Bone Reports 13 (2020) 100300

Contents lists available at ScienceDirect

Bone Reports

journal homepage: www.elsevier.com/locate/bonr

Effect of dihydrotestosterone, 17-β-estrogen, genistein and equol on remodeling and morphology of bone in osteoporotic male rats during bone healing

Philipp Kauffmann[a,*], Anna Rau[c], Dana Seidlová-Wuttke[b], Hubertus Jarry[b], Boris Schminke[a], Swantje Matthes[a], Karl Günter Wiese[a]

[a] Department of Oral and Maxillofacial Surgery, University Medical Center Goettingen, Georg-August-University Goettingen, Robert-Koch-Str. 40, D-37099 Goettingen, Germany
[b] Department of Endocrinology, University Medical Center Goettingen, Georg-August-University Goettingen, Robert-Koch-Str. 40, D-37099 Goettingen, Germany
[c] Department of Department of Anesthesiology, University Medical Center Goettingen, Georg-August-University Goettingen, Robert-Koch-Str. 40, D-37099 Goettingen, Germany

ARTICLE INFO

Keywords:
Male osteoporosis
Phytoestrogens
Histomorphometry
Mineral apposition rate
Bone healing
Sex steroids

ABSTRACT

Introduction: The aim of this study was to investigate the effect of dihydrotestosterone (DHT), 17-β-estrogen (E2), genistein (GEN) and equol (EQ) on bone remodeling and bone morphology during healing of osteoporotic male rat tibiae.

Materials and methods: 180 Sprague-Dawley male rats were divided in 5 groups of 36 animals. After orchidectomy (ORX) and development of osteoporosis, trepanation of the tibia was performed. Until the time of trepanation all groups received soya free food (SF), then food change occurred and treatment started. At day 95, 102 and 151, samples were taken and histomorphometry was performed to analyze changes in bone structure under treatment. At day 33 and 70 all animals received calcein respective alizarin for polychrome bone labeling.

Results: The cortical bone was particularly affected. Treatment with DHT and E2 led to a significant long-term expansion of the thickness of the diaphyseal cortical bone, while the phytoestrogens EQ and GEN only had a positive short-term effect in this area. Only E2 preserved the trabecular bone for a limited time. In all groups, periosteal and endosteal bone areas showed the highest bone formation activity. The osteoporotic male injured bone shows a shift in mineral apposition rate (MAR) from periosteal to endosteal bone in the SF, DHT and E2 groups but not in the GEN and EQ phytohormones groups. An MAR decrease in trabecular bone formation was observed at day 70 in all groups except the E2 group.

Conclusion: We conclude from our results that healing of cortical bone defects in a rat model of male osteoporosis are mainly influenced by the estrogen pathway. Nevertheless, effects via purely androgenic mechanisms can also be demonstrated. The role of a phytohormone therapy is only marginal and if only useful for a short-term supportive approach. The role of the periosteal to endosteal shift during male osteoporotic bone healing needs to be further examined.

1. Introduction

In recent decades, osteoporosis has received increasing attention from scientists. Previously, osteoporosis was seen as a physiologically unavoidable part of aging. Meanwhile, there have been established expert groups who dedicated themselves to exploring osteoporosis, not least because of its economic consequences and frequency (Pfeilstifter, 2008). Seven million eight hundred thousand Germans suffer from osteoporosis (Haussler et al., 2007). Osteoporosis is one of the 10 most-frequently occurring and therefore most expensive diseases worldwide (Seeman, 2003). Adequate prevention, diagnosis and therapeutic concepts for the treatment and avoidance of the extensive consequences of this disease are therefore absolutely needed (Pfeilstifter, 2008).

Animal studies have demonstrated that estrogen and testosterone have stimulatory effects on bone in male rodents (Khosla et al., 2011). In the past, it was believed that the preservation of bone mass in women was controlled by estrogen and in men by testosterone. This belief has been relativized over the years and it has been suggested that bone

* Corresponding author at: Robert-Koch-Strasse 40, 37099 Göttingen, Germany.
E-mail address: philipp.kauffmann@med.uni-goettingen.de (P. Kauffmann).

https://doi.org/10.1016/j.bonr.2020.100300
Received 8 February 2020; Received in revised form 20 July 2020; Accepted 23 July 2020
Available online 28 July 2020

preservation in men is also mainly influenced by estrogen (Riggs et al., 1998).

Observational studies attribute the lower incidence of osteoporosis among women in the Eastern world to a diet rich in phytoestrogens (Coxam, 2008). These phytoestrogens are plant-derived compounds that have affinity for the estrogen receptor and are able to act as either estrogen agonists or antagonists. Phytoestrogens interact with ERα and ERβ receptors. Their effects are comparable to selective estrogen receptor modulators (SERMs). Phytoestrogens interfere with estrogen biological pathways in various ways (Sehmisch et al., 2010a).

Plant-derived phytoestrogens are considered to be an alternative therapy option without many side effects (Vatanparast and Chilibeck, 2007). Genistein (GEN) and equol (EQ) are the most well-known and examined phytoestrogens, especially in female studies. GEN is found in soybeans and binds to estrogen receptors in various tissues such as bone and the uterus. GEN inhibited osteoclasts by increasing osteoprotegerin (OPG) and lowering Receptor activator of nuclear factor kappa-B ligand (RANKL) in serum (Bitto et al., 2008). EQ is the active metabolite of daidzein, a phytoestrogen also found in soybeans, and produced in the intestine by bacteria. As the metabolism of daidzein to EQ differs from individual to individual in studies, the active metabolite EQ is frequently used (Frankenfeld et al., 2005). EQ binds to ERα and ERβ and has a stronger estrogen effect than does genistein (Tousen et al., 2011). Significant increases in bone mineral density (BMD) of osteopenic mice were shown in earlier studies made by Fujioka 2004 (Fujioka et al., 2004).

Although the morbidity and mortality of osteoporosis-associated fractures are higher in men than in women, past research has focused on female osteoporosis, having a higher prevalence through post-menopause. The mechanisms of the male osteoporosis are not as well understood as they are in females. Therefore, further research on the male osteoporosis is needed.

Bone formation almost always occurs in close proximity to blood vessels and as a reaction to bone injury (Almeida et al., 2017). We chose trepanation to see how bone injury trauma affects the remodeling process in cortical and cancellous osteoporotic bone in male rats during healing.

The positive effects of female sex steroids in the treatment of female osteoporosis are known (Khosla et al., 2011; Riggs et al., 1998; Sehmisch et al., 2010a; Sehmisch et al., 2010b). Do male sex steroids in male osteoporosis during healing also have bone protective effects via the pure androgenic pathway? Is the estrogen pathway leading in its osteoprotective effect in male osteoporosis defect healing as well? How is the remodeling influenced in the cortical and in the trabecular bone by sex steroids and plant derived compounds?

To shed more light on the above-mentioned questions we have compared the pure androgenic and the estrogenic pathway during the remodeling of defects in osteoporotic male rat tibiae.

To study the pure androgenic pathway, we used 5α-dihydrotestosterone (DHT), which is not converted to estrogen by aromatase, so as not to falsely attribute positive estrogenic effects to the androgenic path (Riggs et al., 1998). The classical substance groups 17-β-estrogen (E2) and the phytohormones genistein (GEN) and equol (EQ), which act via the estrogen pathway and are known from osteoporosis research in women, were compared to a substance-free (soya free, SF) control group SF.

2. Materials and methods

We used male orchiectomized Sprague-Dawleys rats as they have been seen as an adequate model for male osteoporosis research (Erben, 2001; Kalu, 1991). The predicted bone healing time in osteoporotic Sprague-Dawley rats is 90.54 days and delayed (Chen et al., 2016). That is why we have chosen a long trial period to investigate the effects of the test substances DHT, E2, GEN and EQ.

2.1. Animals and food

The present study was approved by the animal research committee and permitted by the local authorities of Lower Saxony, Brunswick, Germany (Permission No. G 43.08). Experiments were performed with 180 three-month-old male Sprague-Dawley rats (380 g) obtained from Co. Winkelmann (Winkelmann, Germany). Animals were castrated at the age of three months and 3 weeks after delivery. The rats were exposed to CO_2 and were anesthetized via an intraperitoneal injection of xylazine (Rompun®; Bayer, Germany) and ketamine (Hostaket®, Hoechst, Germany). The used dose per kilogram (kg) body weight (bw) was 10 mg xylazine and 45 mg ketamine. For 4 weeks after castration, the rats received phytoestrogen-free pelleted food (SF) with potato proteins added (ssniff SM V1354–000, ssniff, R-Z. 10 mm, Soest, Germany) in order to develop osteoporosis. A monocortical, bilateral trepanation of the distal tibia in the diaphyseal region was performed using a 1.5 mm diameter bone drill to evaluate the influence on healing. Until the time of trepanation all animals received soya free (SF) food. After trepanation, animals were divided into 5 treatment groups, and food change occurred. This was the time when the treatment started (see also Fig. 2 for the complete schedule of trial). Group 1, the vehicle control received the same soya free (SF) food as before. Group 2 received dihydrotestosterone (DHT: 1 g/kg food). Group 3 (E2) received a diet supplemented with estradiol benzoate (10 mg/kg food). Group 4 received supplementation with genistein (GEN: 1 g/kg food; 98.5% purity), supplied by Chemos (Regenstauf, Germany). Group 5 received soy-free food with the addition of a racemic mixture of equol (EQ: 400 mg/kg food; 98% purity, Changzhou Dahua Imp. & Exp. Group, China). The diet was ordered custom-made from the company. We determined the average intake of animal food per day by weighing the provided food at the beginning and end of the day. The choice of test substance dosage was based on the established concentrations of other study groups and was performed identically (Sehmisch et al., 2010a; Seidlova-Wuttke et al., 2003; Seidlova-Wuttke et al., 2008; Rachon et al., 2007; Hanada et al., 2003). At day 95, 102 and 151 twelve animals in each group were sacrificed under anesthesia.

2.2. Bone labeling

At day 33 bone labelling with calcein (BLC, 20 mg/kg, Merck, Darmstadt, Germany) and at day 70 bone labelling with alizarin-complexone (BLA; 30 mg/kg; Fluca, Germany) was performed by subcutaneous injection (see Fig. 2 for time schedule). The mineral apposition rate (MAR) was calculated by the method described by Frost and others (Frost and Villaneuva, 1960; Frost et al., 1961; Epker and Frost, 1966). Additionally, we used the diameter of the calcein and alizarin fluorochrome band to evaluate the effect of ORX before and after treatment on the mineral apposition rate (MAR). To calculate the time-dependent apposition rate (MAR = μm/day) of new bone using the width of fluorochrome band one must know how long the subcutaneously injected depot of calcein and alizarin releases these fluorochromes. To test how long the fluorochromes are available subcutaneously for labeling, we explored how long alizarin dyes the color of the rat's urine visible red. Calcein and Alizarin are eliminated by renal filtration. Alizarin dyes the color of urine visible red and so it's presence or absence in the circulation system is indicated by the urine color. So, we housed the animals after alizarin injection on white paper fleece, changed it every day and were able to see how long the fleece was dyed red (Frost and Villaneuva, 1960; Frost et al., 1961; Epker and Frost, 1966).

2.3. Histomorphometric evaluation

The rat tibiae were dehydrated in an alcohol series, embedded in methyl methacrylate and polymerized. The samples were cut into 7-μm-thick sections in a sagittal direction using a rotating desk microtome

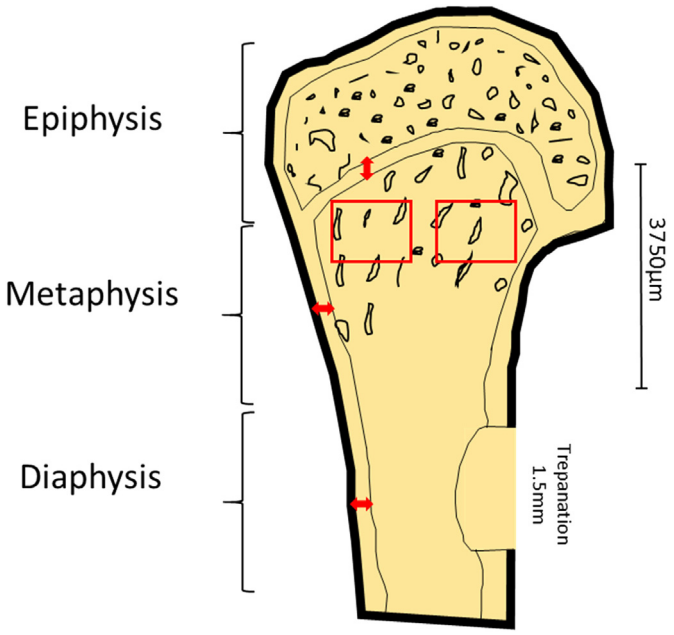

Fig. 1. Anatomic scheme of a rat tibia.
The anatomical regions are shown, the red arrows indicate the measured structures (width of the epiphyseal plate, cortical thickness of the metaphysis and cortical thickness of the diaphysis); in addition, the red boxes mark the areas of cancellous bone that were histomorphometrically evaluated. The trepanation area is also shown.

(Leica RM 2165, Germany).

The samples were placed in 96% alcohol, fixed on objective plates and stained according to Goldner (Goldner, 1938). Additionally, undyed, undecalcified sections were examined using fluorescence microscopy to acquire a series of images in order to visualize fluorochrome labelled bone areas.

We used a digitizing morphometric system consisting of a microscope with an installed camera (Axioskop 2 plus with Axio Cam MRc5, Zeiss GmbH, Germany) for digitalization. Cancellous and cortical bone was examined. Using the histomorphometry program Sigma Scan Pro 5 (SPSS Inc., USA) we analyzed and measured bone histomorphometric parameters as cancellous bone area per image and trabecular connectivity (number of trabecular nodes/area) (Figs. 1, 4A, B). From the cancellous bone area per image section we calculated the relative cancellous bone area. For this purpose, all cancellous bone surfaces per image were added and the sum was divided by the sum of all image areas and an absolute percentage value was calculated and graphically displayed as single bars without confidence intervals and as it is an absolute value did not show error bars (Fig. 4D).

Furthermore, we analyzed the cortical thickness in the metaphysis and diaphysis as well as the epiphyseal width (Figs. 1, 3A, 4A, B).

Cortical thickness was measured at 1.25× magnification. To identify the diaphyseal region, a distance of 3750 µm from the epiphyseal fugue was measured. Ten thickness measurements were taken in the diaphyseal region, another 10 in the metaphyseal region per side and finally 20 measurements in the epiphyseal fugue region (Figs. 1, 3A). To assess the cancellous bone we used a 10-fold magnification of the metaphyseal area at a distance of 500 µm from the epiphyseal plate (Fig. 1). The Sigma Scan Pro software offers the function of placing a so-called overlay (red overlay Fig. 4B) over the bone parts stained by the Goldner staining. This serves as a calculation basis for the program.

2.4. Statistics

For statistical analysis, we calculated the mean value and/or confidence intervals (95% or 98%) for each parameter using the SigmaPlot (SPSS Inc., Chicago). The effect of groups treated with hormones was tested using one-way ANOVA and a multiple comparison test versus the SF control group (Holm-Sidak-test). The significance level was chosen to be $\alpha = 5\%$.

3. Results

3.1. Treatment doses and body weight

Before the beginning of hormone treatment, the average food intake of the animal per day and rat was 21.83 g ($n = 180$). After the start of treatment, the food intake was slightly reduced (DHT = 4%; Gen = 4%; EQ = 6%; each group $n = 36$) with the exception of the E2 group that shows a food intake reduction of 20%. The SF group had no reduction. To take this into consideration the average hormone intake per animals and day was 21.2 mg DHT, 0.17 mg E2, 20.7 mg GEN and 8.2 mg EQ. The time-dependent body weight of the animals during the trial can be seen in Fig. 2.

3.2. Effects on cortical bone of the diaphysis and metaphysis

After 95 days, all test groups showed a significant increase in cortical bone thickness of the diaphyseal and metaphyseal tibia ($p \leq 0.05$). After 102 days DHT, E2, and GEN showed significantly smaller

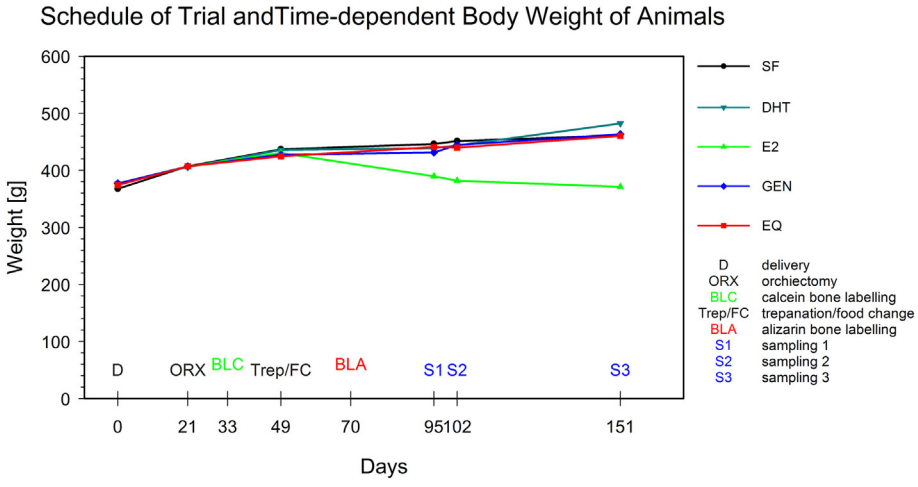

Fig. 2. Overview of study trial.
Schedule of trial and the time-dependent mean body weight of the animals during the investigation. The measurement of the body weight starts with the delivery to our facility (D) of the rats. 21 days after delivery the animals were orchiectomized (ORX) and at day 49 trepanation (Trep) of the tibial bone was performed. At that day the food was changed (FC) by adding dihydrotestosterone (DHT), 17-β-estrogen (E2), genistein (GEN) and equol (EQ) to the food of the different groups treated. The soya free (SF) group continued to receive the soya free food represents the vehicle control group. Before and after trepanation bone labelling with calcein (BLC) and alizarin-complexone (BLA) was performed at day 33 and day 70, respectively. From each of the groups samples ($n = 12$) were drawn 95, 102 and 151 days after delivery of the rats. At day 0, 21 and 49 each symbol of every group represents the mean weight of 36 animals, whereas at day 95, 102 and 151 the mean is based on n = 12 rats.

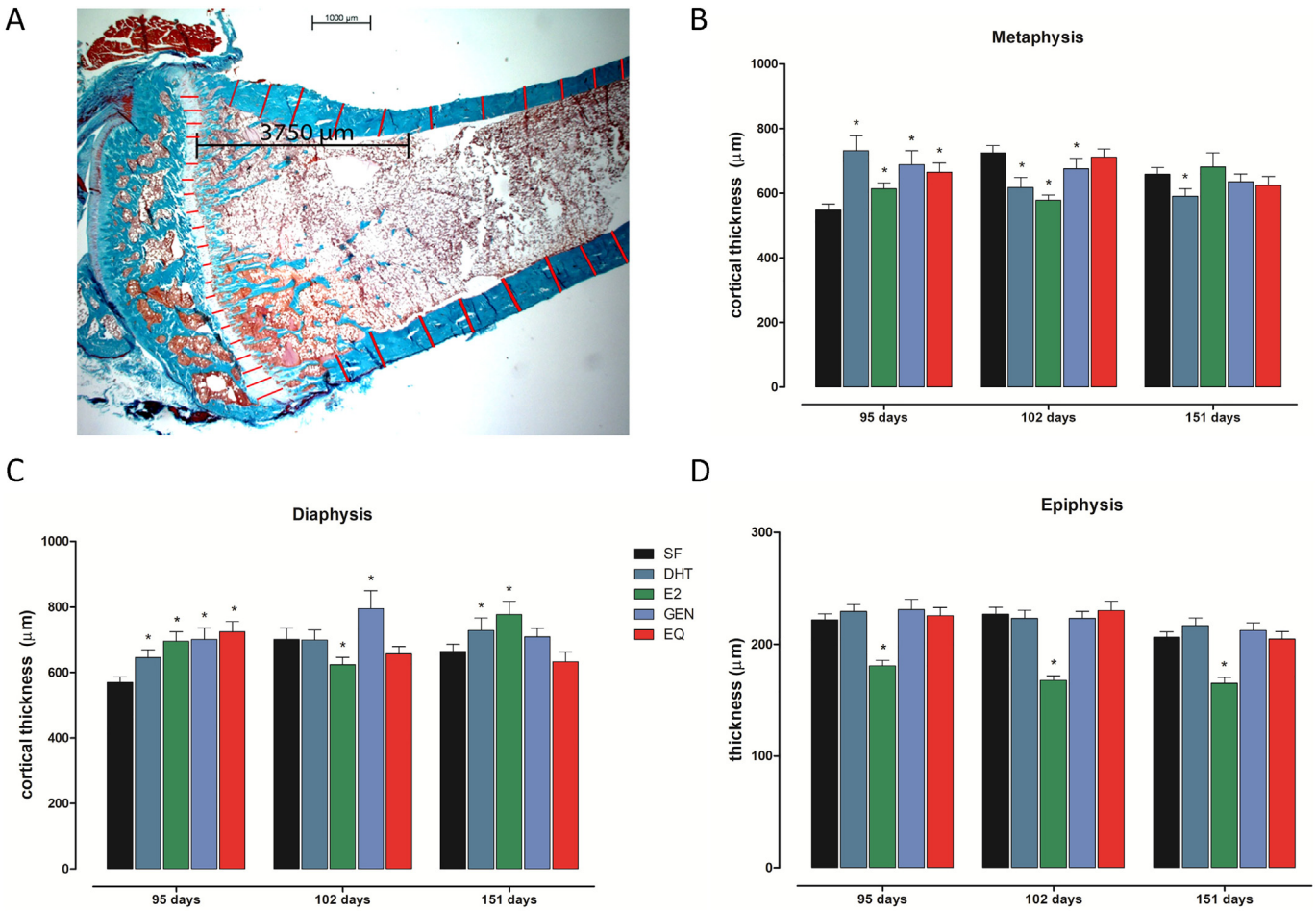

Fig. 3. Cortical and epiphysial measurements.
A) Measurements of the diaphysis, metaphysis and epiphysis with an image analysis program (see also scheme Fig. 1). Illustration of the tibia in 1.25-fold magnification in longitudinal section. The red lines mark the measuring distances (explained in the material and method section) ($200 \leq n \leq 240$ measurements). The 3750-µm zone marks the metaphysis, the zone outside the diaphysis ($200 \leq n \leq 240$ measurements). B) Mean values and 95% confidence interval of the cortical thickness of the metaphysis after 95, 102, 151 days. C) Mean values and 95% confidence interval of the cortical thickness of the diaphysis after 95, 102 and 151. D) The mean value and 95% confidence interval of the width of the epiphyseal gap after 95, 102 and 151 days ($200 \leq n \leq 240$ measurements) (*$p \leq 0.05$, ANOVA and multiple comparison of each autopsy group against the SF reference according to Holm-Sidak).

thickness of cortical bone in the metaphysis; in the diaphysis E2 showed a smaller thickness of cortical bone and GEN a thicker one. At 151, only the sex steroid groups DHT and E2 had a positive effect on the cortical bone thickness in the diaphyseal part. In the metaphyseal region, we could not find this anabolic effect of sex steroids. Moreover, the DHT group had significantly thinner cortical bone in the metaphysis. The bone thickness of the other test groups did not differ compared to the control (Fig. 3).

3.3. Effects on epiphyseal width

Analysis of the epiphysis showed a significant smaller width at all times within the E2 group ($p \leq 0{,}05$). The other test groups did not differ compared to control (Fig. 3D).

3.4. Effects on the cancellous bone

Regarding to the histomorphometric analysis relative cancellous bone area and number of trabeculae did not show an anabolic effect on bone growth during the entire treatment time of 151 days. Interestingly, at day 95, E2 and EQ had a significant higher number of trabeculae. At day 102, E2 and EQ showed a lower number of trabeculae. The relative cancellous bone area did not show a positive effect of any of the other agents regarding day 95 and 151 (Fig. 4D). In comparison to the SF control group only the E2 group showed a higher remodeling activity in cancellous bone at day 102 of the experiment but without statistical significance. DHT, GEN and EQ decreased this activity (Fig. 4D).

3.5. Bone labeling and mineral apposition rate (MAR)

Two days and a half after injection alizarin dyes the urine slightly red and afterwards the color disappears completely. Therefore, under the conditions of our experiment, the fluorochromes were available for labeling at the most for three days and the width of the calcein and alizarin band represents the apposition of new mineralized bone within these three days. At day 33 all animals were medically untreated and without bony lesions. Therefore we regarded all animals at that time as control group, so we used the green calcein band of all rats ($n = 180$) to calculate the means ($N = 2001$ measurements) of the periosteal (1.62 µm/day), endosteal (1.48 µm/day) and the trabecular (1.07 µm/day) apposition rate (Fig. 5A, B). At this time the highest remodeling can be seen in the periosteal bone area. All apposition rates differ significantly from each other. Between day 70–72 the periosteal and endosteal mineral apposition rate (MAR) increases significantly in all untreated animals of the SF control group ($p \leq 0.001$, periosteal 14%

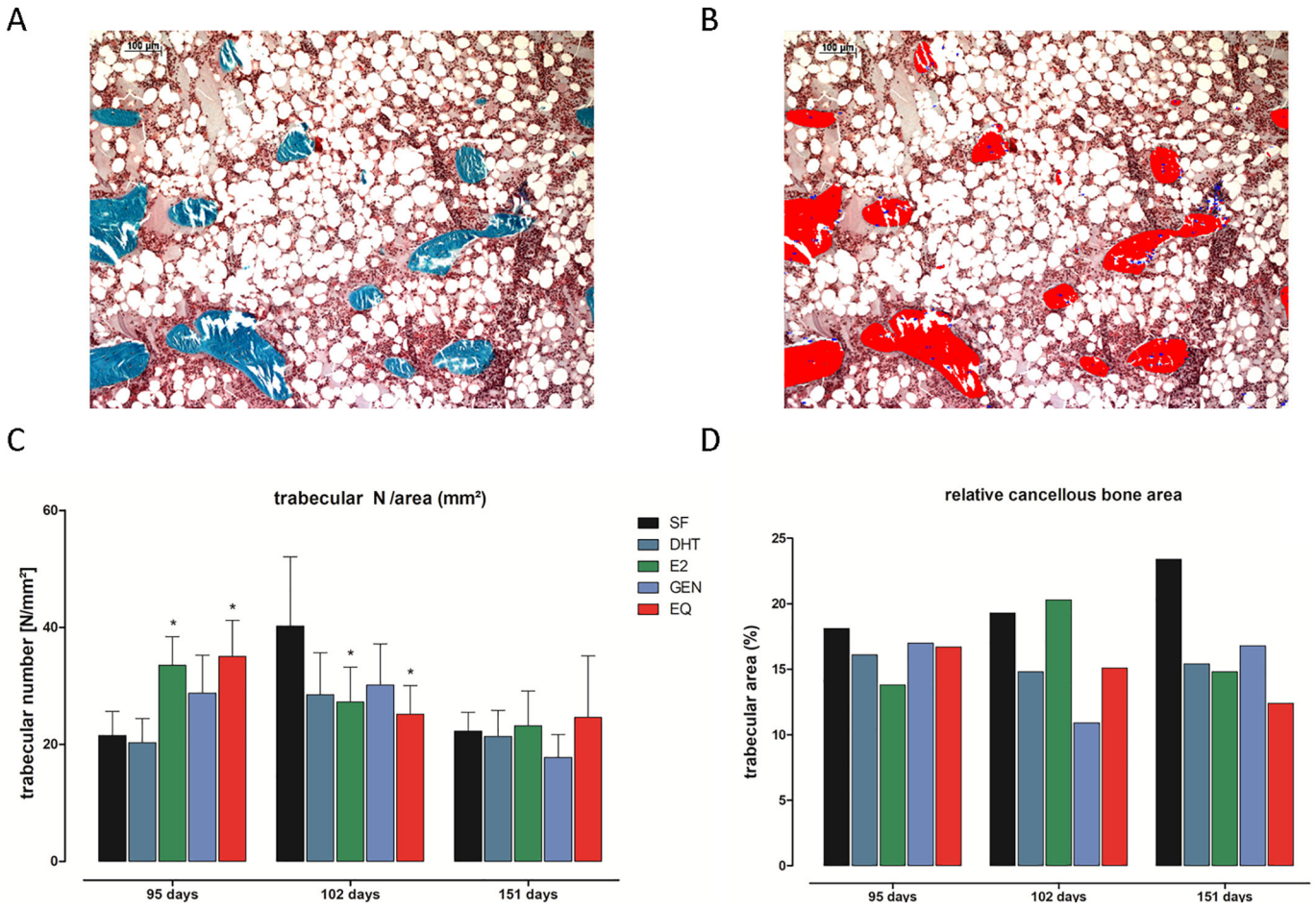

Fig. 4. Evaluation of the spongiosa.
A, B) Extract from the image analysis software. Representation of spongiosa at 10xmagnification (see also Fig. 1 for area definition represented by the red boxes), A) without red overlay, B) with red overlay. C) Mean value and 95% confidence interval of the trabecular N/area mm^2 after 95, 102 and 151 days ($20 \leq n \leq 24$ areas) D) Presentation of the relative cancellous bone area in % at day 95, 102 and 151 ($20 \leq n \leq 24$ images) (*$p \leq 0.05$, ANOVA and multiple comparison against the SF reference group according to Holm-Sidak).

increase and endosteal 25% increase) and the highest rate shifts from the periosteal to endosteal bone, whereas the trabecular rate significantly ($p \leq 0.001$) decreases from 1.07 to 0.69 µm/day of about 36% (Fig. 5B, C). Treatment using DHT, E2, GEN and EQ during bone healing after trepanation reduces the MAR in cortical bone. DHT, GEN and EQ had reduced significantly the MAR of trabecular bone. Only E2 raises significantly the MAR of trabecular bone compared to the SF control group (Fig. 5C).

4. Discussion

4.1. Animals and food

Osteoporosis is considered to be a women's disease and is therefore underdiagnosed in men (Cawthon, 2011; Lambert et al., 2011; Solimeo, 2011). Hormone replacement therapy (HRT) with estrogens, androgens and plant-derived compounds such as GEN or EQ have been well-examined in women (Sehmisch et al., 2010a; Sehmisch et al., 2010b; Kolios et al., 2009; Kostelac et al., 2003; Riesco et al., 2011). The male orchiectomized rat has been seen as an adequate model for male osteoporosis research and was therefore used here (Erben, 2001; Kalu, 1991). The proximal tibia in rats, is affected by osteoporosis in particular. Therefore, this area was the focus of our study (Wronski et al., 1985).

The most potent androgen sex hormone DHT, which is not converted by α-aromatase, was used in our study to examine only the androgenic effect on bone. Studies have shown an osteoprotective effect of DHT in cortical as well as in cancellous bone in male rats (Tramontana et al., 2001; Vandenput et al., 2002; Wakley et al., 1991; Ucer et al., 2015). In bone, ERα and ERβ receptors for estrogen are expressed (Hou et al., 2006). According to literature estrogen stimulates bone growth, leads to a closure of the epiphysis and has an antiresorptive effect on bone (Kostelac et al., 2003; Krum et al., 2008; Kuiper et al., 1998). It reduces bone loss in cancellous and cortical bone and has positive influence on bone healing (Kolios et al., 2009; Vandenput et al., 2002; Takano-Yamamoto and Rodan, 1990; Vidal et al., 2000). GEN is a plant-derived compound with a similar chemical structure as estrogen. This leads to a capacity to bind to ERα and ERβ (Rickard et al., 2003). The affinity to ERβ is stronger (Kuiper et al., 1998). Studies have shown antiresorptive effects on bone (Fanti et al., 1998). Bone quality is influenced in a positive way by increasing OPG in serum and reducing RANKL (Bitto et al., 2008). The estrogenic effects on the breast and uterus seem to be weak. Therefore, it is thought to be an alternative therapy option to hormone replacement therapy for the treatment of the female osteoporosis (Sehmisch et al., 2010a; Wuttke et al., 2010). EQ is the second plant-derived compound we used and is a metabolite of daidzein. As the metabolism rate and therefore bioactivity varies interindividually (Frankenfeld et al., 2005), we avoided the metabolism process by using the bioactive agent EQ. EQ binds to ERα and ERβ in a stronger way than does GEN and is said to have a stronger

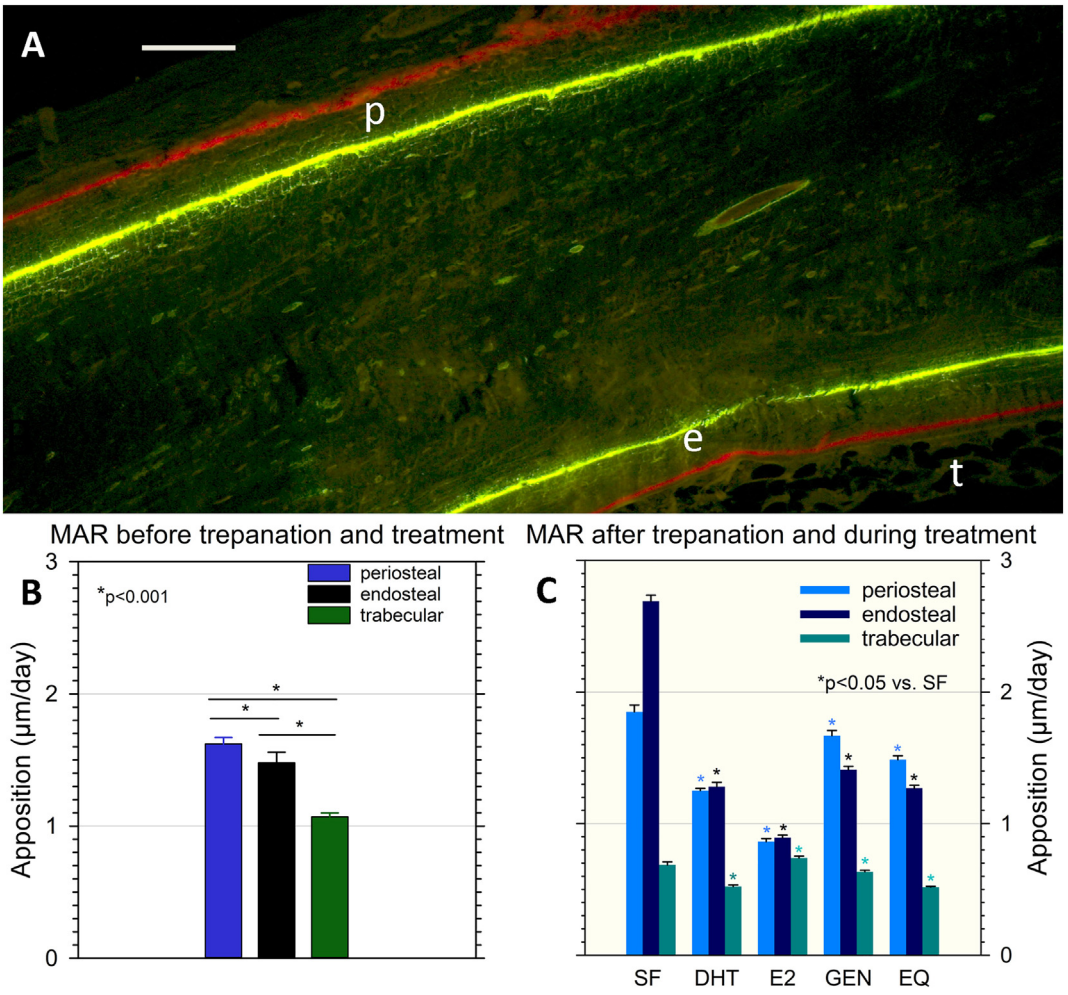

Fig. 5. Bone labeling and mineral apposition rate.
A) Fluorescence bone labeling with calcein and alizarin red, periosteal (p), endosteal (e) and trabecular (t). B) Presented are the mean values and the 99% confidence interval of the diameter of calcein bands before treatment. The vertical bars represent the apposition rate per day before trepanation and treatment ($n = 2001$, $^*p \leq 0.05$, ANOVA and multiple comparison within the SF Reference group according to Holm-Sidak). C) Apposition rate after trepanation and treatment (µm/day) of the SF, DHT, E2, GEN and EQ group ($322 \leq n \leq 402$, $^*p \leq 0.05$, ANOVA and multiple comparison against the corresponding category of SF Reference group according to Holm-Sidak).

estrogenic effect than does GEN (Tousen et al., 2011).

4.2. Body weight

Considering the development of body weight, two core observations can be made. First, the DHT group showed the highest weight gain of all groups over the entire trial period. The weight gain after testosterone application has already been described in literature and could be confirmed in our study (DHT) as well (Gentry and Wade, 1976; Vanderschueren et al., 2014). Second, the E2 group showed a steady loss of body mass. The catabolic effect of E2 in our study can be explained by the observed food intake reduction. An appetite suppressant effect of E2 was also observed by Vandenput et al. (Vandenput et al., 2002) and has been described identically in other studies (Stuermer et al., 2009). Furthermore, postmenopausal studies have shown that estrogen deficiency or estrogen resistance lead to marked obesity or weight gain as the appetite suppressant effect of E2 is missing (Genazzani and Gambacciani, 2006; MacGillivray et al., 1998). The other groups (GEN and EQ) behaved similarly to the reference group SF. They steadily increased in body mass. Consideration of the weight development confirms that the phytohormones in male rats have no appetite suppressing effect. GEN and EQ do not appear to have an additional anabolic effect compared to the control SF. This was already observed in 2008 by Santollo et al. (Santollo and Eckel, 2008).

4.3. Effects on cortical bone of the diaphysis and metaphysis

DHT had an osteoprotective effect in the cortical diaphysis and increased the cortical thickness of the diaphysis throughout the entire study period which is in accordance to other results (Vanderschueren et al., 2014). In the metaphysis, this positive effect of DHT could only be observed in the beginning at day 95. The effect of DHT on day 102 is catabolic in the metaphysis and diaphysis and thus identical to E2, which can be interpreted as increased bone remodeling under DHT and E2 influence. Throughout the whole study period in the metaphysis, DHT led to thinner cortical bone. Laurent et al. 2016 showed in osteoporotic male mice that DHT cannot prevent bone loss in the cortical bone either (Laurent et al., 2016). What we know from other studies is that fracture healing and response to sexual steroids vary by location (Thormann et al., 2014). Therefore, we saw long-term osteoprotective effects in the cortical bone of the diaphysis but not in the metaphysis. We do not consider direct stimulation of E2 receptors to be causal because we used non-aromatase-converted DHT (Vanderschueren et al., 2014). Previous studies have also suggested site-specific effects and indirect actions of DHT on some other cell types or tissues which do not require AR or ERα signaling in any cell type across the osteoblast or

osteoclast differentiation lineage (Ucer et al., 2015).

In our study, E2 reduced bone loss in cortical bone, especially in the diaphysis, and had even a stronger effect than DHT in this area. In the metaphysis, E2 only showed short-term positive effects analogous to DHT as we have seen them in the diaphysis before but not as strong. ER receptors have been reported to be localized in fracture callus (Cheung et al., 2016). The explanation for the particularly high effect of E2 in the diaphysis can be seen in the ER receptor expression during bone healing, since we performed the trepanation in the diaphysis (Vanderschueren et al., 2014; Cheung et al., 2016). Osteoblasts, osteocytes and osteoclasts are mainly involved in the remodeling process of bone. They are influenced by sexual steroids and can lead to bone loss and also to bone formation. This complex process is controlled by osteocytes and their mechanoreceptors. This process has already been described by Almeida et al. 2017 (Almeida et al., 2017). An imbalance in this system leads to pathological states of the bone. Bone formation almost always occurs in the immediate vicinity of blood vessels. In addition, perivascular cells serve both as mesenchymal skeletal stem cells and for establishing the hematopoietic stem cell niche (Almeida et al., 2017). Mesenchymal skeletal stem cells are the precursors for osteoblasts and the hematopoietic stem cells serve as precursors for osteoclasts. An inflammatory irritation or an irritating stimulus as our trepanation in the diaphysis can, under additional hormonal influence, trigger anabolic as well as catabolic effects in the vicinity of the bone injury (Almeida et al., 2017). This could explain the partly anabolic, partly catabolic effect of the E2 in the metaphysis and diaphysis.

The plant compounds that bind to ER receptors also appear to have a stimulatory effect, which is limited to day 95 and 102 of our study. GEN is more effective than EQ on day 102 and the E2 effect in the diaphysis and metaphysis on day 102 is temporarily even catabolic in contrast to the phytohormones. This result shows the dynamic and precise timing of regulatory proteins such as the sexual steroid E2 for bone metabolism in the healing and remodeling phase.

In our study, GEN had a short-term positive influence on the cortical diaphysis and metaphysis. For EQ we observed an osteoprotective short-term effect, especially in the cortical bone of the diaphysis and metaphysis. Other working groups found a positive influence on bone healing in female rats, especially in the cortical metaphysis (Kolios et al., 2009).

4.4. Effects on epiphyseal width

The closure of the epiphysis could also been seen in our study and is a control for the effectiveness of E2 in this area (Vidal et al., 2000; Nilsson and Baron, 2005). DHT did not have any effect on the epiphysis because DHT was not converted by α-aromatase into estrogen and there for does not bind to ERα receptors. GEN und EQ did not show any effect on the epiphyseal width either. The ER receptors of the epiphysis seem to be very specific for estrogen and not for not for analogues.

4.5. Effects on cancellous bone

DHT did not influence positively cancellous bone in our study. This is in contrast to other studies that reported osteoprotective effects even in cancellous bones (Ucer et al., 2015; Khosla et al., 2006).

In cancellous bone, E2 led to a higher number of trabecular nodes but could not prevent bone loss in this area either. However, it should be noted that the interpretation of the E2 effect on cancellous bone can be obscured by weight loss. This could lead to an underestimation of the positive E2 trend, since despite weight loss and thus less stress on the bone, the bone surface remains the same and the branching temporarily even increases in the sense of an increased remodeling. Without weight loss, a more positive influence of E2 would have been easier to observe. Vandenput et al. observed an appetite-suppressing effect, as mentioned above, but without weight loss and was therefore able to observe the positive effects on cancellous bone more easily (Vandenput et al., 2002).

The cancellous bone was not influenced by GEN. It seems as if GEN does not have a stimulating effect on the ERα receptors in these areas. EQ is said to have a stronger effect on cancellous bone (Rachon et al., 2007). In our study, this stronger effect on the cancellous bone could not be seen. Only a short-term positive effect on the trabecular structure was seen at day 95 but changed to a negative one at day 102 which could be a sign for a higher remodeling through EQ.

4.6. Bone labeling and mineral apposition rate

The fluorochromes calcein and alizarin are chelating agents and form complex molecules with Ca^{2+}. The intensity of their fluorescence light is an indirect measure of the calcium ion concentration in the new area of mineralisation and, therefore, the diameter of the fluorochrome band allows measuring the time-dependent apposition of new formed bone. In our case fluorochrome labeling of new formed bone only lasts three days, i. e. as long as the subcutaneously injected depot releases calcein or alizarin into the circulation system. These findings allow calculating the MAR not only from the area intermediate between the fluorochrome bands but also from the bands itself, which makes it easier to study bone remodeling (Frost et al., 1961; Epker and Frost, 1966). The circumstance of the time-limited fluorescence marking of 3 days is important for the interpretation of the apparent contradiction of a lowered MAR in the test groups DHT, E2, GEN and EQ compared to the control group SF. The determination of the MAR after trepanation was only performed between days 70–72, but the first sample collection for histomorphometric examinations was obtained much later, on day 95. During this period of time, especially the sex hormones seem to cause a significant increase in cortical thickness despite the lower MAR in the period of 3 days between days 70–72. Again, we see a strong difference in the time of measurement during remodeling. This again shows the dynamic of the remodeling process and the partially diverging effects of the test substances DHT and E2 (Almeida et al., 2017).

In osteoporotic bone, defect healing alters bone remodeling as our results show. In comparison to the uninjured status of bone and in the initial phase of the developing osteoporosis bone formation is higher in cortical and lower in trabecular bone areas. In uninjured bone periosteal bone has the highest MAR followed by endosteal and trabecular bone. If injury, like our trepanation of the tibial bone, occurs remodeling increases and the highest MAR now shifts to endosteal bone and trabecular bone formation decreases to a remarkable extent. Under treatment with sex steroid like DHT or E2 this shift stays the same but the phytohormones do not show this shifting.

4.7. Limitations

The limitations of our study are that we do not present bone turnover makers or micro CT data and did not present data on seminal vesicle and levator ani weights of the rats.

Furthermore, we saw different effects of DHT in our study on cortical and cancellous bone compared to others.

5. Conclusion

We conclude from our results that osteoporotic long bones are also mainly influenced by the estrogen pathway in male healing. Nevertheless, effects via purely androgenic mechanisms can also be demonstrated. By using testosterone the positive androgen effects and the positive estrogen effects through an α aromatase conversion of testosterone could be used, but cardiovascular events, polycythemia and stimulation of prostate tissue must be considered.

The role of a phytohormone therapy is only marginal and if only useful for a short-term supportive approach. Furthermore, the role of the periosteal to endosteal shift during male osteoporotic bone healing needs to be further examined. Further CCTs and RCT's are needed to

examine the effects in human patients, and the hormone-related adverse effects.

CRediT authorship contribution statement

Philipp Kauffmann: Investigation, Data curation, Writing - original draft, Writing - review & editing. **Anna Rau:** Investigation, Writing - original draft. **Dana Seidlová-Wuttke:** Methodology, Writing - original draft. **Hubertus Jarry:** Methodology, Writing - original draft. **Boris Schminke:** Investigation, Writing - original draft. **Swantje Matthes:** Investigation, Data curation. **Karl Günter Wiese:** Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

We gratefully acknowledge Sabine Lüdemann, Heidi Brüggemann-Meyer and Antje Ahrbecker for their technical assistance. We are indebted to Adrian Cogill for reviewing the manuscript in his native language. The study was supported by the grant No. JA398/10-1 of the German Research Society (DFG). We acknowledge support by the Open Access Publication Funds of the Göttingen University.

References

Almeida, M., Laurent, M.R., Dubois, V., Claessens, F., O'Brien, C.A., Bouillon, R., Vanderschueren, D., Manolagas, S.C., 2017. Estrogens and androgens in skeletal physiology and pathophysiology. Physiol. Rev. 97 (1), 135–187.

Bitto, A., Burnett, B.P., Polito, F., Marini, H., Levy, R.M., Armbruster, M.A., Minutoli, L., Di Stefano, V., Irrera, N., Antoci, S., Granese, R., Squadrito, F., Altavilla, D., 2008. Effects of genistein aglycone in osteoporotic, ovariectomized rats: a comparison with alendronate, raloxifene and oestradiol. Br. J. Pharmacol. 155 (6), 896–905.

Cawthon, P.M., 2011. Gender differences in osteoporosis and fractures. Clin. Orthop. Relat. Res. 469 (7), 1900–1905.

Chen, L., Yang, L., Yao, M., Cui, X.J., Xue, C.C., Wang, Y.J., Shu, B., 2016. Biomechanical characteristics of osteoporotic fracture healing in ovariectomized rats: a systematic review. PLoS One 11 (4), e0153120.

Cheung, W.H., Miclau, T., Chow, S.K., Yang, F.F., Alt, V., 2016. Fracture healing in osteoporotic bone. Injury 47 (Suppl. 2), S21–S26.

Coxam, V., 2008. Phyto-oestrogens and bone health. Proc. Nutr. Soc. 67 (2), 184–195.

Epker, B.N., Frost, H.M., 1966. Periosteal appositional bone growth from age two to age seventy in man. A tetracycline evaluation, Anat Rec 154 (3), 573–577.

Erben, R.G., 2001. Skeletal effects of androgen withdrawal. J. Musculoskelet. Neuronal Interact. 1 (3), 225–233.

Fanti, P., Monier-Faugere, M.C., Geng, Z., Schmidt, J., Morris, P.E., Cohen, D., Malluche, H.H., 1998. The phytoestrogen genistein reduces bone loss in short-term ovariectomized rats. Osteoporos. Int. 8 (3), 274–281.

Frankenfeld, C.L., Atkinson, C., Thomas, W.K., Gonzalez, A., Jokela, T., Wahala, K., Schwartz, S.M., Li, S.S., Lampe, J.W., 2005. High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart. Br. J. Nutr. 94 (6), 873–876.

Frost, H.M., Villaneuva, A.R., 1960. Tetracycline staining of newly forming bone and mineralizing cartilage in vivo. Stain. Technol. 35, 135–138.

Frost, H.M., Villanueva, A.R., Roth, H., Stanisavljevic, S., 1961. Tetracycline bone labeling. J New Drugs. 1, 206–216.

Fujioka, M., Uehara, M., Wu, J., Adlercreutz, H., Suzuki, K., Kanazawa, K., Takeda, K., Yamada, K., Ishimi, Y., 2004. Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. J. Nutr. 134 (10), 2623–2627.

Genazzani, A.R., Gambacciani, M., 2006. Effect of climacteric transition and hormone replacement therapy on body weight and body fat distribution. Gynecol. Endocrinol. 22 (3), 145–150.

Gentry, R.T., Wade, G.N., 1976. Androgenic control of food intake and body weight in male rats. J Comp Physiol Psychol 90 (1), 18–25.

Goldner, J., 1938. A modification of the masson trichrome technique for routine laboratory purposes. Am. J. Pathol. 14 (2), 237–243.

Hanada, K., Furuya, K., Yamamoto, N., Nejishima, H., Ichikawa, K., Nakamura, T., Miyakawa, M., Amano, S., Sumita, Y., Oguro, N., 2003. Bone anabolic effects of S-40503, a novel nonsteroidal selective androgen receptor modulator (SARM), in rat models of osteoporosis. Biol. Pharm. Bull. 26 (11), 1563–1569.

Haussler, B., Gothe, H., Gol, D., Glaeske, G., Pientka, L., Felsenberg, D., 2007. Epidemiology, treatment and costs of osteoporosis in Germany—the BoneEVA study. Osteoporos. Int. 18 (1), 77–84.

N.N. Hou, Y.M. Zhu, H.F. Huang, [The expression of estrogen receptor alpha and beta in the intervention of different estrogens in rat bone metabolism], Fen Zi Xi Bao Sheng Wu Xue Bao 39(4) (2006) 289–96.

Kalu, D.N., 1991. The ovariectomized rat model of postmenopausal bone loss. Bone and mineral 15 (3), 175–191.

Khosla, S., Riggs, B.L., Atkinson, E.J., Oberg, A.L., McDaniel, L.J., Holets, M., Peterson, J.M., Melton III, L.J., 2006. Effects of sex and age on bone microstructure at the ultradistal radius: a population-based noninvasive in vivo assessment. J. Bone Miner. Res. 21 (1), 124–131.

Khosla, S., Melton III, L.J., Riggs, B.L., 2011. The unitary model for estrogen deficiency and the pathogenesis of osteoporosis: is a revision needed? J. Bone Miner. Res. 26 (3), 441–451.

Kolios, L., Sehmisch, S., Daub, F., Rack, T., Tezval, M., Stuermer, K.M., Stuermer, E.K., 2009. Equol but not genistein improves early metaphyseal fracture healing in osteoporotic rats. Planta Med. 75 (5), 459–465.

Kostelac, D., Rechkemmer, G., Briviba, K., 2003. Phytoestrogens modulate binding response of estrogen receptors alpha and beta to the estrogen response element. J. Agric. Food Chem. 51 (26), 7632–7635.

Krum, S.A., Miranda-Carboni, G.A., Hauschka, P.V., Carroll, J.S., Lane, T.F., Freedman, L.P., Brown, M., 2008. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. EMBO J. 27 (3), 535–545.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139 (10), 4252–4263.

Lambert, J.K., Zaidi, M., Mechanick, J.I., 2011. Male Osteoporosis: epidemiology and the pathogenesis of aging bones. Curr. Osteoporos. Rep. 9, 229.

Laurent, M.R., Jardí, F., Dubois, V., Schollaert, D., Khalil, R., Gielen, E., Carmeliet, G., Claessens, F., Vanderschueren, D., 2016. Androgens have antiresorptive effects on trabecular disuse osteopenia independent from muscle atrophy. Bone 93, 33–42.

MacGillivray, M.H., Morishima, A., Conte, F., Grumbach, M., Smith, E.P., 1998. Pediatric endocrinology update: an overview. The essential roles of estrogens in pubertal growth, epiphyseal fusion and bone turnover: lessons from mutations in the genes for aromatase and the estrogen receptor, Horm Res 49 (Suppl. 1), 2–8.

Nilsson, O., Baron, J., 2005. Impact of growth plate senescence on catch-up growth and epiphyseal fusion. Pediatr. Nephrol. 20 (3), 319–322.

Pfeilstifter, J., 2008. Leitlinienbasierte Prävention. Diagnostik und Therapie, Schattauer Verlag, Stuttgart.

Rachon, D., Seidlova-Wuttke, D., Vortherms, T., Wuttke, W., 2007. Effects of dietary equol administration on ovariectomy induced bone loss in Sprague-Dawley rats. Maturitas 58 (3), 308–315.

Rickard, D.J., Monroe, D.G., Ruesink, T.J., Khosla, S., Riggs, B.L., Spelsberg, T.C., 2003. Phytoestrogen genistein acts as an estrogen agonist on human osteoblastic cells through estrogen receptors alpha and beta. J. Cell. Biochem. 89 (3), 633–646.

Riesco, E., Choquette, S., Audet, M., Tessier, D., Dionne, I.J., 2011. Effect of exercise combined with phytoestrogens on quality of life in postmenopausal women. Climacteric 14 (5), 573–580.

Riggs, B.L., Khosla, S., Melton 3rd, L.J., 1998. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. J. Bone Miner. Res. 13 (5), 763–773.

Santollo, J., Eckel, L.A., 2008. Estradiol decreases the orexigenic effect of neuropeptide Y, but not agouti-related protein, in ovariectomized rats. Behav. Brain Res. 191 (2), 173–177.

Seeman, E., 2003. Invited review: pathogenesis of osteoporosis. J. Appl. Physiol. 95 (5), 2142–2151.

Sehmisch, S., Erren, M., Kolios, L., Tezval, M., Seidlova-Wuttke, D., Wuttke, W., Stuermer, K.M., Stuermer, E.K., 2010a. Effects of isoflavones equol and genistein on bone quality in a rat osteopenia model. Phytotherapy research: PTR 24 (Suppl. 2), S168–S174.

Sehmisch, S., Uffenorde, J., Maehlmeyer, S., Tezval, M., Jarry, H., Stuermer, K.M., Stuermer, E.K., 2010b. Evaluation of bone quality and quantity in osteoporotic mice—the effects of genistein and equol. Phytomedicine: international journal of phytotherapy and phytopharmacology 17 (6), 424–430.

Seidlova-Wuttke, D., Hesse, O., Jarry, H., Christoffel, V., Spengler, B., Becker, T., Wuttke, W., 2003. Evidence for selective estrogen receptor modulator activity in a black cohosh (Cimicifuga racemosa) extract: comparison with estradiol-17beta. Eur. J. Endocrinol. 149 (4), 351–362.

Seidlova-Wuttke, D., Jarry, H., Jager, Y., Wuttke, W., 2008. Bone development in female rats maintained with soy-free or soy-containing food as determined by computer-assisted tomography and serum bone markers. J. Bone Miner. Metab. 26 (4), 321–327.

Solimeo, S.L., 2011. Living with a `women's disease': risk appraisal and management among men with osteoporosis. J Mens health 8 (3), 185–191.

Stuermer, E.K., Sehmisch, S., Tezval, M., Tezval, H., Rack, T., Boekhoff, J., Wuttke, W., Herrmann, T.R., Seidlova-Wuttke, D., Stuermer, K.M., 2009. Effect of testosterone, raloxifene and estrogen replacement on the microstructure and biomechanics of metaphyseal osteoporotic bones in orchiectomized male rats. World J. Urol. 27 (4), 547–555.

Takano-Yamamoto, T., Rodan, G.A., 1990. Direct effects of 17 beta-estradiol on trabecular bone in ovariectomized rats. Proc. Natl. Acad. Sci. U. S. A. 87 (6), 2172–2176.

Thormann, U., El Khawassna, T., Ray, S., Duerselen, L., Kampschulte, M., Lips, K., von Dewitz, H., Heinemann, S., Heiss, C., Szalay, G., Langheinrich, A.C., Ignatius, A., Schnettler, R., Alt, V., 2014. Differences of bone healing in metaphyseal defect fractures between osteoporotic and physiological bone in rats. Injury 45 (3), 487–493.

Tousen, Y., Abe, F., Ishida, T., Uehara, M., Ishimi, Y., 2011. Resistant starch promotes equol production and inhibits tibial bone loss in ovariectomized mice treated with daidzein. Metabolism 60 (10), 1425–1432.

Tramontana, J., Benghuzzi, H., Tucci, M., Tsao, A., Hughes, J., 2001. Morphometric analysis of cortical bone upon the exposure to sustained delivery of anabolic promoting agents using adult male rats as a model. Biomed. Sci. Instrum. 37, 293–298.

Ucer, S., Iyer, S., Bartell, S.M., Martin-Millan, M., Han, L., Kim, H.N., Weinstein, R.S., Jilka, R.L., O'Brien, C.A., Almeida, M., Manolagas, S.C., 2015. The effects of androgens on murine cortical bone do not require AR or ERalpha signaling in osteoblasts and osteoclasts. J. Bone Miner. Res. 30 (7), 1138–1149.

Vandenput, L., Boonen, S., Van Herck, E., Swinnen, J.V., Bouillon, R., Vanderschueren, D., 2002. Evidence from the aged orchidectomized male rat model that 17beta-estradiol is a more effective bone-sparing and anabolic agent than 5alpha-dihydrotestosterone. J. Bone Miner. Res. 17 (11), 2080–2086.

Vanderschueren, D., Laurent, M.R., Claessens, F., Gielen, E., Lagerquist, M.K., Vandenput, L., Borjesson, A.E., Ohlsson, C., 2014. Sex steroid actions in male bone. Endocr. Rev. 35 (6), 906–960.

Vatanparast, H., Chilibeck, P.D., 2007. Does the effect of soy phytoestrogens on bone in postmenopausal women depend on the equol-producing phenotype? Nutr. Rev. 65 (6 Pt 1), 294–299.

Vidal, O., Lindberg, M.K., Hollberg, K., Baylink, D.J., Andersson, G., Lubahn, D.B., Mohan, S., Gustafsson, J.A., Ohlsson, C., 2000. Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proc. Natl. Acad. Sci. U. S. A. 97 (10), 5474–5479.

Wakley, G.K., Schutte Jr., H.D., Hannon, K.S., Turner, R.T., 1991. Androgen treatment prevents loss of cancellous bone in the orchidectomized rat. J. Bone Miner. Res. 6 (4), 325–330.

Wronski, T.J., Lowry, P.L., Walsh, C.C., Ignaszewski, L.A., 1985. Skeletal alterations in ovariectomized rats. Calcif. Tissue Int. 37 (3), 324–328.

Wuttke, W., Jarry, H., Seidlova-Wuttke, D., 2010. Plant-derived alternative treatments for the aging male: facts and myths. Aging Male 13 (2), 75–81.