

Effect of daidzein, 4-methylbenzylidene camphor or estrogen on gastrocnemius muscle of osteoporotic rats undergoing tibia healing period

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Abstract

The effect of daidzein (D), 4-methylbenzylidene camphor (4-MBC) or estradiol-17 β -benzoate (E₂) on muscle of osteoporotic rats during fracture healing was studied. After performing a metaphyseal tibia osteotomy in 96 osteoporotic 5-month-old female Sprague-Dawley rats, they received daily 50 mg D, 200 mg 4-MBC or 0.4 mg E₂ per kg body weight, or soy free (SF) diet up to 36 and 72 days. Mitochondrial activity, fiber area, and capillary density were analyzed in *M. gastrocnemius*. Osseous callus bridging of fracture was observed in half of the rats after 36 days. By day 72, fracture was healed in most of the animals. State 3 mitochondrial respiration significantly enhanced in E₂, 4-MBC and D groups versus SF after 36 days (30, 32 and 32 vs 23 pmol O₂/s per mg). It declined after 72 days,

however, in E₂ group it was still at a higher level versus SF (25, 23 and 21 vs 20 pmol O₂/s per mg). Size of fast oxidative glycolytic (FOG) and fast glycolytic (FG) fibers, capillary density did not differ significantly between the groups, however, at day 36 an increase in D and 4-MBC groups was detectable. FOG diameter was 64, 66, 68, and 58 μ m and FG diameter was 88, 98, 95, and 89 μ m in SF, D, 4-MBC, and E₂ groups. The ratio of capillaries to muscle fiber was 1.1, 1.4, 1.3, and 1.1 in SF, D, 4-MBC and E₂ groups by day 36. D and 4-MBC react similar to estrogen thereby improving oxidative cell metabolism in severe osteoporotic rats. The level of mitochondrial activity was higher, though no significant morphological differences could be shown.

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Introduction

Today it is still unclear which role the skeletal muscle plays in the main problem of osteoporosis, the traumatic event (fall), the osteoporotic fracture, and its healing process. A decline in estrogen production at menopause is associated with the failure to maintain muscle mass (Aloia *et al.* 1991). Loss in muscle strength appears to occur more rapidly than does the loss in bone mineral density after the onset of menopause (Marcus *et al.* 1994). Loss in muscle strength leads to a physical frailty, increasing risk of osteoporotic fracture, and may impair the fracture healing process to some extent.

Since bone and muscle are functionally linked, evaluation of muscle function in osteoporotic organisms is also important, particularly in situations where bone is adversely affected and the organisms are treated with osteoprotective substances to facilitate bone healing. Bone healing period is associated with an inactivity of the muscles that may cause their atrophy (Machida & Booth 2004). It was demonstrated that ovarian hormone deficiency could play a role in the

failure to recover muscle mass after a period of inactivity of the muscle (Sitnick *et al.* 2006). The presence of estrogen receptors in skeletal muscle indicates that skeletal muscle function is also regulated by estrogen (Piccone *et al.* 2004). Changes in hormone concentration have profound influence on muscle fibers. Ovariectomy in growing rat resulted in an increase of the diameter of all fiber types in muscles, whereas, estrogen replacement reduced the size of all types of fibers (Piccone *et al.* 2004). In muscles of mature female rodents, estrogen has been associated with modulation of muscle fiber types (Kadi *et al.* 2002, Feng *et al.* 2004), whereas, the cross-sectional areas of all types of fibers were not affected by estrogen status (McClung *et al.* 2006, Moran *et al.* 2007). Moreover, estrogen has been reported to promote angiogenesis in skeletal muscles of estrogen deficient animals (Kyriakides *et al.* 2001).

Ovarian hormones have consistently been indicated in modulating mitochondrial function, such as ATP production, mitochondrial membrane potential, and calcium concentration (Wang *et al.* 2001, Nilsen & Brinton 2003).

The presence of mitochondrial estrogen receptors α and β suggests that estrogens may act directly on the mitochondria (Yang *et al.* 2004, Stirone *et al.* 2005). Given the importance of mitochondria in basic cellular functions as well as the critical role of mitochondrial dysfunction in the development of various diseases (e.g. Stirone *et al.* 2005) an important question is whether osteoprotection through estrogenic substances also exerts effects on the mitochondria in muscle.

Post-menopausal estrogen replacement therapy (ERT) was often recommended for prevention and treatment of osteoporosis (Glazier & Bowman 2001) as well as for maintaining muscle strength and mass (Phillips *et al.* 1993, Sorensen *et al.* 2001). However, ERT is associated with potential side effects on reproductive tissues, such as breast cancer and refused as being unnatural by women (Glazier & Bowman 2001). This has driven a search for substances that produce only beneficial effects without undesirable effects, especially on the reproductive organs.

Recently, much attention has been focused on phytoestrogens, especially isoflavones, as a potential safe alternative for pharmaceutical ERT (Glazier & Bowman 2001). Soy and soy products are the richest source of dietary isoflavones in humans. Daidzein (D) is the prevalent isoflavone in soy and has attracted the most clinical interest because of its weak estrogenic effect when consumed by animals or humans (e.g. Picherit *et al.* 2000). D was demonstrated to prevent bone loss without any adverse effects on the uterus in the ovariectomized (Ovx) rat model of post-menopausal osteoporosis (Picherit *et al.* 2000). Nowadays, phytoestrogens containing D are commercially available as dietary supplements.

Several u.v. filters frequently used in cosmetics represent new classes of endocrine active chemicals that have been found to have estrogenic effect if applied in animals (Schlumpf *et al.* 2001, 2008). 4-Methylbenzylidene camphor (4-MBC) exhibited the highest estrogenic activity *in vivo* in the group of u.v. filters studied (Schlumpf *et al.* 2001). It has been shown that 4-MBC prevented bone loss and exerted a very weak estrogenic effect on the uterus and the vagina after a 3-month treatment of Ovx rats (Seidlová-Wuttke *et al.* 2006).

Whereas the beneficial effect of D or 4-MBC treatment on osteoporotic bones has been reported (Picherit *et al.* 2000, Seidlová-Wuttke *et al.* 2006), the effect on skeletal muscle of estrogen deficient organisms has not been studied so far. Therefore, the aim of the present study was to investigate the effect of D, 4-MBC and estrogen on mitochondrial function and structure of the M. gastrocnemius (MG) in osteoporotic rat during fracture-healing period.

Materials and Methods

Animals and treatments

Ninety six 3-month old Sprague-Dawley rats (Harlan Winkelmann, Borchon, Germany) were bilaterally Ovx

under i.p. ketamine (Medistar, Holzwickede, Germany) and xylazine (Riemser, Greifswald-Insel Riems, Germany) anesthesia (40 mg and 10 mg per kg of body weight (BW) respectively) and then randomly assigned into four groups, each consisting of 24 rats. Rats were housed 3–4 in standard cages under 12 h darkness:12 h light regimes at a constant temperature of 22 ± 1 °C. Rats received soy free pelleted diet containing potato protein. Soy proteins were substituted by potato proteins to eliminate an estrogenic effect of isoflavones presented in soy. After 8 weeks, when osteoporosis was established (Kalu 1991), a metaphyseal osteotomy and T-plate fixation of the tibiae was done under ketamine/xylazine anesthesia as described previously (Stuermer *et al.* 2008). Along with anesthesia, rats received s.c. 5 mg Decentan (Merck) and 4 mg Rimadyl (Pfizer, Karlsruhe, Germany) per kg of BW respectively. Rimadyl was given two times per day during two post-operative days.

After operation (day 0), animals were fed with special diets (Sniff special diet GmbH, Soest, Germany). Group 1 received soy free (SF) diet. Group 2 was given a diet supplemented with 5 g 4-MBC per kg of diet. Group 3 received diet supplemented with 1 g D (purity of 98%, Changzhou Dahua Imp. and Exp. (Group) Corp. Ltd, Jiangsu, China) per kg of diet and Group 4 received a diet containing 10 mg estradiol-17 β -benzoate (E₂; Merck) per kg.

The daily intake was calculated by weekly weighing of food. All animals had free access to food and water throughout the experiment. Animals were weighed on the day of ovariectomy and weekly during 72 days of administration of supplemented diet. A daily dosage of 4-MBC averaged 200 mg per kg BW (Schlumpf *et al.* 2001). Daily dosages of D and estrogen were on average 50 mg and 0.4 mg per kg BW respectively (Vortherms 2006). The doses of 4-MBC and D were selected on the basis of previous studies showing beneficial effects on osteoporotic bones (Picherit *et al.* 2000, Seidlová-Wuttke *et al.* 2006). The daily intake of 0.4 mg estrogen per kg BW results in serum estrogen concentration of about 40 pg/ml (Vortherms 2006). This is within the physiological range reported for healthy female rat (Krinke 2000).

Four rats died during the study (one each of SF and E₂ groups day 36 and two of the E₂ group day 72). A reduction in physical activity of the rats was observed subjectively during the first two days after osteotomy.

After 36 and 72 days of supplemented feeding, 12 animals of each group were decapitated under carbon dioxide anesthesia. Immediately, MG was extracted randomized from either the left or right limb of the rats. The MG was chosen because of its location at a fracture site and its large size that enabled us to collect samples from the same (middle) part of the muscle for both mitochondrial and histological analyses. From the middle part of the MG a piece of muscle, about 80 mg, was immersed in ice cold storage medium, pH 7.1 (15 mM phosphocreatine, 49 mM KMES, 20 mM taurine, 20 mM imidazole, 5.2 mM ATP, 9.5 mM

MgCl₂×6H₂O, 3 mM KH₂PO₄, 0.5 mM dithiothreitol (DTT), 1.9 mM Ca-K-EGTA water solution (3.804% EGTA, 1.122% KOH, 1% CaCO₃ dissolved at 80 °C, pH adjusted to 7.0 with KOH), and 8.1 mM K-EGTA solution, same as Ca-K-EGTA solution without CaCO₃) for up to 1 h before respirometric measurements were performed. A block of muscle (1×1×1 cm³) was removed from the same region of the MG, frozen directly in liquid nitrogen after coating it with talcum (Moline & Glenner 1964) and stored at -80 °C until analyses of muscle fibers and capillaries. Uterine wet weight was recorded. Tibia randomized from either right or left limb was dissected free of soft tissues and after extraction of plate and screws were stored at -20 °C until histomorphological analyses.

The animal study protocol was approved by the local regional government and conformed to German animal protection laws (permission from 12/05/03, Az: 509.42502/01-53.03 District Government of Braunschweig).

All chemicals were obtained from Sigma–Aldrich Chemie GmbH, unless otherwise indicated.

Respirometric measurements

Muscle samples were prepared as described previously (Trumbeckaite *et al.* 2001) with some modifications. Ten to 20 mg samples were carefully dissected and permeabilized for 20 min in 1 ml storage medium (stated above) supplemented with 50 µg saponin per ml. Permeabilized muscle fibers were washed in three 5-min changes of incubation medium (pH 7.4) consisting of 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 0.5 mM EDTA, 20 mM Tris–HCl, 5 mM MgCl₂×6H₂O, 10 mM KH₂PO₄, 0.5 mM DTT, and 1 mg per ml fatty acid free BSA. All procedures were performed on ice.

Respirometric measurements were made using an Oroboros Oxygraph (Oroboros, Innsbruck, Austria), a two chamber respirometer with integrated electromagnetic stirrers. Bundles of fibers (3–7 mg) were carefully dried on a filter paper and added to the oxygraph chambers. The measurements were performed at 30 °C in 2.1 ml incubation medium using the added substrates pyruvate (9.5 mM) and malate (1.9 mM). The weight specific oxygen consumption rate (pmol O₂/s per 1 mg sample weight) was calculated as the time derivative of the oxygen concentration (DatLab 4.0 analysis software, Oroboros). The rate of state 3 respiration was determined following the addition of 5 mM ADP. The effect of ADP was recorded and state 4 respiration was initiated by the addition of 26 µM carboxyatractyloside (Calbiochem, Darmstadt, Germany; EMD Chemicals Inc., Darmstadt, Germany; Merck), an inhibitor of the ADP/ATP translocation. Physiological respiratory activity of mitochondria was estimated by calculating the respiratory control ratio (RCR) defined as ADP-stimulated (state 3) respiration rate divided by the resting (state 4) respiration rate (Renner & Gnaiger 2003). At least five replications per treatment group were done.

Analyses of muscle fibers

Serial cross sections of 12 µm were cut in a cryostat at -20 °C and mounted on glass slides. Sections were fixed for 1 min in 1% (v/v) paraformaldehyde solution (pH 6.6) containing 1% (w/v) CaCl₂ and 6% (w/v) sucrose (Meijer 1970) followed by two 5-min washing in distilled water. A modified procedure of Horák (1983) was used to stain the sections. Sections were subjected to the following consecutive actions: a) incubation in Diaphorase solution consisting of 2.4 mM NADH, 27 mM phosphate buffer (pH 7.4), 0.4 mM nitro blue tetrazolium chloride at 37 °C under humidified atmosphere for 60 min; b) washing in distilled water for 15 min; c) pre-incubation in 18 mM CaCl₂ with addition of 0.4% (v/v) glacial acetic acid, pH 4.2 for 15 min; d) washing in 100 mM Tris buffer (Tris (hydroxymethyl) aminomethane), pH 7.8 containing 18 mM CaCl₂ for 2 min; e) ATPase incubation (pH 9.4) consisting of 0.1 M glycine (pH 9.4), 18 mM CaCl₂, 48.8 mM KCl, 2.8 mM ATP at 37 °C for 30 min; f) washing in three 30-s changes of 68 mM CaCl₂; g) treatment in 84 mM cobalt chloride for 3 min; h) washing in three 30-s changes of distilled water; i) incubation in 0.08% (v/v) ammonium sulfide solution for 3 min; j) rinsing in running water for 10 min followed by immersing in distilled water for 5 min. Thereafter, samples were air-dried and fixed with cover slips using Canadian balsam. Representative sections were used for final measurements of fast-twitch oxidative glycolytic (FOG) and fast-twitch glycolytic (FG) fibers classified according to Peter *et al.* (1972). Slow-twitch oxidative (SO) fibers were rarely represented among the samples, therefore were not included in the analyses. The fiber cross sectional area and diameter of each histochemical fiber type were measured after transfer of representative section of the muscle with the aid of a digital camera (DVC 1310 C, DVC Company, Maidenhead, UK) and a microscope (Eclipse E 600, Nikon, Tokyo, Japan) at a magnification of ×10 using a computerized image analysis program (Lucia G, Laboratory Imaging, Version 4.82). The outline of the fiber contour was manually defined on a digital image. At least 50 FOG and FG fibers in each rat muscle were typed and measured. Ten animals per treatment group were analyzed.

Analyses of muscle capillaries

Periodic acid-Schiff (PAS) staining method (Andersen 1975) was applied with some modifications to stain the blood capillaries. Serial cross sections of 12 µm were fixed for 1 h in ethanol/chloroform/glacial acid (16:3:1) at 4 °C followed by 10-min fixation at room temperature and subsequent 10 changes of distilled water. Thereafter, sections were treated with 0.3% (w/v) water solution of α-amylase (from porcine pancreas) for 25 min at 37 °C, and after ten changes of distilled water, with 1% (w/v) periodic acid for 30 min. Washing in ten changes of distilled water was repeated and the sections were placed in Schiff's Reagent solution

(Roth, Karlsruhe, Germany) for 35 min followed by treatment with 10% (w/v) potassium sulfite/1 N HCl/water (1:1:20) for 30 min. Finally, sections were rinsed in running water for 10 min followed by immersing in distilled water for 5 min. Air-dried samples were fixed with cover slips using Canadian balsam. Capillaries and fibers found in five randomly selected fields (1 mm² each) from the muscle section were counted at a magnification of $\times 10$ (microscope Eclipse E 600) using a digital camera (DVC 1310 C, DVC Company) and image analysis program (Lucia G, Laboratory Imaging, Version 4.82). The ratio of capillaries to muscle fiber was taken in the analyses. At least five replications per treatment group were done.

Analyses of bone healing

The labeling of new bone formation during fracture healing was performed *in vivo* according to Rahn (1976) using fluorescence dyes xylenol orange (XO; 90 mg/kg BW), calcein green (CG; 10 mg/kg BW), alizarin complexone (AC; 30 mg/kg BW) and tetracycline (T; 25 mg/kg BW) injected subcutaneously. The dyes were applied on day 13 (XO), on day 18 (CG), on days 22 and 24 (AC), and on day 36 (T) in animals treated up to 36 days. Rats kept for up to 72 days received XO on day 36, CG on day 47, AC on days 56 and 59, and TC on day 72. Tibia was thawed and subjected to the sequential ascending concentrations of ethanol followed by embedding in methylmethacrylate (Merck). Longitudinal sections 150 μ m thick were cut at right angles to the plate position in the center of the specimens using Leica SP 1600 diamond saw microtome (Leica Instruments GmbH, Nussloch, Germany). Three representative sections from the center of each tibia were mounted on glass slides with stable medium (Eukitt, O Kindler GmbH, Freiburg, Germany) for fluorescence microscopy. The mounted sections were digitalized using Leica MZ75 microscope equipped with Leica DC 200 digital camera (Bensheim, Germany) and analyzed qualitatively. The osteotomy gap was divided into three regions of interest: 1) plate side; 2) opposite side; 3) the medullar part of the tibia. Time of the earliest callus and new bone bridging of fracture gap was determined by analyses of fluorochrome labeling according to the regions of interest.

Statistical analyses

Statistical analyses were conducted using SAS program (Version 8). ANOVA (*F*-test, $P < 0.05$) were applied to reveal the impact of two fixed effects (diets, duration of their administration) on the food intake and weight of the animals. Analyses of covariate with weight of the rats as a covariate (*F*-test, $P < 0.05$) was used to study the effects of 'diets' and 'duration' on mitochondrial respiration, muscle fibers, and capillaries. The weight of the animals had no significant effect on the respective variables (*F*-test, $P > 0.05$). Differences between individual means were estimated using Schéffe-test ($P < 0.05$).

Results

Food intake

Food intake significantly increased during the first 22 days of feeding among the groups, but during further feeding changes were not significant (Fig. 1).

Average food intake in groups SF and D was higher (15 g/rat per day in both groups) compared with that in 4-MBC and E₂ groups (14 and 13 g/rat per day respectively). Rats of the 4-MBC group consumed on average significantly more than those in E₂ group, however, it was less ($P < 0.05$) than that in SF and D groups. The differences are shown in Fig. 1 in detail.

Body weight

BW of the rats increased significantly among the groups (Fig. 2) after ovariectomy. During the first 2 weeks after osteotomy and supplemented feeding, the BW significantly decreased in all groups. Thereafter, it continuously rose approaching in groups SF, D and 4-MBC the level measured at the date of osteotomy (Fig. 2). In group E₂, it remained at a lower level throughout the experiment.

The BW was significantly higher in SF group than that in group 4-MBC and E₂ from the second week onward (Fig. 2). In group D, the BW was lower compared with that in SF group during experiment, however, the differences were not significant. The lowest ($P < 0.05$) BW was observed in group E₂. The BW of the group D and 4-MBC differed not significantly (Fig. 2).

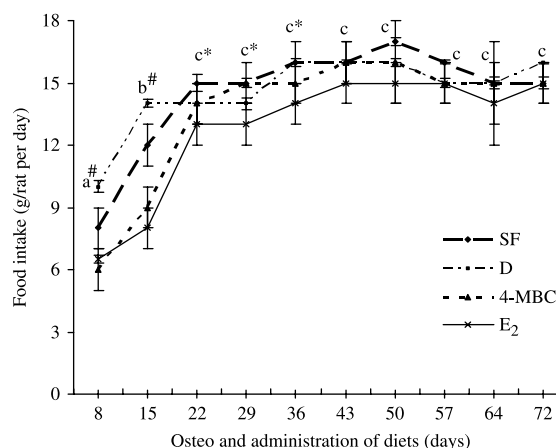


Figure 1 Food intake (g/rat per day) of osteoporotic rats subjected after osteotomy of tibia (day 0, osteo) either to soy free diet (SF) or daidzein (D), 4-MBC, estradiol-17 β -benzoate (E₂) supplemented diet during tibia healing for up to 72 days. Data are presented as means and S.E.M. Between days means with different letters differ ($P < 0.05$, Scheffé-test). *SF versus E₂ differ, #SF versus 4-MBC and E₂, D versus 4-MBC and E₂ differ ($P < 0.05$, Scheffé-test).

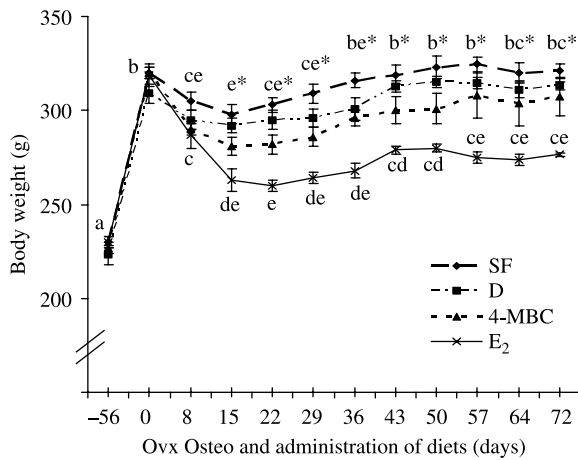


Figure 2 Body weight of the rats (g) ovariectomized at day 56 (Ovx) subjected after osteotomy of tibia (day 0, osteo) either to soy free (SF) diet or diet supplemented with daidzein (D), 4-MBC, estradiol-17β-benzoate (E₂) for up to 72 days. Data are presented as means and s.e.m. Between days, means with different letters differ ($P < 0.05$, Scheffé-test), showed combined for groups SF, D, and 4-MBC. *SF versus 4-MBC and E₂, 4MBC versus E₂ differ ($P < 0.05$, Scheffé-test).

Uterus weight

The uterus weight significantly differed between the treatment groups during experiment. In Ovx rats treated with estrogen, the mean uterus weight was significantly higher compared with that observed in Ovx rats in groups SF, D and 4-MBC (585 mg versus 233, 217 and 239 mg respectively). The differences in uterus weight measured in SF, D and 4-MBC groups were negligible ($P > 0.05$). Average s.e.m. was 22.

Respirometric measurements

State 3 and state 4 respiration significantly declined between day 36 and 72 of supplemented feeding. RCR ratio tended to

decline with the time as well (Table 1). In groups D and 4-MBC, state 3 respiration decreased significantly from 32 to 21 or 23 pmol O₂/s per mg sample weight respectively, reaching the level observed in SF group (20 pmol O₂/s per mg sample weight) that remained unchanged. In group D state 4 respiration decreased significantly with prolonged treatment from 6 to 3 pmol O₂/s per mg sample weight respectively.

Between the groups, the significant difference was revealed only in state 3 respiration rate (Table 1). After 36 days, state 3 respiration rate was significantly higher in animals fed with supplemented diet compared with those fed with unsupplemented diet (SF). As the bone healing was completed at day 72, only in group E₂ did state 3 respiration remain on a higher level (Table 1).

Analyses of muscle fibers

The cross sectional areas and diameter of FG and FOG fibers did not change ($P > 0.05$) during the period of supplemented feeding among the groups (Table 2). Between the groups, the differences in the cross-sectional area and the diameter of both fiber types were insignificant ($P > 0.05$). After 36 days of treatment period, cross sectional area and diameter of fibers were slightly, though not significantly enlarged in groups D and 4-MBC compared with others (Table 2). After 72 days, it was less pronounced. No sign of degeneration was detected in muscle fibers during preparation of samples.

Analyses of muscle capillaries

The density of capillaries did not significantly change between the treatment groups (Fig. 3). After 36 days, the ratio of capillaries to muscle fiber was slightly, though not significantly enhanced in groups D and 4-MBC (1.4 and 1.3) compared with that in groups SF and E₂ that was 1.1. During

Table 1 Mitochondrial respiration rates (pmol O₂/s per mg sample weight) in saponin-permeabilized fibers of gastrocnemius muscle of osteoporotic rats fed during tibia healing either with soy free (SF), daidzein (D), 4-methylbenzylidene camphor (4-MBC) or estradiol-17β-benzoate (E₂) supplemented diet up to 36 and 72 days measured with substrates: 9.5 mM pyruvate and 1.9 mM malate in the presence of 5 mM ATP (state 3) and 26 μM carboxyatractyloside (state 4)

Days	Diets	No	State 3		State 4		RCR	
			Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
36	SF	5	23 ^{Ax}	3	3 ^A	1	10	3
	D	8	32 ^{Ay}	3	6 ^A	1	8	2
	4-MBC	5	32 ^{Ay}	5	5 ^A	1	9	3
	E ₂	8	30 ^{Ay}	1	5 ^A	1	9	3
72	SF	10	20 ^{Ax}	2	3 ^A	0.4	6	1
	D	8	21 ^{Bxy}	1	3 ^B	1	7	1
	4-MBC	9	23 ^{Bxy}	2	4 ^A	0.3	7	1
	E ₂	9	25 ^{Ay}	3	4 ^A	1	7	1

RCR: state 3 divided by state 4. At least five replications per treatment were conducted. ^{xy}Between diets means with different letters differ on day 36 or 72 ($P < 0.05$, Schéffe-test). ^{AB}Means of the diet with different letters differ on 36 vs 72 d ($P < 0.05$, Schéffe-test).

Table 2 Cross sectional area (μm^2) and diameter (μm) of fast glycolytic (FG) and fast oxidative glycolytic (FOG) fibers in gastrocnemius muscle of osteoporotic rats exposed during tibia healing period either to soy free (SF) diet or daidzein (D), 4-methylbenzylidene camphor (4-MBC), estradiol-17 β -benzoate (E_2) supplemented diet up to 36 or 72 days. Treatments were conducted with 10 replications

Days	Diets	Cross sectional area, FG		Diameter, FG		Cross sectional area, FOG		Diameter, FOG	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
36	SF	6204	409	88	3	3277	333	64	3
	D	7685	487	98	3	3548	287	66	3
	4-MBC	7120	218	95	2	3691	259	68	2
	E_2	6367	728	89	5	2759	344	58	4
72	SF	6969	594	93	4	2978	274	61	3
	D	6794	530	92	4	3170	185	63	2
	4-MBC	6471	568	90	4	2806	216	59	2
	E_2	6636	426	91	3	3100	244	62	3

The differences were not significant ($P > 0.05$, *F*-test).

further treatment (day 72), this was not detectable (1.2, 1.1, 1.2, and 1.0 in groups SF, D, 4-MBC and E_2 respectively).

Analyses of bone healing

Qualitative analyses of fracture healing showed that after 36 days post-fracture, bone healing was not complete and fracture gap could be clearly observed in all groups. Callus bridging was observed in about half of the animals (Table 3). The bridging occurred during the fourth week of healing predominating endosteally and at the side opposite to the plate. At the side of the plate, less callus bridging was observed. In animals of SF, 4-MBC, and D groups prevailed periosteal callus bridging formed at the side opposite to the plate, whereas, in estrogen treated animals endosteal bridging was superior (Table 3). High-trabecular connectivity was observed among the groups.

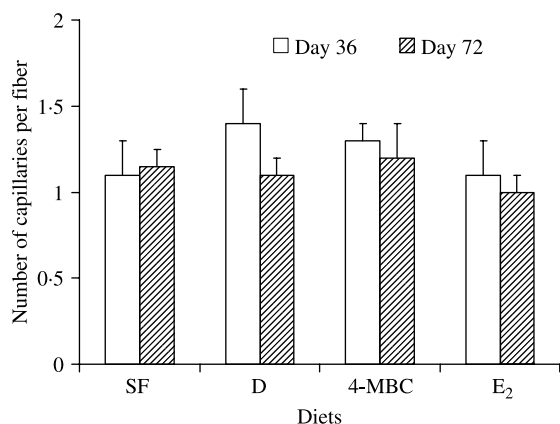


Figure 3 Number of capillaries per muscle fiber measured in gastrocnemius muscle of osteoporotic rats exposed during tibia healing period either to soy free (SF) diet or daidzein (D), 4-MBC, estradiol-17 β -benzoate (E_2) supplemented diet up to 36 and 72 days. Data are presented as means and S.E.M. The differences were not significant ($P > 0.05$, *F*-test).

By day 72, the fracture gap was united in 92% of animals in both SF and D groups. In groups 4-MBC and E_2 , united fracture gap was observed in 67 and 60% of animals respectively (Table 3). Periosteal callus appeared to be homogeneously filled with newly formed bone, whereas original cortical bones tended to resorb. New bone bridged the fracture gap during the sixth week of the healing. Endosteal callus was partially or completely resorbed. The SF, 4-MBC, and D groups showed poor-trabecular continuity.

Discussion

Mature Ovx rats are often used as an animal model for studying the effect of post-menopausal ovarian hormone deficiency on various organs and systems (Kalu 1991). The intent of our study was to evaluate the therapeutic effect of phytoestrogen D, u.v. filter 4-MBC and estrogen on the structure and mitochondrial function of skeletal muscles of severe osteoporotic rats during bone fracture healing period. We tried to mimic clinical situation the best, when a typical patient (female) with osteoporotic fracture, before being traumatized, suffers for years from post-menopausal changes in muscle and bone and receives no fracture preventive treatment (Haeussler *et al.* 2007). Therefore, a metaphyseal osteotomy of tibia was performed after severe osteoporosis had been established (Wronski *et al.* 1985, Stuermer *et al.* 2008) and the animals received the substances orally to facilitate bone healing. Finally, the comparisons were made with osteoporotic rats either untreated serving as an osteoporotic control or properly treated with estrogen to meet the goal of the present experiment.

Analyses of muscle

In the present study, we showed that D, 4-MBC as well as estrogen treatment beneficially affected mitochondrial activity in MG of osteoporotic rat after 36 days of their administration (31 vs 23 pmol O_2 /s per mg sample weight in osteoporotic

Table 3 Qualitative analyses of tibia fracture healing in osteoporotic rats exposed either to soy free (SF), daidzein (D), 4-methylbenzylidene camphor (4-MBC) or estradiol-17 β -benzoate (E₂) supplemented diet up to 36 and 72 days post-fracture determined using fluorochrome-labeled sections according to the plate side, opposite side, and endosteally

	Histological event	Treatment groups			
		SF	D	4-MBC	E ₂
Days					
36	Total number of animals	11	12	12	11
	Osseous callus bridging (number of animals)	6	9	7	5
	Periosteal bridging plate side	3	4	4	1
	Periosteal bridging opposite side	6	9	7	3
	Endosteal bridging	3	8	6	5
	Osseous callus bridging (days after fracture)	27	25	26	26
	Periosteal bridging plate side	28	26	24	26
	Periosteal bridging opposite side	25	25	27	27
	Endosteal bridging	27	23	26	24
72 ^a	Total number of animals	12	12	10	9
	United fracture gap (number of animals)	11	11	6	6
	Periosteal plate side	9	8	5	6
	Periosteal opposite side	11	9	6	6
	Endosteal	6	6	5	6

^aNew-bone bridging was on average on day 47 of healing in all treatment groups.

group respectively). It was assumed that osteotomy of tibia may result in an activation of regenerative mechanisms increasing vascularization around the injury place after 36 days and decreasing it as the bone healing is completed at day 72. According to Walsh *et al.* (1997), bone fracture is able to completely heal within 6 weeks in osteoporotic rats. Indeed, in the animal model used in the present study, fractured bones were healed in most of the animals by day 72. Although estrogen has been shown to stimulate angiogenesis in skeletal muscles (Kyriakides *et al.* 2001), we observed a slight increase in capillary density in D and 4-MBC treated rats, but not in the estrogen supplemented animals after 36 days of fracture healing. On the other hand, the estrogenic effect of the substances may facilitate the animals to restore their physical activity more rapidly. A drop in physical activity in Ovx rats has been observed (Wade 1972, Kadi *et al.* 2002), whereas, estrogen substitution is associated with a normalization of the level of physical activity (Kadi *et al.* 2002). The maintenance of state 3 respiration rate in the estrogen group at a significantly higher level throughout the experiment suggests that this was caused by action of estrogen on mitochondrial function via the mitochondrial estrogen receptors. The presence of mitochondrial estrogen receptors α and β has been reported suggesting that estrogens may act directly on the mitochondria (Yang *et al.* 2004, Stirone *et al.* 2005). Stirone *et al.* (2005) showed that estrogen is able to modulate mitochondrial function in cerebral blood vessels that resulted in a greater energy production capacity. They reported that estrogen could act through both mitochondrial and nuclear genomes involving multiple mechanisms to enhance mitochondrial function. In skeletal muscles of rat, ovariectomy decreased activity of mitochondrial fat oxidative enzymes, while maximal activity of these enzymes was

restored by estrogen treatment (Campbell & Febbraio 2001). D and 4-MBC have been reported to have binding affinity to estrogen receptors acting as weak estrogens (Picherit *et al.* 2000, Mueller *et al.* 2003, Schlumpf *et al.* 2004). Thus, it is possible that D and 4-MBC react similar to estrogen on mitochondria improving oxidative cell metabolism of the animals. A slightly increased blood supply to the muscle and a more rapid return to the normal physical activity in the D, 4-MBC, and estrogen treated animals might have enhanced the mitochondrial respiration rate at day 36. Further investigations are under progress to elucidate these assumptions.

The combination of NADH enzyme for aerobic oxidative metabolism reaction and myofibrillary ATPase histochemistry showed that the diameter and cross-sectional area of FOG and FG fibers were unaltered irrespective of food supplements or duration of their administration in mature female rat. Our results are in agreement with Moran *et al.* (2007) who found, that the cross-sectional areas of all fiber types were not affected by estrogen status in mature female rodents. We rarely observed slow oxidative fibers across the samples, as also found in MG by Armstrong & Phelps (1984).

D and 4-MBC affected slightly, though not significantly, the muscle fibers, enhancing their size at the early stage of fracture healing (day 36 after osteotomy). During aging up to 7 months (day 72 after osteotomy), these changes could not be detected. A general body growth including muscle growth may also contribute to these changes. According to Berg & Harmison (1957) linear growth of Sprague–Dawley rat was rapid until 170 days and then declined markedly. The difference in the BW of rats found between the treatment groups seems to have occurred independently of changes in muscle fibers. Significant changes in physical activity and

metabolism in Ovx rats, discussed above are responsible for the increase in adipose tissues and the BW, consequently. In other studies, increased wet muscle mass in Ovx versus estrogen replaced adult female rodents was explained by an extracellular fluid accumulation because the cross-sectional area of all fiber types was not affected by estrogen status (McClung *et al.* 2006, Moran *et al.* 2007).

In a previous study, it was stated that estrogen replacement in Ovx rats benefited atrophied muscle recovery from disuse atrophy (Kawano *et al.* 1997). McClung *et al.* (2006) reported that a cross-sectional area of muscle fibers was able to fully recover after 28 days, regardless of the rats were intact, Ovx or estrogen replaced. Our results corroborate these findings displaying no significant differences in the size of muscle fibers after 36 and 72 days of bone-healing period.

Animal model

The atrophied uterine horns observed in SF, D, and 4-MBC groups confirmed successful ovariectomy. Neither D nor 4-MBC given orally exhibited estrogenic activity on the uterus confirming the results obtained previously (Picherit *et al.* 2000, Seidlová-Wuttke *et al.* 2006). Oral administration of estrogen induced an uterotrophic activity increasing uterine weight in Ovx rat (Mohamed & Abdel-Rahman 2000). The advantage of the oral administration of food supplements instead of being injected is that this method is non-invasive and painless for the animals and does not require specialized consumables (syringes etc.) or personnel training. It is near to clinical practice as well. Moreover, 4-MBC has been administered orally or per dermal application in rat so far (reviewed by Schlumpf *et al.* 2008). Although during the first 3 weeks dosages of the substances enhanced with increasing food intake, during further feeding the dosages remained unchanged at a desirable level (Schlumpf *et al.* 2001, Vortherms 2006). Measurements of food intake were limited to the period after osteotomy was done. However, it could be suggested that food intake decreased during the first days after osteotomy and then rose recovering to its level (from 8 to 15 g/rat per day in average respectively). This assumption is supported by other results showing that food intake of the intact and Ovx Sprague–Dawley rats of 3 to 6 months of age ranged from 16 to 18 g/rat per day (Wronski *et al.* 1987, Vortherms 2006).

The lower food intake observed during the first weeks after osteotomy presumably resulted in the weight loss of the rats. Additionally, a reduced physical activity can cause disuse atrophy in muscle (Machida & Booth 2004) and bone loss (Everett & Gilligan 1991) that along with lower food intake may also reduce the BW. As previously reported, experimental procedures needed for fracture and fixation in rats are easily tolerated by the animals and they resume normal movement immediately after recovering from anesthesia (Bak & Jensen 1992). However, we observed subjectively the reduction in physical activity during first days after

osteotomy, probably because of the residual pain and/or of the intraoperatively received neuroleptic drug.

Food intake and weight of the rats were observed to be different between the treatment groups. Estrogen fed animals consumed less than others (13 vs 15 g/rat per day respectively) which is in accordance with previous studies (Piccone *et al.* 2004). This may contribute to the lowest BW in these rats, but is not the only reason. According to Toth *et al.* (2001) ovarian hormones affected body composition independently of changes in food intake. A reduced physical activity in Ovx animals (Wade 1972, Kadi *et al.* 2002) may have an impact on the recorded differences in the BW. A significantly lower weight was recorded in 4-MBC group compared with osteoporotic group. In D group, it was also lower, however, not significantly. It may be explained by the mild (Schlumpf *et al.* 2001, Vortherms 2006), but probably different estrogen-like effect of these substances on the metabolism and physical activity. Mueller *et al.* (2003) reported that 4-MBC at low doses is able to induce activity of estrogen receptors α and β *in vitro* with a lower estrogenic potency compared with phytoestrogens. For the rapid increase in BW (about 100 g) after ovariectomy observed in the beginning of our study, amongst other factors, a general growth of the 3-month old animals may be responsible (Berg & Harmison 1957).

The fracture healing can be divided into three stages: the inflammatory, reparative, and remodeling. In the animal model used in the present study, the conditions 36 days after fracture corresponded to the reparative stage and 72 days post-fracture corresponded to the remodeling stage. At the reparative stage, callus bridges and stabilizes fractured bone ends. Endosteal healing associated with slight periosteal callus formation is typical for fracture healing under stable conditions at the metaphyseal site (Claes *et al.* 1998). In the present study, endosteal bridging prevailed in estrogen treated rats. The predominance of periosteal callus bridging at the side opposite to the plate in SF, D, and 4-MBC groups may be due to the increased periosteal growth of cortical bone reported for Ovx rats (Turner *et al.* 1987). Additionally, micromovements may occur despite the biomechanically stable fixation and could stimulate callus formation at this side. Micromovement at the fracture site has been shown to be essential for sufficient callus formation (Stuermer *et al.* 1980). At the end of the experiment, there was an unexpectedly lower rate of complete healing in E₂ and 4-MBC group compared with that in other groups. BW in Ovx and D group was higher than in E₂ and 4-MBC groups from the second post-fracture week through the subsequent weeks of fracture repair, indicating that the calluses were loaded differently. Increased mechanical load has been reported to promote fracture healing and callus formation (Gardner *et al.* 2006). High-trabecular connectivity observed during the early stage of fracture healing (36 days) in all treatment groups indicates the capacity of an organism to compensate the potentially negative effect of osteoporosis in fracture healing. After 72 days, however, this capacity is decreased and osteoporotic animals in groups SF, D, and 4-MBC showed poor-trabecular continuity.

Conclusion

The phytoestrogen D and u.v. filter 4-MBC react in a similar way to estrogen thereby improving oxidative cell metabolism in muscle when applied at the early stage of fracture healing (36 days). For the late period (72 days) only estrogen treated rats maintained a high-mitochondrial respiration rate. D and 4-MBC treatments slightly, though not significantly, enhanced muscle fiber size and capillary density in the early phase, but not in the late phase of fracture healing. Thus, D and 4-MBC exert a positive effect on the mitochondria and microstructure of the skeletal muscle during the early stage of osteoporotic fracture healing. Considering their ability to prevent bone loss (Picherit *et al.* 2000, Seidlová-Wuttke *et al.* 2006) they may be a chance to revive post-fracture mobilization.

Declaration of interest

There is no conflict of interest for the research reported.

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