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Oral Administration of Tamarillo Seeds (*Solanum Betaceum Cav.*) Ethanol Extract Inhibited the Decrease of Leydig Cells and Testosterone Levels in Male Wistar Rats (*Rattus Novergicus*) Exposed to Cigarette Smoke

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Abstract: ***Background and purpose:*** ***Exposure to cigarette smoke can induce oxidative stress. The process of smoking causes the accumulation of free radicals in the body and causes damage in three ways, namely lipid peroxidation, cell damage, and DNA damage. Tamarillo seeds (Solanum betaceum Cav.) contain various kinds of active compounds including flavonoids, phenols, tannins, vitamin C, carotenoids, anthocyanins, and others. This study was conducted to examine the effect of ethanol extract of tamarillo seeds on the number of Leydig cells and testosterone levels of male Wistar rats exposed to cigarette smoke.*** ***Methods:*** ***This study was an experimental study with a post-test control group design. The samples of this research were 36 healthy male Wistar rats, 6 months old, and 180-200 grams weight. Adapted for 7 days and then divided into two groups (18 rats per group). The control group was given a placebo (aquadest) 2ml/day and the treatment group was given ethanol extract of tamarillo seeds 80mg/200gBW/day just before being exposed to cigarette smoke 1 hour/day for 21 days. On day 22 the rats were euthanized and blood was drawn through the medial canthus sinus orbitalis dextra to check testosterone levels using the ELISA method and testicular surgery for microscopic preparation and counting the number of Leydig cells.*** ***Results:*** ***The analysis results of the Leydig cells number showed that the mean in the control group was 17,21±5,14 per one large field of view and the treatment group was 34,46±5,87 per one large field of view (p<0,001). The mean value of testosterone levels in the control group was 10,88±1,34 nmol/ml and the treatment group was 20,24±2,16 nmol/ml (p<0,001). The independent sample t-test showed that the mean number of Leydig cells and the testosterone levels in the treatment group were significantly higher (p<0,001).*** ***Conclusion:*** ***From this study, it can be concluded that oral administration of tamarillo seeds (Solanum betaceum Cav.) ethanol extract can inhibited the decrease of Leydig cells and testosterone levels in male Wistar rats (Rattus novergicus) exposed to cigarette smoke.***

Keywords: tamarillo seeds, Leydig cell, testosterone level, cigarette smoke

1. Introduction

The aging process does not always mean that organ function decreases with age but this process can be inhibited and re-optimized so that the quality and life expectancy can be better. The newest concept in Anti-Aging Medicine (AAM) states that the aging process can be inhibited, treated, and restored because this process is considered similar to the disease. Hormones decrease not due to increasing age but decreased hormone levels can accelerate the aging process. Aging can be influenced by internal and external factors. Internal factors such as excess free radicals, reduced hormone production, cell death, glycosylation, methylation, decreased immunity, and genetics. Meanwhile, external factors are bad lifestyle, wrong habits, pollution, poverty, and stress.[1] One sign of aging that affects the quality of life is sexual problems caused by decreased organ function related to sexual function.[2]

Free radicals are the cause of aging. Cigarette smoke exposure is a substance that can induce oxidative stress.[3-6] Cigarettes contain carbon monoxide, hydrogen cyanide, nitrogen-oxygen, tar, nicotine, and metals such as lead, nickel, cadmium, chromium, arsenic, and iron. Substances in cigarettes or cigarette smoke contribute to accelerating the aging process.[4,7] Damage in the body due to the accumulation of free radicals through three routes, namely: peroxidation of lipid components results in damage to cell membranes, modification of proteins, and triggers damage to DNA.[8] Diffusion and active transport into Leydig cells resulting in the death of Leydig cells. If there is the death of Leydig cells, the steroidogenesis process, particularly the secretion of the hormone testosterone will be inhibited.[8,9]

Tamarillo seeds contains vitamin A, vitamin B_6, vitamin C, vitamin E, carotenoids, magnesium, calcium, phosphorus, carbohydrates, fat, protein, fiber, terpenes, steroids, phenols, saponins, and flavonoids. Those components are classified as a natural antioxidant.[10] Research on the ethyl acetate extract of tamarillo seeds conducted by Dewi et al. (2014) shows that the flavonoid compounds were higher in seeds compared to the skin and pulp extracts. Blood plasma MDA levels of Wistar rats decreased by giving ethyl acetate extract of tamarillo seeds at a dose of 200mg/kgBW which is equivalent to vitamin E 100mg/kg BW because it inhibits lipid peroxidation.

The complex antioxidant component in tamarillo makes interest for this research, especially the ethanol extract of tamarillo seeds to determine its effectiveness in inhibiting the decrease in the number of Leydig cells and testosterone levels of male Wistar rats exposed to cigarette smoke.

The purpose of this study was to proof whether oral administration oftamarillo seeds ethanol extract inhibited the decrease of Leydig cell and testosteron levels in male Wistar rats (*rattus novergicus*) exposed to ciggarette smoke.

2. Method

This research is an experimental study using a post-test control group design and conducted at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, Denpasar, Bali. The sample was 36 healthy, male Wistar rats, 6 months old and weighing 180-200 grams which were divided into two groups, each group of 18 rats. The control group was given a placebo (aquadest) 2ml/day and the treatment group was given ethanol extract of tamarillo seeds 80mg/200gBW/day. Each treatment was given just before exposure to cigarette smoke for 21 days and smoke exposure was given 1 hour daily. After the treatment, blood was drawn to check testosterone levels using the ELISA method and testicular surgery was done for microscopic evaluation and Leydig cell counts.

This research has met the requirements of animal ethics and was awarded a certificate of approval for animal ethics from the Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali.

Testosterone levels examination. Venous blood was drawn from the medial canthus sinus orbitalis dextra, put into an Eppendorf tube, placed at an angle of 45° and left at room temperature, then centrifuged at 3000 rpm for 10 minutes to obtain serum. Testosterone levels were analyzed using the Bioassay Technology Laboratory ELISA Testosterone Rat Kit using the indirect ELISA method.

Leydig cells count. Leydig cells are present in the interstitial tissue between several seminiferous tubules, polyhedral shape, size 15-20 µm, has a round nucleus and eosinophilic granular cytoplasm. Leydig cell count was done by observing left and right testes histologically in 5 fields of view (zigzag direction like the letter Z) with the magnification of 400X. The results obtained are summed and averaged, then divided by 5 fields of view. The number of Leydig cells is expressed as the number per one large field of view.

Analysis Results. Data were analyzed using SPSS version 22.0. Data were analyzed statistically using an independent t-test and considered statistically significant if the p value is <0,001 with 95% confidence.

3. Result

The analysis result of Leydig cell count showed that the mean in the control group was 17,21±5,14 per one large field of view and the treatment group was 34,46±5,87 per one large field of view with p value <0,001. The mean of Leydig cells in the treatment group was significantly higher than the control group.

Table 1: The mean difference of Leydig cell count per one large field of view between the control and treatment group.

Variable	Group	n	Mean ± SD	*p*
Leydig cell	Control	18	17,21± 5,14	< 0,001
	Treatment	18	34,46 ± 5,87	

n = number of samples, SD = standard deviation, *p* = probability

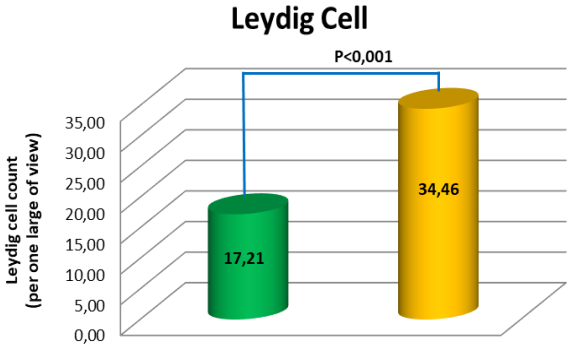


Figure 1: The mean difference of Leydig cell count per one large field of view between the control and treatment group.

The mean of testosterone levels in the control group was 10,88±1,34 nmol/ml and the treatment group was 20,24±2,16 nmol/ml with p value <0,001. The mean testosterone levels in the treatment group were significantly higher than in the control group.

Table 2: The mean difference between testosterone levels (nmol/ml) for each group.

Variable	Group	n	Mean ± SD	*p*
Testosterone level	Control	18	10,88 ± 1,34	< 0,001
	Treatment	18	20,24 ± 2,16	

n = number of samples, SD = standard deviation, *p* = probability

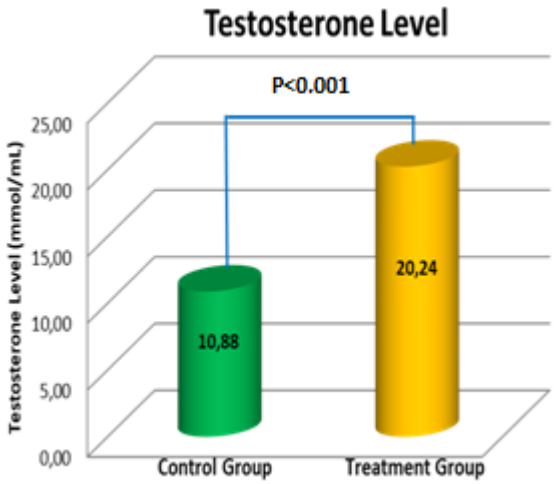
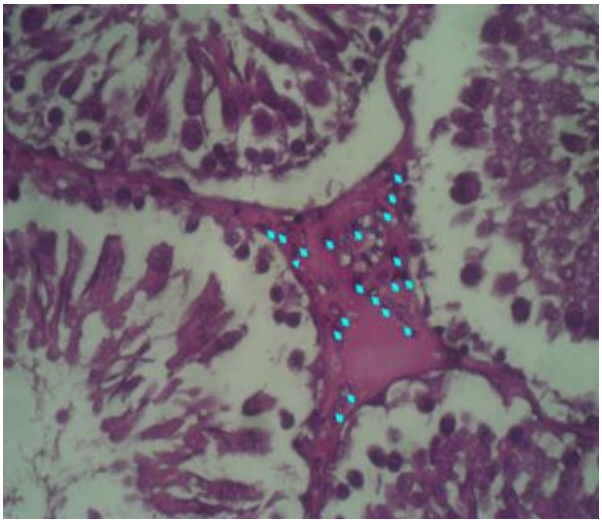
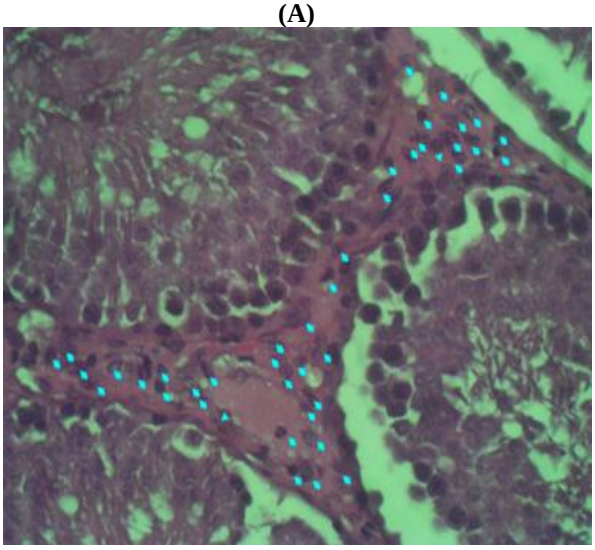


Figure 2: Mean difference of testosterone level (nmol/ml) between control and treatment group.



(A)



(B)

Figure 3: Leydig cells between interstitial tissue in the control group (B) was higher than control (A) (blue arrows).

4. Discussion

Effect of cigarette smoke exposure on the Leydig cells count and testosterone levels

Cigarette smoke consists of various chemical components in the form of gases and particles and is toxic to our cells. Active and passive smoking can lead to the entry of toxic compounds into the lungs and circulate into the blood circulation. There can be a process of diffusion and active transport into Leydig cells which can cause Leydig cell death. The process of steroidogenesis in particular the testosterone formation by Leydig cells is also inhibited.[8,9] Carbon monoxide binds to hemoglobin in red blood cells, causing narrowing and hardening of the blood vessels then resulting in blockages and blood circulation disorders.[12] The spermatogenesis process in the testes requires high oxygen consumption. If the vascularization of the testes is not good, there will be competition for oxygen consumption in the process of spermatogenesis and steroidogenesis, where both of these processes are very susceptible to oxidative stress.[13] Nicotine can change the hypothalamic-pituitary-gonad axis by increasing the release of dopamine and activating the release of opioid peptides in the brain which ultimately inhibits GnRH secretion and decreased LH secretion which causes failure of Leydig cells to secrete testosterone.[14,15] The cadmium content in cigarette smoke illustrates the toxicity in the reproductive system including decreased sperm cell count and sperm abnormalities with DNA fragmentation disorders. Cadmium is anti-steroidogenic in Leydig cells.[16] The metal content of cadmium and nickel in cigarette smoke disrupts the activity of the enzyme adenylyl cyclase on the Leydig cell membrane so that the cell function in secreting testosterone was inhibited.[14,15] At the testicular level, it increases ROS formation which leads to oxidative stress and results in damage to cell membranes, nucleic acids, and inhibits DNA repair which ultimately triggers apoptosis.[17] A study showed that mice exposed to lead have increased GnRH stimulatory response that continued with inhibition of FSH and LH secretion resulting in a reduced ability of Leydig cells in secreting testosterone.[18]Continuousexposure to Benzo(a)pyrene can significantly reduce serum and intratesticular testosterone levels in mice. The insufficiency results in testicular atrophy with massive apoptosis. The decrease in testosterone levels was followed by decreased expression of StAR and 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD) of Leydig cells. StAR is probably the main steroidogenic protein targeted by Benzo(a)pyrene or other Polycyclic aromatic carbons. Toxic substances contained in cigarette smoke can interfere with the spermatogenesis process by damaging the endothelial blood vessels and testicular blood barrier by initiating inflammation in the testes resulting in apoptosis.[19] Smoking can result in an imbalance between antioxidants and ROS, which triggers oxidative stress.[20] Increased ROS also affects cell characteristics, including Leydig cells that cause functional deficits. Leydig cell deficit will result in inhibition of the testosterone synthesis pathway. Increased oxidative stress also increases lipid peroxidation, DNA damage, and disruption of preventive enzymatic antioxidants.[21,22]

The Role of Ethanol Extract of Tamarillo Seeds on Leydig Cell Count and Testosterone Levels

Research on tamarillo seeds conducted by Dewi et al. (2014) stated that the ethyl acetate extract of tamarillo seeds contained flavonoids, terpenes, phenols, and saponins. The flavonoid is higher than the extract of the skin and pulp of tamarillo. Extract with a dose of 200mg/kgBW is equivalent to vitamin E (100mg/kg BW) in inhibiting lipid peroxidation.[11] Tamarillo has complete nutritional content. Several studies on the compounds contained in tamarillo were conducted by Sinaga (2009) and stated that ethanol extract of fresh tamarillo contained alkaloids, flavonoids, tannins, saponins, steroids/triterpenoids, β-carotene, anthocyanins, and fiber.[23] Kadir et al. (2014) stated that the tamarillo extract contained higher antioxidants than yellow cherry tomatoes, red cherry tomatoes, and tomatoes. The administration of tamarillo extract also resulted in increased antioxidant activity of GPx, SOD, antioxidant capacity, and inhibited inflammation.[24,25]

The flavonoids can increase steroidogenesis by increasing cAMP formation so the mitochondrial cholesterol import increase. There is also an increase in the StAR protein levels in Leydig cells. Those effects increase the ability of Leydig cells to secrete testosterone.[26] Flavonoids act as a vasodilator and anti-inflammatory so testicular inflammation is inhibited

and there will be increase blood flow to the testes so that the spermatogenesis and steroidogenesis processes can take place. These processes require very high oxygen consumption.[27] Flavonoids work as an exterminator of free radicals or free radical scavenger by breaking free radical chains.[28] Vitamin C can inhibits free radicals by maintaining the integrity of the cell membrane.[29] Vitamin C also inhibits lipid peroxidation so that the transport of essential ions from inside and outside the cell can take place to inhibited apoptosis. Vitamin C reacts immediately with superoxide anions, hydroxyl radicals, singlet oxygen, and lipid peroxides. Giving vitamin C to normal animals can stimulate both sperm production and testosterone secretion.[13] The phenolic compounds contained in the tamarillo seeds extract act as primary antioxidants due to their ability to stop lipid oxidation so Leydig cell damage can be inhibited.[30] Tannin compounds are useful by binding metals, neutralizing ROS, and prevent oxidative damage to DNA.[31,32] Carotenoids can absorb singlet oxygen and deactivate ROS. β-carotene is a very important carotenoid because it cannot be attacked by free radicals. The function of β-carotene in the testes can improve oxidative stress due to cadmium by suppressing lipid peroxidation and maintaining SOD, GST, and GSH in normal conditions.[13,23,33] Anthocyanins consume derivate from reactive oxygen free radicals such as hydroxyl, peroxyl, and oxygen. Anthocyanins are useful in inhibiting various degenerative diseases caused by the continuous oxidation process in the body which causes cell damage.

The limitation of this study is that our study was done only in 21 days. Further research is needed to be carried out with a longer duration to identify the effectiveness of the ethanol extract of tamarillo seeds in inhibiting the aging process in humans.

From this research, it has been proven that oral administration oftamarillo seeds ethanol extract can inhibited the decrease of Leydig cell number and testosterone levels in male Wistar rats exposed to cigarette smoke.

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