The Number of Leydig Cells and Testosterone Levels in The Group Additional Vitamin D was Higher Than The Control Group in Male Wistar (Rattus norvegicus) Exposed to Cigarette Smoke

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Abstract

Background : The harmful content contained in cigarettes smoked will cause damage to various organs and cells , especially the leydig cells as the main producer of the hormones testosterone . Vitamin D in the human body has a pleiotropic effect and functions in cell differentiation and proliferation . this study aims to prove that the number of leydig cells and testosterone levels in the group given orally vitamin D were higher than the control group in male wistar rats (Rattus Novergicus) exposed to cigarette smoke.

Methods : This study used a randomized posttest only control group design . The subjects were 36 white rats , Wistar strain , male, healthy , aged 3-4 months , weight 180-200 grams which were divided into two groups (18 rats / group). The controls group was given a placebo (1 ml of distilled water) and exposed to cigarettes smoke , the treatments group was given oral vitamin D 0.5 mg / kg / day in 1 ml distilled water (aquadest) and exposed to cigarettes smoked for 30 minutes every day before treatments for 21 days . After treatment , the number of Leydig cells was examined histopathologically by HE staining and the testosterone level was examined by the ELISA method .

Results : The results showed that the mean number of Leydig cells in the treatments group was higher , 12.1 ± 2.88 cells / field of view , whereas in the control group it was lower , 5.05 ± 2.19 cells / field of views (p = 0.001). Testosterone levels in the treatments group were also higher , 21.2 ± 4.0 nmol /L, while those in the control group were 8.9 ± 1.1 nmol /L (p = 0.001).

Conclusion : It can b concluded that oral vitamin D can prevented aging by increasing the number of Leydig cells and testosterone levels in male Wistar rats (Rattus novergicus) exposed to cigarettes smoked .

Keywords : Vitamin D, Leydig cells , testosterone , exposure to cigarettes smoked

INTRODUCTION

Aging is a phase in life that inevitably occurs as the chronological age of humans increases.[1] The aging factor is based on two causes, namely internally and externally. Internal factors such as reduced hormones, and genetics. One of the main external factors is exposure to UV-B sunlight, unhealthy lifestyles, stress , and environmental pollution.[1]

The factor that causes aging that most often and easily occurs in the environment is high air pollution such as the result of cigarette smoke which can cause an increase in free radicals. [2] Cigarette smoke contains more than 10^{17} harmful compounds which are carcinogenic and mutagenic which of course will accelerate the aging process. [2] These compounds will increase oxidative stress which will lead to programmed cell death (

apoptosis) and permanent cell aging without apoptosis (cellular senescence). [2]
Substances that contain free radicals in cigarettes will be absorbed through the lungs until they experience precipitation through diffusion and active transport to all tissues and organs. The testes are one of the affected target organs. Leydig cells contained in the testes will die and it will certainly be difficult to proliferate new cells to replace cells damaged by these radical substances. [3] Leydig cell damage can be caused by exposure to cigarette smoke which contains cadmium and nickel metals. These metals can inhibit the activity of the adenycyclase enzyme in the Leydig cell membrane.[3]

Leydig cells are in the interstitial tissue of the testis between tubule seminiferous . Leydig cells are an important organ in the body found in the testes where its function is as the main producer of the hormone testosterone. The hormone testosterone is needed by the body, especially considering its role in anabolic and androgenic.[4]

Increasing age will cause the body to be unable to optimally produce new cells, which will affect the decrease in the number of leydig cells . The reduced number of leydig cells will certainly have an impact on decreasing levels of the hormone testosterone in the body.[4]

Aging Medicine medicine assume that aging is something that can be prevented , slowed down, and treated. One thing that can be done to prevent aging is by giving vitamin supplementation . Vitamin D is generally used as a food supplement as an immune booster. A micronutrient that is fat-soluble, vitamin D is important for calcium and phosphate balance, vascular health, cell differentiation, and proliferation. Vitamin D is mostly produced in the skin and found in diet, but food supplements are also a significant source.. In recent years, vitamin D has provided evidence that vitamin D can be excreted and has a positive effect on male reproduction through its synthesis process. [6] There is evidence that vitamin D can increase germ cell proliferation in the testis. [7]

Regarding the function of vitamin D in the male reproductive organs, additional and focused research is required, especially in increasing the production of leydig cells and the hormone testosterone which is exposed to free radicals, namely cigarette smoke, bearing in mind that previous studies have shown that vitamin D plays an important role in sperm capacitation , sperm motility , and increase sperm survival. So, research on the correlation between vitamin D administration and leydig cells and testosterone levels needs to be tested further.

METHODS

This study used a posttest only control group design in a laboratory experimental study utilizing experimental animals. This study was carried out in the Faculty of Medicine, Integrated Biomedical Laboratory, Udayana University in Bali from September 2022 to December 2022.

36 male Wistars between the ages of 2-4 months, weighing 150-200 grams, and in good health served as the study's subjects. The process of acclimating to the experimental animals was done first, before the experiment was conducted. There were two groups of mice. Rats in each group were exposed to cigarette smoke for one hour and two sticks per day for 21 days before to therapy. After that, on the same day, the rats in the control group received aquadest and were daily administered 0.5 mg/kg of vitamin D that was dissolved in aquadest. Vitamin D supplement in the form of tablets trademark Comvit D 1000 (NOVAPHARIN) 1 vitamin D tablet contains 10 mg / 1000 IU. The doses given follow the research doses that have been previously carried out by other researchers (Al- Charak et al. , 2021).

Leydig Cell Examination

Leydig cell levels in this study were determined by histopathological methods using hematoxylin-eosin (HE) staining. Observations he was made at 40X, 100X, and 400X magnifications and calculated at 400X magnification. Calculated data on a numerical scale are the number of Leydig cells/LPB (per patch, magnification x400). A number of Leydig cells are found in the interstitial part of the testis between the seminiferous tubules and are circular with a central nucleus marked by a red arrow.

In descriptive analyses, mean levels of Leydig cells and testosterone were higher in the treatment group. The Shapiro-Wilk data normality test gives data on the number of normally distributed Leydig cells.

A comparison test of the mean number of Leydig cells with T-independence concluded that there was a statistically significant difference in the number of Leydig cells between the groups, with a higher number of Leydig cells observed in the treatment group. ($p<0.01$).

Testosterone Examination

Each test subject's blood was drawn for 1 ml in order to measure their testosterone levels. In this work, the measurement of testosterone levels in experimental animals was done using an ELISA kit with the manufacturer's Cat. No. E0259Ra, Bioassay Technology Laboratory. The inspection process adheres to the guidelines provided in the KIT.

The results of the Shapiro-Wilk normality test indicated that the testosterone control group's data were not normally distributed. In order to modify the data, a non-parametric test was used. The data was then retested using the Mann-Whitney test, and significant findings were obtained, specifically $p = 0.001$.

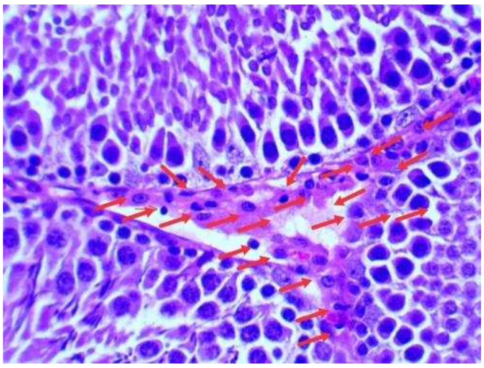
Data analysis

The data obtained were analyzed and processed using the SPSS Version 2.0 for windows . Data analysis went through the stages of descriptive analysis, normality test and comparison test.

RESULTS

In this investigation, testicular tissue preparations were used to histologically assess Leydig cell count. Averaging the results was done after calculations were completed by counting the Leydig cells in the right and left testes in each of three zig-zag fields of view under a 400 X magnification microscope.

When the average number of leydig cells was compared between the groups, the treatment group had more than the control group (K 5.05 ± 2.19 vs P 12.15 ± 2.88). Additionally, the treatment group outperformed the control group in terms of mean testosterone levels (K 8.9 ± 1.1 vs. P 21.2 ± 4.0). All group comparisons statistically differed significantly ($p< 0.05$).



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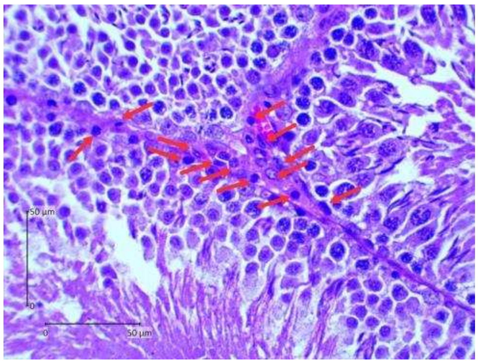

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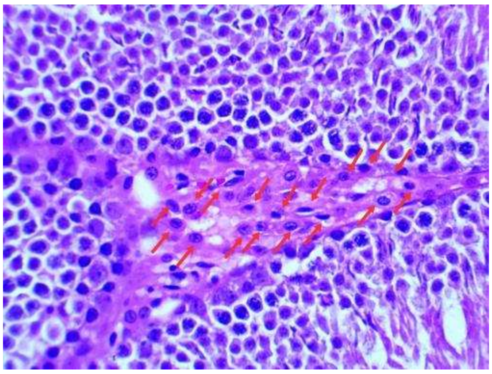
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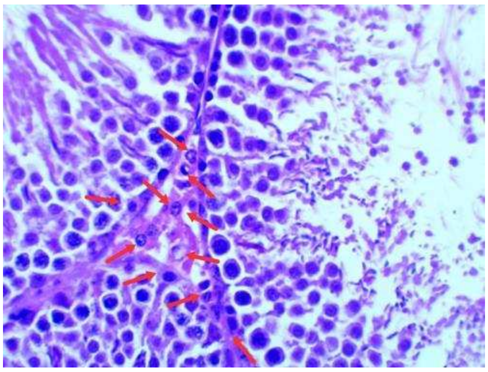
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Fig. 1. (a,b) Leydig Cell Histopathology Picture of Treated Group **Mice** ; (d,e)Control Group (red arrows indicate Leydig cells ; magnification 400x)

Table 1. Comparison of Mean Leydig Cell Counts (cells/field of view) between groups after being given oral vitamin D

Group	n	Mean±SB	p
Control	18	5.05 ±2.19	<0.001
Treatment	18	12.15 ± 2.88	

Table 2. Average Comparison Testosterone Levels **(** nmol /L) Between Groups After Oral Vitamin D Administration

Group	n	Median (Min;Max)	p.s
Control	18	9.1 (5.5;10.3)	<0.001
Treatment	18	20,3 (16.4; 28.6)	

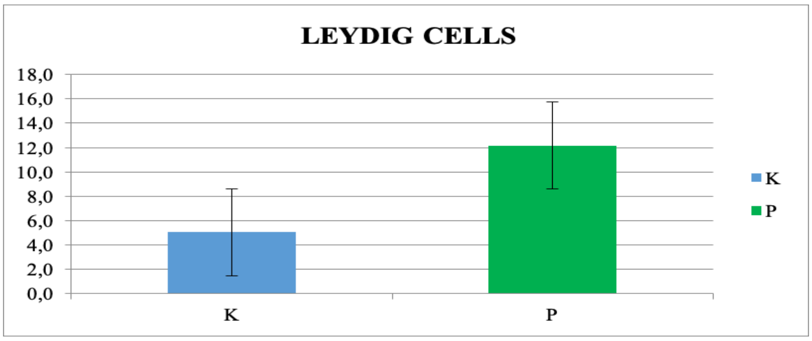

Fig. 2. Comparison of mean leydig cells between groups

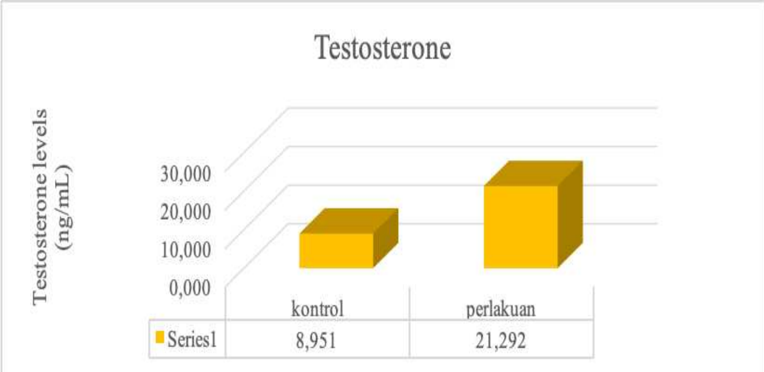

Fig. 3. Comparison of mean testosterone levels between groups

DISCUSSION

Given that vitamin D is thought to be able to regulate the process of apoptosis and cell differentiation, the goal of this study is to determine whether vitamin D has a positive influence on increasing the number of leydig cells. The results showed that the treatment group that was given oral vitamin D experienced a significant increase in the average number of leydig cells compared to the control group. In the treatment group, the mean results obtained after administration for 21 days were 12.1 ± 2.88 cells per field of view, while in the control group, only 5.05 ± 2.19 cells per field of view.

In the comparative test with T -independent showed a p value <0.001 which means that the mean number of leydig cells in the two groups after treatment was significantly different. This shows that giving oral vitamin D at a dose of 0.5 mg/ kg BW rats/day can increase the number of leydig cells exposed to cigarette smoke for 21 days.

increase in the number of Leydig cells is due to the mechanism of vitamin D biosynthesis in which VDR and calcitriol will form heterodimers with RXR/RAR to form transcription factor complexes in target genes. Furthermore, through the regulation of these target genes will affect the vascular endothelium, inflammation, and cell proliferation. [9]

The results of this study are supported by several studies which state that there is indeed a relationship between vitamin D and the function of the male reproductive system. A cross-sectional study to analyze the relationship between vitamin D levels and seminal parameters and sex hormones in 119 infertile men showed positive results. Semen volume, sperm count, total and morphology, testosterone levels were higher in the group with high serum vitamin D. [10]

Systematic research reviews with the aim of knowing the role of vitamin D as a functional steroid hormone, the results obtained from studies that have been conducted on animals and humans indicate that vitamin D has a role in male reproduction, especially in semen parameters and male androgen levels. [11] Observational studies with subfertile male subjects aged 20-50 years indicated that there was a significant relationship between VDR levels and sperm motility . [12] Administration of vitamin D at a dose of 40 IU/Kg and a high dose of 400 IU/kg had a significant effect on increasing germ cell proliferation in old rat testes. [13] The results of this study also strengthen the theory that several enzymes that play a role in vitamin D metabolism in the liver and kidneys as activators of vitamin D are indeed expressed in the male reproductive tract and will have a positive effect on the male reproductive system. In addition, observational research with systematic a review of male fertility, especially sperm motility with vitamin D does have a significant role. Vitamin D receptors (VDR) as well as enzymes that play a role in vitamin D metabolism are simultaneously expressed in germ cells, Sertoli cells , Leydig cells , spermatozoa, and in the epithelial cells lining the male reproductive tract. [14]

The findings also demonstrated that testosterone levels in Wistar rats (Rattus norvegicus) exposed to cigarette smoke increased following oral vitamin D delivery. The outcomes of a statistically significant comparison of the means of the control and treatment groups provide evidence in favor of this. The control group's testosterone levels were 8.9 ± 1.1 nmol/L, while the treatment group's levels were 21.2 ± 4.0 nmol/L. A score of $p < 0.001$ in the analysis of the comparison indicates that there is a significant difference in testosterone levels between the groups after treatment (posttest).

This research is also reinforced by other studies where there is a correlation between 25(OH)D and total testosterone, it is stated that a lack of vitamin D in the body causes a deficiency of androgen hormones which is a marker of a weak body condition. [15]

Research that analyzes the relationship between vitamin D and testosterone by doing Mendelian random results showed low levels of straight line 25(OH)D with low total testosterone. This indicates that there is indeed a relationship between low vitamin D and low levels of the hormone testosterone. The results of this study are supported by the theory where Vitamin D metabolism consists of 2 sources, namely exogenous (UVB) and endogenous (food intake / supplements). In exogenous metabolism, vitamin D in supplementation forms chylomicrons (D2/D3) and is synthesized into a lipoprotein. Lipoprotein which consists of cholesterol is the precursor for the formation of steroid hormones, especially testosterone. [16]

Vitamin D in Anti Aging medicine

There are two most important preparations of vitamin D, namely vitamin D2 or called ergocalciferol and vitamin D3 namely cholecalciferol . Cholecalciferol is a vitamin that is easily found in food or in the form of food supplements. [17] In people with old age / andropause and menopause, vitamin D in the body will certainly be low. This is caused by a decrease in the amount of 7- dehydrocholesterol in the skin as a precursor of vitamin D and low intake of foods containing vitamin D. In addition, exposure to UVB is also limited considering that old age must experience limitations in daily activities . [18] This decrease in vitamin D in the body will certainly cause complaints in the musculoskeletal , cognitive, cardiovascular systems and various other complaints. Vitamin D in the form of supplements , will work in elderly people with its synthesis and expression in various target organs that will play a good role according to the stimulation site that triggers gene transcription. Furthermore, this interaction will affect the regulation of cell growth, angiogenesis, to the regulation of calcium and phosphorus. [19]

On study this used dose of vitamin D 0.5 mg/ kg / day (in study this used preparation of 10 mg of vitamin D = 1,000 iu). Population used _ on study this is mouse wistar male with heavy body 180-200 grams. Conversion dose from mouse to man use formula conversion [20] : HED (mg/kg)= Animal dose (mg/kg) x animal Km/ Human Km . So acceptable dose of oral vitamin D used or consumed on man per day is 486 IU if converted with average weight mice 200 grams and heavy 60 kg human . Oral vitamin D is expected to contribute to the development of preventive therapy in the anti- aging field medicine especially in preventing aging by increasing the number of leydig cells and the hormone testosterone.

CONCLUSION

In rats (Rattus norvegicus) of the male Wistar strain exposed to cigarette smoke, oral vitamin D supplementation can enhance the number of Leydig cells by daily doses of 0.5 mg/kg BW. Rattus norvegicus male Wistar strain rats exposed to cigarette smoke can have their testosterone levels raised by taking vitamin D orally when given a daily dose of 0.5 mg/kg BW. It is necessary to check gonadotropin levels, especially LH, to determine the target mechanism for increasing testosterone . In future studies, it is better to use pure vitamin D as a treatment material so that it is not contaminated by other active substances in non-pure vitamin D preparations

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