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Exposure to pastures fertilised with sewage sludge disrupts bone tissue homeostasis in sheep

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ABSTRACT

The femurs of male and female sheep (*Ovis aries*), aged 18 months, bred on pastures fertilized twice annually with sewage sludge (2.25 tonnes dry matter/ha; Treated; T) or on pastures treated with inorganic fertilizer (Control; C) were studied, using peripheral Quantitative Computed Tomography (pQCT) and the three-point bending test. Males were maintained on the respective treatments from conception to weaning and then maintained on control pastures while the females were maintained on the respective treatments until slaughter.

T rams exhibited increased total bone mineral density (BMD) at the metaphyseal part of femur (+10.5%, $p < 0.01$) compared with C rams but had a reduced total cross sectional area (CSA, −11.5%, $p < 0.001$), trabecular CSA (−17.1%, $p < 0.01$) and periosteal circumference (−5.7%, $p < 0.001$). In the mid-diaphyseal part, T rams had an increased total BMD (+13.8%, $p < 0.0001$) and stiffness (+6.4%, $p < 0.01$) but reduced total CSA (−12.1%, $p < 0.0001$) and marrow cavity (−25.8%, $p < 0.0001$), relative to C rams.

In ewes although pQCT analysis of neither the metaphyseal nor the mid-diaphyseal part of the female femur bones showed any significant differences with treatment, the biomechanical method revealed a reduction in load at failure (−17.3%, $p < 0.01$) and stiffness (−10.7%, $p < 0.05$) amongst T ewes. It is concluded that exposure to pollutants present in sewage sludge can perturb bone tissue homeostasis in sheep, but particularly in males.

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1. Introduction

The application of sewage sludge to land is likely to increase in both Europe and the United States as a dumping at sea is banned and land-fill sites become less available (Rhind et al., 2005b; Swanson et al., 2004). However, sludge contains high concentrations of many environmental pollutants including endocrine disrupting compounds (EDCs) such as alkyl phenols, phthalates, polychlorinated biphenyls (PCBs), polybro-

minated diphenyl ethers (PBDE), polycyclic aromatic hydrocarbons (PAH), dioxins, pesticides and toxic metals (Brunner et al., 1988; Ghanem et al., 2007; Giger et al., 1984; Stevens et al., 2003). Since these are known to exert adverse effects on animal and human physiology and health (IEH, 1999; Toppari et al., 1996), potential effects on wildlife, domestic animals and humans are of concern.

Theoretical and empirical studies, designed to investigate the issue of accumulation of pollutants in domestic animal

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products, generally indicate that exposure to sewage sludge is associated with only limited tissue accumulation (Rhind, 2005; Rhind et al., 2005a; Rhind et al., 2005b; Smith, 1995) and so the risks to humans of exposure to pollutants through the consumption of animal products is likely to be small. On the other hand, previous studies of sheep grazing pastures fertilized with either sewage sludge or inorganic fertiliser have shown that exposure to EDCs and other pollutants through the sewage sludge can disrupt the development and function of the foetal testis (Paul et al., 2005), ovary (Fowler et al., 2008) and adult offspring behavior (Erhard and Rhind, 2004). These observations suggest that human health could be at risk, from exposure to environmental concentrations of a mixture of pollutants, not only through the application of sludges to land but also more generically, through routes other than the consumption of contaminated domestic animal products such as through diet or inhalation.

One significant human health issue is the reduction in bone formation at the tissue level (Seeman, 2008) associated with declining oestrogenic influence, particularly in post-menopausal women. Since many EDCs have oestrogenic effects, the potential significance of increasing exposure to environmental oestrogens with respect to bone density is of public health interest. Evidence of effects on bone structure of exposure to a range of pollutant classes has been derived, in part, from empirical studies designed to investigate effects of exposure and mechanisms of action using laboratory animals (Hermesen et al., 2008; Jamsa et al., 2001; Lind et al., 1999; Lind et al., 2000b; Miettinen et al., 2005; Yilmaz et al., 2006) and domestic animals (Lundberg et al., 2006). Most of these studies concern exposure to abnormally high concentrations of the chemicals concerned. Studies of wildlife species (Fox et al., 2008; Lind et al., 2003; Lind et al., 2004; Sonne et al., 2004) and of humans (Alveblom et al., 2003; Glynn et al., 2000; Hodgson et al., 2008) based on observational data show effects of exposure but, owing to the fact that the populations studied have been exposed to a mixture of pollutants, cannot identify the causal agents.

The aim of this study was to study effects on bone tissue of rearing sheep on pastures fertilized with sewage sludge so that they were exposed to a mixture of organic pollutants and heavy metals, for a prolonged period, but at concentrations that were only slightly elevated relative to background. In view of the different circulating steroid profiles of male and female animals, both sexes were studied. However, owing to the fact that male and female animals could not be maintained on the same pastures, to avoid uncontrolled matings and requirements of other components of the experimental programme, it was not possible to treat the two sexes identically. Thus, the groups of male and female animals were regarded as two separate experiments and could not be directly compared, in the statistical analysis.

2. Material and methods

All animal procedures were approved by the ethical committee of the Macaulay Institute.

2.1. Animals and exposure

Two groups of Texel ewes (*Ovis aries*) were mated with Texel rams on pastures fertilized with sewage sludge (Treated; T) or inorganic fertilizer (Control; C), at the Macaulay Institute's Research Station in Hartwood, Scotland. The patterns of sludge and fertilizer application have been described previously (Rhind et al., 2002). Briefly, the T pastures were fertilized by surface application of thermally-dried pellets of sewage sludge (2.25 tonnes/ha, twice annually) and the C plots with conventional inorganic fertilizer containing the same amount of nitrogen. The sludge was applied, each year, before the seasonal onset of pasture growth (March; late gestation) and before the final month of significant growth (September; approximately two months before mating). The plots were managed so that the pasture height was maintained at an optimal height, as far as possible; this was achieved by adding and removing non-experimental animals from the plots, as required, to manage herbage mass and to ensure that the nutritional state of the animals of the respective treatments remained similar at all times.

In total, 24 male (12 T; 12C) and 24 female (12 T; 12C) offspring from the respective treatments were used in the study. T animals were exposed to the pollutants in sewage sludge from conception until weaning at approximately 4 months of age via their dams, *in utero* and through milk, and through exposure to sludge-treated pasture from when they began to consume herbage. After weaning, owing to the need to avoid uncontrolled matings and to other requirements of the experimental programme, male lambs were kept on control pastures until slaughter while female lambs remained on the respective T or C pastures. All experimental animals were slaughtered at 18 months of age, femurs removed and blood samples were collected and plasma separated and stored at -20°C .

2.2. Sludge collection and analysis

Sub-samples of the sludge and inorganic fertiliser applied to the T and C pastures, respectively, were collected and analysed for selected EDCs. The concentrations of selected PCB and PBDE congeners (AccuStandard, USA) and 16 USEPA priority PAHs (Greyhound Chromatography, UK) were determined as follows:

For PCBs and PBDEs, sludge samples (approximately 1 g dry weight) were spiked with 0.01 mg internal standards of ^{13}C PCB-138 and ^{13}C PBDE-99 (Greyhound Chromatography, UK) and 8 ml ethanoic potassium hydroxide (1 M) was added. It was heated at approximately 90°C for 8 h. The samples were allowed to cool and 2 ml of Milli-Q water added. Samples were extracted into 3×10 ml iso-hexane and the extracts were then concentrated to 0.5 ml under a gentle stream of nitrogen. Sample clean-up was achieved using absorption chromatography to remove lipids, primarily, using in-house prepared columns (25 cm length, 15 mm i.d, fitted with PTFE taps). The extracts were loaded on to a 5 g silica (Merck, UK), 10 g of acid modified (2:1, silica:sulphuric acid) and 1 g anhydrous Na_2SO_4 column, conditioned with 40 ml iso-hexane (Rathburn Chemicals, UK). Sample was loaded and eluted by 20 ml discarded iso-hexane, and then eluted by 80 ml of iso-hexane which was

collected. For PAHs, 1 g of dry samples were spiked with 7 deuterated internal standards (d8-naphthalene, d10-acenaphthene, d10-fluorene, d10-phenanthrene, d10-anthracene, d10-pyrene and d12-chrysene; 0.3 µg; Greyhound Chromatography, UK) and then were saponified and extracted using the method described for PCBs and PBDEs. For clean-up of PAH extracts, a column comprising 10 g activated silica gel and 1 g anhydrous Na₂SO₄ was used. It was conditioned with 40 ml iso-hexane and, for collection, was eluted with 50 ml of 1:1 DCM:iso-hexane. The fractions collected were concentrated to 0.1 ml by rotary-evaporation and under a gentle stream of nitrogen and were then ready for analysis using gas chromatography linked to mass spectrometry (GC-MS).

Thermo Electron Trace MS (Hemel Hempstead, UK) linked to a Trace 2000 GC with an AS2000 autosampler, was used for PCBs, PBDEs and PAHs analysis with selected ion mode (SIM; Table 1). The capillary column was ZB-5MS (30 m × 0.25 mm i.d. × 0.25 µm film thickness, Phenomenex, UK). The tempera-

Table 1 – Mass to charge ratio of ions monitored for PCBs, PBDEs and PAHs

	m/z ^a
PCB-28	256
PCB-52	220
PCB-101	326
PCB-118	326
PCB-138	360
PCB-153	360
PCB-180	394
¹³ C 138 ?	372
PBDE-28	406
PBDE-47	326
PBDE-99	566
PBDE-100	566
PBDE-153	644
PBDE-154	644
PBDE-183	722
¹³ C 99	416
Naphthalene	128
Acenaphthalene	152
Acenaphthene	153
Fluorene	166
Phenanthrene	178
Anthracene	178
Fluoranthene	202
Pyrene	202
Benzo[a]anthracene	228
Chrysene	228
Benzo[b]fluoranthene	252
Benzo[k]fluoranthene	252
Benzo[a]pyrene	252
Indeno[1,2,3-cd]pyrene	276
Dibenzo[a,h]anthracene	278
Benzo[ghi]perylene	276
d-8 Naphthalene	136
d-10 Acenaphthene	164
d-10 Fluorene	176
d-10 Phenanthrene	188
d-10 Anthracene	188
d-10 Pyrene	212
d-12 Chrysene	240

^a m/z: mass to charge ratio; a relationship between the mass of a given ion and the number of elementary charges that it carries.

Fig. 1 – Schematic overview of the inserted screw and measure points as used for pQCT analysis.

ture program for PCB started at 120 °C for 1 minute; the temperature was then ramped at 4 °C/min to 280 °C and held for 1 min and then ramped to 320 °C at 30 °C/min and held for 5 min. The operating temperature for PBDE was programmed from 70 °C (1 min) to 170 °C at 30 °C/min and then ramped to 300 °C at 8 °C/min and held for 5 min. The temperature for PAH was programmed from 70 °C (3 min) to 250 °C at 5 °C/min and held for 1 min, then ramped to 300 °C at 6 °C/min and held for 6 min and finally ramped to 325 °C at 10 °C/min and held for 5 min. The carrier gas was helium and samples were injected in splitless mode with a surge. The injector and mass spectrometer were held at 250 °C and 200 °C respectively. The electron impact energy was set 70 eV for mass spectrometer.

Before sample analysis, relevant standards were analyzed to check column performance, peak height and resolution, and the limits of detection (LoD). LoD was calculated to be 0.02 µg/kg for all PCBs, 0.02 µg/kg for PBDE 28, 47, 99 and 100 and 0.50 µg/kg for PBDE 153, 154 and 183. The LoD was 1 µg/kg for all PAHs except phenanthrene, fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene and dibenzo[a,h]anthracene for which they were 5 µg/kg and pyrene for which it was 15 µg/kg. With each set of samples analysed, a standard mixture, quality control samples, and a procedural blank were run in sequence to check for contamination, recovery, peak identification and quantification. Compounds were identified mainly by selected ion and their retention times. All results for sludge samples were reported on a dry-weight basis.

2.3. Bone preparations

Femur bones were cleaned and stored at –20 °C. Before analysis, using peripheral Quantitative Computed Tomography (pQCT), bones were moved to a refrigerator (+8 °C) and thawed for approximately 24 h. The preparation of the bones

for the pQCT analysis has been described in previous studies (Hermesen et al., 2008; Lundberg et al., 2006). In brief, femur lengths were measured to the nearest 0.1 mm, using a slide calliper. A screw (2.5 × 12 mm; DynaPlus, zinc plated) inserted dorsal to the *trochlea ossis femori* was used as a reference point for the pQCT analyses (Fig. 1). The bones were covered with gauze bandage (Molleplast, elastic, Lohmann & Rauscher AG, Switzerland), moistened with Ringers solution (pH 7.4; Tris (0.3 g/l), NaCl (9 g/l), CaCl₂·H₂O (0.24 g/l), KCl (0.4 g/l)) and then wrapped in polyethylene plastic foil to prevent drying.

2.4. pQCT measurements

pQCT (Stratec XCT 960 A with software version 5.21; Norland Stratec Medizintechnik, Pforzheim) was used to estimate dimensions and densitometric variables of the femurs. The pQCT instrument was calibrated once each week with a hydroxyapatite phantom of known density (Gasser, 2003). The femurs were placed horizontally in the pQCT instrument with the distal end at the front and the anterior side facing upwards. The position of the bone was examined with the scout view of the pQCT. The femurs were analysed using voxelsize 590 µm. The settings used were: peel mode 2, contour mode 1, threshold 270 mg/cm³, inner threshold 500 mg/cm³ and outer threshold 690 mg/cm³.

2.4.1. Metaphyseal measurements

The metaphysis (Fig. 2) of the bone was analysed by examining a 3 mm thick slice at 3.6% of the total bone length from the reference point located at the *trochlea ossis femori* in the distal part of the femur; at this site there is a continuous layer of cortical bone surrounding the inner trabecular bone. The variables evaluated were total bone mineral content (BMC, mg/mm), total bone mineral density (BMD, mg/cm³), trabecular (trab) BMC (mg/mm), trab BMD (mg/cm³), total cross sectional area (CSA, mm²), trab CSA (mm²), and periosteal circumference (PERIC, mm).

2.4.2. Diaphyseal measurements

The diaphysis of the bone was analysed by examining a 1 mm thick slice at 50% of the total bone length; this site consists,

mainly, of cortical (cort) bone. The variables evaluated were total BMC (mg/mm), total BMD (mg/cm³), total CSA (mm²), cort BMC (mg/mm), cort BMD (mg/cm³), cort CSA (mm²), cort thickness (cort THKC, mm), PERIC (mm), endosteal circumference (ENDOC, mm), polar moment of inertia (mm⁴) and marrow cavity (mm²).

2.4.3. Reproducibility

To evaluate the reproducibility of the pQCT measurements, the coefficients of variation (CV %) for the different variables were calculated from 10 repeated measurements with a single sample which was repositioned before each measurement. The CV for the ten different pQCT measurements at the metaphyseal measure point were: 0.4% (total BMC), 0.3% (total BMD), 1.5% (trab BMC), 1.5% (trab BMD), 0.6% (total CSA), 1.1% (trab CSA) and 0.3% (PERIC). The CV for the ten different pQCT measurements at the diaphyseal measure point were: 0.2% (total BMC), 0.2% (total BMD), 0.2% (total CSA), 0.2% (cort BMC), 0.2% (cort BMD), 0.3% (cort CSA), 0.5% (cort THKC), 0.1% (PERIC), 0.3% (ENDOC), 0.3% (Polar moment of inertia) and 0.2% (marrow cavity).

2.4.4. Biomechanical testing

The three-point bending test (mid-part bent until fracture) measurements were conducted at the mid-diaphyseal part of the femur. A servo-hydraulic material testing machine (MTS 858 Mini Bionix with Teststar II data acquisition with software Testware SX version 3.1B, MTS corp, Minneapolis, MN, USA) was used for the tests. A load cell with maximum capacity of 10000 N was used. The span length was 100 mm and the loading speed 1 mm/s. Following thawing, the load was applied on the point that was used in the diaphyseal pQCT measurement (50% of the total bone length). The load (N) applied and the displacement (mm) of the crosshead was recorded at a sampling rate of 50 Hz. Based on the load and displacement data the stiffness (N/mm) and energy until failure (N × mm) were calculated. The maximal stiffness was defined, based on the steepest part of the load-displacement curve. The load displacement values collected with 50 Hz during the testing were used to calculate energy to failure by applying a formula that calculated the cumulative area under

Fig. 2 – Representative images from the pQCT scan at the 50% measure point from male sheep. The white outer part is cortical bone and the inner darker part is the marrow cavity. To the left control and to the right treated (T). Note the small area of the marrow cavity, the shorter endosteal circumference and periosteal circumference and the increased cortical thickness in the T specimen.

the load displacement curve. By using such a formula a fairly precise assessment of the energy to failure could be obtained irrespective of the degree of non-linearity of the load displacement curve. The same equipment and methodology was used in several studies (Fox et al., 2008; Lind et al., 2000a; Lundberg et al., 2006) with slight adjustments to accommodate the specific bones being tested.

2.4.5. Analysis of bone biomarkers

The analyses of the levels of the biochemical marker BAP (bone specific alkaline phosphatase, Ostase BAP Immunoenzymetric Assay) for bone formation (osteoblast activity) was performed according to the manufacturer's instructions (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). Briefly, 50 µL of standards, controls and plasma specimen (duplicates) were incubated with 100 µL of conjugate (primary antibody) for 1 h at 18–25 °C. After washing, the specimens were incubated for 15 min at 18–25 °C. After stopping the reaction, the absorbance was measured at 450 and 595 nm (Molecular Devices Kinetic Reader). The results were analysed using point-to-point curve fit (SoftMax Pro). A standard curve was retrieved and used for the determination of the concentration (µg BAP/L) in the plasma samples.

The levels of the biochemical marker CTX (carboxyterminal telopeptide of type 1 collagen) for bone resorption (osteoclast activity) were analysed according to the manufacturer's instructions (Serum Crosslaps ELISA, Nordic Bioscience Diagnostics A/S, Herlev, Denmark). This is an *in vitro* enzyme immunological test for the quantification of degradation products of CTX. Briefly, 50 µL (duplicates) of standards, controls and specimens were incubated with 150 µL of antibody solution were incubated at 18–22 °C on a 96-well plate for 2 h. After washing and adding 100 µL of substrate solution, the plate was incubated for 15 min at 18–22 °C in the dark. After quenching the reaction, the absorbance was measured at 450 and 595 nm (Tecan Magellan Plate Reader). The results were analysed using quadratic curve fit (Assay Zap software 3.1, Biosoft, Cambridge, UK) with absorbance values from the standards and the CrossLaps concentrations. Plasma concentrations (ng/mL) were determined by the equation of the curve.

Concentrations of vitamin D were analysed according to the manufacturer's instructions (25-Hydroxy Vitamin D (25-OH D) EIA, Immunodiagnostic Systems Ltd). This is an enzymatic immunoassay for determination of 25-OH D and other hydroxylated metabolites. Briefly, 200 µL of calibrators and specimen were incubated at 18–25 °C on a 96-well plate for 2 h. After washing, 200 µL enzyme conjugate was added and the plate was incubated at 18–25 °C for 30 min. After a second washing, 200 µL TMB (tetramethylbenzidine) was added and the plate was incubated at 18–25 °C for 30 min to quench the reaction. After quenching the reaction, the absorbance was measured at 450 and 595 nm (Tecan Magellan Plate Reader). A standard curve with percent binding (B/B % = (mean absorbance / (mean absorbance for 0 calibrator) × 100) of each calibrator was calculated retrieved using four parameter logistic curve fit (4PL, Assay Zap software 3.1, Biosoft, Cambridge, UK). The standard curve was used for the determination of the concentration of 25-OH D (nmol/L) in the samples.

2.5. Statistical analysis

The results were evaluated using t-test (Graph Pad Prism, version 4.03) and, thereafter, analysis of covariance (ANCOVA, StatView, version 5.0; SAS Institute Inc., Cary, NC, USA) was applied to adjust for differences in bodyweight, since bodyweight may influence the different outcome parameters. $P < 0.05$ was considered to be statistically significant.

3. Results

Concentrations of selected PCBs, PBDEs and PAHs in sludge and inorganic fertilizer are given in Table 2.

3.1. Bone measurements

3.1.1. Rams

Fig. 2 and Table 3 present the results obtained from the pQCT measurements and the biomechanical testing of ram femurs. Sewage sludge exposed animals (treated, T) exhibited significantly a greater total BMD (+10.5%, $p < 0.01$) and lower total CSA (−11.5%, $p < 0.001$), trab CSA (−17.1%, $p < 0.01$), and PERIC (−5.7%, $p < 0.001$) at the metaphysis of the femurs, compared to C animals.

In addition, at the diaphysis, sewage sludge exposure was associated with a significantly increased total BMD (+13.8%,

Table 2 – Concentrations (µg/kg) of selected PCB, PBDE and PAH in single pooled samples of sludge and inorganic fertilizer applied to T and C pastures

Pollutant	Sludge	Inorganic fertilizer
PCB-28	32.57	N/D
PCB-52	10.11	N/D
PCB-101	2.08	0.04
PCB-118	9.34	0.25
PCB-138	5.58	N/D
PCB-153	10.75	0.03
PCB-180	4.55	N/D
PBDE-28	6.71	N/D
PBDE-47	162.1	N/D
PBDE-99	202.2	0.94
PBDE-100	9.46	N/D
PBDE-153	20.03	N/D
PBDE-154	151.0	8.17
PBDE-183	7.45	N/D
Naphthalene	21.0	N/D
Acenaphthalene	33.9	N/D
Acenaphthene	109	N/D
Fluorene	210	N/D
Phenanthrene	1773	N/D
Anthracene	1155	N/D
Fluoranthene	1493	N/D
Pyrene	1493	N/D
Benzo[a]anthracene	1071	N/D
Chrysene	601	N/D
Benzo[b]fluoranthene	2169	N/D
Benzo[k]fluoranthene	375	N/D
Benzo[a]pyrene	425	N/D
Indeno[1,2,3-cd]pyrene	2171	N/D
Dibenzo[a,h]anthracene	786	N/D
Benzo[ghi]perylene	359	N/D

N/D = not detectable.

Table 3 – Results of pQCT analysis and three-point bending test (biomechanical testing) of the femurs of the rams

		RAM			
		Control	Treated	p-value	
		(n=12)	(n=12)	t-test	ANCOVA
pQCT	Metaphysis, 3.6%				
	Weight (kg)	77.9±1.0	80.3±1.8	ns	–
	Total BMC (mg/mm)	432.9±14.0	427.6±9.4	ns	ns
	Total BMD (mg/cm ³)	459.7±15.7	508.0±11.1	<0.0001	<0.01
	Trab BMC (mg/mm)	152.2±9.8	140.0±8.1	ns	ns
	Trab BMD (mg/cm ³)	264.6±18.4	281.6±10.1	ns	ns
	Total CSA (mm ²)	945.2±21.1	847.8±29.6	<0.0001	<0.001
	Trab CSA (mm ²)	583.8±24.7	498.6±26.5	<0.0001	<0.01
	PERIC (mm)	108.9±1.2	103.0±1.8	<0.0001	<0.001
	Diaphysis, 50%				
	Total BMC (mg/mm)	380.4±10.0	384.6±8.5	ns	ns
	Total BMD (mg/cm ³)	643.6±16.9	732.3±15.9	<0.001	<0.0001
	Total CSA (mm ²)	592.9±14.2	528.8±19.1	<0.01	<0.0001
	Cort BMC (mg/mm)	338.7±10.0	346.3±7.8	ns	ns
	Cort BMD (mg/cm ³)	1315.7±3.9	1321.6±4.0	ns	ns
	Cort CSA (mm ²)	257.4±7.4	262.1±6.2	ns	ns
	CortTHKC (mm)	3.4±0.1	3.8±0.1	<0.01	<0.05
	PERIC (mm)	86.2±1.0	81.4±1.4	<0.05	<0.0001
	ENDOC (mm)	64.8±1.3	57.7±1.6	<0.01	<0.0001
	Polar moment (mm ⁴)	39493.3±1728.7	34550.9±2170.5	ns	<0.01
	Marrow cavity (mm ²)	335.6±12.9	266.7±14.7	<0.01	<0.0001
Biomechanics	Diaphysis, 50%				
	Displacement (mm)	4.2±0.4	4.4±0.2	Ns	ns
	Load (N)	5440.7±320.3	6396.7±153.6	<0.05	ns
	Energy (N×mm)	15823.9±17744.6	16663.1±1316.6	ns	ns
	Stiffness (N/mm)	2570.4±84.9	2734.4±124.2	ns	<0.01

Values are mean±SE. n = number of individuals; BMC = bone mineral content; BMD = bone mineral density; trab = trabecular; CSA = cross sectional area; PERIC = periosteal circumference, cort = cortical; THKC = thickness; ENDOC = endosteal circumference. Differences between groups were evaluated by ANCOVA with adjustments for body weight since body weight may influence the different outcome parameters. ns = non-significant.

$p<0.0001$) and cort THKC (+11.8%, $p<0.05$) but significantly decreased total CSA (–12.1%, $p<0.0001$), PERIC (–5.9%, $p<0.0001$), ENDOC (–12.3%, $p<0.0001$) and marrow cavity (–25.8%, $p<0.0001$) relative to C animals (Fig. 2). The polar moment of inertia, a theoretical prediction of the bone's ability to resist torsion was also reduced in the treated animals (–9.4%, $p<0.0001$). Sewage sludge exposure was associated with significantly increased femoral stiffness (+6.4%, $p<0.01$) relative to C rams.

3.1.2. Ewes

The results of the pQCT and the three-point bending test on the ewe femurs are given in Table 4. In ewes although pQCT analysis of neither the metaphyseal nor the mid-diaphyseal part of the female femur bones showed significant differences with treatment, the biomechanical method revealed a reduction in load at failure (–17.3%, $p<0.01$) and stiffness (–10.7%, $p<0.05$) amongst T ewes.

3.2. Analysis of bone biomarkers

There were no significant differences between T and C animals in any of the biomarker measurements in either rams (Table 5) or ewes (Table 6).

4. Discussion

The present study is the first reported study exploring the effects on bone tissue of breeding sheep on pastures fertilized with sewage sludge. The analyses of the sludge and inorganic fertiliser indicate that the sludge contained significant quantities of organic pollutants which were virtually absent in the inorganic fertiliser. While the precise pattern of animal exposure cannot be deduced from these data, it is clear that all T animals were exposed to above-background levels of pollutants through the sludge, from conception until weaning, via their dams, *in utero* and through milk, or through consumption of grass. Accurate assessment of pollutant burden in the target tissue is logistically extremely difficult, firstly because several thousand chemicals may be involved, each of which has different characteristics and is present at low concentrations, often close to the limits of detection. Furthermore, exposure depends on the rate of environmental degradation and availability of these chemicals, their concentration in ingested feed, rates of uptake, excretion and metabolism by the animal and the efficiency with which it is assimilated in the tissue (Rhind, 2008). Finally, each of these processes is likely to change with both time and stage of development of the animal (Rhind, 2008). Risks of tissue

Table 4 – Results of pQCT analysis and three-point bending test (biomechanical testing) of the femurs of the ewes

		EWE			
		Control	Treated	p-value	
		(n = 12)	(n = 12)	t-test	ANCOVA
pQCT	Metaphysis, 3.6%				
	Weight (kg)	63.6 ± 1.0	61.2 ± 1.2	ns	–
	Total BMC (mg/mm)	363.7 ± 7.2	346.0 ± 9.6	<0.05	ns
	Total BMD (mg/cm ³)	510.7 ± 15.5	473.7 ± 13.6	<0.01	ns
	Trab BMC (mg/mm)	201.3 ± 8.7	234.6 ± 12.2	<0.001	ns
	Trab BMD (mg/cm ³)	719.3 ± 25.2	736.5 ± 27.3	ns	ns
	Total CSA (mm ²)	371.9 ± 22.8	442.7 ± 27.2	<0.001	ns
	Trab CSA (mm ²)	94.9 ± 1.6	96.0 ± 1.8	ns	ns
	PERIC (mm)	63.6 ± 1.0	61.2 ± 1.2	ns	–
	Diaphysis, 50%				
	Total BMC (mg/mm)	305.2 ± 6.7	285.5 ± 6.8	ns	ns
	Total BMD (mg/cm ³)	671.1 ± 14.7	659.0 ± 17.4	ns	ns
	Total CSA (mm ²)	456.5 ± 11.7	434.7 ± 9.1	ns	ns
	Cort BMC (mg/mm)	271.5 ± 6.7	254.0 ± 6.8	ns	ns
	Cort BMD (mg/cm ³)	1322.3 ± 8.8	1316.2 ± 5.9	ns	ns
	Cort CSA (mm ²)	205.2 ± 4.6	192.9 ± 4.8	ns	ns
	CortTHKC (mm)	3.1 ± 0.1	3.0 ± 0.1	ns	ns
	PERIC (mm)	75.7 ± 1.0	73.9 ± 0.8	ns	ns
	ENDOC (mm)	56.1 ± 1.1	55.0 ± 1.0	ns	ns
	Polar moment (mm ⁴)	23979.2 ± 1056.0	21456.6 ± 813.7	ns	ns
	Marrow cavity (mm ²)	251.3 ± 9.7	241.8 ± 8.9	ns	ns
Biomechanics	Diaphysis, 50%				
	Displacement (mm)	3.5 ± 0.2	4.0 ± 0.3	ns	ns
	Load (N)	4851.4 ± 139.3	4134.5 ± 194.0	<0.05	<0.01
	Energy (N × mm)	10236.9 ± 583.2	11884.9 ± 970.3	ns	ns
	Stiffness (N/mm)	2183.1 ± 125.4	1971.8 ± 110.7	ns	<0.05

Values are mean ± SE. n = number of individuals; BMC = bone mineral content; BMD = bone mineral density; trab = trabecular; CSA = cross sectional area; PERIC = periosteal circumference, cort = cortical; THKC = thickness; ENDOC = endosteal circumference. Differences between groups were evaluated by ANCOVA with adjustments for body weight since body weight may influence the different outcome parameters. ns = non-significant.

accumulation of several pollutant groups have been assessed for ruminants grazing pastures treated with sludge (Fries and Marrow, 1977; Smith, 1996) and concentrations of selected organic pollutants and heavy metals in sheep maintained on the same pastures as those in the present study have been reported (Rhind et al., 2007; Rhind et al., 2005a; Rhind et al., 2005b). Both predicted and actual concentrations of various pollutants in tissue, following exposure to treated pastures,

including heavy metals and organic pollutants were low and not consistently increased, relative to control pastures. However, these measurements may not provide an index of exposure at the many stages of development before slaughter at which bone development may be affected. Increases in exposure as a result of grazing sludge treated pasture may be transient and variable, depending on climatic and pasture conditions.

Table 5 – Mean (±SE) plasma concentrations of bone-specific alkaline phosphatase (BAP), carboxyterminal telopeptide of type 1 collagen (CTX) and 25-OH D=25-hydroxy vitamin D (25-OHD) in rams

RAM				
	Control	Treated	p-value	
	(n = 12)	(n = 12)	t-test	ANCOVA
BAP (ng/ml)	14.5 ± 1.1	17.7 ± 2.4	ns	ns
CTX (ng/ml)	1.2 ± 0.2	1.1 ± 0.1	ns	ns
25-OH D (nmol/l)	19.4 ± 2.1	19.6 ± 2.1	ns	ns

Differences between groups were evaluated by ANCOVA with adjustments for body weight since body weight may influence the different outcome parameters. ns = non-significant.

Table 6 – Mean (±SE) plasma concentrations of bone-specific alkaline phosphatase (BAP), carboxyterminal telopeptide of type 1 collagen (CTX) and 25-OH D=25-hydroxy vitamin D (25-OHD) in ewes

EWE				
	Control	Treated	p-value	
	(n = 12)	(n = 12)	t-test	ANCOVA
BAP (ng/ml)	15.1 ± 1.4	18.1 ± 1.9	ns	ns
CTX (ng/ml)	0.6 ± 0.1	0.6 ± 0.1	ns	ns
25-OH D (nmol/l)	24.3 ± 2.8	25.0 ± 3.4	ns	ns

Differences between groups were evaluated by ANCOVA with adjustments for body weight since body weight may influence the different outcome parameters. ns = non-significant.

Notwithstanding the difficulties of defining exposure dose, the results of the studies indicate that exposure to pollutants via sewage sludge can perturb bone tissue homeostasis in sheep. This effect was most pronounced in male offspring (rams). The exposed rams exhibited marked effects especially in the diaphysis of the femur, including reduced CSA and marrow cavity but increased cortical thickness (Fig. 2). In contrast, the female offspring (ewes) exhibited no changes in pQCT variables but the three-point bending test, however, showed that the exposed ewes in the present study had more fragile bone (lower load at failure and reduced stiffness) than the C ewes. Similar reductions in bone strength have been reported previously in studies of rats exposed to dioxin and dioxin like PCBs (Jamsa et al., 2001; Lind et al., 2000b; Miettinen et al., 2005) and a PCB mixture (Andrews, 1989).

The differences in responses between rams and ewes in the present study could be a reflection of the difference in the duration of the period of exposure to sludge. However, during development *in utero* and neonatal life, the most vulnerable period of development, the ram and ewe lambs of respective treatments were exposed to sludge-treated pastures, or not, in an identical manner. It is noteworthy that the animals exposed for the longer period (ewes) exhibited a lesser treatment difference. Thus, it seems unlikely that the difference in the duration of exposure is the cause of the apparent sex difference in responses. Apparently, the bone tissue is more sensitive in rams than in ewes to pollutants present in sewage sludge. The mechanisms behind this are not known.

Comprehensive quantification of animal exposure to EDCs from sludge is logistically extremely difficult owing to variation in the characteristics of individual pollutants and changing climatic and pasture conditions. However, sludge is known to contain large amounts of a wide range of EDCs (Brunner et al., 1988) and the results of the chemical analysis in the present study (Table 2) indicate that application to land might result in some increase in the soil concentrations of selected EDCs, suggesting that animal exposure was likely to be increased. Since sludge contains a large number of EDCs of different classes and with different mechanisms of action, it is not possible to identify the agent(s) responsible for the observed effects on bone tissues. However, previous studies have shown that bone tissue homeostasis can be perturbed by exposure to either mixtures of EDCs in wildlife species (Fox et al., 2008; Lind et al., 2003; Lind et al., 2004; Sonne et al., 2004) or humans (Alveblom et al., 2003; Glynn et al., 2000; Hodgson et al., 2008) or single compound studies in laboratory (Hermesen et al., 2008; Jamsa et al., 2001; Lind et al., 1999; Lind et al., 2000a) or domestic animals (Lundberg et al., 2006).

For the male animals, the absence of differences between T and C animals in plasma concentrations of the bone markers (BAP, CTX, and 25-OH D) on the day of slaughter can be explained by the long period of time between exposure to EDCs and measurement of the effect or by the fact that bone homeostasis was already stable at this stage. Clearly, the absence of treatment differences in ewes cannot be explained by the absence of exposure to sludge since they were exposed until slaughter. There are at least two possible explanations. Firstly, it is possible that the degree of exposure had declined

by the time of slaughter, as a result of loss of pollutant from the sludge to the atmosphere and to the soil so that any changes in marker expression which may have been induced were reduced accordingly. Alternatively, the physiological processes responsible for the altered bone structure may have been active only at earlier stages of development, in which case alteration of bone marker expression would not be expected at later times.

The consequences of the observed effects, from animal and human health perspectives, are difficult to assess. However, the fact that quantifiable changes in bone tissue were detected in sheep grazing pastures fertilized with sewage sludge is of concern because any deviation from the normal development must be regarded as sign that exposure, even to low concentrations of a mixture of environmental pollutants, may interfere with normal physiological processes.

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