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Effect of the lipoxygenase-inhibitors baicalein and zileuton on the vertebra in ovariectomized rats

D. Saul [a,*], S. Gleitz [a], H.H. Nguyen [a], R.L. Kosinsky [b], S. Sehmisch [a], D.B. Hoffmann [a], M. Wassmann [c], B. Menger [a], M. Komrakova [a]

[a] Department of Trauma, Orthopaedics and Reconstructive Surgery, Georg-August-University of Goettingen, Goettingen, Germany
[b] Department of General, Visceral and Pediatric Surgery, University Medical Center Goettingen, 37075 Goettingen, Germany
[c] Medical Institute of General Hygiene and Environmental Health, University of Goettingen, 37075 Goettingen, Germany

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ABSTRACT

Osteoporosis is one of the most common diseases worldwide. In osteoporosis, vertebral fractures represent a major burden. Lipoxygenase (LOX) inhibitors such as baicalein and zileuton may represent a promising therapeutic option owing to their antioxidative effects and suppression of various inflammatory processes in muscle and bone. The effect of these LOX inhibitors on the spine was studied in osteopenic rats.

Female Sprague-Dawley rats were divided two times into five groups: four groups each were ovariectomized (OVX) and one control group was non-ovariectomized (NON-OVX). Eight weeks after ovariectomy, three concentrations of baicalein (1 mg/kg body weight [BW], 10 mg/kg BW, and 100 mg/kg BW) were administered subcutaneously daily in three OVX groups for 4 weeks. Similarly, zileuton was administered in three concentrations via food for 5 weeks. In vivo computed tomography (pQCT) of the spine was performed before the treatments and at the end of the experiment. Lumbar vertebrae were subjected to a compression test, micro-CT, and ashing analyses.

After baicalein treatment, cortical bone mineral density (BMD) was improved; trabecular connectivity and trabecular BMD were diminished at high dose. After zileuton treatment, the total BMD, anorganic weight, trabecular nodes, and trabecular area were improved.

The in vivo stress-strain index was increased and alkaline phosphatase activity in serum was enhanced after both treatments. A dose-dependent effect was not clearly observed after both treatments. The treatments using baicalein for 4 and zileuton for 5 weeks were not sufficient to change the biomechanical properties and bone volume fraction (BV/TV).

Overall, baicalein improved the cortical bone parameters whereas zileuton had a favorable effect on the trabecular structure. Moreover, both treatments increased the bone formation rate. Longer trials, a combination of both LOX inhibitors, and their effect at the cellular and molecular levels should be investigated in further studies.

1. Introduction

Osteoporosis is a common disease in humans, with a prevalence of ~10.3% in Germany [1]. Osteoporosis increases mortality and reduces the quality of life [2]. Vertebral fractures indicative of injury are a major burden of this disease [3]. One therapy that is currently used is calcium and vitamin D supplements [4]; however, this is not sufficient for the treatment of severe osteoporosis. Other therapeutic options include a limited range of medications such as bisphosphonates, human parathyroid hormone (hPTH), selective estrogen receptor modulators (SERMs), strontium ranelate, anti-receptor activator of NF-κB ligand (RANKL) antibodies, and newly investigated drugs that are based on the inhibition of cathepsin K or of sclerostin [5–8].

However, all recently prescribed anti-osteoporosis drugs have serious negative side-effects ([12–15], and therefore, new therapeutic alternatives need to be developed. For example, lipoxygenase (LOX) inhibitors were found to improve bone density and strength [9]. In particular, 5-lipoxygenase (5-LOX) inhibitors suppressed osteoclast formation [10], and they can potentially enhance bone formation [11] and help to treat bone resorption diseases [16].

Baicalein is a phytochemical agent extracted from the plant *Scutellaria baicalensis Georgi*. It acts as a LOX (especially cyclooxygenase [COX-I]) inhibitor, and it is a potent inhibitor of 12-LOX and 15-LOX, thereby producing an antioxidative effect. Baicalein also inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and this, in turn, suppresses the function of tumor necrosis factor

* Corresponding author.
E-mail address: Dominik.Saul@med.uni-goettingen.de (D. Saul).

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alpha 8 (TNFα) and interleukin 6 (IL-6), all of which are mediators of inflammation cascades. Baicalein affects degenerative bone diseases such as rheumatoid arthritis [17,18]. It also activates alkaline phosphatase by the mTORC1-pathway, thereby inducing osteoblast-differentiation markers in osteoblasts and even increasing bone parameters in distal femurs [19]. Baicalein also inhibited the bone resorptive activity of osteoclasts by inhibiting osteoclast differentiation and promoting osteoclast apoptosis [20,20]. Mechanistically, baicalein inhibits 5-LOX translocation to the nucleus and p38 phosphorylation, whereas zileuton does not [21].

Zileuton (Zyflo®) is a therapeutic drug approved for treating asthma since 1997 in the USA [22,23]. As 5-LOX downregulates fracture healing [24], zileuton, in a manner similar to 5-LOX inhibitors, is expected to have a positive effect on bone. Zileuton has been found to lead to reduced bone resorption in the maxilla [25]. However, it enhances fracture repair in femoral fractures with increased callous size and earlier bone formation [26].

The effects of the LOX inhibitors baicalein and zileuton on osteoporotic vertebrae have not yet been examined, although LOX inhibitors could be potential candidates for treating bone resorptive diseases [10,25,27]. Thus, this study aims to examine their effects on both the cortical and trabecular bones of lumbar vertebrae in ovariectomy-induced osteoporotic rats.

2. Materials and methods

2.1. Animals and treatment

For this study, 129 three-month-old virgin female Sprague-Dawley rats (Winkelmann Company, Borken, Germany) were housed at 20 °C and relative humidity of 55% in Makrolon IV® cages. Two experiments were performed in conformity with the ethical standards of animal care and with the approval of the local district government.

The baicalein experiment involved 60 female rats, with 12 rats per group. The zileuton experiment involved 69 rats with 13 rats each in the non-ovariectomized (NON-OVX) group and ovariectomized (OVX) groups, 14 rats each in the C1 (1 mg/kg body weight [BW]) and C2 (10 mg/kg BW) groups, and 15 rats in the C3 (100 mg/kg BW) group.

After acclimatization for 1 week, bilateral OVX was performed or the ovaries were left intact, as described by Iwasa et al. [28]. Surgery was performed under ketamine/xylazine (ketamine: 62.5 mg/kg BW; Hostaket®, Hoechst, Bad Soden, Germany; xylazine: 7.5 mg/kg BW; Rompun®, Bayer, Leverkusen, Germany) anesthesia (0.1 ml/100 g BW, intraperitoneal [i.p.]). After shaving, anesthesia, and disinfection, the skin was incised on both sides of the lower abdomen, and the adnexa were dissected, clamped, and removed before the wound was closed.

After 8 weeks, an osteotomy of tibia metaphysis with plate osteosynthesis [29] was performed as a part of other studies. Based on previous studies [29–31], we assumed that at around this time point, that is, eight weeks after OVX, the rats would have developed osteoporosis. One day after the osteotomy, baicalein and zileuton treatments were started. For injections, baicalein (98%; Sigma-Aldrich Chemie Gmbh, Munich, Germany) was dissolved in 100% dimethyl sulfoxide (DMSO). Both control groups (NON-OVX and OVX, each $n = 10$) received only DMSO.

Baicalein was injected subcutaneously, as described previously [32,33], at different concentrations (C1: 1 mg/kg BW, C2: 10 mg/kg BW, and C3: 100 mg/kg BW, respectively) in three groups with 10 animals in each group. The injections were administered every 24 h for 4 weeks.

Zileuton was administered with food for 5 weeks after osteotomy. A soy-free diet (ssniff® special diet; GmbH, Soest, Germany, ingredients in Table 2) was supplemented with zileuton (Zyflo ®; Cornerstone Therapeutics Inc., Cary, NC, USA) at three different concentrations. The rats received demineralized water throughout the experiments.

Body weight and food intake were recorded weekly (Fig. 1). The average daily food intake was calculated. The zileuton dosages of 1, 10, and 100 mg/kg BW were administered [34,35]. The effective dose calculated based on the daily food intake was, on average, 0.71, 7.72, and 64.44 mg/kg BW for C1, C2, and C3, respectively. The OVX and NON-OVX groups received a soy-free diet without zileuton, as mentioned above.

At completion, the rats were anesthetized using CO_2 and decapitated, and the weight of the rats and their uteri were recorded. The lumbar vertebral bodies were isolated and stored at −20 °C until further analysis. The second vertebral body (L2) was used in an ashing analysis, L3 was subjected to a compression test, and L4 was analyzed by in vivo peripheral quantitative computed tomography (pQCT) and micro-computed tomography (micro-CT).

2.2. In vivo quantitative computed tomography (pQCT)

pQCT was performed in isoflurane-anesthetized rats ($n = 5$ per group) using the pQCT device (XCT Research SA, Stratec Medizintechnik GmbH, Pforzheim, Germany), as described in a previous study [36]. L4 was scanned before the treatment (8 weeks after OVX) and at the end of the experiment. The total bone mineral density (BMD, mg/cm^3) and stress-strain index (SSI, mm^3) were evaluated using XCT-6.20C software (Stratec Medizintechnik GmbH, Pforzheim, Germany).

2.3. Compression test

The biomechanical properties of the vertebra were tested according to Sehmisch et al.'s protocol [37]. A Zwick machine (145 660 Z020/TND; Ulm, Germany) was used to measure the mechanical resistance of lumbar vertebrae.

The dissected vertebrae, located on the base of the Zwick machine, were fixed. Next, a slender stamper was dipped onto the vertebra at a rate of 50 mm/min and with initial force of 1 N to fix the vertebra on the plate. Subsequently, measurements were obtained with accuracy of 0.2–0.4% over 2–500 N, as described previously by our group [38].

Live testing showed a linear increasing curve, and testing was stopped when the curve declined by >10 N. The test was recorded using testXpert software (Zwick GmbH & Co. KG, Ulm, Germany). The stability was measured in increments of 0.1 mm. We quantified the maximum load (Fmax), yield load (yL), and stiffness (S) as described by Sehmisch et al. [39] and Stuermer et al. [40]. Fmax is the highest force that the ground plate can withstand. yL is the bending point from elastic to plastic deformation. Stiffness measures the bone's elasticity [38].

2.4. Micro-CT analysis

The vertebral bodies were scanned using Quantum FX micro-CT (Caliper Sciences, Hopkinton, MA, USA). The scan protocol was as follows: 70 kVp, 200 μA, 2-min exposure time, 360° rotation, 3600 projections, 20 × 20 mm^2 field of view, 512-pixel matrix, and 40-μm resolution. A phantom block with five hydroxyapatite elements of several mineral densities was scanned with each vertebra to interpret the gray scale in terms of density (g/cm^3). Fig. 2A shows the lumbar vertebrae scanned with the phantom three dimensionally (3D). 3DOsteoAnalyze developed in our laboratory was used to assess the bone parameters according to the American Society for Bone and Mineral Research (ASBMR) criteria [41,42]. Trabecular, cortical, and total bone densities (g/cm^3); tissue and total volume (mm^3); and bone volume fraction (BV/TV) were assessed. The cortical area (mm^2) was measured at the dorsal and ventral segments of the vertebral body cut on the sagittal plane of the 3D images (Fig. 2B).

Structural analyses were performed using 2D images (transformation with sectional plane is shown in Fig. 2C). Four images of sagittal cut vertebral bodies were analyzed using MetaMorph Basic Acquisition Software (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany, Fig. 2 D–J). The trabecular nodes (N.Nd), trabecular connectivity (N.Nd/mm^2),