Effects of Setting Bone Cement on Tissue-Engineered Bone Graft: a Potential Barrier to Clinical Translation?

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**Background:** Strategies to improve mechanical strength, neovascularization, and the regenerative capacity of allograft include both the addition of skeletal stem cells and the investigation of novel biomaterials to reduce and ultimately obviate the need for allograft altogether. Use of bone cement is a common method of stabilizing implants in conjunction with impacted allograft. Curing cement, however, can reach temperatures in excess of 70°C, which is potentially harmful to skeletal stem cells. The aim of this study was to investigate the effects of setting bone cement on the survival of human adult skeletal stem cells within tissue-engineered allograft and a novel allograft substitute.

**Methods:** Milled allograft and a polymer graft substitute were seeded with skeletal stem cells, impacted into a graduated chamber, and exposed to curing bone cement. Sections were removed at 5-mm increments from the allograft-cement interface. A quantitative WST-1 assay was performed on each section as a measure of remaining cell viability. A second stage of the experiment involved assessment of methods to potentially enhance cell survival, including pretreating the allograft or polymer by either cooling to 5°C or coating with 1% Laponite, or both.

**Results:** There was a significant drop in cellular activity in the sections taken from within 0.5 cm of the cement interface in both the allograft and the polymer (p < 0.05), although there was still measurable cellular activity. Pretreatment methods did not significantly improve cell survival in any group.

**Conclusions:** While the addition of bone cement reduced cellular viability of tissue-engineered constructs, this reduction occurred only in close proximity to the cement and measurable numbers of skeletal stem cells were observed, confirming the potential for cell population recovery.

**Clinical Relevance:** These studies highlight a potential pitfall when translating tissue-engineering strategies, but indicate that the use of bone cement should not necessarily be ruled out during the application of cell populations and biomaterials in tissue regeneration.

There is an ever-expanding requirement for bone graft within orthopaedics, with a wide range of indications. The rate of revision hip arthroplasty, which often requires treatment of bone loss, increased threefold between 2003 and 2010. For procedures involving the replacement of large volumes of bone stock, the main source is usually allograft from either cadavera or donors. The beneficial effects of the addition of skeletal stem cells to allograft have been proven via both in vitro and in vivo impaction bone-graft models to improve aggregate shear strength, increase new bone formation, and improve vascularization. This approach has been successfully incorporated into a core decompression technique for the treatment of patients with osteonecrosis of the femoral head, and clinical trials for patients undergoing revision hip surgery involving impaction bone graft are also planned.

However, problems related to the use of allograft include cost, rejection, and infective transmission. Tissue-engineered constructs combining skeletal stem cells with biodegradable...
polymers have the potential to replace allograft. Recently, skeletal stem cell/polymer composites have been specifically tailored for use in impaction bone-grafting, and both in vitro and in vivo trials have shown encouraging results for potential clinical use.

There remain, however, important differences between experimental models and the actual clinical use of tissue-engineered materials that need to be carefully addressed prior to the incorporation of novel technologies. A possible pitfall to the successful implementation of tissue-engineered constructs in impaction bone-grafting involves the potential damaging exothermic effects of setting bone cement. The purpose of this study was therefore to investigate the effects of setting bone cement on both a tissue-engineered skeletal stem cell/allograft composite and a novel skeletal stem cell/polymer composite designed for translational use in impaction bone-grafting. A second stage of the experiment entailed investigating methods of possibly reducing the damaging exothermic effects of the cement on the skeletal stem cells.

**Materials and Methods**

**Cell Culture**

With approval of our Local Research Ethics Committee (LREC 194/99/w 27/10/10), bone marrow was obtained from fully consenting patients undergoing a routine total hip replacement. The cells used in study 1 were from an eighty-nine-year-old patient (M89), and the cells used in study 2 were from a sixty-six-year-old patient (M66). Progenitor cells were extracted and then cultured on standard tissue culture plastic (T150) flasks in basal media (Sigma-Aldrich, Poole, United Kingdom) and incubated at 37°C in 5% CO2. On confluence (fourteen days), the cells were released with use of trypsin in ethylene diamine tetra-acetic acid (EDTA; Sigma-Aldrich), combined and suspended in basal media, and centrifuged at 1100 rpm for four minutes, after which the supernatant was discarded. The remaining cell pellet was resuspended in basal media to give a concentration of $5 \times 10^{5}$ cells/mL, as determined with a hemocytometer.

**Allograft Preparation**

Femoral heads were also obtained with patient consent and approval of our Local Research Ethics Committee (LREC 194/99/w 27/10/10) from patients undergoing total hip replacement. Under aseptic conditions in an operating room environment, the cartilage, cortical bone, soft tissue, and cysts were removed, prior to decellularization with a standard bone mill (Noviomagus; Spierings Orthopaedics, Nijmegen, the Netherlands [mean particle size, 2.1 mm]). The bone was then defatted via repeated immersion in 6% hydrogen peroxide solution, followed by multiple washes in phosphate-buffered saline solution (PBS) and a twenty-four-hour incubation in 5% antibiotic solution under ultraviolet (UV) light to ensure sterility; it was then subjected to an additional twenty-four hours of incubation in basal media.

**Polymer Preparation**

Previous work performed in our department had identified high-molecular-weight poly(DL-lactide) (PDLA; LA) in combination with 10% hydroxyapatite to have desirable characteristics for potential translation as an allograft alternative in impaction bone-grafting. This material was obtained from Evonik (Birmingham, Alabama), and porous scaffolds were produced with use of a novel supercritical CO2 foaming technique as detailed previously. These scaffolds were then milled and sterilized in 5% antibiotic solution and twenty-four-hours of UV light exposure, followed by another twenty-four hours of incubation in basal media.

**Allograft/Polymer Seeding**

The basal media were removed from both the milled allograft and the polymer, and the cell suspension was added at a ratio of 1 mL of solution to 1 mL of allograft or polymer. The composites were then incubated for two hours (37°C, 5% CO2) in universal tubes, with gentle agitation every twenty to thirty minutes in order to allow diffuse cell adhesion. For study 1, the seeded allograft or polymer was used immediately for experimentation, as would be the case when it is applied clinically. For the second study, the allograft and polymer were seeded with skeletal stem cells in the same manner; they were subsequently transferred to individual wells of six-well plates (approximately 2 to 3 mL of each per well); and were incubated in basal media at 37°C, 5% CO2, with standard PBS washes and media changes every three to four days until the cells reached confluence at approximately fourteen days. Cell numbers involved for experimentation were thus higher, and hence any beneficial effects on cell survival of pretreatment methods would be more pronounced and, critically, detectable and quantifiable.

**Study 1**

A protocol for the methodology used in study 1 is shown in Figure 1. Sixteen 5-mL syringes were customized such that the tips were removed and markings were made at 0 to 5-cm intervals longitudinally. Eight of these syringes were filled up to the 2-cm mark with skeletal stem cell (M89)-seeded allograft (group 1) and eight, with skeletal stem cell (M89)-seeded polymer (group 2). Cement (Surgical Simplex P; Stryker, Newbury, United Kingdom) was then prepared according to the manufacturer’s protocol and was placed in four of the syringes of each group in apposition with the graft prior to setting, such that a cement mantle of 5 mm was created. The thickness of the cement mantle affects the temperatures reached, and 5 mm was deemed to be the upper end of the thickness obtained during impaction bone-grafting.

Thumb pressure was applied to the syringe plungers with an attempt to apply a roughly equal compressive force to the cement against the graft in order to mimic the compression that occurs during cement pressurization (see Appendix). The graft-containing syringes were then placed into a custom-made polystyrene holder, such that the heat from the setting cement was directed toward the skeletal stem cell-seeded graft. The remaining four syringes from each group were used as positive controls. The cement was molded into cylinders, allowed to set and cool, and then placed in apposition with the seeded allograft or polymer (thumb pressure applied), so that the skeletal stem cells were not exposed to the exothermic reaction. The syringes were then submerged in basal media prior to analysis.

**Assessment of Cell Viability**

**WST-1 cell proliferation assay:** A WST-1 assay was used as a measure of remaining cell viability after exposure to setting cement. This assay is a measure of total cell metabolic activity and hence its results are closely related to the number of viable cells. After one hour (the time required for the experimental setup), the contents of the syringes were analyzed. The plunger was used to expel the graft in 0.5-cm incremental distances from the cement-graft interface. Each 0.5-cm section was analyzed separately so that the exothermic effects of the cement could be quantified by distance. This provided a range of distances from the cement at which 50% of cells had survived (LD50). Each graft section was then submerged in 2 mL of a 1:10-dilution WST-1 substrate (Roche, Welwyn Garden City, United Kingdom). Blank WST-1 samples were run in parallel (negative controls).

After four hours, 3 x 100-μL WST-1 substrates were removed from each sample with use of a micropipette (Edu-Lab, Norford, United Kingdom) (n = 4 per 0.5-cm increment) and analyzed with a Bio-Tek KC4 microplate fluorimeter (Bio-Tek, Winooski, Vermont) at 410 nm. An increase in absorbance value (i.e., an increase in the optical density of the substrate) indicated increased cell number and viability. The mean absorbance value was then calculated for both the allograft and the polymer at each 0.5-cm incremental distance from the cement-graft interface, and the mean negative-control WST-1 absorbance value was subtracted.

**Standard Curve**

In order to calculate an LD50 distance, a standard curve for the WST-1 assay was established with use of the same (M89) cell line. Known numbers of cells...
were seeded into individual wells of a twelve-well plate in 1 mL of basal media. One milliliter of 1:10-dilution WST-1 reagent was added, and 3 $\times$ 100 $\mu$L of substrate was removed at four hours, and read as described above; the mean absorbance value was then calculated. After subtraction of the negative control value, a change in the absorbance value could be related to a known cell number and hence the distance (in 0.5-cm increments) from the cement-graft interface at which 50% of the cells survived the exposure could be calculated.

Temperature Assessment

A supplemental test was conducted to assess the temperature profile through the graft as a function of the distance from the cement-graft interface. For this test, 2.5-mL syringes were loaded with graft material up to 0.5 cm from the open end, and instrumented with use of K-type thermocouple wires (RS Components; Corby, United Kingdom). Thermocouples were placed to measure the temperature at four locations throughout bone-cement curing: i.e., in the cement as well as 0.5 cm, 1.0 cm, and 1.5 cm above the cement-graft interface. Data were recorded with use of a MicroMeasurements 7000 Series Data Acquisition System and StrainSmart software (Vishay Precision Group UK, Basingstoke, United Kingdom), at a sample rate of 10 Hz. The cement was mixed and after two minutes, when it was doughy, it was introduced to fill the remaining 0.5 cm of the syringe and pressed in with light thumb pressure. The syringe was then pressed into a block of expanded polystyrene for thermal isolation, and data were recorded for twenty minutes, at which point a temperature peak associated with cement curing had been recorded by all of thermocouples. The experiment was repeated in triplicate for both the polymer and the allograft specimens.

**Study 2**

A protocol for the methodology used in study 2 is shown in Figure 2. Having established the effects of setting bone cement on tissue-engineered constructs, we initiated changes to the pretreatment protocols in an attempt to reduce the toxicity. A modified experiment was designed because of the increase in experimental groups and to keep all graft in close proximity (<1 cm) to the cement-graft interface, after which distance negligible loss of cell viability was observed (established by study 1). Cells were grown to confluence on milled polymer and allograft as described above, before being impacted...
into modified electron-microscopy pots (1-mL volume). There were twenty pots in total for the allograft and twenty pots in total for the polymer, and these were subsequently divided into five groups of four pots each. One group was treated as a negative control and was subjected to cold, preset cement only. In a second group, which acted as a positive control, 0.5 mL of mixed cement was added to the electron-microscopy pots (see Appendix), which was pressurized via thumb compression and subsequently allowed to set via its normal exothermic reaction. A third group of four pots acted as the first of the treatment groups. These pots were precooled to 5°C for one hour prior to exposure of the graft to the cement, which was added as described for group 2. In the fourth group, the allograft or polymer was dipped in 1% Laponite (a bioactive clay, made by Rockwood Additives, Widnes, United Kingdom, with the potential for protecting the cells) prior to exposure to the setting cement. In a final group, the pots were cooled to 5°C for one hour and the allograft or polymer was dipped in 1% Laponite prior to cement exposure.

Cell Viability

Live/dead immunostain: Small portions of polymer were taken from the graft-cement interface and at a 1-cm distance, in order to illustrate visually the effects of the setting bone cement. Samples were incubated for ninety minutes in 5 mL of standard Cell Tracker Green probe (CTG)/ethidium homodimer (EH-1) solution (10 μg/mL of CTG and 5 μg/mL of EH-1, Invitrogen; Life Technologies, Paisley, United Kingdom). They were then fixed in ethanol and stored in PBS prior to imaging. Images were obtained with use of Carl Zeiss Axiovision Software (version 3.0) via an AxioCam HR digital camera on an Axiovert 200 inverted microscope (Carl Zeiss, Cambridge, United Kingdom) under fluorescent light.

WST-1 assay: Cell viability was again tested with use of a WST-1 cell proliferation assay. After one hour of incubation in basal media after cement exposure, the contents of each of the electron-microscopy pots were removed and were placed individually into 1 mL of 1:10-dilution WST-1 substrate (Roche). After four hours of incubation, 3·10−10 L of substrate was removed and was analyzed with use of a Bio-Tek KC4 microplate fluorescent reader at 410 nm. After subtraction of the blank WST-1 absorbance values, the means and standard deviations for optical densities were calculated for each of the treatment groups.

Statistical Methods

All experimental groups were replicated four times, and statistical comparison was performed with use of one-way analysis of variance (ANOVA) with post hoc Bonferroni analysis. A p value of <0.05 was considered to be significant.

Source of Funding

The work in the Bone and Joint Research Group (Southampton) is funded by grants from the Biotechnology and Biological Sciences Research Council (BBSRC). Manufacture of the scaffold (Nottingham) was funded by the Medical Research Council (MRC).

Results

Study 1

The mean cell viability, as measured by an increase in the optical density with use of the WST-1 assay, was observed to increase as the distance from the cement-bone interface increased in both the skeletal stem cell-seeded allograft and the skeletal stem cell-seeded polymer group (see Appendix). The effect was more pronounced in the allograft group, which had a reduction in the mean optical density of 84% (compared with the control group) at a distance of 0 to 0.5 cm from the cement-graft interface and of 48% at a distance of 0.5 to 1.0 cm, although only the reduction at 0 to 0.5 cm was significant (one-way ANOVA, p < 0.01) (Fig. 3).

When the absorbance values were related to the actual cell numbers via the standard curve (see Appendix), the mean control value (optical density = 0.06) was found to be related to approximately 38,000 cells. In comparison, the value for 0 to 0.5 cm from the cement-graft interface (optical density = 0.01) related to approximately 4000 cells, the value for 0.5 to 1.0 cm from the cement-graft interface (optical density = 0.03) related to approximately 18,000 cells, and that for 1.0 to 1.5 cm
(optical density = 0.07) related to >40,000 cells. There was thus no measurable loss of cells at 1.0 to 1.5 cm compared with the controls; hence, in this experiment, the LD50 distance was observed to lie between 0.5 and 1 cm from the cement-graft interface.

In the polymer group, there was a reduction in the mean optical density (compared with the control group) of 32% at a distance of 0 to 0.5 cm from the cement-bone interface and of 16% at 0.5 to 1.0 cm (Fig. 3), although these changes were not significant. When absorbance values were related to actual cell numbers (see Appendix), the mean control value (optical density = 0.13) was found to be related to approximately 90,000 cells and the value for 0 to 0.5 cm from the cement-graft interface (optical density = 0.09) related to approximately 59,000 cells. Thus, in this group, the LD50 distance was noted to lie between 0 and 0.5 cm from the cement-graft interface.

**Temperature Assessment**

A typical graph displaying change in temperature against time for each of the 0.5-cm increments as the cement cured is displayed in the Appendix, and the mean increases in temperature values at these locations are shown in Table I. In keeping with the results of the cellular experiments, there was only a moderate temperature rise (mean and standard deviation, 9.1 ± 3.9°C for the polymer and 8.4 ± 3.8°C for the allograft) at a distance of 0.5 cm from the cement and negligible temperature rises at farther distances.

**Study 2**

**Live/Dead Stain**

Cell viability was displayed visually by using a standard live/dead stain. Samples were taken from the cement-graft interface and from 1 cm distal to it. There was a reduction in the density of viable (green-staining) cells at the cement-bone interface and an increase in the number of dead or compromised (red) cells. One centimeter distal to the cement, there was confluent live-cell coverage and very few dead cells (Fig. 4).
Discussion

The addition of skeletal stem cells to allograft for use in impaction bone-grafting enhances the cohesive strength of the allograft in vitro as well as improves vascularization and bone formation in vivo. However, none of the previous studies on this technique exposed the graft to the exothermic effects of bone cement, a factor used in the majority of clinical cases. In the present in vitro study, we examined the impact of setting bone cement on two skeletal stem cell-seeded constructs, both of which have the potential for clinical use. We demonstrated that there is a loss of cell viability associated with the rise in temperature but it is significant only within 0.5 cm of the cement-graft interface. Only one previous (unpublished) study has, to our knowledge, addressed the question of skeletal-stem-cell heat tolerance; in that study, cell cultures were exposed to media of increasing temperatures for differing time periods. The authors concluded that cells could survive exposure to up to 48°C for 150 seconds but most cells perish when exposed to >58°C for the same incubation period. Our data complement their research but we add a model more consistent with the clinical situation: we used actual bone cement as the toxicant and cell-seeded allograft for the analysis, allowing calculation of a quantitative distance for the harmful effects.

Studies of cadaveric patients who had undergone impaction bone-grafting with use of standard allograft have shown allograft replacement by host bone around the periphery of the construct, but the central areas in close proximity to the cement consisted of necrotic bone and fibrous tissue. The current study indicates that, despite the addition of skeletal stem cells to any construct, this is still a possibility due to the loss of cell viability in these regions. However, our experiments did demonstrate some measurable cell viability within close proximity (0.5 cm) of the cement-graft interface (especially in the polymer graft substitute), although the viability was reduced. This finding of continued viability indicates that the cellular components of the constructs are not completely destroyed, hence leaving the potential for cell colony recovery via subsequent proliferation of the surviving cells. Temperatures experienced at the bone-cement interface were found to rise to a maximum of 48°C in one study and 56°C in another, both of which temperatures were significantly less than the temperature of the actual cement layer. Reasons cited for this phenomenon include the presence of blood and moisture, the large surface area, and poor heat conductivity of methylnethacrylate. The findings of both our cell study (continued cell viability close to the cement) and our temperature analysis are in keeping with those of these studies.

One limitation of our study involved the estimate of the total cell number and the LD50 distance via the relation to the standard curve. The WST-1 assay is a measure of total cell metabolism; hence, the activity of a known number of cells seeded on tissue culture plastic does not necessarily represent the same cell number in the three-dimensional environment. It is also not known whether the surviving cells’ ability to divide or differentiate is affected by the heat exposure. Another limitation includes the fact that the cells from only one donor were used in...
References


