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Nano-chitosan mitigates lead acetate-induced histological changes in rat testicular seminiferous tubules

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ABSTRACT. Lead is a toxic metal known to be harmful once within the body. Chronic lead accumulation causes the emergence of free radicals, thereby causing damage to organ systems, one of which is the reproductive system. Chitosan can chelate lead metal ions which behave as free radicals. In nanostructures, chitosan's ability to chelate heavy metal ions is expected to be more effective. The use of nano-chitosan is expected to facilitate its distribution so that its ability to chelate lead ions is more effective. This study aims to analyze the effect of nano-chitosan on the histology of seminiferous tubules and spermatogenesis of rats induced by lead acetate (PbAc). Animals are divided into 3 groups; Naive Group (NG: aquadest), Positive Control (PC: PbAc 200 mg kg^{-1} BW), Treatment Group (TG: PbAc 200 mg kg^{-1} BW and nano-chitosan 64 mg kg^{-1} BW) with a treatment duration of 35 days, and 6 rats in each group. Rats were exposed to lead by oral administration. At the end of the treatment period, animals were euthanized by dislocation of the cervical spine after being lightly anesthetized using 10% chloroform. Then, testicular organs were dissected, and histological preparations of the seminiferous tubules were analyzed. Measurements include epithelial thickness, tubule diameter and spermatogenesis scoring based on the Johnson criteria. The results obtained proved that administration of nano-chitosan after PbAc induction increased epithelial thickness, seminiferous tubule diameter and spermatogenesis score. In conclusion, nano-chitosan can overcome lead toxicity in the rat reproductive system induced by PbAc with indicators of improvement in the histology structure of seminiferous tubules and increased spermatogenesis scores.

Keywords: Exposure; fertility; lead-induced rat; male reproduction.

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Introduction

Lead is one of the heavy metals that at certain levels will pollute the environment. The use been widely used by humans for thousands of years, becoming widespread in the environment. Lead exist naturally existing on the Earth, it has a soft metal form, high density, it is corrosion resistant, stable, and has conductivity with a very long half-life (Fu & Xi, 2020). Coal-fired power plants, industrial waste, and industry emissions are some of the main causes of lead pollution (Lee, Payus, Ali, & Vun, 2017). Lead contaminated paints, cosmetics, children's toys, and food are also sources of lead pollutants (Swaringen et al., 2022).

Lead exposure mainly occurs through the respiratory and digestive systems (Shukla et al., 2018). However, it can be also occur through the skin. Long-term lead accumulation will raise the body's level of free radicals, which presence can cause oxidative stress in tissues. Free radicals from lead ions can attack protein compounds, lipids, lipoproteins, carbohydrates, unsaturated fatty acids, DNA and RNA (Martemucci et al., 2022).

The testicular are among the organs affected by the accumulation of lead in the body. The chronic oxidative stress caused by lead makes the testes endogenous antioxidant defenses of unable to neutralize the damaging effects caused by Reactive Oxygen Species (ROS) (Dutta, Sengupta, Slama, & Roychoudhury, 2021). Lead can bind to sulfhydryl groups, thus reducing the level of endogenous antioxidants such as glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). Lead ions are also able to bind to calmodulin (CaM) in the second messenger system, thereby inhibiting the release of Gonadotropin-releasing Hormone (GnRh) (Flora, Gupta, & Tiwari, 2012).

In addition, lead can penetrate the Blood testes barrier (BTB) using passive transport due to its lipophilic properties (Zhu et al., 2017). Lead is also suspected of using a transporter facility on the surface of Sertoli cells to cross BTB more quickly. SRC family kinases (SFKs), which are known to control a variety of cellular processes like cell migration, differentiation, proliferation, survival, and apoptosis, are the unexpected

transporters (Yu et al., 2023). Furthermore, SFK function in the testes regulates spermatogenesis, particularly the adhesion of Sertoli-Sertoli and Sertoli-germ cells. Once lead crosses BTB and enters the seminiferous tubules, it can exert toxic effects on developing sperm cells.

Antioxidant are molecules that can prevent free radicals in the body. Antioxidants neutralizing free radicals by donating their electrons, thereby satifying the electron deficiency in free radicals, stabilizing them, and inhibiting their chain reaction (Munteanu & Apetrei, 2021).

One compound that has the potential to neutralize free radicals is chitosan. Chitosan is a polymer compound derived from chitin that undergoes a deacetylation process under chitosan base conditions widely used in pharmaceutical and biomedicine products. Figure 1 presents the molecular structure of chitin and its deacetylated derivative, chitosan, with the formation of the amine group (NH_2) and hydroxyl group (OH) in chitosan, which play important roles in enhancing solubility, reactivity, antioxidant activity, and chelating agents compared to chitin. The amine group and the OH group owned by chitosan play an important role in its antioxidant activity and in its ability as a chelator agent for metal ions (Rimu & Rahman, 2022).

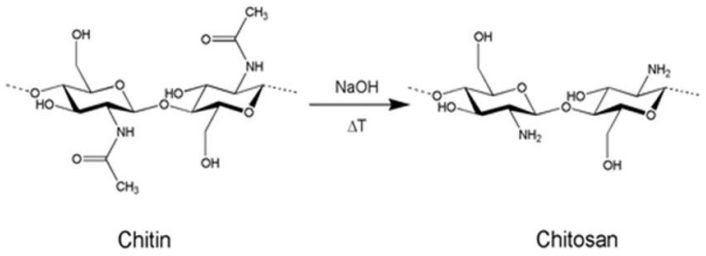

Figure 1. Structure of chitin and chitosan (Hahn et al., 2020).

Marianti, Anatiasara, and Ashar (2017) reported that chitosan was able to protect the kidneys and liver of rats from poisoning caused by heavy metal lead. Chitosan was also able to lower the level of Lead concentration in the testes so that the quality of rat sperm improved. The decrease in lead concentration is due to the chelating activity of chitosan. This chitosan activity lowers the level of free radicals in the blood, providing a protective effect against lead-induced damage to male reproductive organs (Marianti, Isnaeni, Setiani, & Sumadi, 2020). Due to its non-toxic nature, chitosan has the potential to be further developed as an alternative material for overcoming the negative effects of lead exposure.

In biological functions, it is observed that nano-chitosan has antibacterial, antifungal, anti-inflammatory, antitumor, immunostimulant and antioxidant effects. This natural material presents excellent physicochemical and biological properties, making it bioactive without harm to humas, while being environmentally friendly (Sowjanya, Dhivya, Meenakshi, & Vedhanayakisri, 2013).

The use of nanotechnology offers several advantages in drug delivery. Nanoparticles have distinctive properties and can penetrate a variety of spaces that other conventional particles cannot reach. Nanoparticle technology can be conjugated with other substances, resulting in a new drug delivery system with more complete specifications. Furthermore, the use of nano-chitosan as an antidote to free radicals induced by lead in the male reproductive system has not been widely studied. Hence, investigating the impact of nano-chitosan on males exposed to lead metal offers intriguing insights into its effect on reproductive system.

Material and methods

Animals Maintenance

This research used eighteen (18) male Wistar rats with an average weight of 150-200 g, obtained from the Animal House, Biology Laboratory, Universitas Negeri Semarang, Indonesia. Animals were fed with standard rat pellets and water *ad libitum*. This research has fulfilled the research ethical requirements issued by Commission on Medical/Health Research Bioethics of the faculty of Medicine Universitas Sultan Agung Semarang, Indonesia with number of Ethical Clearance No. 305/VII/2022/Komisi Bioetik.

Preparations of materials research

The lead used was Lead (II) Acetate-3-Hydrat Pro Analysis MERCK (Pb $(CH_3COO)_2$ $3H_2O$, which was dissolved in distillate water. Nano-chitosan ($C_6H_{11}NO_4$) used in this study has a particle size of 80-100 nm produced by Nanoshel. For administration, it was dissolved into 2% glacial acetic acid.

Experimental design

This study employed a true experimental research design with a post-test control group design approach carried out in the laboratory. This research was carried out at the Biology Laboratory of Universitas Negeri Semarang, where rats were bred, solutions were prepared, and treatments involving lead acetate and nano-chitosan were administered, alongside surgical procedures.

Histological preparations of rat testicular seminiferous tubules were made at Animal Health Laboratory. Histological readings of the seminiferous tubules of rat testicles were carried out at the Pathology Laboratory of RSI Sultan Agung Semarang. Histological images of the seminiferous tubules were taken at the Biology Multimedia Laboratory, Universitas Negeri Semarang.

Eighteen rats were acclimated for 1 week, then weighed, marked and divided into three groups with six rats in each group: Naive Group (NG: aquadest), Positive Control (PC: PbAc 200 mg kg^{-1} BW), Treatment Group (TG: PbAc 200 mg kg^{-1} BW and nano-chitosan 64 mg kg^{-1} BW). The treatment period lasted 35 days, during which oral administration was conducted using a sonde to facilitate ingestion and subquent processing through the digestive system. On the 36^{th} day, all male rats from the three treatment groups were euthanized by dislocation of the cervical spine, after being lightly anesthetized using 10% chloroform (Shubina & Dudenkova, 2016). Subsequently, abdominal dissection was performed to retrieve the testicles, which were then placed in vials containing a 10% formalin buffer for fixation.

Histological preparations

Histological preparations of testicular seminiferous tubules were made using the paraffin method, with an organ section thickness of 5 µm and hematoxylin-eosin (HE) staining (Bidanchi et al., 2022).

Measurement of the thickness of the seminiferous tubule epithelium

The Measurement of the seminiferous tubule epithelium thickness followed the method outlined by Nur, Ilahika, and Andari (2023) with observation at 200x magnification across 4 fields of view. In one seminiferous tubule, measurements are taken on all four sides of the tubule. Selected visual fields were taken at 4 points, specifically at 12, 3, 6 and 9 o'clock positions. The thickness was assessed using Motic Images Plus 2.0 program.

Measurement of the diameter of the seminiferous tubule

The measurement of the seminiferous tubules diameter followed the method described by Prastyaningtyas et al. (2021), using a microscope magnification of 200x. Measurements were carried out in 5 fields of view. In 1 field of view, 4 seminiferous tubules were measured at an angle of 90^{o} from each, and the result were averaged. The diameter measurement was analyzed using the Motic Images Plus 2.0 program.

Spermatogenesis scoring

Spermatogenesis scoring, based on the Johnsen score criteria and adapted from Chang et al. (2023), was carried out at 400x microscope magnification in 5 random fields of view within each preparation. The spermatogenesis score assessments were facilitated using the motic.net software application.

Statistical analysis

The data analysis technique used SPSS 26 software with the following data analysis methods: (1) normality test, using the Shapiro-Wilk test with a normal distribution of $p>0.05$; (2) the homogeneity test uses Levene's test $p>0.05$ to determine uniformity; (3) if the evaluation is homogeneous and normally distributed, the One Way ANOVA (Analysis of Variance) statistical test is used to find differences in group means; (4) The final test used the Duncan Multiple Range Test/DMRT which aims to see any real differences between treatment groups; (5) if the data is not normally distributed and homogeneous, then non-parametric analysis was carried out using the Kruskal-Wallis test. $P<0.05$ was accepted as the level of statistical significance, and all data are presented as mean ± SD.

Result

After being induced by lead acetate for 35 days and given 64 mg kg^{-1} BW nano-chitosan treatment, epithelial thickness and seminiferous tubule diameter was measured, and the spermatogenesis score was performed. Table 1 showed the results of the normality and homogeneity test of the epithelial thickness and

seminiferous tubule diameter. The result showed that the data is normally and homogeneously distributed, so one-way ANOVA statistical analysis can be used. The results of one-way ANOVA showed that there are significant differences between treatment groups (Table 1). Since the One-way ANOVA results showed significant differences, DMRT analysis was conducted. The results of the DMRT analysis indicate significant variations among each treatment group (Table 1).

Table 1. Results of analysis of epithelial thickness and seminiferous tubule diameter.

Variables	Group	Normality	Homogeneity	F value	Significance (p<0.05)	Mean ± SD
Epithelial thickness	NG	0.381	0.151	82.459	0.000	24.07 ± 2.04^{b}
	PC	0.345				18.76 ± 0.86^{a}
	TG	0.867				27.19 ± 1.74^{c}
Seminiferous tubule diameter	NG	0.177	0.317	873.256	0.000	73.63 ± 2.28^{c}
	PC	0.128				63.79 ± 2.26^{a}
	TG	0.087				86.91 ± 1.89^{b}

Values with different letters in the same column are different by DMRT (p<0.05). Based on table 1, there was a significant decrease (P<0.05) in the epithelial thickness and seminiferous tubule diameter of positive controls compared to naive group. There was a significant increase (P<0.05) in the epithelial thickness and seminiferous tubule diameter of the treatment group induced by lead acetate and given nano-chitosan, when compared to the naive group and positive control.

Another important indicator is spermatogenesis scoring based on the Johnsen score criteria, which assessed both the quality and quantity of sperm production. Images depicting spermatogenesis stained with the Hematoxylin-eosin for each treatment group are shown in Figure 2 and Figure 3.

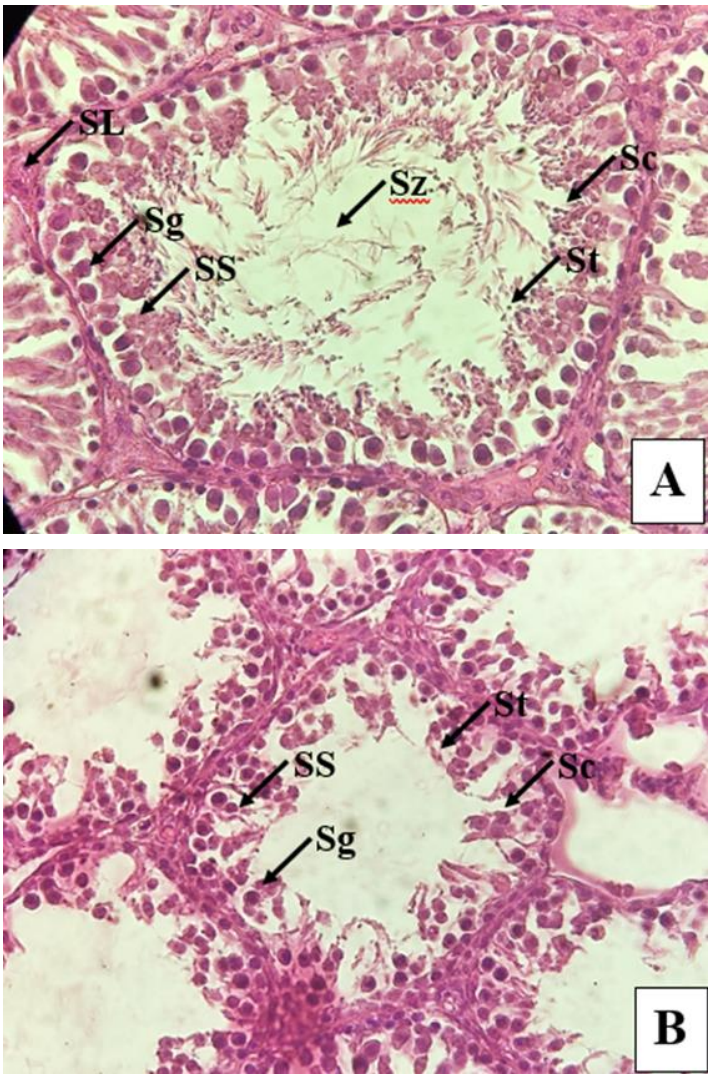

Figure 2. A) Naive Group, spermatogenesis score 10; B) Positive Control, spermatogenesis score 7; Sg: spermatogonia; Sc: primary spermatocyte; St: spermatid; Sz: spermatozoa; SS: Sertoli cell; SL: Leydig cell. HE: 400x.

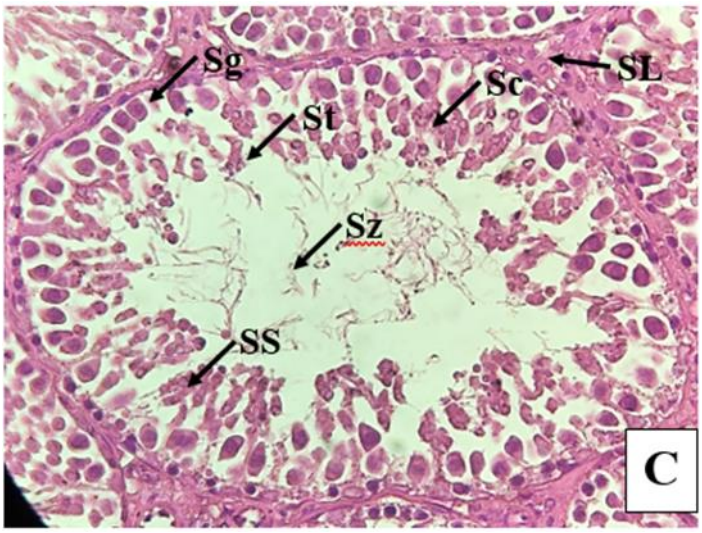

Figure 3. C) Treatment Group, spermatogenesis score 10; Sg: spermatogonia; Sc: primary spermatocyte; St: spermatid; Sz: spermatozoa; SS: Sertoli cell; SL: Leydig cell. HE: 400x.

The normality and homogeneity test of spermatogenesis scoring data indicates non-normal distribution and lack of homogeneity (P>0.05). Therefore, non-parametric analysis using the Kruskal-Walli's test was employed, revealing a significant difference among treatment groups ($p < 0.05$, $p = 0.000$). To determine the real difference between treatment groups, the Mann-Whitney Post Hoc test was conducted. The results of the non-parametric analysis of the Kruskal-Walli's test and Mann Whitney Post Hoc are shown in Table 2.

Table 2. Results of analysis of spermatogenesis scoring.

Group	Mean ± SD	Kruskal-Wallis	Mann-Whitney
NG	10.00 ± 0.00^{a}	0.000	1.000
PC	7.33 ± 66.19^{b}	0.000	0.000
TG	10.00 ± 0.00^{a}	0.000	1.000

Numbers accompanied by different letters (^{a}, ^{b}) indicate significant differences based on the Mann-Whitney test.

Discussion

The results of the seminiferous tubules diameter and epithelial thickness, as well as the spermatogenesis scores indicated that the positive control exhibited the smallest measurements compared to the naive group and treatment groups. This was also reported by Al-Okaily and Murad (2021), Kadhim (2022), Massányi, Massányi, Madeddu, Stawarz, and Lukáč (2020), indicating that lead induction significantly decrease seminiferous tubule diameter, epithelial thickness and spermatogenesis score.

The mechanism of lead toxicity involves a second messenger system at the cellular level, namely Ca^{2+} substitution, which affects all physiological and biochemical processes in cells that require calcium. Lead has a high affinity so that it can replace the position of Ca^{2+} in binding to CaM, which is a protein carrier (Gorkhali, Huang, Kirberger, & Yang, 2016). This can have an impact on disrupting GnRH stimulation which functions to stimulate the pituitary gland to produce the hormones FSH and LH, as a result of damage to the working function of the hypothalamus.

Lead compounds that enter the body are degraded releasing Pb2+ ions which inhibit the work of proteolytic enzymes, thereby causing cell injury. Since lead can bind with sulfhydryl groups, it can affect the body's antioxidant system by reducing GSH, SOD, and CAT. Superoxide radical elimination may be hampered by a drop in SOD concentration in the body, whereas superoxide radical binding may be compromised by a drop in CAT (Flora et al., 2012).

The significant decrease observed was attributed to interference with FSH and LH secretion, mediated by ROS produced by lead induction (Al-Okaily & Murad, 2021; Kadhim, 2022; Massányi et al., 2020). These two hormones play a crucial role in the normal function of the testicles, specifically in the spermatogenesis process. Excessive free radicals in the body can damage DNA, proteins, and lipid membranes through lipid peroxidation, thereby disrupting the spermatogenesis process (Dare, Oyeniyi, & Olaniyan, 2014).

Changes in spermatogenesis can increase the thickness of the seminiferous tubule epithelium (Suede, Malik, & Sapra, 2020). The presence of free radicals in excess can cause the process of spermatogenesis to be disrupted, thus affecting the thickness of the epithelium of the seminiferous tubules. In the seminiferous tubules epithelium, apoptosis can occur spontaneously as well as in response to hormonal changes, high temperature, and chemotherapeutic agents (Utomo, Daningtia, Yuliani, & Yuniarti, 2019).

A decrease in Sertoli cells or a blockage in the spermatogenesis process is also indicated by the reduced size of the seminiferous tubules (Saputra, Sitasiwi, & Saraswati, 2020). This reduction in diameter is likely also due to the large number of germ cells undergoing apoptosis. The diameter of the seminiferous tubules is determined by the hormonal interaction between FSH and LH. This interaction is determined by FSH levels, where if FSH levels are low or not even produced, LH cannot maintain the normal diameter of the seminiferous tubules, so the seminiferous tubules will become smaller (Oduwole, Huhtaniemi, & Misrahi, 2021).

Suppression of the secretion of the hormone testosterone and gonadotropin hormones leads to a decrease and change in the arrangement of the spermatogenic cell layer. The Lumen of the seminiferous tubule may become reduced or enlarged, and the decrease in the number of interstitial cells could result from their degeneration due to inadequate stimulation by gonadotropin hormones.

This study shows that the administration of nano-chitosan to lead acetate-induced rats can significantly increase epithelial thickness and diameter of seminiferous tubules, as well as spermatogenesis scores. The increase occurred due to the activity of nano-chitosan as a chelator of heavy metal ions and antioxidants. Structural improvements are related to the viability of the constituent cells of the seminiferous tubules. This is also reported by Kadhim (2022), who uses the flavonoid compound quercetin to mitigate the effects of lead-induced free radicals. The antioxidant activity of quercetin can increase the diameter of seminiferous tubules due to its ability as a chelating agent. In addition, Marianti et al. (2020) reported that chitosan can lower the level of lead-induced free radicals, thereby preventing damage to the spermatogenic layer in the testes. Free radicals can cause the mechanism of sperm production to be disrupted, leading to a decrease in the number of germ cells (Asadi, 2017).

Nano-chitosan has an amine group (NH_2) on its chain, which is crucial for its antioxidant activity. This group served as a ligand, enabling it to bind metal ions and function as a metal absorbent (Marianti et al., 2017; Sheth, Dharaskar, Khalid, & Sonawane, 2021). Additionally, the NH2 group acts as a chelating agent capable of binding to lead metal (Tayel, Elzahy, Moussa, Al Saggaf, & Diab, 2020), in order to form (Pb(NH2)2), thus reducing its toxicity.

The small size of nano-chitosan and its high surface area also contribute to its enchanced adsorption capacity for heavy metals (Seyedmohammadi, Motavassel, Maddahi, & Nikmanesh, 2016). The adsorption mechanism of nano-chitosan on heavy metals is based on the electrostatic attraction between positively charged amino groups on the chitosan surface and negatively charged pollutant ions (Pang, Tan, Lim, & Chong, 2021). In addition, the nitrogen lone pair in the amino and N-acetyl amino groups can form coordinated covalent bonds with transition metal ions (Kumar, Kumar, Chopra, & Sindhu, 2023). Chitosan nanoparticles have been proven to effectively absorb lead, cadmium and copper ions (Kumar et al., 2023).

The water insolubility of chitosan can be addressed through the use of chitosan nanoparticles. By reducing chitosan to nano size, its surface area increases significantly, thereby enhancing its absorption capacity. The increased absorption capability of nano-chitosan for Pb metal is attributed to its expanded surface area facilitated by nanotechnology, enabling greater biding of Pb ions and reducing resultant toxicity.

Nano-chitosan exhibits reparative effect, including immunomodulatory and enhanced antioxidant activity (El-Megharbel, Al-salmi, Al-Harthi, Alsolami, & Hamza, 2021; Suwignyo, Hersanti, & Widiantini, 2022). Susilawati and Soewondo (2022) stated that small particle sizes can produce a large surface area, facilitating enhanced absorption of antioxidant activity on the nanoparticle surface, and thereby amplifying overall antioxidant effectiveness.

Nanoparticles have garnered significant attention in research and patents due to their large surface area, strong resistance to heat and chemicals, and high adsorption capacity to remove both organic and inorganic contaminants. The use of nanotechnology offers several advantages in drug delivery, as nanoparticles have distinctive properties enabling them to penetrate spaces that cannot be reached by other conventional particles (Modena et al., 2019; Yetisgin et al., 2020).

In the pharmaceutical industry, nano-chitosan is highly appropriate for increasing the therapeutic efficacy and oral bioavailability of chitosan and other water-insoluble medications (Agrawal & Patel, 2011). Furthermore, nanoparticles possess a larger surface area, enhancing their absorption capacity (Agrawal &

Patel, 2011). Al-Eisa (2018) demonstrated that nano-chitosan improved the antioxidant capacity of treated rats by reducing markers of lipid peroxidation (MDA). Nano-chitosan has a strong effect in counteracting testicular toxicity caused by lead. In biological functions, nano-chitosan is observed to have antibacterial, antifungal, anti-inflammatory, antitumor, immunostimulant and antioxidant properties. The result of the group that received nano-chitosan administration may be related to a structural improvement associated with the viability of the cells that make up the seminiferous tubules.

Conclusion

Nano-chitosan can provide an improvement effect on the structure of rat seminiferous tubules affected by lead acetate induction. Nano-chitosan significantly mitigates the lead-induced damage by improving seminiferous tubules structure and enhancing spermatogenesis score.

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