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# Effects of Chitosan on Sperm Quality of Lead Acetate-Induced Rats

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**Abstract** Escalation in blood lead level may decrease sperm quality. Chelating agent, such as chitosan, is known to reduce the detrimental effects from lead exposure. This research aims to analyze the effect of chitosan on sperm quality of lead acetate-induced rats. 30 rats were divided into six groups. All treatment groups, aside from control group, were induced by 175 mg/kg weight of lead acetate. Positive control group was treated with Ca<sub>2</sub>EDTA, while negative control group was only treated with lead acetate. Treatment group 1, 2, and 3 were treated with chitosan in gradual dose; 64, 32, and 16 mg/kg weight respectively. On the 31<sup>st</sup> day, the sperm quality was analyzed and the rats were terminated. Statistical result showed that positive control group and treatment groups had no significant difference from control group but was significantly different from negative control group. Research parameters, which were sperm count, motility of progressive sperm, immotile sperm, and normal sperm, were significantly different among the all treatment groups. However, another research parameter, which were nonprogressive sperm motility and sperm viability were not significantly different. Treatment group 1 was not different significantly from control and positive control groups. To sum up, chitosan improves the sperm quality of lead acetate-induced rats.

## 1. Introduction

Escalation in blood lead (Pb) level that surpasses normal threshold (10 µg/dl) may cause male reproductive problems. Common problems that occur in male reproductive system are decreased libido, abnormal sperms; indicated by decreased sperm count and motility, chromosomal defect, infertility, abnormalities in prostate function, and testosterone serum alteration. Functional disruption in reproductive system is directly correlated to the exposure concentration and duration. Escalation in blood lead level of 12,5 µg/dl causes infertility in men, while 6 µg/dl increase does not show any symptoms of infertility [1].

Chronic lead exposure is commonly found in industrial labours who work in companies that use lead as its raw materials. Most of the craftsmen in a brass factory in Pati [2], an accumulator smelting industry in Tegal [3], and several metal smelting factories have more than 10 mg/dl increase of lead level in their blood. These all cases took place in Central Java with most of the craftsmen being male. Even though there has not been a research focusing on emerging disruptions in reproductive system from the group, the craftsmen are susceptible to the disruption due to high blood lead level.

Male infertility cases are allegedly rising in the past few years. One of the essential factors that may cause this problem is pollution. In China, lead is included in one of main heavy metal contaminants



that poses danger to human and animal health. In its ionic form ( $Pb^{2+}$ ), lead will transform into free radicals that may be accumulated in male reproductive organs. In seminal plasma, lead accumulation reaches  $10 \mu M$ . Accumulation of  $Pb^{2+}$  ions impair spermatogenesis and sperm quality *in vivo*.  $Pb^{2+}$  ions also inhibit sperm functions, although the exact mechanism has not still been found [4]. Reduced sperm density, viability, and motility, is highly correlated to reduced zinc level [5]. The reduced zinc level is also significantly equivalent to increased lead accumulation. Aside from posing detrimental effects in physiological parameters of sperm, lead accumulation can also damage DNA structure and integrity. These occurrences will inevitably lead to significant decrease in male fertility [6].

Lead is not easily eliminated, oxidative, and tends to be accumulated in the body. It also affects physiological processes and takes an essential role in biochemistry, plasma chemistry, and other supporting chemical processes [7]. Sperm will function optimally if ROS and antioxidants are in equilibrium state. High ROS level and low endogen antioxidant level, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidation (GPx), will affect sperm count, viability, and motility [8].

One of the methods to decrease  $Pb^{2+}$  ions accumulation is by binding blood  $Pb^{2+}$  ions. The binding process is called chelation, while the binding agent is called chelating agents. Organic compound which is commonly used as chelating agent is EDTA (ethylene diamine tetra acetic acid). EDTA is administered through intravenous infusion in form of  $Ca_2EDTA$  in the dose of 2 gr/week [9]. EDTA is slowly administrated through intravenous infusion for two hours and in the next 12 hours, the urine is collected for examination. Heavy metals in urine is stated in microgram ( $\mu g$ ) per g of creatinine [10].

For industrial workers, who are continuously exposed to lead in their work environment, decent protections are required to diminish the detrimental effects from lead exposure. A certain compound that reduce the free radicals from lead exposure is needed. In this condition, intravenous EDTA administration for lead chelation will be impractical. Oral administrations preferred for its simplicity. Not only for curative purpose, but also for prevention. One of the most potential chelating agents to bind lead is chitosan. Chitosan is a polysaccharide that is composed of a long chain of glucosamine and is made from chitin deacetylations. Chitosan is also known to chelate divalent cations [11]. Chitosan is safe for consumption due to its nontoxic nature. It can also be orally administrated. Several researches have proven that chitosan is effective in binding  $Pb^{2+}$  ions and increasing endogenous antioxidative enzymes level [12]. Therefore, chitosan potential in protecting male reproductive organs from lead exposure needs to be examined.

## 2. Methods

### 2.1. Animal

Thirty adult male rats weighing 250-300 g and aged 10-12 weeks old were used in present study. The animals kept in wire bottomed cages in a room under standard condition with a 12 light-dark cycle at  $26 \pm 1^\circ C$ . They were provided with tap water and diet ad libitum. At the beginning and end of the experimental period, the body weight of each individual rat was measured. The Study was approved by the ethical committee of Department of Public Health Science on Faculty of Sport Science, Universitas Negeri Semarang.

### 2.2 Preparation of materials research

Chitosan 100 mesh dissolved in  $CH_3COOH$  2%,  $Ca_2EDTA$  dissolved in aquadestilata and Pb acetate dissolved in aquadestilata. All reagent of analytical grade manufactured by Merck. All materials given according the dose schedule by the oral route administration.

### 2.3 Experimental Design

Thirty adult male rats were randomly analyzed into six groups of five rats each. Grouping: 1). Normal Control Group: Rats received water and diet ad libitum. 2). Negative control Group: Rats received lead acetate (175 mg/kg BW), 3). Positive control Group: Rats received lead acetate (175 mg/kg BW) +  $Ca_2EDTA$  (50 mg/kg BW) [13], 4) Treatment 1 : Rats received lead acetate (175 mg/kg BW) +

chitosan (64 mg/kg BW), 5). Treatment 2: Rats received lead acetate (175 mg/kg BW) + chitosan (32 mg/kg BW), 6). Treatment 3: Rats received lead acetate (175 mg/kg BW) + chitosan (16 mg/kg BW). The Treatment were given for 30 days. Lead acetate and chitosan are given orally.

#### 2.4 Collection and preparation of samples

**2.4.1 Blood samples** Twenty-four hours after the last treatment (on 30<sup>th</sup> day), blood samples were collected via plexus retro orbitalis. They were prepared to measurement of blood Pb levels by the AAS (Atomic Absorption Spectrophotometer) method.

**2.4.2 Sperm functions analysis.** Rats were scarified by cervical dislocation on the 31<sup>st</sup> day, reproductive organs; testes, prostate, seminiferous tubules and Vas deferens were dissected out.

**2.4.3 Sperms count.** The epididymis and vas deferens were minced in 1ml of Phosphate Buffer Saline (PBS) (pH 7.2) to obtain a suspension. The sperm count was conducted in the filtrate as per standard method Neubauer's chamber. Briefly, an aliquot from the suspension (up to 0.5) was taken in leukocyte hemocytometer and diluted with PBS up to the mark 11. The suspension was well-mixed and charged in to Neubauer's counting chamber. The total sperm count in eight squares (except the central erythrocyte area) of 1 mm<sup>2</sup> each was determined and multiplied by 5x 10<sup>4</sup> to express the number of spermatozoa/ epididymis

**2.4.4 Sperms Motility.** The epididymal sperm content was obtained by maceration of the tail of the epididymis in 1ml of PBS. An aliquot of this solution was on the slide and percentage of motility was evaluated microscopically at a magnification of 400 x. Motility estimates were performed from the three different fields in each sample. The mean of the three estimations was used as the final motility score.

**2.4.5 Sperms Viability.** A drop of the epididymal content of each rat was mixed with an equal drop of 1% eosin stain prepared in accordance Metwally [14]. Thin films were made by spreading the stained content onto clean slides and quickly dried. Viable sperm remains colorless. One hundred sperm cells per rat were scored for determining the viability percent.

#### 2.5 Histological examination

Testis was taken for histological specimen. Testis was washed in physiological solution (NaCl 0,9%), weighed, and fixated in formalin 10%. Liver tissue specimen (6 µm) was made according to embedding method and stained with haematoxylin and eosin (HE). Liver specimen were made in Research Centre for Animal Disease and Veterinary in Yogyakarta.

#### 2.6 Analysis of Blood Lead Level

Blood sample was put into reaction tube with no anticoagulant. Blood sample was added with 5-10 mL of HNO<sub>3</sub> 65% solution and 2 mL of H<sub>2</sub>O<sub>2</sub> and then was destructed in the microwave. Destructed blood sample was then put into 50 mL beaker glass and was added with matrix modifier solution with deionized water. Lead level was then measured with AAS at the wavelength of 283,3 nm (SNI 2011).

#### 2.7 Analysis of Spermatogenic Layer Amounts in Testis

Testis tissue specimen was examined and analysed under the microscope with five different fields of view in magnification of 400x. In each field of view, the number of spermatogenic cell layer was examined. The data was descriptively analyse. The process took place in Pathology Anatomy Laboratory of Faculty of Medicine UGM.

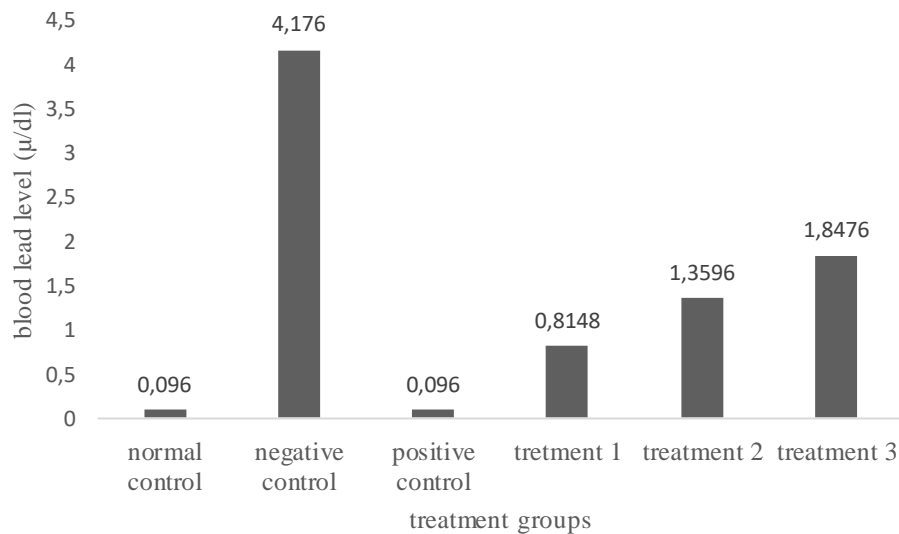
#### 2.8 Statistical Analysis

Sperm quality was analysed from sperm shape, count, and motility. The result of each calculation was analysed with one-way ANAVA. Significant difference was tested using Duncan Multiple Range Test. Data was analysed using SPSS 20 for Windows.

### 3. Result and Discussion

#### 3.1. Blood Lead Level

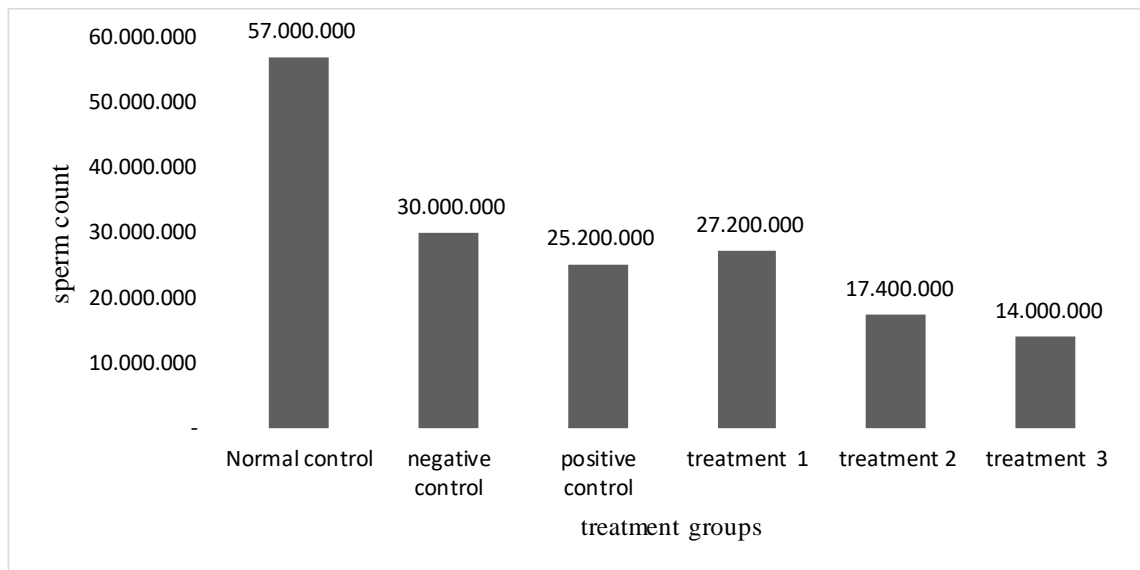
Comparison of blood lead level from each group is available on Fig 1. Difference in blood lead level from each treatment group indicates chitosan ability in chelating lead in the lead acetate-induced rats. Treatment group 1, which had the highest chitosan dose, displays low blood lead level compared to the other groups (except from EDTA group - positive control).



**Figure 1.** Blood lead level between all treatment groups.

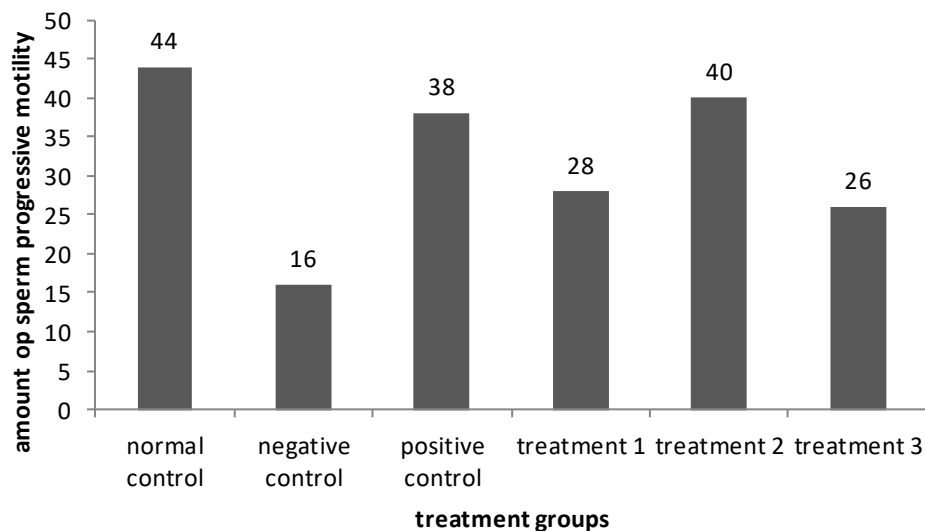
#### 3.2. Sperm Quality

Analysis result of sperm count from all treatment groups is available on Figure 2. According to Figure 2, control group has the highest sperm count, while the negative control group has the lowest count. The sperm count of positive control group is similar to groups treated with chitosan.



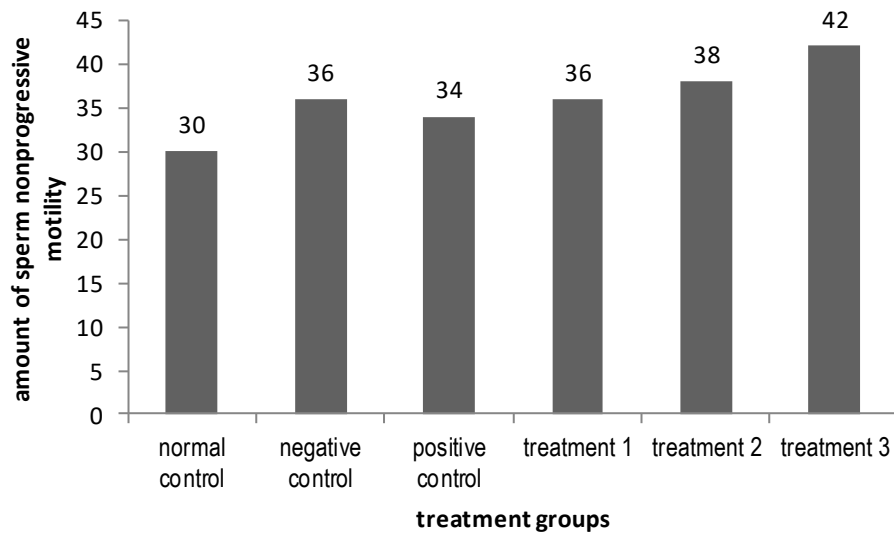
**Figure 2.** Comparison of sperm count from all treatment groups.

Sperm motility is grouped into 3 categories; progressive motility, nonprogressive motility, and immotile. The motility is considered progressive if the sperm moves forward. If the sperm only moves in its current place, the motility is considered to be nonprogressive. Immotile group belongs to sperm which does not move. The comparison of motile sperm amount is available in Figure 3.



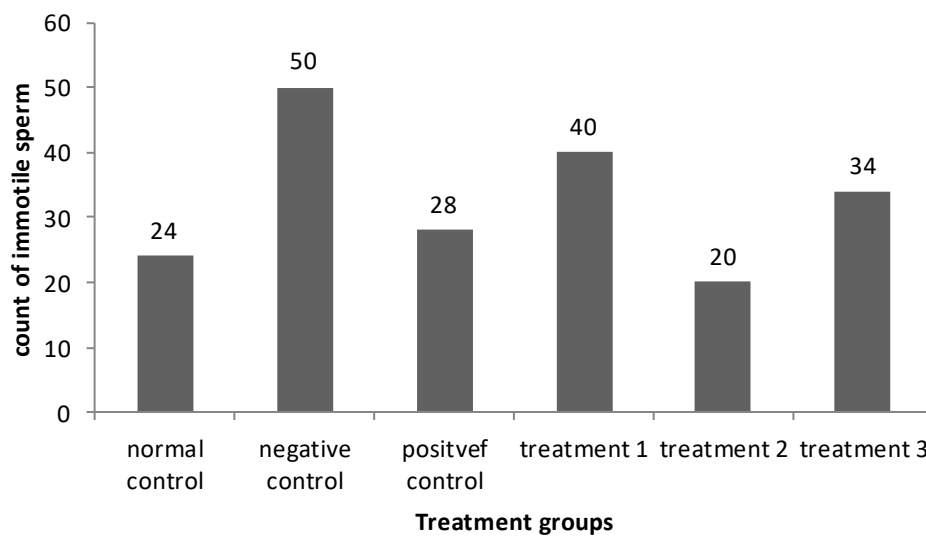
**Figure 3.** Comparison of sperm progressive motility from lead-acetate induced rats.

The result for sperm counts with nonprogressive motility is available in Figure 4.



**Figure 4.** Comparison of sperm count from all treatment groups with nonprogressive motility.

The result for immotile sperm count is available in Figure 5. Figure 5 shows variation in immotile sperm count between all treatment groups.



**Figure 5.** Comparison of immotile sperm count.

In order to ensure the presence of significant difference between all treatment groups, one-way ANOVA was employed. Homogeneity and normality test were performed for all treatment groups. The result was considered significantly different if the significance score  $p < 0,05$ .

**Table 1.** Result for One-Way ANAVA on all research variables.

Research Variable	F value	Sig (p<0,05)
Blood lead level	4.862	0.003*
Sperm count	34.309	0.00**
Sperm viability	3,040	0.029*
Progressive motility	4,699	0.004*
Nonprogressive motility	0,828	0.543
Immotile	6,345	0.01*

Note: \*Significantly different, \*\*Very significantly different

The result of one-way ANAVA as in Table 1 shows that almost all research variables are significantly and very significantly different (aside from nonprogressive motility variable). All variables with significant differences were then tested with Duncan Multiple Range Test. The result is available on Table 2.

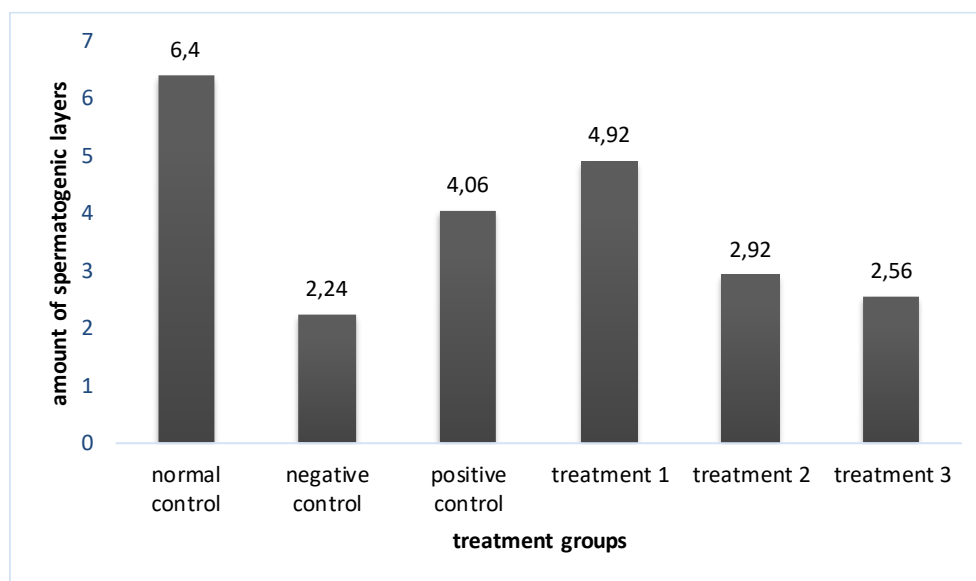
**Table 2.** Result for Duncan Multiple Range Test

Treatment Groups	Research Variables				
	Blood lead level	Sperm count	Sperm viability	Progressive motility sperms	Immotile sperms
Normal control	0,09600 <sup>a</sup>	5.70 x 10 <sup>7</sup> <sup>d</sup>	95.800 <sup>b</sup>	44.00 <sup>d</sup>	24.00 <sup>ab</sup>
Negative Control	4.1760 <sup>b</sup>	1.4 x 10 <sup>7</sup> <sup>a</sup>	79.000 <sup>a</sup>	16.00 <sup>a</sup>	50.00 <sup>d</sup>
Positive Control	0,09600 <sup>a</sup>	3.00 x 10 <sup>7</sup> <sup>c</sup>	86.400 <sup>ab</sup>	38.00 <sup>bd</sup>	28.00 <sup>abc</sup>
Treatment 1	0,81480 <sup>a</sup>	2.04 x 10 <sup>7</sup> <sup>ab</sup>	85.400 <sup>ab</sup>	28.00 <sup>abc</sup>	40.00 <sup>cd</sup>
Treatment 2	1,35960 <sup>a</sup>	2.72 x 10 <sup>7</sup> <sup>bc</sup>	93.400 <sup>b</sup>	42.00 <sup>cd</sup>	20.00 <sup>a</sup>
Treatment 3	1,84760 <sup>a</sup>	1.74 x 10 <sup>7</sup> <sup>a</sup>	84.600 <sup>ab</sup>	26.00 <sup>abc</sup>	34.00 <sup>bc</sup>

Note: Same alphabet code shows no significant difference.

According to DMRT test, the difference in sperm quality from each group is related to the production rate of sperm. In order to examine the histological condition of testis that affect the sperm quality, an analysis on the amount of spermatogenic layers was conducted in Laboratory of Pathology Anatomy in Faculty of Veterinary Universitas Gadjah Mada. The comparison of the amount of spermatogenic layers between all treatment groups is shown below in Figure 7.





**Figure 7.** Comparison of the amount of spermatogenic layers in all treatment groups.

In order to ensure if there is any significant difference, one-way ANAVA test is employed. The result showed the F value of 16, 294 with significance of 0,00. It implies that there is a highly significant difference between all treatment groups. Duncan Multiple Range Test (DMRT) was performed to analyse which group that is the most different or similar among all the groups. The result is shown in Table 3.

**Table 3.** The result for Duncan Multiple Range Test (DMRT) in the amount of spermatogenic layers.

Treatment Groups	DMRT Result
Negative control	2.240 <sup>a</sup>
Treatment 3	2.560 <sup>a</sup>
Treatment 2	2.920 <sup>ab</sup>
Positive Control	4.060 <sup>bc</sup>
Treatment 1	4.920 <sup>c</sup>
Normal control	6.400 <sup>d</sup>

Note: Same alphabet code shows no significant difference.

Result shows that lead induction affect the amount of spermatogenic layers on rat testis. The number of spermatogenic cells become the reference to figure out the sperm quality. Sperm quality can also be identified from the sperm count and viability. In this research, the effect of lead upon sperm quality is known from its effect on sperm count and viability and the amount of spermatogenic layers inside the seminiferous tubules.

Disruption from lead on sperm quality in this research is in accordance with the research reported by Yulianto [15], which showed that lead acetate-induced rats had decreased sperm quality. Lead acetate-induced rats had fertility disruption, indicated by decreased testis weight, sperm count, and viability.

The negative effect from lead upon sperm may be minimalized by blood-testis barrier. It prevents blood from coming into testis, so that the lead exposure will be insignificant. However, chronic lead

exposure will still penetrate the blood testis barrier, proven by decreased amount of spermatogenic layers on rats with high blood lead level [1].

As free radicals,  $Pb^{2+}$  ions can damage DNA on sperms by impairing purine and pyrimidine base. ROS causes single and double strand DNA breaks that furtherly cause cross-links and chromosomal rearrangements [16]. ROS also affects sperm motility. Hyperactivation will increase if sperms are exposed to ROS. ROS stimulates tyrosine phosphorylation in flagellum that causes hyperactivation (Wright et al. 2014). Aside from hyperactive or progressive sperms, there is also immotile sperms. Immotile sperms are derived from the escalation of lipid peroxidation. ROS will induce damage in mitochondrial DNA that decrease ATP and energy availability [17].

In order to overcome ROS, endogenous antioxidative enzymes such as SOD, CAT, and GPx, are produced. High level SOD is found in Sertoli cells that nourish the sperms from spermatogenesis to mature state. SOD is also found in epididymis and seminal plasma. Decreased SOD level is an indication of oxidative stress, DNA damage, and inhibited sperm motility [8].

Detrimental effects from lead exposure upon sperm quality may be minimized by chitosan. The result showed that chitosan is able to protect the testis that will preserve the sperm quality. Chitosan is also able to overcome free radicals by chelating  $Pb^{2+}$  ions that may decrease the negative effects. This research shows that chitosan chelating properties in on par with EDTA. This is also proven by inexistent significant difference of decreased lead level between EDTA-treated group (positive control) and all groups that are treated with chitosan. It also lowers down the lead level in testis and other surrounding male reproductive organs that also reduce the disruption in testis. To conclude, this research proves that chitosan provides protective effects upon male reproductive organs that are exposed to lead.

As commonly known, EDTA is widely used to overcome metal poisoning, including lead. However, in several cases, EDTA in certain doses may cause hypersensitivity, symptomatic hypocalcaemia, and thrombophlebitis [18]. Therefore, chitosan, which is also nontoxic, has the potential for further development as alternative natural compound to overcome the negative effects of lead exposure.

#### 4. Conclusion

Lead induction in rats decrease the amount of spermatogenic layers on testis that affects sperm quality. Sperm quality is identified from sperm count, viability, and motility. Chitosan treatment escalates the rat's sperm quality. Chitosan chelating activity decreases the free radical's level in blood. Decreased free radicals level prevents damage in spermatogenic layers in testis that may improve the sperm quality from lead acetate-induced rats.

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