**CLINICAL AND EXPERIMENTAL FORUM**

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**Filling the bone defect with osteogenic material**

**An experimental study**

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**Abstract** In this experimental study with bone defects, we focussed on the one hand on external and internal osteogenic callus formation after filling the defect and on the other on the osteochondrogenic differentiation capacity of 4-day-old fibrous-like callus grafts and 12-day-old woven bone grafts in an osteogenic environment. A standard cortical bone defect of the femur was created in 95 young rats. The defect was filled with a cortical bone graft and 4- and 12-day-old callus grafts. The grafts were transplanted as such or in Nucleopore chambers. Follow-up was done at 1, 2, 3 and 6 weeks. The osteochondrogenic tissue formed was studied histologically and histomorphometrically. The results suggest that the filling of the bone defect had no influence on the primary external and internal osteogenic callus formation at 1 and 2 weeks. At 3 and 6 weeks in the chamber groups the persisting internal bridging woven bone was converted into more compact lamellar bone whereas periosteal callus remained at the edges of the defect. In the other groups at 3 and 6 weeks the normal shape of the cortex was reconstituting. Four-day-old fibrous-like callus formed bone in the Nucleopore chamber, indicating that fibrous-like callus tissue at 4 days contains osteogenic cells. Twelve-day-old callus consisting of woven bone was partially differentiated to cartilage, showing that woven bone contains cells capable of chondrogenic differentiation.

**Introduction**

General callus formation of tubular bone after a fracture varies with the degree of injury [17] and the treatment used [3] according to the law of Wolff [20]. The primary callus reaction after trauma has been considered identical in fractures, amputation stumps and bones injured without producing a fracture, with environmental factors having little effect on primary callus formation [11]. In our previous studies, however, the primary osteochondrogenic callus formation was also related to the degree of bone injury [8] and an external factor: re-trauma significantly increased primary osteogenic callus formation [7].

In a bone defect the area is first filled by haematoma and then replaced by fibrous-like tissue and finally by woven bone advancing from the medullary side as an osteogenic front [4, 8, 12, 18]. The artificially produced bone defect offers a tool to study the pattern of osteochondrogenic callus formation and differentiation in a standardized environment [8].

The purpose of the present study were to evaluate (1) the influence of filling the bone defect on external and internal osteogenic callus formation and (2) the osteochondrogenic properties of 4 and 12-day-old callus grafts in an osteogenic environment.

**Materials and methods**

Experimental animals and operative technique

The series consisted of 95 young Wistar rats of both sexes weighing 190–250 g. Thirteen rats were anaesthetized with an intramuscular (0.3 ml/kg) injection of flunisone fentanyl citrate (Hynnorm; Philips-Duphar, Amsterdam, The Netherlands) and intraperitoneal (2.5 mg/kg) injection of diazepam (Diapam; Orion, Espoo, Finland). Eighty-two rats were anaesthetized with an intraperitoneal (50 mg/kg) injection of pentobarbital (Mebumal; Orion). The rats were divided randomly into six groups (Table 1).

Group 1: Empty Nucleopore chamber (10 rats). A lateral approach was made to the left diaphysis of the femur, and a hole of 1.8 x 7 mm reaching the medulla was sawn with a dental circular saw (RA 231, ISO No 040; Hager & Meisinger, Düsseldorf, Germany). Slow velocity was used, and the bone was moistened with saline to prevent overheating. In the chamber a polymethylmethacrylate frame (DAF, Hedehusene, Denmark) was closed on both sides with polycarbonate membranes (Nucleopore, Pleasanton, Calif. USA) with a porosity of 0.6 μm. The membranes were fixed to the frame with cyanacrylate glue (Loctite, Ireland).
The outer size of the capsule was $1.2 \times 6$ mm and 1 mm in height, and the inner volume of the chamber was 3.5 mm$^3$. The empty chambers were inserted in the hole of the femur with the membranes facing externally and medullary to the bone. The muscle and skin were closed with 4–0 polyglycolic acid sutures (Dexon; Davis and Geck, Portsmouth, UK).

Group 2: Four-day-old callus in Nucleopore chamber (20 rats). An anteromedial approach was made to the proximal diaphysis of the left tibia. A hole of $1.2 \times 8$ mm in the cortical bone reaching the medulla was sawn with a circular dental saw. The wounds were closed. Four days later the animals were reoperated. A lateral approach was made to the diaphysis of the left femur, and a hole of $1.8 \times 7$ mm reaching the medulla was sawn with a dental circular saw. The tibial wound was re-opened, and the callus from the tibial bone defect was excised with a scalpel and transplanted into the Nucleopore chamber in the hole of the femur. The operation was accomplished similarly to that for the previous group. In the pilot study with 7 control defects, the 4-day-old reparative tissue of the defect area consisted of fibrous-like tissue when studied histologically.

Group 3: Twelve-day-old callus in Nucleopore chamber (20 rats). The bone defects to the bone were made as in the previous group. Twelve days after the first operation, the callus was transplanted into the Nucleopore chamber in the hole of the femur. Twelve-day-old callus from the tibial bone defect consists of woven bone [8].

Group 4: Twelve-day-old callus in the bone defect (15 rats). The bone defects were made as in the previous groups. Twelve days after the first operation, woven bone was transplanted into the hole in the femur.

Group 5: Cortical bone graft in the bone defect (15 rats). After sawing a similar diaphyseal defect in the femur as in the previous groups, the piece of cortical bone of $1.2 \times 6$ mm in diameter was turned 180 deg in plane and placed back in the hole.

Group 6: Bone defect (15 rats). A similar bone defect as in the previous groups was created in the left femur and was left empty.

During the operations each rat received an intramuscular injection (30 000 IU/kg) of procaine penicillin (Procapen: Orion). After the operations, the rats were returned to their cages and fed and watered ad libitum. No immobilization was used. The rats were killed by an overdose of diethyl ether. The study followed the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [5], and the study was approved by the ethical committee for animal experiments of Helsinki University Central Hospital.

Examination methods

The whole tibia with the surrounding muscles was fixed in 70% ethanol, dehydrated in increasing concentrations of ethanol, and embedded in methylmetacrylate [16].

Table 1 The number of rats in the different groups and follow-up periods

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Type</th>
<th>Follow-up in weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1  2  3  6</td>
</tr>
<tr>
<td>1</td>
<td>Empty chamber</td>
<td>5  5  5  5</td>
</tr>
<tr>
<td>2</td>
<td>4-day-old callus in chamber</td>
<td>5  5  5  5</td>
</tr>
<tr>
<td>3</td>
<td>12-day-old callus in chamber</td>
<td>5  5  5  5</td>
</tr>
<tr>
<td>4</td>
<td>12-day-old callus</td>
<td>5  5  5  5</td>
</tr>
<tr>
<td>5</td>
<td>Cortical bone</td>
<td>5  5  5  5</td>
</tr>
<tr>
<td>6</td>
<td>Defect</td>
<td>5  5  5  5</td>
</tr>
</tbody>
</table>

For the histological and histomorphometric analysis, 5-μm thick transverse sections were cut from the middle section of the defect with a microtome (Polycut S; Reichert-Jung, Nussloch, Germany) and stained by Masson-Goldner's method [6]. In the semiautomatic quantitative histomorphometric analysis the microscope was linked via a camera to a MOP-Videoplan (Kontron, Munich, Germany). The computer gave a magnification of × 90. The error of this histomorphometric method is shown by the coefficient of variation, 1.3%. To evaluate callus formation of the host the following variables were measured: the transverse area of the cortex and the defect. The areas of woven bone and cartilage were measured separately from defect area, excluding the inside of the Nucleopore chamber, the external callus and the internal callus (Fig. 1). To evaluate the osteochondrogenic callus formation of the grafts, the areas of bone and cartilage were measured from the inside of the Nucleopore chamber. The results for the groups were expressed as means and standard deviations (SD). In the histomorphometric analysis of the host osteochondrogenic callus formation, the values were presented as percentages of the transsectional cortical bone area [14], and statistical evaluation of the different subgroups with the corresponding values of the defect group were performed with Student’s t-test. $P$ values less than 0.05 were considered statistically significant.

Fig. 1 Osteochondrogenic callus formation of the host was calculated in different areas of the bone: A defect area, excluding the inside of the chamber; B external, outside cortical bone; and C internal, inside cortical bone

Fig. 2 A bone defect filled with 4-day-old callus in the Nucleopore chamber (group 2) at 6 weeks. The transplant inside the chamber shows bone formation. The internal woven bone of the host is being converted into a more compact lamellar-like bridging structure. External bone formation has reached the edges of the defect, but does not bridge it. (Original magnification × 10)