Introduction

Colon cancer is one of the leading causes of cancer-related deaths worldwide [9, 15, 19, 27]. In the United States, this figure is 10% of all cancer deaths annually [9], and in Ukraine - 13.2% among men and 15.3% among women [29]. According to recent studies, various mechanisms contribute to the progression of colorectal cancer, including mutations in the cell cycle, apoptotic pathways, angiogenesis, invasion and metastasis, and the ability of tumor cells to avoid the immune response [3, 15, 27, 28].

Bcl-2 family proteins perform various critical functions during the body development and functioning [3, 14]. In accordance with numerous studies, it has been demonstrated that in cancer, the regulation of genes that encode proteins of the Bcl-2 family changes [11, 21]. Their overexpression inhibits cell death caused by various biological stimuli, particularly if such a stimulus is an
antitumor cytotoxic drug. In account of this Bcl-2 family proteins can be a potential target in cancer diagnosis and have a prognostic value in treating cancer with chemotherapeutic drugs [3, 11, 21].

Spleen is the largest peripheral organ of hematopoiesis and the immune system [4, 2, 16]. L. M. Sun et al. [24] described that people with splenectomies have a higher risk of developing cancer, especially of non-traumatic origin. Animal studies displayed that the role of spleenectomy in the progression of non-small cell lung cancer depended on the time of spleenectomy [28]. The tumor microenvironment includes myeloid suppressor cells, tumor-associated neutrophils, and tumor-associated macrophages that promote cancer progression and are generated from splenic hematopoietic stem cells and progenitor cells [13].

Many factors cause obstacles to the development of effective cancer treatment. In particular, one of the main problems is the effective delivery of drugs to the tumor and the insufficient selectivity of chemotherapy for tumor cells [6, 30]. Advances in nanotechnology have led to the development of various nanomaterials for therapeutic and diagnostic purposes [1, 6]. There are already more than eight hundred different products made based on nanotechnology. Among them are polymer micelles, liposomes, inorganic nanoparticles, etc. These nanomaterials have many unique advantages over traditional anticancer treatments [1, 5]. One of the first nanoparticles known for a long time are nanometals and nanoclusters formed by them, in particular nanoparticles of iron, gold and silver [18, 23, 26]. Despite the growing number of studies on nanoparticles of different metals, there is still no reliable information on their ability to work together as a composition.

Therefore, our work aimed to investigate the histological changes and expression of Bcl-2 family proteins in the spleen of rats with N,N-dimethylhydrazine-induced colon adenocarcinoma followed by protective administration of Au/Ag/Fe nanometals.

**Material and methods**

**Animals**

The research was accomplished on 72 white outbred male rats with a body weight 190±5 g. The animals had been retained in standard conditions of vivarium. Body weights and survival had been supervised all through. Experimental animals had unfastened access to drinking water and basal food regimen ad libitum. All manipulations with animals throughout this experiment have been conformed according to internationally accepted requirements and accredited by the Bioethical Committee of Ternopil National Medical University (protocol № 75, 01.11.2023). All experiments were carried out in accordance with the requirements of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Different Medical Functions" [7].

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**Colorectal cancer model**

N,N-dimethylhydrazine-induced (DMH-induced) colon adenocarcinoma was modelled by introducing N,N-dimethylhydrazine hydrochloride (Sigma-Aldrich Chemie, Japan, series D161802) dissolved in an isotonic sodium chloride solution. The chemical carcinogen was administered subcutaneously into the interscapular area once per week for a duration of 30 weeks, with a single dose of 7.2 mg/kg of body weight (primarily based on the active substance). Animals in the control group received 0.1 ml of physiological saline following the same frequency and procedure. At 30 weeks of N,N-dimethylhydrazine hydrochloride administration, colon adenocarcinoma in situ was histologically diagnosed in all rats.

**Nanoparticle's dosage and administration**

In this study, the composition of spherical silver (d=30 nm), gold (d=30 nm) and iron (d=40 nm) nanoparticles with a concentration per 1 ml: 1.6 mg Ag; 0.1 mg Fe; 3.088 μg Au was used. Silver nanoparticles’ initial water dispersion was synthesized by the tannin (tannic acid) reduction of silver nitrate (AgNO₃) in the presence of potassium carbonate (K₂CO₃). The synthesis of gold nanoparticles was performed via reduction of the tetra chloroauric (III) acid (HAuCl₄) by sodium citrate tribasic dehydrate in the presence of potassium carbonate. Iron nanoparticles were synthesized via reduction of iron (III) chloride by sodium borohydride (NaBH₄). The nanoparticle Au/Ag/Fe composition for this research was acquired by the mechanical mixing of the silver, gold and iron nanoparticle water dispersions. Metal nanoparticles used to receive the experimental mixture, as well as the obtained composition, was characterized as safe according to the criteria of cytotoxicity (MTT-test), genotoxicity (comet assay), mutagenicity (Allium-test) and immunotoxicity under in vitro tests [22].

Animals received nanoparticle Au/Ag/Fe water dispersion intragastrically once a day for 21 days at a dose of 0.842 mg Ag/0.0526 mg Fe/1.625 μg Au per 1 kg of rat body weight. The initial nanoparticle Au/Ag/Fe mixture was diluted with sterile distilled water at a ratio of 1:10 before the intragastric administration.

**Histological and immunohistochemical study**

Splenic tissue was collected from animals and fixed...
overnight in 10% neutral buffered formalin (Biognost, Croatia). The tissue was processed in a histoprocessor LOGOSone (Milestone, Italy). For histological analysis, spleen paraffin sections (5 μm thickness) were made with manual rotary microtome AMR 400 (Amos Scientific, Australia), stained with Hematoxylin and Eosin (H&E) (Biognost, Croatia), and analyzed with a light microscope Nikon Eclipse Ci (Nikon, Japan), and fixing photos with a digital camera Sigeta M3CMOS 14000.

Immunohistochemical analysis was performed on thin formalin paraffin-embedded sections of the rat spleen. The sections were stained with a rabbit monoclonal antibody against Bcl-2 (Cat. № ab32124). Bcl-2 detection was accomplished using a Mouse/Rabbit PolyVue Plus HRP/DAB Detection System (Diagnostic Biosystems). The sections were then counterstained with Hematoxylin M (Biognost, Croatia) and mounted under coverslips.

**Results**

Histological studies of the spleen after 30 weeks of exposure to N,N-dimethylhydrazine established destructive and degenerative changes in it. Disorders of blood circulation in the organ with uneven blood supply of red and white pulp was detected. In some fields of view, vessels were found without blood-formed elements; in others, they were filled with erythrocytes, indicating stasis and, in some places, thrombosis. Microscopically, macrophages with accumulation of hemosiderin in the cytoplasm were noted. During this observation period, the reduction of lymphatic nodules and the loss of their zonality are pronounced. The lumen of the central artery is narrowed, and its wall is thickened. In the red pulp’s stroma, the thickening of trabeculae, growth of the population of fibroblastic cells and, consequently collagen formation were noticed. A particularly noticeable location of the concentration of the fibrous component was the perivascular spaces around arteries and capillaries. These changes indicate sclerotic processes in the organ (Fig. 1).

Immunohistochemical study of Bcl-2 expression in the rat’s spleen with DMH-induced colorectal cancer at the 30th week of the experiment revealed an intense positive reaction in the cytoplasm of cells of the spleen’s white and red pulp. The development of chronic neoplastic endotoxemic syndrome and destructive-degenerative changes in vascular beds and lymphatic nodules are accompanied by an increase in the number of Bcl-2 cells in the red and white spleen pulp, which indicates a malfunction of the organ, and that can significantly affect both the progression of the tumour process and its subsequent correction.

The use of the Ag/Au/Fe nanometals composition showed a positive effect on the histological structure of the spleen components and the expression of the Bcl-2 in the organ.

At the microscopic level, it was found that the use of nanometals Ag/Au/Fe in DMH-induced carcinogenesis lead to the less pronounced morphological manifestations of structural changes in the spleen compared with the N,N-dimethylhydrazine-only group. Lymphatic nodules of the white pulp contained an enlarged germinal center, represented by lymphoblasts with normochromic nuclei. Hemosiderophages were observed in some places, which may indicate the presence of hemorrhages in this area in the previous stages of the experiment. Lymphocytes in the periarterial pulp were compact and their nuclei were hyperchromic. The number of lymphocytes and macrophages in the marginal areas and periarteriolar lymphoid sheaths was moderate. The mantle and marginal areas were expanded. The latter often came into contact with neighboring follicles. At the same time, the manifestations of hemodynamic disorders persisted, namely the plethora of red pulp sinusoids (Fig. 2).
Under the conditions of nanoparticle administration, a moderately pronounced (++) positive reaction against Bcl-2 in the cytoplasm of cells of germinal centers and marginal zones of lymphatic nodules of white pulp and a pronounced (+++) cytoplasmic reaction in red pulp cells were detected immunohistochemically (Fig. 3).

To assess the biosafety of the composition of nanometals, we examined their effect on the histological state of the spleen of unaffected animals. Manifestations of the pathological changes in the organ were not detected. The structure of the red and white pulp corresponded to the normal histological structure of the organ (Fig. 4).

Immunohistochemical examination against Bcl-2 of the spleens of unaffected animals injected with nanoparticles revealed a moderately expressed (++) positive reaction in the cytoplasm of the cells of the germinal centers of lymphatic nodules of the white pulp and a pronounced (+++) cytoplasmic reaction in their marginal zones in the cells of the red pulp (Fig. 5).

**Discussion**

Our research aims to elucidate histopathological changes and detection of Bcl-2 expression in the rat’s spleen during DMH-induced oncogenesis and the corrective effect of Ag/Au/Fe nanometal compositions. The spleen is the largest secondary lymphoid organ that performs essential immune functions and participates in the formation of cells that create the tumor microenvironment [2, 4, 16, 24]. That is why the study of changes in the structure of this organ during carcinogenesis can have significant prognostic and immunotherapeutic value. The red pulp of the spleen is a depot of blood, particularly erythrocytes and platelets, as well as a depot of iron. Blood is filtered in the red pulp of the spleen, and in fetal and newborn rodents, it is the site of hematopoiesis [2, 4, 16]. It can be assumed that under the conditions of its destruction, there will be a decrease in the elimination of cellular debris and the ageing of erythrocytes, increasing general intoxication. The white pulp of the spleen, which surrounds the central arteries, includes a quarter of the body's lymphocytes and stimulates the immune response to antigens [4, 13, 28].

The results of microscopic studies after 30 weeks of N,N-dimethylhydrazine hydrochloride administration proved the presence of destructive-degenerative changes in the spleen with the phenomena of sclerosing of the organ and delymphatization of the white pulp. These data are consistent with the results of research by Caprezo N. O. et al. [12], observed the appearance of dystrophic zones in the spleen and damage to the nuclear apparatus of its...
cells when exposed to N,N-dimethylhydrazine hydrochloride. Research by Svitina H. M. et al. [25] demonstrated a decrease in the proliferative activity of splenocytes under the influence of N,N-dimethylhydrazine hydrochloride. According to the analysis conducted by Li B. et al [17], the spleen plays a dual role in cancer incidence and progression.

Detection of the expression of Bcl-2 family proteins is important in oncogenesis because anti- and pro-apoptotic mechanisms are among the main ones in cancer progression [11, 14, 21]. An increase in the expression of Bcl-2 by parenchymal cells of the spleen under the influence of N,N-dimethylhydrazine hydrochloride was established. These data are confirmed by other researchers, who testify to their increased expression in various types of cancer [20]. Abnormalities in the expression of anti-apoptotic or pro-apoptotic members of the BCL-2 protein family can promote tumor development and make malignant cells resistant to anticancer therapy [10].

When studying the effect of metal nanoparticles, the main studies are devoted to changes at the cellular level, while morphological data describing changes at the tissue level are generally insufficient [1, 18]. An open problem of nanotoxicology remains a large number of studies using nanoparticles of different sizes, shapes and compositions, the results of which are contradictory, unreliable and unregulated. Currently, the entire aspect of the long-term residence of nanoparticles in the human body remains unexplored [30]. Our choice of components of the Ag/Au/Fe nanocomposite was connected with the already proven effectiveness of each nanometal separately.

Since nanoparticles of Ag/Au/Fe metals have antimicrobial, antifungal, antiviral, catalytic, photocatalytic, and antioxidant properties [18, 23, 26], this allowing us to understand our positive results.

To adequately assess the effect of nanoparticles, a group of unaffected N,N-dimethylhydrazine hydrochloride experimental animals with a 21-day administration of Ag/Au/Fe nanoparticle composite was also selected. Our studies demonstrated the absence of pathological changes in the spleen of white rats of this group.

Conclusions

1. Induced carcinogenesis in the colon causes evident reactive changes in the spleen: remodeling of its structural elements, hemodynamic disorders, sclerosis, deterioration of the white pulp, and increased expression of the Bcl-2 gene by parenchymal cells.

2. The use of the composition of metal nanoparticles contributed to a significant improvement in the histological structure of the spleen: the balance of parenchymal ratios of red and white pulp was restored, as well as the zoning of lymphatic nodules, thrombi in the vessels of the microcirculatory bed were undiscovered. Immunohistochemically confirmed a decrease in the level of Bcl-2 expression by white pulp cells.

References


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Імуногістохімічно виявлено меншу виражену експресію Bcl-2 у білій пульпі селезінки тварин, яким проводили ін'екцію наночастинок металів Au/Ag/Fe після 30-тижневого випливу N,N-диметилгідразину гідрохлориду, порівняно з тваринами без корегуючого впливу композиції нанометалів.

Ключові слова: селезінка, колоректальний рак, N,N-диметилгідразину гідрохлорид, Bcl-2, гістопатологічні зміни.