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Long-term effects of the rhapontic rhubarb extract ERr 731® on estrogen-regulated targets in the uterus and on the bone in ovariectomized rats

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ABSTRACT

The efficacy of ERr 731®, a commercially available extract isolated from *Rheum rhaponticum*, in terms of menopausal complaints like hot flushes, depression, anxiety and vaginal dryness has been proven in a two-year clinical study. Further a recent preclinical study excluded unwanted side effects on the endometrium by showing a lack of stimulation of proliferation marker genes by ERr 731® or its constituents in the 3-day uterotrophic assay. The present study aimed at further substantiating the safety of ERr 731® in terms of endometrial hyperplasia and at the same time test for potential estrogenic effects in the bone. Therefore, ovariectomized (ovx) rats were treated in a dietary long-term administration for 90 days. Hence, the modulation of proliferation in the uterus was investigated by examining the effects on the mRNA expression of proliferation marker genes (*Mki67*, *Pcna*), on the estrogen-responsive gene *C3* and on the estrogen receptors ERα and ERβ. We additionally performed densitometry analysis of the proximal tibia metaphysis using peripheral computed tomography (pQCT) and quantified bone homeostasis markers in the serum to examine potential effects on the bone.

In this study design, neither an uterotrophic response nor a modulation of proliferation marker genes on mRNA level has been observed as response to the long-term application of the rhapontic extract. Furthermore, no impact of the two administered ERr 731® doses on the E2 deprivation-induced bone loss has been evident at the end of the study.

In conclusion, the observations from previous trials regarding the endometrial safety of ERr 731® have been supported by our experimental findings that exclude a stimulatory activity on proliferation in the uterus in a long-term administration in the young adult rat but no effect on the bone mineral density could be observed.

1. Introduction

The menopausal transition of women results from a cessation of the ovarian function leading to a continuous decline of estrogen and progesterone secretion. Complaints that are associated with low serum estradiol (E2) levels are hot flushes, depression, anxiety and vaginal dryness [1]. Moreover, decreased estrogen levels have an impact on bone homeostasis by changing the balance in formation and resorption which is changed in favour of bone resorption. Due to the subsequent bone loss, the decline of endogenous E2 can result in osteoporosis. This systemic skeletal disorder is characterized by low bone mass and micro-architectural deterioration of the bone tissue which leads to a high bone fragility [2].

Beside, hormone replacement therapy (HRT) has been described to alleviate climacteric complaints and to be effective in preventing or reducing postmenopausal bone loss, however, on the other side the Million Women Study showed an increased breast cancer incidence linked to HRT treatment [3,4].

Therefore, the interest to find effective and safe alternatives for the treatment of climacteric symptoms has been grown in recent years [5,6]. In this context, plant-derived polyphenolic compounds like soy isoflavones are more and more discussed as alternatives because of their structural similarity to estrogens and their ability to initiate estrogen-dependent transcription by binding to the estrogen receptor subtypes [7].

Since the beginning of the 1990s, ERr 731®, an extract isolated from the roots of the Siberian rhubarb (*Rheum rhaponticum*), has been used regularly for the treatment of women suffering from oligomenorrhoea or amenorrhea (trade names Phyto-Strol®, Müller-Göppingen and femi-loges®, Loges). Furthermore, it has been described to be effective in the relief of menopausal symptoms in a 12-week double-blind, placebo-controlled trial in 109 perimenopausal women [8]. The total score of the Menopause Rating Scale II (MRS) was found to be significantly reduced compared to the placebo group.

The safety in regard to endometrial hyperplasia has been proven in a long-term clinical study [9].

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The main compounds of ERr 731® (drug-to-extract ratio 16–26:1 with the extraction solvent calciumoxide-to-water 1:38, mass/mass) are the hydroxystilbene glycosides rhaponticin and desoxyrhaponticin as well as their aglycones desoxyrhapontigenin and *trans*-rhapontigenin as minor constituents (both about 5%).

In recent mechanistic studies, ERr 731® was analyzed in two human cell culture models on its selectivity towards the two estrogen receptor subtypes (ERα and ERβ). The rhubarb extract caused an ERβ mediated activation of the reporter gene in human endometrial adenocarcinoma cells HEC-1B and human osteosarcoma cells U2OS. By contrast, only a weak stimulation of the ERα coupled reporter gene assay by ERr 731® could be detected [10,11].

Moreover, the estrogenic potential of ERr 731® has been tested in an uterotrophic assay in the ovariectomized (ovx) rat [12]. In these studies, subcutaneously administered ERr 731® exhibited no estrogenic potential on recovering ovariectomy-induced loss of the uterine wet weight. Beside this the observed ERβ selectivity of ERr 731® *in vitro* has been supported by the results of co-treatment with E2 [12]. The observed counteracting properties of the extract on some E2-induced responses in the uterus are in line with the Yin-Yang hypothesis of ER action suggested by Gustafsson et al. [13,14].

In addition to the utilization of the ovx rat to test for uterotrophic effects of substances it is also used as an experimental model in osteoporosis research in a modified version [15–17]. Though the rat skeleton lacks the Haversian remodeling system (intracortical remodeling) found in humans, the removal of the ovaries induces bone loss by enhancing the cancellous and endocortical bone turnover [17]. As this situation resembles human osteopenia found in peri- and postmenopause, the ovx rat can be seen as an appropriate model system in terms of osteoporosis. Bone homeostasis can be also monitored by quantification of osteocalcin and collagen I degradation fragments in the serum that are markers for bone formation and degradation respectively [18].

Peripheral quantitative computed tomography (pQCT) is a 3-dimensional imaging method that allows for densitometric analysis of the bone. Furthermore, this technique is able to distinguish between cortical and cancellous bone.

Until now, no data exist about the influence of orally administered ERr 731® on the rat uterus. Hence, the aim of the present study was to evaluate potential risks of a long-term administration of ERr 731® in terms of the endometrial hyperplasia. Therefore, the impact on the modulation of growth and proliferation in the uterus after treatment of 90 days was investigated. In parallel, we examined the potency of the rhubarb extract with regard to prevention of ovariectomy-induced bone loss in the experimental model system for postmenopausal osteoporosis.

2. Materials and methods

2.1. Substances

β-Estradiol 3-benzoate (E2benzoate) was purchased from Sigma–Aldrich (Hamburg, Germany). The Chemisch-Pharmazeutische Fabrik, Carl Müller Apotheker GmbH & Co. KG (Göppingen, Germany) provided the extract ERr 731®.

2.2. Animals

Thirty-one young female Wistar Unilever rats (150–200 g) obtained from Harlan were housed under controlled conditions (20 ± 1 °C, 50–80% of relative humidity; 12:12-h light–dark-cycles) with free access to water. Two weeks after bilateral ovariectomy (ovx, $n = 25$) or sham operation ($n = 6$) respectively, animals were randomly split into feeding groups. The sham operated animals received a phytoestrogen-free diet (Ssniff SMR/M-H, 10 mm, PE-free) purchased from Ssniff GmbH (Soest, Germany). The ovariectomized rats were divided into groups receiving a PE-free diet (unsubstituted control group), a diet containing 10 mg/kg E2benzoate (positive control group), respectively a diet containing either 1 mg/kg ERr 731® or 1 g/kg ERr 731®. After 90 days on the respective diet, animals were sacrificed by CO_2-inhalation subsequently to a light O_2/CO_2-inhalation anesthesia. Uteri were weighted and snap frozen in liquid nitrogen. Right tibiae were collected and stored in 70% ethanol after the removal of associated tissue. All animal handling and all experimental conditions were licensed and carried out according to the Institutional Animal Care and Use Committee guidelines as regulated by the German federal law governing animal welfare.

2.3. RNA preparation and mRNA quantification

Total RNA was extracted from the rat uteri using peqGOLD TriFast™ according to the manufacturer's instructions (PEQLAB Biotechnologie GmbH, Erlangen, Germany). RNAs from the same treatment group were pooled and DNA contamination was eliminated by subsequent enzymatic digestion (RQ1 DNase, Promega, Karlsruhe, Germany). Success of the DNA elimination was checked by PCR.

First-strand cDNA synthesis was performed by mixing 3 μg of digested RNA with MMLV reverse transcriptase (Promega Corp., Madison, USA) and Oligo $(dT)_{12-18}$ primers.

Quantitative real-time PCR was applied for mRNA amplification with SybrGreen I (Sigma–Aldrich, Chemie GmbH, Steinheim, Germany) as detection dye using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, München, Germany).

Expression of all genes was normalized to the housekeeping gene *cytochrome c-oxidase subunit I* (1A). The primers used for the quantitative PCR are shown in Table 1. The $\Delta\Delta C_T$ method was used for the evaluation of the relative gene expression levels [19]. The expression data of the animals of the ovx control group were defined as 1, gene expression levels of the other groups were normalized to this control group.

2.4. Measurement of bone mineral density (BMD)

BMD of the right tibiae were measured by peripheral quantitative computed tomography (pQCT; XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany).

Two slices in the proximal tibial metaphysis and one slice located in the mid-diaphysis were measured using a voxel size of 0.07 mm.

For calculations of trabecular density, 280 mg/cm^3 and 710 mg/cm^3 were used as lower and upper density thresholds. Values measured in the cross sections being higher than 710 mg/cm^3 have been considered as cortical bone.

BMD values of the proximal metaphysis were calculated as the mean of the two slices.

2.5. Quantification of bone markers

At the time of necropsy, blood samples were collected and serum was subsequently separated and stored at −20 °C. Enzyme-immunoassays were performed to determine the serum concentration of the bone formation marker osteocalcin (Rat-MID™ Osteocalcin EIA, Immunodiagnostic Systems GmbH, Frankfurt, Germany) and degradation fragments of type I collagen (RatLaps™ EIA, Immunodiagnostic Systems GmbH, Frankfurt, Germany) to assess bone resorption.

Table 1
Primer pair sequences and amplicon sizes: 1A, cytochrome-c-oxidase subunit 1A; *C3*, complement C3; *CaBP9k*, calcium-binding protein D-9k; *Clu*, clusterin; *Esr*1, estrogen receptor 1; *Esr2*, estrogen receptor 2; *Igf*1, insulin-like growth factor 1; *Igf*1*r*, insulin-like growth factor 1 receptor; *Mki67*, antigen identified by monoclonal antibody Ki67; *Pcna*, proliferating cell nuclear antigen.

Primer	Direction	Sequence	Amplicon size [bp]
1A	fwd	5′-TGA GCA GGA ATA GTA GGG ACA GC-3′	260
	rev	5′-GAG TAG AAA TGA TGG AGG AAG CA-3′	
C3	fwd	5′-ACA GCC TTC CCG GGA GCA TCA ACA-3′	275
	rev	5′-AGC GCA CCA CAG GAG GCA CAG AGT C-3′	
CaBP9k	fwd	5′-TGT CTG ACT CTG GCA GCA CTC ACT G-3′	180
	rev	5′-CCT TCA GGA GGC TGG GGA ACT CTG-3′	
Clu	fwd	5′-CCC TCC AGT CCA AGA TGC TCA ACA C-3′	302
	rev	3′′-CCA TGC GGC TTT TCC TGC GGT ATT C-3′	
Esr1	fwd	5′–GGA AGC ACA AGC GTC AGA GAG AT-3′	382
	rev	5′-AGA CCA GAC CAA TCA TCA GGA T-3′	
Esr2	fwd	5′-CTA CAG AGA GAT GGT CAA AAG TGG A-3′	215
	rev	5′-GGG CAA GGA GAC AGA AAG TAA GT-3′	
*Igf*1	fwd	5′-CTG CTT GCT CAC CTT TAC CAG-3′	212
	rev	5′-TAC ATC TCC AGC CTC CTC AGA-3′	
*Igf*1*r*	fwd	5′′-GTG GAG GAG GTG ACA GAA AAT C-3′	156
	rev	5′-CAA AGA TGG AGT TGT GAA GGA A-3′	
Mki67	fwd	5′-AAC CAG GAC TTT GTG CTC TGT AA-3′	208
	rev	5′-CTC TTT TGG CTT CCA TTT CTT C-3′	
Pcna	fwd	5′-GAG CAA CTT GGA ATC CCA GAA CAG G-3′	157
	rev	5′-CCA AGC TCC CCA CTC GCA GAA AAC T-3′	

2.6. Statistical analysis

The data of the uterine wet weight and the pQCT data are presented as mean ± standard deviation (SD). Statistical analysis included one way analysis of variance (ANOVA) followed by Bonferroni post hoc test in order to determine significant differences ($p \leq 0.05$). Statistically significant differences of the uterine gene expression profiles were tested using Student's *t*-test ($p \leq 0.05$).

3. Results

3.1. Effects on uterus and body weight

The relative uterine wet weight (UWW) in animals treated with E2benzoate through the diet was significantly higher compared to that of the ovx control animals (1482 ± 361 mg/kg vs. 230 ± 35 mg/kg bw, $p = 0.05$, Fig. 1A) while the average relative UWW of the intact animals remained almost ten times higher compared to the ovx group (2640 ± 700 mg/kg bw) after 90 days of hormonal decline. In contrast, neither of the two administered doses of ERr 731® significantly affected the relative UWW in ovx animals.

At the time of the bilateral ovariectomy, the body weight of all animals was comparable (138–146 g). Fig. 1B shows the percentage change of the body weight relative to the onset of the feeding experiment. E2benzoate reduced the ovariectomy-related hyperphagia leading to a significant lower increase in body weight compared to the control animals (22% vs. 62% increase relative to baseline). The body weight of the intact animals also increased, but to a significantly lower degree than to the ovx group (Fig. 1B). In contrast, dietary ERr 731® had no effect on the ovx-induced gain of body weight.

3.2. Uterine expression of genes associated with proliferation

The dietary administration of E2benzoate (10 mg/kg diet) resulted in a significant decrease of the mRNA expression of *Esr1* and *Esr2* in the uterus (Fig. 2A and B).

Compared to the ovx group, reduced expression rates of both ERs could also be detected in the intact animals (Fig. 2). In contrast, dietary ERr 731® did neither affect the *Esr1*- nor the *Esr2*-mRNA-expression level.

As expected, the uterine *Mki67*- and *Pcna*-mRNA-expression levels were significantly elevated in the sham animals as well as in the E2benzoate-treated animals (Fig. 3). Both ERr 731® doses had no effect on the mRNA-expression levels of these proliferation marker genes.

The uterine *C3*-mRNA-expression was significantly up-regulated in response to the E2benzoate treatment (Fig. 3C).

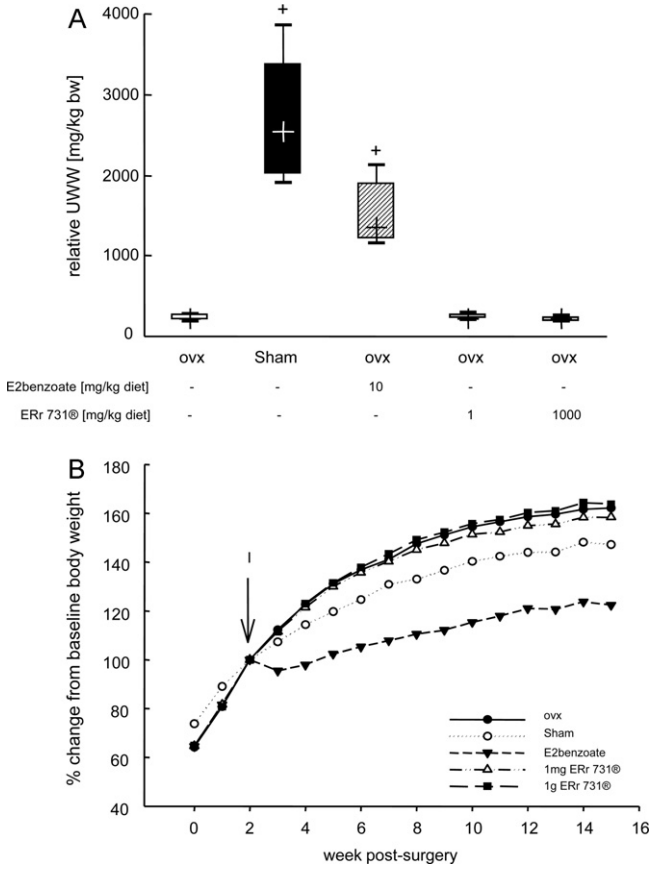

Fig. 1. Relative uterine wet weight and body weight change. (A) Uterine wet weights were determined 90 days after the onset of the feeding experiment. (B) Body weight percent change from baseline, I marks the time point when the groups were put on the accordant diet. $^{+}p \leq 0.05$ denotes statistical significance from the ovx group.

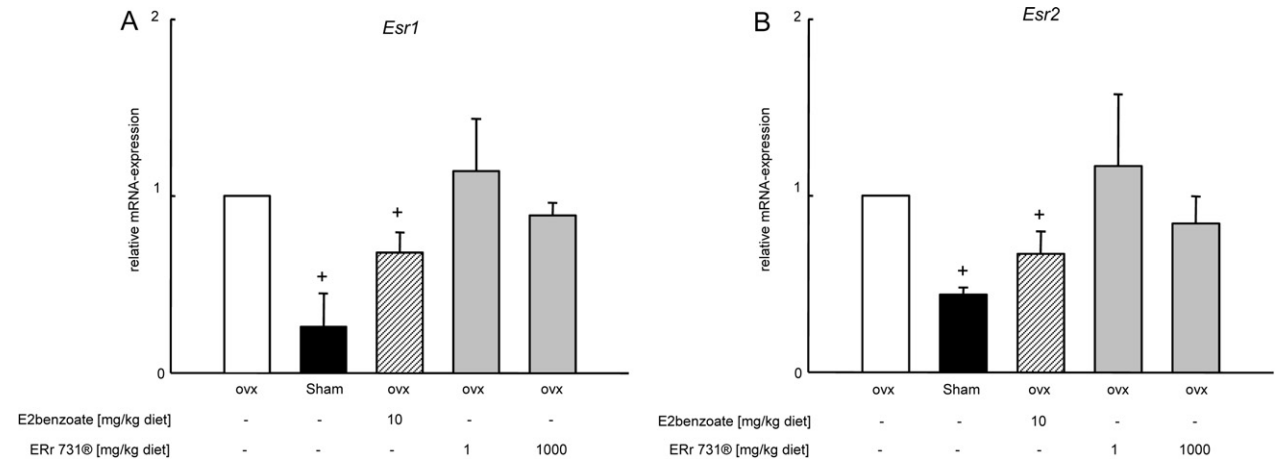

Fig. 2. Relative mRNA expression of the estrogen receptors. Uterine expression levels of *Esr1* (A) and *Esr2* (B). Statistical significance was tested by Student's *t*-test, + denotes statistical significant differences from the untreated ovx group, $p \leq 0.05$.

In contrast neither dose of the rhubarb extract influenced the uterine *C3*-mRNA-expression level compared to the untreated ovx control group. Furthermore, the mRNA expression rates of additional estrogen response genes have not been altered by ERr 731® treatment (Table 2).

3.3. Effects on the bone

Peripheral quantitative CT was used to assess mineral content and mineral mass in the tibia after the three-month feeding period to investigate potential bone sparing effects of the rhubarb extract.

The mineral density and the mineral content of the trabecular bone in the tibial metaphysis were significantly reduced 90 days after ovariectomy compared to the intact animals (Table 3). In contrast, the cortical BMC was significantly higher in the ovx control animals.

E2benzoate inhibited the ovx-induced bone loss as the BMD and the BMC of the trabecular as well as the cortical bone were significantly higher compared to the ovx control group. However, the trabecular BMD and BMC were significantly lower compared to the intact animals (326 mg/cm^3 vs. 437 mg/cm^3 and 3.90 mg vs. 5.13 mg, $p \leq 0.05$).

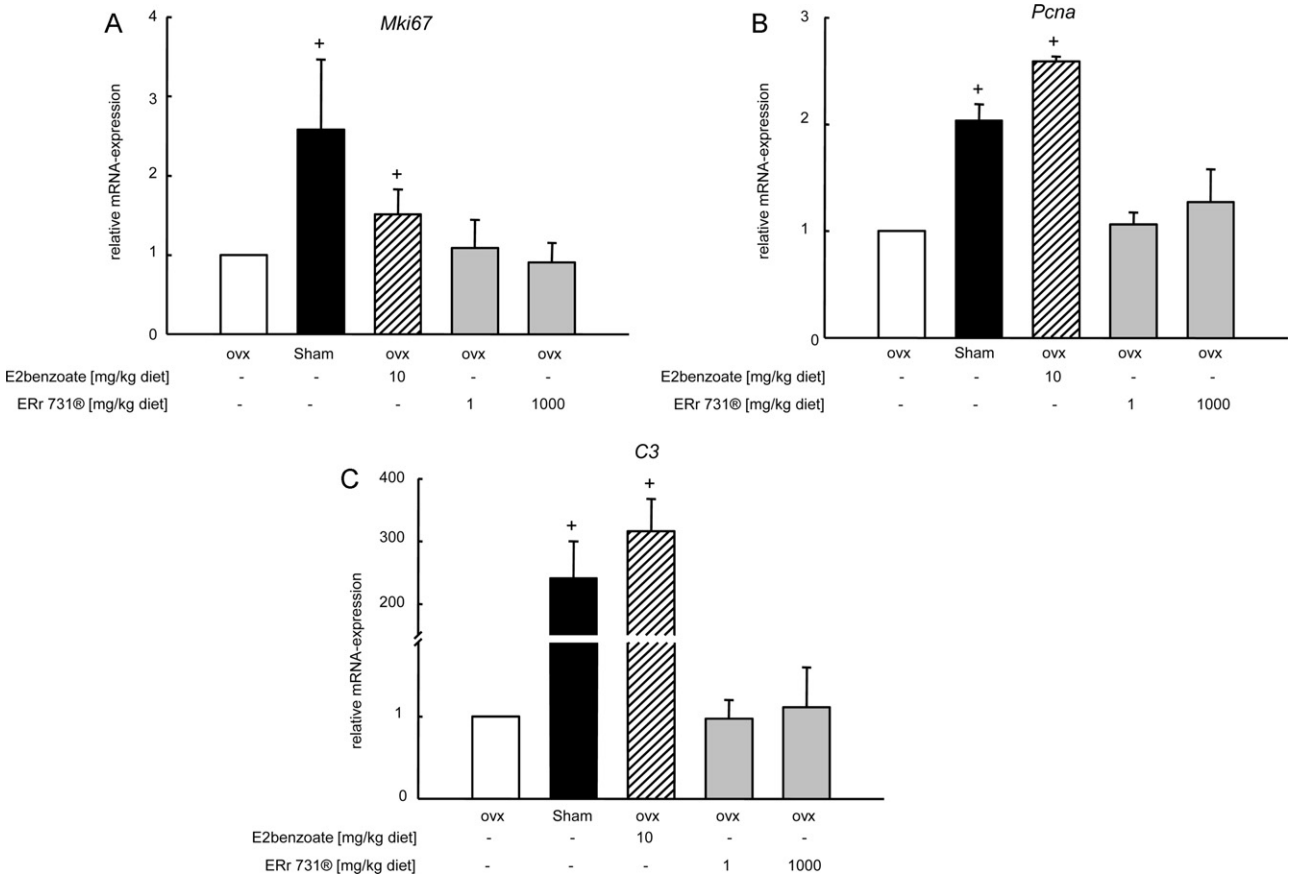

Fig. 3. Relative expression levels of proliferation markers. Effects of dietary E2benzoate (10 mg/kg diet) and ERr 731® (1 mg/kg diet and 1 g/kg diet) on the uterine mRNA expression levels of *Mki67* (A), *Pcna* (B) and complement *C3* (C). $p \leq 0.05$ indicates statistically significant differences compared to the ovx group (Student's *t*-test).

Table 2
Relative uterine expression rates of the estrogen sensitive genes. Statistical significance was tested by Student's *t*-test. *CaBP9k*, calcium-binding protein D-9k; *Clu*, clusterin; *Igf*1, insulin-like growth factor 1; *Igf*1*r*, insulin-like growth factor 1 receptor.

Genes	Sham	E2benzoate	1 mg/kg ERr 731®	1 g/kg ERr 731®
CaBP9k	82.1 ± 4.7*	118.9 ± 16.0*	0.98 ± 0.04	0.99 ± 0.4
Clu	0.41 ± 0.20*	0.39 ± 0.20*	0.88 ± 0.28	0.84 ± 0.32
*Igf*1	4.48 ± 0.97*	7.44 ± 2.87*	1.38 ± 0.52	1.10 ± 0.54
*Igf*1*r*	0.08 ± 0.08*	0.04 ± 0.01*	0.71 ± 0.23	0.54 ± 0.26*

* Statistical significance compared the untreated ovx group, $p \leq 0.05$.

Table 3
Bone mineral density (BMD [mg/cm^3]), bone mineral content (BMC [mg]) and length [mm] of the right tibia 90 days after the groups were put on PE-free diet (ovx/sham) or diet containing either 10 mg/kg E2benzoate, 1 mg/kg ERr 731® or 1 g/kg ERr 731®. pQCT was used to measure BMD and BMC of trabecular bone in the tibial metaphysis and of the cortical bone in the diaphysis. Data are shown as mean ± SD. *p*-Values shown in square brackets ([]) were calculated by one-way ANOVA and represent significant change with respect to the ovx control group ($^+p \leq 0.05$).

	ovx	Sham	E2benzoate	1 mg ERr 731®	1 g ERr 731®
Trabecular BMD [mg/cm^3]	187.94 ± 42.9	436.78 ± 43.6 [<0.001$^+$]	325.62 ± 30.3 [<0.001$^+$]	164.98 ± 55.5 [0.7085]	201.73 ± 49.3 [0.5999]
Trabecular BMC [mg]	2.42 ± 0.61	5.13 ± 1.04 [<0.001$^+$]	3.90 ± 0.55 [<0.001$^+$]	1.88 ± 0.59 [0.1283]	2.52 ± 0.63 [0.7956]
Cortical BMD [mg/cm^3]	1302.7 ± 13.7	1317.3 ± 7.5 [0.648]	1297.5 ± 25.9 [0.041$^+$]	1299.6 ± 13.1 [0.686]	1309.3 ± 8.4 [0.333]
Cortical BMC [mg]	7.05 ± 0.39	6.40 ± 0.37 [0.011$^+$]	6.31 ± 0.14 [0.001$^+$]	6.85 ± 0.46 [0.428]	7.32 ± 0.47 [0.281]
Tibial length [mm]	39.9 ± 0.7	37.7 ± 0.8 [<0.001$^+$]	38.3 ± 0.5 [<0.001$^+$]	39.5 ± 0.6 [0.329]	40.5 ± 0.8 [0.1567]

Furthermore, the tibial length was significantly reduced in the sham as well as in the E2benzoate treated animals (Table 3).

Regardless of the dose, dietary ERr 731® did not cause an effect on the bone mineral of trabecular and cortical bone examined after 90 days of administration.

The serum levels of bone homeostasis markers were comparable in ovx and sham animals, while the osteocalcin concentration was slightly reduced in the group having received E2benzoate (Table 4). Furthermore, E2benzoate significantly reduced the serum concentration of the collagen degradation marker. The serum levels of osteocalcin and RatLaps™ were not altered in the groups having received ERr 731® in comparison to the unsubstituted control animals.

4. Discussion

The safety of the rhapontic extract ERr 731® in terms of proliferative alterations in the uterus of ovariectomized rats has recently been shown by a three-day uterotrophic assay [12]. In the present study we aimed to investigate the safety of a long-term dietary administration of ERr 731® as the extract has been administered as tablets in the clinical trials [9,20–22]. Potential estrogenic effects on the uterine gene expression were tested after treatment of rats with two doses of ERr 731® over a 90-day period.

The ovx-induced estrogen deprivation was characterized by a significantly decreased uterine wet weight compared to the intact animals [23]. In contrast, the wet weight was significantly up-regulated in the E2benzoate-substituted animals which is consistent with previous findings [24].

In accordance with the three-day *s.c.* application of ERr 731® in an uterotrophic assay [12], none of the dosages chosen in this study design had an impact on the uterus wet weight. This is supported by the lack of an increase in the mRNA expression of the proliferation marker genes *Mki67* and *Pcna*. Hence, the long-term treatment with ERr 731® did not induce estrogenic growth responses in the uterus which in turn emphasizes the safety in terms of endometrial hyperplasia.

In comparison with the castrated rats, the expression levels of both estrogen receptor subtypes ERα and ERβ were significantly lower in the animals producing endogenously E2 or being substituted with E2benzoate. This effect described in literature is a consequence of a negative feedback mechanism [25].

In contrast, the treatment with the rhapontic extract did not change the uterine mRNA expression levels of the *Esr1* and *Esr2* genes significantly. This observation is in agreement with absent effects of the therapeutic doses of ERr 731® on the mRNA expression of the estrogen receptors after the three-day treatment where only pharmacological doses changed the expression [12].

By determining the mRNA level of *C3*, a classical estrogen-responsive gene whose expression remained significantly elevated in sham operated animals (240-fold) is significantly up-regulated in E2benzoate (315-fold) treated animals in this study, the estrogenic potency of a substance could be tested. In the case of ERr 731®, the mRNA expression of *C3* remained unaffected by ERr 731® application providing further evidence for excluding an estrogenic potency in the uterus.

The absence of induction of genes related to cell proliferation and growth underlines the lack of uterine wet weight gain by ERr 731®.

E2benzoate delivered through the diet was able to counteract the significant decrease of the trabecular BMD as a result of the estrogen deprivation three-months after bilateral ovariectomy.

We observed no effect of the rhubarb extract on the trabecular BMD after an administration period of 90 days. The lack of a protective effect on bone could be explained by the estrogen receptor subtype selectivity of the compounds found in ERr 731®. As shown by the usage of estrogen receptor subtype specific agonists, the bone-protective effect of Estradiol is accomplished via the ERα [26,27]. Although a weak induction of ERα-coupled luciferase activity by the rhapontic extract and its constituents has been shown in bone derived U2OS cells [11], data from that mentioned publication and another *in vitro*-study suggest that ERr 731® and its constituents are ERβ-selective [10,11]. This selectivity has been supported by the observation of the partial

Table 4
Serum levels of bone homeostasis markers osteocalcin and collagen I degradation fragments. Data are shown as mean ± SD. *p*-values are reported for the one-way ANOVA in square brackets ([]) representing significant differences from the ovx group ($p \leq 0.05$).

	ovx	Sham	E2benzoate	1 mg ERr 731®	1g ERr 731®
Osteocalcin	228.5 ± 37.6	222.9 ± 21.8 [0.7567]	187.8 ± 41.9 [0.09]	254.8 ± 17.1 [0.1456]	251.7 ± 19.5 [0.2033]
RatLaps™	16.8 ± 1.4	16.5 ± 1.5 [0.7562]	14.1 ± 2.3 [0.0262$^+$]	15.8 ± 1.0 [0.1671]	18.3 ± 1.8 [0.1088]

abolishment of the E2 induced uterotrophic effect in a three-day co-treatment [12].

The observed 62% increase of body weight 90 days post ovariectomy can be counteracted by E2benzoate (22% increase). This effect is as well described to be regulated by the activation of the ERα subtype of receptor [28,29]. In this study, the application of the rhapontic extract did not modulate the increase of body weight, which indicates furthermore a lack of ERα activating capacity as seen in the uterus and the bone. Due to the young age of the rats of about three months, even the intact animals gained about 47% of their weight. While the increase was significant compared to the unsubstituted control animals, the gain observed in the E2 depleted group was significantly higher compared to that of the sham animals.

Comparing the bone mineral parameters of the intact animals with that of the E2benzoate substituted ones it is conspicuous that the trabecular BMD and the BMC are significantly higher in the intact group ($p < 0.001$ and $p = 0.03$ respectively). A potential explanation for this could be that the intervention started two weeks after the ovariectomy meaning that the bone homeostasis had already been altered. Furthermore, as the peak bone mass of the rat is reached around the age of 10 months, the growth of the intact animals leads to higher values of BMD and BMC at the end of the study [16]. The quantification of the bone formation and resorption markers in the serum after the three-month study time also showed no significant difference between the groups of intact and the E2 deprived rats. The ovx-induced increase of both, markers of bone formation and bone resorption can be ascribed to the higher rate of bone turnover that leads to bone loss as the balance is shifted in favour of the resorption. The protective effect of the oral E2benzoate administration could be seen as both biochemical markers were significantly reduced compared to the untreated ovx group. In contrast to the ovx group, the high serum marker levels in the sham group are a sign of a higher bone turnover, in which the balance is rather in favour of bone formation as a result of growth [30].

In accordance with the failure in protecting against the ovx-induced decrease of the BMD and the BMC, ERr 731® had no impact on the serum markers of bone homeostasis.

A further response to ovariectomy was observed looking at the tibial length that was found to be enhanced compared to the sham group. An induction of the longitudinal growth rate subsequent to ovariectomy, that could be inhibited by E2 substitution has been described previously [31]. This is in accordance with our data, indicating a significantly decreased tibial length in the E2benzoate substituted group compared to the carrier treated animals ($p < 0.001$). In contrast none of the test compounds or their metabolites reduced the rate of longitudinal bone growth. The impact of the E2benzoate substitution on the tibial length shows that the animals had not reached skeletal maturity. The rat skeleton is described to be mature at the age of 10 months, thus the skeletally mature rat is described as an appropriate model for postmenopausal osteoporosis [16]. The age of the rats of the current study was approximately 2.5 months, thus they had reached sexual but not skeletal maturity at the time of the ovariectomy. The young age of the animals is one limitation of this study as the ovariectomized skeletally immature rat is rather a model for the influence of nutritional or endocrine factors on peak bone mass.

In conclusion, the data in terms of the uterine and endometrial safety of the rhapontic extract ERr 731® described in recent trials are supported by the results of the dietary long-term administration in this study. Furthermore, this study investigated ERr 731® regarding bone effects for the first time and did not detect a protective effect on ovariectomy-induced bone loss in young adult rats. Whether or not there is a potential age-related effect of ERr 731® in skeletally mature rats is subject of additional studies.

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