Egypt. J. Vet. Sci. **Vol. 56, (Special issue),** pp. 725-733 (2025)

Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Immunomodulatory Effect of Chitosan Nanoparticles on Ehrlich Ascites Carcinoma Mice

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Abstract

ENHANCING the immune response while minimizing cancer-related damage is crucial for improving treatment outcomes in cancer therapy. We recently reported that chitosan nanoparticles (CNPs) could improve Sorafenib therapeutic potential and reduce EAC-triggered hepatic damage. This study aimed to investigate the immunomodulatory effects of CNPs on mice bearing Ehrlich ascites carcinoma (EAC) and evaluate their potential to alleviate EAC-induced spleen injury. A total of 21 female Swiss albino mice were randomly divided into 3 groups (7 mice per group): Group I (normal control) orally received PBS, Group II (EAC) was injected intraperitoneally with a single dose of 1 million EAC cells, and Group III (CNPs) consisted of EAC mice treated orally with CNPs from Day 4 to Day 14. Oxidative/antioxidant status was measured spectrophotometrically, CD4+ and CD8+ cell populations in the spleen were assessed by flow cytometry, and gene expression was analyzed through real-time polymerase chain reaction (qPCR). CNP treatment stimulated an immune response in EAC-bearing mice, as evidenced by increased globulin level, a higher count of CD4 and CD8 expressing cells, and enriched lymphocytes in the spleen. CNPs also restored oxidative balance, indicated by lower MDA levels and increased SOD, CAT, and GPx activities. Additionally, they reduced apoptosis (downregulation of *Bax* and upregulation of *Bcl2*), and decreased inflammation (downregulation of *IL1β* and *TNFα*) in the spleen. These findings suggest that CNPs not only restored spleen structure and function but also exhibited significant immunomodulatory effects in EAC-bearing mice.

Keywords: Ehrlich ascites carcinoma, chitosan nanoparticles, immunomodulatory effect, spleen.

Introduction

Ehrlich ascites carcinoma (EAC) is a widely used *in vivo* experimental model for studying tumor biology and testing anticancer and immunomodulatory potentials [1-4]. Although EAC cells are typically localized in the abdomen, they can migrate to remote organs causing their damage [3-6]. EAC, as a rapidly growing tumor model, also significantly affects the host's immune system by inducing immunosuppression and altering immune cell functions. This compromised immune system allows for unchecked tumor progression and metastasis, highlighting the importance of therapies that can restore immune balance in EAC-bearing hosts [7-9].

Chitosan is a biodegradable polymer made from chitin that has powerful anti-cancer, immune-boosting, and antibacterial characteristics [10]. Studies indicate that CNPs exert these effects by improving antioxidant status and decreasing oxidative stress [11,12]. Research has also shown that CNPs have anticancer properties against EAC [4,13-15]. CNPs have been shown to enhance host immunity by modulating both innate and adaptive immune responses. Due to their biocompatibility and immunostimulatory properties, CNPs can activate macrophages, increase cytokine production, and enhance the activity of antigen-presenting cells (APCs), thereby promoting a more robust immune response [16]. CNPs are also known to stimulate T-cell proliferation and improve the functionality of CD4+ and CD8+ T cells, which are critical for mounting an effective immune response against tumors [17]. Furthermore, CNPs have antioxidant

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(Received 28 October 2024, accepted 24 November 2024)
DOI: 10.21608/EJVS.2024.331998.2455

properties that reduce oxidative stress, which is often elevated in cancer patients, thus helping to restore the redox balance and protect immune cells from damage. By boosting immune surveillance and promoting apoptosis in tumor cells, CNPs help in counteracting tumor-induced immunosuppression, making them a promising adjuvant in cancer therapies [12].

We recently reported that CNPs could enhance the therapeutic efficacy of Sorafenib while reducing hepatic damage caused by both EAC and Sorafenib [4]. However, the immunomodulatory effects of CNPs on EAC have not yet been fully elucidated. Therefore, this study aimed to evaluate these immunomodulatory effects.

Material and Methods

Prior to commencing this experiment, ethical approval was obtained from the research ethics committee at Kafrelsheikh University in Egypt, under the license number KFSIACUC/154/2023. All procedures adhered to the standards outlined by ARRIVE.

Nanoparticle Preparation and Analysis

In order to create chitosan nanoparticles (CNPs), the powdered chitosan (Marine Hydrocolloids Company in Meron, India) was treated with sodium TPP according to the methods previously detailed [18]. The CNPs' size and shape were studied with a 100 kV JEOL transmission electron microscope (TEM, JEM-2100) as previously reported [4].

Experimental Design

The 21 female Swiss albino mice (measuring 19-24 g and 9-11 weeks old) were housed in a controlled environment with an unlimited supply of water and a baseline diet. Three groups of seven mice each were randomly assigned to the animals. Oral administration of 300 µl of phosphate buffer saline (PBS) was given to each mouse in Group I (Control). Group II (EAC) injected 1 million EAC cells intraperitoneally [3]. From Day 4 to Day 14 of the injection, EAC mice in Group III (CNPs) were given 300 µl of CNPs orally, at a dosage of 2.5 mg/kg body weight [4]. On day 15, the blood samples were collected, centrifuged at 3500 rpm for 10 min, and the collected serum was kept at -20 °C. Following euthanization by overdose of anaesthesia, spleens were removed, divided into four sections: the first was fixed in 10% formalin for histological examination, the second was lysed for flow cytometry, the third was homogenized for spectrophotometry, and the fourth was frozen at -70 °C for real-time qPCR analysis.

Biochemical Assays

Total protein and albumin levels were assessed using Spinreact kits (Spain) following the manufacturer's guidelines. The serum globulin level was determined by subtracting the measured serum albumin concentration from the total serum protein concentration [19]. To assess oxidative and antioxidant status, spleen homogenates were prepared according to the method of Mohamed, *et al.* [20]. The splenic levels of oxidant MDA and antioxidant enzymes (SOD, CAT, and GPx) activities were detected by commercial kits purchased from Biodiagnostics (Egypt). SOD levels were determined by measuring the inhibition of adrenochrome formation at a pH of 10.2, while CAT activity was assessed by monitoring the degradation of H_2O_2 at 240 nm as previously detailed [21,22].

Flow Cytometry

Splenocyte suspensions were prepared and counted. Cell viability in spleen samples was assessed using the trypan blue exclusion method, with a cell density adjusted to 1×10^6 cells/ml. The expression of anti-mouse CD4 mAb Fluorescein isothiocyanate (FITC)-labelled (clone: H129,19), and FITC-labelled anti-mouse CD8 mAb (clone: M3-6,7, Sigma, St Louis, MO, USA) surface markers was quantified and analyzed using flow cytometry FACScan flow cytometer (BD Biosciences, USA), and data were analyzed using FlowJo V.10 software (BD Biosciences).

Histopathological Examination

Following overnight fixation, spleen tissues were dehydrated using a graded series of ethanol. Afterward, they were cleared in xylene, embedded in paraffin wax, and sectioned into 5 µm slices using a microtome. The tissue sections were stained with hematoxylin and eosin (H&E) and examined under a microscope equipped with an automatic camera (Olympus, Japan) for analysis and imaging.

Gene Expression Analysis by qPCR

To analyze the relative expression levels of *Bax*, *Bcl2*, *IL1β* and *TNFα* in the spleen, quantitative PCR (qPCR) was performed. Total RNA was isolated using the Trizol reagent (Invitrogen, USA), followed by cDNA synthesis using reverse transcriptase containing Kit (Thermo Scientific, USA). PCR reaction (20 µl) was prepared with Syber Green (Thermo Scientific), cDNA, primers (listed in Table 1). The amplification conditions followed the manufacturer's instructions. Gene expression was normalized to *GAPDH* and calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were expressed as mean ± standard error mean (SEM) and analyzed using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Statistical significance was defined as $P < 0.05$. All analyses were performed using GraphPad Prism 8 software.

Results

Characterization of CNPs

TEM analysis revealed that CNPs were spherical in shape and exhibited a consistent, uniform size distribution. The diameters of the CNPs ranged from 160 to 300 nm, as depicted in Figure 1, confirming their structural homogeneity and suitability for experimental applications.

CNP Stimulated an Immune Response in EAC Mice

Serum levels of globulin were significantly reduced ($P < 0.05$) in untreated EAC mice compared to the control group (Table 2). However, CNPs treatment led to a significant ($P < 0.05$) improvement in globulin levels compared to the untreated EAC group. Histopathological analysis of spleen tissues from untreated EAC mice revealed significant lymphocyte depletion, accumulation of pleomorphic hyperchromatic EAC cells compared to the normal control group (Fig. 2). However, CNPs treatment restored lymphocyte levels in comparison to untreated mice. To further evaluate the immunological profile changes in the spleen, flow cytometry was used to assess CD4+ and CD8+ cell expression (Table 2). EAC-bearing mice had significantly ($P < 0.05$) reduced CD4+ and CD8+ cell counts in the spleen compared to normal control animals. Mice treated with CNPs had significantly ($P < 0.05$) higher CD4+ and CD8+ cell counts than the EAC mice.

Impact of CNPs on Redox Balance, Inflammation, and Apoptosis

EAC mice had a significant ($P<0.05$) increase in splenic MDA levels, along with a notable ($P<0.05$) reduction in SOD, CAT, and GPx activities compared to all other groups (Fig. 3). These changes were associated with a significant ($P<0.05$) upregulation of *IL1β*, *TNFα*, and *Bax* and a significant ($P<0.05$) downregulation *Bcl2* in the EAC group relative to other groups (Fig. 4). CNPs treatment restored these markers to levels comparable to the normal control group.

Discussion

This study was conducted to explore the effects of CNPs on the immune response, redox balance, inflammation, and apoptosis in EAC-bearing mice. We found that CNPs treatment positively influenced both immune and antioxidant responses in these mice, while also reducing inflammation and restoring normal apoptotic activity in spleen cells.

TEM confirmed that the CNPs synthesized in this study were spherical and uniform in size, ranging from 160 to 300 nm. The uniformity of CNP size is crucial for their functional application in biological systems, as nanoparticle size affects cellular uptake, biodistribution, and the release of active ingredients. Previous studies have shown that NPs within this size range are ideal for enhanced cellular internalization, improving drug delivery efficiency and therapeutic efficacy [12,23-25]. The results from TEM analysis suggest that the CNPs produced in this study are structurally consistent and suitable for further applications in cancer treatment.

In the present study, the EAC group exhibited significantly lower host's immune response as revealed by reduced globulin serum levels, a lower count of CD4 and CD8 expressing splenocytes, and depleted lymphocytes in the spleen. Consistent with our findings, previous studies have shown that EAC leads to a decrease in T-cell proliferation and impairs the function of various immune cells, including macrophages and natural killer cells [7,9]. On the other hand, our results demonstrate that CNPs treatment significantly improved immune function in EAC-bearing mice. Specifically, CNPs led to an increase in serum globulin levels, which had been significantly reduced in untreated EAC mice. Globulin levels serve as an indicator of immune status, as they are composed of various immunoglobulins that are vital for humoral immunity. This restoration in globulin levels following CNP treatment suggests that CNPs can enhance the immune response by potentially stimulating the production of antibodies or improving the immune system's capacity to respond to cancerous cells. Tumors, including EAC, are known to impair host immunity by inducing a state of chronic inflammation and promoting immune evasion [26]. CNP treatment, however, reversed this lymphocyte depletion, restoring immune competence in the spleen. Flow cytometry analysis revealed a significant increase in CD4+ and CD8+ T cells in the spleen of CNP-treated mice. CD4+ helper T cells and CD8+ cytotoxic T cells play essential roles in orchestrating and executing immune responses against tumor cells [8,27]. The restoration of these cell populations further supports the hypothesis that CNPs exert immunomodulatory effects that can help combat tumor-induced immune suppression. These findings are consistent with previous studies that have shown the ability of CNPs to modulate immune responses [16]. Chitosan and its NPs have been reported to activate macrophages, enhance antigen presentation, and stimulate cytokine release, all of

which contribute to an enhanced immune response [28]. Additionally, CNPs may function by protecting lymphocytes from oxidative damage, thus preserving their function in cancer-impaired immune environments [14,17].

Oxidative stress plays a pivotal role in cancer progression, as elevated reactive oxygen species (ROS) levels promote tumor growth and survival by damaging cellular components, inducing genetic mutations, and impairing immune function [29,30]. In this study, untreated EAC-bearing mice exhibited significantly elevated levels of the lipid peroxidation marker malondialdehyde (MDA) and decreased activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). This imbalance in the oxidative/antioxidant status is a hallmark of cancer-induced oxidative stress [3,31]. Cancer cells including EAC are known to induce oxidative stress, which exacerbates immune dysfunction by damaging immune cells and reducing the activity of antioxidant enzymes [4,8,9]. Similarly, our data revealed higher oxidation as evidenced by high MDA levels and low activities of SOD, CAT, and GPx. Excessive oxidative stress could induce apoptosis. Indeed, we also found high apoptosis in the spleen of the untreated EAC group, as revealed by upregulated *Bax* and downregulated *Bcl2* gene expression. In contrast, CNP treatment restored redox balance in the spleen by significantly reducing MDA levels and increasing the activity of SOD, CAT, and GPx, bringing them closer to control group levels. The antioxidant activity of CNPs can be attributed to their ability to scavenge free radicals and inhibit lipid peroxidation. Previous studies have demonstrated that CNPs can enhance the expression and activity of endogenous antioxidant enzymes, thereby protecting cells from oxidative damage [4,12,32,33]. By restoring antioxidant enzyme activity, CNPs may protect both immune cells and non-cancerous tissues from oxidative damage, potentially enhancing their ability to mount an effective immune response against tumor cells. Moreover, the antioxidant properties of CNPs have important implications for the prevention of oxidative stress-induced apoptosis. Indeed, we found significantly decreased expression of the apoptotic *Bax* gene and the increased expression of the anti-apoptotic *Bcl2* gene in the spleen of EAC mice treated with CNPs. This suggests that CNPs promote cell survival in immune cells by inhibiting apoptosis. This is in line with previous reports indicating that CNPs can inhibit apoptosis by reducing oxidative stress and modulating pro-apoptotic signaling pathways [4,12,18]. By restoring redox balance, CNPs may mitigate the oxidative damage that often leads to the dysfunction or death of immune cells in the tumor microenvironment. This reduction in oxidative stress likely contributed to the improved immune function observed in CNP-treated mice, as demonstrated by the restoration of lymphocyte levels in the spleen and the increase in CD4+ and CD8+ T cell populations.

EAC immunosuppressive effect is primarily attributed to the tumor's ability to create a pro-inflammatory environment that facilitates its survival and growth. Tumor cells secrete cytokines such as IL1β, TNFα and TGFβ, which inhibit the activation of cytotoxic T cells and promote regulatory T cell activity, further dampening the host's immune response [7]. In agreement we also found upregulation of *IL1β* and *TNFα* genes in the spleen of the untreated EAC mice. Chronic inflammation is a key factor in tumor development, promoting both tumor growth and metastasis [20,34]. In the present study, untreated EAC-bearing mice exhibited significantly elevated *IL1β* and *TNFα* expression. These cytokines are known to facilitate tumor progression by promoting an immunosuppressive tumor microenvironment and enhancing tumor cell survival [35]. CNP treatment, however, significantly reduced the expression of *IL1β* and *TNFα*, bringing their levels closer to those observed in healthy control mice. This suggests that CNPs possess anti-inflammatory properties that can help counteract the tumor-promoting effects of chronic inflammation. The anti-inflammatory and anti-apoptotic effects of CNPs are likely interrelated. Chronic inflammation in the tumor microenvironment can induce oxidative stress, which in turn promotes apoptosis in immune cells. By reducing both inflammation and oxidative stress, CNPs may protect immune cells from apoptosis, thereby enhancing immune function and improving the host's ability to fight the tumor.

The results of this study suggest that CNPs hold significant promise as an adjunct therapy for cancer treatment. Their ability to modulate immune responses, restore redox balance, reduce inflammation, and inhibit apoptosis in immune cells positions them as a potential candidate for enhancing the efficacy of existing cancer treatments. CNPs could be particularly useful in combination therapies, where they may enhance the immune system's capacity to recognize and eliminate tumor cells, while simultaneously protecting healthy tissues from the deleterious effects of oxidative stress and inflammation. Additionally, the ability of CNPs to modulate the tumor microenvironment is of great importance in the context of immunotherapy. Immunotherapies, such as checkpoint inhibitors, rely on a functional immune system to mount an effective response against tumors. By restoring immune function and reducing the immunosuppressive effects

of the tumor microenvironment, CNPs may enhance the efficacy of such therapies.

Conclusion

The present study demonstrates that CNPs exert a range of beneficial effects in EAC-bearing mice, including the stimulation of immune responses, restoration of antioxidant enzyme activity, reduction of inflammation, and inhibition of apoptosis. These findings suggest that CNPs may be a valuable therapeutic tool for enhancing cancer treatment by mitigating tumor-induced immunosuppression and oxidative stress. Further research is needed to explore the mechanisms underlying these effects and to determine the potential of CNPs as part of combination therapies for cancer.

Acknowledgement

Not applicable.

Funding statement

Authors did not receive any external funds.

Conflicts of Interest

The authors declare no conflict of interest.

Author`s contributions

Authors contribute equally in this work.

TABLE 1. Primers used in qPCR.

Gene	Forward	Reverse	Reference
Bax	GGCTGGACACTGGACTTCCT	GGTGAGGACTCCAGCCACAA	[36]
Bcl2	TTCGCAGAGATGTCCAGTCA	TTCAGAGACAGCCAGGAGAA	
TNFα	GACAAGGCTGCCCCGACTACG	CTTGGGGCAGGGGCTCTTGAC	[37]
IL1β	AAATCTCGCAGCAGCACATCAA	CCACGGGAAAGACACAGGTAGC	
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA	[1]

TABLE 2. Effect of CNPs on globulin serum levels and CD4 % and CD8 % in the spleen

Groups	Globulin (g/dl)	CD4 %	CD8 %
Cnt	3.36±0.15 [a]	23.13±0.52 [a]	21.22±0.48 [a]
EAC	2.05±0.08 [c]	11.00± 0.31 [c]	12.20±0.19 [c]
CNPs	2.70±0.11 [b]	16.81±0.37 [b]	13.50±0.14 [b]

Columns marked with different letters indicate statistically significant differences at the $P \leq 0.05$ level. Data are expressed as mean values ± SEM, with n = 7 for each group.

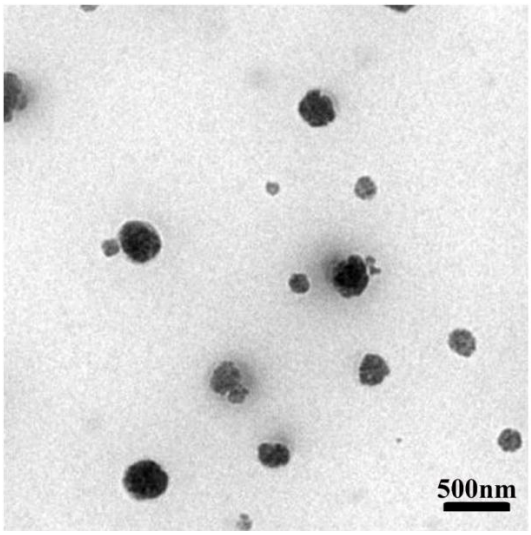

Fig. 1. Transmission electron microscopy (TEM) analysis reveals that the synthesized chitosan nanoparticles (CNPs) exhibit a size range of 160 to 300 nm.

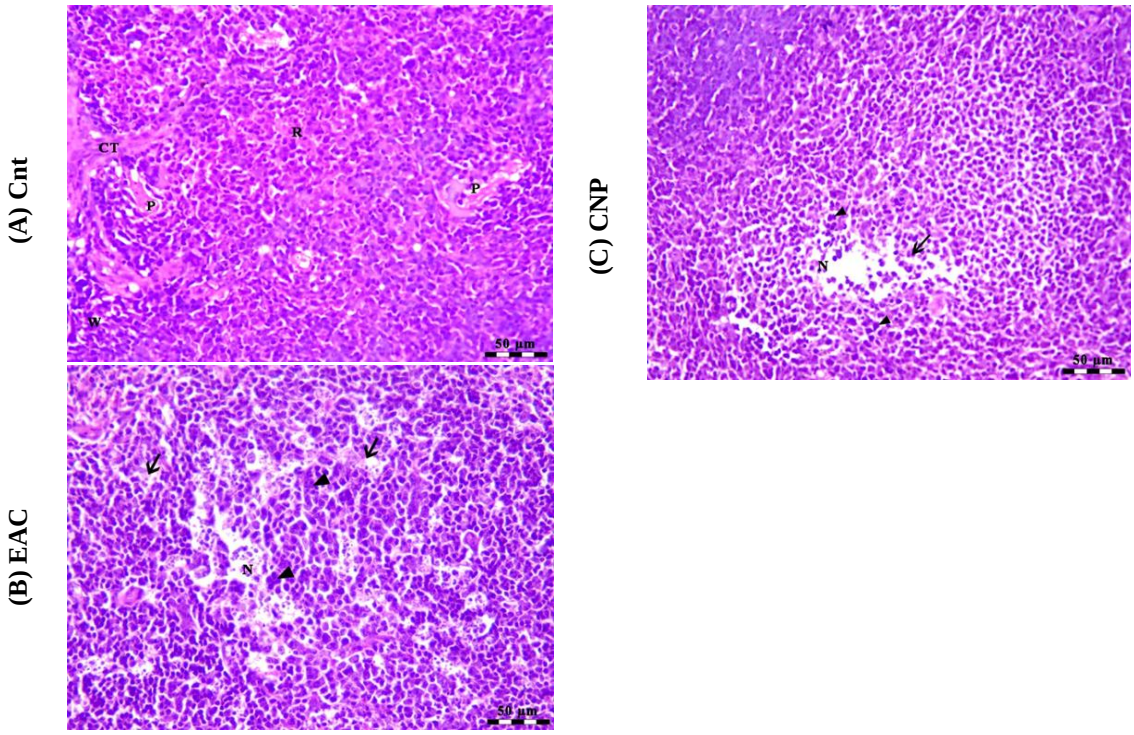

Fig. 2. Representative light micrograph showing spleen histopathology of EAC mice after treatment with CNPs. (A) Spleen of normal control (Cnt) showing mixed white (W) and red (R) pulp with peri-arterial lymph sheath (P) and connective tissue septa (CT). (B) Spleen of the EAC group showing accumulation of pleomorphic hyperchromatic EAC cells (arrowheads), necrosis (N), and depletion of white pulp cells (black arrow). (C) Spleen of the CNPs group showing sporadic pleomorphic hyperchromatic EAC cells (arrowheads), necrosis (N), and mild depletion of white pulp cells (black arrow). The sections were stained with H&E, Scale bar = 50 µm.

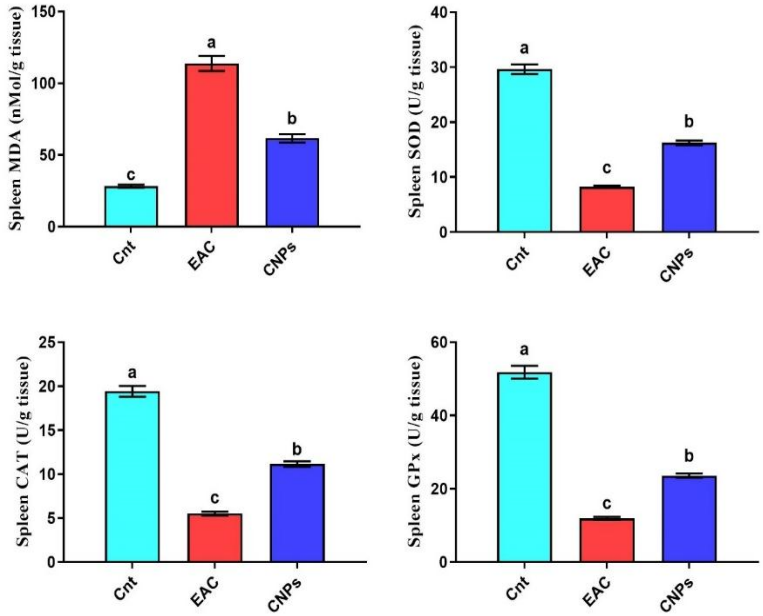

Fig. 3. Impact of CNPs on oxidative (MDA) and antioxidant (SOD, CAT, GPx) markers in the spleens. Data are shown as mean ± SEM (n = 7/group). Significant differences (P < 0.05) are indicated by different letters [a (highest) to c (lowest)] with comparisons made between all groups.

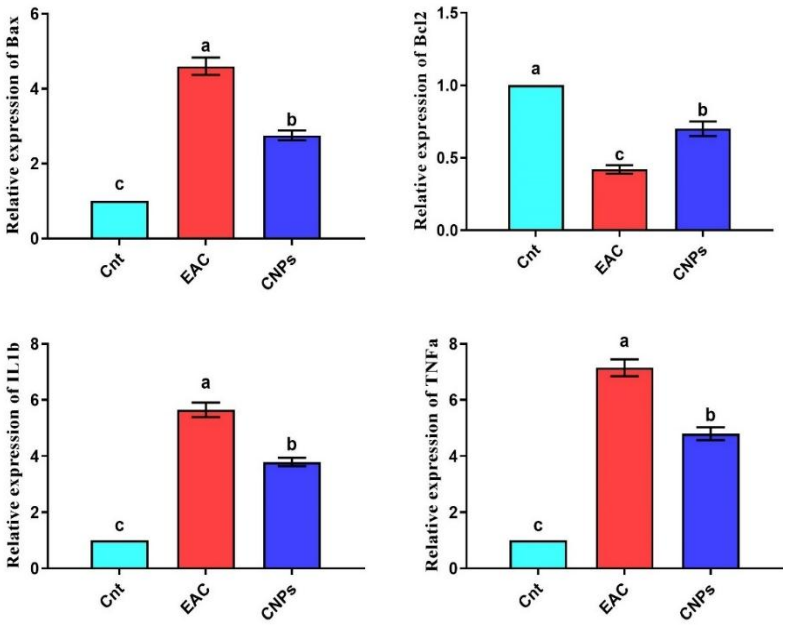

Fig.4. QPCR analysis illustrates the effect of CNPs on *Bax*, *Bcl2*, *IL1b*, and *TNFa* gene expression in the spleens. Results are presented as fold change means ± SEM (n = 7/group). Distinct letters [a (highest) to c (lowest)] denote statistically significant differences (P < 0.05) in comparisons across groups.

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التأثير المناعي لجزيئات الكيتوزان النانوية على الفئران المصابة بسرطان إيرليخ السائل

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الملخص

تعزيز الاستجابة المناعية مع تقليل الأضرار المرتبطة بالسرطان أمر بالغ الأهمية لتحسين نتائج علاج السرطان. يمكن لجزيئات الكيتوزان النانوية (CNPs) أن تعزز من الفعالية العلاجية لعقار سورافينيب وتقلل من الأضرار الكبدية الناجمة عن سرطان إيرليخ السائل (EAC). هدفت هذه الدراسة إلى التحقيق في التأثيرات المناعية لجزيئات الكيتوزان النانوية على الفئران المصابة بـ EAC وتقييم قدرتها على تخفيف إصابات الطحال الناجمة عن المرض. تم تقسيم 21 فأرًا من إناث الفئران السويسرية البيضاء عشوائيًا إلى 3 مجموعات (7 فئران لكل مجموعة): المجموعة الأولى (الضابطة الطبيعية): تلقت فمويًا محلولPBS , المجموعة الثانية :(EAC) حُقنت داخل الصفاق بجرعة واحدة من مليون خليةEAC ,المجموعة الثالثة :(CNPs) ضمت فئران EAC التي عُولجت فمويًا بجزيئات الكيتوزان النانوية من اليوم الرابع إلى اليوم الرابع عشر. تم قياس حالة الأكسدة/مضادات الأكسدة باستخدام التحليل الطيفي، وتم تقييم خلايا CD4 و CD8في الطحال بواسطة التدفق الخلوي، وتحليل التعبير الجيني باستخدام تقنية تفاعل البلمرة المتسلسل اللحظي.(qPCR) حفزت معالجة CNPs استجابة مناعية في الفئران الحاملة لـEAC ، حيث لوحظ زيادة في مستوى الجلوبيولين، وارتفاع عدد الخلايا الطحالية المعبرة عن CD4 وCD8، وثراء في الخلايا اللمفاوية في الطحال. كما استعاد العلاج بجزيئات الكيتوزان النانوية التوازن التأكسدي، والذي تجلى في انخفاض مستويات MDA وزيادة نشاط SOD و CATو .GPxبالإضافة إلى ذلك، قللت الجزيئات من الاستماتة) من خلال تقليل تعبير Bax وزيادة تعبير (Bcl2 وخفضت الالتهاب) من خلال تقليل تعبير IL1β و (TNFαفي الطحال. تشير هذه النتائج إلى أن جزيئات الكيتوزان النانوية لم تستعد فقط بنية ووظيفة الطحال، بل أظهرت أيضًا تأثيرات مناعية ملحوظة على الفئران الحاملة لـ.EAC

الكلمات المفتاحية: سرطان إيرليخ السائل، جزيئات الكيتوزان النانوية، التأثير المناعي، الطحال.