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MORPHOLOGICAL AND FUNCTIONAL STATE OF THE MACROPHAGAL LINK OF THE SPLEEN UNDER CONDITIONS OF OXIDATIVE-NITROSATIVE STRESS CAUSED BY PROLONGED ADMINISTRATION OF A COMPLEX OF CHEMICAL FOOD ADDITIVES

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The study investigates the effect of long-term administration of a dietary supplement complex on the state of the macrophage link in the spleen of rats. The experiment was conducted on 70 male rats that received a supplement mixture for 1–20 weeks. The levels of malondialdehyde, nitrites, and the immunomorphology of CD68+ macrophages were assessed. A biphasic reaction of the macrophage population was established. In the early stages, an increase in cell number was observed, peaking at week 4, at 1.79 times the control. Chronic exposure led to a marked reduction in cell numbers by week 20, with cell numbers 4.48 times lower than the initial values. Morphological analysis revealed progressive cell fragmentation, atrophy of the marginal zone, and decreased immunohistochemical staining intensity. Structural degradation of the macrophage apparatus closely correlated with the accumulation of lipid peroxidation products. The study demonstrates the mechanism of depletion of the spleen's immune barrier under prolonged xenobiotic stress.

Key words: immune system, morphological changes, oxidative stress, nitrosative stress, spleen, white pulp, red pulp, food additives, CD68, malondialdehyde, monosodium glutamate, sodium nitrite, ponceau 4R.

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МОРФОФУНКЦІОНАЛЬНИЙ СТАН МАКРОФАГАЛЬНОЇ ЛАНКИ СЕЛЕЗІНКИ ЗА УМОВ ОКСИДАТИВНО-НІТРОЗАТИВНОГО СТРЕСУ, ВИКЛИКАНОГО ТРИВАЛИМ ВВЕДЕННЯМ КОМПЛЕКСУ ХІМІЧНИХ ХАРЧОВИХ ДОБАВОК

Дослідження присвячено вивченню впливу тривалого введення комплексу харчових добавок на стан макрофагальної ланки селезінки щурів. Експеримент проведено на сімдесяти щурах-самцях, які отримували суміш добавок протягом 1–20 тижнів. Оцінювали рівні малонового діальдегіду, нітритів та імуноморфологію CD68+ макрофагів. Встановлено двофазну реакцію макрофагальної популяції. На ранніх термінах спостерігалося зростання кількості клітин із піком на 4-му тижні, що у 1,79 раза перевищувало контроль. Хронічний вплив призвів до вираженої редукції чисельності клітин до 20-го тижня у 4,48 раза порівняно з початковими значеннями. Морфологічний аналіз виявив прогресуючу фрагментацію клітин, атрофію маргінальної зони та зниження інтенсивності імуногістохімічного забарвлення. Структурна деградація макрофагального апарату тісно корелювала з накопиченням продуктів пероксидного окиснення ліпідів. Робота демонструє механізм виснаження імунного бар'єру селезінки під дією тривалого ксенобіотичного навантаження.

Ключові слова: імунна система, морфологічні зміни, оксидативний стрес, нітрозативний стрес, селезінка, біла пульпа, червона, харчові добавки, CD68, малоновий діальдегід, глутамат натрію, нітрит натрію, понсо 4R.

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The modern development of the food industry is characterised by the widespread use of synthetic compounds designed to improve the organoleptic properties of products. The most common among them are the flavour enhancer monosodium glutamate, the preservative sodium nitrite and the colouring agent ponso 4R. Recent scientific data indicate that these substances, when ingested over a long period of time, become a source of xenobiotic stress, initiating oxidative-nitrosative stress [2, 3]. The spleen, as a key organ of immune defence and biological filter, is the first to respond to systemic metabolic changes. Of particular importance is the state of resident macrophages, which are identified by the expression of the CD68+ receptor and ensure tissue homeostasis [1, 4, 12].

An analysis of recent publications shows that prolonged consumption of dietary xenobiotics leads to restructuring of the alveolar apparatus of the lungs and changes in the testicles and small intestine tissues [1, 9, 10]. However, the morphological transformation of the macrophage link of the spleen under the combined influence of these additives remains insufficiently studied. The relevance of the problem is due to the need to clarify the structural basis of immunological deficiency, which develops under the influence of common food components through the mechanisms of lipoperoxidation [8, 11].

The purpose of the study was to establish the features of structural reorganization of the macrophage link of the rat spleen in the dynamics of prolonged administration of a complex of food additives (monosodium glutamate, sodium nitrite, Ponceau 4R) and its relationship with indicators of oxidative-nitrosative stress.

Materials and methods. The study was conducted on 70 sexually mature white rats weighing 190±1.34 g, which were kept under standard conditions in the vivarium of Poltava State Medical University. They were kept in standard cages (90cm (L) x 60cm (D) x 120cm (H), 4 rats/cage, with corn cob bedding. In cage, they had 3 cardboard tunnels and nesting material from wood and wool. Rats had a controlled temperature in g=cages (22±2°C), middle humidity (50±10 %) and 12 hours light/dark cycle. The experiment lasted for half a year, from March 2024 to September of 2024. The animals were divided into a control group and six experimental groups, with 10 animals in each group (1 control group (n=10) and 6 experimental groups (n=60)). The animals in the control group received a physiological solution, while the experimental groups received a complex of sodium glutamate 20 mg/kg (Meihua Holdings Group Co., Ltd., China), Ponso 4R 5 mg/kg (Ajanta Chemical Industries, India) and sodium nitrite 0.6 mg/kg (BASF, Germany) in 0.5 ml of distilled water orally for 1, 4, 8, 12, 16 and 20 weeks. These doses were half the maximum permissible levels for food products. At other times, the rats had free access to water and standard vivarium feed. The experimental animals were removed from the experiment by overdosing with thiopental sodium under ether anaesthesia, followed by collection of experimental material from the spleen tissue for biochemical and immunohistochemical analysis. The level of free malondialdehyde (MDA) in 10 % homogenate was determined by spectrophotometry (Ulab 101, Ukraine) using a reaction with 1-methyl-2-phenylindole (Sigma Aldrich, USA) at a wavelength of 586 nm. The state of nitrosative stress was assessed by the level of nitrites using the Griss–Ilosvaya reagent (Ukraine) and NO synthase (NOS) activity. The activity of the inducible form of NOS (V3) was calculated by the difference between total and constitutive activity using the inhibitor aminoguanidine (Sigma, USA).

For immunohistochemical examination, spleen fragments were fixed in 10 % neutral formalin. Rabbit recombinant antibodies to CD68 (ab303565, Abcam, USA) and the PolyVue™ HRP/DAB imaging system (USA) were used. Counterstaining was performed with Mayer's haematoxylin. The number of CD68+-positive macrophages was counted in 10 fields of view (40x magnification) using a Levenhuk D740T microscope. Statistical analysis was performed using IBM SPSS Statistics 26.0 (2020). Since the data had a non-parametric distribution (Shapiro–Wilk criterion, $p>0.05$), the Kruskal–Wallis and Mann–Whitney criteria with Bonferroni correction were used. The results are presented as M±m. Spearman's correlation coefficient was considered significant at $p<0.05$.

The study was conducted in accordance with the Rules for the Use of Laboratory Experimental Animals (2006, Appendix 4) and the Helsinki Declaration on the Humane Treatment of Animals, the Law of Ukraine 'On the Protection of Animals from Cruel Treatment' (No. 3447-IV of 21.02.2006.) in accordance with the requirements of the Bioethics Commission of Poltava State Medical University (Protocol No. 208 of 22.09.2022), in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986).

Results of the study and their discussion. Analysis of biochemical indicators revealed a pronounced dynamic accumulation of free radical oxidation products. Statistically significant intergroup differences in MDA levels were found depending on the observation period ($p<0.001$). The analysis showed a significant increase in MDA concentration compared to the control group, starting from the 4th week, with maximum differences at weeks 12–20.

For nitrite concentration, according to the Kruskal–Wallis criterion, general intergroup differences were also found ($p<0.001$), but statistically significant pairwise differences were found only between individual groups, namely: between the control and week 20 ($p=0.020$), between the 1st and 8th weeks ($p=0.007$), between the 1st and 12th weeks ($p=0.015$), and between the 4th and 16th weeks of observation ($p=0.048$), indicating the fluctuating nature of changes in nitrite levels. (Table 1.)

Table 1

Indicators of oxidative-nitrosative stress in rat spleen tissue

Indicator	Control	1 week	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	p-value
MDA, μmol/g	0.40	0.80	1.71	1.37	3.13	1.76	2.28	P<0.001; p_{0-4}=0.007; p_{0-12}<0,001; p_{0-16}=0.002; p_{0-20}<0.001
Nitrites, nmol/g	0.043	0.067	0.053	0.079	0.071	0.050	0.059	p<0.001; p_{0-20}=0.020; p_{1-8}=0.007; p_{1-12}=0.015; p_{4-16}=0.048

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The morphological picture of the spleen of control rats revealed a typical structure. CD68+-positive macrophages were located mainly in the marginal zone and red pulp, with clear contours and

intense brown cytoplasm colouring. The average number of cells was 47.91±1.79 in 10 fields of view. After the first week of supplementation, reactive activation of the macrophage link was observed. The average number of CD68+-positive cells increased by 1.38 times compared to the control. The cells showed signs of cytoplasmic hypertrophy. After the fourth week of the experiment, the average number of macrophages reached a maximum of 85.71±3.09 in 10 fields of view, which was 1.79 times higher than the similar results in the control group of animals. (Fig. 1)

After the 8th week, the phase of structural involution began. The average number of CD68+-positive cells decreased sharply to 37.11±2.47 in 10 f.v. Morphologically, this was accompanied by the appearance of cells with signs of cytoplasmic fragmentation. After the 12th week, the average number of CD68+-positive macrophages decreased to 24.92±1.34 in 10 f.v. Cells in the marginal zone showed signs of destruction: uneven plasma membrane contours and oxyphilic cytoplasm staining. After week 20, the average number of macrophages was only 10.7±1.11 in 10 f.v. This corresponds to a 4.48-fold decrease in cell number compared to the control and an 8.01-fold decrease compared to the peak values at week 4. (Fig. 2)

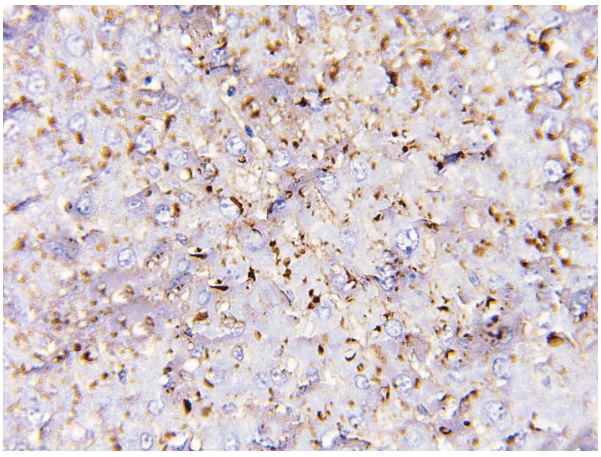

Fig. 1. Distribution of CD68+-positive structures in the spleen parenchyma after the 4th week of the experimental study. Paraffin section. Staining: immunohistochemical reaction with CD68+-positive antigen with Mayer's haematoxylin counterstaining. Collection: ca. 10, vol. 40.

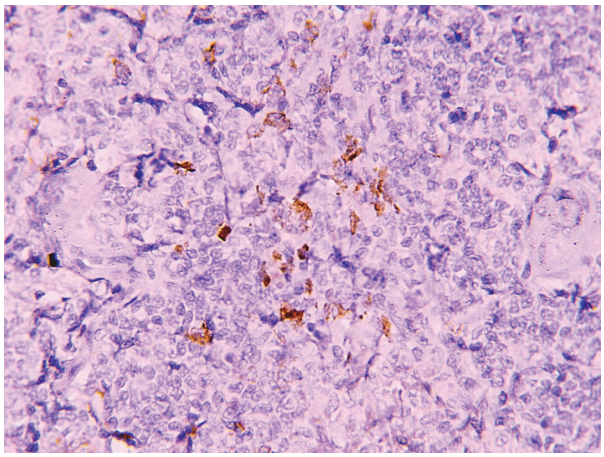

Fig 2. Distribution of CD68+-positive structures in the spleen parenchyma after the 20th week of the experimental study. Paraffin section. Staining: immunohistochemical reaction with CD68+-positive antigen with Mayer's haematoxylin counterstaining. Collection: ca. 10, vol. 40.

Long-term administration of a complex of dietary supplements initiates progressive oxidative stress in the spleen with a peak increase in MDA after 12 weeks – 3.13±0.21 µmol/g. The correlation analysis confirmed that the structural degradation of macrophages is directly related to the accumulation of MDA (rho=-0.643; p=0.001). (Figs. 3A, B.)

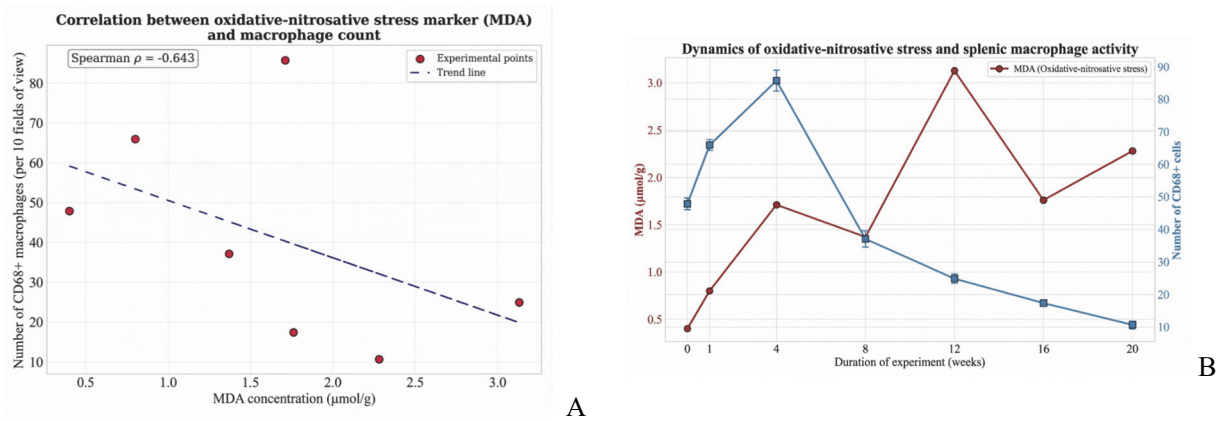

Fig. 3. Oxidative-nitrosative stress and splenic macrophage activity. A – dynamics of oxidative-nitrosative stress and splenic macrophage activity. B – Correlation between MDA levels and the number of CD68+-positive macrophages.

Morphological analysis indicates profound structural changes in the spleen. Initial hyperplasia of CD68+ macrophages (1–4 weeks) is interpreted as a reactive morphological response. However, further cell destruction after the 8th week indicates depletion of regenerative potential. The key morphological substrate of damage is cytoplasmic fragmentation and decreased immunoreactivity. This directly correlates with the accumulation of MDA, which causes disorganisation of the macrophage membrane bilayer.

The pathogenetic basis for these changes is a probable imbalance in the NF-kB and NRF2 regulatory pathways. Chronic activation of the pro-inflammatory factor against the background of depletion of the antioxidant pathway initiates cytolytic processes. The reduction of the macrophage pool in the marginal zone is critical, as these cells initiate the immune response. Thus, morphological degradation of macrophages is a key substrate of immunosuppression [5, 6, 7].

The results obtained are consistent with existing data indicating that long-term use of dietary supplements may cause systemic oxidative stress and tissue remodeling in various organs. However, this study expands current knowledge by providing detailed evidence of time-dependent remodeling of the spleen's macrophage system and its association with impaired redox balance [2].

Overall, the results suggest that chronic exposure to widely used dietary supplements may compromise the spleen's immune barrier through oxidative damage-mediated macrophage exhaustion, highlighting the importance of redox homeostasis in maintaining immune competence.

Limitations. Among the study's limitations is the use of only an experimental animal model, which limits the direct extrapolation of the results to humans. Another factor is that standardized animal housing conditions may not capture the effects of additional stressors that modulate the immune response in real-world conditions.

Conclusions

1. Long-term administration of a complex of dietary supplements initiates progressive oxidative stress in the spleen with a peak increase in MDA after 12 weeks – 3.13±0.21 μmol/g.

2. The reaction of the macrophage link (CD68+) is staged: the phase of reactive activation, peaking at week 4 (85.71±3.09) cells, is followed by a phase of destruction with a 4.48-fold decrease in cell count by the end of week 20 10.7±1.11 cells.

3. A moderate inverse correlation (rho=-0.643) was found between MDA accumulation and macrophage count, confirming the membrane-destructive mechanism of spleen immune barrier degradation.

Prospects for further research. The results of this study suggest promising research direction that is essential for deepening understanding of the mechanisms underlying immune system impairment under chronic xenobiotic exposure. Furtehr translational studies are necessary to evaluate the clinical relevance of these findings in humans, especially in populations with high processed food consumption (China, South Korea, USA etc.). Identifying circulating biomarkers like MDA and nitrites could contribute to the early diagnosis and risk stratification of immune dysfunction associated with dietary factors.

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Conflict of interest. The authors have no conflicts of interest to declare.

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