No influence of alimentary zinc on the healing of calvarial defects filled with osteopromotive substances in rats

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SUMMARY Zinc has been demonstrated to play an important role in bone metabolism and is required for normal growth. However, no studies have investigated the influence of zinc on calvarial bone healing in aged or adult rats. The aim of the study was to evaluate whether alimentary zinc supplementation and depletion affect bone healing of calvarial defects implanted with osteopromotive substances in adult rats. Two 5 mm full thickness critical size bone defects were trephined in the central part of each parietal bone of 60 six-month-old male Wistar rats. The bone defects were filled with demineralized bone matrix (DBM), autogenous bone chips, or were left as unfilled controls. The rats were divided into three groups of 20 rats each and received a semi-synthetic diet containing 20, 60, or 120 mg zinc/kg. After 4 months, the biomechanical integrity of the healing defects was evaluated by a punch out test and the healed defects were examined with histomorphometry. Statistical analysis of the data was carried out by two-way analysis of variance and Wilcoxon's non-parametric signed rank test.

Biomechanical testing revealed that the maximum load was significantly higher in DBM-filled defects than in those filled with autogenous bone, and that the defects filled with autogenous bone were stronger than the unfilled controls. The biomechanical findings indicated that the alimentary zinc content did not influence the healing of calvarial defects. No significant difference in maximum load could be established between the three diet groups for any of the filling materials, whereas the highest zinc supplement resulted in an increase in the relative extension on mineralizing surfaces in the control group. Thus, healing of adult rat calvarial defects is not influenced by alimentary zinc supplementation or depletion. Defects filled with DBM were significantly stronger and exhibited significantly more new bone formation than defects filled with autogenous bone or unfilled controls.

Introduction

An increasing number of surgical interventions involving the craniofacial skeleton are being performed. However, repair and reconstruction of bone, especially when the defects to be repaired surpass a certain size, presents a significant problem. Tissue engineering is essential for healing of large bone defects, for example secondary to clefts, tumours, and trauma. Intraorally autogenous bone grafts from the mandible, or, if insufficient, from the iliac crest have been the preferred method for enhancing closure of major defects, although not without complication such as donor site morbidity (Perry, 1999). For these reasons, research has continued to be directed towards the development of biomaterials that provide osteoinductive and osteoconductive qualities. Other methods for resolving bone defects are allografts, demineralized bone matrix (DBM), hydroxyapatite, bone morphogenetic proteins, and several related growth factors (Giannoudis *et al.*, 2005).

Independent of the interest in grafting materials, the influence of trace elements on the healing of bone has attracted increasing attention (Dollwet and Sorenson, 1988; Benderdour *et al.*, 1998; Barceloux, 1999). Among those, zinc has attracted the most interest, as it has been shown to influence both growth and healing of experimental bone defects in animals (Calhoun *et al.*, 1970; Chesters, 1978; Ovesen *et al.*, 2001). These studies on the influence of zinc on bone have focused on growth and healing of experimentally produced defects of endochondral bone (Cha and Rojhani, 1997). On the other hand, the influence of zinc on the healing of intramembranous bone has not been investigated. Craniofacial surgery is mainly performed on intramembranous bone and a direct extrapolation of the findings from endochondral bone cannot be made, as both the growth process and the mineralization differ between endochondral and intramembranous bone.

Therefore, the aim of the study was to investigate whether alimentary zinc supplementation would lead to enhanced healing of critical size intramembranous bone defects filled with either DBM or autogenous bone in a mature rat model.

Materials and methods

The handling and care of the animals was approved by the Danish Animal Experiment Inspectorate.

Animals and diets

Sixty 6-month-old male Wistar rats (Møllegaard, Lille Skensved, Denmark) were randomly allocated to three

equal groups. The animals were housed in pairs in metal-free cages in rooms with a controlled temperature (21 ± 2°C) and a 12 hour light/dark cycle. The animals were given free access to distilled water and to a semi-synthetic diet (Altromin, Brogaarden, Gentofte, Denmark) with different amounts of zinc added. Diet 1 ($n = 20$) contained 20 mg zinc/kg, diet 2 ($n = 20$) 60 mg zinc/kg, and diet 3 ($n = 20$) 120 mg zinc/kg. Earlier studies have established that a normal zinc diet contains approximately 40–60 mg zinc/kg (Swenerton and Hurley, 1968; Ovesen *et al.*, 2001, 2004). Consequently, a diet containing 120 mg zinc/kg was considered to be above normal, a diet containing 60 mg zinc/kg as normal, and a diet containing 20 mg zinc/kg as subnormal in zinc content.

Anaesthesia and surgical procedures

Critical size calvarial defects were created bilaterally as previously described (Bosch *et al.*, 1995; Jones *et al.*, 2007). In brief; the rats were anaesthetized with a combination of etorphine–acepromazine 1.25 ml/kg (Immobilon, Pharmacia A/S, Hillerød, Denmark) and atropine 0.11 mg/kg administered subcutaneously. The dorsal part of the cranium was shaved and aseptically prepared for surgery through an incision approximately 15 mm long in the skull to expose the temporal bone on both sides of the sagittal suture.

Two full thickness bone defects, 5 mm in diameter, were drilled using a trephine (No. 227–811 001, Messinger, Düsseldorf, Germany) in the centre of the parietal bone under constant irrigation with sterile physiologic saline in order to prevent overheating of the bone margins (Figure 1). During the surgical procedure, care was taken not to damage the dura mater or to puncture the superior sagittal sinus.
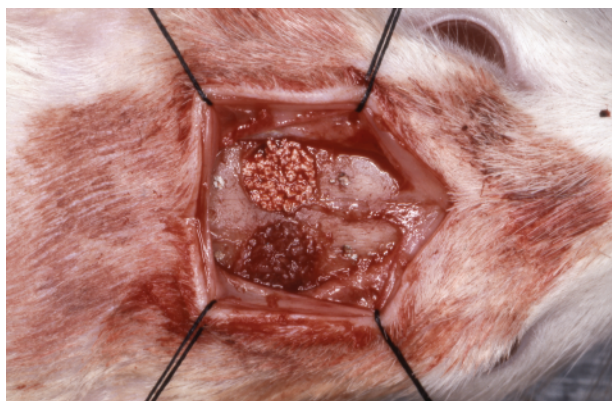

Figure 1 Photograph of an adult rat cranium showing two full thickness bone defects, 5 mm in diameter, trephined in the centre of the parietal bone, and amalgam markers. The right defect is filled with demineralized bone matrix and the left unfilled as a control.

From each diet group, seven animals were assigned to have their calvarial defects filled with DBM, seven with autogenous bone, while the remaining six had their defects left unfilled. The DBM was prepared in-house from intramembranous bone obtained from two additional rats (Nidoli *et al.*, 1999). The trephined bone segments were crushed into small chips and used as autogenous intramembranous bone filler in the defects. The bone chips were packed as densely into the defect as their irregular shape would allow without changing the calvarial gross morphology, similar to the method currently used in orthognathic surgery. The periosteum and muscles were then sutured back in place, using catgut 4.0 (Ethicon, Norderstedt, Germany), and the scalp was sutured with silk 4.0 (Ethicon). After drilling the control defect, the wound was closed in a similar way. To observe wound healing, a clinical evaluation was performed daily for the first three post-operative days and thereafter, on a weekly basis in order to assess healing, any development of infection, and the general condition of the animals.

All animals were injected intraperitoneally with 15 mg/kg tetracycline (Sigma-Aldrich, St Louis, Missouri, USA) 7 days before sacrifice and with 20 mg/kg of calcein (Sigma-Aldrich) 2 days before sacrifice (Allain *et al.*, 1995).

The animals were killed with an overdose of carbon dioxide, 4 months after surgery. Following sacrifice, the heads were detached and the calvarial skin was dissected to facilitate the removal of the defect sites with surrounding bone, soft tissues, and brain. The two parietal bones were separated along the sagittal suture with a parallel-precision saw (Exakt, Apparatebau, Norderstedt, Germany). The specimens were trimmed, leaving 2–3 mm of intact bone around the defect. The right parietal specimen was wrapped in saline soaked gauze and stored at −20°C until subsequent biomechanical testing, whereas the left parietal specimen was fixed in 70 per cent ethanol and prepared for histological evaluation.

Biomechanical testing

On the day of mechanical testing, the bone specimens were slowly thawed to room temperature. The mechanical strength of the healed defects was determined with a modified punch out test in a material testing machine (Alwetron TCT5, Lorentzen and Wettre, Stockholm, Sweden) as previously described in detail (Jones *et al.*, 2007). In brief, the specimens were carefully positioned in a custom-made aluminium jig, which was then transferred to the materials testing machine. The load was applied with a 4 mm diameter steel piston at the centre of the defect at a constant deflection speed of 2 mm/minute. During mechanical testing, load-deflection values were recorded and stored using the computer software supplied with the testing machine. The ultimate load F_{max} (the highest point on the load-deflection curve) was considered as the strength of the healed defect.
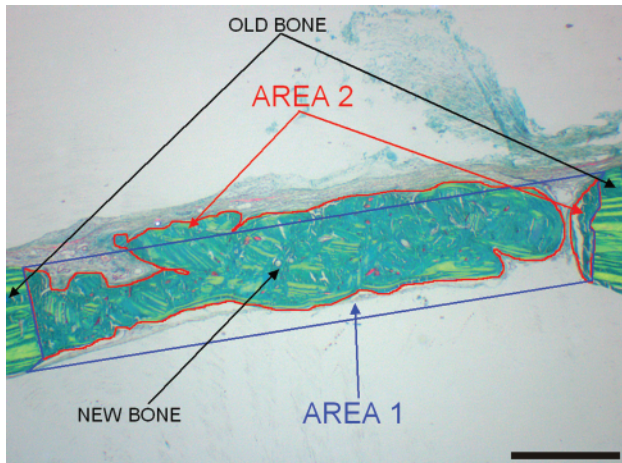
Figure 2 A representative section stained with Masson Goldner trichrome, which stains osteoid tissue red and mineralized tissue green. The images were obtained with a light microscope under polarized light, which accentuates the lamellae in the bone to facilitate the discrimination of new bone from old. Area 1 depicts the original pre-surgical calvarial bone and area 2, the new bone formed within the defect. Bar = 1 mm.

Preparation of histological sections

The left calvarial bone specimens were embedded, undecalcified, in methyl methacrylate. Sections, 7 μm thick, were cut parasagittally through the centre of the healed defect using a hard tissue microtome (Polycut E, Leica Instruments GmbH, Nussloch, Germany). First six sections were obtained, then 15 sections were discarded, and then another set of six serial sections were cut. The first four sections of each set of six sections were stained with Masson Goldner trichrome, whereas the last two sections of each set of the six sections were left unstained for fluorochrome-based analysis.

Histomorphometry

Digital images of the sections were obtained using an Olympus, BH2 light microscope (Olympus, Tokyo, Japan) equipped with a ColorView I digital camera (Olympus) and an integrated 10 × 10 line grid in the eyepiece. The microscope was also equipped with polarized light in order to allow newly formed woven bone to be distinguished from old lamellar bone.

The outline of the tissue (bone + marrow) developed in the original defect was demarcated by following the outer contour and the area of this region was determined using a computer program for digital image analysis (Olympus DP-Soft version 3.2, Soft Imaging System GmbH, Münster, Germany). Likewise, a region representing the pre-surgical outline was drawn and the corresponding area calculated (Figures 2 and 3). The healed tissue area was expressed as a fraction of the original area. This fraction was further corrected for the porosity of the newly formed tissue. The porosity was obtained at a magnification of ×250 using the eyepiece grid.
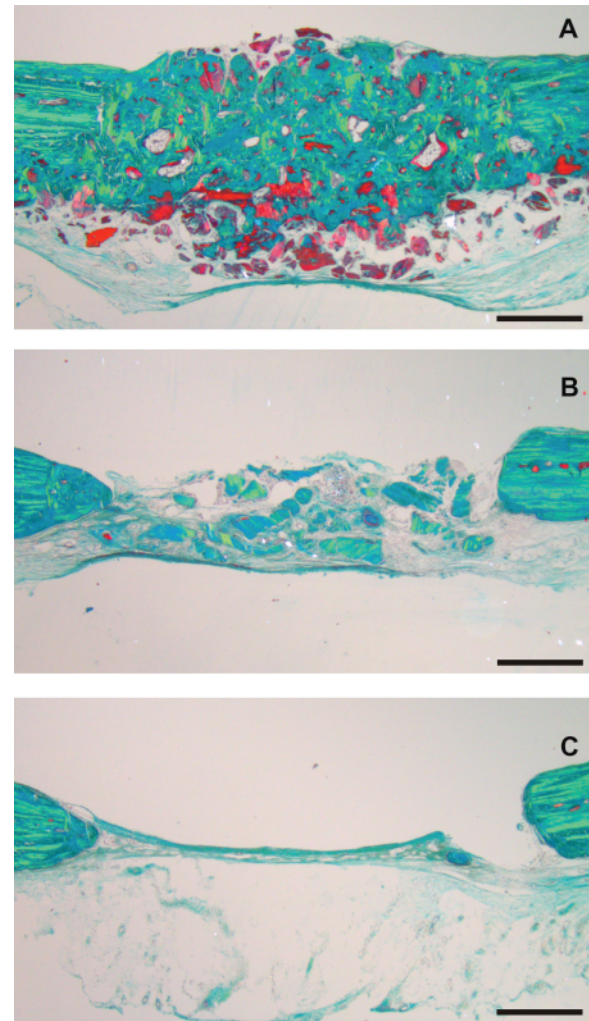

Figure 3 Photograph of calvarial defects filled with (A) demineralized bone matrix (DBM), (B) bone chips, and (C) unfilled controls. The sections were stained with Masson Goldner trichrome and were obtained with a light microscope under polarized light. The DBM (A) shows bone tissue regeneration beyond the original edge of the defect. Areas of diploë were present in the centre of the defect indicating that remodelling of woven bone to cancellous mature bone was occurring. Bar = 1 mm.

Fluorochrome labelling allowed for localization of new mineralizing surfaces laid down at the time of the injection. The intersections between the lines of the grid and the bone surface in the healing zone were evaluated at a magnification of ×100. The relative extent of mineralizing surfaces was determined as the number of grid intersections with labelled surfaces divided by the number of grid intersections with either labelled surfaces or non-labelled surfaces (Wu and Frost, 1969). The entire healing area was covered and the grid was rotated at random between each field of view. As the total number of active surfaces was dependent on the total number of surfaces, a correction for the difference in the total number of surfaces present in the different groups was performed by counting the number of grid intersections per field and expressing the mineralizing surfaces as a ratio in relation to the control group.

Statistical analysis

Descriptive statistics were applied to both the parameters obtained by the mechanical and histomorphometric evaluations. The effect of zinc was assessed by a Wilcoxon's non-parametric signed rank test within each treatment regimen.

The interaction of the different treatments on the defects and the amount of zinc in the diet was analysed by two-way analysis of variance. For all tests, a significance level of $P = 0.05$ was assumed (Campbell and Gardner, 1988).

In addition, the correlation between the mechanical strength and the healing expressed as bone volume was evaluated by Wilcoxon's non-parametric signed rank test (Table 1).

Results

During the experiment, three animals died due to excessive anaesthesia, and one during surgery. All the remaining rats recovered well during the post-operative period. No significant changes in body weight were observed between the beginning and end of the study in any of the animals. No infection was seen in the area of the defect in the skin, which completely healed over the site.

Biomechanical testing

The dietary zinc concentrations did not have a significant impact on the strength of the healed defects regardless of the implant material used (Table 1, Figure 4). In contrast, the material used for filling the calvarial defects had a substantial influence on the strength of the healed defects. The strength of the defects filled with autogenous bone was higher than that of the control defects but not significant for any dietary group. The ultimate load to failure seen in the DBM-filled defects was significantly higher ($P < 0.05$) than the defects filled with autogenous bone or in the unfilled controls.

Subsequent stereomicroscopic inspection of the defects revealed that all failures occurred within the defect itself and not within the surrounding normal bone.

Histomorphometry

The amount of bone in the healing defects varied between 14.2 and 119.8 per cent of the original defect (Tables 2–4, Figure 3). Low values were found in the control group and high values in the DBM group. In some cases, the amount of bone exceeded 100 per cent, indicating that the newly formed bone was thicker than the original bone. No difference between the dietary groups could be verified regarding the amount of bone in the defects.

The cortical porosity for the DBM-filled healed defects was 15.6, 18.0, and 18.3 per cent for the zinc diets of 20, 60, and 120 mg/kg, respectively, whereas the porosity of the defects filled with autogenous bone was 2.0, 1.9, and 2.9 per cent for the three zinc diets, respectively. The cortical porosity of the DBM-filled defects were significantly higher than that for the defects filled with autogenous bone, independent of dietary zinc content.
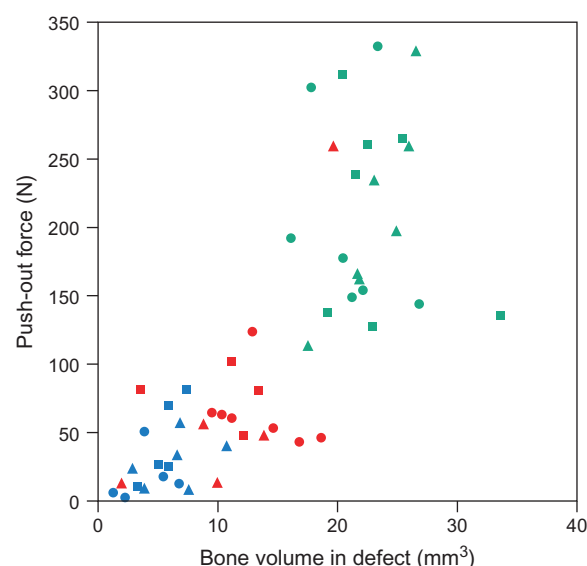

Figure 4 Relationship between biomechanical push out strength and the amount of bone within the defect. Squares: 20 mg/kg zinc; circles: 60 mg/kg zinc; and triangles: 120 mg/kg zinc. Blue symbols: control defects; red symbols: autogenous bone chips; and green symbols: demineralized bone matrix.

Table 1 Maximum force values (F_{max}) for the two different filling materials at the three different zinc diet concentrations. Defects filled with each material were tested biomechanically. The values are given as to means ± standard error of the mean.

	F_{max} (n)			
	Group 1 (20 mg/kg)	Group 2 (60 mg/kg)	Group 3 (120 mg/kg)	Total
Control	42.89 ± 31.02	18.00 ± 19.25	28.95 ± 18.93	29.87 ± 24.05
Bone chips	78.10 ± 22.29	65.04 ± 27.26	43.15 ± 30.30	61.46 ± 28.86
Demineralized bone matrix	210.94 ± 75.51*	207.37 ± 77.49*	209.06 ± 71.73*	209.12* ± 71.12

*Significantly ($P < 0.05$) different from control animals and autogenous bone chips.

Table 2 Bone volumes for the two different filling materials at the three different zinc diet concentrations. The values are given as the means ± standard error of the mean.

	Volume of regenerated tissue (%)			
	Group 1 (20 mg/kg)	Group 2 (60 mg/kg)	Group 3 (120 mg/kg)	Total
Control	14.24 ± 6.09	14.29 ± 11.55	23.81 ± 12.97	17.45 ± 5.51
Bone chips	37.07 ± 21.21	51.19 ± 17.31	34.83 ± 23.98	41.03 ± 8.87
Demineralized bone matrix	119.82 ± 24.34	90.13 ± 19.98	114.06 ± 12.84	108.0 ± 15.74

Table 3 Cortical porosity of the bone formed in the defects. Ten random samples were tested for each alimentary zinc concentration. The values are given as the means ± standard error of the mean.

	Cortical Porosity of repaired tissue		
	Group 1 (20 mg/kg)	Group 2 (60 mg/kg)	Group 3 (120 mg/kg)
Control	2.46 ± 1.33	7.72 ± 15.61	4.8 ± 4.94
Bone chips	1.97 ± 0.75	1.87 ± 1.06	2.85 ± 2.44
Demineralized bone matrix	15.56 ± 8.07	18.04 ± 14.28	18.25 ± 9.07

Table 4 New mineralized bone surfaces expressed as a percentage of the total bone tissue formed within the defect. The values are given as means ± standard error of the mean.

	Mineralized bone surfaces/total bone tissue (%)		
	Group 1 (20 mg/kg)	Group 2 (60 mg/kg)	Group 3 (120 mg/kg)
Control	25.98 ± 15.35	27.57 ± 10.61	47.60 ± 15.28*
Bone chips	46.79 ± 16.33	36.66 ± 10.37	34.83 ± 23.98
Demineralized bone matrix	46.45 ± 10.02	55.08 ± 8.99	56.53 ± 7.77

*Significantly ($P < 0.05$) different from diet groups 1 and 2.

The defects filled with DBM showed extensive new bone ingrowth and a good interface between the new and old bone at the edges of the defect. No old bone was seen across the entire defect space (Figure 3A). In addition, no ectopic bone formation was observed in any of the animals implanted with DBM. The defects filled with autogenous bone consisted mostly of old inert bone from the graft with empty osteocyte lacunae, whereas new bone was sparser (Figure 3B). The control defects showed only limited or no bone formation at the edges of the defect and only a connective tissue layer between the periosteum on the surface of the dura mater, thus confirming the critical size of the defects (Figure 3C).

The animals in the DBM group had a higher percentage of bone surfaces undergoing mineralization than the control animals for the two lowest dietary zinc concentrations, whereas no differences in mineralizing surfaces was observed for the highest alimentary zinc group.

When correcting for the difference in the amount of bone available or the number of bone surfaces present, the DBM group exhibited nine times more bone formation activity than the control and 1.5 times more bone formation activity than the autogenous bone group.

Discussion

Current techniques for surgical reconstruction of large osseous defects are either autogenous bone (Heiple *et al.*, 1987), DBM (Mulliken and Glowacki, 1980), or various calcium phosphate cements (Bucholz *et al.*, 1987). Autogenously harvested bone from the iliac crest is considered the gold standard among available graft materials; however, it has several disadvantages such as post-operative morbidity, the grafts are difficult to contour to a desired shape, and they often undergo unpredictable resorption (Younger and Chapman, 1989). DBM, however, has been used with considerable clinical success in craniofacial reconstruction (Glowacki *et al.*, 1981; Tiedeman *et al.*, 1995). After mild acid extraction of the transplant bone, the majority of proteins are removed; however, a cross-linked osteoconductive collagen matrix with insoluble adherent proteins is preserved (Hardin, 1994). Once the mineral phase is removed, the remaining proteins, called 'bone morphogenic proteins', are more bio-available, i.e. more osteoinductive than the mineralized graft (Urist *et al.*, 1975; Fleming *et al.*, 2000). The role of DBM in craniofacial surgery is based on its unique handling properties. When completely demineralized, DBM is extremely flexible and can easily be shaped, while partially demineralized, it maintains a greater structural rigidity (Hardin, 1994). The major advantage of DBM is its ability to induce bone formation where there is a functional need for bone reconstruction, for example after trauma. On the other hand, unpredictable resorption of the implants results when the functional matrix does not support bone induction (Ousterhout, 1985). When DBM was combined with autogenous bone for repair of alveolar defects, it resulted in new bone formation similar to that achieved with guided

tissue regeneration (Levine *et al.*, 1992). The role of calcium phosphate ceramics in reconstructive orthopaedics is primarily osteoconduction, serving as a scaffold for the osteoprogenitor cells and a reservoir of hydroxyapatite necessary for the formation of new bone (Bucholz *et al.*, 1987). The clinical drawbacks related to the use of ceramic implants are that they are brittle and have poor tensile strength (Bucholz *et al.*, 1987).

Apart from the transplant material itself, the healing process might be enhanced by the addition of trace elements such as manganese, iron, copper, and zinc, which are bound to proteins forming metalloproteins. Many of the metals in metalloproteins are part of enzyme systems that have important structural and storage functions (Fraga *et al.*, 2005). Zinc is involved in the activity of approximately 100 enzymes and its deficiency in humans is common in underdeveloped countries and mainly associated with malnutrition affecting the immune system, wound healing, and impaired DNA synthesis.

The importance and necessity of zinc for tissue engineering after injury has been known for some time (Milachowski *et al.*, 1980); however, the exact mechanism by which it affects osteogenesis is still unknown. Zinc has been found to increase bone mineralization both *in vivo* (Yamaguchi *et al.*, 1987) and *in vitro* (Chen *et al.*, 1999; Ovesen *et al.*, 2001). In contrast, only very limited information is available on the effect of zinc on bone healing and that has mainly been limited to growing animals (Ovesen *et al.*, 2001).

The decision to combine a nutritional factor such as zinc with known graft materials, autogenous bone, and DBM in the present study was made in order to observe whether zinc ions would cause a stimulation of healing in critical size defects in intramembranous created bone. However, the positive effect of DBM found by Jones *et al.* (2007) was not further enhanced by increased dietary zinc. The findings were thus not what could be anticipated from earlier research (Ovesen *et al.*, 2001). That study suggested that increased levels of zinc ions could result in increased bone strength in growing rats. Those authors also verified that zinc had a stimulatory effect on bone growth. The possible role of zinc in bone healing was suggested by a significant lowering of zinc concentration in the tibia of rats following fracture (Milachowski *et al.*, 1980). However, while no stimulation of the healing process was observed in the present study, the results confirmed that the biomechanical strength of the DBM-filled defects was significantly greater than the defects filled with autogenous bone or control defects (Jones *et al.*, 2007).

The lack of a tissue engineering effect from zinc supplementation could be ascribed to the type of bone tested. All previous reports of positive impact of zinc ions on bone growth and healing have referred to bone of endochondral origin, where matrix vesicles are involved in the induction of calcification (Ovesen *et al.*, 2001). In contrast, healing of bone of intramembranous origin seems not to be dependent on the levels of zinc ions.

The dose of zinc used in the present study was 20 mg zinc/kg in the zinc deficient animals, 60 mg zinc/kg in the controls, and 120 mg zinc/kg in the supplemented diet, in parallel with the study of Ovesen *et al.* (2001). However, the recommendations regarding dosage of zinc supplement is controversial. Swenerton and Hurley (1968) reported requirements of 100 ppm as a satisfactory level of zinc, while Williams and Mills (1970) stated that 5–13 ppm was sufficient. Based on a review of the scientific literature, the dietary zinc requirements for weaning and adult rats is 12 mg zinc/kg and 25 mg zinc/kg for lactating rats. Therefore, the highest zinc dosage used in the present study was more than adequate for being considered as a zinc supplementary diet. Moreover, even if the zinc dosages selected were slightly higher than those used by Ovesen *et al.* (2001), the two lowest are still well within the dose response range shown in that research. Consequently, the dosages used in the present study cover a large range and it is unlikely that selecting different alimentary zinc dosages would have changed the outcome.

The lower amount of bone formed in the defects filled with autogenous bone when compared with that seen in the DBM grafted sites indicates a slower graft incorporation in the autogenous bone-filled defects as also found by Burchardt (1983). An attempt was made to assess the mineralization rate by means of intravital staining injected 7 and 2 days before sacrifice. However, it was not possible to distinguish the two fluorochrome labels from each other in the autogenous bone-filled defects as they appeared as one broad line. This indicates a slower formation of bone on the surface of the autogenous bone chips than that seen in the DBM-treated animals.

Although a direct positive effect from zinc supplementation on bone strength was not observed in the present study, a zinc supplemented diet may still be indirectly beneficial to skeletal health. Clinical studies on increased zinc loss in middle-aged men via urinary excretion have been shown to be associated with developing osteoporosis (Atik, 1983) leading to an increased risk of bone fractures (Elmståhl *et al.*, 1998).

Conclusion

Alimentary supplementation with zinc does not substantially enhance healing of calvarial defects in adult rats. The exact role that zinc ions play in bone metabolism and tissue engineering is still not fully known.

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