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Zinc Sulfate and α-Tocopherol Supplementation Enhance Reproductive Performance in Male Albino Rats (*Rattus norvegicus*) With Lead Acetate Toxicity

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ABSTRACT

Metal toxicity from lead affects reproductive organ function by activating reactive oxygen species processes. This study aims to see how α-tocopherol and zinc sulfate (ZnSO4) affect gonads, liver, follicle-stimulating hormone, luteinizing hormone, spermatogenesis (the amount of spermatogonia, spermatocytes, and spermatids), and Leydig cells in male albino rats (*Rattus norvegicus*) exposed to lead acetate $Pb(CH_3COO)_2$. The samples used were 25 male Wistar rats aged 4 months, separated into five groups. All treatment groups were exposed to $Pb(CH_3COO)_2$ at a level of 50 mg/kg body weight (BW). The T1 group was given a dosage of 100 mg/kg BW of α-tocopherol. The ZnSO4 was given to the T2 group at a dose of 0.54 mg/kg BW. Meanwhile, the T3 group was given a mixture of ZnSO4 at 0.54 mg/kg BW and α-tocopherol at 100 mg/kg BW. All treatment is given orally for 30 days. ELISA test was carried out to determine the level of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in a blood plasma sample. Histopathological observations made on the liver included counting damaged cells and seminiferous tubules including counting the amount of spermatogonia, spermatocytes, spermatids, Sertoli cells, and Leydig cells. Using SPSS 20 software, the collected data were analyzed using analysis of variance, followed by Duncan's test with a 95% simultaneous confidence level. The highest average levels of FSH and LH in the T3 group were 3.6162 mIU/mL and 14.9658 mIU/mL. The finding showed that $Pb(CH_3COO)_2$ caused disruptions in the spermatogenesis and Leydig cell processes. Exogenous antioxidants in combination with ZnSO4 and α-tocopherol had a significant effect on enhancing reproductive performance in animals exposed to $Pb(CH_3COO)_2$.

Keywords: Albino Rats, Antioxidant prevention; Lead acetate toxicity; Oxidative stress; Reproductive health

INTRODUCTION

Lead (Pb) has toxic effects and causes health problems even at very low concentrations (Dorostghoal *et al.*, 2011). Its effect on the body depends not only on the concentration but also on its structure and solubility, the ability to form redox and complexes, the route taken into the body, and the exposure amount in the environment. The main reason for their toxic effects on the body is disturbances in intracellular metabolic processes, which lead to DNA damage, apoptosis, and lipid or protein degradation (Bhardwaj *et al.*, 2021). Lead has the most influential toxicity among other heavy metals that have a tendency to catalyze oxidation reactions and give rise to the formation of reactive oxygen species (ROS) (Wiyasihati *et al.*, 2016). Direct exposure to heavy metals via any route like oral, per inhalation, or parenteral in laboratory animals has been shown to have a negative influence on the male reproductive system (Elsheikh *et al.*, 2020).

A number of previous reports have shown that lead exposure affects semen quality at levels of >40 μg/dL in the blood. Increased Pb levels are associated with decreased libido and increased semen disorders. Exposure to inorganic lead of >40 μg/dL in the blood impairs male reproductive function by reducing sperm count, volume, and density or altering sperm motility and morphology (Awadalla *et al.*, 2011). The decrease in total sperm count with increased blood lead levels and the Pb concentration in semen is inversely proportional to the total sperm count, ejaculatory volume, and serum testosterone but not with sperm concentration (Gandhi *et al.*, 2017). Moderate exposure to Pb (BPb < 400 μg/L) and cadmium (Cd) (BCd < 10 μg/L) can significantly reduce semen quality without conclusive evidence of impaired male reproductive endocrine function (Famurewa & Ugwuja, 2017). Lead affects reproductive function by inducing ROS, which is associated with infertility in 30%–80% of cases (Li *et al.*, 2013). Oxidative stress is caused by an increase in ROS production as a result of Pb accumulation in the body (Lee *et al.*, 2020). Animal studies have shown that environmental pollutants such as Pb are reproductive toxins, and they accumulate in the testes and/or epididymides and impair endocrine and reproductive functions (Kumar, 2018). It has been reported that various damages such as decreased sperm count, motility, viability, testosterone levels, antioxidant enzyme activity, and spermatogenic function and an increase in abnormal sperm rate, lipid peroxidation (LPO), and apoptosis occur as a result of the toxicity of the heavy metal testicles, which have an important role in reproductive toxicology (Leidens *et al.*, 2018).

The hypothalamus is affected by oxidative stress, and Pb has a toxic gonadal effect that suppresses the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Asadi, 2017). Both of these hormones are essential for spermatogenesis and reproductive capacity. The LH role is to stimulate Leydig cells to produce testosterone. When LH secretion decreases, so does Leydig cell function (Jungwirth *et al.*, 2001). Since Leydig cells play a crucial role in the process of spermatogenesis and have very active cell activity, they are very sensitive to Pb contamination (Diana *et al.*, 2017). The FSH stimulates Sertoli cells to release ABP (androgen-binding protein), which is responsible for binding testosterone. When FSH is disrupted, the Sertoli cells are also disrupted, which results in a decreased ABP secretion. As a result, ABP is unable to bind to testosterone. Any disruption in the interaction of testosterone, FSH, and LH will disrupt the spermatogenesis process. A disruption in the spermatogenesis process is one of the causes of infertility or reproductive health problems (Mirania, 2019).

Antioxidant induction against the oxidative stress effects of Pb acetate is an approach to reduce toxicity (Flora *et al.*, 2012). The α-tocopherol is an organic compound that is needed as an antioxidant and maintains fertility. It works as an antioxidant in the process of spermatogenesis, which neutralizes free radicals produced by aerobic metabolism (Ogbuewu *et al.*, 2010). Silalahi *et al.* (2016) found that α-tocopherol supplementation can increase the spermatozoa quality after cigarette smoke exposure. Moreover, Jegede *et al.* (2015) found that α-tocopherol supplementation improves the spermatozoa quality in albino rats (*Rattus norvegicus*) exposed to Pb.

Zinc (Zn) is a protective antioxidant against the reproductive toxicity associated with Pb toxicity (Hasanein *et al.*, 2018). It is a component of superoxide dismutase, an enzyme that protects cells from free radical damage. It produces an enzyme system that helps in the neutralization of free radicals. It also regulates the stability of chromatin and spermatozoa cell membranes, protects the testes from degenerative changes, and serves as an antioxidant (Murarka *et al.*, 2015). Silalahi *et al.* (2016) discovered that supplying α-tocopherol and Zn improved the spermatozoa quality. Furthermore, Zn can improve spermatozoa quality because of its function and role in the

reproductive system, especially as an antioxidant that increases the androgen hormone (testosterone) in Leydig cells. Thus, it increases the normal process of spermatogenesis. Zinc sulfate (ZnSO4) has an important role in the maturation process of spermatozoa in the epididymis (Prasad & Bao, 2019). The purpose of this study is the improvement of reproductive performance due to the combined effect of both antioxidants that have a simultaneous mechanism of action.

MATERIALS AND METHODS

Animal ethics

Animal ethics has been carried out with certificate number 502/HRECC.FODM/VIII/2021. Animal ethics testing is required to ensure that all acts on experimental animals are in accordance with the standard operating procedures. The medical ethics test was administered at Universitas Airlangga A Campus, Surabaya, Indonesia.

Chemicals and reagents

Pro-analytical grades lead acetate ($Pb(CH_3COO)_2$), ZnSO4, and α-tocopherol were obtained from Sigma–Aldrich, Germany. The ELISA kits used were commercial kits purchased from Hufeng Chemical Co., Ltd, Nantong, China, with cat. nos. F14574-A, F14573-A, and F4421-A.

Experimental animal

A total of 25 male albino rats with a body weight (BW) of 150–200 g and an age of 4 months were used in the study. The maximum treatment of the experimental animals is taken to ensure that there are no other factors affecting the study other than the treatment given. The rats were adapted for a week for the acclimatization phase of experimental animals to the environment, cage, and feed. They were placed in cages with free access to water and food using ad libitum feeding. The temperature in the cage is set to room temperature.

Treatment design

The $Pb(CH_3COO)_2$ dose used in this study was 50 mg/kg BW dissolved in 0.5 mL aquades, thus causing albino rats to be exposed to oxidative stress. The effective dose of α-tocopherol was 100 mg/kg BW dissolved in 0.5 mL corn oil and the effective dose of ZnSO4 was 0.54 mg/kg BW dissolved in 0.5 ml aquades. A total of 25 rats were randomized into five groups: control (without treatment), group T0 ($Pb(CH_3COO)_2$ at 50-mg/kg BW), group T1 ($Pb(CH_3COO)_2$ at 50-mg/kg BW and α-tocopherol at 100 mg/kg BW), group T2 ($Pb(CH_3COO)_2$ at 50 mg/kg BW and ZnSO4 at 0.54 mg/kg BW), and group T3 ($Pb(CH_3COO)_2$ at 50 mg/kg BW and combination of ZnSO4 at 0.54 mg/kg BW with α-tocopherol at 100 mg/kg BW). The dose is orally administered (gastric probe) for 30 days.

Sample collection

On the 31st day, before sacrified, the animals were enumerated, and 2 mL of blood was taken from the lateral saphenous vein using a 23-gage sterile needle and injector. Blood flow is stopped by applying pressure with sterile gauze to achieve hemostasis. The blood sample was used for the ELISA test to measure FSH and LH levels. After blood collection, the rats will be sacrificed for the liver organ, testicular organ, and epididymal tissue sample collected. Euthanasia was administered using an intraperitoneal mixture of xylazine 10 mg/kg BW and ketamine hydrochloride 80 mg/kg BW followed by cervical dislocation. The euthanized rats were placed on a board in a dorsal recumbency position. The rats were then dissected, and the liver and testicular organs were cleansed with physiological sodium chloride before being put in a 10% formalin buffer for 24 h to make histological preparations. The epididymal tissue sample was used for sperm examinations.

FSH and LH measurements

Blood samples were taken as much as 2 mL in each rat and centrifuged at 3000 rpm for 15 min to obtain blood plasma. The storage of blood plasma collection is at −80°C. Analysis of FSH and LH levels in the blood plasma of male albino rats is done using ELISA kits (Hufeng Chemical Co., Ltd, Nantong, China, with cat. nos. F14574-A, F14573-A, and F4421-A, respectively) according to the manufacturer's instructions (Mozaffari *et al.*, 2020).

Histopathology of organs

Histopathological preparations are done using the liver and testicular organs collected from sacrificed rats. The process of making histopathology slides begins with fixation, trimming, pre-embedding, embedding, sectioning, staining, and coverslipping, and ends with step paraffin blocks. The paraffin method was used to prepare testicular microanatomy specimens, which were then stained with hemotaxylin-eosin (Elrich). Observations were made on the seminiferous tubules using a 400× magnification microscope

with five fields of view, which comprised of spermatogenic cells (spermatogonia, spermatocytes, and spermatids), Sertoli cells, and Leydig cells. The spermatogenic activity was observed in the seminiferous tubules from spermatogenic cells. Spermatogonia cells are found at the bottom with large cell shapes with large nuclei and pale cytoplasm. On the other hand, spermatogonia cells are small in size. Primary spermatocytes are larger than spermatogonia cells, have a circular shape, without walls in the nucleus, and have clearly marked chromosomes. Spermatid cells have a small cell size, have little cytoplasm, and are located more in the middle. The calculation is done by observing the number of Leydig cells found in the interstitial part of the testis or between the seminiferous tubules. The calculation is performed by counting the number of cells in three to four seminiferous tubules in five fields of view. Leydig cells have a round histopathological appearance with a dark nucleus (nucleolus) in the center. It is found in the interstitial seminiferous tubules. Leydig cells are polymorphic cells clustered around blood vessels, with a polyhedral-shaped nucleus (Suvana *et al.*, 2019). The average diameter of seminiferous tubules was determined for histomorphometric analysis by randomly measuring ten circular tubules in the transverse sections of each animal.

Liver histopathology preparations were observed with pathological changes. Although the liver is not a concern of this study, it was used as a reference in determining any cytotoxic effect of the $Pb(CH_3COO)_2$ doses applied (Daoud *et al.*, 2021).

Sperm examinations

Sperm motility was evaluated in a Tris buffer suspension using a light microscope heated at 38°C. A 50-ml suspension was added on the slide and covered with a cover glass. Three microscopic fields were examined for each sample, and the average of them was used as the final motility score (%). The prepared peripheral smear slide is used to assess sperm morphometrics, sperm abnormalities, and sperm viability levels. For this, 25-ml Tris buffer-sperm suspension was mixed with 50-ml eosin-nigrosine stains (1.67% eosin, 10% nigrosine, and 0.1-m sodium citrate), and a peripheral smear slide was prepared. The slide is then viewed under a light microscope at 400× magnification. A total of 200 sperm were examined per slide and expressed as percent (Güner *et al.*, 2020).

Data analysis

The data obtained from the treatment results were analyzed using SPSS V20 (v. 22.0, IBM Corp., Armonk, NY, USA). For data that was normally distributed and had homogeneous variance, the one-way analysis of variance (ANOVA) test was used to assess observations. If there is a significant difference, Duncan's test is performed. The results are presented to be mean ± standard error of means (SEM). A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

FSH and LH levels

Based on the results of data analysis using the one-way ANOVA test (Table I), there was a significant difference in FSH and LH levels between the control and T0 groups. The control group that was given a treatment in the form of aqueous and corn oil showed a higher average amount of FSH and LH levels when compared to the T0 group with the lowest FSH and LH levels. The T0 group is the group with the lowest average FSH (0.89 ± 0.10) and LH (3.96 ± 1.04) levels. The T3 treatment group was the treatment group with the highest average FSH (3.62 ± 0.08) and LH (14.96 ± 0.44) compared to the other group. In this study, it was proven that the activity of α-tocopherol, ZnSO4, and its combination can affect FSH and LH levels in the blood plasma of male albino rats (*Rattus norvegicus*) exposed to $Pb(CH_3COO)_2$. Results showed that the T3 treatment group given ZnSO4 and α-tocopherol and exposed to $Pb(CH_3COO)_2$ had higher FSH and LH levels than the T0 treatment group with $Pb(CH_3COO)_2$ exposure only. The contents of α-tocopherol and ZnSO4 which are antioxidants are able to provide a therapeutic effect on increased FSH and LH levels.

Liver histopathology

The scoring results showed that liver damage varied from severe to no changes. In the histopathological analysis of the liver, the control treatment showed no histopathological changes. Meanwhile, T0 treatment showed quite severe changes in the form of congestion, infiltration of inflammatory cells, parenchymatous degeneration, hydropic degeneration (cloudy swelling), lipid degeneration, and necrosis. Improvements in the histopathological picture were seen in the section obtained from rats treated with $ZnSO_4$, α-tocopherol, and their combinations.

Table I. Average FSH and LH levels in the control and treatment group

Treatment	FSH ± SEM (mIU/mL)	LH ± SEM (mIU/ml)
Control (K)	3.17 ± 0.13^{c}	12.69 ± 1.31^{b}
T0	0.89 ± 0.10^{a}	3.96 ± 1.04^{a}
T1	1.67 ± 0.20^{b}	4.12 ± 0.51^{a}
T2	3.24 ± 0.06^{c}	13.44 ± 0.59^{b}
T3	3.62 ± 0.08^{d}	14.96 ± 0.44^{b}

N: ^{a,b,c} Different superscript column indicate significant differences among means
K: Not given lead acetate, zinc sulfate, and α-tocopherol
T0: Administered lead acetate without zinc sulfate and α-tocopherol
T1: Administered lead acetate and α-tocopherol
T2: Administered lead acetate and zinc sulfate
T3: Administered lead acetate, zinc sulfate, and α-tocopherol

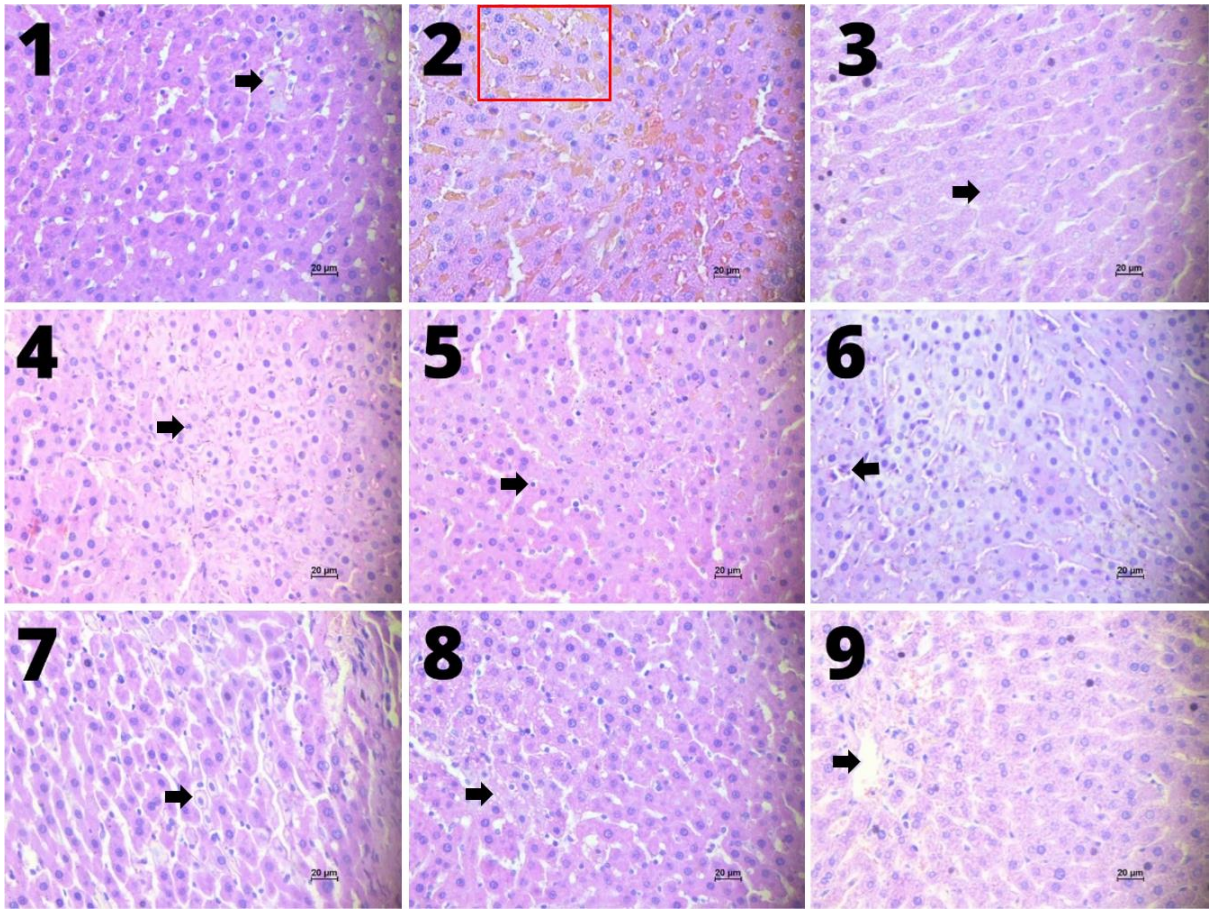

Figure 1. Liver histopathology. The numbers on the images represent pathological abnormalities that have been discovered. (**1**) Liver section, arrow showing necrosis of hepatocytes with marked nuclear hypertrophy (Group T0), (**2**) liver section, red square boxes showing congestion (Group T0), (**3**) liver section, arrow showing mild necrosis (Group T0, T1, and T2), (**4**) liver section, arrow showing apoptotic cells (Group T0, T1), (**5**) liver section, arrow showing cytoplasmic degeneration and some aggregation of inflammatory cells (Group T0), (**6**) liver section, arrow showing apoptotic cells as well as polyploid hepatocytes (Group T0), (**7**) liver section, arrow showing fatty degeneration (Group T0), (**8**) liver section, arrow showing cloudy swelling (all Treatmen Group), and (**9**) liver section, arrow showing congestion (Group T0).

Table II. The average number of rat tubules seminiferous diameter, spermatogenic cells, Leydig cells, and Sertoli cells exposed to $Pb(CH_3COO)_2$ in the control and treatment groups

Treatment	Seminiferous tubule Diameter ± SEM (µm)	Spermatogonia ± SEM (n)	Spermatocyte ± SEM (n)	Spermatid ± SEM (n)	Leydig cells ± SEM (n)	Sertoli cells ± SEM (n)
K	153.26±5.77^{ab}	73.56±0.72^{ab}	76.80±0.92^{ab}	108.80±0.98^{ab}	44.60±0.05^{ab}	6.00±0.44^{ab}
T0	147.84±5.71^{a}	70.00±0.43^{a}	72.60±0.27^{a}	103.60±0.50^{a}	42.00±0.67^{a}	4.60±0.51^{a}
T1	169.34±5.39^{b}	76.60±0.18^{abc}	77.80±0.58^{b}	110.00±0.44^{ab}	47.20±0.38^{bc}	8.00±0.77^{bc}
T2	210.69±10.53^{c}	78.60±0.70^{bc}	79.40±0.79^{b}	116.20±0.59^{bc}	47.60±0.30^{bc}	8.80±1.49^{c}
T3	214.74±5.32^{c}	80.80±0.76^{c}	84.80±0.20^{c}	123.40±0.54^{c}	49.60±0.72^{c}	9.00±0.31^{c}

N: ^{a,b,c} Different super script column indicate significant differences among means
K: Not given lead acetate, zinc sulfate, and α-tocopherol
T0: Administered lead acetate without zinc sulfate and α-tocopherol
T1: Administered lead acetate andα-tocopherol
T2: Administered lead acetate and zinc sulfate
T3: Administered lead acetate, zinc sulfate, and α-tocopherol

The T3 treatment provides the best therapeutic effect even though it is not yet close to the normal histopathological picture (control) (Figure 1).

Testicular organ histopathology

Based on the results of statistical analysis using ANOVA, there was no significant difference in the number of spermatogonia, spermatocytes, and spermatids between groups K (control) and T0. The group K (control), which got no treatment, had a larger mean number of spermatogenic cells than group T0, which had the lowest number of spermatogenic cells. The T0 group had the lowest average spermatogenic cell values, including spermatogonia (70.00), spermatocytes (72.60), and spermatids (103.60). The T3 treatment group had the highest average value, with an average of 80.80, 84.80, and 123.40 for spermatogonia, spermatocytes, and spermatids, respectively. The values listed (Table II) show that there are differences in the results of each treatment group. Treatment groups T1, T2, and T3 showed an increase in spermatogenic cells when compared to groups K and T0. The statistical analysis based on the ANOVA test revealed a significance level of 0.021 for spermatogonia, 0.000 for spermatocytes, and 0.004 for spermatids ($P < 0.05$), which indicates that there was a significant difference in the increase in the number of spermatogenic cells between the treatment groups.

Seminiferous tubule in research treatments given single and combination therapies showed an increase in diameter, which showed a significant difference compared to T0, namely, mice given lead alone without antioxidant treatment. The number of Leydig and Sertoli cells in groups K and T0 was not significantly different based on statistical analysis. However, the K group had a larger average number of cells compared to the T0 group, which had the lowest average number of Leydig and Sertoli cells. The T3 treatment group has the highest average value. Table 2 shows that each treatment group obtains different results. Treatment groups T1, T2, and T3 showed an increase in Leydig and Sertoli cells when compared to groups K and T0. The results of statistical analysis obtained based on the ANOVA test revealed a significance level of 0.012 ($P < 0.05$), indicating that there was a significant difference in the increase in the number of Leydig and Sertoli cells between the treatment and control groups. Duncan's test was used for additional testing. The results of Duncan's test showed that the K group was not significantly different from the T0 group. On the other hand, it was significantly different from the treatment groups T1, T2, and T3. The T3 group had results that gave a good therapeutic effect.

Sperm examinations

Based on the results of ANOVA statistical analysis, the average morphometric sperm rate, motility, viability, and abnormalities (Table III). The T3 group presented with $Pb(CH_3COO)_2$ that was given ZnSO4 and α-tocopherol treatment had the highest average tail length and head length of spermatozoa and had significant differences in control and other treatment groups (T0, T1, and T2). In addition, in the T3 group, the total length of the spermatozoa had a high number, significantly different from the T0 treatment, and no significant difference compared to the control and T1 and T2 treatment groups.

Table III. The average number of sperm morphometric, motility, viability, and abnormalities of rat exposed to lead acetate in the control and treatment groups

Treatment	Sperm head length ± SEM (µm)	Sperm tail length ± SEM (µm)	Total sperm length ± SEM (µm)	Sperm motility ± SEM (%)	Sperm viability ± SEM (%)	Sperm abnormalities ± SEM (%)
K	16.31±0.33^{a}	157.52±2.92^{a}	174.07±3.12^{ab}	73.56±1.72^{ab}	30.80±1.74^{a}	9.20±1.65^{bc}
T0	16.01±0.51^{a}	156.98±4.34^{a}	167.40±7.65^{a}	70.00±1.43^{a}	30.00±1.09^{a}	11.20±1.24^{c}
T1	15.87±0.31^{a}	162.23±2.17^{a}	178.10±1.98^{ab}	76.60±1.18^{abc}	36.60±1.46^{bc}	8.60±0.92^{bc}
T2	15.98±0.41^{a}	163.07±2.83^{a}	178.21±1.63^{ab}	78.60±1.70^{bc}	32.00±2.93^{ab}	6.80±0.86^{ab}
T3	18.26±0.27^{b}	172.45±1.24^{b}	190.23±2.16^{b}	80.80±1.76^{c}	38.20±1.01^{c}	4.80±1.24^{a}

N: ^{a,b,c} Different superscript column indicate significant differences among means
K: Not given lead acetate, zinc sulfate, and α-tocopherol
T0: Administered lead acetate without zinc sulfate and α-tocopherol
T1: Administered lead acetate and α-tocopherol
T2: Administered lead acetate and zinc sulfate
T3: Administered lead acetate, zinc sulfate, and α-tocopherol

The T0 group exposed to $Pb(CH_3COO)_2$ that was given aquadest and corn oil was the group with the lowest average of the tail length, head length, and total length of the spermatozoa. The results of statistical analysis obtained based on the ANOVA test showed significant results on the tail length of spermatozoa (0.008), length of the head of spermatozoa (0.001), and overall length of spermatozoa (0.011). Based on these data, there were differences in the length of the head, tail length, and overall length of spermatozoa between treatment groups ($P < 0.05$).

The results of the observations of spermatozoa motility showed that the T3 group had the highest percentage (80.80 ± 1.76) and significantly differed from groups K and T0. However, it did not significantly differ from groups T1 and T2. The table of results of the percentage viability of spermatozoa in group K did not significantly differ against T0. Group K significantly differed from groups T1 and T3. There were differences between groups K and T2, but it was not significant. The T0 group significantly differed from the T1 and T3 groups. The was a difference between groups T0 and T2, but it was not significant. The T1 group significantly differed from groups K and T0. There was a difference between group T1 and groups T2 and T3, but it was not significant. The T2 group significantly differs from the T3. There were differences between group T2 and groups K and T0, but it was not significant. The T3 group significantly differed against groups K, T0, and T2. The was a difference between groups T3 and T, but it was not significant.

The table of results of the percentage of spermatozoa abnormalities in the K group was significantly different against the T0 group. There were differences between group K and groups T1, T2, and T3, but it was not significant. The T0 group significantly differed against groups K, T2, and T3. The was a difference between groups T0 and T1, but it was not significant. The T1 group significantly differed from the T3 group. There were differences between the T1 group and groups K, T0, and T2, but it was not significant. The T2 group did not significantly differ from the K group. There were differences between group T2 and groups T1 and T3, but it was not significant. The T2 group significantly differed from the T0 group. There were differences between group T3 and groups K and T2, but it was not significant. The group T3 significantly differed from groups T0 and T1.

The $Pb(CH_3COO)_2$ is a heavy metal compound that can cause decreased growth in albino rats (*Rattus norvegicus*) by oral administration. The effects of $Pb(CH_3COO)_2$ can lead to continuous weight loss resulting in a significant increase in testicular weight loss. Weight loss of albino rats (*Rattus norvegicus*) occurs due to a decrease in Leydig cells, germ cells, and elongated spermatids. This will result in decreased testosterone concentrations (Chen *et al.*, 2020). Testosterone is the main hormone to regulate spermatogenesis. If the testosterone concentration decreases, it can affect the spermatogenesis process so that motility, viability, and abnormalities can be disturbed. The testosterone secretion by Leydig cells depends on the secretion of LH by the pituitary gland. This occurs because $Pb(CH_3COO)_2$ induces pathological changes in Leydig cells in the interstitial tissue. The

$Pb(CH_3COO)_2$ level accumulated in the body in large quantities can be toxic, which causes damage to body tissues or organs (Dutta *et al.*, 2020). The ROS compounds are magnetic, reactive, and oxidative which can cause cell damage or death. If ROS continues to form, continuous exposure to ROS can trigger the mechanism of germ cell apoptosis. Exposure to nonessential toxic heavy metals like Pb has been reported to decrease Zn levels in body fluids. Moreover, copper and Zn are essential heavy metals having antioxidant effect and are required for many body functions including male reproduction (Fang *et al.*, 2020). It is known that some heavy metals cause damage in testes by disrupting the blood testis barrier by inducing defragmentation of actin filaments of Sertoli cells and upregulating transforming growth factor β3 (Akarsu *et al.*, 2017).

The Pb that has been absorbed by the body takes a very long time to be excreted. The Pb exposure in the long term can cause damage to the testicular organs (Huang *et al.*. 2018). The testes are highly sensitive to Pb toxicity, and germ cells are the main site of Pb-induced damage (Leidens *et al.*, 2018). According to Hasanein *et al.* (2018), Pb exposure can reduce the quantity of spermatogonia, Leydig cells, and testosterone, the amount of spermatozoa, and the motility of spermatozoa. The Pb exposure increases free radicals in the body and can interfere with the process of germ cell division from early spermatogenesis, causing many spermatogonia cells to fail to proliferate to the next stage (Wang *et al.*, 2020). The Pb causes an excessive production of ROS that attacks the cell's antioxidant defense system, disrupting the cell's pro-oxidant/antioxidant equilibrium and causing oxidative stress. As for the rat testes, Pb causes ROS generation and inhibits the activity of antioxidant enzymes (Jegede *et al.,* 2015). The ROS-induced oxidative stress affects spermatogenesis and spermatozoa function, which results in infertility (Prastiya *et al.*, 2021).

The testes are reproductive organs that produce the hormone testosterone and form spermatozoa. The testes are made up of 900 seminiferous tubules where spermatogenesis occurs. The testes are poorly vascularized and have low oxygen tension, which is a crucial component of the testicular mechanism for protecting itself against free radical damage (Treuting *et al.*, 2017). The testes are one of the organs that contain fat. This is because the structure of the plasma membrane of sperm in the testes contains polyunsaturated fats containing double bonds susceptible to free radical attack by lipid peroxidation (Gandhi *et al.*, 2017).

High concentrations of heavy metals cause an increase in ROS, resulting in reduced enzyme activity and lipid peroxides and a reaction with proteins or deoxyribonucleic acid (Fu & Xi, 2019). Increased ROS can trigger an imbalance between oxidants and antioxidants, causing the level of α-tocopherol in the testes to change. Changes in α-tocopherol levels that decreased in the testes give an indication of oxidative stress caused by increasing ROS. The Pb ions have fat-soluble characteristics; therefore, Pb easily penetrates the testicular cell membrane and accumulates in cells (Riana &Yusuf, 2021).

The Pb that accumulates in cells can reduce antioxidant levels while increasing free radical generation, resulting in an imbalance between antioxidants and free radicals and oxidative stress, which causes cellular and tissue damage in organs and some chronic disorders (Marreiro *et al.*, 2017).

In accordance with the results of this study, the α-tocopherol administration in albino rats (*Rattus norvegicus*) exposed to Pb showed a significant increase in FSH and LH levels and a recovery effect on sperm quality (Ayinde *et al.*, 2012). The α-tocopherol induction in the reproductive system of male animals has an effect in the form of increasing the activity of several antioxidant enzymes, reducing the content of nitric oxide and lipid peroxidation products in the testes (Zhu *et al.*, 2017). The α-tocopherol also has a protective role in the testicles of rats because it is the main chain-breaking antioxidant in the sperm membrane that acts as a catcher of free radicals, superoxides, hydrogen peroxides, and hydroxyl radicals. The $ZnSO_4$ administration can significantly prevent oxidative stress caused by exposure to $Pb(CH_3COO)_2$ by increasing the activity levels of superoxide dismutase (SOD) and catalase. The $ZnSO_4$ affects both the accumulation of $Pb(CH_3COO)_2$ tissue and the toxicity susceptibility of $Pb(CH_3COO)_2$. The $ZnSO_4$ increases the activity of antioxidant enzymes including SOD and protects rat erythrocytes against oxidative damage (Sakamoto & Imai, 2017).

The liver, especially hepatocytes, is the main place for drug biotransformation and a detoxification center. The biotransformation reaction in the liver uses oxidative pathways mainly through the cytochrome P-450 enzyme system pathway. This reaction requires energy obtained from the intracellular transport of

mitochondrial electrons that utilize molecular oxygen. This process pretty much produces ROS derived from Pb exposure (Levin *et al.*, 2021). The increase in the Pb amount in the body produces oxidative stress through the accumulation of extreme free radicals and can reduce the action of antioxidant capacities such as SOD and increase the volume of hydrogen peroxide and malondialdehyde. The Pb exposure greatly impacts the liver because of its performance that can reduce the proliferation activity of hepatocyte cells and suppression of hepatocyte growth factor expression. Too much amount of ROS causes oxidative stress that can lead to cell death (Rajaraman *et al.*, 2007). The Zn exhibits strong antioxidant activity that regulates enzymes present in the liver, such as glutathione and metallothionein, which neutralize free radicals and reduce the degree of hepatocyte damage. The Zn pretreatment might potentially preserve the liver from heat-induced damage in mice and be used for the purpose of additives for livestock feed at high temperatures (Kazmi, 2019)

Sperm morphometry is significantly used as a parameter for predicting the fertility of males. Spermatozoa with a normal head size, normal midpiece size, and a long tail has a tendency to get to the ovum faster in the fertilization process. The morphometric characteristics of spermatozoa are related to assessments in the evaluation of spermatozoa quality and stud fertility. The shape and size of the head to the tail of spermatozoa are considered normal if it is included in the classification for a particular species (Viquez *et al.*, 2020). Soler *et al*. (2017) found that most of the research studies of the basic morphometry of spermatozoa so far have only focused on the head of the spermatozoa while the dimensions of the tail of the spermatozoa are significant with the function as a modulator of spermatozoa. Therefore, it should not neglect the role of the tail. The basic morphometry of spermatozoa generally only measures the head, neck, and tail of spermatozoa. The head of a spermatozoa is shaped like an umbrella and has the ability to penetrate the ovum membrane for the fertilization process (Valverde *et al.*, 2019). In the results of our study, there are differences in the morphometry rate of spermatozoa where the combination of Zn and α-tocopherol treatment is able to increase the length of the head, tail, and total spermatozoa length.

According to Caldeira *et al.* (2018), the greater the percentage of motility of immotile sperm (motion in place and not moving), the greater the possibility of infertility or infertile. Therefore, motility is very necessary so that sperm can reach the ovum and fertilize. Ibanescu *et al*. (2020) found that the motility of spermatozoa is strongly influenced by the normal morphological structure and the state of the environment. The normal morphological structure has to do with spermatogenesis which produces normal sperm cells to support their mobility so that they can enter the female reproductive organs. The Pb toxicity is manifested in the male reproductive system by the Pb deposition in the testes, epididymis, vas deferens, seminal vesicles, and seminal ejaculation. The Pb slows down the motility of live sperm (Madhavi *et al.*, 2007).

Antioxidants can protect spermatogenic cells from damage caused by free radicals. Antioxidants are molecules capable of slowing or preventing the oxidation of other molecules. Free radicals are produced during oxidation reactions, which can start chain reactions that damage cells. Antioxidants such as α-tocopherol can block chain reactions by complementing electron-deficient radicals and preventing oxidation processes (Prastiya *et al.*, 2021). The antioxidant defense system works by preventing the formation of free radicals, converting oxidants into less toxic compounds, preventing the secondary formation of toxic metabolites or inflammatory mediators, blocking the propagation of secondary oxidant chains, repairing free radical-induced molecular damage, and enhancing the antioxidant defense system. These defense mechanisms work together to protect the body from oxidative stress. Treatment with α-tocopherol protects rat testicles exposed to heavy metals, which is evidenced by the improvement of the histological structure toward normal, lowering GSH and SOD levels (Al-attar, 2011). The antioxidant α-tocopherol is stronger compared to vitamin C, which is useful in reducing the damage and inhibiting the growth of organs such as the liver, kidneys, and testicles exposed to Pb (Alasia *et al.*, 2020).

The Zn is an essential mineral for health since it acts as a cofactor for over 300 enzymes and 2000 transcription factors. The Zn is an important mediator of cellular signaling (Roshanravan & Alizadeh, 2015). It also works as a cofactor for essential enzymes that help the antioxidant defense system function properly. Furthermore, Zn can protect cells from oxidative damage by acting as a membrane stabilizer, inhibiting the pro-oxidant enzyme nicotinamide adenine dinucleotide oxidase, and inducing metallothionein production.

Metallothionein is involved in the reduction of hydroxyl radicals and the absorption of ROS produced during stress. Because Zn promotes the conversion of two superoxide radicals to hydrogen peroxide and molecular oxygen, it reduces ROS toxicity by converting highly reactive species to less damaging species. Another mechanism by which Zn acts as an antioxidant is by influencing the expression of glutamate cysteine ligase, a rate-limiting glutathione enzyme for novel synthesis. The Zn has a dual effect in this method, either directly neutralizing free radicals through glutathione or indirectly as a cofactor of glutathione peroxidase (Jurowski & Szewczyk, 2014).

The Zn has a protective role as a pro-antioxidant agent or mediator by reducing the production and accumulation of ROS with Zn-binding protein mechanisms such as nuclear factor κB, Zn-containing transcription factor (A20), peroxisome proliferator-activated receptors, tristetraprolin (TTP), hepatocyte nuclear factor 4α, nuclear factor erythroid 2-related factor 2, Kruppel-associated box, and metallothionein/ regulatory transcription factor metal 1 (MTF-1) (Prasad & Bao, 2019). The Zn is not a direct antioxidant agent itself and is a redox-inert ion that cannot oxidize or reduce other substances in the body (Maret, 2019). Furthermore, Zn can bind sulfur (thiolate) donor cysteine to shape Zn thiolate, which converts Zn into a redox-active. Oxidants can interact with thiolate and release Zn in free. Such oxidative Zn release from cysteine residual thioclate donors produces Zn signals that trigger an antioxidant response to ROS/oxidative stress. Physiological or adequate levels of Zn have a protective effect against ROS/oxidative stress (Rahman & Karim, 2018).

CONCLUSION

In addition, in the results of our study, the treatment of α-tocopherol, Zn, or their combination has triggered the synthesis of FSH and LH, increased the number of Leydig cells that affect testosterone synthesis, and increased the number of Sertoli cells that will nourish and treat spermatogenic cells formed in the seminiferous tubules, which ultimately reduce the effects of oxidative stress caused by Pb toxicity. The α-tocopherol and Zn act as free radical scavengers improve sperm quality, and decrease the testicular dysfunction with enhancement in the sexual hormones dependent on time of treatments. The most noticeable improvement occurred in the group of animals that were treated with antioxidants (α-tocopherol and Zn).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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