Published in final edited form as:

Wound Repair Regen. 2019 July ; 27(4): 335–344. doi:10.1111/wrr.12710.

Sub-clinical dose of bone morphogenetic protein-2 does not precipitate rampant, sustained inflammatory response in bone wound healing

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Abstract

Large bone injuries, defects, and chronic wounds present a major problem for medicine. Several therapeutic strategies are used clinically to precipitate bone including a combination therapy delivering osteoinductive *bone morphogenetic protein 2* (rhBMP-2) via an osteoconductive scaffold (*absorbable collagen sponge*, ACS) (i.e. INFUSE). Adverse side effects reportedly associated with rhBMP2 administration include rampant inflammation and clinical failures. Although acute inflammation is necessary for proper healing in bone, inflammatory cascade dysregulation can result in sustained tissue damage and poor healing. We hypothesized that a subclinical dose of rhBMP2 modeled in the murine calvarial defect would not precipitate alterations to inflammatory markers during acute phases of bone wound healing. We utilized the 5mm critical size calvarial defect in C57BL6 wild-type mice which were subsequently treated with ACS and a subclinical dose of rhBMP2 shown to be optimal for healing. Three and seven-day

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⁸Declaration of Conflicting Interests

Medtronic Inc., the proprietor of Infuse rhBMP-2 tested here, currently employs R. Nicole Howie. James Cray currently has an executed Confidential Disclosure Agreement with Medtronic Inc. the proprietor of Infuse rhBMP-2. Both of these relationships are unrelated to the current study and occurred after completion of this work.

post-operative time points were used to assess the role that rhBMP-2 plays in modulating inflammation versus ACS alone by cytokine array and histological interrogation. Data revealed that rhBMP-2 delivery resulted in substantial modulation of several markers associated with inflammation, most of which decreased to levels similar to control by the seven day time point. Additionally, while rhBMP-2 administration increased macrophage response, this peptide had little noticeable effect on traditional markers of macrophage polarization (M1-iNOS, M2-Arg1). These results suggest that rhBMP-2 delivered at a lower dose does not precipitate rampant inflammation. Thus, an assessment of dosing for rhBMP-2 therapies may lead to better healing outcomes and less surgical failure.

Keywords

Bone Healing; rhBMP2; Inflammation; Macrophage Activity

1. Introduction

While bone possesses the innate capacity for spontaneous regeneration, trauma or surgery resulting in large portions of ablated bone often do not heal properly and present a major clinical problem (1–3). In such cases, therapeutic intervention is necessary. In the United States alone it is estimated that over 100,000 fractures develop non-unions each year, resulting in a significant financial burden (4, 5). While the gold standard intervention for these cases is the autologous bone graft, limited tissue available for harvest, creation of a second surgical site, and donor site co-morbidity have necessitated the search for alternative therapeutic strategies (5–14). One strategy that has become particularly prominent is the application of exogenous recombinant human bone morphogenetic protein 2 (rhBMP-2) via an absorbable collagen sponge (ACS) (15, 16). This product currently has four United States Food and Drug Administration (FDA) approved clinical indications: spinal fusions, tibial shaft fractures, maxillary sinus lift, and alveolar ridge augmentations. However, the use of rhBMP-2 has far surpassed these limits and now, off-label application is reported to represent over 90% of rhBMP-2 use (13, 17). While this protein has revolutionized the field of bone biology, adverse side effects associated with rhBMP-2 application continue to be reported in an ever-growing body of literature (15, 16, 18–23). While rhBMP-2 is extremely successful in precipitating robust bone fill, concerns remain about the quality of the regenerate (15, 16), the production of ectopic bone, as well as the formation of rampant inflammation and severe edema.

Delivery of rhBMP-2 largely drives bone wound healing through a process that most closely resembles endochondral ossification (indirect fracture healing) (24, 25). During this process, a progression of fracture site rigidity occurs initiated by acute inflammation and hematoma formation in response to trauma or surgical intervention. Tight regulation of early inflammation is critical to proper healing as it initiates signal cascades that will lead to repair and/or regeneration. Of particular importance are macrophages as they are one of the earliest responders to injury in bone and their activity (polarization) is essential for inflammatory cascade signaling (3, 26–30). As early acute inflammation is the foundation on which bone

wounds heal, disruption or alteration of this early process will likely lead to aberrant signaling and poor healing outcomes.

Patient morbidity as a result of poorly controlled inflammation and edema formation following osteoinductive peptide delivery presents a large clinical problem in the field of bone tissue engineering. Very little work has been done to characterize how, why, or if rhBMP-2 alters inflammatory cascades. Characterization has relied on gross clinical observation and acquisition of clinical samples (e.g. interrogation of seroma fluid) (15, 31). There exists a large gap in our understanding of the mechanisms through which rhBMP-2 produces clinical side effects. As such, a comprehensive evaluation of the effect of rhBMP-2 on cytokine signaling and inflammation is necessary. The present study aimed to investigate alterations in the inflammatory cascade following the application of a sub-clinical dose of rhBMP-2, which has been shown to produce substantial bone fill without ectopic bone formation (25, 32, 33). We hypothesized based on previous work with rhBMP-2 (20, 33–36), that inflammatory cascades would not be dramatically affected following application of this rhBMP-2 dose, evidenced by normal inflammatory cytokine signaling and un-altered macrophage polarization.

2. Material and Methods

2.1 Animals and Surgeries

Forty-eight 8-week old, mixed sex C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) were evenly distributed into two surgical groups (ACS or ACS + rhBMP-2) and two postoperative endpoints (3 or 7 days). Critical-sized calvarial defects were created as previously described (32, 36, 37). Briefly, animals were anesthetized and a midline scalp incision was performed to expose calvaria. A slow speed hand drill equipped with a 5mm trephine was used to create a craniectomy defect. Careful attention was given to not disturb the underlying dura mater. Defects were filled with either ACS alone (serving as a control) or ACS loaded with 325ng of rhBMP-2 prepared per manufacturer's instruction. Incisions were closed with 6×0 polypropylene suture. Animals were monitored daily for signs of pain or distress. Euthanasia was performed at 3 and 7 days post-operation via CO₂ asphyxiation and cervical dislocation. At time of sacrifice, animals were randomly sub-divided into groups based on downstream analysis; either protein isolation or histological staining. All animal use protocols were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (AR3452). Husbandry and related services were performed by the Division of Laboratory Animal Resources in an Assessment and Accreditation of Laboratory Animal Care accredited facility for the duration of the study. Animals were housed 5 per cage, separated by sex, and allowed access to food and water *ad libitum*. All procedures and the reporting thereof are in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines (38).

2.2 Protein Isolation and Cytokine Array

For specimens used in protein extraction, an 8 mm biopsy of tissue around the defect and regenerate was taken and placed in ice cold Phosphate Buffered Saline (PBS) at sacrifice. Biopsy punches for protein extraction were cut into smaller pieces, wrapped in aluminum

foil, and pulverized with a liquid nitrogen cooled mortar and pestle. Homogenate was transferred to a centrifuge tube containing 300 μ L of radioimmunoprecipitation (RIPA) buffer and agitated for two hours at 4°C. Following agitation, tubes were centrifuged for 20 minutes at 12,000 rpm at 4°C. The supernatant was removed and placed into a new tube. Standard Bradford's assay (Thermo Fisher Scientific, Waltham, MA) was used to calculate protein concentrations for downstream analysis. Samples were stored at -80°C.

Extracted protein was screened for modulation of relevant inflammatory markers using the Invitrogen™ Cytokine 20-Plex Mouse Panel (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions. Four animals from each group and time point were selected based on highest protein yields (n=4). Briefly, all reagents were warmed to room temperature. Samples were thawed on ice. Lyophilized protein standard was reconstituted in assay diluent and serial dilutions followed to create a standard curve. Magnetic antibody beads were added to the plate and allowed to incubate, followed by washing. Standards and samples were added to appropriate wells and allowed to incubate for 2 hours in the dark with agitation. Following washes, biotinylated detector antibody was added to wells and allowed to incubate for an additional hour. Samples and standards were incubated with streptavidin-RPE for an additional 30 min at room temperature. Plates were washed several times before being read on a Luminex 200™ Array Analyzer (Luminex Corporation, Austin, TX). Data were averaged and graphed. Table 1 provides an outline of targets found to be key in this study due to known effects or associations in bone wound healing.

2.3 Histological Staining and Analysis

For specimens used in histological analysis, calvariae were carefully removed and placed in 3.7% formalin for 48 hours at sacrifice. Following fixation, specimens were transferred to 70% ethanol for 24 hours, then moved to a 0.25 M ethylenediaminetetraacetic acid (EDTA) solution at pH 7.4 for decalcification. Samples were decalcified for 3–4 weeks with bi-weekly EDTA switches. Decalcified specimens were coronally bisected through the center of the defect and transferred to 70% ethanol for short-term storage. Specimens were washed, dehydrated in graded ethanol (70–100%), cleared in xylene, and infiltrated with paraffin overnight (Tissue Tek® VIP, Sakura Finetek USA Inc., Torrance, CA). Bisected samples were embedded in paraffin wax with defects 90° to the face, sectioned at 7 μ m, and mounted on slides. Three slides from each sample, at least 40 μ m apart, were chosen for histological staining and analysis (n=4, with n=3 for 3 day ACS Control histological investigation due to poor processing of a sample).

Sections were stained following a standard Harris' hematoxylin & eosin (H&E) staining protocol. Stained specimens were imaged at 10x magnification using an Inverted Microscope with attached camera (Motic, British Columbia, Canada) and images encompassing the entire defect area were stitched using Image Composite Editor (Microsoft®, Redmond, WA) for analysis. Stitched images were subjected to cell count analysis to assess relative differences in cell number and infiltration in the healing defect. The Deconvolution Plugin (ImageJ Software, NIH) was used to separate hematoxylin stained nuclei from other tissue and converted to a binary color scheme for analysis. Particle size parameters were set to minimize background and the number of particles representing

cell nuclei were quantified. Additionally, pathological scoring for histological manifestations of inflammation was performed by an experienced veterinary pathologist (KLH). Specifically, macrophage and neutrophil infiltration, as well as edema and fibrosis, were scored on an arbitrary 0–4 scale. Data were averaged by group and compared by treatment and time point (n=4).

Markers for macrophages as well as macrophage polarization were interrogated. F4/80 a pan-macrophage marker, iNOS classically associated with M1 (pro-inflammatory) macrophages, and Arg1 involved in collagen synthesis and classically associated with M2 (pro-repair) macrophages, were studied via immunohistochemistry. For F4/80 (PA5–32399, 1:100 Thermo Fisher Scientific, Waltham, MA), inducible nitric oxide synthase (iNOS; ab15323, 1:100 Abcam, Cambridge, MA) and arginase-1 (Arg1; PA5–29645, 1:350 Thermo Fisher Scientific, Waltham, MA) immunohistochemistry (IHC), slides were deparaffinized in xylene, rehydrated in graded ethanol (100–70%), moved to diH₂O, and placed in 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were incubated in 1% goat serum with 1% bovine serum albumin to block non-specific binding before adding primary antibody overnight at 4°C. Slides were washed with PBS and incubated with a secondary antibody (Goat anti-rabbit IgG; ab6721, 1:250 Abcam, Cambridge, MA) for one hour at room temperature. Diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) detection method was used to detect immunoreactivity. Slides were counterstained in diluted Harris' hematoxylin, serially dehydrated, and mounted for imaging. Specimens were imaged as above. Specific to IHC analysis, representative 10x images were taken at the both surgical margins and the center of the defect. Images were compared by ImageJ Software and the IHC Profiler Open Source Plugin for automated scoring of immunohistochemical staining to quantify percent positive staining (39). The ratio of iNOS:Arg1 was calculated based on quantification of positivity.

2.4 Statistics

Standard two-way Analysis of Variance (ANOVA) with post-hoc Bonferroni analyses were conducted for all comparisons based on means where appropriate. Violations of homogeneity of variance and normality were corrected through transformations of the data where needed. Differences were considered significant where $p < 0.05$. Continuous data are represented by boxplots with a line indicating the median value, boxes indicating the 25% and 75% percentiles and whiskers showing maximum and minimum values. All discrete data are presented as means $\pm$ standard error of the mean (SEM).

3. Results

3.1 Inflammatory Signaling in the Early Healing Defect

Analysis of modulation of important inflammatory markers following the administration of rhBMP-2 compared to an ACS alone control revealed that rhBMP-2 treatment did alter proinflammatory cytokine levels, however expression was only marginally increased relative to control levels (Figure 1A). Largely, increases in pro-inflammatory cytokine expression resolved to control levels by 7 days and for some of the markers, rhBMP-2 decreased expression relative to control. Specifically, monocyte chemoattractant protein 1 (MCP-1 or

CCL2) showed a similar trend in being significantly up-regulated at the early time point ($p<0.001$). However, this increase was not dependent on the addition of rhBMP-2 which resulted in less MCP-1 as compared to control ($p<0.001$) (Figure 1 A1). Similarly, a significant increase tumor necrosis factor alpha (TNF-alpha) levels was noted with rhBMP2 treatment at 3 days but were observed to return to levels similar to control by 7 days evidenced by a significant interaction of time by treatment ($p=0.010$) (Figure 1 A2). Both interferon gamma (INF-gamma) at the later time point and interleukin 6 (IL-6) at the earlier time point showed increasing trends following addition of rhBMP-2. These increases were not significantly different from the control (Figure 1 A3, A4). INF-gamma was, however, significantly up at seven days compared to three days ($p=0.01$). Interestingly, interleukin 12 (IL-12) was decreased with rhBMP-2 administration compared to control at both three and seven days, albeit not significantly (Figure 1 A5). Overall, IL-12 was significantly increased at the late time point compared to the three days ($p<0.001$). A significant interaction effect was noted with interleukin 1 beta (IL-1 beta) with an increase at three days and a significant decrease by seven days with growth factor delivery ($p=0.021$; Figure 1 A6).

Similar results to that of the pro-inflammatory markers were noted in anti-inflammatory and pro-repair factors, with modulation largely resolving to control levels by the later time point (Figure 1B). Interleukin 4 (IL-4) and interleukin 13 (IL-13) were both up-regulated in response to growth factor delivery at the three-day time point (Figure 1 B1, B2). This was a significant increase for IL-13 ($p=0.019$). A cytokine secreted by M2 macrophages, interleukin 10 (IL-10), appeared to be up regulated in response to rhBMP-2 delivery early, but resolved to levels similar to control by seven days (Figure 1 B3). Interleukin 5 (IL-5) remained unchanged in response to rhBMP-2 treatment (Figure 1 B4). Vascular endothelial growth factor (VEGF) production was shown to have a significant interaction effect with rhBMP-2 treatment (Figure 1 B5). Administration of rhBMP-2 drove a significant increase in VEGF at the early time point but was significantly reduced by seven days ($p<0.001$). Additionally, more VEGF was present at 3 days overall ($p<0.001$). Relatedly, rhBMP-2 administration resulted in a significant increase in basic fibroblast growth factor (FGF) at three days which resolved back to levels similar to control by day seven ($p=0.026$; Figure 1 B6).

3.2 Morphological Characterization of Cellular Infiltrate

Based on histological sections, it is apparent that rhBMP-2 administration has a profound impact on cellular infiltration into the defect area. Increases in cellular infiltrate were noted from three to seven days in the control only group, while the addition of rhBMP-2 appeared to frame shift this cellular infiltration, with three-day rhBMP-2 sections appearing morphologically similar to seven-day control samples (Figure 2A). Additionally, growth factor administration resulted in a noticeable increase in cellular presence by seven days. Infiltrate appeared most abundant at the surgical margins, where native bone interfaces with the defect, and endocranially corresponding with the sagittal sinus. Quantification of nuclei revealed that the presence of rhBMP-2 resulted in a substantial and significant increase in cellular infiltrate at both three and seven days ($p=0.004$; Figure 2B). It should also be noted that time appears to play an important role in infiltration, as the seven day time point had a significant increase in nuclei count over three days ($p=0.004$).

Stained sections were used to assess other signs of inflammation in the defect following rhBMP-2 administration. Pathological scoring of neutrophil presence indicated that rhBMP-2 reduced neutrophil abundance at three days despite a lack of statistical significance (Figure 2C). Furthermore, by seven days, neutrophil levels remained unchanged compared to the control. Signs of edema were also scored based on histological manifestations. At both three and seven days, our results indicated that rhBMP-2 did not result in any differences in local tissue swelling as compared to the control samples however, an increase in edema over time was noted ($p<0.001$) (Figure 2D). Growth factor delivery did appear to have at least some effect on fibrosis. Samples treated with rhBMP-2 indicated that there was slightly more fibrous matrix presence over that of the control, though a significant difference from the control was not found ($p=0.067$, Figure 2E). It should be noted that timing was important in fibrous matrix deposition, with significantly more fibrosis present at seven days compared to three days ($p<0.001$).

3.3 Macrophages in Response to Injury and Treatment

Immunohistochemistry for F4/80, a pan-macrophage surface marker of macrophages, was performed to determine the effect of rhBMP-2 on macrophage presence and localization within the calvarial defect (Figure 3A). Most F4/80 positive cells were noted along the surgical margin and dura mater. Interestingly, rhBMP-2 treated defects appeared to have overall less F4/80 positive staining at the three-day time point compared to control. This was particularly true at the surgical margin. By seven days, F4/80 positivity was dramatically reduced in the control, while rhBMP-2 treated samples showed a marked increase in positively stained cells. Quantification and analysis of F4/80 positivity revealed that there was a significant interaction effect with rhBMP-2 delivery ($p=0.050$; Figure 3B). The administration of rhBMP-2 resulted in a significant decrease in F4/80 positivity at three-days compared to control and a significant increase in F4/80 positivity by the later time point ($p=0.050$). In addition to IHC quantification, hematoxylin and eosin stained sections were also scored for macrophage presence. Scoring of the samples indicated that macrophage abundance was low compared to the control at three days but resolved to levels similar to control by seven days (Figure 3C). Significantly more macrophages were present overall at the late, seven-day time point ($p<0.001$).

3.4 Macrophage Markers Following rhBMP-2 Treatment

In order to investigate the effects of rhBMP-2 on macrophage activity, immunohistochemistry was performed on defect sections for markers commonly associated with phenotypic changes in macrophages (Figure 4). Staining for inducible nitric oxide synthase (iNOS), a factor commonly associated with a pro-inflammatory or M1 phenotype, revealed positively stained cells with expression patterns mimicking those of F4/80; positivity was most apparent along the surgical margin and endocranially (Figure 4A). From three to seven days, positively stained cells appeared to be more present throughout the entire span of the defect. Addition of rhBMP-2 to the defect resulted in slightly more iNOS positive staining when compared to the control. This was particularly true at seven days. To interrogate pro-reparative or M2 macrophages, immunohistochemistry for arginase 1 (Arg1) was performed on defect sections. Again, positively stained cells were localized to the surgical margin and the dural side of the defect (Figure 4B). Arg1 positivity appeared to

increase from three to seven days in the control, following a similar trend as iNOS staining. Conversely, the addition of rhBMP-2 resulted in a noticeable increase in Arg1 positivity at three days which tapered off by day seven.

Administration of rhBMP-2 resulted in an increase of iNOS positivity at both time points, though this difference was not statistically different from the control ($p=0.059$; Figure 4C). Additionally, quantification of Arg1 positivity suggested that rhBMP-2 resulted in increased Arg1 expression at three days which ultimately resolved by the later time point (Figure 4D). There was no significant difference in Arg1 expression by time or treatment. As macrophage polarization tends to fall on a spectrum, the ratio of iNOS to Arg1 was calculated to see if treatment was affecting relative amounts of pro-inflammatory and pro-reparative (M1:M2) macrophages. Interestingly, rhBMP-2 treatment appeared to decrease pro-inflammatory M1 phenotype expression at the early time point compared to the control and remained neutral (1:1) by seven days (Figure 4C).

4. Discussion

Here we interrogated inflammatory cascade modulation following administration of a sub-clinical dose of rhBMP-2 in bone wound healing; testing the central hypothesis that a lower dose of rhBMP-2 would not dramatically increase or prolong normal post injury inflammation. As the endogenous activity of BMP-2 results in a positive feedback loop, discerning where and to what extent rhBMP-2 delivery is acting presents a significant challenge. Our data suggest that the effect of rhBMP-2 is mixed, activating both pro-inflammatory (e.g. TNF- α , INF- γ , IL-6) and anti-inflammatory pathways (e.g. IL-4, IL-13, IL-10). Further complicating interpretation, rhBMP-2 reduced expression of two potent pro-inflammatory cytokines, IL-12 and IL-1 β . In high concentrations, rhBMP2 may overwhelm resident bone cells and cells responding to injury, resulting in production of inflammatory cytokines in a non-specific manner. However, at lower doses, the inflammatory cells may be capable of more readily responding to the mixed signaling precipitated by rhBMP-2 delivery.

Evidence presented here suggests the majority of the inflammatory modulation is acute, resolving within one week which does not reflect the clinically described inflammatory response. While both direct and indirect bone formation are involved, rhBMP-2 largely activates endochondral bone wound healing as its primary mechanism of precipitating bone fill (1). In this process, a progression of increasing rigidity about the wound occurs through deposition of several different matrices, one of which being a fibrous matrix which is deposited via infiltrating fibroblasts (3, 40). Though fibrosis is typically referred to as a disease process, early resolution to this phenotype may well be beneficial in promoting fibrous callous formation and accelerating endochondral bone formation. Our histological data indicated that rhBMP-2 may result in earlier and more substantial fibrotic healing compared to the control. The mechanism by which this is occurring was not directly interrogated, though some insight can be gleaned from the cytokine array data. Delivery of rhBMP-2 resulted in a significant increase in the concentration of fibroblast growth factor (FGF), a potent factor involved in fibroblast recruitment and activation. Thus, the rhBMP-2

dependent response of cells to express FGF could be beneficial in promoting bone wound healing rather than promoting inflammation at this sub-clinical dose.

The concept of macrophage polarization has garnered increasing interest of late, particularly as it relates to proper healing. Additionally, it is becoming more accepted that resident macrophages play a key role in bone homeostasis. Through pathological scoring of defect samples, data here suggest that the addition of rhBMP-2 resulted in a marked reduction in macrophages at three days that resolved to levels similar to control by the later, seven-day time point. Corroborating this data, staining for F4/80 indicated that there was a significant interaction effect that followed a similar pattern. rhBMP-2 administration resulted in less macrophages early and more macrophages later, suggesting a delay in macrophage response. Despite this effect, little change was noted in our ratio of single markers iNOS:Arg1, suggesting that rhBMP-2 is not directly resulting in altered macrophage polarization and thus, supporting our hypothesis that this dose of rhBMP-2 does not change macrophage response. An obvious extension of these studies is to investigate these phenomena *in vitro* where markers can be expanded upon and all variables can be controlled. There is a paucity of literature studying the effects of the rhBMP2 on non-mature bone cells. One study using fetal osteoblasts did establish rhBMP-2 can recover M-CSF expression after bisphosphonate treatment (41). As M-CSF is also important in the monocyte/macrophage lineage, specifically for proliferation and differentiation, it is possible rhBMP-2 may act directly on macrophages. Thus, much more work on the effect of rhBMP-2 on macrophages is necessary to completely rule out this specific cell population as being implicated in the heightened inflammation associated with the clinical rhBMP2 dose.

Here there are few indications that this delivered dose of rhBMP2 resulted in a heightened, damaging, or prolonged inflammatory response corroborating previous data collected by our group (20, 25, 36, 42). Where markers of inflammation were increased, there were few indications that this increase was maintained to the seven day time point. Additionally, treatment also appeared to up-regulate several markers associated with anti-inflammatory/pro-repair phenotypes. While appearing contrary to the current literature, these results were not unexpected. Our group has studied a variety of clinical and sub-clinical dosing schemas using rhBMP-2 in conjunction with a variety of delivery platforms (25, 32, 33, 36, 43, 44). It is only at the supraphysiological doses, scaled doses matching the clinical recommendation, that we have seen signs of the caustic effects associated with this treatment (i.e. ectopic bone and inflammation). Additionally, in a previous murine calvarial defect gene array study, our group found that rhBMP-2 administration resulted in the activation of anti-inflammatory genes, which is substantiated by the data shown here at the protein level (20). Given these findings, reevaluation of how rhBMP-2 is dosed may yield benefit for the patient. Currently, rhBMP-2 is dosed through rudimentary sizes (e.g. small, medium, large, etc.) based on the defect and size of the collagen sponge with a standard concentration of the peptide delivered. While much more research is necessary to hone down the exact specifications that will result in optimum healing, there exists strong evidence to at least amend current procedures to make this potent osteoinductive molecule even more effective.

5. Conclusions

In summary, these findings show that a lower rhBMP-2 dose does not overtly increase or prolong the acute inflammatory marker expression as often cited with clinical rhBMP-2 therapy and may result in earlier fibrous matrix deposition, leading to better healing outcomes. This study, accompanied by our previous studies and work from other groups, suggests that modifications in the rhBMP-2 dosing schema and a more individualized therapeutic approach could hold the key to ameliorating adverse side effects and unlocking the full potential of exogenous rhBMP-2 delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Products provided by *Medtronic Sofamore Danek USA* were used in this research. This study utilized the facilities and resources of the Medical University of South Carolina Center for Oral Health Research (COHR).

7. Funding

This work was supported by the AO Foundation [S-16–108C], the National Institute of Health NIDCR [5T32DE017551] [F31DE026684 to ELD], and NIGM [P30GM103331].

Abbreviations

rhBMP-2	Bone Morphogenetic Protein 2
ACS	Absorbable Collagen Sponge
FDA	United States Food and Drug Administration
RIPA	Radioimmunoprecipitation Buffer
EDTA	Ethylenediaminetetraacetic Acid
H&E	Harris' Hematoxylin & Eosin
IHC	Immunohistochemistry
PBS	Phosphate Buffered Saline
DAB	Diaminobenzidine
ANOVA	Analysis of Variance
SEM	Standard Error of the Mean

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interaction effect in IL-1 beta expression was noted, with greater expression with rhBMP-2 delivery at three days and less expression at seven days compared to control (A6). (B) Relevant anti-inflammatory or pro-repair cytokine expression. No significant difference in IL-4 expression was noted with time or treatment (B1). Addition of rhBMP-2 resulted in a significant increase in IL-13 (B2). More IL-10 expression was noted at three days with rhBMP-2 delivery, despite lack of a statistical difference (B3). IL-5 expression was not significantly altered with rhBMP2 administration (B4). Administration of rhBMP-2 resulted in an increase in both vascular endothelial growth factor (VEGF, B5) and fibroblast growth factor (FGF, B6) at the early time point. A significant interaction effect in VEGF expression was noted, with rhBMP-2 administration leading to an increase at three days and a decrease at seven days. Statistical analysis by two-way ANOVA. **= $p<0.01$ ***= $p<0.001$ n=4

Figure 2. Histological characterization of cellular infiltrate and inflammation.

(A) Hematoxylin and eosin staining of calvarial defect sections. Increases in cellular infiltrate were noted from three (top) to seven days (bottom). Addition of rhBMP-2 resulted in increased cellular infiltrate at the margins and endocranially. High-mag inset images of surgical margin (left) and endocranial aspect (right). (B) Cell counts normalized to tissue area indicated a significant increase in cellular infiltrate with rhBMP-2 and with time. (C) Pathological scoring of neutrophils indicated no significant difference with time or treatment. (D) Pathological scoring of edema indicated no difference with treatment but a significant increase in edema by the late time point. (E) Pathological scoring for fibrosis revealed an increase in fibrosis by seven days but no notable difference with rhBMP-2 administration. Statistical analysis by two-way ANOVA. **= $p < 0.01$ ***= $p < 0.001$ $n = 4$. Stitched images at 10x magnification. Inset images at 20x magnification.

Figure 3. Macrophage infiltration in response to injury and treatment.

(A) F4/80 panmacrophage marker immunohistochemistry was performed on calvarial defect sections. Positive staining (brown) was most noticeable around the surgical margins and endocranially. High-mag inset images of surgical margin (left) and endocranial aspect (right)

(B) Quantification of F4/80 positivity via ImageJ software indicates an interaction effect with rhBMP-2 administration. The addition of rhBMP-2 resulted in less positivity at the early time point compared to control and more at the later time point. (C) Pathological scoring of macrophage presence in hematoxylin and eosin stained calvarial defect sections (Figure 2) indicates an increase in macrophages correlating with time. More macrophages were present in the ACS alone group than in the rhBMP-2 treated group which is most apparent at three days. Statistical analysis by two-way ANOVA. ***= $p<0.001$ $n=4$ Stitched images at 10x magnification. Inset images at 20x magnification

Figure 4. Macrophage polarization in response to injury and treatment.

(A and B) M1 macrophage identification via inducible nitric oxide synthase (iNOS, A) and M2-macrophage identification via arginase 1 (Arg1, B) immunohistochemistry. Brown coloration indicates positive staining. High-mag inset images of surgical margin (left) and endocranial aspect (right). (C) Addition of rhBMP-2 resulted in an increase iNOS positivity at both three and seven days compared to control. (D) Quantification of Arg1 positivity indicated that rhBMP-2 administration had no significant effect on Arg1 staining compared to the control. (E) The ratio of iNOS to Arg1 was calculated based on total iNOS and Arg1 positive staining. Red line represents a ratio of 1:1 or a net neutral macrophage polarization. Statistical analysis by two-way ANOVA. $n=4$. Stitched images at 10 $\times$ magnification. Inset images at 20 $\times$ magnification.

Table 1:

Pro and Anti -Inflammatory Targets Associated with Bone Wound Healing

	Target	Association / Known Effect(s)
Pro-inflammatory	MCP1 (CCL2)	Secreted by neutrophils; macrophage recruitment to fracture site
	TNF-alpha	Classical pro-inflammatory cytokine; critical to proper fracture healing
	INF-gamma	Potent pro-inflammatory cytokine; activates macrophages
	IL-6	Secreted by macrophages and T cells; stimulates immune response to injury
	IL-12	M1 polarization and NK cell activation, stimulates TNF- α and INF- γ production
	IL-1 beta	Secreted by M1 macrophages; pro-inflammatory
Anti-inflammatory	IL-4	Secreted by anti-inflammatory T cells (Th2); M2 polarization
	IL-13	Secreted by anti-inflammatory T cells (Th2); M2 polarization
	IL-10	Secreted by M2 macrophages; pro-repair cascades
	IL-5	Recruits eosinophils; plays a role in osteogenesis; drives fibrosis
	VEGF	Secreted by M2 macrophages; angiogenesis
	FGF	Fibroblast proliferation and maturation