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Research Paper

Protective Effects of Chitosan Nanoparticles against Hepatic and Renal Damage in Rats with Chronic Lead Poisoning

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ABSTRACT

Background: Lead exposure inflicts severe damage to various body organs. The present study sought to assess the effect of chitosan nanoparticles (NPs) on the prevention of hepatic and renal damage in rats with chronic lead poisoning. The indicators were the levels of oxidative stress and proinflammatory cytokines expression.

Methods: We used *Rattus norvegicus* as the animal model. They were assigned to three groups: negative control, positive control, and treatment (n=8 each). The negative control and treatment groups were the models for chronic lead poisoning, and the serum lead levels were determined by atomic absorption spectrometry. The treatment group was orally administered chitosan NPs at 64 mg/kg for 30 days. The reactive oxygen species (ROS) were measured using 2',7'-dichlorodihydrofluorescein diacetate flow cytometry. The hepatic and renal TNF-α and IL-6 gene expressions were also analyzed.

Results: The mean serum lead level was 0.52 mg/L, validating the rats as the lead poisoning model. The results indicated that the liver and kidneys in the treatment group had the lowest ROS and TNF-α levels compared to those in other groups. The treatment rats had a lower hepatic IL-6 level compared to those in positive controls, although this was higher than that in the negative controls. The renal IL-6 level in the treatment group was the highest among all groups.

Conclusion: As evidenced by the results of this study, chitosan NPs had a protective effect on the liver and kidneys of rats during chronic lead poisoning by reducing the ROS and TNF-α levels; nonetheless, it did not suppress the renal IL-6 expression.

Keywords: Chitosan nanoparticles, Cytokines, IL-6, Lead poisoning, ROS, TNF-α

Introduction

Excessive lead exposure is widely recognized as the main contributor to various health problems in numerous developed and developing countries. It induces organ damage that may eventually lead to death [1]. In 2021, the World Health Organization (WHO) reported that the worldwide death toll resulting from lead poisoning had reached more than two million cases. Most of these cases took place in developing countries, including Indonesia [2]. The Institute for Health Metrics has stated that 10.3%, 5.6%, and 6.2% of cases of lead poisoning induce hypertension, ischemic heart disease, and stroke, respectively [3]. To shed more light on the aim of this study, we wish to present a brief review of the relevant literature on the subject before discussing the various sections of this article.

Lead poisoning: the chronic presence of lead ions in the blood and tissues raises the reactive oxygen species (ROS) levels, exerting disruptive effects on the immune system [4] and suppression of T-cell activity [5]. Conversely, the rise in ROS level also activates proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α). Increasing inflammatory levels due to lead poisoning promotes oxidative stress that damages many organs, such as the heart, liver, testes, kidneys, and pancreas [6-9]. This toxic effect springs from a combination of the sulfhydryl group and lead inside the protein that inhibits the enzymatic activities [10, 11]. Lead disrupts hemoglobin synthesis by inhibiting the activity of sulfhydryl-dependent enzymes (e.g., ferro chelatase and aminolevulinic acid dehydratase) with significant roles in generating heme molecules [8]. This situation also increases the number of free erythrocytes protoporphyrins. Other sulfhydryl enzymes inhibited by lead include superoxide dismutase (SOD), G6PD, and catalase.

Oxygen radicals: The oxidative stress is a crucial aspect of lead toxicity since it leads to an imbalance between free radicals and endogenous antioxidant enzymes [9]. The oxygen species that commonly exist in the human body include hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), superoxide anions (O_2^-), and nitrite oxide [10]. Above the threshold limits, ROS initiates oxidative stress that critically damages many cellular macromolecules. Further damage resulting from oxidative stress may bring about pathological consequences, such as cancer, atherosclerosis, diabetes, and other disease conditions [11]. The oxygen radicals also increase lipid peroxidation and lower the activities of antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase (GSH-Px). These, in turn, contribute to further cellular damage in organ tissues [12].

Oxygen radicals are common by-products of metabolism consisting of either free oxygen radicals or partially reduced non-radical oxygen derivates [13-18]. These radicals are highly reactive compared to oxygen due to their uncoupled electrons [14]. This property makes the free radicals bind to various macromolecules and induce the release of various proinflammatory cytokines, such as tumor TNF-α and IL-6, and activate the mitogenic protein kinase pathway [15]. The free radicals and cytokines initiate cellular signaling and prepare the immune system to counter the antigens.

Excessive levels of ROS and proinflammatory cytokines damage DNA components and activate the apoptosome protein complex, inducing cellular apoptosis secondary to mitochondrial damage [16]. These events lead to oxidative stress in the cellular DNA and RNA, causing various human inflammatory conditions, such as abnormal aging [17]. Despite being normal by-products, free oxygen radicals may be generated due to other factors, including prolonged exposure to heavy metal ions [18]. Some ions, including lead, significantly increase the number of ROS that damage the liver tissue [19]. The human body predominantly inhibits the free oxygen radicals by producing antioxidant enzymes. Nonetheless, if free radicals cannot be contained, the oxidative damage disrupts the GSH-Px/GSSG balance [20].

Chitosan therapy: This intervention has been widely employed to diminish or completely inhibit lead poisoning [21-27]. Recent research has reported that antioxidant treatment can effectively prevent excessive ROS production and delay cell oxidation caused by lead [22, 28]. Studies have reported on the activity of chitosan as an antioxidant through free radical scavenging and chelating mechanisms [29-31]. Chitosan also inhibits ROS generation and lowers the lipid oxidation level. The majority of these mechanisms are linked to hydroxyl groups and active amino chains in chitosan [24].

Treatment with chitosan significantly decreases lead, malondialdehyde (MDA), hydroxy-deoxyguanosine (8-OHdG), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). On the contrary, chitosan increases catalase (CAT) activity in lead-exposed hepatic tissue in rats. The combination of chitosan and vitamin C has been demonstrated to increase the activity of SOD, CAT, GSH-Px enzymes, and hemoglobin in rats poisoned with lead [16]. The ability of chitosan to reduce blood creatinine and increase CAT levels is suggestive of its tendency to promote normal kidney function [6, 32, 33]. Nevertheless, it has been reported that the activity of chitosan is less effective compared to that of Ca2EDTA [35]. Moreover, chitosan has illustrated statistically insignificant effects on the levels of chelating glutathione (GSH), ceruloplasmin (Cp), and high-density lipoprotein (HDL) compared to those achieved in controls [33, 34].

Nanoparticle chitosan: The antioxidant property of chitosan is attributed to its molecular weight and deacetylating effect. Chitosan, with lower molecular weight and higher deacetylation capacity, is more effective as a free radical scavenger [36-38]. Ineffective chitosan activity in the protection of organic tissues is supposedly caused by its high molecular weight (10^{-6}-10^{-7}) and low deacetylation capacity (70-85%). These conditions decrease chitosan distribution, chelation, and antioxidant activities, which may be optimized by using chitosan NPs (10^{-9}). At the cellular level, chitosan NPs crosslink with glutaraldehyde, protecting it from being degraded by lysozymes and maintaining its normal activity [23].

Aim of the study: There is controversy over the inflammatory and antioxidant role of chitosan NPs in the protection of lead-exposed hepatic and renal tissues in rats. Due to the hazards posed to humans by lead exposure, the role of chitosan NPs in inhibiting inflammatory cytokines needs to be assessed. In light of the aforementioned issues, the present study aimed to evaluate the effect of chitosan NPs on the protection of rats' liver and kidneys against chronic lead poisoning.

Materials and Methods

Research design: A total of 24 male Wistar rats weighing approximately 250-300 grams were used in this study. All rats were acclimated at room temperature (28°-29°C & 60%-70% humidity) for seven days prior to starting the treatments at the Laboratory of Biology FMIPA UNNES, in Semarang, Indonesia. All food and water supplies were available to the rats *ad libitum*. This study was designed as an experimental project using post-test data. The rats were assigned to three groups: negative control, positive control, and treatment. Each group consisted of eight rats, based on the Federer formula. All treatments were administered after the issuance of an ethical clearance from the Medical/Health Research Bioethics Ethics Commission, Faculty of Medicine, Universitas Islam Sultan Agung, Indonesia (Approval No.: 305/VIII/2022).

Animal models for lead poisoning: Out of 24 rats,

16 cases were included in the positive control and treatment groups. These rats were treated daily for 30 days with lead acetate diluted in distilled water at 200 mg/kg. The blood lead levels were validated by atomic absorption spectrometry (AAS).

Chitosan nanoparticles therapy: Chitosan NPs ($C_6H_{11}NO_4$) were obtained from Nanoshel at a purity of >99%, molecular weight of 161 g/mol, and an APS of 80-100nm. The chitosan compound was dissolved in 1% (v/v) acetic acid solution at 21 g/L. Prior to treatment, the chitosan compound was sterilized and diluted in distilled water to make the stock solution. The chitosan NP was orally administrated to rats in the treatment groups at 64 mg/kg [6]. All treatments were administered to each rat group daily over 30 days.

Sacrifice and organ extraction: Rats were sacrificed using a lethal cocktail dose [24], and their liver and kidneys were dissected and stored in an RNAase-free cryotube. This tube was stored in an RNA solution (Sigma Aldrich; St. Louis, MO, USA) at -80ºC while preparing for the analyses.

Free radical assessment and flow cytometry: Blood samples were mixed with FACS lysing solution (BD Biosciences) and centrifuged at 10,000 rpm for 5 min to obtain the sera. Each serum sample was stained using dichlorofluorescin diacetate

reagent for 30 min at 37ºC in a dark room. The intracellular ROS level was analyzed using the flow cytometer FACS Calibur [29].

Proinflammatory cytokines gene expression: The RNA from tissue samples collected from the liver and kidneys of rats was isolated using Triazole (Sigma Aldrich) reagent, and the RNA was quantified using Nanodrop. The isolated RNA samples were then reacted with Kappa Cyber Mix (SmoBio) reagent to synthesize cDNA. The proinflammatory cytokine gene expression analysis was conducted on reverse transcription-polymerase chain reaction (qRT-PCR) using primers based on the target gene (Table 1). The quantification of qRT-PCR was performed in triplicate using ECO study software [26]. This study used primers from NCBI Primer-BLAST. The annealing process was performed at 95ºC for 15 or 40 sec. As for elongation, the process was performed in 40 cycles at 60ºC for 30 sec. In the termination process, the temperature was set at 65ºC for 5 sec and 10°C for 15 sec at the end.

Data analyses: The data for ROS levels were analyzed in SPSS software (version 25) using one-way ANOVA and Duncan's multiple range test (DMRT). The TNF-α and IL-6 levels were determined using a descriptive method.

Table 1. Primer sequences for proinflammatory cytokine analysis

mRNA	Sequences (5'-3')	Size (bp)
TNF-α	Sense CCGCTCGTTGCCAATAGTGATG Antisense CATGCCGTTGGCCAGGAGGG	235
IL-6	Sense CTTGGGACTGATGCTGGTGACA Antisense GCCTCCGACTTGTGAAGTGGTA	118

Results

Lead poisoning model: Blood samples administered with lead-acetate at 200 mg/kg for 30 days as the toxication model were analyzed using the AAS method in the Laboratory of Chemistry in Universitas Negeri Semarang (UNNES). The mean blood lead level was 52 µg/dL. As illustrated by the obtained result, the blood lead level had surpassed the threshold limit (10 µg/dL). Based on the findings, the rats were poisoned with lead and then used in the study on chitosan NPs treatment protocol. They were also subjected to the measurement of ROS and inflammatory cytokine levels in hepatic and renal tissues. The data for the ROS are presented in Figure 1.

ANOVA analysis: The ***F*** value obtained from the one-way ANOVA analysis was 145.769 (P=0.00). The finding highlights the significant difference in ROS levels in lead poisoning of the animal model among the study groups. Due to the statistical difference, a further test was performed based on DMRT analysis (Table 2). The levels of TNF-α and IL-6 in the samples from the liver and kidneys of rats are presented in Figures 3, 4, and 5.

Tissue TNF-α level: The TNF-α level from the liver tissue of the animals treated with lead nanoparticles is displayed in Figure 2. The results indicated that the rats treated with the nanoparticles had the lowest level of TNF-α. In addition, the TNF-α levels from the kidneys of rats poisoned with lead are demonstrated in Figure 3. The rat group treated with chitosan NPs had the lowest TNF-α level as compared to those in other groups.

Table 2. Results of Duncan's multiple range test

Duncan				
	N	Subset for alpha=0.05		
Treatment		1	2	3
NPs	8	23.8725		
Control (-)	8		35.7275	
Control (+)	8			61.8663
Sig.		1.000	1.000	1.000

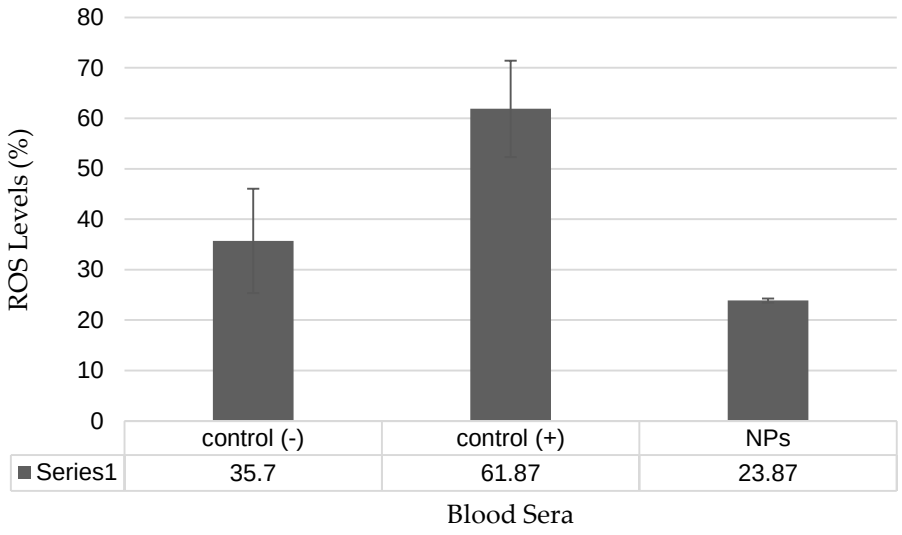

Figure 1. Reactive oxygen species level in blood sera from lead toxicities animal model treated with chitosan nanoparticles

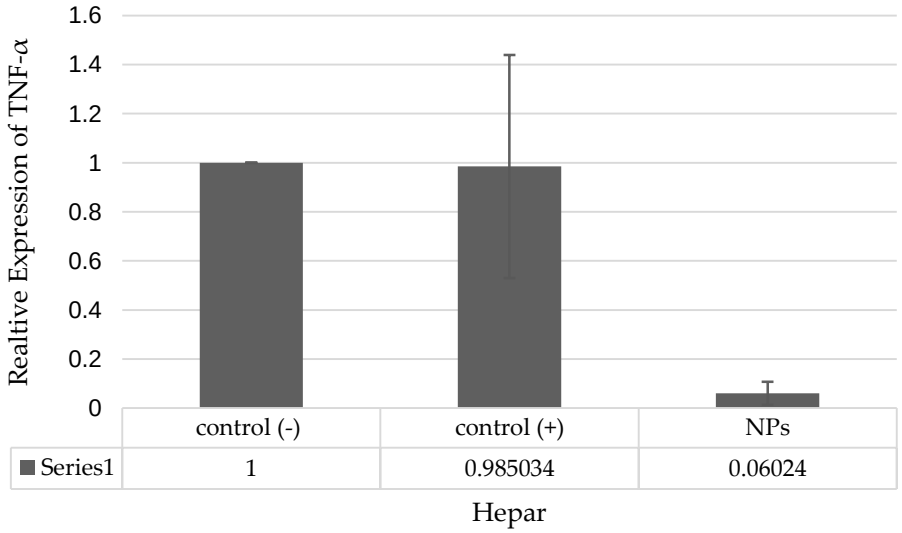

Figure 2. TNF-α level from hepatic tissue of lead toxicities animal model treated with chitosan nanoparticles

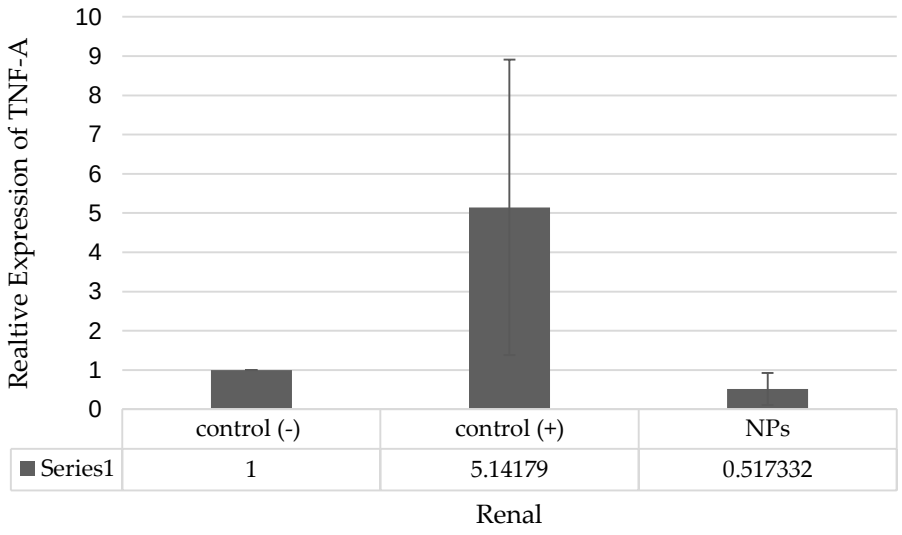

Figure 3. TNF-α level from renal tissue of lead toxicities animal model treated with chitosan nanoparticles

Blood lead and tissue Il-6 levels: The blood samples collected from rats, which were administered with lead-acetate at 200 mg/kg orally for 30 days, were analyzed using the AAS method in the laboratory of chemistry in UNNES. The result of blood lead level was 52 µg/dl. Based on the result, the blood lead level had surpassed the threshold limit (10 µg/dl). Based on this result, the rats were said to be poisoned with lead. These rats were then used as the model for this research employing chitosan NP treatment. The rats were also subjected to ROS and inflammatory cytokine level measurements in their liver and kidney tissue samples. As depicted in Figure 4, the administration of chitosan NPs reduced the Il-6 levels in the liver of lead-poisoned rats; nonetheless, the levels of Il-6 did not reach that of the healthy rats (negative control). Furthermore, the administration of chitosan NPs did not reduce Il-6 levels in the kidneys of the lead-poisoned rats (Figure 5).

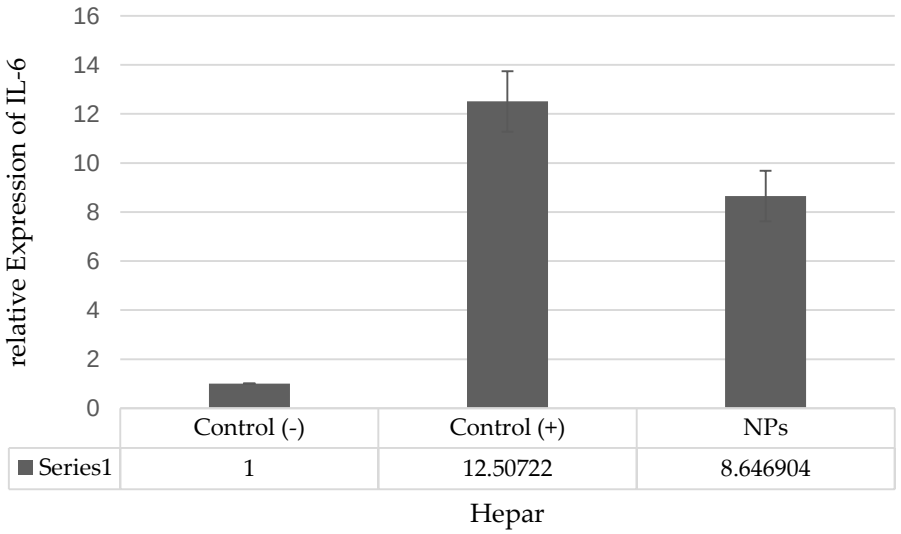

Figure 4. IL-6 level from hepatic tissue of lead toxicities animal model treated with chitosan nanoparticles

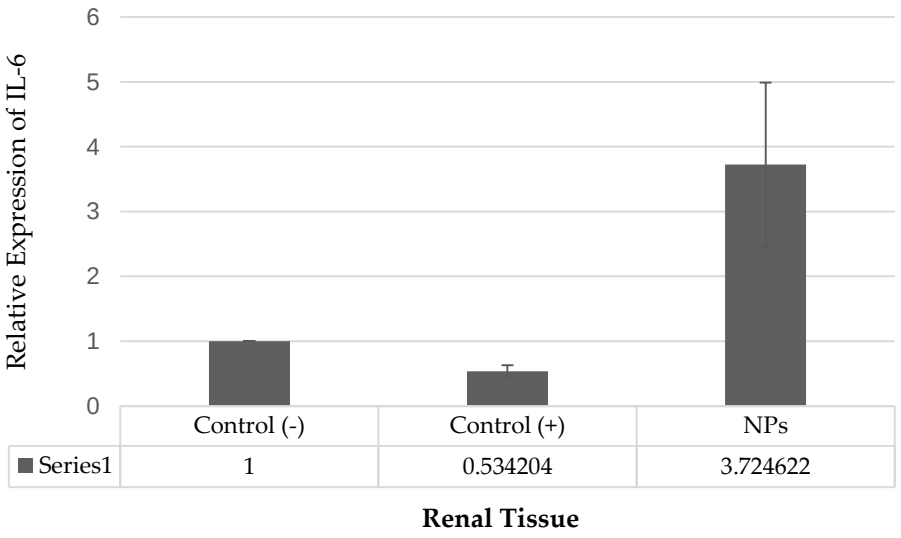

Figure 5. IL-6 level from renal tissue of lead toxicities in animals treated with chitosan nanoparticles

Discussion

Based on the results presented in Figure 1, the rats in the lead poisoning group that were not administered with chitosan NPs had higher ROS levels compared to the healthy ones in the negative controls. The data indicated that blood lead level increases the ROS levels, signifying oxidative stress in rats' tissues. Lead exposure increases ROS production [31]. Excessive ROS will lead to cellular oxidative stress [28, 32] and inhibit antioxidant enzymes, potentially initiating oxidative damage against organ tissues [33, 34]. Lead acetate is an oxidant that disrupts cell redox balance and generates ROS [29]. Free oxygen radicals induce inflammation by modifying gene expression, altering kinase cascades, and activating proinflammatory transcription factors, such as nuclear factor-κβ(NF-κβ) [30]. The activation of the NF-κβ signaling pathway promotes proinflammatory

cytokines that modulate inflammation, including TNF-α, IL-6, and nitric oxide [31].

The ROS level was lower in the rats administered with chitosan NPs than that of the positive controls. The chitosan NPs with access to various molecules bind ROS through a scavenging mechanism and stabilize the redox status in cells [35]. Exposure to oxidants stimulates bodily cells to generate proinflammatory cytokines, including IL-6, IL-1β, and TNF-α [36]. These cytokines prepare the cells for eliminating foreign substances and pathogens that invade the body. The TNF-α is produced by macrophages as a defense against antigens [37]. Associated with lead acetate exposure, the number of rat macrophages increases the production of TNF-α, causing oxidative stress and damaging tissues [38]. This event prevents any detrimental effect that is caused by sudden oxidant exposure resulting from an imbalanced redox status [39]. In this study, lead acetate was the main oxidant that caused oxidative damage in the liver and kidneys.

Cells in the kidneys and liver tissues are subjected to damage due to lead acetate exposure [40]. Kidneys and liver are correlated in detoxification and excretion of lead. In this context, kidneys contain a significant number of immune cells, which are susceptible to lead. Therefore, it undergoes morphological alterations following lead exposure, which causes a subsequent inflammatory response. This event triggers the production of various inflammatory cytokines and the influx of ROS [34]. Heavy metals trigger oxidative stress that interferes with normal hepatic function. The rise in ROS and MDA production stimulates the liver to produce increased levels of Aspartate aminotransferase and Alanine transaminase, manifested as hepatotoxicity [33].

Figures 2 and 3 demonstrate that the amounts of TNF-α in the liver and kidneys were relatively increased in positive control rats compared to that of the negative controls. However, the chitosan NPs treatment led to a marked decline in TNF-α. This event is thought to be linked to the nature of chitosan NPs, which bind to oxidants and prevent oxidative stress. In addition, chitosan is also known to modulate macrophages and dendritic cell activity, which are pivotal to the immune system. Macrophages present antigen fragments to T-cells for immune response and come in M1 and M2 types. The M1s promote proinflammatory conditions, whereas M2s increase anti-inflammatory reactions [41,42]

As evidenced by cytokines levels and surface markers, chitosan induces macrophages into the M2 types. This agent significantly down-regulates the expression of major histocompatibility complex-II (MHC-II) and presents antigen to T-cells. In addition, chitosan lowers the generation of proinflammatory cytokines, such as TNF-α and IL-1β [43]. In supporting the M2 polarization, chitosan promotes the upregulation of IL-10 as a major anti-inflammatory cytokine [44]. It also induces the production of TGF-1β as an anti-inflammatory cytokine. The macrophage polarization is vital to maintaining redox balance, preventing damages caused by hyper-inflammation, and supporting the immune system in defending the body against antigens [45].

The IL-6 level was also examined in the liver and kidneys of the rats exposed to lead acetate. The production of IL-6 increased under stress conditions and exposure to lead [46]. As exhibited in Figure 4, the amount of IL-6 significantly increased in the positive controls, indicating a rise in liver inflammation. Chitosan NPs had a significant impact on reducing the IL-6 level, demonstrating the activity of chitosan NPs in binding to free radicals. However, the IL-6 level in the kidneys of rats treated with chitosan increased, probably due to accumulated lead acetate that was not entirely excreted, which in turn caused oxidative stress and inflammation in that organ.

During exposure to chitosan NPs, IL-6, and TNF-α are down-regulated since it decreases the production of both cytokines at the transcription level and lowers the inflammation in the tissue. Chitosan also protects organs prone to oxidative stress, such as kidneys, by lowering the glycerol-induced inflammatory response [47]. Chitosan down-regulates the expression of inflammatory genes dependent on NF-kβ through lipopolysaccharide-induced mechanisms [48].

Chitosan NPs have hydroxyl (-OH) and amino (-NH_2) groups at carbons 2, 3, and 6 with a wider distribution. These ions bind free heavy metal ions, such as lead, which are chemically unstable and induce oxidative stress. It also donates hydrogen or lone pairs of electrons to stabilize heavy metal ions. Chitosan deactivates the catalytic function of Fe^{2+} and Fe^{3+}. These properties enable chitosan to provide anti-oxidative protection in various organs [49]. As opposed to oxidative stress, chitosan regulates the expression of NF-kβ that modulates inflammatory reactions. It also reduces the generation of MDA and promotes the production of CAT, SOD, and GSH-Px [50]. The findings of this study provide hope in favor of chitosan NPs to inhibit oxidative stress caused by exposure to lead. Its properties can prevent the rise in proinflammatory factors, such as TNF-α and IL-6. This, in turn, causes chitosan NPs to offer the potential to overcome chronic exposure to heavy metal toxins.

Conclusions

As evidenced by the results of this study, chitosan NPs inhibited inflammation in rat models *in vivo* due to lead acetate poisoning by decreasing oxidative stress secondary to the reduced levels of ROS in the rat sera. This event was induced by reduced inflammation based on the expression of the inflammatory cytokine and tumor necrosis factor-α in the liver and kidneys of rats, as well as a decline in IL-6 in the liver but not in the kidneys.

Conflict of Interests

The authors declare that they have no conflict of interest regarding the publication of this article.

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Compliance with Ethical Guidelines

The ethical clearance was issued by the Medical/Health Research Bioethics Commission, Faculty of Medicine, Universitas Islam Sultan Agung, No. 305/VIII/2022/Ethics Commission.

Authors' Contributions

AM: conceptualization, formal analysis, investigation, writing-original draft preparation, writing review, editing, visualization, project administration, funding acquisition. ***NDA:*** conceptualization, methodology, validation, resources, writing - original draft preparation. ***SCD:*** Conceptualization, writing the original drafts, review, editing, visualization, and study supervision. ***RA:*** investigations, resources, and formal analyses. ***MDDR:*** investigations, resources, and data generation. ***MBR:*** investigations, resources, and writing the original manuscript drafts.

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