# Can Au/Ag/Fe nanoparticle composition restore blood cell counts in terms of DMH-induced colon adenocarcinoma?

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## Abstract

Scientific interest in nanomedicine nowadays is constantly growing, and nanomaterials have found wide application in diagnosis and treatment of various diseases. The most promising seem to be metal nanoparticles (NP). Some of them are actively studied in separate and there are favorable results considering their ability to normalize blood cell counts, but their co-work as a composite is still not well known. This opens a great perspective for studying NP as the blood homeostasis corrector, which could help in developing treatment schemes for many threatening diseases followed by the blood cell count disorders, especially malignant tumors. As colorectal cancer is third most commonly diagnosed, this study was focused on evaluation of blood cell counts changes in rats with DMH-induced colon adenocarcinoma *in situ* along with the assessment of Au/Ag/Fe NP composition corrective effect. Colon adenocarcinoma was induced by introducing N,N-dimethylhydrazine hydrochloride during 30 weeks. After pathohistological verification of developing colon adenocarcinoma *in situ* in DMH-treated rats Au/Ag/Fe NP composition was administered during 3 weeks. Introducing NP to the DMH-injured rats lead to increasing RBC and HGB and decreasing pathologically high-leveled MCV, MCH and MCHC to normal references. Assessed NP composition normalized the neutrophils rate, LMR and gave us a PLT rate particularly the same, as in the control group animals. Taking into account the previously proven biosafety of gold, silver and iron NP on their own and as a result the predicted biosafety of their composition we can consider further amplification of preclinical study on this NP composition of high significance, as it might be possibly used as following therapy of non-metastatic forms of colon cancer.

**Keywords:** Experimental carcinogenesis, nanoparticles, blood cell count, rat.

## Introduction

The achievements of nanotechnology have significantly affected the development of nanomedicine and nanobiotechnology, where nanomaterials have found wide application

in the treatment and diagnosis of diseases of various etiologies, in biotechnological industries and sensory technologies[1-3]. The key property of the substance in the nanoscale range lays in increased pharmacological and biological activity. The most promising seem to be metal nanoparticles (NP), which can be used as vectors for targeted therapy in oncology and cardiology, antimicrobial drugs in medicine and veterinary medicine, components of immunobiological drugs (probiotics and vaccines), stimulants in biotechnological industries (for example, in the processes of lyophilization - rehydration of bacterial strains-producers). At the current stage of development of nanomedicine and nanobiotechnology, the creation of a bank of biosafe nanomaterials, in particular, metal NP, is especially relevant[4].

Metal NP possess high bioactivity and recently have become of great perspective for diagnosing and treating different ethiology diseases, especially cancer[5-9]. Among the studied NP of metals are recommended as biosafe: iron NP (INP) of size 77 nm, gold - 30 and 45 nm, silver - 30 nm[4]. Gold NP (GNP), in comparison with other metals, are characterized by unique physical, chemical, biological properties and functional activity[10-14]. The nanoparticle size and shape substantially define their properties[15-19].

High affinity to tumor cells, surface modification ability and special optical properties create the basis for effective usage of GNP as vectors for target antitumor drug delivery[20, 21], in cancer phototermal therapy[22-24], as contrasting agents in magnetic resonance and computer tomography[25, 26]. It was found, that GNP in form of water dispersions possess significant and specific size-dependent bioactivity *in vitro* and *in vivo*. Synthesized GNP *in vitro* expressed size-dependent modulation of $Na^+K^+$-ATP-ase activity in U937 tumor cells membranes. *In vivo* GNP introduced via i.v. injection have shown high affinity to tumor cells. These *in vitro* and *in vivo* results open great perspectives of using GNP in cancer treatment and diagnostics, although the size-dependent biological safety level of GNP concerning normal organs should be taken into account[27].

Drugs and diagnostics based on INP and their oxides are actively created. Among the areas in which INP are used, a special place is occupied by the development of drugs with antianemic action[28]. Recent studies have shown that after a ten-day course of oral administration of INP at a dose of 12 mg / kg / day in experimental animals, normalization of hemoglobin concentration, transferrin saturation and serum iron saturation was observed[29]. According to the data obtained, NSAIDs when administered orally to rats did not cause an increase in serum urea and creatinine compared to controls, which indicates their safety for the kidneys - one of the key organs - targets of toxic effects of nanomaterials[30]. The test substance NSAID was also characterized as safe for the kidneys and liver in terms of total bilirubin concentration and activity of LF, ALT and LDH[29]. The results of another study indicate a high antianemic activity of the substance of NP of zero-

valent iron in the experimental treatment of model iron deficiency anemia under intravenous administration. Of particular note is the fact that INP showed antianemic activity both in the conditionally therapeutic dose and in 1/10 of the conditionally therapeutic dose. This will contribute to the achievement of rapid therapeutic efficacy of the antianemic drug, its high therapeutic safety and prolonged preservation of the antianemic effect[28].

Despite the constantly growing number of recent research papers on various metal NP, it was not yet investigated, how they could work together as a composite. Promising results considering normalizing blood cell counts open a great opportunity for using NP as the blood homeostasis corrector, which could help in developing and widening treatment schemes for many threatening diseases that are followed and worsened by the blood cell count disorders, especially malignant tumors.

It was investigated, that due to many factors of a complicated cascade of tumor pathogenesis it is often accompained by anemia. In addition, growing evidences have emerged in recent years that inflammation may be the origin of many malignancies. Being third most often diagnosed malignant tumor in the world, colorectal cancer (CRC) represents a growing number of cancers associated with inflammation. Thus, CRC is characterized by infiltration of heterogeneous immune cells and peripheral hematologic profile disorder, which configure the complicated microenvironment affecting tumor development[31].

It some recent studies the prognostic impact of peripheral blood leukocyte in the context of intra-tumoral immune profile was investigated. Leukocytosiswas validated as a prognostic factor predicting survivals and tumor response to adjuvant chemotherapy in patients with colorectal cancer[32].

Given all mentioned above this study was devoted to exploring the changes of blood cell counts in rats with DMH-induced colon adenocarcinoma and evaluating the Au/Ag/Fe NP composition influence on these indicators.

## Materials and Methods

### Animals

The research was carried out on 160 white mature outbred male rats with body weight 190 ± 5 g. The experimental animals were kept in standard conditions of vivarium. Animal survival and body weights were monitored throughout. Rats were provided with free access to drinking water and basal diet *ad libitum*. All animal experiments of this study conformed to internationally accepted standards and were approved by the Bioethical Committee of Ternopil National Medical University. All manipulations with animals were performed according to the requirements of the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986)[33]. The rats

were randomly allocated into 4 groups: 1st – 80 control animals, 2nd – 80 animals with modeled colorectal adenocarcinoma *in situ*. Afterwards 30 of injured animals received NP Au/Ag/Fe intragastrically for 21 day (3rd group). 4th group – 10 control animals received NP Au/Ag/Fe in the same manner. At the end of the experimental period, colon adenocarcinoma *in situ*was histologically identified in all DMH-treated rats.

## Colorectal Cancer Model

N,N-dimethylhydrazine (DMH) is known as well-known and widely used model of chemically induced colon cancer in animals. It has several morphological and molecular characteristics with human sporadic CRC. DMH-induced colon adenocarcinoma was modeled by introducing N,N-dimethylhydrazine hydrochloride (Sigma-Aldrich Chemie, Japan, series D161802) dissolved in isotonic sodium chloride solution. The carcinogenic substance was subcutaneously injected into the interscapular region at a dose of 7.2 mg/kg body weight (based on active substance) once a week for 30 weeks. Animals of the control group obtained subcutaneous injections of 0.1 ml physiological saline with the above frequency to simulate the possible stress effects[34].

## NP dosage and administration

Composition of spherical silver (d=30 nm), gold (d=30 nm) and iron (d=40 nm) NP with a concentration in 1 ml: 1.6 mg Ag; 0.1mg Fe; 3.088 μg Au was used in the study. Initial water dispersion of the used silver NP was synthesized via reduction of silver nitrate (AgNO3) by tannin (tannic acid) at the presence of potassium carbonate (K2CO3); gold NP were synthesized via reduction of the tetrachloroauric (III) acid (HAuCl4 · 3H2O) (≥99.9% trace metals basis, Sigma-Aldrich) by sodium citrate tribasic dehydrate at the presence of potassium carbonate; iron NP were synthesized via reduction of iron (III) chloride by sodium borohydride. Composition of the NP NP Au/Ag/Fe used in the work was obtained via the mechanical mixture of the water dispersions of silver, gold and iron NP. Metal NP used to obtain the experimental composition as well as the received mixture were characterized as biosafe according to the criteria of genotoxicity (comet assay), cytotoxicity (MTT-test), mutagenicity (Allium-test) and immunotoxicity under in vitro tests.

Animals received water dispersion of NP Au/Ag/Fe intragastrically one time a day for 21 days at a dose 0.842 mg Ag/0.0526 mg Fe/ 1.625 μg Au per 1 kg of rats body weight. Before the intragastric administration initial water mixture of NP Au/Ag/Fe was diluted by sterile distilled water at a ratio 1:10.

## Blood samples collection and analyzation

Blood samples collecting was provided a day after the last DMH administration to DMH-only treated rats, three days after the last NP administration to the rats that underwent nanocorrection along with the control animals of the same age. Experimental animals were

deeply anesthetized with Thiopental (50 mg/kg, intraperitoneally, Arterium, NUA/3916/01/02) and sacrificed by cervical displacement and exsanguination. Blood samples were collected into EDTA and proceeded using the Yumizen H500 CT automatic hematology analyzer.

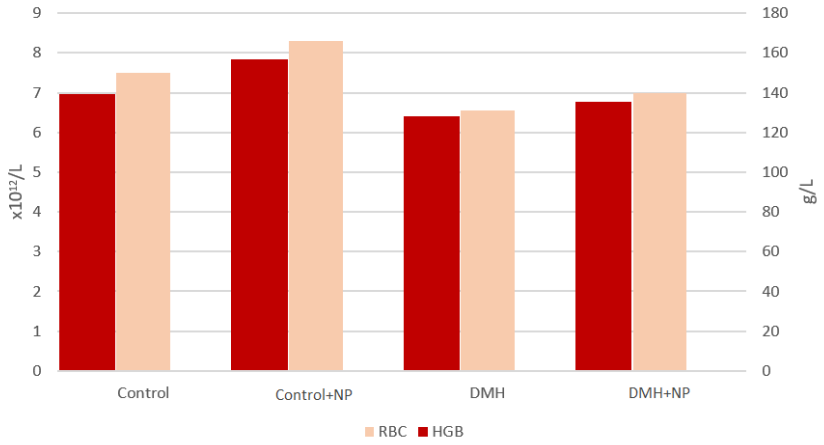
## Results and Discussion

To check the effect of introducing Au/Ag/Fe NP on the blood cell count in healthy animals and to exclude any negative influence of this combination, we administered it to the healthy animals, which obtained only saline. It was investigated that the RBC count and the HGB rate was credibly increased in the nano-treated rats (Pic. 1). It can be suggested that the nanoparticle composition was an additional source of iron that promoted heme synthesis and RBC production in a natural way. The group of animals that received only Au/Ag/Fe NP had a slightly higher (6.94 %) PLT number, than the control group rats. We have estimated an increase in WBC number with the administration of the NP, mainly due to the neutrophils fraction that in this group is 1.64 times higher than the control (Pic. 3). One more subgroup, which is significantly higher, is the eosinophils – 1.42 times higher than the control. This can be explained as a physiological reaction to the introduction of foreign substance, which were the Au/Ag/Fe NP.

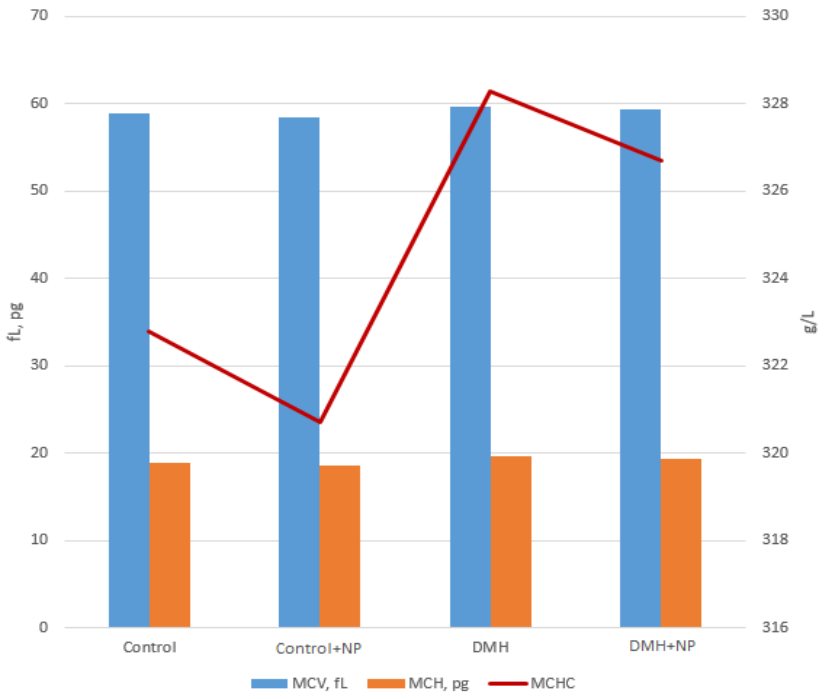
Modeling the CRC by introducing DMH after 30 weeks lead to the decrease of RBC and HGB. All three qualitative indicators – MCV, MCH and MCHC – slightly increased, probably as an adaptation reaction to the decreased number of RBC and general HGB rate (Pic. 2). Those animals that were treated with DMH had a credibly lower (59.4 %) level of PLT, comparing to the control. The WBC count, as well as its subgroups, has undergone significant changes. DMH treatment lead to the increase in WBC number mostly with the loss of lymphocytes. Thus, the WBC rate was 1.54 times lower in the DMH injured rats than the same indicator in the control group animals with the 1.73 times decrease in the number of lymphocytes (Pic. 3). In addition, the LIC count has tripled in comparison to the control group. NLR clearly shows the shift towards the neutrophils in DMH-injured animal group and LMR in the same group is 15.5 % higher comparing to the control (Pic. 4).

Nanoparticle correction helped in slightly increasing the erythrocyte number and returning the HGB rate almost to the level of control animals. Introducing NP to the DMH-injured animals lead to the decrease in MCV, MCH and MCHC, however, they did not return to the control group level (Pic. 2). Moreover, administration of NP gave us a PLT rate particularly the same, as in the control group animals. The Au/Ag/Fe NP correction has normalized the neutrophils rate. Moreover, the lymphocyte number is 26% higher in DMH+NP group rats, than in the DMH-only. However, the lymphocyte rate is still lower comparing to the control and the total WBC count in the group of DMH+ NP resumed 19 % lower, than in the control group animals. NLR remains increased even after the NP

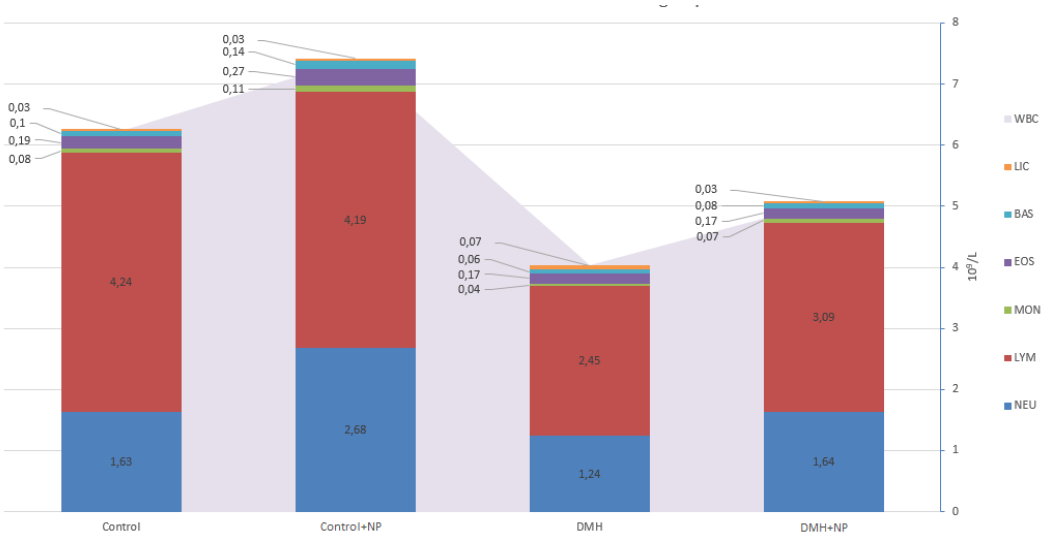
correction despite the normalization of the absolute number of the white cells (Pic. 3). On the other side, Au/Ag/Fe NP correction decreased the LMR and in this group it is even lower, than in the group of control animals (Pic. 4).



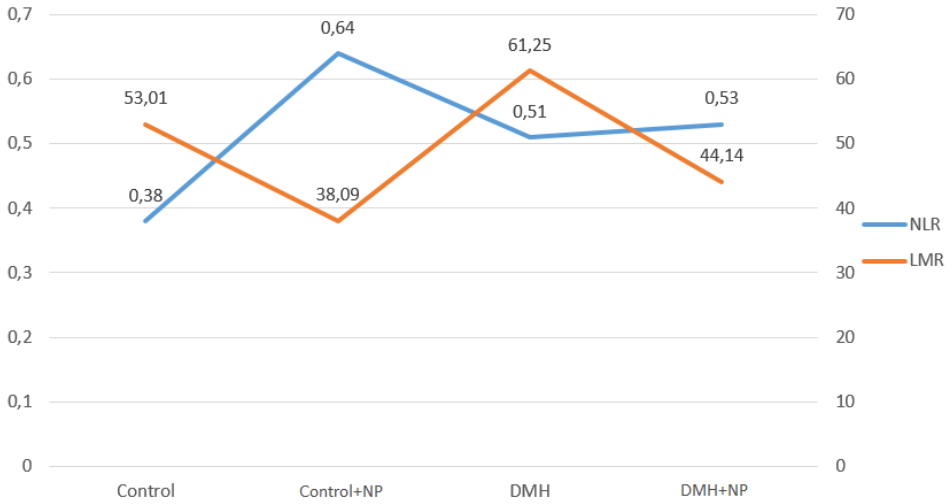

Pic. 1. RBC count and HGB rate in different experimental groups.




Pic. 2. MCV, MCH, MCHC in different experimental groups.




Pic. 3. Redistribution of WBC subgroups.




Pic. 4. NLR and LMR in different experimental groups.

Neutrophils are the most majority of peripheral leukocyte and react first to the sites of inflammation, playing an essential role in tumor development and progression. In fact, at the early stage of colorectal tumorogenesis, infiltration of neutrophils has been detected, as neutrophils infiltrated in colorectal adenomas much more than in adjacent normal mucosa and the count of neutrophils was positively correlated with the size of tumor. In addition, it was established that increased levels of these intra-tumor leukocytes correlated with poor prognosis through inducing metastasis. Neutrophils may be considered responsible as they are producing N-nitrosamines, known to be carcinogenic[31]. Additionally, activated neutrophils can induce replication errors in colon epithelial cells via hMSH2-dependent G2/M checkpoint arrest[35]. These findings are consistent with other observations, indicating leukocytes as an adverse prognostic factor in cancer progression. In addition, inflammation leading to immunosuppression provided a preferred niche for tumorogenesis. Besides the

external causes of inflammation in cancer patients like bacterial and viral infections, there are internal causes like cancer mutations through the malignant transition in way of inflammatory cytokines. Key mediators of inflammation-induced cytokines included NF-kappa B, STAT3 pathways, reactive oxygen/nitrogen species and prostaglandins[32,35].

Other recent findings demonstrated that in CRC patients who underwent curative resection preoperative MCV ≥ 80.5 fL, NLR ≥ 5.5, and LMR < 3.4 has been determined as predictors of poor RFS in II and III stage of the disease[36]. The volume of RBCs indicated by MCV and is often used for diagnosing megaloblastic or iron-deficiency anemia. Meanwhile, recent studies found a prognostic implication of MCV in esophageal and liver cancer[37]. It was previously reported that MCV was a prognostic factor for RFS in patients who underwent $R_0$ resection for stage I/II/III CRC, independent of the tumor stage[38]; microcytosis (MCV < 80 fL) was associated with better outcomes. It was reported that Hb and MCV lowered in short terms before diagnosing the CRC, and association of low MCV and CRC patients survival was weak[39]. CRC often plays along with iron-deficiency anemia that leads to decreased MCV. The mechanism responsible for the association between MCV and disease relapse is unknown, although several hypotheses have been proposed. The first is about oxidative stress, and the antioxidant capacity of the body had been related with the size of the circulating RBCs. Because an elevated MCV or macrocytosis may reflect structural or functional disorders of RBCs, a misbalance in antioxidant capacity can explain poor relapse outcomes after $R_0$ resection for CRC. In addition, affected RBCs deformability because of oxidative stress can damage the microcirculation and tissue oxygenation. On the other hand, macrocytosis may be a sign of disturbed hematopoesis due to the dysfunction of bone marrow. It was investigated that mesenchymal stem cells out of bone marrow play crucial role in the repair of several damaged vital organs[38, 39, 40]. A relatively high MCV is a marker of deficiency in folic acid or vitamin B12. Moreover, tumor location can be related with MCV. J.P. Väyrynen et al. (2018) reported that proximal tumor location was associated with predominant microcytic anemia[41]. T. Ueda et al. (2013) investigated the blood cell components in patients with acute decompensated heart failure and showed that the counts of WBC and PLT were credibly lower in the group with macrocytosis than in the non-macrocytic group[42].

It was shown that the NLR was the second important prognostic factor among the blood cell markers[36]. Inflammatory cytokines and mediators associated with carcinogenesis may stimulate inflammatory responses, which leads to tumor growth, infiltration, and formation of metastases. Lymphopenia can serve as a strong marker for misbalanced cell immunity, and neutrophilia is a response to systematic inflammation[43]. Lymphocytes participate in cytotoxic cell death and inhibition of tumor cell proliferation and migration[44]. The NLR had been reported to be an indicator of systemic inflammatory response in CRC. Many studies showed that a high NLR, with cutoff values ranging between 2 and 5, was

associated with poor long-term outcomes in patients with CRC[45-50].

Monocytes can be responsible for tumor progression and metastasis as well. Tumor-associated macrophages (TAM) that evolve from circulating monocytes possess ability to suppress adaptive immunity along with the angiogenesis, invasion, and migration promotion[47]. Increased level of circulating monocytes reflect the increased rate of TAM and is associated with worse prognosis. However, the level of circulating monocytes had not been widely used as a biomarker of CRC. The LMR is the ratio of lymphocyte to monocyte count in absolute number in blood. Recent studies have indicated that low LMR between 3.0 and 4.8 was associated with unfarorable long-term outcomes in CRC patients[45, 51, 52].

## Conclusion

In this research it was established, that introducing the nanoparticle Au/Ag/Fe composition increased RBC count and the HGB rate in only-nano treated rats, which is more likely because the nanoparticle composition was an additional source of iron that promoted heme synthesis and RBC production in a natural way. Also, this group of experimental animals developed an increase in eosinophil rate being a physiological reaction to the introduction of foreign substance, which were the Au/Ag/Fe NP. Introducing NP to the DMH-injured animals lead to increasing the erythrocyte number and HGB rate, as well as to decreasing in MCV, MCH and MCHC to normal references. Moreover, administration of NP has normalized the neutrophils rate, LMR and gave us a PLT rate particularly the same, as in the control group animals. Thus, introducing the nanoparticle Au/Ag/Fe composition lead to normalizing the blood cell homeostasis in experimental animals with modelled non-metastatic colon adenocarcinoma. Taking into account the previously proven biosafety of the NP of gold, silver and iron on their own and as a result the biosafety of their composition we can consider further amplification of preclinical study on this nanoparticle composition of high significance, as it might be possibly used in future in curation of non-metastatic forms of colon cancer.

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## შეიძლება თუ არა Au/ Ag /Fe ნანონაწილაკებმა აღადგინონ სისხლის უჯრედების რაოდენობა დიმეთილჰიდრაზინით გამოწვეული მსხვილი ნაწლავის ადენოკარცინომის შემთხვევაში?

ნატალია ლისნიჩუკი, სვიტლანა დიბკოვა, ლიუდმილა რიზნიჩენკო, ზორიანა ვივჩარი

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### აბსტრაქტი

ნანომედიცინის მიმართ სამეცნიერო ინტერესი მუდმივად მზარდია. დღეისათვის ნანომასალებს ფართო გამოყენება აქვთ სხვადასხვა დაავადებების მკურნალობასა და დიაგნოსტიკაში; როგორც ჩანს, ყველაზე პერსპექტიული ლითონის ნანონაწილაკებია (NP). ზოგიერთი მათგანი ცალკეა შესწავლილი და

მიღებულია იმედის მომცემი შედეგები, რომლის მიხედვითაც მათ აქვთ სისხლის უჯრედების რაოდენობის ნორმალიზების უნარი, თუმცა კომბინაციაში ჯერ კიდევ არ არიან შესწავლილი. ეს კი იძლევა ნანონაწილაკების შესწავლის პერსპექტივას, როგორც სისხლის ჰომეოსტაზის მარეგულირებელნი. მრავალი დაავადების დროს, რომლებიც სისხლის უჯრედების შემადგენლობის დარღვევებითაა გამოწვეული, შესაძლებელია მათი გამოყენება მკურნალობის სქემებში, განსაკუთრებით კი ავთვისებიანი სიმსივნეების დროს.

ვინაიდან, კოლორექტალური კიბო რიგით მესამე, ყველაზე ხშირად დიაგნოსტირებადი კიბოა, ჩვენ შევისწავლეთ ვირთაგვებში Au/Ag/Fe ნანონაწილაკების კომბინირებული ეფექტი სისხლის უჯრედების შემადგენლობაზე *in situ,* DMH-ით გამოწვეული მსხვილი ნაწლავის ადენოკარცინმის დროს. ნაწლავის ადენოკარცინომა გამოწვეული იყო დიმეთილჰიდრაზინის ჰიდროქლორიდის შეყვანით 30 კვირის განმავლობაში. როდესაც პათოჰისტოლოგიურად დადასტურდა DMH-ით გამოწვეული *in situ* მსხვილი ნაწლავის ადენოკარცინომა ვირთაგვებში, მოხდა Au/Ag/Fe ნანონაწილაკების კომპოზიციის ადმინისტრირება 3 (სამი) კვირის განმავლობაში. ამ ნანონაწილაკების კომბინირებულმა გამოყენებამ გამოიწვია HGB-ის და RBC უჯრედების გაზრდა, ხოლო პათოლოგიურად მაღალი დონის MCV-ის, MCH-ის და MCHC-ის შემცირება ნორმასთან მიმართებაში. აგრეთვე, მოახდინა ნეიტროფილების, LMR შემადგენლობის ნორმალი ზება, ხოლო PLT იყო იგივე, როგორც საკონტროლო ჯგუფის ცხოველებში. იმის გათვალისწინებით, რომ ადრე ჩატარებულ კვლევებში ოქროს, ვერცხლის და რკინის ნანონაწილაკების დამოუკიდებლად გამოყენება იყო უსაფრთხო, მათი კომბინაციაც შეგვიძლია ჩავთვალოთ ბიოლოგიურად უსაფრთხოდ და მათი კომბინირებული გამოყენება შეიძლება განვიხილოთ როგორც მსხვილი ნაწლავის კიბოს არამეტასტაზური ფორმების თერაპიის საშუალება.

**საკვანძო სიტყვები:** სისხლის უჯრედების რაოდენობა, ნანონაწილაკები, ექსპერიმენტული კანცეროგენეზი, ვირთხა.

**Может ли композиция наночастиц Au / Ag / Fe восстанавливать количество клеток крови при аденокарциноме толстой кишки, вызванной DMH?**

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## Абстракт

Научный интерес к наномедицине в настоящее время постоянно растет, и наноматериалы нашли широкое применение в диагностике и лечении различных заболеваний. Наиболее перспективными представляются наночастицы металлов (НЧ). Некоторые из них активно изучаются по отдельности, и есть положительные результаты, учитывая их способность нормализовать количество клеток крови, но их совместная работа в качестве составной части все еще недостаточно известна. Это открывает большие перспективы для изучения НП в качестве корректора гомеостаза крови, что может помочь в разработке схем лечения многих угрожающих заболеваний, за которыми следуют нарушения подсчета клеток крови, особенно злокачественных опухолей. Поскольку колоректальный рак является третьим по частоте диагностированием, это исследование было сосредоточено на оценке изменений количества клеток крови у крыс с DMH-индуцированной аденокарциномой толстой кишки in situ, а также на оценке корректирующего эффекта состава Au / Ag / Fe NP. Аденокарциному толстой кишки вызывали введением N, N-диметилгидразина гидрохлорида в течение 30 недель. После патогистологического подтверждения развития аденокарциномы толстой кишки in situ у крыс, получавших ДМГ, в течение 3 недель вводили композицию Au / Ag / Fe NP. Введение NP крысам с DMH приводит к увеличению RBC и HGB и снижению патологически высокоуровневых MCV, MCH и MCHC до нормальных значений. Оцененный состав NP нормализовал уровень нейтрофилов, LMR и дал нам частоту PLT, в частности, такую же, как у животных контрольной группы. Принимая во внимание ранее доказанную биобезопасность НЧ золота, серебра и железа как таковую, и, как результат, прогнозируемую биобезопасность их состава, мы можем рассматривать дальнейшее усиление доклинических исследований этого состава НЧ, имеющее большое значение, поскольку оно может быть использовано в качестве после терапии неметастатических форм рака толстой кишки.

**Ключевые слова:** экспериментальный канцерогенез, наночастицы, количество клеток крови, крыса.