Research Updates in Medical Sciences (RUMeS)
2013, Volume 1; Issue 1 page 7-12

Research Article

Combined effects of *Eurycoma longifolia* and Calcium on Bone Metabolism of Orchidectomised Male Rats

Nadia Mohd Effendy, Ahmad Nazrun Shuid

Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abd.Aziz, 50300 Kuala Lumpur, Malaysia.

Correspondence should be addressed to Assoc Prof Dr Ahmad Nazrun Shuid, anazrun@yahoo.com

Received 05-12-2012; Accepted 01-01-2013

How to cite:
Nadia ME, Shuid AN, 2013, Combined effects of *Eurycoma longifolia* and Calcium on Bone Metabolism of Orchidectomised Male Rats. Research Updates in Medical Sciences 1(1) : 7-12

ABSTRACT

Osteoporosis is characterized by low bone mass and destruction of bone microarchitecture. Androgen deficiency or hypogonadism is one of the major risk factors of osteoporosis in men. Testosterone replacement therapy (TRT) is the main treatment for hypogonadal osteoporosis but may cause side effects such as prostate cancer and cardiovascular diseases. An alternative treatment is required, with similar efficacy but minimal side effects. *Eurycoma longifolia* is a herbal plant that may have potential in treating osteoporosis, especially if it is supplemented with calcium. This study was conducted to determine the combinational effects of *Eurycoma longifolia* and calcium on the bone markers and biomechanical strength of orchidectomised male rats. A total of 48 rats were randomly divided into 6 groups; sham-operated control (SHAM), orchidectomised control (ORX), orchidectomised + 8mg/kg testosterone (TES), orchidectomised + 15mg/kg *Eurycoma longifolia* (EL), orchidectomised + 1% calcium (Ca) and orchidectomised + combination of 15mg/kg EL and 1% calcium (EL+Ca). Treatment was given for a period of 6 weeks and blood samples were taken prior and after the treatment to measure osteocalcin and CTX levels. The rats were euthanized after 6 weeks and femora were dissected for biomechanical analysis. The osteocalcin levels did not show any significant changes, while the CTX levels were significantly increased in the ORX group but decreased in the other groups. The strain parameter of the EL+Ca was significantly lower than the EL group. In conclusion, single supplementation of EL was better than combination with calcium in terms of the bone remodeling and strength of orchidectomised rats.

Keywords: *Eurycoma longifolia*, orchidectomy, osteoporosis, androgen-deficient

1. Introduction

Osteoporosis and osteoporosis-related fractures are increasing and now becoming a major public health issue. According to World Health Organization (WHO), osteoporosis occurs when the bone mineral density is more than 2.5 SD below the peak bone mass reference standard for young women [1]. If this criterion is used, namely the 2.5 SD below the peak bone reference standard for young men, approximately 1 to 2 million men have osteoporosis and another 8 to 13 million have osteopenia [2]. In both sexes, age-related bone loss begins at about age 50 [3]. Men have greater bone mass, hence most of them presented with osteoporotic fracture about 10 years later than women [4]. Osteoporosis in men is now recognized as an increasingly important public health issue because they are prone to higher rates of morbidity and mortality [5,6].

Osteoporosis in men is mainly caused by androgen deficiency (hypogonadism) [7,8]. Circulating testosterone in men declines progressively by 0.4–2% per year from the age of thirties onward [9,10]. According to the Endocrine Society, approximately 4 million men have hypogonadism, of whom less than 200,000 were treated [11]. Hypogonadism can be due to primary and secondary factors. Primary factors are associated with normal aging and testicular disorders such as undescended testes, orchitis, tumour of testes and orchidectomy [12,13]. Secondary factors are characterized by deficient release

of Gonadotrophin-releasing hormone (GnRH) due to hyperprolactinemia, lesions of hypothalamus and prolonged use of medications [14,15].

Androgens, mainly testosterone, play an important role in the development and maintenance of bone mass. The presence of aromatase in bone allows conversion of testosterone to estradiol, which can stimulate the estrogen receptors located throughout bone [16,17]. Testosterone may also exhibit direct effects on bone via androgen receptors on osteoblasts [18]. Previous studies reported that alterations in circulating androgen have been associated with low bone mass and impaired bone strength [19]. Androgen level decreases with increasing age where elderly men secrete approximately half of testosterone secreted by young men. This lack of androgen may cause an increase in bone resorption, which indirectly affects the bone remodelling mechanism [20,21]. Thus, aging male with androgen deficiency is at high risk of developing osteoporosis [22]

According to the Food and Drug Administration (FDA) and World Health Organization (WHO), orchidectomised rat has been proposed to simulate male osteoporosis due to hypogonadism [23]. Testicular production is the primary source of circulating testosterone in men. Hence, orchidectomy (ORX) will induce a decline in testosterone which leads to higher bone resorption and lower bone formation. Previous studies reported that bone volume and trabecular structure deterioration occurred between two to three weeks post-ORX [24]. Trabecular bone loss is associated with increased bone remodelling which affects normal function of osteoblasts and osteoclasts [25].

Testosterone replacement therapy (TRT) is the main treatment for androgen deficient-osteoporosis which can be given orally, via intramuscular injection or patches. Intramuscular injection gives the most rapid and best result in increasing testosterone level. However, this administration of testosterone is very painful and prone to cause infection. Its prolonged use may result in many adverse effects such as prostate cancer, liver failure and cardiovascular diseases [26]. Other forms of treatment for osteoporosis in men are bisphosphonates and strontium ranelate. These drugs may also result in adverse effects with prolonged use [27,28]. Despite the effectiveness of available treatments, the search for alternative anti-osteoporotic agent in men is highly desirable. This alternative agent should ideally be free of side effects and safe to be taken as supplement.

Previous studies on *Eurycoma longifolia Jack* (EL) reported that it has potential in preventing bone calcium loss [29]. However there is paucity in studies on the anti-osteoporotic effects of EL. EL is commercially known as *Tongkat Ali* in Malaysia, p*asak bumi* in Indonesia and *tung saw* in Thailand [30]. It is known for its aphrodisiac effect and widely used by men to improve their sexual health [31]. The main active compounds in EL are *eurycomanone, eurycomalactone* and *eurycomanol* which can be found in the roots. The root is believed to have the medicinal values to treat a wide range of diseases such as infections, cough, high blood pressure, headache and malaria [32]. EL is known to be an effective aphrodisiac agent which helps in increasing testosterone level and enhance male sexuality and fertility [33]. Previous studies have shown that male rats supplemented with EL mated more frequently than the control rats [34].

Calcium is the most abundant mineral in bones. Calcium supplementation is known to prevent bone loss and helps in treating osteoporosis. Therefore, this study was done to determine the combinational effects of EL and calcium on bone metabolism of orchidectomised rats.

2. Design and Methods

Animal model

A total of 48 male Sprague-Dawley rats, aged between 8-10 months were obtained from Universiti Kebangsaan Malaysia (UKM) Animal House and randomly divided into six groups. The rats were housed in a cage at temperature of 29±3 ºC under natural day/night cycle. They were fed with commercial food pellets (Gold Coin, Port Klang, Malaysia) and deionised water *ad libitum*. They were allowed to adjust to the new environment for a week before the study was started.

EL extract and testosterone

Eurycoma longifolia Jack was supplied by Phytes Biotek Sdn Bhd (Malaysia). It was extracted from the plant's root using a patented high pressure water extraction (US Patent no: US7, 132, 117 B2). It contained bioactive 22.0% eurypeptide, 41.1% glycosaponin and 1.6% eurycomanone. Aqueous EL solution was prepared by diluting the EL brownish powder form in deionised water. Testosterone was purchased from Sigma Chemicals (USA). It was diluted in normal saline and given via intramuscular injection at the dose of 8.0 mg/kg daily for 6 weeks. Calcium was given to rats via drinking water. One percent calcium phosphate was prepared by diluting 1.0 g calcium phosphate in 99 ml deionised water.

Study design

Rats of the first group were sham-operated control (Sham) while rats in the other groups were orchidectomised. The second group was the orchidectomised-control group (ORX), the third group was supplemented with 8.0 mg/kg testosterone (TES) via daily intramuscular injection. Other groups were supplemented with 15.0mg/kg EL via daily oral gavages (EL), 1% calcium *ad libitum* (Ca) or combination of EL and calcium (EL+Ca). Treatments were given 6 days per week for 6 weeks. The study duration was thought to be adequate to induce changes in bone metabolism after orchidectomy [35]. Body weights were measured before the start of treatment and then weekly until the end of the study.
Blood samples were collected prior and after treatment via retro orbital bleeding after anesthetizing the rats with ether. After 3 hours, the blood was centrifuged at 3000 rpm for 10 minutes and the serum was separated prior to

storage at temperature of -70 ºC. At the end of the study, the rats were euthanized with diethyl ether overdose. Femora were dissected out, cleaned and wrapped with aluminium foil prior to freezing at -70ºC before being used for biomechanical bone testing.

Bone markers analysis

Bone biochemical markers of serum osteocalcin and C-terminal telopeptide of type I collagen (CTx) were measured before and after treatment using ELISA technique. The kits used were Rat Osteocalcin ELISA (Biosource Technologies Inc., Nivelles, Belgium) and Rat CTX ELISA (Biomedical Technologies, Herley, Denmark).

Biomechanical analysis

Biomechanical bone testing was done following the 3-point bending method where a significant pressure was loaded on the centre of the femur. This test was performed using an Instron Universal Testing Machine (Model 5848; Instron Corp; Canton, MA) equipped with the Bluehill® 2 software and the parameters recorded can be categorised into extrinsic and intrinsic parameters. Extrinsic parameters are load, displacement and stiffness, while, intrinsic parameters are stress, strain and Young's modulus. Young's modulus value can be obtained from the slope of the stress versus strain graph.

Statistical analysis

The statistical significance of the data was determined using paired T-test and one way analysis of variance (ANOVA) which was then followed by post hoc Tukey's test. The software used was the Statistical Package for Social Sciences (SPSS) version 18.0. The level of significance taken was as $p < 0.05$. All results were expressed as mean ± SEM.

Results

Body weights

The mean body weight for all the groups were significantly different after 6 weeks of treatment compared to their pre-treatment weights [F (9.085), p = 0.000]. The pre-treatment weights were not significantly different between the groups. The post-treatment weight of the ORX group was significantly lower than Sham group. All the treatment groups had significantly higher body weighst compared to the ORX group. The post-treatment weight of the EL group was significantly higher that the ORX and Sham group (Figure 1).

Bone markers analysis

Results of the osteocalcin levels showed no significant differences in the pre- and post-treatment levels within or between the groups (Figure 2). As for the serum CTx levels, there were no significant differences in the pre- and post-treatment levels between the groups. There was a significant increase in the post-treatment level of CTx for the ORX group compared to its pre-treatment level. There were significant reductions in the post-treatment levels of CTx compared to the pre-treatment levels for the TES, EL, Ca and EL+Ca groups (Figure 3).

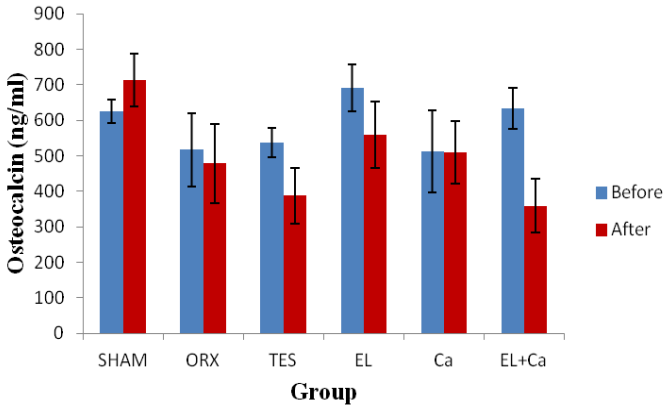

Figure 2 Mean serum osteocalcin levels for all the groups before and after treatment. Data presented as mean±SEM ($p<0.05$). No significant differences were seen within and between the groups.

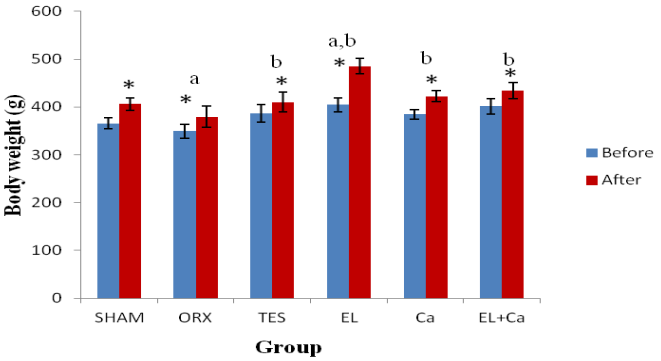

Figure 1 Mean body weight comparison within and between groups. Data presented as mean±SEM ($p<0.05$). '*' indicates significant difference before and after treatment. 'a' indicates significant difference compared to the Sham group. 'b' indicates significant difference compared to the ORX group.

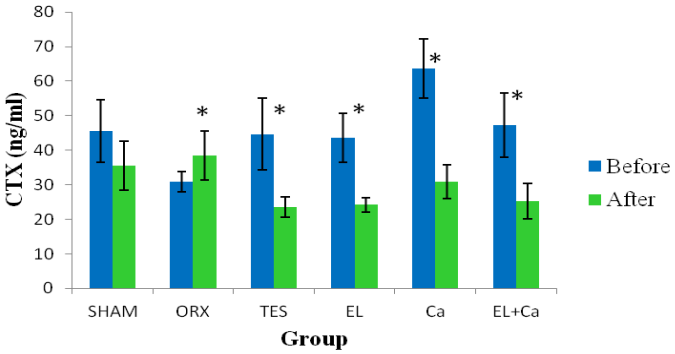

Figure 3 Mean serum C-terminal telopeptide of type 1 collagen for all the groups before and after treatment. Data presented as mean±SEM ($p<0.05$). '*' indicates significant difference before and after treatment within the group.

Biomechanical analysis

Biomechanical testing did not show any significant difference for the maximum load, maximum stress and Young modulus parameters. (Figures 4,5,7). However, for the strain parameter, there was a significant reduction in the strain value of the EL+Ca group compared to the EL group (Fig. 6).

Discussion

Osteoporotic fractures which are highly associated with women especially post-menopausal women are now becoming a serious health issue among men as well. Due to the higher density and stronger bone in men, they begin to experience fragility fractures 10 years later than women at the senescent age [36]. Once hip fractures have occurred, men have higher rates of morbidity and mortality than women [37]. This can lead to devastating effects on their daily life. Hence, effective treatments are vital in alleviating this problem.

The main cause of osteoporosis in men is androgen deficiency which is associated with aging and testicular diseases. Androgen deficiency can be treated effectively with testosterone replacement therapy via intramuscular injections. However, this method of treatment is not only painful but may cause adverse effects. Therefore, an alternative agent to testosterone which is effective but with minimal adverse effects is required to treat androgen deficiency. EL has been proven to exert aphrodisiac effects on men and therefore was chosen for this study. The bone metabolism of rat resembles to that of human. Orchidectomy was shown to cause testosterone deficiency which may result in bone loss [38,39]. Hence, orchidectomised rat can be used as the model of testosterone deficiency or hypogonadal osteoporosis in men. Calcium is an important and major mineral in bone, which affects bone growth and metabolism. Theoretically, combined supplements of EL and calcium should produce better effects on bone than either supplement alone.

At the end of the study, all the treatment groups showed significantly higher body weights than the orchidectomised control (ORX) group. This meant that orchidectomy has stunted the weight gains of the rats. This result was supported by previous studies which reported that orchidectomy was not only able to reduce testosterone levels but also reduce muscle mass, which caused

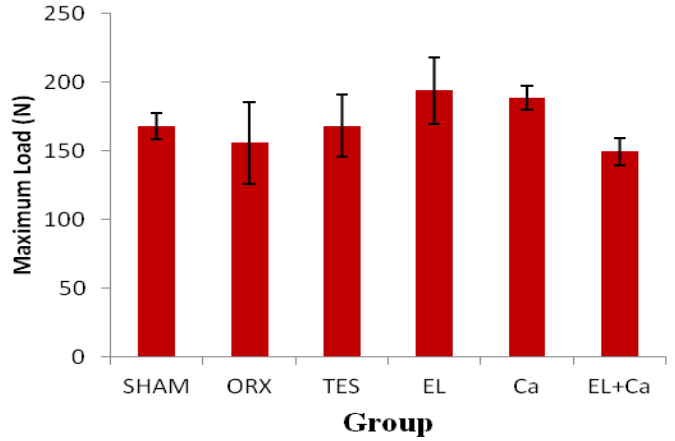

Figure 4 Mean maximum load for all the groups. Data presented as mean±SEM ($p<0.05$). No significant differences were seen within and between the groups.

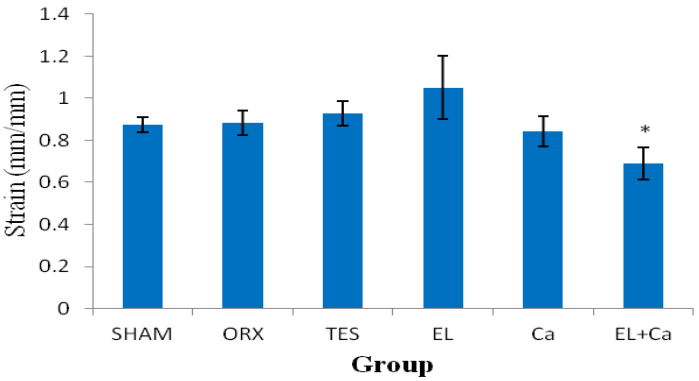

Figure 6 Mean strain of all groups. Data presented as mean±SEM ($p<0.05$).'*' indicates significant difference compared to the EL group.

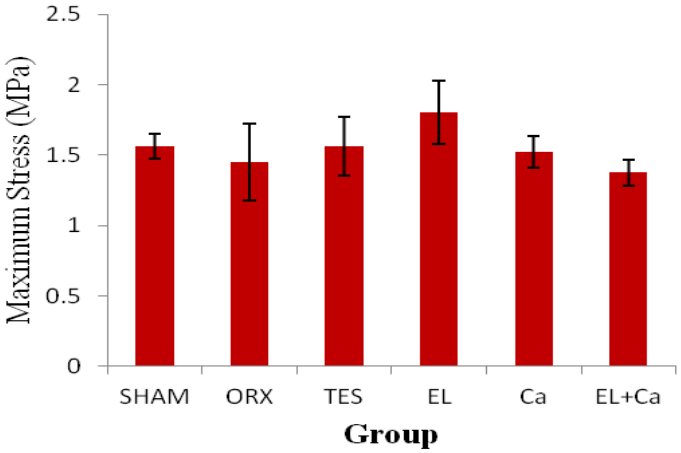

Figure 5 Mean maximum stress of all groups. Data presented as mean±SEM ($p<0.05$). No significant differences were seen within and between the groups.

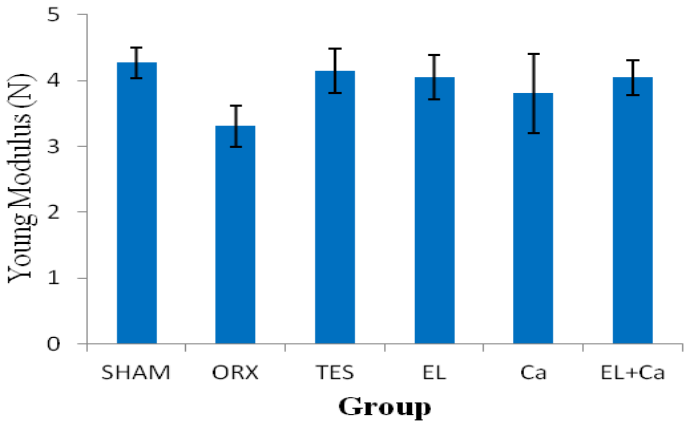

Figure 7 Mean Young modulus of all groups. Data presented as mean±SEM ($p<0.05$). No significant differences were seen within and between the groups.

significant reduction in body weight [40]. Supplementations with EL or calcium or both were able to maintain the weight gains of the orchidectomised rats. This was probably achieved by increases in the muscle mass as reported by previous studies [41,42].

As for the serum analysis of the bone markers, osteocalcin, which is the marker of bone formation, did not exhibit any significant changes. This meant that the bone formation in the orchidectomised rats remain unchanged with treatments. The post-treatment levels of CTx, the marker of bone resorption, were found to be significantly elevated in the ORX group compared to the treatment groups. These results have shown that testosterone deficiency has only affected bone resorption but not bone formation. This was confirmed by previous studies which reported that testosterone exerted significant effects on bone resorption but not on bone formation [43,44]. In the present study, all the supplements given were able to reduce the bone resorption induced by orchidectomy, therefore preventing bone loss.

Based on the bone biomechanical testing, there were no significant changes in the bone strength except for the strain parameter which was significantly lower in the EL+Ca group compared to the EL group. Biomechanical testing is a direct measurement of the bone strength which reflects the bone function. The strain parameter measured the relative deformation of the femur caused by the stress before it fractured. Although single-EL or calcium supplementation or combined EL and calcium supplementation were able to maintain the body weight and reduce bone resorption of orchidectomised rats, the bone of the rats given combined supplementation seemed to be weak. This has raised questions on whether it is ideal to combine EL with calcium and how the interactions between these two supplements would result in weaker bone.

There are several hypotheses on how EL may protect bone from the deleterious effects of androgen deficiency. EL is an aphrodisiac agent which can stimulate dehydroepiandosterone (DHEA), an important endogenous steroid hormone. This hormone is a weak partial agonist of the androgen receptors and functions as an endogenous precursor to testosterone and dihydrotestosterone [45]. Elevated levels of these hormones will stimulate the androgen receptors on bone cells which indirectly reduces bone resorption [46,47]. Therefore, supplementation with EL would be helpful in androgen deficiency by reducing bone loss and improving bone strength.

Previous studies have demonstrated that calcium supplementation increased the bone mass and density [48]. Calcium is known to stimulate calcitonin which inhibits the osteoclasts activity and eventually reducing bone resorption [49]. Despite the benefits of the EL and calcium on bone, the present study found that their combination failed to exert better effects than EL or calcium alone. This may be explained by the fact that EL could increase free testosterone in the blood. In comparison to bound testosterone, free testosterone is an unesterified fatty acid [50] which can disrupt calcium absorption, therefore resulting in weaker bones [51]. In order to overcome this, higher dose of calcium should be used for the combination of EL and calcium, to exert beneficial effects on the bone during androgen deficiency. In future studies, EL may also be combined with calcium and its metabolically active regulator, vitamin D to produce better effects on bone. Further studies on the other bone parameters such as bone density and microarchitecture are warranted to determine the effects of EL on bone.

In conclusion, EL supplementation was able to reduce bone resorptive activity as well as increasing the bone strength of hypogonadal osteoporosis model. It has potential as an alternative agent to testosterone replacement therapy in treating hypogonadal osteoporosis in men. Combination of EL and calcium did not produce better effects than either single- EL or calcium supplementation in the same model.

References

1. World Health Organisation.1994. Assessment of Fracture Risk and its Application to Screening for Postmenopausal Osteoporosis. *WHO Technical Report Series* 843. Geneva: WHO.
2. Kanis J.A, McCloskey E.V, Johansson H, Oden A, Melton III L.J & Khaltaev N. 2008. A Reference Standard for The Description of Osteoporosis. *Bone* 42(3): 467-475.
3. Gilsanz V, Loro M.L, Roe T.F, Sayre J, Gilsanz R & Schulz E. 1995. Vertebral Size in Elderly Women With Osteoporosis. *Journal of Clinical Investigation* 95: 2332–2337.
4. Bilezikian J. P. 1999. Osteoporosis in Men. *The Journal of Clinical Endocrinology & Metabolism* 84(10): 3431-3434.
5. Looker A.C, Orwoll E.S & Johnston Jr C.C. 1997. Prevalence of Low Femoral Bone Density in Older US adults from NHANES III. *Journal of Bone Mineral Research* 12: 1761-1768.
6. Campion J.M. & Maricic M.J. 2003. Osteoporosis in Men. *American Family Physician* 67(7): 1521-1526.
7. Cooper C. & Melton L.J. 1992. Epidemiology of Osteoporosis. *Trends Endocrinology Metabolism* 3: 224-229.
8. Orwoll E.S. 1998. Osteoporosis in Men. *Endocrinology Metabolism Clin North Am* 27: 349-367.
9. Orwoll E, Lambert LC, Marshall LM, Phipps K, Blank J, et al. 2006.Testosterone and Estradiol Among Older Men. *J Clin Endocrinol Metab* 91:1336–1344.
10. Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G et al. 2008. Hypothalamic-Pituitary-Testicular Axis Disruptions in Older Men are Differentially Linked to Age and Modifiable Risk Factors: the European Male Aging Study. *J Clin Endocrinol Metab* 93:2737–2745.
11. Anonymous. 2000. Endocrine Society 82nd Annual meeting, June 21-24.
12. Christina C. 2004. Diagnosis of hypogonadism: Clinical Assessments and Laboratory Tests. *Reviews in Urology* 6 (6): S3-S8.
13. Seftel A. 2006. Male Hypogonadism. Part II: Etiology, Pathophysiology and Diagnosis. *International Journal of Impotence Research* 18(3): 223-228.
14. Morales A.J & Lunenfeld B. 2001. Androgen Replacement Therapy in Aging Men with Secondary Hypogonadism. *The Aging Male* 4(3): 151-162.

15. Frederick C.W, Abdelouahid T, Jennifer M.B, Stephen R.P, Alan J.S, et al. 2010. Identification of Late-Onset Hypogonadism in Middle-Aged and Elderly Men. *The New England Journal of Medicine* 363(2): 123-135.
16. Yanase T, Suzuki S & Goto K. 2003. Aromatase in Bone: Roles of Vitamin D3 and Aandrogens. *Journal of Steroid Biochem Molecular Biology* 86: 393-397
17. Khosla S. 2010. Update on Estrogens and the Skeleton. *Journal of Clinical Endocrinology and Metabolism* 95(8): 3569–3577
18. Riggs B.L, Khosla S & Melton L.J. 2002. Sex Steroids and the Construction and Conversation of the Adult Skeleton. *Endocrine Reviews* 23(3): 279- 302
19. Clarke B.L & Khosla S. 2009. Androgens and Bone. *Steroids* 74(3): 296–305
20. Harman S.M, Metter E.J, Tobin J.D, Pearson J & Blackman M.R. 2001. Longitudinal Effects of Aging in Serum Total and Free Testosterone Levels in Healthy Men. Baltimore Longitudinal Study of Aging. *The Journal of Clincal Endocrinology & Metabolism* 86(2):724–731.
21. Khosla S, Melton III L.J & Riggs B.L. 2011. The Unitary Model for Estrogen Deficiency and the Pathogenesis of Osteoporosis: is a Revision Needed. *Journal of Bone and Mineral Research* 26(3): 441–451.
22. Ebeling P.R. 2010. Androgens and Osteoporosis. *Current Opinion in Endocrinology, Diabetes and Obesity 17*(3): 284-292.
23. Audran M, Chappard D, Legrand E, Libouban H & Basle M.F. 2001. Bone Microarchitecture and Bone Fragility in Men: DXA and Histomorphometry in Humans and in the Orchidectomised Rat Model. *Calcified Tissue International* 69(4): 214-217.
24. Libouban H, Moreau M.F, Legrand E, Audran M, Base M.F & Chappard D. 2002. Comparison of Histomorphometric Descriptors of Bone Architecture with Dual-Energy X-ray Absorptiometry for Assessing Bone Loss in the Orchidectomised rat. *Osteoporosis International* 13(5): 422-428.
25. Blouin S, Libouban H, Moreau M.F & Chappard D. 2008. Orchidectomy Models of Osteoporosis. *Methods in Molecular Biology* 455: 125-134.
26. Lane N.E. 2001. The Osteoporosis Book: A Guide for Patients and Their Families. Oxford University Press. New York, USA.
27. Favus M.J. 2010. Biphosphonates for Osteoporosis. *New England Journal of Medicine* 363(21): 2027-2035.
28. O'Donnell S, Cranney A, Wells G.A, Adachi J.D & Reginster J.Y. 2006. Strontium Ranelate for Preventing and Treating Postmenopausal Osteoporosis. Cochrane Database of Systematic Reviews 3: Article ID CD005326.
29. Nazrun A.S, Mohd Firdaus A.B, Tajul Ariff A.S, Norliza M, Norazlina M & Ima Nirwana S. 2011. The Anti-osteoporotic Effect of *Eurycoma longifolia* in Aged Orchidectomised rat model. *The Aging Male* 14(3): 150-154.
30. Bhat R & Karim A.A. 2010. Tongkat Ali (Eurycoma longifolia Jack): A Review on its Ethnobotany and Pharmacological Importance. *Fitoterapia* 81(7): 669–679.
31. Ang H.H, Ikeda S & Gan E.K. 2001. Evaluation of the Potency Activity of Aphrodisiac in Eurycoma longifolia Jack. *Phytotherapy Research* 15(5): 435-436.
32. Ang H.H., Ngai T.H & Tan T.H. 2003. Effects of Eurycoma longifolia Jack on Sexual Qualities in Middle Aged Male Rats. *Phytomedicine* 10(6-7): 590-593.
33. Ang H.H, Cheang H.S & Md Yusof A.P. 2000. Effects of Eurycoma longifolia Jack (Tongkat Ali) on the Initiation of Sexual Performance of Inexperienced Castrated Male Rats. *Experimental Animals* 49(1): 35-38.
34. Ang H.H, Lee K.L & Kiyoshi M. 2004. Sexual Arousal in Sexually Sluggish Old Male Rats After Oral Administration of Eurycoma longifolia Jack. *Journal of Basic Clinical Physiological Pharmacology* 15: 303-309.
35. Wahab N.A, Mokhtar N.M, Halim W.N.H.A & S. Das. 2010. The effect of Eurycoma longifoliaJack on Spermatogenesis in Estrogen-Treated Rats. *Clinics* 65(1): 93–98.
36. Cooper C & Melton L.J 3d. 1992. Epidemiology of Osteoporosis. *Trends in Endocrinology & Metabolism* 3(6): 224–229.
37. Kalleen Barham, Arthur C & Catherine A. 2007. Osteoporosis in Men. *Hospital Physician* 43(1): 17-21.
38. Gunness M. & Orwoll E. 1995. Early Induction of Alterations in Cancellous and Cortical Bone Histology after Orchiectomy in Mature Rats. *Journal of Bone and Mineral Research* 10(11): 1735-1744.
39. Pavlos P.L, Theodoros T.X, Sofia E.T George P.L & Ismene A.D. 2008. The laboratory Rat as an Animal Model for Osteoporosis Research. *Comparative Medicine* 58(5): 424-430
40. Ke H.Z, Crawford D.T, Qi H, Chidsey-Frink K.L, Simmons H.A, Li M, Jee W.S.S & Thompson D.D. 2001. Long-term Effects of Aging and Orchidectomy on Bone and Body Composition in Rapidly Growing Male rats. *Journal of Musculoskeletal and Neuronal Interactions* 1(3): 215-224.
41. Goreja W.G. 2004. *Tongkat Ali: The tree that cures hundred diseases*. New York: Amazing Herb Press.
42. Bonjour J.P, Carrie A.L. & Ferrari S. 1997. Calcium-enriched Foods and Bone Mass Growth in Prepubertal girls: a Randomised, double-blind, placebo-controlled trial. *Journal of Clinical Investigation*: 99:1287–1289.
43. Anderson F.H., Francis R.M., Selby P.L. & Cooper C. 1998. Sex Hormones and Osteoporosis in Men. *Calcified Tissue International* 62: 185-188.
44. Falahati-Nini A, Riggs B.L, Atkinson E.J, O'Fallon W.M, Eastell R & Khosla S. 2000. Relative Contributions of Testosterone and Estrogen in Regulating Bone Resorption and Formation in Normal Elderly Men. *Journal of Clinical Investigations* 106: 1553-1560.
45. Tambi M.I, Imran M.K & Henkel R.R. 2011. Standardised water-soluble extract of Eurycoma longifolia, Tongkat Ali as Testosterone Booster for Men with Late-onset Hypogonadism. *Andrologia* 44: 226-230.
46. McGriff-Lee N, Pucino F & Anton Calis K. 2005. Effects of Androgens on Bone in Men and Women. *Clinical Reviews in Bone and Mineral Metabolism* 3(1): 51-66.
47. Dustin M.H., Amy C.B., Peterson P., Sunil S., Krista M.D., Nirupama K.S. & Pike J.W. 2001. Androgens Suppress Osteoclast Formation Induced by RANKL and Macrophage-colony Stimulating Factor. *Endocrinology* 142(9): 3800-3808.
48. Bakary D, Ann P., Mustapha C., Dorothy M.S., Tim J.C. and Elizabeth M.E. 2000. Effect of Calcium Supplementation on Bone Mineral Accretion in Gambian Children Accustomed to a Low-calcium Diet. *American Journal of Clinical Nutrition* 71(2): 544-549.
49. Yamamoto Y, Noguchi T & Takahashi N. 2008. Effects of Calcitonin on Osteoclast. *Clinical Calcium* 15(3): 147-151
50. Gerald A.L, Meredith P.G & Robin M.S. 2005. Testosterone -fatty acid Esterification: A Unique Target for the Endocrine Toxicity of Tributylin to Gastropods. *Integrative & Comparative Biology* 45(1): 81-87
51. López-López A, Castellote-Bargalló A.I, Campoy-Folgoso C, Rivero-Urgël M, Tormo-Carnicé R, Infante-Pina D & López-Sabater M.C. 2001. The Influence of Dietary Palmitic Acid Triacylglyceride Position on the Fatty acid, Calcium and Magnesium Contents of at Term Newborn Faeces. *Early Human Development* 65: S83–S94.