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

Philipp Kauffmann⁎, Anna Rau, Dana Seidlová-Wuttke, Hubertus Jarry, Boris Schminke, Swantje Matthes, Karl Günter Wiese

⁎ 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

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 (Pfeilschifter, 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 (Pfeilschifter, 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
2. Materials and methods

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 (Permit 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 CO2 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 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 equal (EQ: 400 mg/kg food; 98% purity, Chargezhou 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 calcine (BLC, 20 mg/kg, Merck, Darmstadt, Germany) and at day 70 bone labelling with alizarin-complexone (BLA; 30 mg/kg; Fluka, 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 calcine 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 calcine 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. Calcine 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 for 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 (Frankenberg et al., 2005). EQ binds to ERα and ERβ and has a stronger estrogen effect than does genistein (Touсен 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 17β-estradiol (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 orchietomized Sprague-Dawley 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.
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 α = 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 ≤ 0.05). After 102 days DHT, E2, and GEN showed significantly smaller...
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 calcine 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 (Fig. 3).

![Figure 3](image-url)
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 \( \mu \text{m/day} \) 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 orchietomized 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 \( \alpha \)-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, ER\( \alpha \) and ER\( \beta \) 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 ER\( \alpha \) and ER\( \beta \) (Rickard et al., 2003). The affinity to ER\( \beta \) 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 ER\( \alpha \) and ER\( \beta \) in a stronger way than does GEN and is said to have a stronger
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 stem cell seems to be 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 the control group SF. The determination of the MAR after trepanation was obtained much later, on 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 fluorochrome calcein and alizarin are chelating agents and form complex molecules with Ca²⁺. 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 bands 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 circumference 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 Kauffman:** 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 Gänzler 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 Brügge mann-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. JIA398/10-1 of the German Research Society (DFG). We acknowledge support by the Open Access Publication Funds of the Göttingen University.

**References**


