

## Effects of an isopropanolic extract of *Cimicifuga racemosa* on urinary crosslinks and other parameters of bone quality in an ovariectomized rat model of osteoporosis

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**Abstract** A potential bone-sparing effect of *Rhizoma actaeae* (= *cimicifugae*) *racemosae* (black cohosh) was evaluated in ovariectomized Sprague–Dawley rats. The rats were ovariectomized at 12 weeks of age (body weight, 219–226g) and placed on a soy-free diet 6 days after surgery. Animals were randomly assigned the following groups: control ( $n = 10$ ), soy-free diet only; RAL ( $n = 10$ ), soy-free diet plus raloxifene 3mg/kg intragastrically; and REM ( $n = 10$ ), soy-free diet supplemented with an isopropanolic black cohosh extract (Remifemin) with a daily intake of 4500µg triterpeneglycosides. Urinary levels of pyridinoline (PYR) and deoxypyridinoline (DPY), specific markers for bone loss, were measured at baseline and at weekly intervals. At the end of the study, the animals were killed and bone loss was determined by volumetric bone mineral density (BMD) measurements and peripheral quantitative computed tomography (pQCT). Mechanical resistance to fracture was also determined. Results demonstrated that an isopropanolic extract of black cohosh significantly diminished the urinary content of PYR and DPY and the morphometric correlates of bone loss associated with ovariectomy in rats. Reversal of the effects of ovariectomy on bone loss began 2–5 weeks after the start of treatment and continued through at least 7 weeks. Results similar in quality and magnitude were obtained in the group treated with raloxifene, a known selective estrogen receptor modulator (SERM). Because extracts of black cohosh are already recognized as safe and effective in the treatment of certain gynecological disorders, a longer-term clinical trial of this herbal remedy for the treatment of osteoporosis is warranted.

**Key words** osteoporosis · phytotherapy · urinary crosslinks · *Cimicifuga racemosa* · animal model

### Introduction

Postmenopausal osteoporosis is a major age-related health problem for women. The condition is characterized by an increase in bone resorption relative to bone formation and a concomitant increase in the rate of bone turnover. Hormone deficiency resulting from the cessation of ovarian function is the most important contributing factor to age-related bone loss [1,2]. Other etiological factors include heredity, dietary habits, and lack of physical activity. The currently available pharmacological interventions for the prevention of fractures in patients with osteoporosis involve one of two strategies: reducing bone resorption with bisphosphonates, calcitonin, calcium, estrogen, estrogen derivatives, or selective estrogen receptor modulators (SERMs), or stimulating bone formation with fluoride salts or parathyroid hormone [3–5].

Hormone replacement therapy (HRT) has an established benefit in the prevention of fractures in postmenopausal women [6,7], and over the past several years HRT has been under intensive investigation as a preventive treatment for coronary artery disease (CAD). Long-term follow up of women receiving HRT has, however, failed to show a continuation of cardiovascular benefits seen in the initial phase of treatment [8]. Initial reports from another long-term, large-scale study of HRT suggest that benefits from HRT in preventing disease, long-term disability, or death in postmenopausal women may be outweighed by a higher incidence of treatment-related thromboembolic complications [9]. Given that HRT has long been associated with an increased risk for breast cancer [10], the medical complications of menopause may be better treated with more specific therapies (e.g., beta-blockers, aspirin, or lipid-lowering agents to prevent coronary disease and stroke, and bisphosphonates or raloxifene to prevent fractures associated with osteoporosis). HRT remains the treatment of choice for the relief of symptoms of

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menopause, but even here the proven benefits of HRT can potentially be achieved with safer herbal treatments such as black cohosh.

*Actaea (=Cimicifuga) racemosa* (black cohosh) is a well-known Native American herbal remedy with an established record in European herbal medicine for the treatment of gynecological disorders such as irritation and congestion of the uterus, vagina, and cervix, as well as for promoting uncomplicated births, uterine involution, and recovery. More recent clinical research with a preparation of black cohosh (Remifemin, a standardized isopropanolic extract of black cohosh obtained from *Rhizoma actaeae racemosae*), suggests that at least some of these benefits are obtained through non-estrogenic mechanisms [11].

Li et al. [12] described a beneficial effect of the extracts of *Rhizoma actaeae heracleifoliae* and *Rhizoma actaeae foetidae* on ovariectomy (OVX)-induced bone resorption in a rat model of osteoporosis in humans. Both these extracts are important traditional Chinese herbal remedies with established uses as anti-inflammatory, analgesic, and antipyretic agents. On the basis of the data of Li et al., we wondered if an extract of *Rhizoma actaeae racemosae* lacking the phytoestrogen formononetin (Remifemin) might be similarly effective in a rat model of the human postmenopausal state.

Using ovariectomized Sprague–Dawley rats, we compared the rate of bone loss in an untreated OVX control group to the bone loss in OVX animals treated with either *Rhizoma actaeae racemosae* (Remifemin) or the SERM raloxifene, two agents potentially capable of preventing bone loss following OVX. Efficacy during 12 weeks of treatment was determined by tracking two urinary metabolites that reflect bone loss rate in osteoporosis, pyridinoline (PYR) and deoxypyridinoline (DPY) [13–17]. Confirmatory data were collected at the end of the study through postmortem morphometric analyses of bone loss and fracture resistance [18].

## Materials and methods

### *Animals and treatments*

The 30 ovariectomized female Sprague–Dawley rats employed in this study, obtained from the Schaper & Brümmer breeding colony, were approximately 12 weeks old and weighed 216–229 g at the start of treatment. Rats were housed by treatment group, 3 animals per macrolon cage, in a room providing alternating 12-h periods of light and dark. Prepared diets and water were permitted ad libitum. All conditions of husbandry were in accordance with local regulations, and experimental procedures were approved and conducted under the auspices of a local German animal care and oversight committee.

The three groups of animals in this study were provided with a special soy-free diet obtained from Altromin (Lage, Germany) to avoid interference with bone development measurements that might result from naturally occurring phytoestrogens present in rodent chow containing soy protein. The base diet contained 0.9 g calcium and 0.7 g phosphorus per 100 g chow. Remifemin was added to the soy-free diet of one group (REM group), providing a daily intake of 4500 µg/kg body weight of triterpeneglycosides, standardized as 27-deoxyacteine. High performance liquid chromatography (HPLC) was employed to standardize the level of 27-deoxyacteine in the diet as well as to confirm the absence of soy phytoestrogens. Another group of animals was given raloxifene intragastrically (RAL group; 3 mg/kg daily) along with the soy-free base diet. Finally, another group of animals was maintained on the soy-free diet with no other treatments (control group). Each group contained ten animals and was maintained on its respective regimen for 12 weeks.

### *Analytical methods*

All analytical methods employed in this study were derived from published methods and optimized in our laboratory with specimens from various standard treatment regimens. These standard treatments included phytoestrogen-containing and phytoestrogen-free diets, orally administered raloxifene or ethinyl estradiol, or subcutaneously administered 17β-estradiol. Measurements were performed in a blinded fashion by a single individual. To avoid detrimental effects of storage, bone specimens were stored in formaldehyde, and urine samples were stored at –20°C for not longer than 6 months before analysis.

### *Urinary crosslinks*

Six days after ovariectomy, i.e., 6 days before the onset of treatment, each rat was housed individually in a metabolic cage for 24 h to obtain a baseline 24-h urine sample. Additional 24-h urine samples were collected at weekly intervals thereafter. Urinary PYR and DPY, representing breakdown products of collagen during bone resorption, were determined by HPLC after complete acid hydrolysis, according to the method of Black et al. [19,20].

### *Bone densitometry*

At necropsy, both femurs as well as the third lumbar vertebra of each animal were freed of soft tissue using small scissors, forceps, and cotton gauze. Each femur was then put into a vial containing distilled water and subjected to a vacuum for 90 min to permit trapped air

to diffuse out of the bone. Each bone was then removed from the vial, patted dry, weighed, and placed into a water-filled 20-ml pycnometer, in which it was weighed again. Density was then calculated from these measurements according to Archimedes' principle [21,22]. Using this method, precision was  $\pm 4.0 \mu\text{g}/\text{cm}^3$  and reproducibility was  $\pm 1.9\%$ .

Bone mineral density [(BMD): total BMD – cortical/subcortical BMD – trabecular BMD] was determined in units of  $\text{mg}/\text{cm}^3$  using the L3 vertebra from each animal and a peripheral quantitative computed tomography (pQCT) instrument (Stratec model XCT Research M+; Stratec Medizintechnik, Pforzheim, Germany). Vertebrae were positioned ventrodorsally to permit transverse measurements. Tomographic scans (three per vertebra) had a thickness of 0.2mm. The middle scan was positioned in the center of the vertebra, and two additional scans were positioned 1.6mm cranially and 1.6mm caudally. Mean BMD was calculated from the results of these three scans.

### Mechanical testing

Mechanical testing of the femoral head was performed with a material testing device (Pharmatron model 6D; Schleuniger, Solothurn, Switzerland) [23]. The femoral epiphyseal region of each sample was positioned mediolaterally between the two press heads and compressed until fracture occurred. The rate of deformation was uniform across samples. The peak force was recorded automatically at the time of fracture (maximum fracture resistance) and then corrected for specimen cross section. The cross-sectional area was estimated from the distance between press heads at the point of maximum applied pressure. Reproducibility of mechanical testing was  $\pm 14.4\%$ .

### Statistical evaluation

In addition to the individual measures of bone development upon which the assessment of treatment effect was based, we developed an algorithm of overall bone quality (bone quality score, BQS), as follows:

$$\text{BQS} = d_{\text{py}n} * 100/\Delta_{\text{py}} + d_{\text{bd}n} * 100/\Delta_{\text{bd}} + d_{\text{bc}n} * 100/\Delta_{\text{bc}} \\ + d_{\text{bt}n} * 100/\Delta_{\text{bt}} + d_{\text{bf}n} * 100/\Delta_{\text{bf}}$$

where measured *max* and *min* values are those attained irrespective of treatment group, and where

$$\Delta_{\text{py}} = \text{max pycnometric bone density} - \text{min pycnometric bone density}$$

$$\Delta_{\text{bd}} = \text{max pQCT total bone density} - \text{min pQCT total bone density}$$

$$\Delta_{\text{bc}} = \text{max pQCT (sub)cortical bone density} - \text{min pQCT (sub)cortical bone density}$$

$$\Delta_{\text{bt}} = \text{max pQCT trabecular bone density} - \text{min pQCT trabecular bone density}$$

$$\Delta_{\text{bf}} = \text{max peak breaking force} - \text{min peak breaking force}$$

For each individual animal, the defined variables are

$$d_{\text{py}n} = \text{pycnometric bone density of animal } n - \text{min pycnometric bone density}$$

$$d_{\text{bd}n} = \text{pQCT total bone density of animal } n - \text{min pQCT total bone density}$$

$$d_{\text{bc}n} = \text{pQCT (sub)cortical bone density of animal } n - \text{min pQCT (sub)cortical bone density}$$

$$d_{\text{bt}n} = \text{pQCT trabecular bone density of animal } n - \text{min pQCT trabecular bone density}$$

$$d_{\text{bf}n} = \text{peak breaking force of animal } n - \text{min peak breaking force}$$

The sum of scores was then compared between groups by means of a one-sided Student's *t* test, and significance was determined using  $P < 0.05$ .

## Results

### Metabolic parameters

All animals, irrespective of treatment group, had high urinary crosslink levels at the beginning of treatment, which is indicative of an OVX-induced increase in bone turnover. After 9 weeks following OVX, the control group showed a decline in urinary PYR and DPY of 18% compared to peak excretion levels. After 7 weeks of raloxifene treatment (i.e., 9 weeks after OVX), the RAL group exhibited markedly decreased urinary DPY and PYR levels (Fig. 1). The REM group exhibited a decline in urinary crosslink levels comparable to the RAL group (DPY, 27%; PYR, 35%). After 5 weeks of treatment, the DPY and PYR levels of the RAL and REM groups were significantly lower than those of the controls ( $P < 0.05$  by Student's *t* test), whereas at 7 weeks urinary DPY levels were still increasing in the control group.

### Morphometric parameters

Direct groupwise comparisons of BMD showed only modest differences in respect to total BMD or cortical BMD using the pQCT method (Fig. 2). However, trabecular BMD was significantly higher in the RAL group compared to control animals using this method ( $P < 0.05$  by Student's *t* test). Similarly, the REM group showed relatively small differences in the individual BMD parameters when compared to those of the control group using pQCT-based measurements. BMD loss was significantly less in the REM and RAL groups com-

pared to the control group when the direct, volume-based method of Archimedes was used (Fig. 3).

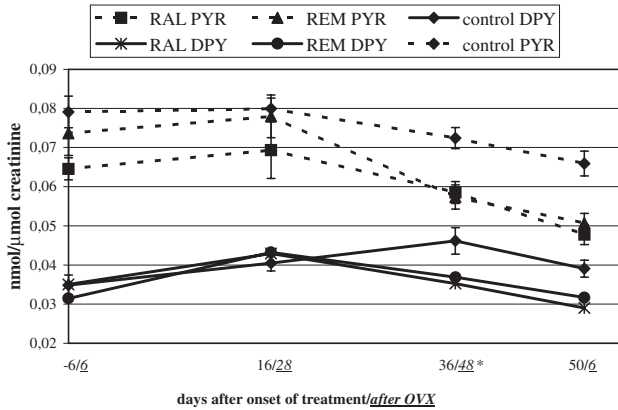
Mechanical breaking force data produced somewhat mixed results: a slightly reduced femur head cross section at fracture point was noted in the REM group, but modest improvements in breaking force needed to cause a fracture were found in both active treatment regimens. None of these differences attained statistical significance (data not shown). However, when parameters of femoral and vertebral bone mineral density and fracture resistance were combined into a single parameter of mechanical stability (overall bone quality score), both active treatment groups showed a statistically significant improvement in overall bone quality compared to the OVX control animals (Fig. 4).

Treatments were generally well tolerated. All groups experienced body weight gain during the 12 weeks of

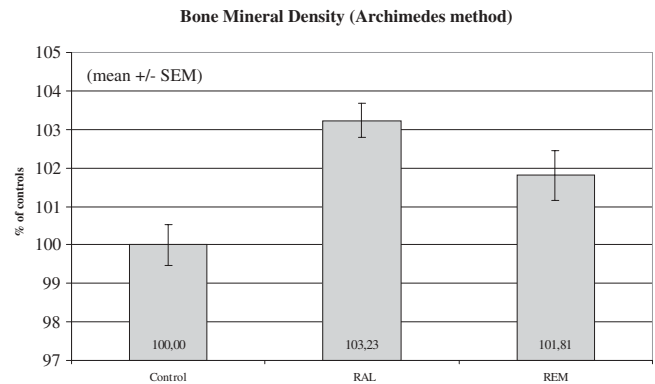
observation, with the RAL group gaining somewhat less weight than the other two groups (Table 1).

**Discussion**

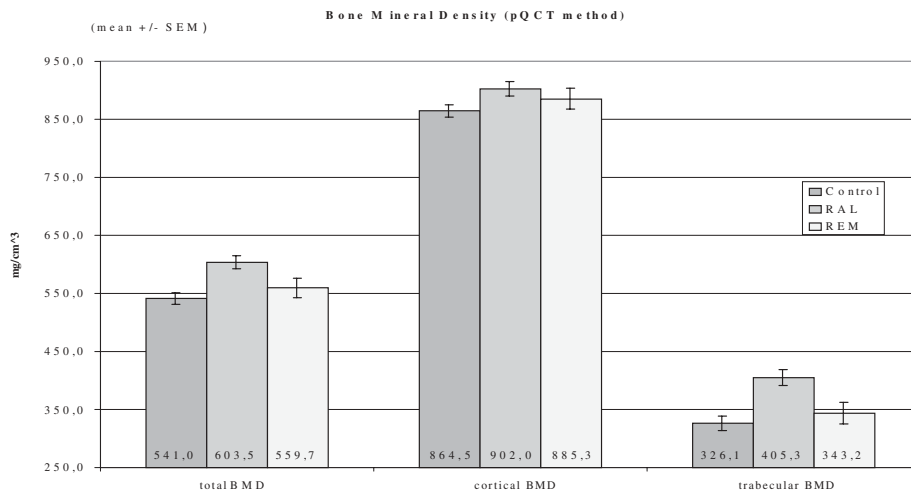
The results presented in this study demonstrate that an isopropanolic extract of black cohosh (*Rhizoma actaeae racemosae*) diminishes the urinary content of PYR and DPY and the morphometric correlates of bone loss associated with ovariectomy in rats. Data from the in vivo portion of the study indicate that reversal of the effects of OVX begins between 2 and 5 weeks after the start of treatment and continues through at least 7 weeks. An improvement in these highly specific markers for bone loss following black cohosh treatment indicates prompt and effective reversal of bone-loss mechanisms associated with OVX. Data confirming a beneficial effect were noted in morphometric parameters of bone loss as well as in bone fracture resistance, and overall bone quality score (BQS).



**Fig. 1.** Urinary excretion of pyridinoline (PYR) and deoxypyridinoline (DPY) following ovariectomy. RAL, raloxifene group; REM, Remifemin group; OVX, ovariectomy. \*Student's t-test:  $P < 0.05$



**Fig. 3.** Bone mineral density (Archimedes method)

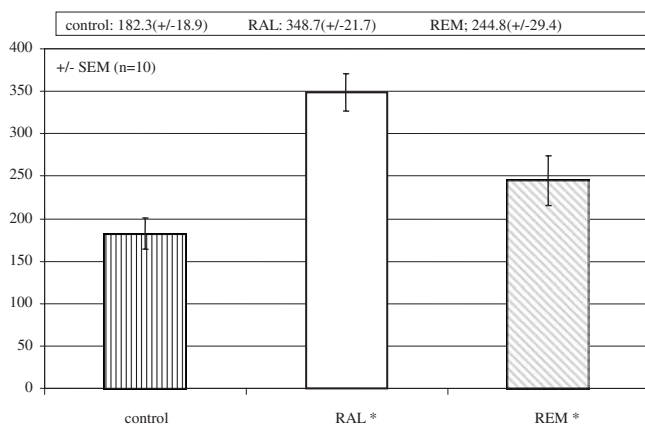


**Fig. 2.** Bone mineral density [peripheral quantitative computed tomography (pQCT) method]. BMD, bone mineral density

**Table 1.** Body weight gain during treatment

Weeks	Body weight (g) $\pm$ SEM									
	0	4	5	6	7	8	9	10	11	12
Control (n = 10)	379 $\pm$ 27	386 $\pm$ 25	398 $\pm$ 21	409 $\pm$ 18	420 $\pm$ 20	426 $\pm$ 20	433 $\pm$ 20	442 $\pm$ 22	449 $\pm$ 22	456 $\pm$ 24
RAL (n = 10)	398 $\pm$ 19	395 $\pm$ 20	385 $\pm$ 20	385 $\pm$ 20	381 $\pm$ 20	382 $\pm$ 21	385 $\pm$ 20	389 $\pm$ 22	398 $\pm$ 23	403 $\pm$ 24
REM (n = 10)	368 $\pm$ 21	363 $\pm$ 18	376 $\pm$ 16	388 $\pm$ 16	403 $\pm$ 16	404 $\pm$ 17	408 $\pm$ 17	413 $\pm$ 18	423 $\pm$ 18	429 $\pm$ 18

SEM, standard error of the mean; RAL, raloxifene group; REM, Remifemin group



**Fig. 4.** Bone quality score ( $\pm$ SEM). \*Student's unilateral t-test:  $P < 0.05$  (vs. control)

We introduced BQS to assess the bone protective potential of experimental drugs and standard treatments in an algorithm comprising parameters of femoral as well as vertebral bone and parameters of mechanical stability. Thus, differences in the extent of therapeutic benefit, as measured in pQCT versus the Archimedes method, can be interpreted as probably a consequence of different regulatory mechanisms prevailing at different locations. The observed differences can, however, also be due to the young age of our experimental animals, which at the time of OVX had not yet attained their maximum peak bone mass. The then still prevailing physiological bone formation [24,25], possibly in combination with a treatment regimen too short for both purposes [26], might be responsible. Results similar in quality and magnitude were obtained in the group treated with raloxifene, a known SERM; this effect of raloxifene is in agreement with a previous report [27]. Inclusion of this positive control demonstrates the ability of our monitoring methods to detect a potentially clinically meaningful difference from untreated controls. In addition, the approximate qualitative and quantitative equivalence of the two active agents sug-

gests that black cohosh may have similar clinical potential to raloxifene.

Predicting efficacy and potential for adverse reactions for an estrogenic agent is dependent to some degree on the mechanism of action by which the agent exerts its beneficial effect. Estrogen and its two known receptors [estrogen receptor- $\alpha$  (ER- $\alpha$ ) and ER- $\beta$ ] play an important role in regulating bone development, maintaining bone integrity and architecture, as well as in innumerable other functions throughout the mammalian organism. Both ER- $\alpha$  and ER- $\beta$  are widely distributed in mammalian tissues and, relevant to our study results, have been described in cultured human fetal cells of osteoblast lineage, in mature human osteoblasts, and in osteocytes [28–30].

Estrogen receptor binding has also been demonstrated for various phenolic constituents of an extract of black cohosh (*Rhizoma actaeae racemosae*) [31], a preparation traditionally used to alleviate menopausal hot flashes. However, there appears to be no evidence for a systemic estrogenic effect for Remifemin [11,32–34]. Thus, the apparent efficacy of black cohosh preparations in the treatment of hormone deficiency symptomatology [35,36] and possibly the bone-sparing effect of black cohosh that we observed may in fact be mediated via SERM pathways rather than by a direct action on estrogen receptors. If further substantiated in clinical studies, Remifemin may prove useful in preserving bone without the undesired side effects of HRT.

Cessation of the ovarian function in humans is associated with an increase in bone turnover, a negative bone balance, and a net decrease in BMD; these changes are also evident in surgically ovariectomized rats [37,38]. The moderate nature of OVX-induced changes that we observed in our study, compared to previously published data, is most probably due to the young age of our experimental animals. Our monitoring methods produced data similar in character to the data obtained in clinical studies of osteoporosis. For example, osteoporosis can be tracked clinically by the appearance

in urine of either PYR or DPY. Reductions in the urinary levels of these major trivalent crosslinks of collagen, which are elevated during a state of net bone resorption, can also provide an early indicator of successful therapeutic intervention in osteoporosis [13–17,39]. In addition, epidemiological studies have demonstrated a close association between decreased femoral or lumbar BMD and an increase in fracture incidence using either double-energy X-ray absorptiometry (DEXA) or pQCT [13,18]. On the strength of our data and the correspondence of our analytical methods with clinical methodologies, we believe that black cohosh may be of benefit in ameliorating the bone-loss complications associated with menopause. Considering that extracts of black cohosh are already recognized as safe and useful in the treatment of certain gynecological disorders, a longer-term clinical trial of this herbal remedy in osteoporosis seems warranted.

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