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Development of an Osteogenic Bone-Marrow Preparation*

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ABSTRACT: The osteogenic effect of bone marrow was tested in rabbits, using chambers that had been implanted in the peritoneal cavity (ectopic site) and in a delayed-union model (orthotopic site). Osteogenesis was accelerated in both sites after concentration of marrow elements by centrifugation, but not after unit gravity sedimentation. Chambers that were implanted with marrow that had been processed by simple and isopyknic centrifugation demonstrated a more pronounced increase in deposition of calcium compared with whole-marrow implants of equal volume (101 compared with 193 per cent). Orthotopic grafting of a rabbit delayed-union model with whole marrow and marrow that had been processed with simple centrifugation significantly increased osteogenesis, as measured biomechanically and biochemically. Significantly improved healing was evident radiographically at five weeks after grafting with bone marrow that had been concentrated by simple centrifugation.

Clinical Relevance: Bone marrow has been used clinically to stimulate osteogenesis in conjunction with bone-inducing agents such as bone morphogenetic protein and with bone-conducting material such as Kiel (animal) bone. Marrow may also effect clinical healing of delayed unions and non-union when it is injected percutaneously into the site of a fracture. Techniques that maximize osteogenesis by the injection of bone marrow offer numerous potential advantages and less morbidity compared with presently used standard methods of bone-grafting.

The ability of red marrow to form bone was first suggested by Goujon, as early as 1869. A number of subsequent studies on animals have shown that autologous bone marrow contains osteogenic precursor cells that contribute to the production of bone. Several investigators have used autologous marrow clinically to augment the osteogenic response to implanted allografts, to bone-inducing or bone-conducting agents such as bone morphogenetic protein, or to Kiel (animal) bone. Connolly and Shindell were, to our knowledge, the first to report on the percutaneous injection of bone marrow to bring about healing of an infected united tibia. In preparation for further clinical trials, the present study was conducted to develop an injectable bone-marrow preparation that would be useful for treating delayed unions and non-unions. The preparation had to be simple to prepare and it had to optimize osteogenic potential.

Freidenstein reported a possible association between the effectiveness of ectopic osteogenesis and cell concentration. Budenz and Bernard also suggested that rabbit bone-marrow cells that were separated over a continuous Ficoll-Paque gradient (Pharmacia, Piscataway, New Jersey) that had a density of 1.050 to 1.056 and 1.064 to 1.067 grams per milliliter produced greater amounts of bone cartilage than did whole marrow. With this work in mind, an investigation was undertaken to determine if different cell-concentration techniques could enhance the osteogenic potential of an injectable marrow preparation.

Simple centrifugation, unit gravity sedimentation, and isopyknic centrifugation were evaluated to determine their ability to improve ectopic calcification using a rabbit intraperitoneal-chamber model. Our purpose was to evaluate the degree of osteogenic enrichment that was achieved by these different cell-concentration techniques and to decide which method was most feasible for clinical application at an orthotopic site. The method of concentration that seemed best was then tested in a rabbit delayed-union model to assess its ability to improve osteogenesis in an orthotopic site.

Materials and Methods

Preparation of Bone Marrow

Bone marrow was obtained by percutaneous aspiration from the posterior iliac crest of adult New Zealand White rabbits that weighed two to three kilograms. With the animal under general anesthesia, a 16-gauge bone-marrow needle was inserted into the posterior iliac crest and was rotated gently into the marrow cavity. The stylet was removed from the needle, and a twenty-milliliter glass syringe that had been moistened with a solution of eight milliliters of 1:1000 preservative-free heparin (pH 7.0) that had been derived from porcine intestinal mucosa (SoloPak Laboratories, Franklin Park, Illinois), diluted to eighty units per milliliter, was attached. Bone marrow was aspirated by sudden retraction of the plunger of the syringe with simultaneous rotation of the needle to avoid back-filling by venous blood. After two to three milliliters of marrow was collected, the

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684 THE JOURNAL OF BONE AND JOINT SURGERY
needle was repositioned until a total of seven to ten milliliters of marrow was obtained from each rabbit. To reduce clumping, cell aggregates were dispersed by passing marrow sequentially through 19 and 22-gauge needles. The resultant marrow suspension was then prepared as follows.

Whole marrow: The suspension was gently vortexed. The nucleated cell count and viability of each aliquot were determined in a hemocytometer, using the trypan-blue exclusion method. The average whole-marrow concentration that was achieved was $2.0 \times 10^6$ nucleated cells per milliliter $\pm 20$ per cent (Table I).

Simple centrifugation: Specimens of marrow were placed in a swinging bucket (Dynac II table-top centrifuge, model-0103, with eight by fifteen-milliliter tubes, rotor model 0109; Clay Adams, Parsippany, New Jersey) and centrifuged at 400 times gravity for ten minutes. The supernatant was removed and used as a control implant material. The sedimented marrow cells were vortexed. The red blood-cell pellet, including the buffy coat, was vortexed. The nucleated cell count and viability were determined on the final cell suspension with a hemocytometer. Using this concentration technique, the nucleated cell count averaged $3.6 \times 10^6$ cells per milliliter (Table I), with cell recovery approaching 100 per cent.

Isopyknic centrifugation: Seven and a half milliliters of bone marrow was added to an equal volume of 0.01-molar phosphate-buffered saline and gently vortexed. The mixture was layered over thirty milliliters of undiluted Ficoll-Paque, which had a density of 1.075 grams per milliliter, and was centrifuged for thirty-five minutes at 400 times gravity. The band of light-density cells and the red blood-cell pellet were removed separately, washed, and resuspended. Before implantation, the final cell-suspension concentration and viability were determined as was described. Nucleated cell recovery from the light-density layer was 70 $\pm$ 10 per cent and averaged $8.0 \times 10^6$ cells per milliliter (Table I).

Unit gravity sedimentation: Seven and a half milliliters of 0.01-molar phosphate-buffered saline and an equal amount of bone marrow were mixed and layered over thirty milliliters of Ficoll. Unit gravity sedimentation was allowed to proceed at room temperature for four hours. The band of light-density cells and the red blood-cell pellet were recovered separately, washed, and resuspended. The final cell suspension concentration and viability were determined as was already described. The final cell-suspension concentration averaged $8.6 \times 10^6$ cells per milliliter (Table I).

**Diffusion Chamber Model**

The osteogenic potential of the different marrow preparations was assessed using the intraperitoneal diffusion-chamber model that was described by Owen and by Ashton et al. Control preparations, including saline solution and acellular supernatants of centrifuged marrow, were used. A total of ten rabbits was used, and each rabbit received twenty chambers. Each chamber contained exactly 150 microliters of the autogenous marrow preparation, diluted to the cell concentration that is shown in Table I. A saline-solution or acellular supernatant control, whole marrow, or marrow that had been prepared by one of the concentration techniques was implanted in each rabbit. No animal received marrow that had been prepared by all three techniques of cell concentration.

The diffusion chambers were constructed from PlexiGlas rings (Rohm and Haas), faced with 0.45-micrometer Millipore filters (Millipore, Bedford, Massachusetts). Ten chambers, spaced at two-millimeter intervals, were joined with Lexan crossbars (Lexan, General Electric, Fairfield, Connecticut). The chamber stacks were wrapped with the peritoneal omentum, ensuring spontaneous encapsulation. Replicate chambers containing whole marrow, centrifuged marrow, isopyknic centrifuged marrow, and unit gravity-sedimented marrow were recovered after forty days to assess the osteogenic response. In a separate experiment, the degree of osteogenesis that occurred in chambers that were filled with whole or centrifuged marrow was evaluated after twenty, forty, and sixty days. Each preparation from each time-interval was tested in at least eight replications that had been implanted in a minimum of two animals.

**Analysis**

One representative chamber from each of the preparations (rabbits) was placed in 95 per cent ethanol at 4 degrees Celsius and later was cold-processed and embedded in JB-4 glycomethacrylate (LKB-Produkter AB, Bromma, Sweden). Three-micrometer sections were cut and stained with Dahl calcium stain, Goldner trichrome, Ackerman's azo dye method for alkaline phosphatase, and hematoxylin and eosin.

The contents of the remaining chambers were collected by carefully removing one of the Millipore filters with a scalpel and collecting the contents of the chamber in a five-milliliter test tube. The contents were mixed with one-half milliliter of demineralized water. The collected specimens were immediately chilled on ice. Each specimen was then

**TABLE I**

**Preparation of Diffusion Chambers**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Nucleated Cells per Milliliter in Final Cell Suspension</th>
<th>Nucleated Cells per Diffusion Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Range ($\times 10^6$)</td>
</tr>
<tr>
<td>Whole marrow</td>
<td>$2.0 \times 10^6$</td>
<td>1.4-2.6</td>
</tr>
<tr>
<td>Simple centrifugation</td>
<td>$3.6 \times 10^6$</td>
<td>3.0-3.7</td>
</tr>
<tr>
<td>Isopyknic centrifugation</td>
<td>$8.0 \times 10^6$</td>
<td>7.7-8.6</td>
</tr>
<tr>
<td>Unit gravity sedimentation</td>
<td>$8.6 \times 10^6$</td>
<td>8.2-8.9</td>
</tr>
</tbody>
</table>
subjected to high-speed homogenization to ensure that all calcified tissue was in suspension. Alkaline phosphatase activity in the chamber was determined using p-nitrophenol phosphate substrate\textsuperscript{13}, and calcium content was determined by atomic absorption. The data were expressed as micromoles of phosphate per hour and micrograms of calcium per chamber.

**Injection of Percutaneous Marrow in a Non-Union Model**

Twenty-three adult male New Zealand White rabbits, weighing three to five kilograms, were used to investigate the osteogenic properties of whole and concentrated bone marrow in a non-union. The model was created by surgically removing a one-centimeter-long osteoperiosteal segment (two to three times the diaphyseal diameter) from the right radius, using a double-blade oscillating saw. This standard animal model of a non-union has been widely used since Heiple et al. used segmental defects of twice the diameter of the diaphysis in their 1963 study of osseous transplants. Radial stabilization was not necessary due to synostosis of the radius with the ulna. The marrow within the cut ends of the bone was washed clear, and the wound was closed in layers with 3-0 Vicryl (Ethicon, Somerville, New Jersey).

Forty-eight hours after osteotomy, the rabbits were anesthetized again and were prepared to receive the bone-marrow grafts. Five milliliters of marrow was collected percutaneously from each rabbit, as described already. Nine control animals received no injection (Group I), seven animals received two milliliters of whole marrow (Group II), and seven received two milliliters of concentrated marrow that had been prepared by simple centrifugation (Group III). Nucleated cell counts of aliquots of whole and centrifuged marrow were not determined before injection. The marrow preparations were percutaneously injected into the osseous defect through a 22-gauge needle that had been placed by directly palpating the defect and then inserted until the needle met the resistance of the cut ends of the bone. As the marrow was injected, the needle was slowly withdrawn to ensure grafting along the entire length of the defect.

**Analysis**

After five weeks, the osteogenic response was measured as follows.

**Radiographic:** Radiographs of each surgically treated limb were made immediately after the operation and at the time when the animal was killed. Three individuals evaluated the radiographs and assigned grades for bone formation, union, and remodeling, according to the protocol that was described by Lane and Sandhu.

**Biomechanical:** After the animals were killed, the ends of the bones of the osteotomized and contralateral radioulnar composites were embedded in methylmethacrylate and

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**Fig. 1:** Representative histological section of marrow-filled chambers, forty days after implantation. The arrows indicate areas of active cartilage calcification. F = fibrous tissue and C = cartilage (undecalcified section, Goldner trichrome. \( \times 80 \)).

**Fig. 2:** Higher magnification of Fig. 1, showing an area of calcification (CA) and hypertrophic chondrocytes (HC) (Goldner trichrome. \( \times 280 \)).
were identically aligned for three-point bend-testing on an Instron materials-testing machine (Instron, Canton, Massachusetts). At a cross-head speed of one millimeter per minute, the load-deformation curve was measured for each specimen. Loading continued until the composite exhibited graphic evidence of plastic deformation, which was represented by a sudden change in the slope of the load-deformation curve. From these data, the rigidity of the composite was determined as the maximum load that had been absorbed by the sample while it underwent a 0.2-millimeter deformation. The rigidity index for the experimental composite (exp) was expressed as a percentage of the control limb:

\[
\text{rigidity index} = \frac{\text{maximum load absorbed (exp) per 0.2-millimeter deformation}}{\text{maximum load absorbed per 0.2-millimeter deformation}} \times 100
\]

**Biochemical:** The degree of calcification of the matrix at the osteotomy site was assessed by determination of buoyant tissue density, ash weight, and mineral-to-matrix ratio. A five-millimeter-wide section was taken through the center of the osteotomy site, and an equivalent section was taken from the control limb. For each sample, we recorded the hydrated weight, submerged weight, dry weight (twenty-four hours at 110 degrees Celsius), and ash weight (eighteen hours at 600 degrees Celsius). Tissue density was determined as:

\[
\text{hydrated weight of specimen} \div \text{volume of specimen}
\]

where volume equals hydrated weight minus submerged weight.

The mineral-to-matrix ratio was determined as:

\[
\text{ash weight} \div \text{dry weight} - \text{ash weight}
\]

Variability among individual rabbits was minimized by expressing the data as a percentage of that for the control limb. The Student t test was used to compare data between subgroups.

**Results**

Each of the chambers that was injected with whole marrow received 2.1 to 3.5 \( \times 10^6 \) cells, and each of the chambers that was injected with centrifuged marrow received 4.5 to 5.6 \( \times 10^6 \) cells. Over-all, simple centrifugation consistently increased the nucleated cell count of the chamber implants by 50 to 70 per cent, and in these concentrated specimens the cell viability was always more than 90 per cent. Chambers that were implanted with reconstituted nucleated cells after isopyknic centrifugation or unit gravity sedimentation received 11.5 to 12.9 \( \times 10^6 \) and 12.3 to 13.3 \( \times 10^6 \) nucleated cells, respectively. Studies revealed 92 per cent cell viability after isopyknic centrifugation and only 80 per cent cell viability after unit gravity sedimentation. Over-all, unit gravity sedimentation and isopyknic centrifugation allowed four to five times as many nucleated cells to be implanted for each chamber.

**Ectopic Osteogenesis**

Histological examination of marrow-filled chambers that were recovered after forty days revealed a mixture of bone, cartilage, and fibrous tissue (Fig. 1). Figure 2 demonstrates differentiation of the tissue into cartilage and bone, including calcified cartilage at the bottom of the photomicrograph. Control chambers that were filled with venous blood or with the acellular supernatant from marrow contained an amorphous, mucoid-like substance. Dense staining for alkaline phosphatase was seen in marrow-filled chambers, localized in areas adjacent to calcifying cartilage (Fig. 3). Extensive calcium deposition was also observed in and around hypertrophic chondrocytes (Fig. 4). Localized nodules of safranin-O-positive cartilaginous tissue were present throughout the tissue in the marrow-filled chambers (Fig. 5).

Biochemical analyses for calcium and alkaline phosphatase activity were normalized and are presented in Table II as the activity of \( 1 \times 10^5 \) cells in each chamber. This made possible a comparison of the calcification activity using different methods of cell concentration. After a forty-day incubation period, the chambers that contained whole or spun marrow had accumulated 229.3 and 460.9 micro-

![Fig. 3](image-url)
grams of calcium, respectively. The chambers that had been implanted with the light-density fraction from isopyknic centrifugation had accumulated 671.1 micrograms of calcium and the cells that had been prepared by unit gravity centrifugation, 94.6 micrograms. When the light-density fraction that had been prepared by isopyknic separation was diluted to match the nucleated cell count of spun marrow, it still had a 50 per cent increase in calcium deposition (671 compared with 460 micrograms), which was statistically significant.

In studies in which the duration of incubation ranged from twenty to sixty days (Table II), the samples that had been subjected to simple centrifugation deposited greater amounts of calcium than did whole-mar row implants at all intervals that were tested. Alkaline phosphatase activity was also greater in the centrifuged specimens. As the time after implantation increased, alkaline phosphatase activity gradually decreased.

**Orthotopic Osteogenesis**

The osteogenic response of the various marrow-cell preparations in the rabbit non-union model was examined five weeks after injection, using radiographic, biomechanical, and biochemical techniques. Radiographs were assessed using the method of Lane and Sandhu, which included quantitative indices for formation of bone, fracture union, and remodeling of the harvested radii. Eleven per cent of the controls (Group I), 28 per cent of the osteotomy gaps that were injected with whole marrow (Group II), and 70 per cent of those that were injected with centrifuged marrow (Group III) were found to be completely filled with mineralized tissue when the animals were killed. Formation of bone, union, and remodeling were significantly greater for fractures that had been injected with centrifuged marrow than for the controls (Table III).

The data from the testing of rigidity (Table IV) indicated less variability between replicates in a group than did our other indices of formation of bone. There was a statistically significant difference between both marrow-injected groups and the controls. More bending rigidity was found in Group III (centrifuged marrow), but the value was not significantly different from that of Group II (whole marrow).

Biochemical analyses of calcification at the osteotomy gap included mineral-to-matrix ratio, ash weight, and buoyant density (Table V). All measurements were significantly greater in the specimens from marrow-injected rabbits than in those from the control animals. Specimens from the animals that had received centrifuged marrow had higher ash weight and mineral-to-matrix ratio than did those from the animals that had been injected with whole marrow. Over-
all, the use of marrow that had been concentrated by centrifugation definitely improved the results, although they were not statistically different from the results with the use of uncentrifuged marrow.

Discussion

The search for new methods for repair of bone derives its impetus from the perceived limitations of autologous bone-grafting, which has been standard treatment for almost fifty years. chemister’s classic technique for autologous bone-grafting involves two operative procedures, with potential morbidity at both the donor and the recipient site\textsuperscript{11,16}; yet skeletal healing is primarily a biological process, dependent on cellular response. The most productive source of cells that influence osteogenesis is considered to be autologous marrow\textsuperscript{11}.

The purpose of our study was twofold: to evaluate, by ectopic implantation, the degree of osteogenic enrichment that was achieved by different cell-concentration techniques, and to decide which method was technically applicable at an orthotopic site. Evaluation of chambers that had been implanted in the abdominal cavity showed that osteogenesis can be increased by increasing cell concentration. The origin of this osteogenicity has been said to be the stromal or endosteal cells within the marrow\textsuperscript{8}.\textsuperscript{18}

In the present study, the marrow cells were concentrated using simple centrifugation, unit gravity sedimentation, or isopyknic centrifugation. All three techniques increased the cell population and, with the exception of gravity sedimentation, proportionately increased osteogenesis and formation of calcium in the implanted chambers. In the case of unit gravity sedimentation, the prolonged time for preparation probably adversely affected cell viability.

Alkaline phosphatase activity did not increase with the time after implantation. Levels of this enzyme are known to be highest during the initial stages of formation of bone and to diminish after twenty to thirty days\textsuperscript{9}. However, the results of our experiment using intraperitoneal chambers showed that injection of marrow that had been prepared by isopyknic centrifugation achieved the greatest cell concentration in each chamber and, consequently, the greatest accumulation of calcium. Simple centrifugation also increased osteogenic activity, as measured by accumulation of calcium, but not to the same degree as did isopyknic centrifugation.

Clinically, the two major factors determining the technical feasibility of these concentration procedures are the time for preparation and the maintenance of sterility.

TABLE II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Duration of Incubation (Days)</th>
<th>No. of Chambers</th>
<th>Accumulation of Calcium* (µg)</th>
<th>Alkaline Phosphatase Activity* (µM/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple centrifugation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>20</td>
<td>16</td>
<td>82.6 ± 11.5</td>
<td>11.5 ± 2.8</td>
</tr>
<tr>
<td>Spun</td>
<td>20</td>
<td>16</td>
<td>140.4 ± 27.3</td>
<td>13.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.025</td>
<td>N.S.</td>
</tr>
<tr>
<td>Whole</td>
<td>40</td>
<td>20</td>
<td>229.3 ± 36.3</td>
<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>Spun</td>
<td>40</td>
<td>20</td>
<td>460.9 ± 17.2</td>
<td>9.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Whole</td>
<td>60</td>
<td>9</td>
<td>403.8 ± 53.8</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>Spun</td>
<td>60</td>
<td>8</td>
<td>649.1 ± 90.2</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Isopyknic centrifugation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light density</td>
<td>40</td>
<td>27</td>
<td>671.1 ± 80.8</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>Heavy density</td>
<td>40</td>
<td>20</td>
<td>95.7 ± 18.8</td>
<td>4.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.025</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Unit gravity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light density</td>
<td>40</td>
<td>6</td>
<td>94.6 ± 6.6</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Heavy density</td>
<td>40</td>
<td>3</td>
<td>15.8 ± 3.6</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venous blood</td>
<td>40</td>
<td>10</td>
<td>62.0 ± 25.3</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Empty chambers</td>
<td>40</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
* Values represent the mean and standard deviation. All samples were normalized to a cell concentration of 1 × 10⁶ cells per chamber. Significance was determined by the Student t test. N.S. = not significant.

TABLE III

<table>
<thead>
<tr>
<th>Group</th>
<th>Bone Formation (Points)</th>
<th>Union (Points)</th>
<th>Remodeling (Points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.78 ± 0.49</td>
<td>0.89 ± 0.48</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>2.86 ± 0.34</td>
<td>2.29 ± 0.52</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>3.29 ± 0.47</td>
<td>3.14 ± 0.59</td>
<td>1.43 ± 0.72</td>
</tr>
<tr>
<td>I vs. II</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>I vs. III</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.04</td>
</tr>
<tr>
<td>II vs. III</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* The radiographic findings were graded on a 0 to 4-point scale\textsuperscript{9}. The values represent the mean and standard deviation. Significance was determined by the Student t test. N.S. = not significant.

\textsuperscript{9} | = control, II = whole marrow, and III = centrifuged marrow.

VOL. 71-A, NO. 5. JUNE 1989
**TABLE IV**

**RESULTS OF BIOMECHANICAL TESTING**

<table>
<thead>
<tr>
<th>Group*</th>
<th>Bending Rigidity†</th>
<th>Significance‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>12.50 ± 2.61</td>
<td>p &lt; 0.05 (I vs. II)</td>
</tr>
<tr>
<td>II</td>
<td>13.75 ± 2.02</td>
<td>p &lt; 0.01 (I vs. III)</td>
</tr>
<tr>
<td>III</td>
<td>14.98 ± 4.77</td>
<td>N.S. (II vs. III)</td>
</tr>
</tbody>
</table>

* I = control, II = whole marrow, and III = centrifuged marrow.
† The results of three-point bend-testing are expressed as a percentage of the value for the contralateral limb. The values represent the mean and standard deviation.
‡ Significance was determined by the Student t test. N.S. = not significant.

**TABLE V**

**RESULTS OF BIOCHEMICAL ANALYSIS**

<table>
<thead>
<tr>
<th>Group†</th>
<th>Buoyant Density</th>
<th>Ash Weight</th>
<th>Mineral:Matrix Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>17.5 ± 2.9</td>
<td>39.3 ± 10.1</td>
<td>38.6 ± 10.4</td>
</tr>
<tr>
<td>II</td>
<td>30.4 ± 2.5</td>
<td>77.6 ± 7.6</td>
<td>70.3 ± 6.7</td>
</tr>
<tr>
<td>III</td>
<td>80.4 ± 3.0</td>
<td>95.6 ± 13.3</td>
<td>68.9 ± 9.6</td>
</tr>
<tr>
<td>I vs. II</td>
<td>p &lt; 0.015</td>
<td>p &lt; 0.015</td>
<td>p &lt; 0.015</td>
</tr>
<tr>
<td>I vs. III</td>
<td>N.S.</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>II vs. III</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* The results are expressed as a percentage of the value for the contralateral limb. The values represent the mean and standard deviation. Significance was determined by the Student t test. N.S. = not significant.
† I = control, II = whole marrow, and III = centrifuged marrow.

Percutaneous centrifugation requires one hour compared with fifteen minutes for simple centrifugation. In addition, maintaining sterility for the former technique is more arduous. These technical problems prompted us to choose simple centrifugation to enhance osteogenesis in our experiment on orthotopic grafting. This study demonstrated that percutaneous grafting of autologous bone marrow significantly improved the results in a delayed union model. Paley et al. and Werntz et al. also studied fracture-healing that was augmented with bone marrow alone. Paley et al. reported that two-millimeter-long defects in rabbit radii that had been injected with marrow elements five days after osteotomy healed with a greater volume of callus, and union was biomechanically stronger and radiographically better than it was in controls.

Our study confirmed the finding of Paley et al. and also showed a difference between the results of the use of whole marrow and centrifuged marrow. The specimens that had been injected with centrifuged marrow had significantly improved radiographic healing compared with controls. They also had better union-over-all, as measured radiographically, biomechanically, and biochemically, compared with specimens that had been injected with whole marrow, although the improvement was not statistically significant.

The lack of statistical significance may be due either to the number of animals that were studied, the small amount (two milliliters) of marrow that was injected, or the time (five weeks) that was chosen for the analysis.

The lack of a statistically significant difference between the data for centrifuged marrow and uncentrifuged marrow suggests that concentration of marrow is indicated primarily to treat problems with healing when space is limited (for example, for non-union of the scaphoid). When the area around the fracture site is large (for example, the tibia), the benefits of concentration of marrow are outweighed by the increased time for preparation and the chance of contamination.

Our present study supports the concept that osteogenesis is stimulated by marrow and that the stimulus is related to cell concentration. In other studies of delayed-union and non-union models, the osteogenic capacity of bone marrow has been utilized in combination with osteoinductive materials, such as bone morphogenetic protein, chips of cancellous bone, and demineralized bone. Many, if not all, of these studies indicated that bone-induction agents are most effective when supplemented with marrow as composite grafts. In our study, the beneficial effects of treating non-union by percutaneous injection indicate that this technique could have considerable advantages and less morbidity compared with presently accepted clinical methods. We think that further investigations of the osteogenic effect of marrow combined with stimulatory factors and injected percutaneously into skeletal defects is now warranted.

**References**