Anatomic Level of Periosteal Injury in a Non-Rodent Model Affects Biological Response

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INTRODUCTION: Surgical or traumatic resection of the periosteum from the proximal metaphysis of long bones is known to trigger an accelerated longitudinal bone growth[1,2]. Previous studies have shown that growth acceleration can occur in many animal species after periosteal procedures[3-7]. The underlying mechanism for this growth acceleration has been attributed to the periosteum acting as a simple tether, mechanically restricting the longitudinal expansion of the growth plate[6]. However, data generated by our group both support and challenge this proposed mechanism. The hypothesis of the current work is that periosteal resection at different anatomic levels will evoke comparable growth responses in both the proximal and distal physes of the treated bone, thus validating the hypothesis that the periosteum functions as a straightforward mechanical tether.

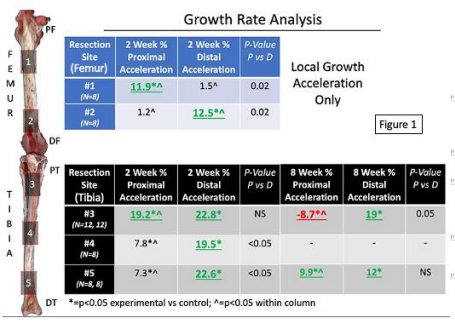
METHODS: Six femurs and seven tibiae were carefully removed from four, seven week old New Zealand White female rabbits and the resected? periosteal fibers imaged *on the bone* using an Ultima In Vivo Multiphoton Microscopy System (SHG) (Bruker; Madison, WI) and computationally analyzed using CurveAlign software to assess differences in collagen fiber alignment. Sixty-four, additional seven-week old NZW female rabbits underwent 10 mm circumferential periosteal resection at one of five anatomic levels in the lower extremity (**Figure 1**) and harvested at two or eight weeks post-op. Pulsed fluorochrome labeling was performed 96 hours (Alizarin Red) and 24 hours (Oxytetracycline) prior to harvest. The femurs and tibiae of the rabbits were collected, regional periosteal strips removed for transcriptional analysis, the bones were then radiographically imaged using high resolution Faxitron (Tucson, AZ), and then fixed in 70% ethanol solution. Bones were coronally sectioned into 1 mm thick slabs utilizing an Isomet Precision saw (Buehler Isomet 2000; Lake Bluff, IL), and visualized using a Nikon Optiphot (Nikon Instruments; Melville, NY) microscope set-up for epifluorescence. Alizarin complexone was optimally viewed with 510-560 nm excitation filter and 590 nm barrier filter, while oxytetracycline was viewed optimally with a 405 nm excitation filter and 470 nm barrier filter. Measurements were made at 2 mm intervals across the width of each growth plate excluding the 2 mm closest to perichondrium to reduce effects of the groove of Ranvier. These measurements were averaged to give a final growth rate. Growth rates of the physis from the bone of interest for the experimental and control limbs were measured for each animal. Paired Student t-tests were used to compare the differences in growth rates between the experimental and sham limbs at each growth plate. Unpaired Student t-tests were then used to compare the change in percent growth rates [(experimental-control)/control X 100] between the proximal and distal growth plates in each bone for each of the resection sites for each time point. A sub-set of the animals had their experimental and control bones harvested and carefully dissected free of soft tissue and two periosteal strips were collected (one proximal and one distal) from the femur along the length of each tibia for both experimental (right) and control (left) sides, and immediately frozen for later mRNA analysis. Total RNA was extracted from harvested tissues using the NucleoSpin RNA II Kit (Clontech Laboratories, Mountain View, CA, USA). The quality and quantity of total RNA were measured by Nanodrop 1000 (Thermo Fisher Scientific) followed by reverse-transcription into complementary DNA (cDNA) strands using the High-Capacity cDNA Reverse Transcription kit (AB Applied Biosystems, Carlsbad, CA, USA). The mRNA expressions from each sample were determined by the qRT-PCR analysis using iQ™ SYBR Green Supermix (Bio-Red, Hercules, CA, USA) with specific primers to detect *Bmp2*, *Bmp6*, *Fgf9*, *Fgf18*, *CNP*, *Gli1*, *Igf*, *Patched*, *Ihh*, *PTHrP*, *Tgfb1*, *Tgfb2*, and*Tgfb3*. All experiments were performed following the manufacturer's instructions, and the level of mRNA expression was calculated using the $2^{-\Delta Ct}$ method by referencing the control *Gapdh*. Relative mRNA expression of *Bmp2*, *Bmp6*, *Fgf9*, *Fgf18*, *Cnp*, *Gli1*, *Igf*, *Patched*, *Ihh*, *Pthrp*, *Tgfb1*, *Tgfb2*, and *Tgfb3* were measured in those samples following the overall pattern of growth response for the anatomic level of resection and summarized as proximal and distal changes.

RESULTS: Distinct regional differences in periosteal collagen alignment within the same bone were observed ($p<0.05$). Both the femur and tibia exhibited greater periosteal fiber alignment in the diaphysis, with lesser alignment observed in the distal metaphysis. The proximal tibia demonstrated the lowest alignment (0.377± 0.123), whereas the proximal femur exhibited the highest alignment (0.841±0.082) among all regions. Notably, the alignment differed significantly between the two bones ($p<0.0001$) **(Figure 2)**. The growth acceleration observed in the femur was a "local" response, indicating that growth acceleration was confined to the physis adjacent to the resection cite. Proximal resection led to a significant proximal growth acceleration of 11.9% ± 8.7% ($p<0.01$)], while distal growth remained unaffected. On the other hand, resection on the tibia triggered growth acceleration in both proximal and distal tibial physes. The most robust growth response, around 20%, occurred in a local and proximal to distal manner. Specifically, only proximal tibial resection induced the greatest proximal growth acceleration (19.2% ± 5.2% vs. 7.8% ± 3.8%, $p<0.001$); 7.3% ± 6.1%, $p<0.001$). However, all three anatomic levels of resection resulted in similar growth acceleration distally (22.8% ± 4.6% vs. 19.5% ± 9.3%, $p=0.4$; 22.6% ± 12.9%, $p=0.8$) two weeks post-operatively. The local growth response in the proximal tibia was transient, as growth was significantly inhibited (-8.7 % ± 6.9%, $p<0.002$) at 8 weeks but continued to be similarly ($p=0.2$) accelerated distally following proximal (19% ± 19%, $p=0.002$) and distal (12.1% ± 11.7%, $p=0.02$) resections **(Figure 1)**. The continued accelerated growth following the distal resections resulted in greater overall Faxitron tibial lengths at 8 weeks (3.3% ± 0.7% vs. 2.1% ± 0.8%, $p=0.01$). Further details on significant differences in regional growth factor transcription qRT-PCR results are provided below **(Figure 3).**

DISCUSSION: Transcriptional and growth responses exhibit variations depending on the bone and anatomic level of injury. These variations cannot be fully explained by the regional distinctions in periosteal fiber structure within the rabbit's lower extremity. This incongruence raises doubts about the mechanical tether theory as the sole regulator of periosteal growth. The observed disparities in growth responses present a model system for further studying the molecular mechanisms that underlie growth acceleration following periosteal injury.

SIGNIFICANCE: These findings highlight regionally specific responses to injury, revealing that different bones and their respective regions respond differently to an equivalent "dose" of injury. These findings hold direct clinical significance for scenarios such as post-traumatic limb overgrowth, growth suppression following conventional limb lengthening, and the application of therapeutic periosteal procedures in skeletally immature patients. These findings potentially extend to much extensive implications within orthopedic research, as they clearly demonstrate that injury responses within a non-rodent model are intricately linked to specific anatomical locations.

REFERENCES: [1]Chaudhary+, 2016; [2] Halanski+ 2016; [3]Dimitrou+ 1988; [4]Lynch+ 1987; [5]Read+ 2002; [6]Taylor+ 1987; [7]Wilde+ 1987



Growth Rate Analysis

Resection Site (Femur)	2 Week % Proximal Acceleration	2 Week % Distal Acceleration	P-Value P vs D
#1 (N=8)	11.9*^	1.5^	0.02
#2 (N=8)	1.2^	12.5*^	0.02

Local Growth Acceleration Only

Figure 1

Resection Site (Tibia)	2 Week % Proximal Acceleration	2 Week % Distal Acceleration	P-Value P vs D	8 Week % Proximal Acceleration	8 Week % Distal Acceleration	P-Value P vs D
#3 (N=12, 12)	19.2*^	22.8*	NS	-8.7*^	19*	0.05
#4 (N=8)	7.8*^	19.5*	<0.05	-	-	-
#5 (N=8, 8)	7.3*^	22.6*	<0.05	9.9*^	12*	NS

*=p<0.05 experimental vs control; ^=p<0.05 within column

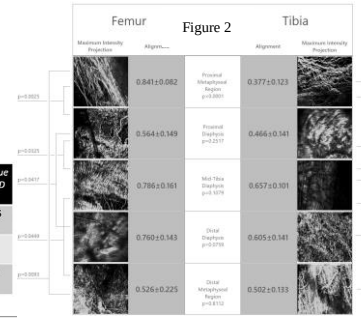


Figure 2

Figure 3

	Proximal Femoral Resection	Distal Femoral Resection
	2 Week Proximal Growth ↑	2 Week Proximal Growth
Proximal Periosteal Strip	↑IL1A, BMP6, MAP2, ↓VEGF	NC
	2 Week Distal Growth	2 Week Distal Growth ↑
Distal Periosteal Strip	NC	NC

	Proximal Tibial Resection		Distal Tibial Resection	
	2 Week Proximal Growth ↑↑↑	8 Week Proximal Growth ↓	2 Week Proximal Growth ↑	8 Week Proximal Growth ↑
Proximal Periosteal Strip	↑ BMP6, IGF ↑PTHRP, TGFB3	↑ BMP6, BMP2, ↑PTHRP, TGFB1	↑ BMP6, FGF9	↑BMP6, BMP2, IGF1, FGF18 ↑ Gli, MAP2, PTHrP2, ↑ IL1A, VEGF
	2 Week Distal Growth ↑↑↑	8 Week Distal Growth ↑↑	2 Week Distal Growth ↑↑↑	8 Week Distal Growth ↑↑
Distal Periosteal Strip	↓ CNP, FGF9, FGF18 ↓BMP2, BMP6 ↓TGFB2, TGFB3	↓ IHH	↓ CNP	↑ BMP6, BMP2, TGFB1 ↑ GH, IGF1, FGF18 ↑ Gli, MAP2, PTHrP2, ↑ IL1A, VEGF, BT

Figure 1. Locations of Periosteal Resection and resultant effects on growth rate. (PF/DF=Proximal/Distal Femur)(PT/DT=Proximal and Distal Tibia). **Figure 2.** Baselin regional differences in periosteal collagen alignment determined via SHG of the Femur (left) and Tibia (right). **Figure 3.** Regional changes in periosteal growth factor/inhibitor transcription at 2 and 8 weeks post-injury.