IJMS
Vol 36, No 4, December 2011

Original Article

Histological Changes in the Fracture Callus Following the Administration of Water Extract of *Piper Sarmentosum* (Daun Kadok) in Estrogen-Deficient Rats

Mohamed Abdalla Estai[1], Ima Nirwana Soelaiman[2], Ahmad Nazrun Shuid[2], Srijit Das[1], Aishah Mohd Ali[3], Farihah Hj Suhaimi[1]

[1]Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.
[2]Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.
[3]Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

Correspondence:
Farihah Hj Suhaimi PhD,
Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, Zip Code: 50300, Kuala Lumpur, Malaysia.
Tel: +60 3 92897263
Fax: +60 3 26989506
Email: farihah@medic.ukm.my
Received: 1 February 2011
Revised: 6 June 2011
Accepted: 3 July 2011

Abstract

Background: The fracture healing is impaired in osteoporosis. *Piper sarmentosum* is a plant, which contains potent antioxidant, naringenin that may enhance fracture healing. The present histological study aimed to determine the effects of water extract of *Piper sarmentosum* on the late phase of fracture healing in estrogen-deficient rats.

Methods: Twenty four female Sprague-Dawley rats (200-250 gm) were obtained. Six rats underwent sham operation and the rest were ovariectomized. Six weeks post-ovariectomy all the rats were fractured at the mid-diaphysis of the right femur and a K-wire was inserted for internal fixation. The sham group was given vehicle (normal saline) and the ovariectomized group was randomly subdivided into three groups: (i) ovariectomized-control group supplemented with vehicle; (ii) ovariectomized+estrogen replacement therapy group treated with estrogen (100 µg/kg/day) and (iii) ovariectomized+*Piper sarmentosum* group treated with *Piper sarmentosum* water extract (125 mg/kg). Following six weeks of treatment, the rats were sacrificed and the right femora were harvested for histological assessment of fracture callus.

Results: The ovariectomized-control group showed a significant delay in fracture healing compared to the sham, ovariectomized-estrogen replacement therapy and ovariectomized-*Piper sarmentosum* groups. The median callus score for the ovariectomized-*Piper sarmentosum* group was 4.50 (range, 4-5), which was significantly higher than the median callus score 3.50 (range, 3-4) for the ovariectomized-control group (P=0.019). However, there was no significant (P>0.05) difference in the callus score among the sham, ovariectomized-estrogen replacement therapy and ovariectomized-*Piper sarmentosum* groups groups.

Conclusion: Treatment with water extract of *Piper sarmentosum* proved beneficial in the fracture healing in estrogen-deficient rats.
Iran J Med Sci 2011; 36(4): 281-288.

Keywords ● Antioxidant ● callus ● fracture healing ● histology ● osteoporosis ● ovariectomy

Introduction

Bone is the only tissue capable of regeneration without leaving a scar following trauma.[1] Fracture healing is a complex process, involving a series of cascade of events. The stages of tissue differentiation during fracture healing resemble that of fetal skeletal development.[2] Osteoporosis is a major worldwide health problem, which leads to an increase in risk of fractures.[3] Postmenopausal estrogen deficiency results in an increased bone remodelling and uncoupling between resorption by osteoclasts and formation by osteoblasts which results in bone loss.[4] Influence of osteoporosis on fracture healing is still not well understood. Earlier studies on animals showed that osteoporosis delayed fracture healing process.[5]

According to earlier research reports, majority of the therapeutic agents used to treat osteoporosis, act to inhibit bone resorption rather than to induce bone formation.[6] The main drugs used for treatment of osteoporotic fractures include: bisphosphonates, estrogen, selective estrogen receptor modulators and vitamin D.[7] Estrogen replacement therapy (ERT) had beneficial effects on osteoporotic fracture healing. However, long-term unopposed estrogen therapy has been proved to be strongly associated with estrogen dependent cancer such as endometrial carcinoma.[8] Considering the high costs incurred, side effects observed and the risk of malignancy following long-term use of these agents, it is needed that natural products with less side effects be tried in addition to conventional treatment.

Piper sarmentosum (P.s) belongs to the family of Piperaceae. It is widely distributed in South East Asia and is usually used as flavoring agent in food.[9] In Malaysia, plant P.s is known as Daun Kadok, and its extract has been used for the treatment of toothache, fungal infection of the skin and cough.[10] It has been reported that extracts of different parts of P.s plant possess antioxidant, antimicrobial, anti-inflammatory and anticarcinogenic properties.[11] Methanolic extract of P.s is rich in phenolic compounds such as naringenin. Naringenin belongs to the flavonoid groups, which exhibit high free radical-scavenging activity.[12] Flavonoids rutin was reported to prevent ovariectomy-induced bone loss in rats.[13] Isoflavones and soy food have been reported to prevent bone loss induced by menopause in women.[14] Parhami concluded that the estrogen deficiency lead to an increase in the level of reactive oxygen species (ROS). Reactive oxygen species induce the release of the cytokines, which is involved in osteoclastogenesis.[15] Earlier studies showed that estrogen deficiency induced oxidative stress by increasing the level of ROS and hydrogen peroxide (H_2O_2), which induced osteoclasts activity.[16] Hence, ROS may increase bone resorption and influence fracture healing. Water, methanol and hexane extracts of P.s were reported to prevent H_2O_2-induced cells apoptosis in human umbilical vein endothelial cells through its antioxidative action.[17]

Ima -Nirwana et al. observed that the P.s water extract reduced bone resorption by decreasing the cortisol level in blood in the adrenalectomised rats.[18] Thus, free radical-scavenging activities of the P.s flavonoids may play an important role in reducing ROS and preventing oxidative stress during fracture healing. Therefore, the main aim of the present study was to determine the effects of P.s water extract on the late phase of fracture healing of rats in estrogen deficient state.

Materials and Methods

Preparation of Water Extract of P.s

Approximately five kg of P.s fresh leaves were obtained from a supplier. The plant was identified by a botanist from Furley Marketing Sdn. Bhd, Malaysia. Water extraction process of P.s was done by Furley Marketing Sdn. Bhd, Malaysia. The water extract was then sent to the Biotechnology Science Faculty for freeze drying process (Freeze Dryer, Labconco, Italy). The freeze dried extract was then kept at 4°C, until used.

Experimental Design

The ethical approval was obtained from the Institutional Animal Ethics Committee, Universiti Kebangsaan Malaysia (UKM). Twenty four female Sprague-Dawley rats weighing 200-250 g were purchased from the Laboratory Animal Resource Unit, UKM. The rats were housed individually in a clean cage at 22°C with adequate ventilation and normal 12-hour light-dark cycle. They were allowed free access to water and rat chow *ad libitium*. They were acclimatized for two weeks before the intervention. Rats were randomly assigned into sham-operated (SO) (n=6) and ovariectomy-operated (OVX) groups (n=18). The SO group underwent sham operation while the OVX group underwent bilateral ovariectomy at the beginning of the study as per previous protocol.[19] Rats were kept six weeks after ovariectomy to develop osteoporosis. The structural histomorphometry of bone using modified Von Kossa method was performed in order to confirm

the development of osteoporosis in rats.

Following six weeks of ovariectomy, the right femur of all rats was subjected to closed fracture. Prior to the fracture procedures rats were anaesthetized with a mixture of Xylazin and Ketamine (1:1) at a dose of 0.1 ml/100g (Troy Laboratories, Australia), which was given intramuscularly. Surgical procedures were done under aseptic technique, a two cm transverse incision was made on the right knee and the patella was laterally dislocated to approach the anterior intercondylar notch. An introducer (16 G needle) was appreciated to create the entry point. A1.0 mm Kirschner wire (K-wire) (Jorgensen laboratories, USA) was then inserted into the right femoral medullary canal toward the greater trochanter of the femur. The ends of the wire were then cut and buried under the skin. Following the insertion of K-wire, a steel guillotine-like blade device weighing 500 g was released from a height of 30 cm on the mid-diaphysis of the rat femur to produce a transverse mid-femoral closed fracture as per previous protocol.[20] Following the fracture, the soft tissues and skin were stitched using appropriate sutures.

Following right femora fracture, x-ray images were immediately obtained by using X-ray machine (Proteus XR/a, GE, UK) to confirm both the intramedullary placement of the K-wire and the fracture (figure 1). Each rat was then separately housed in a clean cage. To prevent infection antibiotic Baytril 5% at a dose of 10 mg/kg (Bayer, Thailand) was administered intramuscularly daily for seven days as well as daily dressing of the incision wound with Povidone iodine solution. On following day after femora fracture, the sham (SO) group (n=6) was started on supplement with normal saline as vehicle, while the ovariectomizedgroup (n=18) was further randomly assigned into three groups: (i) ovariectomized-control (OVXC) group treated with normal saline as vehicle; (ii) ovariectomized+estrogen replacement therapy (ERT) group, treated orally with conjugated equine estrogen (Premarin-Wyeth, Canada) at dose of 100 µg/kg/day,[21] (iii) ovariectomized+ P.s (P.s) group, treated orally with P.s water extract at dose of 125 mg/kg.[18] Following fracture, all the rats received the above treatment by oral gavage for another six weeks. After treatment, the rats were sacrificed with over dose of diethyl ether. The right femora were dissected from the hind limbs and stained with Hemaetoxylin and Eosin (H&E) for histological assessment.

Histological Analysis Using H&E Stain
Tissue Preparation

After the sacrifice, the right femora were dissected from the hind limbs of the rats, cleaned from soft tissues and the K-wires were removed. Neutral buffered formalin 10% was prepared and specimens of right femora were taken and fixed in that solution for at least 24 hours. Ethylenediaminetetracetic acid (EDTA) is a chelating agent, and has been the preferred decalcifying agents due to the facts that EDTA is gentle and slow acting, and preserves tissue components as compared to formic acid. All samples were decalcified by using EDTA 10% solution for 12 weeks as per previous protocol.[22] In addition, the EDTA solution was changed every five days. The samples were placed in a warm place and agitated daily to accelerate decalcification process. Decalcified bone samples were assessed by pricking with a sharp needle. As per previous protocols, following decalcification all bone samples were dehydrated to remove the water content using increasing 70% of Ethanol solution.[23] Samples were immersed in equal parts of Alcohol: Toluene and samples were then cleared by Toluene. Finally bone samples were embedded in suitable containers with melted paraffin wax and stored at -4°C. Paraffin blocks were sectioned longitudinally by microtome (Leica RM 2235) at 5 µm thickness. The sections were stained with

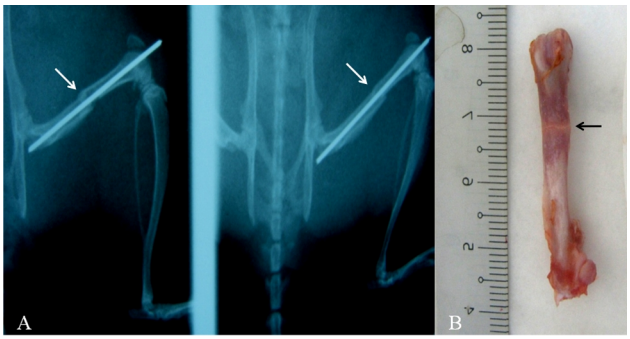

Figure 1: Radiograph image of right femur after fracture (A), and right femur sample harvested after sacrifice (B). Arrows indicate the fracture area.

H&E stain and assessed by image analyzer.

Measurement of Fracture Healing

Fracture healing was measured using modified Allen's grading system. The slides were examined blindly and three sections per specimen were interpreted. Computerized image analysis system with Pixelink color camera (USA) and light microscope (Leica DM RXA2, Germany) were used for the qualitative measurement. Microscopic images were then obtained by using video T-Morphology 5.1 software (VT, Russia). As per previous protocol, the sections were examined by using modified scoring system was adopted from Allen et al. (1980), which is a seven-point scoring system was used to assess the fracture healing.[24] Grading was done as follows: -Grade '0' non union (fibrous tissues), '1' incomplete cartilage union (cartilage with some fibrous tissues), '2' complete cartilage union (entirely cartilage), '3' incomplete bony union with early ossification phase (predominantly cartilage with some trabecular bone), '4' incomplete bony union with intermediate ossification phase (equal amounts of cartilage and trabecular bone), '5' incomplete bony union with late ossification phase predominantly trabecular bone with some cartilage), '6' complete bony union (entirely bone) (table1).[24] All the slides were subjected to blind study by two independent pathologists who were unaware of the treatment.

Table1: Allen's fracture healing scoring system

Healing staging	Score
Non union	0
Incomplete cartilage union	1
Complete cartilage union	2
Incomplete bony union with phase of ossification	3
Incomplete bony union with intermediate phase of ossification	4
Incomplete bony union with late phase of ossification	5
Complete bony union	6

Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS, version 17). Normality of distribution of all variables was examined by Shapiro-Wilk test. Since seven-point scoring system was used to analyze the results, all results were ordinal data and were considered as non parametric. Non parametric data were analyzed by using Kruskal Wallis followed by Mann Whitney U test. Data were presented as median values with the range (minimum-maximum values) in parentheses. Level of significance was considered at $P<0.05$.

Results

Following fracture of the femora, all the rats were observed daily. They started movement 24 hours post-fracture. Weight bearing on the fractured leg started 10 days post fracture. Following six weeks of treatment after the right femora fracture, H&E stained sections of the fractured femora were subjected to seven-point scoring system using modified Allen's grading. The median fracture healing score was higher in the P.s group compared to the OVXC group ($P=0.019$). On the other hand, there was no significant difference in the median fracture healing scores for the SO, ERT and P.s groups ($P=0.078$) (table 2).

Table 2: Fracture callus scores (n=6) based on Allen's fracture healing scoring system of sham-operated (SO) and ovariectomized rats (OVAX), and rats receiving estrogen replacement therapy (ERT) or water extract of *Piper sarmentosum* (P.s)

Group	Allen's fracture healing score
SO	4.75 (range; 4-5)*
OVXC	3.50 (range; 3-4)
ERT	4.25 (range; 4-5)**
P.s	4.50 (range; 4-5)***

Values are expressed as median and (range). The SO rats were treated with normal saline for six weeks. The OVXC group was treated with normal saline for six weeks. The ERT group was treated with conjugated equine estrogen (100 µg/kg/day) for six weeks. The P.s group was treated with P.s water extract (125 mg/kg/day) for six weeks.
* indicates significant difference ($P=0.011$) compared to the OVXC group. ** indicate significant difference ($P=0.020$) compared to the OVXC group. *** indicate significant difference ($P=0.019$) compared to the OVXC group.

Fracture callus sections stained with H&E showed that fracture healing in the SO and the P.s groups were identical suggesting improvement in osteoporotic fracture healing (figure 2). In the OVXC group, there was an abundance of hyaline cartilage (soft callus) which presented with small amount of woven bone, indicating early stage of endochondral ossification (figure 3). The presence of large amount of the hyaline cartilage within the callus at this period suggested a delay in the endochondral ossification of soft callus. The fracture callus in the ERT and P.s group were made up mainly of woven bone whereby most of the soft callus (hyaline cartilage) were replaced with hard callus (woven bone) through endochondral ossification (figure 4 and 5). There were also few scattered hypertrophied chondrocytes trapped within the calcified matrix, which may indicate endochondral ossification at the late stages. In addition, small areas of lamellar bone were dispersed between woven bones of the callus, which may indicate the beginning of remodeling process.

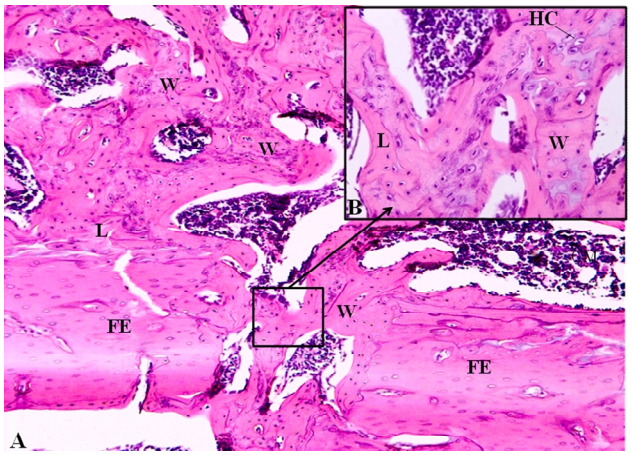

Figure 2: Micrograph section of a fracture callus taken from the sham-operated group and stained with H & E at low magnification (×50) (A). It shows the formation of woven bone (W), which filled the gap between the fracture ends (FE), and areas of woven bone was remodeled to lamellar bone (L). The inset part (B) shows a higher (x200) magnification in which the callus shows spicules of newly formed woven bone (W) that is lined by osteoblasts. It shows few numbers of hypertrophic chondrocytes (HC) trapped within the calcified matrix.

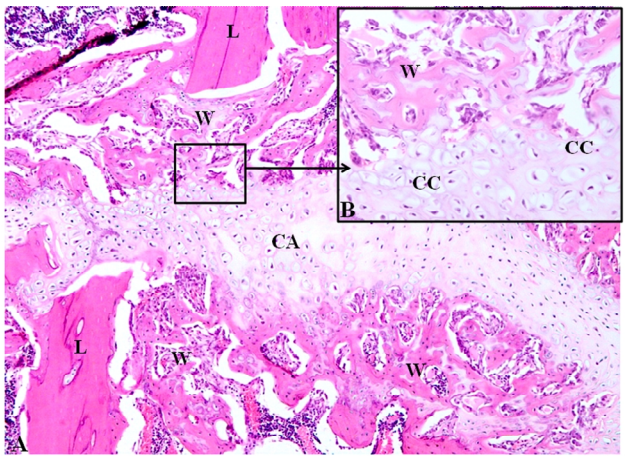

Figure 3: Micrograph section of a fracture callus taken from the ovariectomized group and stained with H & E at low magnification (×50) (A). It displays central mass of hyaline cartilage (CA) within the callus. In addition, there is vascular invasion of cartilage associated with endochondral ossification, which resulted in woven bone formation (W). At higher (x200) magnification (B), the fracture callus shows the presence of large number of mature chondrocytes (CC). It also reveals vascular invasion of cartilage with deposition of osteoid by osteoblasts on the calcified matrix of cartilage.

Discussion

The guillotine fracture technique to generate standardized fracture with minimal soft tissue damage was adopted from the study by Shuid et al.[20] Earlier studies proved that estrogen deficiency influenced the late phase of fracture healing in the ovariectomized rats.[25] Hence, our study was conducted to investigate the effects of administration of P.s extract on the late phase of fracture healing in osteoporotic rat model. Based on histological observations, fracture healing (secondary healing) in human occurs in four overlapping phases including the hematoma formation phase; early inflammatory phase (2-4 weeks); repair phase (proliferation and differentiation, which is within 1-2 months); and late remodeling phase, which lasts for months or years.[26] A seven point scoring (modified Allen's scoring) system was used to assess fracture healing.

In this study, the fracture callus score in the OVXC group was lower compared to the SO group. The lower fracture healing score, as shown by the high content of cartilage in the callus, indicated that the repairing phase was still on-going. Estrogen-deficient state impaired fracture healing by inducing excessive cartilage formation and delaying endochondral ossification. Sartori and colleagues concluded that the period of six weeks post-fracture was regarded as the remodelling phase of fracture healing.[27] The presence of large amounts of cartilage in this phase indicates delayed min-

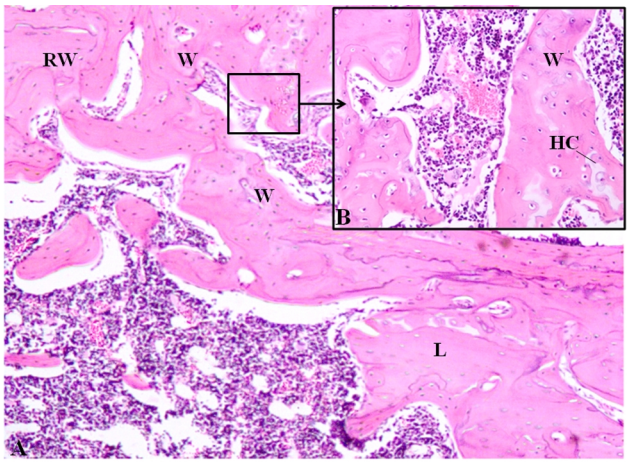

Figure 4: Micrograph of section of a fracture callus taken from the group receiving estrogen replacement therapy and stained with H & E at low magnification (×50) (A). It displays the formation of network of woven bone (W) (immature bone) associated with initiation of remodeling of woven bone (RW). Small areas of woven bone underwent remodeling. At higher magnification (x200) (B), the callus reveals randomly arranged trabeculae of woven bone, which are lined by osteoblasts. There are few hypertrophied chondrocytes (HC) seen embedded within the calcified matrix.

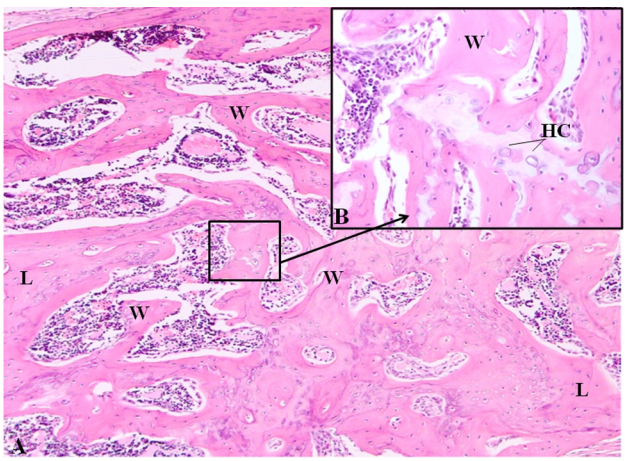

Figure 5: Micrograph section of a fracture callus taken from the group treated with water extract of Piper sarmentosum and stained with H & E at low magnification (×50) (A). The fracture callus shows the formation of spicules of woven bone (W), which filled the entire space of the callus. There are also areas of woven bone remodeled to the lamellar bone (L). At higher magnification (x200) (B), the fracture callus reveals spicules of newly formed woven bone with decreased number of hypertrophied chondrocytes (HC) which are trapped within the calcified matrix.

eralization and endochondral ossification of soft callus. Qiao et al. and Xu et al. observed that the fracture callus in the ovariectomized-control group contained mainly of soft callus (cartilage) compared to the sham group.[5,28] This indicates a delay in fracture healing with appearance of osteoporotic changes. The same pattern was observed by Arslan et al. who found that the fracture callus in the ovariectomized-control group of rabbits had immature bone compared to that in the sham group.[29]

The histological analysis of the ovariectomized rats fed with P.s extract, revealed that the fracture callus score was higher compared to the OVXC group. Higher fracture healing score indicated that P.s extract might have enhanced the healing of osteoporotic fracture of the femur. Ovariectomized rats treated with P.s have shown mature woven bone with some scattered cartilage cells. In some parts of the fracture callus, woven bone remodeled to lamellar bone. Treatment with P.s had beneficial effects on endochondral ossification, whereby most of the soft callus was replaced by woven bone or hard callus.

The fracture callus score in the P.s and ERT groups were consistent. Treatment with P.s extract and ERT had beneficial effects on osteoporotic fracture healing by inducing the mineralization and accelerating endochondral ossification of soft callus. The fracture callus was mainly made up of woven bone and the callus was remodeled toward mature callus. The presence of woven bone in the callus

suggested the completion of endochondral ossification and the initiation of the remodeling process. Estrogen acted mainly by inhibiting bone resorption and preventing overproduction of cytokines, which is involved in osteoclastogenesis.[30] This was similar to an earlier study by Liu et al., which concluded that fracture callus in the ovariectomized+ERT group contained mainly of mature bone and was identical to the control group at eight weeks post-fracture.[31] These researchers suggested that treatment with ERT promoted osteoporotic fracture healing by inducing the expression of transforming growth factor-beta1 (TGF-β1) in the estrogen-deficient state.[31] Estrogen acted most probably by decreasing bone resorption rather than increasing bone formation.

A preliminary study performed by Prasad et al. concluded that the ROS level was increased at the fracture site following the formation of hematoma after a fracture. The increased level of ROS tends to react with cell membrane phospholipids, which resulted in production of lipid peroxide.[32] It was reported that the level of malondialdehyde was significantly higher in rats after fracture, compared to normal.[33] This was also similar to an earlier study, which showed that the administration of antioxidants may prevent bone loss and is beneficial in the acceleration of fracture healing in osteoporotic patients.[34] It is suggested that the antioxidative action of P.s through its flavonoids content prevented lipid peroxidation at the fracture callus by reducing the level of ROS. Treatment with P.s extract and ERT exhibited similar effects on osteoporotic fracture healing but with different mechanism of action.

Conclusion

The current study, suggests that oral administration of P.s water extract (125 mg/kg/day) was as beneficial as ERT in promoting the late phase of osteoporotic fracture healing, as assessed by histological study, in ovariectomized rats. Treatment with P.s extract improved fracture healing, which was achieved by inducing endochondral ossification and accelerating the replacement of soft callus by hard callus (mature callus) as well as preventing osteoporotic changes. Treatment with P.s had advantage over ERT in that long-term treatment of P.s does not have the potential to cause endometrial carcinoma or breast cancer. Hence, the use of P.s extract may be safer than ERT as an antioxidant supplements in patients suffering from osteoporotic fractures. Further studies with different design and sample size may be required to illuminate the issue.

Acknowledgment

The authors thank Universiti Kebangsaan Malaysia for providing the financial support to conduct this study.

Conflict of Interest: None declared

References

1 Hernandez-Gil IFT, Gracia MAA, del Canto Pingarrn M, et al. Physiological bases of bone regeneration I. Histology and physiology of bone tissue. *Med Ora*l 2006; 11: E47-51.
2 Goldhahn J, Scheele WH, Mitlak BH, et al. Clinical evaluation of medicinal products for acceleration of fracture healing in patients with osteoporosis. *Bone* 2008; 43: 343-7.
3 Giannoudis P, Tzioupis C, Almalki T, et al. Fracture healing in osteoporotic fractures: Is it really different? A basic science perspective. *Injury* 2007; 38: 90S-9S.
4 Wronski TJ, Dann LM, Scott KS, et al. Long-term effects of ovariectomy and aging on the rat skeleton. *Calcif Tissue Int* 1989; 45: 360-6.
5 Qiao L, Xu KH, Liu HW, et al. Effects of ovariectomy on fracture healing in female rats. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2005; 36: 108-11.
6 Turner CH. Biomechanics of bone: determinants of skeletal fragility and bone quality. *Osteoporos Int* 2002; 13: 97-104.
7 Fu L, Tang T, Miao Y, et al. Effect of 1,25-dihydroxy vitamin D3 on fracture healing and bone remodeling in ovariectomized rat femora. *Bone* 2009; 44: 893-8.
8 Garrett A, Quinn MA. Hormonal therapies and gynaecological cancers. *Best Pract Res Clin Obstet Gynaecol* 2008; 22: 407-21.
9 Burkill IH. A dictionary of the economic products of the Malay Peninsula. 2nd ed. Kuala Lumpur: Ministry of Agriculture and Cooperative, 1966. p. 132-2347.
10 Perry LM. Medicinal plants of East and Southeast Asia: attributed properties and uses. Cambridge: MIT Press, 1980. p. 314-5.
11 Ariffin Z, Hisham S, Omar W, et al. Intrinsic anticarcinogenic effects of Piper sarmentosum ethanolic extract on a human hepatoma cell line. *Cancer Cell Int* 2009; 9: 6.
12 Subramaniam V, Adenan MI, Ahmad AR, et al. Natural antioxidants: Piper sarmentosum (Kadok) and Morinda elliptica (Mengkudu). *Mal J Nutr* 2003; 9: 41-51.

13 Horcajada Molteni MN, Crespy V, Coxam V, et al. Rutin inhibits ovariectomy induced osteopenia in rats. *J Bone Miner Res* 2000; 15: 2251-8.
14 Potter SM, Baum JA, Teng H, et al. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am J Clin Nutr* 1998; 68: 1375S-9S.
15 Parhami F. Possible role of oxidized lipids in osteoporosis: could hyperlipidemia be a risk factor? *Prostaglandins Leukot Essent Fatty Acids* 2003; 68: 373-8.
16 Lean JM, Jagger CJ, Kirstein B, et al. Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. *Endocrinology* 2005; 146: 728-35.
17 Hafizah AH, Zaiton Z, Zulkhairi A, et al. Piper sarmentosum as an antioxidant on oxidative stress in human umbilical vein endothelial cells induced by hydrogen peroxide. *J Zhejiang Univ Sci B* 2010; 11: 357-65.
18 Ima-Nirwana S, Elvy-Suhana MR, Faizah O, et al. Effects of Piper sarmentosum on bone resorption and its relationship to plasma cortisol in rats. *Bone* 2009; 44: 79S-80S.
19 Zhang R, Liu ZG, Li C, et al. Du-Zhong (Eucommia ulmoides Oliv.) cortex extract prevent OVX-induced osteoporosis in rats. *Bone* 2009; 45: 553-9.
20 Shuid AN, Mohamad S, Mohamed N, et al. Effects of calcium supplements on fracture healing in a rat osteoporotic model. *J Orthop Res* 2010; 28: 1651-6.
21 Hayward MA, Kharode YP, Becci MM, et al. The effect of conjugated equine estrogens on ovariectomy-induced osteopenia in the rat. *J Inflamm Res* 1990; 31: 152-6.
22 Anderson HC, Hodges PT, Aguilera XM, et al. Bone morphogenetic protein (BMP) localization in developing human and rat growth plate, metaphysis, epiphysis, and articular cartilage. *J Histochem Cytochem* 2000; 48: 1493-502.
23 Bancroft JD, Gamble M. Theory and practice of histological techniques. 6th ed. Philadelphia, PA: Churchill Livingstone Elsevier, 2008.
24 Allen HL, Wase A, Bear WT. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. *Acta Orthop Scand* 1980; 51: 595-600.
25 Kubo T, Shiga T, Hashimoto J, et al. Osteoporosis influences the late period of fracture healing in a rat model prepared by ovariectomy and low calcium diet. *J Steroid Biochem Mol Biol* 1999; 68: 197-202.
26 Harwood PJ, Newman JB, Michael ALR. (ii) An update on fracture healing and non-union. *J Orthop Trauma* 2010; 24: 9-23.
27 Sartori AR, Moreira JA, Santos AMM, et al. Bone repair process in normal and osteopenic female rats' tibiae: a comparative study. *Acta Ortop Bras* 2008; 16: 37-40.
28 Xu SW, Yu R, Zhao GF, et al. Early period of fracture healing in ovariectomized rats. *Chin J Traumatol* 2003; 6: 160-6.
29 Arslan H, Ketani A, Gezici A, et al. The effects of osteoporosis on distraction osteogenesis: an experimental study in an ovariectomized rabbit model. *Acta Orthop Belg* 2003; 69: 67-73.
30 Chen FP, Wang KC, Huang JD. Effect of estrogen on the activity and growth of human osteoclasts in vitro. *Taiwan J Obstet Gynecol* 2009; 48: 350-5.
31 Liu H, Xu K, Qiao L. Effects of estrogen on the expression of TGF-B in early fracture healing of ovariectomized rats. *Bone* 2008; 43: 54S-5S.
32 Prasad G, Dhillon MS, Khullar M, et al. Evaluation of oxidative stress after fractures. A preliminary study. *Acta Orthop Belg* 2003; 69: 546-51.
33 Yeler H, Tahtabas F, Candan F. Investigation of oxidative stress during fracture healing in the rats. *Cell Biochem Funct* 2005; 23: 137-9.
34 Sheweita SA, Khoshhal KI. Calcium metabolism and oxidative stress in bone fractures: role of antioxidants. *Curr Drug Metabol* 2007; 8: 519-25.