

Remineralization of demineralized bone matrix in critical size cranial defects in rats: A 6-month follow-up study

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Abstract: The key drawback of using demineralized bone matrix (DBM) is its low initial mechanical stability due to the severe depletion of mineral content. In the present study, we investigated the long-term regeneration of DBM in a critical size bone defect model and investigated the remineralization after 6 months. Bone defects were created in the cranium of male Wistar rats which were filled with DBM or left empty as negative control. *In vivo* bone formation was monitored with computed tomography after 11, 19, and 26 weeks postoperatively. After 6 months, parietal bones were subjected to micro-CT. Mineral content was determined with spectrophotometric analysis. After 11 weeks the DBM-filled bone defects were completely closed, while empty defects were still open. Density of the DBM-treated group increased significantly while the controls remained unchanged. Quantitative analysis

by micro-CT confirmed the *in vivo* results, bone volume/tissue volume was significantly lower in the controls than in the DBM group. The demineralization procedure depleted the key minerals of the bone to a very low level. Six months after implantation Ca, P, Na, Mg, Zn, and Cr contents were completely restored to the normal level, while K, Sr, and Mn were only partially restored. The remineralization process of DBM is largely complete by the 6th month after implantation in terms of bone density, structure, and key mineral levels. Although DBM does not provide sufficient sources for any of these minerals, it induces a faster and more complete regeneration process. © 2015 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 00B: 000–000, 2015.

Key Words: DBM, bone, mineralization, critical size defect

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INTRODUCTION

Demineralized bone matrix (DBM) is a well-known bone substitute that is gaining popularity in orthopedic and spinal surgery indications. Even though autografting is still the gold standard method for bone substitution, DBM has been developed to increase the availability and to avoid donor site morbidity. It is also safe to use due to the elimination of immunogenic surfaces.¹ The other key feature of DBM is also its key drawback: the lack of mineral content. The demineralization process unmasks the osteoconductive and osteoinductive extracellular matrix factors such as collagen and bone morphogenetic proteins at the expense of losing

mechanical strength.^{2–4} Indeed, this is still the main concern among surgeons who use DBM that initial strength is negligible and it is largely unknown when and to what extent shall the mineral structure build-up to load-bearing levels. Until regenerated bone is readily recognizable with imaging techniques by virtue of its opacity, its functional value will always be questioned by surgeons. In other words, if it looks different on radiographs, it cannot be fully relied on. Meta-analyses of studies in spinal fusion showed that DBM in various formats can significantly improve fusion rates when combined with autografts, but its effect is less convincing when applied alone.^{5–7} Thus, the current clinical

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consensus leans toward using DBM as an extender to autografts rather than a standalone bone substitute.

Animal studies has shown that DBM can induce bone formation, however, the resulting bone quantity and quality falls short of native bone or even that can be achieved with supra-physiological doses of bone morphogenetic protein 2 (BMP-2). Alaei used an elegant mouse model of multiple osteogenic reporter genes and observed that although DBM had osteoinductive properties, its effect was rather limited.⁸ When DBM is used as a bone filler for critical size defects, its effects are even less convincing. In rat models, for example, DBM showed significant, but limited bone formation capacity at 8 weeks.^{9–11} Studies with longer timeframes of 90 days also failed to show a complete regeneration by DBM.¹² In addition, in a rabbit cranial defect model it was shown that although DBM putty achieved more bone formation than a synthetic substitute, implant residues were still observed after 3 months.¹³ Mixing DBM with multipotent cells *in vitro* seems to restore the missing osteogenic properties of the material, however, regulatory and reimbursement hurdles significantly limit the spread of such cell therapies.^{14–16} Another promising way is to use DBM as a vehicle for bioactive molecules. For example, serum albumin coated DBM was shown to induce faster ossification since it provides a convenient milieu for stem cell function *in vivo*.¹⁷

As the search for physiological alternatives to recombinant BMPs intensifies, DBM derivatives are becoming the subject of mainstream research again, since they provide a source of osteoinductive proteins at a physiological ratio.¹⁸ However, one may hypothesize that if DBM is used in a concentrated fashion, it may suffer from the same side effects as recombinant BMP-2, namely overinduction of osteogenesis resulting in ectopic bone formation.¹⁹ Although the primary concern when dealing with DBM is still the slow bone formation capacity, the potential for a long-term, uncontrolled bone buildup should not be overlooked. Since most of the experimental studies are terminated at an early to mid-term phase of bone regeneration, it is largely unknown if DBM-induced bone formation intrinsically stops at a desired level or keeps going on toward hyperdens or heterotopic bone.

In the present study we aimed to test the long-term bone formation capacity of DBM in the rat cranial defect model in order to observe whether it is capable of remodeling into a bone structure which equals the physiological bone stock of the cranium.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250 to 300 g were used. The animals were maintained on lab chow and tap water ad lib with 12 h day/night cycle in the animal facility of the Institute of Human Physiology and Clinical Experimental Research in Budapest. The investigation was approved by the local Animal Research Committee according to the guidelines for animal experimentation. (Date of issue: 2009.10.07., Registration number: 22.1/2960/003/2009.)

DBM preparation

Male Wistar rats were euthanized with urethane (5 mg/mL). Parietal bone was identified and cortical bone pieces were harvested with a 4 mm internal diameter trephine bur. The DBM was prepared by following the classical method originally described by Urist et al.²⁰ (1) defatting by methanol for 24 h. (2) Washing with PBS for 5 min. (3) Antigen removal by partial autolysis in 0.1 M PBS containing 10 mM/L sodium azide and 10 mM/L iodic acetic acid at 37°C for 48 h. (4) Decalcination in 0.6N hydrochloric acid at room temperature for 24 h. (Ca content in DBM was 0.1 ± 0.0 m/m%, untreated control 34.1 ± 0.2 m/m%). (5) Washing with PBS for 5 min. (6) Whitening in 3% hydrogen peroxide at room temperature for 36 h. (7) Washing in distilled water. (8) Freeze drying: grafts were frozen at -80°C , freeze dried at -50°C overnight. All chemicals were purchased from Sigma Aldrich Co., Budapest, Hungary.

Surgical procedure

The surgical procedure was performed as described in Nature Protocols²¹ Under ketamine-xylazine anesthesia (100–10 mg/kg, Richter Gedeon Plc., Budapest, Hungary—Sigma Aldrich Co., Budapest, Hungary) and proper disinfection, the skin was incised over the parietal bone of male Wistar rats. Periosteum was carefully elevated from the bone. Bone defects were created using a 4 mm trephine bur (external diameter) on both sides. DBMs were used to fill the defects and the periosteum was united in the midline with a thin absorbable suture (6-0 Vicryl, Johnson & Johnson, Janssen-Cilag Ltd., Hungary). The wound was disinfected and the animals were placed back into their cages (two rats/cage) and soon regained consciousness.

Mineralization status

In order to determine the mineralization status of treated bone allograft and regenerated bone after 26 weeks, ion content was measured by inductively coupled plasma optical emission spectrophotometer (ICP-OES). Grafts were dried under an infrared lamp for 2 h at $\sim 50^{\circ}\text{C}$. After weighing, the grafts were dissolved in 300 μL cHNO₃ and the obtained solutions were diluted with distilled water to an overall volume of 3.0 mL each. Regenerated and untreated bones were tested by harvesting circular bone pieces with a trephine bur with 4 mm internal diameter.

Experimental groups

Bilateral parietal bone defects were created in five rats. DBMs were used to fill the defects in a random order ($n = 5$). Defects filled with blood clot only were used as negative control (Sham treated, $n = 5$). Computed tomography was performed 11, 19, and 26 weeks after the operation. After 26 weeks animals were sacrificed and parietal bones were harvested for micro-CT scans and remineralization measurements.

In vivo follow-up imaging

Computed tomography. Under ketamine-xylazine anesthesia, computed tomography (CT) was performed with a Phillips

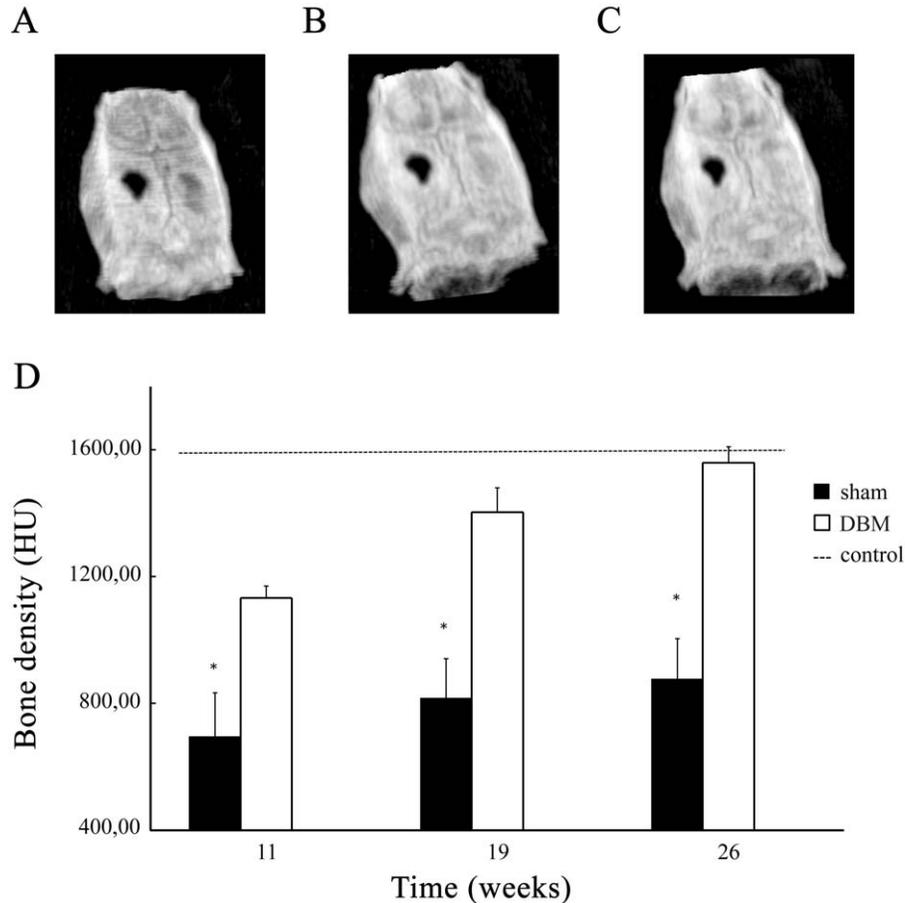


FIGURE 1. *In vivo* CT analysis of bone formation. A–C, Three-dimensional reconstructions of the rat crania. On the left side an unfilled defect is seen as sham treatment and on the contralateral side a defect filled with DBM. The untreated defect is persistent even after half a year, at which timepoint the DBM-treated defects are completely filled. D, The bone density values in the defect site. Control is measured at an unaffected location of the rat cranium. * $p < 0.05$ between the two groups.

Brilliance 16 Slice CT machine (Philips International B.V. Amsterdam, Netherlands). Axial slices were obtained with 120 kV and 300 mA (slice thickness: 0.8 mm, increment 0.4 mm, collimation 16×0.75 mm, rotation time 0.75 second, pitch: 0.4). Images from the CT scan were analyzed to determine bone density. Circular region of interest (ROI) was set on the 11th postoperative week for each defect. Reference points

were used to set the same ROI at each time point. Area of the remaining bone defect was measured from the CT scans. Windows were set to visualize rat bone without soft tissue background (2572/1595 Window/Centrum). The remaining bone defect was calculated on an enlarged reconstructed image with free hand technique. The area of the bone defect (mm^2) was compared between the DBM and control groups.

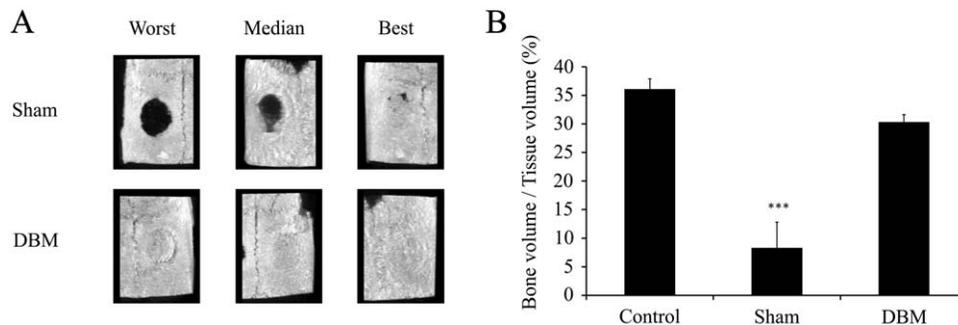


FIGURE 2. *Ex vivo* micro-CT analysis of calvaria defects at 26 weeks follow-up. A, Representative images of the worst/median/best healed defects in both treatment groups. B, Qualitative analysis of regenerated bone in the defect site. While a defect left empty showed only a limited regeneration, the DBM treated defects regained the same density as the control, unaffected cranium. *** $p < 0.001$ between the two groups.

TABLE I. Elements that were Retained in DBM After the Demineralization Process (μg)

| | Al | As | B | Ba | Cu | Fe | Ni | Pb | S | Si |
|---------|------------------|------------------|------------------|------------------|------------------|------------------|-----------------|------------------|-----------------|-----------------|
| Control | 0.224 \pm 0.02 | 0.079 \pm 0.02 | 0.149 \pm 0.01 | 0.424 \pm 0.02 | 0.095 \pm 0.01 | 0.297 \pm 0.07 | 0.05 \pm 0.01 | 0.038 \pm 0.02 | 8.25 \pm 0.81 | 0.64 \pm 0.09 |
| DBM | 0.27 \pm 0.01 | 0.092 \pm 0.0 | 0.15 \pm 0.0 | 0.437 \pm 0.01 | 0.127 \pm 0.01 | 0.262 \pm 0.07 | 0.058 \pm 0.0 | 0.039 \pm 0.01 | 5.95 \pm 0.8 | 0.82 \pm 0.03 |

Ex vivo micro-CT. The harvested parietal bones were scanned using a micro CT scanner (Skyscan 1172 X-Ray microtomograph, Kontich, Belgium) at 59 kV, 167 μA with a 23 μm^3 isovolumetric pixel size. Image analysis was performed with CTAn software in the following way: the centrum of the bone defect was determined on the horizontal and coronal images. On coronal images a circular ROI with 80 pixels of radius were drawn and 100 slices were selected cranial and caudal from the centrum. Bone volume/tissue volume (BV/TV) was calculated to determine the relative bone content (%) in the defect area.

Statistical analysis

All of the values are reported as the means \pm SEM. The statistical analysis was performed with one-way ANOVA and Bonferroni multiple comparison tests using the GraphPad Prism statistical software. Probability values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significant.

RESULTS

In vivo CT

CT scans on the 11th, 19th, and 26th postoperative weeks were completed in order to follow bone regeneration. The density values in the DBM treated group was already significantly higher at the 11th week compared with the untreated controls (DBM: 1132 \pm 76 HU, control: 695 \pm 124 HU) (Figure 1). The density of the sham operated group did not improve significantly any further, while significant improvement was seen in the DBM treated group. By the 26th week the density values of the DBM treated defects were 1559 \pm 37 HU, which is comparable to the intact rat calvarium (1620 \pm 16 HU). Every bone defect in the DBM treated group showed complete closure already at the 11th postoperative week (Figure 1). In contrast, only one defect showed complete closure in the untreated group, the remaining bone defect in this group was 5 \pm 1.2 mm². Some reduction was seen throughout the experiment, but by the 26th week the remaining bone defect was still 3.6 \pm 1.1 mm².

Ex vivo micro-CT

Micro-CT scans were performed at the 26 postoperative week which allowed investigation of the mineral structure in much higher resolution than *in vivo* imaging. The DBM treated group showed complete closure in every case and was not significantly different from the control group in bone volume/tissue volume (BV/TV) values (Figure 2). In contrast, open bone defects and significantly lower BV/TV values were observed in the sham operated group (Figure 2). In this study group only one out of five defects showed complete closure. New bone formation was only observed in

the defect area in each experimental animal, neither ectopic bone formation, nor hyperdens bone was observed throughout the study.

Mineralization status

Detectable amounts were observed in Al, As, B, Ba, Cu, Fe, Ni, Pb, S, Si contents, but we found no differences between fresh bone and DBM, indicating that the demineralization process did not affect these elements (Table I). In contrast, demineralization significantly reduced the Ca, Na, Mg, K, P, Cr, Mn, Sr, and Zn contents (Figures 3 and 4). Namely, Ca, Mg, P, Sr nearly disappeared in the demineralized bone after the preparation, while significantly lower amounts were detected from Na, K, Cr, Mn, and Zn (Figures 3 and 4).

Remineralization was measured by comparing the bone pieces after 6 months *in vivo* regeneration, which we also compared with the element levels of the regenerated tissues filling the untreated bone defects. Calcium buildup in the DBM group reached 80% of the control measurements, whereas the sham group was only 46% (Figure 3). We also found that DBM treatment nearly completely restored the Na, Mg, P, and Cr content, while the sham operated group showed significantly lower levels. Only the Zn content was comparably restored in both the DBM and sham groups (Figure 4). Another group of elements showed a different buildup pattern. Potassium and Strontium were higher after being implanted for 6 months, but neither element was completely restored to the normal cranial bone level (Figure 4). Manganese content was significantly reduced by the demineralization process and failed to be restored in either experimental group (Figure 4).

DISCUSSION

DBM was able to achieve complete bone healing and built up a mineral content and bone structure largely matching that of normal bone at 6 months without any excessive, heterotopic, or hyperdens bone. While most elements have been restored to the normal level after 6 months of implantation, K, Sr, and Mn are still lagging behind indicating that bone remodeling is still underway even when the structure of bone appears to be completely rebuilt (Figure 5). This study confirms that although DBM is slow in inducing bone formation, it is capable of generating a normal bone structure in a critical size defect.

Cranial bone defects of 5 mm in diameter which are left untreated are considered "critical" since they do not heal during the lifetime of the animal. Designing studies with endpoints of end-of-lifetime of the rats is imprecise and impractical, and it was shown that the defects that are not healed by 6 months do not show significant further changes

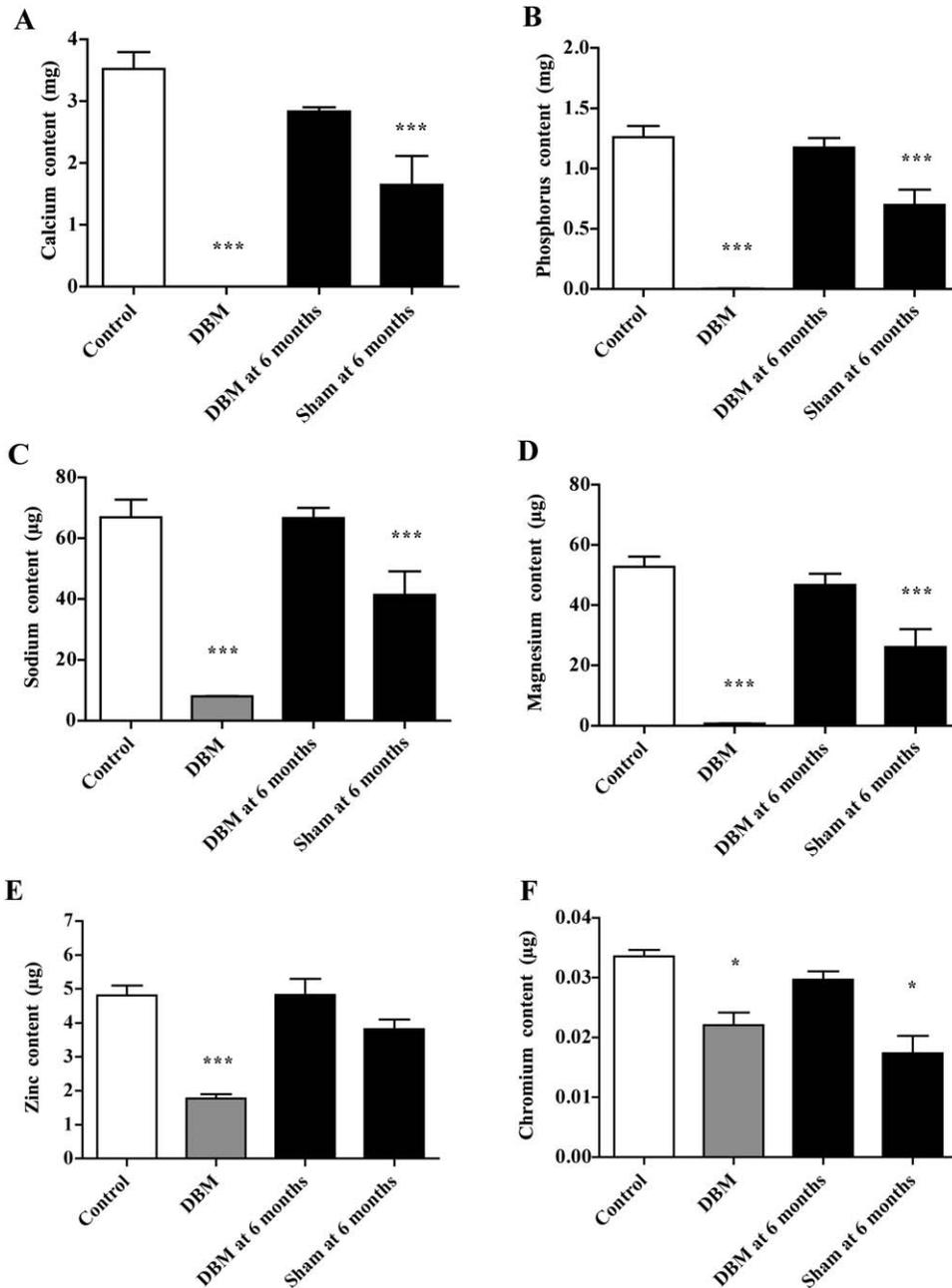


FIGURE 3. Elements that are completely restored at 6 months. A–F, The total amounts of each element in a 4 mm circular cranial tissue. Control is unaffected bone, DBM stands for the prepared graft before implantation and at 6 months after. Sham is tissue in an untreated defect at 6 months regeneration. Each one of these elements were significantly reduced by the DBM preparation procedure and completely rebuilt after 6 months of implantation *in vivo*. The sham operated regenerative tissue is significantly less mineralized in each measurement with the exception of zinc. Also note that the demineralization process affects the various elements to widely differing levels. * $p < 0.05$, *** $p < 0.001$ compared with control.

at 12 months.²² Thus, investigation of bone formation at 6 months is an optimal time point for evaluating long-term effects. A study from Jones have shown that DBM-filled cranial defects were indeed stronger than sham operated or bone chips filled defects at 6 months, however, they did not provide data on how does it compared with healthy bone, that is, whether the bone defect can be considered completely healed at this time point.²³ As we have also shown

in the present study, the untreated bone defect was filled with scar tissue at 3 months, which did not remodel into bone at 6 months either. In contrast, DBM was completely incorporated into the osseous tissue and the mineral content largely matched that of normal bone at 6 months.

Demineralization of bone tissues is usually determined by the remaining calcium content. Although the exact calcium level is not uniform throughout the literature or the

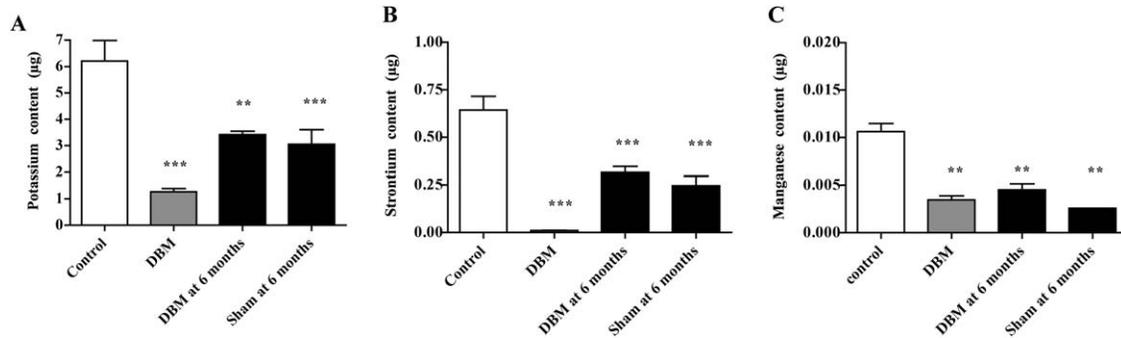


FIGURE 4. Elements that are incompletely restored at 6 months. The levels of K, Sr, Mn contents in DBM were significantly reduced and only partially restored during the 6 months *in vivo* implantation period. ** $p < 0.01$, *** $p < 0.001$ compared with control.

available product ranges, it is generally accepted that about 2% residual calcium can remain in DBM.²⁴ In the current study we followed the classic protocol of Urist²⁰ and achieved an even lower residual calcium which is probably due to the small physical sizes of the rat calvaria samples which are then easily accessible by the applied acids. After implantation into the animals the buildup of calcium was easily monitored by the density measurements on the CT images, which was cross-checked by the actual Ca measurements of the samples *ex vivo*. The calcium content and the remaining visible defect sizes correlated well among the samples, so it is concluded that DBM sufficiently remodeled into a calcified bone tissue at 6 months after implantation. Several key ions, such as sodium and magnesium, as well as phosphate (the most common form of phosphorus measured in biological systems) followed the same pattern like that of calcium, that is, depletion by the demineralization process, partial buildup in sham treatment and complete buildup in DBM-treated defects. Although the microelements

zinc and chromium were not completely depleted to start with, they also followed the same buildup regime. One would expect that potassium, a key element in intracellular homeostasis must also be rebuilt to the same level as the normal bone. In contrast, we measured that potassium content was still significantly lower at 6 months after implantation, although there was no difference between the empty or the DBM-filled defects. Manganese and strontium followed a similar pattern, that is, incomplete buildup and no difference between the treatment groups. Strontium is widely used as a marker of bone regeneration and it is used as a treatment option in severe osteoporosis.²⁵ Despite the numerous studies on the physiology of strontium in bone tissue, its mode of action is still not completely clear.²⁵ Our current observation highlights that morphological remodeling and general mineralization of the majority of key components correlate well with each other, and that strontium follows a somewhat different route. Thus, it is of importance to use several measures for monitoring bone regeneration in order to obtain a complete picture.

We conclude that the remineralization process of DBM is largely complete by the 6th month after implantation in terms of bone density, structure, calcium, and other key mineral levels. Although DBM does not provide sufficient sources for any of these minerals, it induces a faster and more complete regeneration process which results in the restoration of mineral content which is lacking in bone defects that are left untreated.

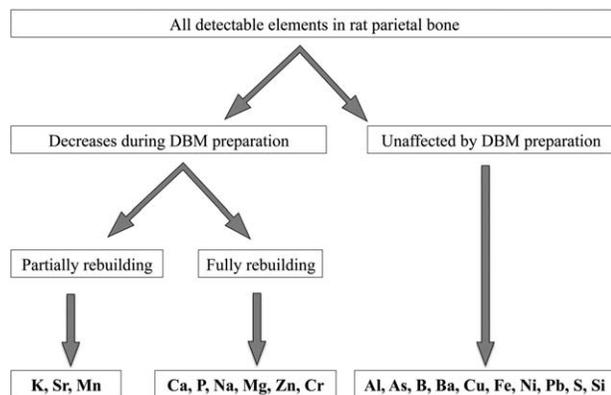


FIGURE 5. The fate of minerals during DBM processing and after implantation. All elements were analyzed in the periodic table and those that had a detectable amount are shown in this figure. The preparation protocol decreased the most abundant elements, mainly calcium and those that are the key building blocks of inorganic matter. In contrast, sulfur and most metals are left unaffected. After implantation of the bone grafts and incorporation into the host environment most depleted elements were fully rebuilt with the exception of K, Sr, and Mn, indicating that although mineral density on the CT returned to normal levels there are still some specific differences between the remodeled graft and the unaffected bone.

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