

# Growth Hormone and Mild Exercise in Combination Markedly Enhance Cortical Bone Formation and Strength in Old Rats\*

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## ABSTRACT

The effects of a combination of mild exercise and GH injections on bone were studied in old female rats. Biosynthetic human GH, 2.7 mg/kg/day, was injected sc for 73 days. Exercised rats ran 8 m/min on a treadmill for 1 h/day. All rats (age 21 months old) were labeled with a tetracycline injection 56 days and a calcein injection 11 days before killing. The GH injections resulted in an 11-fold increase in femoral middiaphyseal bone formation rate and a 12% increase in cross-sectional area compared with the saline-injected group. The mild exercise doubled the mineralizing surface but did not influence the bone formation rate significantly. The combination of GH injections plus exercise, however, resulted in a further increase of 39% in bone formation rate, primarily at the anterolateral aspects, and an increase of 5% in cross-sectional area compared with the group injected

with GH only. The femur ultimate breaking load was increased by 37% and the stiffness by 42% in the group injected with GH compared with the saline-injected group. Exercise alone did not influence the femur mechanical properties. The combination of GH injections plus exercise induced a 4% further increase in ultimate breaking load and 7% further increase in stiffness compared with the group injected with GH alone. The GH injections induced a 117% increase in serum insulin-like growth factor I. The GH-insulin-like growth factor I axis stimulates recruitment of osteoblast precursor cells, resulting in increased bone formation at the periosteal surface. GH injections and mild exercise in combination modulate and increase further the formation and strength of cortical bone in old female rats. (*Endocrinology* 139: 1899–1904, 1998)

IN experimental research on pharmacological products that can induce formation of new bone, injections with GH or PTH have been shown to result in substantial formation of new bone with full biomechanical competence. In old rats, injections with GH resulted in a marked increase in formation of new bone at the periosteal surface (1, 2), and with PTH a substantial formation of new bone at the endocortical surface was found (3, 4). For both GH and PTH, insulin-like growth factor I (IGF-I) seems to be one of the mediators stimulating osteoblasts and thereby bone formation (5–10). The mechanisms behind the observation that GH induces bone formation from the periosteum and PTH primarily induces bone formation from the endocortical and cancellous bone surface are not known. GH increases the turnover of bone (11–14), and the bone formation at the periosteum overrules the bone degradation, resulting in a total increase in bone mass. Exercise has been shown to increase bone formation from the periosteum and increase serum IGF-I and the concentration of IGF-I in the long bones of exercised rats (15). Mechanical loading from weight bear-

ing (gravity) is decisive for bone modeling and remodeling (16–19). Furthermore, suppression of the osteogenic response has been shown in the aging skeleton (20, 21) and impaired bone activity in aged rats (22). GH secretion declines with age both in human individuals and in rats (23–26).

The purpose of this study was to delineate whether mild exercise of old rats could influence the bone formation and mechanical strength of cortical bone, and furthermore, whether its ability to do so could be enhanced by GH injections in old rats. A schedule of tetracycline and calcein labeling was used, followed by examination of bone apposition at the different aspects of the rat femur to elucidate the influence of GH and exercise. Serum IGF-I was measured. The mechanical competence of the femur cortical bone was analyzed by a three-point bending procedure.

## Materials and Methods

Seventy female Wistar rats, 18 months old at the start of the experiment, were randomly divided into five groups. The start control group was killed at the beginning of the experiment. The four remaining groups were all injected twice daily for 73 days in the nape of the neck. Group 2 was injected with isotonic sodium chloride. Group 3 was injected with saline and exercised. Group 4 was injected with biosynthetic human GH in a dose of 2.7 mg/kg per day (Norditropin, Novo Nordisk, Gentofte, Denmark; specific activity, 1 mg = 3 IU). Group 5 was injected with GH and exercised. The exercised rats ran on a treadmill 5 days a week, 8 m/min, 1 h/day during the experimental period of 73 days. The rats were weighed once a week, and the hormone dose was adjusted in relation to the actual body weight. All rats were injected sc with 20 mg tetracycline/kg of body weight (Sigma Chemical Co., St. Louis, MO) 56 days before killing and with 15 mg of calcein/kg (Sigma Chemical Co.) 11 days before killing. The rats were caged separately and had free access to tap water and pellet food (Altromin diet containing 0.9% calcium and 0.7% phosphorous, Chr. Pedersen Ltd., Ringsted, Denmark). The rats

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were then anesthetized with pentobarbital 60 mg/kg ip and killed at the age of 21 months by exsanguination. The blood was centrifuged and serum was stored at  $-20^{\circ}\text{C}$  until analysis. The hind legs were excarticulated in the hip joints and stored airtight at  $-80^{\circ}\text{C}$  until examination. The femurs were dissected free without damaging the periosteum. The length from the top of the femur head to the distal point of the medial condyle was measured by a sliding caliper. The study was approved by the Danish Animal Experiment Inspectorate.

### Mechanical three-point analysis

Femurs were analyzed in a materials testing machine (Alwetron 250, Lorentzen & Wettre, Stockholm, Sweden) using a standardized three-point bending procedure (2, 3, 27, 28). The femurs were placed on the posterior surface on two rounded supporting bars with a distance of 15.1 mm between the bars. The load was applied at the anterior surface by lowering a third rounded bar. The loading point was at a distance of 55% of the total length of the femur from the top of the caput femoris. The femur was deflected at a constant speed of 5 mm/min until failure and load-deflection curves were registered simultaneously on an x-y recorder by transducers coupled to measuring bridges. The load-deflection curves were read into a computer, and load values were calculated for each deflection increment of 10  $\mu\text{m}$ . The ultimate breaking load (maximum load value) and stiffness (slope of the load-deflection curve) were calculated.

Two 300  $\mu\text{m}$  thick transversal sections were cut from both femurs 3 mm below the loading point by means of a precision bone saw (Exakt-Apparatebau, Otto Herrmann, Norderstedt, Germany). One of the sections was placed on a slide and projected onto a screen by means of a projection microscope, magnification 20 $\times$ . The outer and inner circumference and diameters were read into a computer by a digitizer, and the total cross-sectional areas and the diameters were calculated. The ultimate breaking load and stiffness were then normalized to the diameters of the femur diaphysis giving the corrected mechanical data: bending stress and Young's modulus (29).

### Bone formation and histomorphometry

The other section was embedded in acylacetate (Entellan, Merck, Darmstadt, Germany) on a glass slide and used for determination of mineral apposition rates and bone formation rates by fluorescence microscopy (2, 30). A translucent star-shaped plastic grid was placed on top

of the section with the center of the star in the midpoint of the marrow cavity and the 16 lines radiating out from the center point. Since only the center of the star was fixed, the 16 radiating lines intersected the periosteal and endocortical surface at random. At the point of intersection, the distance between the middle of the tetracycline label and the middle of the calcein label was measured at a magnification 200 $\times$ . An aspect with no labeling was classified as a point of no mineral apposition. No distance between the tetracycline and calcein labels was classified as a point of no measurable mineral apposition, *i.e.* zero mineral apposition. The mineral apposition rate was calculated by dividing the distance between the two labels by the interlabeling period in days. One rat in the saline- and one in the GH-injected groups had no calcein labeling. This was interpreted as missed calcein injections.

### IGF-I

Serum IGF-I was measured by a RIA technique (31).

### Statistical analysis

Median values with 95% confidence limits in parenthesis are given. The Kruskal-Wallis test was used for analysis of differences between the groups. In case of differences, the Mann-Whitney's U-test was applied.  $P < 0.05$  (two-tailed) was considered statistically significant (32).

## Results

Data calculated from the tetracycline and calcein labelings of femur middiaphyseal cross-sections are given in Table 1. In the start control group the bone mineral apposition was zero at the anterior and medial aspects and highest at the lateral aspect. In the 73-days older saline control group, the mineral apposition rate was decreased to approximately zero at all aspects compared with the start control group. The exercise regimen did not induce a significant increase in the mineral apposition rate compared with the saline control group. The GH injections resulted in a marked increase in the mineral apposition rates at all aspects compared with the saline control group. Likewise, GH plus exercise resulted in

**TABLE 1.** Femur middiaphyseal histomorphometry

	Start control (n = 14)	Saline control (n = 13)	Exercise (n = 14)	GH (n = 13)	GH + exercise (n = 14)	Kruskal-Wallis P value
Periosteal surface						
Mineral apposition rates ( $\mu\text{m}/\text{day}$ )						
Anterior	0 (0-0)	0 (0-0)	0 (0-0)	0.64 <sup>a</sup> (0.43-0.92)	1.14 <sup>b,c</sup> (0.57-1.42)	<0.000
Lateral	0.21 (0-0.35)	0 (0-0.10)	0 (0-0.21)	1.15 <sup>a</sup> (0.92-1.63)	1.99 <sup>b,c</sup> (1.35-2.13)	<0.000
Posterior	0 (0-0.30)	0 (0-0.10)	0 (0-0.21)	0.99 <sup>a</sup> (0.71-1.07)	0.92 <sup>c</sup> (0.35-1.14)	<0.000
Medial	0 (0-0)	0 (0-0)	0 (0-0)	0.71 <sup>a</sup> (0.35-1.07)	0.92 <sup>c</sup> (0.21-1.10)	<0.000
Mineralizing surface (% of periosteal surface)	47 (38-75)	16 <sup>d</sup> (12-31)	32 <sup>e</sup> (16-44)	100 <sup>a</sup> (81-100)	100 <sup>c</sup> (88-100)	<0.000
Bone formation rate ( $10^3 \mu\text{m}^3/\text{day}$ )	4.3 (3.1-6.3)	1.5 <sup>d</sup> (0.8-2.3)	2.1 <sup>f</sup> (1.6-4.1)	16.8 <sup>a</sup> (12.9-21.4)	23.3 <sup>b,c</sup> (16.8-24.9)	<0.000
Endocortical surface						
Mineralizing surface (% of endosteal surface)	6 (0-18)	3 (0-12)	9 (0-38)	6 (0-19)	0 (0-0)	0.351

The rats were injected with GH daily for 73 days; tetracycline was injected at day 17 and calcein at day 62. Median with 95% confidence limits are shown in parentheses.

<sup>a</sup>  $P < 0.000$  against saline control.

<sup>b</sup>  $P < 0.05$  against GH.

<sup>c</sup>  $P < 0.000$  against exercise.

<sup>d</sup>  $P < 0.001$  against start control.

<sup>e</sup>  $P < 0.05$  against saline control.

<sup>f</sup>  $P < 0.05$  against start control.

FIG. 1. Mineral apposition rates were measured from the tetracycline and calcein labels and ascribed to the different aspects (eight periosteal and eight endosteal aspects) of the middiaphyseal cortical bone cross-section of rat femurs. Median values in micrometers/day are given at each aspect of a GH-injected rat and a GH plus exercise rat. 0, No measurable distance between the labels; -, no label.

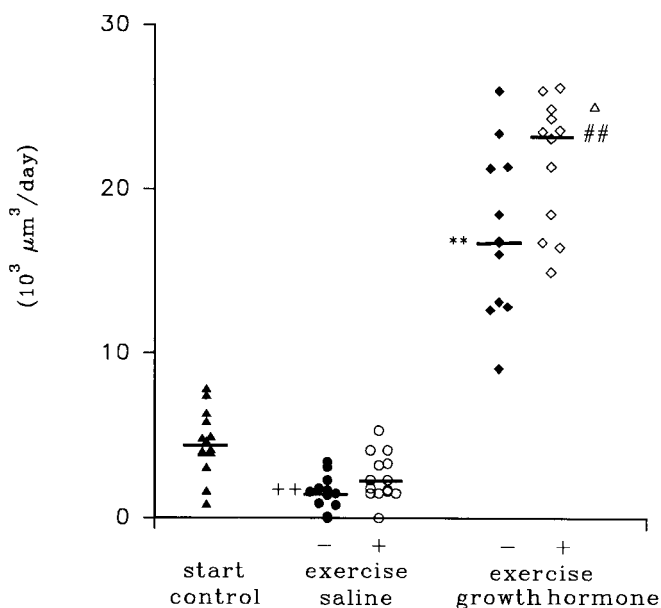
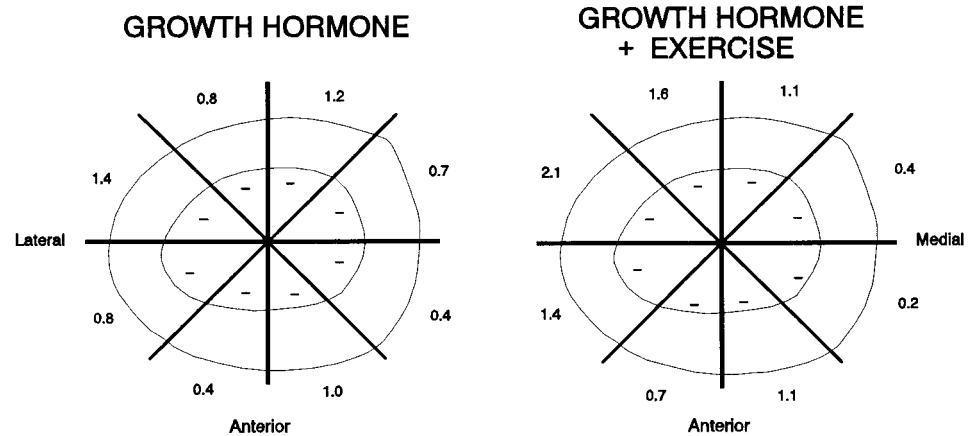


FIG. 2. Bone formation rates were calculated from values of mineral apposition rates at the different aspects and circumferences of middiaphyseal cortical bone cross-sections from rat femurs. The median values are marked by bars. \*\*,  $P < 0.000$  against saline without exercise; ##,  $P < 0.000$  against saline + exercise;  $\Delta$ ,  $P < 0.05$  against GH without exercise.

marked increases in mineral apposition rates compared with the group submitted to exercise alone. In addition, GH plus exercise resulted in an increased mineral apposition at the anterior and lateral aspects compared with the group given GH alone (Fig. 1). The mineralizing surface as a percentage of the total surface decreased to one third during the experimental period of 73 days, estimated from the start control group and the saline control group. Exercise doubled the mineralizing surface compared with the saline control group. GH and GH plus exercise resulted in a 100% mineralizing surface. The bone formation rate (Fig. 2) was reduced to one third in the saline control group compared with the start control group. The GH injections resulted in an 11-fold increase in the bone formation rate compared with the saline control group. The GH-injected plus exercised group also had an 11-fold increase in bone formation rate compared

with the exercise-only group and had a 39% increase in bone formation rate compared with the group injected with GH alone. At the endocortical surface, only a minor part of the surface showed labeling. No measurable mineral apposition was observed at any aspect of the endocortical surface.

The cross-sectional area (Table 2) of the femoral middiaphyseal sections was increased in the group injected with GH and in the GH-injected plus exercised group. No significant differences were observed in the cross-sectional area of the medullas. The femur length was increased by 7% in both the GH-injected group and the GH-injected plus exercised group. Serum IGF-I values (Table 2) were increased by 117% in the group injected with GH compared with the saline control group and increased by 124% in the GH-injected plus exercised group compared with the exercise-only group. Exercise as such did not change the values of serum IGF-I compared with the saline control. The values of serum IGF-I decreased in the saline control group compared with the start control group. The body weight of the GH-injected group increased by 39% and that of the GH + exercised group by 30% (Table 2).

The biomechanical analysis of the femur is summarized in Table 3. The uncorrected ultimate breaking load (Fig. 3) was increased by 37% in the GH-injected group compared with the saline control group and increased by 47% in the GH-injected plus exercised group compared with the exercise-only group. The uncorrected mechanical stiffness of the femur diaphysis was increased by 42% in the GH-injected rats compared with the saline control and increased by 51% in the GH-injected plus exercised group compared with the exercise-only group. The combination of GH injections and exercise induced a 4% increase in ultimate breaking load and a 7% increase in stiffness compared with the group injected with GH alone. When these data were corrected for differences in the middiaphyseal diameters, *i.e.* second moment of area, no differences were found between the groups in bending stress or in Young's modulus. The deflection capacity did not differ between the groups. The second moment of area was increased by 27% in the group injected with GH compared with the saline control and increased by 35% in the GH-injected plus exercised group compared with the exercise-only group. Exercise alone did not affect the second

**TABLE 2.** Femur middiaphyseal cross-sectional area, length, IGF-I, and body weights

	Start control (n = 14)	Saline control (n = 14)	Exercise (n = 14)	GH (n = 14)	GH + exercise (n = 14)	Kruskal-Wallis P value
Cross-sectional area (mm <sup>2</sup> )	9.0 (6.0–9.4)	8.9 (8.1–9.5)	9.1 (8.8–9.3)	10.0 <sup>a</sup> (9.7–10.6)	10.5 <sup>b</sup> (10.0–10.9)	<0.000
Medullary area (mm <sup>2</sup> )	3.9 (3.6–4.2)	3.8 (3.6–4.4)	4.1 (3.7–4.3)	3.6 (3.3–3.8)	3.7 (3.6–4.0)	0.073
Femur length (mm)	32.8 (32.4–33.1)	32.5 (31.8–33.2)	32.6 (32.4–33.0)	35.0 <sup>a</sup> (34.4–35.5)	35.0 <sup>b</sup> (34.7–35.6)	<0.000
Serum IGF-I (μg/liter)	315 (284–335)	280 <sup>c</sup> (235–306)	279 (270–301)	607 <sup>a</sup> (546–724)	624 <sup>b</sup> (576–716)	<0.000
Body weight at start	291 (241–305)	288 (255–305)	288 (270–310)	285 (265–310)	290 (270–308)	0.933
Body weight at end		271 (243–291)	260 (245–279)	392 <sup>a</sup> (315–409)	378 <sup>b</sup> (370–407)	<0.000

Median with 95% confidence limits *in parentheses*. n, Number of rats.

<sup>a</sup> P < 0.000 against saline control.

<sup>b</sup> P < 0.000 against exercise.

<sup>c</sup> P < 0.03 against start control.

**TABLE 3.** Femur biomechanical analysis: three-point bending

	Start control (n = 14)	Saline control (n = 14)	Exercise (n = 14)	GH (n = 14)	GH + exercise (n = 14)	Kruskal-Wallis P value
Uncorrected mechanical data						
Bending strength (N)	134 (125–140)	128 (119–136)	123 (119–136)	175 <sup>a</sup> (154–180)	181 <sup>b,c</sup> (174–187)	<0.000
Stiffness (N/mm)	479 (428–514)	419 (357–475)	425 (401–456)	596 <sup>a</sup> (555–688)	640 <sup>c</sup> (581–689)	<0.000
Corrected mechanical data						
Bending stress (N/mm <sup>2</sup> )	184 (164–204)	185 (161–200)	171 (160–191)	184 (164–203)	183 (170–218)	0.469
Young's modulus (10 <sup>2</sup> N/mm <sup>2</sup> )	86 (80–105)	83 (73–96)	82 (70–89)	83 (75–93)	82 (75–100)	0.562
Deflection (mm)	0.46 (0.42–0.53)	0.50 (0.46–0.61)	0.48 (0.41–0.54)	0.44 (0.37–0.56)	0.45 (0.39–0.55)	0.261
Second moment of area (mm <sup>4</sup> )	3.8 (3.6–4.5)	4.1 (3.1–4.6)	4.0 (3.5–4.2)	5.2 <sup>a</sup> (4.7–6.1)	5.4 <sup>c</sup> (4.3–6.7)	<0.000

The rats were injected with GH daily for 73 days. Median with 95% confidence limits *in parentheses*. n, Number of rats.

<sup>a</sup> P < 0.000 against saline control.

<sup>b</sup> P < 0.05 against GH.

<sup>c</sup> P < 0.000 against exercise.

moment of area or the mechanical properties of the femur. No differences in the biomechanical parameters were found between the start control and the saline control group, and no differences were observed in the biomechanical parameters of the saline control group and the exercise-only group.

### Discussion

GH injections to old female rats resulted in a 39% increase in body weight, a 7% increase in femur length, an 11-fold increase in middiaphyseal bone formation rate, and a 12% increase in cross-sectional area accompanied by a 37% increase in ultimate breaking load and 42% in bending stiffness. The pronounced changes in mechanical properties induced by GH were caused by the increased thickness of the cortical bone. When the ultimate breaking load and stiffness were corrected for the increase in cortical bone thickness, no differences were found between the saline control and GH-injected groups, indicating normal biomechanical properties and full biomechanical competence of the new bone. This is in agreement with a previous study (2). Concerning the middiaphyseal mineral apposition, the fluorescence microscopy revealed that only a minor part (13%) of the mineralizing

surface located posteromedially and posterolaterally showed labeling in the saline control group. In the GH-injected group, 100% of the periosteal surface showed labeling, and the mineral apposition was especially pronounced posteromedially and posterolaterally. Middiaphyseally endocortical mineral apposition was seen neither in the saline control group nor in the GH-injected group, which is in agreement with previous findings in old rats (2).

The mild exercise regimen resulted in a 2-fold increase in mineralizing surface compared with the saline control group but no significant differences concerning mineral apposition rate, bone formation rate, or biomechanical parameters. The combination of mild exercise and GH injections to these old female rats resulted in increased mineral apposition rate at the anterior and lateral aspects and increased bone formation rate, accompanied by an increase in ultimate breaking load values compared with the group injected with GH alone. The body weight of the exercised plus GH-injected rats did not differ from the GH-injected rats. Therefore, the increased bone formation and increased ultimate breaking load and stiffness of the femoral diaphysis in the GH-injected plus exercised rats are induced by the combination of GH and

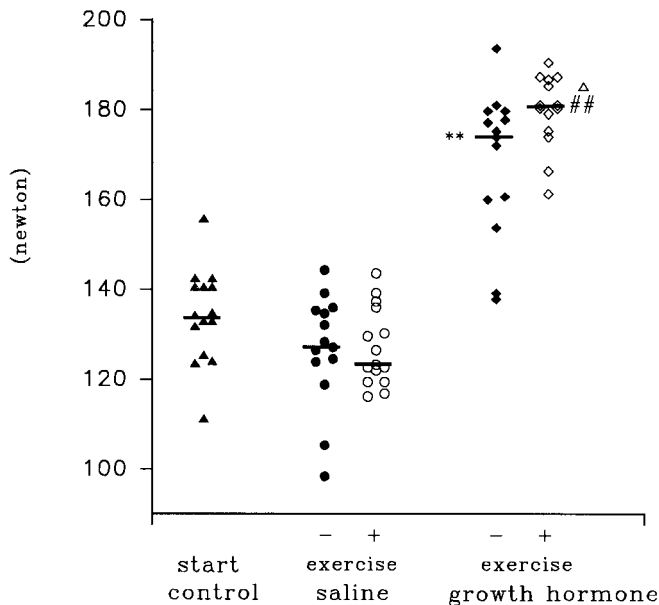


FIG. 3. The mechanical properties of rat femur diaphyses were analyzed in a materials testing machine using a three-point bending procedure. The bending strength for each rat is given. The median values are marked by bars. \*\*,  $P < 0.000$  against saline without exercise; ##,  $P < 0.000$  against saline + exercise; ( $\Delta$ ):  $P < 0.05$  against GH without exercise.

exercise and not simply by an increased load imposed by enhanced body weight. The combined effects of exercise plus GH injections on rat tibia of 14-month-old rats were studied by Yeh *et al.* (33). They used a somewhat more strenuous exercise program (treadmill speed: 17 m/min, 1 h/day) and a lower dose of GH (bovine GH: 0.5 mg/kg/day, 5 days per week). This combination of exercise and GH had no significant effect on tibial cross-sectional area. The exercise alone resulted in a 10% increase in cortical bone cross-sectional area. The exercised plus GH-injected rats had increased bone formation rate both from the periosteal and from the endocortical surfaces after 9 weeks, an effect that was not observed after 16 weeks. The exercise induced an increase in bone formation rate only from the periosteum. The authors interpreted these data as follows: Increased bone formation rate, but failure to increase bone mass in the tibia by the combined intervention of exercise plus GH, might be due to an equal stimulation of both bone formation and resorption. In dose-response studies on rats, however, we have not been able to show any effect of GH on cortical bone dimensions when using a dose of less than 2 mg/kg per day (34, 35). In the present study biosynthetic human GH, 2.7 mg/kg/day injected sc, induces osteoblastic recruitment at the periosteum resulting in increased mineral apposition all around the middiaphysis, but primarily at the posteromedial and posterolateral aspects, and this increase in bone formation rate is reflected in enhanced cortical bone thickness. Mild exercise plus GH injections result in a further increase in bone mineral apposition at the lateral and anterior aspects. Mild exercise seems to superimpose an increased strain on the anterolateral surface of the middiaphysis, increasing the GH effects.

During the experiment an age-related decrease in bone formation rate was observed. The middiaphyseal mineral-

izing surface and bone formation rate of the saline control group decreased to one third when compared with the start control group. The mild exercise increased the mineralizing surface, but could not induce a significant increase in bone formation rate. The GH injections, however, resulted in an activation of the osteogenic activity all around the periosteal surface and an 11-fold increase in bone formation rate. Exercise plus GH injections resulted in a further 39% increase in bone formation rate, suggesting that GH induces a renewal of the osteogenic activity in these old rats, with the result that mild exercise can now modulate and increase bone formation. The result is increased cortical bone mass with full biomechanical competence.

A close relationship between serum IGF-I and bone mass in rats has been demonstrated previously (36). GH increases the production of IGF-I in the liver, which is reflected in the serum IGF-I data of the present study. GH secretion declines with age both in humans (23) and in rats (25). IGF-I also decreases with age in humans (37) and in rats (2). In agreement with this, serum IGF-I of the saline control group decreased by 11% compared with the start control group, reflecting the age-related decrease in IGF-I. The GH injections increased the serum IGF-I concentrations by 117%. In the GH-injected plus exercised group the concentration of serum IGF-I was increased by 127%. Exercise has been shown to increase the IGF-I concentration in serum (15, 38) and, furthermore, to increase the concentration of IGF-I locally in the long bones of exercised rats (15). Increased IGF-I mRNA expression has been found in osteocytes of rat bone in response to mechanical stimulation (39). The mild exercise of the present study did not influence serum IGF-I. The exercise procedure of our study was, however, considerably milder compared with that used by Yeh *et al.* (15). The more strenuous exercise procedure of Yeh *et al.* (treadmill speed being twice as high as the speed used in our study) increased serum IGF-I and the local concentration of IGF-I in bone, suggesting that the IGFs might well be both systemic and local mediators of exercise-induced bone formation in GH-injected rats.

The GH-IGF-I axis stimulates recruitment of osteoblast precursor cells, resulting in increased bone formation at the periosteal surface. GH injections and mild exercise in combination modulates and increases further the formation and strength of cortical bone in old female rats.

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