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Original article

The Molecular Mechanism of Eurycoma Longifolia in The Protection Against Osteoporosis in Orchidectomized Rats

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Abstract
Previous research on orchidectomized rats, a model for androgen-deficient osteoporosis, has demonstrated that *Eurycoma longifolia* (EL) protects against bone calcium loss. Although the exact mechanism is unknown, it might be connected to its capacity to raise testosterone levels or have a direct impact on bone remodeling. This study aims to determine the mechanism involved by investigating the effects of EL extract on gene expression of Receptor Activator of Nuclear Factor kappa-B ligand (RANKL), Osteoprotegerin (OPG), and Macrophage-Colony Stimulating Factor (MCSF) in orchidectomized rats. Thirty-two male Sprague-Dawley rats were divided into four groups of eight rats each, and each group received a different treatment for six weeks, namely the sham-operated group, the orchidectomized-control group, the orchidectomized group treated with EL (15 mg/kg), and the orchidectomized group treated with testosterone (8 mg/kg). Three genes were measured by dissecting the left and right tibia: RANKL (receptor activator of nuclear factor kappa B ligand), OPG (osteoprotegerin), and M-CSF (macrophage colony-stimulating factor). They were measured using the branched DNA technique (bDNA). For the expression of RANKL, OPG, and M-CSF, there was significant upregulation of OPG in the group of rats treated with *Eurycoma Longifolia* compared to the other group. In summary, supplementing the orchidectomized rats with EL extract upregulated OPG gene expression. These actions may be responsible for the protective effects of EL extract against bone resorption due to androgen deficiency.
Keywords. *Eurycoma Longifolia*, Osteoporosis, Orchiectomy, OPG, RANKL

Introduction

One of the main characteristics of osteoporosis, a long-term, degenerative condition that increases the risk of bone fracture, is low bone mass. In the United States, osteoporosis affects about 2 million males, of whom 1.5 million are older than 65. Unfortunately, among older men, osteoporosis is increasingly the leading cause of morbidity and death. Numerous studies indicate that men are more likely than women to die from hip fractures. It is estimated that 31% of men with hip fractures die within a year of the fracture, compared to only 17% of women [1]. The main cause of osteoporosis in men is androgen deficiency due to natural aging [2]. Partial androgen deficiency is common in aged men, as 20–30% of them suffer from testosterone deficiency, exposing them to a high rate of bone loss, which may lead to osteoporosis [3]. Testosterone replacement therapy has been proven in human trials to decrease bone resorption.

Indicators while increasing calcium absorption and bone growth [4-5]. A previous study by Elgalfat (2025) showed that EL extract might increase testosterone production in male orchidectomized rats (androgen deficiency osteoporotic model). Another study discovered that the bone calcium content of orchidectomized rats could be maintained by supplementing them with EL. EL might have accomplished this by modifying osteoclasts' bone resorption activity. Although the exact mechanism is unknown, it might be connected to its capacity to raise testosterone levels or have a direct impact on bone remodeling. Thus, by assessing the expression of three genes—RANKL, OPG, and M-CSF—this work seeks to identify the molecular mechanism by which *Eurycoma Longifolia* protects rats against osteoporosis caused by orchidectomy. An approved animal model for representing androgen deficiency is the orchidectomized rat model. [6]. Osteoprotegerin (OPG) and Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) are the most important agents influencing osteoclastogenesis. Osteoblasts release both factors. By attaching to the membrane-bound RANK receptor on the osteoclast precursor, RANKL enhances osteoclast growth and survival [9].

Numerous variables, including parathyroid hormone, vitamin D, and estrogen, control the expression of RANKL by osteoblasts. By interacting with RANKL and inhibiting RANKL from attaching to RANK receptors, OPG can stop osteoclast development and bone resorption. Consequently, this gene suppresses the process of osteoclastogenesis [10]. The expression of this gene is down-regulated by 1, 25$(OH)_2D_3$ and up-regulated by calcium ions. Osteoblasts also express Macrophage-Colony Stimulating Factor (M-CSF), which is necessary for osteoclastogenesis. It is currently unclear how M-CSF modulates osteoclastogenesis by binding to M-CSF receptors on osteoclasts [11].

Material and methods

Study design

Thirty-two male Sprague-Dawley rats, aged 12 months, were used in this study. They were acquired from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia, Malaysia.

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Animal model

Rats were kept two per cage under 12-hour natural light–dark cycles and were permitted to acclimatize to the surroundings for 1 week. Rats were treated at 9 am 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, orchidectomized-control group, orchidectomized and treated with EL (15 mg/kg), and orchidectomized and treated with testosterone (8 mg/kg). Orchidectomized middle-aged rats were used as the model for androgen-deficient osteoporosis [12]. Before orchidectomy, the weight of the rats was measured and used to calculate the intraperitoneal injection dose of anesthetic drugs (0.1 ml/100 g). Iodine and 70% alcohol were employed to sterilize. The rats were then kept in an environment with adequate lighting. The scrotal skin was opened by 1-2 cm, and then the cremaster muscle was exposed, which was around 0.5 cm wide. Next, a special thread called Tangsi was used to bind the spermatic cord, which contained the epididymis, artery, and vein (blood vessels). Using a surgical knife, the left and right testes were removed from the spermatic cord, and the skin and inner layer of the scrotum were closed back. Each rat was then given an antibiotic (Baytril) at a dose of 0.1 ml once daily intramuscularly for a week after being placed in a separate cage for seven days to allow the wounds to heal.

Eurycoma longifolia extract

In this study, *Eurycoma Longifolia* (EL) was administered at a dose of 15 mg/kg body weight. The weights of rats were between 400 and 500 g (approximately 450 g). This extract was a light brown fine powder. Protein (31.75%), glycosaponins (41.08%), and eurycomanone (1.604%) were the main chemical constituents. Deionized water was used to dilute the material. The stock solution was made by adding 30 mg EL to 20 mL of deionized water. Oral gavage dosages were administered based on the rats' weekly body weight measurements. This homogeneous solution was kept at -4°C in dark bottles. For six weeks, EL was administered orally once a day, six days a week, at a dose of 0.1 ml/100 g. 400 mg or 0.4 g of testosterone was dissolved in 50 mL of normal saline as a stock solution. After that, it was homogenized and preserved at -4°C in glass bottles with labels. For six weeks, testosterone was administered by injection (IM) at a dose of 0.1 ml/100 g once daily, six days a week.

Gene expression

The tibia bones were ground into fine powder with a mortar and pestle by adding liquid nitrogen. Proteinase K was added to liberate ribonucleic acid (RNA) and processed in accordance with instructions provided by Panomics (Fremont, CA). In order to quantify multiple mRNA targets simultaneously in the same sample without the amplification errors of RT-PCR, the technique combines bDNA RNA signal amplification with microspheres that have distinct fluorescent signatures. This allows for the discrimination of highly homologous messages. Specific oligonucleotide capture and extender probe sets (3 per target), designed to anneal exclusively to each mRNA of interest and each housekeeping mRNA, were designed by Panomics to target unique sequences within each message sequence. Each mRNA species' RANKL (NM_057149), OPG (NM_012870), and M-CSF (NM_023981) sections contained probe sets. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_017008), beta-glucuronidase (NM_017015), and hypoxanthine phosphoribosyltransferase 1 (HPRT1; NM_012583) were the housekeeping genes.

In order to record probe-extender probe interactions, particular mRNA transcripts were hybridized to particular fluorescent beads. Signal from each hybridized unit was amplified by attachment of biotinylated label probes at multiple binding sites on the complexes, which in turn bound to streptavidin-conjugated R-phycoerythrin (SAPE) to produce fluorescence. A Luminex 100 IS system (made by i-DNA Biotechnology (M) Sdn Bhd) was used to read the fluorescent signals connected to individual capture beads. The SAPE signal indicated abundance, while the bead signature indicated the RNA target. The geometric mean of the fluorescence of the three housekeeping genes in each well was used to standardize the total fluorescence from each unique bead type (corresponding to individual mRNA species) minus background fluorescence for that bead type. For each mRNA species being examined, a single value was obtained by averaging the normalized signals for individual mRNAs from triplicate wells.

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 treatment group. The Kolmogorov-Smirnov test was used to determine normality. To identify and compare the significant differences between the means of different treatment groups, ANOVA followed by Tukey's HSD was utilized. Significant differences were defined as $P < 0.05$.

Results

The RANKL expressions for the ORX group and SHAM group were similar. RANKL expression for the ORX+EL group was similar to the ORX and SHAM groups. Similarly, when compared to other groups, testosterone replacement therapy (ORX+T) had no discernible impact on RANKL expression. All treatment groups had comparable RANKL expression (Figure 1A). The OPG gene expression of ORX significantly decreased in comparison to SHAM groups, indicating that the OPG gene expression of the tibial bone was reduced following orchiectomy. The OPG gene expression was able to return to SHAM levels with EL supplementation (ORX + EL). When compared to the other groups, testosterone therapy (ORX + T) did not

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significantly alter the expression of the OPG gene (Figure 1B). No significant differences in M-CSF expression were noted in any of the groups (Figure 1C).

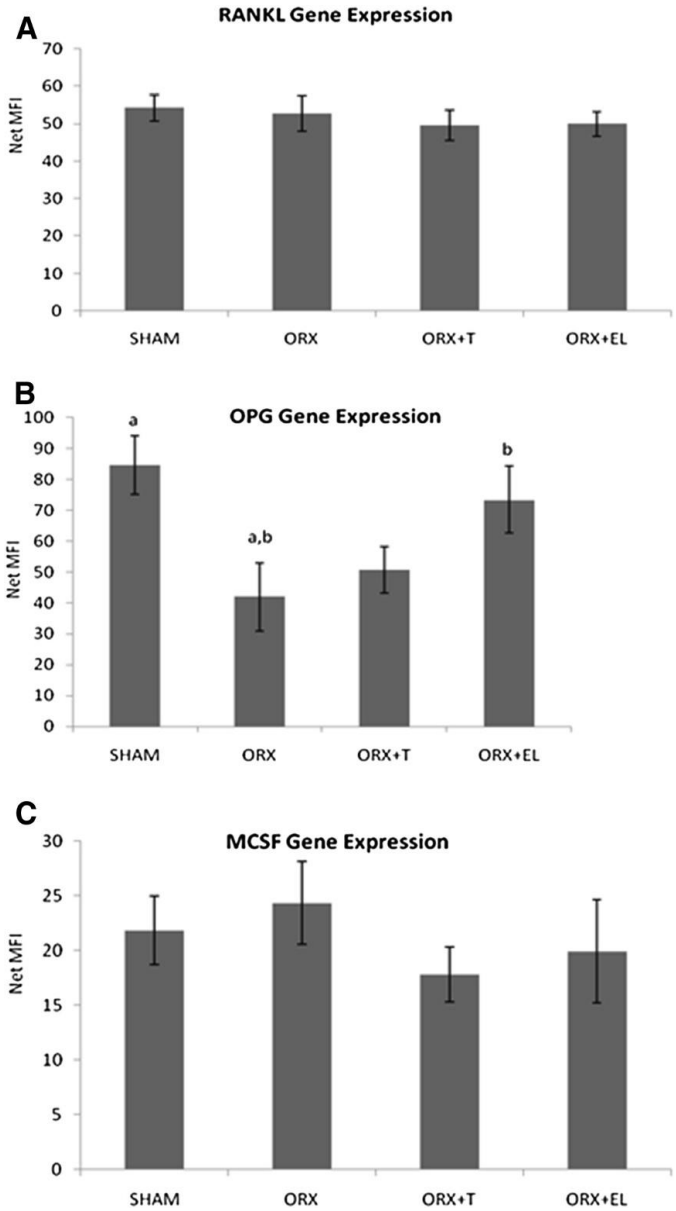

Figure 1A1A, B, and C: RANKL, OPG, and M-CSF gene expression for all groups. The same letter indicates a significant difference between the groups after treatment: SHAM: Sham-operated group, ORX: Orchidectomized control group, ORX+EL: Orchidectomized group treated with EL, ORX+T: Orchidectomized group treated with testosterone

Discussion

A previous study found that EL or testosterone replacement might stop the increase of the bone-resorptive marker C-terminal telopeptide of type I collagen following orchidectomy [13]. This means that both EL and testosterone replacement were able to inhibit bone resorption, but the mechanism is not known. The osteoclast, the bone cell in charge of bone resorption, may be directly impacted by EL and testosterone since it involves bone resorptive actions. The RANKL/OPG pathway's activation and MCSF secretion determine the osteoclastic bone resorptive activity. In order to ascertain if EL may have shielded bone from testosterone shortage by influencing these gene expressions, we have directly assessed these gene expressions in the tibia of our rat model. The branched DNA technique (bDNA), which can identify and analyze several RNA targets at once, was used to measure gene expression.

The hybridization reactions that underpin this signal amplification method are quite susceptible to complete automation, which reduces the amount of work needed to carry out this kind of study. When comparing bDNA to real-time RT-PCR, it was discovered that bDNA is a simpler and more sensitive method of measuring gene expression. Moreover, bDNA requires less time and is less costly than RT-PCR. Thirty genes' expressions can be directly monitored at the same time utilizing bDNA [14]. It is well accepted that osteoblast-derived RANKL and OPG play a significant role in determining osteoclast biology and bone resorption. It was discovered that several factors that impact bone resorption do so by changing the production

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of OPG and RANKL. A crucial cytokine for osteoclast development and activation is RANKL [15]. Serum RANKL was shown to stimulate osteoclast development and activation in mice, which resulted in osteoporosis. Additionally, a human study discovered that while there was no correlation between RANKL and BMD in women, higher RANKL levels were linked to reduced bone mineral density (BMD) in men [16]. They proposed that the inverse RANKL-BMD connection in men could be linked to testosterone levels. When RANKL gene expression was evaluated in this study, there was no significant difference across the groups. A previous study discovered that 8 weeks following orchidectomy, RANKL expression increased in the bone marrow of aged rats. In the same study, subcutaneous testosterone at 6 mg/kg once weekly was able to restore RANKL gene expression in orchidectomized rats to that of sham controls [17]. However, two more in vitro experiments supported our findings. Hofbauer et al. (2002) found that RANKL mRNA was not consistently detectable in mature osteoblasts and marrow stromal cells and was not affected by androgens. This suggested that androgens' effects on the RANKL/OPG system may not be primarily mediated via modification of RANKL synthesis [18]. Our in vivo research was more likely to rule out a connection between EL or testosterone and RANKL gene expression.

OPG is another cytokine that binds to and prevents RANKL from interacting with the RANK receptor on osteoclasts [19]. This inhibits osteoclast development, activation, and survival, as observed in OPG knock-out mice, who were found to be osteoporotic. In this study, orchidectomy reduced OPG gene expression, whereas EL restored it to sham levels. However, testosterone therapy failed to increase OPG gene expression, as evidenced by EL. Chen et al. (2004) found that testosterone was able to amplify OPG mRNA expression in mouse bone-cell cultures and an osteoblastic cell line. In another in vitro study, an opposite effect was found [21]. The inhibitory effects of testosterone on OPG synthesis by osteoblastic lineage cells differ from those of estrogens. Furthermore, testosterone and estrogen have been demonstrated to have direct inhibitory effects on osteoclasts [22]. Thus, the opposing modulation of OPG by testosterone as compared to estrogens provides a plausible mechanism for the clinical findings [23]. In a study on elderly men who were rendered abruptly hypogonadal and then supplemented with estrogen alone, testosterone alone, or both, it was discovered that estrogen and testosterone have opposing effects on circulating OPG levels in vivo [24]. The serum OPG levels were also found to be greater in women than men. Thus, both in vitro and in vivo results indicate that estrogens and testosterone regulate OPG synthesis in opposing directions and that testosterone's anti-resorptive actions may be mediated independently of the RANKL/OPG system. OPG can be regulated by a variety of variables in the in vivo system, including vitamin D3 and calcium ions, which may explain why no effects were observed with testosterone. If EL had worked by increasing the testosterone levels to affect OPG expression, then testosterone therapy should have the same effect on OPG. As a result, EL may alter OPG gene expression by means other than raising testosterone levels. M-CSF promotes the proliferation and survival of osteoclast progenitors by binding to its receptors and increasing RANK expression. As a result, it activates RANKL for osteoclast differentiation.

An in vitro investigation discovered that M-CSF can promote bone resorption [25]. However, one study found that adding exogenous M-CSF to a co-culture of mouse spleen cells reduced the amount of osteoclasts generated . This study found no substantial changes in M-CSF gene expression following orchidectomy. Furthermore, there were no significant variations in M-CSF gene expression across the groups. These findings contradict Huber et al, 2001 study finding that testosterone reduced RANKL- and M-CSF-induced osteoclast development in vitro from bone marrow cytocytes. These activities were carried out by inhibiting c-Jun N-terminal kinase activity and lowering c-Jun expression levels. These decrease Activator protein-1 (AP-1) functional activity, which is required for osteoclast development [26]. Again, the prior study was conducted in vitro, which may account for the significant findings when contrasted to this study.

Conclusion

Our findings support that consuming EL increased testosterone levels. In terms of bone metabolism, this may inhibit bone resorption by osteoclasts, protecting against androgen-deficient bone loss. The only significant finding was in OPG gene expression. Orchidectomy was demonstrated to reduce OPG expression; however, EL was able to increase OPG expression to sham levels. This could be the mechanism by which EL protects bone against androgen-deficient osteoporosis. However, testosterone therapy had no substantial influence on OPG expression. As a result, the OPG increase may not be due to the testosterone-raising effects. Future research investigating the regulation of OPG synthesis by EL and testosterone may provide additional insight into the processes by which EL has slightly distinct effects on bone resorption.

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Conflicts of interest. Nil

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