Encapsulation of Glycogen Synthase Kinase 3 Beta (GSK3β) Phosphorylation Inhibitor, 6-Bromoindirubin-3'-Oxime (BIO), in a Chitosan Based Scaffold for the Treatment of Critical Size Bone Defects

Celine J. Agnes[1,2], Ling Li[1], David Bertrand[1,2], Antoine Karoichan[1], Monzur Murshed[1,2], Bettina M. Willie[1,2], and Maryam Tabrizian[1]
[1]McGill University, Montreal, Quebec, Canada, [2]Shriner's Hospital for Children, Montreal, Quebec, Canada
Email: celine.agnes@mail.mcgill.ca

INTRODUCTION: One of the major unmet clinical challenges in bone healing involves the effective treatment of critical size bone defects arising from bone loss due to varying conditions[1]. To address some disadvantages of available clinical treatments, researchers have focused on designing biomaterial alternatives using a polytherapy framework termed the "Diamond Concept"[2]. By optimizing design aspects to include specific materials, cell types, and growth factors, while also considering the mechanical environment and angiogenic potential, one can more effectively mimic endogenous bone healing. The objective of our research is to use this framework within the scope of a previously designed guanosine diphosphate (GDP) crosslinked chitosan scaffold to encourage bone healing *in vitro* and *in vivo*. We investigate a formulation of this scaffold that incorporates an indirubin derivative, 6-bromoindirubin-3'-oxime (BIO), which acts as a GSK3 inhibitor, thereby mimicking Wnt signaling[3]. Thus, we hypothesize that the addition of this component will aid in the osteogenic differentiation of myoblastic C2C12 cells, both alone and in combination with osteogenic promoter, bone morphogenic protein 2 (BMP2). Furthermore, we hypothesize that this will translate to significantly improved bone regeneration *in vivo* which is currently being assessed.

METHODS: Incorporation of two different doses (1 and 10 µM) of BIO within the scaffold was confirmed using nuclear magnetic resonance (NMR). Various material characterization techniques including gelation time measurements, microCT imaging for scaffold architecture, and rheological studies were conducted to determine whether the addition of BIO significantly alters beneficial elements of the scaffold. Biocompatibility of the new scaffold formulation was assessed through the encapsulation of myoblastic C2C12 cells with and without supplemental BMP2 addition (300 ng/mL). An AlamarBlue assay was used to measure metabolic activity of all groups up to 28 days, and Ki67 staining was done to qualitatively examine cellular proliferation. The influence of BIO with and without BMP2 on osteogenic differentiation was evaluated through measurement of ALP secretion, qPCR and von Kossa staining for mineralization.
To assess the healing capacity of the new scaffold formulation *in vivo*, a mouse critical size femoral segmental defect model is used with experiments currently ongoing. The McGill University Animal Care and Use Committee approved all *in vivo* studies.10-week-old female C57BL6J mice (Jackson Labs) undergo a 2 mm osteotomy at the left femoral midshaft stabilized by a unilateral external fixator (RIsystems) (n = 8 mice/group). Femurs are scanned using an *in vivo* microCT at days 1, 7, 28, 42, and 56 and resulting images are analyzed. Statistical analysis is conducted using a one-way ANOVA; $p < 0.05$. In vitro studies used at least 3 replicates in each group.

RESULTS: NMR spectra of the new scaffold formulation confirmed the successful incorporation of BIO in the scaffold with the presence of a new concentration dependent peak at 41 ppm resulting from an interaction between chitosan and BIO. The BIO-incorporated scaffold demonstrated similar viscoelastic properties and gelation time measurements (less than 1.6 seconds) to the native control scaffold with a highly porous and well-connected internal pore structure. The scaffold's biocompatibility with myoblastic C2C12 cells indicated no significant effect of BIO incorporation at both doses on cellular metabolic activity and viability. However, a dose response of BIO was observed when evaluating osteogenic differentiation with increased ALP production and *RUNX2* gene expression in BIO 1 µM (CHB1) groups compared to scaffold alone and BIO 10 µM groups. The addition of BMP2 alone (CHBMP2) or in combination with BIO 1 µM (CHB1BMP2) did not significantly alter the cellular metabolic activity. The patterns were generally similar amongst the early time points with differences observed at later stages between CHB1, CHBMP2, and CHB1BMP2 groups. As expected, the addition of BIO 1 µM or BMP2 alone resulted in increased ALP secretion at both day 3 and day 7 compared to the scaffold alone. Interestingly, these groups also showed a significant increase compared to the combination group at day 7, suggesting that BIO could act alone in promoting early differentiation markers without the necessity for BMP2.

DISCUSSION: The objective of this study was to comprehensively evaluate the fabrication and beneficial effects of the BIO incorporated GDP crosslinked chitosan scaffold with respect to bone regeneration. Results demonstrated successful scaffold formulation with incorporation of BIO through an interaction with chitosan. The BIO incorporated scaffold shows minor changes in material properties but based on biocompatibility experiments with C2C12 cells, these changes do not significantly affect the encapsulated cells' functionality. While BIO's beneficial influence on osteogenic differentiation is evident at low dosages (1 µM) and the combination with BMP2 was shown to be unnecessary, this study only evaluates this at early differentiation stages. Therefore, further work to examine the synergistic effect of BIO and BMP2 at later stages is ongoing. In addition, work is currently underway to assess the BIO 1 µM scaffold's ability to promote bone healing using the mouse critical size femoral segmental defect model.

SIGNIFICANCE/CLINICAL RELEVANCE: The new scaffold formulation designed within this research, is a promising alternative treatment for critical size bone defects that mimics bone healing and promotes osteogenic differentiation. This is done to overcome some of the disadvantages of autologous grafts including long hospitalization times and donor site morbidity.

REFERENCES: [1] Nayef, L., Mekhail, M., Benameur, L., Rendon, J. S., Hamdy, R., Tabrizian, M., *Acta Biomaterialia* **2016,** *29*, 389-397. [2] Agnes, C. J., Karoichan, A., Tabrizian, M., *ACS Applied Bio Materials* **2023,** *6* (7), 2515-2545.[3] Li, J., Khavandgar, Z., Lin, S.-H., Murshed, M., *Bone* **2011,** *48* (2), 321-331.

ACKNOWLEDGEMENTS: The authors wish to acknowledge the project financial support provided by the Collaborative Health Research Program (CHRP) through CIHR and NSERC, in addition to the Canada Research Chair – Tier 1 in Regenerative Medicine and Nanomedicine. In addition, the authors wish to thank the FRQS for the scholarship to CA.

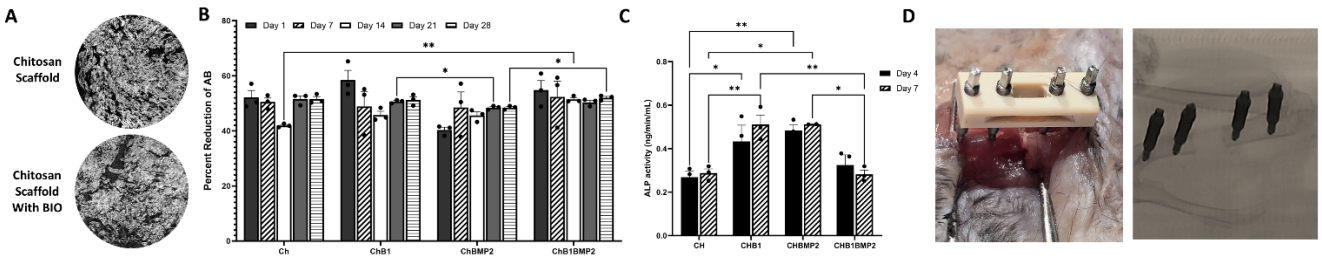

Figure 1. (A) Cross-sections of reconstructed microCT images showing internal architectural differences between control and BIO 1 uM scaffolds. (B) Metabolic activity and (C) ALP secretion of encapsulated C2C12 cells in scaffolds containing either BIO, BMP2 or both. (D) Image of surgical procedure and MicroCT scan showing 2 mm osteotomy and external fixator placement.