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Administration of Green Tea (*Camellia sinensis*) Leaves Ethanol Extract Increased the Number of Leydig Cells and Testosteron Levels in Cigarette Smoke-Exposed Male Wistar Rats (*Rattus norvegicus*)

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Abstract: ***Background: There are many factors that cause aging, one of which is exposure to cigarette smoke because it contains free radicals. Free radicals can cause the death of Leydig cells thereby decreasing testosterone production. To prevent the negative effects of cigarette smoke, it is necessary to provide antioxidants such as green tea extract which contains phenols, flavonoids, tannins and antioxidants. The purpose of this study was to prove that administration of green tea (Camellia sinensis) leaves ethanol extract increases the number of leydig cells and testosteron levels in cigarette smoke-exposed male Wistar rats (Rattus norvegicus). Methods: This study used a post test only control group design. Subjects were 40 male rats, Wistar strain, healthy, weighing 180-200 grams, aged 3-4 months. The control group (20 rats) was given a2 ml/200gBW placebo (aquadest), the treatment group (20 rats) was given a 40mg/200gBW green tealeaves ethanol extract. Each treatment was given 2 hours before exposure to cigarette smoke for 30 days. Blood serum was drawn to examine testosterone levels using the ELISA method. Afterward, the testiswere collected to assess the number of Leydig cells using Hematoxylin-Eosin (HE) staining. Results: The mean number of Leydig cells in the control group was 11.23±1.79 cells/field of view, while the treatment group was 38.29±5.41 cells/field of view (p <0.001). The median testosterone level in the control group was 2.17 nmol/mL (1.70; 3.40). Median testosterone in the treatment group was 6.79 nmol/mL (3.14; 7.36) (p <0.001). Conclusion: It can be concluded that administration of green tea (Camellia sinensis) leaves ethanol extract increased the number of leydig cells and testosteron levels in cigarette smoke-exposed male Wistar rats (Rattus norvegicus).***

Keywords: Green tea leaves ethanol extract, Leydig cells, testosterone, cigarette smoke, male Wistar rats

1. Introduction

Exposure to cigarette smoke is one of the causes of aging, that until now cannot be completely avoided. Many mechanisms are involved in the accelerating aging effects of cigarettes. First, cigarette smoke contains free radicals with oxygen and carbon dioxide on their basis molecule.[1]In accordance with the theory of free radicals on aging,[2] free radicals caused by cigarette smoke can cause oxidative damage which underlying the pathogenesis of degenerative diseases. Second, cigarette smoke causes telomere shortening.[3] This is also in accordance with the genetic theory of aging which causes cellular aging and apoptosis.[2] Third, smoking causes inflammation in the respiratory system which causes degradation of extracellular matrix proteins and tissue damage.[4]

Study found that exposure to cigarette smoke for 2 weeks caused a decrease in the number of Leydig cells.[5] Cigarette smoke contains free radicals that cause oxidative stress which attacks DNA, proteins and cell membranes, thereby stimulating Leydig cell death through both necrosis and apoptosis pathway.[6]Decreasing number of Leydig cells leadstoreduce testosterone levels, which can cause hypogonadism.[7] Testosterone is an important hormone, especially for men, because of its role in reproductive function and regulation of anabolic processes. Decreased testosterone is associated with the emergence of aging phenotypes such as loss of muscle mass, increased fat distribution, insomnia and fatigue, low concentration and short-term memory.[2]

Because of the fact that cigarette smoke increases the levels of free radicals in the body, and causes a decrease in the number of Leydig cells and testosterone, it is necessary to provide antioxidants to prevent this effect. Recently, a source of antioxidants that has been widely studied is natural ingredients, including green tea (*Camellia sinensis*). Green tea extract contains phenol of 20532.48 mg/100g, flavonoids of 75413.22 mg/100g, tannins of 23168.43 mg/100g, and antioxidant of 69198.50 mg/L with IC50% of 1.2858 ppm. In addition, it also contains steroids with levels of 1149.47 mg/kg of beta sitosterol equivalent.

Previously, it has been proven in other studies that green tea extract at a dose of 200 mg/kgBW rats can improve the quality of spermatozoa in rats exposed to cigarette smoke for 30 days.[8] However, until now there has been no research linking the positive effects of green tea extract to prevent a decrease in the number of Leydig cells and testosterone levels in rats exposed to cigarette smoke. The aim of this study was to prove that administration of green tea (*Camellia sinensis*) leaves ethanol extract increases the number of leydig cells and testosteron levels in cigarette smoke-exposed male Wistar rats (*Rattus norvegicus*).

2. Methods

This research was an experimental study using a post test only control group design. Subjects were 40 male rats, Wistar strain, healthy (active and willing to eat), weighing 180-200 grams, aged 3-4 months. Rats were allowed to adapt with laboratory condition for 7 days prior to the treatment. Next, subjects were divided randomly into two groups. The control group (20 rats) was given a2 ml/200gBW placebo (aquadest), and the treatment group (20 rats) was given a 40mg/200gBW green tealeaves ethanol extract. Each treatment was given 2 hours before exposure to cigarette smoke for 30 days. In this study, 2 rats in the control group and 3 rats in the treatment group died (drop out). After 30 days of treatment, all rats were euthanized (control = 18 rats, treatment = 17 rats). Blood serum was drawn to examine testosterone levels using the ELISA method. Afterward, the testiswere collected to assess the number of Leydig cells using Hematoxylin-Eosin (HE) staining.

The data obtained in this study were analyzed with descriptive analysis, normality testusin Shapiro-Wilk, homogeneity test by the Levene'stest, and comparison testusing independent *T*-test and Mann-Whitney test for Leydig cells and testosterone levels, respectively.

3. Results

Descriptive statistic

Descriptive analysis in this study includes mean, standard deviation (SD), median, minimum value (Min.) and maximum value (Max) (Table 1).

Table 1: Descriptive Statistic

Variable	Group	n	Mean	SD	Median	Min.	Max
Leydig cells (cells/field of view)	Control	18	11.23	1.79	11.25	8.80	15.20
	Treatment	17	38.29	5.41	38.80	29.70	52.20
Testosterone (nmol/mL)	Control	18	2.27	0.41	2.17	1.70	3.40
	Treatment	17	6.52	1.08	6.79	3.14	7.36

Note: n= number of replications; SD= standard deviation; Min= minimum value; Max= maximum value

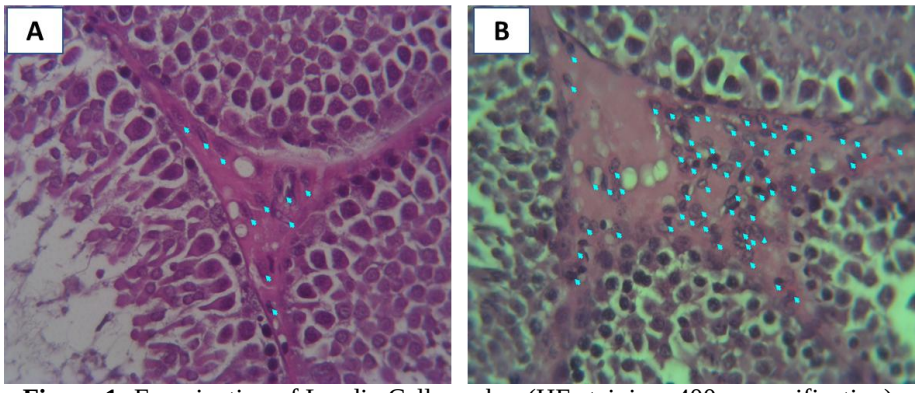

Figure 1: Examination of Leydig Cell number (HE staining, 400x magnification)
(A) The number of Leydig cells in the control group. (B) The number of Leydig cells in the treatment group. It can be seen that the number of Leydig cells in the treatment group was more than the control group.

Normality and Homogeneity of Data

Normality test using Shapiro-Wilk shwed that the Leydig cell data were normally distributed (p> 0.05), testosterone levels of the control group were normally distributed (p> 0.05), while the testosterone levels of the treatment group were not normally distributed (p <0.05). Because there are data that are not normally distributed, the transformation is carried out using the square root method. However, after being transformed, the testosterone data for the treatment group was still abnormal (p <0.05). Furthermore, the homogeneity test with the Levene test showed that the Leydig cells data were not homogeneous (p <0.05), while the data on testosterone levels were homogeneous (p> 0.05) (Table 2).

Table 2: Normality and homogeneity of the data

Variable	Group	Shapiro-Wilk test (*p;* interpretation)	Levene test (p; interpretation)
Leydig cells	Control	0.427; normal	0.026; not homogenous
	Treatment	0.173; normal	
Testosterone	Control	0.160; normal	0.103; homogenous
	Treatment	0.000; not normal	
SQRT_ Testosterone	Control	0.442; normal	Not calculated
	Treatment	0.000; not normal	

Comparison analysis

Because the distribution of Leydig cells data wasnormal, the comparative analysis was conducted with independent T-test. In contrast, testosterone data were not normally distributed; hence, the Mann-Whitney test was performed.The mean number of Leydig cells in the control group was 11.23±1.79 cells/field of view, while the treatment group was 38.29±5.41 cells/field of view (p <0.001). The median testosterone level in the control group was 2.17 nmol/mL (1.70; 3.40). Median testosterone in the treatment group was 6.79 nmol/mL (3.14; 7.36) (p <0.001) (Table 3).

Correlation analysis

In this study, a correlation analysis was carried out to determine the relationship between the two observed dependent variables using Spearman's Rho test (Table 4). The results of the correlation test showed that there was a strong relationship between the Leydig cells and testosterone levels (p <0.05).

Table 3: Comparison Analysis

Variable	Control	Treatment	*p*
Leydig cells (mean±SD) (cell/field of view)	11.23±1.79	38.29±5.41	<0.001
Testosteron [Median(min;max)] (nmol/ml)	2.17 (1.70; 3.40)	6.79 (3.14; 7.36)	<0.001

Table 4: Correlation analysis

Variable	Coeficient correlation	*p*
Leydig Cells	0,846	<0,001
Testosterone		
Note: *p= Spearman's Rho test*		

4. Discussion

Effects of Green Tea Extract on Leydig Cells and Testosterone

Green tea extract in this study was proven to increase the number of Leydig cells and decrease testosterone levels due to exposure to cigarette smoke. Green tea has a high polyphenol content.[9] In this study, we proved that green tea extract used contains phenols, flavonoids, tannins, antioxidants and steroids (*unpublished*).

Phenolic compounds are bioactive compounds that are known as antioxidants[10], so they can reduce free radicals[6], then cause an increase in Leydig cells and increase testosterone levels.[11] Flavonoids are chain-breaking antioxidants and activators of the production of SOD, glutathione and catalase.[12,13] Flavonoids as antioxidants can reduce free radicals[14], inhibit Leydig cell death and inhibit the decrease in testosterone.[15] Tannins have anti-apoptotic activity that prevents a decrease in the number of Leydig cells.[16]

In addition to preventing a decrease in the number of Leydig cells and preventing a decrease in testosterone levels due to cigarette smoke, the content of green tea extract in this study also has the potential to increase these variables. Flavonoids and tannins can activate the extracellular signal-regulated kinases (ERK1/2) pathway, also known as the MAPK p42/p44 pathway, which is a major determinant of cell growth, cell differentiation, and survival of Leydig cells, underlying the involvement of green tea in Leydig cells maturationand accelerate the testosterone production.[17-19] In addition, the content of steroid can activate cAMP and cAMP response element binding protein (CREB), which in turn activates steroidogenic acute regulatory protein (StAR) and GATA-4 followed by testosterone synthesis.[20]

The results of this study are supported by several previous studies using various other extracts such as *Momordica charantia*and *Jurenia dolomiaea* root extract. However, green tea leaf ethanol extract is likely to be better because of the better bioactive compound content. For instance, *M. charantia* extract which has been shown to increase the number of Leydig cells in hyperglycemic rats[21], only contains phenol of 39.76 mg/100g, flavonoids of 14.53 mg/100g, and antioxidants of 300 mg/L.[22]The *J. dolomiaea*root extract contains only 41.1 mg/L of antioxidants can increase testosterone levels in rats induced by oxidative stress with CCl_4.[23,24]

Green Tea Extract and Anti-Aging Medicine

Exposure to cigarette smoke, both active and passive, will accelerate the aging process.[25] This is because cigarette smoke contains very high free radicals.[1] A decrease in the number of Leydig cells which then results in a decrease in testosterone levels can cause aging, and vice versa.[2]Based on the fact that cigarette smoke increases the levels of free radicals in the body, and causes a decrease in the number of Leydig cells and testosterone, which then leads to the aging process, efforts to prevent a deterioration of these variables due to exposure to cigarette smoke can be categorized as an Anti-Aging Medicine measure. Based on this study, green tea extract can be used as an anti-aging effort.

5. Conclusion

It can be concluded that administration of green tea (*Camellia sinensis*) leaves ethanol extract increased the number of leydig cells and testosteron levels in cigarette smoke-exposed male Wistar rats (*Rattus norvegicus*).Thus, it can be used as an antioxidant supplement for active and passive smokers (cathecin content in green tea has 5 times more potential as antioxidant than vitamin C). As a suggestion, toxicity test is important to reveal the potential toxic effect of green tea extract. It is also necessary to do the clinical trial.

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