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Effect of Alendronate on Bone Formation during Tooth Extraction Wound Healing

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Appendix

Materials and Methods

In the study, the soft and hard tissue components of tooth extraction wound healing were carefully analyzed to assess the effect of alendronate (ALN) on bone formation. The growth of ectopically implanted bone was then assessed to substantiate the findings. The experimental animal protocol was approved by the University Committee on Use and Care of Animals.

Animals, Tooth Extractions, Injections, and Vossicle Model

C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at 22 °C in 12-h light/12-h dark cycles and allowed free access to water and a standard rodent diet. Male mice (n = 105) were randomly divided into 15 groups (n = 7/group). At the age of 8 wk, the maxillary right first molars were extracted under general anesthesia (ketamine and xylazine cocktail) using a dental explorer. Either ALN (100 μg/kg/d) (Sigma-Aldrich, St. Louis, MO, USA) or parathyroid hormone (PTH) (40 μg/kg/d) (Bachem, Torrance, CA, USA) was administered daily subcutaneously for 3, 5, 7, 10, and 21 d after extractions to assess the effect of ALN and PTH on tooth extraction wound healing. Saline was used as the vehicle control (VC).

Another 36 C57BL/6J mice underwent tooth extractions and received subsequent injections for 5, 10, and 21 d in the same manner as described above. The tissues at the extraction sites were harvested at sacrifice under a dissecting stereoscope and used for gene expression analysis.

Fresh vertebrae were harvested and implanted into young adult mice. Neonatal mice (C57BL/6J, 3 d old) were used as vertebrae donors and male adult C57BL/6J mice as recipients. The lumbar vertebrae were removed and aseptically sectioned into single vertebral bodies. The vertebral bodies were subcutaneously implanted in the dorsal surface of anesthetized recipients, and ALN (100 μg/kg/d), PTH (40 μg/kg/d), or saline was administered daily for 10 d. At 10 d, the implanted vertebrae (vossicles) were removed.

Histology and Immunohistochemistry

The maxillae, tibiae, and vossicles were dissected at euthanasia, fixed in 10% formalin, and decalcified in 10% ethylenediaminetetraacetic acid. The maxillae and vossicles were processed for cryosectioning. The tibiae were paraffin embedded. Hematoxylin and eosin staining on the maxillae, tibiae, and vossicles was performed. Tartrate-resistant acid phosphatase staining was conducted to visualize osteoclasts using a commercial kit (Sigma-Aldrich). Masson's trichrome staining was performed for the detection of collagen fibers following the manufacturer's instructions (HT15; Sigma-Aldrich). Immunofluorescent staining of lymphatic and blood vessels and neutrophils was performed as follows. Sections were fixed and rehydrated and nonspecific proteins blocked. The sections were incubated in a mixture of primary antibodies. Lymphatic endothelial hyaluronan receptor-1 (LYVE-1) (ab14917; Abcam, Cambridge, MA, USA) and CD31 (MCA2388GA; AbD Serotec, Dusseldorf, Germany) were used as a pair. Ly6G (ab6640; Abcam) was used to label neutrophils. Following incubation with the primary antibodies, fluorescent-conjugated secondary antibodies were applied. DAPI staining was performed to visualize cell nuclei. Alkaline phosphatase (ALP) staining was performed on vossicle sections to detect osteogenic activity. Sections were preincubated in 1% $MgCl_2$ buffer overnight, incubated in ALP substrate solution (naphthol AS-MX phosphate and fast red TR) for 2 h, and then counterstained with methyl green.

Histomorphometric Analysis

Stained sections were photomicrographed and histomorphometrically analyzed using Image-Pro Premier (Media Cybernetics, Bethesda, MD, USA). The following parameters were assessed: collagen area (%), new bone area (%), blood vessel area (%), lymphatic vessel area (%), osteoclast number (Oc.N/BS), $Ly6G^+$ cell number, and number of empty osteocyte lacunae in healing wounds. As for the proximal tibiae and vossicles, osteoclast number (Oc.N/BS) and bone area (BA/TA) were quantified. In addition, ALP activity was assessed in the vossicles.

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Gene Expression Assessment

The soft and hard tissues at the extraction sites were harvested, homogenized in TRIZOL (Invitrogen, Grand Island, NY, USA), and processed for RNA isolation. Total RNA was extracted by the phenol/chloroform method, reverse transcribed, and analyzed using the Mouse Osteogenesis PCR Array (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

Serum Chemistry and Complete Blood Count (CBC)

Blood was collected at sacrifice and CBC performed using Hemavet 950FS (Drew Scientific, Dallas, TX, USA). Serum was also prepared and kept at −80 °C until use. Serum tartrate–resistant acid phosphatase 5b (TRAcP5b) and procollagen type I N-terminal propeptide (P1NP) were measured using commercially available kits (MouseTRACP Assay; IDS, Boldon, UK) (Rat/Mouse P1NP; IDS) to assess osteoclasts and bone formation/remodeling, respectively.

Statistics

Statistical analyses were performed using analysis of variance with a Tukey post hoc test. The drug effect on the wound closure was assessed using the Fisher exact test. All statistical analyses were conducted with SYSTAT (Systat Software, Chicago, IL, USA). An α level of 0.05 was used for statistical significance. Results are presented as mean ± standard error unless otherwise specified.

Appendix Table 1. Results of Complete Blood Count at 5, 10, and 21 d.

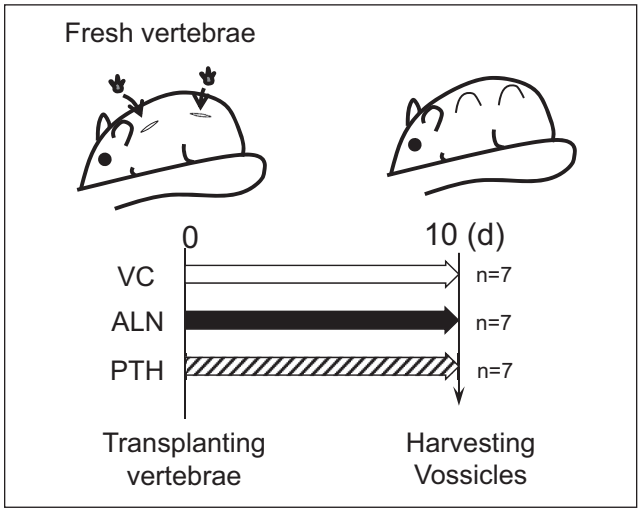
					P Value (Tukey Test)		
5 d	VC	ALN	PTH	*P* Value (1-way ANOVA)	ALN v. VC	PTH v. VC	ALN v. PTH
WBC, K/μL	2.67 ± 1.17	3.33 ± 2.25	5.15 ± 2.06	0.063			
NE#, K/μL	0.36 ± 0.21	0.47 ± 0.28	0.67 ± 0.19	0.061			
LY#, K/μL	2.19 ± 0.95	2.75 ± 1.96	4.32 ± 1.79	0.064			
RBC, M/μL	7.70 ± 1.44	9.05 ± 0.79	9.74 ± 0.72	0.006	0.063	0.005	0.442
HB, g/dL	10.77 ± 1.55	12.50 ± 0.87	13.25 ± 0.52	0.001	0.019	0.001	0.107
HCT, %	34.67 ± 5.43	40.52 ± 3.63	43.52 ± 2.62	0.002	0.038	0.002	0.371
PLT, K/μL	185.8 ± 82.8	343.0 ± 267.0	701.5 ± 357.0	0.011	0.564	0.010	0.076
					P Value (Tukey Test)		
10 d	VC	ALN	PTH	*P* Value (1-way ANOVA)	ALN v. VC	PTH v. VC	ALN v. PTH
WBC, K/μL	4.12 ± 1.50	3.85 ± 1.55	5.16 ± 1.48	0.257			
NE#, K/μL	0.66 ± 0.23	0.79 ± 0.36	1.28 ± 0.91	0.137			
LY#, K/μL	3.35 ± 1.30	2.92 ± 1.16	3.70 ± 1.40	0.538			
RBC, M/μL	9.43 ± 0.38	9.91 ± 0.65	9.69 ± 0.48	0.245			
HB, g/dL	12.75 ± 0.55	13.30 ± 0.95	13.18 ± 0.69	0.372			
HCT, %	42.93 ± 1.96	44.45 ± 2.65	43.10 ± 2.46	0.439			
PLT, K/μL	684.7 ± 43.8	548.2 ± 273.6	769.4 ± 59.5	0.062			
					P Value (Tukey Test)		
21 d	VC	ALN	PTH	*P* Value (1-way ANOVA)	ALN v. VC	PTH v. VC	ALN v. PTH
WBC, K/μL	4.04 ± 0.68	4.34 ± 1.45	3.24 ± 1.57	0.341			
NE#, K/μL	0.72 ± 0.28	0.83 ± 0.22	0.49 ± 0.31	0.122			
LY#, K/μL	3.09 ± 0.62	3.27 ± 1.27	2.59 ± 1.20	0.536			
RBC, M/μL	9.36 ± 0.84	9.41 ± 0.99	8.52 ± 1.84	0.432			
HB, g/dL	12.30 ± 1.20	12.45 ± 1.28	11.22 ± 2.45	0.430			
HCT, %	39.73 ± 3.92	40.62 ± 4.13	36.27 ± 8.17	0.406			
PLT, K/μL	512.2 ± 235.0	471.7 ± 232.7	406.0 ± 193.3	0.709			

Peripheral blood was collected at euthanasia, and complete blood count was performed at 5, 10, and 21 d. Significant differences were observed in RBC, HB, HCT, and PLT between PTH- and VC-treated mice at 5 d. Also, there were significant differences in HB and HCT between ALN- and VC-treated mice at 5 d. However, at 10 and 21 d, statistically significant differences were no longer observed between groups. Results are presented as mean ± standard deviation. ALN, alendronate; HB, hemoglobin; HCT, hematocrit; LY#, lymphocyte count; NE#, neutrophil count; PLT, platelet count; PTH, parathyroid hormone; RBC, red blood cell count; VC, vehicle control; WBC, white blood cell count.

Appendix Table 2. Results of Gene Expression Assessment.

VC		PTH		ALN	
10 v. 5 d	21 v. 5 d	10 v. 5 d	21 v. 5 d	10 v. 5 d	21 v. 5 d
	Bmp2[a]	Bgn[b]	Bmp2[c]	Cdh11[b]	Bmp2[b]
	Twist1[b]	Cdh11[b]	Twist1[c]	Vdr[b]	Twist1[b]
	Bmp4[b]	Igf1[b]	Vdr[c]		Bmpr2[b]
	Col2a1[b]	Tgfbr3[b]	Bgn[b]		Cdh11[c]
	Dlx5[b]	Twist1[c]			Chrd[b]
	Igf1[b]	Vdr[b]			Col2a1
	Nog[c]				Dlx5[b]
	Phex				Gli1[b]
	Sost[a]				Igf1r[b]
	Bglap[b]				Mmp2[b]
	Vcam1[b]				Nog[b]
					Smd3[b]
	(Col4a1[c])				Sost[c]
	(Csf2[b])				Acvr1[b]
	(Gdf10[b])				Vegfa[b]

Osteogenesis-related gene expression was assessed using Mouse Osteogenesis PCR Array (PAMM-026Z; Qiagen). Genes that were significantly activated by treatment are listed. Downregulation of genes in parentheses.
Acvr1, activin A receptor, type 1; ALN, alendronate; Bglap, bone gamma carboxyglutamate protein; Bgn, biglycan; Bmp2, bone morphogenetic protein 2; Bmp4, bone morphogenetic protein 4; Bmpr2, bone morphogenic protein receptor, type II (serine/threonine kinase); Cdh11, cadherin 11; Chrd, chordin; Col2a1, collagen, type II, alpha 1; Col4a1, collagen, type IV, alpha 1; Csf2, colony stimulating factor 2 (granulocyte-macrophage); Dlx5, distal-less homeobox 5; Gdf10, growth differentiation factor 10; Gli1, GLI-Kruppel family member GLI1; Igf1, insulin-like growth factor 1; Igf1r, insulin-like growth factor 1 receptor; Mmp2, matrix metallopeptidase 2; Nog, noggin; Phex, phosphate-regulating gene with homologies to endopeptidases on the X chromosome (hypophosphatemia, vitamin D–resistant rickets); PTH, parathyroid hormone; Smd3, MAD homolog 3 (*Drosophila*); Sost, sclerostin; Tgfbr3, transforming growth factor, beta receptor III; Twist1, twist homolog 1 (*Drosophila*); VC, vehicle control; Vcam1, vascular cell adhesion molecule 1; Vdr, vitamin D receptor; Vegfa, vascular endothelial growth factor A.
[a]$P < 0.001$.
[b]$P < 0.05$.
[c]$P < 0.01$.



Appendix Figure. The vossicle bone transplant system. Fresh vertebrae were harvested from neonatal pups and subcutaneously implanted in the back of the recipient mice. Mice received alendronate (ALN), parathyroid hormone (PTH), or saline (vehicle control [VC]) for 10 d before euthanasia. At euthanasia, vossicles were harvested, fixed, and processed for cryosectioning.