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Pantoprazole increases cell viability and function of primary human osteoblasts *in vitro*

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ABSTRACT

Proton pump inhibitors (PPIs) are a class of drugs that irreversibly inhibit the H^+/K^+-ATPase in gastric parietal cells. Since an association between PPI use and increased fracture risk has been found, the aim of this study was to detect potential adverse effects of pantoprazole, a representative of the PPIs, on primary human osteoblasts *in vitro*. The isolated cells were stimulated with pantoprazole concentrations ranging from 0 μg/ml to 10 μg/ml. Changes in proliferation, total cell number, viability, cytotoxicity, alkaline phosphatase activity, total protein synthesis and gene expression on mRNA level were determined over a period of 7 days. Pantoprazole stimulation resulted in increased viability and decreased cytotoxicity in the osteoblasts. The proliferation rate was stable and so was the relative cell number. Only at the highest pantoprazole concentration on day 7, a slight decrease of the cell number was detected. Alkaline phosphatase activity increased over the tested period under exposure to pantoprazole ($p < 0.05$ at 3 μg/ml and 10 μg/ml pantoprazole). Osteoblast-specific gene expression was increased through pantoprazole stimulation compared to the control on day 3. Towards day 7, gene expression returned to baseline levels or decreased slightly compared to unexposed cells. Interestingly, this *in vitro* experiment detected no evidence of adverse effects of PPIs on primary human osteoblasts. Osteoblasts were rather more viable with increased mitochondrial activity, gene expression and protein synthesis under pantoprazole stimulation. Therefore, these *in vitro* results do not suggest that impaired osteoblast function is the cause of an increased fracture risk in patients under PPI therapy.

Introduction

Proton pump inhibitors (PPIs) are a group of drugs that irreversibly inhibit the H^+/K^+-ATPase (proton pump) in gastric parietal cells and thus result in reduced stomach acidity. They are commonly used to treat gastroesophageal reflux disease and to counteract the adverse effects of non-steroidal anti-inflammatory drugs (NSAID) on the gastric mucosa [1,2]. After their introduction, PPIs were deemed almost risk-free which led to a rapid increase in their prescription. Consequently, today's use of PPIs is inflationary and in numerous cases PPIs are administered prophylactically for no evident reason.

The potentially detrimental effects of PPIs on bone metabolism and the resulting changes in bone density with increased fracture risk have been discussed before, leading to contradicting conclusions. Yu et al. performed a meta-analysis of 11 observational studies that evaluated a possible association between the use of PPIs and the risk of bone fracture in humans, finding an increased fracture risk in both male and female patients [3]. However, the authors could not completely exclude the possibility of unnoticed confounding factors. In contrast, Kaye et al. found no significant correlation between the use of PPIs and increased fracture risk without concomitant major risk factors [4]. A potential explanation for the detrimental effects of PPIs on bone density could be decreased calcium absorption due to less acidic stomach fluids as hypothesised by Insogna et al., although long-term studies regarding this matter are yet to be performed [5].

Additionally to their effects on gastric parietal cells, PPIs seem to inhibit a proton pump (V-ATPase) in osteoclasts [6,7]. This could potentially lead to decreased bone resorption and therefore have a positive effect on bone density [8]. Furthermore, Hyun et al. found an osteopetrosis-like effect on bone density through increased activity of osteoblasts (OBs) and decreased activity of osteoclasts in

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a murine cell model [9]. Targownik et al. used a multivariate linear regression model on a set of data from the Canadian Multicentre Osteoporosis Study to determine whether there is a relevant decrease in bone mineral density (BMD) after administration of PPIs [10]. The analysis revealed that there was no association between PPI usage and accelerated BMD loss over a ten-year period, although PPI users had a lower average baseline BMD.

Bone metabolism is ultimately regulated through two interacting types of cells, namely osteoclasts and osteoblasts. Together they form so-called "basic multicellular units", which are responsible for bone homeostasis [11].

Osteoclasts are multinucleated, bone-specific macrophages that derive from hematopoietic stem cells. Once activated, osteoclasts bind to bone matrix and form so-called Howship's lacunae, which are the site of active bone resorption. Contrary, OBs derive from mesenchymal stem cells, synthesise bone matrix and ultimately differentiate into osteocytes, which sustain matrix integrity.

As stated above, an association between PPI use and fracture risk has been hypothesised. Since the underlying mechanisms remain unknown, it was our goal to investigate the effects of pantoprazole (PP) on primary human OBs regarding their metabolic activity, proliferation potential and specific gene activity *in vitro*.

Materials and methods

Plastic labware was purchased from Sarstedt (Nümbrecht, Deutschland). Culture plates were purchased from GE Healthcare (Little Chalfont, United Kingdom). Chemicals, unless stated otherwise, were purchased from Sigma–Aldrich (St. Louis, MO, United States).

Isolation and culture of primary human OBs

Femoral heads, which otherwise would have been disposed as residue after surgery, were obtained from patients undergoing total endoprosthetic hip replacement ($N = 4$). The age of the recruited patients ranged from 75 to 91 years and the cohort consisted of three female and one male donor. Patient recruitment as well as realisation of the experiments was conducted in accordance with the Declaration of Helsinki and the principles of good clinical practice. Informed consent was obtained from each of the patients. The study was approved by the ethics committee of the Technical University of Munich. Neoplasia and infection were defined as exclusion criteria in this study.

Briefly, cancellous bone fragments were collected from the centre of the femoral heads using Luer's pliers and subsequently washed in Dulbecco's phosphate buffered saline (DPBS) in order to remove unwanted tissue fractions (*e.g.* blood and fat). The fragments were transferred into 175 cm^2 culture flasks. The culture medium consisted of 500 ml low glucose Dulbecco's modified eagle medium, 0.05 M L-ascorbic acid, 10% foetal calf serum (FCS) and 100 U/ml penicillin and 10 μg/ml streptomycin. The cells were incubated at 37 °C and 5% CO_2. The isolated cells were expanded until reaching the third passage to ensure the desired purity. The osteoblastic phenotype was confirmed by alkaline phosphatase (AP) activity measurement and characteristic gene expression (*i.e.* osteocalcin, among others). Finally, the cells of each individual donor were plated separately at a density of 10,000/cm^2 for the experiments.

Experimental setup

Primary human OBs were cultivated using a differentiation medium containing 500 ml low glucose Dulbecco's modified eagle medium, 10 mM β-glycerol phosphate, 1.56 mM $CaCl_2 \cdot 2H_2O$, 5% FCS, 100 U/ml penicillin and 10 μg/ml streptomycin, 0.2 mM L-ascorbic acid, 100 nM dexamethasone and 0.025 M HEPES buffer. After 24 h, detached cells were removed. Subsequently, the cells were incubated with PP (Sigma–Aldrich, St. Louis, MO, United States). In addition to a negative control containing 0 μg/ml PP, the following concentrations of the drug were administered: 0.1 μg/ml, 1 μg/ml, 3 μg/ml and 10 μg/ml. The medium was changed every other day including freshly prepared PP. The effects of PP on the metabolism, proliferation and gene expression of primary human OBs were analysed after 1, 3, and 7 days.

5-Ethynyl-2'-deoxyuridine (EdU) fluorescence stain

Proliferation was analysed using the thymidine analogue EdU in order to label the DNA of dividing cells in our cell culture experiments. The fluorescence stain was performed according to the manufacturer's protocol, using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies, Carlsbad, CA, United States). Briefly, the cells were incubated with medium containing EdU at 37 °C for 24 h. Afterwards, the cells were fixated and the click reaction initiated. Finally, a DNA stain was performed by incubating the cells with Hoechst 33342 for 30 min. The fluorescence signals were documented at room temperature using the BZ-9000 fluorescence microscope (Keyence, Osaka, OSK, Japan). For analysis, a cell counting template was created using the BZ-II Analyzer (Keyence, Osaka, OSK, Japan). Fluorescence signals that were to be counted as cells were defined according to size and shape of OBs. For each representative field of view, all cells that exhibited proliferation activity (EdU stain, green fluorescence) were counted. Then the total cell number (Hoechst stain, blue fluorescence) in each field of view was determined. The proliferation rate was calculated by dividing the proliferating cells by the total amount of cells.

Cell count

The relative cell count was obtained using the CyQuant® NF Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA, United States). The cells were washed once with DPBS and then incubated with the dye reagent for 60 min at 37 °C. The reagent was prepared according to the manufacturer's protocol. The fluorescence signal was photometrically quantified at $\lambda = 530$ nm. The results were expressed relative to the control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was used to determine the effects of stimulation with PP on cell viability and metabolic (mitochondrial) activity. In short, the plated cells were washed twice with DPBS and then incubated with MTT solution containing 1.2 mM thiazolyl blue for 120 min at 37 °C and 5% CO_2. The formazan production was determined photometrically at $\lambda = 570$ nm. The measurements were normalised with the optical density at $\lambda = 690$ nm. The results were expressed relative to the control.

Sulforhodamine B (SRB) staining

In order to obtain the complete amount of cellular protein, SRB staining was performed. Bound SRB was resuspended in unbuffered Tris(hydroxymethyl)aminomethane (TRIS) solution and photometrically quantified at $\lambda = 565$ nm. The protein content was then calculated according to a standard curve that was obtained by plotting known protein quantities to their respective absorptions. The results were expressed relative to the control.