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AAO Foundation Final Report Form
(a/o 1/3/2018)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Please prepare a report that addresses the following:

Type of Award: **Research Aid Award**

Name(s) of Principal Investigator(s): **Dr Farnaz Younessian, Dr Shiva Khatami**

Institution: **Nova Southeastern University**

Title of Project: **Original research**

Period of AAOF Support: **07-01-19 to 07-01-20**

Amount of Funding: **$5,000**

Summary/Abstract

Introduction: As orthodontic tooth movement happens following alveolar bone remodeling, pharmacological agents that modify osteoclasts functions could promote controlled tooth movement during and after orthodontic treatment. The purpose of this study was to compare the effectiveness of locally administration of two-antiresorptive agents including Anti-RANKL Monoclonal Antibody (mAb) and Clodronate (Bisphosphonate) to decrease orthodontic tooth movement.
Methods: Sixty-four Sprague-Dawley rats were divided into 4 groups (n=16/group): 1) Bisphosphonate (Clodronate), 2) rat anti-RANKL mAb (equivalent to human Denosumab), 3) control rat mAb (isotype matched), and 4) control (saline). After initiation of orthodontic movement using a NiTi coil spring (30 cN), agents were locally injected at 0, 7, and 14 days. The rats were euthanized at days 14 and 21 by CO2 inhalation. The bone mineral density (BMD) and tooth movement, as well as bone remodeling biomarkers (Collagen C-telopeptide [CTX], RANKL and Osteocalcin) in local tissues, were measured using microCT and ELISA, respectively. H&E staining were performed for histology sections.
Results: The amount of orthodontic tooth movement demonstrated a statistically significant decrease in both Clodronate and anti-RANKL monoclonal antibody groups compared to control mAb group at 14 days (p values <0.05). Anti-RANKL mAb showed significantly diminished CTX production at 14 days (P value <0.05). No significant differences in microCT measures of alveolar bone (BV/TV and BMD) were seen among groups at 14 and 21 days (p value> 0.05).
Conclusion: Local administration of Anti-RANKL Monoclonal Antibody (mAb) could affect the rate of orthodontic tooth movement and the level of resorptive cytokines in a rat model.

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript OR
2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis and interpretation of results should be included.

Results

Animals follow-up

Two experimental rats failed to survive due to adverse reaction to anesthesia and were excluded from the study. These two samples were replaced immediately. The rest of the rats tolerated the experimental procedures well, with no significant change in their weight and overall health status.
Our pilot study results demonstrated that anti-mouse RANKL mAb (rat IgG2a) reacted to both recombinant mouse- and rat-RANKL antigens, while control rat IgG2a mAb did not show any binding activity to either mouse- or rat-RANKL antigen. The results showed that anti-mouse RANKL mAb, but not control mAb, can neutralize recombinant rat RANKL.

Orthodontic tooth movement (OTM) inhibition

A summary of the OTM monitorization (T1, T2) in the four experimental groups is presented in table 1. At 14 days (T1 time point), maximum amount

of tooth movement (253 micrometer) was recorded at control monoclonal antibody group. The minimum amount of tooth movement was observed in anti-RANKL group (156.73 micrometer). Based on microCT measurements, no statistically significant reduction of the amount of tooth movement was observed at day 14, when compared to the control group (p value> 0.05). In contrast, there was significant reduction in the mesial movement of the molars when animals received injections of Clodrontae or anti-RANKL compared to the control monoclonal antibody at this time point (p value: 0.038 and 0.006, respectively).

At 21 days (T2 time point) the maximum amount of tooth movement (380 micrometer) was recorded for the control group. The minimum amount of tooth movement was observed in Clodronate group (324 micrometer). Local administration of Clodronate, anti-RANKL and sham antibody didn't demonstrate statistically significant reduction in the mesial molar tooth movement at day 21th when compared to the control group, (p value: 0.708, 0.978 and 0.350, respectively). In contrast to day 14, there was no statistically significant difference between the amounts of reduction in tooth movement of anti-RANKL or Clodronate groups with control monoclonal antibody group (p value: 0.575 and 0.336, respectively). The rate of OTM increased during first 2 weeks and slightly decreased from day 14 to day 21. Control monoclonal antibody and control group demonstrated not statistically higher rate of OTM, followed by bisphosphonate and anti-RANKL mAb groups.

Table 1. Mean and standard deviation (μm) of tooth movement in experimental groups using microCT measurement at day 14 (T1) and 21 days (T2)

Time point	**T1 (14 days)**				**T2 (21 days)**			
Group (n=8)	**Mean ± SD**	**SE**	**Min-Max**	**P value**	**Mean ± SD**	**SE**	**Min-Max**	**P value**
Clodronate	182 ± 44	0.015	108-240	0.378¥	324 ± 131	46.60	103-516	0.708
Anti-RANKL mAb	156 ± 63	0.023	98-275	0.140€	346 ± 136	48.14	108-481	0.978
Control mAb	253 ± 68	0.024	137-378	0.190¥€	371 ± 163	57.93	137-653	0.350
Control group	208 ± 43	0.015	137-247	-	380 ± 145	51.44	240-722	-

* Indicates statistical significance when compared with the control group

¥ Indicates statistical significance when compared with another group with same letter (p value: 0.038)

€ Indicates statistical significance when compared with another group with same letter (p value: 0.006)

RANKL, collagen C-telopeptide and Osteocalcin cytokines levels

The local administration of anti-RANKL mAb significantly suppressed the production of CTX in the tooth that received orthodontic forces. Although anti-RANKL mAb demonstrated the trend of suppression on the local production of RANKL and Osteocalcin, there was no significant difference when compared to the group that received tooth movement alone (Table 2). Levels of RANKL, CTX and Osteocalcin were all elevated in the group that received orthodontic tooth movement alone at day 14 compared to that observed in Anti-RANKL mAb and control monoclonal antibody.

According to the available results, the amount of RANKL cytokine was highest in the Clodronate group with the mean amount of 24.2 pg/mg, and lowest in the control group without orthodontic tooth movement (7.3 pg/mg). The amount of RANKL cytokine was higher ($p < .05$) in Clodronate group compared to control group. The amount of CTX cytokine was highest in the Clodronate group with the mean amount of 1.6 pg/mg and lowest in the control group without orthodontic tooth movement (0.68 pg/mg). The amount of CTX cytokine was statistically lower in the anti-RANKL monoclonal antibody than control group with orthodontic movement. The amount of Osteocalcin cytokine was statistically lower in the control group without orthodontic tooth movement compared to control group with orthodontic tooth movement (Table 2).

Table 2. RANKL, CTX and Osteocalcin levels (pg/mg tissue) in all experimental and control groups.

Group	**RANKL Mean ± SD**	**Collagen C-telopeptide Mean ± SD**	**Osteocalcin Mean ± SD**
Clodronate	24.2 ± 8.0*	1.6 ± 0.31	2.9 ± 1.0
Anti-RANKL mAb	10.9 ± 6.3	0.77 ± 0.36*	2.2± 0.9
Control mAb	10.2 ± 7.2	1.12 ± 0.37	2.5 ± 0.8
Control group without tooth movement	7.3 ± 3.0	0.68 ± 0.05 *	1.6 ± 0.3*
Control group with tooth movement	13.0 ± 6.5	1.33 ±0.57	2.8 ± 0.7

*Indicates statistical significance when compared with the control group with orthodontic tooth movement

Alveolar bone microarchitecture response to interventions (BMD, BV, TV and BV/TV ratio)

In order to determine how local injection of agents affects the bone microstructure during tooth movement, we examined the overall bone structure and mineral density by microCT. At the first maxillary molar region, there was no significant difference in the BV/TV ratio between groups at 14 days, (p value > 0.05). However, the BMD measure was significantly lower in all experimental groups including Clodronate, anti-RANKL and control mAb compared to the control group at this time point (p values: 0.021, 0.011 and 0.045, respectively) (Table 3). At 21 days, there was statistically significant difference between bone volume and tissue volume measurement of the Anti-RANKL mAb group with the control group (p value<0.05). However, the BV/TV ratio didn't show differences with control group at 21th day ($p > .05$). Additionally, BMD values did not show any statistically significant difference among all the experimental groups, including Clodronate, RANKL, control mAb and control group at T2 (Table 3).

The changing in the BV/TV in time is detailed in Table 3. Although a decreasing trend might be observed in all experimental groups, this reduction was not statistically significant in any group ($p > 0.05$). Parallelly, control group was the only group showing increasing trend in overall BV/TV ratio from day 14^{th} to 21^{st}. Although, this increasing trend in control group was also not statistically significant (Table 3). Regarding, BMD changes in the alveolar bone, a mixed decreasing and increasing trends is observed in all intervention groups. However, these changes were not statistically significant ($p > 0.05$) except for control group that showed a decreasing trend ($p < 0.05$) in overall BMD value from day 14 to day 21 (Table 3).

Table 3. Bone volume, tissue volume, bone/tissue volume fraction and bone mineral density values and changes in all experimental and control groups at 14 days (T1), and 21 days (T2).

Time point	T1				T2				BV/TV changes			BMD changes		
Group	**BV Mean ± SD**	**TV Mean ± SD**	**BV/TV %**	**BMD Mean ± SD**	**BV Mean ± SD**	**TV Mean ± SD**	**BV/TV %**	**BMD Mean ± SD**	**Mean Difference**	**95% C.I.**	**P value**	**Mean Difference**	**95% C.I.**	**P value**
Clodronate	15.96 ± 2.10	30.09 ± 2.24	54.81 ± 5.20	0.65 ± 0.03*	17.09 ± 1.414	31.59 ± 2.32	0.62 ± 0.16	0.62 ± 0.16	-0.581	-4.92-6.08	0.821	-0.0275	-0.11-0.16	0.865
Anti-RANKL mAb	15.76 ± 1.82	28.07 ± 2.49	56.16 ± 4.34	0.65 ± 0.03*	12.07 ± 3.79*	21.46 ± 3.74*	0.68± 0.07	0.68± 0.07	-1.205	-5.07-7.48	0.664	0.0375	-0.051-0.126	0.325
Control mAb	15.71 ± 2.34	28.54 ± 3.08	55.01 ± 5.02	0.66 ± 0.08*	13.69 ± 3.57	26.38 ± 4.65	0.66 ± 0.05	0.66 ± 0.05	-0.602	-10.63-11.84	0.921	0.0730	-0.001-0.149	0.993
Control group	16.49 ± 3.12	29.4 ± 3.38	48.27 ± 9.01	0.73 ± 0.50	16.60 ± 1.65	32.24 ± 1.83	0.67 ± 0.03	0.67 ± 0.03	4.512	6.04-15.06	0.328	-0.067	0.0385-0.095	0.001 *

**Indicates statistical significance when compared with the control group*
BV: Bone Volume (mm3), TV: Tissue Volume (mm3), BMD: Bone Mineral Density (mg/cc)

Histological evaluation

Histomorphometric analysis with H&E allowed for evaluation of osteoclast counts. The histologic sections were evaluated at three different vertical levels of cervical, mid-root and apical of the first molar. The number of osteoclasts was quantified on H&E by defining a fixed size rectangle for the tension and compression sides that enclosed the alveolar bone, PDL and tooth structure of the mesial root of the first molar for each rat on both experimental and contralateral side. The compression side of orthodontic tooth movement sides of all groups showed a greater number of osteoclasts than the control side across all animals in the alveolar bone (Table 4). There was higher number of osteoclasts in the alveolar bone of the control and monoclonal antibody groups compared with the Clodronate and RANKL groups. Widened PDL space and loss of supporting alveolar bone was observed in all samples due to presence of orthodontic force. Isolated areas of root resorption were also seen in all samples, especially in the midroot and apical areas.

Table 4. Comparison of number of osteoclasts-like cells per μm2 at 21 days.

Group	Osteoclast counts (*Mean ± SD)*							
	Cervical area		Midroot area		Apex area		Overall	
	Mesial	Distal	Mesial	Distal	Mesial	Distal	Mesial	Distal
Bisphosphonate (Clodronate)	5.33 ± 1.52	3.33 ± 0.57	1.42 ± 0.89	1.36 ± 1.03	4.55 ± 1.01	3.11 ± 0.78	4.28± 1.14*	3.28± 1.23*
Anti-RANKL mAb	3.33± 0.57	2.66± 0.57	3.50 ± 0.54	3.00 ± 0.63	3.83± 0.40	2.83 ± 0.40	3.6 ± 0.50*	2.86 ± 0.51*
Control monoclonal antibody	6.01± 0.55	4.33±0.57	4.11± 0.92	3.33± 0.86	3.88±0.92	2.88± 0.53	4.83 ± 2.04	3.22 ± 0.80*

Control group	5.66 ± 0.81	4.16 ± 0.75	6.00 ± 0.53	4.50 ± 0.41	5.5± 0.28	4.5± 0.28	5.38 ± 0.28	4.38 ± 0.23

*Indicates statistical significance when compared with the control group

Response to the following questions:

1. Were the original, specific aims of the proposal realized?
2. Were the results published?
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
    b. Was AAOF support acknowledged?
    c. If not, are there plans to publish? If not, why not?

The work is published as post graduate thesis at Nova Southeastern University. However, we would like to publish the results as an original research manuscript in AJODO journal in near future.

3. Have the results of this proposal been presented?
    a. If so, list titles, author or co-authors of these presentation/s, year and locations
    b. Was AAOF support acknowledged?
    c. If not, are there plans to do so? If not, why not?

This research was accepted to be presented at AAO 2020 Atlanta as Oral Presentation. However, due to COVID-19 pandemic, oral presentation is postponed to next year.

3. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

The AAOF grant was essential to this project as it provided adequate funding to evaluate the immune-histochemical differences (level of cytokines) between the groups. The support from the AAOF provided the opportunity to develop and conduct this research project during my residency. I would like to re-join academia after graduation as a faculty member who focus on research.