Genetic regulation of the biology-based acceleration of orthodontic tooth movement

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SUMMARY OF THE
DOCTORAL THESIS

Stomatology Dept,
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Sevilla, March 2014

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1.

1. INTRODUCTION

The evolution of knowledge regarding the cellular basis underlying the orthodontic tooth movement (OTM) together with the notorious advancement in biomolecular techniques have triggered a new investigation field: biology-based modulation of orthodontic tooth movement. Shortening treatment time in orthodontics has been a long-time desired goal for both patients and orthodontists, and with this in mind, a number of techniques have been published to date, including surgical procedures such as corticotomy. It is however impossible to think about an articulated, science-based acceleration method without strengthen what we already know about the genetic behavior related to OTM.

To achieve this, an extensive literature review has been done, going through the already existing methods to biologically modify OTM, in order to evaluate them on a molecular level, regarding their probable clinical use. At the same time, and with the objective of obtaining as much data as possible for further study, a comparison between gene expression on both normal and surgically accelerated OTM has been performed experimentally. An animal study on 28 male Wistar rats have been performed, appliying orthodontic force between the molars and the incisors of both their upper hemimaxillas and performing in half of them the surgical procedure of corticotomy. Measurements of tooth movement were taken, and bone samples were extracted for RNA isolation and posterior microarray analysis.

The aim of this investigation project is to establish a clear ‘time line’ of what happens, genetically speaking, after an external force is applied to the teeth and to the surrounding bone tissue; what molecules participate in the process and what is the role of the stem cells involved in bone metabolism and turnover. Finally, based on the preexisting background, it could be possible to design a new, more accurate technique that would let us safely accelerate orthodontic tooth movement without the hazards and side effects of surgery.

2. HYPOTHESIS

The supporters of alveolar corticotomy as an acceleratory technique of OTM argue that the enhancement in movement achieved with this particular surgical technique is due to a decrease in the physical resistance of bone against the dental unit, a fact that would occur at the same time than

the regional acceleratory phenomena (RAP). The latter has been explained as an inflammatory process induced by a non specific surgical aggression to tissue, which triggers a raise in inflammatory mediators amongst other molecules, resulting in increased bone resorption. Nevertheless, in spite of these theories, the truth is that the real molecular basis of this OTM enhancement secondary to corticotomy remains still unclear. At the same time, recent literature (1) evidences the existence of selective cellular progenitors right after surgical intervention in cases of bone distraction,; and activation, survival and differentiation of osteoclastic cells have been stated closely linked to regulatory procedures from mesenchymal stem cell colonies. (2)

When taking this two facts in consideration, a number of questions arise: could we apply that to corticotomy? Is it possible that the surgical intervention would result on the release of selective stimuli for the colony of mesenchymal stem cells in the region of the alveolar bone, modulating their cellular activity? Would that modulation be positive over the differentiation of osteoclastic precursors having therefore a direct clinical effect over OTM?

If the molecular base underlying corticotomy is different than normal OTM, their genetic profile may differ in quantity and/or type of genes involved and therefore we could also find differences in their protein expression pattern. Getting to know the exact sequence of genes involved and the amount and exact kind of proteins that mediate both normal and surgically accelerated OTM would help us identify the cellular and molecular response triggered after the application of external force with orthodontic purpose, a matter that has been largely discussed until now.

Moreover, if there is a real connection between the activation of mesenchymal stem cells responsible for the increase of osteoclastogenesis after the inflammation triggered by surgical damage to the tissues and we are able to actually prove it by clearing out the genetic pattern involved, it might be possible in the near future to use stem cell therapy as a safe, very specific technique in order to accelerate OTM individually, avoiding the aggressive nature, side effects and general hazards of other surgical methods that can be found in literature.

3. OBJECTIVES

According with this initial hypothesis, this experimental project is aimed to the following objectives:

3.1. General Objectives

- To support the theories concerning the molecular sequence resulting tooth movement after systematic literature review.
- To review the biological methods existing nowadays in order to both enhance and decrease orthodontic tooth movement.
- To perform a critical analysis of the effectivity of the methods published, as well as the quality of their clinical essays or animal experimentation.
- To determine the advantages and disadvantages of the biologically-based methods for OTM modification as well as their possible use for clinical practice.
- To gather and analyze the data found in literature regarding this topic, summing up the existing knowledge of these methods in a comprehensive way.
- To describe the cellular basis of OTM, how does the cell unit respond to mechanical stress and what are the reactions on a tissular level.
- To update the biological substrate of osteoclastogenesis and bone metabolism.
- To clarify the genetic profile and therefore the protein expression pattern involved in OTM, opening new lines of investigation and research in this field.

3.2. Specific Objectives

- To validate a new experimental orthodontic model for animal use in investigation, easy to reproduce and compare in further similar investigations.
- To use a new, very useful technique such as microarray assay, in order to make clearer the underlying biological basis of tooth movement.
- To determine the clinical suitability of alveolar corticotomy as a surgical method to accelerate OTM.
- To compare statistically, histologically and clinically the modulation of tooth movement with and without an acceleratory technique.

- To study the genetic expression along orthodontic tooth movement.
- To compare the genetic profiles of both standard and surgically accelerated OTM.
- To prove in a molecular level the biological base of acceleratory methods, bringing up the possibility of proposing a new, safer one in future research without the perks and possible contraindications and side effects of surgery.
- To link the tissue reactions after the application of orthodontic force with the response of bone stem cells.

4. MATERIAL AND METHODS

4.1. Literature Review

A systematic literature review was performed using the following search strategy: using the MedLine Database (Entrez PubMed, www.ncbi.nim.nih.gov) without time limits and updated until February 2014. Other databases were also consulted, such as SCOPUS, Embase, Ebscohost and SCIRUS. Furthermore the main JCR-indexed orthodontic journals were hand searched to identify potential studies not included under the above mentioned criteria. Grey literature was searched by exploring OpenGrey database of the EAGLE (European Association for Grey Literature Explotation).

We used the MeSH Terms (Medical Subject Heading) "tooth movement" and “time factors” combined with the following terms: “gene therapy”, “drug”, “piezocision”, “corticotomy”, “electromagnetic fields”, “low-level laser therapy” and “bioelectric energy sources”. The search was based on the guidelines of the National Health Service Centre for Reviews and Dissemination (3), as well as the PRISMA guidelines set by Liberati et al in 2009. (4)

Papers selected for this study fulfilled the following criteria for inclusion, according to PICO format:
Population: Animal; any experimental study, clinical or in vitro investigation that included at least one experimental group with a minimum of 5 animals or samples per experimental group. Intervention: biologic-based method of modifying tooth movement with application of force by an orthodontic or orthopaedic device.

Comparison: control group without biologic-based method and/or without orthodontic tooth movement.
Outcome measure: rate of OTM.

Case reports, case series, descriptive studies, review articles, opinion articles, letters, articles that did not correspond with the objectives of this review or with no adequate description of technique, administration dose or regime were excluded.

Independent quality assessment of the included studies was performed according to a modified Newcastle-Ottawa scale by 2 investigators. (M.CP and A.I.L) Adapted from Chen et al. (5), the variables analyzed were: study design, representativeness of sample of more than twenty-five subjects/specimens per group, adequacy of statistical provision, statement of blinded assessment, previous power calculation, report of dropouts from study. Based on this checkpoint analysis, risk of bias and the quality of each paper was scored as low, medium or high. Briefly, as described previously (5), one point was given to each criterion if fulfilled. Half a point was granted if part of the criterion was met. Studies with less than 2 points were considered to be at high risk for bias; from 2 to less than 4 points, the risk for bias was considered moderate; and for 4 points and above, the risk of bias was considered low.

4.2. Experimental study design

All interventions were previously approved by the Ethic Comitee of Animal Experimentation (CEEA) of the University of Seville. The animal model used was 4 to 5-week old male Wistar rats, performing an initial test on 5 animals and a final one with 28, using both their upper hemimaxillas, making a total sample size of 56, being half of them controls (only with orthodontic force) and the other experimental (corticotomy plus orthodontic force).

We monitored the animals and analyzed the gene expression pattern on the bone in four different time points, depending on the moment of sacrifice: T1: 24 hours, T2 :3, T3: 7 and T4: 12 days after the initial intervention. They were held in groups of four, under continuous temperature (23º C), a light/darkness cycle of 12/12 hours and food/water available *ad libitum*. Daily surveillance was performed in order to notice any changes in weight and to guarantee normal feeding. Prior to any procedure, the animals were held under anesthesia (intra peritoneal injections of 45 mg/kg ketamine

and subcutaneous injection of 5-10 mg/kg diazepam and 0.04-0.10 mg/kg atropine.) After the interventions, analgesic and antibiotic therapy was prescribed for 3 days (0.05 mg/kg subcutaneous application of buprenorphine and tetracycline).

The appliance selected to execute orthodontic force was an adaptation of Ren's 2004 original design. (6). (Fig. 1). The first, second and third molar were tied together with a 0,2 mm stainless steel ligature (Dentaurum) and a 1.5 mm Sentalloy closed coil spring (GAC, Dentsply Int.) was introduced, exerting a force of approximately 25 g.

Teeth were previously conditioned with 37% phosphoric acid etching gel (DGM, Hamburg, Germany) for 10 seconds, after that, the acid was carefully removed using cotton q-tips and washing it afterwards with water applied with a micro syringe. The surgical bed where the rats were placed was slightly inclined so that the water would drain outside, avoiding it to pass to the animals' airways. The surface of the teeth was then thoroughly air blown until it was dry enough to place the bonding material (3M ESPE). At this point the ligatures were tied using pliers and surgical clamps and restorative blue composite material (3M ESPE) was light-cured around the ligatures and on top of the occlusal surfaces of the teeth to avoid the appliance to get loose as a result of chewing.

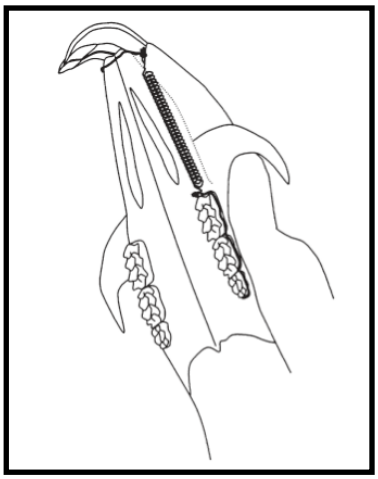

Figure 1. Ren's appliance (7)

Once secured around the molars, the ligatures were introduced in one end of the Sentalloy closed coil springs and on the other side, another ligature was inserted, which was placed around the incisors, previously prepared as described before in order to be covered with restorative composite material as well. All sharp edges were also covered with composite, in order to avoid damage to the soft tissue.

Inside of every group, half of the animals were used as controls and the other half received surgery, always performed on the right maxillary quadrant. Controls were only placed a closed coil spring in order to achieve orthodontic force. The surgical procedure of corticotomy was performed by two operators (MCP and DB) following the guidelines proposed by Sanjideh (8) Oblique incision with a scalpel and a number 15 blade (GmbH, Solingen, Germany) was made along the gingiva mesial to the first molar, and continued parallel to the first, second and third molar on the palatal side. The soft tissue was separated and kept undamaged during the procedure using a surgical clamp.

Vertical decortication was made mesial and distal to the first molar, under saline irrigation, together with direct multiple drilling on the maxillary bone, using a tungsten carbide bur (H141 010 Komet, GmbH, Lemgo, Germany). Suture was applied on the wound afterwards using monofilament synthetic absorbable suture. (Caprosyn® 6-0, 30 inches) Due to the atropine, salivary secretions were minimal, and the bleeding after the incision was controlled by drying and pressuring the wound until it was gone. After finishing the surgery and placing the coil, the rats were sacrificed in 24 hours, 3, 7 and 12 days. From each of the 24 h and 3 days groups, 2 animals were kept for further histology treatment, one control and one surgery respectively, making a total amount of four animals for histology and 24 samples for the Taqman® assay.

In order to obtain the records of orthodontic tooth movement, a high accuracy digital caliper (Digimatic-Mitutoyo, Telford, UK) was used, together with 20x magnifying glasses. The distance from the incisors to the mesial side of the first molar was measured, obtaining the total movement of the average of both the values measured by two different observers, in both the control and the experimental groups, determining afterwards the measuring method error.

Alveolar bone tissue on the maxilla, surrounding the area where the orthodontic external force had been applied to, was extracted after sacrificing the animals with CO_2 inhalation. The right side of the maxilla was kept, quickly eliminating all traces of soft tissue and teeth with the help of a scalpel, immediately freezing the remaining alveolar bone in liquid nitrogen (-125º) and storing the bone samples at -80ºC for an average of a month before isolation of the mRNA was performed.

Samples were transported to the laboratory facilities in hermetically sealed refrigeration boxes, inside of which the Eppendorf tubes containing the bone tissue samples were surrounded by

dry ice. We made sure that the time spent in transport was as short as possible to avoid damaging the samples integrity.

4.3. Sample Processing and analysis

4.3.1. RNA extraction

Isolation and extraction of RNA from bone cells was performed by Genyo (Center for Genomics and Oncological Research, University of Granada, Spain) using QIAamp RNA miRNeasy Minikit (Qiagen, Valencia, CA, USA) and manual extraction with Triazol reagent (Trizol, Invitrogen). Posterior quantification and quality control of the resulting RNA was performed through spectrophotometry (Nanodrop, Fisher) in order to determine the absorbance and fluorímetry. (Qubit). After digestion with DNAse, RNA concentrations were measures, aliquoting and preserving it under -80°C for further analysis.

4.3.2. Microarray analysis

Gene expression was analyzed using a Taqman® Assay. (Life Technologies Corp., Carlsbad, CA, USA) We chose a custom-made Open Array format, able to analyze 224 genes. For the analysis, a list of 224 Wistar rat was carefully designed. Genes for mesenchymal stem cells, hematopoietic stem cells and hypoxia were included.

4.3.3. Histology

Immediately after the sacrifice, the upper maxillary of 4 individuals was kept for histological analysis, 2 form the 24 hours group and 2 from the 3 days group, one control and one surgery each. They were processed the as follows:

- Fixation in 4% para formaldehyde and 4% formaldehyde to obtain a proper tissue fixation never over 24 hours.
- Decalcification a watery solution with 45% formic acid and 20% sodium citrate, renewing it every 21 days.

- Dehydration in increasing alcoholic concentration and alcoholic extraction with 100% Xylan and paraffin.
- Multiple 7µm cuts from the paraffin blocks and fixation in microscope slides.
- Once the paraffin is off, the cuts will be stained with Hematoxylin-Eosin and non-watery solution. After that the samples will be covered and observed through inverted optical microscope.
- The histomorphometric tests will be performed with the image analysis software "Image J" (NIH, Bethesda).

4.4. Bioinformatics and Statistics

For the systematic literature review, all data was gathered in Excel and imported to SPSS program (versión 17.0), performing non parametric statistics (Kruskal-Wallis test followed by paired Mann-Whitney's U test with Bonferroni's adjustment to analyze left and right maxillary data). At the same time, the Wilcoxon's test for paired samples was used to compare between groups, and the inter-observer matching test and the accuracy of the method will be validated with Dahlberg's formula. (9) For every calculation, $p<.05$ was considered as statistically relevant.

For the gene expression analysis, samples were previously normalized based on the expression of endogenous genes (10) and afterwards, models from lineal analysis were taken (11) as well as proportional analysis, following the previously described methodology. (2) At the same time, *clustering analysis expander package* (http://acgt.cs.tau.ac.il/ expander/) was used for the cluster expression analysis.

5. RESULTS

5.1. Results from the literature review

The electronic search identified 218 titles. Ninety-one complete articles were retrieved for review and finally, 74 of these fulfilled the criteria for inclusion, 4 of them were added as a result of a manual search, 1 article was incorporated after contacting the author and 19 of the articles included were published during this review and included on the updates (13-19)

From the 74 articles, only 19 were randomized controlled trials, and according to the point system followed, 12 articles were determined as High Quality, 22 as Medium Quality and the rest as Low Quality, with high risk of bias.

Most of the sample comprised experimental animals, with the rat being the most frequent model (44 out of 74 articles, 29 Wistar and 15 Sprague-Dawley) followed by dogs (6 from 74, beagles and fox hounds, all of them used to test surgical techniques) and in seven cases, rabbits, guinea pigs, monkeys and mice. Only 16 articles included human, and from these, 13 were randomized clinical trials, and the remainder—both human and animal—were comparative studies or evaluation whose sample size seldom exceeded 25 subjects per group . (10 out of 74 articles) We found blinding in 21 papers only: 17 single and 4 double-blind. In brief, the studies about tooth movement modulation using biological methods to alter the OTM rate fall into five categories: electromagnetic fields; low energy laser; SFOTs; pharmacological methods; gene therapy.

5.2. Clinical records of dental movement

An increase of 46,24% was found on the hemimaxillas corresponding to orthodontic force plus corticotomy, compared to controls. (Fig. 2)

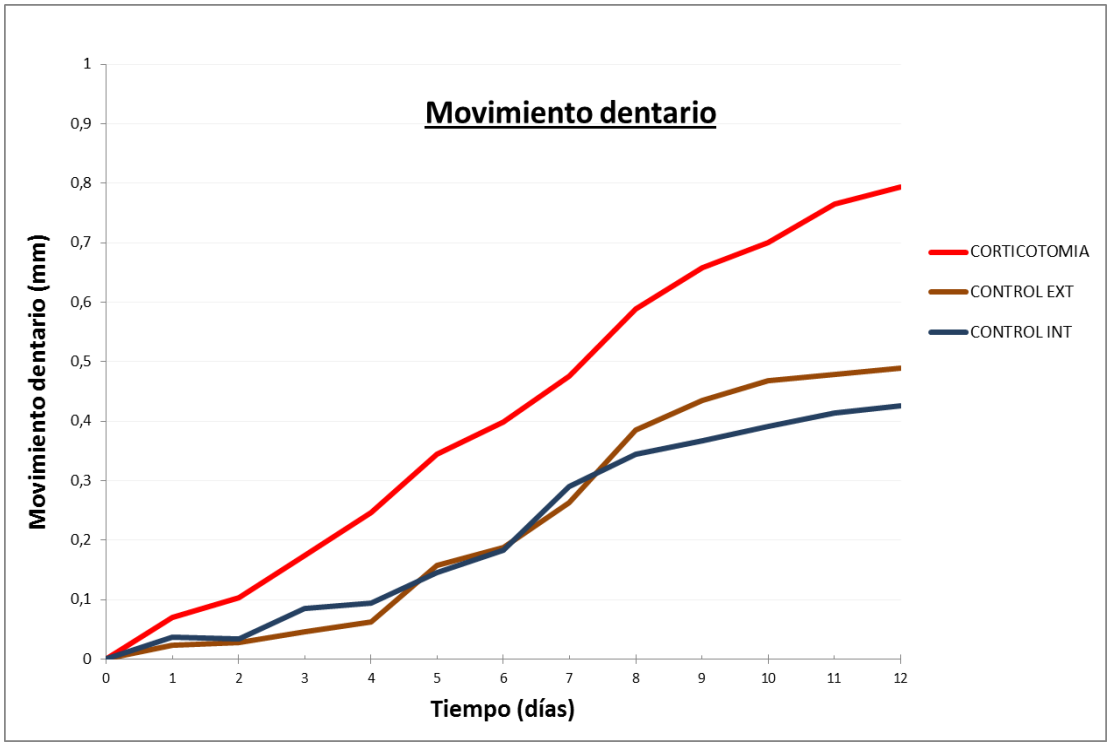

Fig. 2 OTM Quantification in mm.

On the first, third and seventh day of orthodontic force, we found 38% more OTM on the surgical group, compared to the external and internal control groups. ($p<0.001$). No statistically significant differences were observed between control groups. ($p>0.05$) (Fig.2)

5.3. In vitro results and sample preparation

5.3.1.Isolation, Quantification and Quality determination of RNA

We obtained an average of 8.18 µg RNA per sample and an average of 6.44 and 5.70 µg in the 24 hours control and surgery groups respectively; 5.27 and 9.59 µg in the 3 days control and surgery groups; 12.62 and 8.32 µg in the 7 days control and surgery groups and finally 9.38 and 8.08 µg in the 12 days control and surgery groups. The concentration of RNA per sample is described in the attached ANNEX (9.3. RNA Quality Report). Analyzing those data per group, we found an average RNA concentration of 275 and 210.3 µg/µl for the control and surgery groups on the 24 hours group; 244 and 336.33 µg/µl for the control and surgery groups on the 3 days group; 207.33 and 190.67 µg/µl for the control and surgery groups on the 7 days group and 277.67 and 246 µg/µl for the control and surgery groups on the 12 days group.

The RNA integrity number (RIN) on the 24 hours group was an average of 2.3 and 2.4 for the control and surgery groups respectively, 2.3 and 2.6 on the 3 days group for control and surgery; 2.3 and 2.4 on the 7 days group for control and surgery and finally, 2.3 and 2.6 on the 12 days group for control and surgery. This numbers leave us with a RIN= 2.4 in the 24 samples as a whole, 2.4 in all the controls and 2.5 in tall he surgeries.

5.3.2. Gene expression array results after inducing orthodontic tooth movement

On the Figure 3, we can observe the changes in expression of different genes, previously cribbed and selected, normalizing the groups for the same experimental days, including those with significant differences ($p< .05$) between controls and experimental group, showing homogeneous behavior with less than 36 cycles on the 75% of the ones replicated. On Fig 3 therefore, we can see how many times more the gene is expressed or inhibited compared to controls on every time point, following a color code: blue for T1 (24h), red for T2 (3 days), green for T3 (7 days) and purple for T4 (12 days).

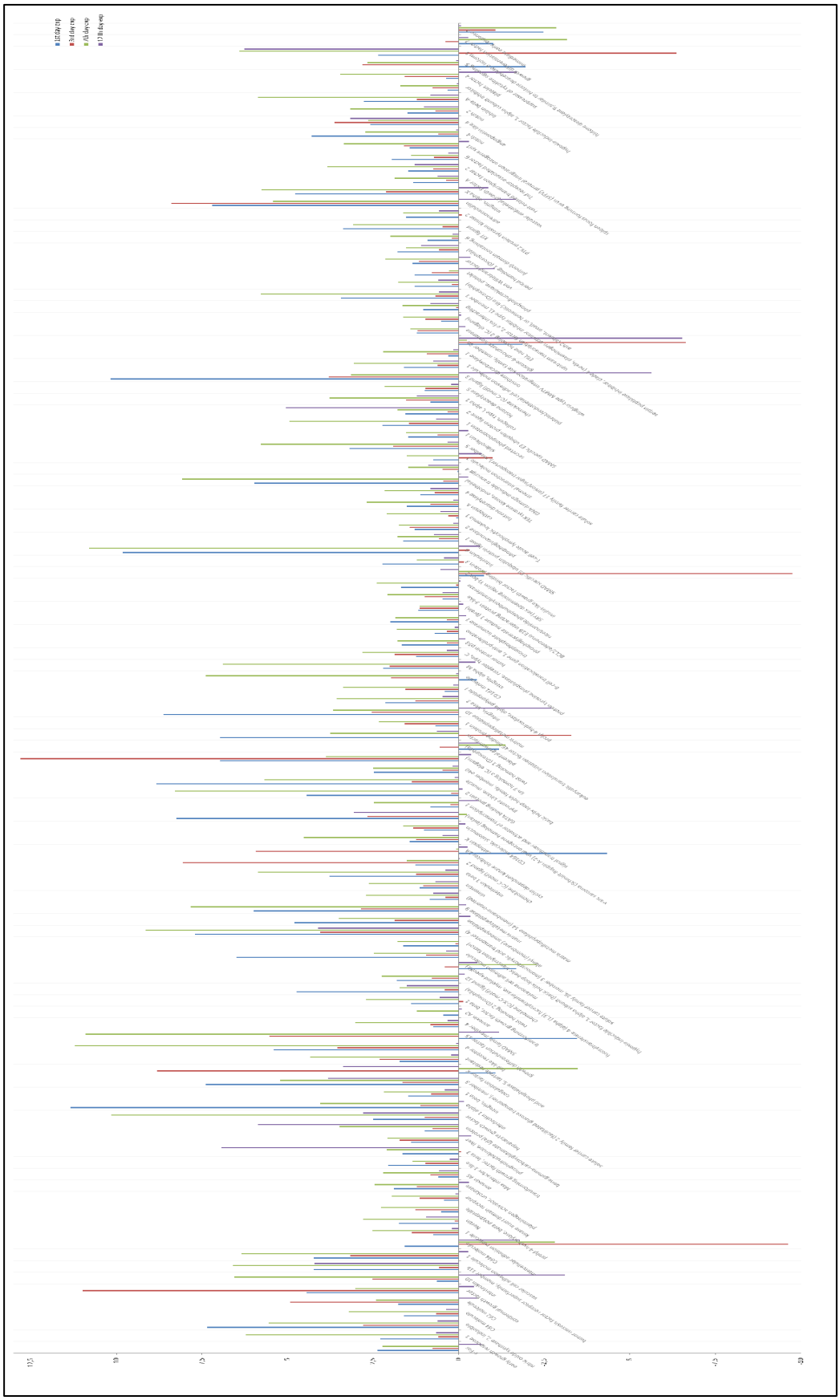

Fig. 3. Changes on gene expression compared to controls on every time point

5.3.2.1. Gene expression after 24 hours

After 24 h from the application of the force, we find that the corticotomy group had increased expression compared with controls of more than 7,5-fold on the following genes: *Interleukine 1 α (IL1α), Viral Oncogene Homolog, Pyruvate Kinase (muscular), MMP 10 (MMP10), Interleukine 6 (IL6) and Chemokine ligand 3 (CC Motif).*

Genes that increased their expression between 5 and 7,5-fold compared to controls were: *Nitric Oxide Syntetase 2 (Nos2), Toll-like receptor 4, Melanoma cell adhesion molecule, Solute carrier family 16 member 3, Matrix metalloproteinase 9 (MMP9), Lin 7 Homolog A (C Elegans), Placental Growth factor (PGF), tyrosine kinase endothelial (TEK) and adrenomedullin.*

Finally, genes with decreased expression were: *Coagulation factor X, Growth Differentiation Factor 15 y 7 (GDF15, 7), Fucosyltransferase 4α, Cyclin-dependent Kinase Inhibitor 1A, Twist homolog 1, Cd1d1 molecule, Sex-determining region Y (SRY) box2, Wingless Type MMTV y Supressor of cytokine signaling 5.*

5.3.2.2. Gene expression on day 3

On day 3, we find that the corticotomy group had increased expression compared with controls of more than 7,5-fold on the following genes: *Epidermal Growth Factor (EGF), Coagulation Factor X, Chemokine ligand 2 (CC motif), Lin 7 Homolog A (C Elegans) and adrenomedullin.*

Genes that increased their expression between 5 and 7,5-fold compared to controls were: *Growth Differentiation Factor 15 (GDF15)* y *Cyclin-dependent Kinase Inhibitor 1A.* genes with decreased expression were: *Cd44 molecule* y *Sex-determining region Y (SRY) 2* (on more than 10-fold) *Placental Growth Factor (PGF), Stromal Interaction Molecule 3*, *Wingless Type MMTV, Histone deacetylase 9* and *Immediate Early Response 2.*

5.3.2.3. Gene expression on day 7

On day 7, we find that the corticotomy group had increased expression compared with controls of more than 7,5-fold on the following genes: *Hepatic Growth Factor (HGF), Toll-like receptor 4, Growth Differentiation Factor 15 (GDF15), Solute carrier family 16 member 4, Matrix Metalloproteinase 9 (MMP9), GATA binding protein 1, Interleukin 6 (IL6) and tyrosine kinase endothelial (TEK).*

Genes that increased their expression between 5 and 7,5-fold compared to controls were: *Early growth response 2, Nos2, IL10, TNF11b, Vascular Cell Adhesion Molecule 1, IL1β, Pyruvate Kinase (muscular), Integrin αM, adrenomedullin, Inhibin βA, Histone deacetylase 9 y Serpin Peptidase Ihibitor 1.*

On the contrary, *GDF7, Immediate Early response 2, Sex-determining region Y (SRY) 2, Fucosyltransferase 4α, Intercel Adhesion Molecule 1 y Coagulation Factor X* were downregulated.

5.3.2.4. Gene expression on day 12

On day 12, there is a general decrease aon the rate of OTM, associated to a lower increase of the majority of the genes analyzed. There is no increased expression compared with controls of more than 7,5-fold on any gene, and only three of them are expressed between 5 and 7,5-fold: *Transforming Growth Factor β3, Gla Protein and Histone deacetylase 9.*

The most significally downregulated genes at this point are*: Early Growth Response 1, Cd2 molecule, Epidermal Growth Factor (EGF), Vascular Cell Adhesion Molecule 1, Cd44 molecule, GDF 15, Fucosyltransferase 4α, SMAD1, Stromal Interaction Molecule 3, adrenomedullin, Integrin αX, Platelet Factor 4.* They are all under 2.5-fold, except *IL10 and Chemokine Ligand 3* that go under 7,5-fold.

5.4. Histological results

There is evidence of bone resorption in both groups, with structural changes on the bone tissue, finding more resorptive lacunae on the corticotomy group, consistent with the increase in osteoclastic activity. There is clear inflammatory evidence triggered by both groups.

6. DISCUSION

The array analysis offers a huge amount of information regarding the genetical sequence of biologically-accelerated OTM. Among our results, the expression Interleukins, IL10, 6, 1α and β particularly, is quite representative. This last two have been described as inflammatory mediators secondary to bone damage (21) and have been found increased in surrounding tissues after applying orthodontic force. (22) After 24 h, IL6 increased over 7,5- fold on the corticotomy group compared to controles, and so did IL 1α. On day 7, IL6 and IL 1β continued being increased, while IL10's expression grew between 5 and 7,5-fold to fall dramatically after that until almost reaching inhibition on day 12.

On the other hand, MMPs have been described as triggered by mechanical stress (23) and bone resorption during tooth eruption and OTM. (24) In particular, MMP9 regulates the angiogenic response from stem cells After 24h, MMP10 increased more than 7,5 -fold and so did MMP9, also on day 7.

Adrenomedullin, a vasodilator that enhances angiogenesis form hematopoietic stem cells (25), shows increased expression on T1, T2 and T3, however decreasing drastically on day 12.

While some genes appear to be upregulated in every time point, such as: *Fucosyltransferase 4α, Stromal Interaction Molecule 3, Histone deacetylase 9, Epidermal Growth Factor (EGF), Immediate Early response 2, Sex-determining region Y (SRY) 2, Wingless Type MMTV.*

On the contrary, some other genes seem to be downregulated on every one of them, such as: *Pyruvate kinase, Nos2, Toll-like receptor 4, Solute carrier family 16 member 4, Lin 7 Homolog A (C Elegans), Tyrosine kinase endothelial (TEK).*

Microarrays allow us to analyze expression profiles (mRNA and microRNA levels) and structural variation (DNA copy number) on a genome-wide level. (26) High density mRNA microarrays are able to analyze up to 50.000 genes, but they require low-degradation RNA samples that need to be previously treated with paraffin and even then, normally between 10 and 15% of the samples are lost. (27) Bone is one of the organic tissues more prone to RNA degradation, and this can be appreciated in our samples. Because of this, we finally chose to make a Taqman® assay, that consists in an exponential qPCR which is replicated 5 times, having statistically higher safety on the results. (28) Up to 224 genes can be analyzed with Taqman® assay, and after exhaustive literature review, we could approximately choose a list of genes that would most likely be involved in tooth movement.

Because of the above mentioned reasons, we paid special attention in keeping the integrity of the RNA when taking the samples. In bone, the quantity of RNA expression in the tissues is already limited and degradation occurs quickly. Samples were taken as fast as possible after the sacrifice of the animals and bone pieces were immediately frozen with liquid nitrogen and kept under -80ºC. Even though, after extraction, the quantity and quality of RNA was small.

When reading the results, it must be taken into account that our study has been conducted on animal model, and therefore, data cannot be exactly transferred to human. In our literature review, very few of the 74 papers finally chosen were in humans (16 from 74), and those who were made on animal models used different species, being mostly rats (44 out of 74 articles, 29 Wistar and 15 Sprague-Dawley) followed by dogs (6 from 74, beagles and fox hounds, all of them used to test surgical techniques) and in seven cases, rabbits, guinea pigs, monkeys and mice. It is well known that doses, metabolism and reaction to chemicals widely vary between species which makes comparison between studies difficult, and transfer of the results from them to human are hard to establish. However, even with limitations, animal models, especially in mammals, are able to mimic complex cellular processes that also happen in human, and have obvious advantages over *in vitro* studies where a limited number of cells are isolated from a bigger environment that can also influence results. Moreover, cause and effect relationships can be safely established through activators or inhibitors of and through genetically modified animals. (29)

Rats, which seem to be the most popular animal model in literature and it is also the one we used in this experiment, show a physiological distal drift of the molars that could interfere with the

measurements and histological analysis. They also appear to have a continuous eruption of the incisives that could modify the direction of the force, making unreliable the anchorage used. In spite of using the appliance proposed by Ren et al (7) specifically designed to minimize the possible side effects, it is difficult to control the amount and direction of the force applied from the coil springs, that may not be constant or decay during the experiment.

To avoid this, we tried to calculate previously the ideal amount of force we should use in this kind of rodents to avoid tissue damage, but the range of the force application seen on the reports consulted was also very wide, going from 15 grams in those articles that use magnetic force to 400 grams in some corticotomy techniques. Many studies did not mentioned the force used or mentioned it only at the beginning of the experiment, without quantification of possible force decay. A rat molar is 50 times smaller than a human's, therefore, to compare force range, root surface areas should be estimated. According to *Ren et al,* (30) 20 g force in rats could be translated to 1kg force in humans, and most papers use forces overwhelmingly over that range.

In our experiment, a force of approximately 25 g was used, in the average with the studies consulted, (31,32) where range of force for rats vary widely from 10 (33) to 100 grams (34).

Even though, we could consider that small differences in magnitude and direction of the force do not directly affect the purpose of our study, as, even if we measured and compared differences in tooth movement, the main objective was to study the gene expression map between normal and accelerated tooth movement by corticotomy. Nevertheless, an initial test was made to validate the orthodontic appliance used, as well as the experimental procedures and side effects that could emerge.

We chose a closed coil spring to exert the orthodontic force because as seen in literature, the orthodontic devices used are mostly closed coil springs made of Nickel-Titanium or Sentalloy, although there is also a wide variety in this, as screw wrenches, springs or elastic rubbers, hand made orthodontic devices and palatal expanders have also been used. With coil springs, direction and magnitude of the force cannot always be controlled as it may not be constant during the entire experiment due to force decay. Elastics under watery conditions show a force decay from 45 to 0 N within the first 0,2 mm decompression (7) and direction of the force may also vary.

The alveolar bone is also denser in this kind of rodents, without marrow gaps and the changes over the tissues created by orthodontics occur faster than in humans. The size of the teeth, as we said, is also smaller. To avoid mistakes, all procedures were performed by two different operators (M.C and D.B) using adapted magnifying glasses, and supervised afterwards by a different one (A.I).

The experimental phase with the animals lasted 12 days, with sacrifices after 24 h, 3, 7 and 12 days. We chose this experimentation times because, as was mentioned before, the metabolism of rats is very fast, chemical reactions and general turnover occur in a short time, so we wanted to avoid force decay to influence the results on gene expression after 12 days. It's also been proved that after 7 days, evidence of bone loss has already been clearly found in rats after placing ligatures and executing tooth movement. (35)

In the literature included on the systematic review, there is a wide range of study times, being 5 days the shorter (36, 37) and 90 the longest. (38) The average experimentation time in rats (including Wistar and Sprague-Dawley) is 26.2 days, (39-41) but the techniques studied are quite varied. Among the gene therapy studies, average is 30.75 days and among the surgical techniques (including only corticotomy studies that would resemble better our purpose of study) average was 36.6 days.

7. FINAL CONCLUSIONS

Taking into account the information extracted form the exhaustive and specific literature research on the current biologically-based methods to accelerate tooth movement, and after analyzing the experimental data obtained, we can state the following conclusions:

1. There is a wide variety in literature of "biologically-based" methods to accelerate tooth movement, but due to the lack of consensus regarding the experimental protocol (animal model, type of appliance to deliver the force, force range for each species), it is very complex to establish absolute comparisons between studies or to extract solid conclusions.

2. The measures of tooth movement in both groups (controls, only with orthodontic force and experimental group, also with corticotomy) show that this surgical intervention significantly

increases the rate of orthodontic tooth movement when compared with controls. (46,2% more OTM on the surgery group)

3. About the molecular regulation of the acceleration mechanism of corticotomy, after the gene expression array, we can conclude that on the corticotomy group, there is an increased expression of genes related to inflammatory mediators such as Interleukins (1, 6 and 10) on the early days, bone resorption activators such as MMPs 9 and 10, and angiogenic enhancers from hematopoietic stem cells. On the other hand, a decrease can be observed on those molecules that promote bone formation from mesenchymal stem cells. On the forth and last time point, twelve days after the interventions, there is an obvious decrease on gene expression related to bone healing and inflammation.

4. Because of all the above, we could state that our initial hypothesis is confirmed; there is a real connection between the activation of mesenchymal stem cells responsible for the increase of osteoclastogenesis after the inflammation triggered by surgical damage to the tissues. Therefore, it might be possible in the near future to use stem cell therapy as a safe, very specific technique in order to accelerate OTM individually, avoiding the aggressive nature, side effects and general hazards of other surgical methods that can be found in literature.

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