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Original Article

Effectiveness of Moringa Leaf Extracts (*Moringa Oleifera*) on Testosterone Levels in Wistar Rats (*Rattus Norvegicus*)

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ABSTRACT

Background: *Moringa oleifera* is most notable for its potent antioxidant properties that neutralize harmful free radicals within the body. Free radicals are produced as natural by products of metabolic processes during aging. Testosterone, a crucial hormone in male development experiences a gradual decline as men age associated with various physiological and psychological changes. One of the factors that contribute to this decline is oxidative stress which impairs the function of leydig cells that synthesize testosterone. Therefore, this study aims to identify the effectiveness of different doses of Moringa oleifera extract on testosterone levels in aged male Wistar rats.
Methods: This is an experimental study with a post-test-only control group design. The data was collected from the total samples of 27 rats and randomized into 3 groups. 9 samples of a control group, 9 samples of a group given a dose of 250mg/kg, and 9 samples of a group given a dose of 500mg/kg of Moringa oleifera extract. The extract was given through oral gavage for 21 days and blood was collected to analyze the total testosterone levels.
Results: The results of the study showed the mean value of the treatment group tends to be lower than the control group. Testosterone levels between the 3 groups obtained a p-value of 0.598 where the value was > 0.05, which means there was no significant difference between the 3 groups based on testosterone levels.
Conclusions: Moringa leaf extract was not significantly effective on testosterone levels in aging male Wistar rats. The effectiveness of Moringa leaf extract at a dose of 500mg/kg was not significantly different from a dose of 250mg/kg on testosterone levels in male Wistar rats.

Key Words: *Moringa oleifera extract, Testosterone, Wistar Rat, Aging*

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INTRODUCTION

Measurement of serum testosterone levels is essential for the evaluation of hypogonadism in men and androgen excess in women. Testosterone (T) plays an important role in male sexual differentiation, sexual maturation, reproductive health, as well as men's general health and quality of life[1]. Testosterone deficiency syndrome is also called hypogonadism which involves a deficiency of androgens, especially the hormone Testosterone which is produced by the Leydig cells of the testicles. Hypogonadism not only inhibits the spermatogenesis process but also disrupts normal reproductive physiology in men[2].

The onset of hypogonadism can be congenital from birth or occur later in life. As with all endocrine organs, there is a decline in the function of the hypothalamic-pituitary-testicular axis with aging. This condition is often called late-onset hypogonadism (HOL), but a more appropriate term is functional hypogonadism (HF) because this condition is more often caused by chronological aging, with obesity and comorbidities. Male aging is characterized by a decrease in circulating T levels. gradually. When T decreases below the reference range for young men, HOL/HF may be suspicious[3].

Testosterone is responsible for promoting primary sexual development, which includes testicular descent, spermatogenesis, penis and testicular enlargement, and increased libido. Testosterone is involved in regulating secondary male characteristics responsible for masculinity. These secondary sex characteristics include anabolic effects that include pubertal growth and skeletal muscle growth (testosterone stimulates protein synthesis). Testosterone levels generally decrease with age; Therefore, men tend to experience decreased testicular size, decreased libido, decreased muscle density and mass, increased fat production, and decreased erythropoiesis, which may lead to anemia[4].

Several epidemiological studies have shown that community-dwelling men over the age of 30 years are characterized by a 0.5-15% annual decrease in circulating testosterone concentrations and a 2-3% decrease in free

testosterone concentrations. About 40% of men over 45 years and 50% of men over 80 years are found to be hypogonadal[5].Men's testosterone concentrations continue to decline by 1–2% per year, beginning after the third decade of life. Additionally, obesity, metabolic syndrome, and chronic diseases, such as type 2 diabetes, renal insufficiency, and chronic lung disease, are associated with lower serum testosterone concentrations among men[6].Testosterone deficiency has a prevalence of up to 20% among adolescent and young adult men, defined as men aged 15-39 years according to the National Cancer Institute. Using baseline data, the National Health and Nutrition Examination Surveys (NHANES) found a significant decline in average Total Testosterone (TT) levels among adolescent boys and young adult men in the United States in 1999 and 2016.

Reactive oxygen species (ROS) are reactive molecules that contain oxygen in the body. They are maintained at optimal concentrations by various antioxidant enzymes. However, high levels of ROS can disrupt the oxidative balance, thereby impacting the HPT axis resulting in reduced secretion of reproductive hormones. Oxidative stress occurs either due to increased ROS production or reduced antioxidant availability. During oxidative stress, biomolecules are damaged, causing loss of function and even cell death, and become free radicals containing reactive oxygen causing molecular cascade damage. Testicular oxidative stress causes a decrease in testosterone production, either due to injury to the Leydig cells or other endocrine structures such as the anterior pituitary[5].

Natural traditional medicine is actively practiced throughout the world and has been a part of many lives for generations. Many pharmaceutically active drugs are being developed based on natural resources such as plants, as they provide many bioactive components. The use of native plants has increased in recent years due to their originality, relative accessibility, and minimal side effects in treating common diseases worldwide[8].

Moringa oleifera (MO) belongs to the unique Moringaceae family with great potential highlighted by the National Institute of Health and is used traditionally to treat bronchitis, infections, and fever among several other diseases[7]. M. oleifera is consumed not only for its nutritional value but also for its health benefits. Rich in beta-carotene, vitamin C, vitamin E, and polyphenols, M. oleifera leaves are a good source of natural antioxidants. Currently, M Oleifera has been reported to increase various biological activities including antioxidant, antibacterial, antifungal, anti-inflammatory, anticancer, hepatoprotective, cardioprotective, and neuroprotective activities. Additionally, Moringa Oleifera Extract (MOE) can modulate the immune system and many studies have shown therapeutic benefits including diabetes, rheumatoid arthritis, atherosclerosis, infertility, pain relief, depression, and regulation of diuretics and thyroid. Due to these reported functions, M. oleifera's bioactivity has received tremendous attention over the past decade, leading to increased exploration and comprehension of its pharmacological functions and underlying mechanisms[8].

Based on theory, the oxidative stress process in Leydig cells in older men can cause a decrease in testosterone levels and the emergence of clinical symptoms of late-onset hypogonadism. Thus, it raises the question of whether Moringa oleifera extract, a strong antioxidant, has an effect on serum testosterone levels to treat aging-induced hypogonadism in men.

METHODS

This type of research is experimental research using a post-test-only control group design carried out on male Wistar rats. In this study, the rat used were healthy Wistar rat, weighing 160 - 320 grams, and aged 2 years old. The sample size used in this study was 9 animals per group. Therefore, the number of samples required for this experiment is 27rats. This research was conducted at the Animal Housing and Research Laboratory at the Faculty of Medicine, Ciputra University, July - November 2023.

Acclimatization was carried out on experimental animals within a period of 7 days to adapt to the laboratory environment. Experimental animals that die or become sick during acclimatization will be excluded from the study. The sample groups were divided using 27 male rat aged 2 years old and weighing 160-320 grams, then randomly divided into three groups, namely groups K0, K1, and K2.

Experimental animals of each group were given a different treatment. Group K0 was given sweet corn and mineral water. Group 1 was given sweet corn and mineral water, then given Moringa leaf extract at 250mg/kg/day for 21 days. Group K2 was given sweet corn and mineral water, then given Moringa leaf extract at 500mg/kg/day for 21 days. Standard rat feeding was carried out throughout the research.

In making a moringa leaf extract, 96% ethanol was used for the extraction process which is a critical point in extracting compounds from plants so it needs to be done carefully. The materials that need to be prepared are simply the moringa leaf and 96% ethanol as the solvent[9].Then moringa leaf extract was given through oral gavage every day for 21 days. At the end of the treatment, rats were sacrificed to take blood samples by cardiac puncture. This procedure requires termination with anesthesia.

Rats were given ketamine HCl 50mg/kg intraperitoneally to facilitate euthanasia[10]. Dead ratwere characterized by negative pupillary reflexes. Next, a 5 ml blood sample was taken. Anesthetize the rat deeply and check for anesthesia by lack of spontaneous movements, slow breathing rate, and lack of response to stimuli (such as toe pinching). The blood obtained is put into an EDTA tube. Using a centrifuge spun at 2200–2500 RPM for at least 15 minutes yields both serum and plasma. Testosterone levels were examined using the Quantitative Fluorescence Immunoassay (FIA) method with serum obtained in EDTA tubes in the Animal Housing and Research laboratory at the Faculty of Medicine, Ciputra University, Surabaya.

RESULTS

This research was carried out at Universitas Ciputra Surabaya, Indonesia. In this study, 27 male Wistar rats were divided into 3 groups, namely the control group, treatment group one, and treatment group two. The data used consisted of a total of 27 rat blood samples and 9 rat blood samples for each group. To analyze the data, descriptive statistical tests, data normality tests, homogeneity tests, and non-parametric statistics have been described. Based on descriptive statistical analysis results, the mean value of the treatment group tended to be lower than that of the control group. The mean value of testosterone in the group given a dose of Moringa leaf extract 500 mg/kgBW, tended to be lower than the group given a dose of Moringa leaf extract 250mg/kgBW (Table 1 and Figure 1).

The normality test used in this study is the Shapiro-Wilk test. This is because the sample size used in the research is less than 50 data (a total of 27 samples). The decision on normality testing can be determined based on the significance value, where the Shapiro-Wilk test indicates a normal distribution if $p > 0.05$. Based on the results of the normality test, it was found that the Testosterone level data for the K2 group was declared not normally distributed because the siq value or p-value was 0.011 which was <0.05 (Table 2).

Testosterone data between groups was tested for homogeneity using Levene's Test. The results show homogeneous data ($p>0.05$). Based on the results of the homogeneity test, it was found that the testosterone level data was declared homogeneous because the siq value or p-value was 0.668, which was > 0.05 (Table 3).

After obtaining the results from the data normality test, the next stage was to carry out a non-parametric statistical test, namely the Kruskal-Wallis test which aims to determine whether there are significant differences between the three groups of data. The comparison test for testosterone levels between the 3 groups used the Kruskal Wallis test because, during the normality test, there was 1 group that was declared abnormal. Based on the results of the comparison test for testosterone levels between the 3 groups, a p-value of 0.598 was obtained, where this value was > 0.05, which means there was no significant difference between the 3 test groups based on testosterone levels, although, in terms of the mean value, the testosterone levels of the treatment group tended to be lower than those of the treatment group. control but the results were declared not statistically significant. (Table 4 and Table 5).

DISCUSSION

The decrease in the two treatment groups given Moringa leaf extract at a dose of 250mg/kgBW and at a dose of 500mg/kgBW may have been caused by a decrease in the ability to regulate testosterone levels in the blood due to the aging process coupled with the impact of stress given every day during the procedure of administering of Moringa leaf extract. Oral probes induce activation of the stress response, as shown by increased adrenal corticosterone output[11]. Administering doses to rats and rat via oral probes can cause increase of stress level significantly, including increases in blood pressure, heart rate, and plasma corticosterone levels[12]. In addition, it is proven that an increase in cortisol results in a decrease in circulating testosterone levels[13]. This suggests that method of administering treatment and stress must also be considered as the factors that can impact the result of testosterone level and further research is required to also include the group of rat with young adult ages to compare the ability to regulate testosterone levels.

Another possible cause of the decrease in the two treatment groups given Moringa leaf extract at a dose of 250mg/kgBW and a dose of 500mg/kgBW was the insufficient dose of Moringa leaf extract given to elderly rat which experienced a decreased ability to regulate testosterone levels. The dose of Moringa seeds given to middle-aged rat (46 weeks) that had not yet experienced andropause was given 750mg/kgBW/day to produce significant changes[14]. Meanwhile in this study, the age of the rats used are 2 years old and the decrease of testosterone level might be due to an inadequate dose. This suggests carrying out further research on a larger dose of Moringa leaf extract for aging Wistar rats.

The Moringa plant has many beneficial nutritional components, one of which has been proven to be beneficial for mothers who breastfeed their babies by increasing prolactin levels in the blood. Moringa leaves increase breast milk volume by increasing prolactin and providing important nutrients[15]. However, prolactin and testosterone levels tend to have a negative correlation, where higher prolactin levels correlate with lower testosterone levels in women and men[16]. Hence, it can be seen that the decreased testosterone levels after administering Moringa leaf extract can be caused by the effect of increasing prolactin levels from Moringa leaves.

In many mouse strains, hypothalamic-pituitary changes occur with age and cause a decrease in LH levels thereby reducing Leydig cell stimulation. Aged Leydig cells experience deficits in the number of LH receptors, cAMP production, STAR and TSPO cholesterol transport, and the number of steroidogenic enzymes in the mitochondria and smooth endoplasmic reticulum[17]. Senescent cells are relatively insensitive to LH and thus reduced cAMP production may account for the age-related reduction in steroidogenesis by senescent cells. Thus, aging can weaken the testicular response to LH and administration of the LH substitute, hCG, in pharmacological amounts fails to stimulate maximal testosterone concentrations in young adults in many elderly men. Five experimental paradigms show impaired feedback regulation of LH production due to endogenous the concentrations in elderly men[18]. It can be seen that there is damage to testosterone-mediated negative feedback in elderly men with impaired GnRH and LH outflow in the brain. At this age point, administration of Moringa leaf extract which contains antioxidant and anti-inflammatory components is not able to increase testosterone levels due to axial changes in the hypothalamus-pituitary and indirectly results in testicular weakness in LH.

In this research, the Moringa leaves used came from the UPT Herbal Materia Medica Laboratory in Batu. Knowing the origin of herbal plants is very important as a consideration factor that can influence the experiment results because different plant growth locations have different chemical and nutritional components for each plant. In addition, the results of the Moringa leaf extract that has been processed should also be tested for the content profile where different chemical compositions can also have different results from the Moringa leaf extract that is processed alone compared to the results of administering Moringa leaf extract in other studies.

Apart from the influence of the Moringa leaf content, the process of making Moringa leaf extract can affect testosterone levels due to different chemical solvents used for extractions such as extraction with water, ethanol, or methanol. In the process of making Moringa leaf extract, the ethanol that has been used for extraction has been evaporated in a rotary evaporator for 7 hours to ensure that all the ethanol is evaporated. However, this process cannot guarantee 100% cleanliness of Moringa leaf extract free from ethanol.

Although these findings contribute to the existing literature, several other journals with similar experiments should be considered for comparison (Table 6). The observed changes are within the range of natural variability, and further research is needed to validate theconfounding factors in need to provide more comprehensive understanding of the results.

CONCLUSION

In conclusion, our study observed a decrease in mean testosterone levels after administration of Moringa leaf extract, however was not significantly effective on testosterone levels in aging male Wistar rats. The effectiveness of Moringa leaf extract at a dose of 500mg/kg was not significantly different from a dose of 250mg/kg on testosterone levels in male Wistar rats.

DECLARATIONS

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Conflict of interest: No conflict of interest to be disclosed

Ethical approval: This research has obtained a permit from the National Unity and Politics Agency (Badan KesatuanBangsa dan Politik) with approval number 070/7145/209/2023. All procedures involving research subjects were conducted following the guidelines specified in the ethical clearance from the Health Research Ethics Committee of the Faculty of Medicine Ciputra University (Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Ciputra. All participants provided written consent before participating in this study.

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Table 1: Descriptive Analysis of Testosterone Levels

Group	N	Min	Max	Mean	Median	Std. Deviation
K0	9	19	5,01	2,34	2,31	1,67
K1	9	19	4,22	2,06	1,80	1,22
K2	9	19	6,75	1,89	1,37	2,05
Total	27	19	6,75	2,10	1,75	1,62

Table 2: Shapiro-Wilk Normality Test

Group	Statistic	df	Sig.	Interpretation
K0	0.951	9	0.700	Normal
K1	0.990	9	0.996	Normal
K2	0.775	9	0.011	Not Normal

Table 3: Levene Homogeneity Test

Kadar Testosteron	Levene Statistic	df1	df2	Sig.
Based on mean	0.410	2	24	0.668
Based on median	0.239	2	24	0.789
Based on Median with adjusted df	0.239	2	16.566	0.790
Based on trimmed mean	0.277	2	24	0.760

Table 4: Ranks Kruskal-Wallis

	Group	N	Mean Rank
Testosterone Level	K0	9	15.56
	K1	9	14.56
	K2	9	11.89

Table 5: Statistic Test Kruskal-Wallis

	Testosterone Level
Kruskal-Wallis H	1.030
df	2
Asymp Sig.	0.598

Table 6: Comparison of Other Studies regarding the Effectiveness of Moringa Leaf Extract on Testosterone Levels

No.	Journal Title	Result
1	Effects of aqueous extract of Moringa oleifera seed on cadmium-induced reproductive toxicity in male Wistar rats[19]	Moringa seeds (100 and 500 mg/kg) caused a decline in serum levels of testosterone, and these were significantly lower than the control. Testosterone levels were also lower in the two groups that received both cadmium and Aqueous Extract Moringa Seeds, but the decline was not statistically significant
2	Sex hormones and oxidative stress biomarkers of male Wistar rats treated with Moringa oleifera seed fractions[20]	Serum testosterone and LH were significantly reduced in Methanol Fractioin Moringa Seed -treated (50 and 100 mg/kg) rats. Hexane Fraction Moringa Seed had no effect on testosterone, LH, or FSH.
3	Effect of Ethanol Extract Moringa oleifera Leaves on Fertility Hormone and Sperm Quality of Male Albino Rats[21]	Mean values of rats treated with M. oleifera leaves showed significant ($P \leq 0.01$) increased in fertility hormone (testosterone, FSH and LH)
4	Pre and Post-Treatment Effects: Estimation of Serum Testosterone and Lipid Peroxidation Levels on Moringa olifera Extract Induced Cadmium Exposed Rats[22]	Highly significant increase in testosterone levels were observed when group IV (CdCl + MOE) was compared to group III (CdCl)
5	Ameliorative Effect of Moringa oleifera Extract on Male Fertility in Paroxetine Treated Rats [23]	Administration of moringa did not reveal any significant change in serum reproductive hormones level except FSH when was compared with the control group. However, co-administration of moringa hydro alcoholic extract and paroxetine induced a significant ($p<0.05$) increase in the level of serum reproductive hormones (FSH, LH, testosterone and estrogen) when compared with paroxetine group

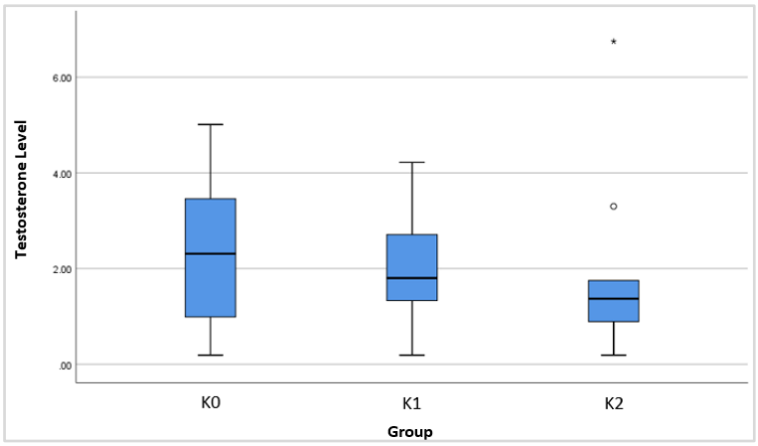

Figure 1: Testosterone Level Comparation Diagram