Effects of hypothyroidism on the structure and mechanical properties of bone in the ovine fetus

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Abstract

Thyroid hormones are important for normal bone growth and development in postnatal life. However, little is known about the role of thyroid hormones in the control of bone development in the fetus. Using computed tomography and mechanical testing, the structure and strength of metatarsal bones were measured in sheep fetuses in which thyroid hormone levels were altered by thyroidectomy or adrenalectomy. In intact fetuses, plasma concentrations of total calcium and the degradation products of C-terminal telopeptides of type I collagen increased between 100 and 144 days of gestation (term 145 ± 2 days), in association with various indices of bone growth and development. Thyroid hormone deficiency induced by thyroidectomy at 105–110 days of gestation caused growth retardation of the fetus and significant changes in metatarsal bone structure and strength when analyzed at both 130 and 144 days of gestation. In hypothyroid fetuses, trabecular bone was stronger with thicker, more closely spaced trabeculae, despite lower bone mineral density. Plasma osteocalcin was reduced by fetal thyroidectomy. Removal of the fetal adrenal gland at 115–120 days of gestation, and prevention of the prepartum rises in cortisol and triiodothyronine, had no effect on bodyweight, limb lengths, metatarsal bone structure or strength, or circulating markers of bone metabolism in the fetuses studied near term. This study demonstrates that hypothyroidism *in utero* has significant effects on the structure and strength of bone, with different consequences for cortical and trabecular bone.

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Introduction

Thyroid hormones play an important role in normal skeletal growth and development both before and after birth (Williams 2009). In postnatal animals, triiodothyronine (T_3) stimulates bone growth and ossification by a number of mechanisms, including differentiation of osteoblasts and hypertrophic chondrocytes, angiogenesis, synthesis of bone matrix, and mineralization (Bassett & Williams 2003). Thyroid hormone deficiency after birth causes suppression of bone growth, abnormalities in the organization of the epiphyseal growth plates, and delays in endochondral and intramembranous ossification (Stevens *et al.* 2000, Flamant *et al.* 2002, Freitas *et al.* 2005). However, much less is known about the consequences of thyroid hormone deficiency for bone development in the fetus.

Congenital hypothyroidism (CH) is a condition of thyroid hormone deficiency that affects 1 in 3000–4000 births in the UK and is commonly due to abnormal development of the thyroid gland in the fetus (Kratzsch & Pulzer 2008). If left untreated, CH causes neurological disability and growth retardation of the skeleton and other tissues. Before birth,

there is limited transfer of maternal thyroid hormones across the human placenta to the fetus, which may partially compensate for the deficiency in thyroid hormone production in the fetus with CH (Vulsma *et al.* 1989). Despite this, abnormalities in bone structure are evident in neonates with CH, which suggest that bone development is sensitive to reduced thyroid hormone levels *in utero* (Sack *et al.* 1993). Indeed, the bone age of the newborn infant is used as a clinical marker of the severity of hypothyroidism before birth and is one of the best predictors of the long-term consequences of CH for neurological function (Rovet *et al.* 1987, Virtanen 1988).

Developmental changes in the availability of the thyroid hormones occur before birth, and in the fetus as well as in the adult animal, the circulating and local levels of thyroid hormones are largely determined by the activities of deiodinase enzymes in specific tissues (Polk 1995). For most of gestation, thyroxine (T_4) is metabolized to reverse-T_3, which is biologically inactive, by deiodinase type 3 (D3) activity in the placenta and fetal organs. However, close to term, thyroid hormone metabolism changes such that T_4 is preferentially deiodinated to T_3 by D1 and D2. Maturation of

the thyroid hormone axis in the fetus near term and the rise in circulating T_3 closely parallel the prepartum rise in plasma cortisol and are known to be induced by glucocorticoids *in utero*. In sheep fetuses, surgical removal of the adrenal gland prevents the prepartum rise in both plasma cortisol and T_3, while exogenous administration of cortisol or dexamethasone in immature fetuses causes a premature increase in plasma T_3 (Forhead *et al.* 2006, 2007). Furthermore, changes in glucocorticoids and thyroid hormones near term are associated with changes to the growth rate of the fetus (Fowden *et al.* 1996), although the effects of these maturational hormones on bone development near term are unknown.

In the sheep fetus, surgical removal of the thyroid gland causes growth retardation of the skeleton and a delay in ossification of the epiphyseal centers of the limbs (Hopkins & Thorburn 1972, Erenberg *et al.* 1974, Ayromlooi *et al.* 1983). However, little is known about the role of thyroid hormones in the development of bone structure and strength *in utero*. Therefore, this study sets out to examine the effects of hypothyroidism induced by thyroidectomy on aspects of the structure and mechanical properties of bone in the sheep fetus as determined by computed tomography and strength testing. This study also examined bone development in fetuses where the prepartum rise in cortisol and T_3 was abolished by adrenalectomy. The sheep fetus is a good experimental model for the human fetus as the timing of the development of the thyroid hormone axis is similar in the two species (Polk 1995). The ovine placenta appears to be impermeable to the transfer of maternal thyroid hormones, at least during late gestation (Hopkins & Thorburn 1971), which means that thyroid hormone deficiency *in utero* can be investigated by fetal thyroidectomy.

Methods and Materials

Animals

A total of 45 Welsh Mountain sheep fetuses of known gestational age were used in this study: 20 were singletons and 25 were twin fetuses. There were 19 female and 26 male fetuses. The ewes were housed in individual pens and were maintained on 200 g/day concentrates with free access to hay, water, and a salt-lick block. Food, but not water, was withheld for 18–24 h before surgery. All surgical and experimental procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the local animal ethics committee.

Surgical procedures

All surgical operations were carried out under halothane anesthesia (1·5% in O_2–N_2O) with positive pressure ventilation. Using surgical techniques described previously (Hopkins & Thorburn 1972, Barnes *et al.* 1977), 11 fetuses were thyroidectomized (TX) between 105 and 110 days of gestation (term 145 ± 2 days) and five fetuses were adrenalectomized (AX) between 115 and 120 days of gestation. A further 12 fetuses were sham operated with exposure of either the thyroid ($n=4$) or the adrenal gland ($n=8$). At surgery, all fetuses were administered 100 mg ampicillin i.v. (Penbritin; Beecham Animal Health, Brentford, UK) and 2 mg gentamicin i.v. (Frangen-100; Biovet, Mullingar, Ireland). The ewes were given antibiotics i.m. (procaine penicillin, Depocillin; Mycofarm, Cambridge, UK) on the day of surgery and for 3 days thereafter.

Tissue collection

Seventeen unoperated fetuses were delivered at either 100 ($n=5$) or 114–116 (mean 115 days; $n=5$) or 127–131 (mean 130 days; $n=7$) days of gestation, whereas six TX fetuses were delivered at 127–131 days (mean 130 days). A further 12 intact, five TX, and five AX fetuses were delivered at 141–146 days (mean 144 days). All of the fetuses were delivered by cesarean section under general anesthesia (20 mg/kg sodium pentobarbitone i.v. to the ewe). At delivery, 5 ml blood samples were taken by venipuncture of the umbilical artery and placed into EDTA-containing tubes. The samples were centrifuged for 5 min at 1000 g and 4 °C, and the plasma aliquots were stored at -20 °C until analysis. The fetuses were administered with a lethal dose of barbiturate (200 mg/kg sodium pentobarbitone) and then weighed. Measurements of crown–rump length (CRL) and hind-limb (femur, tibia, metatarsus/phalanges) and fore-limb (humerus, radius, and metacarpus/phalanges) lengths were taken. For each fetus, the metatarsal bone from one hind limb was immediately frozen in liquid nitrogen and stored at -80 °C until analysis. In the sheep, each hind limb has one elongated metatarsal bone that is a fusion of metatarsals III and IV. Metatarsals I and V are not present and metatarsal II is a small vestigial bone. At delivery, there was no evidence of adrenal remnants in any of the AX fetuses, or thyroidal tissue in any of the TX fetuses.

Biochemical analyses

Plasma cortisol concentration was measured by RIA validated for use with ovine plasma as described previously (Robinson *et al.* 1983). The lower limit of detection was 1·5 ng/ml and the inter-assay coefficient of variation (CV) was 11%. Total plasma T_3 and T_4 concentrations were also measured by RIA using commercial kits validated for ovine plasma (ICN Biomedicals, Thame, UK; Fowden & Silver 1995). The lower limits of detection were 0·14 ng/ml for T_3 and 7·0 ng/ml for T_4. The inter-assay CVs were 10% for each assay. Plasma levels of total osteocalcin and the degradation products of C-terminal telopeptides of type I collagen (CTX) were determined by ELISA (Immunodiagnostics Systems Ltd, Boldon, UK). The lower limits of detection of osteocalcin and CTX were 0·5 and 0·02 ng/ml, respectively, and all

measurements were made in a single assay. Total plasma calcium was measured by a Siemens Dimension RXL autoanalyzer using Siemens reagents and calibrators (Siemens Healthcare, Camberley, UK).

3D computed tomography

Following dissection, the metatarsi from all fetuses were measured (length and diameter) using digital calipers (Mitutoyo, Andover, UK) and scanned using an Xtek Benchtop 160Xi scanner (Xtek Systems Ltd, Tring, UK) equipped with a Hamamatsu C7943 x-ray flat panel sensor (Hamamatsu Photonics, Welwyn Garden City, UK). All scans were taken at 150 kV, 60 µA using a molybdenum target with an exposure time of 534 ms and 4× digital gain. Image resolution was 29 µm. Reconstructed volume images were analyzed using VGStudio Max 1.2.1 Software (Volume Graphics GmbH, Heidelberg, Germany) to give values for bone volume to total volume ratio (BV/TV), bone surface to BV ratio (BS/BV), trabecular thickness, and spacing. Using standards of known density, all the voxels that formed the structure were automatically assigned bone mineral density in grams per cubic centimeter. Additional calculations were made of porosity, Euler number (a measure of connectivity), structural model index (SMI, a measure of surface convexity where an ideal plate, cylinder, and sphere have SMI values of 0, 3, and 4 respectively), trabecular pattern factor (an index of relative convexity or concavity of the total BS, where concavity indicates connectivity and convexity indicates isolated, disconnected structures), average object area and average object number (indicators of structural connectivity, where high connectivity results in few and large objects, while fragmentation results in large numbers of smaller objects), and degree of anisotropy (a measure of how highly oriented trabeculae are) using a custom written package and the Visilog Quantification+package (both Noesis, Crolles, France) within the Amira 4.1.2 package (Mercury Computer System, Inc., Chelmsford, MA, USA). Cross-sectional moment of inertia (CSMI) was calculated as $CSMI = (\pi/4) \times (r_1^4 - r_2^4)$, where r_1 is the mean radius of the midshaft and r_2 is the mean radius of the lumen at the same position.

Mechanical bone strength testing

Mechanical strength was carried out on the bones collected from fetuses at 144 days of gestation using a Bose Electroforce 3200 electromagnetic test instrument (Bose Corporation, Eden Prairie, MN, USA). The midshaft strength of the metatarsal bone was tested using a three-point bend test. Bones were placed anterior surface down on two supports equidistant from the ends and 40 mm apart. Samples were centrally loaded at a constant rate (6 mm/min) up to failure. To test the trabecular bone, a small block of trabecular bone (3×3×6 mm) was cut from the distal end of the developing bone using a Buehler Isomet low-speed saw and a Buehler

diamond wafering blade (Buehler UK Ltd, Coventry, UK). The block was then placed between two small platens and then loaded at a constant rate (1 mm/min) until failure. Load–displacement curves were used to calculate maximum load at failure, maximum displacement at failure, stiffness, energy, and stress. Stiffness was calculated as the slope of the linear portion of the load–displacement curve. Energy was determined as the area under the curve. Stress was determined as the maximum load divided by the cross-sectional area as determined by computed tomography.

Statistical analysis

Developmental changes in variables measured in the intact fetuses were determined by one-way ANOVA followed by the Tukey test, or by one-way ANOVA on ranks followed by Dunn's test, as appropriate. The effect of TX at 130 and 144 days of gestation was assessed by two-way ANOVA with treatment and gestational age as factors, followed by the Tukey test. Differences between variables measured in intact and AX fetuses at 144 days of gestation were determined by unpaired t-test. At 144 days of gestation, measurements of mechanical strength were compared among intact, TX, and AX fetuses by one-way ANOVA followed by the Tukey test. For the bone density graphs, data were compared every $0·13$ g/cm^3 over the range shown on each graph. Data were compared by two-way ANOVA with density and treatment as factors followed by the Tukey test. Data are presented as mean±s.e.m.; significance was determined with a P level of $0·05$ or lower.

Results

Plasma hormone concentrations

In the intact fetuses, plasma concentrations of cortisol, T_3, total calcium, and CTX increased between 100 and 144 days of gestation ($P < 0·01$ in all cases; Table 1, Fig. 1a and b). There was no significant change in plasma osteocalcin over the gestational period studied (Fig. 1f).

Fetal TX abolished the prepartum rise in fetal plasma T_3, but not cortisol, concentration (Fig. 1a and c). Between 130 and 144 days of gestation, significant increments in plasma cortisol were observed in both intact and TX fetuses, and there was no significant difference in plasma cortisol concentration between the two groups of fetuses at each gestational age (Fig. 1a). In the TX fetuses, plasma concentrations of T_4 and T_3 were below, or close to, the limit of assay detection at 130 and 144 days of gestation (Fig. 1b and c). No significant difference in plasma T_3 was observed between the intact and the TX fetuses at 130 days of gestation, but at 144 days, plasma T_3 in the TX fetuses was lower than in the intact fetuses ($P < 0·05$; Fig. 1c). Plasma osteocalcin was lower in the TX fetuses compared with intact fetuses at both 130 and 144 days of gestation ($P < 0·05$ in both cases; Fig. 1f). Fetal TX had no effect on plasma total calcium or CTX (Fig. 1d and e).

Table 1 Mean (±S.E.M.) measurements of bodyweight, crown–rump length (CRL), and limb lengths in the fetuses of each experimental group at tissue collection

Gestational age (days)	100	115	130		144		
Treatment	Intact	Intact	Intact	TX	Intact	TX	AX
Number of fetuses	5		7	6	12	5	5
Body weight (kg)	0·76±0·03[a]	1·42±0·08[a]	2·68±0·19[b]	2·46±0·14	3·57±0·16[c]	2·64±0·17*	4·10±0·40
CRL (cm)	29·0±1·0[a]	36·4±1·2[b]	42·9±1·0[c]	42·7±1·3	48·6±1·5[d]	42·2±1·0*	50·6±2·0
Metatarsus/phalanges (cm)	9·0±0·2[a]	12·2±0·1[b]	15·5±0·4[c]	13·7±0·4*	17·8±0·3[d]	14·2±0·4*	18·3±0·9
Tibia (cm)	7·9±0·1[a]	11·3±0·2[a]	13·7±0·5[ab]	12·0±0·4*	15·6±0·3[b]	12·8±0·5*	15·0±0·6
Femur (cm)	6·2±0·1[a]	8·9±0·3[b]	11·6±0·4[c]	9·3±0·6*	13·0±0·5[c]	10·4±0·4*	12·3±1·2
Hind-limb length (cm)	23·1±0·2[a]	32·4±0·5[b]	40·8±1·2[c]	35·2±1·5*	45·1±1·5[c]	37·4±0·9*	45·7±0·9
Metacarpus/phalanges (cm)	7·3±0·3[a]	10·1±0·2[b]	13·1±0·3[c]	11·8±0·3*	14·6±0·3[d]	11·8±0·2*	15·7±0·3
Radius (cm)	6·2±0·2[a]	8·5±0·2[b]	10·9±0·3[c]	9·9±0·3*	12·3±0·2[d]	10·4±0·2*	11·7±1·2
Humerus (cm)	5·2±0·2[a]	7·4±0·2[b]	9·0±0·3[c]	8·4±0·4	10·7±0·3[d]	8·7±0·2*	11·7±1·3
Fore-limb length (cm)	18·7±0·7[a]	26·0±0·6[b]	33·0±0·8[c]	30·2±0·8*	37·6±0·7[d]	30·9±0·6*	39·0±2·6

Within groups of intact fetuses and for each parameter measured, values with different superscript letters are significantly different from each other ($P<0·05$). *Significant difference from intact fetuses of the same gestational age ($P<0·05$).

Fetal AX prevented the normal prepartum increments in both plasma cortisol and T_3 concentrations (Fig. 1a and c). At 144 days of gestation, plasma cortisol and T_3 concentrations in the AX fetuses were lower than those observed in the intact fetuses and similar to those seen in the intact fetuses at 130 days of gestation ($P<0·05$ in both cases; Fig. 1a and c). Fetal AX had no effect on plasma concentrations of T_4, total calcium, CTX, or osteocalcin at 144 days (Fig. 1).

Fetal morphology

In the intact fetuses, significant increments in bodyweight, CRL, and limb lengths were observed between 100 and 144 days of gestation ($P<0·001$ in all cases; Table 1). At 130 days of gestation, TX had no effect on fetal bodyweight or CRL, but fore- and hind-limb lengths were reduced in the

TX compared with intact fetuses ($P<0·05$ in all cases; Table 1). By 144 days of gestation, bodyweight, CRL, and limb lengths in the TX fetuses were lower than those in the intact fetuses ($P<0·05$ in all cases; Table 1). No significant differences in bodyweight, CRL, or limb lengths were seen in the TX fetuses between 130 and 144 days of gestation (Table 1). No significant differences in the indices of morphology measured were observed between intact and AX fetuses at 144 days of gestation (Table 1).

Bone structure

In the intact fetuses, cortical thickness, diameter, and CSMI at the metatarsal midshaft increased between 100 and 144 days of gestation ($P<0·05$ in all cases; Table 2; Fig. 2). The trabecular bone showed no consistent changes in trabecular thickness,
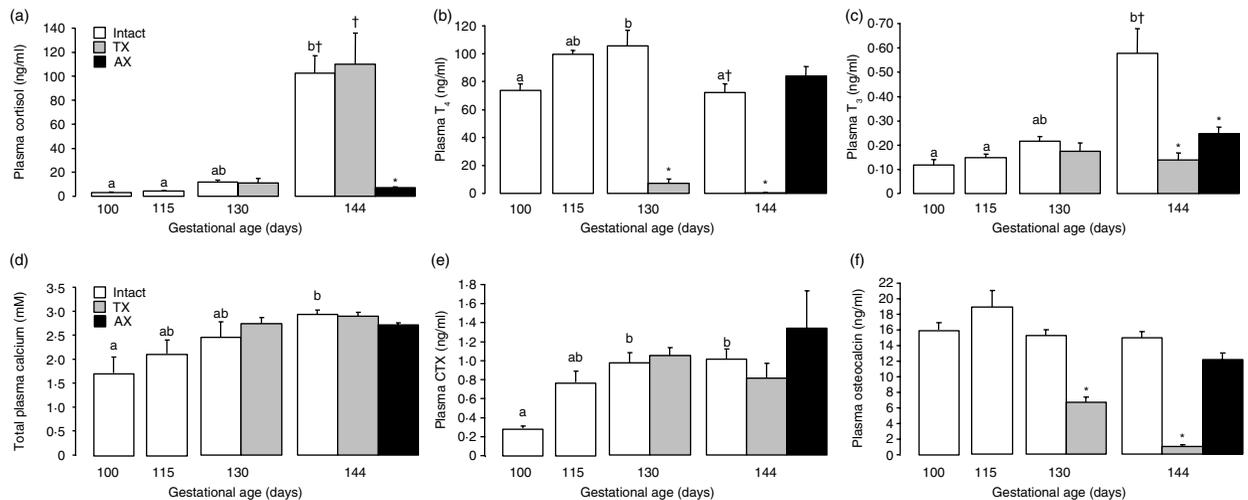
Figure 1 Mean (±S.E.M.) plasma concentrations of (a) cortisol, (b) T_4, (c) T_3, (d) total calcium, (e) CTX, and (f) osteocalcin in the fetuses of each experimental group at tissue collection. Within intact fetuses, columns with different letters are significantly different from each other ($P<0·05$). *Significant difference from intact fetuses at the same gestational age ($P<0·05$). †Significant difference from fetuses of the same treatment at 130 days of gestation ($P<0·05$).

porosity, SMI, average object number and area, Euler or degree of anisotropy between 100 and 144 days of gestation (Table 2). However, there were increases in trabecular spacing and the trabecular pattern factor (indicating an increasingly disconnected trabecular structure) between 115 and 144 days of gestation ($P<0.05$ in both cases; Table 2). This change in spacing lowered the BV/TV ratio but not to a significant level (Table 2).

Fetal TX impaired the growth of the metatarsal bone as shown by the reductions in the length and the midshaft diameter at both 130 and 144 days of gestation ($P<0.05$ in all cases; Table 2) and delayed ossification (Fig. 2). There were also significant differences in trabecular bone structure at both gestational ages. First, compared with control animals, the samples from the TX fetuses at 130 days of gestation showed a lower BS/BV ratio as a consequence of increased trabecular thickness ($P<0.05$ in both cases; Table 2; Fig. 3). At 144 days of gestation, the reduction in BS/BV seen in the TX fetuses was not significant, although there was a significant decrease in BV/TV ratio, which was associated with increases in both trabecular thickness and spacing ($P<0.05$ in all cases; Table 2; Fig. 3b).

Secondly, at both gestational ages, TX caused a significant increase in porosity and altered trabecular connectivity ($P<0.05$; Table 2). In the TX fetuses, the higher SMI indicated a more rod–like appearance of the bone trabeculae, whereas the lower object number and increased object area indicated that there were fewer but larger trabeculae per slice, compared with the intact fetuses ($P<0.05$ in all cases; Table 2; Fig. 3). Fetal TX was associated with increases in both the Euler value and the degree of anisotropy, which indicated a less connected structure and a higher level of directionality, although the change in Euler did not reach significance in the TX fetuses studied at 144 days of gestation (Table 2).

Most of the effects of TX on bone structure observed at 130 days of gestation were maintained at 144 days (Table 2). In the TX fetuses, there were no significant differences in any of the structural measurements made in trabecular bone between 130 and 144 days of gestation (Table 2). Significant decreases in midshaft diameter and CSMI were observed in the TX fetuses over the last 2 weeks of gestation ($P<0.05$ in both cases; Table 2). There were no significant differences between intact and AX fetuses in any of the parameters of bone structure measured at 144 days of gestation (Table 2).

Table 2 Mean ($\pm$s.e.m.) metatarsal length, midshaft cortical bone, and distal trabecular bone characteristics in the fetuses of each experimental group at tissue collection

Gestational age (days)	100	115	130		144		
Treatment	Intact	Intact	Intact	TX	Intact	TX	AX
Number of fetuses	5	5	7	5	12	5	5
Metatarsal length (mm)	69.6 ± 1.0^a	95.2 ± 1.7^b	112.2 ± 2.6^{cd}	$102.6\pm1.5^*$	114.7 ± 4.3^d	$103.4\pm3.4^*$	118.9 ± 3.5
Metatarsal midshaft cortical bone							
Wall thickness (mm)	1.15 ± 0.08^a	1.43 ± 0.04^{ab}	1.43 ± 0.09^{ab}	1.49 ± 0.09	1.62 ± 0.13^b	1.89 ± 0.18	1.71 ± 0.12
Diameter (mm)	5.22 ± 0.16^a	6.78 ± 0.15^{bc}	7.76 ± 0.18^c	$6.71\pm0.21^*$	8.92 ± 0.41^d	$7.65\pm0.31^{*,\dagger}$	9.38 ± 0.36
Cross-sectional moment of inertia (mm^4)	33.7 ± 4.6^a	92.9 ± 7.2^a	151.8 ± 14.2^{ab}	$92.3\pm11.8^*$	279.6 ± 55.0^b	$163.3\pm26.8^\dagger$	329.7 ± 45.0
Metatarsal distal trabecular bone							
BV/TV	0.45 ± 0.02	0.45 ± 0.02	0.40 ± 0.05	0.48 ± 0.05	0.35 ± 0.03	$0.49\pm0.06^*$	0.33 ± 0.03
BS/BV	24.0 ± 1.0	23.1 ± 2.2	20.4 ± 1.2	$16.7\pm0.9^*$	20.6 ± 2.4	16.2 ± 2.4	17.1 ± 0.4
Trabecular thickness (mm)	0.08 ± 0.003	0.09 ± 0.01	0.11 ± 0.01	$0.17\pm0.01^*$	0.10 ± 0.01	$0.15\pm0.02^*$	0.117 ± 0.002
Trabecular spacing (mm)	0.11 ± 0.01^a	0.12 ± 0.02^a	0.40 ± 0.03^b	0.40 ± 0.10	0.52 ± 0.02^c	$0.40\pm0.07^*$	0.60 ± 0.06
Porosity (%)	1.8 ± 0.9	4.0 ± 1.1	2.1 ± 0.7	$9.1\pm1.8^*$	2.2 ± 0.6	$8.1\pm2.4^*$	1.8 ± 0.4
Structural model index	0.12 ± 0.02	0.16 ± 0.01	0.17 ± 0.03	$0.30\pm0.05^*$	0.10 ± 0.02	$0.22\pm0.07^*$	0.05 ± 0.03
Trabecular pattern factor	-7.6 ± 0.8^{ab}	-9.6 ± 2.1^a	-3.6 ± 0.6^{bc}	-5.4 ± 1.1	-2.0 ± 0.4^c	$-4.0\pm1.1^*$	-0.9 ± 0.5
Average object number	38 ± 7	24 ± 2	49 ± 13	$15\pm3^*$	54 ± 10	27 ± 13	36 ± 4
Average object area (mm^2)	0.04 ± 0.02	0.10 ± 0.02	0.09 ± 0.03	$0.31\pm0.05^*$	0.07 ± 0.01	$0.34\pm0.13^*$	0.08 ± 0.01
Euler	-386 ± 34^a	-1072 ± 188^{ab}	-1365 ± 191^b	$-733\pm110^*$	-1091 ± 201^{ab}	-583 ± 233	-623 ± 101
Degree of anisotropy	0.11 ± 0.03^{ab}	0.21 ± 0.02^b	0.14 ± 0.03^{ab}	$0.28\pm0.03^*$	0.11 ± 0.01^a	$0.20\pm0.04^*$	0.12 ± 0.01

Within the groups of intact fetuses and for each parameter measured, values with different superscript letters are significantly different from each other ($P<0.05$). *Significant difference from intact fetuses of the same gestational age ($P<0.05$). $\dagger$Significant difference from fetuses of the same treatment at 130 days of gestation ($P<0.05$).
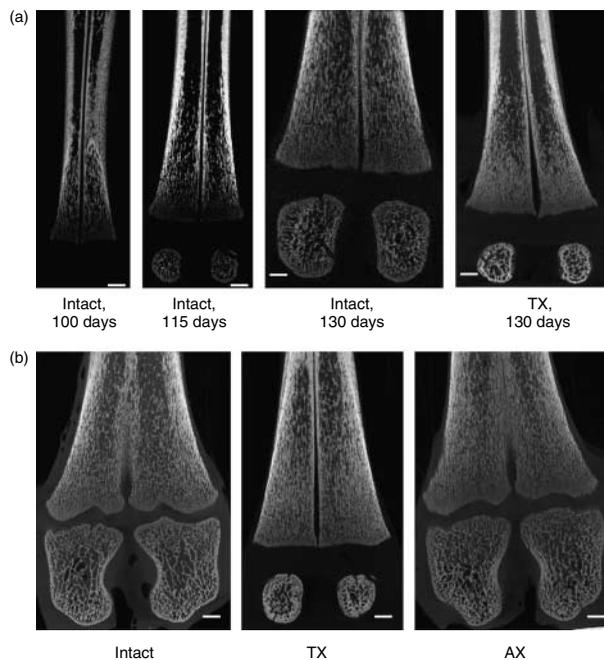
Figure 2 Representative images of transverse section of distal metatarsal bone showing trabecular structure in (a) intact fetuses at 100, 115, and 130 days of gestation, and TX fetuses at 130 days of gestation, and (b) intact, TX, and AX fetuses at 144 days of gestation. Bars represent 2 mm.

Bone strength

Table 3 shows that, compared with the intact fetuses, there were no significant differences in the maximum load, maximum displacement, stiffness, energy absorbed, or stress in the metatarsal midshaft bones from the TX and AX fetuses at 144 days of gestation. In contrast to the cortical bone at the midshaft, the distal trabecular bone in the TX fetuses failed at a higher maximum load compared with that in the intact fetuses ($P<0.05$; Table 3). In addition, the trabeculae in the TX fetuses showed higher stiffness and stress than in the intact fetuses ($P<0.05$ in both cases; Table 3). There were no differences between intact and AX fetuses in any of the parameters studied (Table 3).

Bone mineral density

No differences were found in bone mineral density at the proximal, midshaft, or distal portions of the metatarsal between any of the groups at 130 days of gestation (data not shown). There were also no differences in bone mineral density in the midshaft of the metatarsal (a region of purely cortical bone) between the groups of fetuses at 144 days of gestation (Fig. 4a). At 144 days of gestation, the distal end of the metatarsal showed significant differences in bone mineral density between the treatment groups (Fig. 4b). Lower bone density ($P<0.05$) was observed in the TX fetuses compared with the intact and AX fetuses for the range 0·25–0·6 g/cm^3

(the range corresponding to trabecular bone; Fig. 4b). In addition, there were no differences in bone mineral density between the intact and the AX fetuses for any of the sites studied (Fig. 4).

Discussion

In this study, thyroid deficiency *in utero* affected both the structure and the mechanical properties of metatarsal bone. Abnormalities in bone structure were observed, and several
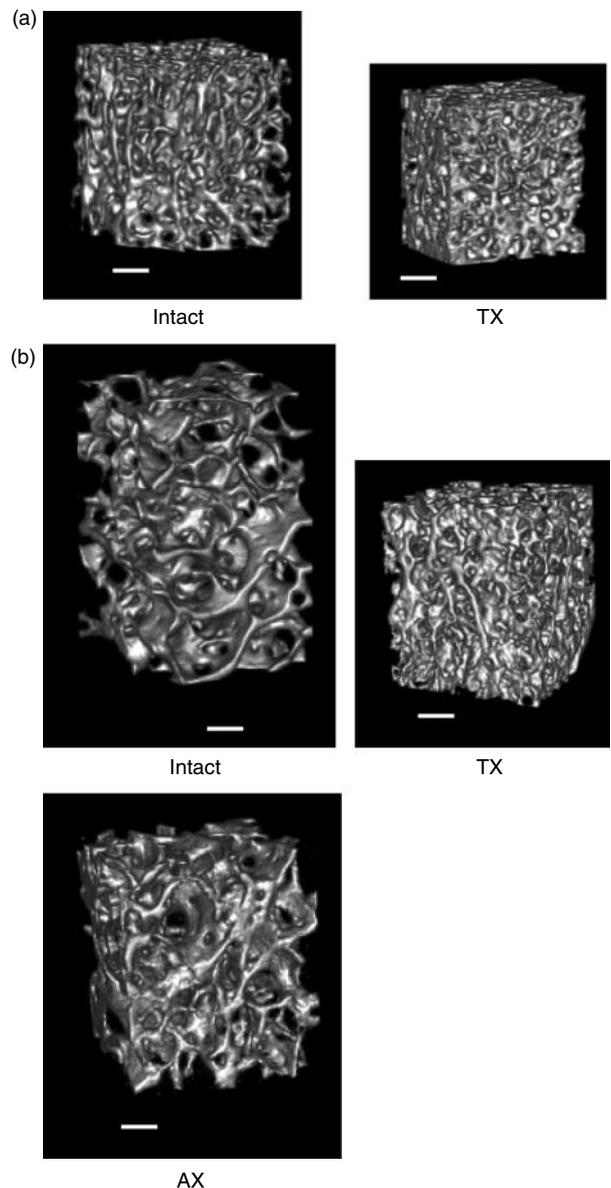
Figure 3 Representative 3D images of metatarsal trabecular structure in (a) intact and TX fetuses at 130 days and (b) intact, TX, and AX fetuses at 144 days of gestation. Bars represent = 500 μm.

Table 3 Mean ($\pm$S.E.M.) metatarsal midshaft cortical bone and distal trabecular bone strength characteristics in the fetuses of each experimental group at tissue collection at 144 days of age

| Treatment	Intact	TX	AX
Number of fetuses	12	5	5
Metatarsal midshaft cortical bone			
Maximum load (N)	$147 \cdot 6 \pm 2 \cdot 0$	$132 \cdot 9 \pm 24 \cdot 4$	$145 \cdot 1 \pm 8 \cdot 4$
Maximum displacement (mm)	$2 \cdot 8 \pm 0 \cdot 1^{ab}$	$2 \cdot 1 \pm 0 \cdot 3^{a}$	$3 \cdot 0 \pm 0 \cdot 2^{b}$
Stiffness (N/mm)	$64 \cdot 1 \pm 4 \cdot 4$	$90 \cdot 1 \pm 19 \cdot 1$	$71 \cdot 7 \pm 16 \cdot 3$
Energy (N/mm)	260 ± 15	178 ± 46	273 ± 5
Stress (N/mm^2)	$2 \cdot 3 \pm 0 \cdot 3$	$3 \cdot 1 \pm 0 \cdot 7$	$1 \cdot 9 \pm 0 \cdot 1$
Metatarsal distal trabecular bone			
Maximum load (N)	$5 \cdot 1 \pm 1 \cdot 0^{a}$	$18 \cdot 8 \pm 2 \cdot 9^{b}$	$8 \cdot 9 \pm 1 \cdot 5^{a}$
Maximum displacement (mm)	$0 \cdot 52 \pm 0 \cdot 15$	$0 \cdot 47 \pm 0 \cdot 12$	$0 \cdot 47 \pm 0 \cdot 09$
Stiffness (N/mm)	$15 \cdot 0 \pm 4 \cdot 6^{a}$	$74 \cdot 3 \pm 18 \cdot 2^{b}$	$24 \cdot 3 \pm 6 \cdot 5^{a}$
Energy (N/mm)	$0 \cdot 7 \pm 0 \cdot 08$	$3 \cdot 5 \pm 1 \cdot 5$	$1 \cdot 5 \pm 0 \cdot 4$
Stress (N/mm^2)	$56 \cdot 3 \pm 11 \cdot 4^{a}$	$208 \cdot 8 \pm 32 \cdot 0^{b}$	$98 \cdot 8 \pm 16 \cdot 8^{a}$

For each parameter measured, values with different superscript letters are significantly different from each other ($P<0 \cdot 05$).

developmental changes normally seen during the last third of gestation were impaired, in the fetuses with undetectable circulating levels of T_4 and T_3. At the midshaft, a region of purely cortical bone, hypothyroidism led to a reduction in cortical diameter without any change in cortical thickness or bone mineral density. In the distal region of the metatarsal, fetal hypothyroidism caused the development of thicker and more closely spaced trabeculae. Near term, the trabecular structure in the TX fetuses was stronger, i.e. sustained a greater mass per unit area before fracture, and yet was more stiff and brittle, i.e. bent less before fracture, than in the intact fetuses. These changes were associated with a reduction in bone mineral density in the range corresponding to trabecular bone. It is not known whether the increased bone strength observed in the TX fetuses was due to this trabecular arrangement and/or the composition of individual struts. In human patients with hypothyroidism in adulthood, trabecular bone thickness was found to be increased by $\sim 30\%$ (Eriksen *et al*. 1986). In both regions of metatarsal bone, the alterations in structure seen in the hypothyroid fetuses appeared, largely, to take place between the time of TX (at 105–110 days) and 130 days of gestation as there was little difference between the TX fetuses studied at 130 and 144 days. Previous studies in ovine fetuses have shown that circulating levels of T_4 become undetectable from within a week of TX (Hopkins & Thorburn 1971).

No differences in bone structure or mechanical strength, or circulating markers of bone metabolism, were observed in the AX fetuses in which the prepartum rises in cortisol and T_3 were prevented. These findings indicate that the prepartum rises in circulating cortisol and T_3 have little influence on bone development near term. Previous studies in ovine fetuses have demonstrated that linear skeletal growth declines near to term in association with the prepartum rise in cortisol (Fowden *et al*. 1996). In addition, fetal AX has been shown to prevent the normal decrease in the rate of CRL growth in individual fetuses studied longitudinally (Fowden *et al*. 1996);

although in this study, the number of AX fetuses studied may have been too small to identify changes in body and limb lengths at a single time point near term across a population of animals. Taken together, the observations from the TX and AX fetuses indicate that circulating levels of T_4, rather than the prepartum rise in plasma T_3, are important for normal bone development in the fetus during late gestation. However, local concentrations of T_3, generated from T_4 by deiodinase activity within bone, are likely to be essential for normal bone development. Indeed, it is possible that during hypothyroidism, transport of T_4 into bone cells and local conversion of T_4 to T_3 may be upregulated in an attempt to maintain the important actions of thyroid hormones in the control of bone growth and development. In mice, maternal hypothyroidism causes increases in D2 mRNA and activity and decreases in D3 mRNA and activity, in the bones of fetuses near term (Capelo *et al*. 2008). Deiodinase activity has not been measured in the bones of fetal sheep, although hypothyroidism has previously been shown to increase D2 activity in the cerebral cortex of the sheep fetus in order to preserve local production of T_3 important for normal brain development (Polk *et al*. 1988).

The changes in bone structure seen in sheep fetuses normally during the last third of gestation and in response to thyroid hormone deficiency were accompanied by changes in circulating markers of bone metabolism. Plasma levels of CTX and osteocalcin were used as indicators of osteoclast and osteoblast activities respectively. In control animals, plasma osteocalcin concentration and osteoblast activity remained high throughout the study period, as might be expected with rapid bone development. Plasma CTX and the level of osteoclast activity increased over the study period, possibly to remodel the developing bone as shown by the ontogenic alterations in bone structural parameters. In contrast, in the TX fetuses, plasma osteocalcin and osteoblast activity decreased in association with the reduction in bone development. These results suggest that the changes in bone growth and structure
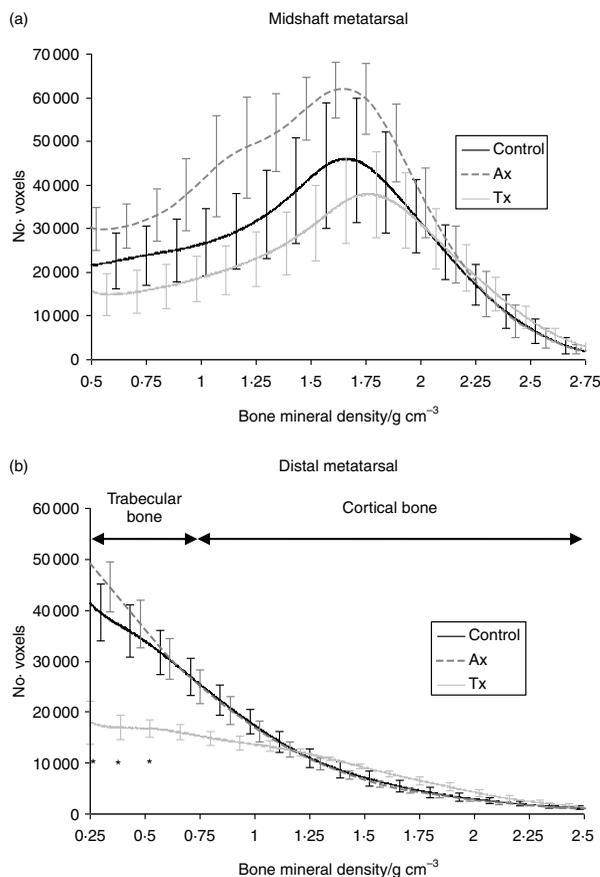
Figure 4 Bone mineral density plots for (a) the midshaft and (b) the distal regions of the metatarsal bone in intact, TX, and AX fetuses at 144 days of gestation. Plots are mean ± s.e.m. *Significant difference of TX fetuses from intact fetuses ($P < 0.05$).

induced by fetal hypothyroidism are associated with impaired bone deposition rather than bone resorption.

Thyroidectomy of the sheep fetus leads to the removal of C-cells, and internal parathyroid glands, within the thyroid glands while leaving the superior parathyroid glands intact. Therefore, this procedure may have consequences for the levels of calcitonin, parathyroid hormone (PTH), and PTH-related peptide (PTH-rP) in the fetal circulation and, in turn, bone growth and development in the TX fetus. In sheep, PTH is undetectable in the fetal circulation from 80 to 145 days (term) of gestation, and calcitonin and PTH-rP are present at stable levels over this period of gestation (Collignon *et al*. 1996). However, the effect of TX *in utero* on the circulating levels of these hormones is unknown. Total plasma calcium concentration remained normal in the TX fetuses in this study, and previously, a reduction in total plasma calcium, and a reversal of the placental calcium gradient between the mother and fetus, was observed in sheep fetuses after removal of both the thyroid and the parathyroid glands, but not following TX alone with T_4 replacement (Care *et al*. 1986,

Rodda *et al*. 1988). In addition, mutation of the calcitonin/calcitonin gene-related peptide gene in fetal mice has no effect on skeletal weight, or growth plate morphology or gene expression, although it causes a reduction in skeletal magnesium, but not calcium, content (McDonald *et al*. 2004). Therefore, it appears unlikely that deficiencies in hormones other than the thyroid hormones are responsible for the changes in bone structure and strength seen in this study, although this could be confirmed by T_4 replacement in the TX fetus.

Thyroid hormones may regulate normal bone growth and development before birth by a number of mechanisms that may be direct and/or indirect via other endocrine systems. For instance, thyroid hormone deficiency may influence the circulating and local production of growth factors in the fetus with consequences for tissue growth and bone development.

In fetal sheep, TX alters the expression of the genes for the growth hormone receptor, insulin-like growth factor 1 (IGF1), and IGF2 in liver and skeletal muscle (Forhead *et al*. 1998, 2000, 2002). Hypothyroidism in postnatal rats also reduces serum IGF1 and IGF1 protein in the growth plate (Freitas *et al*. 2005). In addition, thyroid hormones have been shown to influence the gene expression of PTH-rp and receptors for fibroblast growth factor in rodent chondrocytes (Stevens *et al*. 2000, Barnard *et al*. 2005). Furthermore, thyroid hormone deficiency *in utero* may affect general body metabolism with consequences for growth and development. In the ovine fetus, TX has previously been shown to reduce umbilical oxygen uptake and glucose oxidation (Fowden & Silver 1995). Therefore, the hypothyroid fetus appears to have less energy available to support normal growth of bones and other non-essential tissues.

In conclusion, this study demonstrates that hypothyroidism *in utero* causes significant changes to the structure and strength of bone in the fetus. Critically, the long-term consequences of these effects for bone development and strength in postnatal life, however, remain unknown. The delay in bone age seen in neonates diagnosed with severe CH is still evident at 12 months of age despite treatment (Dubuis *et al*. 1996). Furthermore, bone strength and risk of fracture in adulthood are determined both by bone turnover and by the peak bone mass acquired in pre- and postnatal development (Williams 2009). There is also evidence to suggest that the intrauterine environment can influence skeletal growth and final bone structure after birth. For example, the offspring of rats fed a low-protein diet during pregnancy have lower bone mineral content and changes to the epiphyseal growth plate and the structural and mechanical properties of the skeleton in adult life (Lanham *et al*. 2008). Poor intrauterine nutrition is associated with reduced circulating concentrations of thyroid hormones in the fetus (Dwyer & Stickland 1992, Rae *et al*. 2002), and therefore, thyroid hormone activity may contribute to the long-term consequences of the intrauterine environment on bone development, structure, and integrity before and after birth.

Declaration of interest

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