UNIVERSITÀ DI PARMA
ARCHIVIO DELLA RICERCA

University of Parma Research Repository

Aptamers recognizing fibronectin confer improved bioactivity to biomaterials and promote new bone formation in a periodontal defect in rat

This is the peer reviewd version of the followng article:

Original
Aptamers recognizing fibronectin confer improved bioactivity to biomaterials and promote new bone formation in a periodontal defect in rat / Parisi, Ludovica; Rivara, Federico; Alves Costa, Camila; Paolo Flores Abuna, Rodriguo; Bazan Palioto, Daniela; Macaluso, Guido Maria. - In: BIOMEDICAL MATERIALS. - ISSN 1748-6041. - (2020). [10.1088/1748-605X/abb6b2]

Availability:
This version is available at: 11381/2911768 since: 2022-01-17T12:48:36Z

Publisher:

Published
DOI:10.1088/1748-605X/abb6b2

Terms of use:
openAccess

Anyone can freely access the full text of works made available as "Open Access". Works made available

Publisher copyright

(Article begins on next page)

19 November 2022

Biomedical Materials

ACCEPTED MANUSCRIPT

Aptamers recognizing fibronectin confer improved bioactivity to biomaterials and promote new bone formation in a periodontal defect in rat

To cite this article before publication: Ludovica Parisi *et al* 2020 *Biomed. Mater.* in press https://doi.org/10.1088/1748-605X/abb6b2

Manuscript version: Accepted Manuscript

Accepted Manuscript is "the version of the article accepted for publication including all changes made as a result of the peer review process, and which may also include the addition to the article by IOP Publishing of a header, an article ID, a cover sheet and/or an 'Accepted Manuscript' watermark, but excluding any other editing, typesetting or other changes made by IOP Publishing and/or its licensors"

This Accepted Manuscript is **© 2020 IOP Publishing Ltd**.

During the embargo period (the 12 month period from the publication of the Version of Record of this article), the Accepted Manuscript is fully protected by copyright and cannot be reused or reposted elsewhere.
As the Version of Record of this article is going to be / has been published on a subscription basis, this Accepted Manuscript is available for reuse under a CC BY-NC-ND 3.0 licence after the 12 month embargo period.

After the embargo period, everyone is permitted to use copy and redistribute this article for non-commercial purposes only, provided that they adhere to all the terms of the licence https://creativecommons.org/licences/by-nc-nd/3.0

Although reasonable endeavours have been taken to obtain all necessary permissions from third parties to include their copyrighted content within this article, their full citation and copyright line may not be present in this Accepted Manuscript version. Before using any content from this article, please refer to the Version of Record on IOPscience once published for full citation and copyright details, as permissions will likely be required. All third party content is fully copyright protected, unless specifically stated otherwise in the figure caption in the Version of Record.

View the article online for updates and enhancements.

This content was downloaded from IP address 130.92.100.2 on 11/09/2020 at 13:56

Aptamers recognizing fibronectin confer improved bioactivity to biomaterials and promote new bone formation in a periodontal defect in rat

Ludovica Parisi[1,2,3,*], Federico Rivara[1,2,*], Camila A. Costa[4,5,*], Rodriguo PF. Abuna[6,7], Daniela B. Palioto[4], Guido M. Macaluso[1,2,8]

[1] Centro Universitario di Odontoiatria, University of Parma, Via Gramsci 14, 43126 Parma, IT

[2] Dipartimento di Medicina e Chirurgia, University of Parma, Via Gramsci 14, 43126 Parma, IT

[3] Laboratory for Oral Molecular Biology, Dental Research Center, Department of Orthodontics and Dentofacial Orthopedics, School of Dental Medicine, University of Bern, Freiburgstrasse 3, 3010 Bern, CH

[4] Department of Oral & Maxillofacial Surgery, and Periodontology, School of Dentistry of Ribeirão Preto, University of São Paulo, Av. Do Cafè, 14040-904, Ribeirão Preto, SP, BR

[5] Department of Stomatological Sciences, School of Dentistry, Federal University of Goias, Avenida Arumã, 74835-320 Goiâna, GO, BR

[6] Cell Culture Laboratory, School of Dentistry of Ribeirao Preto, University of São Paulo, Av. Do Cafè, 14040-904, Ribeirão Preto, SP, BR

[7] Fiocruz-Bi-Instituional Translational Medicine Project, Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Rua dos Técnicos, 14040-030, Ribeirão Preto, SP, BR

[8]Istituto dei Materiali per l'Elettronica ed il Magnetismo, Consiglio Nazionale delle Ricerche, Parco Area delle Scienze 37/A, 43124 Parma, IT

[*] Equal contribution of the authors

Corresponding Author:

Guido Maria Macaluso

Centro Universitario di Odontoiatria

Dipartimento di Medicina e Chirurgia

University of Parma

43126 Parma, PR, Italy

e-mail: guidomaria.macaluso@unipr.it

tel: +39 0521033659

fax: +39 0521347076

Accepted Manuscript

Abstract

The use of alloplastic materials in periodontal regenerative therapies is limited by their incapacity to establish a dynamic dialog with the surrounding milieu. The aim of the present study was to control biomaterial surface bioactivity by introducing aptamers to induce the selective adsorption of fibronectin from blood, thus promoting platelets activation *in vitro* and bone regeneration *in vivo*.

A hyaluronic acid/polyethyleneglycole-based hydrogel was enriched with aptamers selected for recognizing and binding fibronectin. *In vitro*, the capacity of constructs to support osteoblast adhesion, as well as platelets aggregation and activation was assessed by chemiluminescence within 24h. Matrices were then evaluated in a rat periodontal defect to assess their regenerative potential by microcomputed tomography (µCT) and their osteogenic capacity by Luminex assay 5, 15 and 30 days postoperatively.

Aptamers were found to confer matrices the capacity of sustaining firm cell adhesion ($p=0.0377$) and to promote platelets activation ($p=0.0442$). *In vivo*, aptamers promoted new bone formation 30 days post-operatively ($p<0.001$) by enhancing osteoblastic lineage commitment maturation.

Aptamers are a viable surface modification, which confers alloplastic materials the potential capacity to orchestrate blood clot formation, thus controlling bone healing.

Keywords: dna aptamers - fibronectin - activation, platelet - bone regeneration - bone loss, periodontal;

Accepted Manuscript

1. Introduction

The periodontium, consisting of alveolar bone, cementum, periodontal ligament and gingiva, is a highly organized tissue that surrounds, supports and nourishes teeth. This apparatus creates a developmental, biologic and functional unit that can change due to age, functional modifications and oral environment alterations.

Periodontitis is a chronic inflammatory condition of bacterial origin, further worsened by environmental and behavioral factors, that affects the structures that support the tooth. As a result of a dysbiosis process on the host immune/inflammatory response and microbial upload, a loss of connective tissue attachment to the root surface and the formation of periodontal pockets takes place. This disruptive process might ultimately lead to tooth loss by provoquing the destruction of the alveolar bone mass and the periodontium[1].

Over the last decades, numerous bone grafting materials have been used for the management of critical periodontal bone defects. Grafts of natural origin (e.g. autografts, allografts and xenografts) are good osteoconductive materials, serving as scaffolds to stabilize the clot and to support new osteogenesis in the early healing phases[2-6]. However, problems and challenges related to their harvesting (autografts) as well as limitations in their regenerative potential (allografts and xenografts) still make their use questionable[7]. As such, a number of alloplastic synthetic materials have been recently developed to serve as bone substitutes[8]. These materials have the main advantages of being available in large amounts, lacking any risk of disease transmission and being moldable on specific-tissue requests[9]. Still, a major limitation of alloplastic materials is the lack of bioactivity, which is created by their inability to establish an effective crosstalk with the surrounding environment. In particular, for bone grafting materials the ability to influence the initial coagulum organization and stabilization has been shown to be of pivotal importance for a new bone formation[10].

After graft positioning and barriers fixation, blood clot formation and stability is controlled by proteins adsorbed at the bone-graft interface, which might hamper cells to directly experience material surface[11]. As such, the control of spontaneous protein adsorption to the graft material may represent a reliable way to capture and concentrate specific bioactive mediators from patients' own blood. This approach allows the tailoring of host specific reactions, which include coagulum organization.

To this purpose, we have recently described the possibility to improve the adsorption of fibronectin (FN) at the material-bone interface, with the intention to further ameliorate osteoblasts and epithelial cell response, by means of specific aptamers[12-14]. Aptamers are small (<100bp), single or double-stranded oligonucleotides, which possess a unique capacity to recognize and bind their target from a great pool of molecules[15]. FN, which in its soluble and inactive form is one of the major components of blood plasma (300µg/ml), is considered to be an optimal target candidate for tailoring the bioactivity of alloplastic bone substitutes for many reasons[9, 16, 17]. First of all, FN in injured tissues is organized into a fibrillar form that promotes clot formation, which provides a substratum for platelet adhesion and the formation of the granulation tissue[17,

Accepted Manuscript

[18]. Additionally, the possibility of adsorbing FN from patient's blood on the material surface certifies that the physiological FN isoforms are present, avoiding the cost-intensive production and manual deposition of specific FN isoforms. Eventually, by promoting adhesion and migration of osteoblasts on the material surface, FN stimulates a more rapid healing[19].

The aim of this *in vivo* pre-clinical study has been to improve the performance (clot maturation and new bone formation) of alloplastic materials in periodontal fenestration defect rat models using aptamers to selectively recognize FN.

2. Materials and Methods

2.1 Biomaterial preparation

A hyaluronic acid (HA)/polyethyleneglycole diacrylate (PEGDA)-based hydrogel (HG) (HyStem™ Hydrogel Kit, ESI-BIO, San Francisco, CA, USA) was used as a matrix vehicle for aptamer immobilization. Aptamers were ssDNA oligonucleotides, 40bp long and 12597.4g/mol heavy, which were selected against human fibronectin. Aptamers were further modified with a short carbon chain containing a disulfide bond on their 3'-end (ATW0008, Base Pair Biotechnologies, Pearland, TX, USA). These thiol groups were exploited to bind aptamers to the polyethyleneglycole component acrylate groups through a Michael's addition and thus to immobilize them at the interface of the scaffolding material.

Selective Fibronectin HG preparation (sFN-HG) - Prior to matrix preparation, the S-S bond at the aptamer's 3'-end was reduced with a 2mM solution of Tris(2-carboxyethyl) phosphine hydrochloride pH 7.4 (TCEP, Sigma-Aldrich, Saint-Louis, CA, USA) for 2h at room temperature (RT). The excess of TCEP was then removed on a chromatographic column (mini Quick Spin Oligo Columns, Roche, Basel, CH) following the manufacturer's recommendation.

Reduced aptamers were then mixed with polyethyleneglycole to a final concentration of 14µM and incubated at RT for 20min prior to the addition of hyaluronic acid in a 1:1 ratio with PEGDA. sFN-HG matrices were transfered into an insulin-syringe and allowed to jellify for 1 h prior to use.

HG preparation – Bare HG was used as control. HA and PEGDA components were combined in a 1:1 ratio, then transfered to an insulin-syringe and allowed to settle for 1h prior to use.

2.2 *In vitro* assays

Accepted Manuscript

Prior to animal experiments, the capacity of sFN-HG to support cell colonization was confirmed by imaging and viability (see below). Furthermore, to investigate the capacity of the aptamer to promote blood clot formation, the activation of platelets was quantified by chemiluminescence.

Cell culture – Human osteoblasts (hOB) were obtained from Sigma-Aldrich and cultured in complete DMEM (Thermo Fisher Scientific, Carlsbad, CA, USA) supplemented with 10% FBS (Thermo Fisher Scientific) and 1% Penicillin and Streptomycin (PenStrep, Thermo Fisher Scientific). Cells were plated on HG or sFN-HG settled in 96-well plates at a final concentration of 5000cells/well.

Cell imaging and viability – 24h after seeding, cells were rinsed twice in PBS (Thermo Fisher Scientific) and either fixed in 4% PFA (Sigma-Aldrich) for 20min, stained with methylene blue, and imaged for cell colonization or assayed for cell viability with a chemiluminescent assay (CellTiterGLO, Promega, Madison, WI, USA). In brief, cells were incubated with 200µl of a 1:1 solution of culture medium and CellTiterGLO Lysis Buffer for 2min on an orbital shaker. Sample luminescence was stabilized for 10min in the dark at RT and measured with a GLOMAX 20/20 luminometer (Promega).

Platelet activation – 500µl aliquots of whole plasma were collected and centrifuged at 3000xg for 5min. The supernatant, rich in platelets, was then collected and seeded on hydrogels in the presence or in the absence of aptamers. To quantify platelet activation, the release of ATP was evaluated after 5, 15 and 30min by chemiluminescence as described above.

2.3 *In vivo* experiments

Animals - The study was conducted in accordance to Committee of Ethics in Animal Research of the School of Dentistry of Ribeirao Preto, University of Sao Paulo, which approved all animal procedures performed (2014.1.494.58.0).

This article was further written in accordance with the ARRIVE guidelines[20].

Fifty-four old male Whistar Kyoto rats weighing 250-300 g were used in this study. Animals were randomly assigned to one of the 3 experimental groups: i) spontaneous healing, ii) HG (control), iii) sFN-HG (test). Each group was further divided in 3 randomized subgroups according to the healing time point 5, 15 and 30 days.

Surgical procedure – 15min prior to the surgical procedure, all animals were anesthetized with an intra-muscular administration of 70mg/kg ketamine (Agener União, Embu-Guaçu, SP, Brasil) and 6mg/kg xylazine (Calier, Juatuba, MG, Brasil). Afterwards, the surgical site on the left mandible was shaved off and disinfected. All the surgical procedures were executed under a 10-40x magnifying stereomicroscope (Nikon SMZ800,

Accepted Manuscript

Nikon Instruments Inc, Tokyo, Japan) to identify the anatomic landmarks. A superficial extra-oral incision was made at the base of the mandible to expose the bone. Using a round bur with high-speed instrumentation a bony defect of 2 mm, 4 mm and 1 mm of height, length and depth, respectively, was created to denude the distal and buccal roots of the first molar and the mesial root of the second molar[21]. After the application of HG or of sFN-HG in the defect area, muscle and skin were repositioned in layers and a resorbable suture carried out. An intramuscular injection of 24.000 unities of Penicillin G-benzathine (Zoetis, NJ, USA) and 0,08 ml of analgesic 0,5g/ml (Lema-injex Biologic, SP, BR) were performed.

To reduce the risk of bias, the same trained operator performed all the surgeries.

At the end of each experimental period, the rats were anesthetized as previously described and euthanized in a 100% CO_2-filled chamber. The mandibular blocks were then obtained and fixed in formaldehyde 4% (Sigma-Aldrich) for 24h to procedure micro computed-tomography (µCT) analysis. Before, soft tissues near by the surgical area were also collected preserving adjacent periosteum, immediately stocked in cold PBS with 2 µl of protease inhibitor (1:200 – cOmplete™, Roche, Basel, Switzerland) and processed as previously described for immunoenzymatic assay analysis[22].

Micro computed-tomography analysis - Non-demineralized specimens were scanned with a cone-beam µCT system (Micro-CT Skyscan 1174 scanner, Bruker, MA, USA). The x-ray generator was operated at an accelerated potential of 60kV with a beam current of 165µA and an exposure time of 650ms per projection, in 180°. Images were recorded with a voxel size of 6*6*6µm. Tomographic projections were rebuilt (N.Recon, Version, 1.66, SkyScan, Bruker, Billerica, MA, USA) to generate 3D images.

A volume of interest (VOI) was outlined from analyze of scanned images in 2D in coronal, transaxial and sagittal axis (DataViewer, Bruker), which was chosen individually following the limit of distal margin of mesial root from first molar until distal margin of mesial root of second molar. Recorded parameters for µCT analysis (3D analyze, CTAn v.1.10, Bruker) were total bone volume (BV), as well as bone volume/tissue volume (BV/TV), corresponding to the amount of mineralized bone, bone surface/tissue volume (BS/TV), total porosity, trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th).

Luminex assay - Cytokines related to bone regeneration, namely osteoprotegerin (Opg), osteopontin (Opn) and osteocalcin (Ocn), were quantified with Luminex xMAP assay using Milliplex® kits (TGFBMAG-64K-01REAGMILLIPLEX TGF-beta 1 Single Plx MAGNETIC Bead Kit and RBN2MAG-31K-04 Rat Bone Magnetic Panel 2, MerckMillipore, Burlington, MA, USA) following the manufacturer's recommendations for soft tissues biopsies. The amount of each cytokine expressed as pg/mg was normalized by the amount of total protein of each sample, using a commercial kit (BioRad, Hercules, CA, USA) according to manufacturer's instructions.

2.4 Statistical analysis

Accepted Manuscript

For cell experiments, data were performed three times with multiple replicates.

Animal sample size was determined with G*Power software (Heinrich Heine Universität Düsseldorf) taking into consideration previous studies with similar regeneration periodontal defects, and considering bone volume as a primary outcome[23-26]. A sample size of 6 animals was used.

Data were analyzed using Prism 7 (GraphPad, La Jolla, CA, USA) and reported as mean ± SEM. Differences among the groups were evaluated with a t-Test or with a two-way analysis of variance (ANOVA) statistical test and with the Tukey post-test. Differences among the groups were considered significant when $p<0.05$.

3. Results

3.1 Aptamers promote early and firmly osteoblasts adhesion – To confirm the hypothesis that sFN-HG could promote cell adhesion, hOB cells were seeded on hydrogels in the presence or in the absence of anti-FN aptamers and cultured for 24h.

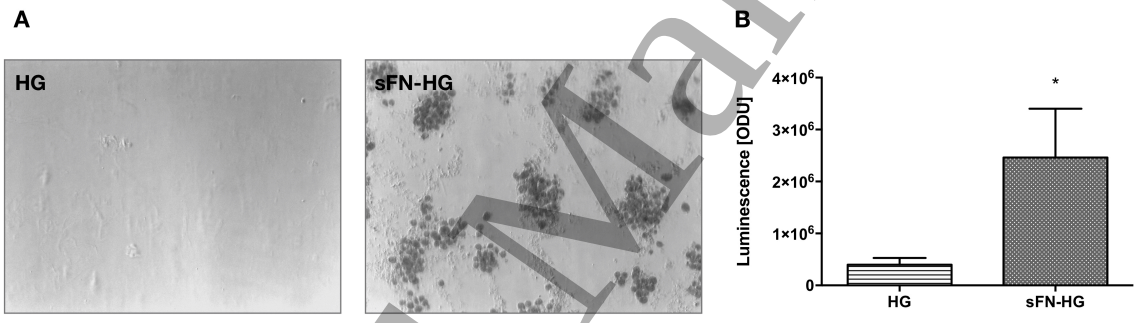


Figure 1: Cell attachment. (A) Representative images of firmly attached hOB cells stained with methylene blue after 24h of culture on bare hydrogel (HG) in the presence or in the absence of aptamers. (B) Histogram reporting cell viability expressed as luminescence of attached hOB cells after 24h of culture on HG or on sFN-HG. Aptamers affected the amount of firmly attached hOB cells. *=$p<0.05$.

Interestingly, control hydrogel (HG) only supported little osteoblasts adhesion. In contrast, osteoblasts firmly attached to and formed colonies on HG containing the aptamer (**Fig.1A**). The presence of firmly adhered cells was confirmed by the analysis of cell viability through chemiluminescence (**Fig.1B**). 24h after plating the cell viability was 6.2-fold higher on sFN-HG than on HG ($p=0.0377$).

3.2 Aptamers promote platelet activation – Blood clot formation requires the adhesion and the activation of platelets soon after material implantation, which is accompanied by the release of molecules, including ATP, in the surrounding milieu. Therefore, to understand if anti-FN aptamers could be potentially involved in promoting blood clot formation, platelets activation was investigated by measuring the ATP released on HG and on sFN-HG 5, 15 and 30min after seeding.

Accepted Manuscript

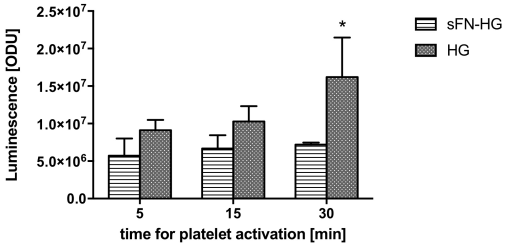


Figure 2: Platelet aggregation and activation. Histograms reporting the amount of ATP released from platelet cultured on HG or on sFN-HG after 5, 15 and 30min. Aptamers affected the activation of platelets. *=p<0.05.

The amount of ATP released, expressed as luminescence, was higher on sFN-HG than on control at each time point (**Fig.2**), suggesting a higher aggregation of platelets. Furthermore, after 30min, which is a reasonable timeframe for a proper clot formation, the differences among the groups were found to be significant (p=0.0442).

3.3 Aptamers promote new bone formation – According to the µCT analysis no significant differences in term of bone formation were detected up to 15 days among the different experimental groups. However, according to the three-dimensional reconstructed image when spontaneous healing occurred, evidence of root exposure was still evident after 15 days. After 30 days the new-formed BV (**Fig.3B**) was found to be the highest for the sFN-HG group (Spontaneous Healing vs. sFN-HG p<0.0001; HG vs. sFN-HG p<0.0001). Differences were found to be significant also for the HG if compared to spontaneous healing (p<0.0001). Consistently, the ratio BV/TV (**Fig.3C**) was the highest for the aptamer group (Spontaneous Healing vs. sFN-HG p<0.0001; HG vs. sFN-HG p=0.0008), followed by the HG group (Spontaneous Healing vs. HG p=0.0005) and the spontaneous healing; while the total porosity (**Fig.3E**) was minimal for sFN-HG (Spontaneous Healing vs. sFN-HG p<0.0001; HG vs. sFN-HG p=0.0008). Regarding the BS/TV (**Fig.3D**), aptamers did not significantly influence this parameter, which was found to be affected by the presence of a scaffolding material (Spontaneous Healing vs. sFN-HG p=0.0019; Spontaneous Healing vs. HG p=0.0372).

Focusing on the parameters describing bone morphometry, the presence of aptamers did not affect the changes of these parameters (Tb.Th, Tb.N and Tb.Sp). On the other hand Tb.Th (**Fig.3F**) and Tb.N (**Fig.3G**) were influenced by the presence of the material and differences were found to be significant for both the groups when compared to the spontaneous healing (Tb.Th: Spontaneous Healing vs. sFN-HG p=0.0059; Spontaneous Healing vs. HG p=0.0068 - Tb.N: Spontaneous Healing vs. sFN-HG p<0.0001; Spontaneous Healing vs. HG p<0.0001).

Accepted Manuscript

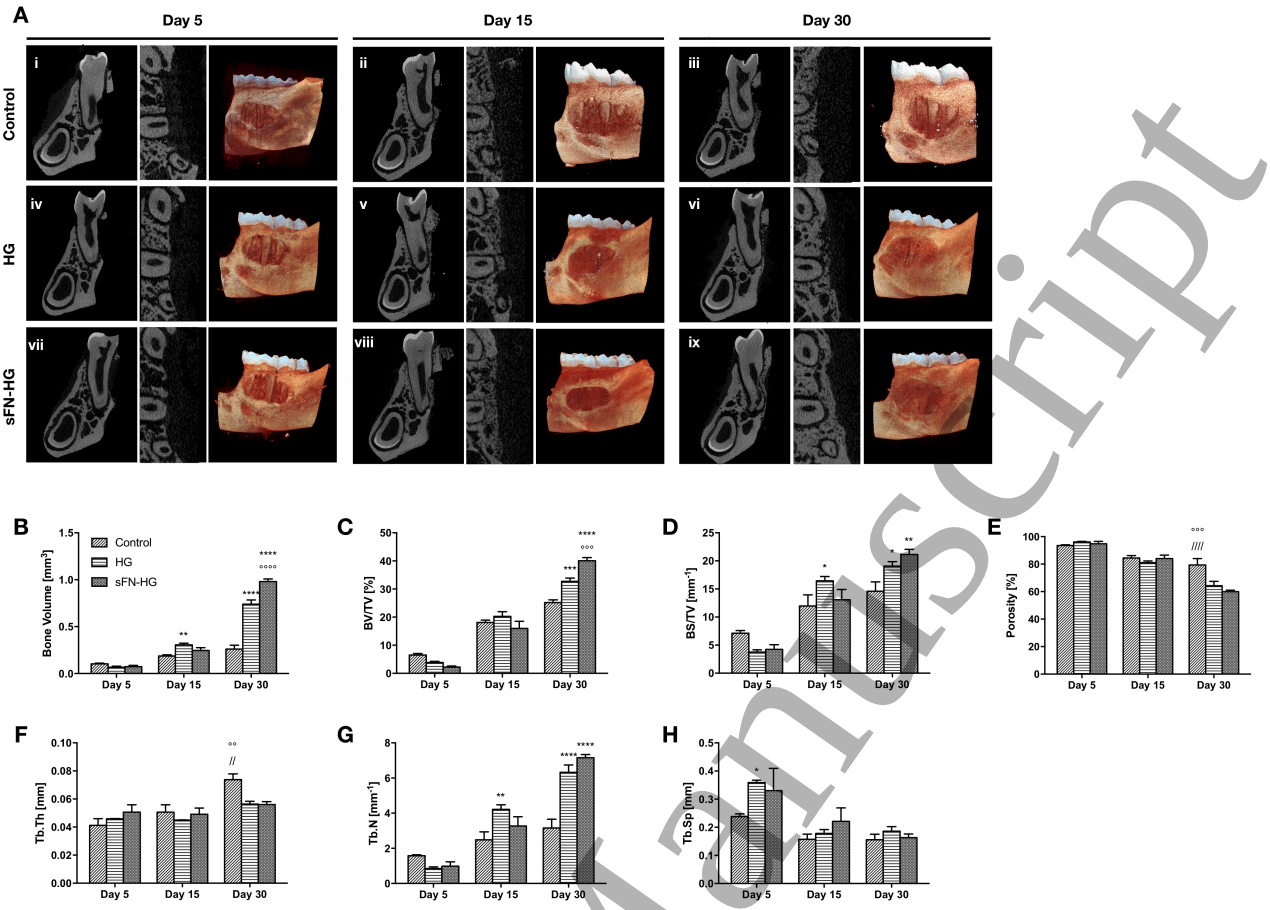
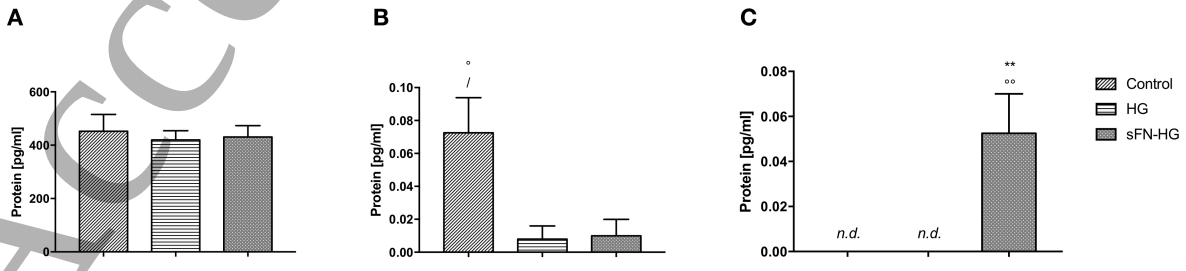


Figure 3: Micro-computerized tomography (µCT) results of HG and of sFN-HG in the regeneration of fenestration periodontal defects 5, 15 and 30 days postoperatively. (A) µCT images in 2D longitudinal and coronal axis and 3D reconstructed images in 5, 15 and 30 days of spontaneous healing (i, ii, iii), HG (iv, v, vi) and sFN-HG (vii, viii, ix) groups. (B) Histograms reporting total bone volume (BV). (C) Histograms reporting the ratio between bone volume and total tissue volume (BV/TV). (D) Histograms reporting the ratio between bone surface and total tissue volume (BS/TV). (E) Histograms reporting bone total porosity. (F) Histograms reporting trabecular thickness (Tb.Th), namely the mean thickness of bone trabeculae. (G) Histograms reporting trabecular number (Tb.N), namely the average number of bone trabeculae. (H) Histograms reporting trabecular separation (Tb.Sp), namely the mean distance between bone trabeculae. After 30 days of healing the presence of aptamers significantly affected BV, BV/TV and porosity parameter. *=p<0.05 vs. Spontaneous healing. °=p<0.05 vs. HG. /=p<0.05 vs. sFN-HG.

3.4 Aptamers enhance mature bone phenotype – To assess the phenotype of cells involved in graft consolidation an immunoenzymatic assay was performed on the last time point (day30).



Accepted Manuscript

Figure 4: Cell phenotype investigation. (A) Histograms reporting osteocalcin (Ocn) levels after 30 days of healing. (B) Histograms reporting osteoprotegerin (Opg) levels after 30 days of healing. (C) Histograms reporting osteopontin (Opn) levels after 30 days of healing. *=p<0.05.

Ocn, which is a marker of mature bone-deposing osteoblasts, was highly expressed among all the groups and no significant differences were detected after 30 days (**Fig.4A**), suggesting that new-bone deposition was still ongoing for each experimental group. However, the analysis of Opn and Opg expression levels revealed a different stage of osteoblastic lineage commitment among the various experimental groups. In the control group (spontaneous healing), a strong expression of Opg was detected (**Fig.4B**) with significant differences both against HG (p=0.0148) and sFN-HG groups (p=0.0178). These data highlight an earlier commitment of osteoblastic population in the control group. On the other hand, the expression of Opn, which was detected only in the sFN-HG group (**Fig.4C** - Spontaneous Healing vs. sFN-HG p=0.0015; HG vs. sFN-HG p=0.0020), indicates a phenotype close to mature bone in the presence of aptamers.

4. Discussion

Blood clot formation has been recognized to be critical for proper tissue regeneration. Its maturation is strictly dictated by blood plasma proteins adsorbed at interface, which establish a pivotal biological talk among the material and the surrounding milieu[10]. However, protein adsorption, which is the first event occurring after material implantation, is mostly an unspecific and random phenomenon that affects platelet adhesion and activation, thus rendering coagulation largely unpredictable[16, 27]. Therefore, the precise control of blood protein adsorption at the interface may represent a viable approach to tailor the success of alloplastic material for tissue regeneration[9, 28].

In our previous studies, we have repeatedly described the capacity of aptamers to control plasma protein adsorption on material surface both quantitatively and qualitatively[12-14, 29]. As such, the aim of the present study has been to investigate if the control of protein adsorption by blood stream at the interface of biomaterials by means of aptamers could promote platelets response and thus new-bone deposition in a periodontal defect *in vivo*.

On an *in vitro* level, when the HG was enriched with aptamers, significantly more cells were found to be attached and organized in clusters after 24h (**Fig.1**). Furthermore, when HG or sFN-HG were incubated with the medium rich in platelets obtained from whole blood, an increasing release of ATP was observed over the time in the presence of aptamers (**Fig.2**). Since FN is one of the main components of this supernatant, as well as it is involved in clot maturation[30], we can assume that aptamers, by promoting FN adsorption, are responsible for a higher platelet aggregation and activation on sFN-HG.

In vivo experiments revealed that bone formation started from the residual walls of the defect (**Fig.3A**) and equally proceeded for all the groups for up to 15 days of healing. However, quantitatively more new-formed

Accepted Manuscript

bone was detected in the defect area 30 days after the surgery when the HG or the sFN-HG were applied (**Fig.3B-E**). Since the periodontal fenestration defect is not a critical defect, these results indicate a key role of the scaffolding material as a temporary matrix to provide support for faster osteoblasts ingrowth, proliferation and commitment. Intriguingly, the presence of aptamers (sFN-HG) further promotes the amount of total bone volume filling in the original defect area after 30 days (**Fig.3B-C**). On the other hand, no differences were determined by the presence of aptamers when morphometric parameters were evaluated (**Fig.3F-H**).

The results of the µCT evaluation were consistent with the gene expression of putative markers in the defect area after 30 days (**Fig.4**). While Ocn (**Fig.4A**) was robustly expressed in all the experimental groups, indicating the presence of active mature bone deposing osteoblastic cells, Opg and Opn levels correlate with the amount of new-formed bone detected. In particular, low Opg secretion (**Fig.4B**) was observed for both the HG and the sFN-HG group. Opg is known to be an antagonist of RANKL in osteoclasts activation, thus suggesting an early activation of bone remodeling when the matrix was applied. Additionally, the expression of Opn (**Fig.4C**) was detected only for the aptamer group (sFN-HG). This observation leads us to speculate that a more mature bone phenotype, richer in osteocytes, was promoted by the presence of aptamers.

Therefore, our model used in this study is recommended for the investigation of the early healing processes that occur after blood coagulation as a consequence of a material or device implantation. These effects are appreciable from 2 to 6 weeks after material implantation. Furthermore, they benefit the extraoral approach of the surgery, which leads to the isolation of the defect from the oral cavity, thus preventing any negative effect such as contamination or infection by saliva and resident oral flora[31]. Based on these observations and our own results, we can speculate that the increment in new-formed bone and in the expression of a more mature phenotype in the sFN-HG group is related to the use of aptamers. A major explantation could be connected to the capacity of aptamers to promote fast platelet aggregation and activation during blood coagulation, thus favoring the proliferation and the migration of mesenchymal progenitors from the host bone.

To the best of our knowledge, this is the first work in the literature that aims to test the *in vivo* effects of a surface modification capable to selectively control the adsorption of specific autologous cues on the surface of alloplastic materials, thus improving their interaction with the biological surrounding milieu. A similar approach proposed by Bartold and co-workers[32-33] investigated the regenerative potential of a polycaprolactone (PCL) scaffolds coated with a decellularized periodontal ligament cell (PDL) extracellular matrix (ECM). However, even if the use of decellularized constructs possesses a lot of clinical potential harnessing the potentially negative immunogenic effects of the cellular material and conferring bioactivity to the scaffold[32], no significative effects in new bone formation were detected among PCL and ecmPDL-PCL after 28 days in this study[33].

Accepted Manuscript

It has to be stated, that one of the major limitations of this study is the lack of histological evaluations, which would provide insights into the possibility of using aptamers to promote true periodontal regeneration. Therefore, histological and histomorphometric analysis will be the object of future studies.

A limitation of the vehicle could be also raised, since the mechanical characteristics of the HG used are far from that of bone tissue, this material could be considered as non-ideal for this specific application. However, this matrix has been chosen for different reasons. First of all, the HA/PEGDA hydrogel is a 3D matrix thought for the culture of stem cells, which thus offers scant adhesion sites for cells making more appreciable the aptamer effect. Secondly, this hydrogel possesses functional groups easily accessible and thus prone to bind secondary molecules. Third, since the hydrogel possesses mechanical properties no close to that of native bone, any positive effects could be solely reconducted to the use of aptamer.

All considered, the findings derived from the present study provide evidence on the possibility of exploiting aptamers as a novel modification to promote blood coagulation and thus new bone formation.

To conclude, the selective control of FN adsorption at material interface by means of aptamers increased the capacity of platelets to be activated and promoted bone new deposition and maturation in a periodontal fenestration defect in rat. Future deep investigations are needed to clarify the molecular mechanism behind aptamer-improved healing.

Acknowledgements

The authors would like to thank Luiz Gustavo de Souza and Paulo Batista de Vasconcelos (School of Dentistry of Ribeirão Preto) for their assistance in animal surgeries and Adriana Luisa Gonçalves Almeida (School of Dentistry of Ribeirão Preto) for her assistance in µCT analysis.

The author would also like to thank Dr. Martin Degen (Department of Orthodontics and Dentofacial Orthopedics, University of Bern) for critical reading and editing of the manuscript.

This study has been supported by a 2013 grant by the American Academy of Implant Dentistry Foundation and 2014 grant by Sao Paulo Research Foundation (FAPESP No. 2014/03516-0). Also, the authors acknowledge the Osteology Foundation for the grant that supported Ludovica Parisi and the Coordination of the Improvement of Graduated Personnel (CAPES) for the scholarship that supported Camila A. Costa.

The authors declare they have no conflict of interests related to this study.

Reference

1. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005;366:1809-1820.
2. Janner SFM, Bosshardt DD, Cochran DL, et al. The influence of collagen membrane and autogenous bone chips on bone augmentation in the anterior maxilla: a preclinical study. *Clinical Oral Implants Research* 2017;28:1368-1380.
3. Lumetti S, Galli C, Manfredi E, et al. Correlation between Density and Resorption of Fresh-Frozen and Autogenous Bone Grafts. *Biomed Research International* 2014.
4. Chaushu L, Chaushu G, Kolerman R, Vered M, Naishlos S, Nissan J. Anterior atrophic mandible restoration using cancellous bone block allograft. *Clin Implant Dent Relat Res* 2019.

Accepted Manuscript

5. Rivara F, Negri M, Lumetti S, et al. Maxillary Sinus Floor Augmentation Using an Equine-Derived Graft Material: Preliminary Results in 17 Patients. *Biomed Research International* 2017.
6. Ivanovic A, Bosshardt DD, Mihatovic I, Schwarz F, Gruber R, Sculean A. Effect of pulverized natural bone mineral on regeneration of three-wall intrabony defects. A preclinical study. *Clinical Oral Investigations* 2014;18:1319-1328.
7. Tomford WW. TRANSMISSION OF DISEASE THROUGH TRANSPLANTATION OF MUSCULOSKELETAL ALLOGRAFTS. *Journal of Bone and Joint Surgery-American Volume* 1995;77A:1742-1754.
8. Kunert-Keil C, Scholz F, Gedrange T, Gredes T. Comparative study of biphasic calcium phosphate with beta-tricalcium phosphate in rat cranial defects-A molecular-biological and histological study. *Annals of Anatomy-Anatomischer Anzeiger* 2015;199:79-84.
9. Parisi L, Toffoli A, Ghiacci G, Macaluso G. Tailoring the Interface of Biomaterials to Design Effective Scaffolds. *Journal of Functional Biomaterials* 2018;9:E50.
10. Shiu HT, Goss B, Lutton C, Crawford R, Xiao Y. Formation of Blood Clot on Biomaterial Implants Influences Bone Healing. *Tissue Engineering Part B-Reviews* 2014;20:697-712.
11. Andrade J, Hlady V. Protein adsorption and materials biocompatibility - a tutorial review and suggested hypotheses. *Advances in Polymer Science* 1986;79:1-63.
12. Galli C, Parisi L, Piergianni M, et al. Improved scaffold biocompatibility through anti-Fibronectin aptamer functionalization. *Acta Biomaterialia* 2016;42:147-156.
13. Parisi L, Galli C, Bianchera A, et al. Anti-fibronectin aptamers improve the colonization of chitosan films modified with D-(+) Raffinose by murine osteoblastic cells. *Journal of Materials Science-Materials in Medicine* 2017;28.
14. Parisi L, Toffoli A, Bianchi MG, et al. Functional Fibronectin Adsorption on Aptamer-Doped Chitosan Modulates Cell Morphology by Integrin-Mediated Pathway. *Materials (Basel)* 2019;12.
15. Mascini M, Palchetti I, Tombelli S. Nucleic Acid and Peptide Aptamers: Fundamentals and Bioanalytical Aspects. *Angewandte Chemie-International Edition* 2012;51:1316-1332.
16. Morris AH, Kyriakides TR. Matricellular proteins and biomaterials. *Matrix Biology* 2014;37:183-191.
17. Pankov R, Yamada KM. Fibronectin at a glance. *Journal of Cell Science* 2002;115:3861-3863.
18. Barker TH, Engler AJ. The provisional matrix: setting the stage for tissue repair outcomes. *Matrix Biology* 2017;60-61:1-4.
19. Karp JM, Sarraf F, Shoichet MS, Davies JE. Fibrin-filled scaffolds for bone-tissue engineering: An in vivo study. *Journal of Biomedical Materials Research Part A* 2004;71A:162-171.
20. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010;8:e1000412.
21. King GN, King N, Cruchley AT, Wozney JM, Hughes FJ. Recombinant human bone morphogenetic protein-2 promotes wound healing in rat periodontal fenestration defects. *Journal of Dental Research* 1997;76:1460-1470.
22. de Oliveira P, Silveira E Souza A, Novaes AJ, et al. Adjunctive effect of antimicrobial photodynamic therapy in induced periodontal disease. Animal study with histomorphometrical, immunohistochemical, and cytokine evaluation. *Lasers Med Sci* 2016;31:1275-1283.
23. Benatti BB, Neto JBC, Casati MZ, Sallum EA, Sallum AW, Nociti FH. Periodontal healing may be affected by aging: a histologic study in rats. *Journal of Periodontal Research* 2006;41:329-333.
24. Correa MG, Campos MLG, Marques MR, Casati MZ, Nociti FH, Sallum EA. Histometric Analysis of the Effect of Enamel Matrix Derivative on the Healing of Periodontal Defects in Rats With Diabetes. *Journal of Periodontology* 2013;84:1309-1318.
25. Correa MG, Campos MLG, Marques MR, et al. Outcome of Enamel Matrix Derivative Treatment in the Presence of Chronic Stress: Histometric Study in Rats. *Journal of Periodontology* 2014;85:E259-E267.
26. Rodrigues TL, Nagatomo KJ, Foster BL, Nociti FH, Somerman MJ. Modulation of Phosphate/Pyrophosphate Metabolism to Regenerate the Periodontium: A Novel In Vivo Approach. *Journal of Periodontology* 2011;82:1757-1766.
27. Clause KC, Barker TH. Extracellular matrix signaling in morphogenesis and repair. *Current Opinion in Biotechnology* 2013;24:830-833.
28. Parisi L, Toffoli A, Mozzoni B, et al. Is selective protein adsorption on biomaterials a viable option to promote periodontal regeneration? *Medical Hypotheses* 2019;132.
29. Saccani M, Parisi L, Bergonzi C, et al. Surface modification of chitosan films with a fibronectin fragment-DNA aptamer complex to enhance osteoblastic cell activity: a mass spectrometry approach probing evidence on protein behavior. *Rapid Communications in Mass Spectrometry* 2019;33:336-342.
30. Wang YM, Carrim N, Ni HY. Fibronectin orchestrates thrombosis and hemostasis. *Oncotarget* 2015;6:19350-19351.
31. Rios F, Giannobile W. Preclinical protocols for periodontal regeneration. In: Quintessence, ed. *Osteology guidelines for oral and maxillofacial regeneration - preclinical models for translational research*: Quintessence, 2011:77-102.
32. Farag A, Vaquette C, Theodoropoulos C, Hamlet SM, Hutmacher DW, Ivanovski S. Decellularized Periodontal Ligament Cell Sheets with Recellularization Potential. *Journal of Dental Research* 2014;93:1313-1319.
33. Farag A, Hashimi SM, Vaquette C, Bartold PM, Hutmacher DW, Ivanovski S. The effect of decellularized tissue engineered constructs on periodontal regeneration. *Journal of Clinical Periodontology* 2018;45:586-596.

Accepted Manuscript