



### THE EFFECT OF CYCLOPHOSPHAMIDE COMBINED WITH ZINC ON MALE MICE *Mus musculus* SPERMATOZOA

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#### ABSTRACT

**Background:** The usage of Cyclophosphamide (Cp) leads to infertility of reproductive system caused by acrolein. Acrolein can itself cause oxidative damage by depletion of cellular glutathione (GSH) by conjugation, leading to membrane disruption, DNA and mitochondrial damage and can exacerbate apoptosis, which may affect spermatogenesis. Zinc (Zn) which is constituents of superoxide dismutase, has a protective effect towards free radicals from physiological or pathologic effects to minimize the cell's damage.

**Objective:** The purpose of this research was to know the effect of Zn on the spermatozoa count of *Mus musculus* that given Cyclophosphamide intraperitoneally (ip).

**Methods:** In the present study, Cyclophosphamide was administered in saline 200 mg/kg 1x weekly for 5 weeks by ip route, whereas Zn was supplemented by oral route with doses of 25, 50, 100 mg/Kg/day for 5 weeks. The data were analyzed with Anova and followed by Bonferroni Test at a significant level of 5%.

**Results:** The result of this research revealed that high Zn diet and Cp administration decrease sperm count simultaneously. It showed by the decrease of sperm count from 1490 (1 x 10<sup>3</sup>)/ml sperm in control group becomes 240 (1 x 10<sup>3</sup>)/ml sperm in treatment group with 100 mg/Kg of oral Zn and 200 mg/kg of Cp

**Conclusion:** This research show that Zn supplement to prevent Cyclophosphamide toxic effect in spermatogenesis doesn't have a protective effect, in fact its reduce sperm count by excess of Methallothine production and alter the spermatogenesis by reduce Cu intake from intestine.

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**Keywords :** Acrolein, Cyclophosphamide, Oxidative Stress, Spermatozoa Count, Zinc

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## **INTRODUCTION**

Cyclophosphamide (Cp) is an alkylating agent for cancer and for the most part used in lymphoma, leukemia and for immunosuppressive effects. Its usage is correlated with severe side effects, in which infertility is one of the major interest for the younger patients. As well as obesity that leads to an altered reproductive hormonal profile [1], reproductive damage induced by Cyclophosphamide especially due to the establishment of oxidative stress, lipid peroxidation, DNA disturbance and decreased glutathione (GSH) levels.[2] The main function of GSH in spermatogenesis is associated to its interactions with other systems as a preventive mechanism against ROS. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), afterwards converted in lipid peroxide. Lipid peroxide was scavenged to H<sub>2</sub>O by GSH. From that mechanism, GSH prevents imbalance of ROS in spermatogenesis process.[3,4]

Acrolein, composed in the degradation of Cyclophosphamide to phosphoramidate mustard, is a highly reactive aldehyde and may reinforce Cyclophosphamide induced reproductive cell damage, probably with reduction of cellular GSH by conjugation. It forms a conjugate with GSH and is excreted into the urine as 3-hydroxypropylmercapturic acid.[5] There's many toxicity effect of acrolein such as protein adduction, mitochondrial dysfunction, DNA adduction, inflammation and immune alterations.[6]

Zinc (Zn) is well known to be an important trace element in the organism, with three major biological roles, as catalyst, structural, and regulatory ion. Zn has critical effect in homeostasis, immune function, oxidative stress prevention, apoptosis mechanism and aging process.

There's a significant disorders of great public health interest are associated with Zn deficiency.[7] In biochemical systems, the antioxidant role of Zn has been clearly determined and, for the most part, appear to be independent of Zn metalloenzyme activity. Generally, the mechanism of antioxidant reaction from Zn can be separated into acute and chronic effects.[8]

The effect of the chronic administration of Zn can be called by an indirect effect. Intrinsically all of the constructive effects from long-term administration of Zn can be linked to the induction of some other substance that serves as the ultimate antioxidant. In this regard, at the very studied effectors are the metallothioneins.[8,9] Metallothioneins play two important roles in the regulation of apoptosis. The first role of metallothioneins is regulation of intracellular Zn concentration, and the second role is interaction of metallothioneins with some proteins involved in apoptosis. Metallothioneins (MT) preserve against apoptosis by distributing cellular Zn. Zn is an intracellular mediator of apoptosis, which can interfere the action of Ca<sup>2+</sup>. Zn inclusion prevents DNA fragmentation and inhibits many proteins connected to apoptosis, such as caspases and calcium-magnesium-dependent proteases.[7]

The acute antioxidant effects of Zn are broadly manifested in the presence of a demonstrable short-term increase in levels of this metal. Zn has been exhibited in numerous systems to alienate the catalytic properties of the redox-active transition metals iron (Fe) and copper (Cu) with pertain to their abilities to stimulate formation of •OH from H<sub>2</sub>O<sub>2</sub> and superoxide.[9]

From the potency of Zn as the alternative to reduce ROS, this research conducted to observe the sperm count as the parameters of sperm quality in male mice affected from Cp administration and in the exist of Zn.

## **MATERIAL AND METHODS**

### **Animal Experiment**

All the animal experimental protocols were approved by The Health Research Ethics Committee of UPN Veteran Jakarta with ethical clearance No: B/2200/X/2019/KEPK. Outbreed Swiss webster mice with approximately weight 20-30g were obtained from Advanced Biomedical Laboratory of Padjajaran University, Bandung. The animals were kept at room temperature ( $23 \pm 2^{\circ}\text{C}$ ), with controlled 12 hours light and dark cycle. Standard laboratory animal feed (CP551 concentrate) and water were provided ad libitum. Mices were acclimatized for one week before initiation of experiment.

### **Dose Administration and Animal Treatment**

The study have total 6 experimental groups with 3 control group and 3 treatment group, each group consisting of 5 mice divided randomly, (1) Negative Control; (2) Zn control (Lecozinc Tablet from Ifars Pharmaceutical Laboratories, Indonesia, 100 mg/kg/day for 5 weeks, Po); (3) Cyclophosphamide control (Cyclophosphamide 100mg stock from Kalbemed, Indonesia, in saline 200 mg/kg weekly for 3 weeks, Ip); group 4 (Cyclophosphamide + Zn 25 mg/Kg/day), 5 (Cyclophosphamide + Zn 50 mg/Kg/day) and 6 (Cyclophosphamide + Zn 100 mg/Kg/day) receiving Cyclophosphamide (same as group 3). Cyclophosphamide

dose application in this experiment is in toxic amount rate that can interfere the spermatogenesis directly.[10] The treatment was developed for five weeks to embrace, at least one spermatogenic cycle in mice.[11]

### **Evaluation of Sperm Count**

Sperm was collected from cauda epididymis and 1:20 dilution was made for each well-mixed sample by diluting 50  $\mu\text{l}$  of liquefied semen with 950  $\mu\text{l}$  of diluent consisting of 50g  $\text{NaCHO}_3$ , 10 ml of 35% (V/V) formalin, and 5 ml of saturated genital violet made up to a final volume of 1000 ml with distilled water [12]. Gilson automatic pipettes was used for pipetting semen and diluent. The diluted specimen was evaluated with Neubauer counting chamber. The hemocytometer was allowed to stand for 5 minutes to allow the cells to silt before the counting of spermatozoa with 5 boxes in the central grid was done under a light microscope with x40 objective.

### **Statistical Analysis**

Results was expressed as mean  $\pm$  SEM for each group. Statistical analysis was performed using International Business Machines - Statistical Product and Service Solutions version 22 (IBM - SPSS) Software. For multiple comparisons, One-way Anova was used and post hoc analysis was performed with Bonferroni test. P values  $\leq 0.05$  was considered significant.

## **RESULTS**

In this study, data was collected after 35 days period of time described in table 1. Table 1 showed that the negative control group had the highest sperm count

compared to the other groups with average amount 1490 ( $1 \times 10^3$ )/ml. Treatment 3 group had the lowest sperm count compared to the others. The result from this study was disparate to the previous research from Maremanda[2] that Zn can reduced the side effect of Cyclophosphamide and preserve sperm count.

**Table 1 : Average amount of mice spermatozoa count**

Group	Average amount of mice spermatozoa ( $1 \times 10^3$ ) / ml
Negative control	1490
Zn Control (Zn 100 mg/Kg/day, Po)	790
Cp Control (Cp 200 mg/Kg/ week, Ip)	580
Treatment 1 ( Zn 25 mg and Cp)	370
Treatment 2 (Zn 50 mg and Cp)	380
Treatment 3 (Zn 100 mg and Cp)	240

*Note : Cp administration in every treatment group was same as Cp control group (200 mg/Kg/week)*

From the figure 1, it can be seen that there's a progressive decline of sperm count in every group compared to negative control group. According to previous research conducted by Payaran[13] said enhancement of Zn supplement can increase the sperm count, yet in this study Zn control group had lower sperm count compared to negative control group. From this data, Zn can be assumed to decrease the sperm count with Cyclophosphamide in certain dose level and consumption time period. The data was obtained using One-way Anova, showed the significance different with decrease of sperm count between group ( $p \leq 0.05$ ,  $p = 0.02$ ). Then post hoc Bonferroni test was carried out to find out which groups were significantly different. The results obtained from the test showed that only the negative control group was significantly different to treatment group with Cyclophosphamide and Zn administration showed by the decrease of sperm count ( $p \leq 0.05$ ,  $p = 0.008$ ;  $p = 0.008$ ;  $p = 0.002$ ).

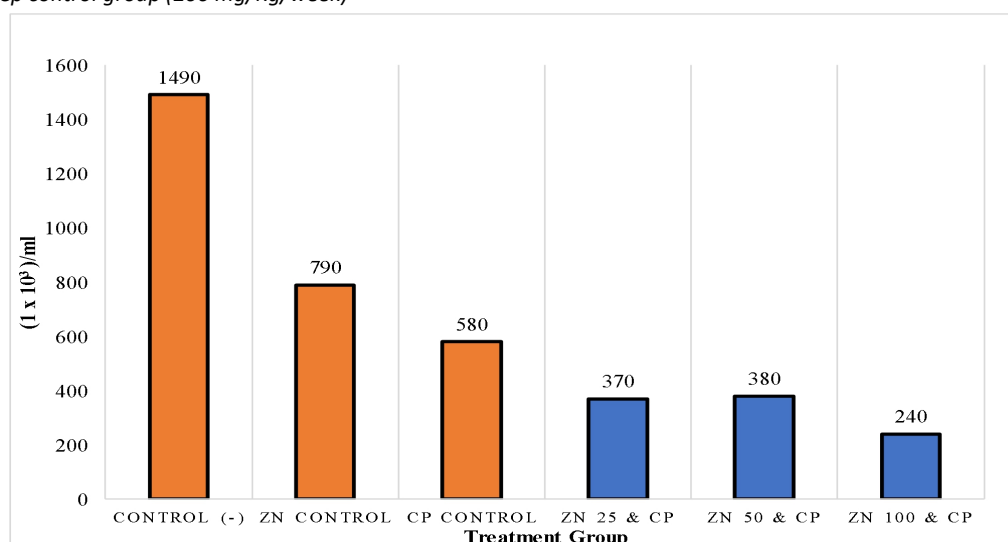


Figure 1. Effect of Zn Supplementation and Cyclophosphamide on sperm count. Sperm count was calculated using hemocytometer Neubauer counting chamber, The comparison graphic of sperm count showed a significance deflation from control group to treatment group (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , 'a' vs. control (-) and 'b' vs. CP Control)

## **DISCUSSION**

The gonadal tissue, with affluence of highly unsaturated fatty acids, high rates of cell division, and variety of testis enzymes results very vulnerable to the overexpression of ROS.[4] From this condition, Cyclophosphamide treatment provoke the spermatogenesis and lead into infertility, especially to be concern in young adult. The present experiment reported a Cyclophosphamide-induced oxidative stress decreases sperm count correspond to research conducted by Maremanda et.al[1] and Kanno et.al[10].

Cyclophosphamide treatment will decrease the feed intake which resulted in mitigate body weight gain. This circumstance will alleviate testes and epididymis weight, which might be the reasons for postponed testicular growth in mice and interfere the spermatogenesis. Further Zn supplementation improved the feed intake, body and testicular weight.[2] The influence of Zn for male fertility only appear recently, being propelled in part by consumer interest in nutritional supplements containing ionic trace minerals.[14]

In our experiment, Zn was found don't have any particular protective effect to increase sperm count or maintain it, but in some reason, its decrease sperm count progressively. It's be evidenced by the data wherein negative group control had the high sperm amount compared to Zn control group with Zn dose 100 mg/Kg/day. Our result obtained there is a distinctive outcome compared to preciding research by Payaran et.al[13] that immense of Zn intake lead to escalation sperm characteristic include sperm count. The underlying existance of Zn induced sperm reduction had been reported. The case report study by Hoffman et.al and Willis et.al[15] found that Zn had an effect to

reduce Copper (Cu) intake in body through MT production. Excess Zn levels induce the synthesis of the intracellular ligand MT in enterocytes, which then binds Zn. The excess Zn bound to MT then is excreted in the feces through enterocyte shedding. However, Cu, with its higher affinity For MT, displaces Zn and also excreted, reducing the amount of Cu delivered to the enterocyte.[15] Fe and Cu were important components of Superoxide Dismutase (SOD) and Catalase (CAT), two essential antioxidant enzymes preventing fluctuations in ROS and protecting the cellular structure against oxidative damage. SOD and CAT activities were positively associated with semen quality parameters in mammals including rapid progressive motility, nonprogressive motility, viability and spermatozoa concentration.[16]

In other hands, Cu have a major contribute in spermatogenesis via meiosis process. Meiosis is a specialized cell division process by which diploid germ line cells generate haploid gametes, which are required for sexual reproduction. In Cu insufficient zygote, there will be a meiotic arrest at metaphase 1 and decrease of mfc1 protein that crucial for meiosis completion, this condition culminate to degression of spermatid production and lead into infertility.[17]

## **CONCLUSION**

This research show that Zn supplement to prevent Cyclophosphamide toxic effect in spermatogenesis doesn't have a protective effect (aproximately with Zn dose  $\geq 25$  mg/Kg/Day), in fact its reduce sperm count by excess of Methallothine production that inhibit Cu intake in intestine and leads to Cu insufisient state and alter the spermatogenesis.

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## **Author Contribution**

M.F.A and Y.N. conceived the project and designed the experiments. MFA developed the theory and performed the computations. Y.N.A. and C.F. verified the analytical methods. M.F.A and Y.N. executed the calculation of Zn and CP to investigate the effect of Zn on the spermatozoa count of *Mus musculus* that given Cyclophosphamide, directed by Y.N.A., M.F.A., and C.F. interpreted the data and wrote the manuscript and all authors reviewed the manuscript

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