Histomorphometric Analysis of the Effects of Standard Heparin on Trabecular Bone In Vivo

By Jeffrey M. Muir, Maureen Andrew, Jack Hirsh, Jeffrey L. Weitz, Edward Young, Paula Deschamps, and Stephen G. Shaughnessy

Long-term heparin treatment causes osteoporosis through an as yet undefined mechanism. To investigate this phenomenon, we treated rats with once daily subcutaneous injections of heparin (in doses ranging from 0.25 to 1.0 U/g) or saline for 8 to 32 days and monitored the effects on bone both histomorphometrically and by serial measurements of urinary type I collagen cross-linked-pyridinoline (PYD) and serum alkaline phosphatase, markers of bone resorption and formation, respectively. Histomorphometric analysis of the distal third of the right femur in the region proximal to the epiphyseal growth plate showed that heparin induces both a time- and dose-dependent decrease in trabecular bone volume, with the majority of trabecular bone loss occurring within the first 8 days of treatment. Thus, heparin doses of 1.0 U/g/d resulted in a 32% loss of trabecular bone. Heparin-treated rats also showed a 37% decrease in osteoblast surface as well as a 75% decrease in osteoid surface. In contrast, heparin treatment had the opposite effect on osteoclast surface, which was 43% higher in heparin-treated rats, as compared with that in control rats. Biochemical markers of bone turnover showed that heparin treatment produced a dose-dependent decrease in serum alkaline phosphatase and a transient increase in urinary PYD, thus confirming the histomorphometric data. Based on these observations, we conclude that heparin decreases trabecular bone volume both by decreasing the rate of bone formation and increasing the rate of bone resorption.

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Materials and Methods

Materials. Specific pathogen-free female Sprague-Dawley rats (180 to 185 g) were purchased from Charles River Laboratories (St Constant, Quebec, Canada). Unfractionated heparin was provided by Rhone-Poulenc Rorer (Montreal, Quebec, Canada). Urinary type I collagen-derived cross-linked pyridinoline (PYD) and creatinine were assayed using a commercially available enzyme immunoassay (Metra Biosystems, Palo Alto, CA), whereas serum ALP was measured using an assay from Sigma Chemical Co (St Louis, MO). Tetracycline was purchased from MTC Pharmaceuticals (Cambridge, Ontario, Canada).

Experimental design. To examine the effect of heparin on bone morphology, a total of 32 rats were studied. The animals were arbitrarily randomized on arrival into one of four treatment groups, each consisting of 8 rats. Three groups were administered daily subcutaneous injections of unfractionated heparin at concentrations of 0.25 U/g, 0.5 U/g, or 1.0 U/g for a total of 32 days. The fourth group served as a control, and the rats were administered equivalent volumes of saline in place of heparin. To permit measurements of bone apposition rates, all animals received 2 intraperitoneal injections of tetracycline (26 mg/kg). The first was administered 2 days before starting the study, whereas the second was administered 30 days after initiation of treatment. On day 32, all rats were killed with 5% isoflurane, and, after exsanguination, the right femur was removed for histological evaluation.

In a second study that included 36 rats, we examined the effects of unfractionated heparin on bone morphology as a function of time. A total of 18 rats were treated with daily subcutaneous injections of heparin (1.0 U/g), whereas the remaining 18 rats served as controls and were administered an equivalent volume of saline. On days 8, 16, and 32, 6 rats from each of the control and treatment groups were killed, and their right femurs were removed for histomorphometric analysis. As in the previous study, all animals received 2 intraperitoneal injections of tetracycline (20 mg/kg). The first was administered 2 days before starting the study, whereas the second was administered 2 days before exsanguination with 5% isoflurane.
Biochemical markers of bone turnover. Tail-vein blood samples were collected from each rat before the start of treatment and weekly thereafter. After sedimentation of the red blood cells at 2000g, the serum was removed and assayed for ALP activity, as an index of bone formation. Urine that was collected daily between 2 and 4 PM was assayed for PYD as an index of bone resorption. Creatinine concentrations also were measured in the same urine samples, and the levels of PYD were then expressed as nanomoles PYD per milliliter of urinary creatinine.

Bone histomorphometry. Bone histomorphometry was performed as described by Kostenuik et al.21,22 Briefly, after embedding the undecalcified distal third of the right femur of each rat in glycol-methacrylate (JB-4 embedding medium; Analychem, Toronto, Canada), 6- to 8-μm sections were obtained. The mounted sections were stained with either 1% toluidine blue or hematoxylin and eosin and subjected to morphometric analysis. In addition, unstained mounted sections were examined using UV microscopy to evaluate tetracycline labeling. In each case, a region 60 μm below the epiphysial growth plate and including the entire metaphysis was subjected to light microscopy under oil immersion (1,000×) using a Mertz grid. 21–23 This encompassed a total tissue area of approximately 10 to 15 mm². The parameters determined are as follows: (1) trabecular bone volume, (2) osteoblast surface, (3) osteoid surface, and (4) osteoclast surface. For each section, trabecular bone volume was calculated from a total of greater than 1,600 point measurements (45 fields) that were selected at random using the Mertz grid. The percentage of osteoblast, osteoid, or osteoclast surface was calculated by recording the presence or absence of each where the hemispherical grid of the Mertz radicule crossed trabecular bone. All histological analysis was performed by a single investigator who was blinded to treatment allocation.

Bone mineralization was quantified in a similar fashion. Thus, using the Mertz radicule, trabecular bone surface was scored, at 400× magnification, as either labeled or unlabeled, depending on the presence or absence of fluorescence at the trabecular bone surface. Fluorescent bone surface was further characterized as having either single or double label, according to the number of distinct lines that were observed on the labeled surface. In the case of a double label, the distance between the labels was measured to calculate the mineral apposition rate (MAR).

Statistical analysis. Analysis of variance was used to compare the results between the experimental and control groups. If a significant difference between experimental and control groups was detected, an unpaired Student’s t-test was performed at each point. Significance levels were adjusted by a Bonferroni correction factor for multiple comparisons.

RESULTS

Effect of heparin on ALP and PYD levels. During the 32 days of this study, both control and heparin-treated rats gained weight equally. No significant differences with respect to weight gain were found between the rats treated with heparin at a dose of 1.0 U/g and control rats (80.8 ± 7.1 v 78.8 ± 2.4 g, respectively). However, heparin treatment did result in both a time- and dose-dependent decrease in serum ALP activity (Fig 1). Maximum inhibition was observed in rats treated with heparin at a dose of 1.0 U/g (Fig 1, inset). When administered in this dose, heparin produced a 25.5% ± 0.5% decrease in serum ALP levels (P < .005). In contrast to ALP, the urinary excretion of PYD was transiently increased in heparin-treated rats in a time- and dose-dependent fashion (Fig 2). By day 8, the excretion of urinary PYD in rats treated with 1.0 U/g heparin had increased by 151% ± 10% (P < .005). However, by day 32, PYD levels were at or below control values (Fig 2).

Effect of heparin on trabecular bone. Sections obtained from the right undecalcified femur of heparin-treated and nontreated rats were subjected to morphometric analysis. A region 60 μm below the epiphysial growth plate and including the entire metaphysis was analyzed for trabecular bone. As shown in Fig 3, heparin treatment produced a significant decrease in trabecular bone volume. This effect was both time- and dose-dependent, with the majority of trabecular bone loss occurring within the first 8 days of heparin treatment (Fig 3). In control rats, trabecular bone volume did not change significantly over the course of the experiment (data not shown). When administered at a dose of 1.0 U/g, heparin decreased trabecular bone volume by 31.9% ± 5.2% (mean of pooled data from both experiments; P < .001).

Because reduced trabecular bone volume can result from a reduction in either trabecular bone width and/or trabecular number, we examined the effect of heparin on these parameters. Table 1 shows that both trabecular width and number were significantly decreased by heparin treatment. Control rats had a mean of 12.2 ± 0.8 trabecuela/mm with a mean width of 15.8 ± 1.7 μm. Over the course of 32 days, heparin treatment (1.0 U/g) reduced the number of trabeculae to a mean of 7.0 ± 0.4 trabecuela/mm, with the remaining trabeculae having a mean width of 9.28 ± 0.8 μm. This resulted in a 69.6% ± 12.7% decrease in the distance between adjacent trabeculae (Table 1).

Effect of heparin on surface-based data. Sections were stained with toluidine blue to quantify surface-based data. The parameters measured were (1) percentage of osteoblast surface, (2) percentage of osteoid surface, and (3) percentage of osteoclast surface. Figure 4A shows that heparin produces a dose-dependent decrease in both osteoblast surface and the percentage of trabecular bone covered by osteoid. At a dose of 1.0 U/g, heparin caused a 37.2% ± 2.6% decrease in osteoblast surface as well as a 74.9% ± 2.8% decrease in the amount of osteoid (Fig 4A; P < .001). These changes occurred within the first 16 days of heparin treatment (data not shown). Osteoid thickness also was decreased by heparin from 2.75 ± 0.49 μm to 1.66 ± 0.34 μm (P < .005). In control rats, the parameters of osteoblast surface, osteoid surface, and osteoid thickness remained unchanged throughout the course of the experiment (data not shown).

In contrast to heparin’s effect on osteoblast and osteoid surface, a dose-dependent increase in the percentage of trabecular bone covered by osteoclasts was observed in heparin-treated rats (Fig 4B). Rats administered 1.0 U/g heparin showed a 42.9% ± 7.6% (mean of pooled data from both experiments; P < .001) increase in osteoclast surface. Similar to the changes in osteoblast number or the amount of osteoid, the effects of heparin on osteoclast number occurred within the first 16 days of treatment (data not shown).

Effect of heparin on bone mineralization. Bone mineralization was measured by dual tetracycline labeling over an 8-day period. Table 2 shows that single-labeled surface decreased by a mean of 18.3% ± 2.1% (P < .005) in rats.
The effect of unfractionated heparin on serum ALP levels. Rats were injected with vehicle alone or unfractionated heparin at a concentration of 1.0 anti-Xa U/g. Serum samples were obtained at days 0, 8, 16, and 32 from both control and heparin-treated rats and assayed for ALP activity. Inset: Serum ALP levels at day 32 in rats injected with increasing concentrations of unfractionated heparin. Data are expressed as mean ± SEM. *P < .005 when compared with control values.

Fig 2. The effect of unfractionated heparin on the excretion of urinary PYD. Rats were injected with vehicle alone (●) or unfractionated heparin at concentrations of 0.25 U/g (△), 0.5 U/g (▼), and 1.0 U/g (□), and the excretion of PYD was determined. To account for variations in urine concentration, results are calculated in terms of nanomoles per milliliter of urinary creatinine and are expressed as a mean ± SEM. *P < .01 when compared with control values.

administered 1.0 U/g of heparin. Similarly, the percentage of trabecular bone covered by double tetracycline labels also decreased with heparin treatment. Thus, there was a 34.9% ± 12.7% decrease in the percentage of double-labeled surfaces in rats administered 1.0 U/gm heparin (from 6.3% ± 1.0% to 4.1% ± 0.8%; P < .01). However, neither the mineral apposition rate nor the percentage of osteoid mineralizing (derived from double-labeled surface only) was significantly affected by heparin treatment (Table 2). Similar results were obtained in rats that were administered daily subcutaneous injections of unfractionated heparin (1.0 U/g) for a total of either 16 or 32 days (data not shown).

DISCUSSION

Although heparin has been shown to decrease bone density in patients and in experimental animals, the mechanism responsible for bone loss has not yet been elucidated. To address this issue, rats were treated with pharmacologically relevant doses of heparin for 8 to 32 days, and its effects on bone were examined both histomorphometrically and by obtaining serial measurements of serum ALP and urinary PYD, as indices of bone formation and resorption, respectively. Here we report that (1) heparin causes bone loss in a dose- and time-dependent fashion; (2) both osteoblast and osteoclast number, as well as activity, are altered by heparin treatment; and (3) heparin-induced bone loss is the result of both a decrease in bone formation and an increase in bone resorption.

Heparin treatment decreases osteoblast activity, as evidenced by its ability to produce a dose-dependent decrease...
in both the levels of serum ALP (Fig 1 inset) and the percentage of trabecular surface covered by osteoid (Fig 4A). Consistent with these observations, heparin was also found to cause a dose-dependent decrease in the number of osteoblasts lining the trabecular surface. Similar to the changes in osteoid, the decrease in cell number began early in the course of heparin treatment and reached a plateau by day 16. This suggests that at least part of heparin's ability to decrease serum ALP and/or osteoid results from a reduction in osteoblast numbers. The inhibitory effect of heparin on osteoblast activity is consistent with reports that heparin reduces collagen synthesis in cultured rat calvariae. In contrast, heparin has been shown to stimulate collagen synthesis by cultured osteoblasts. Although the explanation for these conflicting results is unclear, findings in a whole bone organ culture system are more likely to reflect events in vivo than those that occur in isolated osteoblast cultures.

The mechanism responsible for heparin-induced reduction in osteoblast activity is unknown. However, it is possible that this reflects the propensity of heparin to bind to critical growth factors, such as basic-fibroblast growth factor (bFGF), that regulate osteoblast activity. In support of this concept, heparin has been shown to prolong the half-life of bFGF, and, in cultured rat calvariae, the combination of heparin and bFGF suppressed collagen synthesis to a much greater extent than did either heparin or bFGF alone.

Because bone turnover reflects the dynamic balance between the activity of osteoblasts and osteoclasts, we also used histomorphometry to examine the effects of heparin on osteoclast number (Fig 4B). In contrast to its effect on osteoblast number, heparin was found to increase the number of osteoclasts. These changes, similar to the changes in osteoblast number, began after 8 days of heparin treatment and reached a plateau by day 16. The heparin-induced increase in osteoclast numbers was associated with a transient increase in osteoclast activity, as evidenced by an elevation in the urinary levels of PYD (Fig 2). The elevation in urinary PYD is disproportionately greater than the increase in osteoclast number (Fig 4B), suggesting that heparin not only increases osteoclast number but also enhances their activity. These findings conflict with those of previous studies using fetal mouse or rat calvariae, in which heparin was reported either to have no effect on bone resorption or to inhibit bone resorption at high concentrations. In addition, low-dose heparin was found not to cause a change in biochemical

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**Table 1. The Effect of Unfractionated Heparin (1.0 U/g) on Trabecular Thickness, Number, and Separation**

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<tr>
<th>Variable</th>
<th>Control</th>
<th>Heparin-Treated</th>
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<tbody>
<tr>
<td>Trabecular width (μm)</td>
<td>15.8 ± 1.7</td>
<td>9.2 ± 0.8*</td>
</tr>
<tr>
<td>Trabecular number (no./mm)</td>
<td>12.2 ± 0.8</td>
<td>7.0 ± 0.4*</td>
</tr>
<tr>
<td>Trabecular separation (μm)</td>
<td>18.1 ± 2.0</td>
<td>30.7 ± 2.3*</td>
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Rats were injected daily with either vehicle alone or unfractionated heparin, at a dose of 1.0 U/g, and the region extending from the epiphyseal growth plate and including the entire metaphysis was analyzed at day 32 for (1) trabecular width, (2) trabecular number, and (3) trabecular separation. Data are expressed as mean ± SEM. *P < .005 when compared with control values.
markers of bone resorption when administered to 6 healthy male volunteers for a period of 10 days. However, the current findings are consistent with those of our studies using fetal rat calvariae, in which we reported that heparin increases bone resorption in a concentration-dependent fashion.

The mechanism responsible for the transient increase in urinary PYD levels is uncertain. Osteoclast function is known to be controlled by the osteoblast. One possible explanation is that the osteoblast controls both the formation and the activity of the osteoclast through the release of unknown stimulatory factors and that heparin increases the production of these stimulatory factors, thereby explaining the initial increase in osteoclast number (Fig 4B) and activity (Fig 2). However, this effect would be transient because heparin also reduces osteoblast number in a time-dependent fashion. With the decrease in osteoblast number, levels of these stimulatory factors would no longer be sufficient to sustain the increase in osteoclast activity, resulting in a subsequent decrease in urinary PYD levels.

To examine the effects of heparin on bone mineralization, both the amount of mineralizing bone surface and the MAR were measured. As shown in Table 2, heparin decreases the amount of bone undergoing mineralization, as evidenced by a reduction in both single- and double-tetracycline labeled trabecular surface. In contrast, however, neither the mineral apposition rate nor the percentage of osteoid undergoing mineralization were changed by heparin treatment. This apparent discrepancy between the reduction in tetracycline labeling and the unchanged MAR suggests that the former results from a decrease in available osteoid rather than from a direct inhibition of the mineralization process itself.

As shown in Fig 3, heparin produces a dramatic decrease in trabecular bone. Heparin-induced bone loss is dose-dependent and occurs after as little as 8 days of treatment (Fig 3, inset). The observation that prolonged heparin administration causes bone loss is consistent with previous reports that used densitometry rather than histomorphometry to monitor the effect of heparin on bone. However, unlike densitometry, the use of histomorphometry allows us to make a number of conclusions as to how heparin causes bone loss in our rat model. Thus, although we observed that maximal trabecular bone loss occurs by day 8, neither the number of osteoblasts nor the number of osteoclasts show a significant change until

Table 2. The Effect of Unfractionated Heparin (1.0 U/g) on Tetracycline-Based Measurements

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Heparin-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Single-labeled surface</td>
<td>62.9 ± 1.8</td>
<td>51.4 ± 1.3*</td>
</tr>
<tr>
<td>% Double-labeled surface</td>
<td>6.3 ± 1.0</td>
<td>4.1 ± 0.8*</td>
</tr>
<tr>
<td>MAR (μm/d)</td>
<td>1.11 ± 0.14</td>
<td>1.03 ± 0.16</td>
</tr>
<tr>
<td>MS/OS</td>
<td>27.5 ± 7.2</td>
<td>28.9 ± 3.2</td>
</tr>
</tbody>
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Animals were randomized into one of two treatment groups each consisting of six rats. One group was given daily subcutaneous injections of unfractionated heparin at a concentration of 1.0 U/g for a total of 8 days, whereas the remaining 8 rats served as controls and were given an equivalent volume of saline. All animals received IP injections of tetracycline 2 days before beginning heparin/saline injections and again 2 days before termination. The percentage of trabecular bone covered by either single or double labels was then determined using a Metz grid. The mineral apposition rate was calculated as described in Materials and Methods. The % of osteoid mineralizing was calculated by dividing the % double labeled surface by the % osteoid surface and multiplying by 100. Data are expressed as mean ± SEM.

Abbreviation: MS/OS, percentage of osteoid mineralizing.

* P < .05 when compared with control values.
day 16. At day 8, only osteoclast activity, as evidenced by an increase in urinary PYD output, and the percent osteoid surface had changed significantly (data not shown). Thus, the rapid loss of trabecular bone can only be attributed to a change in osteoblastic and osteoclastic activity and not to a change in osteoblast or osteoclast cell numbers.

Spinal fractures have been reported in patients treated with long-term (≥3 months) subcutaneous heparin at doses as low as 10,000 IU/d.11,12 Doses as high as 50,000 IU/d are often used when treating pregnant women with venous thromboembolism.5,10,12 In the current study, we used heparin doses of 0.25 U/g/d to 1.0 U/g/d, which are equivalent in a 70 kg patient to doses ranging from 17,500 IU/d to 70,000 IU/d. These doses were chosen because they span the range of heparin doses used for both prophylaxis and treatment. Whether our results are predictive of heparin’s effects on bone turnover in patients undergoing long-term anticoagulant therapy is not known. Histomorphometry has only been performed on bone biopsy specimens obtained from 2 patients who developed clinically overt osteoporosis during long-term heparin therapy.33,34 The first case was that of a pregnant woman receiving heparin at a dose 40,000 IU/d who developed multiple fractures of the vertebrae.33 Histomorphometric analysis showed considerable trabecular bone loss accompanied by an increase in osteoclast number and a decrease in the number of osteoblasts. The second case was of a patient who also developed vertebral fractures while on long-term heparin therapy. In this patient a bone biopsy specimen obtained 18 days after discontinuing heparin therapy failed to show similar histomorphometric abnormalities, suggesting that the effects of heparin on bone may be reversible.33

In summary, our studies show that heparin has profound effects on bone turnover. Thus, heparin increases both the number and the activity of the osteoclasts while reducing the number and activity of the osteoblasts. This results in bone loss that begins early in the course of heparin treatment. Although the effects of heparin on bone turnover in humans may be quantitatively different from those in rats, it is likely to act through the same mechanism. This would explain why patients treated with heparin have measurable decreases in bone density that can lead to symptomatic fractures.1−12

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Histomorphometric analysis of the effects of standard heparin on trabecular bone in vivo

JM Muir, M Andrew, J Hirsh, JI Weitz, E Young, P Deschamps and SG Shaughnessy