Strain imparted during impaction grafting may contribute to bony incorporation

AN *IN VITRO* STUDY OF THE RELEASE OF BMP-7 FROM ALLOGRAFT

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In order to investigate the osteoinductive properties of allograft used in impaction grafting and the effect of strain during impaction on these properties, we designed an *in vitro* experiment to measure strain-related release of bone morphogenetic protein-7 (BMP-7) from fresh-frozen femoral head allograft. A total of 40 10 mm cubes of cancellous bone were cut from ten samples of fresh-frozen femoral head. The marrow was removed from the cubes and the baseline concentrations of BMP-7 were measured. Specimens from each femoral head were allocated to four groups and subjected to different compressive strains with a material testing machine, after which BMP-7 activity was reassessed. It was present in all groups. There was a linear increase of 102.1 pg/g (95% confidence interval 68.6 to 135.6) BMP-7 for each 10% increase in strain. At 80% strain the mean concentration of BMP-7 released (830.3 pg/g bone) was approximately four times that released at 20% strain. Activity of BMP-7 in fresh-frozen allograft has not previously been demonstrated. This study shows that the freezing and storage of femoral heads allows some maintenance of biological activity, and that impaction grafting provides a source of osteoinductive bone for remodelling.

We have shown that BMP-7 is released from fresh-frozen femoral head cancellous bone in proportion to the strain applied to the bone. This suggests that the impaction process itself may contribute to the biological process of remodelling and bony incorporation.

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Impaction grafting is a successful technique for restoring the lost bone stock in both the femur and the acetabulum during revision hip arthroplasty.¹⁻⁴ Up to 100% survival of the implant has been reported at ten years using this technique.² The procedure involves progressive compaction of morcellised cancellous bone chips into the femoral canal or acetabular cavity. The bone most commonly used to create the morcellised graft is fresh-frozen femoral head allograft.⁵ In order to achieve long-term survival of the prosthesis, two requirements must be met. First, initial stability must be achieved at the time of surgery, and second, the allograft must be incorporated into the host skeleton and undergo remodelling in response to the stress applied to it. If either of these steps fails, the implant will subside and further revision will be required. It has been shown in histological studies that impaction grafted bone does incorporate, but to a variable degree.⁶⁻⁹ We wished to investigate the potential contribution of the impaction process itself to the incorporation of allograft bone. It was hypothesised that, during impaction, the microfractures created in the bone chips may

allow the exposure and release of factors in the non-collagenous matrix that would help to stimulate the subsequent incorporation and remodelling. We decided to investigate the release of bone morphogenetic protein-7 (BMP-7) or osteogenic protein-1 (OP-1), as this is a well-studied member of the transforming growth factor (TGF)- β superfamily, and in recombinant form has been used to stimulate healing in spinal fusions,¹⁰ segmental defects of long bone¹¹ and nonunions of the scaphoid.¹² The use of additional recombinant BMP-7 in revision arthroplasty of the hip has also been studied.¹³⁻¹⁶

Materials and Methods

Preparation of the bone cubes. The bone used was taken from fresh-frozen femoral heads which had been cleared for use in research by the quality control team of Tissue Services at the National Blood Service, Liverpool, United Kingdom. The consent from donors included use for research and development. The heads were not suitable for clinical use, usually because of incomplete serological tests. Ten femoral heads were chosen at random and

Fig. 1

Bone cubes following different levels of strain: from left to right 0%, 20%, 40%, 60% and 80%. All cubes showed a degree of recoil after strain, as demonstrated by the final resting heights.

Table I. Summary statistics of each strain group

Strain group	Mean (SD) baseline (pg/g bone)	Mean (SD) after strain (pg/g bone)	Difference (pg/g bone)
80%	425.2 (435.7)	830.3 (466.2)	405.1 (455.7)
60%	373.5 (287.8)	570.7 (507.9)	197.2 (499.6)
40%	299.1 (214.9)	333.2 (261.2)	34.2 (265.1)
20%	317.3 (247.2)	216.6 (199.1)	-100.7 (102.8)

thawed at room temperature for two hours prior to dissection of the soft tissue. They were then cut into 10 mm cubes using a low-speed handsaw and a modified mitre block. The cubes were cut in line with the axis of the femoral neck. The orientation of each bone cube was noted, so that subsequent strain could be applied in the vertical direction. A total of 40 bone cubes were produced.

In order to remove the marrow content from the cubes they were first washed in 200 ml water at room temperature. They were then placed individually in universal tubes and centrifuged at 1850 g for 15 minutes. They were then removed from the tubes and placed in 30 ml distilled water at 37°C in clean universal tubes. These tubes were agitated at 200 rpm in a desktop orbital incubator, maintaining a constant temperature of 37°C, for one hour. The centrifuge step was repeated and the cubes were then weighed accurately, placed in clean universal tubes, and frozen at -20°C until required. Throughout this washing procedure the tubes were labelled to maintain the identity of each cube.

Experimental method. On the morning of the experiment the cubes were allowed to thaw at room temperature for one hour. To each universal tube, 4 ml of sterile distilled water was added (at 37°C). The tubes were then agitated at 200 rpm in the desktop incubator for two hours. The wash fluid was then removed, placed in labelled tubes and stored at -20°C until required. These tubes were labelled 'wash 1'. The cubes were then allocated to one of four groups, with

one cube from each head in each group, giving ten cubes in each of the four groups. A universal testing machine (Lloyds Instruments, Fareham, United Kingdom) was then used to impart a predefined strain to the individual cubes. The testing machine was set up so that the base consisted of a flat metal block and the cross-head attachment was a similarly flat metal plate, parallel to the base-plate. All the cubes in a group received the same level of strain: either 80%, 60%, 40% or 20% (Fig. 1). The cubes were strained individually after accurate measurement of all dimensions using the vernier callipers. The universal testing machine was set to a preload of 9 N and a strain rate of 10 mm/min. Following the application of strain, the cubes were returned to clean universal tubes and a further 4 ml of distilled water at 37°C was added. The tubes were agitated at 200 rpm for a further two hours. The fluid was removed and stored in separate, universal containers. These were labelled 'wash 2' and stored at -20°C until required.

BMP-7 assay. The concentration of BMP-7 in all wash samples was determined using a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (RayBio-tech Inc, Norcross, Georgia). All samples were run in duplicate. Where the BMP-7 levels were below the limit of detection by the ELISA (10 pg/ml) a standard value of 5 pg/ml was assigned to prevent negative values interfering with the statistical calculations.

Statistical analysis. Statistical analysis was performed using Excel (Microsoft Corporation, Redmond, Washington) and SPSS 14.0 (SPSS Inc., Chicago, Illinois). The differences between groups were assessed using mixed modelling. Fixed (strain group and baseline BMP-7 value) and random (head) effects were estimated using restricted maximum likelihood.

Results

BMP-7 was present in all samples of femoral heads tested. The mean baseline concentration of BMP-7 for all cubes was 353.8 pg/g. The SD of 300.3 (11.8 to 1442.1) reflects the wide variation in concentrations between samples. The release of BMP-7 in relation to strain was calculated by subtracting the calculated release after strain from the baseline values. The mean, SD and the change in the level of BMP per strain group are shown in Table I. The mean and variability of the BMP-7 showed differences between the four groups.

There was a statistically significant effect of strain on change in BMP-7 ($p < 0.0001$), in the form of a linear increase of 102.1 pg/g (95% confidence interval (CI) 68.6 to 135.6) for each 10% increase in strain. There was no evidence of any non-linearity in the effect ($p = 0.36$). The between-head SD was estimated to be 289.7, whereas the estimated within-head SD was 234.9.

Discussion

We have shown that BMP-7 is present in fresh-frozen femoral head allograft. Although BMPs have been shown to be

present in demineralised bone matrix,¹⁷ this has not previously been demonstrated in fresh-frozen femoral head allograft and is a reflection of the osteoinductive properties of this type of graft.

The results also indicate that strain or microfracture induces the release of BMP-7 from fresh-frozen femoral head allograft. This is a phenomenon that has not previously been described in the literature. We observed a significant increase in the release of BMP-7 with increasing strain ($p < 0.0001$). This increase was 102.1 pg/g bone per 10% increase in strain.

Bone morphogenetic proteins are sequestered in the bone matrix during osteogenesis and are bound to various types of collagen, including type I, IV and IX.¹⁸ The proposed mechanism of release as observed in this study is that during strain, microfractures are created, exposing fresh areas of bone matrix. Each microfracture exposes some new collagen strands to the surrounding micro-environment. The BMPs present on the collagen are also exposed. The open-pore nature of the cancellous bone used in these experiments allows fluid to flow around these newly-exposed surfaces, washing off any cytokines that have been exposed into either solution or suspension. This mechanism is passive and is as equally applicable to fresh bone as any other form of bone in which BMPs are present.

In impaction grafting the process of impaction produces a biomechanically stable bed by creating interdigitation between bone particles. The aim of this experiment was to define whether the impaction process contributes to the biological incorporation of the graft bone. Having shown that BMP-7 is released following strain and microfracture, we can now postulate that the process itself may be beneficial to incorporation of the bone. Typical dosing regimens for *in vivo* use of recombinant BMPs are of the order of milligrams,¹⁹ rather than the picogram level found in our study. However, it has been noted that the osteoinductive properties of recombinant BMPs are reduced compared with purified BMPs,²⁰ and although this study only measured BMP-7, this is a reflection of all the other BMPs that will be present in the matrix. Indeed, a study into the use of human BMPs for nonunion in long bones indicated that 100 ng of endogenous BMP effected union in 20 of 30 patients.^{21,22} A femoral head weighing 80 g would yield approximately 66 ng of BMP-7 after impaction, and combined with the other BMPs present, this dose may have a significant effect. In addition to the release of growth factors after impaction, the compacted graft will also act as a slow-release depot for BMPs. As the bone is resorbed and remodelled, so the matrix will be broken down, releasing growth factors.

In terms of the observed quantity of BMP-7 measured in the samples, it is currently not known how much BMP-7 is present within a given amount of bone. However, one study showed that 20 µg of highly purified BMP could be produced from 10 kg of bovine bone by heparin-sepharose chromatography and reverse-phase high-pressure liquid chromatography.

²³ The largest release of BMP-7 in our experiment was 830.3 pg/g bone after the application of 80% strain. If we take 20 µg per 10 kg bone to be indicative of BMP content, this equates to 2 ng/g bone. The 830.3 pg/g would therefore represent 42% of the total BMP present in the bone, which seems unlikely. However, the technique used to quantify the BMP-7 in this study, namely ELISA, is highly sensitive and the guanidinium chloride extraction process used by Wang et al²³ may underestimate the total amount of BMP present. Furthermore, recent studies of BMP in demineralised bone matrix have indicated levels of BMP-7 of 227 ng/g.²⁴ As the non-organic mineral content of bone is known to be 60% of the weight, extrapolation indicates that there should be 90 ng BMP-7 per gram of bone. The observation of 830.3 pg/g bone therefore only accounts for 0.92% of the total BMP-7, a figure that seems more realistic.

The bone used for this experiment was taken from fresh-frozen femoral heads procured from live donors at the time of primary hip replacement. Although many of these donors are elderly, whose bone is not always of good quality in terms of bone mineral content, and the articulating surface itself is, by definition, osteoarthritic, these heads are most commonly used in impaction grafting, which is the focus of this research. It is therefore logical to use this bone in these cytokine release experiments.

We have shown that the change in BMP-7 is markedly variable between femoral heads, even having accounted for the baseline value and strain effects (SD 289.7). However, the variability between individual cubes of bone is of a similar magnitude (SD 234.9). There are a number of possible explanations for this effect. There may be a relationship between age and cytokine sequestration in the matrix. However, as all of the femoral heads were procured from patients undergoing primary total hip replacement, this group is from a relatively narrow age range, precluding this analysis. Alternatively, the presence of disease such as osteoarthritis may have a significant effect on cytokines in the matrix. Although all the femoral heads showed evidence of osteoarthritis, both the extent and the cause of degeneration were variable. The variability between individuals observed in all the tests, from the haemoglobin levels to the bone mass, is unlikely to be the sole explanation for the large variation observed, as the mean concentration of BMP-7 for the femoral heads varied by a factor of 10. No information on this variation in cytokine sequestration between individuals, or in different disease states, is available in the literature, although two recent studies used similar ELISA techniques to show marked variability in different BMPs in preparations of demineralised bone matrix.^{17,24} Other confounding factors, such as differences in the treatments of the 40 bone cubes, were kept to a minimum by the simultaneous processing of all cubes in the marrow removal and incubation wash stages.

If our hypothesis is correct and growth factors are released following microfracture of bone particles, it could be suggested that 'large' bone morsels should be used when under-

taking this technique. Using larger particles will naturally create more microfracture when the bone is impacted. This assumes that it is beneficial to release growth factors in this way, an assumption that is easy to make in light of the recent clinical applications of recombinant BMPs to fracture repair. This recommendation is in line with current biomechanical knowledge of impacted bone stability.²⁵

The final practical implication of this work is in relation to washing of the graft. Provided the graft has been washed after morcellisation, as suggested by biomechanical²⁶⁻²⁸ and bony ingrowth studies,²⁹ washing should not be repeated following impaction, otherwise some of the released growth factors may be lost.

Supplementary Material

A table showing the full data set for each of the 40 cubes is available with the electronic version of this article on our website at www.jbjs.org.uk

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