connective tissue, prepared specimens were stained with iron hematoxylin according to Weigert's and with picrofuchsin according to van Gieson techniques, and reticular fibers

**Table 4**
Reagents used for histochemical and immunohistochemical staining of the mouse gastrointestinal tract.

| Dyes | Catalog number | Provider | Dilution | Manufacturer |
|---|---|---|---|---|
| Rabbit monoclonal antibody [EPR17673] to Collagen III | ab184993 | Abcam | 1:100 | Abcam |
| Masson's trichrome stain (Goldner variant) | 1.00485 | Sigma–Aldrich Co. | Ready to-use | Sigma–Aldrich Co., Germany |
| Silver impregnation | 21–026 | Biovitrum | Ready to-use | ErgoProduction LLC, Russia |

**Table 5**
Secondary antibodies and other reagents.

| Antibodies and Other Reagents | Source | Dilution | Label |
|---|---|---|---|
| AmpliStain™ anti-Mouse 1-Step HRP (#AS-M1-HRP) | SDT GmbH, Baesweiler, Germany | ready-to-use | HRP |
| DAB Peroxidase Substrat Kit (#SK-4100) | Vector Laboratories, Burlingame, CA, USA | ready-to-use | DAB |
| Mayer's hematoxylin (#MHS128) | Sigma–Aldrich | ready-to-use | w/o |

were detected by impregnation with silver nitrate [45–47]. This selectivity of the applied techniques arises from immunomorphological approaches to study fibrous collagens [48–50]. In particular, van Gieson-stained fibrous structures are predominantly type I collagen, while impregnated fibers contain a higher content of type III collagen [51–53]. As is known, reticular fibers can be a direct continuation of collagen fibers [24,54,55]. Thus, the histochemical techniques used in the study allowed the differentiation of reticular fibers containing type III collagen from fibrous structures formed by other fibrillar collagens (types I, II, V, XI, etc.) [24,56]. Rabbit polyclonal antibodies and other reagents were used for immunohistochemical detection of type III collagen on paraffin Sections 2 μm thick (Tables 4 and 5).

### *4.5. Image Acquisition*

The topography and tinctorial features of collagen fibers in the gastric and intestinal interstitium were evaluated using a hardware-software complex for biological investigations with a documentary system based on a direct research microscope ZEISS Axio Imager.Z2 (Carl Zeiss Microscopy, Germany). To obtain quantitative data on the state of the fibrous component of the interstitium, the authors applied a planimetric approach and determined the index of the fibrous content in the field of view in standard units using a x100 objective using the QuPath software product [57]. The representativeness of the sample was achieved by evaluating at least 50 fields of vision.

### *4.6. Assessment of type III collagen expression in the muscular membrane of the digestive organs*

In the muscularis externa of the stomach and jejunum of experimental animals, the pericellular matrix of at least 500 smooth myocytes was evaluated, from which the relative amount with the expression of type III collagen was calculated. From the number of smooth muscle cells, cells with high, moderate, weak or no expression were conditionally identified, and their relative contents were calculated as percentages. The intensity of staining was independently reviewed by three pathologists (vs., IB, and DA) using a × 100 objective lens, and upon reaching consensus, scoring was assigned from (+++) to (+). A consensus score of (+++) represented the highest content of type III collagen around the smooth muscle cells, and weakly noticeable staining was assigned as (+). The quantification of the cell content was performed using a counting program incorporated in AxioVision software (Carl Zeiss Vision GmbH, Zeppelinstr. 485399, München-Hallbergmoos, Germany).

### *4.7. Statistical analysis*

Statistical analysis was performed using the SPSS software package (Version 13.0). The results are presented as the mean (M) ± m (standard error of the mean). To assess the significance of the differences between the two groups, Student's *t*-test or Mann–Whitney *U* test in the case of a nonparametric distribution was used to compare two samples with a confidence interval $p < 0.05$.

## 5. Conclusions

Thus, the collagen fibrous structures of the extracellular matrix of the gastrointestinal tract can be considered a system with pronounced gravisensitivity, and its state will determine the adequacy of remodeling processes in the absence of the Earth's gravity and the integrity of the adaptive reactions of the digestive system. This thesis poses new challenges in space gastroenterology related to the prevention of the adverse effects of weightlessness on digestive organs.

## Funding

This work was supported by the Ministry of Science and Higher Education of the Russian Federation within the framework of the state assignment FSSF-2023-0046.

## Data availability statement

All data and materials are available upon reasonable request. Address to D.A. (email: atyakshin-da@rudn.ru).

## Institutional review board statement

The study was approved by IACUC of MSU Institute of Mitoengineering (Protocol N№ 35, 1 November 2012) and of Biomedical Ethics Commission of IBMP (protocol N№ 319, 4 April 2013) and conducted in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

## Informed consent statement

Not applicable.

## CRediT authorship contribution statement

**Viktoriya Shishkina:** Writing - original draft, Investigation, Formal analysis, Data curation. **Andrey Kostin:** Data curation. **Nataliya Alexeeva:** Investigation. **Svetlana Klochkova:** Methodology. **Dmitry Nikityuk:** Project administration. **Artem Volodkin:** Methodology, Formal analysis. **Igor Buchwalow:** Writing - review & editing. **Markus Tiemann:** Project administration. **Dmitrii Atiakshin:** Writing - review & editing, Writing - original draft, Investigation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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