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ORIGINAL ARTICLE

Pre-coating deproteinized bovine bone mineral (DBBM) with bone-conditioned medium (BCM) improves osteoblast migration, adhesion, and differentiation in vitro

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Abstract
Objectives Autogenous bone grafting has remained the gold standard for bone augmentation procedures with ability to release growth factors to the surrounding microenvironment. Recent investigations have characterized these specific growth factors released by autogenous bone chips with further isolation into a "bone-conditioned medium" (BCM). The aim of the present investigation was to utilize autologous growth factors from bone chips (BCM) in combination with deproteinized bovine bone mineral (DBBM) and investigate the ability for BCM to enhance osteoblast behavior.
Materials and methods Mouse ST2 cells were seeded on (1) DBBM particles alone or (2) DBBM + BCM. Thereafter, samples were compared for cell recruitment, adhesion, proliferation, and real-time PCR for osteoblast differentiation markers including Runx2, collagen 1 alpha 2 (COL1A2), alkaline phosphatase (ALP), and osteocalcin (OCN). Alizarin red staining was used to assess mineralization.
Results Coating BCM on DBBM particles improved cell migration of ST2 cells and significantly enhanced a 2-fold increase in cell adhesion. While no significant increase in cell proliferation was observed, BCM significantly increased mRNA levels of COL1A2, ALP, and OCN at 3 days post seeding. Furthermore, a 3-fold increase in alizarin red staining was observed on DBBM particles pre-coated with BCM.
Conclusion Pre-coating DBBM with BCM enhanced the osteoconductive properties of DBBM by mediating osteoblast recruitment, attachment, and differentiation towards bone-forming osteoblasts. Future animal study is necessary to further characterize the added benefit of BCM as an autogenous growth factor source for combination therapies.
Clinical relevance The application of BCM in combination with biomaterials may serve as an autogenous growth factor source for bone regeneration.

Keywords Bone-conditioned media · Guided bone regeneration · Bone grafting · Barrier membranes · Growth factors

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Introduction

Despite numerous attempts that have been made in recent years with the development of novel bioactive biomaterials, the use of autogenous bone has remained the gold standard for a variety of bone augmentation procedures due to its excellent combination of osteogenesis,

osteoinduction, and osteoconduction [1]. Advantages of locally harvesting autogenous bone chips for guided bone regeneration procedures include (1) the use of an autogenous source with no issues of immune system compatibility, (2) a bone graft containing living progenitor cells within the scaffold capable of differentiating into and/or stimulating bone forming osteoblasts, as well as (3) the ability for the graft to release a wide array of osteoinductive growth factors capable of speeding new bone formation [2]. For these reasons, favorable long-term results in implant dentistry requiring bone augmentation procedures are routinely augmented using autogenous bone chips adjacent to implant surfaces to favor faster implant osseointegration [3, 4].

Recently our laboratory has been involved in a series of in vitro experiments aimed at discovering the key elements that make autogenous bone grafts so favorable for bone augmentation procedures. It was first discovered that harvesting technique could significantly vary the survival of bone cells contained within the matrix of autogenous bone chips and subsequently alter their release of potential osteoinductive growth factors [2, 5, 6]. Thereafter, proteomic analysis of the growth factors released from autogenous bone chips were characterized into 43 different growth factors such as gelsolin, pleiotrophin, alpha-2-HS-glycoprotein, annexin A2, biglygan, decorin, transforming growth factor (TGF)-beta-induced protein ig-h3, latency-associated peptide, and TGF-beta2 [7]. The role of each of these growth factors and extracellular matrix molecules and their contribution to bone remodeling is further discussed by Caballé-Serrano [7]. Interestingly, these proteins may be harvested and collected into a so called "bone-conditioned medium" (BCM) which has been the basis of a number of in vitro studies from our laboratory [5, 8–11]. The primary objective of these studies was to reveal the potential for BCM to affect a wide variety of cell types implicated in graft consolidation. It has been shown that BCM induces osteoclastogenesis [8] and improves oral fibroblast cell activity through transforming growth factor-beta activity both from autogenous bone chips as well as demineralized bone chips [12, 13].

Based on these preliminary findings, the possibility exists that local collection of growth factors from autogenous bone chips over a period of time into BCM may potentially be utilized as a fresh source of growth factors derived specifically from bone tissues for future bone regeneration. Therefore, the aim of the present study was to collect BCM from autogenous bone chips and utilize this freshly prepared bone growth factor source to subsequently investigate if the regenerative potential of an osteoconductive deproteinized bovine bone mineral (DBBM) could be improved following coating with BCM.

Methods

BCM and bone grafting material source

The bone was obtained from adult pigs (Metzgerei Balsiger, Wattenwil, Switzerland), harvested from the buccal-sided mandibular cortical bone with a "bone scraper" (Hu-Friedy, Rotterdam, Netherlands), and placed into sterile plastic dishes as previously described [9]. An in vivo ethical approval was not necessary as the animals were euthanized at the local butcher for non-scientific reasons, and thereafter, the bone samples were immediately collected and transported for use in this study. BCM was harvested by placing 10 mL of culture media with 5 g of autogenous bone chips. After 24 h of incubation at 37 °C in a humidified atmosphere and 5 % carbon dioxide, BCM was filtered sterile and kept frozen at −80 °C and thawed during experimental cell seeding as previously described [9]. For all in vitro cell experiments, cells were exposed to culture media containing 20 % BCM at time point 0 until the end of the experiment. DBBM (BioOss, Geistlich, Switzerland) was utilized as the material of choice for the present study due to our laboratories previously handling with its use [14, 15]. For in vitro experiments, 8 mg of DBBM particles were placed in the bottom of 24-well dishes and thereafter coated with BCM for 5 min prior to cell seeding.

Cell culture system

Undifferentiated mouse cell-line ST2 stromal bone marrow cells were used for this study.

Cells were detached from the tissue culture plastic using trypsin solution. During cell seeding for differentiation experiments, α-MEM medium (Invitrogen, Basel, Switzerland) was supplemented with 50 μg/mL ascorbic acid (Invitrogen) and 2 mM β-glycerophosphate (Invitrogen) to promote osteoblast differentiation as previously described [2]. Osteoblasts were seeded at a density of 10,000 cells in 24-well culture plates for cell adhesion and proliferation experiments and 50,000 cells per well in 24-well dishes for real-time PCR and alizarin red experiments. For experiments lasting longer than 5 days, medium was replaced twice weekly.

Cell migration assay

The migration assay of ST2 cells was performed with a Boyden chamber using a 24-well plate and polyethylene terephthalate filters with a pore size of 8 μm according to the manufacturer's protocol (ThinCert™, Greiner Bio-One GmhH, Frickenhausen, Germany). Briefly, DMEM containing 10 % FBS were filled over NBM alone or NBM + BCM into the lower compartment of the wells. After cells were starved in DMEM containing 0.5 % FBS for 12 h, 10,000 cells were re-suspended in medium with 0.5 % FBS and

seeded into the upper compartment. The cells were allowed to migrate for 8 h at 37 °C in a humidified 5 % CO_2 atmosphere. After 8 h, cells were fixed with 10 % neutral buffered formalin for 2 min. After washing with PBS twice, cells on the insert membrane were permeabilized by methanol for 20 min and stained with Giemsa solution (MERCK, Darmstadt, Germany) for 15 min. Excess Giemsa solution was removed by merging the insert in ddH_2O three times, and the upper side of the filter membrane was gently wiped by a cotton swab to remove the cell debris. The number of cells on the lower side of the filter was counted under a microscope. Data were analyzed for statistical significance using Student's *t* test (*p values <0.05 was considered significant).

Adhesion and proliferation assay

Osteoblasts were seeded in 24-well plates at a density of 10,000 cells per well either control DBBM particles or DBBM particles + BCM. Cells were quantified using fluorescent MTS assay (Invitrogen) at 1, 3, and 5 days for cell proliferation as previously described [16]. At desired time points, cells were washed with phosphate-buffered solution (PBS) and quantified using a fluorescence plate reader (Infinite 200, Tecan Group Ltd. Männedorf, Switzerland). Experiments were performed in triplicate with three independent experiments for each condition. Data were analyzed for statistical significance using two-way analysis of variance with Bonferroni test (*p values <0.05 was considered significant).

Real-time PCR for growth factors and differentiation markers

Real-time RT-PCR was used to investigate the expression of genes encoding osteoblast differentiation markers. Total RNA was isolated using High Pure RNA Isolation Kit (Roche, Basel, Switzerland) at 3 and 14 days. Primer and probe sequences for genes encoding alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), collagen 1 alpha 2 (COL1A2), osteocalcin (OCN), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were fabricated with Primer sequences according to Table 1. Reverse transcription was performed with Transcriptor Universal cDNA Master. Real-time RT-PCR was performed using Roche FastStart Universal SYBR Green Master and quantified on an Applied Biosystems 7500 Real-Time PCR Machine (Biosystems, Life Technologies Corporation, Carlsbad, CA). A Nanodrop 2000c (Thermo, Wilmington, DE) was used to quantify total RNA levels. All samples were assayed in duplicate with three independent experiments were performed. The ΔΔCt method was used to calculate gene expression levels normalized to total RNA values and calibrated to control samples. Data were analyzed for statistical significance using two-way analysis of variance with Bonferroni test (*p values <0.05 was considered significant).

Table 1 PCR primers for genes encoding Runx2, ALP, BSP, OCN, and GAPDH

Gene	Primer sequence
mRUNX2 F	agggactatggcgtcaaaca
mRUNX2 R	ggctcacgtcgctcatctt
mBSP F	gcatcgaagagtcaaaatag
mBSP R	ttcttctccattgtcttctc
mALP F	ggacaggacacacacacaca
mALP R	caaacaggagagccacttca
mOCN F	cagacaccatgaggaccatc
mOCN R	ggactgaggctctgtgaggt
mGAPDH F	aggtcggtgtgaacggatttg
mGAPDH R	tgtagaccatgtagttgaggtca

Alizarin red staining

Alizarin red staining was performed to determine the presence of extracellular matrix mineralization. After 14 days, cells were fixed in 96 % ethanol for 15 min and stained with 0.2 % alizarin red solution (Sigma-Aldrich) in water (pH 6.4) at room temperature for 1 h as previously described [2]. Alizarin red staining was performed using images captured on a Nikon D610 camera with a Heerbrugg M400 ZOOM microscope (Wild Heerbrugg, Switzerland). ImageJ software was used to quantify data using set parameters for color intensity staining of red using color threshold including parameters for hue, saturation, and brightness. The same threshold values were used for all analyzed. Means and standard deviations (SE) were calculated, and the statistical significance of differences was examined by Student's *t* test between both groups (*p values <0.05 was considered significant).

Results

Influence of BCM on osteoblast migration, adhesion, and proliferation

The influence of BCM was first investigated on osteoblast cell recruitment in a Boyden chamber (Fig. 1). It was found that BCM in combination with DBBM was able to significantly increase osteoblast progenitor cell recruitment when compared to DBBM alone (Fig. 1). Thereafter, the effects of BCM were investigated on early cell adhesion to bone grafting particles (Fig. 2). It was found that coating with BCM significantly improved cell adhesion at 4 h approximately 2-fold when compared to cells seeded on DBBM alone (Fig. 2). Cells seeded on both DBBM alone and BCM + DBBM demonstrated no significant difference in cell

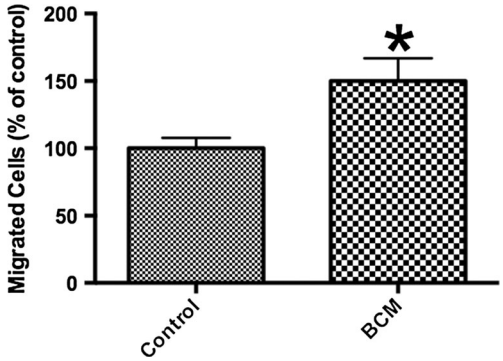

Fig. 1 Migration assay of ST2 osteoblasts seeded on (1) control DBBM particles and (2) DBBM particles coated with BCM. It was observed that BCM was able to significantly upregulate osteoblast recruitment (*asterisk* denotes significant difference, $p < 0.05$)

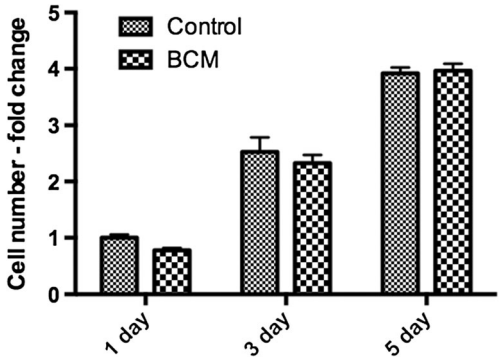

Fig. 3 Proliferation assay of ST2 osteoblasts seeded on DBBM alone and DBBM + BCM particles. No significant difference was observed between groups at 1, 3, and 5 days post seeding

proliferation as quantified by an MTS assay at 1, 3, or 5 days post seeding (Fig. 3).

Influence of BCM on osteoblast gene expression and mineralization

The effects of BCM were then investigated on osteoblast differentiation. It was first observed that BCM in combination with DBBM significantly enhanced early mRNA levels of COL1A2, ALP, and OCN at 3 days post seeding (Fig. 4). No significant difference was observed for Runx2 (Fig. 4a). By 14 days post seeding, the effects of BCM on mRNA levels returned to baseline levels equivalent with DBBM alone (Fig. 4). The effects of BCM on osteoblast mineralization potential were then investigated using alizarin red staining (Figs. 5 and 6). It was first observed that DBBM particles pre-coated with BCM demonstrated more intensely stained DBBM particles (Fig. 5b) when compared to DBBM alone (Fig. 5a). Quantitative analysis via color thresholding software demonstrated significantly higher alizarin red staining for DBBM particles pre-coated with BCM (Fig. 6).

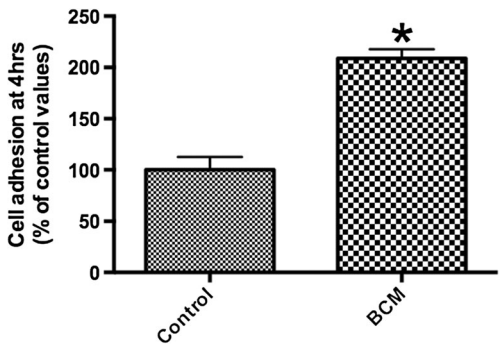

Fig. 2 Effect of BCM on the attachment of ST2 osteoblasts to DBBM particles. Significantly more cells attached to DBBM particles coated with BCM at 4 h when compared to BCM alone (*asterisk* denotes significant difference, $p < 0.05$)

Discussion

A great deal of research over the past decade has been performed with respect to combination approaches utilizing growth factors with either collagen barrier membranes or bone grafting materials. Growth factors commonly utilized in regenerative dentistry include bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), enamel matrix derivative (EMD), and fibroblast growth factor-2 (FGF2) [17–21]. Despite attempts that have been made to combine grafting materials or barrier membranes with recombinant growth factors, autogenous bone has remained the gold standard of bone grafting materials due to its excellent combination of osteogenesis, osteoinduction, and osteoconduction. Some of the reported drawbacks of utilizing recombinant growth factors include their costs, bioactivity over time caused by protein instability, fast dissolution rates, short half lives, and side effects caused by high supra-physiological doses [22–24]. For these reasons, our group has recently been interested in employing new strategies whereby harvesting of autogenous growth factors derived from bone chips may present possible advantages over recombinant growth factors.

The aim of the present study was to investigate the use of BCM as a possible autogenous growth factor source for bone regeneration. Simply, BCM hypothetically resembles other previously utilized autogenous growth factor such as platelet-rich plasma (PRP) or platelet-rich fibrin (PRF). Whereas the concentration of growth factors in PRP/PRF are derived from whole blood and have been shown to be successfully utilized for certain regenerative procedures in dentistry [25, 26], the possible use of BCM is a similar collection of growth factors from an autogenous source, however, derived specifically from bone tissues. This collection of growth factors is hypothesized to contribute more specifically to bone regeneration since the factors released into BCM are known contributors of bone remodeling [6, 7]. Previous studies performed by our group found that BCM contains over 40 different growth factors and extracellular matrix molecules released

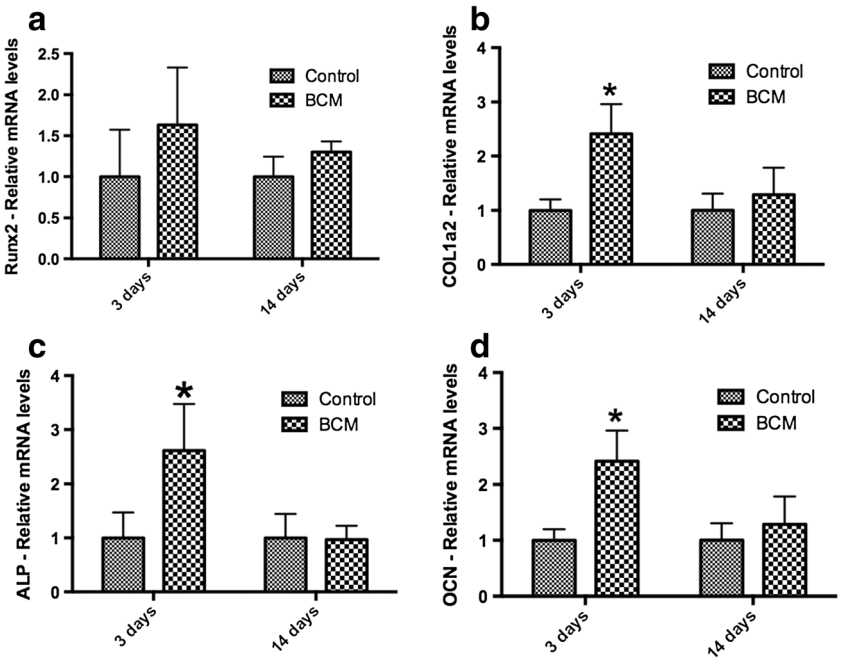

Fig. 4 Real-time PCR of osteoblast differentiation markers for genes encoding **a** Runx2, **b** collagen 1 alpha 2 (COL1A2), **c** alkaline phosphatase (ALP), and **d** osteocalcin (OC) at 3 and 14 days post seeding. DBBM particles pre-coated with BCM demonstrated significantly higher levels of COL1A2, ALP, and OCN at 3 days post seeding when compared to DBBM alone (*asterisk* denotes significant difference, $p < 0.05$)

from bone chips into BCM [7]. This mixture of proteins has furthermore been shown to induce gingival cell activity and modulate osteoclastogenesis [6, 7]. In vitro research by our group later found that BCM induces a strong response of TGF-beta-dependent pathways by significantly altering the expression of ADM, IL11, IL33, NOX4, PRG4, and PTX3, all known downstream regulators of TGF-beta pathways [27]. Based on the combination of these previous studies, it was hypothesized in the present investigation that BCM could potentially contribute to osteoblast activity by favoring osteoblast recruitment, adhesion, and differentiation towards bone-forming osteoblasts.

To date, no study has investigated the possible use of autogenous growth factors derived specifically from bone tissues in combination with a bone-grafting material. In the present study, we found that BCM in combination with DBBM was able to significantly upregulate osteoblast cell migration towards bone particles, favored their cellular attachment, and enhanced mRNA levels of early osteoblast differentiation markers including COL1A2, ALP, and OCN. Investigation of mRNA levels at 14 days demonstrated that these factors returned to baseline at a later time point, thus demonstrating that most of the effects of BCM seem to favor early time periods (Fig. 4). Nevertheless, the in vitro mineralization was quantified at 14 days using alizarin red staining confirming that the addition of BCM to DBBM particles promoted in vitro mineralization (Fig. 6). While these preliminary findings demonstrate a positive effect for BCM in combination with a bone grafting material, it remains to be investigated what effect BCM may carry in vivo with a much greater regenerative potential needed.

Furthermore, there remain some key questions that need to be addressed prior to the possible clinical utilization of growth factors derived from bone chips. First, the stock solution of BCM used in the present investigation was derived from a collection of culture medium harvested from bone chips after a 24-h period.

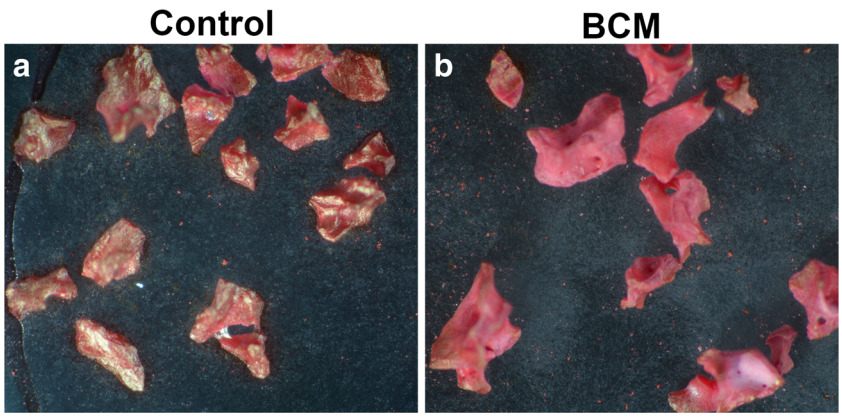

Fig. 5 Visual microscopic images of **a** DBBM and **b** DBBM + BCM particles stained with alizarin red. Particles that were pre-coated with BCM demonstrated more intensely stained surfaces for alizarin red at 14 days post seeding

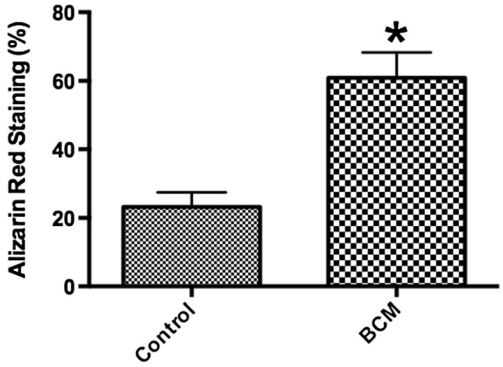

Fig. 6 Percentage of alizarin red staining of DBBM alone and DBBM particles coated with BCM (*asterisk* denotes significant difference, $p < 0.05$)

Since the average guided bone regeneration procedure during clinical practice typically lasts 45 min to 1 h, it remains to be investigated what concentration of accumulated growth factor would be derived from this shorter period and what effect this might have on cell activity. Furthermore, it is known that the use of recombinant growth factors typically require a much lower growth factor concentration for in vitro use when compared to animal or clinical use [16]. Therefore, the ideal concentration for in vivo use remains to be investigated. It is also unknown at present how patient genetic variability and age might affect the osteopromotive potential of BCM. It is known in the literature that patient variability affects the osteoinductive potential of demineralized freeze-dried bone allografts [28, 29]. Therefore, it remains to be determined how patient age, systemic conditions, or gender may affect the regenerative potential of BCM. Nevertheless, the findings derived from the present study reveal a possibility for future harvesting of autogenous growth factors derived specifically from bone chips.

Conclusions

In summary, coating DBBM particles with BCM significantly increased cell migration, attachment, and differentiation of ST2 cells towards bone formation osteoblasts. Although many questions remain to be investigated prior to utilizing this strategy as an autogenous growth factor source for bone regeneration, these preliminary results suggest that BCM promotes the cellular activity of osteoblasts in vitro. Future animal testing is now necessary to further evaluate the regenerative potential of BCM for bone regeneration.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest DBBM particles were kindly supplied by Geistlich AG, Switzerland. All authors declare no conflict of interest.

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Informed consent For this type of study, informed consent was not required.

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