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Effect of Ovariectomy, Malnutrition and Glucocorticoid Application on Bone Properties in Sheep: A Pilot Study

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Abstract. The demographic changes in the human population continue to lead to an increasing incidence of osteoporosis. The main clinical symptom of osteoporosis is fracture. Fracture fixation in osteoporosis is frequently complicated by failure of fixation. There is a great need for a large-animal model of osteoporosis for controlled studies, which allows the investigation of fracture healing and fracture treatment in weak bone. Eight swiss mountain sheep, 7–9 years old, were divided into four treatment groups of two animals each. Group 1 was ovariectomized and fed a calcium/vitamin D-restricted diet (O+D). Group 2 was ovariectomized and given a daily intramuscular injection of 25 mg methylprednisolone (O+S). Group 3 was ovariectomized, fed a calcium/vitamin D-restricted diet and injected with 25 mg intramuscular methylprednisolone per day (O+D+S). Group 4 was used as an untreated, not sham operated control group. At the beginning of the study and every 2 months for 6 months the bone mineral density (BMD) was determined using quantitative computed tomography (pQCT) at the distal radius. Biopsies were taken after 6 months from vertebral bodies and femoral heads and the bone structure, i.e. trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), bone surface fraction (BS/BV) and bone volume fraction (BV/TV), was determined by micro-CT. In vitro compression testing of the biopsies was performed to determine failure load and stiffness. The control group showed no changes in BMD. The greatest decrease in BMD was seen in group 3 (O+D+S), which had a decline of 58% in cancellous bone and 22% in cortical bone. In the vertebral body biopsies a prominent change in structural parameters was observed (Tb.N, –53%; Tb.Th, –63%, Tb.Sp, +150%). The changes were less pronounced in the femoral head biopsies. In the compression test the vertebral body biopsies of group 3 (O+D+S) had stiffness values 40% lower failure load 70% lower compared with the control group. The most effective method of inducing osteoporosis in sheep was found to be the combined treatment. These results need to be confirmed in a larger number of animals.

Keywords: Mechanical testing; Micro-CT; Osteoporosis; Osteoporosis induction; pQCT; Sheep model

Introduction

Postmenopausal osteoporosis is a health problem of major proportions. Fractures commonly associated with osteoporosis are located at the proximal femur, lumbar vertebrae and distal radius. Fractures of the hip and vertebrae pose the most serious health risks associated with the disease, causing prolonged hospital care, greater morbidity and mortality rates [1]. While the best defence against the disease would be prevention, there is also a great need for appropriate treatment of osteoporotic fractures.

Bone and mineral metabolism are known to change with age and hormonal imbalance, e.g., in postmenopausal women [2]. Systemic reduction of bone mineral density (BMD) may be induced by malnutrition (e.g., reduced calcium intake) and drugs (e.g., corticosteroids) in both humans [3,4] and animals [5,6]. Studies of

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fracture fixation have sometimes ignored or poorly documented the quality and quantity of bone in the vicinity of implants. In the clinical setting orthopedic implants are frequently placed in aged patients, but most studies have used young animals, or animals of unknown age, to investigate fracture fixation, fracture healing and prosthetic fixation.

The effects of estrogen deficiency on the histomorphometry and mechanical competence of bone have been documented in humans as well as in various animal species [7,8]. An experimental animal model to study the mechanisms of bone loss and to test possible solutions for fracture treatment in osteoporotic bone could eliminate the difficulties associated with studying the disease in humans, i.e., inhomogeneous and small study groups, ethical constraints and the lack of control groups. Currently experimental studies on bone metabolism and bone healing in osteoporosis could be improved by such an animal model.

The most commonly used laboratory animal for osteoporosis research is the rat because it is cost-effective and convenient to handle. However, there are differences between the human skeleton and that of rodents with respect to bone and mineral metabolism, lack of lamellar bone and limited capacity for bone remodeling. Experimental procedures such as sampling of body fluid, removal of tissue and performance of surgical procedures are difficult due to the small size of the rat [9]. Studies on ovariohysterectomized dogs have shown that changes in cancellous bone remodeling were a series of transient phenomena. The duration of these changes did not appear to be sufficient to effect a sizeable or significant reduction in bone volume [10]. Non-human primates, while providing an excellent model on theoretical grounds, are not cost-efficient. Pigs have a thicker cortical bone and higher BMD in trabecular bone compared with humans [11].

The ideal animal model for osteoporosis research should be large enough to facilitate serial sampling and experimental procedures to investigate fracture repair, have mineral metabolism and bone structure similar to humans, be readily adaptable to the laboratory environment and be available at reasonable cost.

Sheep seem to be a promising model for the study of fracture healing and treatment in osteoporotic bone for various reasons; they are docile, easy to handle and house, relatively inexpensive, available in large numbers, ovulate spontaneously, and have hormone profiles similar to women [9]. Haversian remodeling is seen in older animals. Studies have demonstrated, that biomechanical incompetence of bone follows ovariectomy [11]. There are data available on the long-term assessment of changes in BMD after ovariectomy in sheep showing a decrease after 4–6 months [12]. The sheep has been used to study the effects of ovariectomy on biochemical and histologic indices of bone turnover [13]. Biomechanical testing of the femur has been performed earlier in ovariectomized sheep with placebo and salmon calcitonin treatment [14]. Lill et al. [15] demonstrated that BMD and mechanical properties of sheep tibiae are significantly reduced after a combined treatment of ovariectomy, calcium/vitamin D-restricted diet and steroid application. It has also been shown that histomorphometric parameters of iliac crest biopsy specimens changed significantly. In this study we report on the same animals.

The size and gross anatomy of the sheep skeleton generally represents the human. Its bone histology and bone remodelling activity are similar to human bone [13]. Physiologic influences and the effects of hormones on the production of osteocalcin in sheep and humans are comparable [16].

For the above reasons, we have embarked on studies to evaluate the sheep as a model for severe human osteoporosis and for further investigations on fracture healing and fracture treatment in osteoporotic bone.

The goal of this pilot study was to compare three different osteoporosis induction modalities with respect to BMD, structural parameters and mechanical properties of bone achieved by the three modalities. This study aimed to complement the data published earlier and to find the regimen to induce maximal bone loss. The sheep model of osteoporosis will not primarily provide new information about the pathophysiology of different types of osteoporosis but be used for the development and in vivo testing of new strategies for fracture treatment in mechanically weak bone. In a first step a study on bone healing in osteoporotic and nonosteoporotic sheep will be conducted.

Materials and Methods

Approval for this animal study was obtained according to relevant state and federal guidelines.

Eight Swiss mountain sheep, identically bred with a mean age of 8 ± 1 years, were investigated in this study. The mean weight was 65 ± 5 kg and did not change over 6 months.

Group 1 was ovariectomized and fed with a calcium/vitamin D-restricted diet (O+D). Group 2 was ovarietomized and injected with steroids (O+S). In group 3 the animals were ovariectomized, fed a calcium/vitamin D-restricted diet and injected with steroids (O+S+D). Group 4 was used as an untreated control group. Each group consisted of two animals.

The diet contained 1.5 g calcium and 100 IU vitamin D_3 per day (Firma Eberle Nafag, Gossau, Switzerland). The animals of group 2 and 4 received a standard feed with normal calcium and vitamin D intake (5 g calcium and 1000 IU vitamin D per day). The steroid application for groups 2 and 3 consisted of a daily intramuscular injection of 25 mg methylprednisolone (Strenzli Pharmazeutika, Uzach, Switzerland).

The treated sheep (groups 1–3) were ovariectomized bilaterally under general anesthesia. One animal from group 3 had to be excluded after 2 months because of a generalized infection.

Bone mineral density (BMD) was determined every 2 months at the distal radius on both sides by peripheral

quantitative computed tomography (pQCT) using a Densiscan 1000 Scanner (Scanco Medical, Bassersdorf, Switzerland). The in vivo measurements were performed with the sheep under general anesthesia to avoid artifacts due to movement. The limbs of the sheep were fixed in the CT scanner gantry to prevent motion artifacts due to breathing. To define the axial position a projectional scout view of the region of interest (distal radius) was obtained first. The distal end plate of the bone was marked with a reference line to ensure the same starting position for each measurement. Ten consecutive slices were obtained in high-resolution mode. In order to locate identical cross-sections for analysis, a matching algorithm based on identical bone area was employed. The effective energy of the X-ray beam was set to 40 keV and 0.5 mA. The slice thickness was 1.5 mm (in-plane resolution of 0.3 mm) with an increment of 1.5 mm. A matrix of 512×512 pixels was used. In each cross-section the outer and inner contour of the cortex was detected. BMD (g/cm^3) of the cortical bone was determined between these two contours; BMD of the cancellous bone was determined inside the inner contour. The mean values of 5 slices were calculated. The precision of pQCT is below 0.3%, the reproducibility in healthy individuals below 0.3% and in osteoporotic individuals below 1%.

After 6 months the animals were killed and biopsies of lumbar vertebrae were harvested (L3, L4). The biopsies were taken in the craniocaudal direction from the center of the vertebral bodies, about 3 mm anterior to the spinal canal. A trephine with an inner diameter of 10.6 mm was used. The specimen consisted of an upper and a lower cortical layer and cancellous bone in between. The cortical end plates were removed by means of a diamond-coated band saw (Exakt, Norderstedt, Germany) to produce a purely cancellous cylinder of 10 mm height with plane-paralleled surfaces. Similar specimens were obtained from femoral heads. With a trephine (inner diameter 10.6 mm) drilling was performed over the ligamentum capitis femoris perpendicular to the femoral head surface through the femoral neck. A specimen of 10 mm length was taken 5 mm below the cortex from the biopsy of approximately 30 mm length. The samples were fixed immediately in phosphate-buffered 10% formalin and analyzed using the μCT 20 (Scanco Medical, Bassersdorf, Switzerland). This three-dimensional micro-CT system is based on a fan-beam and works in a multislice mode. An X-ray tube with a microfocus is used as a source, a CCD array as a detector. Spatial resolution was 28 μm in all directions. The volume of interest was set to $4 \times 4 \times 4$ mm^3 and was represented in $14 \times 14 \times 14$ μm^3 voxels. Trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), bone surface/bone volume (BS/BV) and bone volume/total volume (BV/TV) were determined based on the plate-like model. The precision of micro-CT is below 0.3%, the reproducibility below 1%.

Subsequent to micro-CT analysis, the cancellous bone cylinders were subjected to mechanical testing to determine the compressive stiffness and strength. The specimen was placed in a materials testing machine (Instron 4302, Instron series IX automated materials testing system, Instron, High Wycombe, UK). The cylinders were compressed between two parallel plates in the craniocaudal direction at a rate of 1 mm/min and kept wet during the whole procedure. The load/displacement curve was documented.

Results

QCT

The mean BMD values from the right and the left distal radius were determined. In all treatment groups (1–3) the decrease in BMD in cortical bone was less prominent than in cancellous bone. After 6 months, the decrease in BMD in cortical bone of the two sheep in group 1 (O+D) was 3.9 ± 0.5% and 5.5 ± 0.1% respectively. The BMD of cancellous bone in this group declined by 7.1 ± 0.4% and 7.4 ± 0.2% respectively. In group 2 (O+S) the density of cortical bone decreased by 12.9 ± 0.2% and 20.3 ± 0.6% respectively. Cancellous bone density decreased in this group by 29.6 ± 0.5% and 32.4 ± 2.1% respectively. In treatment group 3 (O+D+S) a decline of 22 ± 1% was seen in cortical bone and of 57.7 ± 1.4% in cancellous bone. There was no significant change in BMD of cortical bone in the control group (decrease of 0.4 ± 0.7% and increase of 0.1 ± 1.3%). BMD of cancellous bone in the control group decreased by 0.25 ± 0.9% and 0.1 ± 1.2% respectively.

Micro-CT

Visual inspection of the three-dimensional micro-CT reconstructions demonstrated the differences between the control group and group 3 (O+D+S) (Figs. 1, 2).

The structural parameters of vertebral biopsies of group 1 (O+D) were not different from the control group (Table 1). In group 1 Tb.N was 0.5% higher, Tb.Th 0.3% higher and Tb.Sp 2% lower than in the control group. Group 2 showed a decrease of 13% in Tb.N, a decrease of 38% in Tb.Th and an increase of 41% in Tb.Sp compared with the control group. The sheep of group 3 (O+D+S) had a 53% lower Tb.N, a 63% lower Tb.Th and an increase of 150% in Tb.Sp relative to the control group.

The ratio of bone surface to bone volume (BS/BV) in group 1 (O+D) was 1.2% higher than the control group. The ratio of bone volume to total volume (BV/TV) was 2% higher in group 1. In group 2 (O+S) BS/BV increased by 72% and BV/TV declined by 51% in comparison with the control group. The animal of group 3 (O+D+S) showed an increase of 167% in BS/BV and a decrease of 82% in BV/TV compared with the control group.

The structural changes in femoral head biopsies are shown in Table 1. Tb.N did not differ between group 3 (O+D+S) and the control group. Tb.Th in group 3 was

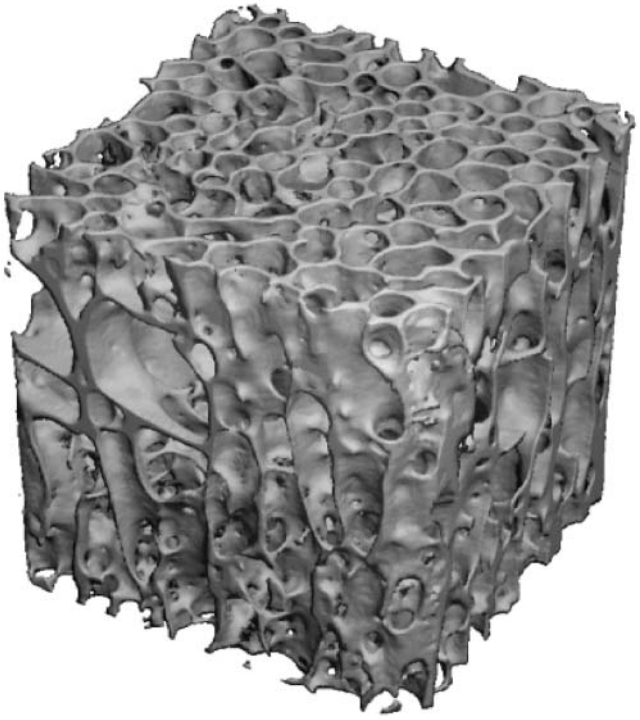

Fig. 1. Three-dimensional reconstruction of a sheep vertebral body biopsy (L4) from the control group (sheep no. 1).

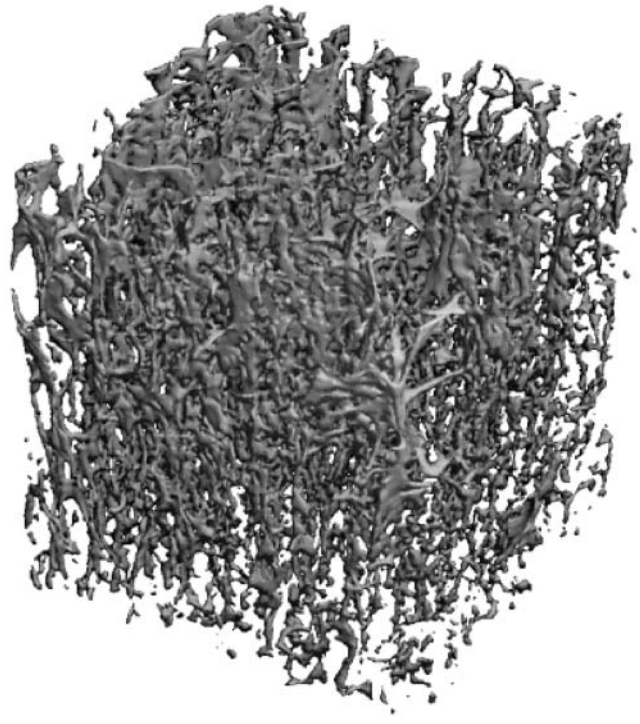

Fig. 2. Three-dimensional reconstruction of a sheep vertebral body biopsy (L4) after 6 months of osteoporosis induction with ovariectomy, steroid application and a calcium/vitamin D-restricted diet (sheep no.7).

Table 1. Histomorphometric parameters from femoral head and vertebral body (L4) biopsies from micro-CT measurements 6 months after osteoporosis induction

Sheep no.	Group	Tb.N (1/mm)	Tb.Th (mm)	Tb.Sp (mm)	BS/BV (%)	BV/TV (%)
Femoral head						
1	Control	2.41	0.17	0.24	11.7	0.41
2	Control	2.08	0.12	0.36	16.7	0.25
3	O+D	2.45	0.14	0.27	14.7	0.33
4	O+D	2.27	0.17	0.27	11.8	0.38
5	O+S	2.56	0.11	0.28	18.7	0.27
6	O+S	2.58	0.13	0.25	15.1	0.34
7	O+D+S	2.28	0.09	0.35	23.6	0.19
L4						
1	Control	2.02	0.12	0.38	17.2	0.23
2	Control	1.94	0.10	0.41	19.2	0.20
3	O+D	2.08	0.10	0.38	19.9	0.21
4	O+D	2.27	0.11	0.33	17.4	0.26
5	O+S	1.56	0.06	0.58	32.7	0.10
6	O+S	1.90	0.07	0.54	30.0	0.12
7	O+D+S	0.94	0.04	1.02	48.6	0.04

40% below that in the control group, while Tb.Sp was 20% greater. BS/BV in group 3 was 70% greater compared with the control group and BV/TV was 40% below the control.

Mechanical Testing

In compression testing of vertebral body biopsies (L3, L4) of each animal the failure load and stiffness were determined. Average values were calculated and compared with the control group.

There was a slight difference in the failure load between group 1 (O+D) and the control group (2.32 ± 0.14 kN and 2.63 ± 0.44 kN respectively). In group 2 (O+S) the failure load was 1.54 ± 0.23 kN, which is 40% less than in to the control group. The sheep of group 3 (O+D+S) showed a decrease of 70% in failure load (0.77 kN; Table 2).

The stiffness of the vertebral body biopsies under compression had changes comparable to those in the failure load and displacement. Group 1 (O+D) and the control group had similar values for stiffness. Group 2 (O+S) was 10% below and group 3 (O+D+S) 40% below these values (Table 2).

In compression testing of femoral head biopsies the failure load in group 1 (O+D) was 15% less and in group 2 (O+S) 31% less compared with the control group. In group 3 the maximum load was 53% less than the control (Table 2).

Stiffness in group 1 (O+D) showed a reduction of 43% compared with the control group. Group 2 (O+S) and group 3 (O+D+S) had a similar stiffness, which was about 70% less than in the control group (Table 2).

The mechanical properties of cancellous bone of vertebral body biopsies and femoral head biopsies showed group-specific changes in compression testing.

Table 2. Failure load and structural stiffness in compression testing of vertebral body (L3, L4) and femoral head (Fh) biopsies according to different treatment regimens

Group	Sheep no.	Biopsy location	Max. load (kN)	Stiffness (N/mm)
Control	1	L3	2.15	8209
		L4	2.37	8447
		Fh	1.17	328
	2	L3	3.11	9377
		L4	2.88	9227
		Fh	1.69	547
O+D	3	L3	2.12	8283
		L4	2.39	8891
		Fh	1.13	298
	4	L3	2.32	8661
		L4	2.44	8262
		Fh	1.30	202
O+S	5	L3	1.39	6981
		L4	1.30	7319
		Fh	0.65	135
	6	L3	2.07	8390
		L4	1.89	8768
		Fh	1.03	140
O+D+S	7	L3	0.80	5421
		L4	0.75	5355
		Fh	0.57	126

Discussion

For the investigation of changes in bone remodeling following drug treatment or ovariectomy old sheep have been identified as a potential model [11]. In 1994 the US Food and Drug Administration (FDA) produced draft guidelines [17] on the treatment and prevention of osteoporosis. The sheep was accepted as a large animal model for osteoporosis.

Chavassieux et al. [18,19] and Pastoureau et al. [13] found this animal to be an excellent model for bone growth and remodeling assessment as shown by bone histomorphometry. A decrease in bone mass was found in young sheep 6 months after ovariectomy. Analysis of radiographs of the proximal femur showed a significant loss of cancellous bone in ovariectomized compared with age-matched control sheep. There was also a decrease in bone volume, a decrease in Tb.Th and an increase in Tb.Sp in specimens from the iliac crest [12]. In the iliac crest of sheep treated with ovariectomy, calcium/vitamin D-restricted diet and steroid application Lill et al. [15] found a significant decrease of Tb.N, Tb.Th and a significant increase in Tb.Sp. To prove the usefulness of the sheep model for the investigation of fracture treatment in osteoporotic bone it is necessary to know whether there are any site-specific differences at relevant sites for osteoporotic fractures concerning the degree of BMD reduction.

The examination of BMD at the distal radius is a routine procedure to determine information about the level of osteoporosis in humans. By using pQCT it is possible to examine cortical and cancellous BMD separately [20]. In this study greater changes in BMD of cancellous bone were found, in agreement with the literature.

Turner et al. [12] showed a decrease in BMD 6 months after ovariectomy. At the vertebral bodies they demonstrated a decrease in BMD, but no change in BMD was seen at the distal radius and calcaneus. In this study a decline of 7% at the distal radius was seen after ovariectomy and the feeding of a calcium/vitamin D-restricted diet. From this finding we assume that there is an additional effect of malnutrition on osteoporosis induction after ovariectomy.

The sheep is described as a large animal which has a bone remodeling process similar to that of humans, and the effects of fluoride and high doses of glucocorticoids on bone tissue have been found to be similar to those observed in humans [18,19]. A thinning of the trabeculae without any change in the connectivity has been reported after corticosteroid therapy in humans [21]. In this study a decrease in the number of trabeculae and a loss of connectivity, which is characteristic for postmenopausal osteoporosis, was found.

The mechanisms underlying corticosteroid-induced osteoporosis in humans consist mainly of a defect of bone formation combined with an increase in bone resorption. The increased resorption is due to secondary hyperparathyroidism as a result of the inhibitory effects of corticosteroids on intestinal calcium absorption [21]. The defect of bone formation consists in a depression of osteoblast activity with a reduced bone formation rate at the tissue and cell levels. Furthermore it has been reported that the active formation period was reduced [4]. In vitro studies have confirmed the direct effect of corticosteroids on osteoblasts and receptors of glucocorticoids have been demonstrated in osteoblast-like cells.

In the present study the additional application of 25 mg methylprednisolone after ovariectomy decreased BMD another 30% in cancellous bone. After 4 months of daily injection of 15 mg methylprednisolone in 16 old sheep without ovariectomy Deloffre et al. [22] found no differences between treated and untreated animals with respect to BMD at the femur and lumbar spine. Chavassieux detected a decline in bone formation in sheep after 3 months of daily injection of 16 mg methylprednisolone without ovariectomy but did not find a significant change in bone volume. Similar results were found after injection of 30 mg methylprednisolone for 2 months followed by a daily application of 16 mg for 1 month [5,18].

From these findings the idea was to combine ovariectomy, a calcium/vitamin D-restricted diet and steroid application to achieve significant bone loss in sheep. We [15] reported a significant decrease in BMD at the distal tibia of about 50% in cancellous bone and 7% in cortical bone after osteoporosis induction with ovariectomy, calcium/vitamin D-restricted diet and steroid application.

To demonstrate structural changes in the three-dimensional structure of cancellous bone according to different degrees of osteoporosis we used microtomo-

graphy. Ito et al. [23] investigated bone samples from the iliac crest of humans and compared osteoporotic and nonosteoporotic individuals. In trabecular number (Tb.N) they found a decrease of 12% in osteoporotic patients, while we detected a decline of 13% in group 2 (O+D) and 53% in group 3 (O+D+S) in vertebral body biopsies. In femoral heads no significant change was seen. Trabecular separation (Tb.Sp) increased by 20% in osteoporotic humans. In this study similar changes were found in the femoral heads of osteoporotic sheep from group 3. In group 2 an increase in Tb.Sp of 11% was seen. In vertebral bodies of osteoporotic sheep from group 2 an increase of 41% was evident. In group 3 Tb.Sp increased 150% compared with the control group. We found a decrease in the ratio of bone volume to total volume (BV/TV) at vertebral bodies of 51% in group 2 and 82% in group 3 compared with the control group, while Ito et al. [23] found a decrease of 22% in osteoporotic patients. In femoral head biopsies a decrease of 40% in BV/TV occurred in group 3 (O+D+S) and a decline of 7% in group 2 (O+S). We chose to use the femoral head for the investigation of microstructure and mechanical properties of bone because failure of implants frequently occurs in the femoral head (e.g., the cutting out phenomenon of the Dynamic Hip Screw). The comparison of three-dimensional stereologic bone indices between human data and our data indicates that in this study a more severe osteoporosis was induced in sheep. In sheep, greater changes were seen in vertebral bodies compared with femoral heads. In humans, vertebral fractures can be a first clinical sign of osteoporosis. The relatively wide variation in structural properties of femoral head biopsies is probably due to the small number of specimens and the difficulty of harvesting the biopsy from exactly the same area due to anatomic variations between sheep regarding size and angulation of femoral heads and femoral necks.

We saw a decrease in BMD at the distal radius of 4% and 5.5% in the two animals from group 1 treated with ovariectomy and diet The BMD reduction could be documented in repeated measurements in one animal each. In group 1 there was no difference in bone volume of biopsies from vertebral bodies and femoral heads compared with the control group. These measurements could be performed only once after the sheep had been killed. We could not compare the data within one animal as in the BMD measurements of the radius, so we had to compare the bone volume fraction with the control group. This is most probably the reason for the incongruent results.

In this study mechanical properties of cancellous bone from vertebral bodies have been related to different degrees of bone loss in sheep. The increased incidence of spine fractures in elderly women has been associated with osteoporosis-related reductions in trabecular bone density that occur in the vertebral body. For compression testing of cancellous bone cylindrical specimens with a height of 10 mm and a diameter of 10 mm were used according to the recommendations of Burstein and Frankel [24].

It has been well established that trabecular bone strength has a power relationship to apparent bone density. Bone strength also depends on the structure of the trabecular network. In this study the findings of Mitton at al. [25], who found a close relation between density and bone strength (r = 0.86), were confirmed. The anisotropy of the weight-bearing vertebral trabecular bone is accentuated with age and osteoporosis because of a loss of thin horizontal trabeculae and an increase in diameter among the remaining vertical trabeculae. The present study has demonstrated that with decreasing BMD and osteoporosis associated structural changes, the mechanical properties in compression testing change.

As this study was designed as a pilot study we used only a small number of animals and cannot report statistically meaningful data. Together with the previously published data [15] it was possible to demonstrate that induction of severe osteoporosis in sheep is possible. A significant BMD reduction was seen at the relevant sites for osteoporosis-associated fractures. Certainly these results need to be confirmed in a larger study. We find that the data presented can be used as a basis for future studies. Ovariectomized sheep treated with steroids and fed a calcium/vitamin D-restricted diet showed a remarkable decrease in BMD. Bone structural parameters from vertebral bodies and femoral heads changed according to the degree of osteoporosis and can be compared with the human situation. There was a close relation between BMD, bone structure and strength. For further judgement of the type of osteoporosis and comparison with the human situation, histomorphometry and histomorphology needs to be performed.

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