Linking local fracture mechanics with systemic biological response

Jan Barcik[1], Manuela Ernst[1], Tim Buchholz[1], Caroline Constant[1], Stephan Zeiter[1], Sophie Verrier[1]
AO Research Institute Davos, Davos, Switzerland
Sophie.verrier@aofoundation.org

Disclosures: Jan Barcik (N), Manuela Ernst (N), Tim Buchholz (N), Caroline Constant (N), Stephan Zeiter (N), Sophie Verrier (N).

Introduction: It is well-known that bone healing path and outcome are highly defined by the fracture's initial mechanical environment [1]. Mechanical stimuli promote callus formation during bone fracture repair. While the mechanical stimulation is undoubtedly important, recent animal studies have pointed out that the resting periods between stimulation cycles might be a crucial — but, so far, underestimated — parameter impacting the fracture healing [2-3]. In a previous work [3] we observed that the stiffness of fracture repair tissue increased predominately during the pause between mechanical stimulation events and fluctuated during the daily stimulation period. Moving from observations to mechanistics, in the present study, we investigated a possible correlation between the bone alkaline phosphatase (BALP) level in circulating blood with the callus stiffness regulation and the role of the mechanical stimulation pause herein.

Methods: *Animal model*: Upon local ethics committee approval (TVB2020/26 Canton of Grisons, CH) a partial osteotomy was created in the tibia of 5 female Swiss White Alpine sheep and instrumented with a custom-made active fixator equipped with a pneumatically driven apparatus [4] applying controlled mechanical stimulus to the osteotomy. The fixator applied 1,000 loading cycles per day, evenly distributed from 9 am to 9 pm (Stimulation Period, SP), followed by a 12-hour of Resting Period (RP). During loading cycles, the active fixator measured callus stiffness continuously during SP. *Blood samples and BALP quantification*: On day 22 and day 29 post-surgery, blood samples were taken from each animal at key time points: 9 am (start of SP), 11 am, 9 pm (start of RP) and 9 am next day (start of a new stimulation period, 9am+). For pre-operative baseline measurements, blood samples were taken following the same schedule. After plasma separation, Bone Alkaline Phosphatase (BALP) content was analyzed by enzyme-linked immunosorbent assay (ELISA, neobiolab, #SB0145). *Statistical analyses*: Firstly, the level of BALP and stiffness were correlated (Spearman R) for each animal. Secondly, we evaluated the daily variation of stiffness by calculating the percentage of stiffness difference between SP and RP. The same calculation method was applied for BALP measurements. The changes in BALP concentration and stiffness during stimulation- (SP) and during resting-period (RP) were statistically investigated using the pared T-test.

Results: All animals tolerated the active fixation and the stimulation protocol well during the full length of the study.

Effect of circadian cycle: All sheep showed the same profile of stiffness regulation over 24 hours measurement with special emphasis on 9 am, 11 am, 9 pm, and 9 am+ (Fig. 1A) at both 22- and 29-days post-op. A decrease of the stiffness was observed between 9 am and 9 pm, while an increase was detected between 9 pm and 9 am+ (next day). Results presented in Figure 1B showed a stable BALP expression over 24 hours in the base line samples (Pre-op). On the other hand, 22 days, and 29 days after surgery the BALP plasma concentration decreased between 9 am and 9 pm while a slight increase was observed between 9 pm and 9 am+, following less pronounced but similar pattern as the stiffness measurements. In three out of five animals the levels of BALP positively correlated with callus stiffness (Figure 1C).

Effect of resting period: Figure 2 depicts the effect of the resting period (RP) versus stimulation period (SP) on the callus stiffness (Fig. 2A) and BALP concentration (Fig. 2B). At both, day 22 and day 29 post surgery, a clear increase in the callus stiffness was measure during RP compared to SP (Fig. 2A). Looking at the BALP concentration (Fig. 2B) the baseline level of BALP remained stable (grey zone). On this other hand, upon mechanical stimulation a clear increased of BALP concentration was observed during the RP, when compared to the SP.

Discussion-Conclusion: In the absence of bone injury, BALP levels detected in the sheep blood samples were not affected by the circadian cycles (pre-op samples). On the other hand, upon fracture and during the healing process, fluctuations of the BALP concentration at different times of the day were observed over 24 hours. Interestingly, these fluctuations showed similar profile to the short-term (24 hours) fracture stiffness progression. Likewise, both BALP plasma concentration and callus stiffness increased when no mechanical stimulation was applied.

Altogether, our data suggests that the bone formation process – characterized here by BALP plasma concentration and bone stiffness – occurs mainly during the resting periods. Looking at each sheep individually, stiffness and BALP profiles were similar and positive correlations were observed for 3 out of 5 animals. These preliminary findings emphasize further the importance of resting periods between stimulatory events as a crucial parameter impacting fracture healing progression, but also the possible use of blood BALP levels as an indicator of the bone healing process.

Significance: Our unique callus-stiffness monitoring system opens a wide range of study opportunities aiming to explore the dynamic relationship between the local mechanical conditions at the fracture gap and the biochemical circulatory response. This will enable a deeper understanding of the underlying mechanobiological mechanisms of bone healing, but also the identification and validation of healing markers in the bloodstream.

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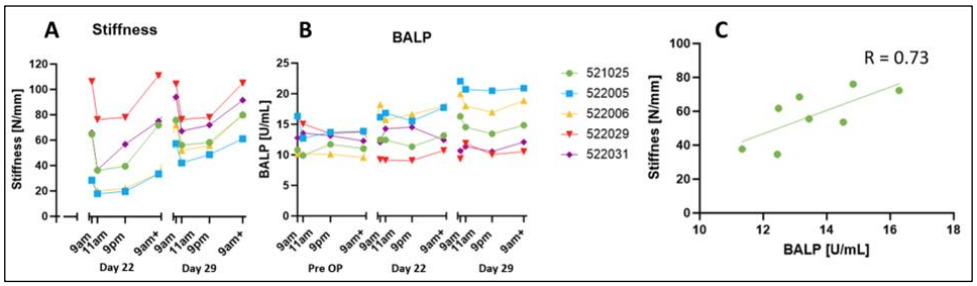

Figure 1: Effect of circadian cycle on bone healing markers. (A) Callus stiffness measured at different time points over 24 hours.
(B) ELISA quantification of BALP in plasma.
(C) Spearman R analysis of correlation between stiffness and BALP concentration for one representative animal.

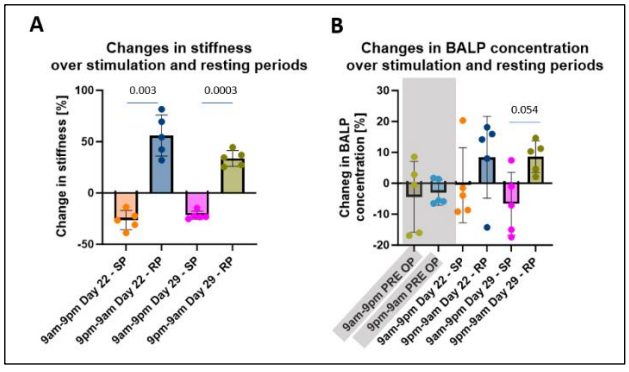

Figure 2: Effect of resting on bone healing markers. In (A) and (B) results are presented as percentage of changes over SP and RP were calculated as follow:

$$\Delta_{stimulation} = \frac{(x_{9_{pm}} - x_{9_{am}})}{x_{9_{am}}} 100\%$$

$$\Delta_{Resting} = \frac{(x_{9_{am\_next\_day}} - x_{9_{pm}})}{x_{9_{pm}}} 100\%$$

(A) Percentage of stiffness changes at day 22 and 29 over SP and RP.
(B) Changes of BALP concentration during the resting period versus the stimulation period. Grey zone underlines the pre-op base line values.