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Spatial mapping of mineralization with manganese-enhanced magnetic resonance imaging^{☆,☆☆}

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Abstract

Paramagnetic manganese can be employed as a calcium surrogate to sensitize the magnetic resonance imaging (MRI) technique to the processing of calcium during the bone formation process. At low doses, after just 48 h of exposure, osteoblasts take up sufficient quantities of manganese to cause marked reductions in the water proton T1 values compared with untreated cells. After just 24 h of exposure, 25 μM MnCl_2 had no significant effect on cell viability. However, for mineralization studies 100 μM MnCl_2 was used to avoid issues of manganese depletion in calvarial organ cultures and a post-treatment delay of 48 h was implemented to ensure that manganese ions taken up by osteoblasts is deposited as mineral. All specimens were identified by their days in vitro (DIV). Using inductively coupled plasma optical emission spectroscopy (ICP-OES), we confirmed that Mn-treated calvariae continued to deposit mineral in culture and that the mineral composition was similar to that of age-matched controls. Notably there was a significant decrease in the manganese content of DIV18 compared with DIV11 specimens, possibly relating to less manganese sequestration as a result of mineral maturation. More importantly, quantitative T1 maps of Mn-treated calvariae showed localized reductions in T1 values over the calvarial surface, indicative of local variations in the surface manganese content. This result was verified with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). We also found that $\Delta R1$ values, calculated by subtracting the relaxation rate of Mn-treated specimens from the relaxation rate of age-matched controls, were proportional to the surface manganese content and thus mineralizing activity. From this analysis, we established that mineralization of DIV4 and DIV11 specimens occurred in all tissue zones, but was reduced for DIV18 specimens because of mineral maturation with less manganese sequestration. In DIV25 specimens, active mineralization was observed for the expanding superficial surface and $\Delta R1$ values were increased due to the mineralization of small, previously unmineralized areas. Our findings support the use of manganese-enhanced MRI (MEMRI) to study well-orchestrated mineralizing events that occur during embryonic development. In conclusion, MEMRI is more sensitive to the study of mineralization than traditional imaging approaches.

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Keywords

Manganese-enhanced magnetic resonance; imaging; Osteoblasts; Mineralization; Calvaria; Laser ablation inductively coupled plasma; mass spectrometry

Introduction

At present, detailed analyses of bone apposition rates *in vivo* require bone to be fluorescently labeled at two time points, biopsied, embedded, and destructively sectioned for bone histomorphometry studies. This technique, while important for calculating bone turnover rates, is very invasive and the preparation of bone sections can disrupt some of the labile mineral phases [1]. Non-invasive microcomputed tomography (microCT) can be used to study the dynamics of bone development [2,3] but this modality does not have the required sensitivity to detect sub-resolution thickness changes in skeletal elements or the presence of low density mineral deposits within extruded matrix vesicles. Typically, treatment protocols lasting 6–12 weeks are required such that microCT or microMRI images can detect subtle changes in the trabecular architecture. We would like to present an alternative approach to the study of bone mineralization, namely, manganese-enhanced MRI (MEMRI). In our earlier work, we found that the addition of manganese to the culture medium can sensitize the MRI technique to newly deposited manganese-containing mineral formed during the mineralization process [4–6]. In this work, we propose to use MEMRI to map the spatial variation in mineralizing activity for calvarial skull bones maintained in organ culture.

Manganese (Mn^{2+}) with an ionic radius similar to that of calcium (Ca^{2+}) is known to enter cells through voltage-gated calcium channels [7]. Additionally, radioactive $^{54}Mn^{2+}$ can be taken up and transported by olfactory neurons [8], providing ample evidence to support of the use of paramagnetic Mn^{2+} as a surrogate for Ca^{2+} for studying axonal transport in dedicated neuronal pathways [9–11], calcium-stimulated insulin secretion by pancreatic β cells [12,13], and cardiac function in stimulated myocytes [14–16]. Our group has shown that manganese can be used to sensitize the MRI technique to the mineralization process because it can be taken up by voltage-gated calcium channels on osteoblasts [6]. However, the putative toxicity of high doses (1mM) of manganese precludes its use in studying the dynamics of the mineralization process. For this reason, this work has focused on (i) the effect of short exposure times and lower manganese doses on the viability of osteoblasts and (ii) whether low manganese doses are sufficient to cause notable changes in the water proton longitudinal relaxation time (T_1) of Mn-treated cell pellets compared with untreated cells. Next, we determined whether organ cultured calvariae treated with a low dose of manganese for just 24 h can sequester sufficient manganese to reduce its T_1 value compared with untreated specimens. Finally, quantitative T_1 maps were used to (i) estimate the level of mineralizing activity in organ cultured calvariae and (ii) map the spatial variation in mineralizing activity over the calvarial surface. The reported enhancement in tissue T_1 values due to the deposition of manganese-containing mineral was validated against manganese elemental maps acquired with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).

Methods

Cell pellet studies

Fertilized White Leghorn chicken eggs were purchased from CBT Farms (Chestertown, MD) and maintained in a humidified commercial egg incubator. Calvariae were harvested with the approval and strict adherence to the guidelines of the Institutional Animal Care and Use Committee at the AFIP. Chick embryos were removed from the egg, decapitated, and

the calvarial bones that comprise the vault of the skull were aseptically dissected free of periosteal membranes. Primary osteoblasts were obtained from the third population of cells released by serial collagenase digestion of the calvarial bones of 16-day-old chick embryos [17]. For the final digestion, minced calvariae were treated with 3 mg/mL collagenase (Roche Molecular Biochemicals, Indianapolis, IN) for 40 min at 37 °C with agitation. Isolated osteoblasts were resuspended in a known volume of culture medium and counted with a hemocytometer.

To study the uptake of manganese by primary osteoblasts, cells were plated at a density 1.5×10^7 cells per T-75 flask (Corning, Corning, NY) and cultured in 20 mL of low-glucose Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS), L-glutamine, Fungizone™, and gentamicin. Tissue culture supplies were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise specified. The flasks were maintained at 37 °C in a 5% carbon dioxide incubator. When the cells were confluent, the culture medium was supplemented with 3 mM calcium (II) chloride (Sigma-Aldrich, St Louis, MO) and a series of flasks were treated with 0, 25, 100, 250, and 500 μM manganese (II) chloride (Sigma-Aldrich) for 48 h. After manganese treatment, the medium was decanted from the flasks, and the cells were washed twice with 5 mL of phosphate buffered saline (PBS). The flasks were then treated with 5 mL of 0.25% trypsin in 1 mM EDTA (Invitrogen) and incubated at 37 °C for 5 min to release the adherent osteoblasts. The resulting cell suspension was transferred to 15 mL centrifuge tubes. Each tube was centrifuged for 5 min at 200×g and the supernatant removed. The resulting cell pellets were washed twice with PBS and resuspended in 500 μL of PBS. The final cell suspensions were transferred to 800 μL Eppendorf tubes and centrifuged for 5 min at 1075×g to form the final pellet. After centrifugation, the supernatant was decanted and replaced with Fluorinert™ (FC-77, Oakwood Products, Inc., West Columbia, SC), a fluorinated hydrocarbon used to prevent desiccation of the pellets during the MRI experiment.

For MRI studies, Eppendorf tubes with manganese-free and manganese-treated cells pellets were imaged directly in a 10-mm radio-frequency (RF) probe. Quantitative maps of water proton T1 relaxation times were extracted from MRI images of the cell pellets. Two-dimensional cell pellet images had a slice thickness of 1 mm and an in-plane resolution of 109 μm. Cells pellets from parallel cell culture experiments were submitted for inductively coupled plasma optical emission spectrometry (ICP-OES).

Cell viability assay

Primary osteoblasts were isolated from embryonic chick calvarial bones as described above. Cells were grown to confluence in T-75 flasks and first-passage cells were plated onto an opaque solid bottom 96-well plate with 25,000 cells per well. The cells were allowed to attach overnight, then the culture medium was removed and replaced with 100 μL culture medium containing 3 mM CaCl₂. The Mn-treated wells had the following MnCl₂ doses, 25, 100, 250, 500, and 1000 μM. Separate plates were incubated for 24 and for 48 h at 37 °C. At the end of the incubation period, cell viability was determined using a Cell titer 96 Non-Radioactive Cell Proliferation Assay (MTT) Kit (Promega, Madison, WI.) Briefly, 15 μL of Dye Solution (kit reagent) was added to each well and the plate was incubated for 4 h at 37 °C. The MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was reduced by metabolically active cells to insoluble purple formazan dye crystals. After the incubation period, 100 μL of Stop Solution (kit reagent) was added to the plate to lyse the cells and dissolve the dye crystals. After an overnight incubation at 37 °C, the plate was read at 570 nm with a reference wavelength of 670 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnydale, CA.) Wells with culture medium and kit reagents were used for background subtraction. Absorbance data were analyzed using the SoftMax Pro 4.8

(Molecular Devices) software package and a calibration curve generated from wells with known numbers of viable cells.

Calvarial organ cultured studies

Calvariae, harvested from 16-day-old chick embryos, were washed with sterile PBS and cultured in 6-well dishes for up to 25 days [18]. Tissue culture medium was supplemented with 3 mM CaCl_2 , 1% β -glycerophosphate (Sigma), and 50 $\mu\text{g/mL}$ ascorbic acid (sodium salt, Sigma) to promote mineralization [19]. Typically, Mn-treated specimens were exposed to 100 μM MnCl_2 for just 24 h followed by an additional 48 h of culture with control medium. This delay was to ensure that excess, unbound manganese got removed from the sample and the manganese ions, taken up by the cells, got deposited as mineral. Specimens treated on days 1, 8, 15, and 22, were imaged on days 4, 11, 18, and 25, respectively. We refer to all specimens by their days in vitro (DIV), specifically DIV4, DIV11, DIV18, and DIV25. All Mn-treated specimens were subjected to MRI together with a control specimen that was cultured for the same length of time in control medium.

In preparation for the MRI experiments, specimens were washed with a large excess of PBS and the frontal bone was removed and the remaining bone was flattened between two glass slides (width 12 mm, height 75 mm) separated by spacers above and below the calvarial specimens. The whole assembly was immersed a glass tube (o.d. 15 mm) filled with PBS and imaged in a 15-mm RF probe at 37 °C. High-resolution MRI images of mineralizing chick calvariae were acquired with a nominal in-plane resolution of 117 μm and slice thickness equivalent to the space between the two glass slides. Water proton T1 relaxation times were measured for DIV4, DIV11, DIV18, and DIV25 specimens. After MRI, calvaria were fixed with 100% ethanol (Aldrich) prior to further analysis by LA-ICP-MS. Calvaria from parallel organ culture experiments, fixed with 100% ethanol, were submitted for elemental analysis by ICP-OES.

Magnetic resonance imaging studies

All MRI experiments were performed at 37 °C on a Bruker DMX spectrometer (Bruker Biospin, Inc. Billerica, MA) coupled to a vertical-bore magnet (diameter=89 mm) operating at 9.4 T (400.13 MHz for protons). Quantitative T1 relaxation maps were calculated from two-dimensional slice images acquired using a saturation recovery sequence with variable repeat times ($\text{TR}=0.1\text{--}8$ s) and a fixed echo time ($\text{TE}=10$ ms). The total acquisition time of this saturation recovery sequence was ~3 h. To calculate the T1 relaxation time, image intensities were fitted on a pixel-by-pixel basis with a single exponential decay function of the form: $I=I_0[1-\exp(-\text{TR}/\text{T1})]+\text{offset}$, where I_0 is the T2-weighted water proton signal intensity, TR is the repeat time, T1 is the longitudinal relaxation time to be determined, and the offset is the noise level in the image. All image analysis was accomplished using Bruker Paravision software.

Inductively coupled plasma optical emission spectrometry studies

Cell pellets and ethanol-fixed calvarial specimens were digested in a mixture of concentrated nitric acid and 30% hydrogen peroxide (all chemicals were purchased from Fisher Scientific, Pittsburgh, PA) using an accelerated microwave digestion system (Anton Paar, Ashland, VA). The resulting digest was diluted with ultra pure water to 5 mL and the solutions were analyzed for Ca, P, and Mn using ICP-OES (PerkinElmer Optima 5300DV, Shelton, CT). The ICP-OES analysis conditions are summarized in Table 1. An external calibration curve with lutetium as an internal standard was used for quantification. A certified reference material (NIST CRM 1577b, Bovine Liver, NIST, Gaithersburg, MD) was used as a control specimen. Control specimen recoveries were within the certified ranges.

Laser ablation inductively coupled plasma mass spectrometry studies

For LA-ICP-MS studies, calvariae fixed with 100% ethanol were air-dried between two pieces of glass to ensure a flat geometry. Dry specimens were attached to a glass slide using double sided tape in order to eliminate sample motion during the ablation process. Spatial elemental maps were acquired using a laser ablation (NewWave UPMacro, NewWave, Fremont, CA) inductively coupled plasma mass spectrometry system (PerkinElmer Elan 6000, PerkinElmer, Shelton, CT). The laser ablation and ICP-MS parameters are summarized in Table 1. Calibration was performed using an in-house prepared calcium phosphate micro-analytical standard spiked with multiple elements (MAPS1, USGS, Denver, CO). The levels of the elements of interest in MAPS1 are Ca=28.5%, P=23%, Mn=14.5 mg/kg. Data reduction analysis was performed using Excel (Microsoft Corp., Redmond, WA) and spatial elemental concentration maps were generated using Surfer 8 (Golden Software, Golden, CO).

Statistics

The mean and standard deviation of each data point was estimated from a minimum of 4 repeat experiments. A one-way analysis of variance using a post *ad hoc* Bonferroni correction was used to determine whether the difference in the means of Mn-treated and untreated specimens was significant. A difference with $p<0.05$ was regarded as significant and $p=NS$ indicates no significant change. All analyses were performed using SigmaStat (Systat, San Jose, CA).

Results

Cell pellet studies

To establish whether MRI can distinguish between untreated and Mn-treated cell pellets, cells were exposed to the following doses of $MnCl_2$, 0, 25, 100, 250, 500, and 1000 μM . After 48 h of treatment, the cells were prepared for MRI. A representative T1 map of four tubes with cells treated with 0, 25, 100, and 1000 μM manganese, from the bottom to the top of the stack, is shown in Fig. 1A. A summary of the T1 results, for at least 4 repeat experiments, is presented in Fig. 1B for $MnCl_2$ doses up to 500 μM . There was a steady and significant reduction in water proton T1 values compared with control cell pellets as the manganese dose increased. This decline in T1 was attributed to increasing levels of intracellular manganese with higher manganese doses. ICP-OES data, averaged for at least 4 experimental runs, confirmed that the manganese content of the cell pellets increased as a function of manganese dose (Fig. 1C).

The effect of different doses of manganese on osteoblast cell viability was measured after 24 and 48 h of exposure using a MTT cytotoxicity assay (Fig. 2). After 24 h of manganese treatment, cell viability was not impacted by 25 μM $MnCl_2$ but was significantly reduced to $76\pm2\%$ ($p<0.001$) by 1 mM $MnCl_2$. After 48 h, the viability of cells treated with 25 μM $MnCl_2$ was significantly reduced to $89\pm1\%$ ($p<0.001$) compared with control cell pellets and it was even lower ($61\pm2\%$, $p<0.001$) for cells treated with 1 mM $MnCl_2$. This reduction in cell viability was consistent with that reported for osteoblast-like cells after 48 h of exposure to 1 mM $MnCl_2$ [20].

ICP-OES of organ cultured calvaria

According to our ICP-OES results, presented in Fig. 3A and B, control and Mn-treated calvariae continued to lay down mineral while in culture. The calcium content of control calvariae increased from $19.8\pm0.6\%$ for DIV4 to $20.8\pm0.2\%$ ($p<0.001$) for DIV11. Both DIV18 ($21.9\pm0.5\%$) and DIV25 ($22.1\pm0.3\%$, $p=NS$) had comparable levels of calcium that were higher than that of DIV11 ($p<0.001$). The phosphorus content of DIV4 controls

($9.9 \pm 0.3\%$) was comparable to that of DIV11 ($10.2 \pm 0.1\%$, $p = \text{NS}$) and DIV18 ($10.7 \pm 0.3\%$) and DIV25 ($10.7 \pm 0.2\%$) had comparable levels of phosphorus ($p = \text{NS}$) which was significantly higher than that of DIV11 ($p < 0.03$). The calcium (and phosphorus) content of Mn-treated calvariae was comparable to age-matched controls, except for DIV4 where the phosphorus content of Mn-treated specimens was slightly lower ($9.5 \pm 0.2\%$; $p = 0.05$) than that for control specimens. The manganese content of organ cultured calvariae (Fig. 3C) did not exceed 15 ppm, but with manganese supplementation there was a marked increase in the manganese content of all treated specimens (Fig. 3D). The manganese content of Mn-treated DIV4 (965 ± 56 ppm) and DIV11 (950 ± 28 ppm) were comparable ($p = \text{NS}$) and significantly higher than that of DIV18 (710 ± 32 ppm; $p < 0.001$) and DIV25 (688 ± 38 ppm; $p < 0.001$) specimens. Notably, these manganese contents were lower than what was previously reported for calvariae treated continuously with 1 mM doses of MnCl_2 [6].

MRI of organ cultured calvaria

To test our hypothesis that organ cultured calvariae treated with a low dose of manganese can sequester sufficient manganese to alter measured MRI parameters, we cultured a series of calvariae from 16-day-old chick embryos. Typically Mn-treated specimens were exposed to 100 μM MnCl_2 for just 24 h followed by an additional 48 h of culture with control medium. We selected this dose so that manganese depletion would not occur during our organ culture experiments. A 48-h delay was implemented to ensure that excess, unbound manganese got removed from the sample and the manganese ions, taken up by the cells, got deposited as mineral. All Mn-treated specimens were subjected to MRI together with a control specimen that was cultured for the same length of time in control medium.

Representative T1-weighted images of DIV4, DIV11, DIV18, and DIV25 calvariae are shown in Fig. 4. Mn-treated specimens were located above the untreated control specimens. In all cases, the signal intensity of the Mn-treated specimens was higher than the control specimens. Given that the fraction of free water between the microscope slides is greater than the fraction of intracellular or bone interstitial water, the observed image contrast was attributed to the interaction of free water molecules with the manganese-containing mineral deposited on the calvarial surface. There was some signal enhancement on the edge of the control specimens closest to the Mn-treated specimen. This was attributed to the uptake of manganese ions released by metastable mineral disrupted during the preparation of the treated specimens for MRI. Notably, the calvarial surface of the treated specimens was not uniformly enhanced. In some localized areas the T1 values were not enhanced as much as the surrounding bone. In all specimens, however, the lowest signal intensity was localized along the calvarial ridge which had the thickest bone or the least volume of water available to interact with the calvarial surface. With increasing culture times, the calvarial bones grew in size and many of the sharp angles observed in DIV4 specimens became less prominent, possible due to the different growth rates of the 4 tissue zones indicated in Fig. 4, specifically, the orbital surface and S1, S2, and S3 of the superficial surface. Signal loss at the top and bottom of the MRI images was dictated by the limited detection volume of our RF probe.

To test our hypothesis that MEMRI can be used to estimate the level of mineralizing activity in organ cultured calvariae, we analyzed the local variation in T1 values of water protons situated over the 4 tissue zones shown in Fig. 5. Next, we calculated $\Delta R1$ by subtracting ($1/T1_M$), the relaxation rate ($R1_M$) of Mn-treated specimens, from ($1/T1_C$), the relaxation rate ($R1_C$) of its age-matched control. Then using the standard equation for contrast agent relaxivity, we estimated $\Delta R1$ to be proportional to the surface concentration of manganese [Mn] if we assume (i) the relaxivity of the manganese-containing deposits is constant and (ii) the volume of water above each zone is constant. Thus, high $\Delta R1$ values suggest high levels of manganese sequestered at the calvarial surface, which implies high levels of

mineralizing activity. To explore the utility of $\Delta R1$ values for mapping levels of mineralizing activity, we plotted $\Delta R1$ for the 4 time points (4, 11, 18, 25 days) for each tissue zone. These results are presented in Fig. 5. We found that for the orbital surface mineralization remained fairly constant for the days we investigated. While in the S1 zone mineralization tapered off by day 18 but increased significantly on day 25 ($p=0.005$). In zones S1, S2, and S3, the least mineralization occurred on day 18 and the most mineralization occurred on day 25. To be able to compare changes in the surface [Mn] content for the different zones, we need to take into account the differences in tissue thickness. We can, however, report that on days 4 and 11 mineralization occurred in all zones but was dramatically reduced on day 18. On day 25, mineralization was localized to the S3 surface. It should be noted that some of the reported $\Delta R1$ values may be somewhat attenuated because the control sample absorbed manganese from the Mn-treated sample during the MRI experiment which could explain the variability in the $\Delta R1$ values presented in Fig. 5.

LA-ICP-MS of organ cultured calvaria

After our MRI studies, we subjected DIV4 and DIV25 specimens to LA-ICP-MS, in which a laser ablation unit is coupled to the ICP-MS system to allow the generation of spatially dependent trace element maps [21]. Representative T1 maps of DIV4 and DIV25 specimens are shown in Fig. 6A and B, respectively. These T1 maps confirm that more manganese is sequestered on day 4 compared with day 25. Additionally, regional changes in T1 values with respect to age-matched controls confirmed that the DIV4 sequestered more manganese on all surfaces while DIV25 sequestered more manganese on the posterior regions of the superficial surface. These MRI results were validated against manganese elemental maps acquired using LA-ICP-MS for DIV4 and DIV25 specimens, shown in Fig. 6C and D, respectively. Elemental manganese maps supported our MRI findings that DIV4 specimens sequester more manganese than DIV25 specimens and that reduced levels with preferential uptake of manganese by the posterior regions of the superficial zone occurred on day 25. Within each zone, we found pockets where the T1 values were higher than the surrounding bone (white arrows). These zones were found to have lower levels of manganese on LA-ICP-MS maps than the surrounding bone of comparable thickness. This result was consistent with localized mineralizing events occurring concurrently with the areal expansion of the underlying bone. The calcium and phosphorus elemental maps (data not shown) confirmed that the levels of calcium and phosphorus at the calvarial surface was higher for DIV25 compared with DIV4, possibly due to the maturation of mineralized deposits.

Discussion

In an earlier publication, we established that manganese added to culture medium is taken up by primary osteoblasts and is deposited in the mineral phase found on the surface of organ culture calvariae [6]. The manganese taken up by the cells was detectable by MRI due to the effective relaxation sink afforded by manganese ions bound to cytoplasmic proteins. For mineralizing calvariae, maintained in culture medium supplemented with 1 mM $MnCl_2$, the MRI signal was virtually absent in those locations containing high surface concentrations of Mn-containing mineral. The composition of the surface mineral was verified by electron probe microanalysis. Our previous experiments, however, used very high doses of manganese (1 mM) and chronic exposure to demonstrate the feasibility of using manganese as a surrogate for calcium to sensitize the MRI experiment to the formation of mineral deposits. Owing to the putative cytotoxic effects of high manganese doses, we decided to establish the lowest concentration of manganese that can be tolerated by osteoblasts to generate a reproducible deposition pattern in calvarial skull bones after just 24 h of exposure.

Cell pellet studies were performed using low manganese doses (25 to 500 μM) to assess the uptake of manganese by treated cells. In these experiments, the confluent cell layer was treated for 48 h with Mn-containing culture media with excess calcium (3 mM) to maximize the uptake of cations by the cells. We found that the manganese content of the cell pellets increased with manganese dose but it was not monotonic, possibly due to manganese depletion at the lower doses and cell death with cell loss at the higher doses. Importantly, the T1 of the cells treated with the 25 μM dose (2.04 ± 0.06 s) was significantly lower than that of untreated cells (2.82 ± 0.27 s; $p < 0.001$), despite the relatively small difference in their manganese contents, 58 ± 8 and 11 ± 4 ppm ($p = \text{NS}$), respectively. This result supports our hypothesis that the relaxivity of Mn^{2+} is significantly enhanced in the presence of cytosolic proteins [22–24]. Thus, very low levels of cytosolic manganese can have a marked effect on cell pellet T1 values.

Cell viability studies confirmed that cells were marginally impacted by the 25 μM MnCl_2 dose after just 24 h. However, to avoid concerns about manganese depletion, we increased our dose to 100 μM for organ cultured calvarial studies. Elemental data derived from ICP-OES studies confirmed the continued deposition of mineral by control and Mn-treated calvariae with increasing time in culture. The resulting Ca/P molar ratio for control calvariae increased from 1.54 on day 4 to 1.60 on day 25 which was consistent with the substitution of HPO_4^{3-} with CO_3^{2-} ions during the maturation of the mineral phase [25]. With Mn-treatment, there was no significant difference in the calcium and phosphorus contents of the Mn-treated specimens compared with age-matched controls, except for DIV4 where the phosphorus content of the Mn-treated specimens was slightly lower than that of controls ($p = 0.05$). It is not clear if our selected manganese dose inhibited the action of alkaline phosphatase, a metal dependent enzyme responsible for degrading the mineralization inhibitor pyrophosphate and increasing the local concentration of inorganic phosphate for mineralization [26]. The transient nature of this effect suggests that manganese may interfere with this important process during the earliest phases of mineralization. Additional studies will need to be done to understand the inhibitory effect of manganese on this enzyme.

The manganese content of control specimens did not exceed 15 ppm and it did not increase with culture time. We attributed the observed variability in our controls to variable trace levels of manganese either supplied by the egg shell during development or from the fetal bovine serum used to supplement the culture medium. For Mn-treated specimens, there was no significant difference in manganese content between DIV4 and DIV11 or between DIV18 and DIV25 specimens. However, there was a significant decline in manganese content going from DIV11 (950 ± 11 ppm) to DIV18 (710 ± 32 ppm; $p < 0.001$). This decline may be attributed to (i) mineral maturation with less manganese sequestration in already mineralized zones, (ii) new manganese-containing mineral deposits in smaller, previously unmineralized areas arising from an increase in the areal size of the calvarial bones, or (iii) reduced manganese diffusion into the bone due to the mineral accumulation within its interstitial spaces. This may also explain the comparable levels of manganese for DIV18 (710 ± 32 ppm) and DIV25 (688 ± 38 ppm; $p = \text{NS}$) specimens.

Manganese sequestration in mineral deposits at the calvarial surface had a marked effect on water proton T1 values compared with age-matched controls. We found that by subtracting R1 values of age-matched controls from those of Mn-treated specimens, the mineralization activity for each zone can be assessed in terms of its surface [Mn] content. This basic linear relationship between ΔR1 and [Mn] assumes that the relaxivity of the manganese-containing mineral is constant. If manganese replaces a fixed number of calcium ions in the hydroxyapatite crystal structure, it is safe to assume that the relaxivity of hydroxyapatite mineral with trace amounts of manganese is a constant. However, the manganese concentration used in the relaxivity equation can be assumed to be proportional to its surface

concentration if we can assume a constant volume of water above the tissue zones being compared. Given this assumption, our MEMRI results have yielded valuable insights into the spatial and temporal mineralization sequence of calvarial bones and confirmed that this highly regulated process proceeds in culture without the added mechanical stimulus of an expanding brain. However, a small change in the surface [Mn] content in the orbital zone with the thickest bone, overlaid with the lowest volume of water, may have a larger effect than a large change in [Mn] in the S3 zone with the thinnest bone and overlaid with the largest volume of water. Thus, our comparisons of mineralizing activity must occur within tissue zones of comparable thickness. In those zones with similar surface [Mn] contents, small increases in tissue thickness can manifest as augmented surface [Mn] content. Consequently, this amplification strategy may prove useful when trying to measure sub-voxel differences in bone appositional rates in response to various treatment paradigms.

Our T1 maps of mineralizing activity were verified using LA-ICP-MS to extract manganese surface concentration maps of samples previously submitted for MRI. A limitation of the LA-ICP-MS technique is that only elements located on the ablated surface are detected and their quantity is a function of the laser ablation efficiency. With MRI, manganese deposited on both the intra- and extracranial surfaces influence water proton T1 values. MRI images closely resemble the manganese elemental maps because the extracranial surface with its calvarial ridge was in contact with nearly all of the water located between the slides used to hold the specimen; while the relatively flat intracranial surface was in contact with mostly glass. Despite the high overall mineral density of the calvarial ridge, only a very thin line of manganese was found at this location possibly due to low levels of active mineralization or reduced laser efficiency in this highly calcified zone.

We suspect that in very thin tissue zones, the overall [Mn] content on LA-ICP-MS maps was lower because there was less material to ablate. Therefore, a better measure of the surface [Mn] content was calculated by normalizing [Mn] to the phosphorus content of the ablated bone. Various ROIs drawn on T1 and LA-ICP-MS maps were used to extract the T1 and new percent [Mn] for the different tissue zones in the Mn-treated DIV4 specimen. We found that R1 was linearly correlated with [Mn] with a slope of $0.37 (\% \text{ s})^{-1}$ and $R^2=0.76$. For the DIV25 specimen the slope was $0.17 (\% \text{ s})^{-1}$ and $R^2=0.80$. The differences in the relaxivity of the Mn-containing mineral for DIV4 compared with DIV25 may be attributed to differences in the amount of calcium substituted by manganese in immature compared with mature mineral deposits. Variation in mineral relaxivity as a function of maturation may also explain the error in our regression analysis. Thus, $\Delta R1$ can be used to estimate the level of mineralizing activity in zones with the comparable mineral compositions and tissue thickness.

With this insight, we revisited the $\Delta R1$ data present in Fig. 5. We found that the lower $\Delta R1$ values for S1, S2, and S3 in DIV11 compared with DIV4 specimens might be attributed to tissue thinning due to tissue expansion as these specimens had similar whole specimen [Mn] contents. The notable reduction in $\Delta R1$ values for S1, S2, and S3 for DIV18 compared with DIV4 and DIV11 specimens was likely due to less manganese sequestration as a result of mineral maturation and from the inclusion of thinner, less mineralized tissue in our ROI measurements. This result supports the reduced whole specimen [Mn] content of DIV18 compared with DIV4 and DIV11 specimens presented in Fig. 3D. The significant increase in the $\Delta R1$ values for the S1, S2, and S3 tissue zones for DIV25 compared with DIV18 specimens might be attributed to the sequestration of more manganese in immature mineral deposits localized to small previously unmineralized areas.

In conclusion, we found that T1 maps of mineralizing calvariae exposed to $100 \mu\text{M MnCl}_2$ for just 24 h was sufficient to highlight those zones within the developing skull undergoing

active mineralization. Moreover, we found that $\Delta R1$ values were proportional to the surface [Mn] content and thereby yielded an important measure of mineralizing activity within each tissue zone. These $\Delta R1$ values were augmented by subtle increases in bone thickness or attenuated due to bone thinning during tissue expansion or due to mineral maturation resulting in less manganese sequestration. To further understand the carefully orchestrated dynamics of skull mineralization, we intend to monitor manganese uptake by calvarial surfaces and the processing of this cation into mineralized deposits by acquiring a series of T1 images as a function of time post manganese treatment. These studies are expected to yield important insights into the timing of various calcium requiring processes during mineralization.

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Fig. 1. Quantitative T1 map (A) of cell pellets treated with culture medium supplemented with 0, 25, 100, and 1000 mM MnCl_2 (bottom to top) for 48 h. Average T1 values (B) and ICP-OES-derived manganese levels (C) for 4 experimental runs plotted against different manganese doses. * $p < 0.05$ vs. control cell pellets are indicated on plots.

Fig. 2.

Bar graph of cell viability for osteoblasts treated with 0, 25, 100, 250, 500, and 1000 μM MnCl₂ after 24 (white) and 48 (filled) hours of exposure. $p < 0.05$ vs. control cell pellets for 24 (*) and 48 (#) hours of exposure are indicated.

Fig. 3.

Averaged quantities of calcium (A), phosphorus (B), and manganese (C, D) for control (white) and Mn-treated (filled) calvariae. Each bar represents a minimum of 5 repeat experiments. Calvariae were treated with culture medium containing 3 mM CaCl_2 and on days 1, 8, 15, and 21 the culture medium was supplemented with 100 μM MnCl_2 for 24 h and after an additional 48 h DIV4, DIV11, DIV18, and DIV25 specimens were submitted for ICP-OES analysis. Control experiments were run in parallel. Significant differences ($p < 0.05$) among specimens from the same group (treated vs. control) are indicated with (*) if compared with a DIV4 specimen or (#) for a DIV11 specimen from the same group.

Fig. 4. T1-weighted images of manganese-treated specimens exposed to culture medium containing 100 μM MnCl_2 for just 24 h. MRI images of manganese-treated (top) and control (bottom) DIV4 (A), DIV11 (B), DIV18 (C), and DIV25 (D) specimens were acquired with a nominal in-plane resolution of 117 μm , 48 h post-treatment. The calvarial ridge (white arrows), midline suture (black arrows), and the orbital (O) and superior S1, S2, and S3 zones of the calvaria are indicated. Scale bar=1 mm.

Fig. 5.

Bar graph of $\Delta R1$ values for the orbital, S1, S2, and S3 tissue zones, calculated by subtracting the relaxation rate of Mn-treated DIV4, DIV11, DIV18, and DIV25 specimens from that of age-matched controls. Each bar represent the average $\Delta R1 \pm$ its standard deviation. Within a specific tissue zone, $\Delta R1$ is proportional to the surface [Mn] content which is an indirect measure of mineralizing activity. $p < 0.05$ vs. DIV25 (*) or vs. DIV18 (#) specimens for each tissue zone are indicated on the bar graph.

Fig. 6. Representative T1 (A, B) and LA-ICP-MS derived Mn elemental maps (C, D) of Mn-treated DIV4 and DIV25 calvariae, respectively. Scale bars represent 1 mm.

Table 1

Experimental parameters for ICP-OES and LA-ICP-MS experiments.

<i>ICP-OES</i>	
Plasma power	1300 W
Cool gas flow	15 L/min
Auxiliary gas flow	0.4 L/min
Nebulizer gas flow	0.8 L/min
Signal monitored	317.929 nm for Ca 213.616 nm for P 257.607 nm for Mn
Replicates	3
Acquisition time	5 s
<i>LA-ICP-MS</i>	
Laser ablation	
Laser wavelength	266 nm
Laser pulse rate	10 Hz
Laser spot size	100 μ m
Laser line scan rate	30 μ m/s
Line spacing	150 μ m
Sweep gas	He, 0.8 L/min
ICP-MS	
Plasma power	1300 W
Cool gas flow	15 L/min
Auxiliary gas flow	1.0 L/min
Sample gas flow	0.9 L/min
Skimmer and sampler cones	Ni
Monitored masses	m/z 30.97, 41.96, 54.94
Dwell time	50 ms
Sweeps	1
Reading/replicate	1