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Osteoprotective effects of *Cimicifuga racemosa* and its triterpene-saponins are responsible for reduction of bone marrow fat

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ABSTRACT

Purpose: Elderly people often develop visceral obesity accompanied by osteoporosis. Visceral adipocytes secrete a number of adipokines and cytokines which augment the development of arteriosclerosis and type 2 diabetes. Bone marrow fat cells also secrete these pro-inflammatory cytokines which stimulate osteoclast and inhibit osteoblast activity. Ovariectomized (ovx) rats also develop general and bone marrow obesity and osteoporosis both of which can be partially prevented by estradiol (E2) and the special extract of *Cimicifuga racemosa* (CR) BNO 1055. Whether this extract or the thereof isolated triterpene-saponins or polar substances can also prevent bone marrow obesity and thereby the development of osteoporosis was compared with the effects of estradiol (E2).
Methods: Rats were ovx and fed with food containing either CR BNO 1055 or its triterpene-saponin or polar constituents or with E2 for 4 weeks. Histomorphometry and STRUT analyses were applied to histological preparations to determine the amount of trabecles, hematopoietic and fat tissue in the bone marrow.
Results: Ovx rats lost significant amounts of trabecular BMD, surface and nodes while the number of free trabecular ends and fat load in the marrow increased. This was totally prevented by E2 and partially by CR BNO 1055 and the triterpene-saponin but not by the polar fraction. High serum osteocalcin and CrossLaps levels were reduced by E2 and the S-fraction.
Conclusions: It is well established that E2 prevents osteoporosis. It is also known that CR BNO 1055 does not contain estrogenic substances. CR BNO 1055 and the triterpene-saponin-fraction reduced the development of osteoporosis most likely by a reduction of the bone marrow fat load and possibly by reducing the secretion of pro-inflammatory cytokines. Hence, the triterpene-saponin-fraction may serve as a basis for a new osteoporosis preventing preparation also in human patients.

Introduction

Many elderly people develop obesity. Particularly, the visceral fat depots cause severe diseases as their adipocytes secrete pro-inflammatory adipo- and cytokines (Espinola-Klein et al. 2011; Lemieux et al. 2007; Potenza and Mechanick 2009) which cause increased serum lipids. The high serum levels of low density lipoproteins (LDL) and triglycerides increase the risk to develop arteriosclerosis, heart attacks and strokes. Also insulin receptor sensitivity decreases and this eventually results in type II diabetes (Lemieux et al. 2007; Potenza and Mechanick 2009). Development of obesity and osteoporosis is postmenopausally augmented by the lack of estrogens (Poledne et al. 2009). This can be prevented by classical hormone replacement therapy (HRT) which however, increases the risk of breast cancer and of cardiovascular diseases (Rossouw et al. 2002).

Bone marrow also contains fat cells which secrete – as the visceral fat cells – pro-inflammatory cytokines which stimulate osteoclast and inhibit osteoblast development and function (Cao 2011; Syed and Melim 2011). On the basis of negative side effects of HRT preparations scientists and patients are looking for alternatives. One alternative is estrogenic isoflavone-containing soy or red clover extracts which are vigorously advertised even though they have mild if any effects to prevent osteoporosis (Levis et al. 2011; Wuttke et al. 2007).

Ovariectomy (ovx) of rats is known to cause obesity and osteoporosis (Seidlova-Wuttke et al. 2003; Zoth et al. 2010) and the ovx rat is considered as an excellent model to study the development and treatment possibilities of these diseases (Kalu 1991; Lelovas et al. 2008; Seidlova-Wuttke et al. 2003). We previously observed

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Table 1
Food intake and bodyweights of the treated animals.

Group	Number of animals (n)	Food intake (g/day/animal)	Intake of test extracts (mg/day/animal)	ΔBW (g) increase from initial BW
Co (ovx)	10	17	No	94.48
ovx CR BNO 1055	10	16.45	8.22	74.41*
ovx S-fraction	10	17.42	2.05	87.47
ovx R-fraction	10	18.49	7.07	81.94
ovx E2	10	10.81*	0.108	−15.51

* $p < 0.05$ vs Co (ovx).

that an extract of *Cimicifuga racemosa* (CR) BNO 1055 was able to reduce ovariectomy induced obesity and this was associated with reduced loss of bone mineral density (BMD) as determined by quantitative computer tomography (qCT) (Seidlova-Wuttke et al. 2003).

In order to identify whether the polar or unpolar constituents of the CR BNO 1055 extract were responsible for the prevention of obesity and for the expected reduction of ovx induced accumulation of fat cells in the bone marrow and for the prevention of bone loss we treated rats with CR BNO 1055 or with its triterpene-saponin containing (S-fraction) or the water soluble rest fraction (R-fraction) over a period of 4 weeks following ovx. Quantitative histomorphometric analyses were performed in the metaphysis of the tibia which allowed quantification of trabecular surfaces and of the amounts of fat and hematopoietic tissue in the bone marrow.

Furthermore, STRUT analysis for determination of the trabecular infrastructure was performed. With this analysis the number of trabecular cross sections (nodes) and of free ends (termini) were counted and the quotient of these values is an index for stability of the spongious bone (Chappard et al. 2008, 2001).

Serum leptin levels were determined as a measure whether the different treatments had effects on the total fat load of the body. Similarly, the effects of E2, CR BNO 1055 and its fractions on total skeletal osteoblast and osteoclast activities were determined by quantification of the osteoblast product osteocalcin and of the breakdown product of bone collagen, the CrossLaps as markers for osteoclast activity.

Materials and methods

Permission to perform the experiments was obtained from the district authorities of Braunschweig, Germany (permission No. Az.33.425002-082/06). A total of 72 three months old female SD-rats (Winkelmann, Borken, Germany), weighing 230–280 g were used in the present study. To get adjusted to the animal facilities of the University Medical Center Göttingen they were kept in groups of 4–5 in Makrolon cages (type 4) for 3 weeks under a 12 h light, 12 h dark cycle at room temperature of 22–24 °C and relative humidity of 50–55%. Animals had access to soy-free food (V 1355 R-Z, 10 mm, poor phytoestrogens, ssniff, Soest, Germany) and water ad libitum. After adjustment period the rats were anesthetized with Isoflurane (Forene, Abbot, Wiesbaden, Germany), weighed and ovx. For the following weeks body weights and food intake of the animals were recorded once per week.

Production of the extract/fractions

The special extract of CR BNO 1055 and a triterpene-saponin-containing (S-) and a rest (R-) fraction were produced as described in detail earlier (Seidlova-Wuttke et al. in press). In short: via liquid/liquid extraction using dichloromethane and water as solvents, the CR BNO 1055 extract was separated into 2 fractions, a lipophilic fraction rich in triterpene-saponins and a hydrophilic fraction rich in sugars and phenylpropanoids. The fractions were characterized by thin layer chromatography and high performance liquid chromatography and UV detection, evaporative light scattering detection and mass spectrometric detection.

The extracts were used to prepare the food for the ovx animals. From preliminary experiments it was known that a daily intake of 17 ± 1.2 mg of the CR BNO 1055 extract had osteoprotective effects. It was also known that the food intake of our rats approximated is in the range of 18 ± 1 g/day/animal. Separation of the special extract CR BNO 1055 into the triterpene-saponin (S-) and the rest (R-) fraction also allowed calculation of the amounts of each of these fractions needed to be added to the food in order to achieve comparable amounts of triterpene-saponins, sugars, phenylpropanoids, etc. in the food fed as part of the CR BNO 1055-containing diet. Based on this information the food was prepared and animals were fed immediately after ovx with these diets. Estradiol (E2) was commercially available (Estradiolbenzoat, ordering no. E 9000, Sigma–Aldrich Chemie GmbH, Munich, Germany).

The daily food intake per animal was approximated from the weekly measured food consumption per cage divided by 7 and the number of animals per cage (Table 1). After a treatment period of 4 weeks the animals were sacrificed under CO_2 anesthesia, blood samples collected from the trunk, uteri and the upper part of the tibiae were collected for hormone analyses and histomorphometry, respectively.

Quantitative histomorphometry

From all sacrificed animals specimens containing the upper tibiae were collected from both hind legs and cleaned from adjusting muscle and connective tissue and prepared for histological preparations as follows: After dehydration in ascending alcohol concentrations (70%, 80%, 90%, 100%) the specimens were embedded in a mixture of 100 ml methyl-methacrylate (Merck No. 800590), 200 ml di-butylphthalate (Merck No. 12487) and 29 g benzoylperoxide (Merck No. 801641). Following hardening 5 μm thick longitudinal preparations were cut with the Leica Jung Polycut S microtome. Preparations were placed on chrome-alaun gelatine coated glass slides and extended with 96% ethanol.

Following exposure to 100%, 70% and 40% alcohol the preparations were placed into an aqueous medium and stained with tri-chrome according to Goldner. Fig. 1 shows a representative histological preparation in which the STRUT analysis to determine parameters of the trabecular apparatus is indicated (Chen et al. 2008; Chen and Heiman 2001; Mellish et al. 1991). The STRUT analysis is a measure of trabecular strength. It quantifies the 2-dimensional structural pattern of cancellous bone. Application of the STRUT analysis allows counting of free trabecular ends and of the cross sections of trabecles, i.e. the nodes. It has been shown in a number of publications that strength of the trabecular apparatus is highest when the number of nodes is high and lowest when the number of free ends is high (Chappard et al. 2001; Kasukawa et al. 2004).

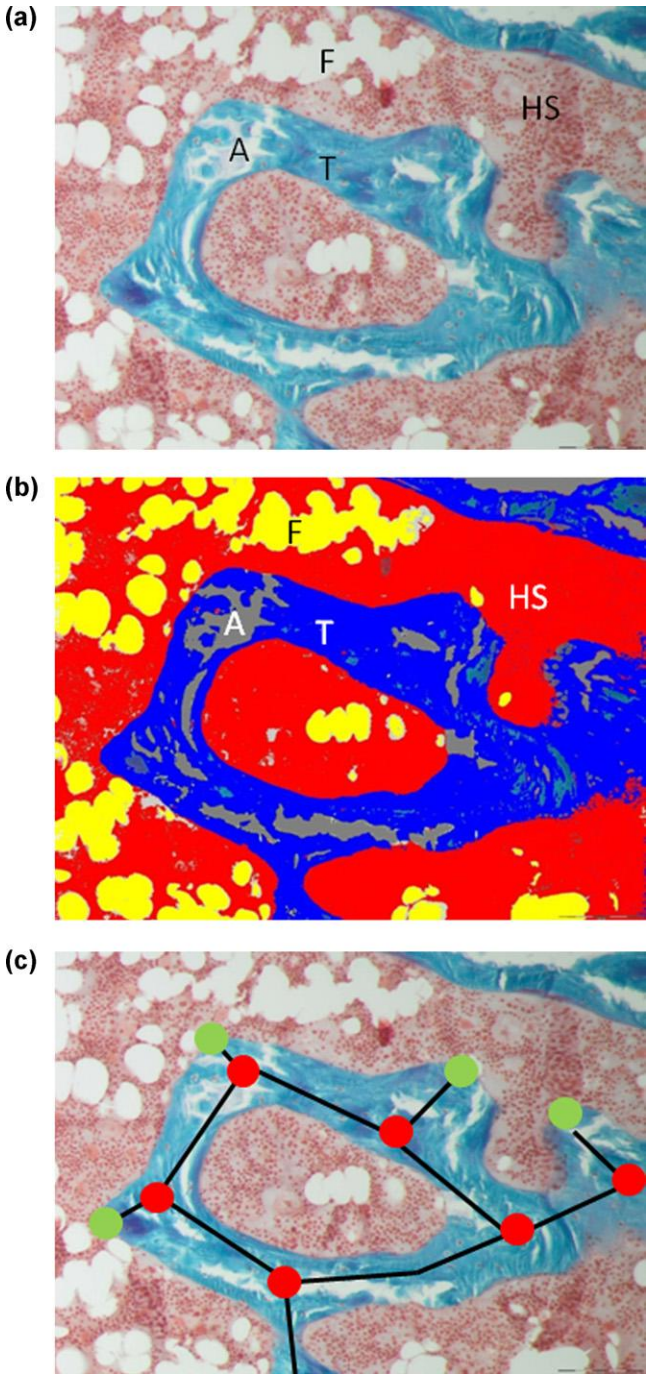

Fig. 1. Original Goldner stained histologic preparation of parts of the upper metaphysis of the tibia (a). The computer assisted digitalized conversion into computer readable colors (b) allowed quantification of the surface of the trabecular (T in blue), fat (F in yellow) and hematopoietic (HS in red) systems. Cutting artifacts are colored in gray. For STRUT analysis (c) cross sections of 2 or more trabecles (nodes) are indicated by red dots, free trabecular ends (termini) by green dots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Typical histological views for determining bone parameters were selected 100 μm below the epiphysis in the medial part of the metaphysis of the tibia. From each tibia 2 preparations (5 μm thick) were prepared. In each of the 20 preparations an area of 1.7 mm^2 was evaluated. Fig. 1a shows a typical example of a histological view of a metaphysis of the tibia of an ovx animal. Bone surface is stained in green, hematopoietic (erythro- and lymphopoietic) tissue in red and fat tissue in white. During the cutting procedure artifacts may occur particularly in bony parts of the preparation which are marked in gray. These artifacts were not evaluated. The surfaces of the bone marrow compartments were determined with a computer assisted program (Imaging Software Cell, Olympus, Münster, Germany). Fig. 1b shows the computer assisted conversion of the stained features in digitalized and then color-coded pictures. This allowed quantification of the surface of trabecular, fat and of the hematopoietic system in the bone marrow. For STRUT analysis cross sections of trabecles (nodes) were indicated by red and free trabecular ends (termini) by black dots.

Statistical analysis

From all measures arithmetic means ± standard errors of the means (SEM) were calculated and subjected to ANOVA followed by Dunnet's test. $p < 0.05$ was considered to be statistically significant.

Results

Table 1 details food intake and bodyweights of the differently treated animals. Animals fed with estradiol (E2)-containing food ate significantly less and were therefore lighter in comparison to the ovx animals. Those fed with the control diet or the CR BNO 1055 or its S- or R-fraction-containing food ate similar amounts of food as the ovx control group but this resulted in significantly less weight gain.

Fig. 2a details histomorphometric analysis of the view area covered by trabecles. Coverage of the endosteal area with fat tissue is shown in Fig. 2b and coverage by hematopoietic tissue in Fig. 2c. Ovx animals had lowest trabecular surfaces whereas the treatment with E2 yielded significantly larger values. CR BNO 1055 and the S- but not the R-fraction resulted also in higher values of trabecular surfaces in comparison to the ovx animals. The amount of bone marrow fat tissue of the differently treated animals was highest in the ovx and lower in the E2 treated animals (Fig. 2b) while the hemato-/lymphopoietic amount of tissue was only marginally affected by the E2 treatment (Fig. 2c). Treatment with CR BNO 1055 and the S- and R-fraction reduced fat tissue in the bone marrow. Interestingly the amount of erythropoietic/lymphopoietic tissue was only marginally and not significantly stimulated by CR BNO 1055. However, the S- and R-fraction stimulated this tissue type significantly (Fig. 2c).

Fig. 3 shows the inverse correlation between trabecular surface and marrow fat tissue, i.e. the more the marrow fat tissue the lower the trabecular surface. This correlation was statistically significant.

The number of trabecular cross sections (nodes) and of free trabecular ends (termini) was affected by treatment with E2 and the S- and R-fraction such that the quotient struts/termini was significantly higher in comparison to the ovx animals (Fig. 4).

Serum leptin levels (Fig. 5) were highest in the ovx controls and in the animals treated with the R-fraction. Lowest leptin levels were observed in the E2-treated animals. The animals treated with CR BNO 1055 and the S-fraction had significantly lower leptin serum concentrations than the controls but higher than the E2-treated animals.

Finally, we found in the ovx control group high serum osteocalcin levels which were significantly reduced after E2 treatment (Fig. 6a). In contrast, feeding with the S-fraction increased serum osteocalcin levels significantly. Serum CrossLaps were found to be high (Fig. 6b) in the ovx controls and were slightly reduced after treatment with E2 and the triterpene-saponin fraction.

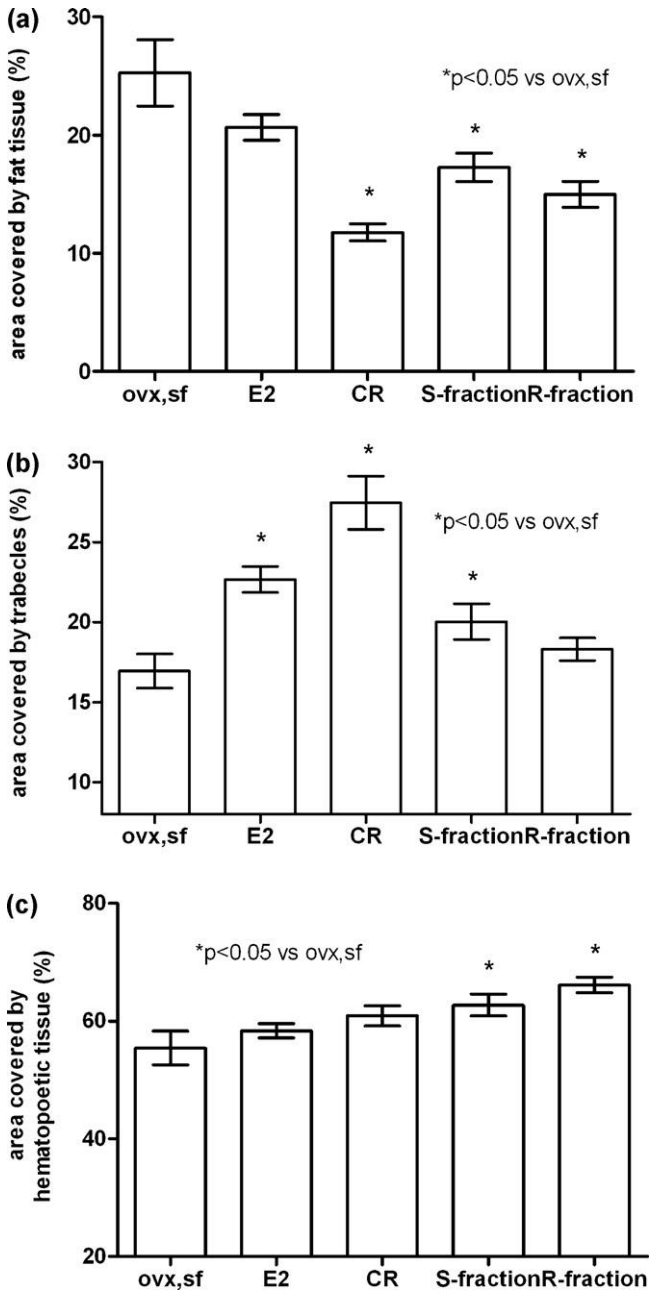

Fig. 2. Percentage of endosteal view area covered by fat tissue (a), trabecles (b) and by hematopoietic tissue (c). Ovx, sf = ovariectomized animals fed with soy free food, E2, CR, S-fraction and R-fraction indicate type of substance added to the food. $^{*}p < 0.05$.

Discussion

Obesity is increasing worldwide (Potenza and Mechanick 2009); particularly, visceral obesity is harmful in many aspects (Russell et al. 2010). For review see Sheu and Cauley (2011). Visceral adipocytes secrete pro-inflammatory cytokines which increase cholesterol triglycerides and this result in hypertension, arteriosclerosis, heart attacks and strokes (Bruce and Byrne 2009; Takada et al. 2009; Zhao et al. 2008). Furthermore, insulin receptors desensitize and this causes the final stage of visceral obesity, namely type 2 diabetes. All of these symptoms represent the metabolic syndrome (Aucott et al. 2011; Hajer et al. 2008).

It is well accepted that the ovx rat represents a good model to study development and preventive measures of obesity (Campbell and Febbraio 2001; Zoth et al. 2010) and osteoporosis (Kalu 1991). Following ovx of rats particularly visceral fat depots are largely increased (Seidlova-Wuttke et al. 2003). In previous experiments it was also shown that the metaphysis of the tibia of ovx rats is an ideal model to study the development of osteoporosis and its prevention (Lelovas et al. 2008). Particularly the microarchitecture and therefore the stability maintaining properties of the trabecular apparatus deteriorate rapidly and this can be prevented by E2 (Kapur et al. 2010a,b; Seidlova-Wuttke et al. 2003). Therefore, this structure was investigated. The ultrastructural integrity of the trabecular apparatus was determined by quantitative histomorphometry and STRUT analysis. The histomorphometric analyses allowed quantification of the amount of fat tissue in the bone marrow. With these tools

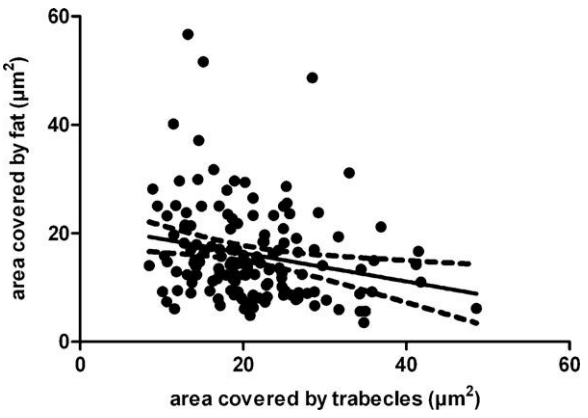

Fig. 3. The negative correlation between bone marrow area covered with fat tissue and trabecular surface is statistically significant.

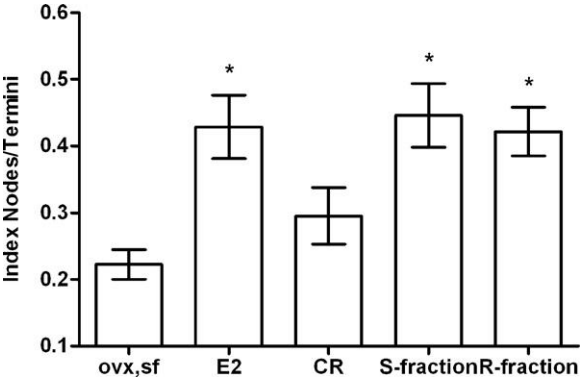

Fig. 4. The quotient between nodes/termini is an indicator of bone stability. For further details see legend to Fig. 1. $^{*}p < 0.05$.

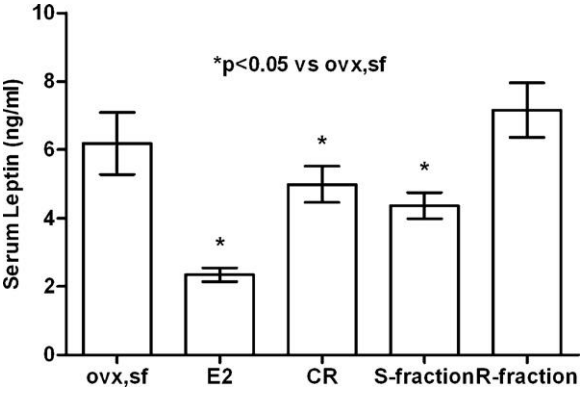

Fig. 5. E2, CR BNO 1055, the S- but not the R-fraction reduce serum leptin levels. $^{*}p < 0.05$.

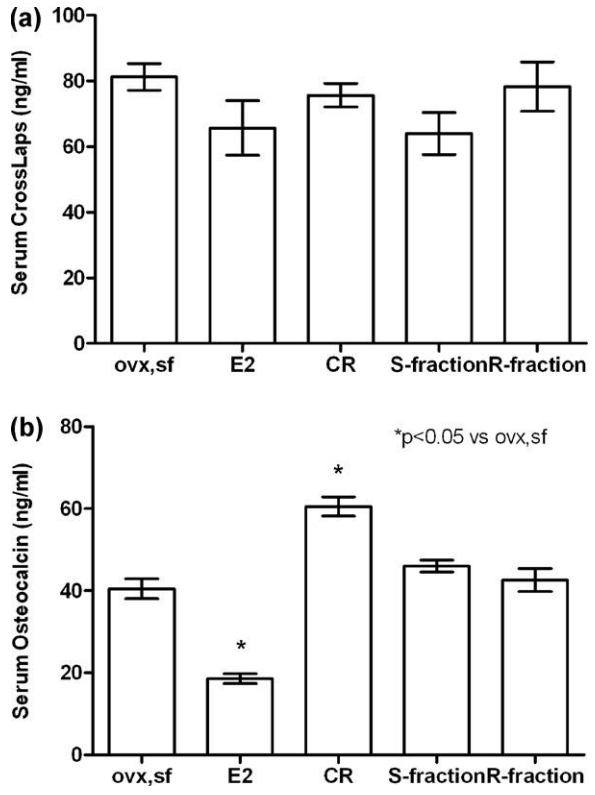

Fig. 6. Serum CrossLaps (a) are significantly reduced by E2 and the S-fraction. E2 decreases but CR BNO 1055 increases serum osteocalcin (b) concentrations. *$p < 0.05$.

we demonstrated that the amount of adipocytes in the bone marrow increases markedly following ovx and this correlated with the reduction of trabecular surface.

The amount of fat tissue in the bone marrow was significantly higher following ovx in comparison to the E2-treated animals, enforcing the idea that the adipocytes secrete less cytokines which is desired for the prevention of osteoporosis.

With the present results we also gave evidence that CR BNO 1055 had bone protective effects in ovx rats. In this well accepted model we showed previously that the mother extract CR BNO 1055 prevented loss of bone mineral density (Seidlova-Wuttke et al. in press). With the present results we demonstrated that CR BNO 1055 and the triterpene-saponin-S- and less R-fraction were able to conserve the trabecular surface significantly. It was interesting to observe that both the S-fraction and the R-fraction were able to exert beneficial effects on trabecular surface indicating that more than 1 substance is present in CR BNO 1055 to exert the antiosteoporotic effects.

With STRUT analysis the number of nodes and free ends were counted by a histomorphometric approach. It is well established that the more cross sections (nodes) are present in the trabecular apparatus the more stable is the bone whereas a high number of free trabecular ends (termini) indicates less bone stability (Chappard et al. 2008, 2001; Kasukawa et al. 2004; Mellish et al. 1991). In the present experiments STRUT analysis indicated that the trabecular infrastructure in the ovx animals was severely disturbed: trabecular surface was decreased, the number of nodes was decreased and of termini increased. This resulted in a reduction of the quotient between nodes and termini which is indicative for less bone stability. Treatment with E2 was largely able to prevent the deleterious effects of ovx. Treatment with E2, with the mother extract CR BNO 1055 and with both CR fractions was able to exert beneficial effects on these bone parameters.

It has also been established that following ovx of rats the bone marrow fat load increases (Cao 2011; Syed and Melim 2011). Our observation that E2 decreases fat load in the bone marrow suggests that the lack of estrogens is a factor responsible for the high fat load. The marrow fat load in the CR BNO 1055 treated ovx rats was massively and significantly more reduced in comparison to the E2 treated animals and also administration of the S- and R-fraction reduced fat tissue more efficiently than E2. This may give hints to the mechanisms of action of the compounds present in CR BNO 1055. In earlier experiments it was shown that bone marrow fat cells secrete pro-inflammatory cytokines, particularly tumor necrosis factor (TNF), interleukin 6 and others. Hence, they share many properties of visceral fat cells (Cao 2011; Syed and Melim 2011). These cytokines are known to inhibit osteoblast and to stimulate osteoclast formation and function (Syed and Melim 2011). Therefore, in addition to the direct adverse effects of lacking estrogens on bone cells the most likely increased cytokine production due to a high bone marrow fat load has additional deleterious effects on bone integrity. Such adverse effects of adipocytes are also clinically supported by the observation that patients suffering from the metabolic syndrome have an increased risk of bone fractures (Migliaccio et al. 2011; Zhao et al. 2008).

The chemical structures of these bone protective principles remain to be determined. A number of polyphenols were previously shown to have antiosteoporotic effects (Trzeciakiewicz et al. 2009) and many of them including actein are present in *Cimicifuga racemosa* (Cicek et al. 2010; Einbond et al. 2006). An actein derivate present in *Cimicifuga racemosa* was shown to inhibit pro-inflammatory cytokine (Schmid et al. 2009).

This view is supported by our demonstration of a statistically significant inverse correlation between bone marrow fat load and trabecular density and trabecular surface which does not prove, but is strongly suggestive that a causal relation exists between these parameters, i.e. it is likely that the cytokines secreted by the large number of marrow adipocytes in ovx animals augment the development of osteoporosis. Consequently, their reduction by E2, CR BNO 1055 and its fractions results in less local cytokine production in the bone marrow which prevents development of osteoporosis. We showed earlier a high increase of fat load in ovx rats (Seidlova-Wuttke et al. 2003) and that this is supported by the high serum leptin concentrations which indicate a high total body fat load. The reduction of leptin by E2 and CR BNO 1055 and the S-fraction is indicative for a reduction of total fat load of the body. This may bring up the possibility that CR BNO 1055 and the S-fraction may be beneficial for the prevention and treatment of the metabolic syndrome.

In comparison to ovx rats the circulating levels of osteocalcin and of the CrossLaps in the E2 treated animals were decreased indicating that this steroid reduced the activity of both bone-forming osteoblasts and bone-resorbing osteoclasts. This resulted obviously in a new healthy equilibrium of the activity of both the cell types and therefore, osteoporosis did not develop. The mechanism of action of CR BNO 1055 appears to be different from that of E2. Serum CrossLaps levels in the CR BNO 1055 treated animals remained as high as those in the ovx animals indicating that the activity of osteoclasts remained high. Under CR BNO 1055 osteocalcin was even higher than that in the ovx animals which is in agreement with earlier observations in ovx rats and in postmenopausal women (Raus et al. 2006; Wuttke et al. 2003) which points to an osteoblast stimulatory effect of this extract. This is also reflected by the higher trabecular surface observed in the metaphysis of the tibia. Such effects were not seen under administration of the S- or R-fraction which supports the concept of each other synergizing effects present in the 2 fractions. In each case, the high activities of osteoclasts and osteoblasts appear to be maintained in ovx animals by CR BNO 1055 and its fractions at an equilibrium which is healthy for the bone.

In summary, we showed a marked correlation of bone marrow adipose tissue with the deterioration of trabecular architecture in ovx animals. The higher the bone marrow fat load the larger was the development of osteoporosis. CR BNO 1055 appears to stimulate osteoblast activity and does not mimic the effects of E2 to reduce the high activity of osteoblasts and osteoclasts following ovx. In each case a new healthy equilibrium between osteoclast and osteoblast activity appears to be stabilized by CR BNO 1055. Therefore, this extract may be used to prevent osteoporosis in elderly human beings.

Conflict of interest

MK, GS and JH are employed by the Bionorica SE (Neumarkt, Germany). WW is advisor to this company. The other authors have no conflict to disclose.

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