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Original article

Eurycoma longifolia Elevates Testosterone Levels in Androgen-Deficient Osteoporosis Rat Model

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Abstract
Osteoporosis is a chronic, progressive illness characterized by low bone mass and strength of the bone, resulting in increased risk of bone fracture. Testosterone replacement therapy (TRT) is one of the main treatments for male osteoporosis. However, TRT causes side effects, mainly prostate cancer and liver dysfunction. So, an alternative agent is required. This study was conducted to determine the effects of *Eurycoma Longifolia* (EL) on serum testosterone in orchidectomized rats, the androgen-deficient osteoporosis model. Thirty-two male *Sprague Dawley* rats were divided into 4 groups, with 8 rats in each group, which followed different treatments for 6 weeks, namely the sham-operated group, orchidectomized-control group, orchidectomized group and treated with EL (15 mg/kg), and orchidectomized group and treated with testosterone (8 mg/kg). Body weights of rats were taken weekly, and blood samples were taken before and after treatment to measure serum testosterone using the Testosterone ELISA technique. The result showed that EL and testosterone therapy have led to an increase in rat body weights. The levels of testosterone in the serum were considerably higher ($p < 0.05$) for the orchidectomized group treated with testosterone compared to the orchidectomized control and sham group. The orchidectomized group treated with EL also showed a significant increase in serum testosterone ($p < 0.05$) compared to the same group before treatment, but there was no significant difference between the other groups. In conclusion, EL treatment was successful in raising the level of serum testosterone in orchidectomized rats
Keywords. *Eurycoma Longifolia*, Osteoporosis, Orchidectomy, Testosterone.

Introduction

Low bone mass is a defining feature of osteoporosis, a chronic, progressive disease, and a weakening of the bone resulting in increased risk of bone fracture. Approximately 2 million men in the United States suffer from osteoporosis [1], and 1.5 million of these patients are more than 65 years old [2]. Unfortunately, osteoporosis is becoming the main cause of morbidity and mortality in older men. According to many studies, the mortality associated with hip fractures is higher in men than in women [3]. Men are twice as likely to die in the hospital following a hip fracture (14% versus 6% for women), and it is estimated that 31% of men with hip fractures died within 1 year after the fracture occurred, but only 17% of women died after the fracture [4]. The main cause of osteoporosis in men is androgen deficiency, which may occur in old age due to normal aging or in young men with undescended testes, malignancy in the testis, or any abnormality in the chromosomes [5]. 20-30 % of elderly men suffered from testosterone deficiency compared to younger men. There were several reports that partial androgen deficiency is common in aged men [6].

Elderly men with androgen-deficient osteoporosis can be treated with testosterone replacement therapy. Many clinical trials have concluded that testosterone replacement therapy has led to a decrease in bone resorptive markers [7]. Furthermore, there have been reports of testosterone being used to treat osteoporosis in hypogonadal men with testosterone increases calcium absorption and bone formation [8].

However, patients may reject the treatment due to the painful injection of testosterone and its dangerous side effects, such as prostate cancer. So, there is a requirement to treat and prevent osteoporosis in men with a much safer and more convenient oral intake, such as herbal medicine. In Asia, traditional and herbal medicines are commonly used to treat infertility and low libido and improve sexual performance and muscle strength [9].

A Previous study by Nazrun et al, on male orchidectomised rats (androgen deficiency osteoporotic model), found that *Eurycoma Longifolia (EL),* a plant with androgen effects, was able to maintain the bone calcium content. *Eurycoma Longifolia Jack* is used as traditional medicine in Southeast Asian countries, including Myanmar, Indochina, Thailand, Laos, Cambodia, and Malaysia [10]. *E. Longifolia is* identified locally as Tongkat Ali in Malaysia, Pasakbumi or BidaraPahit in Indonesia, Ian-don in Thailand, and “Cay babin`h” in Vietnam. The plant name is translated as a tree in whose roots are used to enhance or improve sexual performance and libido, and treatment of infertility, malaria, and cancer [11-12].

Therefore, this study aims to point out the potential of EL for the treatment of male osteoporosis by determining the mechanism of *Eurycoma Longifolia* in the protection against osteoporosis in orchidectomized rats by measuring the testosterone level. Orchidectomized rat model is an accepted animal model to represent androgen-deficient osteoporosis in men. According to previous studies, this rat model reflected the skeletal effects of androgen deficiency.

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Material and methods

Study design

This study involved thirty-two male Sprague-Dawley rats, aged 12 months were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia**,** Malaysia.

Animal model

Rats were kept two per cage under 12-hour natural light–dark cycles and were permitted to acclimatize to the surrounding for 1 week. Rats were treated at 9am every morning for 6 consecutive weeks. During the treatment, a standard diet (rat pellets) and deionized water were given to all the rats. There were four groups of rats with each group consisting of 8 rats; sham-operated group, orchidectomize-control group, orchidectomized and treated with EL (15mg/Kg) and orchidectomized and treated with testosterone (8 mg / Kg). Orchidectomized middle-aged rats were used as the model for androgen-deficient osteoporosis [13].
The orchidectomy was performed for rats in groups 2, 3, and 4. The surgical instruments were sterilized by washing with 70% alcohol before and after each use. Before orchidectomy, the rats' weights were recorded and used to determine the dose of anesthetic agents, which were injected into rats intraperitoneally (0.1 ml /100 g). 70% alcohol and iodine were used for sterilization. Then, the rats were operated under suitable lighting conditions. The skin of the scrotum was opened (1-2 cm), followed by the muscle (cremaster muscle) with an estimated width of 0.5 cm. After that, the spermatic cord, which contained the epididymis, artery, and vein (blood vessels), was tied by using a special thread (Tangsi) to block the arterial and venous blood flow. The left and right testes were taken out by separating them from the spermatic cord using a surgical knife, and the inner layer and skin of the scrotum were closed back. After that, each rat was put in a separate cage for 7 days for the wounds to heal and received an antibiotic (Baytril) at the dose of 0.1 ml once daily intramuscularly for a week. The rats were weighed once a week and blood samples were taken before and after the treatment.

Eurycoma longifolia extract

In this study, the dose of *Eurycoma Longifolia* (EL) was 15 mg/kg body weight. And the weights of rats were between 400-500 g (approximately 450 g). This extract was in light brown fine powder. Their major chemical compounds were protein (31.75 %), glycosaponins (41.08 %), and eurycomanone (1.604%). The substance was diluted in deionized water.30 mg EL were added to 20 mL of deionized water to make a stock solution. The doses of oral gavage were given according to the body weight of rats, which was measured weekly. This solution was homogeneous and stored in dark bottles at a temperature of - 4 ° C. EL was given orally 0.1ml /100 g once daily, 6 days a week for 6 weeks.
400 mg or 0.4 g of testosterone was dissolved in 50 ml of normal saline as a stock solution. Then, it was mixed until it became homogenous and stored in labeled glass bottles and kept at – 4 ° C. EL was given orally 0.1ml /100 g once daily, 6 days a week for 6 weeks.
The blood samples were taken from the orbital sinus twice, prior the start and after treatment. The rat was anesthetized with diethyl ether inhalation. A capillary tube placed in the eye dorsally, laterally or medially. Capillary action was used to let blood enter the capillary tube or pipette. The samples were left for 3-4 hours to get the serum, and they were centrifuged at a speed of 3000 rpm for 10 minutes. After that, the supernatant was pipette in labeled micro-tubes and kept at -70□C.

Bone biochemical markers

The testosterone levels were measured using the Testosterone ELISA Kit (i-DNA Biotechnology, Hamburg, Germany).

Statistical analysis

Analysis of variance (ANOVA) was conducted using SPSS Version 19.0 (IBM, USA) to determine the treatment effects. Data analysis involved the mean for each of the treatment groups. The normality was tested by using the Kolmogorov-Smirnov test. Paired students' test and ANOVA followed by Tukey" 's HSD were used to determine and compare the significant differences among the means of various treatment groups. Differences with $P < 0.05$ were considered significant.

Results

Body weight

In all groups**,** a paired student's test revealed a significantly higher body weight at the end of the treatment than the baseline body weight. At the end of treatment, all groups had noticeably greater body weights than orchidectomized control group at the end of treatment. (Figure 1).

Serum testosterone levels

Before treatment, no significant difference in the serum testosterone levels was noted in all treatment groups (Figure 2). The serum testosterone level of the OrxC group after six weeks of treatment was considerably less than its level before treatment. This means that orchidectomy has resulted in

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testosterone reduction, as testes are the main producers of testosterone. As expected, testosterone replacement therapy in orchidectomized rats (Orx + T) caused a significant increase in the level of testosterone compared to before treatment. Supplementation with EL to the orchidectomized rats (Orx + EL) had also significantly increased the testosterone level compared to before treatment. When the groups were compared after treatment, the testosterone levels of Orx+T group were significantly higher than SHAM and OrxC groups, but not significantly different compared to Orx +EL.

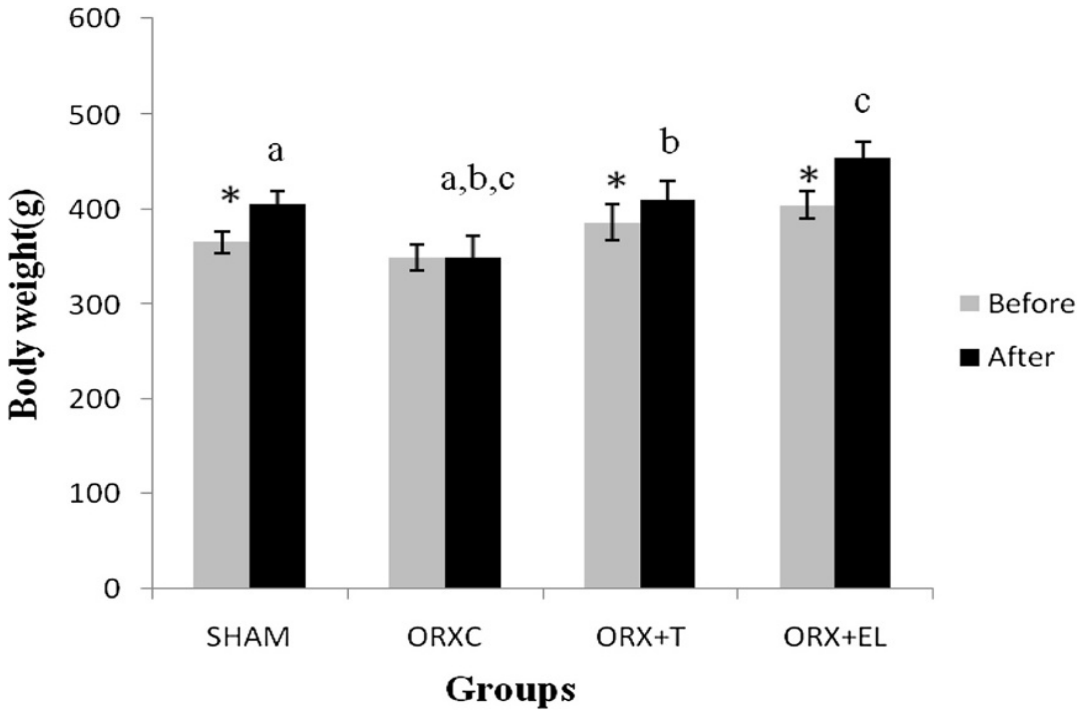

Figure 1: Mean rat weight before and after treatment.

**indicate significant difference before and after treatment Same alphabet indicates significant difference between the groups after treatment SHAM : Sham- operated group OrxC : Orchidectomized control group .Orx + T : group that underwent orchidectomy and received testosterone treatment .Orx+ EL: Orchidetctomized group received EL treatment.*

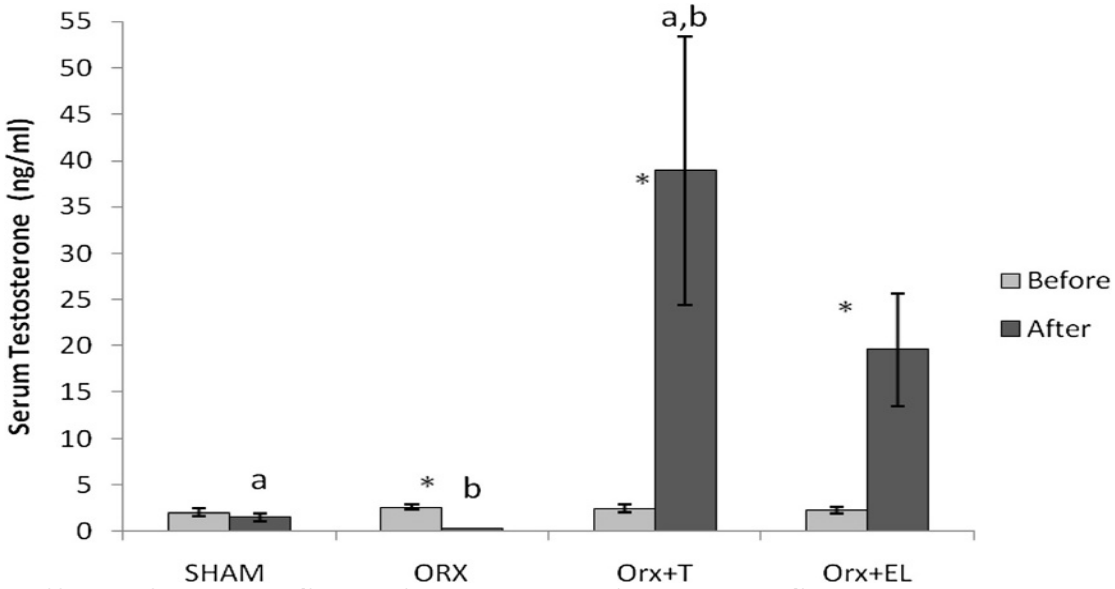

Figure 2: Effects of EL (15mg/kg) and testosterone therapy (8mg/kg) on serum testosteronelevels.

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Discussion

Based on the results of this study, all treatment groups showed a significant increase in body weight compared to the orchidectomized control group. Orchidectomy is known to reduce testosterone levels, which might lead to a reduction in muscle mass. Orchidectomy also reduced bone mass without affecting

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the fat mass [14]. This study showed that testosterone therapy leads to weight gain. Testosterone therapy in hypogonadal men restores lean body mass while simultaneously decreasing fat mass. Testosterone therapy enhanced the muscle mass, resulting in increased body weight [15].

The orchidectomized rat model has been used in many studies as it reflected well the skeletal effects of androgen deficiency in hypogonadal men [16]. Orchidectomy promotes bone loss, which can lead to osteoporosis by inducing a testosterone-deficient state. state. Significant alterations in bone density and micro-architecture can be seen just 4 weeks following the orchidectomy, with these changes worsening after 4 months of the procedure. In this research, the treatment period lasted for 6 weeks, consistent with earlier study by Nazrun et al., which should be sufficient to observe all the bone changes induced by orchidectomy [17].

In the current study, orchidectomised rats had noticeably lower testosterone levels, while intramuscular injections of testosterone significantly raised the testosterone levels. Study found that male rats' serum testosterone levels were reduced by 80% after rorchidectomy [18]. While testosterone replacement therapy was able to raise the testosterone levels in orchidectomised rats [19]. EL supplementations at the dose of 15 mg/kg for 6 weeks were shown to increase serum testosterone levels significantly in orchidectomized rats. This finding was supported by Zanoli et al, who found that supplementation of EL to adult male Sprague–Dawley rats at the dose of 500 mg/kg for 6 days significantly raised the testosterone levels compared to unsupplemented rats [20].

EL appeared to have a similar testosterone-raising action as testosterone replacement therapy. The mechanism of EL in raising the testosterone level is still unclear, but it may enhance the production of testosterone by leyding cells in the testis by increasing luteinizing hormone. It may also enhance the 17-hydroxylase enzyme, which is responsible for the biosynthesis of testosterone. Another possible mechanism is that it may promote the testosterone dissociation from sex hormone binding globulin, causing an increase in the free testosterone level [20-21]. All these results showed that even in the absence of testes, testosterone production would continue. It is possible that EL extract was able to upregulate this extra testicular testosterone production in orchidectomised rats. More significantly, the bone protective effect of EL extract in orchidectomised rats may be due to its testosterone-raising ability.

In a study that measured the testosterone levels in mice, there was a notable individual variation, with results in mice of the same age and strain kept in identical conditions ranging from less than 1 ng/ml to 30 ng/ml. Samples taken from the same animal at different times showed variation in plasma testosterone levels ranging from two to five times [22]. The wide range of testosterone levels may also be true for our rat model as seen by the wide error bars, demonstrating large variations in the testosterone levels within the group.

Throughout the day, the testosterone levels fluctuate, peaking in the morning and then progressively declining throughout the day. By obtaining the blood samples simultaneously at 9 am, when the testosterone levels were high. The testosterone levels also deteriorated with aging, whereby its level dropped to half in a 60-year-old man compared to a young man. Typically, during youth (14-20years of age), the testosterone levels are at their highest. The normal human male testosterone levels are widely ranged between 250 to 850 ng/dl [23]. However, as men age, the testosterone levels generally diminish with time, resulting in less vigor, weakness, and impaired health profiles. We have attempted to reduce the age factor by making sure our rats were around 10 months of age at the start of the study.

The potential of EL to treat male osteoporosis has been highlighted by the current study. Since EL has long been utilized as a remedy for male wellbeing, it stands to reason that the frequency of male osteoporosis would be lower in Southeast Asian nations. There are no reliable statistics on the prevalence of osteoporosis in men in Asian nations that utilize EL, such as Indonesia and Malaysia. In Malaysia and Thailand, the incidence of hip fractures in men is 88 and 114 per 100,000, respectively [24]. The incidence is 362 and 311 per one hundred 000 in Norway and Denmark, respectively. Consequently, the incidence is much lower in countries that use EL. However, the correlation with EL use is difficult to determine as the percentage of men taking EL is not known. Assuming that a great wide variety of fellows were taking EL in South East Asia in comparison to Scandinavia, it'd explain why the prevalence of hip fracture in men in South East Asia turned into simply one fourth to one third in comparison to Scandinavia [25].

Conclusion

Our study has supported claims that consumption of EL caused a rise in testosterone levels, which may additionally explain the bone-protecting effects of EL. In terms of bone metabolism, this may reduce bone resorption by osteoclasts and therefore offer protection against androgen-deficient bone loss. Further studies assessing the regulation of testosterone production by EL may provide additional insight into the mechanisms whereby EL has somewhat different effects on bone resorption.

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Conflicts of interest. Nil

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