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Green Tea Kombucha Increases Leydig Cell Numbers and Testosterone Level in Male Wistar Rats Exposed to Nicotine Electric Cigarette Vapor

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ABSTRACT

Background: Nicotine as a free radical reduces male fertility. Green tea, as an antioxidant, has contributed to protecting testes function; however, green tea kombucha has never been studied for such benefits. This study demonstrated the effect of green tea kombucha in increasing Leydig cells number and testosterone levels in male Wistar rats exposed to nicotine from electric cigarettes.

Methods: A post-test-only control group trial was performed on 28 male Wistar rats randomly divided into control, untreated, treatment groups 1 and 2. The control group was exposed to e-cigarette vapor + 1 ml of distilled water. Treatment groups 1 and 2 were exposed to e-cigarette vapor + 0.5 ml/day and 1 ml/day of green tea kombucha, respectively - the e-cigarette vapor exposure occurred daily for 30 minutes. After 28 days, Leydig cell and testosterone levels were measured and compared between groups.

Results: The control group's mean value of Leydig cell number is lower than untreated group ($p=0.004$). Only treatment group 2 had a higher mean of Leydig cell number than control ($p<0.001$). The control group's mean value for testosterone levels was lower than untreated group ($p<0.001$). The mean testosterone level in treatment groups 1 and 2 was higher than in untreated and control ($p<0.001$). In addition, treatment group 2 had higher mean testosterone levels than group 1 ($p<0.001$).

Conclusions: Green tea kombucha has been shown to inhibit the harmful effects of nicotine. These results serve as a basis for the administration of green tea kombucha to prevent free radical-induced testosterone decline.

Keywords: Kombucha, Leydig Cells, Testosterone, Nicotine, Antioxidant

INTRODUCTION

As a new device, the existence of the electric cigarette arouses curiosity. Even among conventional smokers, many have tried to switch to electric cigarettes because they are considered safer and have a modern style without compromising the smoking experience of conventional cigarettes. Electric cigarettes contain varying amounts of key ingredients such as propylene glycol (PG), vegetable glycerine (VG), flavourings, and nicotine.[1]

Cigarette vapor has a number of negative effects on the reproductive organs, including a reduction in sperm quality and number and inhibition of Leydig cells, leading to a decrease in testosterone production and damage to the vas deferens in the testes.[2] Smoking for a long period of time can lead to an increase in reactive oxygen species (ROS), which can interfere with the release of luteinizing hormone (LH).[3] Leydig cell development is hindered when the secretion of luteinizing hormone (LH) decreases.

Smoking has effects on spermatogenesis, sperm quality, and testosterone levels. Chronic exposure to cigarette vapor leads to changes in the stage of the testicular

epithelium at various stages of spermatogenesis and to the death of spermatogenic cells, resulting in a decrease in testosterone levels, testicular weight, and the total number of spermatogenic cells. The decline in Leydig cells and total testosterone due to exposure to electric cigarette vapor causes men to enter the andropause phase.[4] Andropause is one of the signs of aging that can be treated and restored to its original state according to the concept of anti-aging medicine, and one of the ways is to use antioxidants to fight free radicals in electric cigarettes that cause a decline in Leydig cells and testosterone.[5]

Antioxidants are found in kombucha. Kombucha produces polyphenols, gluconic acid, vitamins, and lactic acid during fermentation.[6] Kombucha contains free phenolic compounds formed during fermentation that increase antioxidant activity because the more phenols obtained, the higher the antioxidant activity.[7] Total polyphenols in green tea kombucha were 299.6±3.1 (mg/L), but continued to increase to an optimal value of 320.1 ± 3.5 (mg/L) on the fourteenth day, after which they decreased.[8]

In addition to antioxidants, kombucha is a traditional natural tea that contains beneficial compounds such as organic acids, minerals, vitamins, proteins, polyphenols, and various anions. Kombucha has anticancer, antimicrobial, antifungal, and hepatoprotective effects. However, studies on the specific effects of green tea kombucha on male fertility, especially on Leydig cells and testosterone levels, have yet to be extensively conducted. The biochemical constituents of kombucha are expected to reduce the negative effects of oxidative stress and suppress ROS, which protects the testes from cell damage. The aim of this study was to demonstrate the effect of green tea kombucha administration on increasing Leydig cell number and testosterone levels in male Wistar rats (*Rattus norvegicus*) exposed to nicotine from electric cigarette vapor.

MATERIALS & METHODS

Materials

The green tea brand Brew Me from PT. Bali Cahaya Amerta, grown in Angseri village, Bali, was used to prepare green tea kombucha. The preparation was done in Mengwi using a modified recipe from the literature.[8,9] In this study, eVOD and liquid electric cigarettes with a propylene glycol (PG)/VG 50/50 and a nicotine content of 3 mg were used and vapored for 30 minutes daily. To ensure that the vapor of the electric cigarette is stable, a ventilation tube was used at the base of the electric cigarette. Green tea kombucha phytochemical examination was conducted at the Faculty of Agricultural Technology Laboratory Service Unit, Udayana University. Test procedures on animals and examining Leydig cell number, and testosterone levels were conducted at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University, Bali, and Sentra Laboratory, Denpasar, Bali. This research has been approved by the ethics commission of Udayana University, Bali, (B/178/UN14.2.9/ PT.01.04/2022).

Study design and experimental animals

This study was a true experimental study with a randomized post-test only control group design. The experiment was conducted at the Integrated Biomedical Laboratory Unit, Udayana University. The experiment was conducted for four weeks. The sample required for this experiment consisted of 24 male Wistar rats (n=6), 8 to 10 weeks old and weighing 170-200 grams. To avoid failures, 10% of the total sample was added. A total of 28 Rats were divided into four equal groups: Control group, untreated group, and treatment groups 1 and 2 (n=7).

Preparation of green tea kombucha

Preparation began by boiling 1 liter of water (temperature of 70°C), which was then poured into a flask containing 100 grams of white cane sugar (90.1 mg/mL) and 8 grams of green tea (7.2 mg/mL). The warm brewed

tea was then poured into a glass jar, covered and stored at room temperature (25-30 °C). Kombucha can be made with a microbial starter called a "symbiotic culture of bacteria and yeast" (SCOBY) and then fermented for 14 days. The starter was purchased commercially. When the temperature of the tea was equal to room temperature, the SCOBY was added to the jar and stirred sufficiently. The jar was covered with thick gauze, tied with a rubber band and stored in a clean, dry room at room temperature and fermented for 14 days. On day 14, the gauze was removed and the kombucha was poured into a glass bottle and strained.

Intervention procedure

A total of 28 male Wistar rats were adapted for seven days. After the eighth day, the rats were divided into the control group, the untreated group, and treatment groups 1 and 2. In the untreated group, the rats were neither administered kombucha nor exposed to cigarette vapor. In the control group, rats were exposed to 3 mg of nicotine vapor from an e-cigarette and received 1 ml of distilled water. In treatment groups 1 and 2, rats received 3 mg of nicotine vapor from the e-cigarette and 0.5 ml and 1 ml of green tea kombucha daily, respectively. During the 28-day treatment period, the rats were placed in a special cage with holes for the e-cigarette vapor tube using a plastic tub and exposed to the e- cigarette vapor for 30 minutes daily.[10,11] Distilled water and green tea kombucha were administered orally via a feeding tube.

After day 28, euthanasia was performed by administering 5 mg/kg ketamine 10% and 20 mg/kg xylazine 2% via intramuscular injection into the posterior thigh. Male Wistar rats were then analyzed for testosterone levels using a blood sample collected from the medial canthus orbitalis and placed in a labeled tube. To check Leydig cell count, samples were taken from the left and right testes and placed in formalin-soaked and labeled test tubes. The male Wistar rats were appropriately incinerated and then the data were analyzed.

Examination of testosterone level and Leydig cells

Testosterone level assay was performed using an enzyme-linked immunosorbent assay (ELISA) kit for rats (BT-LAB, Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd, Shanghai, China). The absorbance value of the color density associated with the spectrum at a wavelength of 450 nm is determined using a spectrophotometer.[12]

The number of Leydig cells was the parameter in the histological examination stained with haematoxylin-eosin (HE).[13] The number of Leydig cells was observed and counted under the microscope (OptiLab, Miconos, Yogyakarta, Indonesia) by plotting the total number of Leydig cells from the 5 best high-power fields (HPF) of the right and left testis in a zigzag direction (like the letter Z). The total number of Leydig cells from the 5 best high-power fields (HPF) of the right and left testis was counted under the microscope. These observations were stained under the microscope at 400× magnification on the right and left testis using the HE method, after which they were averaged to obtain the number of Leydig cells per HPF using ImageRaster software, version 4.0 (Miconos, Yogyakarta, Indonesia).

STATISTICAL ANALYSIS

Statistical analysis was performed with IBM SPSS statistics for Mac version 26 (IBM Corp, NY). Normality test was assessed using Shapiro-Wilk test and homogeneity test was assessed with Levene's test. Comparability test was assessed using One Way ANOVA and continued with Post Hoc Test to examine the difference in each group.

RESULT

Normality and homogeneity tests on Leydig cell numbers and testosterone levels for each group show that each group's Leydig

cell numbers and testosterone levels are normally distributed and homogenous. A comparability test on Leydig cell number and testosterone level was done to compare the average of each variable in the untreated group, control group, and treatment groups 1 and 2. The comparative analysis is presented in Table 1.

Table 1. Comparative Analysis of Leydig Cell Number and Testosterone Level Between Groups

Variable	Mean ± SD				
	Control	Untreated	T1	T2	p
Leydig cell number (/HPF)	8.33 ±1.98	4.42 ± 1.49	5.61 ± 1.67	9.79 ± 2.15	0.001
Testosterone level (ng/mL)	6.32 ± 0.25	2.08 ± 0.05	12.38 ± 0.17	18.99 ± 0.21	0.001

p = significance; T1 = treatment group 1; T2= treatment group 2

The comparative analysis in Table 1 shows a significant difference in the mean number of Leydig cells and testosterone levels between groups ($p<0.05$). Furthermore, in the Post Hoc analysis, we found that the mean value of Leydig cell count in the control group decreased significantly compared to the untreated group ($p=0.004$). Meanwhile, even though treatment groups 1 and 2 have insignificant differences in the mean Leydig cell count compared to the untreated group ($p>0.05$), only treatment group 2 has a significantly higher mean value when compared to the control group ($p<0.001$). The difference in the mean of Leydig cell count in treatment groups 1 and 2 can be seen in Figure 1.

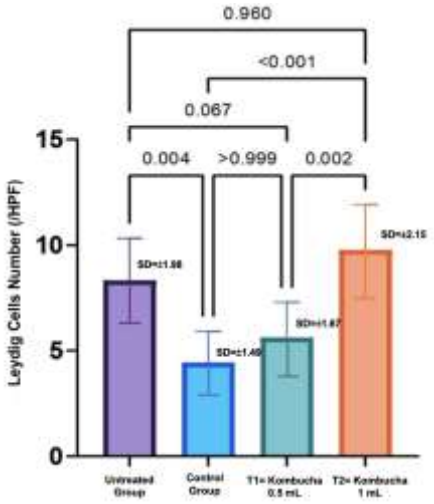

Figure 1. Comparison of Leydig Cell Numbers (/HPF) between Groups.

Post Hoc analysis of testosterone levels shows that the mean number in the control group is significantly lower than the untreated group ($p<0.001$). Meanwhile, the mean testosterone levels in treatment groups 1 and 2 are significantly higher than the untreated or control groups ($p<0.001$). Furthermore, treatment group 2 has a significantly higher mean testosterone level than treatment group 1 ($p<0.001$). The difference in mean testosterone levels between groups is presented in Figure 2.

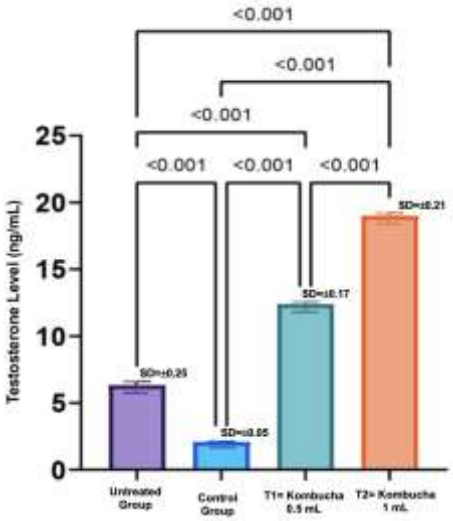

Figure 2. Comparison of Testosterone Level (ng/mL) between Groups

Figure 3 shows the comparison of the histology of seminiferous tubules between groups. The yellow arrow in the picture marks Leydig cells. The number of Leydig cells appeared to be higher in the untreated group and treatment group 2 compared to treatment group 1. The number of Leydig cells appeared to be higher in treatment group 1 than in the control group.

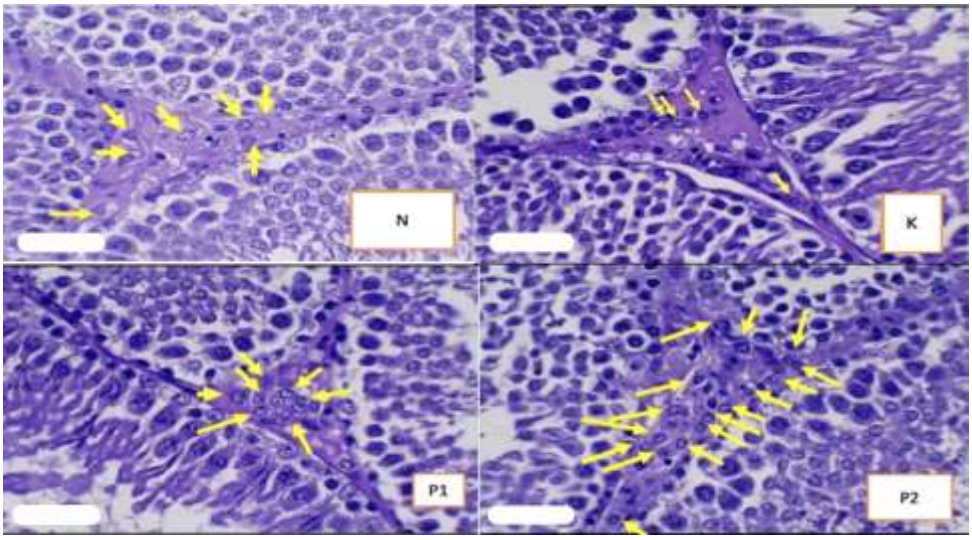

Figure 3. Histology of Leydig Cells in Seminiferous Tubules. N = Control Group; K = Untreated Group; P1 = Treatment 1; P2 = Treatment 2; White Bar = 50 μm.

DISCUSSION

In this study, the mean number of Leydig cells in the untreated group was 8.33±1.98/HPF, whereas the mean number of Leydig cells in the control group was 4.42±1.49/HPF ($p<0.05$), which was 47% lower. Similar to the testosterone level, the mean of the untreated group was 6.32±0.25ng/mL, while the control group was 67% lower, 2.08±0.05ng/mL ($p<0.05$). This result shows that 28-day exposure to electric cigarette smoke significantly decreases the number of Leydig cells and testosterone levels in male Wistar rats. The results in line with another study showing that rats exposed to 3 mg of nicotine and a nicotine-free electric cigarette were affected by the decrease in Leydig cells in the testes.[14]

Nicotine, one of the main components of the electric cigarette, is toxic and is rapidly absorbed through the mucous membranes of the respiratory tract, mouth, and skin when smoked.[11] Negative effects have also been observed in passive smokers when they inhaled the smoke of burning cigarettes.[15,16] The StAR promoter region is damaged by nicotine and alters epigenetic regulation, disrupting testosterone biosynthesis in Leydig cells due to inhibition of cholesterol transfer to mitochondria. Leydig cells, located around the seminiferous tubules, play an important role in the formation of testosterone and the development of secondary sexual characteristics.[17]

Moreover, testosterone levels significantly decreased in nicotine-exposed rats, whereas autophagy gene expression increased after nicotine exposure, as shown by single transcriptome data.[11] Similarly, chemical and immunological staining showed that autophagy of Leydig cells increased after nicotine administration compared with the apoptotic process. Apoptotic processes are mainly found in spermatids. Autophagy-related gene expression (e.g., Beclin1 and LC3) is overexpressed after nicotine exposure. In addition, the methylation level of the TCL1 promoter region was increased and TCL1 expression was decreased in the nicotine-exposed group compared with the control group.[11]

In this study, the mean number of Leydig cells in treatment group 2 (which received green tea kombucha 1ml/day for 28 days) was significantly higher than that in the control group ($p<0.05$). While treatment group 1 (0.5 ml/day of green tea kombucha) showed no significant difference from the control group. These results suggest that the effect of green tea kombucha is dose-dependent, with the higher dose of 1 ml/day showing a better effect than that of 0.5 ml/day.

In this experiment, green tea kombucha fermented for 14 days was used. A study in

which green tea kombucha fermented for 14 days shows that the kombucha has the highest antioxidant content, as evidenced by the results of DPPH assay and total polyphenols of 320.1 mg/L.[8] In addition, green tea kombucha from green tea has an antioxidant effect on superoxide radicals.[18] Compared with black tea kombucha, the phenolic content of green tea kombucha was found to be higher.[19] The mechanism for the increase in the number of Leydig cells as a result of green tea kombucha administration may be largely due to the total polyphenol content, which provides a protective mechanism against excessive oxidative stress.[20]

The result of this study also shows that oral administration of green tea kombucha resulted in an increase in serum testosterone level in male Wistar rats exposed to nicotine from electric cigarette vapor compared with the untreated and control groups ($p<0.001$). This result is consistent with the effect of green tea kombucha increasing the number of Leydig cells, because Leydig cells are the main testosterone-producing cells in men and male animals, so they have a direct effect on increasing testosterone levels.[14,21]

Increased ROS can cause oxidative stress, which leads to DNA damage and apoptotic processes that cause a decrease in the number of Leydig cells and ultimately in testosterone levels.[22,23] Therefore, the administration of antioxidants is considered a potential strategy for treating male infertility.[24,25] This study showed that administration of green tea kombucha, specifically at 1 ml/day for 28 days, was shown to increase Leydig cell numbers and testosterone levels in male Wistar rats exposed to electric cigarette vapor. Green tea kombucha contains several active compounds such as polyphenols and vitamin C, which act as antioxidants. The antioxidant capacity of green tea kombucha in this study was 976.2 mg/L GAE, which is consistent with previous studies that assumed 955 mg/L GAE, which are known to have a protective effect on liver and kidney function.[26]

The dose used in the study was 1 ml of green tea kombucha per day, which was half the dose that caused the adverse reaction. In this study, green tea kombucha was shown to inhibit the harmful effects of free radicals on Leydig cell number and testosterone levels. These results can be used as a basis for administering green tea kombucha to men to prevent a drop in testosterone levels due to excessive free radical exposure. However, the optimal dose for humans needs further study, as do the side effects of long-term use.

We acknowledge the limitations of our study. Antioxidant biomarkers were not measured in this study, so further investigation is needed to determine the mechanism of action of the antioxidants in green tea kombucha in inhibiting the decline of Leydig cells and testosterone levels in the reproductive system. Another limitation is that only two different concentrations of kombucha green tea were used in this study, and the first objective is to assess whether there is an effect. Therefore, we cannot evaluate the dose trend.

CONCLUSION

Oxidative stress, autophagy, and apoptosis in Leydig cells are closely associated with testicular function. Elevated levels of ROS can cause oxidative stress, which leads to DNA damage and apoptotic processes that cause a decrease in the number of Leydig cells and ultimately affect testosterone levels. Administration of antioxidants is a possible strategy for treating male infertility. Green tea kombucha is a beverage that is commonly used for health purposes. Green tea kombucha contains phenolic and alkaloid compounds that act as antioxidants. This study showed that administration of green tea kombucha, especially at a concentration of 1 ml/day for 28 days, increased the number of Leydig cells and testosterone levels of adult male Wistar rats exposed to e-cigarette vapor. Green tea kombucha has been shown to inhibit the harmful effects of free radicals, which reduce Leydig cell numbers and testosterone

levels. These results could serve as a basis for administering green tea kombucha to men to prevent a decline in testosterone levels caused by exposure to excessive free radicals. However, the optimal dose in humans needs further study, as do the side effects of long-term ingestion.

Declaration by Authors

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