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

N. Morozova, E¹, N. Morozov, V¹, V. Tverskoi, A¹, N. Perepelkina, S¹, P. Konshina, V¹

1. Belgorod State University, 308015, Russia, Belgorod, Pobeda St. 85
Corresponding author: morozov_v@bsu.edu.ru

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 which lead to the diffuse nodular lymphoid hyperplasia. For instance Giardia lamblia and Helicobacter pylori are the major pathogens causing 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 aim of the current study was to investigate morphological features of the small intestine’s aggregated lymphoid nodules after the Imunofan (IM) administration following Cyclophosphamide-induced immunosuppression. Seventy two Wistar male rats randomly divided into 2 groups (n=36). Group I was considered as the control group. Group I was administered to intramuscular injections (needle 21 G) of 0.2 ml of normal saline following the Cyclophosphamide-induced immunosuppression on the 2nd, 4th, 6th, 8th, 10th 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 administered to intramuscular injections (needle 21 G) of the 0.2 ml IM at a dose of 0.7 μg/kg body weight on the 2nd, 4th, 6th, 8th, 10th days of the experiment. The results of the study showed that on the 7th day in group II the length, width of the aggregated lymphoid nodules increases, 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 30th days of the experiment, the linear dimensions of the aggregated lymphoid nodules exceed, but to a lesser extent than on the 7th days 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 and also the lymph nodes.
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 exposed to long-term exposure to exogenous antigens, and contact with them during the prolonged passage of food through the digestive tract is very close, which explains the presence of lymphoid tissue in it, which largely determines the degree of human health and its adaptive capabilities(4). Small nodules 2 to 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). Compensating for the lack of lymphoid tissue in the gut may be the cause of this immunodeficiency. Intestinal infections may even repeatedly trigger immune immunity in the intestinal lymphoid tissue and cause these lymphoid nodules. Nodular lymphoid hyperplasia (DNLH) is associated with immunodeficiency, including common variable immunodeficiency, selective IgA 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 body, both humans and 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 secrete 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 for 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, microscopic levels(9).

Cyclophosphamide is an alkylating agent (Oxazaphosphorines group), and in addition to its anti-toxic and anti-inflammatory effects, it has immunosuppressive properties as well as immune regulatory properties(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 for 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 is one of the new generation drugs that are used as an immunomodulator for prophylactic or therapeutic purposes in Russia and Ukraine(13-15). Considering that the available literature is incomplete or practically absent data on the effect of Imunofan on the lymphoid tissue of the small intestine, the work aimed to study aggregated lymphoid nodules at the macroscopic, microscopic levels after the administration of Imunofan 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. Materials and Methods
2.1. Animals and treatments
Seventy two Wistar male rats randomly divided into 2 groups (n=36). Group I was considered as the control group. Group I was administered to intramuscular injections (needle 21 G) of 0.2 ml of normal saline following the Cyclophosphamide-induced immunosuppression on the 2nd, 4th, 6th, 8th, 10th 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 administered to intramuscular injections (needle 21 G) of the 0.2 ml IM at a dose of 0.7μg/kg body weight on the 2nd, 4th, 6th, 8th, 10th days of the experiment. The keeping and manipulation of the animals was 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 taken out of the experiment on days 7th (n=12), 30th (n=12), 90th (n=12). Under the deep anesthesia the small intestine was isolated using a special instrument (15), and its length was determined. Using a caliper, 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. Then the small intestine was fixed in 10% formalin solution, after which it was subjected to standard histological wiring. Sections 5-6 μm thick were stained with hematoxylin-eosin. Using an automated morphometric complex, including an Olympus CX41 light microscope, a digital camera, and a personal computer with a set of applied programs, the height and width of the lymphoid nodules (LN) and internodular zones in each accumulation of lymphoid tissue were measured (13).

2.2. Statistical analysis
The digital data were processed using the program "STATISTICA 5.11" (the Student's t-test was
3. Results

Microscopic observations in rats showed that aggregated lymphoid nodules have the same color as the surrounding tissues; protrude above the wall of the small intestine from the side of the free edge of the organ, oval, round, 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, the last - in the area of the ileocecalfjunction from the side of the ileum.

![Figure 1. Aggregated lymphoid nodule of the small intestine of a mature male rat on the 30th day.](image1)

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).

![Figure 2. Small intestine (1) with aggregated lymphoid nodules (2) and a section of the large intestine (3) of mature male rats on the 30th day of observation after the Imunofan correction of](image2)

determined, the differences with a level of significance at p <0.05 were considered significant).
Cyclophosphamide-induced immunosuppression.

On the 7th day in the control and treatment groups, compare the mean of length and width of the ALN showed that significant increased 18.71% and 16.94% (Table 1), the distance from the ileocecal junction to the first of them decreased by 6.15%, which was not significant(p<0.05).

Table1.Linear parameters of the small intestine and ALN of immunosuppressed rats after treatment with Imunofan in the experimental and control groups at different periods of observation (n = 36)

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

Note: * - significant differences from the control data at p<0.05.

Comparison of the mean in the two experimental groups on the 30th day after injection indicated that, statistically significant differences were observed in increasing of the linear dimensions ALN 3.56% and 24.22%, as well as, the distances from the ileocecal junction to the first of them decreased by 19.80% (p<0.05). Morphometric measurement on 90th day, showed that control and treatment group data no statistical differences.

Findings of histological studies (Figure 3 a, b), ALN of mature male rats explained that consists of groups of lymphoid nodules from 4 to 15. Their surface is smooth and faces the intestinal lumen, and the adjacent areas of the intermodular zone are covered with villi that partially cover the surface of the lymphoid nodules. Lymphoid nodules is located in the lamina propria of the mucosa and submucosa, arranged in one row.
Figure 3. ALN in the wall of the small intestine of mature rats (a – control, b - on the 30th day after injection of Imunofan Cyclophosphamide-induced immunosuppression): 1 - ALN, 2 - crypt, 3 - villus, 4 - dome, 5 - germinal center, 6 - peripheral zone, 7 - internodular zone. Staining: hematoxylin-eosin. Approximation: Zoom 162. Lens: Plan C N 4x / 0.25∞ / / FN22.

Statistical analysis of 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 control rats by 29.79% and 12.73% (7 days) and 10.49% and 12.46% (30 days). The linear dimensions of the internodular zones increased by 39.35%, 10.33% (day 7) and by 4.77%, 4.25% (day 30), in contrast to the control parameters, which were all significant. By day 90, the height and width of the lymphoid nodules and internodular zones approached those of 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, a germinal center, and is delimited 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)**

<table>
<thead>
<tr>
<th>Linear parameters (μm)</th>
<th>Mature rats</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7th day</td>
<td>30th day</td>
<td>90th day</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Experimental group</td>
<td>Control</td>
<td>Experimental group</td>
<td>Control</td>
<td>Experimental group</td>
</tr>
<tr>
<td>Lymphoid nodule height</td>
<td>1393.00±5 6.30*</td>
<td>978.00±31.50</td>
<td>1115.00±32.30*</td>
<td>998.00±38.30</td>
<td>998.00±13.10</td>
</tr>
<tr>
<td>Lymphoid nodule width</td>
<td>1100.00±2 7.00*</td>
<td>960.00±41.60</td>
<td>859.00±42.00*</td>
<td>752.00±13.90</td>
<td>973.00±13.90</td>
</tr>
<tr>
<td>Internodular zone height</td>
<td>709.00±28.00*</td>
<td>430.00±8.40</td>
<td>398.00±25.50*</td>
<td>379.00±7.60</td>
<td>288.00±17.80</td>
</tr>
<tr>
<td>Internodular zone width</td>
<td>455.00±12.70*</td>
<td>408.00±9.20</td>
<td>400.00±29.60*</td>
<td>383.00±7.00</td>
<td>516.00±6.13</td>
</tr>
</tbody>
</table>

Note: * - 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, less often macrophages, 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, less often medium and small lymphocytes are detected (Figure 4 a-d).
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 30th day (a - dome, b - germinal center, c - peripheral zone, d - internodular zone): 1 - lymphocyte, 2 - macrophages, 3 - reticulocyte, 4 - intraepithelial lymphocyte, 5 - mucosal epithelium, 6 - vessel. Staining: hematoxylin-eosin.

Approximation: Zoom 162. Lens: Plan C N 4x / 0.25∞ / - / FN22.

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.

4. Discussion

Analysis of effect of Imunofan in immunosuppressed rat 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). Thus, the use of Imunofan against the background of an immunosuppressive 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 their significant increase in comparison with the data of the control group during the month (p<0.05).

According to data of other authors, the use of Imunofan against the background of immunosuppression restores the linear dimensions of the thymus to the control parameters within a
month(17). At the same time for a given period of time in the lymph nodes of rats when using the Imunofan, not only the leveling of linear data occurs, but also their increase in comparison with the control group(18), which corresponds to the above changes obtained in the study of ALN of the small intestine. Based on this, the use of Imunofan 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 exceed the values of the control group on the 7th and 30th days after the correction with Imunofan Cyclophosphamide-induced immunosuppression (p <0.05). By day 90, the experimental data are leveled with the control parameters. Similar changes were observed after oral administration of P. Multicida B2 to Spraque-Dawley rats weighing 200-250 g(19). The data obtained may indicate a pronounced reaction of ALN of the small intestine to the introduction of Imunofan against the background of an immunosuppressive state for a month, while after three months the linear dimensions and structure of the organ are restored.

The pharmacological action of the peptide immunooxidant is based on the achievement of the correction of the body’s immune and redox systems(20). It was found that on the 7th day, the immunoregulatory effect of this drug is manifested - the restoration of disturbed parameters of cellular and humoral immunity. This pharmacological action is explained by the ability of the Imunofan to restore the production of the thymic hormone thymulin to values characteristic of normal animals (21). This results in an increase of the population of T-lymphocytes in the thymus of rats. The above 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, which, allows the active proliferation of immunocompetent cells in the germinal center to be maintained. It should also be noted that the action of the Imunofan is aimed at restoring damaged cells(10), which contributes to the preservation of the population of lymphocytes in the organ and 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 90th day of observation.

In conclusion, the use of Imunofan after experimental immunosuppression did not affect the length of the small intestine. At the same time, a significant change in morphometric sizes was observed (an increase in lymphoid nodules and internodular zones, as well as the size of the latter in general, while the distance from the ileocecal junction to the first accumulation of lymphoid nodules decreased) on days 7 and 30 of the experiment, compared to the control. By the 90th day of observation, these changes in parameters were leveled.
References