



***Original Article***

# **Evaluation of Morphological and Histological Changes of Aggregated Lymph Nodes in the Small Intestine after Imofan Treatment in Immunosuppressed Rats**

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## **Abstract**

Diffuse nodular lymphoid hyperplasia is a rare gastrointestinal disease that can be diagnosed by multiple nodules in the small intestine, large intestine, or both. Immunodeficiency and infections are the common situations that lead to the diffusion of nodular lymphoid hyperplasia. For instance, *Giardia lamblia* and *Helicobacter pylori* are the major pathogens leading to this disorder. Diffuse nodular lymphoid hyperplasia leads to allergic reactions, immunodeficiency, and autoimmune diseases. Imunofan-RDKVYR Peptide-is a potential agent in regenerative medicine. The present study aimed to investigate morphological features of the aggregated lymphoid nodules of the small intestine after the Imunofan (IM) administration following Cyclophosphamide-induced immunosuppression. In total, 72 Wistar male rats were randomly divided into two groups (n=36). Group I was considered the control group, and group II was subjected to intramuscular injections (needle 21 G) of 0.2 ml of normal saline following the Cyclophosphamide-induced immunosuppression on the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 10<sup>th</sup> days of the experiment. The animals in group II were injected with Cyclophosphamide at a dose of 200 mg/kg bodyweight to induce immunosuppression. The animals in the experimental group (n=36) were subjected to intramuscular injections (needle 21 G) of the 0.2 ml IM at a dose of 0.7 µg/kg body weight on the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup> days of the experiment. The results of the study indicated that on the 7<sup>th</sup> day in group II, the length and width of the aggregated lymphoid nodules increased, as well as the height and width of the lymphoid nodules and internodular zones as structural components of the lymphoid formations in the small intestine. In group I, by the 30<sup>th</sup> day of the experiment, the linear dimensions of the aggregated lymphoid nodules exceeded, but to a lesser extent than on the 7<sup>th</sup> day of the experiment which explains the ability of IM to neutralize the effects of Cyclophosphamide. It should also be noted that the IM was performed to regenerate damaged cells which helped maintain the population of lymphocytes in the limb and led to an increase in linear dimensions (length and width) not only between the joint but also in the lymph nodes.

**Keywords:** Aggregated lymphoid nodules, Cyclophosphamide, Imunofan, Rats, Small intestine

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## **1. Introduction**

The human body is influenced by various exogenous factors associated with the deterioration of the ecological situation on the planet in recent years (1, 2). The mucous membrane of the digestive tube is one of the most significant surfaces of the body in terms of area, which is in constant interaction with the external

environment (3). The small intestine is under long-term exposure to exogenous antigens and has close contact with them during the prolonged passage of food through the digestive tract. This explains the presence of lymphoid tissue in it, which largely determines the degree of human health and its adaptive capabilities (4).

Small nodules of 2-10 mm in diameter in the stomach, small intestine, large intestine, and rectum are called nodular lymphoid hyperplasia of the gastrointestinal tract, and the pathogenesis of this disease is largely unknown (5). Compensation for the lack of lymphoid tissue in the gut may be the cause of this immunodeficiency. Intestinal infections may even repeatedly trigger immunity in the intestinal lymphoid tissue and cause these lymphoid nodules. Nodular lymphoid hyperplasia is associated with immunodeficiency, including common variable immunodeficiency, selective Immunoglobulin A deficiency, and human immunodeficiency virus infection (6).

These nodules contain lymphocytes and macrophages, which protect against penetrating bacteria and other pathogens that enter these passages along with food, air, or urine. Macrophages initiate the immune response by ingesting foreign matter and kill the invaders. When foreign substances enter the bodies of either humans or animals, the functions of the lymphoid tissue of the small intestine may be impaired. This leads to allergic reactions, immunodeficiency states, and autoimmune diseases (7).

The immune system does not exist in a specific organ but is a collection of cells in the body that are distributed throughout the body. Lymphocytes are the most influential cells involved in immune responses, which are secreted by the lymphatic system (more than 90%) (8). In practical medicine, both immunomodulators and immunosuppressants are used to correct the revealed disorders (9, 10). For the purpose of safety and human health, the use of drugs of each group has strict indications, and the effectiveness of their use should be confirmed by studying the state of target organs at the macroscopic and microscopic levels (9).

Cyclophosphamide is an alkylating agent (oxazaphosphorine group) which has immunosuppressive properties and immune regulatory properties as well as anti-toxic and anti-inflammatory

effects (10). Cyclophosphamide is widely used among immunosuppressive drugs, which is included in many anticancer therapy regimens, is used for the prevention of transplant rejection, as well as the treatment of autoimmune diseases. Inhibition of the functional activity of cells of the immune system is one of the main reasons for the toxic effect of Cyclophosphamide; therefore, the problem of reducing its side effects is very urgent (11, 12).

Imunofan (IM) is one of the new generation drugs that are used as an immunomodulator for prophylactic or therapeutic purposes in Russia and Ukraine (13-15). The available literature is incomplete or that data is practically absent on the effect of IM on the lymphoid tissue of the small intestine. Therefore, this study aimed to investigate the aggregated lymphoid nodules at the macroscopic and microscopic levels after the administration of IM against the background of Cyclophosphamide-induced immunosuppression in the experiment. Since the structure and functioning of the human and rat immune systems have much in common (4), rats were used as experimental animals in the experiment.

## 2. Material and Methods

### 2.1. Animals and Treatments

In total, 72 Wistar male rats were randomly divided into two groups (n=36). Group I was considered the control group and was administered to intramuscular injection (needle 21 G) of 0.2 ml of normal saline following the cyclophosphamide-induced immunosuppression on the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 10<sup>th</sup> days of the experiment. The animals in group II were injected with cyclophosphamide at a dose of 200 mg/kg body weight to induce immunosuppression. The animals in the experimental group (n=36) were subjected to intramuscular injections (needle 21 G) of the 0.2 ml IM at a dose of 0.7 $\mu$ g/kg body weight on the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, and 10<sup>th</sup> days of the experiment. The keeping and manipulation of the animals were carried out in accordance with the rules for keeping

experimental animals established by Directive 2010/63/EU of the European Parliament and the Council of the European Union (14).

## 2.2. Morphometric Measurement and Histological Analysis

Animals from both groups were removed from the experiment on days 7<sup>th</sup> (n=12), 30<sup>th</sup> (n=12), and 90<sup>th</sup> (n=12). Under deep anesthesia, the small intestine was isolated using a special instrument (15), and its length was determined. The length and width of the aggregated lymphoid nodules (ALN), as well as the distance from the ileocecal junction to the first of them, were measured using a caliper. Next, the small intestine was fixed in 10% formalin solution, after which it was subjected to standard histological wiring. Sections 5-6- $\mu$ m-thick was stained with hematoxylin-eosin. An automated morphometric complex was used to measure the height and width of the lymphoid nodules (LN) and internodular zones in each accumulation of lymphoid tissue. The automated morphometric complex included an Olympus CX41 light microscope, a digital camera, and a personal computer with a set of applied programs (13).

## 2.3. Statistical Analysis

The digital data were processed using Statistica software (version 5.11). It should be mentioned that the

student's t-test was determined, and the differences with a level of significance of less than 0.05 were considered significant.

## 3. Results

Microscopic observations in rats showed that aggregated lymphoid nodules have the same color as the surrounding tissues. Moreover, it was founded that these nodules protrude above the wall of the small intestine from the side of the free edge of the organ and are oval and round and less often of irregular shape (Figure 1). The first accumulation of lymphoid tissue was determined, as a rule, in the distal part of the duodenum, while the last was determined in the area of the ileocecal junction from the side of the ileum.

Comparison of the length of the small intestine during the study period showed no statistically significant difference between treatment and control groups ( $P < 0.05$ ) (Figure 2).

On the 7<sup>th</sup> day in the control and treatment groups, a comparison of the mean length and width of the ALN showed a significant increase by 18.71% and 16.94%, respectively (Table 1). Moreover, the distance from the ileocecal junction to the first of them decreased by 6.15%, which was not significant ( $P < 0.05$ ).

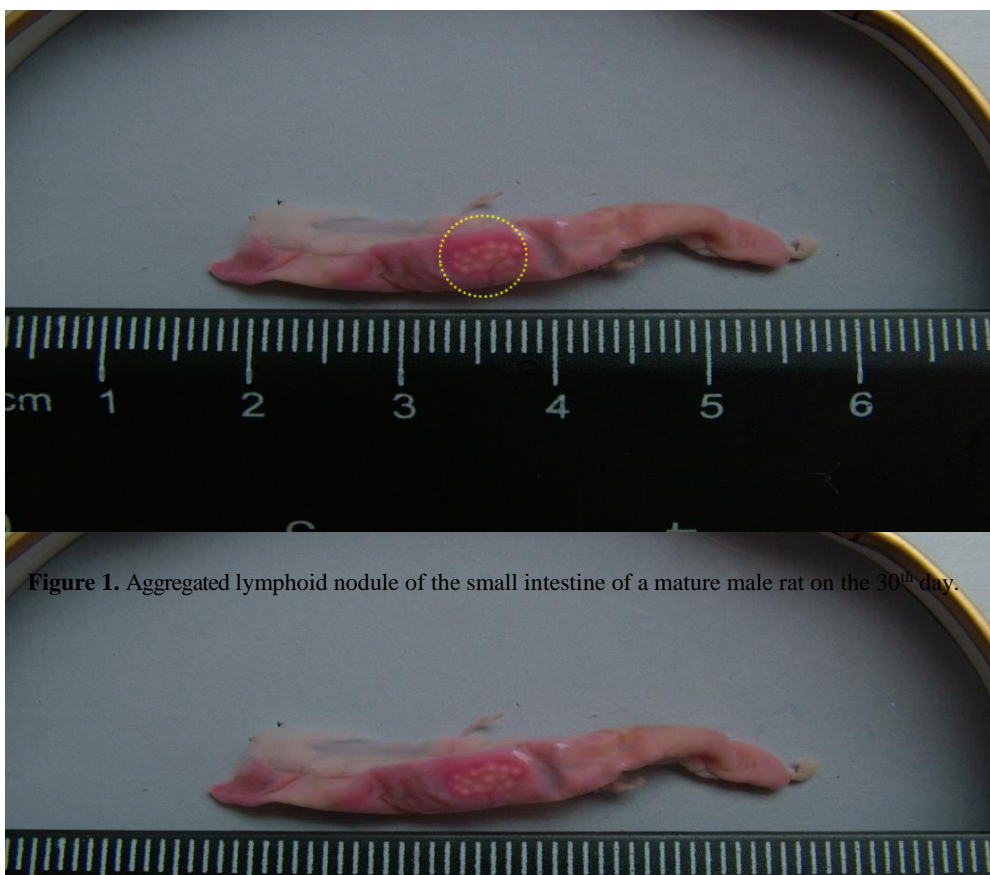
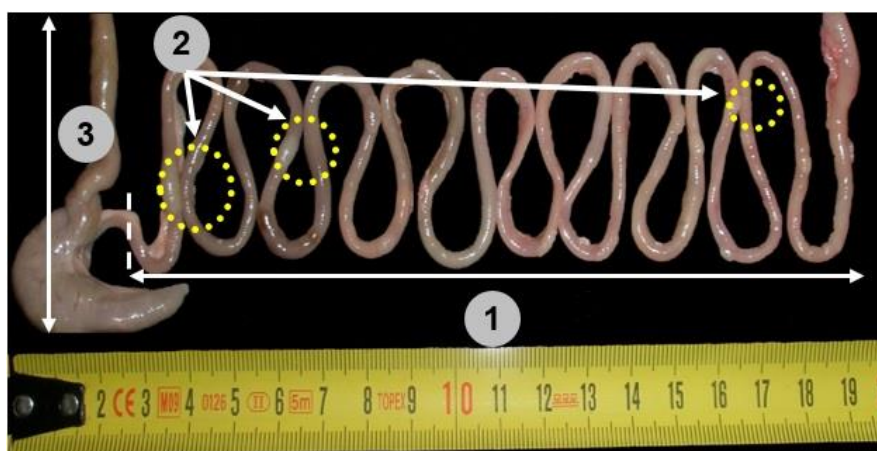


Figure 1. Aggregated lymphoid nodule of the small intestine of a mature male rat on the 30<sup>th</sup> day.



**Figure 2.** Small intestine (1) with aggregated lymphoid nodules and (2) a section of the large intestine (3) of mature male rats on the 30<sup>th</sup> day of observation after the Immunofan correction of cyclophosphamide-induced immunosuppression.

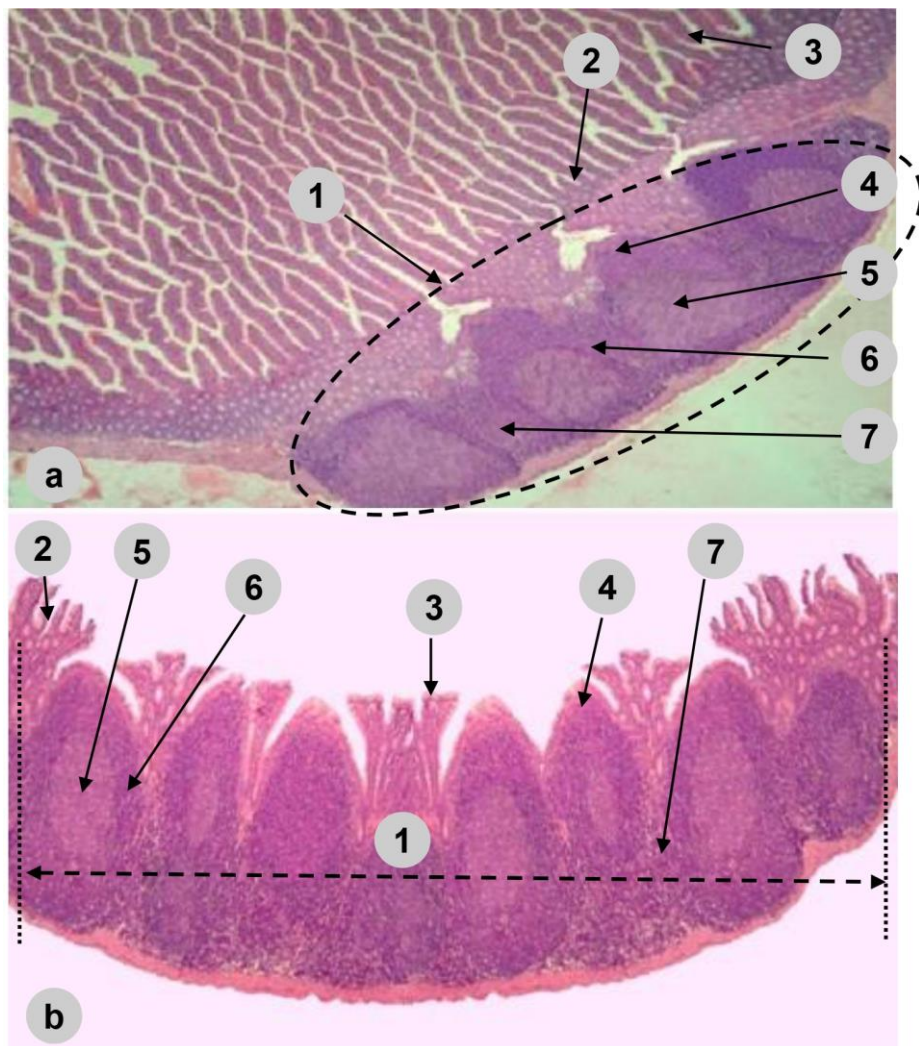
**Table 1.** Linear parameters of the small intestine and aggregated lymphoid nodules of immunosuppressed rats after treatment with Immunofan in the experimental (n=36) and control (n=36) groups at different periods of observation.

Linear parameters(mm)	Mature rats					
	7 <sup>th</sup> day		30 <sup>th</sup> day		90 <sup>th</sup> day	
	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group
Small intestine length	842.50 +25.00	836.00 +26.60	915.30 +31.00	900.00 +24.40	1105.00 +30.40	1100.00 +30.00
ALN length	5.33 +0.32*	4.49 +0.20	5.23 +0.17*	5.05 +0.08	4.90 +0.17	4.87 +0.15
ALN width	2.83 +0.15*	2.42 +0.09	2.77 +0.12*	2.23 +0.05	2.25 +0.12	2.24 +0.05
Distance from the ileocecal junction to the first LN cluster	48.80 +1.73	52.00 +2.58	39.70 +2.81*	49.50 +1.07	49.00 +1.20	49.50 +1.07

\*Significant differences from the control data at  $P < 0.05$ .

Comparison of the mean values of the two experimental groups on the 30<sup>th</sup> day after injection indicated a statistically significant increase in the linear dimensions of ALN by 3.56% and 24.22%. Furthermore, the distances from the ileocecal junction to the first of them decreased by 19.80% ( $P < 0.05$ ). Morphometric measurement on the 90<sup>th</sup> day showed no statistical differences between the data collected from control and treatment groups.

Findings of histological studies on (Figure 3 a, b) the ALN of mature male rats revealed that it consists of 4-15 lymphoid nodules. Their surface was smooth and faced the intestinal lumen, and the adjacent areas of the internodular zone were covered with villi that partially covered the surface of the lymphoid nodules. Lymphoid nodules were located in the lamina propria of the mucosa and submucosa, arranged in one row.



**Figure 3.** Aggregated lymphoid nodules in the wall of the small intestine of mature rats. (a. control, 6 - on the 30<sup>th</sup> day after injection of Imunofan cyclophosphamide-induced immunosuppression): 1. aggregated lymphoid nodules, 2. crypt, 3. villus, 4. dome, 5. germinal center, 6. peripheral zone, and 7. internodular zone. Staining: hematoxylin-eosin. Approximation: zoom 162. Lens: plan C N 4x/0.25 $\infty$ /- /FN22.

Statistical analysis of the data showed that in mature animals of the experimental group, the height and width of the lymphoid nodules increased in comparison with the data of the control rats. Accordingly, the height and width increased by 29.79% and 12.73% until day 7 and 10.49% and 12.46% until day 30, respectively. Moreover, the height and width of the internodular zones increased significantly by 39.35% and 10.33% until day 7 and 4.77% and 4.25% until day

30, in contrast to the control group. By day 90, the height and width of the lymphoid nodules and internodular zones approached those of the control group ( $P < 0.05$ ) (Table 2). Microscopic examination of lymph node tissue sections revealed that each lymphoid nodule in an accumulation of lymphoid tissue consists of a dome, a peripheral zone, and a germinal center, and is separated from adjacent nodules by an internodular zone.

**Table 2.** Morphometric parameters of Lymphoid nodule in the small intestine of mature animals of the experimental and intact groups at different periods of observation after treatment with Imunofan cyclophosphamide-induced immunosuppression M+m (n=36).

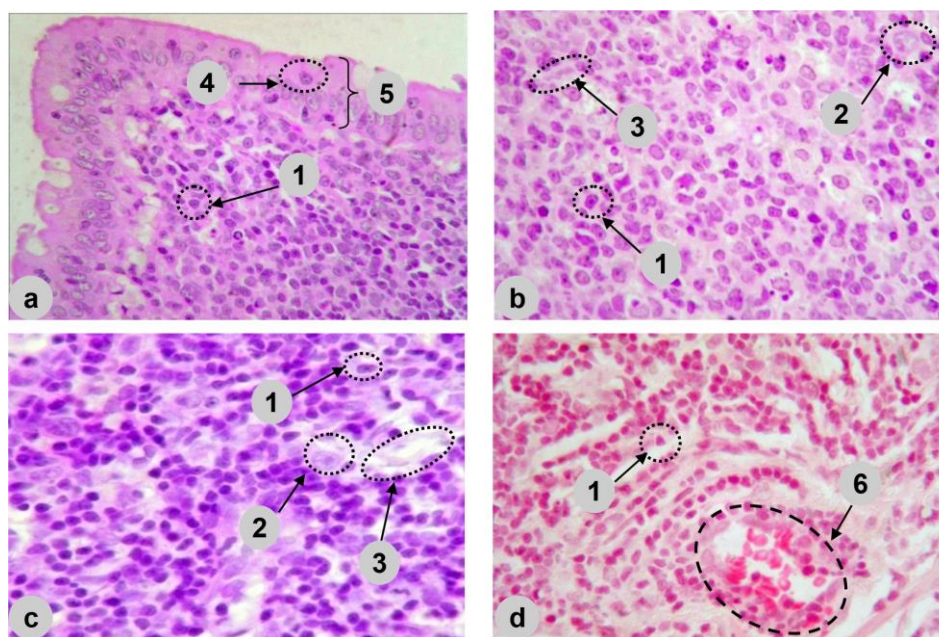
Linear parameters ( $\mu\text{m}$ )	Mature rats					
	Day 7		Day 30		Day 90	
	Experimental group	Control	Experimental group	Control	Experimental group	Control
Lymphoid nodule height	1393.00+56.30*	978.00 +31.50	1115.00 +32.30*	998.00 +38.30	998.00 +13.10	1000.00 +33.60
Lymphoid nodule width	1100.00+27.00*	960.00+41.60	859.00 +42.00*	752.00+30.00	973.00+13.90	970.00 +48.20
Internodular zone height	709.00 +28.00*	430.00+18.40	398.00 +25.50*	379.00+17.60	288.00 +17.80	285.00 +13.40
Internodular zone width	455.00 +12.70*	408.00+19.20	400.00 +29.60*	383.00+17.00	516.00 +6.13	511.00 +25.20

\*Significant differences from the control data at  $P < 0.05$ .

The dome of lymphoid nodules, facing the lumen of the small intestine, is covered with epithelial cells and contains small and medium lymphocytes and less often macrophages and plasma cells. In the peripheral zone, the cells are located more compactly and are represented mainly by small lymphocytes. Macrophages, reticulocytes, and single plasma cells are rarely found. In the germinal center, cells with patterns of mitosis, large

lymphocytes, plasma cells, macrophages, reticulocytes, and less often medium and small lymphocytes are detected (Figure 4 a-d).

The internodular zone is well pronounced and differs in a lower density of cells than in the nodules. On histological sections, a moderate number of blood vessels of the microcirculatory bed, small, medium, and large lymphocytes, macrophages and reticulocytes are revealed in it.



**Figure 4.** Zones of the lymphoid nodules in the group accumulation of lymphoid tissue of the small intestine of rats of the experimental group on the 30<sup>th</sup> day (a. dome, b. germinal center, c. peripheral zone, and d. internodular zone): 1. lymphocyte, 2. macrophages, 3. reticulocyte, 4. intraepithelial lymphocyte, 5. mucosal epithelium, and 6. vessel. Staining: hematoxylin-eosin. Approximation: zoom 162. Lens: plan C N 4x/0.25 $\infty$ /-FN22.

#### 4. Discussion

Analysis of the effect of IM on the immunosuppressed rats showed that the length of the small intestine in male rats of the experimental group does not significantly exceed the control parameters ( $P>0.05$ ). Therefore, the use of IM against the background of the immunosuppression state does not affect the length of the organ. The study of the linear parameters (length and width) of ALN in mature male rats showed a significant increase in comparison with the data of the control group during the month ( $P<0.05$ ).

According to the results of other studies, the use of IM against the background of immunosuppression restores the linear dimensions of the thymus to the control parameters within a month (16). At the same time, for a given period, the usage of IM in the lymph nodes of rats leads not only to the leveling of linear data but also to their increase in comparison with the control group (17). This is in line with the above-mentioned changes obtained in the study of ALN of the small intestine. Based on this, the use of IM changes the linear dimensions of the accumulations of the lymphoid tissue of the small intestine.

Microscopic examination showed that the height and width of the lymphoid nodules and internodular zones exceeded the values of the control group on the 7<sup>th</sup> and 30<sup>th</sup> days after correction with IM cyclophosphamide-induced immunosuppression ( $P<0.05$ ). By day 90, the experimental data were leveled with the control parameters. Similar changes were observed after oral administration of *Pasteurella multocida*B2 to Sprague-Dawley rats weighing 200-250 g (18). The obtained data may indicate the pronounced reaction of ALN of the small intestine to the introduction of IM against the background of an immunosuppressive state for a month, while after three months the linear dimensions and structure of the organ were restored.

The pharmacological action of the peptide immunoxidant is based on the achievement of the correction of the immune and redox systems of the

body (19). On the 7<sup>th</sup> day, the immunoregulatory effect of this drug was manifested which was the restoration of disturbed parameters of cellular and humoral immunity. This pharmacological action is explained by the ability of the IM to restore the production of the thymic hormone, thymulin, to values characteristic of normal animals (20). These results increase the population of T-lymphocytes in the thymus of rats.

The above-mentioned data allows explaining the increase in the linear dimensions of the internodular zones (T-dependent zones) of the ALN of the small intestine in comparison with the control parameters during the month. The activity of lymphocytes in the ALN of the small intestine largely depends on the antigenic load, which stimulates the synthesis of tumor necrosis factor by lymphocytes. This, in turn, allows the active proliferation of immunocompetent cells in the germinal center to be maintained.

It should also be noted that the action of the IM is aimed at restoring damaged cells (10), which contributes to the preservation of the population of lymphocytes in the organ. Furthermore, it leads to an increase in the linear dimensions (length and width) of not only internodular zones but also lymphoid nodules. The effect of the drug lasts up to 3-4 months; this explains the leveling of the experimental parameters with the control values on the 90<sup>th</sup> day of observation.

In conclusion, the use of IM after experimental immunosuppression did not affect the length of the small intestine. At the same time, a significant change was observed in morphometric sizes on days 7 and 30 of the experiment, compared to the control group. Accordingly, there was an increase in lymphoid nodules and internodular zones, as well as the size of the latter in general. However, the distance from the ileocecal junction to the first accumulation of lymphoid nodules underwent a decrease. By the 90<sup>th</sup> day of observation, these changes were leveled in parameters.

#### Authors' Contribution

Study concept and design: E. N. M.

Acquisition of data: V. N. M.

Analysis and interpretation of data: A. V. T.

Drafting of the manuscript: S. N. P.

Critical revision of the manuscript for important intellectual content: V. P. K.

Statistical analysis: E. N. M.

Administrative, technical, and material support: E. N. M.

### Ethics

The present study was approved by the Ethics Committee of the Belgorod State University, Belgorod, Russia.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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