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The influence of a bisphosphonate on bone generation determined using a chick-femur model

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Abstract

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Aim To determine the direct influence of a bisphosphonate (pamidronate) delivered by one of two carriers, on bone generation in chick-femurs supported by chick egg chorio-allantoic membranes.

Methodology Twenty chick femurs freshly harvested from fertilized eggs were randomly allocated to two groups: (i) Affi-Gel blue bead carrier (n = 10); and (ii) hydroxyapatite bead carrier (n = 10). The femurs in each group were further randomly divided into control (n = 4) and experimental (n = 6) subgroups. Carriers charged with PBS solution or 0.1 M pamidronate were delivered into the bone marrow of each femur at its mid-portion through a needle puncture. Each femur was then grafted onto the chorio-allantoic membrane of a chick egg and incubated for 7 days. Each experimental and control subgroup femur yielded four histological sections at the puncture site, constituting the test and *inter*-bone controls. In addition, two histological sections were also obtained from 400 to 450 μm away from each end of the experimental puncture site to act as the *intra*-bone controls. Bone generation was quantified and the ratio of cross-sectional area of bone marrow to circumference of bone (outcome measure) was determined using a software package, Image-Pro® Plus. The data were analysed using Mann–Whitney tests and Wilcoxon signed rank tests.

Results The outcome ratio in the test group was significantly ($P < 0.001$) smaller than both the *inter*-bone and *intra*-bone control groups. There was evidence of increased bone formation directly over the pamidronate-charged carriers.

Conclusions The test model established that pamidronate had a positive effect on bone generation over a period of 7 days, regardless of the carrier type.

Keywords: bisphosphonates, bone regeneration, chick-femur model, endodontics.

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Introduction

Bisphosphonates fall within a class of drugs that has been used in patients with osteoporosis and other bone diseases since 1982 (Fleisch 1982). Its main benefit is to retain bone (Masarachia *et al.* 1996) but has recently gained notoriety for its reported side effect of localized osteonecrosis of the maxilla and/or mandible (Marx 2003). The management of exposed bone after non-surgical and surgical root canal treatment, following systemic bisphosphonate therapy has become a topical issue (Sarathy 2005, Katz 2005).

In contrast, recent studies have also shown the potential of topical application of bisphosphonates in periodontics (Tenenbaum *et al.* 2002), implant dentistry (Eberhardt *et al.* 2007) and bone grafting (Aspenberg & Astrand 2002) to aid retention or regeneration of bone. The same potential may have applicability for endodontic problems since periapical disease involves bone loss and periapical healing, its regeneration. There is, however, a lack of information on the potential effect of bisphosphonate therapy on the outcome of endodontic treatment; and no published

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research on the potential use of bisphosphonates to aid regeneration of bone in problems of endodontic origin.

Perhaps this is because the mechanisms of action of bisphosphonates are still far from clear. There is some evidence that bisphosphonates fulfill their function by inhibition of osteoclasts but reports of their effect on osteoblasts are conflicting (Fleisch *et al.* 1982, Tenenbaum *et al.* 2002, Woo *et al.* 2006, Orriss *et al.* 2008, Uveges *et al.* 2008). Once the precise mechanism of action of this class of drug has been elucidated, dentists and endodontists may form a clearer understanding of the potential biological applications for therapeutic use in bone regeneration as well as methods for prevention and management of the adverse effects of bisphosphonate therapy.

The aim of this preliminary study was to determine the direct influence of a bisphosphonate (pamidronate) delivered by one of two carriers, on bone generation in chick-femurs supported by chick egg chorio-allantoic membranes (CAM). This is a relatively simple and rapid *ex vivo* model that requires no extensive or expensive mammalian animal laboratory to gauge a biological response (Ribatti *et al.* 1997, Yang *et al.* 2004, Ribatti 2008, Saw *et al.* 2008, Mangieri *et al.* 2009). The approach is free of the administrative burden associated with ethical and legal aspects of mammalian animal experiments and provides an intermediate step between *in vitro* cellular tests and pre-clinical animal experiments (Vargas *et al.* 2007). The method has been used for studying angiogenesis, evaluating pharmaceutical formulation development and bone formation (Yang *et al.* 2004). The method enables easy bone implantation, followed by a simple controllable harvest of the entire femur for processing and microscopic evaluation. The femur can be incubated on the CAM of a host egg acting as a natural bioreactor, supplying it with required nutrients through a viable blood supply. This delivery has been proven in preliminary experiments where calcein added onto the CAM on day 1 could be detected in the histological sections of the embedded femur after 7 days of incubation. As the defect is made in the middle of the long bone and the evaluation is calculated on the width of the bone (bone : marrow ratio), the model is representative of intramembranous rather than endochondral bone formation. This is akin to the mandibular scenario where intramembranous bone formation is predominant and presumably biologically more relevant to the endodontic situation.

In this study, the CAM model was adopted for studying bone generation associated with the local delivery of a bisphosphonate (pamidronate) using two different carrier systems with different affinities and therefore release characteristics.

Materials and methods

All small animal experiments were carried out as described in project license PPL 70/6269 under the supervision of or by researchers with a personal license (K Gellynck: PIL 70/20356), both according to the Animals (scientific procedures) act 1986, Home Office, UK.

Preparation of fertilized chick eggs to act as donors or host

Fertilized *donor* chick eggs were incubated at 37 °C for 14 days to allow embryo development. The fertilized *host* eggs were placed in the incubator at 37 °C on day 7 and windowed after 3 days; their shells were punctured with a sterile scalpel following surface disinfection with 90% alcohol spray (BDH Laboratory Supplies, Poole, UK). 5 mL of albumen was removed with a 19G needle to help detach the developing CAM from the shell. A small rectangular window was created in the shell with a diamond disc, exposing the CAM. The window was sealed with adhesive tape (3M, Bracknell, UK) and the egg was incubated at 37 °C for a further 4 days. On the 7th and 14th day, respectively, the host and donor eggs were ready for femur isolation and their grafting onto the CAMs of the *host* chick eggs.

Preparation of the carriers

Two types of carrier beads were used: Affi-Gel blue (Bio-Rad Laboratories, Richmond, California, USA) and hydroxyapatite (HA). Calcium phosphate biomaterials have already been shown to be good release vehicles for bisphosphonates *in vitro* (Faucheux *et al.* 2009) because of their affinity to bisphosphonates. Affi-gel blue beads are inert allowing an immediate release of the Pamidronate solution; hence the rationale for comparison of the two bead systems. The HA beads were custom prepared; 5 g hydroxyapatite (Sigma-aldrich, Dorset, UK) was mixed with 10ml of 2% chitosan (Sigma Chemical Co., St Louis, MO, USA) solution in 2% acetic acid. The resulting solution was poured into sunflower-oil and stirred to dispense the chitosan–HA solution into small globules. Gluturaldehyde (Sigma Chemical Co.) was added to form cross-links with chitosan. The formed beads were filtered, washed with acetone (BDH Laboratory Supplies) and sintered at

1 300 °C for 2 h. The chitosan and gluturaldehyde were burned away to leave pure porous HA beads (Paul & Sharma 1999). Half of the Affi-Gel blue and HA beads were immersed in PBS (PAA Laboratories, Haidmannweg, Germany) and the other half in 0.1 M pamidronate (Sigma Chemical Co.) solutions for at least 24 h prior to femur extraction on the 14th day.

Extraction of the femurs from donor chick eggs

On the 14th day, the surface-disinfected (as before) shell of the donor chick egg was removed and the chick embryo isolated using sterile forceps. The head of the embryo was cut off with a pair of scissors and the embryo transferred to a sterile Petri dish. All the tissues attached to the femur were removed using micro-forceps and a scalpel under a light microscope (Leitz DMRB; Leica Microsystems Ltd, Wetzlar, Germany). The extracted femurs were transferred to separate sterile Petri dishes in readiness for impregnation with test and control carriers. A total of 20 femurs were randomly assigned to either the Affi-Gel blue (group 1) or HA (group 2) carrier. The 10 femurs in each group were further randomly subdivided into two subgroups: four femurs were allocated to a control subgroup and six to an experimental subgroup.

Impregnation of donor femurs with carriers

A needle puncture (Microlance™ 3 19G, BD, Drogheda, Ireland) was created in the middle of each femur (Fig. 1a) under the light microscope (×2.5) (Leitz DMRB, Leica Microsystems Ltd, Wetzlar, Germany). An equal amount of control or test Affi-Gel blue or HA bead carriers was first placed on the femur surfaces adjacent to the puncture wounds and then pushed into the femur with a 19G needle (Fig. 1b) under the stereo microscope.

Grafting of impregnated femurs onto the CAM of the host chick eggs

The pre-prepared host egg shells were surface-disinfected as before; the adhesive tape was removed and the eggs checked for signs of infection (odour, fungal growth and change in colour to milky white or black); infected eggs were discarded. The prepared femurs were carefully placed onto host egg CAMs near blood vessels to maximize nutrient supply (Fig. 1c). The windows were resealed with adhesive tape (3M, Bracknell, UK) and the host eggs incubated at 37 °C for a further 7 days.

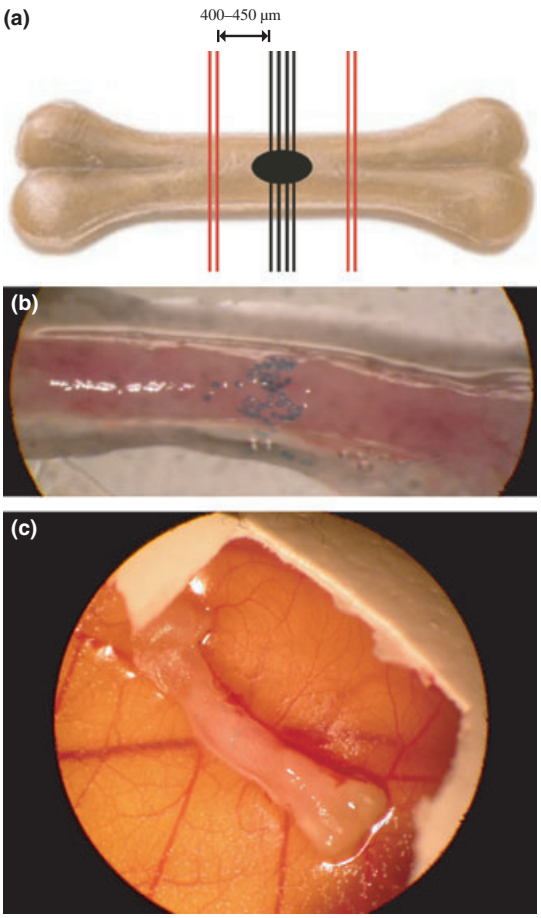

Figure 1 (a) Four consecutive sections centred on the puncture wound were chosen as the test sections (black lines). At a distance of 400–450 μm away from both ends of the puncture wound, two consecutive sections were chosen as the intra-bone control sections (red lines). (b) The Affi-Gel blue bead carriers placed on the femur (×2.5). (c) The femur placed on the CAM of host egg (×2.5).

Histological processing of the femurs

After a further 7 days of incubation (21st day), the femurs were removed from the host eggs and checked for infection. If uninfected, they were fixed in 4% paraformaldehyde (VMR International Ltd, Poole, UK) for 1 day. Decalcification before wax embedding was not necessary as the embryo bone is thin and immature, making it possible to cut it without the need for decalcification. This made it possible to retrieve the HA beads and use the Von Kossa method to stain for bone and HA beads. They were then washed in PBS solution (PAA Laboratories) for 30 min, followed by a series of graded solutions of alcohol (70%, 70%, 70%, 90%,

95% and 100% – BDH Laboratory Supplies) for 30 min each. The final wash was in xylene (Solmedia Laboratory Suppliers, Essex, UK) for 30 min. The femurs were embedded in paraffin wax inside individual cassettes for 1 h and then stored at −20 °C for 1 day.

Serial sections (~1 000/femur), 7 μm in thickness were cut and mounted onto glass slides (BDH Laboratory Supplies). Each glass slide held 7–9 sections mounted in consecutive order. The slides were placed in a heated oven (Sandrest Ltd, Windsor, UK) followed by two 5-min xylene washes to remove the wax. The sections were rehydrated using a reverse alcohol series (100%, 100%, 50%, distilled water, respectively, for 1 min each) to remove the xylene and allow staining.

The sections were stained with von Kossa's and van Gieson stains; the former to stain bone and calcified tissue black and the latter to stain connective tissue red and muscle tissue yellow. Following the Von Kossa method, the rehydrated sections were placed under dark into 0.5% aqueous silver nitrate solution (Sigma Chemical Co.) until they turned pale yellow (5–20 min) and then washed with distilled water to remove excess silver nitrate. The sections were then immersed in freshly prepared 0.5% aqueous hydroquinone (Sigma Chemical) to reduce the silver until they turned black (5–15 s), followed by rinsing in distilled water. They were then fixed in 2.5% aqueous sodium thiosulphate (Hopkin & Williams, Essex, UK) for 5 min, followed by washing under running tap water. The sections were then counterstained with Van Gieson solution for 5 min, blotted dry with absorbent paper and secured with a cover-slip (VMR International Ltd, Poole, UK) and adhesive (DPX mountant; BDS, Poole, UK).

All sections from both groups 1 and 2 were examined under the light microscope (Leitz DMRB; Leica Microsystems Ltd, Wetzlar, Germany). The femurs were discarded if over 30% of the integrity of the femur sections was impaired.

Determination of outcome measures

After analysing all the sections from each femur, the sections with the puncture wound were identified and those with Affi-Gel blue or HA bead(s) were selected. Four consecutive sections centred on the puncture wound were chosen; from the control subgroups as the inter-bone controls and from the test sections as experimental sites (Fig. 1a). Two consecutive sections were taken at 400–450 μm from each extremity of the test puncture wound as intra-bone control sections.

Qualitative assessment of bone generation

Under the light microscope (×10) (Leica Microsystems Ltd, Wetzlar, Germany), mineralized trabecular bone appeared black, newly formed sub-periosteal bone red, bone marrow stained yellow or red and was either centred in the bone or lay slightly eccentrically, surrounded by mineralized or newly formed sub-periosteal bone. The puncture wound appeared as a break in the bone boundary with soft tissue ingrowth extending to the marrow or as a 'U'-shaped deflection of the bone outline with soft tissue ingrowth without 'connection' with the bone marrow. The Affi-Gel blue beads appeared as blue or green circles and HA beads as black or brown particle clusters.

Quantitative assessment of bone generation

Images of the selected sections were taken using a digital camera (EOS D300, Canon Inc., Tokyo, Japan) adapted to the microscope lens (Leica Microsystems Ltd, Wetzlar, Germany). The Image-Pro® Plus software (Media Cybernetics Inc., Silver Spring, USA) was used for image analysis. Measurements were calibrated using a 100 μm ruler. The cross-sectional area of the bone marrow and the outer circumference of the bone were measured by plotting their boundaries and the areas calculated as a routine function of the software. The ''area of bone marrow'' divided by ''area of the whole bone'' (marrow : bone ratio) was calculated. The measurement procedure was repeated one week later for all sections to determine the level of intra-observer agreement. The measurements were also performed by an independent observer for one third of the sections (randomly selected) to determine the inter-observer agreement.

Data and statistical analysis

The mean values and their standard deviations were calculated for the cross-sectional areas of bone marrow and whole bone; the marrow : bone ratios were also calculated.

The intra-observer and inter-observer agreements for the marrow : bone ratios were calculated first by plotting the difference between pairs of readings against the means of the pairs. The 'limit of agreement' was calculated and a decision was made if these limits were acceptable in the context of the present study (Bland & Altman 1986). The one-sample Kolmogorov–Smirnov Test revealed that data on marrow : bone ratios were not normally distributed therefore non-parametric tests were used for analyses. Mann–Whitney tests were used

to compare the marrow : bone ratios between the inter-bone and intra-bone control groups; and the inter-bone control and test groups. Wilcoxon signed rank tests were used to compare the marrow : bone ratios between the intra-bone control and test groups.

Results

Number of femurs and sections selected for qualitative and quantitative analyses

The 20 femurs yielded around 20 000 serial sections at around a 1 000 per femur. Two femurs from group 2 (HA bead carrier) were discarded because more than 30% of the section integrity was impaired. A total of 112 sections centred at the relevant sites were selected, with 64 for group 1 (Affi-Gel blue bead carrier) and 48 for group 2 (HA bead carrier) (Table 1).

Intra- and inter-observer agreements

A total of 40 out of the 112 sections were randomly selected for assessment of the inter-observer agreement in assessing the marrow : bone ratio. The limits of agreement were ±0.037 and ±0.033 for intra-observer and inter-observer, respectively.

Qualitative assessment of bone generation for Affi-gel blue carrier

Inter-bone control group

The cross-sectional area of bone marrow (mean area: 398 461.3 ± 30 332.4 μm^2) was large compared to that of the whole bone (mean area: 698 618.8 ± 44 830.0 μm^2). In half of the femurs, the bone marrow was centred within the bone but in others, it was eccentrically situated. All Affi-Gel blue beads (Fig. 2a) were inside the bone marrow and lay eccentrically. Direct contact between the bone and the bead(s) was not evident. Mineralized bone (Fig. 2a) was always evident but newly formed sub-periosteal bone was *never* evident.

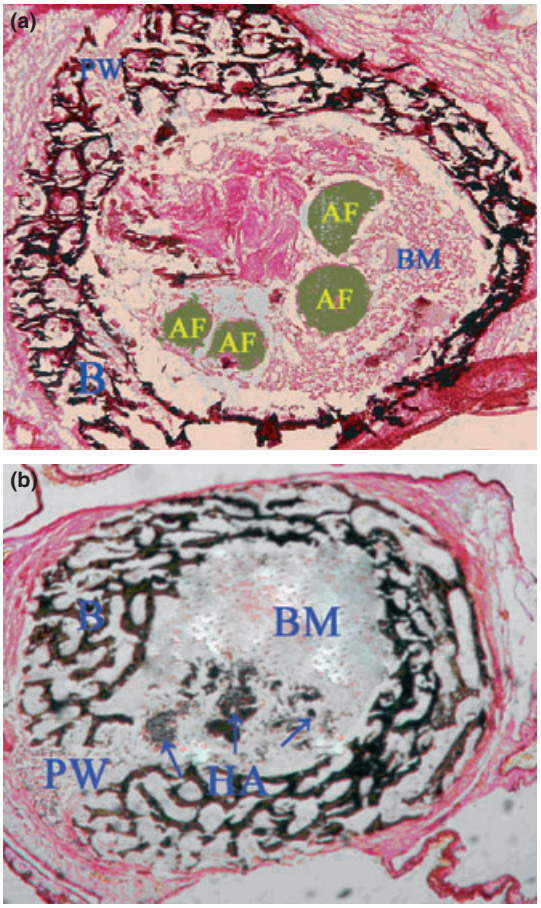

Figure 2 (a) One of the Affi-Gel blue inter-bone control sections. The Affi-Gel blue beads (AF) were situated inside the bone marrow (BM). There was mineralized bone (B) deposited around the bone marrow. (PW): puncture wound (×10). (b) HA inter-bone control sections. HA beads (HA) were situated inside the bone marrow (BM). (B): bone, (PW): puncture wound (×10).

Table 1 Summary of femurs and sections selected

	Subgroup	Section group	No. of femurs included	No. of femurs discarded	No. of sections selected
Group 1	Control	Inter-bone control	4	0	16
Affi-Gel blue	Experimental	Intra-bone control	6	0	24
		Test			24
Group 2	Control	Inter-bone control	4	0	16
HA	Experimental	Intra-bone control	4	2	16
		Test			16
				Total no. of sections selected:	112

Intra-bone control group

The cross-sectional area of bone marrow (mean area: 404 775.6 ± 64 229.1 μm^2) was large compared to that of the whole bone (mean area: 734 910.1 ± 67 090.8 μm^2). The bone marrow in *all* femurs lay eccentrically. Half of the femurs showed newly formed sub-periosteal bone. All the newly formed sub-periosteal bone was on one side of the bone (NB in Fig. 3b). There was a gradual transition from mineralized bone to newly formed sub-periosteal bone (GT in Fig. 3c). Affi-Gel blue beads could not be detected.

Test group

The cross-sectional area of bone marrow (mean area: 127 560.0 ± 196 92.1 μm^2) was small compared to that of the whole bone (mean area: 824 731.6 ± 34 043.6 μm^2). The bone marrow in *all* femurs lay slightly eccentrically as did all the Affi-Gel blue beads. Half of the femurs showed newly formed sub-periosteal bone; these were the same femurs showing new sub-periosteal bone in the intra-bone control groups. There was a transition from mineralized bone to newly formed sub-periosteal bone. Mineralized bone was deposited in between the bone marrow and the puncture wound in *all* femurs. In five out of the six femur samples, mineralized bone formation was evident directly on the beads (Fig. 4i).

Quantitative assessment of bone generation for Affi-gel blue carrier

The mean marrow : bone ratios, in descending order were: 0.57 (±0.04), 0.55 (±0.04) and 0.15 (±0.03) for the inter-bone control, intra-bone control and test groups, respectively. The marrow : bone ratios of the inter-bone group was significantly (P = 0.04) greater than the intra-bone control group, although the difference (mean difference 0.03; 95% CI 0.01, 0.05)

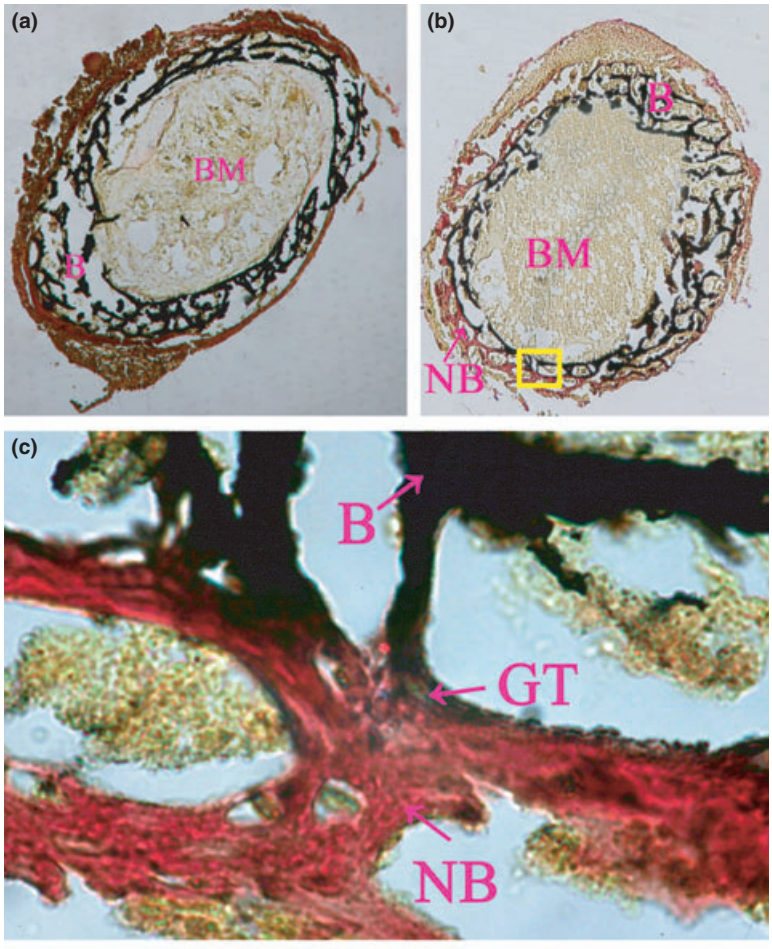

Figure 3 Two of the intra-bone control sections (a) (×10) section with no newly formed sub-periosteal bone (B). (b) Section with newly formed sub-periosteal bone (NB) which was only on one side of the bone (×10). The demarcated area in (b) was magnified in (c) (×40) and showed the gradual transition (GT) from mineralized bone to newly formed sub-periosteal bone. BM, bone marrow.

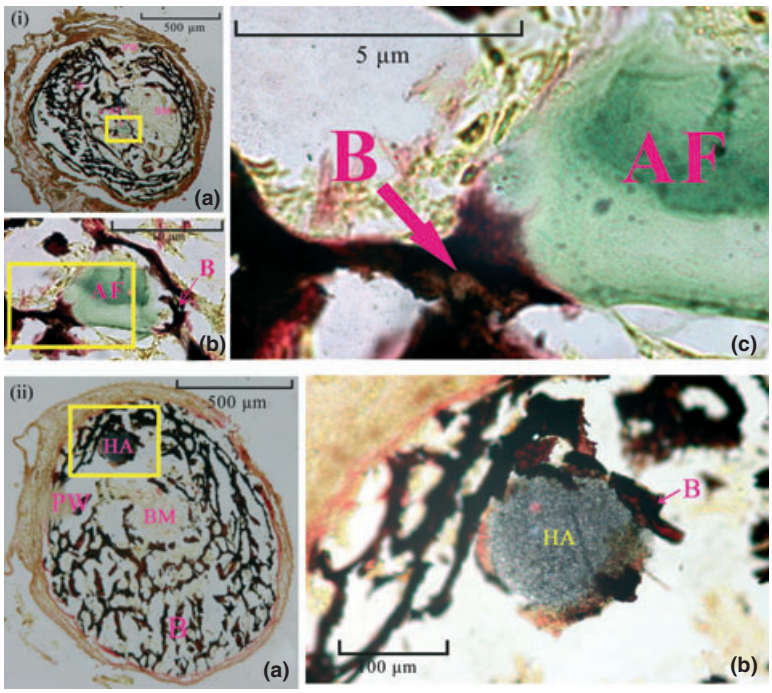

Figure 4 (i) The demarcated area in (a) was magnified in (b) and showed that the Affi-Gel blue bead (AF) was in direct contact with the surrounding mineralized bone (B). The demarcated area in (b) was magnified in (c) and showed the bone formation directly on the bead. (ii) There was mineralized bone (B) deposition in between the puncture wound (PW) and the bone marrow (BM). The demarcated area in (a) was magnified in (b) and showed that the HA bead (HA) was in direct contact with the mineralized bone and there was direct deposition of bone on the bead.

Table 2 Mann–Whitney tests for comparison of marrow : bone ratios of inter-bone and intra-bone control groups; and inter-bone control and test groups. Wilcoxon signed rank test for comparison of marrow : bone ratio of intra-bone control and test groups

	Mean difference (95% CI) in marrow : bone ratio	*P*-value
(a) Affi-Gel blue bead carrier		
Inter-bone–intra-bone control groups	0.03 (0.01, 0.05)	0.04
Inter-bone control – test groups	0.42 (0.40, 0.44)	<0.001
Intra-bone control – test groups	0.39 (0.37, 0.41)	<0.001
(b) HA bead carrier		
Inter-bone–intra-bone control groups	−0.03 (−0.05, −0.01)	0.002
Inter-bone control – test groups	0.29 (0.28, 0.31)	<0.001
Intra-bone control – test groups	0.32 (0.30, 0.34)	<0.001

was small (Table 2). The marrow : bone ratios of inter-bone control and intra-bone control groups were significantly ($P < 0.001$) greater than the test group. The mean differences in ratio were 0.42 (95% CI 0.40, 0.44) and 0.39 (95% CI 0.37, 0.41), respectively (Table 2).

Qualitative assessment of bone generation for HA bead carrier

Inter-bone control group

The cross-sectional area of bone marrow (mean area: 326 496.1 ± 13 325.0 μm^2) was large compared to that of the whole bone (mean area: 707 333.5 ± 30 764.9 μm^2). The bone marrow was centred within the bone in 3/4 femurs, whilst one was eccentrically situated. All HA beads (Fig. 2b) were visible inside the bone marrow and lay slightly eccentrically. Direct contact between the bone and the bead(s) was not evident. All the bone (Fig. 2b) surrounding the bone marrow was mineralized with an absence of newly formed sub-periosteal bone.

Intra-bone control group

The cross-sectional area of bone marrow (mean area: 355 619.8 ± 43 281.6 μm^2) was large compared to that of the whole bone (mean area: 707 063.4 ± 67 555.3 μm^2). The bone marrow lay eccentrically in 3/4 femurs, whilst the final one was centred within the bone. There was no evidence of intact beads. All the bone surrounding the bone marrow was mineralized with an absence of newly formed sub-periosteal bone.

Test group

The cross-sectional area of bone marrow (mean area: 132 285.5 ± 13 298.8 μm^2) was small compared to that of the whole bone (mean area: 773 145.4 ± 33 203.8 μm^2). The bone marrow in *all* femurs lay slightly eccentrically as did the HA beads; they were in the bone marrow in 3/4 femurs. In one femur, the HA beads showed direct contact with the mineralized bone with evidence of deposition of bone on the bead (Fig. 4ii). One femur showed no mineralized bone between the bone marrow and puncture wound; there was an absence of newly formed sub-periosteal bone.

Quantitative analysis of bone generation for HA bead carrier

The mean of marrow : bone ratios, in descending order were: 0.49 (±0.03), 0.46 (±0.02) and 0.17 (±0.02) for the intra-bone control, inter-bone control and test groups, respectively.

The marrow : bone ratios of the inter-bone group was significantly ($P = 0.02$) smaller than the intra-bone control group, although the difference (mean difference −0.03; 95% CI −0.05, −0.01) was small (Table 2). The marrow : bone ratios of inter-bone control and intra-bone control groups were significantly ($P < 0.001$) greater than the test group. The mean differences in ratio were 0.29 (95% CI 0.28, 0.31) and 0.32 (95% CI 0.30, 0.34), respectively (Table 2).

Discussion

The aim of this study was considered to have been achieved. A major effort was expended in developing the sectioning technique as the utility of each histological section depended on its physical integrity. Causes of impaired integrity of sections at the pilot stage included the relative difference in hardness between the wax and bone (although the bone was not difficult to section); as well as the prolonged interval between sectioning and mounting onto glass slides. Decalcification was rejected partly as this would also decalcify the HA beads, the location of which was one of the desired outcomes. The use of resin embedding, which is harder than bone, was considered but abandoned as it was unnecessary and merely prolonged sectioning. Mollifex (VMR International Ltd, Poole, UK) was tried in an attempt to soften the bone in the pilot study but was unsuccessful. Instead re-cooling of the paraffin embedded specimens after every 50–60 sections, as well as maintaining a low room temperature proved the most effective solution. Pilot studies also established the influence of time interval between the sectioning and mounting (1-day, 1-h, 1-min); the percentage of useful sections decreased as the time interval increased, with 95% retention of sections at 1-min. Two femurs from the HA group were discarded due to integrity issues in the early stages.

Pamidronate was selected as it is commonly delivered by injection, albeit intravenously (Marx 2005). The estimated incidence of osteonecrosis in patients taking pamidronate was 4% (Durie *et al.* 2005). The present study only evaluated the short term local response (7 days) to pamidronate delivery but its half-life in bone upon intravenous delivery is more than 10 years (Marx 2005); the immediate, middle and long-term effects on osteoblast biology are a matter of conflicting insights from different studies (Fleisch *et al.* 1982, Tenenbaum *et al.* 2002, Woo *et al.* 2006, Orriss *et al.* 2008, Uveges *et al.* 2008). This study did not set out to resolve these mechanism conflicts but merely to evaluate the final outcome measure.

Although the status of the femur before implantation of the beads was unknown, the study design incorporating intra-bone controls should account for this effect and also serve to ensure that the marrow : bone ratios among the inter-bone control, intra-bone control and test groups would be similar at the commencement of the experiment. The use of micro-CT, as a non-invasive measure of activity was considered during protocol development but rejected because of the potential effect of radiation on chick embryo development as well as problems of low resolution with low dosage and scan times with the available instruments.

The marrow : bone ratio was adopted as the outcome measure instead of absolute area measurements because of the greater variation of absolute values compared to ratios in different femurs. The natural variation in marrow : bone ratios along the length of the femurs was known and taken into account; that at the ends of the femurs would be greater than at the centre. The selection of the intra-bone controls at 400–450 μm from the puncture wound fell within a dimensionally non-variable middle portion of the femur. It was expected therefore that the starting ratio should be similar between test and control sites. The location of the intra-bone controls away from the puncture wound would enable these controls to account for the effect of the puncture wound effect.

The image analysis system adopted potentially offered an objective method for measuring the areas

but it was necessary to use manual boundary recognition for accuracy, introducing an element of subjectivity. Consequently, intra- and inter-observer limits of agreements were established to obtain a measure of the validity of the outcomes and found to be acceptable in the context of this study.

In all of the inter-bone control sections, no direct bone contact with either type of carrier could be found. Although there was a significant ($P < 0.05$) difference in the marrow : bone ratios between the inter-bone and intra-bone controls in both groups 1 and 2, the actual difference was small. The precise reasons for the small difference were not clear and could perhaps be attributed to different diffusion effects from the two carrier systems; the pamidronate does not bind to Affi-gel blue, whilst it does bind to the HA beads and to the surrounding bone. In any event, both carrier systems seemed suitable for local bisphosphonate delivery.

The marrow : bone ratios were significantly ($P < 0.001$) smaller in the test groups compared to the intra-bone and inter-bone controls, suggesting that pamidronate could promote bone growth in the short test period (7 days) and this effect was independent of the carrier type. The finding is consistent with recent studies using different animal models (Houshmand *et al.* 2007, Omi *et al.* 2007). The long-term effect of bisphosphonate remains unknown in this model but reported effects have been attributed to high dosage or prolonged delivery resulting in uncontrolled bone growth and impairment of blood supply. A related reason may be the inhibition of capillary angiogenesis (Ribatti *et al.* 2007).

The newly formed sub-periosteal bone found in half of the intra-bone controls and test groups of the same femur for Affi-Gel blue carriers was interesting. The observation suggests that the bisphosphonate caused an increase in circumferential sub-periosteal bone growth. The reliability of the observation is based on both controls as it was not possible to calculate the area of bone cross-section before implantation.

There was no evidence of newly formed sub-periosteal bone in any of the HA carrier sections, which could perhaps be attributed to the different release profiles. One possibility was that any newly formed sub-periosteal bone had already completed mineralization. The eccentric distribution of the newly formed sub-periosteal bone may have been due to the eccentric placement of the Affi-Gel blue beads exerting their effect on bone growth through a diffusion gradient. Any possible differences in the quality of trabeculae apparent in the presented images for the HA and Affi-gel blue groups were not observed consistently in all sections. The difference between the carrier systems was that the lack of binding of Pamidronate to affi-gel blue beads may produce different release profiles and therefore greater subperiosteal effects. In contrast, the binding of Pamidronate to HA beads may explain the relative absence of such subperiosteal effects and the possibly greater likelihood of newly formed bone in contact with the carrier. The different effects related to carrier system may possibly be harnessed to induce either more localized or more diffuse bone generation in periapical repair.

The decreased marrow : bone ratio over this short time frame may be suggestive of a dual effect of the bisphosphonate in not only inhibiting resorption but also aiding bone formation. This likely to be even more so in flat bone formation where there is no need for removal of the initial cartilaginous callus by osteoclastic activity, a process which will be inhibited by the bisphosphonate. The observation of direct bone formation on pamidronate-charged carriers may suggest their biocompatibility and osteoconductive potential for enhancing bone generation, not solely based on osteoclast inhibition, which is supported by *in vitro* studies using MC3t3 cells (Xiong *et al.* 2009).

This present study showed a significant increase in bone generation upon local bisphosphonate application for a short period of time, independent of the carrier used, although the pattern of effect may have been different. The local delivery of bisphosphonates could be beneficial in promoting bone regeneration after endodontic treatment or surgery. In endodontics, bisphosphonates could potentially be delivered locally in conjunction with grafting procedures involving periapical lesions and in root canal filling materials. The osteoconductive property of the bisphosphonate used suggests that it could be used as a surface-coating material for bone grafting materials, root ends and root fillings. Clinical use of bisphosphonates for aiding bone regeneration may only be recommended once the biological basis of their action is fully understood. Future research should focus on clarifying the mechanisms of biological actions, and their critical delivery profiles.

Conclusions

Within the limitations of this study, it may be concluded that a test model for evaluating bisphosphonate delivery in the chick femur using the chorioallantoic membrane model was successfully adapted.

Pamidronate had a significant ($P < 0.001$) effect on bone generation over 7 days, regardless of the type of carrier used.

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References

Aspenberg P, Astrand J (2002) Bone allografts pretreated with a bisphosphonate do not resorb. *Bone* **30**, 37S.

Bland JM, Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **8476**, 307–10.

Durie BG, Katz M, Crowley J (2005) Osteonecrosis of the jaw and bisphosphonates. *New England Journal of Medicine* **353**, 99–102.

Eberhardt C, Habermann B, Muller S, Schwarz M, Bauss F, Kurth AH (2007) The bisphosphonate ibandronate accelerates osseointegration of hydroxypatite-coated cementless implants in an animal model. *Journal of Orthopaedic Science* **12**, 61–6.

Faucheux C, Verron E, Soueidan A *et al.* (2009) Controlled release of bisphosphonate from a calcium phosphate biomaterial inhibits osteoclastic resorption *in vitro*. *Journal of Biomedical Materials Research* **89**, 46–56.

Fleisch H (1982) Bisphosphonates: mechanisms of action and clinical applications. *Bone and Mineral Research Annual* **1**, 319–57.

Houshmand B, Rahimi H, Ghanavati F, Alisadr A, Eslami B (2007) Boosting effect of bisphosphonates on osteoconductive materials: a histologic *in vivo* evaluation. *Journal of Periodontology* **42**, 119–23.

Katz H (2005) Endodontic implications of bisphosphonate-associated osteonecrosis of the jaws: A report of three cases. *Journal of Endodontics* **31**, 831–4.

Mangieri D, Nico B, Colucia AM, Vacca A, Ponzoni M, Ribatti D (2009) An alternative *in vivo* system for testing angiogenic potential of human neuroblastoma cells. *Cancer Letters* **277**, 199–204.

Marx RE (2003) Pamidronate (Adredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. *Journal of Oral and Maxillofacial Surgery* **61**, 1115–8.

Marx RE (2005) Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. *Journal of Oral and Maxillofacial Surgery* **63**, 1567–75.

Masarachia P, Weinreb M, Balena R, Rodan GA (1996) Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. *Bone* **19**, 281–90.

Omi H, Kusumi T, Kijima H, Toh S (2007) Locally administered low-dose alendronate increases bone mineral density during distraction osteogenesis in a rabbit model. *Journal of Bone and Joint Surgery* **89**, 984–8.

Paul W, Sharma CP (1999) Development of porous spherical hydroxyapatite granules: application towards protein delivery. *Journal of Materials Science: Materials in Medicine* **10**, 383–8.

Ribatti D (2008) Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. *Internal review of Cellular Molecular Biology* **270**, 181–224.

Ribatti D, Gualandris A, Bastaki M *et al.* (1997) New model for the study of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane: the gelatin sponge/chorioallantoic membrane assay. *Journal of Vascular Research* **34**, 455–63.

Ribatti D, Nico B, Mangieri D *et al.* (2007) Neridronate inhibits angiogenesis *in vitro* and *in vivo*. *Clinical Rheumatology* **26**, 1094–8.

Rocha M, Nava LE, Vázquez de la Torre C, Sánchez-Márin F, Garay-Sevilla ME, Malacara JM (2001) Clinical and radiological improvement of periodontal disease in patients with type 2 diabetes mellitus treated with alendronate: a randomized, placebo-controlled trial. *Journal of Periodontology* **72**, 204–9.

Sarathy AP, Bourgeois JSL, Goodell GG (2005) Bisphosphonate-associated osteonecrosis of the jaws and endodontic treatment: Two cases reports. *Journal of Endodontics* **31**, 759–63.

Saw CL, Heng PW, Liew CV (2008) Chick chorioallantoic membrane as an in situ biological membrane for pharmaceutical formulation development: a review. *Drug Development in Industrial Pharmacology* **34**, 1168–77.

Tenenbaum HC, Shelemay A, Girard B, Zohar R, Fritz PC (2002) Bisphosphonates and periodontics: potential applications for regulation of bone mass in periodontium and other therapeutic/diagnostic uses. *Journal of Periodontology* **73**, 812–22.

Vargas A, Zeisser-Labouèbe M, Lange N, Gurny R, Delie F (2007) The chick embryo and its chorioallantoic membrane (CAM) for the in vivo evaluation of drug delivery systems. *Advanced Drug Delivery Reviews* **59**, 1162–76.

Woo SN, Hellstein JW, Kalmar JR (2006) Systematic review: Bisphosphonates and osteonecrosis of the jaws. *Annals of Internal Medicine* **144**, 753–61.

Xiong Y, Yang HJ, Feng J, Shi ZL, Wu LD (2009) Effects of alendronate on the proliferation and osteogenic differentiation of MG-63 cells. *Journal of International Medical Research* **37**, 407–16.

Yang XB, Whitaker MJ, Sebald W *et al.* (2004) Human osteoprogenitor bone formation using encapsulated bone morphogenetic protein 2 in porous polymer scaffolds. *Tissue Engineering* **10**, 1037–45.