ANIMAL MODELS

Osteocyte Regulation of Receptor Activator of NF-κB Ligand/Osteoprotegerin in a Sheep Model of Osteoporosis

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Osteoporosis induction in a sheep model by steroid administration combined with ovariectomy recapitulates decreased bone formation and substandard matrix mineralization in patients. Recently, the role of osteocytes has been frequently addressed, with focus on their role in osteoclastogenesis. However, the quantification of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) signaling in osteocytes was not studied in sheep. The current study reproduced the sheep model of osteoporosis to study the RANKL/OPG ratio correlation to the method of osteoporosis induction. We investigated the induction of osteoporosis after 8 months using 31 female merino land sheep divided into four groups: control, ovariectomy, ovariectomy with dietary limitation, and ovariectomy with dietary limitation and steroid injection. In accordance to previous reports, the present study showed trabecular thinning, higher numbers of apoptotic osteocytes, and imbalanced metabolism, leading to defective mineralization. The global RANKL/OPG ratio in the spine after 8 months of steroid and dietary treatment was not different from that of the control. Interestingly, assessment of the osteocyte-specific RANKL/OPG ratio showed that the steroid-induced osteoporosis in its late progressive phase stimulates RANKL expression in osteocytes. Sclerostin is suggested to induce RANKL expression in osteocytes. The findings of this study can contribute to further explain the success of sclerostin antibodies in treating osteoporotic patients despite increased osteocyte-expressed RANKL.

Osteoporosis is the most common metabolic bone disorder,1 which is associated with an increased risk of fractures even after minimal trauma.2 Risk of osteoporotic fractures is diagnosed by >1.5 SDs lower than the mean of age-matched individuals (Z-score). Half of documented osteoporotic fractures are at the spine and have an increased mortality rate up to 20%.3 Bone mineral density (BMD) is governed by bone metabolism that results in bone loss by either increased osteoclast-mediated bone resorption4 or decreased osteoblast-mediated bone mineralization.5–9 In turn, osteocytes are distributed within the bone matrix10 to establish connections with adjacent cells and respond to mechanotransduction.11

Receptor activator of NF-κB ligand (RANKL) is essential for osteoclastogenesis13 through binding to the RANK receptor on the surface of osteoclast precursors. RANK decoy receptor

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Different criteria (race, pregnancy, disease history, infection) osteoporosis. Animals were selected based on several criteria. Female Merino land sheep (F31/36) were used to study the cellular imbalance in osteoporotic large animal model. This study was part of a trans-regional project aimed to deepen the understanding of the behavior of cellular components in an osteoporotic large animal model. This study was performed in accordance with our institutional regulations and German animal protection laws and approved by the ethical committee of the local governmental institution (Regierungsspräsidium Darmstadt, permit number/application number F31/36).

Materials and Methods

This study is a part of a trans-regional project aimed to deepen the understanding of the behavior of cellular components in an osteoporotic large animal model. This study was performed in accordance with our institutional regulations and German animal protection laws and approved by the ethical committee of the local governmental institution (Regierungsspräsidium Darmstadt, permit number/application number F31/36).

Experimental Design

Female Merino land sheep (n = 31), with an average age of 5.5 years, were used to study the cellular imbalance in osteoporosis. Animals were selected based on several different criteria (race, pregnancy, disease history, infection) and then were randomly divided into four groups: i) control group (control, n = 8); ii) bilaterally ovariectomized group (OVX, n = 7); iii) bilaterally ovariectomized and treated with a deficient diet (OVXD, n = 8); and iv) triple treatment group [in addition to OVX and deficient diet, this group received a glucocorticoid treatment (methylprednisolone)] (OVXDS, n = 8).

Two weeks after recovery from bilateral ovariectomy, the deficient diet was given to the OVXD and OVXDS groups. All four groups were examined at 8 months after treatment. Animals received a premedication of 10 mg/kg ketamine hydrochloride (100 mg/mL Ketavet; Bela-Pharm GmbH und Co KG, Vechta, Germany), 0.01 mL/kg xylazine (2% Rompun; Bayer AG, Berlin, Germany), 0.3 mg/kg midazolam (5 mg/mL Midazolam Rotexmedica; Rotexmedica GmbH, Tüttar, Germany), and 0.01 mg/kg atropine (0.5 mg/mL Atropinsulfat; B. Braun Melsungen AG, Melsungen, Germany) before the bilateral ovariectomy. Subsequently, i.v. anesthesia was administered with 2 mg/kg propofol (20 mg/mL propofol; Fresenius Kabi, Bad Homburg vor der Höhe, Germany) and 2 mg/kg ketanyl (50 mg/mL Fentanyl-Hameln; Hameln Pharmaceuticals GmbH, Hameln, Germany). Painkillers were delivered before surgery and up to 5 days after surgery i.m. using 0.01 mg/kg buprenorphine hydrochloride (0.3-mg Temgesic ampoules; RB Pharmaceuticals GmbH, Heidelberg, Germany) and 0.5 mg/kg meloxicam (20 mg/mL Metacam only for veterinary use; Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany) dosages. Daily monitoring revealed no further need for painkillers or antibiotics postoperatively. Animals were euthanized by i.v. administration of 50 mg/kg pentobarbital (Anestesial; Pfizer, Deutschland GmbH, Berlin, Germany) under anesthesia. After euthanasia, LV and biopsy specimens from the IC (Figure 1B) were collected and analyzed.

Animal Diet

The experimental groups control and OVX received a standard diet (catalog number S6189-S010; Sondermischung Schaf, 4-mm pellet; ssniff-Spezialdiäten GmbH, Soest, Germany). The OVXD and OVXDS groups received a diet deficient in calcium and vitamin D2/3. The diet was offered twice each day besides ad libitum hay.

Steroid Treatment

Two weeks after ovariectomy, each sheep in the OVXDS group received a biweekly dosage of 320 mg methylprednisolone (Depot-Medrate only for veterinary use, 40 mg/mL i.m. injection; Pfizer Deutschland GmbH, Berlin, Germany).

Dual-Energy X-Ray Absorptiometry

Dual-energy X-ray absorptiometry serves as a noninvasive and precise method to follow up radiological changes. Alterations in BMD and the percentage of total body fat...
(Fat %) were investigated using a dual-energy X-ray absorptiometry device and software (Lunar Prodigy version 13.40; GE Healthcare, Darmstadt, Germany).

Device calibration was performed according to the manufacturer’s protocol. The sheep were anesthetized and placed under intubation in a prone position with splayed limbs (Figure 1A). The neck and head were adjusted and fixed with attenuation-free accessories. A whole body scan was performed. Subsequently, BMD (g/cm²) and Fat % were measured for IC and LV.

Biomechanical Testing

The maximum strength of the vertebrae was determined using 20-mm slices from the middle part of the L2 vertebrae. The vertebrae were cleaned from muscles, ligaments, and the intervertebral disk. The posterior part, including the pedicles, was removed so that only the anterior part of the vertebrae was left. Then, both the inferior and superior end plates with approximately 10 mm were excised using a band saw to separate standardized 20-mm slices with parallel planes from the middle part of the vertebrae. These specimens were placed on a flat surface in a material testing machine (Zwick/Roell Z020, Ulm, Germany), and a compression force by another flat plunger was applied (Figure 1C), according to the standard DIN-EN-ISO-604. Briefly, the specimens were compressed along their major axis at constant speed until they fractured or until the load reached a predetermined value. From the resulting load-deformation curve, the maximum load peak in the stiffness within linear elastic region was determined.

Histological Staining

Decalcified histological stains were performed on IC and LV samples to investigate structural and cellular changes. IC and LV were harvested, and freed from soft tissue. The IC biopsy specimen was sawn longitudinally into two halves for decalcified and undecalcified histological investigations. One IC half and a slice of LV (5 mm thick) were fixed in 4% paraformaldehyde and later decalcified (4% paraformaldehyde and 14% EDTA at 4°C for 8 weeks). After embedding, paraffin blocks were divided into sections (5 μm thick) to perform the following analyses: trichrome Masson-Goldner staining was performed to investigate the changes in bone matrix mineralization and bone quality; and silver nitrate staining was performed to analyze the morphological and arrangement discrepancies of osteocytes, as described before. Silver nitrate visualizes osteocytes and the canaliculi network by staining acidic protein present in bone matrix.

Enzyme and Immunohistochemical Analysis

Direct and indirect immunostaining was performed on IC and LV decalcified samples to describe the cellular imbalance and regulation. Therefore, the following stains were used: i) Alkaline phosphatase (ALP) enzyme histochemical staining was performed to investigate osteoblast activity.

Figure 1  Effect of multideficient diet, steroid treatment, and ovariectomy on bone quality and functional competence in a sheep model. A: In vivo dual-energy X-ray absorptiometry (DXA) scan. B: Iliac crest (IC) biopsy sample. C: Biomechanical testing on L2 vertebral body. D and E: Z-score evaluation in IC and lumbar vertebrae (LV), respectively. F and G: Evaluation of fat percentage in IC and LV, respectively. H and I: Evaluation of mechanical competence of bone shows significantly lower maximum load at failure (Fmax) and stiffness in OVXDS compared to control, OVX, and OVXD, respectively. One-way analysis of variance with Bonferroni corrections was used for D and E, and U-test was used for for F–I. Data are expressed as boxplots (D and E) or as means ± SEM (F–I). n = 8 per group (A–I). *P < 0.05.
Briefly, sections were deparaffinized and stained with 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium substrate (catalog number 508108; KPL, Gaithersburg, MD) and incubated at 37°C for 90 minutes. After washing, slides were counterstained with nuclear fast red aluminum sulfate solution (catalog number N069.1; Carl Roth GmbH, Karlsruhe, Germany). ii) Tartrate-resistant acid phosphatase enzyme investigated osteoclast activity. Deparaffinized sections were stained with tartrate-resistant acid phosphatase solutions [sodium acetate (catalog number 1.06346.0500), naphthol-AS-TR-phosphate (catalog number N6125), N,N-dimethyl formamide (catalog number D-4551), and sodium tartrate (catalog number 1.06663.0250), all from Merck, Darmstadt, Germany; and fast red (catalog number 89453-69-0) from Sigma-Aldrich, Munich, Germany] and incubated for 50 minutes at 37°C. The slides were counterstained with hematoxylin (catalog number 6765015; Thermo Fischer Scientific, Darmstadt, Germany). iii) Anti-RANKL antibody was used (Ab12A668; Abcam, Cambridge, UK). and iv) Anti-OPG antibody was used (Ab250800; Abcam). Sections were deparaffinized and then blocked with freshly prepared 3% H2O2 for 5 minutes. Afterward, sections were incubated in the primary antibody dilution overnight with Dako buffer (Agilent Technologies GmbH, Ratingen, Germany). Afterward, sections were incubated with secondary antibody diluted in 1% bovine serum albumin in tris-buffered saline with sheep serum in a 1:100 ratio (for 30 minutes at room temperature). Finally, secondary antibodies were visualized by incubating sections with red alkaline phosphatase substrate kit (catalog number SK-5100; Vector Laboratories, Burlingame, CA). The slides were counterstained with silver nitrate to investigate the changes in RANKL and OPG activity in osteocytes. The slides were mounted with Vitro-Clud (R. Langenbrinck GmbH, Emmendingen, Germany).

Fluorochrome Labeling

To investigate the dynamic bone remodeling and mineralization rate, three animals from each group were randomly selected for in vivo double fluorochrome labeling. Fluorochromes were purchased from Sigma-Aldrich (Munich, Germany) and administered s.c. after adjustment to pH 7.2 and filter sterilization. Calcein green (0.05 mL/kg, catalog number C0875) was injected at 14 and 7 days pre euthanasia, whereas alizarin red (1.5 mL/kg, catalog number A3882) was injected 15 days pre euthanasia. The markers were injected in intervals of 1, 7, or 8 days before euthanasia. After euthanasia, collected samples were embedded in polymethylmethacrylate (Technovit 9100 NEU; Heraeus Kulzer, Hanau, Germany) and prepared in grindings (10 to 20 μm thick).

Microscopy Imaging

Images were taken using a Leica microscopy system (Leica DM5500 photomicroscope equipped with a DFC7000 camera and operated by LASX software version 3.0; Leica Microsystems Ltd., Wetzlar, Germany). Visualization of anti-OPG and anti-RANKL was measured under Texas-Red filter (Leica Microsystems Ltd.) at >560-nm emission. The excitation wavelength range for the fluorescent red substrate was set to 365 up to 560 nm. Alizarin red and calcein green labeling were visualized using Texas-Red and Green Fluorescent Protein filters (Leica Microsystems Ltd.), respectively.

Histomorphometry

Histomorphometry was performed using ImageJ software version 2.4 (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

The quantification of different histological stains provides an insight into the changes in bone parameters. Therefore, the following parameters were obtained through histomorphometry: i) Nonmineralized and mineralized matrix portion in IC and LV from triehrome Masson-Goldner staining, using Trainable Weka Segmentation plugin version 1.49 (https://imagej.net/Trainable_Segmentation) on overview images of the whole bone region.

ii) Quantification of toluidine blue staining on LV samples was performed using BoneJ plugin version 1.3.14 (https://bonej.org) to obtain trabecular thickness, trabecular length, and trabecular area normalized to the total area for all four groups. iii) Spindle, spherical, and empty lacunae non-normalized and normalized to trabecular area from silver staining through assessment of 40 randomly selected fields from each sample. Trabecular area was determined after clearing bone marrow and converting images to binary.

Osteocyte types were counted using cell counter plugin after subjectively assigning into one of three categories: i) spindle-shaped osteocytes with a well-marked dendrite network and clearly visible cell core, ii) intermediary-shaped osteocytes (oval shaped) with less marked dendrite network, and iii) spherical osteocytes with a rounded shape, reduced dendrite network, and no visible cell core. Total osteocyte number and numbers per group were calculated and normalized to trabecular area. The subjective analysis was performed by two independent investigators (F.M. and T.E.K) to avoid bias.

iv) RANKL or OPG activity within the trabecular bone. The analysis of trabecular bone for RANKL/OPG included intracortical areas, resulting from three distinctive areas of analysis: the erosion tunnel within the intracortical area, the eroded lacunae on the surface of trabecular bone, and the osteocytes within the trabecular bone. The global OPG or RANKL signal was calculated by the summation of all three areas to correlate with the global relative expression.

Laser-Scanning Confocal Microscopy

IC samples (n = 8 per group) were processed for embedding in PMMA, according to standard protocols. However, preinfiltration and infiltration solutions were modified to contain Rhodamine-6G powder (Sigma-Aldrich, Steinheim, Germany) dissolved in 0.01 w/v%. Rhodamine staining was
performed to investigate the changes in osteocyte network in all four groups. Sections (10 μm thick) were prepared and mounted with florescence mounting medium (S3023; Dako, Santa Clara, CA), and left at 4°C overnight. Imaging was performed using the Axio Imager Z1 imaging system (LSM 710 Confocal Microscope equipped with a 63× glycerin microscope objective operated by Zen 2011 SP3 Imaging software version 8.1; Carl Zeiss, Jena, Germany).

Analysis of Bone Remodeling Markers in Serum

Circulating serum concentrations of biochemical markers reflect the systemic effect of treatment on bone physiology. Intact osteocalcin (OCN) and bone alkaline phosphatase (BAP) were measured as anabolic markers, whereas cross-linked N-terminal telopeptide of type I collagen (NTX) indicated bone catabolism. Commercially available immunoassay kits (OCN: MicroVue Osteocalcin EIA; BAP: MicroVue BAP ELISA; and NTX: EIA OSTEOMARK NTX-Serum, all from TECOMedical GmbH, Buende, Germany) were used to measure serum concentration, according to the manufacturer’s protocol. Samples were run in duplicates. Concentrations were measured using the E-Liza Mat 3000 instrument (DRG Instruments GmbH, Marburg, Germany) and iEMS Reader MF (Labsystems Diagnostics, Vantaa, Finland).

RNA Isolation, cDNA Synthesis, and Real-Time PCR Analysis

Molecular analysis of relative gene expression reflects the cellular changes examined by histological analysis and biochemical markers. Postmortem, L1 biopsy specimens were collected in liquid nitrogen and stored directly at −80°C for RNA isolation. The extraction of total RNA was performed according to the Trizol manufacturer’s protocol (Life Technologies, Darmstadt, Germany) until separation into aqueous and organic phases. The aqueous phase using RNasey protocol was mixed with 70% ethanol and transferred to the RNasey spin column (Qiagen, Hilden, Germany), followed by DNase I digestion (Qiagen). Total RNA (1 μg) was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). Quantitative PCR was performed using GoTaq qPCR master mix (Promega). Real-time analysis was performed with Applied Biosystems 7300 system (Thermo Fischer Scientific, Darmstadt, Germany). β2 Microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L19 (Rpl19), and protein kinase C inhibitor protein-1 (YWHAZ) were used as the housekeeping genes. The relative gene expression of ALP, carbonic anhydrase type II (CA2), OPG, RANKL, type I collagen subunit α 2 (COL1A2), and fibronectin 1 (FN1) were investigated and calculated using DataAssist Software version 3.01 (Thermo Fischer Scientific, Waltham, Massachusetts). The complete list of primer sequences is provided in Table 1.

Statistical Analysis

Descriptive statistics were used to test normal distribution of data sets. Data were analyzed using Statistical Package PASW 21.0 (IBM, SPSS Inc., Armonk, NY). One-way analysis of variance with Bonferroni correction was used for

<table>
<thead>
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<th>Table 1</th>
<th>Primer List of the Genes Used for qPCR Analysis</th>
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<tr>
<td>Rankl (Tnfsf11)</td>
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</tr>
</tbody>
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F, forward; Opg, osteoprotegerin; qPCR, real-time quantitative PCR; R, reverse; Rankl, receptor activator of NF-κB; TNF, tumor necrosis factor.
parametric distribution (dual-energy X-ray absorptiometry). Significance analysis for relative gene expression was performed using t-test. Significance analysis for nonparametric distribution was performed using U-test. Bar graphs were presented as means ± SEM. Z-score was calculated for each experimental group using BMD measurements and normalized on age-matching animals from the control group. Clinically, Z-score < -1.5 indicates higher fracture risk in osteoporotic patients; therefore, the same assumption was applied to the animal model. Analysis of RANKL/OPG ratio in osteocytes in correlation to treatment was performed using exponential regression analysis and F-test for significance.

Results

Radiological and Biomechanical Assessment of OVXDS Reflects Osteoporotic Bone Status 8 Months after Treatment

The Z-score of control and OVX in both IC and LV regions was higher than the cutoff of −1.5 (Figure 1, D and E). However, OVXDS was < −1.5 and statistically significant when compared with either control or OVX in both anatomical regions. However, Z-score was not significantly different between OVXD and OVXDS in the IC (Figure 1D). Interestingly, LV Z-score was significantly higher in the OVXD when compared with OVXDS (Figure 1E). Furthermore, Fat % in IC was lower in the OVX compared with the control, although not statistically significant. The Fat % was exponential to treatment progression. The values were higher in the OVXD compared with the OVX and higher in the OVXDS compared to all groups (Figure 1F). OVXDS showed significantly higher Fat % when compared with control and OVX. However, no statistical significance was seen between control, OVX, and OVXD. Intriguingly, Fat % in the LV showed an exponential pattern correlated to treatment despite lack of statistical significance (Figure 1G).

L2 biomechanical testing indicated strength, elasticity, and deformation of bone through maximum load at failure and stiffness measurements. Both parameters were only significantly lower in the OVXDS when compared to all other groups (Figure 1H and I).

Both parameters biomechanical strength (maximum load at failure) and stiffness are influenced by the microstructure and the apparent bone mineral density. Therefore, histological analysis to differentiate mineralized and nonmineralized bone samples was performed.

Trabecular Thinning in OVXDS Spine and Pelvis Reflects Imbalanced Bone Mineralization

Quantitative analysis of nonmineralized matrix portion and mineralized bone area in all four groups in both IC and LV showed thinning of trabeculae with onset of the treatment (Figure 2, A and B). Noticeably, thin trabecular structure was seen in OVXDS compared to control. IC nonmineralized matrix portion was significantly lower in OVXDS compared to control (Figure 2C). However, no significant differences were seen among OVX, OVXD, and OVXDS. However, LV nonmineralized matrix portion was significantly higher in OVX compared to control, OVXD, and OVXDS groups. Also, OVXD and control groups in LV showed significantly higher nonmineralized matrix portion when compared to OVXDS (Figure 2C). IC mineralized bone area was significantly lower in OVXDS compared to control and OVX. No other significant differences were seen in IC (Figure 2D). On the other hand, LV mineralized bone area was significantly lower in OVXDS compared to control, OVX, and OVXD (Figure 2D). Furthermore, trabecular thickness, trabecular length, and trabecular area normalized to the total area were also measured in the LV samples (Supplemental Figure S1). OVXDS showed significantly lower trabecular thickness when compared to control, OVX, and OVXD. Trabecular length was higher in OVXDS when compared to control, OVX, and OVXD. Furthermore, OVXD showed significantly lower trabecular length when compared to OVXDS group. Trabecular area normalized to total area was significantly lower in OVXDS when compared to control, OVX, and OVXD (Supplemental Figure S1).

Loss of trabecular structure encouraged the serum and gene expression analysis of bone metabolism and matrix degradation markers (Figure 2, E–H). OCN serum concentration was significantly higher in OVXDS when compared to all groups (Figure 2E). However, no significant change in OCN serum levels was seen between control, OVX, and OVXD. Relative expression of ALP was higher in correlation to treatment accumulation from control until OVXDS; however, no statistical significance was seen between the groups (Figure 2F). A similar pattern without statistical significance was also seen in ALP immunostaining in the LV (Supplemental Table S1). Furthermore, BAP serum concentration was higher in OVXDS when compared to the control, OVX, and OVXD (Supplemental Figure S2); however, no significant differences were observed. On the other side, NTX serum concentration was significantly higher in the OVX and control when compared to the OVXD and OVXDS (Figure 2G). No significant differences were observed between control and OVX. NTX serum levels were lower in a trend in OVXDS when compared to OVXD (Figure 2G). The tartrate-resistant acid phosphatase immunostaining was higher in OVXDS compared to all other groups, which showed no significant difference among each other (Supplemental Table S1). However, the OVXDS showed a lower trend when compared to the control and OVX spine. Relative expression of CA2 was significantly lower in the OVX when compared with the control and with the OVXD (Figure 2H). The CA2 relative expression was lower, although not statistically significant, in the OVXDS when compared to OVXD.
Imbalance of bone mineralization caused by multi-deficient and steroid treatment requires detailed study of cellular components present in bone matrix.

**Deterioration of Bone Matrix in OVXDS Reflects on Osteocyte Morphology**

Osteocyte morphology showed the presence of normal (spindle) and abnormal (spherical) shape in control, OVX, OVXD, and OVXDS (Figure 3, A and B). Osteocytes and their networks appeared well arranged and more spindle shaped within the trabecular bone matrix of both IC and LV control groups. However, OVX and OVXD in both IC and LV showed a shorter osteocyte network, more spherical shape, and more empty lacunae within the trabecular bone (Figure 3, A and B). Osteocytes in the OVXDS group of IC and LV showed irregular distribution, spherical shape, and more empty lacunae within the trabecular bone.

Furthermore, lacunae count, spindle-shaped osteocytes, and spindle/spherical-shaped osteocytes were analyzed (Figure 3, C–H). Normalized empty lacunae count in IC correlated positively to treatment severity (Figure 3C). Nonnormalized empty lacunae count in IC was higher in OVX compared with the control, and lower in the OVXD compared with OVX. However, the steroid effect in the OVXDS showed higher empty lacunae in the OVXDS comparable to the values of OVX (Figure 3C). Normalized empty lacunae count was significantly higher in the IC OVXDS group compared to the control, OVX, and OVXD groups. No significant differences were seen in non-normalized empty lacunae count in IC. Normalized empty lacunae count in LV was higher in correlation to treatment severity.

**Figure 2** Alterations in bone matrix mineralization of OVXDS shows inferior bone quality. **A and B**: Trichrome Masson-Goldner shows the deterioration in trabecular structure of iliac crest (IC) and lumbar vertebrae (LV) with treatment, respectively. **C**: Nonmineralized matrix portion is lower in OVXDS in both IC and LV. **D**: Mineralized bone area is lower in OVXDS in both IC and LV. **E**: Osteocalcin (OCN) serum level. **F**: Relative gene expression of alkaline phosphatase (ALP). **G**: N-terminal telopeptide of type I collagen (NTX) serum level. **H**: Relative gene expression of carbonic anhydrase type II (CA2). *t*-test was used for relative expression, and *U*-test was used for histomorphometry and serum analysis. Data are expressed as boxplots (C and D) or as means ± SEM (E–H). *n = 8 per group (A–H). *P < 0.05 (dashed lines show IC; solid lines, LV). Scale bars = 200 μm (A and B). BCE, bone collagen equivalent.
severity from control until OVXDS (Figure 3F). Although nonnormalized empty lacunae count in LV showed no significant change from control to OVX, the lacunae count was sequentially higher throughout to OVXD before decreasing in the OVXDS (Figure 3F). Normalized empty lacunae count was significantly higher in the LV OVXDS group compared to control, OVX, and OVXD. Furthermore, the LV OVXDS group showed significantly higher normalized lacunae count compared to control and OVX. On the other side, nonnormalized lacunae count was significantly higher in LV OVXD compared to control and OVX.

Both normalized and nonnormalized spindle-shaped osteocytes in IC were higher in succession to treatment from control until OVXDS, then lessened in the OVXDS compared to OVXD (Figure 3D). Furthermore, the OVXDS group IC showed significantly lower normalized and nonnormalized spindle-shaped osteocytes compared to control, OVX, and OVXD. However, no significant differences between control, OVX, and OVXDS groups were seen. Normalized spindle-shaped osteocytes in LV showed no change from control until OVXD, despite higher counts in OVXD when compared with OVXDS (Figure 3G). Nonnormalized spindle-shaped osteocytes in LV were lower with treatment severity from control until OVXDS. No significant differences in both normalised and nonnormalised spindle-shaped osteocytes were observed between groups (Figure 3G).
The ratio of spindle- to spherical-shaped osteocytes showed varied patterns in both IC and LV (Figure 3, E and H). The normalized ratio of spindle- to spherical-shaped osteocyte count in IC was higher in response to treatment from control until OVX; however, it declined from OVX to OVXDS (Figure 3E). Nonnormalized spindle- to spherical-shaped osteocytes were higher in the OVX when compared with the control, then again declined from OVX to OVXDS. Both the normalized and non-normalized ratio of spindle- to spherical-shaped osteocytes in OVXDS was significantly lower when compared to control, OVX, and OVXDS. The normalized ratio of spindle- to spherical-shaped osteocytes in LV showed no significant change (Figure 3H). Nonnormalized spindle- to spherical-shaped osteocytes in LV were successively lower from control to OVX and then OVXD; however, a higher ratio was seen in the OVXDS when compared with the OVXD. The decline of normal spindle-shaped osteocytes and the higher empty lacunae in OVXDS suggest compromised extracellular matrix composition that affects bone metabolism. A detailed investigation of changes in bone metabolism is required to understand the cellular components involved.

Higher Osteocyte-Expressed RANKL after Severe Bone Loss Results from Steroid Treatment

Histomorphometry showed changes in RANKL, OPG, and RANKL/OPG activity between all groups (Figure 4, A, B, and F). OPG activity was lower with treatment combination from control throughout to OVXDS (Figure 4A). Control showed significantly higher OPG activity when compared to OVX and OVXDS. However, no significant differences were seen between OVX, OVXDS, and OVXDS. RANKL activity showed a trend of lower activity in OVX compared to control and higher in OVXDS when compared to control.

**Figure 4**  Comparable RANKL/OPG ratio in the OVXDS spine with the control. A: OPG activity on trabecular bone. B: RANKL activity on trabecular bone. C: Relative gene expression of OPG. D: Relative gene expression of RANKL. E: Relative gene expression ratio of RANKL/OPG. F: RANKL/OPG activity on trabecular bone. G and H: Active osteocyte signals visualized in OVXDS via silver nitrate counterstain within RANKL- and OPG-positive areas. Magnified images show the active osteocytes on bone surface. t-test was used for relative gene expression, and U-test was used for histomorphometry. Data are expressed as means ± SEM. n = 8 per group (A–H). *P < 0.05. Scale bars = 20 μm (G and H).
and OVX. However, OVXDS showed lower RANKL activity compared to all three groups (Figure 4B). No significant differences were observed between groups. Interestingly, RANKL/OPG ratio correlated negatively with the treatment severity from OVX until OVXDS (Figure 4F). The ratio of the total RANKL plus area to the total OPG plus area of the LV was 0.7% on average in the control and was thus significantly smaller than that of OVX with 11.1% and OVXD with 6.9%. The RANKL/OPG ratio in the control was slightly lower than in OVXDS with 2.7%.

Relative gene expression of OPG corresponded positively to the treatment combination (Figure 4C). Relative expression of RANKL was highest in the OVXD and lowest in the control, both without significant differences (Figure 4D). Interestingly, relative expression ratio of RANKL/OPG was significantly lower in OVXDS when compared to control, OVX, and OVXD (Figure 4E).

To understand the cell- and area-specific RANKL/OPG ratio within the trabecular bone, active osteocytes were visualized via silver nitrate counterstain after OPG and RANKL immunostaining (Figure 4, G and H). Furthermore, protein signal in osteocytes was captured through fluorescence microscopy (Figure 4, G and H). Collectively, both RANKL and OPG showed abundant signal in osteocytes.

Osteocytes had the highest percentage of total positive RANKL area among all groups (Figure 5A). Osteocyte-specific RANKL signaling amounted to 68% in control, 83% in OVX, 47% in OVXD, and 62% in OVXDS from the total positive RANKL area. The percentage at the erosion tunnel from total positive RANKL area was 18% in control, 42% in OVX, 5% in OVXD, and 5% in OVXDS, whereas erosion lacuna counted a percentage of 14% in control, 12% in OVX, 11% in OVXD, and 33% in OVXDS of total positive RANKL area (Figure 5A). In the control, OVX and OVXD osteocyte-specific RANKL was higher than the erosion area in relation to the trabecular area. All groups were significantly higher than OVXDS in all areas (Figure 5A).

Quantitative analysis of OPG staining showed a fluctuation among the groups (Figure 5B). Nevertheless, the percentage of osteocyte-specific OPG related to total positive OPG area was 19% in control and OVX, 5% in OVXD, and 13% in OVXDS. The percentage in the erosion tunnel was 36% in control, 3% in OVX, 83% in OVXD, and 52% in OVXDS from total positive OPG area. Furthermore, erosion lacuna mounted to 45% in control, 78% in OVX, 12% in OVXD, and 35% in OVXDS when compared to the total positive OPG area (Figure 5B). However, an apparent statistical difference in the lower OVXDS osteocyte signaling normalized on trabecular area was seen compared to all groups. Furthermore, OPG area in the erosion lacuna correlated negatively with treatment severity. In the erosion tunnel, a significantly lower OPG signaling to the trabecular area control was seen in the OVX group (Figure 5B). The osteocytic RANKL/OPG ratio increased in the spine exponentially with each additional treatment. Regression analysis showed a close to exponential increase ($R^2 = 0.999$) and significant ($P = 0$) from control to OVXDS (Figure 5C).

### Steroid Combined Treatment Alters Osteocyte Networking and Matrix mineralization More Than Estrogen Ablation and Dietary Limitation Alone

Osteocyte morphology was incrementally affected with treatment accumulation. Rhodamine staining showed well-arranged, mainly spindle-shaped osteocytes with multiple canaliculi connecting adjacent cells in the control group (Figure 6A). Slight disarrangement was seen in the OVX and OVXD groups, where spherical osteocytes and globule bodies started to appear besides the apparent shorter canaliculi when compared with the control group. However, OVXDS showed disarranged, round-shaped osteocytes with no canaliculi network, and large numbers of globule bodies (Figure 6A).

Relative expression of both COL1A2 and FN1 was higher in the OVX when compared with the control, before becoming lower in the OVXD, and then higher again in the OVXDS (Figure 6, B and C). OVXDS showed significantly higher expression of COL1A2 when compared to control and OVXD (Figure 6B). On the other side, FN1 relative expression showed no significant differences between any of the groups (Figure 6C). The alizarin red and the first calcein green bands were closer to the bone surface in all samples. As the

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**Figure 5** Treatment combination negatively affects osteocyte-expressed RANKL and OPG. **A:** Positively stained RANKL expression per trabecular area in the osteocytes. **B:** Positively stained OPG expression per trabecular area in the osteocytes. **C:** Exponential regression analysis in RANKL/OPG ratio across four groups. Data are expressed as means ± SEM (A and B). Exponential regression, $R^2 = 0.999$, F-test, $P = 0.000$. $n = 8$ (control and OVXD); $n = 7$ (OVX and OVXDS). *$P < 0.05$. 

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lines were laying closely to each other, an orange band appears in the superimposed image (Figure 6, D and E). Nonetheless, the OVXDS only showed one green band, whereas all other groups showed two green bands, indicating a mineralization deficiency in bone matrix (Figure 6E).

Discussion

The present study addressed the cellular discrepancies in RANKL/OPG regulation in control and diseased bone in a large animal model of osteoporosis. The sheep model was reported to develop bone loss after ovariectomy, dietary limitation, steroid therapy, or combination of treatments. Bone loss at both examined anatomical regions (LV and IC) indicated osteoporotic bone status as Z-score is well < −1.5 after 8 months of triple treatment. However, lower Z-score in the LV compared to IC supports the suggestion of systemic, yet anatomically discrepant, osteoporotic bone status. Many studies reported bone loss at various anatomical sites after ovariectomy alone or combined with dietary manipulation in the sheep without reaching a clinically identified osteoporosis. OVX alone in a sheep model after 6 months of treatment failed to show osteoporotic bone status and no significant changes in BMD and biomechanics. Nonetheless, the long-term effect (18 months) of OVX on white alpine sheep showed bone loss and deprivation in bone microarchitecture; however, after a few months, values returned to pre-OVX levels. In Merino land sheep aged 4 to 6 years, OVX alone showed only marginal differences in the reduction of trabecular bone compared to control. Furthermore, a pilot study showed restricted impact of combined treatment of calcium/vitamin D and OVX on bone metabolism. In our current study, the loss of BMD in OVX and OVXDS also showed marginal differences when compared. Taken together, achieving inferior bone quality in sheep using only ovariectomy or in combination with dietary limitation requires long-term treatment.

Furthermore, the understanding of osteocyte role in bone metabolism is crucial. The activity of RANKL and OPG is important for osteoclast and osteoblast to maintain calcium in bone. However, RANKL and OPG expression is important for various cell types.

The current study, to our knowledge, for the first time shows a detailed analysis of RANKL and OPG expression in the osteocytes in an osteoporotic sheep, which revealed a higher RANKL/OPG signal at the osteocytes. Unlike rodent models, which reflect trabecular loss, the described model showed trabecular thinning rather than lower trabecular number or higher trabecular separation. Nonetheless, this finding is in accord with previous studies of osteoporotic sheep: Schorlemmer et al showed a reduced trabecular thickness in tibia and spine of steroid-treated ovariectomized sheep in comparison with control sheep. However, the number of trabeculae was not affected. Furthermore, Augat et al described a 30% lower trabecular thickness after ovariectomy and glucocorticoid treatment for 6 months, whereas trabeculae numbers were not affected. Similarly, reduced trabecular thickness, with no significant change in trabeculae numbers, was reported by Zarrinkalam et al after 6 months of steroid treatment in ovariectomized sheep when compared with controls in lumbar spine and iliac crest.

The cellular balance was affected in all groups under each treatment. Interestingly, OCN serum level was significantly

Figure 6  Imbalance in cellular components alters ECM composition. A: Rhodamine staining performed on IC samples. B: Relative gene expression of type I collagen subunit α 2 (COL1A2). C: Relative gene expression of fibronectin 1 (FN1). D: Lumbar vertebrae (LV) OVX shows one alizarin red band and two distinct calcine green bands. E: LV OVXDS only shows the alizarin red and the subsequent calcine green band. Data are expressed as means ± SEM. n = 8 per group (A–C and E); n = 7 (D). *P < 0.05 (t-test for relative gene expression). Scale bars: 20 μm (A); 100 μm (D and E).
changed only in the OVXDS group, which also had a higher trend in ALP relative expression in the LV. Intriguing, the OVXDS group showed the least nonmineralized portion in both anatomical regions. In contrast, NTX serum level was the highest in the OVX group and lowest in the OVXDS group (Figure 2). This finding accords with the increased NTX levels in postmenopausal women.15 The lower level of cross-linking markers, such as cross-linked C-terminal telopeptide and NTX, was previously reported after 5 months of steroid treatment in OVX sheep.21,45 The collagen catabolic process is indicated by CA2 relative expression in the spine, which was highest in the OVXD, whereas COL1A2 highest expression was in the OVXDS. The higher expression of COL1A2 indicates bone loss in osteoporosis.46 COL1A2 expression levels are in line with the lower osteoid portion in the treated groups, mainly OVXD and OVXDS, at 8 months. The higher NTX serum level and COL1A2 and FN1 expression suggest that the OVX treatment alone was still exerting an effect after 8 months, whereas the OVXDS reached a point of critical bone loss. Furthermore, the lower NTX serum level and lower CA2 relative expression, besides the trend in lower tartrate-resistant acid phosphatase immunostaining and lower osteoid formation in the OVXDS spine, are consistent with the effects of long-term steroid induction. Clinical observation revealed that glucocorticoid-induced osteoporosis occurs in a rapid early phase that reduces BMD by excessive bone resorption, and a slower progressive phase with affected bone formation.47 The data provided herein suggest that after 8 months, the bone resorption effect is stabilizing in favor of inferior bone formation. This finding is best supported by the lack of a second layer of calcium deposition, as seen in the fluorochrome labeling in the OVXDS (Figure 6). Despite the estrogen deficiency through the ovariectomy in the OVX group and the combined calcium and vitamin D deficiency in the OVXD group, both groups showed three lines marking calcium deposition on three different time points. The OVXDS group showed only two lines, reflecting a substandard mineralization rate.

Inferior biomechanical competence and substandard mineral content are typical for the clinical definition of osteoporotic bone status. In correlation, the higher COL1A2 expression reflects higher degradation of bone matrix48 and the lower FN1 expression suggests less anchorage protein quality.48 This finding correlates with the lower bone anabolic markers and the higher number of osteocyte empty lacunae in both loaded and unloaded regions. The lack of osteocyte networking and their disarrangement with globulus formation in the OVXD and OVXDS is a pathological criterion of osteoporotic bone.49 However, the absolute number of spindle-shaped (normal) osteocytes was lower in the IC and LV OVXDS group. However, the effect was more evident in the unloaded region. This suggests that the loading mechanism has an effect in preserving the osteocyte activity. The results confer the previous reports that the bone erosion lacunae correlate negatively with the numbers of active osteocytes and positively with the number of empty lacunae.28 Steroid therapy is reported to increase fracture risk because of increased apoptotic osteocytes, despite lower BMD.50 Osteocytes are involved in bone remodeling by contributing in RANKL/OPG regulation. Studies showed that osteocyte expression of RANKL increases by the presence of microfractures to initiate bone repair.51 The osteocyte-expressed RANKL plays a crucial role on the remodeling of trabecular bone. Osteocyte-specific RANKL knockout was reported to cause 70% trabecular bone loss in mice.13 In this study, the global RANKL/OPG ratio in relative expression and immunostaining was significantly lower in the OVXDS spine trabeculae when compared to all groups. In other words, the RANKL/OPG ratio in all three regions of trabecular bone shows lower bone catabolism. However, the RANKL/OPG ratio only in the osteocytes is exponentially increasing with treatment accumulation. However, this finding was not reflected in the IC, where the pattern of global RANKL/OPG ratio was similar to the osteocyte, erosion lacunae, and erosion tunnel. Previous reports described an increased RANKL expression at the vicinity of apoptotic osteocytes and a reduced OPG expression near the microfractures in fatigue-loaded rat bone.52 Studies suggest that apoptotic osteocyte signals normal osteocytes to increase RANKL expression ergo an increased RANKL/OPG ratio.52,53 This theory supports the higher number of empty lacunae and higher RANKL/OPG ratio in osteocytes of this sheep model. The global RANKL/OPG relative expression reported herein included all RANKL-expressing cell types, among which are the T cells. However, the RANKL/OPG global immunostaining included only bone cells in trabecular and intracortical regions. Nonetheless, the results of both analyses show a globally reduced RANKL/OPG ratio. This finding supports the previous suggestion reviewed by Martin and Sims51 in 2015, which evades the T-cell contribution to osteoclastogenesis. However, the lower expression and signal localization of OPG in the OVXDS group are consistent with the effects of glucocorticoid treatment on OPG,54 which, in turn, reflect on the higher RANKL levels.

Furthermore, the higher RANKL/OPG ratio in osteocytes might correlate to a higher sclerostin (SOST) local expression in osteocytes, as described previously in vitro.54 Taken together, SOST local stimulation of RANKL in vital osteocytes could be a response to the RANKL-induced described apoptotic osteocytes. Interestingly, this theory can further elucidate the success of an SOST antibody treatment as it does not only increase osteoblast-mediated bone formation55 but also inhibits osteocyte-mediated bone resorption by inhibiting RANKL expression on osteocyte apoptosis. Recently, we have also reported a positive correlation of increased SOST expression and apoptotic osteocytes in ovariectomized rats with dietary treatment.51 The exponential increase in RANKL/OPG ratio in osteocytes confers with the role of estrogen deficiency and dietary limitation in the sheep model as well.
Osteocytes incorporate many regulatory mechanisms in bone remodeling. Most described is their mechanosensory function in loaded and unloaded regions and the repair of microfractures. The osteocyte response to local factors, such as phosphate concentration, SOST, and fibroblast growth factor 23 expression, was comprehensively described. Furthermore, the response of osteocytes to systemic hormone alterations, like estrogens, parathyroid hormone, and vitamin D, was fully reviewed. The previous work, reviewed by Atkins and Findlay, elucidates the osteocyte regulatory role in bone remodeling (RANKL/OPG system) and in matrix mineralization through phosphate regulating endopeptidase homolog, X-linked, matrix extracellular phosphoglycoprotein, and dentin axtrix acidic phosphoprotein 1 expression. Many other studies also revealed the role of osteocytes together with osteoblasts in energy metabolism.

Conclusion

This study offers a new perspective about the osteocyte regulation in a sheep model of osteoporosis. The use of osteocyte-specific staining as a counterstain enabled the detailed histomorphometry of RANKL/OPG ratio in osteocytes, thereby reflecting osteocytes’ regulatory role in response to the treatment not as a part of the global pattern of regulation in loaded or unloaded regions. Unfortunately, the availability of specific antibodies for immunostaining or serum level (ie, parathyroid hormone and fibroblast growth factor 23, SOST, and MEPE) measurements in sheep is still limited. Therefore, a deeper understanding of the role of osteocytes in this particular model remains hindered.

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Supplemental Data

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References


