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Effects of *Eurycoma longifolia* on Callous Strength of Male Osteoporosis Fracture Model

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ABSTRACT

Eurycoma longifolia (EL), a medicinal plant with pro-androgenic effects was recently reported to possess anti-osteoporotic activities. However, its effect on osteoporotic fracture has never been studied. This study aimed to provide more insight into the action of EL on healing of osteoporotic fracture by assessing callous strength. Tibial fracture was induced in orchidectomised rat, the model for osteoporotic fracture in androgen-deficiency state. The effects of EL supplementation for 6 weeks on fracture healing was assessed using Instron, a biomechanical testing device. The results were compared with testosterone, the standard treatment for androgen deficiency. Based on the biomechanical parameters, EL did not significantly improve the strength of fracture callous compared to testosterone replacement. These findings were not supportive of the role of EL in promoting osteoporotic fracture healing in orchidectomised rat model. To discover the true potential of EL, studies should be repeated with different castration models with intact testes for testosterone production.

Key words: Eurycoma longifolia androgen, osteoporosis, orchidectomy, fracture, biomechanical strength, callous

INTRODUCTION

Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue (Anonymous, 1993). Recently, there have been studies to explore the potential use of medicinal plants in treating osteoporosis such as *Triticum aestivum* (Banji *et al*., 2014) and *Ulmus wallichiana* (Arya *et al*., 2011). There are also reports that parts of animals such as deer antler could be used to strengthen bone (Kawtikwar *et al*., 2010). *Eurycoma longifolia* Jack (EL) is another medicinal plant with was reported to have anti-osteoporosis activities. This herbal supplement is used for men's health. It is extracted from the root of the medicinal plant which grows along the slopes of hilly territories in the Malaysian rainforest (Burkill and Hanif, 1930). It is used to enhance libido in men and as anti-aging supplements (Cyranoski, 2005; Talbott *et al*., 2013). The herbal product is advertised in the internet and marketed on-line worldwide. Studies on male rat model have demonstrated the pro-androgenic effects of EL (Ang and Cheang, 2001) . In a human study, EL was found to elevate testosterone level and increase semen volume (Tambi and Imran, 2010).

EL may influence testosterone, a principal male hormone which also regulate bone remodeling and bone minerals homeostasis. Low testosterone level or hypogonadism in men is the main cause of osteoporosis, a bone disease characterized by low bone mineral density and high risk of fractures. Once a fracture had occurred in osteoporotic bone, it may heal poorly and is associated with significant morbidity and mortality (Magaziner *et al*., 2003; Tosteson *et al*., 2008; Abrahamsen *et al*., 2009; Haentjens *et al*., 2010). There were several studies done to determine the anti-osteoporotic activities of EL. It was found to prevent bone calcium loss in orchidectomised rats (Shuid *et al*., 2011a; Effendy and Shuid, 2013). However, the bone protective effects of EL were not consistent in histomorphometry and micro-CT studies (Ariff *et al*., 2012; Ramli *et al*., 2012).

Osteoporosis could promote bone fragility and vulnerability to fracture as the bone strength is weak. Once, a fracture has occurred, the bone auto-repairs itself with the help of surrounding tissues such as periosteum, blood, bone marrow, external soft tissue and reinstates its own mechanical loading ability. This process involves a sequence of cellular and molecular events that are mostly similar with soft tissue wound healing (Glowacki, 1998).

To the best of our knowledge, there is no study on the effects of EL supplementation on osteoporotic fracture healing. Therefore, in this study, fracture was induced in orchidectomised rat model and fracture healing with EL supplementation was compared to testosterone replacement, the standard treatment for androgen deficiency.

MATERIALS AND METHODS

Animals and treatment: Forty-eight male Wistar rats weighing between 350-450 g were obtained from the Laboratory Animal Resources Unit, Faculty of Medicine, UKM. The rats were randomly assigned into sham-operated group (n = 12) and orchidectomised group (n = 36).

Orchidectomy was performed through scrotal approach. A small incision was made at the tip of the scrotum. The tunic was opened and the testis, cauda epididymis, vas deferens and spermatic blood vessels were exteriorized. The blood vessels and vas deferens were then ligated with 4-0 absorbable suture. The testis and epididymis were then surgically removed and the remaining tissues were returned into the sac. Similar procedure was repeated for the other testis. The skin incision were closed with a non-absorbable suture (Foley, 2005).

Two weeks following orchidectomy or sham operation, the right tibiae of all the rats were fractured under anesthesia according to Stuermer *et al*. (2010). Osteotomy was executed at the proximal metaphyseal part of the tibia using pulsed ultrasound (Piezosurgery®, Mectron Medical Technology, Carasco, Italy) and the fractures were fixed with plate and screws.

The rats were further divided into four groups consisting of sham-operated (SHAM), orchidectomized-control (ORX), orchidectomized and treated with *Eurycoma longifolia* (EL) and orchidectomized and treated with testosterone enanthate (TEN). The EL group was treated with daily oral gavages of 15 mg kg^{-1} of *Eurycoma longifolia* extract (Phytes Biotek. Sdn. Bhd., Shah Alam, Selangor). The TEN group received an intramuscular injection of testosterone enanthate (Jesalis Pharma, Germany) at the dose of 7 mg per rat once a week (Yarrow *et al*., 2008). The SHAM and ORX control groups received vehicles only.

Following six weeks of treatment, the rats were euthanized, the fixation plate was removed and the tibiae were extracted and stored in 10% formalin at room temperature. The protocol was approved by Universiti Kebangsaan Malaysia (UKM) Animal Ethics Committee (UKMAEC: PP/FAR/2011/NORLIZA/30 NOVEMBER/414-NOVEMBER-2011-JUNE-2012).

Biomechanical analysis: The biomechanical strength of the healed tibiae was measured using Instron Microtester 5848 Model (Instron Corp, USA). The machine was equipped with Bluehill

software for data analysis. Three point bending test was selected since it was suitable for the small metaphyseal tibia measurement (Stuermer *et al.*, 2010). The samples were thawed at room temperature for an hour and continuously moistened with phosphate buffer solution to prevent the bones from drying out.

The right tibiae were placed with three-point contact on the aluminium base. The base was fixed earlier in the Instron Microtester with a distance of 3 mm between the end of the proximal tibia and the center of the load. Load was applied to the fracture callous of the tibia at the speed of 5 mm min^{-1} until it refractured.

Statistical analyses: All the data was expressed as Mean±Standard Error Mean (SEM). Statistical analysis was conducted using Statistical Package for Social Sciences version 18.0 (SPSS 18.0, Chicago, USA). The data was tested for normality using Kolmogorov Smirnov test. Normally-distributed data was analyzed using parametric one-way ANOVA test followed by Tukey's HSD test while not normally-distributed data was analysed using Kruskall-Wallis, Mann-Whitney U and Wilcoxon Signed Rank tests. The level of significance was taken as $p<0.05$.

RESULTS

Maximum load: The Maximum Load is the amount of load that the fracture callous was able to receive before it re-fractures. The Maximum Load of the ORX group (54.10±6.60 N) was significantly lower than the SHAM group (138.23±75.08 N) and TEN group (198.16±25.29 N). The Maximum Load of the TEN group was significantly higher than the SHAM group. Albeit not significant, the EL group (97.89±16.56 N) seemed to have higher Maximum Load than the ORX group (Fig. 1).

Maximum stress: Maximum Stress is the amount of stress that the fracture callous was able to receive before it re-fractures. The Maximum Stress for the TEN group (47.04±13.07 MPa) was significantly higher than the ORX (15.13±3.66 MPa) and EL group (23.24±23.24 MPa) (Fig. 2).

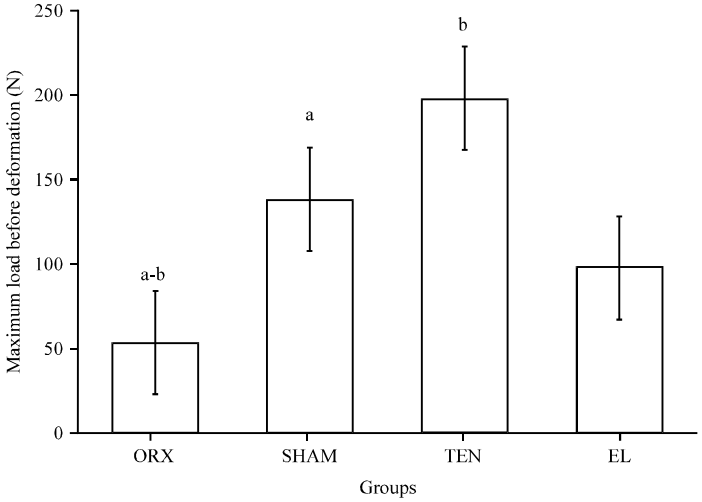

Fig. 1: Maximum load values for all the groups. Results were expressed as Mean±Standard Error Mean (SEM). Similar alphabets indicate significant difference between the groups, SHAM: Sham operated, ORX: Orchidectomy control, TEN: Orchidectomy+testosterone and EL: Orchidectomy+ *Eurycoma longifolia*

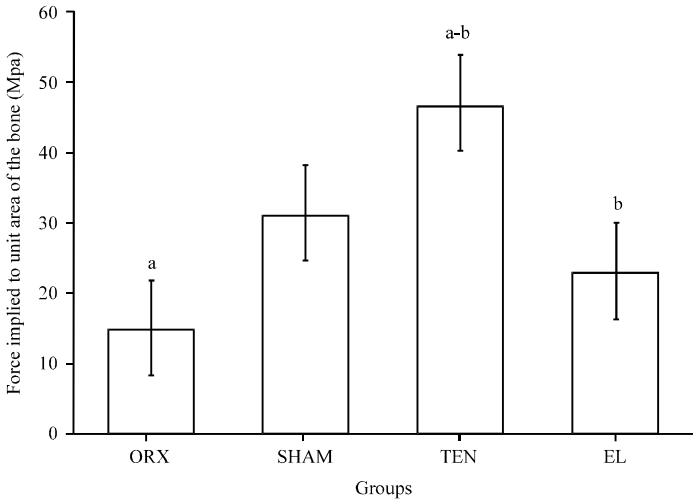

Fig. 2: Maximum Stress values for all the groups. Results were expressed as Mean±Standard Error Mean (SEM). Similar alphabets indicate significant difference between the groups SHAM: Sham operated, ORX: Orchidectomy control, TEN: Orchidectomy+testosterone and EL: Orchidectomy+*Eurycoma longifolia*

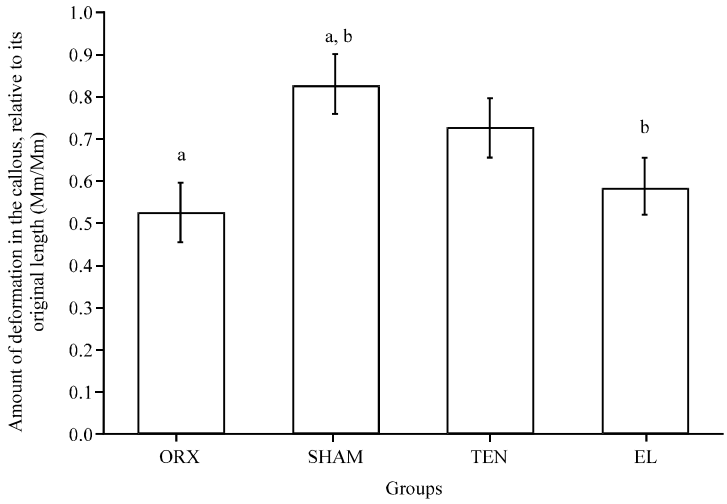

Fig. 3: Strain values for all the groups. Results were expressed as Mean±Standard Error Mean (SEM). Similar alphabets show significant difference, SHAM: Sham operated, ORX: Orchidectomy control, TEN: Orchidectomy+testosterone and EL: Orchidectomy+*Eurycoma longifolia*

Strain: Strain is relative deformation of the fracture callous before it re-fractures. The Strain for the ORX group (0.52±0.13 mm mm^{-1}) was significantly lower than the SHAM group (0.82±0.07 mm mm^{-1}). The Strain of the EL group (0.58±0.14 mm mm^{-1}) was also significantly lower than the SHAM group (Fig. 3).

Young's modulus: Young's Modulus parameter was derived from the slope of the elastic region of the stress-strain curve. Young Modulus of the TEN group (65.38±15.77 N) was

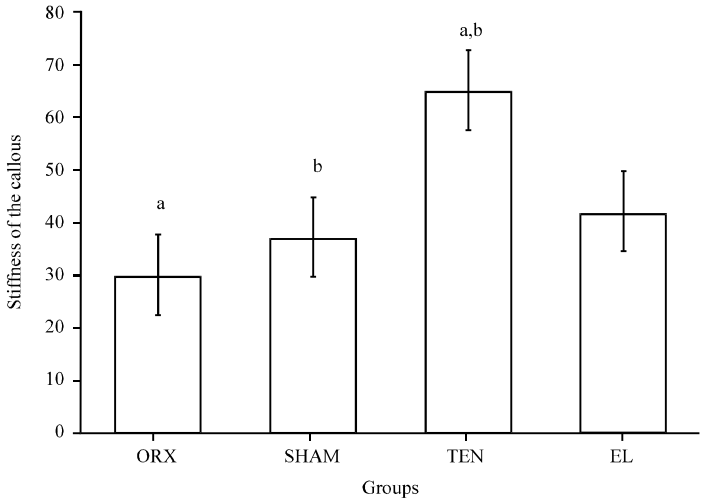

Fig. 4: Young modulus for all the groups. Results were expressed as Mean±Standard Error Mean (SEM). Similar alphabets indicate significant difference, SHAM: Sham operated, ORX: Orchidectomy control, TEN: Orchidectomy+testosterone and EL: orchidectomy+*Eurycoma longifolia*

significantly higher than the ORX (30.13±7.62 N) and SHAM (65.38±15.77 N) groups but not significantly different compared to the EL group (42.26±13.36) (Fig. 4).

DISCUSSION

About 39% of osteoporotic fracture cases involving men were reported every year (Johnell and Kanis, 2006). They require more attention as men were reported to have higher mortality risk after an osteoporotic fracture than women (Bliuc *et al.*, 2009). The most reliable way to assess fracture healing is by assessing the fracture callous strength, as what matters most during healing is the strength it offers to prevent fracture recurrence. However, direct biomechanical testing can only be carried out in animal model as the bone needs to be extracted out and the testing performed until the bone re-fractured. Human bone strength could only be estimated indirectly using special biomechanical software (Shuid *et al.*, 2010).

Fracture healing is a very complicated and poorly understood mechanism, especially with the presence of osteoporosis (McCann *et al.*, 2008). Osteoporotic fracture in men is gaining interest as it was shown that the relative risk of subsequent fracture in men was higher than women (Center *et al.*, 2007). There are already studies on the effects of EL on osteoporosis with some showing potentially good results (Shuid *et al.*, 2011b, 2012; Ramli *et al.*, 2012). However, there is no study yet to investigate the effects of EL on fracture healing of osteoporotic bone.

Orchidectomised rat is a reliable animal model for simulating fracture due to androgen-deficient osteoporosis (Erben *et al.*, 2000; Libouban *et al.*, 2001; Lefort *et al.*, 2005). Removal of the testes resulted in androgen deficiency which led to increased bone resorption and bone loss (Vanderschueren *et al.*, 2004). Fracture was created at the metaphyseal region of tibia based on the fact that osteoporotic fracture occurs more frequently on trabecular bone in human (Thompson *et al.*, 1995). There is only a thin subcutaneous tissue lining the anterior part of the tibia with limited vessels. Therefore, trauma and haemorrhage which could affect fracture healing, could be minimized in the tibial fracture model (Ibrahim *et al.*, 2013). Most studies carried out on

large animals had measured the strength of femur or tibia at the diaphysis. These may not provide accurate information as osteoporotic fracture occurs more frequently at the metaphysis of humans long bones. Therefore, the present study has adopted the biomechanical testing by Stuermer *et al.* (2010) which could accurately measure callous strength at the metaphysis of rat tibia.

In our previous study, EL was found to elevate testosterone levels, reduce bone resorption marker and up-regulate osteoprotegerin gene expression of orchidectomised rats (Shuid *et al.*, 2012). These actions may be responsible for the protective effects of EL against bone resorption due to androgen deficiency. They were consistent with the protective effects of EL from bone calcium loss (Shuid *et al.*, 2011a) but further investigations using bone histomorphometry and micro-CT failed to demonstrate these actions (Ariff *et al.*, 2012; Ramli *et al.*, 2012).

Osteoporosis has been proven to impair and delay bone fracture healing. This was consistent with the findings of the present study that the ORX group with osteoporotic bone had relatively poor fracture healing than the SHAM group. In the latter group, fracture was induced in non-osteoporotic bone to represent traumatic fracture with normal fracture healing.

The control groups (ORX, SHAM, TEN) had behaved expectedly. It was clearly shown that orchidectomised-induced osteoporosis had resulted in weaker callous strength when compared to that of normal bone (SHAM group). Testosterone replacement in the TEN group which acted as the positive control group, displayed a substantially better healing than the ORX group. Therefore, testosterone was able to improve fracture healing by providing better callous strength which was at par with the healing of non-osteoporotic bone of the SHAM group.

As for the EL treatment group, the stress and strain parameters were similar to the ORX group. There were non-significant improvements with the Maximum Load and Young Modulus parameters. The beneficial effects of EL on the fracture healing of osteoporotic bone seem plausible but the evidence is not sufficient to support this statement. There were several possibilities that may contribute to the non-significant findings of EL.

One of the possibilities is that the dose of EL used may not be high enough to produce significant effects on fracture healing. However, this is unlikely as the EL dose used should be adequate according to Shuid *et al.* (2011b). Furthermore, the dose was nearly twice the dose of EL used to increase sperm counts in normal rats (Wahab *et al.*, 2010).

In terms of the possible mechanisms behind the effects of EL on fracture healing, its bioactive product, especially eurycomanone may influence testosterone synthesis through the hypothalamic-pituitary-gonadal axis. Eurycomanone induced anterior pituitary to release Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) into the circulatory system (Low *et al.*, 2005). This would promote more testosterone production by the Leyding cells in the testes. In the orchidectomised model, the eurycomanone action through HPG axis may not be possible with the absence of testes. However, eurycomanone may still be able to stimulate small amount of testosterone production by the liver and kidneys (Nurhanan *et al.*, 2005).

Another testosterone-raising action of EL is via promoting the release of free testosterone from the Sex Hormone Binding Globulin (SHGB) (Talbott *et al.*, 2013; Ang and Cheang, 2001). In the absence of testes, the main producer of testosterone, the effects of whatever testosterone that is available may be utilized via this mechanism.

EL has cytotoxic activities on human lungs and breast cancer (Nurhanan *et al.*, 2005). Ramli *et al.* (2012) suggested that EL may adversely affect bone microstructure, possibly by exerting its cytotoxic effects on bone cells that are involved in bone formation. In terms of toxicity,

acute oral intake of aqueous extract EL did not cause acute toxicity to rats. However, at very high dose of more than 1200 mg kg^{-1}, subacute oral intake of aqueous extract EL may cause liver toxicity in rats (Shuid *et al*., 2011a).

CONCLUSION

Based on the findings of this study, EL was not able to significantly promote the healing of osteoporotic fracture. The testosterone-raising ability of EL may be hampered in the orchidectomy model, due to the absence of testes. Therefore, studies on EL should be repeated with different castration models.

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