Bone and Cellular Immune System of Multiparous Sows are Insensitive to Ovariectomy and Nutritive Calcium Shortage

Abstract
Research in osteoporosis, which is a complex systemic disease, demands suitable large animal models. In pigs, most research has been done in growing minipigs, which probably are not ideal models for postmenopausal osteoporosis. Therefore, our aim was to analyze the effects of ovariectomy (OVX) and nutritive calcium shortage on multiparous Large White sows. 32 animals were randomly assigned to 4 groups in a cross design with OVX vs. sham and physiological calcium supplementation (0.75% calcium) vs. dietary calcium shortage (0.3% calcium). The observation period was 10 months with blood sampling every 2 months for hematological, immunological, and biochemical bone marker measurements. At the termination of the experiment, animals were sacrificed. Samples of trabecular bone of distal radius, proximal tibia, and sixth lumbar vertebra were subjected to micro-computed tomography imaging and ashed afterwards. Dual X-ray absorptiometry scans of the proximal femora were performed with prepared bones being placed in a water bath for mimicking soft tissue. Analyses of bone marker and cytokine profile kinetics, distribution of leucocyte subpopulations, and morphometrical and densitometrical analyses showed no evidence of any impact of OVX or calcium shortage. In conclusion, the skeleton of adult sows of a conventional breed is seemingly protected from effects of OVX and calcium shortage.

Introduction
Osteoporosis is a complex systemic disease [1]. In vitro models are not sufficient to analyze systemic effects, thus, there is a great need for suitable animal models. Rodent models, which have several advantages in terms of laboratory management, are well established and have been widely used in osteoporosis research. However, the US Food and Drug Administration demands also the use of large animal models, mainly due to biomechanical issues, besides rats in preclinical testing of antosteoporotic substances with an experimental time frame of 12 months when using rats and 16 months when using larger species. According to FDA regulations, valid animal models have to develop an osteoporotic phenotype either spontaneously or after ovariectomy (OVX), which mimics postmenopausal estrogen deficiency [2]. Pigs as well as sheep are among the most frequently used large animal species in osteoporosis research. Although there are a lot of similarities of diverse porcine organ systems to their human analogues, the pig’s usefulness as an osteologic model species is still not entirely clear. However, even though porcine femoral compact bone is predominantly plexiforme, it is converted to well-developed osteonal bone earlier than in sheep [3]. Peak bone mass is obtained with an age of 2–3 years. The main body of investigations in this area of research was performed using growing minipigs, which, however, might not appropriately reflect the situation of the postmenopausal osteoporotic woman due to their juvenile age and dwarfism. On the other hand, minipigs achieve sexual maturity earlier than conventional pigs and thus OVX may induce the desired phenotype earlier than in conventional sows. OVX in 10 months old minipigs resulted in a 6% decrease in bone mineral density (BMD), 15% in bone volume (BV), and 13% in trabecular number, and an increase of 15% in trabecular separation after 6 months, whereas OVX in combination with a mild nutritive calcium shortage (0.75% Ca^2+), which had been started already at
Materials and Methods

Animals and group assignment

32 multiparous Large White sows aged 33.5±9.6 months in the mean and all of them approximately 2 months post partum and thus not lactating for over 5 weeks were allocated equally to 4 groups to compare the effects of OVX and dietary calcium short-age: (I) OVX, 0.75% Ca²⁺, (II) OVX, 0.3% Ca²⁺, (III) sham-OVX, 0.75% Ca²⁺, and (IV) sham-OVX, 0.3% Ca²⁺. Ovariectomy was performed from the left flank under full anesthesia with an IV bolus of ketamine and azapenone. Post-surgical treatment was done with enrofloxacin, metamizole, and local oxytetracycline spray. Sows were housed as groups of 5 or 6 in a separate stable under standard conditions. Diets were mainly based on barley, soy, and lignocellulose and produced by a farm animals' feed company (Biomin, Herzogenburg, Austria). One diet contained the usually supplemented calcium levels for nongestating sows, that is, 0.75%, and the other one was a low calcium diet containing 0.3%. For detailed composition of diets see Table 1. Blood samples were collected by venipuncture of the jugular vein every 2 months. All animal experiments were approved by the institutional and the governmental ethics committees. The experiment was terminated after 10 months.

Biochemical bone markers and hormones

Serum levels of following bone metabolism markers were determined by commercially available ELISA kits, which have been validated also for pigs [7,8]: receptor activator of nuclear factor-κB ligand (ampli-sRANKL, ELISA, Biomedica, Vienna, Austria), calcitriol (25-OH-Vitamin D direct ELISA, Immundiagnostik, Bensheim, Germany), PTH (Porcine Intact PTH ELISA, ImmunoDiagnostik, San Clemente, CA, USA), bone specific alkaline phosphatase (Metrà®BAP ELISA, Qidel Corporation, San Diego, CA, USA), osteocalcin (Metrà®Osteocalcin ELISA, Qidel Corporation), CiCP (Metrà®CiCP ELISA, Qidel Corporation), pyridoline (Metrà®Serum PYD ELISA, Qidel Corporation), and crosslaps (Serum CrossLaps®ELISA, ImmunodiagnosticSystems, Frankfurt/ Main, Germany). For measurement of serum estrogens, a home made enzyme immunoassay for the detection of estron and 17β-estradiol was applied [9]. The low detection limit of this test of 2 pg of estrogens/ml is superior to the one of commercial systems.

Table 1  Composition of diets (per kg)

<table>
<thead>
<tr>
<th></th>
<th>Low-calcium diet</th>
<th>Physiological diet</th>
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<tbody>
<tr>
<td>ME (MJ)</td>
<td>11.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>13.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Crude fat (g)</td>
<td>22.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Crude fiber (g)</td>
<td>102.0</td>
<td>102.0</td>
</tr>
<tr>
<td>Vit A (IU)</td>
<td>9900</td>
<td>9900</td>
</tr>
<tr>
<td>Vit D₃ (IU)</td>
<td>1320</td>
<td>1320</td>
</tr>
<tr>
<td>Vit E (mg)</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Ca (g)</td>
<td>3.0</td>
<td>7.5</td>
</tr>
<tr>
<td>P (g)</td>
<td>3.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Na (g)</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Cu (mg)</td>
<td>16.5</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Hematological and immunological analyses

Hematological analyses were performed out of EDTA-blood using an ADVIA®120 (Siemens Health Care Diagnostics, Deerfield, IL, USA) with veterinary software adapted for pigs. Cytokine levels were determined at both the genomic as well as the protein levels. For preparation of total RNA out of peripheral blood mononuclear cells (PBMCs), the pellet was suspended in 1.2 ml of hemolysis buffer (140 mM NH₄Cl, 17 mM Tris, pH 7.2) and incubated at 37°C for 15 min. Samples were then centrifuged and supernatant was removed. Pelleted white blood cells were resuspended in 1 ml of TRIReagent® (Molecular Research Center, Inc., Cincinnati, USA) and frozen at −80°C until analysis. Total RNA was extracted in accordance to the manufacturers’ protocol. Integrity, quantity, and contamination with genomic DNA were analyzed on the Agilent BioAnalyzer 2100 (Agilent Technologies, Palo Alto, USA) using the RNA6000 Nano LabChip® kit (Agilent). 1 μg of total RNA was used to synthesize cDNA using SuperScript™ II RNase H-reverse transcriptase (200U/reaction; Invitrogen, Carlsbad, USA) and anchored oligo-dT-primers (3.5 μM final concentration). To check the generation of amplifiable cDNA in the reverse transcription reaction, a conventional PCR step was performed using GAPDH specific primers. The profiling of the expressions of the cytokine genes interleukin (IL)-1, IL-2, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, as well as inducible NO synthase (iNOS) and heme oxygenase (HO)-1 and the 3 housekeeping genes cyclophilin, GAPDH, and β-actin were measured in PBMCs as described previously using a FACSAria® flow cytometer (Becton Dickinson, San Jose, CA, USA) [11]. Subpopulations of white blood cells including monocytes, plasmacytoid dendritic cells, B cells, naïve, activated, and memory T helper cells, regulatory T cells, cytotoxic T lymphocytes, γδ-T cells, and natural killer cells were discriminated and quantified by FACS. Antibody clones used for distinguishing cell markers as well as surface marker co-expression profiles used for identification of antigen presenting cells and lymphocyte subpopulations were the same as used by Sipos et al. [11].

μCT and DXA analyses of selected bone sites

The following bone sites were considered for μCT imaging and analysis: sixth lumbar vertebra (L6), proximal tibia, and distal radius. Bones were frozen at ~80°C until preparation. After freeing bones from soft tissue, a transverse section was obtained from each site of interest and from each animal (diamond band saw, 300 CP, Exakt GmbH, Germany). Then, one trabecular bone cylinder of 8 mm in nominal diameter and 12 mm in nominal
length was extracted from each section using a diamond coated coredrill. The biopsies were immersed in saline solution, freed of air bubbles by application of vacuum and scanned with 12 μm resolution using a μCT 40 system (ScancoMedical AG, Switzerland) with 70 kV, 114 mA, and 200 ms integration time. The region of interest was restricted to an inner cylinder with 7 mm diameter and 11.5 mm length via contouring to exclude peripheral artefacts. Morphological analysis of each biopsy was performed with the inbuilt software tools provided by the manufacturer (IPL). The images were filtered using a Gaussian filter (sigma=0.7, support=1) and segmented using a global threshold value corresponding to 20% of the maximum gray value intensity. A standard evaluation protocol was used to compute the histomorphometric parameters from the segmented images. 2 biopsies containing a significant part of the growth plate were excluded from the analysis. DXA scans of femoral neck, trochanter, and the total region including head, neck, and trochanter were performed using a Lunar iDXA® bone densitometer with prepared femora being placed in a water bath for mimicking soft tissue.

**Bone ash analysis**
The volume of each trabecular bone biopsy used for μCT analysis was calculated out of 5 consecutive measurements of height and diameter. Ashing was performed for 24 h at 650 °C and ash weight was measured after a consecutive cooling down phase at room temperature for another 24 h.

**Statistics**
Statistical evaluation was performed by PASW-Statistic Software, version 17.0.2. All variables were tested for normal distribution. As most of them fulfilled the requirements for parametric evaluation, ANOVA was applied for group comparisons. In cases where normal distribution was excluded, a Kruskal-Wallis test was used. For post hoc analysis, a Bonferroni test was applied.

**Results**

**Hematology and cellular immune parameters**
Hematological analyses including FACS-based analysis of the distribution of antigen-presenting cells and lymphocyte subpopulations as well as cytokine profile kinetics revealed no meaningful trends over the course of the trial. Also, no differences could be observed between the 4 groups at either time point. Instead, cytokine titer or cell numbers of immunocyte subpopulations were found within the respective reference ranges [11] (data not shown). They only slightly undulated from one time point of measurement to the next and thus gave no evidence of any OVX- or calcium shortage-induced change in hematological or immune status.

**Biochemical bone markers and hormones**
Mean values and standard deviations of investigated biochemical bone markers of the last sampling session are given in **Table 2**. As with hematological and cellular immune parameters, levels were only slightly undulating without any trend of either increasing or decreasing titer for each of the parameters over time. Results of group 3, which was the control group, can be considered as physiological reference values.

**Morphological and densitometrical analyses**
Also morphological as well as densitometrical analyses gave no evidence of any impact of OVX or calcium shortage on bone microstructure or density (Fig. 1). To better demonstrate the uniformity of trabecular bone microstructure of each anatomical site between groups, 2D μCT images of all investigated bone sites of reference animals of each group are given (Table 3, 4). To demonstrate the uniformity of trabecular bone microstructure of each anatomical site between groups, 2D μCT images of all investigated bone sites of reference animals of each group are given (Table 3, 4).

**Discussion and Conclusions**

To date, mostly minipigs are used as large animal biomedical model species. Pure bred minipigs are expensive and have distinct ontogenetic and physiologic features when compared to conventional pigs [12]. These include earlier sexual maturity and a chondrodystrophic phenotype, which is common to most minipig strains. Additionally, most experiments in osteoporosis research were performed with growing minipigs, which may be a drawback when trying to extrapolate data to the situation of postmenopausal women. Therefore, our aim was to investigate the suitability of multiparous conventional sows as a model in osteoporosis research. We analyzed the effects of calcium shortage and OVX over a time span of 10 months. We chose this time frame because changes in bone metabolism as well as bone mass and architecture due to OVX and calcium shortage could have

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Biochemical bone marker levels acquired at the termination of the experiment (10 months post OVX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (OVX)</td>
</tr>
<tr>
<td>sRANKL (pmol/l)</td>
<td>0.7</td>
</tr>
<tr>
<td>BAP (E/l)</td>
<td>13.5</td>
</tr>
<tr>
<td>OC (ng/ml)</td>
<td>194.7</td>
</tr>
<tr>
<td>CICP (ng/ml)</td>
<td>40.0</td>
</tr>
<tr>
<td>PYD (nmol/l)</td>
<td>8.5</td>
</tr>
<tr>
<td>Crosslaps (ng/ml)</td>
<td>1.5</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>16.3</td>
</tr>
<tr>
<td>VitD3 (nmol/l)</td>
<td>170.1</td>
</tr>
</tbody>
</table>
Nevertheless, these authors also did not find any significant changes concerning bone chemistry and histomorphometry. One reason for the observed dynamic rigidity, meaning the inability to respond to estrogen and calcium shortage, might be the notably high BMD of adult pigs, making efforts to artificially reduce bone mass and weakening bone a hardly achievable task. Lactating sows have to nourish litters of up to 13 piglets and thus produce large amounts of milk with 10–11 kg per day containing approximately 50 mmol/l calcium, which corresponds to a 14 times higher calcium concentration than human milk, containing approximately 50 mmol/l calcium, which corresponds to a 14 times higher calcium concentration than human milk, consequently giving evidence of the intense need for huge calcium resources [13]. Despite these tremendous physiologic needs for calcium the skeletal apparatus has to function properly. This may provide some explanation for the peculiarities of bone physiology of adult sows.

In other ungulates investigated so far, that is, sheep, OVX alone reduces bone mass, which can be enforced by an additional nutritive calcium shortage. BMD of L5 and distal radius as evaluated by DXA was significantly changed after 6 months and the one of L4 1 year after OVX, whereas the proximal parts of femur, humerus, and tibia did not exhibit alterations to that extent [14]. However, MacLeay and colleagues [15] were not able to detect

<table>
<thead>
<tr>
<th>Bone site</th>
<th>Group 1 (OVX)</th>
<th>Group 2 (OVX, low Ca)</th>
<th>Group 3 (Sham)</th>
<th>Group 4 (Sham, low Ca)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Neck</td>
<td>1.47</td>
<td>0.08</td>
<td>1.56</td>
<td>0.16</td>
</tr>
<tr>
<td>Trochanter</td>
<td>1.59</td>
<td>0.14</td>
<td>1.58</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>1.67</td>
<td>0.09</td>
<td>1.67</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Bone ash values (mg/cm³)

<table>
<thead>
<tr>
<th>Bone site</th>
<th>Group 1 (OVX)</th>
<th>Group 2 (OVX, low Ca)</th>
<th>Group 3 (Sham)</th>
<th>Group 4 (Sham, low Ca)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Radius</td>
<td>380.0</td>
<td>61.0</td>
<td>393.7</td>
<td>108.9</td>
</tr>
<tr>
<td>Tibia</td>
<td>344.3</td>
<td>95.4</td>
<td>339.3</td>
<td>113.8</td>
</tr>
<tr>
<td>Lumbar vertebra</td>
<td>394.7</td>
<td>55.3</td>
<td>374.9</td>
<td>60.7</td>
</tr>
</tbody>
</table>

Table 3 Bone mineral content at 10 months post OVX

Table 4 μCT data of respective bone sites at 10 months post OVX

Sipos W et al. OVX and Calcium Shortage in Adult Sows ... Horm Metab Res 2011; 43: 404–409
areal BMD changes in lumbar vertebrae in ovariectomized sheep 3 months after surgery. Another study showed a significantly decreased femoral but not lumbar vertebral BMD as well as sig-
ificant effects on cortical bone parameters by 6 months after
OVX [16]. The lesser effects of single OVX treatment on bone
mass in sheep are discussed by their probable ability of extrago-
nadal estrogen production, which has not been reported for pigs
so far. Interesting and seemingly inconsistent with the hypo-
thesis of extraglandular estrogen synthesis is the fact that sheep
experience significant microarchitectural changes in vertebral
cancellous bone (decreased BV/TV by approximately 30%,
trabecular thickness by 13%, and increased trabecular separa-
tion by 46%) 2 years after OVX and show significantly increased
osteoclast numbers already 3 months after surgery [17,18].
Another study reported the advantages of combining OVX and
glucocorticoid administration over combining OVX and calcium
restriction with a higher decrease of BMD of distal radius, distal
tibia, and calcaneus (spongiosa by 25% and corticalis by 17% in
the former group and 10 and 5%, respectively, in the latter) [19].
Combining all 3 measures led to the most pronounced reduc-
tions (60 and 25%). When using corticosteroids, the disadvan-
tage of immunosuppressive side effects causing local and/or
systemic opportunistic infections [20] and of hampering oste-
oimmunological analyses has to be kept in mind.
Sows seem to differently handle loss of ovarian function and
nutritive calcium shortage with respect to bone morphology,
but also their cellular immune system gives no evidence of being
affected by these measures. This is another difference to post-
menopausal women, whose T cells have been shown to be acti-
vated as a consequence of increased IFN-γ, TNF-α, and RANKL
synthesis [21]. Also, postmenopausal women have been shown
to harbor higher levels of CD8⁺CD57⁺ cells and to suffer from a
proinflammatory state [22,23], none of which has been observed
in our model.
Another way to possibly achieve an osteoporosis-like phenotype
in adult sows could be glucocorticoid treatment with all the dis-
advantages discussed above. Glucocorticoid-induced osteopo-
rosis has already been shown as an option in the porcine model,
albeit again minipigs have been used for these experiments.
Scholz-Ahrens and colleagues [24] induced an osteoporotic phe-
ton type in adult (30 months old) primiparous Götttingen mini-
pigs by daily oral prednisolone treatment at a dose of 1 mg/kg for
2 months with a reduction of this dose to 0.5 mg/kg thereafter
until the end of the experiment, which was after 8 months in the
short-term group and 15 months in the long-term group. In the
short term, glucocorticoids reduced BMD at the lumbar spine by
48 mg/cm³ from baseline, whereas in the control group reduc-
tion was 12 mg/cm³. These changes were also evidenced by
plasma BAP levels, which decreased significantly in the gluco-
corticoid group. In the long term, the loss of BMD became more
pronounced, and bone mineral content, trabecular thickness,
and mechanical stability tended to be lower compared with the
control group. There was a negative association between the
cumulative dose of glucocorticoids and BMD, which could be
 traced back to impaired osteoblastogenesis.
The present study has 2 major limitations. First, we analyzed only
8 sows per group. This small number is a consequence of the ani-
mal’s’ dimension with each sow weighing over 300 kg. On the
other hand, comparable large animal studies included even
smaller numbers of pigs [4,25]. The second limitation is the cross-
sectional design instead of a longitudinal one, which was due to
logic reasons. One additional limitation is the impropriety of
serum estrogen measurement for validation of successful OVX in
the pig model, which in the case of large animal surgery might not
be considered a serious drawback as the ovaries are clearly visible
and of a comparably large size. The aforementioned ineligibility is
due to the fact that on the one hand serum estrogens are below
the detection limit in non-ovariectomized sows during diestrus
and on the other hand this study gives evidence of extraglandular
sexual steroid synthesis in ovariectomized sows. Nevertheless,
the percentage of animals with a measurable systemic estrogen
titer was significantly lower in the group of ovariectomized sows
when compared to non-ovariectomized ones.
In conclusion, the skeleton of adult conventional sows is seem-
ingly protected from effects of OVX and calcium shortage, which is
a very interesting finding. Hence, these animals do not appear
to be a suitable model for investigations concerning postmeno-
pausal osteoporosis but could be used to identify factors that
protect bone from calcium or sex hormone deficiency. Moreover, this study additionally provides valuable information about yet unknown physiological data on bone metabolism parameters in adult sows. At the moment we have to accept that bone of some species such as laboratory rodents reacts to generally accepted osteoporosis-inducing stimuli such as O VX and calcium shortage as would human bone, whereas the skeleton of other species, such as the adult pig, seems to be resistant to the development of osteopenia or osteoporosis. The skeleton of bears may serve as another contradictory example, as it remains unaltered in the context of immobilization during hibernation [26]. Future studies should focus on unraveling the endocrinological and perhaps immunological mechanisms, which function as protectors of bone mass and structure in these species.

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