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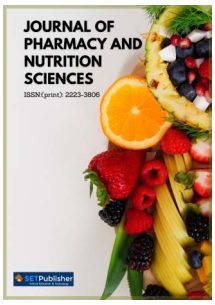

Gadolinium Orthovanadate $GdVO_4:Eu^{3+}$ Nanoparticles Ameliorate Carrageenan-Induced Intestinal Inflammation

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Abstract:

Gadolinium orthovanadate $GdVO_4:Eu^{3+}$ nanoparticles (VNPs) have been shown to scavenge reactive oxygen species (ROS), making them a promising therapeutic agent in inflammation.

This study aims to assess the effects of VNPs administered orally on E407a-induced inflammation.

Materials and Methods: Fragments of the small intestine of 8 rats treated orally with a carrageenan-containing food additive E407a at a dose of 140 mg / kg of weight during 2 weeks, 8 animals orally exposed to both E407a and VNPs at a dose of 20 μg / kg of weight during the same period of time, and 8 control rats were stained routinely and immunostained for CD3 and CD68 with the subsequent immunohistochemical scoring. Moreover, analysis of viability and cell death modes of granulocytes was performed by flow cytometry using Annexin V and 7-aminoactinomycin D (7-AAD).

Results: Oral exposure to the food additive E407a resulted in the development of enteritis associated with altered small intestinal morphology, infiltration of the lamina propria with macrophages and T-lymphocytes, and activation of peripheral blood granulocyte apoptosis. VNPs administered against the background of E407a-induced slight intestinal inflammation improved small intestinal morphology, decreased infiltration rate of the immune cells mentioned above without affecting the intensity of granulocyte apoptosis.

Conclusion: Oral administration of VNPs ameliorates E407a-induced enteritis.

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INTRODUCTION

Nanotechnology has become a promising field of research due to the unique physical, chemical, and biological properties of nanomaterials (NMs) observed solely at the nanoscale level [1]. NMs are defined as materials whose at least one dimension does not exceed 100 nm [2]. According to Jeevanandam *et al*., NMs can be classified into carbon-based, inorganic-based, including metal and metal oxides, organic-based, and composite-based NMs [3]. Properties of many inorganic nanoparticles (NPs) are well characterized, and they are increasingly used for medical purposes, including drug delivery, bioimaging, and biosensing [4-6]. Furthermore, there is evidence that inorganic metal-containing NPs have broad-spectrum antibacterial properties, anti-proliferative, cytotoxic, and, thus, anticancer effects [7, 8]. In addition, NP-based strategies have been investigated to treat inflammatory diseases [9-11]. Redox properties of NPs such as cerium oxide and selenium particles have been reported to underlie their anti-inflammatory effects, acting as so-called *electron sponges* [12, 13]. Europium-doped gadolinium orthovanadate nanoparticles $GdVO_4:Eu^{3+}$ (VNPs) have been demonstrated to scavenge free radicals acting as antioxidants [14]. Besides data concerning their antioxidant properties obtained *in vitro*, animal experiments have shown that VNPs can increase life expectancy in rats [15].

In order to be successfully tested and applied in the clinic, NPs have to be biocompatible and non-toxic. It is important to note that the toxicity of NPs depends not only on their size, structure, and physical properties, but also on the modifications, which NPs undergo in the body. Inorganic NPs have been demonstrated to possess several mechanisms for intracellular toxicity: disruption of cell membranes, reactive oxygen species (ROS) generation, induction of pro-inflammatory cytokines, corrosion of metal-containing NPs in the acidic medium of lysosomes (the so-called “lysosome – enhanced Trojan horse effect hypothesis”), induction of cell death such as apoptosis and autophagy, and others [16-19]. VNPs at the dose used in this study are non-toxic [20]. Thus, taking into account their antioxidant properties and relative safety, VNPs can be assessed for their possible application as anti-inflammatory agents.

This research aims to evaluate the ability of VNPs to affect experimental intestinal inflammation.

MATERIALS AND METHODS

Design

Twenty-four female WAG rats that were 4 to 5 months old, weighing up to 190g were chosen. They were kept in standard laboratory conditions in the local vivarium. The average temperature was 24 ± 2°C, while the relative humidity was 60 ± 5%. Animals were provided with food pellets and drinking water *ad libitum*. All rats were subdivided into three equal groups (n=8). Animals from group A obtained daily a k-carrageenan-containing solution of processed *Eucheuma* seaweed (PES) in drinking water (140 mg/kg of weight) orally during a fortnight. Besides carrageenan, PES is characterized by the presence of approximately 15% of algal cellulose. Rats from group B were treated with a water colloidal solution of VNPs at a dose of 20 μg/kg of weight for 14 days against the background of PES consumption. The control group (group C) consisted of intact rats administered the same volume of drinking water.

The solution of VNPs was prepared according to the method reported earlier [21]. Initially, 10 mL of water solution of rare-earth chlorides (0.01 mol/L) was added to 8 mL of ethylenediaminetetraacetic acid disodium salt (EDTA 2 Na) solution (0.01 mol/L). Then 8 mL Na_3VO_4 (0.01 mol/L) was added to the mixture dropwise (pH=13). After stirring using a magnetic stirrer, a transparent colloidal solution was obtained. Then the mixture was cooled and dialyzed against H_20 for 24 h to remove excessive ions using a membrane with a molecular weight cutoff. Its molecular weight was 12 kDa, whereas the pore size reached 2.5 nm. As a result, spindle-like nanoparticles with the chemical structure of $Gd_{(0,9)}$ $Eu_{(0,1)}VO_4$ and the average size of 8x25 nm were produced. They were used in this study.

The Bioethics Committee of Kharkiv National Medical University approved the study. The cervical dislocation was used as a method for euthanasia. All manipulations were performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

Morphological and Immunohistochemical Methods

Samples of the small intestine were collected from animals of all three groups. Routine staining

procedures were used. Initially, small intestinal specimens were fixed in 10% buffered-neutral formalin. After dehydration in graded alcohol, they were embedded in paraffin and sectioned (4-5 µm) using a microtome. Micro-slides were stained with hematoxylin and eosin, Einarson's gallocyanin chrome alum, as well as with picric acid and acid fuchsin (van Gieson's stain) in accordance with standard histological procedures.

For immunostaining, paraffin-embedded sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked. Then the micro-slides were incubated with anti-rat CD3 and CD68 antibodies. For detection of the antigen-antibody complexes, UltraVision^{TM} Quanto Detection System HPR DAB (*Thermo Fischer Scientific*, USA) was used. A brown color indicated the positive staining.

$CD3^+$ and $CD68^+$ cells were counted in 1 mm^2 areas. Five such fields in the lamina propria were analyzed in each sample (400x). The total amounts of $CD3^+$ and $CD68^+$ cells, as well as $CD68^+$/ $CD68^-$ and $CD3^+$/ $CD3^-$ ratios were determined and compared between groups in order to assess the absolute and relative number of leukocyte types mentioned above [22]. Furthermore, a score varied from 0 to 5 was given based on the average number of either $CD3^+$ or $CD68^+$ cells in the areas of small intestinal lamina propria. The score from 0 to 5 corresponded to 0-2, 3-5, 6-8, 9-11, 12-14, and over 15 immunostained cells per 1 mm^2 area, respectively. Five fields were estimated in each sample [23].

Flow Cytometry Analysis of Viability and Cell Death Modes of Granulocytes

Blood samples were collected from all animals involved in this study. They were used to prepare leukocyte suspensions for flow cytometric analysis according to the lyse/wash protocol. Lysis of erythrocytes was performed by 1x PharmLyse solution (BD, USA). Then the content of tubes was washed twice with phosphate-buffered saline (PBS). Cell pellets were resuspended in 1ml of 1x annexin-binding buffer (BD, USA). Then 100 µl of leukocyte suspension in annexin-binding buffer was transferred to new polystyrene tubes for incubation with 5 µl of FITC Annexin V, 7-aminoactinomycin D (7-AAD, BD Pharmingen™, USA) and antibodies to CD45 (BD Pharmingen^{TM} APC-Cy^{TM}7 Mouse Anti-rat CD45, BD Biosciences, USA). The former can bind to phosphatidylserine (PS) molecules translocated to the outer leaflet of cell membranes in early apoptotic cells, while the latter is a DNA intercalator that cannot pass through intact cell membrane. However, late apoptotic and necrotic cells have their membrane integrity lost, allowing 7-AAD to enter the cell. After adding the FITC-labeled Annexin V and 7-AAD, solutions were gently vortexed and incubated for 15 minutes in the dark. Then 400 µl of 1x annexin-binding buffer was added to each sample, and suspensions were analyzed by a BD FACSCanto™ II flow cytometer (Becton Dickinson, USA) with the acquisition of 5,000 events in each sample.

BD FACSDiva™ software was used to assess the results. The region of granulocyte was gated (Figure **1**) in an SSC and FL6 (APC-Cy^{TM}7) dot plot. Figure **1** was generated using FlowJo™ (v10, BD Biosciences, USA). The percentages of viable (Annexin V^-, 7-AAD^-), early apoptotic (Annexin V^+, 7-AAD^-), late apoptotic/necrotic (Annexin V^+, 7-AAD^+), and dead necrotic (Annexin V^-, 7-AAD^+) granulocytes was analyzed in the corresponding region.

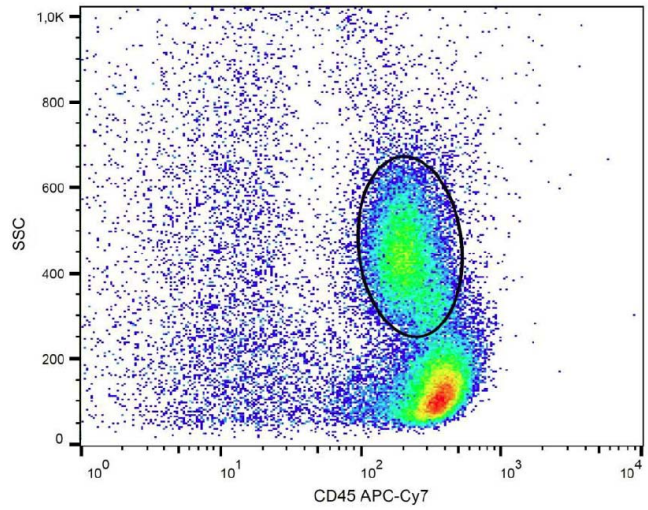

Figure 1: Representative SSC/FL6 (APC-Cy^{TM}7) dot plot that shows the gating strategy for identifying granulocytes among $CD45^+$-cells (i.e. leukocytes) is demonstrated.

Statistical Analysis

Three independent parameters were compared using a non-parametric Kruskal-Wallis ANOVA test. It was followed by a post-hoc Dunn's multiple comparison test. Data were presented as the median and interquartile range (IQR). When comparing the results of CD3 and CD68 immunostaining scoring, analysis of variance (ANOVA) was done with the subsequent application of Bonferroni post-hoc test. Numerical values were presented as the mean ± standard deviation. Differences were considered statistically significant at $p < 0.05$. Data obtained in this research

were analyzed by a GraphPad Prism 5.0 application (GraphPad software, USA).

RESULTS

In this study, we evaluated the effects of E407a on small intestinal morphology. In control animals, the morphological picture was typical for the intact small intestine (Figure **2** a1-a4). Morphological results indicated that administration of this food additive resulted in the development of inflammation. Rats from group A displayed altered small intestinal morphology, namely damaged villi with desquamated epithelial cells, avillous regions or areas with altered villous architecture, inflammatory cell infiltration in the lamina propria, more pink-colored collagen deposits (van Gieson's stain) in the intestinal mucosa compared with controls (Figure **2** b1-b4).

Supplementation of rats with VNPs improved intestinal morphology and relieved inflammation. The native architecture of villi and their epithelium in group B was better preserved than in animals from group A, and visually the infiltration was less pronounced. Evaluation of collagen fibers by van Gieson's stain revealed a decrease in the density of pink-stained deposits in the small intestinal mucosa of rats treated with VNPs compared to the untreated ones (Figure **2** c1-c4). It is important to note that both thick and thin intestinal villi were found among rats from group B. Furthermore, areas with no villi were revealed in this group of animals. However, their number was lower than in group A, i.e., rats obtained no VNPs against the background of E407a-induced enteritis (Figure **2** b2, c2).

It is worth mentioning that $CD68^+$ cells formed groups consisting of several cells in the lamina propria of rats from the control group, while $CD3^+$ cells were distributed more diffusely (Figure **3a**, **e**). It should be emphasized that regions with intact epithelia were characterized by no accumulation of T-lymphocytes in the subepithelial layer (Figure **3b**). This accumulation in rats from group B was observed only at the top of intestinal villi with substantial damage to the epithelial layer integrity (Figure **3c**, **d**). Similarly, macrophage accumulation was associated with damaged regions of villi (Figure **3f**). They were located in subepithelial and interepithelial regions (Figure **3g**, **h**). Positive staining for CD3 and CD68 was assessed quantitatively. The total amount of $CD3^+$ and $CD68^+$ in 1 mm^2 area of the lamina propria in the small intestine of rats was assessed. Moreover, $CD3^+/CD3^-$ and $CD68^+/CD68^-$

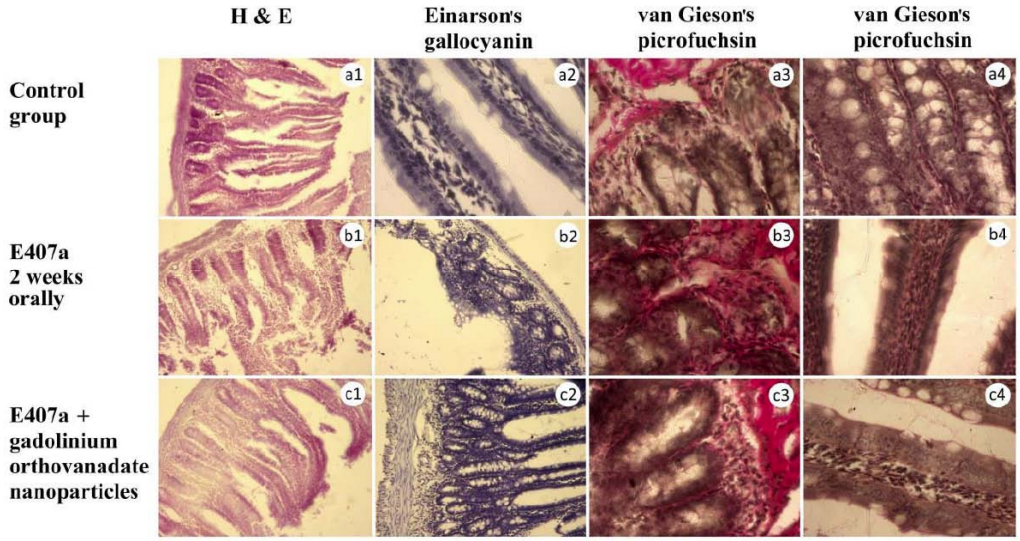

Figure 2: Microslides of the small intestine routinely stained with hematoxylin and eosin, Einarson's gallocyanin stain and van Gieson's picrofuchsin. Thin villi with the well preserved epithelial lining can be seen in the control group (a1). Epithelial cells contain high amounts of RNA. The number of cells in the lamina propria is not high (a2). Fuchsinophilic deposits are located in the submucosal layer of the small intestine, while solitary thin collagen fibers can be seen in the lamina propria (a3, a4). In rats orally exposed to E407a, small intestinal villi are damaged. Epithelial cells are desquamated. There is a lack of villi in some regions (b1). Areas of the lamina propria significantly infiltrated with leukocytes can be noticed (b2). More pronounced fuchsinophilic staining is found in rats treated with E407a. Fuchsinophilic masses can be noticed in the lamina propria. The lamina propria is broader and enriched with interstitial collagen (b3, b4). In animals exposed to E407a and treated with gadolinium orthovanadate nanoparticles, villi, including the epithelial layer, are better preserved compared with the rats treated with E407a only (c1, c2). Multiple goblet cells can be noticed in the epithelial layer. Fuchsinophilic deposits are accumulated in the submucosal layer and lamina propria. However, their amount is reduced compared with rats exposed to the food additive only. Villi are characterized by relatively low fuchsinophilic staining (c3, c4).

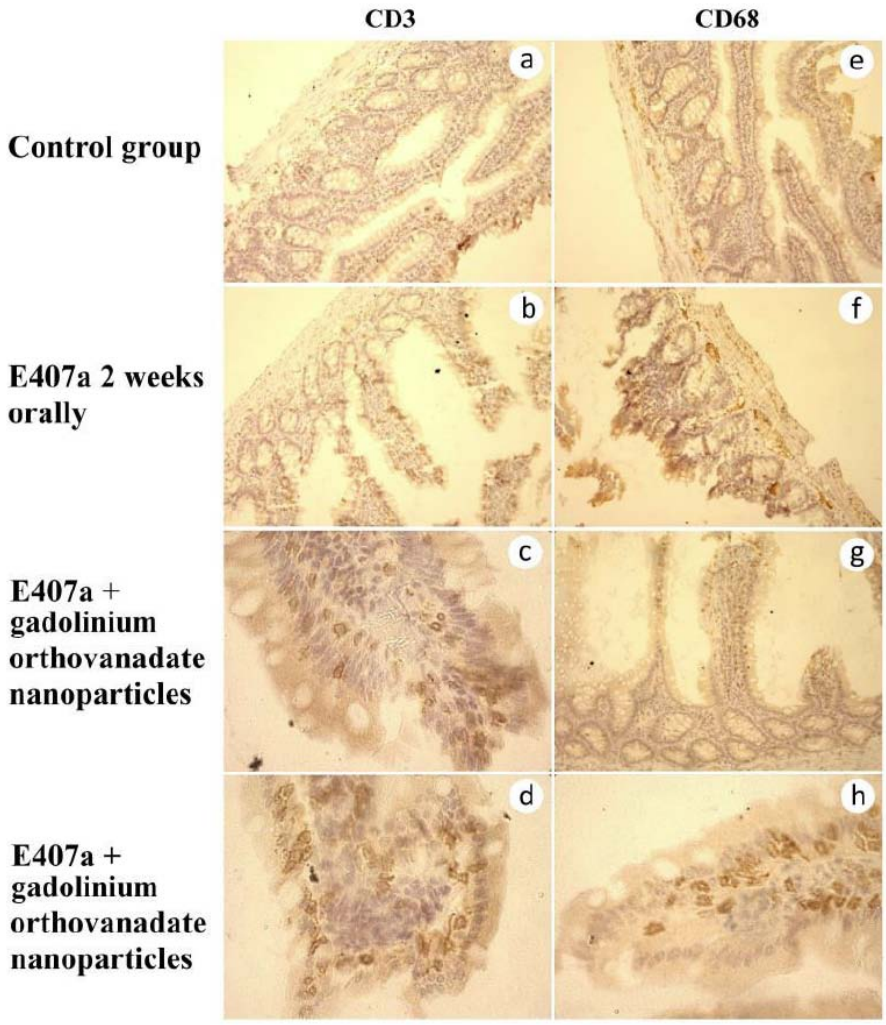

Figure 3: Immunostaining of the small intestine using antibodies to CD3 and CD68. Small amounts of $CD3^+$ cells are found in the lamina propria of villi in the control group. They are located diffusely (a). In the control group, the lamina propria in lower portions of the small intestinal mucosa contains some $CD68^+$ cells. Their amount in the lamina propria of villi is higher (e). In rats exposed to semi-refined carrageenan, more $CD3^+$ cells can be seen in the small intestinal lamina propria compared with controls. They are mainly accumulated in the subepithelial areas (b). In damaged mucosa of rats exposed to E407a, the regions with destroyed villi are characterized by strong staining indicating the presence of high amounts of $CD68^+$ cells prior to destruction (f). In rats treated with E407a and gadolinium orthovanadate nanoparticles, regions of damaged epithelia on the surface of villi are associated with migration of $CD3^+$ cells. (c, d, x400). In addition, villi, which are better preserved than in animals treated with E407a only, have $CD68^+$ cells accumulated in the subepithelial layer and between epithelial cells (g, h).

ratios were determined for a better comparison. Exposure to E407a was found to be associated with infiltration of the lamina propria with macrophages. Evaluation of CD68 expression scoring showed that rats treated with E407a had a higher absolute number of $CD68^+$ cells compared with controls (11.0±2.4 against 4.8±1.9). The difference was statistically significant ($p<0.0001$). The same observation was true for $CD3^+$ cells. The number of cells with positive immunoreaction for CD3 in rats exposed to the food additive was higher in the small intestinal lamina propria than in control animals (8.4±1.8 and 3.9±1.7, respectively, $p<0.0001$). Furthermore, rats from group A had higher values of both $CD3^+/CD3^-$ and $CD68^+/CD68^-$ ratios in comparison with animals from group C. In particular, the $CD3^+/CD3^-$ ratio in the small intestinal lamina propria of rats treated with PES only was 1.71-fold higher than in controls (0.36±0.12 against 0.21±0.10, $p<0.001$). As for CD68, the ratio was 2.21–fold elevated (0.53±0.10 and 0.24±0.08, $p<0.0001$) in group A compared with controls.

It is important to note that a statistically significant decrease in CD3 and CD68 expression, both in terms of the absolute number of positively stained cells and values of ratios, was found. The number of T-lymphocytes in group B was 5.9±1.4, which is statistically significantly different compared with group

A ($p<0.001$). The ratio was 0.28±0.08 ($p<0.001$ compared with group A). The number of macrophages in rats treated with E407a and VNPs was 7.4±2.3 ($p<0.001$). As for the ratio, it was 28% lower (0.38±0.15, $p<0.001$) compared with rats exposed to E407a only.

Analysis of immune cell marker scores revealed that the consumption of E407a was associated with a statistically significant increase in scores for both CD3 and CD68. The values for CD3 were 0.93±0.10 in the control group and 2.45±0.10 in the rodents exposed to E407a ($p<0.0001$), whereas these parameters were 1.30±0.10 and 3.35±0.14 ($p<0.0001$) for CD68, respectively. It should be noted that the application of VNPs reduced the values of CD3 and CD68 marker scores to 1.63±0.09 and 2.13±0.13, respectively. The difference compared with rats exposed to semi-refined carrageenan only was statistically significant in both cases ($p<0.0001$).

Annexin V FITC and 7-AAD staining aims at identifying four populations of cells after annexin V binding to PS on the outer leaflet of membranes observed in early apoptotic and late apoptotic/necrotic cells. Meanwhile, 7-AAD is used to distinguish viable and non-viable cells since the latter have the membrane integrity compromised and, in this event, the dye can penetrate the cells and bind to DNA.

The rats exposed to E407a had statistically significantly lower ($p<0.001$) amounts of viable annexin V^-, 7-AAD^- granulocytes compared with controls. The same was true for the animals exposed to both E407a and VNPs. However, the treatment of rats with VNPs did not affect this parameter in comparison with the animals administered E407a ($p>0.05$).

It is worth noting that exposure to E407a almost 8-fold ($p<0.001$) increased the percentage of early apoptotic annexin V^+, 7-AAD^- granulocytes compared with the control group. In rats administered E407a and VNPs, this index was also statistically significantly higher ($p<0.001$) than in controls. However, there was no difference in the amounts of annexin V^+, 7-AAD^- granulocytes between group A and group B ($p>0.05$).

The percentages of granulocytes double positively stained with annexin V-FITC and 7-AAD did not differ ($p>0.05$) in all three groups studied. Nor the consumption of E407a and E407a with VNPs led to statistically significant changes ($p>0.05$) in the percentages of dead necrotic annexin V^-, 7-AAD^+ granulocytes (Figure **4**).

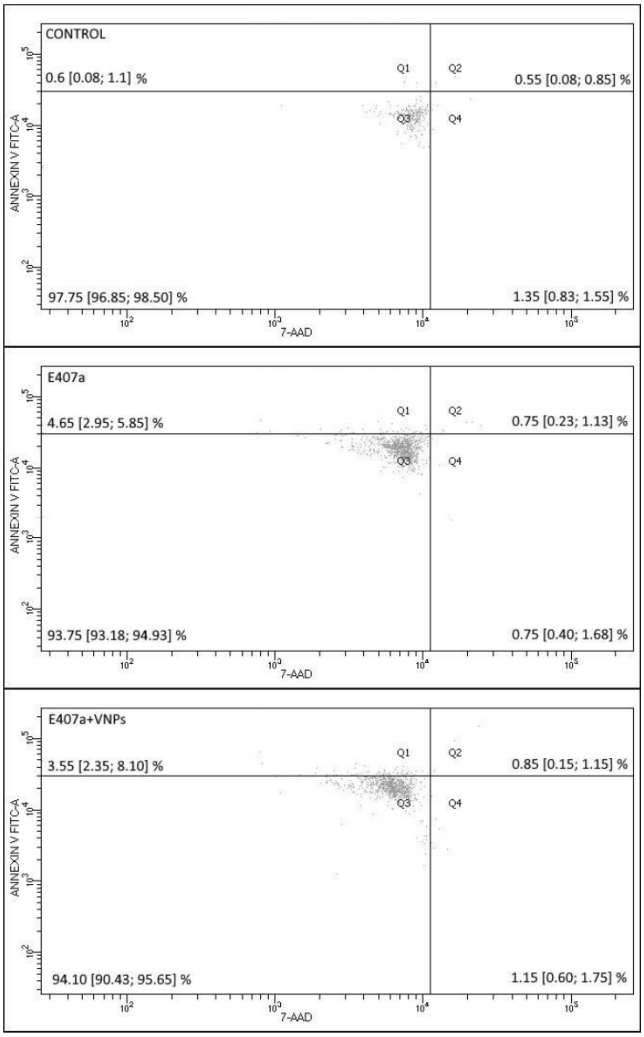

Figure 4: Representative profiles of Annexin V FITC/7-aminoactinomycin D (7-AAD) staining of granulocytes in a control animal (above), a rat exposed to E407a (in the middle) and an animal treated with both E407a and gadolinium orthovanadate nanoparticles (below). Oral exposure to E407a during 2 weeks resulted in a statistically significant decrease in the percentage of viable granulocytes against the background of an increase in early apoptotic cells ($p<0.001$). No statistically significant changes ($p>0.05$) in the amounts of viable, early apoptotic, late apoptotic/necrotic and dead necrotic granulocytes were observed between the rats administered E407a and the animals consumed E407a and nanoparticles.

DISCUSSION

The search for novel substances with anti-inflammatory action is a relevant interdisciplinary research direction [24, 25].

The safety of carrageenans, anionic sulfated polysaccharides of marine origin generally recognized as safe and registered as food additives E407 (food-grade carrageenan) and E407a (semi-refined carrageenan), has been debated for decades.

Numerous both *in vitro* and *in vivo* studies have revealed controversial results [26-33]. Our findings suggest that the carrageenan-containing food additive E407a, which is used in food products as a thickener and gelling agent and widely consumed by the population of developed countries, promotes small intestinal inflammation in rats upon oral exposure, evidenced by altered morphology, infiltration of the intestinal mucosa with macrophages and T-lymphocytes, and fibrosis development. This study corroborates the findings of other authors concerning the pro-inflammatory effects of carrageenans [26, 27, 31-33]. Furthermore, it has been reported that carrageenans cannot be absorbed in the intestine due to their resistance to digestive enzymes and high molecular weight [34, 35]. Thus, their adverse effects are supposed to be limited to the gut. However, data on the induction of granulocyte apoptosis in peripheral blood presented in this study indicate that carrageenans may exert a systemic action on the body. There is evidence that neutrophils and reactive oxygen species (ROS) generated by them play an important role in the development of experimental carrageenan-induced inflammation, in particular, peritonitis [36]. Moreover, we have demonstrated that food-grade carrageenan may enter neutrophils *in vitro* [37]. Therefore, we believe that intense apoptosis of granulocytes found in this study can develop due to excessive ROS generation induced by carrageenans since ROS accumulation is known to trigger apoptosis in neutrophils [38].

As reported herein, the oral administration of VNPs against the background of E407a-induced enteritis resulted in improving the small intestinal morphology and reducing infiltration of the stroma with immune cells. Structural changes in the tissue architecture and lower CD3 and CD68 scores in rats exposed to VNPs indicate amelioration of inflammation. As expected, VNPs were shown to be protective in intestinal inflammation. However, it is important to note that the beneficial effects of VNPs were limited to the intestine. In particular, administration of VNPs does not reduce granulocyte apoptosis, whose activation is observed in response to E407a consumption.

Furthermore, earlier, we demonstrated that VNPs did not improve serum cytokine profile in E407a-induced enteritis [39]. No changes in TNF-α, IL-1β, and IL-10 were revealed in rats treated with both E407a and VNPs and those exposed to E407a only. Thus, VNPs mainly act locally in the intestine without affecting systemic parameters such as granulocyte cell death and circulating levels of cytokines.

The anti-inflammatory action of VNPs can be ascribed to their ROS scavenging ability. Recently it has been revealed that $LnVO_4:Eu^{3+}$ (Ln= Gd, Y, La) NPs inhibit lipid oxidation caused by ROS in aqueous solutions. Moreover, the observed scavenging effect of $LnVO_4:Eu^{3+}$ NPs was comparable to that of the well-known antioxidant CeO_2 NPs [40]. The mechanism of ROS scavenging for $LnVO_4:Eu^{3+}$ NPs is considered to be associated with the features of their crystal structure, namely, with the presence of ions with changeable valence state V^{4+}/V^{5+} [41]. Electrons stored on V^{4+} ions can participate in ROS neutralization reactions resulting in a decrease in their amounts and preventing negative consequences of their action. Data presented herein are consistent with the results discussed. However, it is worth noting that the ROS scavenging ability of $LnVO_4:Eu^{3+}$ NPs is governed by the size of NPs, and consequently, the amount of V^{4+} in the crystal lattice [39]. A stronger ROS scavenging ability was revealed in small $GdYVO_4:Eu^{3+}$ particles (d = 2 nm). Thus, it can be suggested that small $GdVO_4:Eu^{3+}$ NPs should exhibit a more pronounced anti-inflammatory action. However, this hypothesis should be verified experimentally.

Taking all experimental results together, we can assume that VNPs have anti-inflammatory properties. However, it seems that further experimental research is necessary for confirming this conclusion and figuring out possible mechanisms of their anti-inflammatory action and adverse effects.

CONCLUSIONS

VNPs improve altered morphology of the inflamed small intestine and decrease the rate of macrophage and T-lymphocyte infiltration in mucosa ameliorating, thus, E407a-induced experimental inflammation in rats.

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TRANSPARENCY DECLARATION

Conflicts of Interest

None to declare.

AUTHORS' CONTRIBUTIONS

All authors have made substantial contributions to this paper. Conceptualization: Anton Tkachenko, Anatolii Onishchenko; Data analysis: Galina Gubina-Vakulyck, Anatolii Onishchenko, Svetlana Yefimova, Oksana Nakonechna; Original draft writing: Anton Tkachenko, Denys Pogozhykh, Svetlana Yefimova, Valeriy Myasoedov, Leonid Podrigalo, Tetyana Chumachenko; Experimental data acquisition: Volodymyr Prokopyuk, Anatolii Onishchenko, Oksana Nakonechna, Andrii Andrieiev, Nataliya Kavok, Hanna Polikarpova; Interpretation of data: Anton Tkachenko, Svetlana Yefimova, Valeriy Myasoedov, Anatolii Onishchenko, Oksana Nakonechna, Volodymyr Prokopyuk, Vladimir Klochkov, Nataliya Kavok; Statistical analysis: Dmytro Butov, Anatolii Onishchenko; Proofreading: Anatolii Onishchenko, Anton Tkachenko, Svetlana Yefimova, Vladimir Klochkov, Nataliya Kavok; Funding: Anton Tkachenko, Valeriy Myasoedov. All authors read and approved of the final manuscript.

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