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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).