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Role of Chitosan on the Spermatogenesis Disruptionof Rat (*Rattus*sp.) After Plumbum-Acetate Administration

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Abstract: Plumbum as one of the causes of pollution lngkungan can cause human health problems. One of them is the disruption of male reproductive organs. Chitosan is derived from shrimp shells is expected to reduce the negative effects of Pb in experimental rats as an animal model. This study investigates the role of chitosan on the spermatogenesis disturbtion of rat (*Rattus* sp.) after Pb-acetate administration.Thirty male, rat, aged 8-12 weeks, weighed 150-250g were equally divided into control (K1, K2) and experimental groups (P1-P4). The experimental research design using a completely randomized design (CRD) which consists of 6 groups, control (K1), control+Pb (K2), Pb+0.5% chitosan (P1), Pb+0.75% chitosan (P2), Pb+1% chitosan (P3), chitosan 1% (P4). Observation parameters by measuring germ cellsnumber and Johnsen score of geminal cells every seminiferous tubules, Apoptotic index, and viability of sperm.SPSS-20, Kruskal Wallis and Mann-whitney test analyzed all parameters to assess the significance of changes between control and experimental groups. Ethical approval for the study was duly obtained from the responsible Ethical committee in faculty of Mathematics and Natural Sciences Univ. of Sumatera Utara-Medan, Indonisia. The results showed a significant difference ($p<0.05$) between treatment groups on all test parameters (germ cellsnumber and Johnsen score of geminal cells every seminiferous tubules, Apoptotic index, and viability of sperm).The results of this study showed the chitosan can reduce the negative influence of Pb-acetate to germcellsnumber and Johnsen score of geminal cells every seminiferous tubules, Apoptotic index, and viability of sperm.
Keywords: Pb-acetate, Johnsen score, apoptotic index, viability of sperm.

Introduction

Acute heavy metal intoxications may damage central nervous function, the cardiovascular and gastrointestinal (GI) systems, lungs, kidneys, liver, endocrine glands, and bones[1,2]. Chronic heavy metal exposure has been implicated in several degenerative diseases of these same systems and may increase the risk of some cancers[3, 4].

Plumbum is a heavy metal that can cause male reproductive disorders[5]. It is associated with disruption of lead to endocrinesystem[2]. The testes are part of the endocrine system in males and testosterone functions in income as well as the process of spermatogenesis (formation of sperm)[5,6,7]. Al-Harrbyhave been conducted to determine the effect of lead (Pb) on weight of somebody organs as well as male reproduction in albino mice, 20 albino Swiss mice (Balb-c) used in this study, the animals were divided into four groups, treatment with lead at concentration (0, 0.1, 0.3 and 0.5) ppm for 7 days. The results showed no significantly ($P>0.05$) effect of treatment on body organs weight (liver, kidney and adrenal gland), except weight of spleen ($P<0.05$), while weight of testis, epidydmus, seminal vessels and prostate decreased significantly ($P<0.05$) when compared with control especially at concentrations 0.3, 0.5 ppm, and the sperm parameter (grade activity, concentration of

sperm in epidydmus and percentage of sperm abnormalities) showed significant effects (P<0.05) in all treatment groups in comparsion with control group, and this effects may be due to of Pb accumulation in reproductive system and sperm parameter[8].

Chronic exposure to heavy metals has been involved in several degenerative diseases of the same system and can increase the risk of some types of cancer[3,4]. Heavy metals in the environment exist everywhere[9]. Human risk of over exposure to environmental concentrations of naturally occurring (eg. arsenic-rich mineral deposits) or human activities (eg. lead or mercury pollution as a result of industrial pollution[10]. It is not possible to completely avoid exposure to toxic metals[11]. Even people who are not occupationally exposed to bring certain metals in their bodies as a result of exposure from other sources, such as food, beverages, or air[12,13]. It is, however, possible to reduce the risk of metal toxicity through lifestyle choices that reduce the uptake of harmful heavy metals, such as dietary measures that can improve safety metabolism or excretion of heavy metals ingested[14].The lead that enters the body and carried by the blood to enter the organs of the body and then accumulate in certain organs. More than 90% of the total amount of lead in the body is stored in bone tissue[15].

Plumbum in bone attachment there are two phases. First, the storage occurs in the bone surface which usuallyare exchangeable. Second, Pb together bound with hydroxyapatite crystals in bone, and it is more stable[16].The concentration of Pb in soft tissue varies greatly. Accumulation of Pb can be found in the liver, kidney, aorta and in low quantities in the muscles and the brain[15].The content of Pb at 10 ug / g in liver tissue and 25 ug / g in kidney tissue is diagnostic value for most animals[17].

A small number of the total Pb absorbed through the digestive tract, enter the general circulation, most of the Pb enters the portal circulation to the liver and partly eliminated through the gall bladder. Absorption of inorganic Pb at bat experiments reached 55-75% greater than in mice (±16%) and organic lead compounds more quickly absorbed in large amounts (±47%) in rats[18]. Studies in vivo and in vitro can be shown that more than 95% Pb in blood circulation bound to the erythrocytes, while the remaining bound to the plasma (approximately 3%). Plumbum is deposited on the surface of erythrocytes in the form of aggregate Pb phosphate[16].MostPb bound to albumin, α-globulins and other proteins. This bond also serves as a business deionized and detoxification. Protein itself will undergo denaturation by Pb, which then will cause disruption to the work of a particular enzyme. The average lifespan (mean half life) of Pb in blood ranged 36 ± 5 days[19]. Of lead can cross the placental barrier in both animals and humans, with the deposition of Pb in bone tissue, blood and fetal liver[20].

It has been reported that chitosan can absorb Pb before entering into the blood of rats in the surface channel intestinus[21]. Histopathological examination revealed degeneration of hepatocytes of rat liver treated with lead acetate. Chitosan supplement improve the detrimental effects of lead acetate in rats. Therefore research of chitosan as a preventive or curative material of desease is very important to know.

Experimental

This study used 25 albino rats, aged 8-11 wks with a weight of 150-250 g. It was experimental research with a completely randomized design (CRD), the which consists of 6 groups items, namely: K1 = control, control + Pb = K2, P1 = Pb + 0.5% chitosan, P2 = Pb + 0.75% chitosan, P3 = Pb + chitosan 1%, P4 = 1% chitosan. Each treatment was done during the 5-weeks. At the end of treatment, rat were sacrificed by anesthesia and dislocation of the neck. Testes were taken and fixed in Bouin solution 24 hrs,. dehydratedinethanol,embeddedinparaffinandstainedwithhematoxylinandeosin forhistopathologicalevaluation. The testicles sliced with a microtome thickness of 5 µm. Parameters observation was done by (a) measuring germ cells, seminiferous tubules each number. Preparations were stained with eosin and counted Hamatoksilin germinalnya cells (A spermatogonia, primary spermatocytes preleptoten, pachiten, and spermatids) at 100 round seminiferous tubules is counted under a microscope 400x magnification. After histologic preparations completed, the spermatogenic cell population was calculated. Calculation of spermatogenic cell populations carried out on 40 testicular seminiferous tubules (left and right) with Image Processing Software System Vixio connected to a microscope (Zeiss, Germany) equipped with a micrometer size at 10 x 40 magnification and the scale that has been measured (micrometers). To obtain the data spermatogenic cell populations, the actual correction to the Abercrombie correction factor[30]by the following formula:

$$P = A.\left(\frac{M}{L+M}\right)$$

Note: P = The observations that have been corrected, A = Result rough calculation of germ cells, M = Thick pieces preparations (microns), and L = average core diameter of germ cells (microns).(b) Johnsen score of geminal cells each seminiferous tubules. The testicular organ of rats were fixed in Bouin's fixative for 24hrs, dehydrated in ethanol, embedded in paraffin and stained with hematoxylin and eosin for histopathological evaluation. Tubule degeneration was evaluated in each group using Johnsen scores. This scoring system Is principally based on the progressive degeneration of germinal epithelium and subsequent loss of the most mature cell types during testicular damage. Johnsen score was also used to categorize the level of spermatogenesis in the control testes and compared with the scores of the Pb-induced testes. A grade from 1to 10 was given to each tubule cross-section according to the following criteria: 10═complete spermatogenesis and perfect tubules; 9═many spermatozoa present and disorganized spermatogenesis;8═only a few spermatozoa present;7═nospermatozoa but many spermatids present; 6═only a few spermatids present;5═nos permatozoa or spermatids but many spermatocytes present; 4═only a few spermatocytes present;3═only spermatogoniapresent;2═no germ cells but only Sertoli cells present;1═nogerm cells and no Sertoli cells present. (c) Apoptotic index. The apoptotic index in germ cells was determined by the terminal deoxynucleotidyl transferase deoxyuridinetriphosphate nick end labeling(TUNEL)assay.Paraffinsectionsof5-mmthicknessfrom the testes tissues were washed twice in phosphate-buffered solution (PBS) for 5 min. Following the incubation of slides with the permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at 40^{o}C and washing twice with PBS for 5 min, the labeling. reaction was performed using 50 ml of TUNEL reagent for each sample, except for the negative controls, in which the reagent was added without enzyme. The slides were then incubated for 1 h at 37^{o}C. After washing with PBS, the slides were incubated with a converter reagent for 30 min at 37^{o}C. Color development to localize the cells containing labeled DNA strand breaks was performed by incubating the slides with fast red substrate solution for 5 min. The TUNEL assays were conducted using a cell death detection kit (Roche, Mannheim, Germany). The apoptotic index was determined by a Zeiss Axioplan microscope (Thornwood, NY, USA). All seminiferous tubules from each group were observed, and the numbers of TUNEL-positive and TUNEL-negative seminiferous tubules were recorded. The seminiferous tubules with apoptotic cell signals were defined as positive seminiferous tubules. The positive seminiferous tubule rate was calculated by multiplying the ratio of positive tubules to the total number of tubules by 100%.(d) viability of sperm.Evaluation of spermatozoa viability was done by supravital staining, namely 10 µL semen + 10 µL solution of 0.05% eosin-Y and mixed objects on top of the glass and covered with a cover glass. Live spermatozoa colorless but colored dead. Then the observation with a light microscope at 400x magnification and counted against 100 spermatozoa. As a result expressed in terms of percent life obtained from the results for the number of viable spermatozoa with the total number of viable and not viable spermatozoa were multiplied by 100%[22].

Results and Discussion

Based on the results of measurements performed test parameters and results of the analysis of data collected, then the fourth parameter (germcellsnumber and Johnsen score of geminal cells every seminiferous tubules, Apoptotic index, and viability of sperm) there were significant differences when compared between treatments ($p<0.05$).

(a) Measuring germ cellsnumber each seminiferous tubules.

Measuring germ cells, seminiferous tubules each number was studied by using a light microscope. Analisys of germ cells number reported in Figure 1 Germ cells revealed that chitosan can number increase of the number of germ cells after the negative effects of Pb administration was Significantly ($P <0.05$). As in the treatment group P1, P2 and P3, where the administration of chitosan 0.5, 0.75 and 1% after administration of Pb can improve the number of germ cells to the normal state, since P2 and P3 are not significantly different from the control or K1. This is caused by the activity of chitosan that can capture Pb before being absorbed by the intestine through the capillaries in the tuft-tuft small intestine. As research Nadapdap[21], stated that the administration of chitosan can reduce the levels of lead in the blood was significantly ($p <0.05$) compared with administration of Pb alone (control) in rats (*Rattus* sp.). Suharsih[31] also reported that there is a decrease in the content of Pb in the blood so hemoglobin levels be increased when administration of chitosan in stages.

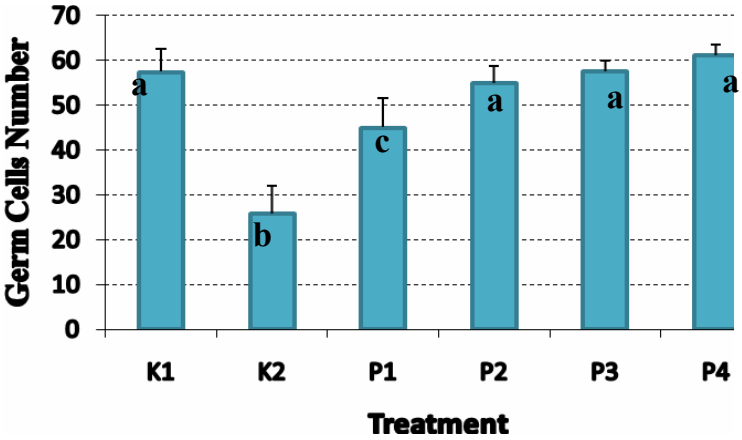

Figure 1. Histogram of the average of Germ cells number after treatment. Description: K1 = control, control+Pb = K2, P1 = Pb+0.5% chitosan, P2 = Pb+0.75% chitosan, P3 = Pb+1% chitosan, P4 = 1% chitosan; Lowercase letters are different at different treatments were different significantly ($p<0.05$) at the 5% level.

The decrease of Pb in the blood causes a negative effect on spermatogenesis will be smaller. So the germ cells produced by the process of spermatogenesis will be more or close to normal as the number of K1. As research Mendiola[23]suggest that the presence of lead and cadmium in the reproductive tract of men may be related to a moderate alteration of reviews their seminal parameters {Seminal volume (mL), Sperm (countx10^6),% motile sperm (A + B),% Normal morphology and total sperm count (x10^6)}.

(b) Johnsen score of geminal cells each seminiferous tubules

Measurement and analysis of apoptotic index can be seen in Figure 3 apoptotic index was tested by ANOVA through bootstrapping the data. The result shows p value of <0.05. Bonferroni test results Bootstrap for Multiple Comparisons showed that the differences were significant ($p<0.05$) between the administration of Pb (K2) with control (K1) and the administration of chitosan (P1-P4). This suggests that, Pb administration has caused the death of the germ cell apoptosis. Finally Pb will be a negative effect on cement products (sperm and semen). As reports Teliiman[24], that the overall Pb study results indicate that even moderate exposures to Pb (Blood Pb<400 pg / L) and Cd (Blood Cd<10 pg / L) can significantly reduce human semen quality without condusive evidence of impairment of male reproductive endocrine function.

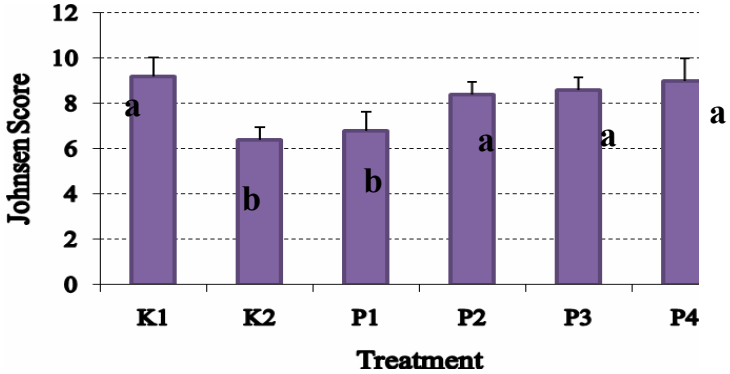

Figure 2.Histogram of the average of Johnsen Score after treatment. Description: K1 = control, control+Pb = K2, P1 = Pb+0.5% chitosan, P2 = Pb+0.75% chitosan, P3 = Pb+1% chitosan, P4 = 1% chitosan; Lowercase letters are different at different treatments were different significantly ($p<0.05$) at the 5% level.

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(c) Apoptotic index

Measurement and analysis of apoptotic index can be seen in Figure 3 apoptotic index was tested by ANOVA through bootstrapping the data. The result shows p value <0.05. Bonferroni test results Bootstrap for Multiple Comparisons showed that the differences were significant (p<0.05) between the administration of Pb (K2) with control (K1) and the administration of chitosan (P1-P4). This suggests that, Pb administration has caused the death of the germ cell apoptosis. Finally Pb will be a negative effect on cement products (sperm and semen). As reports Teliiman[24], that the overall Pb study results indicate that even moderate exposures to Pb (Blood Pb<400 pg / L) and Cd (Blood Cd<10 pg/L) can significantly reduce human semen quality without condusive evidence of impairment of male reproductive endocrine function.

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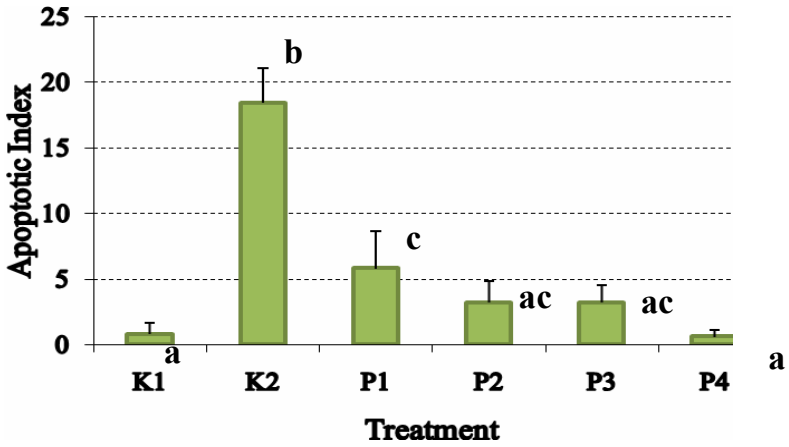

Figure 3.Histogram of the average apoptotic index of rats after multiple treatments. Description: K1 = control, control+Pb = K2, P1 = Pb+0.5% chitosan, P2 = Pb+0.75% chitosan, P3 = Pb+1% chitosan, P4 = 1% chitosan; Lowercase letters are different at different treatments were different significantly (p<0.05) at the 5% level.

Apoptotic index in the P2 group more significantly (administration of Pb), when compared with the control group and the other treatment (P <0.05). The apoptotic index relates the number of germ cells in the testes of mice. Conversely a decrease in apoptotic index will cause an increase in the number of testicular germ cells. According Lamondo[25] that Pb induced significant numbers of germ cells to undergo apoptosis in the seminiferous tubules of the rat. The same thing is stated by Wang[26] and Adhikari[27,] that the provision of Pb in rats give effect to the suppression of spermatogenesis causing widespread germ cell apoptosis.

Administration of chitosan 0.5, 0.75 and 1% in rats exposed to Pb showed a decrease in testicular apoptosis index. This means that more cells were not dying or more germ cell counts after administration of chitosan in this study. In research Silano[28], chitosan was able to depress the gliadin toxicity, and to lower caspase-3 activation and apoptosis and nitric oxide production. Caspase-3, -8, and -9 are active in apoptotic germ cells during the first wave of rat spermatogenesis. The extrinsic pathway of apoptosis may therefore play an important role in germ cell apoptosis in the trat[29].Caspase 3 is a executor for apoptosis of germ cells[6].

(d) Viability of sperm

Measurement and analysis of the viability of sperm can be seen in Figure 4 Viabilitu of sperm was tested by ANOVA through bootstrapping the data. The result shows p value <0.05. Through the Bonferroni test Bootstrap for Multiple Comparisons have shown the existence of significant differences (p<0.05) between the administration of Pb (K2) with control (K1) and the administration of chitosan (P1-P4). This suggests that, Pb administration has led to a decrease in sperm viability. The situation is caused by the presence of Pb effects that influence the supply of nutrients to the Sertoli cells or suppression of activity of the Leydig cell testosterone generated so that little or no maximal function. In accordance with research Nadapdap[21], that the administration of Pb in rats led to a lack of sperm viability and this is associated with a decrease in the amount of sperm produced. Furthermore, the administration of materials that can suppress Leydig cells work will affect spermatogenesis thus produced fewer sperm[7].

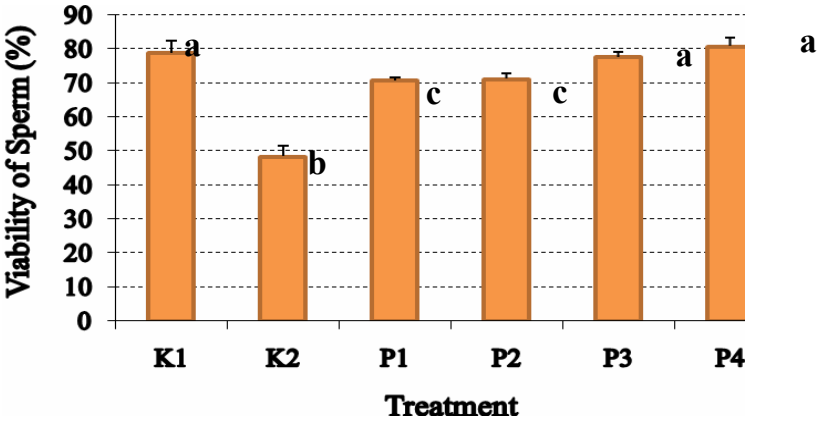

Figure 4.Histogram of the average viability of sperm of rats after multiple treatments. Description: K1 = control, control+Pb = K2, P1 = Pb+0.5% chitosan, P2 = Pb+0.75% chitosan, P3 = Pb+1% chitosan, P4 = 1% chitosan; Lowercase letters are different at different treatments were different significantly (p <0.05) at the 5% level.

Administration of chitosan in mice that had been given Pb (P1-P3), showed a reduction or elimination of negative effects of Pb on spermatogenesis, so that chitosan can increase the viability of sperm from mice. In research Silano[28], chitosan was Able to depress the lactate dehydrogenase release by the cells, whilst improving the cell viability.

Conclusions

It was concluded that chitosan can reduce the negative influence of Pb-acetate against measuring germcellsnumber each seminiferous tubules, Johnsen score of geminal cells each seminiferous tubules, apoptotic index andviability of sperm.

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