The Administration of Zinc Inhibited the Decrease of Leydig Cell and Testosterone Level in Male Wistar Rats (Rattus Norvegicus) that are Exposed to Electric Cigarette Smoke

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Abstract: Electric cigarette smoke is one of the exogenous sources of free radical formation in the body which can cause a decrease in the number of Leydig cells and testosterone levels which can trigger the aging process. Zinc has antioxidant ability to neutralize free radicals and has the ability to work directly on steroidegenesis enzymes so that organ function can be maintained in a physiological state and the risk of premature aging can be avoided. This study was conducted to prove that zinc can inhibit the decrease in the number of Leydig cells and total testosterone levels in male Wistar strain rats (Rattus norvegicus) exposed to electric cigarette smoke. The study was a true experimental study that applied posttest-only controlled group as its research design. The samples in this study were 36 male Wistar rats aged 8-10 weeks, with body weight of 170-200 grams, which were divided into 2 groups, with 18 rats each. The control group was administered with 1 cc placebo (aquadest), while the treatment group was administered with 0.8 mg zinc dissolved in 1 cc of aquadest immediately after being exposed to electric cigarettes. Each group was given the treatment once a day for 28 days. On day 29, the rats were euthanized, then measured testosterone level with ELISA method and testicle surgery was conducted to generate microscopic supply, calculate the number of Leydig cells. The analysis result of the Leydig cells calculation showed that the average total /1 HPF in the control group was 21, 01±1,90, the treatment group was 32,58±2,83 with p<0.001. The average value of testosterone level showed in the controlled group was 2, 27±0, 40 and the treatment group was 3, 44±0, 46 with p<0.001. There were significant differences in the number of Leydig cells and Testosterone levels in the control group compared to the treatment group. The study leads to the concluded that administration of zinc potentially inhibits the decreased of Leydig cells and testosterone level in male Wistar rats (Rattus norvegicus) that were exposed to electric cigarette smoke.

Keywords: zinc, Leydig cell, testosterone level, electric cigarette smoke

1. Introduction

Until now, WHO is still fighting the tobacco epidemic with various strategies, one of the ways is by using nicotine replacement therapy (NRT) such as chewing gum, lozenges, skin patch preparations, inhalers, and nasal sprays. WHO classifies electric cigarettes as battery-based inhalers that can deliver nicotine or also known as Electronic Nicotine Delivery System (ENDS) or nicotine electric delivery systems¹.

Electric cigarettes contain the main components, namely propylene glycol, vegetable glycerine, flavorants (flavors) and nicotine with varying percentages². Other organic compounds found in the vapors produced by electric cigarettes such as Tobacco Specific Nitrosamine (TSNA), carbonyl compounds, Volatile organic compounds (VOCs), particulates, metals. Exposure to electric cigarettes with or without nicotine, results in decreased circulating testosterone levels³. Leydig cells are cells that are very sensitive to reactive oxygen species (ROS). If the number of Leydig cells decreases, the production of the hormone testosterone will also decrease⁴. Decreased testosterone levels will occur with age, this is caused by functional disorders associated with reduced Leydig cells. Aging that occurs in the reproductive organs occurs due to decreased hormone levels, not decreased hormone levels that cause humans to grow old. By understanding the concept of Anti-Aging Medicine, where aging is treated as a disease that can be prevented, treated and can be restored to its original state in various ways. One way is by giving the right antioxidants if the cause is free radicals⁵.

Zinc has antioxidant properties through the mechanism of the synthesis of Metallothioneins (MTs) which inhibit the reaction of the formation of free radicals⁶. In addition, zinc also plays a role in the reproductive system by stimulating the androgen hormone (testosterone) in Leydig cells. If the mineral zinc in the blood increases, the stimulation of the hormone testosterone will increase so that sexual activity, especially the formation of spermatozoa, will increase⁷.

2. Materials and Methods

2.1 Material

The research subjects were 36 healthy male Wistar rats (Rattus norvegicus) aged 8-10 weeks and weighing 170-200 grams from the Integrated Biomedical Laboratory Unit, Faculty of Medicine, University of Udayana, Denpasar, Bali. Zinc preparations were obtained from PT. Indofarma. Electric cigarettes, liquid electric cigarettes, aquadest, 10%
ketamine, 2% xylazine, Hematoxylin-Eosin, ethanol, paraffin, Testosterone Elisa Kit obtained from the Bioassay Technology Laboratory.

Tools
Rat cage, rat scale, microspine, incubator, spectrophotometer, centrifuge, syringe, dropper, surgical instrument, automatic tissue processor machine, histological examination equipment, Eppendorf tube.

2.2 Method
Before being used in this study, 36 rats were acclimatized for 7 days. On day 8, weighting was carried out to determine the average body weight, then divided randomly into two groups, namely the control group and the treatment group. The control group was administered with 1 cc placebo (aquadest), while the treatment group was administered with 0.8 mg zinc dissolved in 1 cc aquadest immediately after being exposed to electric cigarettes. Each group was given the treatment once a day for 28 days. On the 29th day the rats were euthanized, then measured testosterone levels by the ELISA method and performed testicular surgery to determine the histological picture and count the number of Leydig cells. Observation and counting of the number of Leydig cells using a microscope were carried out by adding up Leydig cells from the best 5 fields of view in each testis (right testis and left testis) in a left to right direction or zig zag like the letter Z under a microscope with 400x magnification or objectively 40x on the preparation histology of the right and left testes which had been stained with the Hematoxylin- Eosin (HE) staining method, then averaged to obtain the number of Leydig cells per one high-power field (HPF).

Analysis Outcome
The data processing was conducted using SPSS. The data was analyzed using independent sample t-test.

3. Results
The analysis result of the Leydig cell calculation showed that the total average of /1 HPF in control group was 21, 01±1, 90 and that of treatment group was 32,58±2,83. With an independent sample t-test, the examination of Leydig cell number with p value < 0.001 showed in the treatment group was more significant, that there was an actual difference between control group and treatment group. The average testosterone level of control group was 2, 27±0, 40 and that of treatment group was 3, 44±0, 46. With an independent sample t-test, the examination of testosterone level with p value < 0.001 showed testosterone levels were significantly higher in the treatment group, there is a significant difference between the control group and the treatment group.

4. Discussion
Influence of Electric Cigarette Smoke to Leydig Cell Number and Testosterone Level
Exposure to electric cigarettes induces the formation of free radicals in the body that can damage endothelial cell defenses and alter the antioxidant defense mechanisms in the testes leading to an increase in the activity of antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (Cat), Glutathione s-transferase (GST) and Sulfhydryl (SH)^5. The imbalance between antioxidants in the body and the formation of ROS along with the formation of protein carbonyl and lipid peroxidation products will increase the occurrence of oxidative stress. On the other hand, the formation of aldehyde compounds coupled with the presence of nicotine can cause disturbances in the expression of steroidogenic enzymes, namely 3β-Hydroxysteroid dehydrogenase (3β-HSD) and 17β-Hydroxysteroid dehydrogenase (17β-HSD), which play a role in the process of steroidogenesis. Oxidative stress also causes impaired testosterone secretion and decreased expression of Messenger ribonucleic acid (mRNA) which is responsible for steroidogenesis, which is characterized by histological changes in the testes, namely a decrease in the number of Leydig cells and impaired sperm^2.

Nicotine exposure lowers serum Luitenizing hormone (LH) which functions to stimulate follicles and testosterone and reduces the number of Leydig cells and gene expression levels. Nicotine inhibits androgen production in Leydig cells by decreasing the expression level of the steroidogenesis enzyme^10. Electric cigarettes with nicotine cause caspase-3 cleavage through AxV positivity and Bax expression which have cytotoxic effects that cause programmed apoptosis and necrosis^11. The effect of nicotine on testosterone can be improved by stopping nicotine through several mechanisms in the body, namely by using DNA repair enzymes or antioxidants in the body. With the cessation of exposure will break the chain and will return to the physiological state of the hypothalamic-pituitary-gonadal axis, where there will be an increase in the secretion of Gonadotropin-releasing hormone (GnRH) by the hypohalamus which is expected to increase the secretion of pituitary gonadotropins, especially LH which will stimulate Leydig cells in the testes. To increase testosterone secretion. In addition, the effects of nicotine on reproductive hormones depend on the dose, duration of exposure and the level of damage caused. Physiologically, Leydig cells do not proliferate, but can regenerate when damaged. Leydig cell regeneration is reflected in an increase in serum testosterone levels that return to the normal range. The regeneration mechanism of Leydig cells is influenced by gonadotropic stimulation and local testicular factors from the seminiferous tubules which can stimulate the growth of Leydig cells^12.

Effect of Zinc on Leydig Cell Count and Testosterone Levels
Zinc is able to protect biological structures from damage by free radicals by stabilizing the levels of MTs, including SOD and SH, thereby preventing interactions between chemical groups forming free radicals^13. Zinc absorbed in the testes has the ability to upregulate A20 mRNA, creating Zinc-Finger Protein A20, which has an anti- oxidant role to inhibit ROS. Furthermore, the inhibitory effect of ROS by zinc by reducing the activation of p38 mitogen-activating protein kinase (p38MAPK), p53 and cyclo-oxygenase-2 (COX2) which inhibits oxidative stress, through transporters
In this study, zinc was shown to have the ability to inhibit the decrease in the number of Leydig cells and testosterone levels due to exposure to e-cigarette smoke as evidenced by the ability to increase the number of Leydig cells and testosterone levels in male Wistar (Rattus norvegicus) rats exposed to e-cigarette smoke for 28 days. Where zinc is given at a dose which is a conversion dose from humans at a dose of 50 mg so that the dose for treated rats is 0.8 mg zinc. The dosage of zinc is based on the lowest level of side effects or those that do not cause side effects and can be tolerated by the body and its effect on copper levels or erythrocyte superoxide dismutase activity, namely enzymes that affect zinc and copper status. There is a homeostatic mechanism that keeps zinc levels in the body stable. If zinc intake is low, the synthesis of Zrt-and irtlike protein-4 (ZIP4) will be increased so that more zinc can be absorbed. At the same time, the secretion of endogenous zinc into the intestinal lumen is reduced. Conversely, if the intake is high, then ZIP4 will be degraded rapidly accompanied by an increase in endogenous zinc secretion into the intestinal lumen. Zinc can be used as a modality in the field of Anti-Aging Medicine, namely to prevent, inhibit and return to optimal conditions. This is in accordance with the hormone theory where aging can be prevented by hormonal changes, one of which is by using the right antioxidants if the source is free radicals.

**Image 1:** Histology of the control group. A. Leydig cells between seminiferous tubules (HE 40x). Green arrow line: seminiferous tubule, red arrow line: Leydig cells. B. Leydig cells between seminiferous tubules (HE 400x). Green arrow line: Leydig cells

**Image 2:** Histology of the treatment group. C. Leydig cells between seminiferous tubules (HE 40x). Green arrow line: seminiferous tubule, red arrow line: Leydig cells. D. Leydig cells between seminiferous tubules (HE 400x). Green arrow line: Leydig cells

**Figure 3:** The number of Leydig cells in the control and treatment groups

**Figure 4:** Testosterone levels in the control and treatment groups.
5. Conclusions

Zinc can inhibit the decrease in the number of Leydig cells and testosterone levels in male Wistar rats exposed to electric cigarette smoke. It is necessary to conduct a study to assess the effectiveness of zinc in inhibiting the decrease in the number of Leydig cells and testosterone levels in humans. Long-term longitudinal studies are needed to understand the effectiveness of zinc supplementation in electric cigarette users in the long term. It is necessary to study the effectiveness of zinc on the effect of other reproductive hormones due to exposure to electric cigarettes. It is necessary to study whether zinc in other forms such as zinc citrate, zinc gluconate, zinc oxalate, zinc picolinate, zinc chelated or in the form of fortification in foodstuffs has the same effectiveness as oral supplementation with zinc sulfate monohydrate in experimental animals receiving exposure treatment electric cigarette smoke.

References