Original article

Effects of long-term administration of pantoprazole on bone mineral density in young male rats

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A B S T R A C T

Introduction: Epidemiological studies suggest that long-term administration of proton pump inhibitors (PPIs) may decrease bone mineral density (BMD) and increase the risk of osteoporotic fractures. The aim of the study was to assess the influence of pantoprazole on bone metabolism in growing rats.
Methods: The experiment was carried out on twenty-four young male Wistar rats divided into two groups receiving either pantoprazole at the dose of 3 mg/kg or vehicle for 12 weeks. Femoral bone mineral density (BMD) and bone histomorphometry were assessed. Serum total calcium, inorganic phosphate and markers of bone turnover were measured.
Results: In pantoprazole-treated rats a decreased BMD was detected (0.2618 ± 0.0133 g/cm^2 vs. 0.2715 ± 0.0073 g/cm^2, $p < 0.05$). Bone histomorphometry revealed a decrease in growth plate thickness (G.Pl.RTh.) (161.0 ± 27.8 μm vs. 195.0 ± 20.8, $p < 0.05$) in pantoprazole-treated animals. Serum total calcium level and osteocalcin concentrations were decreased in the pantoprazole-treated group (9.62 ± 0.55 mg/dl vs. 10.15 ± 0.38 mg/dl, $p < 0.05$ and 242.7 ± 44.4 pg/ml vs. 342.5 ± 123.3 pg/ml, $p < 0.05$, respectively).
Conclusion: We observed that PPIs might have a negative impact on bone formation in growing rats mainly due to their inhibitory effects on the gastric proton pump, with probable deterioration of calcium absorption and decrease in growth plate thickness.
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Introduction

Osteoporosis is a highly prevalent systemic bone disease characterized by low bone mass and deterioration of bone microarchitecture, leading to increased bone fragility and a risk of fractures [1]. Osteoporotic fractures decrease patients' quality of life, and may lead to disability and increased mortality rate [2]. High socioeconomic costs of osteoporosis and its complications indicate the importance of prophylaxis and therapy of the disease [3]. Peak bone mass achieved at adolescence belongs to factors that predict bone mass and the risk of osteoporosis later in life.

Proton pump inhibitors (PPIs) are very potent inhibitors of gastric acid secretion, and their clinical usage increases dramatically. To the main indications for their administration are: severe GERD (gastroesophageal reflux), esophagitis, peptic ulcer, dyspeptic symptoms and eradication of Helicobacter pylori. Generally PPIs seem to have a similar clinical efficacy in standard and comparable doses [4]. They inhibit both basal and stimulated gastric acid secretion leading to an increase in gastric pH, that may affect absorption of some other drugs and food components, e.g. calcium [5,6]. On the other hand, acid secreting proton pump is expressed on bone resorbing osteoclasts and it has been reported that PPIs are able to inhibit its activity preventing bone loss in rats [7]

In recent years an increased administration of PPIs even in small children and neonates has been observed [8]. Epidemiological studies suggest that long-term administration of PPIs may decrease bone mineral density (BMD) and increase the risk of osteoporotic fractures [9,10]. However, findings from studies assessing the association between PPIs administration and the risk of osteoporosis development are contradictory [6,9,11], and there are only few studies on influence of a chronic PPIs administration on growing bones [12,13].

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A dynamic balance between resorption of old bone by osteoclasts and bone formation by osteoblasts is observed in bone tissue. The tissue undergoes permanent remodeling. Bone formation prevails during periods of growth and adolescence, allowing achievement of the maximum peak bone mass that protects against osteoporosis development later in life. One of the most important factors influencing bone mineralization and peak bone mass is a sufficient calcium supplementation [14].

Most of studies examine the influence of PPIs administration on the mature skeletal tissue. The aim of our study was to assess the influence of a long-term PPIs administration on bone metabolism in growing rats.

Materials and methods

Drugs and chemicals

Pantoprazole (Controloc, Nycomed Pharma) dissolved in saline solution purchased from Farmacol, sodium pentobarbital (Morbital, Biowet) purchased from Bayleg.

Animals

The experiment was carried out on twenty-four male Wistar rats, aged 50-59 days. Animals were housed individually at the room temperature of 21–23 °C with 12:12-h light-day cycle. They were fed with a standard diet. Food and water were provided *ad libitum*.

Acclimated rats were randomly assigned to one of two groups (twelve animals in each group): group P—receiving pantoprazole at the dose of 3 mg/kg (dosing of pantoprazole as in Ref. [15]) in saline solution 4 ml/kg daily intragastrically (*ig*) for 84 days (time needed to cause significant changes in bone mineral density), or group C—control group (saline solution 4 ml/kg daily *ig* for 84 days).

Body weights were checked once daily throughout the 84-day experimental period. On the day 84 blood samples were collected for serum isolation. Serum was separated by centrifugation (at 1500*g*) and then stored at −70 °C until required for bone metabolic marker assays.

Animals were sacrificed and femurs were collected from each animal. Right femurs were stored at −70 °C until required for dual-energy X-ray absorptiometry (DXA) and left femurs were fixed in 10% neutral-buffered formaldehyde for further histomorphometric examination.

Serum biochemical markers of bone metabolism

Serum total calcium and inorganic phosphate levels were measured in a certificated laboratory using commercial tests performed according to their manufacturers' instructions.

Serum osteocalcin (OC) and C-terminated telopeptide of type I collagen (CTX) levels (both sensitive biochemical markers of bone metabolism) were determined using commercial osteocalcin and CTX ELISA kits (Rat Osteocalcin ELISA Kit, USCN Life Science Inc. and Rat Beta-Crosslaps (bCTX) ELISA Kit, USCN Life Science Inc., respectively). Serum osteoprotegerin (OPG) and Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) levels (both regulatory molecules involved in bone metabolism) were also determined using commercial ELISA kits (Rat Osteoprotegerin ELISA Kit, USCN Life Science Inc. and Rat Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) ELISA Kit, USCN Life Science Inc., respectively). ELISA tests were performed according to their manufacturers' instructions.

Body weight and bone parameters

Body weights were checked once daily throughout the 84-day experimental period. The femoral index defined as the ratio of femur weight and body weight ($\frac{femur\ mass[g]}{body\ mass[g]} \times 100\%$) was calculated. Length of femurs and mid-femoral diameter were measured with elecronic callipers (resolution 0.01 mm).

Bone mineral density (BMD)

Bone mineral density (BMD) of the right femur was measured by trained examiners by dual-energy X-ray absorptiometry (DXA) with Hologic DXA equipment (Hologic Discovery W 81507) using the software for small animals. Results were obtained as grams of mineral content per square centimeter of bone area (g/cm^2). The scanner was calibrated daily using a phantom provided by the manufacturer.

Histological preparations

Rat femurs were fixed in 10% neutral-buffered formaldehyde, then decalcified in 8% formic acid and 5,6% hydrochloric acid solution for 14 days. Next, the bones were embedded in paraffin and fixed, and 4-μm histological sections, stained with hematoxylin and eosin were subsequently prepared.

Histomorphometry

The histomorphometric examination was performed with respect to the 2012 update of the standardized nomenclature, symbols, and units for bone histomorphometry [16].

Histological slides were scanned with the NanoZoomer 2.0. In the NDP.view 2 software a representative tissue area (T.Ar) (of at least 1.7 mm^2) of femoral distal metaphysis trabecular bone, localized at least 400 μm away from the closest edge of a growth plate and the external cortical bone surface have been chosen for the analysis. That area was exported to a .tiff image format and trabecular bone area was thresholded in the ImageJ 1.50b software. Using the ImageJ, the total trabecular bone area (B.Ar) and the trabecular bone perimeter (B.Pm) were measured.

The bone volume to tissue volume ratio (BV/TV) was calculated as B.Ar/T.Ar, as it is numerically identical. Next we have calculated the bone surface to tissue volume ratio (BS/TV) as B.Pm/T.Ar*1.2 and the BS/BV ratio as BS/TV*BV/TV. The mean trabecular thickness (Tb.Th) was calculated as 2/BS/BV.

Furthermore we have measured mean growth plate thickness (G.Pl.Th) in a following way: Using the NDP.view 2 software, the growth plate width has been measured in 3 to 5 randomly chosen points and the mean value has been calculated. Next, the value has been divided by 1.2 to obtain the thickness value, as histological sections were not ideally perpendicular to the plane of the growth plate.

Statistical analysis

The significance of differences between values was estimated by the Student's t-test. P-value of less than 0.05 was considered to indicate statistically significant differences. Results are presented as the mean ± standard deviation (SD) unless otherwise noted.

All statistical analysis were performed using the STATISTICA software (data analysis software system), version 10, from StatSoft, Inc. (2011).