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Whole body vibration during fracture healing intensifies the effects of estradiol and raloxifene in estrogen-deficient rats

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ABSTRACT

Current osteoporosis therapies aim to delay bone destruction and have additional anabolic effects. While they have demonstrated some positive effects on bone healing, more progress is needed in this area. This study used the well-known osteoporotic agents estrogen (E) and raloxifene (R) in conjunction with biomechanical whole body vibration (WBV) at a frequency of 70 Hz twice daily for six weeks to stimulate bone healing. Eighty-four 3-month old female Sprague–Dawley rats (12 per group) were bilaterally ovariectomized to develop osteopenia within eight weeks. Osteotomy of the metaphyseal tibiae was performed and fracture healing was then studied using mechanical tests, histomorphometry, computed tomography (μCT), and gene analysis. We found that E and R improved the structure of osteopenic bones as did WBV alone, although significant levels for WBV were seldom reached. Combination treatments significantly enhanced stiffness (R + WBV; $p < 0.05$), endosteal bone (R + WBV; $p < 0.01$), and trabecular density (E + WBV; $p < 0.05$, R + WBV; $p < 0.05$). In addition, the expression of osteoclast-specific *Trap* was significantly reduced after treatment with E, R, or their combination with WBV ($p < 0.01$). The effects were additive and not inhibitory, leading us to conclude that the combined applications of WBV with E or R may improve the healing of osteopenic bones. The therapies studied are all currently approved for human use, suggesting ready applicability to clinical practice. To better understand the effects of WBV on osteopenic bones, the ideal vibration regime will require further study.

Introduction

Osteoporosis results from an imbalance between anabolic and resorptive processes, leading to decreases in bone mass, mineral content and a deterioration of bone microarchitecture and increasing the risk of osteoporotic fracture. In 2000, the number of osteoporotic fractures was estimated to be approximately 9 million worldwide [1]; this number is expected to further increase with the predicted demographic shift toward an aging of the population.

In an elderly population, early osteoporosis intervention is important in order to prevent secondary disorders such as back pain, fractures and long-term immobility. Current treatment of osteoporotic postmenopausal women mainly consists of bisphosphonates, selective estrogen receptor modulators (SERMs), estrogens or strontium ranelate [2,3]. Hormonal therapies with estradiol (E) or raloxifene (R) improved fracture healing in estrogen-deficient rats in previous studies [4,5]. Preexisting estrogen therapy has also been shown to enhance the healing process in osteoporosis [6].

Moreover, physical activity and mechanical load have been shown to be beneficial in treating osteoporosis by reducing pain and the risk of falls in women suffering from osteoporosis [2]. Evidence suggests that whole-body vibration (WBV) can be an effective training method for the musculoskeletal system [7–9], but this concept requires further research to determine its beneficial effects in humans. Studies on rats have demonstrated that WBV not only affects osteoporotic bones but also stimulates fracture healing [10–12].

Limited research exists on the combined effects of hormonal treatments and WBV; therefore, the present study focused on this therapeutic approach. Anti-osteoporotic drugs and WBV are two completely different methods to improve the structure and strengthen the quality of osteoporotic bone. We have insufficient evidence to date regarding how the combination of these methods may act, whether additively or competitively. Therefore, the aim of the present study was to determine the coactions of two representative anti-osteoporotic drugs, estrogen and raloxifene, combined with WBV using mechanical and histological

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evaluation as well as examining bone-specific gene expression in an animal model of osteoporosis.

Materials and methods

General procedures

Seventy-two 3-month old female Sprague–Dawley rats (Harlan Winkelmann, Borchen, Germany) were bilaterally ovariectomized under intraperitoneal ketamine (Medistar, Holzwickede, Germany) and xylazine (Riemser, Greifswald-Insel Riems, Germany) anesthesia (115 mg and 8 mg per kg of body weight, respectively). An additional twelve rats were left intact. Rats were housed in standard cages under 12 h dark/light regimes at a constant temperature of 22 ± 1 °C. Until osteotomy all rats received phytoestrogen-free pelleted food (SM R/M, 10 mm, Ssniff special diets GmbH, Soest, Germany). They had ad libitum access to food and water during the experiment and were allowed to freely move within the cages at all times. Animals were weighed weekly, and the daily food intake was calculated after osteotomy based on weekly weighing of the food.

Within eight weeks, the ovariectomized animals developed a severe osteopenia [13]. All rats underwent bilateral metaphyseal transverse osteotomy of the tibia under ketamine/xylazine anesthesia as described previously [14]. Both tibiae were osteotomized 7 mm distal to the knee surface and 3 mm distal to the growth plate using a pulsed ultrasound saw (Piezosurgery®, Mectron Medical technology, Carasco, Italy). A 0.5 mm osteotomy gap was generated to create a bridge plating with a 5-hole T-shaped titanium mini plate (thickness: 0.55 mm, length: 15.3 mm, width T-part: 7.1 mm, width: 3.1 mm; 57–05140, Stryker Trauma, Selzach, Switzerland). The distance between the centers of the bone screws was 3.7 mm.

Immediately after the surgical procedure, the osteopenic animals were divided into six groups of 12 rats each. The intact and two ovariectomized groups (OVX and OVX + WBV) continued on phytoestrogen-free pelleted food. Another two ovariectomized groups, referred to as "E" and "E + WBV", received phytoestrogen-free food supplemented with estrogen in the form of 17β-estradiol benzoate (10 mg/kg food). The average food intake per animal per day was approximately 13 g, so that the average E intake was 0.46 mg/kg body weight (BW) and 0.45 mg/kg BW in the E and E + WBV groups, respectively. In the remaining two ovariectomized groups, food was supplemented with raloxifene (167 mg/kg food), with the average intake being 7.6 mg/kg BW in the R group and 7.9 mg/kg BW in the R + WBV group. Animals were kept on these specific diets during fracture healing for a period of six weeks.

Due to the stable osteotomy fixation, an intact fibula and appropriate pain management with subcutaneous injections of Decentan (Merck; 100 mg/kg BW) and Rimadyl (Pfizer, Karlsruhe, Germany; 4 mg/kg BW) within the first 72 h, the rats allowed unlimited movement beginning on the first postoperative day. Five days after the osteotomy, one group on soy-free food alone (OVX + WBV), one group with estradiol supplementation (E + WBV), and one group with raloxifene-supplemented food (R + WBV) were exposed to WBV at a frequency of 70 Hz with an amplitude of 0.4 mm. The rats, in groups of 8 to 10 at a time, were transferred to the WBV device (Vibra Maschinenfabrik Schultheis GmbH & Co, Offenbach, Germany) and vibrated for 15 min 2 times per day for 37 days. A plastic cage was attached to the device to reduce vibration loss and to protect the rats during the vibration sessions. The intact group was not exposed to vibration.

During fracture healing, the labeling of new bone formation was performed in vivo using fluorescent dyes injected subcutaneously [4]. Xylenol orange (XO, 90 mg/kg BW) was injected on day 12, calcein green (CG, 10 mg/kg BW) on day 22 and alizarin complexone (AC, 30 mg/kg BW) on day 32 after osteotomy, respectively. Tetracycline (TC, 25 mg/kg BW) was injected 1 h prior to decapitation, 42 days after osteotomy.

After 42 days, the rats were decapitated under deep CO_2 anesthesia. Blood samples were collected and centrifuged and the serum was stored at −20 °C until analysis. Alkaline phosphatase (AP) was determined by a colorimetric assay at the Department of Clinical Chemistry, University Medical Center, Goettingen, using an automated chemistry analyzer (Modular P Chemistry Analyzer, Roche/Hitachi Diagnostics, Indianapolis, US) and commercially available kits (Roche, see above) according to the manufacturer's instructions (Roche, see above). Each uterus was extracted and weighed.

The plates and screws were carefully removed from the left and right tibiae and were dissected free of the soft tissues. One tibia per rat was chosen randomly for mechanical, histological and computed tomographic (μCT) analyses and stored at −20 °C. From the contralateral tibia, the metaphyseal clip with a newly formed callus was prepared, immediately placed in liquid nitrogen and stored at −80 °C for gene expression analyses. The animal study protocol was approved by the local regional government and conformed to German animal protection laws (permission from 05/09/11, Az: 33.9-42502-04-11/0389, District Government of Oldenburg).

Bone analyses

Micro computed tomography (μCT)

Tibiae were thawed and analyzed using a micro-computer tomograph (eXplore Locus SP-Scanner, GE Healthcare, Ontario, Canada). The following parameters were evaluated: bone mineral density (BMD), callus and cortical density, cortical and trabecular volume, total bone volume (BV) and bone volume fraction (BV/TV). By measuring five hydroxyapatite standards of several mineral densities, the data could be converted into bone mineral density (g/cm^3) using a linear regression equation (BMD = 0.265 × value −30.2) that was formulated. The scan protocol adapted to the guidelines of Bouxsein et al. [15] was as follows: the X-ray tube potential (applied peak electric potential of X-ray tube that accelerates electrons for generating X-ray photons) was 72 kVp, the X-ray intensity (X-ray tube current (mA) or product of current and time) was 90 μA, and the integration time (duration of each tomographic projection) was 1600 ms. Total rotation was 360°, and the reconstructed voxel size (three dimensions defining the basic discrete unit of the mCT image) was 22 × 22 × 22 μm^3. No frame averaging was used because the detector binning of 2 × 2 ensures a sufficient SNR projection (number of tomographic viewpoints used for reconstruction) of 900 numbers of views. With the help of the MicroView-Program (v2.1.2, GE Healthcare) all 3D reconstructions were performed. The measurement area extended 2.5 mm proximally and distally from the osteotomy gap. After reconstruction of the data, the analysis was conducted using an established bone analysis program (3D OsteoAnalyze©) which is based on recognized standards [16,17]. A gray scale value histogram was created by using this program that determined four levels of light intensities corresponding to the different dilutions of X-rays: one peak for cortical bone, the trabecular bone, the soft tissue, and one for the air. According to this the bone parameters were detected by an auto-detection algorithm run of the analyzing program.

Mechanical analyses

Stiffness and yield load of the tibia callus formation were evaluated by a 3-point bending test using a material testing machine (type 145660 Z020/TND, Zwick/Roell, Ulm, Germany) as described elsewhere [14] (Fig. 1). One tibia per rat was randomly chosen for mechanical and morphological analysis and was loaded directly at the previous osteotomy gap. After applying a preload of 1 N, a non-destructive measurement was performed at a feed motion rate of 5 mm/min with the aid of the "testXpert"-software. The test was automatically stopped by the software when elastic deformation changed to plastic deformation, which was visible by a decrease of the curve's rising. The stiffness