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Absence of positive effect of black cohosh (*Cimicifuga racemosa*) on fracture healing in osteopenic rodent model

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2 **Absence of positive effect of black cohosh (*Cimicifuga racemosa*) on fracture**
3 **healing in osteopenic rodent model**
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Abstract

Healing of predominantly metaphyseal fractures in postmenopausal osteoporosis is delayed and comparatively poor. Due to potential side effects of HRT, natural alternatives are appealing. The aim of this study was to determine whether *Cimicifuga racemosa* extract BNO 1055 improves metaphyseal fracture healing in severe osteopenic bone in rats.

Thirty-three twelve-week-old female rats developed severe osteopenia during ten weeks after ovariectomy. After metaphyseal tibial-osteotomy and standardized T-plate-osteosynthesis healing-period in ovariectomized rats(C), 17- β -estradiol-(E) and *Cimicifuga racemosa*-(CR) supplemented diets were assessed for 35 days. Changes in callus-morphology were evaluated qualitatively by biomechanical testing and quantitatively in microradiographies and fluorochrome-labeled histological sections.

CR-supplementation slightly improved callus quality and trabecular bone-formation. It significantly enhanced endosteal callus density (Cl.Dn.e C:59.08 \pm 21.89, E:45.95 \pm 18.39, CR:60.85^A \pm 18.66), though most of the other morphological parameters examined showed no improvement. Time-course of fracture healing did not change due to CR. Estrogen-supplementation enhanced biomechanical properties of fracture-site. Trabecular bone was improved indicating the physiological endosteal healing process.

CR-supplementation did not exhibit positive effects in severe (senile) osteopenic fracture healing as seen in early (postmenopausal) osteoporosis in rats. Callus-formation was slightly improved under CR. Estrogen improved fracture healing in severe osteopenic bone, while the extent of callus formation played a minor role.

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4 **Keywords** fracture healing – *Cimicifuga racemosa* - osteoporosis – estrogen –
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6 phytohormones
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10 11 12 13 14 **Introduction** 15

16
17
18 Osteoporosis has become one of the most prominent health problems due to
19 increased life expectancies and changing population demographics. It is often
20 recognized when the fracture occurs (Eisman et al., 2004), while most patients are
21 not examined (Häussler et al., 2007) and not treated in accordance with the
22 evidence-based clinical guidelines for osteoporosis (Feldstein et al., 2003). In the
23 context of the hormonal changes that occur after menopause, bone turnover
24 increases and an imbalance between bone formation and bone resorption develops
25 (Savoca et al., 2007). Bone mineral density (BMD) is decreased, bone micro-
26 architecture is disrupted, and the amount and variety of non-collagenous proteins in
27 bone are altered (Boonen et al., 2008). These changes occur primarily in the
28 trabecular bone of the metaphyses, resulting in increased fragility (Curtis et al.,
29 2005). Fracture healing process in osteoporotic bone is delayed and qualitatively
30 reduced (McCann et al., 2008; Hao et al., 2007). Bone-improving substances
31 administered at the time of internal fixation could substantially alleviate this problem
32 (Stuermer et al., 2008; Kolios et al., 2009 (1)).
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49 Black cohosh (*Cimicifuga racemosa* (CR)) exerted estrogenic effects in the bone
50 tissue (Seidlova-Wuttke et al., 2003, 2009; Viereck et al., 2005) via an estrogen
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2 receptor-dependent mechanism (Chan et al., 2008). Extracted from the rhizomes of
3
4 *Cimicifuga racemosa* (black cohosh), this substance enhances differentiation and
5
6 increases the OPG-to-RANKL (regulators of bone metabolism) ratio of normal human
7
8 osteoblasts, contributing to the positive skeletal effects of CR (Viereck et al., 2005).
9
10 Extracts have also been proven to reduce climacteric complaints as efficiently as
11
12 conjugated estrogens without exerting estrogenic effects in the uterus (Seidlova-
13
14 Wuttke et al., 2009). The effect of CR on osteoporotic fracture healing remains
15
16 unclear.
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20 Application of HRT for postmenopausal bone changes is continually
21
22 discussed. Estrogen ($17\beta\text{E}$) has shown potential to protect against coronary-artery
23
24 disease (Hsia et al., 2006), diabetes mellitus (Salpeter et al., 2006) and Alzheimer's
25
26 disease (Zandi et al., 2002). However, after long-term administration, HRT increased
27
28 the incidence of breast cancers, thromboembolic events, stroke, and cardiovascular
29
30 disease (Roussouw et al., 2002; Horwitz et al., 2008; Beral et al., 2003). From the
31
32 other hand, it exerted a protective effect on bone increasing BMD and decreasing
33
34 fracture incidence (Tarakida et al., 2007; Gambacciani et al., 2007). According to the
35
36 recent review of the WHI investigators (L'Hermite et al., 2008), HRT seems to be a
37
38 valid option, especially in osteopenic women. During short-term fracture-healing
39
40 period (4 to 10 weeks), the positive effects of estrogen administration may outweigh
41
42 the negative effects observed during long-term treatment (Stuermer et al., 2008;
43
44 Kolios et al., 2009(1, 2)). In recent treatment advances substances like
45
46 bisphosphonates, calcitonin, SERMs (raloxifene) and strontium ranelate are
47
48 recommended more than estrogen. In addition, next to calcium and vitamin D
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50 application, bone stimulating agents like parathyroid hormone or thiazide diuretics
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2 are applied for osteoporosis treatment (National Osteoporosis Foundation 2008,
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4 Theodorou et al. 2002).

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8 In the present study, we examined the effects of Black cohosh extract BNO
9
10 1055 on metaphyseal fracture healing in ovariectomy-induced osteopenia in rats and
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12 compared its effect to that of 17- β -estradiol administered at a physiological dosage.
13
14 The animal model selected possessed severe untreated osteopenia, in which an
15
16 occurring fracture was treated with Black cohosh or estrogen to improve bone
17
18 healing.

21 22 **Materials and Methods**

23 Animals and treatment groups

24
25 Thirty-six three-month-old female Sprague-Dawley rats (Winkelmann, Borcken,
26
27 Germany) were ovariectomized under Xylazine (Rompun®, Bayer Health Care) and
28
29 Ketamine (Ketanest S®, Pfizer Pharma) (3.5 V/V, 1mL/kg) anesthesia. Within ten
30
31 weeks on a diet of phytoestrogen-free food (ssniff SM R/M, 10 mm Pellets; ssniff
32
33 Spezialitäten GmbH, Soest, Germany), they developed severe osteopenia (bone loss
34
35 about 40% BMD in the proximal tibia). After this period, all animals underwent a
36
37 standardized metaphyseal tibia osteotomy and osteosynthesis with a five-hole T-
38
39 shaped titanium plate (XS 57-05140, Stryker Trauma, Selzach, Switzerland), as
40
41 previously described (Stuermer et al., 2008). Rats were housed 3-4 in standard
42
43 cages under 12h darkness : 12 h light regimes [fluorescent tubes (osram, Munich,
44
45 Germany) with a color temperature of warm-white (830=3000K) or neutral-white
46
47 (840=4000K9] at a constant temperature of 22±1 °C and relative humidity of 55%.

1
2 The rats were divided in three groups containing 12 animals each. The first group,
3 the osteopenic control group, received phytoestrogen-free pelleted food (C). The
4 second group as fed with phytoestrogen-free food supplemented with 17- β -estradiol
5 (E), 0.01g/kg rodent chow (Sigma Medical, Apeldoorn, Netherlands). The average
6 daily intake was 0.097g. In the third group (CR), phytoestrogen-free food was
7 supplemented with *Cimicifuga racemosa* which was an aqueous/ethanolic extract of
8 the rhizomes of cultivated plants. The average intake was 24.9 mg/day per rat. The
9 preparation of the CR extract BNO1055 was as follows: ground CR rhizomes were
10 maintained in 5 volumes of 50% ethanol for 48 h and, thereafter, were percolated
11 with a speed of 500kg/h. After filtration, the extract was concentrated under vacuum
12 and dried up at 100-140 mbar according to the patent-protected process (Bionorica,
13 Neumarkt, Germany). The product temperature did not exceed 40°C (Seidlova-
14 Wuttke et al., 2003(1)) (1.66 g/kg rodent chow, BNO 1055, Bionorica, Neumarkt,
15 Germany). Phytoestrogen-free food contained 1000IE of vitamin D per kg chow, 1%
16 of calcium and 0, 7% of phosphorus. The substances were administered at
17 established oral doses (Stuermer et al., 2008; Seidlova-Wuttke et al., 2003) for a
18 fracture-healing period of 35 d (Fig.1). All animals had free access to food and water
19 throughout the experiment.

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40 The animal study protocol was approved by the local regional government and
41 conformed to German animal protection laws (District Government of Braunschweig,
42 permission from 12/5/03, Az: 509.42502/01-53.03).

43 44 45 46 47 Intravital fluorochrome labeling

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49 To visualize the location, the amount and the time-line of bone tissue
50 deposition, fluorochrome stains (Merck, Darmstadt, Germany), which bind
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Deleted: ¶

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2 competitively to the calcium on the surface of hydroxylapatite in newly built bone,
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4 were injected subcutaneously (Rahn et al, 1976). The stains used included: xylenol
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6 (orange, 90 mg/kg) on day 13, calcein (green, 10 mg/kg) on day 18, alizarin (red, 30
7
8 mg/kg) on days 24 and 26, and tetracycline (yellow, 25 mg/kg) on day 35 (three
9
10 hours before decapitation). The additional dose of alizarin red was administered on
11
12 day 26 to intensify the color. With this labeling procedure, the chronological
13
14 sequence of newly built bone, bone resorption and bone remodeling could be easily
15
16 distinguished.

17 18 19 20 Endpoint and specimen preparation

21
22 The animals were decapitated while under deep CO₂ anesthesia on day 35.
23
24 This time point was chosen because callus formation is stable and peripheral
25
26 resorption of the outer callus did not occur at this time. Tibiae were prepared; the
27
28 skin, muscles and tendons of the lower leg were removed carefully. Exarticulation of
29
30 the tibia–fibula-complex and ankle joint followed; plate and screws were removed.
31
32 Tibiae were immediately stored at -80 °C until further analyses. The right and left
33
34 tibiae were randomized and used for biomechanical testing, as well as histological
35
36 and microradiographic analysis.

37 38 39 Radiographic and biomechanical evaluation

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41 Microfocus radiographs in the anterior-posterior and lateral views were taken, and
42
43 fracture healing of each tibia was evaluated in a blinded manner. Particular attention
44
45 was paid to the position of the implants, accidental fracturing of the fibula and axial
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47 deviation. Thereafter a previously described (Stürmer et al., 2006) standardized,
48
49 three-point bending and breaking test device (ZWICK software, Bensheim, Germany)
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51 was used to examine the biomechanical properties of the callus area, including
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2 stiffness (S) and yield load (yL). The force (N) was plotted against the distance of
3
4 tibial bending (mm). This curve shows a linear progression, with the first phase
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6 representing the stiffness of the bone. In the second part, the rise of the curve
7
8 declines. Elasticity changes to irreversible plastic deformation. The onset of force
9
10 declination and the force of first microfracturing, called yield load (yL), were
11
12 calculated.

13 14 15 16 Morphologic evaluation

17 Tibiae were defatted and embedded in methylmethacrylate. The central three
18
19 $120\mu\text{m}\pm 10\mu\text{m}$ thick longitudinal sections were chosen for quantitative evaluation.
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21 Microradiographies were performed as previously described (Schenk et al., 1965). A
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23 light microscope (Leitz DM-RXE, Leica, Bensheim, Germany) with an I3 filter system
24
25 (excitation filter 450-490 nm, dichronic mirror 510 nm, suppression filter 515 nm) was
26
27 used. The quantitative analysis of bone tissue was digitally determined by using the
28
29 Quantimet digital image processing system (Leica DM-RXE, Bensheim, Germany).
30
31 Thus, the same histological sections could be directly compared regarding
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33 microradiography and fluorescence-labeling.
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38 Evaluation and statistics

39 X-rays were analyzed regarding the site and extension of callus formation,
40
41 implant loosening and involvement of the fibula. Based on the bending graph, the
42
43 stiffness and yield load were calculated. In the microradiographs, callus area density
44
45 (amount of calcified tissue per $\text{mm}^2/\text{callus area}$) and width (mean width measured at
46
47 10 parallel lines perpendicularly to cortical bone) as well as cortical structure (density
48
49 and width) and trabecular bone (three locations: ventro-medial, dorsal, endosteal)
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51 were determined digitally (Fig. 2g). Data are shown as the means of three
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1
2 microradiographic sections. For the intravital fluorochrome-labeled histological
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4 sections, the same areas were evaluated for the amount of bound calcein (green),
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6 alizarin (red) and tetracycline (yellow) labeling by using the Quantimet digital image
7
8 processing system (Schenk et al., 1965) (Fig. 2a-f). The system was not able to
9
10 detect xylenol (orange)-stained bone because only small islands of it surrounded by
11
12 intensely green, calcein-labeled bone could be found. In addition, the total callus area
13
14 was calculated [Abbreviations conform to ASBMR Nomenclature Committee (Parfitt
15
16 et al., 1987)].
17

18 19 20 **Results**

21 22 Body weight and food intake

23
24 At the beginning of experiment, body weight of the animals did not differ significantly.
25
26 After ovariectomy BW increased in all groups. BW in E group remained at the lowest
27
28 level compared to the other treatment groups. BW in CR group was lower compared
29
30 to that in C group at the end of the experiment, however, it was significantly higher
31
32 than that observed in E group (Tab. 1). The daily food intake showed no significant
33
34 differences between the groups throughout the experiment.
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37

38 39 Radiographic evaluation

40
41 The tibia osteotomies of all 36 rats healed adequately without implant loosening. In
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43 four rats (two each from the C and E groups), accidental fracturing of the fibula
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45 (probably during internal fixation) with axial deviation and visibly increased callus
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47 formation occurred. This biomechanical factor confounded the fracture-healing
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49 process, interfering with the given test substances; therefore, these tibiae were
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51 excluded from the study.
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Biomechanical and microradiographic evaluation

The stiffness was highest in the E-substituted group, followed by the CR group. This measure was slightly improved in comparison to osteopenic C (Stiffness [N/mm]: C:121.4±47.08, E:147.9±39.38, CR:129.8±44.93). Stiffness showed the same distribution when related to body weight, but differences were more apparent (Stiffness BW-related [N/mm/g]: C:0.36±0.13, E:0.53±0.14, CR:0.42±0.16). Yield load was enhanced in the E group, while CR was on the level of C group C (yL [N]: C:76.41±18.83, E:93.84±29.68, CR:76.21±14.91). The trend observed for body weight highlights the impact of the treatments: we observed significantly enhanced yL in the E group and slightly enhanced yL in the CR group as compared to the C group (Yield load BW-related [N/g]: C:0.23±0.05, E:0.33*±0.1, CR:0.24±0.06) (Fig. 3).

The ventromedial callus width was similar in E- and CR-substituted groups; both groups presented reductions in comparison to the C group. However, this trend was significant only for the CR group. Significantly more callus was built dorsally, as compared to the ventromedial region, in all three groups (Fig.4). Measurements of callus density, both ventromedially and dorsally, showed no differences between the groups. Endosteal callus density was significantly improved in CR group vs. E and was slightly superior to the levels observed in the C group (Fig.5). The trabecular structure, in terms of trabecular width and density, number of nodes and nodular density was clearly improved under E treatment as compared to the C group (Fig.5, Tab.2). In the CR group, trabecular width and number of nodes were slightly improved compared to the C group. Otherwise, the trabecular area density was only

1
2 slightly reduced in comparison to the osteopenic C group. None of the substances
3
4 had any effect on the cortical structure (Fig.4, Tab.2).
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6
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8 Evaluation of intravital fluorochrome labeling

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10 As measured in the fluorochrome-labeled sections, most callus apposition occurred
11
12 in the endosteal region in all three groups (Fig. 6d), followed by the dorsal region.
13
14 Regarding the time course of callus building in the osteopenic C group, a significant
15
16 increase of callus apposition during the 35 days was visible in the ventromedial
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18 (Fig.6a) and endosteal (Fig.6c) regions. In the dorsal region (Fig.6b), levels of newly
19
20 formed bone remained high throughout all three phases. In contrast, callus building in
21
22 the E-supplemented group began with high values in the early CG-labeled period,
23
24 surpassing those of the C group, especially ventromedially and endosteally. Callus
25
26 building decreased during this period. Only in the ventromedial region did callus
27
28 formation remain relatively constant (Fig.6a-c). In the CR group, the time course was
29
30 similar to that observed in the C group, with only a slight increase in the amount of
31
32 newly formed bone observed throughout the period studied (Fig.6a-c).
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34

35 **Discussion**

36
37 Osteoporotic fractures affect the metaphyseal bone; in humans, these fractures occur
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39 mainly at the hip, the vertebrae, the proximal humerus and the wrist. Previous
40
41 experimental studies on fracture healing in osteoporosis have, for the most part,
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43 examined the diaphyseal femur (Cao et al., 2002; Bolander et al., 1992) or tibia
44
45 (Schmidmaier et al., 2001). Research on fracture healing in osteoporosis should be
46
47 done predominantly at the metaphyseal bone, because osteoporosis affects the
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49 metaphyseal bone to the largest extent (Thompson et al., 1995; Wronski et al.,
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51 1985). Additionally, diaphyseal bone heals primarily with periosteal callus formation,
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2 while metaphyseal bone heals predominantly endosteally (Stuermer et al., 2008).
3
4 Therefore, a metaphyseal osteotomy model (Stuermer et al., 2008) was used. When
5
6 osteotomy was performed, the bone structure was similar to that of typical elderly
7
8 female patients, who are largely untreated for osteoporosis. This model should
9
10 represent the severe, senile untreated osteoporosis, which is often seen in elderly
11
12 patients who suffer from acute hip and spine fractures. Therefore, the Black cohosh
13
14 and estrogen were used as immediate therapy after osteotomy (fracture) and not as
15
16 a prophylaxis.
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18
19
20 Herbal alternatives are popular due to the ongoing discussion about HRT and its
21
22 adverse effects. Black cohosh as osteoporosis prophylaxis has shown bone
23
24 protecting effects without estrogen-like effects on uterus in the mature osteopenic rat
25
26 model (Seidlova-Wuttke et al., 2003, 2009; Viereck et al., 2005), with direct effects on
27
28 the enhancement of bone nodule formation in osteoblasts. This action is mediated by
29
30 an estrogen receptor-dependent mechanism (Chan et al., 2008). In a double-blind
31
32 clinical trial with 62 postmenopausal women, researchers observed a bone-protecting
33
34 effect of CR under low estrogen-like adverse conditions (Wuttke et al., 2003). In
35
36 recent years, there have been several reports about hepatic events associated with
37
38 the use of CR, such as acute hepatitis or hepatic failure (Huntley et al., 2003; Whiting
39
40 et al., 2002; Levitsky et al., 2005). But new animal studies (Mazzanti et al., 2008) as
41
42 well as prospective longitudinal clinical trials (Nasr et al., 2009) showed that CR did
43
44 not affect liver morphology or hepatic function.
45

46
47 Previously, we have shown a positive effect of black cohosh on fracture healing
48
49 processes (Kolios et al., 2009 (3)). It was given as a prophylaxis of osteoporosis in a
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51 ovariectomized rat. In the presented study, CR was tested regarding its anabolic
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1
2 effects at severely osteopenic bone. The biomechanical properties of fracture sites
3
4 under CR therapy were slightly improved compared to the C group, although the
5
6 difference was not significant and values did not reach those of the E group.
7
8 Regarding the morphological analysis, most of the calluses in the CR group were
9
10 built in the endosteal region. Although the amount of endosteal callus was not
11
12 enhanced in comparison to the C and E groups (Fig.6d), density was significantly
13
14 improved under CR donation compared to the E group. The enhanced callus density
15
16 contributes to the slightly improved biomechanical properties observed and the
17
18 physiological healing process in the metaphysis. In addition, the trabecular structure
19
20 (Fig.4b, c) was slightly but not significantly improved under CR donation. Regarding
21
22 the time course of newly formed bone in the context of callus formation, CR does not
23
24 ensure the potency of stabilizing fractures in the early healing period, as observed in
25
26 the estrogen group. Compared to the osteopenic C group, the growth of callus tissue
27
28 increased during the three periods examined in this study. Fracture healing is still in
29
30 progress. Interestingly, dorsal area callus apposition decreases slightly in the second
31
32 and third period as compared to the first CG labeled phase. The bridge plating with
33
34 the induced, mainly dorsal-occurring micro-movements, in combination with CR
35
36 therapy may lead to an earlier stabilization, so that in the later fracture healing period
37
38 less new bone is necessary. In sum CR did not exhibit a positive effect on fracture
39
40 healing, as was observed in early (postmenopausal) osteopenic bone. CR is able to
41
42 preserve the bony structure and supports the fracture healing process in the bone
43
44 matrix. However, CR did not exert sufficient anabolic effect to improve fracture
45
46 healing in severe (senile) osteopenic bone. CR exhibited a slight but not significantly
47
48 positive effect.

49 Concerning the mechanisms and the active principles of *Cimicifuga racemosa* in
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51 organisms, previously published data have been largely contradictory. Some authors
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1
2 suggest a direct interaction with estrogen receptors (Chan et al., 2008) or a several
3
4 SERM activity of Black Cohosh contains (Seidlova-Wuttke et al., 2003). In contrast
5
6 other reports negate any estrogen receptor activity of CR using several molecular
7
8 biological assays (Lupu et al., 2003; Liu et al., 2001; Alves et al., 2008). Recent data
9
10 have demonstrated that Black Cohosh may have an effect on dopaminergic and
11
12 serotonergic systems (Kanadys et al., 2008). Some evidence exists for the
13
14 presence of monoaminergic substances in Cimicifuga racemosa extracts, which are
15
16 important in bone homeostasis. Receptors for serotonin, noradrenaline and
17
18 dopamine have been detected in osteoblasts and osteoclasts (Bouxsein et al., 2009;
19
20 Elefterion, 2008; Graham et al., 2008; Haney et al., 2009; Warden and Haney, 2008).
21
22 Serotonin and serotonin reuptake inhibitors have an osteoporosis-promoting effect on
23
24 bone, conducted by osteoblasts. From the other hand, serotonin has an anti-
25
26 osteoporotic effect by inhibiting the sympathetic noradrenalin release via central
27
28 nervous mechanisms. The latter may explain the mechanism of CR action since the
29
30 serotonergic substances were detected in the CR extract (Burdette et al., 2003;
31
32 Powell et al., 2008). Clinical study of Haney (2009) confirmed this coherence.
33
34 Dopamine has anti-osteoporotic properties as shown in dopamine-transporter knock-
35
36 out mice, which had a more fragile bone than the wild-type ones (Bliziotis et al.,
37
38 2000). The CR-extract BNO 1055 analyzed possessed dopaminergic mechanisms
39
40 which have not been yet identified chemically (Jarry et al., 2003). The extract
41
42 contains no estrogen-like substances, because the uterus weight and the estrogen-
43
44 regulated gene expression were not changed under BNO 1055 treatment (Seidlova-
45
46 Wuttke et al., 2003; Wuttke et al., 2003 (2)).
47
48 In addition there are reports about other active principles of Black Cohosh as the
49
50 biologically active constituents of triterpene glycoside and cycloartane glycoside
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52 (Gaubé et al., 2007; Kanadys et al., 2008). These named neocimigenosides A and B
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2 were isolated and identified by chromatographic and spectroscopic analyses by
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4 Mimaki et al., 2006. The triterpenoids are reported to exert inhibitory effect on
5
6 osteoclastic bone resorption through the suppression of OCLs (osteoclast-like cells)
7
8 formation and their resorbing activity and also showed a significant protective effect
9
10 on BMD in OVX mice (Li et al., 2007). It is known that osteoclast activity is necessary
11
12 and requested for fracture healing, because the damaged bone had to be
13
14 metabolized and restructured. As it was observed for Genistein (Kolios et al., (1)
15
16 2009) osteoclast inhibition resulted in a less structured callus with poor
17
18 biomechanical properties. CR seemed to have similar mode of action. Lie et al.
19
20 (2007) showed a positive effect of CR as a prophylactic agent for osteoporosis. We
21
22 confirmed the prophylactic effect of CR during fracture healing in the early stages of
23
24 osteoporosis (Kolios et al., (3) 2009). However, CR given as a therapeutic agent has
25
26 no potency to improve fracture healing in severe osteopenic bone.
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30 Fractured callus treated with estrogen displays excellent biomechanical properties.
31
32 Callus tissue becomes very elastic with significantly increased resistance to
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34 microfracturing. The reasons for this quality of the fracture site may be the enhanced
35
36 amount of endosteal callus (Fig.6d), as well as the fortified trabecular structure
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38 (Fig.4b, c). Both are also signs of the physiological, endosteal fracture-healing
39
40 process that are typical for the metaphysis. As typical for estrogen treatment, the
41
42 ventromedial and dorsal callus levels were not enhanced in comparison to
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44 osteopenic bone; periosteal callus formation was reduced as compared to the
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46 endosteal callus. Endosteal healing is also typical for the metaphyseal healing of
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48 healthy bone. The results of the histological evaluation showed an increased amount
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50 of callus formation in the first CG-labeled period, especially in the ventromedial and
51
52 endosteal regions of the E group as compared to the osteopenic C group. Callus
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1
2 formation decreased with time, which indicates that less bone is necessary during
3
4 fracture healing to stabilize the osteotomy gap.
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8 As previously described (Seidlova-Wuttke et al., 2003; Wronski et al., 1985),
9
10 the influence of osteoporosis on the metaphyseal cortical bone is very low. In this
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12 study, CR and even estrogen did not change the cortical width or density. The
13
14 influence on the diaphyseal bone may be completely different.
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18 Although we focused in this study on bony structure and not on adverse
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20 effects of the substances, the systemic effect of substituted estrogen has to be
21
22 mentioned. We observed that body weight in E group remained at the lowest level
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24 among the groups. CR showed less effect on body weight. As a mild systemic
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26 reaction, body weight was only slightly but not significantly lower compared to the
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28 osteoporotic C group after 5-week treatment.
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32 The results of this study are presented while bearing in mind the methodological
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34 limitations of the work. The design of the study focused only on short-term application
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36 of the substances during the fracture-healing process. The healing and
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38 biomechanical properties might be different with long-term supplementation of
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40 estrogen and *Cimicifuga racemosa*. Aside from body weight, no systemic effects
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42 were analyzed in this study. With regard to the influence of estrogen and CR on the
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44 mammary glands and liver, it would be interesting to examine the possibility of
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46 structural changes due to short-term treatment. However, this study focused on
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48 osteopenic bone fracture healing, and we presume the possible effects of 5-week
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50 treatment would be slight and reversible.
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Conclusions

Cimicifuga racemosa did not display any positive effect on fracture healing in severe (senile) osteopenic bone as in early (postmenopausal) osteoporosis in rats. Callus formation was only slightly improved under CR and healing of severe osteopenic bone was not accelerated. Estrogen as an anabolic agent was able to improve fracture healing in severe osteopenic bone as well. Its positive effects could outweigh possible adverse reactions of short-term treatment. Finally, the metaphyseal tibial osteotomy model in ovariectomized rats produced reliable results regarding the therapeutic effects of antiosteoporotic substances.

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Figure legends

Tab. 1: Changes in bodyweight determined at ovariectomy, osteotomy and obduction. Daily food intake and daily intake of pure 17 β estradiol (group E), or BNO 1055 CR extract (group CR) respectively in the test groups; C presents the control group. Values are expressed as the mean \pm SD (*p < 0.05 vs C, Δ p < 0.05 vs E).

Tab. 2: Quantitative evaluation of the three central microradiographic sections to examine tibial callus formation after treatment with Black cohosh extract BNO 1055 (CR) and 17- β estradiol (E) in severe osteoporotic rats. Values are expressed as the mean \pm SD. (*p < 0.05 vs C, Δ p < 0.05 vs E).

Fig. 1: The three arms of the study with the specific diets and their nutrient composition. 36 ovariectomized rats received phytoestrogen-free rodent chow for 10 weeks while developing osteopenia. After metaphyseal tibia osteotomy they were divided in 3 groups containing 12 rats each, receiving pure phytoestrogen-free chow (C), 17- β -estradiol supplemented chow (E) or cimicifuga racemosa (BNO 1055) supplemented chow (CR) during the fracture healing period of 35d.

Fig. 2: I. Exemplary microradiography with the scheme of the determined parameters of the callus formation [callus density ventromedial/endosteal/dorsal (Cl.Dn.v/e/d), callus width (Cl.Wi. v/e/d)] as well as the cortical situation [cortical density and width ventromedial/dorsal (Ct.Dn/Wi v/d)] and trabecular structure [trabecular density and width (Tr.Dn/Wi), number of trabecular nodes (N.Nd)]. **II.** Example of Quantimet®-based evaluation of fluorescence-labeled sections: **a** native picture, **b** cortical structure colored, **c** CG-labeled callus [fluorochrome labeling with calcein green (CG: day 18)], **d** AK-labeled callus [fluorochrome labeling with alizarin red (AK: day 24 and 26)], **e** TC-labeled callus [fluorochrome labeling with tetracycline (TC: day 35)], **f** entire callus area.

Fig. 3: Stiffness and yield load (sole and BW-related) of the metaphyseal tibiae from the bending test. **a** and **c**: the stiffness (S) and yield load (yL) were highest in the E-substituted group (E), followed by the CR group (CR), being slightly improved in

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2 comparison to osteopenic C. **b** and **d**: S and yL showed the same distribution when
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4 related to body weight, but differences were more apparent. Especially in the E group
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6 yL was significantly enhanced vs. C. Values are expressed as the mean \pm SD (*p <
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8 0.05 vs C).
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11 **Fig. 4:** Quantitative evaluation of microradiographic sections of tibiae under 17- β -
12 estradiol- (E) and cimicifuga racemosa (CR) supplementation vs. osteopenic control
13 group C. Exemplarily shown: **a** Callus width dorsal (Cl.Wi.d), **b** callus density
14 endosteal (Cl.Dn.e), **c** trabecular width (Tr.Wi) and **d** number of trabecular nodes
15 (N.Nd). Values are expressed as the mean \pm SD (*p < 0.05 vs C).
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21 **Fig. 5:** Corresponding microradiographic and fluorescence-labeled sections.
22 Estrogen (E) induced a very dense and compact callus, and maximally increased the
23 trabecular structure compared to the osteopenic C group. Under CR (Cimicifuga
24 racemosa)-donation the callus structure was slightly enhanced as compared to C,
25 but levels did not reach those of the E group.
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28 **Fig. 6:** Quantitative analysis of bone healing at the metaphyseal tibia after intravital
29 fluorochrome labeling with calcein green (CG: day 18), alizarin red (AK: day 24 and
30 26) and tetracycline (TC: day 35) at the **a** ventromedial, **b** dorsal and **c** endosteal
31 region. One-way ANOVA and Tukey-Kramer post-hoc test (PrismTM, GraphPad, San
32 Diego, USA). **d** Average total callus area after 35 days of fracture healing. Values are
33 expressed as the mean \pm SD. (* = p < 0.05 vs. CG-area of same group, Δ = p < 0.05
34 vs. AK-area of same group).
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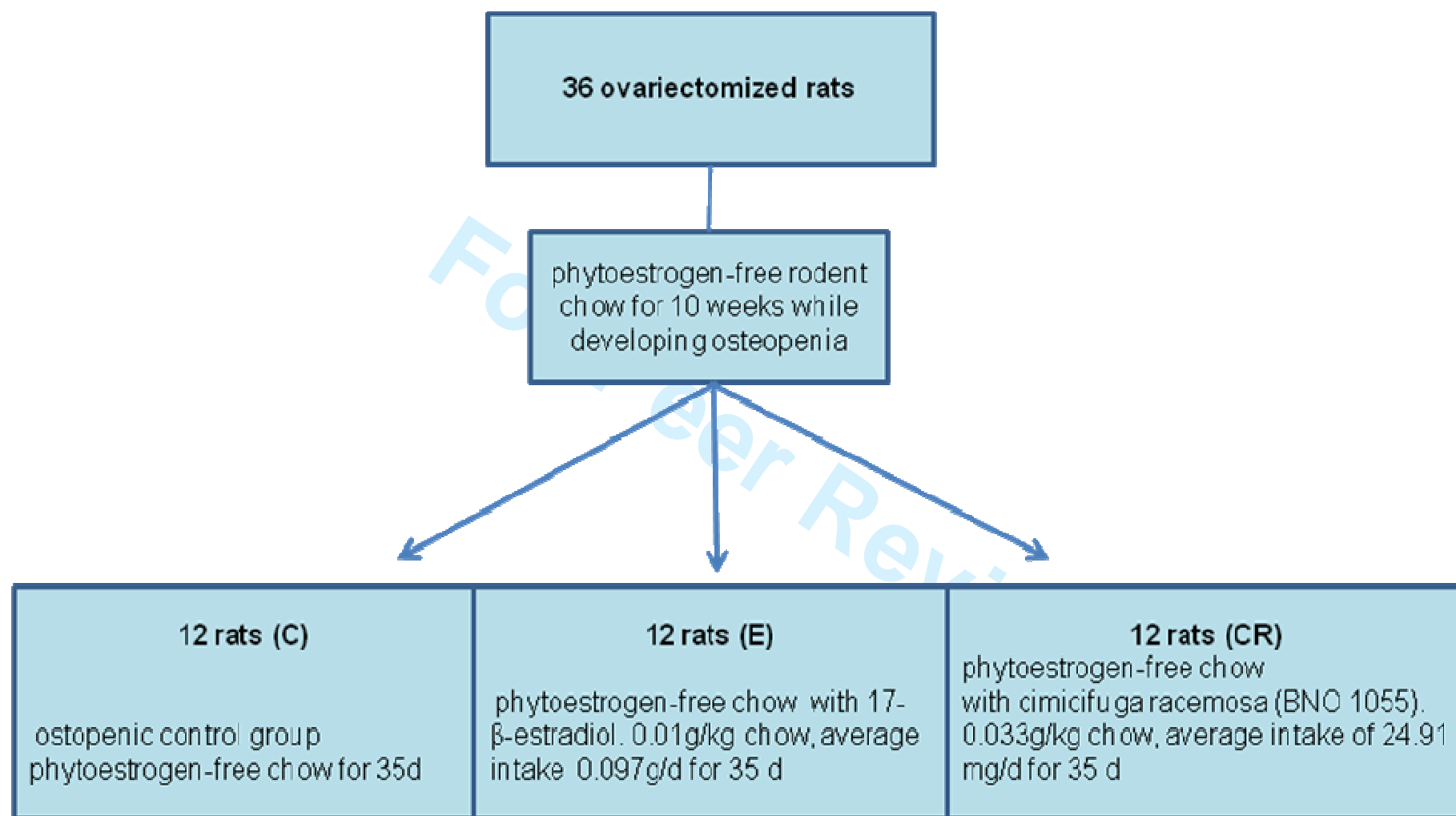
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Table 1: Changes in bodyweight determined at ovariectomy, osteotomy and obduction. Daily food intake and daily intake of pure 17β estradiol (group E), or BNO 1055 CR extract (group CR) respectively in the test groups; C presents the control group. Values are expressed as the mean \pm SD (*p < 0.05 vs C, Δ p < 0.05 vs E).

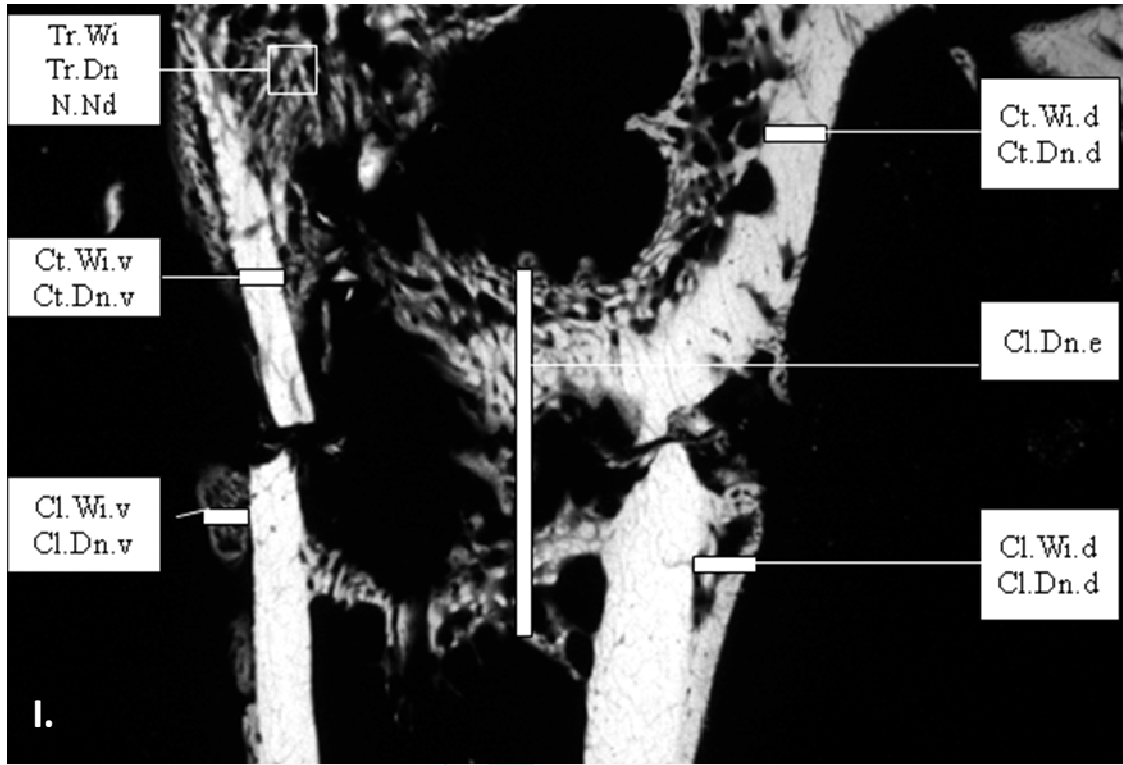
	C Mean \pm SD	E Mean \pm SD	CR Mean \pm SD
Body Weight [g]			
Ovariectomy	228.8 \pm 14.04	238.0 \pm 12.53	235.9 \pm 11.83
Osteotomy	347.6 \pm 19.97	347.8 \pm 16.06	332.5 \pm 26.18
Obduction	342.2 \pm 19.91	280.3 \pm 12.05*	317.3 \pm 23.31 Δ
Food intake after osteotomy [g/d/rat]	17.19 \pm 2.89	11.57 \pm 0.11	15.01 \pm 0.89
Substance intake [mg/d/rat]		0.097 \pm 0.002	24.91 \pm 1.47

Table 2 Quantitative evaluation of the three central microradiographic sections of the tibia callus formation after therapeutically treatment with Black cohosh extract BNO 1055 (CR) and 17- β estradiol (E) in severe osteoporotic rats (average value \pm SD of the mean)

	C Mean \pm SD	CR Mean \pm SD	E Mean \pm SD
Cortical width ventro-medial (Ct.Wi.v) [mm]	0.35 \pm 0.08	0.34 \pm 0.06	0.34 \pm 0.04
Cortical width dorsal (Ct.Wi.d) [mm]	0.49 \pm 0.1	0.45 \pm 0.11	0.44 \pm 0.12
Cortical density ventro-medial (Ct.Dn.vm) [%]	98.38 \pm 1.52	99.04 \pm 1.68	98.88 \pm 1.11
Cortical density dorsal (Ct.Dn.d) [%]	99.11 \pm 0.94	99.14 \pm 0.89	99.59 \pm 0.34
Callus width ventro-medial (Cl.Wi.vm) [mm]	0.35 \pm 0.2	0.22 \pm 0.12	0.23 \pm 0.16
Callus width dorsal (Cl.Wi.d) [mm]	0.72 \pm 0.51	0.49 \pm 0.27	0.49 \pm 0.2
Callus density ventro-medial (Cl.Dn.v) [%]	59.36 \pm 16.24	56.11 \pm 17.72	54.86 \pm 23.62
Callus density dorsal (Cl.Dn.d) [%]	67.59 \pm 14.84	67.16 \pm 17.63	67.61 \pm 15.02
Trabecular density (Tr.Dn) [%]	11.47 \pm 9.81	10.93 \pm 8.49	14.68 \pm 8.62
Nodular density (Nd.Dn) [%]	7.12 \pm 11.65	6.87 \pm 13.67	10.19 \pm 11.93

**Figure 1**

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Peer Review

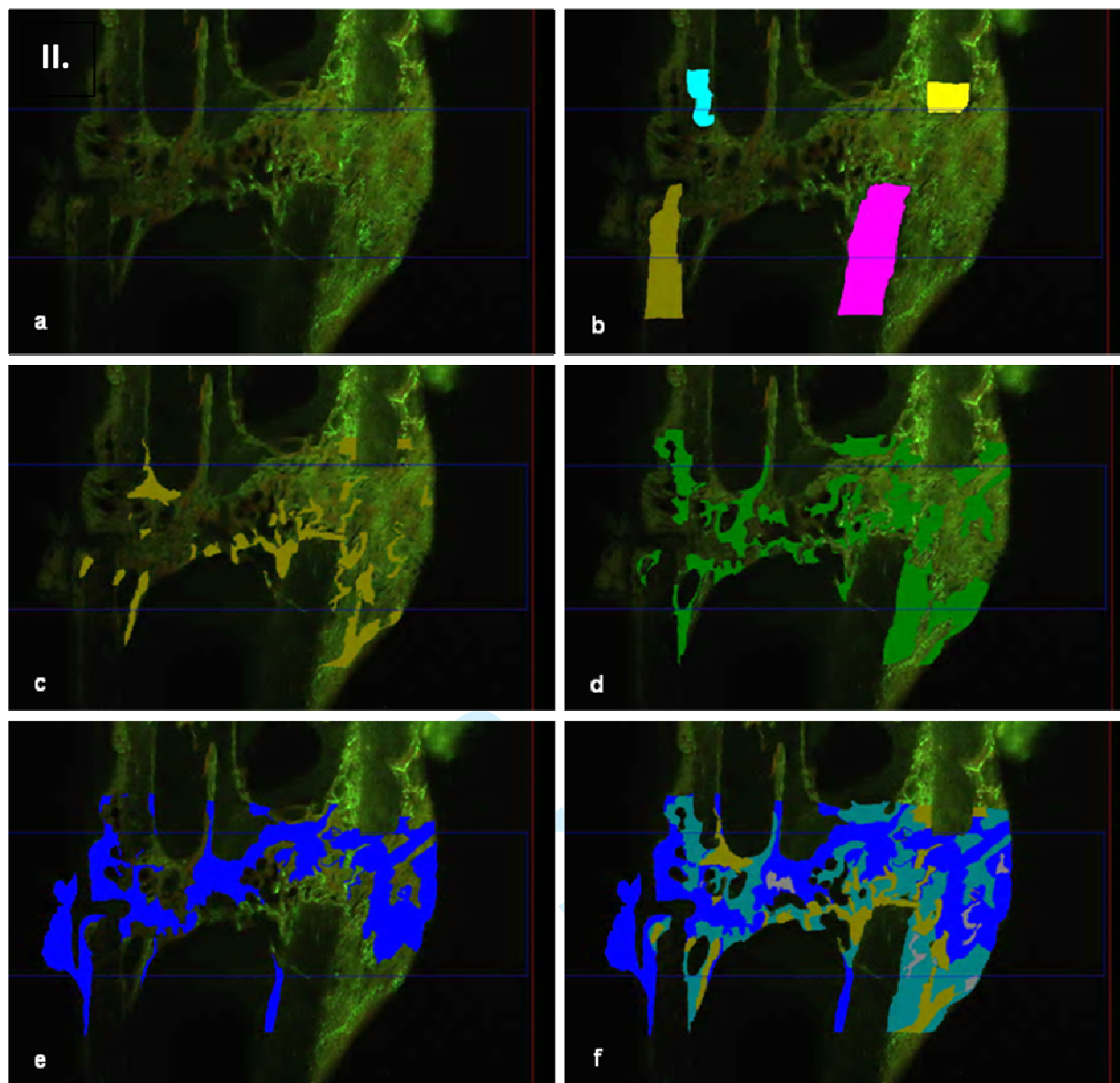


Figure 2

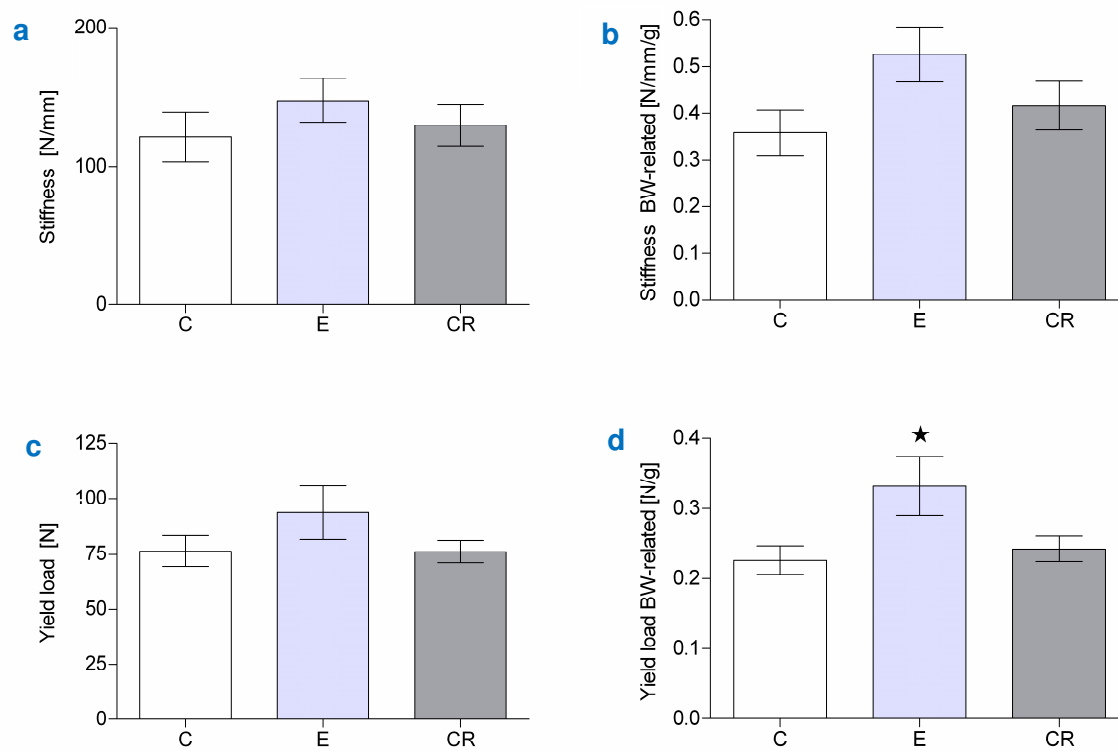


Figure 3

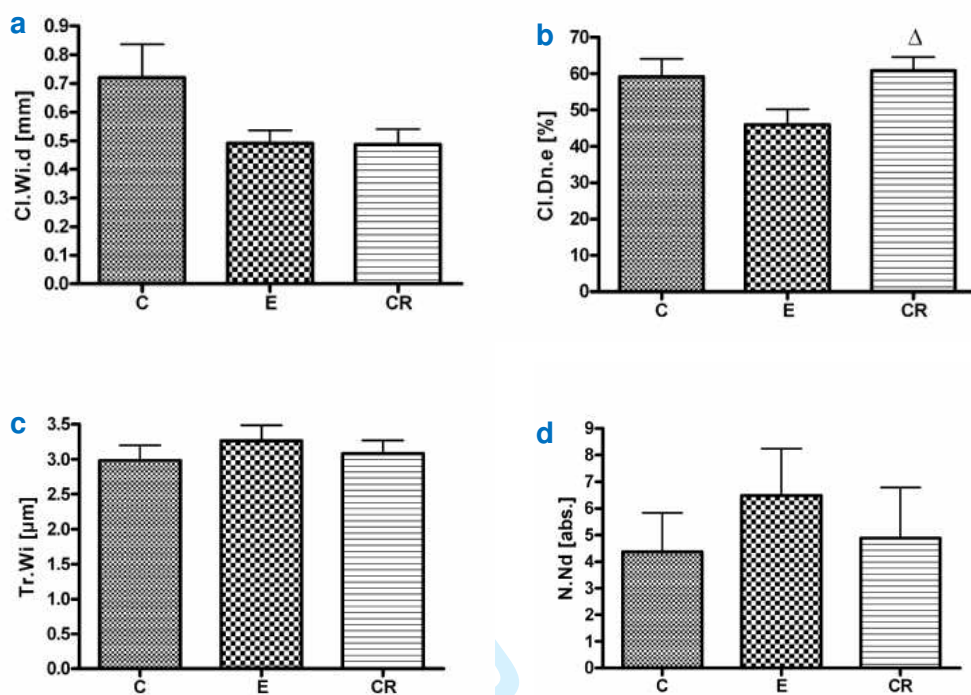


Figure 4

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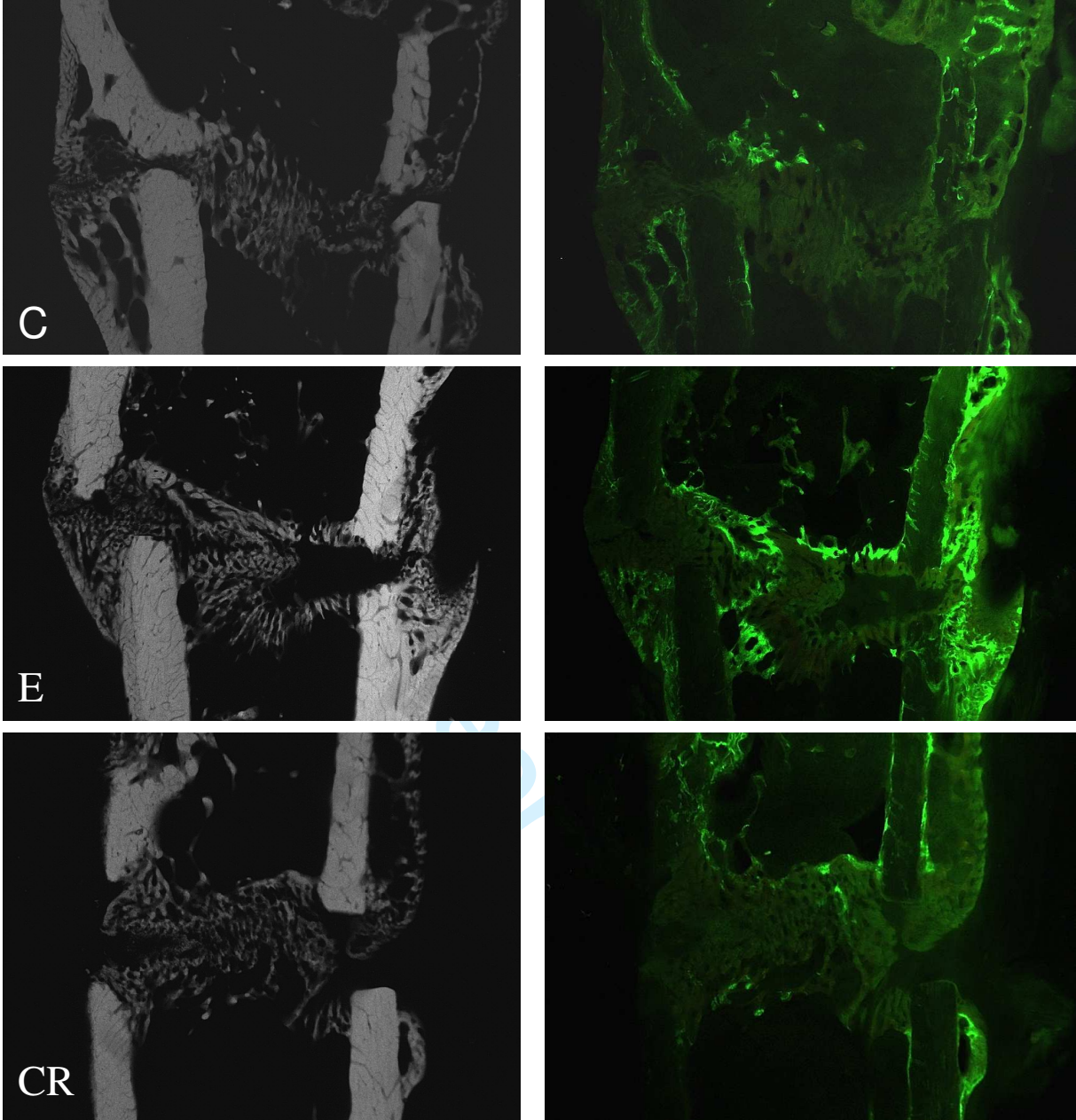


Figure 5

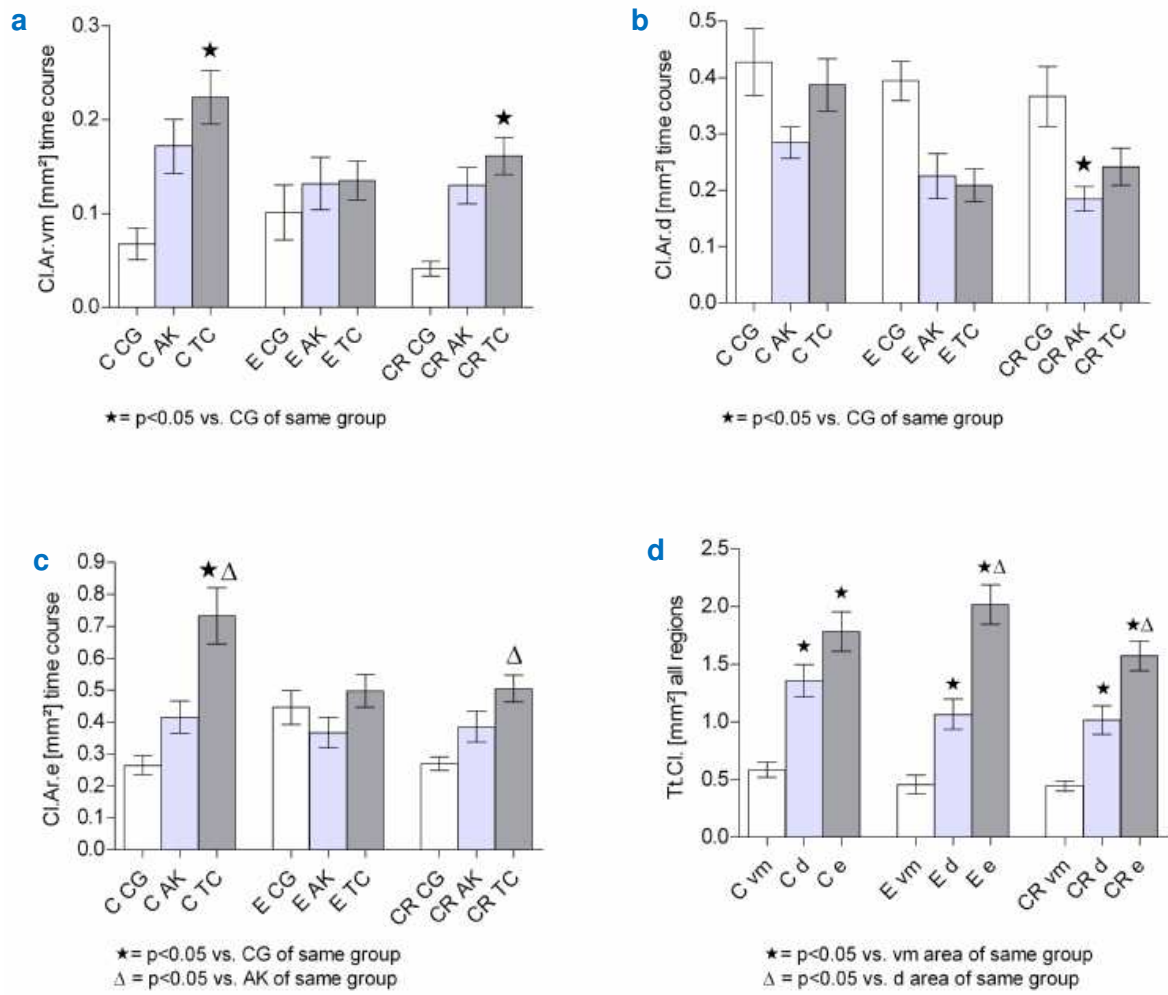


Figure 6 (a, b, c, d)