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# METRICAL INDICES OF THE CECUM WALL IN AREAS OF GUT-ASSOCIATED LYMPHOID TISSUE LOCALISATION UNDER THE INFLUENCE OF FOOD ADDITIVE COMPLEX

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There are insufficient data in the current literature on the effect of food additives on the occurrence of pathological processes in the intestine. A total of 84 sexually mature male non-linear rats were used in the experiment. Rats of the control group consumed drinking water and received oral physiological solution. Animals in the experimental group received food additives at the following doses: sodium nitrite (E250) 0.6 mg/kg, sodium glutamate (E621) 20 mg/kg, ponceau 4R (E124) 5 mg/kg. Sampling for histological examination was carried out at 1, 4, 8, 12 and 16 weeks. A significant reduction in the thickness of the cecum wall of the animals in the areas of localisation of intestinal-associated lymphoid tissue was found at the early stages of observation. A marked lymphoid tissue reaction in the form of intense cellular infiltration of the submucosa and mucosa was recorded. Disturbance of crypt structure and desquamation of epithelium occurred. At the late terms of observation there was a significant increase of the cecum wall thickness in the areas of localization of the lymphoid tissue associated with the intestine due to edema of submucosa, partial restoration of mucosa structures.

**Key words:** food additives, monosodium glutamate, sodium nitrite, ponceau 4R, gut-associated lymphoid tissue.

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# МЕТРИЧНІ ПОКАЗНИКИ СТІНКИ СЛІПОЇ КИШКИ У ДІЛЯНКАХ ЛОКАЛІЗАЦІЇ АСОЦІЙОВАНОЇ З КИШЕЧНИКОМ ЛІМФОЇДНОЇ ТКАНИНИ ЗА ВПЛИВУ КОМПЛЕКСУ ХАРЧОВИХ ДОБАВОК

У сучасних літературних джерелах недостатньо даних щодо впливу вживання харчових добавок на виникнення патологічних процесів в кишечнику. В експерименті використано 84 статевозрілих нелінійних щурів-самців. Щури контрольної групи споживали питну воду та перорально отримували фізіологічний розчин. Тварини дослідної групи отримували харчові добавки в наступних дозах: нітриту натрію (Е250) - 0,6 мг/кг, глутамату натрію (Е621) - 20 мг/кг, понсо 4R (Е124) - 5 мг/кг. Відбір зразків для гістологічного дослідження проводили на 1, 4, 8, 12 та 16 тиждень. На ранніх строках спостереження було встановлено достовірне зменшення товщини стінки сліпої кишки тварин в ділянках локалізації асоційованої з кишечником лімфоїдної тканини. Реєстрували виражену реакцією лімфоїдної тканини у вигляді інтенсивної клітинної інфільтрації підслизової основи та слизової оболонки. Відбувалося порушення структури крипт та десквамація епітелію. На пізніх строках спостереження спостерігалося достовірне збільшення товщини стінки сліпої кишки в ділянках локалізації асоційованої з кишечником лімфоїдної тканини за рахунок набряку підслизової основи, часткового відновлення структур слизової оболонки.

**Ключові слова:** харчові добавки, глутамат натрію, нітрит натрію, понсо 4R, асоційована з кишечником лімфоїдна тканина.

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Food additives are widely used in various branches of the food and pharmaceutical industries. Food additives are used as preservatives, flavour enhancers and colourings. Reviews of the literature indicate that the consumption of food colourings has increased five times since the 1950s. However, the effects of these substances on the human body remain controversial and need to be studied closely. In particular, the question of the intestinal pathology that results from the continued and uncontrolled use of food additives remains poorly studied. The role of food additives, in particular colouring agents, in the occurrence of colorectal cancer due to disruption of the normal intestinal microbiome has been repeatedly reported (1). For example, the use of sausages containing nitrites has been reported to increase the incidence of colorectal cancer in mice with adenomatous polyposis coli. The researchers believe oxidative stress and dysbacteriosis induced by the food additive to be the cause of the pathology [3].

The intestinal mucosa has an extremely developed lymphatic system due to the fact that it is constantly exposed to a high antigenic load through contact with food and pathogenic and opportunistic microorganisms [2, 7]. The total area occupied by lymphoid tissue in the human intestine is known to be about 260-300 $m^2$. [4]. Gut-associated lymphoid tissue of the large intestine includes diffuse clusters of lymphoid cells and organized lymphoid nodules localized in the intestinal mucosa, intraepithelial lymphocytes localized in the epithelium. In contrast to lymph nodes, histologically mucosa-associated

lymphoid tissue is predominantly aggregates of lymphoid and supporting cells, which are not completely encapsulated or lack a capsule altogether. The functional sections of GALT are represented by the lymphoid (the subepithelial dome region) and the overlying follicle-associated epithelium [10].

**The purpose** of the study was to establish the dynamics of changes in the metric parameters of the cecum's gut-associated lymphoid tissue in the normal conditions and under the complex action of food additives.

**Materials and methods.** The experiment involved 84 mature male rats (Rattus norvegicus) weighing 204.5±0.67 g, which were obtained from the experimental-biological clinic of the University. All procedures followed the standard rules established by the commission of Poltava State Medical University on ethical issues and bioethics (order of the rector No. 330 of May 30, 2020) in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The rats of all groups had access to food and water ad libitum. The animals were sacrificed under thiopentone anesthesia overdose. The rats of control group (n=14) consumed drinking water and were administered with saline orally. The rats of the experimental group, with access to water ad libitum, were administered with 0,6 mg/kg sodium nitrite E250 (Uralchem, China), 20 mg/kg monosodium glutamate E621 (Multichem, China) and 5 mg/kg Ponceau 4R E124 (Multichem, China) in 0.5 ml of distilled water once daily orally. Collection of samples for histological examination was carried out at 1, 4, 8, 12 and 16 weeks. After euthanasia the rats were dissected following the method of complete evisceration. The cecum was removed and fixed with a 10 % neutral formalin solution. The material was washed and prepared for paraffin embedding according to standard techniques [11]. Sections of 5-10 μm thick were obtained using the на manual rotary microtome HistoLine. Histological sections were stained with hematoxylin and eosin (H&E). Series of histological slide's photomicrographs from objectives 4x and 10x were captured by a microscope Levenhuk D740T attached to a digital 5.1 Mpx kit camera. Photo fixation and morphometry were performed in Levenhuk Lite software. The data was expressed as the means ± standard error of the mean. Statistical processing of morphometric data was performed using the Excel software, that analyzed using parametric Student's t-test. The data was expressed as the means ± standard error of the mean. Besides, $p<0.05$ was considered to be statistically significant.

**Results of the study and their discussion.** According to the results of morphometric study, the total thickness of the cecum wall in control animals was 617.76±5.57 μm, thickness of mucosa was 181.74±2.63 μm, thickness of submucosa was 263.85±1.5 μm (Table 1).

Table 1

**Results of metric studies of the rat cecum wall at sites of localized gut-associated lymphoid tissue when exposed to a complex of nutritional supplements**

| Week | Total wall thickness, μm | Mucosa thickness, μm | Submucosa thickness, μm |
|---|---|---|---|
| Control | 617.76±5.57 | 181.74±2.63 | 263.85±1.5 |
| 1 | 524.46±2,8 * | 163.79±8.8 * | 266.44±1.59 |
| 4 | 491.32±20.8 *,** | 182.17±1.31 ** | 204.27±9.42 *,** |
| 8 | 504.01±2.47 * | 185.32±1.37 | 236.27±3.19 *,** |
| 12 | 593.71±4.01 *,** | 237.74±1.13 *,** | 261.93±1.85 ** |
| 16 | 777.23±6.93 *,** | 246.09±9.65 * | 279.3±4.35 *,** |

Notes: * – $p<0.05$ compared to the control group; ** – $p<0.05$ compared to the previous time period of the observation.

In control rats, organized lymph nodes and diffuse clusters of lymphoid cells were recorded in the subepithelial submucosa. The submucosal base was a layer of loose connective tissue and contained numerous blood vessels of different calibers. Lymph nodes were an intense accumulation of unencapsulated lymphatic tissue. The lymphoid tissue extended throughout the submucosa and had indistinct borders with a poorly defined light germinal centre and a darker interfollicular mantle zone. Towards the mucosa, the interfollicular (mantle) zone gradually progressed to the subepithelial dome region, which was characterised by a gradual decrease in the number of lymphoid cells in the surrounding connective tissue. The muscle plate was represented by several layers of smooth myositis. On the lumen side, the mucosa was represented by follicle-associated epithelium, which was a layer of columnar epithelial cells. Short crypts, the lumen of which was lined by columnar absorbing epithelial cells and bocalytic exocrinocytes, were visualized. A slight infiltration of the mucosa with single lymphoid cells was observed.

The total thickness of the rat cecum wall at the 1st week of observation was 524.46±2.8 μm, reliably less by 15.1 % in comparison with the control group. The thickness of mucosa at this period of observation was 163.79±8.8 μm and was significantly less than that of the control group by 9.88 %. The submucous membrane thickness was 266.44±1.59 μm and did not reliably differ from the control group of animals.

At the first week of the experiment in the subepithelial submucosa of the rat cecum an active migration of cells from lymph nodes into the surrounding submucosal tissues was recorded. Lymph nodes had a well-defined light germinal centre and a dark interfollicular zone, intensely stained due to a large concentration of lymphoid cells. The boundary between the interfollicular (mantle) zone and the area of subepithelial dome was not clearly visualized due to active migration of lymphocytes from lymph nodes towards the intestinal lumen. As a consequence, cellular infiltration of the lamina propria of the mucosa and mucosa was observed (fig. 1a,1b).

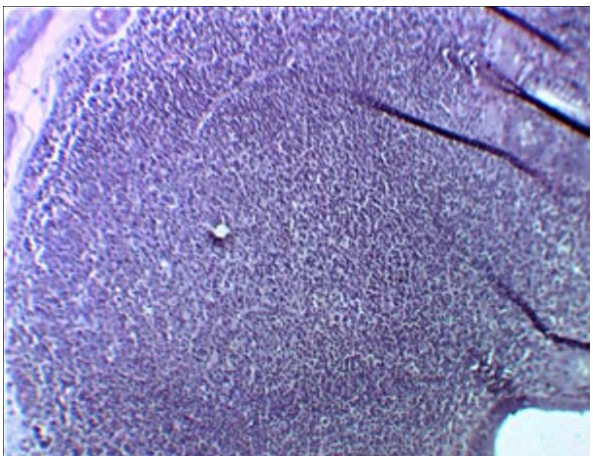

Fig. 1a. Structure of the rat cecum wall at sites of localised gut-associated lymphoid tissue (1st week of experiment). Hematoxylin and eosin staining. x100

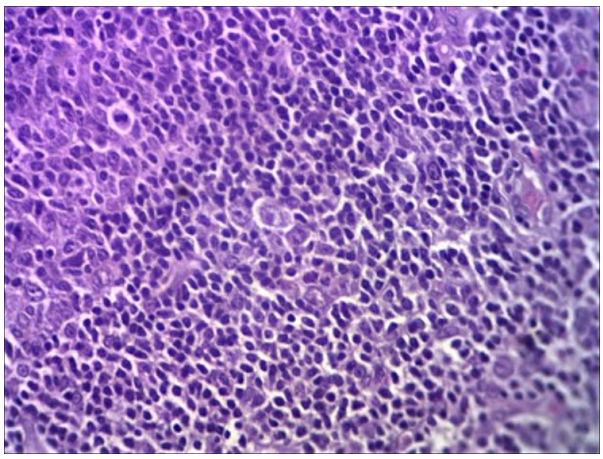

Fig. 1b. A lymph node in the submucosal base of the cecum of rats (1st week of experiment). Hematoxylin and eosin staining. X400.

At the 4th week of experiment the total cecum wall thickness in rats was 491.32±20.8 μm, which was reliably less by 20.47 % than in the control group, and by 6.32 % less than in the previous observation period. The mucosal thickness was within 182.17±1.31 μm, and did not differ significantly from the control group. However, it was 11.22 % greater than the previous observation period. The submucosa thickness was 204.27±9.42 micrometres and differed significantly from the control group and the previous period of observation by 22.58 % and 23.33 % respectively.

At week 4 of the experiment, the lymph nodes of the rat cecum wall had lost their structure. The germinal centre of the nodule, interfollicular zone and subepithelial dome were undifferentiated. The submucosal base in the area of lymphoid tissue localization stained homogeneously. It should be assumed that lymph node obliteration occurred due to edema and increased migration of granulocytes and lymphocytes into lamina propria of mucosa from lymphoid nodules and submucosal base vessels. In the mucosa, desquamation of epitheliocytes both on the surface and in the crypt lumen was recorded (fig. 2a).

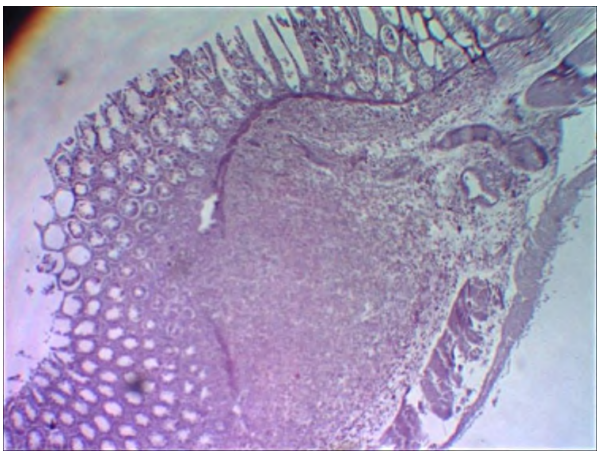

Fig. 2a. Structure of the rat cecum wall at sites of localization of gut-associated lymphoid tissue (4th week of experiment). Hematoxylin and eosin staining. x100.

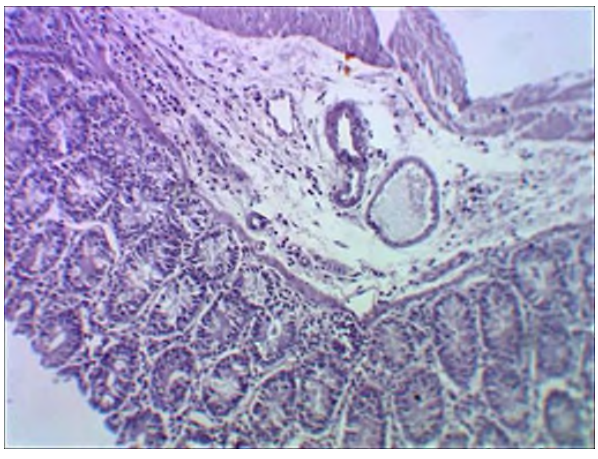

Fig. 2b. Structure of the rat cecum wall at sites of localization of gut-associated lymphoid tissue (8th week of experiment). Hematoxylin and eosin staining. x100.

At the 8th week of experiment the total cecum wall thickness was 504.01±2.47 micrometres, which was 18.41 % less than the control and probably did not differ from the previous period of observation. Mucosa thickness was 185.32±1.37 μm and did not differ significantly from the control or the previous

follow-up. The submucosa thickness was 236.27±3.19 micrometres and was significantly greater than the control by 1.97 %, and the previous follow-up by 15.67 %.

At this time of observation, complete desquamation of submucosal lymph nodes was recorded. Spasmed arterial vessels and blood-filled venous vessels were visualized. The germinal centre of the nodule, interfollicular zone and subepithelial dome were not visualized. A small number of lymphoid cells were registered at the periphery of the nodule, against the background of significant cellular infiltration of the nearby loose connective tissue of submucosal base. Intense cellular infiltration of the mucosa persisted. There was moderate desquamation of epithelial cells in the upper crypts and superficial epithelium (fig. 2b).

At the 12 weeks of observation the total cecum wall thickness in rats was 593.71±4.01 micrometres, which was significantly less by 3,89 % than in the control group, and by 28.29 % more than in the previous observation period. The cecum mucosa thickness in rats was 237.74±1.13 μm, which was 30.81 % more than that in the control group, and 28.29 % more than that in the previous observation period. The submucosa thickness was 261.93±1.85 μm, significantly greater than the previous observation term by 10.85 %.

At week 12, microscopic changes in the submucosa were similar to those of the previous observation period. The germinal centre of the nodule, the interfollicular zone and the subepithelial dome were not visible. A small number of lymphoid cells were visualized at the periphery of the nodule. Intense cellular infiltration of loose connective tissue of submucosa, intrinsic lamina and mucosa was recorded (fig. 3a).

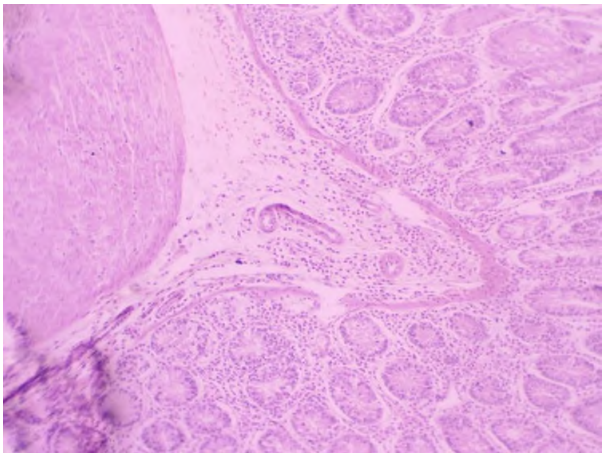

Fig. 3a. Structure of the rat cecum wall at sites of localization of gut-associated lymphoid tissue (12th week of experiment). Hematoxylin and eosin staining. x100.

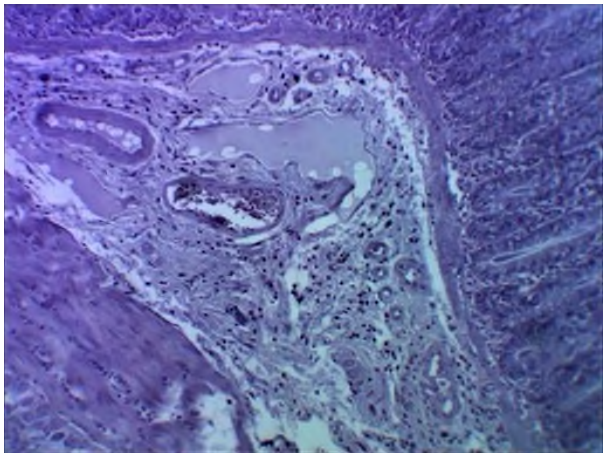

Fig. 3b. Structure of the rat cecum wall at sites of localization of gut-associated lymphoid tissue (week 16 of the experiment). Hematoxylin and eosin staining. x100.

At the 16th week of experiment the total cecum wall thickness in rats was 777.23±6.93 μm and differed significantly from the control and previous period of observation. Thus, this index was 25.81 % higher than the control, and 30.91 % higher in comparison with the previous period of observation. The mucous membrane thickness was 246.09±9.65 micrometres and was 35.41 % greater than the control group. The submucosa thickness was 279.3±4.35 microns. This index was significantly different from the control group and the results of the previous period of observation and was greater by 5.86 % and 6.64 %, respectively.

The microscopic picture at week 16 of the experiment was characterized by “desolation” of the submucosal lymph nodes, the germinal centre of the node, the interfollicular zone and the subepithelial dome were not visualized. In the loose connective tissue of submucosal base, only single lymphoid cells were visualized in the localized gut-associated lymphoid tissue. At the same time, significant cellular infiltration of the adjacent loose connective tissue of submucosal base was preserved. In places of localization of lymph nodes a large number of spasmed arterial vessels and venous blood vessels were registered. There were moderate phenomena of desquamation in the mucosal epithelium against the background of preserved cellular infiltration of its lamina propria (fig. 3b).

During the experiment, it was established that metric indexes of the cecum wall in sites of localization of gut-associated lymphoid tissue under the influence of complex of food additives changed in the direction of decreasing of all the studied indexes up to the 8th week of observation. The decrease in submucosal thickness was probably due to active migration of lymphoid cells from lymph nodes into the surrounding connective tissue and mucosa of the rat cecum. The thickness of the mucosa probably

decreased due to desquamation of the epithelium, up to the disruption of the crypt structure. Starting from the 8th week of observation, the total thickness of the rat cecum wall in the areas of gut-associated lymphoid tissue localization began to increase and exceeded the control values by the 16th week of observation (fig. 4). It is likely that the increase in submucosal thickness was due to edema and vasospasm due to the prolonged action of the altering factor. No recovery of the lymph node structure was observed, in contrast to the mucosa where the phenomena of desquamation became less pronounced by the 16th week of observations and the crypt structure partially recovered.

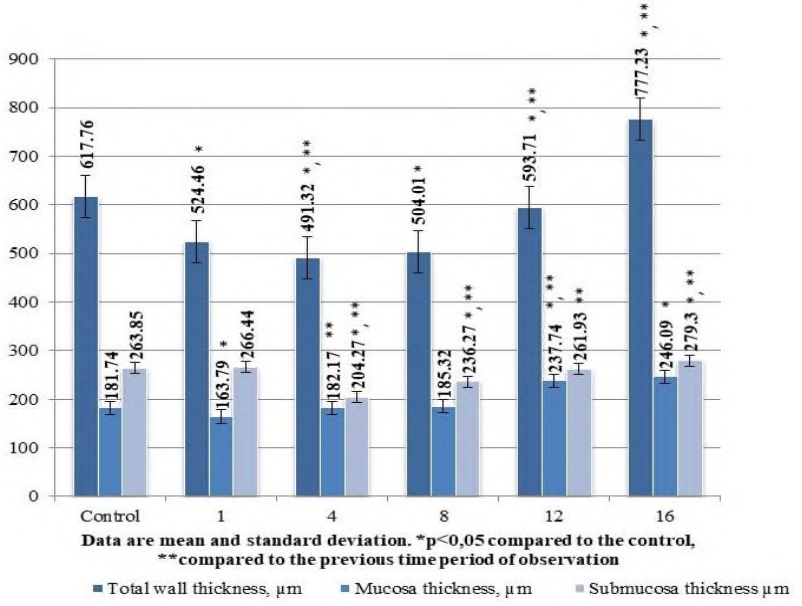


Fig. 4. Dynamics of morphometric indices of the rat cecum wall at sites of localized gut-associated lymphoid tissue.

In our opinion, the metrics of the rat cecum wall in the areas of gut-associated lymphoid tissue localization indicate a direct effect of the food additive complex on the local mucosal immunity response. Normally, the immune response of the intestinal mucosa to food antigens is characterised as tolerant (so-called "oral tolerance"). In the absence of inflammatory substances in the food masses, the presentation of food antigens by dendritic cells in the intestinal mucosa does not activate T-lymphocytes, but rather conditions their tolerance. Intestinal epithelial cells are also involved in the presentation of food antigens and the formation of antigen tolerance. [7]. The cells of the intestinal epithelium act as peculiar sensors for food and microbial antigens; they deliver these antigens to the lymphoid tissue of the own lamina, where an immune response is formed [13]. Consequently, damage to the mucosa in the experiment, as evidenced by epithelioocyte desquamation, disruption of crypt structure and intense lymphoid infiltration, can lead to impaired formation of antigen tolerance, which is consistent with the data of previous research [14]. The presence of an inflammatory reaction in response to the presence of a particular substance in the intestinal contents suggests hypersensitivity or an alternative action of the above-mentioned substance.

When monosodium glutamate, nitrite sodium and ponceau 4R supplements were used, a gradual reduction in the overall thickness of the cecum wall at sites of gut-associated lymphoid tissue localisation was observed in the early stages of follow-up. The morphology of the intestinal wall and gut-associated lymphoid tissue in particular is known to be influenced by pituitary hormones. Thus, total pituitaryectomy in rats led to a decrease in the height of the small intestinal villi and a decrease in the number of cell bodies and in the number of IgA- and IgM-secreting lymphocytes. The area of the intestinal mucosal plate was reduced in animals subjected to anterior and total pituitaryctomy [1]. Consequently, the effect of nutritional supplements on intestinal wall morphology may be mediated, as monosodium glutamate is known to cause disruption of the neuroendocrine function of the hypothalamic-pituitary system [10].

It should be noted that the structure and function of gut-associated lymphoid tissue is provided by normal microflora [8]. At the same time, the consumption of monosodium glutamate leads to a disturbance in the composition of normal gut microflora, a reduction of bifidobacteria and causes dysbiosis [9, 15]. Some members of the normal human gut microflora are sensitive to sodium nitrite and its combinations with other additives [6].

Thus, in our opinion, there are several aspects of the effect of a complex of food additives on the gut-associated lymphoid tissue of the cecum. The first aspect is the direct alterative effect of food additives on the intestinal mucosa through contact with food masses. As a result, the barrier and antigen-presenting functions of colonocytes are impaired and, as a consequence, immune cell function is locally compromised. The second aspect is the effect of food additives, in particular monosodium glutamate, on the central neuroendocrine system, resulting in pathological changes in the intestinal mucosa and gut-associated lymphoid tissue. The third aspect is the effect of food additives on normal gut microflora and the occurrence of dysbiosis, which directly affects gut-associated lymphoid tissues.

## Conclusion

Administration of the monosodium glutamate, sodium nitrite and ponceau 4R complex causes a decrease in the thickness of the cecum wall of the white rats in the localization areas of the gut-associated lymphoid tissue in the early period of observation, which is associated with a marked mucosa-associated submucosa and mucosa reaction, disturbance of crypt structure and epithelium desquamation. At the late terms of observation, an increase in the thickness of the cecum wall of white rats in the areas of localization of gut-associated lymphoid tissue with edema and submucosa base and partial recovery of mucosa structures were observed.

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