Low dose BMP-2 promotes fracture healing in a femur segmental defect model in rats without inducing excessive and prolonged inflammation

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INTRODUCTION: Effective bone healing relies on a robust but timely-restricted pro-inflammatory phase [1]. Clinically applied bone morphogenetic growth factors like BMP-2 show good healing capacity but have been associated with excessive and prolonged pro-inflammatory cytokine release and heterotopic ossification in muscle at high doses [2]. Immunomodulation represents a novel approach to improve the efficiency of lower and saver BMP-2 doses by *e.g.* local administration of the interleukin-1 receptor antagonist (IL-1Ra), which has been shown to enhance bone healing in a rat femur segmental defect model when combined with low dose (1 µg) recombinant BMP-2 [3]. Inhibiting interleukin 1ß (IL-1ß) in the fracture microenvironment with a monoclonal antibody may represent a more effective strategy than targeting IL-1Ra. This study aims to investigate (1) the effect of low dose BMP-2 (1 or 5 µg) alone, delivered locally via a collagen scaffold, on callus formation and pro-inflammatory cytokine production in the fracture repair tissue (hematoma/callus) and adjacent muscle at early time points in a femur segmental defect model in rats and (2) the effect of administration of a monoclonal anti-IL-1ß antibody in combination with low dose BMP-2 (1 µg) on bone healing over 14 weeks in the model.

METHODS: 2 mm internally-fixed femoral defects were created in female Fischer F344 rats (22-27 weeks old; mean body weight at surgery 195 ± 7 g; group housed with enrichment) (animal license: GR/19/2022) under general anesthesia with sevoflurane and with established protocols for analgesia (buprenorphin 0.03 mg/kg subcutaneously (s.c.) + carprofen 5 mg/kg s.c.). To study BMP-2 mediated effects on early fracture healing, animals (n=3/group) received either no treatment (empty defect), a Lyostypt collagen sponge, or Lyostypt + 1/5 µg BMP-2 (InductOs, Medtronic) and were sacrificed at days 3, 7 or 14. Radiographs were taken at surgery and throughout the study. The fracture repair tissue and the adjacent muscle were collected in T-PER tissue protein extraction reagent (Thermo Fisher), and cytokine levels, *e.g.* IL-1β, were determined by ELISA (R&D Systems DuoSet). In a follow up study, animals (n=7/group) received either a collagen sponge or a collagen sponge + 1 µg BMP-2 alone or in combination with a monoclonal anti-IL-1ß antibody (BioXCell, 10 mg/ml). The antibody was administered intravenously under anesthesia, starting from post-operative day 3, and then every third day until day 15. New bone formation within the defect site was accessed using *in vivo* micro-CT immediately after surgery and at 2, 3, 4, 6, 8, 10 and 14 weeks post-OP. The mechanical properties of the operated femurs were assessed by non-destructive 4-point bending (Instron 5866) and compared to the contralateral femurs. Histopathological analysis was performed on Giemsa-Eosin-stained sections (Olympus BX63). Data in Fig. 2 was statistically tested by two-way ANOVA followed by Bonferroni post-hoc analyses, data in Fig. 3 by one-way ANOVA followed by Tukey post-hoc test (GraphPad Prism 8).

RESULTS SECTION: In the early healing phase following the fracture, radiographs demonstrated pronounced new bone formation and cortical bridging 2 weeks post-OP for the BMP-2 groups **(Fig. 1)**. IL-1β levels in the hematoma declined from day 3 (208 pg/mg) to 14 (47 pg/mg) for all groups **(Fig. 2A)**, significantly lower levels were detected in the muscle ($p<0.001$) **(Fig. 2B)**. IL-1β levels in hematoma were reduced on day 7 for 1 µg BMP-2 ($p<0.05$), and even more for 5 ug BMP-2 ($p<0.001$) compared to controls. In the second part of the study, operated femurs treated with 1 µg BMP-2 reached an average mechanical stiffness of 91±37% of contralateral femurs, femurs in the anti-IL-1ß group 111±12% **(Fig. 3)**. Micro-CT scans showed that treatment with collagen sponge led to 33.1% of the defect site being filled with new bone after 14 weeks, 1 µg BMP-2 to 70.2% and 1 µg BMP-2 + anti-IL-1 β to 73.1%. This was confirmed by histopathological evaluation (n=4) which demonstrated that collagen sponge alone led to non-union (4/4 femurs) while complete bicortical bridging was observed for 3/4 femurs in the 1 ug BMP-2 group and 4/4 in the anti-IL-1ß group after 14 weeks.

DISCUSSION: In the early healing phase following the fracture, low dose BMP-2 alone had a strong local effect on new bone formation in the femur segmental defect model in rats without inducing excessive and prolonged cytokine expression. Functional healing outcome assessed by mechanical competence of femurs treated with 1 µg BMP-2 was comparable to the average mechanical stiffness of the by us previously tested 5 µg BMP-2 group (106% of contralateral femur stiffness) after 14 weeks [4]. Combined immunomodulatory treatment with monoclonal anti-IL-1ß in the first two weeks following surgery further increased the mechanical competence of femurs (+ 20% increase in bone stiffness), although not significantly compared to low dose BMP-2 alone. We next aim to explore the potential of immunomodulation targeting IL-1b in combination with clinically applied higher doses of BMP-2 and to assess the efficacy of anti-IL-1β administration for enhancing BMP-2-induced bone healing in a more challenging diabetic healing environment.

SIGNIFICANCE: Our study shows the save and efficient use of low dose of human recombinant BMP-2 to promote bone healing.

REFERENCES: [1] Loi *et al.* Bone. 86 (2016) :119-130 [2] James *et al.* Tissue Eng. Part B Rev 22(4) (2016):284-297 [3] Lackington *et al.* Acta Biomater. 1(149) (2022):149-189 [4] Lackington *et al.* Mater. Today Commun. 29 (2021)

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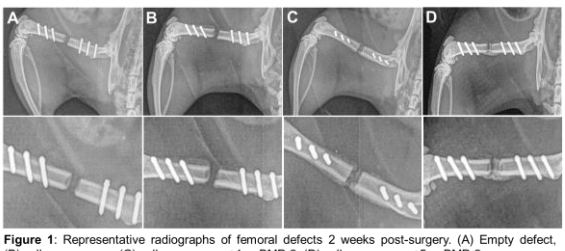

Figure 1: Representative radiographs of femoral defects 2 weeks post-surgery. (A) Empty defect, (B) collagen sponge, (C) collagen sponge+1µg BMP-2, (D) collagen sponge+5µg BMP-2.

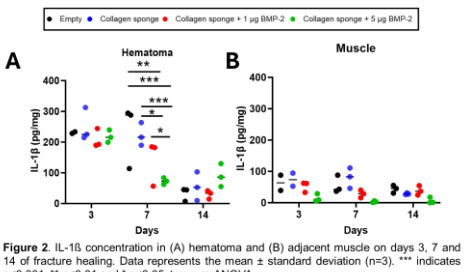

Figure 2. IL-1ß concentration in (A) hematoma and (B) adjacent muscle on days 3, 7 and 14 of fracture healing. Data represents the mean ± standard deviation (n=3). *** indicates $p<0.001$, ** $p<0.01$ and * $p<0.05$, two-way ANOVA.

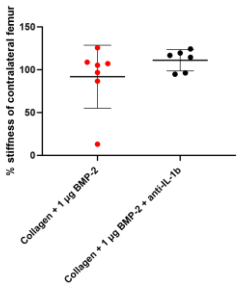

Figure 3: None destructive mechanical testing of operated femurs. Bone stiffness is given in % of contralateral femur stiffness (n=7/6). One animal in the anti-IL-1b group was excluded due to measurement error.