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Growth hormone treatment promotes guided bone regeneration in rat calvarial defects

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SUMMARY This study evaluated the biomechanical strength and bone formation in calvarial critical size bone defects covered with expanded polytetrafluoroethylene (e-PTFE) membranes in rats treated systemically with recombinant human growth hormone (rhGH).

A full-thickness bone defect, 5 mm in diameter, was trephined in the central part of each parietal bone in 40 one-year-old female Wistar rats, which were randomly assigned to two groups of 20 animals each. The bone defects were covered with an exocranial and an endocranial e-PTFE membrane. From the day of operation, the rhGH-treated animals were given 2.7 mg rhGH/kg/day and the placebo-injected rats were given isotonic sodium chloride. The animals were killed 28 days after operation. The biomechanical test was performed by a punch out test procedure placing a 3.5-mm diameter steel punch in the centre of the right healed defect. After mechanical testing, the newly formed tissue inside the defect was removed and the dry and ash weights were measured. The left healed defects were used for three-dimensional (3D) reconstruction by means of micro-computer tomography (micro-CT).

Ultimate load, ultimate stiffness, and energy absorption at ultimate load were significantly increased in the rhGH-treated group ($P < 0.003$). Also, tissue dry and ash weights were significantly augmented in the rhGH-treated group ($P < 0.001$). The 3D reconstruction of newly formed bone showed that there was almost twice as much bone volume present in the rhGH-treated defects compared with the placebo group. The experiment demonstrated that rhGH administration enhances bone deposition and mechanical strength of healing rat calvarial defects, covered with e-PTFE membranes.

Introduction

In normal rats, growth hormone (GH) administration increases cortical bone mass, inducing subperiosteal bone formation, without influencing the endosteal bone surface (Andreassen *et al*., 1995). Correspondingly, an increase in the mechanical strength of the whole bone occurs, whereas the mechanical quality (mechanical strength normalized for dimensions of the bone) of the osseous tissue is equivalent in GH-injected animals and controls (Turner and Burr, 1993; Andreassen *et al*., 1995). GH administration increases bone turnover by enhancing both bone formation and resorption (Ohlsson *et al*., 1998). GH treatment also increases the mechanical strength of healing fractures in both young and old rats (Bak *et al*., 1990a; Bak and Andreassen, 1991).

The rat calvarial defect model has been used for experiments into bone regeneration (Mulliken and Glowacki, 1980; Schmitz and Hollinger, 1986; Dahlin *et al*., 1991; Linde *et al*., 1993; Bosch *et al*., 1995, 1998). The diameter of the defect has varied from 2 to 8 mm (Freeman and Turnbull, 1973; Mulliken and Glowacki, 1980; Dahlin *et al*., 1991) and the amount of newly formed bone depends on its size. The critical size bone defect has been defined as the size of an osseous defect

that does not heal spontaneously with bone during the lifetime of the animal. Such defects fill with fibrous connective tissue and limited bone regeneration occurs at the margins of the defect (Schmitz and Hollinger, 1986). Placement of a barrier membrane reduces massive ingrowth of interfering soft connective tissue and maintains a high concentration of locally produced osteogenesis-stimulatory factors (Dahlin *et al*., 1988; Linde *et al*., 1993). Spontaneous bone healing of 5-mm calvarial defects does not occur in adult rats. However, bone regeneration takes place when the defects are covered with expanded polytetrafluoroethylene (e-PTFE) membranes, although the healing proceeds very slowly (Bosch *et al*., 1995). The purpose of this study was to evaluate guided bone regeneration and mechanical strength in calvarial defects covered with e-PTFE membranes in rats simultaneously given systemic GH.

Materials and methods

Animals and treatment

Forty 12-month-old female Wistar rats (Møllegaard, Lille Skensved, Denmark) were used. The rats were randomly divided into a group treated with recombinant human GH (rhGH) and a placebo-injected group, consisting of 20 animals each. The rats were injected subcutaneously twice a day during the whole healing period of 28 days with either rhGH (Norditropin, Novo Nordisk, Gentofte, Denmark; specific activity: 1 mg = 3 IU), at a dose of 1.35 mg/kg, or with isotonic sodium chloride. The animals were maintained with a light cycle of 12 hours (06.00–18.00 hours). Each rat was kept in a separate plastic cage. The animals had free access to tap water and standard laboratory rat pellets (Altromin diet 1324 containing 0.9 per cent calcium and 0.7 per cent phosphorous; Chr. Pedersen Ltd, Ringsted, Denmark).

Surgical procedure

The rats were anaesthetized with a combination of etorphin-acepromazine 10 per cent, 1.25 ml/kg (Immobilon, Pharmacia AS, Hillerød, Denmark) and atropine 7.4 per cent, 0.11 mg/kg, administered subcutaneously. The dorsal part of the cranium was shaved, and aseptically prepared for surgery through an incision approximately 20 mm long in the scalp along the sagittal suture and skin, musculature, and periosteum were reflected.

Two full-thickness bone defects, 5 mm in diameter, were trephined in the centre of the parietal bone without damaging the dura. Bosch *et al*. (1998) demonstrated that the full-thickness 5-mm calvarial defect fulfils the criteria for a critical size bone defect. A 5-mm trephine bur (No: 227-811001, Meissinger, Düsseldorf, Germany) was used to create the defects under constant irrigation with sterile physiological solution, to prevent overheating of the bone margins. The defect preparation was made in order not to dilacerate the dura.

Equidistant from the centre of each bone defect, two holes were drilled with a fine carbide tungsten bur (ISO 012, no. 9803, Horico, Germany), one anterior and one posterior to the margins of the defect. The holes were filled with amalgam (Dispersalloy, regular set code 2891, Johnson and Johnson, Skillman, NJ, USA) and used as markers to accurately identify the centre of the bone defect after death (Figure 1).

The bone defects were covered with an endocranial e-PTFE membrane (Gore-Tex, W. L. Gore and Assoc., Flagstaff, AZ, USA) between the dura mater and the parietal bone, and an exocranial membrane placed between the periosteum and the parietal bone (Figure 2).

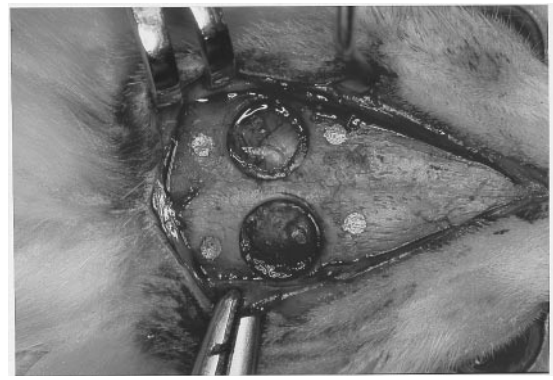

Figure 1 Adult rat cranium showing two full-thickness bone defects, 5 mm in diameter, trephined in the centre of the parietal bone, and amalgam markers.

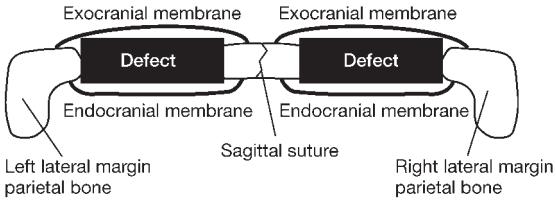

Figure 2 Coronal section of an adult rat cranium showing the two full-thickness bone defects covered with an exocranial and an endocranial membrane.

The periosteum and muscles were sutured in place using catgut 4-0 (Ethicon, Norderstedt, Germany) and the scalp sutured with silk 4-0 (Ethicon). Reversal of anaesthesia was achieved by 10 per cent Diprenorfin (Revivon, Pharmacia AS, Hillerød, Denmark) at a dose of 1.5 ml/kg. The experiment was approved by the Danish Animal Experiment Inspectorate.

The animals were killed with an overdose of carbon dioxide 28 days after surgery. The skin was dissected and the defect sites were removed with surrounding bone, soft tissues, and brain, and rinsed in physiological saline. During subsequent X-ray and biomechanical testing, the specimens were stored in plastic containers immersed in a Ringer's solution (pH 7.4) at 4°C. All these procedures were performed on wet specimens and finished within three hours after death.

X-ray of the specimens

The presence of newly formed bone tissue within the defect was detected radiographically. The skull specimen was placed with the exocranial surface on top of a radiographic film (X.omat, Kodak, Chalon, France) at a standardized distance of 50 cm from the X-ray head (40 mAs, 25 kV). For assessing bone ingrowth, the following scoring system for each animal was used: score 0 for no defects closed; score 1 for one defect closed; score 2 for both defects closed.

Biomechanical test

The two os parietalis were subsequently separated along the sagittal suture using a high precision central cutting saw (Exact-Apparatebau, Otto Herrmann, Nordersted, Germany). The specimen was trimmed leaving 2–3 mm of intact bone around the defect. The right os parietalis specimen was used for biomechanical testing, and the left was embedded in methylmethacrylate and used for micro-computer tomography (micro-CT). In order to measure the mechanical strength of the newly formed tissue inside the defect, a materials testing machine (Alwetron 250, Lorentzen and Wettre, Stockholm, Sweden) was used. The specimens were tested by a modified punch out test procedure using a 3.5-mm diameter steel punch at a constant deflection speed (2 mm/min). The load was applied in the centre of the defect. The specimens were positioned over a 6-mm circular space of the holding device with a stereomicroscope and then transferred to the testing machine. The load-deformation (force-deflection) was recorded continuously until failure on an *x–y* recorder by transducers coupled to measuring bridges.

For further calculations, the load-deflection curves were read into a computer by a graphic tablet (HP 9816S and HP 9178A, Hewlett-Packard, Fort Collins, CO, USA) and load values were calculated for each deflection increment of 10 μm. Ultimate load (maximum load), ultimate stiffness (equal to the maximum slope of the load-deflection curve), and energy absorption at ultimate load (area under the load-deflection curve) were calculated for each specimen.

Dry and ash weights of tissue deposit in the defect

After biomechanical testing, the specimens were placed in a dissection microscope with transparency light and the newly formed tissue inside the calvarial defect was removed. The tissue dry weight was determined after freeze-drying for three days. The ash weight was then determined after incineration in a muffle oven (MRE, Heraeus, Hanau, Germany) at 105°C for two hours, followed by 580°C for 24 hours.

Micro-CT scanning

The specimens embedded in methylmethacrylate were cut down to fit into a cylindrical sample

holder with a diameter of 15.4 mm to be used in connection with a micro-CT scanner (μ-CT20, Scanco Medical, Bassersdorf, Switzerland). While placing the specimens in the sample holder, special care was taken to position the superficial surface of the parietal bone horizontally. The specimens were then scanned with the scanning direction parallel to the superficial surface. Scanning was performed in high-resolution mode corresponding to an in-plane pixel size and slice thickness of 15 μm. In order to ensure that the entire thickness of the calvarial bone and defect would be included, the number of slices was set to 100.

The micro-CT scanner's built-in software was used to make a three-dimensional (3D) reconstruction from the set of scans (Figure 3). For this reconstruction, the lower and upper threshold values for bone were assumed to be 225 and 500 out of a total range of 0–1000 (where 0 corresponds to a linear attenuation coefficient of 0 cm^{-1} and 1000 to a linear attenuation coefficient of 8.0 cm^{-1}). The attenuation process for a beam of photons traversing a slab of matter is an exponential function of the form $I = I_0 e(^{-\mu l})$, where I and I_0 are the intensities of the transmitted beam and the incident beam, respectively, l is the distance travelled in matter, and μ is the linear attenuation coefficient. From the entire 3D data set, a cylindrical region of interest (ROI) was chosen with a diameter of 321 pixels, corresponding to 4.8 mm (in order to fall within the circumference of the original defect) and a height that covered the entire number of scans. The edges of the original defect were still clearly recognizable, and a circle defining the ROI was placed as concentrically to the original defect as possible. The total volume of the newly formed bone in the ROI was calculated by dividing the number of voxels representing bone by the total number of voxels and multiplying this by the known volume of the ROI. The calculated volumes of the newly formed bone inside the defects were correlated to the ash weights of the contralateral defects using linear regression. To assess the relative degree of mineralization of the newly formed bone in the ROI, the grey-values of the voxels were stratified in a histogram running from 225 to 500 with a range of 25. The median, lower, and upper quartiles of those histograms were then calculated for further statistical analysis.

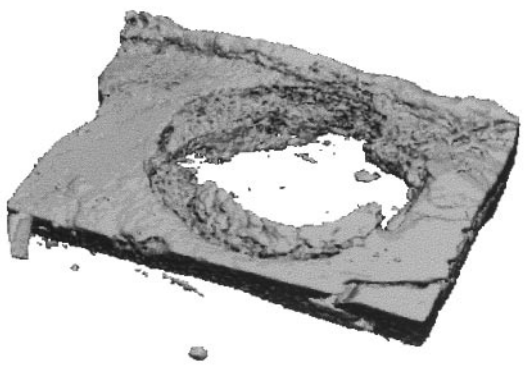

Figure 3 Three-dimensional micro-CT reconstruction of a partially filled calvarial defect with surrounding intact parietal bone.

Statistical analysis

For analysis of differences between the two groups, the Mann–Whitney *U*-test was applied, except for analysis of the radiographic results, where Fisher's exact test was used (no defect closed versus one or both defects closed; Daniel, 1990). $P < 0.05$ (two-tailed) was considered statistically significant.

Results

The results of the mechanical testing are given in Table 1. The ultimate load was increased by 151 per cent in the rhGH-treated group and ultimate stiffness by 97 per cent when compared with placebo-injected rats. Furthermore, energy absorption at ultimate load increased by 218 per cent in the rhGH-treated rats. The analysis of fracture revealed that all failures always occurred within the defect itself and not within the normal bone. The two first tested calvarial defects had to be excluded from the biomechanical results due to a sudden failure of the load cell.

The results of the amount of tissue and bone deposit are given in Table 2. Compared with placebo-injected rats, tissue dry weight was increased by 186 per cent in the rhGH-treated group and ash weight by 250 per cent. Also, ash

Table 1 Mechanical properties of a calvarial defect (diameter 5 mm) after 28 days of healing (mean values ± SD).

	Placebo	Growth hormone (2.7 mg/kg/day)	Mann–Whitney *U*-test (*P*-value)
Number	20	18	
Ultimate load (N)	14.1 ± 5.1	35.4 ± 10.3	0.001
Ultimate stiffness (N/mm)	33 ± 11	65 ± 18	0.003
Energy absorption at ultimate load (N × mm)	5.6 ± 2.3	17.8 ± 4.6	0.002

N = Newton.

Table 2 Amount of tissue and bone deposit in a calvarial defect (diameter 5 mm) after 28 days of healing (mean values ± SD).

	Placebo	Growth hormone (2.7 mg/kg/day)	Mann–Whitney *U*-test (*P*-value)
Number	20	20	
Tissue dry weight (mg)	2.9 ± 1.3	8.3 ± 2.7	<0.001
Ash weight (mg)	1.4 ± 0.7	4.9 ± 1.2	<0.001
Ash weight (% of tissue dry weight)	48 ± 6.2	59 ± 5.8	<0.001

Table 3 Radiographic examination of a calvarial defect (diameter 5 mm) after 28 days of healing.

	Number of rats	
	Placebo	Growth hormone[a] (2.7 mg/kg/day)
No defects closed	18	8
One defect closed	1	10
Both defects closed	1	2

[a]Growth hormone $P = 0.01$ compared with placebo (Fisher's exact test).

weight as a percentage of tissue dry weight was increased (23 per cent) in the rhGH-treated rats.

The results of the radiographic examination are given in Table 3. The rhGH-treated group showed a significantly higher number of animals with one or both defects closed than the placebo-injected group ($P = 0.01$).

Amalgam spillage in the defect appeared to cause some artefacts in the CT images. For this reason, only 10 specimens from the rhGH group and 10 specimens from the placebo group could be used for micro-CT analysis. The 3D reconstruction of newly formed bone in a placebo-treated rat and a rhGH-treated rat is depicted in Figure 4A and B. On average, there was almost twice as much bone volume present in the defects in the rhGH group compared with the placebo group (Table 4); however, this difference was not significant ($P = 0.10$). The median, lower, and upper quartiles of the grey-value histograms are given in Table 4. All three values were lower in the rhGH-treated group, although

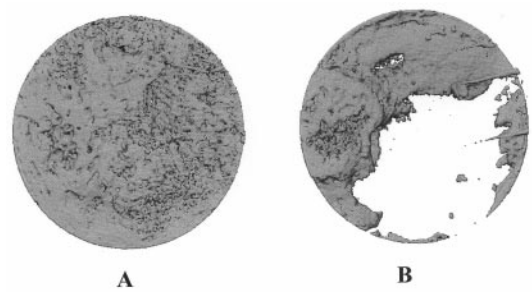

Figure 4 Three-dimensional micro-CT reconstruction of a typical calvarial defect from a GH-treated rat (A) and a placebo-injected rat (B).

Table 4 Volume of new bone inside a calvarial defect (diameter 5 mm) after 28 days of healing. Quartile descriptions of the new bone grey-value distribution (mean values ± SD).

	Placebo	Growth hormone (2.7 mg/kg/day)	Mann–Whitney *U*-test (*P*-value)
Number	10	10	
Bone volume (mm^3)	3.7 ± 2	6.4 ± 1.8	0.10
Grey-value			
Lower quartile	278 ± 12	266 ± 3	0.08
Median	325 ± 18	310 ± 7	0.08
Upper quartile	377 ± 16	360 ± 11	0.06

not significantly (median: $P = 0.08$; lower quartile: $P = 0.08$; upper quartile: $P = 0.06$). Regression analysis revealed a positive correlation between the micro-CT-estimated volume of newly formed bone and ash weight of the new bone inside the contralateral defect ($R = 0.62$, $P = 0.03$).

Mean body weight (± SD) of the rhGH-treated rats and placebo-injected rats at the time of surgery was 269 ± 11 and 264 ± 10 g, respectively ($P = 0.74$). During the healing period, the body weight of the rhGH-treated animals increased by 33 per cent, whereas placebo-injected animals did not change in weight (358 ± 8 and 266 ± 11 g; $P < 0.001$).

Discussion

The present study demonstrates that rhGH treatment increases guided bone regeneration and mechanical strength in critical size rat calvarial defects covered with e-PTFE membranes.

It has been found that systemic administration of GH at 2–200 μg/day for three weeks has a significant stimulatory effect on the healing of critical size rat mandibular defects compared with a saline placebo, as measured by histological and histomorphometric methods (Hedner *et al.*, 1996). The amount of bone formed outside the bone defect, as reflected by the peripheral bone parameter, was also higher in the GH-treated animals. Rats treated for four weeks displayed more bone formation compared with those treated for three weeks in terms of bone union and peripheral bone scores. Enhanced bone formation was also found after four weeks in animals given 0.2, 2, and 20 μg/day of GH locally, compared with local administration of saline. This effect showed a tendency to be dose-related. Their histological sections also showed a pronounced healing with fusing trabeculae of woven bone with signs of active osteogenesis still ongoing in the GH-treated defects, while mainly connective tissue healing and only limited amounts of new bone at the rims of the original defect were seen in the saline-treated rats. Their data agree with the findings of this investigation of increased bone deposition in the defect measured as increased ash content. Short-term rhGH treatment has been found to stimulate bone formation at the intact exocranial surface of the parietal bone (Turner, 1995). The ingrowth of new bone into the defect comes from the walls of the defect, and therefore this activation of the exocranial surface of the skull could be relevant.

Analysis of the micro-CT reconstructed calvarial defects has shown that in the rhGH group more new bone was generated compared with the placebo group and these results are in agreement with the increased amount of ash found in the contralateral defect. The lower grey-values in the new bone could be due to enhanced bone formation, as the mineralization is decreased in newly formed bone.

Earlier studies (Bak *et al.*, 1990a,b, 1991; Bak and Andreassen, 1991; Nielsen *et al.*, 1991) have mainly investigated the effects of GH treatment on long bone fracture healing, and found an increased strength development of healing fractures in both young and old rats, when giving the animals two daily subcutaneous injections of rhGH in the dose range of 2–10 mg/kg/day.

It was not possible to influence fracture healing when using doses of 0.8 mg/kg/day or lower. Subcutaneous rhGH injections had no significant effect on the mechanical strength of intact bone when using doses lower than 1 mg/kg/day (Bak *et al*., 1990a; Jørgensen *et al*., 1991). Hedner *et al*. (1996) used continuous systemic administration of rhGH by means of mini-osmotic pumps, implanted subcutaneously in the back of the animals, and found stimulatory effects using far lower daily doses of rhGH. In the present study, rhGH was administered systemically by giving two daily subcutaneous injections. A dose of 2.7 mg/kg/day was chosen because administration of such a dose has been found to increase the mechanical strength of both healing fractures and intact bone (Bak *et al*., 1991; Nielsen *et al*., 1991; Andreassen *et al*., 1995).

Recently, Raschke *et al*. (1999) evaluated mechanical strength development in a micropig bone-distraction model after 25 days of treatment with recombinant porcine GH. Non-destructive *in vivo* testing showed that torsional stiffness of the regenerate was significantly higher in the GH-treated group than in the placebo group. Final regenerate biomechanical testing showed that both torsional failure load and stiffness were substantially increased. These data show that systemic administration of GH greatly accelerates ossification of bone regenerate in distraction osteogenesis.

The rat calvarial bone defect model has been widely used in healing studies (Freeman and Turnbull, 1973; Mulliken and Glowacki, 1980; Schmitz and Hollinger, 1986; Dahlin *et al*., 1991; Linde *et al*., 1993; Bosch *et al*., 1995, 1998; Colombier *et al*., 1999; Kamakura *et al*., 1999; Wang and Glimche, 1999). The model allows standardized production of defects that enable convenient analysis of the newly formed bone. The mechanical exclusion of undesirable soft tissues from the defect by means of barrier membranes located at the endocranial and exocranial surfaces prevents penetration of surrounding cells, allowing osteogenic cells from the adjacent bone walls to repopulate the defect (Nyman *et al*., 1982; Karring *et al*., 1993). Both local and systemic administration of compounds and growth factors have been applied to the model in order to influence bone regeneration. The combined use of barrier membranes and growth factors, such as bone morphogenetic proteins, insulin-like growth factor 1, transforming growth factors, and fibroblast growth factors, has been found to significantly enhance bone neogenesis inside the defect (Bosch *et al*., 1996; Busch *et al*., 1996; Hedner *et al*., 1996; Khouri *et al*., 1996; Kobayashi *et al*., 1996; Cuevas *et al*., 1997; Wurzler *et al*., 1998).

Conclusions

The present investigation demonstrates that systemic administration of rhGH is able to enhance bone regeneration and mechanical strength of healing critical size rat calvarial defects, covered with e-PTFE membranes.

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