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Role of Phytoestrogen Ferutinin in Preventing/Recovering Bone Loss: Results from Experimental Ovariectomized Rat Models

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1. Introduction

Osteoporosis is a chronic bone disease, caused by an imbalance between bone resorption and bone formation (Riggs & Melton, 1986), in which the skeleton becomes fragile and leads to an increased risk of fractures. In osteoporosis the bone mineral density is rapidly reduced, the bone microarchitecture is disrupted and the amount/variety of non-collagenous bone proteins is altered (Chestnut, 1995; Paschalis et al., 1997). In menopausal women, the rapid decrease of estrogens is the predominant cause of the imbalance between bone formation and bone resorption that results, in turn, in severe bone loss (Riggs & Melton, 1986). Hormone Replacement Therapy (HRT), based on estrogen administration, is a method to recover both bone loss and incidence of skeletal fractures in postmenopausal women (Turner et al., 1994); however, as it is well known, it increases, as negative side effects, the occurrence of cardiovascular diseases and endometrial/breast/ovarian malignant cancers (Beral, 2003; Genant et al., 1998; Lacey et al., 2002; Termine & Wong, 1998). In addition to HRT, other compounds such as bisphosphonates, calcitonin, calcium products, RANK Ligand, stronzium ranelate, PTH 1-34, thiazide diuretics and ipriflavone (Bruhn, 2010; El-Desoky et al., 2009; Lacroix, 2000; Rybchyn et al., 2011; Schoofs, 2003; Wasnich, 1983; Zhang et al., 2010) are currently used as pharmacological approaches to osteoporosis, but they are often associated with negative side effects. Therefore, the need to find safer and more effective bone protective agents is still prominent. A great number of preparations from medicinal plants was shown to reduce bone loss induced by ovariectomy in rats (Occhiuto et al., 2007) and to increase bone density in postmenopausal women (Clifton-Bligh, 2001). Among natural products increasingly used as an alternative therapy, the phytoestrogenic isoflavones have been shown to increase bone density in postmenopausal women following high dietary intake (Mei et al., 2001). Also in animal studies, the administration of isoflavones or their derivatives prevented bone loss in ovariectomized rats. They are structurally similar to estradiol and their estrogenic-like activity may also depend on their affinity for some estrogen receptors (ERs). Phytoestrogens appear to bind preferentially to the ERβ and have been classified as Selective Estrogen Receptor Modulators (SERMs) (An et al., al., 2001; Brzezinski & Debi, 1999; Messina et al., 2006). ERβ may play a protective role in breast breast cancer development by reducing mammary cell growth, as well as inhibiting the

stimulatory effects of ERα (An et al., 2001; Strom et al., 2004). Considering the properties of such such natural compounds, phytoestrogens could be employed as Complementary/Alternative Medicine (CAM) instead of HRT, in order to recover menopausal symptoms (Lee et al., 2000; Morris et al., 2000; Morris et al., 2006). Such evidence that SERMs mime estrogens as osteoprotective substances (Albertazzi, 2002; Wang et al., 2006) without displaying the negative side effects on the etiogenesis of some types of cancer (Duffy et al., 2007; Eason et al., 2005; Gallo et al., 2006; Garcia-Perez et al., 2006; Lian et al., 2001; Limer & Speirs, 2004; Murray et al., 2003; Wu et al., 2002) suggests interesting perspectives in planning alternative treatment strategies.

A great number of preparations from medicinal plants, including red clover, hops and black cohosh, have been tested to investigate their influence on ovariectomy-induced bone loss. Red clover (*Trifolium pratense L.*) was shown to reduce bone loss induced by ovariectomy in rats (Occhiuto et al., 2007) and to increase bone density in postmenopausal women (Clifton-Bligh, 2001). The prenylated flavanone contained in hops (*Humulus lupulus L.*), 8-prenylnaringenin (8-PN), and genistein (found in a number of plants including lupin, fava beans, soybeans, kudzu, and psoralea) seem to protect from ovariectomy-induced bone loss in rats, while exhibiting minimal trophic effects on uterus endometrium (Garcìa-Pérez et al., 2006; Hümpel et al., 2005); in particular isoflavone genistein, by enhancing uterine endometrial glandular apoptosis *in vivo* and *in vitro*, may confer protection against uterine carcinoma (Eason et al., 2005). Moreover, a reduced bone resorption was demonstrated also after black cohosh (*Cimicifuga racemosa L.*) therapy and it was ascribed to the significant binding of its components to estrogen receptors (Wuttke et al., 2003). Despite the huge amount of data published *in vitro* and *in vivo* on another phytoestrogen, Ferutinin, extracted from *Ferula hermonis root* (Fig. 1) (Abourashed et al., 2001), whose effect was investigated on calcium-related cellular processes, few observations are reported in literature concerning ferutinin role on the skeleton, particularly on bone metabolism in both the preventing and curative treatment of osteoporosis. Ferutinin shows high affinity for both subtypes of estrogen receptors (ERs). Even if ferutinin can bind to both ERs, it acts as an agonist for ERα and as agonist/antagonist for ERβ (Ikeda et al., 2002). Thus, this compound may be useful as a selective estrogen receptor modulator (SERM) (Appendino et al., 2002).

Recently, the interest of the authors was to investigate the effects of ferutinin administration on bone metabolism in prevention and in recovery of severe estrogen deficiency-osteoporosis and to compare them with those of estradiol benzoate treatment, in order to propose an alternative solution to the hormone replacement therapy (HRT) commonly used in osteoporotic women. The animal model used, i.e. ovariectomized rat, appears to be an appropriate model for collecting information which could be applied to human postmenopausal osteoporosis, because of the many similarities of the pathophysiological mechanisms (Comelekoglu et al., 2006; Kalu, 1991; Wronski & Yen, 1991).

Further crucial problem correlated to the use of the phytoestrogen ferutinin is to evaluate its side effects, specifically on the organs which are reputed to be the target of estrogen effects, like uterus, vagina, mammary glands. It is well-known that estrogens stimulate endometrial proliferation and their administration in HRT was associated to an increased risk of cancer. Some phytoestrogens are claimed to have beneficial effects with a minor incidence of undesired side effects in comparison with estrogen therapy. Proliferative activity in estrogen-responsive cells can be either enhanced or suppressed by phytoestrogens depending on their concentration and relative potency (Whitten & Patisaul,

2001). Clinical reports about phytoestrogen effect on endometrial cancer are limited to case-controlled observational studies (Johnson et al., 2001). Hence the interest of the authors also in the problem of ferutinin side effects.

Fig. 1. *Ferula Hermonis.*

2. Methods

The authors report the following methods from some animal experiments they performed in the recent past on the topic.

2.1 Experimental animals and treatments

For animal experiments female Sprague-Dawley rats, aged 7 weeks and weighing 170-190 g at the beginning of the experiments, were used, according to the general age-models

reported in literature (Fanti et al.,1998; Kalu, 1991). They were housed two per cage and maintained in standard conditions with a 12:12 light/dark cycle, at the temperature of 22 ± 1°C and 55-60% relative humidity. Commercial rat pellets free of estrogenic substances and drinking water were available ad libitum. After a 7-day adaptation period, the animals were randomly divided in different groups according to two protocols (for prevention and recovery of bone loss, respectively). Animal care, maintenance and surgery were conducted in accordance with the Italian law (D.L. n. 116/1992) and European legislation (EEC n. 86/609). The experimental designs and procedures received the approval of the Bioethical Committee of the Italian National Institute of Health.

2.1.1 Preventing study protocol

The animals were randomly divided in four groups (Palumbo et al., 2009). Rats of group 1 were sham-operated, while rats of other groups were bilaterally ovariectomized (OVX) under ketamine hydrochloride plus xylazine hydrochloride anaesthesia and the ovaries were bilaterally removed; sham-operation was performed in the same way as ovariectomy, but only exposing the ovaries. Starting on the day after the ovariectomy, half of the female rats were submitted to the following treatments for 30 days and the remaining half for 60 days:
Group 1 (SHAM): Sham-operated controls receiving vehicle (5% Tween 80 in water)
Group 2 (C-OVX): Ovariectomized controls receiving vehicle (5% Tween 80 in water)
Group 3 (F-OVX): Ovariectomized treated with ferutinin 2 mg/kg/day
Group 4 (EB-OVX): Ovariectomized treated with estradiol benzoate 1.5 µg/rat twice a week.
Ferutinin, whose formula is showed in Figure 2, was solubilized in Tween 80 (5%) and deionized water and administered in the volume of 5 ml/kg by oral gavage (*per os*). The dosage was chosen taking into account previous studies on rat sexual behavior (Zanoli et al., 2005; Zavatti et al., 2006). Control animals (groups 1 and 2) received the same volume of vehicle solution. Estradiol benzoate, used as a reference compound, was dissolved in peanut oil and subcutaneously injected in the volume of 0.3 ml/rat.

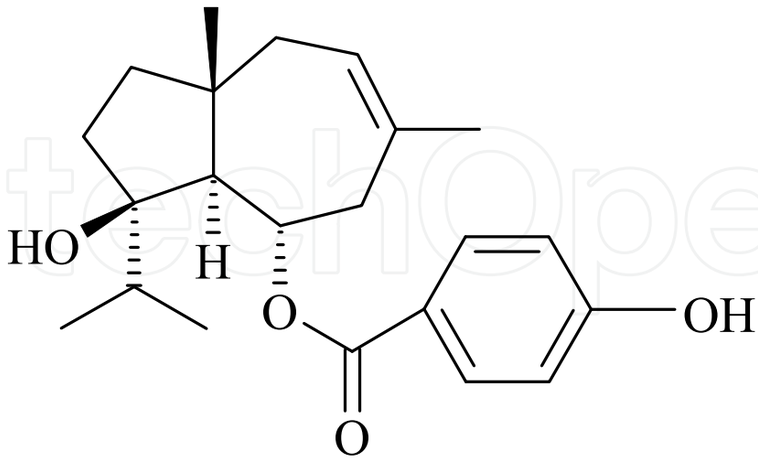


Fig. 2. Chemical structure of ferutinin.

The body weights of all animals were recorded before ovariectomy and after 30 and 60 days of treatment. Half of each rat group was sacrificed after 30 days of treatment and the remaining animals at the end of the experiment.

2.1.2 Recovering study protocol

The rats was randomized into four groups (Ferretti el al., 2010). One group of rats were sham operated, while the rats of the other three groups were ovariectomized. Ovariectomy and sham-operation were performed as above described in the protocol for preventing study. Two months after ovariectomy, namely when osteoporosis was obtained by the consequent estrogen deficiency, half of the rats of each group underwent the following treatments for 30 days and the remaining ones for 60 days:
Group 1 (SHAM): Sham-operated controls receiving vehicle (5% Tween 80 in water)
Group 2 (C-OVX): Ovariectomized controls receiving vehicle (5% Tween 80 in water)
Group 3 (F-OVX): Ovariectomized treated with ferutinin 2 mg/kg/day
Group 4 (EB-OVX): Ovariectomized treated with estradiol benzoate 1.5 µg/rat twice a week.
Ferutinin and Estradiol benzoate were used as above described.
The body weight of each animal was recorded at 4 different times: before ovariectomy (i.e., at the start of the experiment), two months after ovariectomy (namely, at the beginning of treatment), and after 30 and 60 days of treatment. At the end of the treatments, all rats were sacrificed.

2.2 Histology and histomorphometrical evaluation

Soon after the sacrifice, the 4th, 5th lumbar vertebrae and the right femurs were removed, processed and embedded in methyl methacrylate according to standard protocol for light microscopy. Serial sections of 200µm thickness were taken from both lumbar vertebrae and femurs by means of a diamond-saw microtome cutting system. In particular, the 4th lumbar vertebrae were cut according to sagittal planes, whereas the 5th lumbar vertebrae were transversely cut; concerning the femurs, the distal epiphyseal level was sagittally sectioned, whereas the shaft was transversely sectioned at the mid-diaphyseal level (Fig. 3).
Histomorphometric analysis was performed on Fast-Green or Alizarin-Red stained sections using a light microscope equipped with an image analysis system. In histomorphometric evaluations of vertebral bodies, only trabecular bone was taken into account: it was manually selected, outlining the internal surface of the cortical bone (Fig. 3A,B). In femoral sagittal sections, a constant area (3.5mm^2) of trabecular bone was defined by drawing a circular line adjacent to the cartilagineous plate (Fig. 3C). In transversal mid-diaphyseal femoral sections the cortical bone area was measured (Fig. 3D).
The following parameters were calculated:

- the ratio between the *trabecular bone area* (BV) and the *total area* (TV), i.e. the *trabecular bone volume* (BV/TV) expressed in percent values, in trabecular bone;
- the difference between the total cross section area and the medullary canal area , i.e. the *cortical bone area* (Ct-B-Ar), in cortical bone.

Only in preventing study protocol, in order to obtain a more precise evaluation of the collected data (i.e. to eliminate the effects of body weight on bone histomorphometric parameters), both the ratio BV/TV and the value Ct-B-Ar were "normalized" (i.e. corrected) with respect to body weight (dividing the calculated parameters by the body weight) on the basis that ovariectomy implies a considerable weight increase, while the chronic treatment with both ferutinin and estradiol benzoate (starting the day after ovariectomy) avoids such increment. On the contrary, in recovering study protocol the same treatment was performed 2 months after ovariectomy and after such period, the body weights of all OVX animals (C-OVX, F-OVX, EB-OVX) were all similar; for this reason, histomorphometric parameters (BV /TV and Ct-B-Ar) were not normalized with respect to body weight.

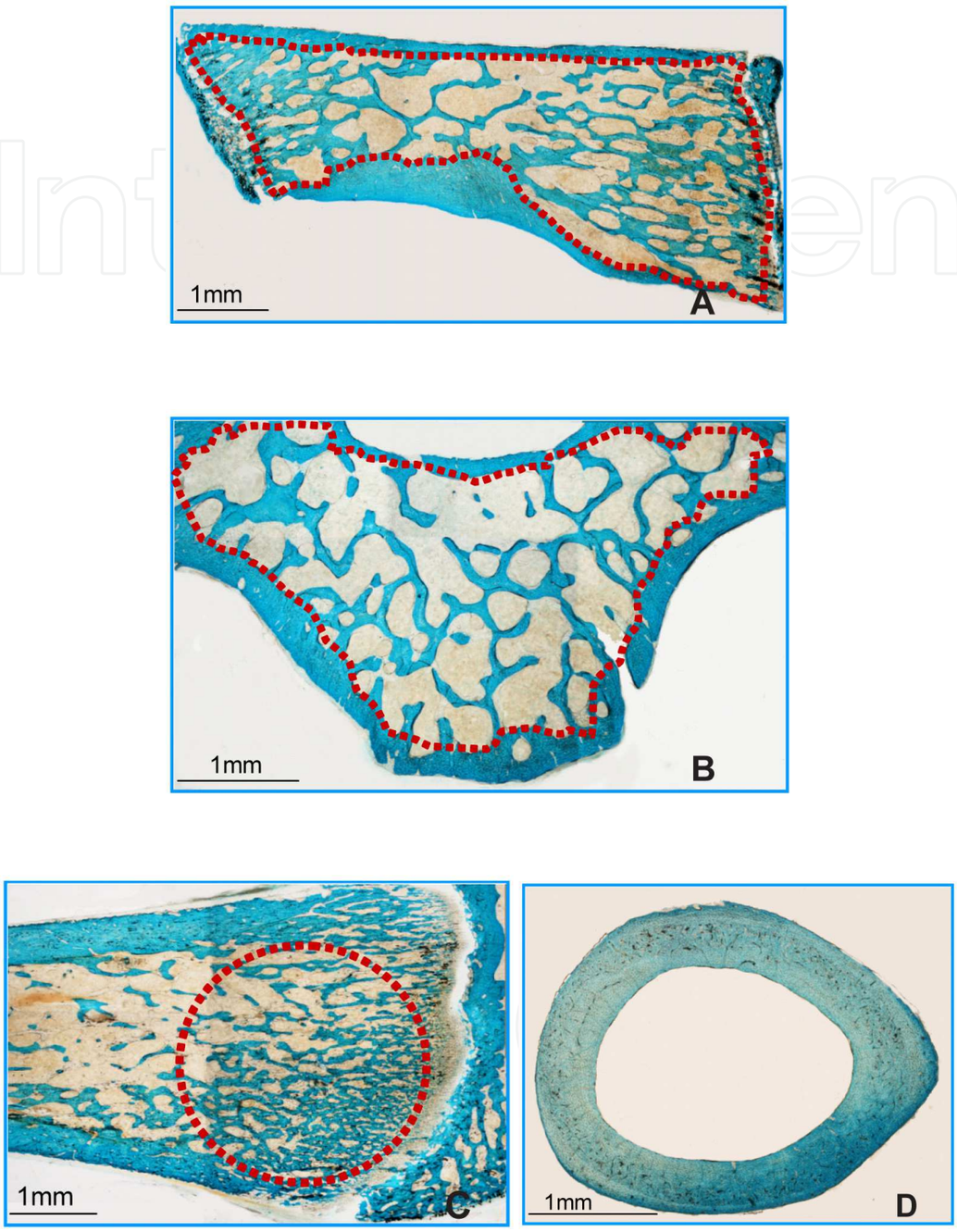


Fig. 3. Histological sections taken from SHAM group in which the histomorphometrical analyses were performed. (A) sagittal section of 4th lumbar vertebra; (B) transversal section of 5th lumbar vertebra; (C) sagittal section of the distal epiphyseal level of femur; (D) transversal section at the mid-diaphyseal level of femur. The dotted lines indicate the areas in which evaluations were recorded. (Figure by Palumbo et al., 2009).

2.3 Biochemical assays

Blood samples from experimental rats were collected in tubes and the serum was immediately separated by centrifugation, aliquoted into small volumes and stored at –20°C for analysis. The serum levels of magnesium, calcium, inorganic phosphorus and alkaline phosphatase (ALP) activity were determined by colorimetry using commercially available test kits.

2.4 Statistical analysis

One-way analysis of variance (ANOVA) with Newman-Keuls test for post-hoc comparisons between individual treatment groups and controls was performed. Student's *t*-test was used where appropriate. Values of $P<0.05$ indicate significant differences between groups.

3. Results

3.1 Body weights

Both in preventive and recovering studies, initial body weights of the four animal groups were similar.

In preventing study (Table 1), as expected, the body weight of C-OVX rats, sacrificed at 30 and 60 days after ovariectomy, was significantly higher than that of SHAM animals. The chronic administration of ferutinin as well as estradiol benzoate significantly counterbalanced body weight increase. It must be stressed that estradiol benzoate (EB) treatment was able to equal the body mass gain of sham-operated control rats, while ferutinin caused a more marked decrease in body weight in comparison to EB.

TABLE 1 - Body weight in Preventing study			
Treatment group	Initial BW	BW at 30th day	BW at 60th day
SHAM	198.9±2.4	249.7±4.1	246.0±5.4
C-OVX	205.1±2.1	308.4±5.6^{a}	335.2±9.8^{a}
F-OVX	196.8±1.7	194.4±4.0^{b,c}	246.0±5.4
EB-OVX	204.7±3.1	229.8±1.9^{b}	246.8±3.4^{b}

Table 1. Effect of ferutinin and estradiol benzoate on body weight of ovariectomized rats. Values represent mean±SEM. Anova followed by Newman-Keuls post test: $^{a}P<0.001$ vs. SHAM, $^{b}P<0.001$ vs. C-OVX, $^{c}P<0.001$ vs. EB-OVX. SHAM: sham-operated controls receiving vehicle; C-OVX: ovariectomized controls receiving vehicle; F-OVX: ovariectomized treated with ferutinin; EB-OVX: ovariectomized treated with estradiol benzoate; BW: body weight.

In recovering study (Fig. 4) two months after ovariectomy (namely, at the beginning of treatment) the body weight of ovariectomized rats (C-OVX, F-OVX and EB-OVX) was significantly higher, as expected, with respect to SHAM, but after both 30 and 60 days of chronic administration of ferutinin as well as of estradiol benzoate the body weight reduces significantly in comparison to C-OVX and it is similar to that of SHAM one.

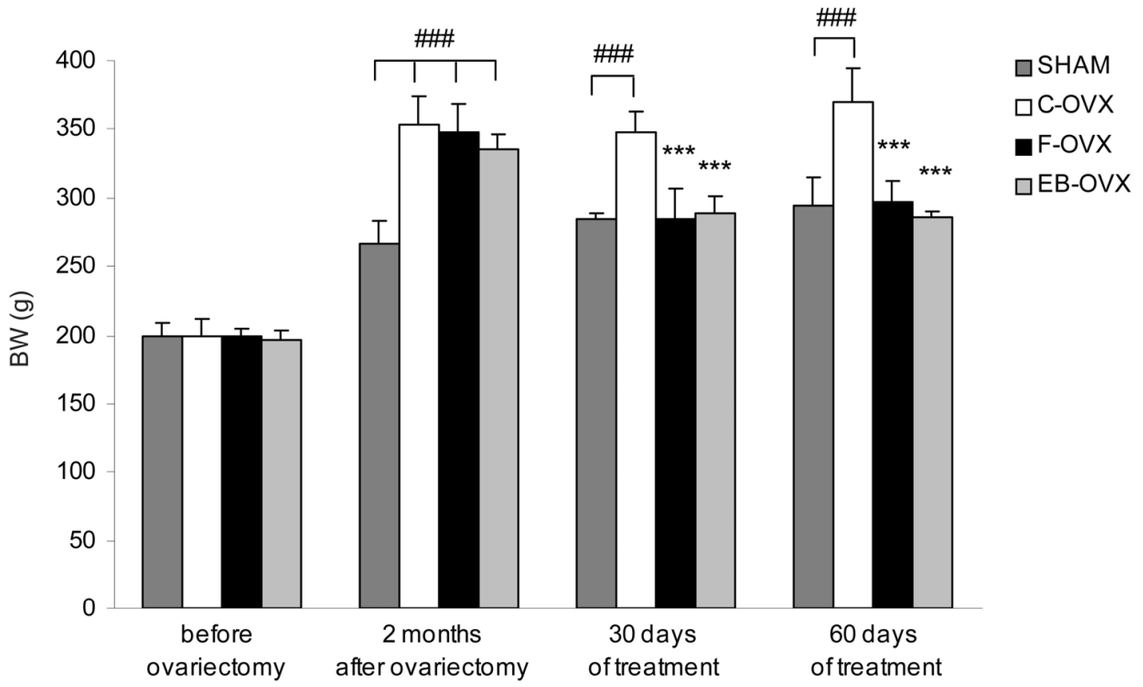


Fig. 4. Recovering study. Histograms showing the mean values of body weights-BW (g) recorded from all animal groups at 4 different times: (1) at the start of the experiment (before ovariectomy), (2) two months after ovariectomy, (3) after 30 days of treatment and (4) after 60 days of treatment. Values are expressed as mean ± SEM. $^{***}P<0.001$ vs. C-OVX; $^{###}P<0.001$ vs. SHAM (Anova followed by Newman-Keuls test). SHAM: sham-operated controls receiving vehicle; C-OVX: ovariectomized controls receiving vehicle; F-OVX: ovariectomized treated with ferutinin; EB-OVX: ovariectomized treated with estradiol benzoate; BW: body weight.

3.2 Histology and histomorphometric analysis

3.2.1 Bone mass in preventing study protocol

Histological observations of bone sections of vertebrae and femurs from treated and control animal groups underlined, as it is expected, that bone mass is clearly lower in C-OVX rats, with respect to SHAM and treated (F-OVX and EB-OVX) animals (Fig. 5).

The histomorphometric results obtained after 30 and 60 days of treatment showed that ovariectomy induced reduction in bone mass of lumbar vertebrae and femur, which is not observed in the animals treated with ferutinin or estradiol benzoate (Figs. 6 and 7); in particular, comparing the two groups of ovariectomized animals treated with ferutinin (F-OVX) and estradiol benzoate (EB-OVX), the mean values are always higher in F-OVX with respect to EB-OVX, sometimes displaying statistical significance.

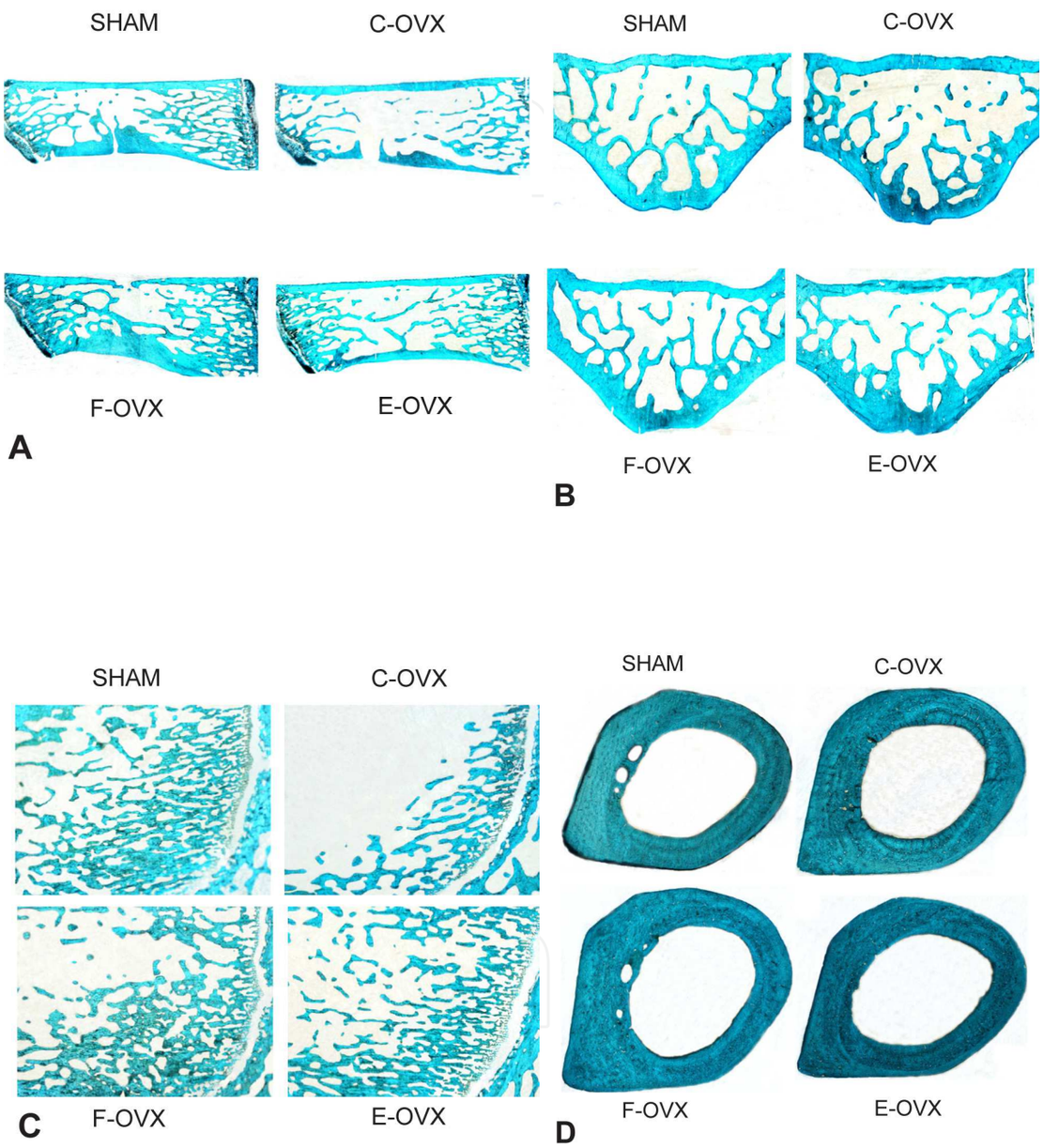


Fig. 5. Preventing study. LM micrographs showing the bone histology from the four experimental animal groups: (A) sagittal sections of 4th lumbar vertebra; (B) transversal sections of 5th lumbar vertebra; (C) sagittal sections of the distal epiphyseal level of femur; (D) transversal sections at the mid-diaphyseal level of femur. (Figure by Palumbo et al., 2009).

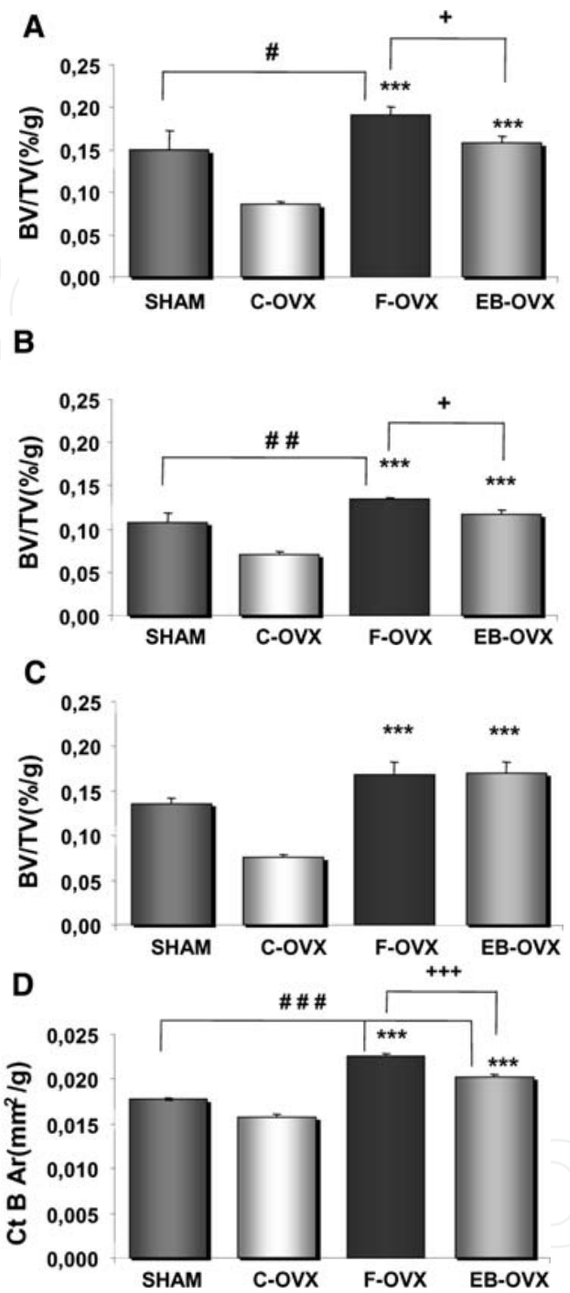


Fig. 6. Preventing study. Mean values of histomorphometric normalized parameters, expressed as BV/TV (%/g) and CT-B-Ar (mm^2/g), in both trabecular and cortical bone of the four animal groups, after 30 days from ovariectomy. (A) sagittal sections of 4th lumbar vertebra; (B) transversal sections of 5th lumbar vertebra; (C) sagittal sections of the distal epiphyseal level of femur; (D) transversal sections at the mid-diaphyseal level of femur. Values are expressed as mean ± SEM. ***P<0.001 vs. C-OVX; ^{+++}P<0.01 vs. EB-OVX; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. SHAM (ANOVA followed by Newman–Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; F-OVX ovariectomized treated with ferutinin; EB-OVX ovariectomized treated with estradiol benzoate.

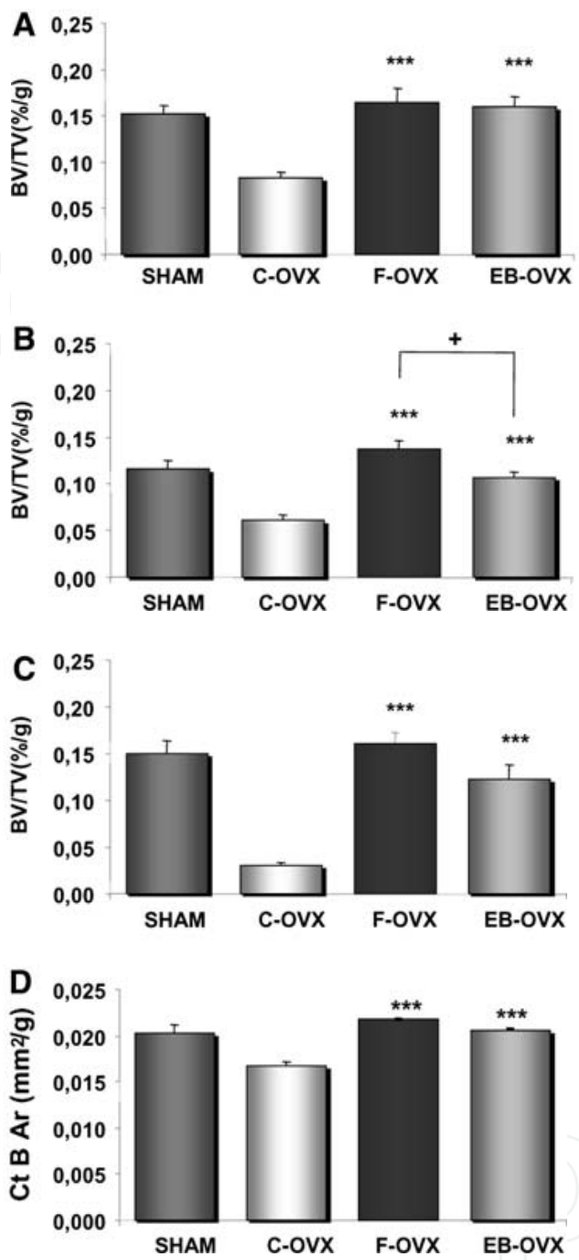


Fig. 7. Preventing study. Mean values of histomorphometric normalized parameters, expressed as BV/TV (%/g) and Ct-B-Ar (mm²/g), in both trabecular and cortical bone of the four animal groups, after 60 days from ovariectomy. (A) Sagittal section of 4th lumbar vertebra; (B) transversal section of 5th lumbar vertebra; (C) sagittal section of the distal epiphyseal level of femur; (D) transversal section at the mid-diaphyseal level of femur. Values are expressed as mean ± SEM. ***P<0.001 vs. C-OVX; +P<0.05 vs. EB-OVX (ANOVA followed by Newman–Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; F-OVX ovariectomized treated with ferutinin, EB-OVX ovariectomized treated with estradiol benzoate.

3.2.2 Bone mass in recovering study protocol

As regards both periods of time (30 and 60 days), the histological sections of vertebrae and femurs from SHAM and treated animal groups showed higher amount of trabecular bone (Figure 8A-B-C) with respect to C-OVX group, while the amount of cortical bone did not show differences among all groups (Fig. 8D). The histomorphometric analyses clearly showed the different results in trabecular and cortical bone: the amount of trabecular bone (Figures 9A-B-C and 10A-B-C) in F-OVX and EB-OVX animals are always higher with respect to C-OVX ones, although they do not reach the values of SHAM animals; as far as cortical bone (Figures 9D and 10D) is concerned, no statistically significant differences were found in bone area among all groups after both 30 and 60 days of treatments.

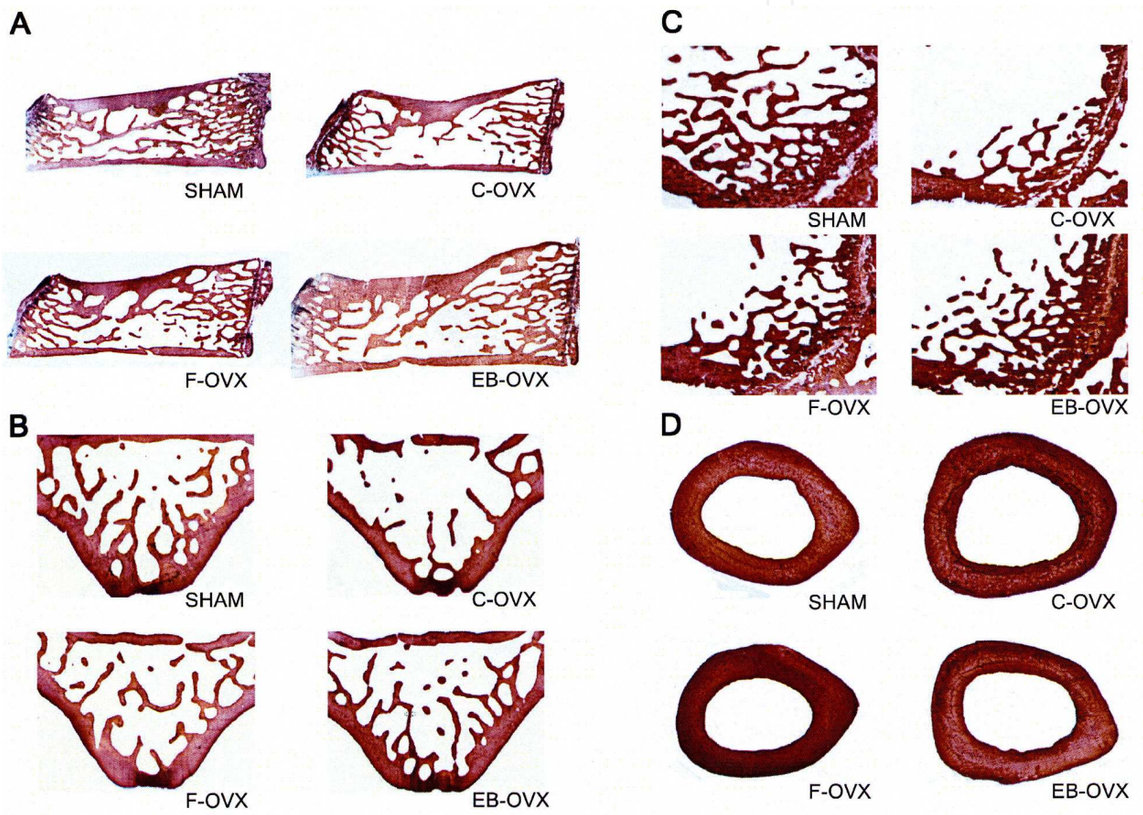


Fig. 8. Recovering study. LM micrographs showing the bone histology from the four experimental animal groups after 30 days of treatment. (A) Sagittal sections of the 4th lumbar vertebra; (B) transversal sections of the 5th lumbar vertebra; (C) sagittal sections of the distal epiphysis of femur; (D) transversal sections at the mid-diaphyseal level of femur. (Figure by Ferretti et al., 2010).

3.2.3 Uterine tissues

Preliminary data, not yet published by the authors, concern also the side effects of the chronic treatment with ferutinin on the uterus of ovariectomized rats, particularly regarding weight, size, morphology and structure. The target was to compare ferutinin side effects with those elicited by estradiol benzoate treatment, both in preventing and recovering protocols. Although data are incomplete, ferutinin would seem to exert the same effect of

estrogen benzoate in increasing uterine weight not only in the preventing study but also in the recovering one. In particular, the morphological and morphometrical preliminary data suggest that ferutinin would act on the uterus in a manner similar to that of estradiol benzoate, stimulating endometrial hypertrophy. Moreover, the treatment with ferutinin is of particular interest because the apoptotic index in both preventing and recovering studies seems to be almost always higher in both luminal and glandular endometrial epithelia with respect to animal groups treated with estradiol benzoate.

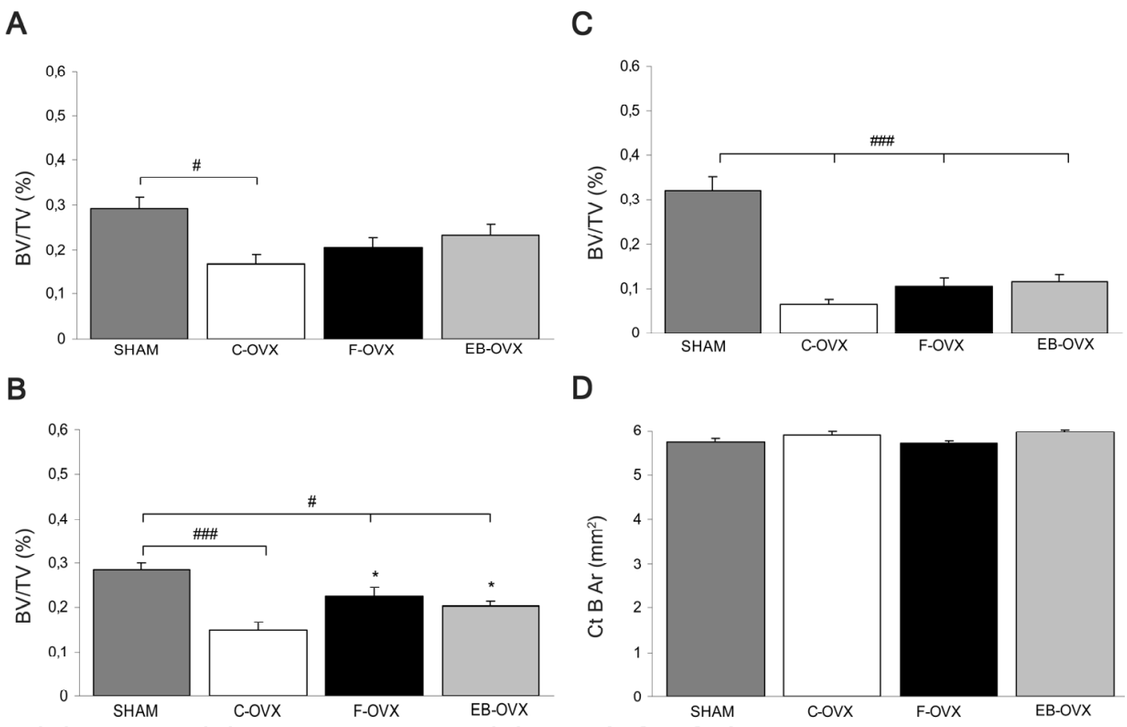


Fig. 9. Recovering study. Mean values of histomorphometric parameters, expressed as BV/TV (%) and Ct-B-Ar (mm^2), in both trabecular and cortical bone of the all animal groups after 30 days of treatment. (A) Sagittal section of the 4th lumbar vertebra; (B) transversal section of the 5th lumbar vertebra; (C) sagittal section of the distal epiphysis of femur; (D) transversal section at the mid-diaphyseal level of femur. Values are expressed as mean ± SEM. *P< 0.05 vs. C-OVX; #P< 0.05, ###P< 0.001 vs. SHAM (ANOVA followed by Newman–Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; F-OVX ovariectomized treated with ferutinin; EB-OVX ovariectomized treated with estradiol benzoate.

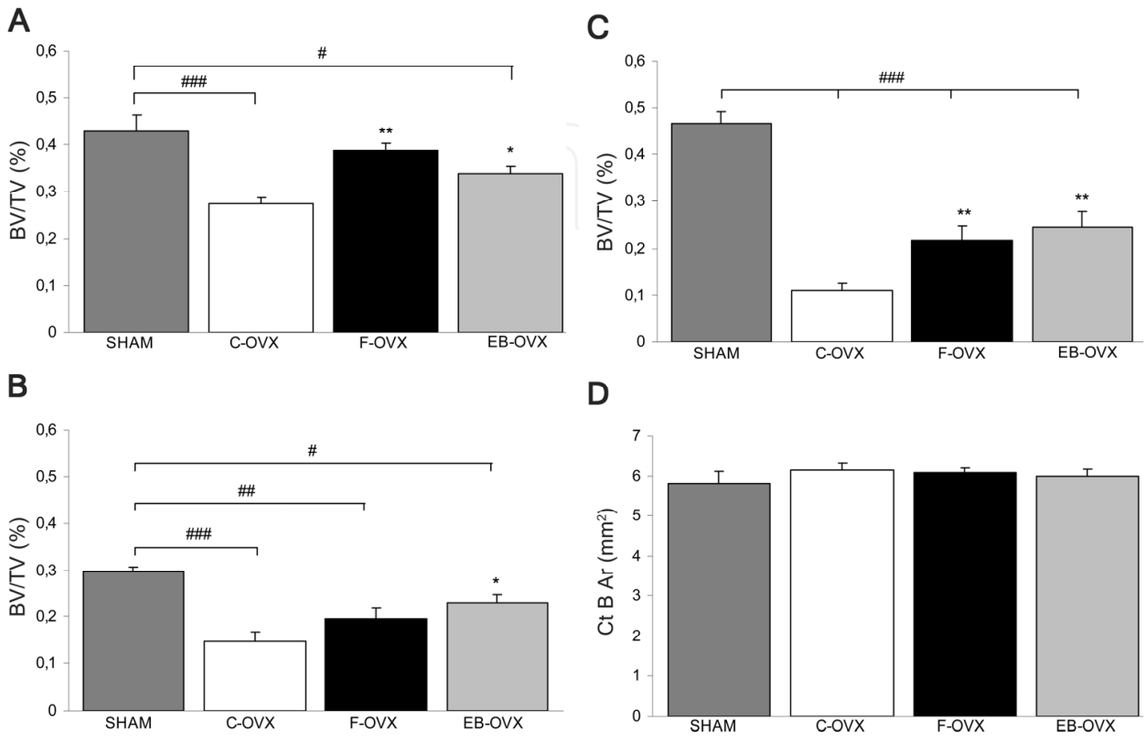


Fig. 10. Recovering study. Mean values of histomorphometric parameters, expressed as BV/TV (%) and Ct-B-Ar (mm^2), in both trabecular and cortical bone of the all animal groups after 60 days of treatment. (A) Sagittal section of the 4th lumbar vertebra; (B) transversal section of the 5th lumbar vertebra; (C) sagittal section of the distal epiphysis of femur; (D) transversal section at the mid-diaphyseal level of femur. Values are expressed as mean ± SEM. **P*< 0.05, ***P*< 0.01 vs. C-OVX; #*P*< 0.05, ##*P*< 0.01, ###*P*< 0.001 vs. SHAM (ANOVA followed by Newman–Keuls test). SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; F-OVX ovariectomized treated with ferutinin; EB-OVX ovariectomized treated with estradiol benzoate.

3.3 Serum biochemical analysis

In preventing study protocol, serum level variations of magnesium, calcium and inorganic phosphorous among the groups were more evident after 30 days of treatment rather than after 60 days (Table 2), while in the recovering study protocol, no significant differences in serum levels were recorded (Table 3). As far as serum alkaline phosphatase is concerned, its levels in F-OVX animal groups were always higher with respect to all other groups both in preventive and recovering study protocols after 30 as well as 60 days of treatment.

TABLE 2	Treatment group	Mg (mg/dl)	Ca (mg/dl)	P inorganic (mg/dl)	ALP (UI/l)
30 day-treatment	SHAM	2.82±0.02	9.8±0.1	9.06±0.22	110.3±12.0
	C-OVX	2.94±0.09	10.2±0.05$^{\#}$	9.85±0.35	106.2±4.9
	F-OVX	2.74±0.04	9.6±0.1$^{**\ ++}$	7.59±0.21$^{***\ \#\#\ ++}$	148.4±17.5
	EB-OVX	2.88±0.05	10.1±0.07	9.07±0.26	111.0±8.4
60 day-treatment	SHAM	2.54±0.01	9.7±0.1	7.81±0.17	90.8±5.1
	C-OVX	2.52±0.05	9.7±0.2	7.76±0.34	102.6±4.2
	F-OVX	2.55±0.05	9.9±0.1	7.05±0.31	118.0±7.1^{++}
	EB-OVX	2.53±0.05	9.7±0.2	7.03±0.33	79.7±7.3

Table 2. Preventing study. Effect of ferutinin/estradiol benzoate on serum biochemical values of ovariectomized rats treated for 30 and 60 days. All values are expressed as mean ± SEM. Anova followed by Newman-Keuls post test: $^{**}P<0.01$, $^{***}P<0.001$ vs. C-OVX; $^{++}P<0.01$ vs. EB-OVX; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. SHAM. SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; F-OVX ovariectomized treated with ferutinin; EB-OVX ovariectomized treated with estradiol benzoate.

TABLE 3	Treatment group	Mg (mg/dl)	Ca (mg/dl)	P inorganic (mg/dl)	ALP (UI/l)
30 day-treatment	SHAM	2.43±0.06	10.6±0.01	6.29±0.43	103±13.65
	C-OVX	2.41±0.07	10.18±0.18	7.18±0.3	81.6±3.98
	F-OVX	2.43±0.04	10.56±0.1	6.09±0.22	144.6±15.4$^{**\ \#\ ++}$
	EB-OVX	2.48±0.05	10.52±0.1	6.57±0.25	75±12.5
60 day-treatment	SHAM	2.57±0.06	10.25±0.16	7.52±0.46	89.5±9.24
	C-OVX	2.86±0.4	10.28±0.02	6.2±0.22$^{\#}$	90.2±8.61
	F-OVX	2.55±0.07	10.58±0.12	6.37±0.27$^{\#}$	109.2±7.19^{+}
	EB-OVX	2.47±0.04	10.58±0.14	6.02±0.17$^{\#}$	72.8±5.91

Table 3. Recovering study. Effect of ferutinin/estradiol benzoate on serum biochemical values of ovariectomized rats (30 and 60 days of treatment). All values are expressed as mean ± SEM. Anova followed by Newman-Keuls post test: $^{**}P<0.01$ vs. C-OVX; $^{+}P<0.05$, $^{++}P<0.01$ vs. EB-OVX; $^{\#}P<0.05$ vs. SHAM. SHAM sham-operated controls receiving vehicle; C-OVX ovariectomized controls receiving vehicle; F-OVX ovariectomized treated with ferutinin; EB-OVX ovariectomized treated with estradiol benzoate.

4. Discussion

The results so far obtained have clearly suggested that ferutinin displays positive effects on bone mass both in preventing and in curative treatment of estrogen deficiency osteoporosis; more precisely, the observations have indicated that ferutinin seems to exert similar effects to estradiol benzoate in curative treatment (Ferretti et al., 2010), and even it seems to be

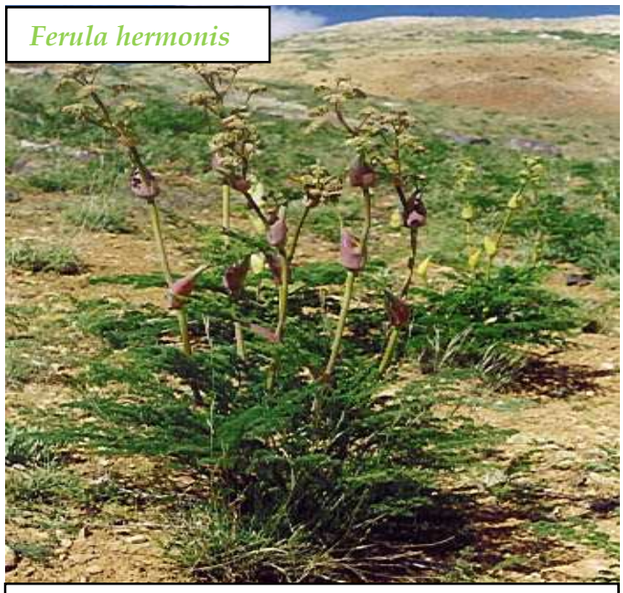
more effective, compared with estradiol benzoate, in preventing bone loss due to estrogen deficiency (Palumbo et al., 2009). It is to be underlined that, comparing the results from the two protocols, in curative study the values of bone mass of treated animals never reach those of SHAM group; this is due to the fact that the treatment started after the occurrence of a severe osteoporosis (as a consequence of estrogen deficiency secondary to two months of ovariectomy).

It is important to emphasise that one of the most important effects of skeletal diseases, like osteoporosis, is the progressive trabecular bone resorption that, in turn, implies enhanced bone fragility and, consequently, an increased frequency of fractures. According to the literature (Kalu, 1991; Wronski et al., 1987) ovariectomy in rats induces different effects on trabecular bone of the axial with respect to the appendicular skeleton, with more marked bone resorption taking place in the latter; for this reason, both vertebrae and femurs were investigated. Moreover, ovariectomy differently affects trabecular and cortical bone, since in OVX animals the bone mass loss observed in trabecular bone was not equally observed in cortical bone; in fact, the values of femur cortical bone areas are similar in OVX and SHAM groups. These data are in line with older ones showing an earlier started decrease in bone mass, more extensive in the spongiosa than in the compacta of rats fed a low-calcium diet (Lozupone & Favia, 1988). This fact is a consequence of the different pattern of distribution of mechanical stresses acting on the two different bony architectures and it is probably related to the different metabolism of the various skeletal regions that, in turn, affect the bone turnover rate of the different skeletal regions, viz. metaphysis compared with diaphyses (Canè et al., 1982). Other authors have also shown that cortical bone is not very sensitive to bone loss due to ovariectomy standing the increased endosteal osteoblasts (Jee et al., 1990; Liu & Kalu, 1990; Turner et al., 1987). All these considerations make the "Bone Organ" a sophisticated system in which metabolic and mechanical demands are actually sensed and integrated in answering both systemic and loading needs.

As previously mentioned the authors wanted to evaluate whether the chronic administration of ferutinin, starting from the day after ovariectomy, is able to prevent estrogen deficiency effects similarly to HRT. The results obtained clearly showed that the phytoestrogen ferutinin displays positive effects in preventing osteoporosis due to estrogen deficiency; more precisely the observations suggest that ferutinin seems to be more effective in preventing bone loss compared with estradiol benzoate. Another positive aspect of ferutinin treatment is to prevent weight gain that typically occurs after ovariectomy. As above mentioned, ferutinin has been shown to interact with estrogen receptors (Appendino et al., 2002; Ikeda et al., 2002). While the majority of phytoestrogens have a higher relative binding affinity for ERβ than ERα, ferutinin displays a higher affinity for ERα (IC_{50}=33.1 nM) than for ERβ (IC_{50}=180.5 nM) (Ikeda et al., 2002). The different roles of specific estrogen receptors ERα and ERβ on body weight regulation were recently investigated by Wegorzewska and co-workers (2008), using the ovariectomized rat model. OVX rats showed a significant increase in body weight, which was reversed by the daily treatment (for 21 days) with estradiol or PPT (propylpyrazoletriol, a selective ERα agonist), but not by the daily treatment with DPN (diarylpropionitrile, a ERβ agonist); these results confirm the major role of ERα in regulating body weight, as it was previously suggested by other authors (Kraichely et al., 2000; Stauffer et al., 2000) by using ER-specific knockout mice.

Regarding the bone turnover-related serum levels, the recorded values of alkaline phosphatase (the most widely recognized biochemical marker for osteoblastic activity -

Evans et al., 1990; Nian et al., 2006) suggest that the process of osteogenesis should be triggered in F-OVX group, because ALP value in F-OVX is higher with respect to the other groups. A positive effect on osteoblast activity *in vitro* by other phytoestrogens, like genistein, has already been published (Liao et al., 2007; Pan et al., 2005).
As far as ferutinin side effects is concerned on the organs commonly targeted by estrogens, the apparent above cited antiapoptotic effect on endometrial epithelia is in line with observations previously recorded for genistein by other authors that administered such phytoestrogen to ovariectomized mice (Eason et al., 2005; Garcìa-Pérez et al., 2006). Since an increased risk of endometrial cancer due to excessive hypertrophy is one of the recognized prejudicial effects of estrogens, the phytoestrogen ferutinin, althought induces thickening of endometrium as well as estrogens, seems to increase the percentage of apoptotic epithelial cells, particularly the glandular ones. This effect might exert a protective role against uterine carcinoma.



Ferula hermonis

C. Palumbo, F. Cavani, L. Bertoni, M. Ferretti-2011

5. Conclusion

On the light of the observations above reported on the effect of ferutinin in preventing/recovering severe osteoporosis secondary to ovariectomy in rats, the authors suggest to enumerate ferutinin among the osteoprotective substances. This fact acquires a more relevant importance in the light of recent tenable evidences, as above cited, reported from some authors concerning the absence of negative side effects by some phytoestrogens (particularly genistein, 8-prenylnaringenin, reveratrol and red clover extract) on the tropism of various organs commonly targeted by estrogens (Burdette et al., 2002; Duffy et al., 2007; Eason et al., 2005; Gallo et al., 2006; Garcia-Perez et al., 2006; Hümpel et al., 2005; Lian et al., 2001; Limer & Speirs, 2004; Murray et al., 2003; Whitsett & Lamartiniere, 2006; Wu et al., 2002).

In conclusion, the results here reported not only provide evidence that ferutinin can significantly prevent/recover ovariectomy-induced bone loss in rats, but also that it could protect against the onset of uterus cancer. Although the putative undesired estrogenic-like side effects on uterus of such phytoestrogen have not yet been fully investigated, ferutinin could be an interesting safer alternative new candidate for HRT in treatment of post-menopausal symptoms, since it seems to protect from bone loss induced by ovariectomy (Ferretti et al., 2010; Palumbo et al., 2009) and in part to mime the ovarian endocrine function during menopause. The authors are aware that additional studies are required to characterize the mechanism by which ferutinin acts both in improving/resolving severe degrees of bone mass loss and in protecting from uterine cancer onset.

6. Acknowledgments

The researches on ferutinin effects in preventing/recovering estrogen deficiency-osteoporosis were supported by 2009-2010 "Fondazione di Vignola" funds and by "Banca Popolare dell'Emilia Romagna".

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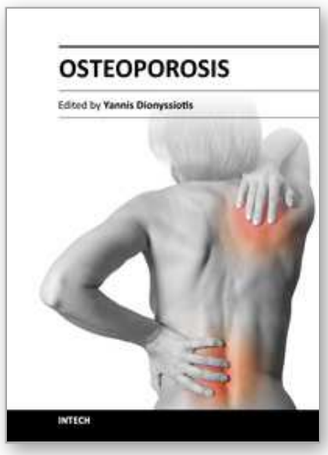

Osteoporosis

Edited by PhD. Yannis Dionyssiotis

ISBN 978-953-51-0026-3
Hard cover, 864 pages
Publisher InTech
Published online 24, February, 2012
Published in print edition February, 2012

Osteoporosis is a public health issue worldwide. During the last few years, progress has been made concerning the knowledge of the pathophysiological mechanism of the disease. Sophisticated technologies have added important information in bone mineral density measurements and, additionally, geometrical and mechanical properties of bone. New bone indices have been developed from biochemical and hormonal measurements in order to investigate bone metabolism. Although it is clear that drugs are an essential element of the therapy, beyond medication there are other interventions in the management of the disease. Prevention of osteoporosis starts in young ages and continues during aging in order to prevent fractures associated with impaired quality of life, physical decline, mortality, and high cost for the health system. A number of different specialties are holding the scientific knowledge in osteoporosis. For this reason, we have collected papers from scientific departments all over the world for this book. The book includes up-to-date information about basics of bones, epidemiological data, diagnosis and assessment of osteoporosis, secondary osteoporosis, pediatric issues, prevention and treatment strategies, and research papers from osteoporotic fields.

How to reference

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Carla Palumbo, Francesco Cavani, Laura Bertoni and Marzia Ferretti (2012). Role of Phytoestrogen Ferutinin in Preventing/Recovering Bone Loss: Results from Experimental Ovariectomized Rat Models, Osteoporosis, PhD. Yannis Dionyssiotis (Ed.), ISBN: 978-953-51-0026-3, InTech, Available from: http://www.intechopen.com/books/osteoporosis/role-of-phytoestrogen-ferutinin-in-preventing-recovering-bone-loss-results-from-experimental-ovariec

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