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A Histomorphometric Study of Cortical Bone of the Iliac Crest in Patients Treated with Glucocorticoids

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Abstract. The effects of glucocorticoids on cancellous bone remodeling and structure are well documented but there are no reported histomorphometric studies in human cortical bone in glucocorticoid-treated patients. We have performed a histomorphometric analysis of iliac crest cortical bone in 14 patients treated with glucocorticoids, 9 females and 5 males, aged 18 to 48 years (34.1 ± 7 years) (mean ± standard deviation [SD]). The underlying disease was cystic fibrosis in 8 patients; asthma 3; and nephrotic syndrome; Crohn disease and inflammatory pseudotumor of the liver in one patient each. Results were compared with an age-matched control group of 10 premenopausal women and 4 men aged 22 to 38 years (30.1 ± 4.8 years) who were not, howerver matched for underlying disease. Cortical bone indices were assessed by image analysis. Cortical width and area were similar in the two groups. However, cortical porosity, Haversian canal number, and density were higher in patients treated with glucocorticoids compared with controls (8.4 ± 8.9% vs. 5.1 ± 3.9%; $P = 0.03$) (45.9 ± 23.2 vs. 31.9 ± 24.4; $P = 0.003$) (13.7 ± 9.4 vs. 6.7 ± 3.3/mm^2; $P = 0.00005$). Haversian canal area did not differ significantly between groups. The mean wall width of the osteons, bone formation rate (μm^2/μm/day) and mineral apposition rate (μm/day) were lower in treated patients compared to controls (48.8 ± 7.1 μm vs. 59.8 ± 12.9 μm; $P = 0.01$) (0.056 ± 0.040 vs. 0.095 ± 0.058; $P = 0.05$) and (0.59 ± 0.12 vs. 0.75 ± 0.11; $P = 0.002$). The proportion of canals with an eroded surface was lower in the treated compared with the control group, although this difference was not statistically significant. These results demonstrate that cortical porosity is increased in patients treated with long-term glucocorticoid therapy, due mainly to an increase in the number rather than size of Haversian canals. This may be because of increased bone resorption during the early stages of glucocorticoid therapy, in combination with long-term impairment of bone formation. Effects of the underlying disease on bone remodeling may also contributed to these changes and could not be excluded in the present study; since control subjects were not matched in terms of disease status.

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Glucocorticoid treatment leads to bone loss and increased fracture risk [1–3]. These adverse effects have been known for more than 70 years [4], but the pathophysiology of bone loss remains to be fully established. Both cortical and cancellous bone are affected and fractures occur particularly in the vertebrae, hip, pelvis, forearm, and ribs [5–7]. Although it is often stated that adverse effects are greater in the axial than in the appendicular skeleton, similar degrees of bone loss have been observed in the spine and proximal femur in the untreated group of randomized controlled trials in glucocorticoid-treated patients [8–10], and in a recent meta-analysis, the reduction in spine and hip bone mineral density in glucocorticoid users was reported to be 89.4 and 88.8%, respectively [11].

Previous histomorphometric studies of glucocorticoid-induced osteoporosis have demonstrated a reduction in bone formation at cellular and tissue level in cancellous bone, resulting in reduced bone volume and reduced trabecular thickness. In addition, an increase in bone turnover and/or resorption has been described in some studies, particularly in association with higher doses of glucocorticoids [12–16]. Therefore, the early rapid bone loss and increase in fracture risk demonstrated in glucocorticoid-treated patients may be attributable to the combination of increased bone resorption and decreased formation, whereas in the later stages of glucocorticoid therapy reduced bone formation predominates. The mechanisms by which glucocorticoids increase bone resorption include increased production of receptor-activated NF-κB ligand (RANKI), other proresorptive cytokines, and, more controversially, hyperparathyroidism secondary to reduced intestinal calcium absorption. Direct effects on osteoblastogenesis, together with increased apoptosis of osteoblasts and osteocytes, are believed to be mainly responsible for reduced bone formation [17, 18].

To date, histomorphometric data in glucocorticoid-treated patients have focused exclusively on cancellous bone. In this study we have investigated cortical bone remodeling in a group of glucocorticoid-treated patients, in order to elucidate the mechanisms underlying cortical bone loss.

Subjects

Biopsies were obtained from 14 patients who were receiving glucocorticoid therapy, 9 females aged 28 to 44 years (mean, 36.8 years) and 5 males aged 18 to 48 years (mean, 29 years). Eight (6 females and 2 males) of these patients had cystic fibrosis and had received 5 to 30 mg prednisolone for between 1 and 10 years. All were prescribed 900 IU daily of vitamin D. None of the patients were receiving gastrostomy feeds and all were in relatively good health at time of biopsy. They had not had recent exacerbations of their chest disease. Their forced expiratory volume at 1 second ($\%FEV_1$) was 36.6 ± 14.5 (mean ± standard deviation [SD]), serum parathyroid hormone (PTH) level of 29.3 ± 12.6 (pg/ml), and serum 25 hydroxy vitamin D levels (25 OHD) of 46.6 ± 14.2 (nmol/L). Their body mass index (BMI) ranged between 16.5 and 25.8, the lumbar spine T score between −0.1 and −3.01, and femoral neck T score between −2.7 and 0.3. The remaining 6 patients (3 females and 3 males), were recruited into this study from the outpatient department at Addenbrooke's Hospital. The underlying disease was asthma ($n = 3$), nephrotic syndrome ($n = 1$), Crohn disease ($n = 1$), and inflammatory pseudotumour of the liver ($n = 1$). They were receiving 7.5 to 60 mg daily of prednisolone at the time of biopsy. The duration of steroid therapy ranged between 2 and 16 years.

The control group consisted of 10 premenopausal women with untreated endometriosis aged 23 to 40 years, who served as controls for an earlier study of the effects of gonadotrophin-releasing hormone agonists [19] (mean 30.5 years) and 4 males aged 23 to 33 years (mean 29.5 years) from a previous study of normal healthy subjects [20, 21], who had consented to undergo bone biopsy during general anesthesia for a minor surgical procedure. None had a history of metabolic bone disease or had taken drugs known to affect bone. All patients received double tetracycline labeling prior to biopsy (300 mg of demeclocycline twice daily for 2 days, followed by a 10-day gap, 300 mg twice daily for 2 days, followed by the biopsy 3 to 5 days after the last dose).

Written informed consent was obtained from all patients and the study was approved by the local research ethics committee.

Bone Biopsies and Sample Preparation

Transiliac crest biopsies were obtained under local anaesthetic using an 8-mm internal-diameter modified Bordier trephine. Iliac crest biopsies were embedded in LR White medium resin (London Resin Co.). Undecalcified sections (8 μm) were stained with 1% toluidine blue. Unstained sections (15 μm) were used for fluorescence microscopy. All biopsies and histologic sections were coded and assessed "blind" by the same observer. In the four male control subjects, examination of tetracycline fluorescence was not technically possible because of the long-time period that had elapsed between the embedding of the samples and the present study, although values for mineral apposition rate had been obtained in the original study [21].

Image Analysis

Composite digital image maps of toluidine blue-stained sections from each biopsy were prepared as described previously by Jordan et al. [22]. For each section, composite digital images of each of the cortices were constructed from multiple image grabs with a magnification of 1.6. The resolution of the composite image was 155 pixels per mm. Using NIH image (version 1.61)[1] the number, location (X-Y coordinates), and area of each Haversian canal were recorded and a mean value (Ha.Ca.Ar) was calculated. Osteonal systems that were affected by artefacts such as tears caused during section preparation were excluded. Total cortical area (Ct.Ar), and cortical width (Ct.Wi: the mean perpendicular length between periosteal surface and endosteal surface at four equidistant points) were also measured. To ensure that multiple measurements were not made on the same osteonal system, only one section was analyzed per biopsy.[2]

The following parameters were derived from the above indices.

$$\text{Cortical porosity (Ct.Po; \%)}: \quad \frac{\sum Ha.Ca.Ar}{Ct.Ar} \times 100$$

$$\text{Haversian Canal Density: (Ha.Ca.Dn; no mm}^2\text{)} \quad \frac{\text{Total Canal No}}{Ct.Ar}$$

Histomorphometry

Further histomorphometric measurements were made using a 'Digicad' digitizing tablet and cursor with an LED point light source (Kontron Ltd.) and an Olympus BHS-BH2 binocular transmitted light microscope with a BH2-DA drawing attachment (Olympus Optical Co. UK Ltd., London).

Primary Measurements

The mean width (W.Wi) of each numbered osteon was measured on toluidine blue-stained sections viewed under polarized light at ×156 magnification. Only osteons with intact cement lines were measured. The wall width was measured as the average of four distances from the Haversian surface to the cement line at four equidistant points around the osteon. The presence or absence of an osteoid seam or an eroded surface in each osteonal system, indicating its remodelling status, was recorded during microscopic examination of the section. Each osteon was viewed under polarized light and if the lamellae were cut at an angle to the surface, then that osteon was considered to have an eroded surface with or without the presence of any osteoclasts. Tetracycline labeling was viewed by fluorescence microscopy on 1 to 3 unstained sections at ×156 magnification. Mineralizing perimeter (Md.Pm) was calculated as follows:

$$\text{Md.Pm/H.Ca.Pm}(\%) = \text{dL.Pm} + (0.5 * \text{sL.Pm})/\text{H.Ca.Pm},$$

where dL.Pm is the doubled labeled perimeter, sL.Pm is the single-labeled perimeter, and H.Ca.Pm is the Haversian canal perimeter.

The mean distance between double labels was measured directly at ×312 magnification using the digitizing tablet and cursor. Measurements were made at approximately four equidistant points along the double labels. All double labels associated with osteonal systems were measured in the three sections. Mineral apposition rate was calculated as:

$$\text{MAR}(\mu\text{m/day}) = \text{L.Wi/LP},$$

Where L.Wi is the interlabel width and LP is the labeling period (12 days).

[1] This software was developed at the U.S. National Institutes of Health and is available on the internet by anonymous FTP from zippy.nimh.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia (part no. PB95-500195GEI)

Table 1. Structural indices in iliac crest cortical bone in glucocorticoid-treated patients and controls.

Structural indices	Glucocorticoid - treated group (n = 14)	Control group (n = 14)	P value
Mean Haversian canal area (μm^2)	0.31 ± 0.26	0.26 ± 0.23	NS
Total cortical area (mm^2)	4.37 ± 2.71	4.72 ± 2.68	NS
Cortical porosity (%)	8.43 ± 8.9	5.1 ± 3.9	0.03
Haversian canal number	45.9 ± 23.2	31.9 ± 24.4	0.003
Haversian canal density (no/mm^2)	13.7 ± 9.4	6.7 ± 3.3	0.0001
Cortical width (μm)	908.0 ± 318.6	916.0 ± 419.7	NS

Results are shown as the mean ± SD

Table 2. Comparison of indices related to bone turnover and remodelling in iliac crest cortical bone of glucocorticoid treated patients and controls

Indices	Glucocorticoid-treated group (n = 14)	Control group (n = 14)	P value
Osteonal mean wall width (μm)	48.8 ± 7.1	59.3 ± 12.0	0.008
Mineral apposition rate (μm/day)	0.59 ± 0.12	0.75 ± 0.11	0.002
Bone formation rate* (at tissue level) ($\mu m^2/\mu m$/day)	0.056 ± 0.04	0.095 ± 0.058	0.05
% Osteons with eroded surface	5.73 ± 7.34	11.52 ± 7.46	NS
% Active osteons (with eroded and/or forming surface)	16.0 ± 7.1	22.0 ± 10.5	NS
Activation frequency* (yr^{-1})	0.439 ± 0.322	0.625 ± 0.488	NS

Results are shown as the mean ± standard deviation (SD)
*Treated group (n = 14) and control group (n = 10)

Derived Indices

The tissue based bone formation rate (BFR/H.Ca.Pm) was calculated as follows: BFR / H.Ca.Pm ($\mu m^2/\mu m$/day) = MAR * (Md.Pm/H.Ca.Pm) %

Activation frequency (Acf) was calculated as:
Acf = BFR/H.Ca.Pm * W.Wi

Statistical Analysis

All measurements are presented as two dimensional values in accordance with the ASBMR nomenclature [23]. Statistical analysis was performed using the Student's t-test (paired or unpaired) after log transformation of the data.

Results

Table 1 shows the comparison of the structural indices of cortical bone in the glucocorticoid-treated patients and control group. Cortical width and area were similar in the two groups but cortical porosity was significantly higher in patients treated with glucocorticoids (P = 0.03). The Haversian canal number was also significantly higher in this group (P = 0.0005) as was the Haversian canal density (P = 0.0001). However, the mean Haversian canal area did not differ significantly between the groups. There was no significant correlation found between the dose or duration of steroid therapy and the cortical porosity.

Indices related to bone turnover and remodeling are shown in Table 2. Osteonal mean wall width was significantly lower in glucocorticoid-treated patients compared with the control group (P = 0.01). Bone formation rate and mineral apposition rate were also both significantly lower in the glucocorticoid treated group (P = 0.05 and P = 0.002, respectively). Both activation frequency and mineralizing perimeter were lower in the glucocorticoid-treated group but these differences were not statistically significant. The proportion of canals with an eroded surface was lower in the glucocorticoid-treated group though it did not reach significance. There was an overall trend toward a lower proportion of actively remodeling canals in the patients on long-term glucocorticoid theraphy.

Discussion

Our results demonstrate increased cortical porosity in patients treated with glucocorticoid therapy, owing mainly to an increase in the number of Haversian canals rather than to an increase in their size. Since all the patients in this study had received continuous oral glucocorticoid therapy for at least 1 year, it is likely that these changes reflect a steady rather than a transitional state, although effects of the underlying disease on bone remodelling may also contribute to the observed changes. In the absence of evidence of increased bone resorption at the time the biopsy was obtained, the observed changes would be consistent with an earlier, transient increase in activation frequency in combination with a long-term reduction in bone formation at the level of the multicellular bone unit, resulting in failure to fill-in previously resorbed cavities. Although canal area

did not differ significantly between the two groups in this study, there was a trend toward increased size in the glucocorticoid treated patients, consistent with an earlier increase not only in osteoclast number but also in activity.

A number of observations support the contention that bone turnover and resorption are increased in the early stages of glucocorticoid therapy, whereas low bone formation predominates at later stages. Therefore, prospective studies have shown rapid bone loss and an increase in fracture risk during the first few months of glucocorticoid therapy [11], which could not be explained on the basis of low bone turnover and formation at the basic multicellular level alone. Secondly, histomorphometric and biochemical assessment of patients undergoing solid-organ transplantation has revealed evidence of increased bone turnover in the first 3 to 6 postoperative months [24, 25]. Thirdly, significant disruption of bone microarchitecture has been reported in patients who have been treated with high doses of glucocorticoids, consistent with increased turnover and resorption [15, 26].

Bone loss at cortical sites has been demonstrated in glucocorticoid-treated patients in a number of studies and appears quantitatively similar to the losses observed in cancellous bone [2, 6, 8, 10]. The results of our study indicate that cortical bone loss is due, at least in part, to an increase in cortical porosity. In agreement with the study of Chappard et al., [26] we were not able to demonstrate a reduction in cortical width in the present study, possibly as a result of the relatively small number of patients studied and the large sampling variance that arises from the unavoidable variations in the precise positioning of the biopsy. Nevertheless, increased endocortical bone resorption during the early stages of glucocorticoid therapy provides a mechanism by which reductions in cortical width may occur, and in one study of glucocorticoid-treated patients with chronic active hepatitis, a significant reduction in cortical width was reported [27].

The patients in our study exhibited considerable heterogeneity in terms of the underlying disease and dose and duration of glucocorticoid therapy. In particular, it could be argued that the underlying disease could, *per se*, induce or contribute to abnormalities in bone remodeling and structure. In patients with cystic fibrosis or inflammatory bowel disease, histomorphometric studies indicated that bone turnover and formation are reduced, with an increase in bone resorption in some cases [28, 29]. Furthermore, severe osteopenia has been reported in mice in which the cystic fibrosis transmembrane conductance regulator (CFTR) gene is inactivated, suggesting a direct effect of the disease bone [30]. In addition, the consequences of the disease such as sex hormone deficiency, low body weight, a systemic inflammatory response, malabsorption, other medications, calcium and vitamin D deficiency, and reduced mobility could also affect bone health. In this study, as in previous histomorphometric studies of glucocorticoid-induced osteoporosis, control data were not available from non-glucocorticoid treated patients with the same range of underlying diseases, and age- and sex-matched reference data were used. It is, therefore, not possible to exclude the contribution of factors other than glucocorticoid therapy to the changes observed in cortical bone structure. Because we were unable to examine tetracycline fluorescence in biopsies from our original control series, which was collected in the late 1970s, we included a group of more recently studied premenopausal women with untreated endometriosis as controls; such women have been shown to have normal bone mineral density, and there are no theoretical grounds to indicate abnormalities of bone remodelling [31, 32]. However, for the male controls, only biopsies from our original study were available, and, thus, bone formation rate and activation frequency could not be calculated.

The changes in bone remodelling observed in our study are similar to those reported in cancellous bone of individuals receiving long-term glucocorticoid therapy [12, 13, 15, 16]. Therefore, mineral apposition rate and wall width, indices of osteoblast activity, were reduced significantly reduced when compared to the controls, and bone formation rate at tissue level was also reduced significantly. Consistent with low bone turnover, the percentage of osteons with an eroded surface was reduced when compared to the control group as was the percentage of osteons undergoing remodelling. However, our finding of a reduced eroded surface in cortical bone conflicts with the study of Carbonare et al. [12] in cancellous bone, in which eroded surfaces were significantly increased in glucocorticoid-treated women when compared to non-glucocorticoid treated women with postmenopausal osteoporosis. This may reflect differences in the duration of glucocorticoid therapy (not stated in the latter study) or to differences in the criteria used for definition of an eroded surface.

In conclusion, this study provides the first detailed information about cortical bone remodelling and structure in patients treated with glucocorticoids. The relative contributions of the underlying disease and glucocorticoid therapy *per se* are uncertain, but both are likely to play a role. The increase in cortical porosity and number of Haversian canals in combination with the observed changes in bone remodeling are consistent with an earlier and transient increase in bone turnover together with a long-term reduction in bone formation at cellular and tissue level. Greater cortical porosity may, therefore, contribute to cortical bone loss and weakness in glucocorticoid-treated individuals. Although we were not able to show a reduction in cortical width in this study, evidence from a previous larger

study [27] suggests that this is also a determinant of cortical bone loss.

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