Characterization of Composition and Structure of Callus Tissue in Fractures Treated with BMP-7 and Zoledronate in an Osteoporotic Rat Model

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Introduction: Successful fracture repair entails the formation of a callus bridging the fracture site. In instances of fracture non-unions, the callus formation process could be augmented through the use of potent anabolic agents such as bone morphogenic protein 7 (BMP-7) or anti-catabolic agents such as the bisphosphonate Zoledronate (ZO) [1]. Use of BMP-7 alone or synergistically combined with ZO has proven to substantially increase callus formation and enhance fracture healing [1]. Our aim is to characterize the molecular composition and mineral structure of the callus tissue formed through the use of pharmacological manipulation in healthy and osteoporotic bone.

Methods: Female Sprague-Dawley rats (n=24) were ovariectomized (OVX) at 12 weeks of age, or kept as healthy controls. At 24 weeks of age, mid-diaphyseal osteotomy was introduced in the right femur and fixed with an intramedullary K-wire as a model of recalcitrant non-union [1]. Subsequently, the rats were randomly allocated to one of four treatment groups: i) Control (BMP-7 only), ii) OVX (BMP-7 only), iii) Control (BMP-7 + ZO), iv) OVX (BMP-7 + ZO). At 6 weeks post-osteotomy, the rats were sacrificed and fracture femurs were harvested, dehydrated and embedded. Bone composition and structure were evaluated using Fourier-transform infrared (FTIR) spectroscopy and small angle X-ray scattering (SAXS) respectively. FTIR: Spectral acquisition was performed on longitudinal sections (4 µm thick) at the D7 beamline of the MAX-IV laboratory (Lund, Sweden) using a Bruker Hyperion 3000, an FPA detector covering 341 x 341µm, and a spectral resolution of 4 cm⁻¹. Spectra of callus and fracture site composition were recorded. Spectra were baseline corrected and the resin subtracted [2]. Mineral/matrix ratio, crystallinity, collagen maturity and acid phosphate substitution were calculated along with the heterogeneity for each parameter [2]. SAXS: Measurements were conducted on longitudinal sections (100 µm thick) at the cSAXS beamline of the Swiss Light Source (Villigen PSI, Switzerland). Areas of 5 x 4 mm covering the fracture site and callus were scanned with a beam size of 20 x 20 µm (exposure time 50 ms, λ = 1 Å, q-range 0.01 - 1.7 Å⁻¹). Mineral plate thickness, predominant orientation (calculated relative to long axis of femur) and degree of alignment of the mineral plates were determined using custom-written MATLAB scripts in three regions of interest (ROI): i. External Callus, ii. Inner Callus, iii. Cortex (Figure 1) [3]. Statistics: The Mann-Whitney U-test was used to compare the groups and the Wilcoxon signed rank test was used to compare between the ROIs (SPSS, v22, SPSS Inc).

Results: FTIR: Overall, differences were not observed in the composition between groups (irrespective of treatment or bone health). However, significant differences were observed when comparing the callus and fracture site cortex composition within the groups. Consistently across all groups, the...
mineral/matrix ratio was significantly higher in the cortex than in the callus \( (p < 0.01; p < 0.05) \) (Figure 2A). Noteworthy also, the heterogeneity was significantly increased in the callus relative to the cortical bone in the BMP-7 + ZO groups \( (p < 0.05) \) (Figure 2B). The acid phosphate parameter consistently yielded significantly increased values in the callus relative to cortices across all groups \( (p < 0.01; p < 0.05) \). This was also found in the heterogeneity \( (p < 0.05) \). Crystallinity was similar in the callus and the cortex except in the BMP-7 only treated controls \( (p < 0.05) \). Conversely, collagen maturity was significantly lower in all calluses \( (p < 0.05) \) except BMP-7 only treated controls.

SAXS: Overall, mineral plate thickness values did not differ between groups. In both healthy and osteoporotic rats treated with BMP-7 only, mineral plate thickness was significantly greater in the External Callus region relative to both the Inner Callus or Cortex regions \( (p < 0.05) \). When BMP-7 was combined with ZO, mineral plate thickness values of the External Callus region was on par with that of the Inner Callus region. Across all groups, variations in plate thickness were significantly higher in the External and Inner Callus regions than in the cortical regions \( (p < 0.05) \). Within the callus region, the variation was significantly lower in the External Callus compared to the Internal Callus in the healthy Control groups \( (p < 0.05) \). In the OVX groups, no such difference was found. The angular distribution of mineral crystals was significantly more ordered in the cortex relative to callus regions across all groups \( (p < 0.05) \). Predominant orientation parameter yielded larger angles in the external callus regions compared to the inner callus regions \( (p < 0.05) \). Greater variation in measurements of this parameter were observed in callus regions compared to cortical regions consistently across all groups \( (p < 0.05) \).

**Discussion:** Overall, our results demonstrate that the efficacy of treating with BMP-7 alone or BMP-7 + ZO is retained irrespective of whether the underlying bone is healthy or osteoporotic. This is in line with our previous work looking at radiological healing rates, microCT parameters and mechanical properties in healthy and OVX fractures subjected to the same treatments [4]. The molecular composition in the callus formed via either treatment had lower degree of mineralization, lower collagen maturity and higher acid phosphate content relative to the cortex which is consistent with expectations of newly formed bone. Observation of significantly greater heterogeneity in the degree of mineralization of the BMP-7 + ZO groups is believed to be due to the very large calluses containing immature, poorly organized and partially remodelled tissue [1]. This was reinforced by microCT data we reported previously which underscored the voluminous effect on callus formation alongside measures of significantly diminished tissue mineral density [4].

To our knowledge, comparable structural analysis of fracture healing at a high resolution has not been reported in the literature previously. Noteworthy, is the distinctly higher mineral plate thickness in the External Callus region relative to the Inner Callus/Cortex regions in the BMP-7 only groups. Although BMPs are potent stimulators of bone anabolism, it is also known that BMPs induce catabolic upregulation leading to premature callus resorption. This is particularly evident in Figure 1 where the effects of this premature resorption is concentrated in the inner callus region. The absence of this phenomenon in BMP-7 + ZO groups is attributed to the anti-catabolic effects of ZO. Findings in the structural analysis of greater heterogeneity in plate thickness and in the orientational parameters in callus regions relative to cortical regions is in line with expectations of the properties of newly formed bone.
Significance: Our findings demonstrate that BMP-7 alone and BMP-7 + ZO retain equivalent efficacy in osteoporotic bone as in healthy bone. However, the respective anabolic-catabolic paradigms of BMP-7 alone and BMP-7 + ZO have significant influence on callus molecular composition and mineral structure.

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