Morphostereometry of heterotopic ossicles in the rat

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The objective of this study was to describe induced heterotopic bone formation in the rat using stereologic methods and to optimize the sampling for toxicologic studies. Bone formation was induced by implants of demineralized bone powder in 24 male Wistar rats. The ossicles formed were removed after 14, 16, 17, 18, and 20 days. From each ossicle, six sections were sampled equidistantly, and seven microscopic fields were analyzed in each section. Ossicle volume and weight decreased from Day 16 to Day 20. Bone volume density increased during the experimental period because of the reduction in ossicle volume, whereas the total bone volume did not change. Bone thickness increased from Day 14 to Day 20. No changes were observed in the surface density and total area of formative surfaces. The major part of the total variation was due to true variation between animals. The described histomorphometric methods may prove useful in quantitative toxicologic studies, e.g., of fluoride effects on cartilage and bone formation.

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The increase in potentially bone-toxic agents has created a need for methods to study bone dynamics. By quantitative histologic procedures, it is possible to evaluate relatively small morphologic alterations in bone; but to date, stereologic methods have mainly been applied to bone remodeling studies (Melsen 1978, Kragstrup et al. 1982, Melsen et al. 1983, Vesterby et al. 1987). Little quantitative morphologic information is available about growth-related bone formation, and only a few methods and experimental models have been described (Seinsheimer and Sledge 1981, Smeets and Buul Offers 1983, Baltadjiev 1987).

Urist et al. (1968) developed a model where bone is formed subcutaneously after implantation of demineralized bone matrix. The heterotopic model has primarily been used for biochemical and qualitative histologic studies (Reddi and Huggins 1972, Reddi 1981, Harakas 1984); but because the model allows sequential removal of induced bone (ossicles) from the same animal and because the ossification is not influenced by spatial and functional limitations, the heterotopic model may prove useful in toxicologic studies.

Our aim has been to describe induced heterotopic bone formation in the rat using stereologic methods and to optimize the sampling for toxicologic studies.

Materials and methods

Two groups of 5–7-week-old male Wistar rats, with an initial weight of 129 (SD 8.5) g, were examined. Group A consisted of 16 and Group B of 8 animals. During the experimental period, the rats were kept in metal cages. The animals were fed a standard diet (Altromin no. 1314) and tap water.

Under Imobillon® anesthesia, (Fisker et al. 1982), a 1-cm-long incision was made in the thoracic midline of all the animals, and two subcutaneous pockets were prepared. Demineralized bone powder (25 [SD 2.3] mg) was inserted into each pouch.

Demineralized bone matrix (DBM) was prepared from the femur and the tibia of 4–6-month-old Wistar rats of both sexes. The diaphyses were cut into chips, washed in water, dehydrated in ethanol, extracted in diethyl ether, and dried overnight at room temperature. The dehydrated bone chips were crushed in a mill and sieved. The final particle size of the powder was 100–500 μm. Demineralization was carried out in 0.5 M HCl (25 meq/g bone) for 3 h. During this period, the acid was renewed three times. Subsequently, the powder was washed in water and ethanol, dried overnight at room temperature, and stored.

The 16 Group A animals were used for a quantitative assessment of heterotopic bone for-

mation as a function of time. On Days 14 and 20 after bone induction, one ossicle was removed from each of 8 randomly selected animals; and on Days 16 and 18, one ossicle was removed from each of the remaining animals.

The 8 animals in Group B were used to evaluate the sampling design. Two ossicles were removed from each animal on Day 17.

Histologic procedures

Fixation was carried out stepwise in a 40–100 percent ethanol series. The ossicles were embedded undemineralized in methyl methacrylate, and 8 µm sections were cut on a heavy-duty microtome (Polycut, Jung). Sections were stained with toluidine blue (Baron et al. 1983) and examined under a light microscope.

Sampling and stereology

The sampling design was evaluated by nested analysis of variance (Sokal and Rohlf 1969). From each ossicle, six sections, spaced approximately 40 µm from each other, were sampled for stereologic analysis. In each section, seven equidistantly spaced microscopic fields were analyzed. To determine the optimal allocation of resources, the optimal number (n) of items per sampling level was calculated using the formula $n = (v_1 \times c_2/v_2 \times c_1)^{1/2}$ (Gundersen 1980), where (v_1) is the variation, (c_1) is the cost of an extra item at the lowest level (e.g., sections), (v_2) is the variation, and (c_2) is the cost of an extra item at the following level (e.g., ossicles). The cost factor was expressed in hours.

The following variables were considered:

Ossicle volume was estimated immediately after removal on the basis of the weight of the displaced fluid (distilled water).

Cartilage volume density: The volume fraction of cartilage in the entire ossicle was **estimated** by point counting (400 points per specimen; Elias and Hyde 1980).

Cartilage volume: The absolute volume of cartilage in the ossicle was calculated as the volume of the ossicle multiplied by the cartilage volume density.

Bone volume density: The volume fraction of bone in the entire ossicle was **estimated** by point counting (400 points per specimen; Elias and Hyde 1980).

Bone volume: The absolute volume of bone in the ossicle was calculated as the volume of the ossicle multiplied by the bone volume density.

Surface density of osteoblast-covered surfaces: The area of osteoblast-covered surfaces per unit volume ossicle was **estimated** by intersection counting (Elias and Hyde 1980).

Area of osteoblast-covered surfaces: The absolute area of osteoblast-covered surfaces was calculated as the volume of the ossicle multiplied by the surface density of the osteoblast-covered surfaces.

Osteoid, cartilage, and trabecular thickness: The three-dimensional thickness was **estimated** as the apparent two-dimensional thickness multiplied by $\pi/4$, measured perpendicular to the surface at random sites (60 measurements per animal; Kragstrup et al. 1982).

Differences were analyzed either by the Student's *t*-test or by the Mann-Whitney's nonparametric rank sum test. $P < 0.05$ was considered significant.

Results

No change of ossicle volume was observed from Days 14 to 16. From Days 16 to 20, the ossicle volume and weight decreased (Table 1). Cartilage was only present on Day 14. The volume density of cartilage was 0.03 (SD 0.02), and the total volume of cartilage was 0.0051 (SD 0.0037) mm^3. The thickness of the cartilage was 42.2 µm (SD 7.41).

The bone volume density increased between Days 16 and 18, whereas the total bone volume did not change. Trabecular thickness was higher on Day 20 compared with previous days, while Days 14, 16, and 18 showed no differences (Table 2). The total area of osteoblast-covered surfaces did not change, but osteoid-seam thickness was higher on Day 14 compared with Day 16 (Table 3).

The major part of the total variation of bone volume density was due to true variation between animals (48 percent) and sections within ossicles (37 percent); on the other hand, the variation between ossicles in 1 animal (14 percent) was of less importance (Table 4). Other parameters exhibited a similar pattern. The contribution to total variation made by true variation between animals ranged between 46 and 58 percent, true variation between sections from 24 to 32 percent, and true variation between ossicles from 15 to 19 percent. The optimal number of sections per ossicle is three, whereas one ossicle per animal must be investigated (Table 4).

Table 1. Volume and weight of ossicles removed 14–20 days after osteoinduction. Mean *SD*

Day	Volume (mm^3)		Weight (g)	
14	0.14	*0.03*	0.15	*0.03*
16	0.14	*0.03*	0.15	*0.03*
18	0.13	*0.05*	0.13	*0.05*
20	0.10	*0.02*	0.10	*0.02*

Table 2. Stereologic estimates of bone volume density, total bone volume, and trabecular thickness in ossicles removed 14–20 days after osteoinduction. Mean *SD*

Day	Bone volume density		Total bone volume (mm^3)		Trabecular thickness (µm)	
14	0.12	*0.029*	0.017	*0.0056*	40	*6.3*
16	0.12	*0.027*	0.017	*0.0058*	44	*5.7*
18	0.16	*0.063*	0.019	*0.0061*	42	*4.2*
20	0.16	*0.034*	0.015	*0.0038*	54	*3.8*

Table 3. Stereologic estimates of surface density of osteoblast-covered surfaces, area of osteoblast-covered surfaces, and osteoid thickness in ossicles removed 14–20 days after osteoinduction. Mean *SD*

Day	Surface density of osteoblast-covered surfaces (cm^{-1})		Total area of osteoblast-covered surfaces (mm^2)		Thickness of osteoid seams (µm)	
14	26.42	*6.10*	38.46	*13.14*	6.17	*0.51*
16	25.40	*15.11*	37.32	*12.82*	5.34	*0.55*
18	30.30	*8.00*	37.37	*14.23*	5.66	*0.61*
20	35.65	*13.21*	33.90	*12.28*	5.79	*0.84*

Table 4. Nested analysis of variance performed on estimates of bone volume density in 8 animals 17 days after osteoinduction

	Sections	Ossicles	Animals
Observed variance	1.18	3.91	22.10
Isolated variance	1.18	0.46	1.51
Items per animal	12	2	–
Contribution to total variance percent	37	15	48
Price per extra animal (hours)	0.5	2	5
Optimal number per level	3	1	–

Discussion

A systematic quantitative description of bone formation in subcutaneous ossicles has not previously been reported. Results from qualitative histologic studies (Reddi and Huggins 1972, Reddi 1981) have suggested that the amount of bone in the ossicle increases from Day 11 to Day 20. In the present study the apparent increase in the amount of bone was due to a reduction in ossicle volume. This emphasizes the need of quantitative histologic methods to avoid misinterpretation of morphologic changes in normal and pathologic conditions.

Ash weight, and calcium and phosphate ratios have been used as indices of new bone formation in ossicles. Urist et al. (1970) demonstrated a tenfold increase in ash weight, total calcium, and phosphorus between Days 14 and 20 after bone induction in intramuscular sites in rabbits coinciding with bone formation, whereas Bauer et al. (1984) reported a fourfold increase in the ash weight of various bone implants in rats between Days 10 and 21. The increase in ash weight between Days 10 and 14 observed in the latter study can be explained by an increase in the amount of new bone during this period. The present quantitative study showed no increase in total bone volume from Day 14 to Day 20, but an increase in ash weight between these two dates may occur owing to a continued incorporation of calcium into already formed bone. Methodologic differences may also play a role in interpreting the results, as heterotopic bone formation is influenced by several factors, such as species, implantation site, implant preparation, and amount of implanted matrix (Urist et al. 1968).

The nested analysis of variance demonstrated that the true variance between animals contributed almost 50 percent of the total observed variance. The major part of the remaining variance was due to variation between sections within the ossicle. The optimal sampling design depends on variance, as well as on cost. These figures may vary from laboratory to laboratory, but it is evident that very little is gained by increasing the number of ossicles per animal. The results indicate that to improve the design more animals must be studied. The optimal number (N) of animals may be determined by assessing the acceptable error (p), applying the formula $N = (SD \times 100/\text{mean} \times p)^2$. If, e.g., a 5 percent error in the mean bone volume density is accepted, 25 animals must be investigated. In the present study, the accepted error was 9 percent á posteriori.

By the methods presented in this study, it was possible to describe quantitative bone parameters, which are generally used in bone remodeling studies, and therefore may prove useful in quantitative toxicologic studies of, e.g., the effect of

fluoride on cartilage and bone formation. Further, the heterotopic model and the stereologic methods can be successfully applied in biomechanical bone studies.

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