

BASIC SCIENCE

Human Spinal Bone Dust as a Potential Local Autograft

In Vitro Potent Anabolic Effect on Human Osteoblasts

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Study Design. *In vitro* Study.

Objective. To evaluate the effect that factors released from human posterior spinal bone dust have on primary human osteoblast growth and maturation.

Summary of Background Data. Bone dust, created during spinal fusion surgeries, has the potential to be used as an autologous bone graft by providing a source of viable autologous osteoblasts and mesenchymal stem cells with osteogenic potential. Till date, no information is available on whether bone dust also provides a source of anabolic factors with the potential to enhance osteoblast proliferation and maturation, which would enhance its therapeutic potential.

Methods. Bone dust was collected from consenting patients undergoing elective posterior spinal fusion surgeries, and primary human osteoblasts were cultured from patients undergoing elective hip or knee arthroplasty. Growth factors and cytokines released by bone dust were quantified using enzyme-linked immunosorbent assay. Primary human osteoblast proliferation and gene expression in response to bone dust were assessed using ³H-thymidine incorporation and real-time polymerase chain reaction, respectively.

Results. Human bone dust released anabolic cytokines (IL-1 β and IL-6) and growth factors (TGF- β , VEGF, FGF-Basic, and PDGF-BB) in increasing concentrations over a 7-day period.

In vitro, the anabolic factors released by bone dust increased osteoblast proliferation by 7-fold, compared with osteoblasts cultured alone. In addition, the factors released from bone dust up-regulated a number of osteoblastic genes integral to osteoblast differentiation, maturation, and angiogenesis.

Conclusion. This study is the first to demonstrate that human posterior spinal bone dust released anabolic factors that potentially enhance osteoblast proliferation and the expression of genes that favor bone healing and bone union. As bone dust is anabolic and its harvest is fast, simple, and safe to perform, spinal surgeons should be encouraged to 'recycle' bone dust and harness the regenerative potential of this free autologous bone graft.

Key words: bone burring, bone dust, bone grafting, bone paste, bone pate, spinal fusion.

Level of Evidence: N/A

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Clinically, a number of bone grafting options can be employed during spinal fusion surgeries to optimize the chance of fusion, with the 'gold standard' being autologous iliac crest bone graft (ICBG).¹ Despite its unrivalled therapeutic value, limitations exist with the use of ICBG. For example, the process of harvesting ICBG can be associated with significant morbidity and the overall complication rate can be as high as 49%.^{1,2} Another limitation of ICBG is the finite amount of graft, which is often insufficient for multilevel fusions, such as scoliosis or revision surgeries.

Over the past decade, the rising incidence of spinal fusions coupled with the intrinsic limitations with ICBG has fuelled the research and development of novel bone graft substitutes. However, till date the only FDA approved bone graft substitute that has demonstrated comparable clinical efficacy with ICBG is recombinant human bone morphogenetic protein-2 (rhBMP-2).³ Unfortunately, the emergence of serious adverse events in relation to the use of rhBMP-2 has sparked a re-evaluation of this product.⁴ Furthermore, the exorbitant cost of BMP is another significant limiting factor precluding widespread clinical use.⁵ Aside from ICBG and BMP, an

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The manuscript submitted does not contain information about medical device(s)/drug(s).

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overwhelming array of bone graft substitutes exists, including allograft bone, synthetic scaffolds, and platelet gels. Each bone graft substitute has perceived advantages but none of the currently available products perform and autologous ICBG.⁶

Bone dust, created by burrs during the surgical approach to the spine is a free source of autologous bone that is usually lost through suction. However, by using a simple suction trap, bone dust can be easily “recycled.” Macroscopically, bone dust collected during spinal fusions resembles a pate, which is malleable and can be grafted back to the surgical site. **Microscopically, spinal bone dust is composed of 65% bone, mixed with blood products and fibrous tissue.**⁷ There are a number of advantages to the use of bone dust as a graft material. First, the autologous nature of bone dust eliminates potential disease transmission and immune rejection associated with some allograft and synthetic products. Second, bone dust harvest is simple and safe to perform, contrary to the high donor site complications associated with the use of ICBG. **Finally, studies have shown that bone dust is a reservoir for viable osteoblasts and mesenchymal stem cells with osteogenic potential.**^{8–10}

From a regenerative point of view, bone dust can potentially release anabolic factors into the host environment and promote bone formation. However, **till date, there is no information in the literature examining whether bone dust is capable of releasing any anabolic factors.** We hypothesized that human posterior spinal **bone dust releases anabolic factors capable of enhancing human osteoblast proliferation and maturation.** Therefore, this study was designed to assess the therapeutic potential of human spinal bone dust through its effect on human osteoblasts.

MATERIALS AND METHODS

Ethical Approval

The New Zealand Northern Regional Ethics Committee approved this study. Informed consent was obtained from patients donating bone dust or trabecular bone tissue used for osteoblast culture.

Reagents and Materials

Dulbecco's Modified Eagle Medium (DMEM) and Penicillin/Streptomycin mixture (10,000U/mL) were purchased from Gibco ThermoFisher Scientific Inc. (MA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (UT). Bovine serum albumin (BSA) was purchased from Immuno-Chemical Products Ltd. (Auckland, New Zealand). L-ascorbic acid-2-phosphate (AA2P) was purchased from Sigma-Aldrich (MI). ³H-Thymidine was purchased from Amersham Biosciences (NJ). Tissue culture plates and inserts (1 μm pore size) were purchased from Greiner Bio-one (Kremsmünster, Austria). Sandwich enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (MN). The specific ELISA kits with the R&D Systems catalogue number in parenthesis include: IL-1β (DY201), IL-6 (DY206), TGF-β (DY240), VEGF (DY293B), FGF-basic (DY233), and PDGF-BB (DBB00).

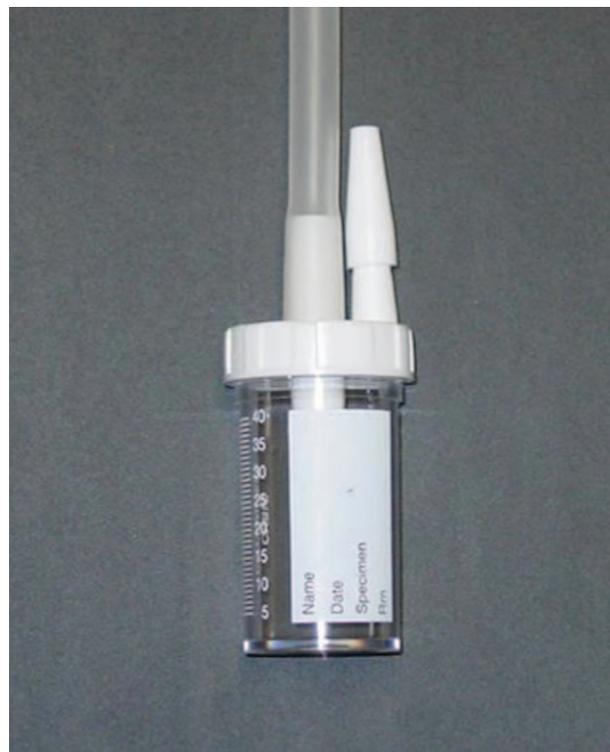


Figure 1. Inline suction trap. Suction trap used for bone dust collection during spinal fusion surgeries.

Human Osteoblast Culture

Primary human osteoblasts were grown from trabecular bone explants obtained from patients undergoing **hip or knee arthroplasty for osteoarthritis.** The trabecular bone explants were harvested intraoperatively by orthopedic surgeons and authors (RG and JM). The osteoblast cultures were carried out within our laboratory by trained senior technicians. The bone was chopped into chips and the bone marrow removed by repeated washes with Phosphate Buffered Saline (PBS). The bone chips were placed in T75 flasks with DMEM containing 10% FBS, 5μg/mL AA2P and antibiotics. When the first sign of osteoblast outgrowths were observed the medium was refreshed and the outgrowing osteoblasts, having twice weekly media changes, were grown to 90% confluence. The osteoblasts were then trypsinized, washed, and cryogenically stored in liquid nitrogen until the cells were needed.

Human Spinal Bone Dust Collection and Processing

Bone dust samples were harvested from patients over 18 years of age undergoing elective posterior lumbar spinal fusions. All patients underwent the spinal surgery as per routine practice. During the surgical approach, a **3 mm burr (6000 rpm) was used to remove bone in preparation for spinal fusion (Midas Rex Legend burr, Medtronic, Minneapolis, Minnesota, USA).** **The burr tip was constantly cooled by normal saline lavage to minimize the risk of thermal damage.** The bone dust generated as part of the burring process was collected using an inline suction trap (Figure 1). Once full, the content within the suction trap was emptied onto gauze to soak up excess fluid. The



Figure 2. Bone dust 'pate.' Image of bone dust collected from one patient during posterior spinal fusion surgery.

remaining product contained a mixture of bone dust and blood, resembling a pate (Figure 2). The bone dust was then transferred to the laboratory where it was washed with PBS and then loaded into tissue culture inserts with 1 μm pore size at 100 mg/insert. The inserts with bone dust were then submerged under the culture media and suspended over human osteoblasts seeded in 24-well plastic tissue culture plates. This novel experimental design prevented direct contact of bone dust with primary human osteoblasts; however, the pores in the inserts permitted factors released from the bone dust to diffuse into the media to act on the human osteoblasts (Figure 3). Each experiment contained six wells of bone dust, and each experiment was repeated with four different patient samples. For each experiment, matching control wells were used with equivalent number of cells seeded per well.

Release of Anabolic Factors

Bone dust in tissue culture inserts was incubated in serum starvation media over a 7-day period (DMEM with 0.1% BSA and 10 $\mu\text{g}/\text{mL}$ AA2P). Conditioned media was collected at days 0, 1, 2, and 7 after incubation, and stored at -20°C until used. Human IL-1 β , IL-6, TGF- β , VEGF, FGF-basic, and PDGF-BB were measured in the supernatants using ELISA according to the protocols of the manufacturer (R&D Systems, MN). Statistical analysis was performed using one-way ANOVA and posthoc Dunnett's test ($*P < 0.05$).

Cell Mitogenesis

Primary human osteoblasts were seeded into 24-well tissue culture plates at a density of 2.5×10^4 cells/well in DMEM

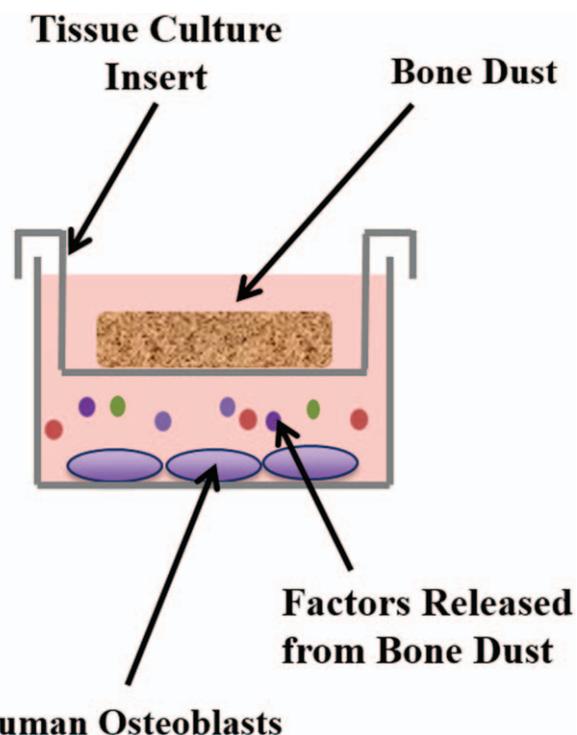


Figure 3. *In vitro* methodology. Schematic diagram demonstrating the experimental set up. Primary human osteoblasts are seeded in 24-well plates, above which bone dust is placed in tissue culture inserts with a 1 μm pore size. The pores in the tissue culture inserts allow factors released from the bone dust to act on primary human osteoblasts.

with 5% FBS and 10 $\mu\text{g}/\text{mL}$ AA2P for 24 hours. Cells were growth arrested in 0.1% BSA for 24 hours. Tissue culture inserts alone (control) or with 100 mg of bone dust were then added. After an overnight incubation period, inserts were removed and cell mitogenesis was measured by determining cell incorporation of ^3H -Thymidine using the Wallac Microbeta Trilux 1450 (PerkinElmer, Waltham, MA) during a 6-hour window at the end of 24 hour of treatment. Statistical analysis was performed using an unpaired *t* test ($*P < 0.05$).

Gene Expression

Human osteoblastic cultures were incubated with bone dust as described for cell mitogenesis. Cell pellets were collected at days 0, 1, 2, and 7 of incubation with bone dust. Total cellular RNA was extracted from cultured cells and purified using the RNeasy mini kit (Qiagen, the Netherlands). Genomic DNA was removed using RNase-free DNase set (Qiagen). The quantity and purity of the RNA were measured using a NanoDrop Lite spectrophotometer (Thermo Scientific, Australia). Reverse transcription (500ng RNA used for each sample) was carried out using SuperScript III (Life Technologies, Auckland, New Zealand); cDNA was used for real-time PCR. Primer-probe sets were purchased as TaqMan Gene Expression Assays from Applied Biosystems, Auckland, New Zealand (Thermo Scientific). Multiplex PCR was performed with FAM-labeled probes specific for

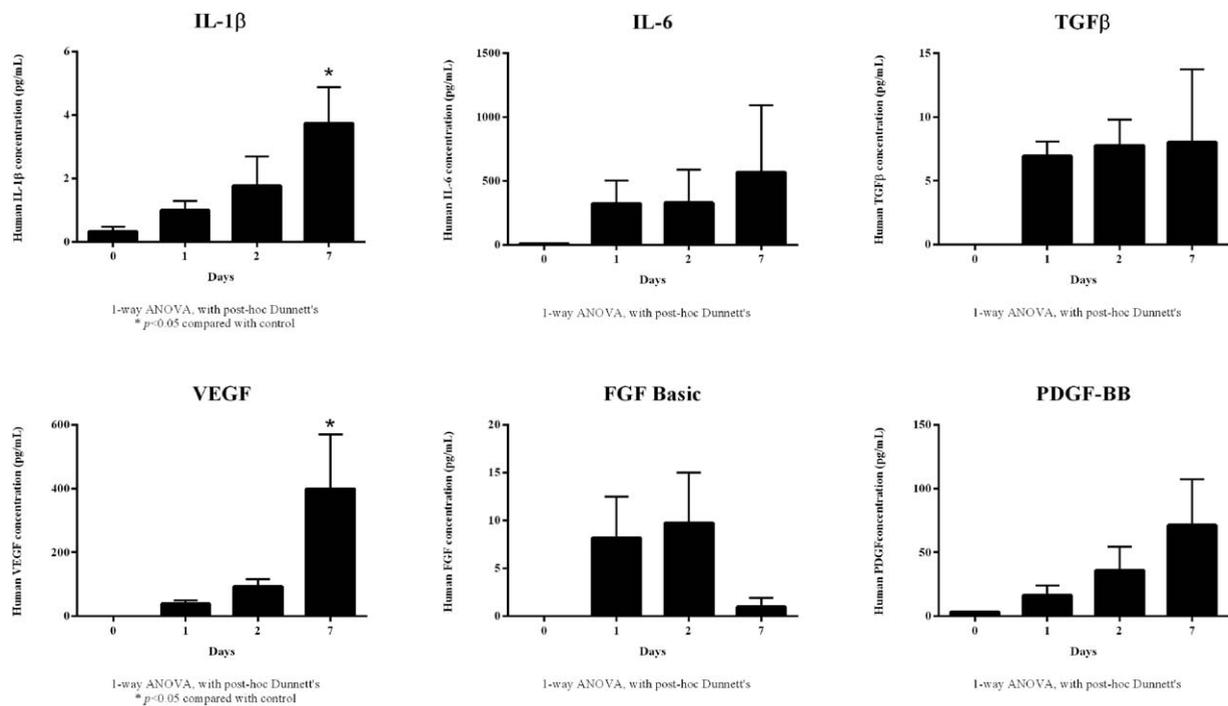


Figure 4. Bone dust acts as a source of growth factors and cytokines. Enzyme-linked immunosorbent assay (ELISA) results demonstrating the presence of a number of anabolic growth factors (TGF- β , VEGF, FGF-Basic, and PDGF-BB) and cytokines (IL-1 β and IL-6) released from human posterior spinal bone dust over a 7-day period. Data presented are mean of four patient samples \pm SEM. Statistical analysis was performed using one-way ANOVA and posthoc Dunnett's test. * $P < 0.05$ ($n = 4$).

the genes of interest, and VIC-labeled 18S rRNA probes according to the company's instructions, using a QuantStudio 12K Flex Real-Time PCR machine (Applied Biosystems). Samples were assayed in triplicates. The relative level of mRNA expression was determined using the $\Delta\Delta C_t$ calculation method, normalized to values of the nontreated cells at day 0. Statistical analysis was performed using two-way ANOVA and posthoc Dunnett's test (* $P < 0.05$).

RESULTS

Bone Dust Releases Cytokines and Growth Factors

The two proinflammatory cytokines (IL-1 β and IL-6) and four growth factors (TGF- β , VEGF, FGF-Basic, and PDGF-BB) tested in our study were detectable using ELISA (Figure 4). Although there was high variability among the four patients, the overall growth factor and cytokine release profile was consistent, with increased concentrations of IL-1 β , IL-6, TGF- β , VEGF, and PDGF-BB, over 7 days. The concentration of FGF-Basic peaked on day 2.

Bone Dust Promotes Osteoblast Proliferation and Induces Changes in Osteoblast Gene Expression

Using ^3H -thymidine incorporation, we found that bone dust treatment increased osteoblast proliferation by approximately 7-fold compared with control ($n = 4$) ($P < 0.05$) (Figure 5).

A number of genes were upregulated in response to bone dust treatment, including Integrin-Binding Sialoprotein

(IBSP) a gene important for osteoblast mineralization and maturation; and genes integral to inflammation and macrophage chemotaxis: Interleukins (IL-6, IL-11) and Monocyte Chemoattractant Protein 1 (MCP1).

Although there were no significant changes in the expression of the early osteoblast differentiation marker Runt-related transcription factor 2 (RUNX2), on days 2 and 7, the expression of the late osteoblast marker IBSP was more than

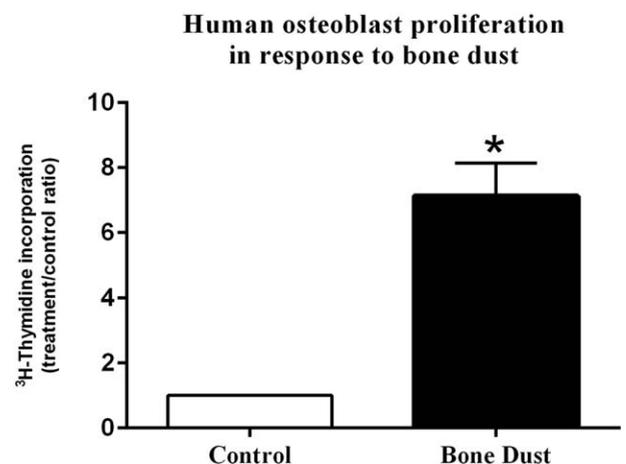


Figure 5. Primary human osteoblast proliferation increased in response to bone dust. ^3H -Thymidine incorporation in primary human osteoblasts cultured in the presence of bone dust for 24 hours. Data presented are mean of four patient samples \pm SEM. Statistical analysis was performed using unpaired t test. * $P < 0.05$ ($n = 4$).

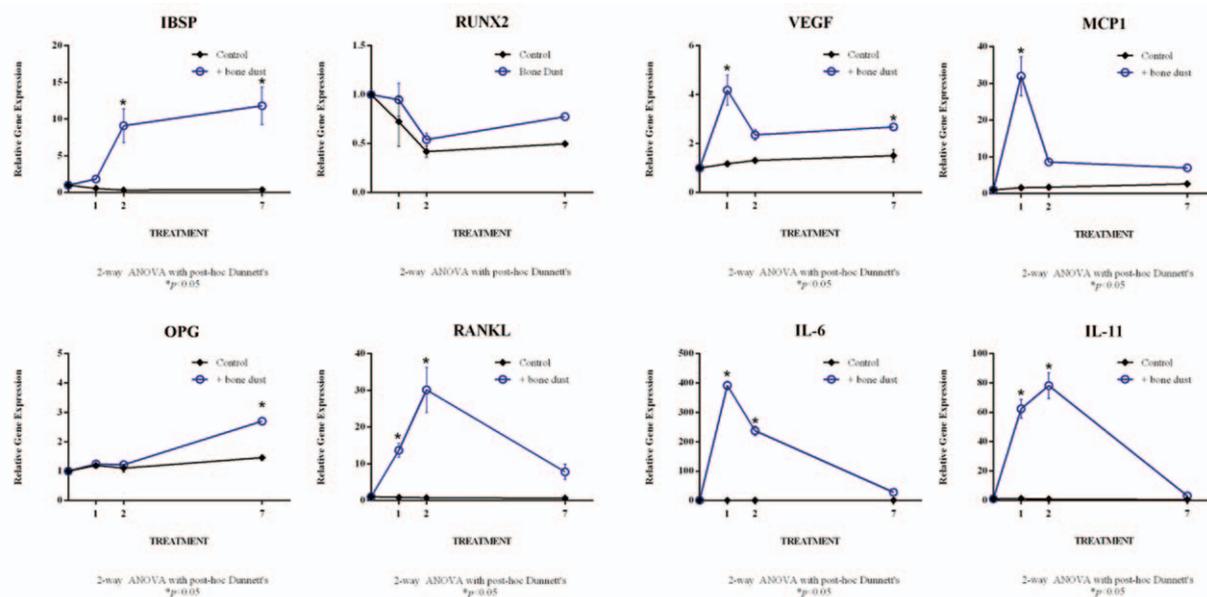


Figure 6. Bone dust increases osteoblast gene expression. Expression levels of osteoblastic marker genes, and genes important in inflammation and angiogenesis, in primary human osteoblasts exposed to bone dust for up to 7 days, as determined by real-time PCR. Results are presented relative to the expression of each gene on day 0 in the control group. The osteoblasts cultured without bone dust (control) are represented by black diamonds; open circles represent osteoblasts treated with bone dust. Data presented are mean \pm SEM. Statistical analysis was performed using two-way ANOVA and posthoc Dunnett's test. * $P < 0.05$. Representative data from one patient shown.

8-fold higher in response to treatment with bone dust compared with control ($P < 0.05$). With regards to genes that play an integral role in the coupling with osteoclasts, the expression of receptor activator of nuclear factor- κ B ligand (RANKL) was higher on days 1 and 2 compared with control ($P < 0.05$), whereas the expression of Osteoprotegerin (OPG) was higher on day 7 in the group treated with bone dust ($P < 0.05$). Finally, the expression of VEGF, a gene critical for angiogenesis, was upregulated in the osteoblasts treated with bone dust compared with control on days 1 and 7 ($P < 0.05$) (Figure 6).

DISCUSSION

Herein, we have demonstrated for the first time that bone dust is capable of releasing growth factors and cytokines with anabolic effects on human osteoblasts. We believe that the presence of osteogenic cells within bone dust, coupled with the anabolic factors demonstrated here acting on the local cell population, will enhance osseous fusion in spinal fusion surgeries.^{8,10} The novel findings from our study provide further evidence to support the clinical application of spinal bone dust as an autologous bone graft.

Successful spinal fusion hinges on the interplay of a number of growth factors and cytokines acting locally on host cells to promote bone formation.¹¹ We showed that bone dust releases the cytokines (IL-1 β , IL-6), and growth factors (TGF- β , VEGF, FGF-Basic, and PDGF-BB), all with known anabolic effects on bone healing. *In vitro*, VEGF, FGF-Basic, and PDGF are all implicated in osteoblast proliferation and differentiation.^{12,13} In the context of bone regeneration, FGF-Basic has been shown to enhance bone

healing in preclinical long bone and calvarial models.^{14,15} In addition, FGF-Basic is involved in angiogenesis, which is a critical step in bone regeneration.¹⁶ Another growth factor that plays pivotal roles in angiogenesis is VEGF. In our study, we detected increasing concentrations of VEGF from day 0 to day 7 ($P < 0.05$), an effect that will likely be beneficial for bone regeneration. In addition to promoting angiogenesis, VEGF has been shown to influence bone formation and remodeling by inducing osteoblast and osteoclast differentiation.¹³ Another important growth factor for bone remodeling and repair is TGF- β .¹⁷ TGF- β stimulates matrix protein synthesis and modulates bone formation. *In vivo*, TGF- β has been shown to be an important growth factor for bone healing in a number of animal models.^{18,19}

In general, proinflammatory cytokines, including IL-1 β and IL-6, are active during the early phase of bone healing. *In vitro*, IL-1 β has been shown to promote osteoblast proliferation and mineralization.²⁰ *In vivo*, IL-1 β is active during the early fracture repair process and promotes osteoblastogenesis.^{20,21} Similarly, IL-6 is involved in the regulation of osteoblast differentiation and mineralization and maturation of fracture callus in murine models.^{20,22}

The ability of bone dust to release anabolic factors is clearly a desirable feature, and one that has the potential to enhance osseous union in spinal fusion surgeries. Clinically, the primary objective of spinal fusion surgeries is to achieve a mechanically solid fusion mass through proliferation and maturation of host osteoblasts into functional bone, an effect that is likely to be augmented by the anabolic factors released from bone dust.^{11,13} Using ³H-thymidine

incorporation, we showed that the anabolic factors released from bone dust increased osteoblast proliferation by approximately seven-fold. The profound increase in osteoblast proliferation highlighted the anabolic effect of anabolic factors released from bone dust, and reiterated bone dust's potential therapeutic value.

To form a mechanically solid fusion mass, the host osteoblasts must be able to differentiate and produce functional bone, a process that follows specific regenerative patterns and involves changes in the expression of a number of osteoblastic genes. Having demonstrated the potent proliferative effect of bone dust on human osteoblasts, the final step of our study was to investigate the effect of the anabolic factors released from bone dust on osteoblast gene expression. Osseous union in spinal fusion begins with an acute inflammatory phase involving hematoma formation and upregulation of a number of corresponding genes.^{11,23,24} In this study, a number of genes implicated in bone healing (IL-6, IL-11, MCP1) were significantly upregulated at the early time points in response to bone dust treatment compared with control ($P < 0.05$).²⁵⁻²⁸ Studies have shown that both IL-6 and IL-11 are important regulatory genes in bone formation;^{26,27} whereas MCP1 functions as an important chemokine that regulates the recruitment of bone forming cells during fracture repair.²⁸

With regards to the genes integral to osteoblast maturation, factors released from bone dust triggered a significant upregulation of IBSP, a late osteoblast marker involved in osteoblast mineralization ($P < 0.05$).²⁹ In bone regeneration, coupling occurs between osteoblasts and osteoclasts. In our study, we demonstrated that bone dust treatment led to an increase in the expression of both OPG and RANKL. Under physiological conditions, the RANKL/RANK signaling pathway regulates the formation and survival of osteoclasts. OPG on the other hand, functions as a decoy receptor to RANKL; hence, OPG protects the skeleton from excessive bone resorption.³⁰ The upregulation of both OPG and RANKL in response to bone dust treatment may enhance and regulate bone remodeling at the site of spinal fusion. Finally, we observed an increase in the expression of VEGF. VEGF expression plays a pivotal role in angiogenesis which is important in bone regeneration. In summary, human osteoblastic genes integral in the early phase of bone healing (IL-6, IL-11, and MCP-1), and osteoblast mineralization (IBSP), angiogenesis (VEGF), and bone remodeling (OPG, RANKL, and VEGF), are upregulated in response to bone dust treatment and may translate to accelerated bone healing in spinal fusion surgeries.

A potential limitation in our study is the use of multiple sources of tissue samples. Primary human osteoblasts were grown from trabecular bone harvested from patients undergoing hip or knee arthroplasty, whereas bone dust samples were harvested from patients undergoing spinal fusion surgery. The primary osteoblasts were cultured for up to 2 weeks before they reach the cell density required for the experiment, whereas bone dust samples were used within 24 hours of surgery. Therefore, the osteoblasts and bone

dust in each experiment were not sourced from the same patient. Although we believe it is very unlikely, we cannot exclude the possibility that cells respond differently to factors released from the patient's own bone dust to those released from a sample taken from a different patient. Reassuringly, the multiple biological repeats in our study have found overall similar profiles of growth factors and cytokines released from bone dust samples, and similar changes in gene expression in the osteoblasts in response to these factors. Although the amount of released factors and the magnitude of changes in gene expression varied, this degree of variability among patients' samples was not unexpected, and is common in studies of this nature.

The strength of our study is the translational nature of our methodology. Here we have utilized the novel approach of suspending the bone dust samples in media to prevent direct contact of the bone dust and the relevant target cell. This allowed anabolic factors from the bone dust to diffuse into the tissue culture media and exert their effect on the human osteoblasts, which *in vivo* would be the cells driving new bone formation. Future studies could adopt this methodology to further understand the effect of bone dust on osteoblast matrix production and elucidate its effect on osteoclast formation and activity.

CONCLUSION

This study is the first to demonstrate that human posterior spinal bone dust is a source of anabolic factors that potently enhance human osteoblast proliferation and gene expression in a favorable pattern for bone healing, regeneration and ultimately, osseous union. Given that bone dust harvest is fast, simple, cheap, and safe to perform, and bone dust releases anabolic factors and contains viable osteoblasts and mesenchymal stem cells with osteogenic potential, spinal surgeons should be encouraged to 'recycle' spinal bone dust and harness the regenerative potential of this free autologous bone graft.

➤ Key Points

- ❑ Human spinal bone dust is an autologous bone graft that can be easily collected during spinal fusion surgeries using cheap suction traps.
- ❑ Bone dust releases anabolic factors that enhance primary human osteoblast proliferation and gene expression in a favorable pattern for bone healing, regeneration and ultimately, bone union.
- ❑ Viewed in the context of currently available evidence, spinal surgeons should be encouraged to 'recycle' bone dust, a free source of autologous osteoblast and anabolic growth factors, during posterior spinal fusion surgeries.

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