Preclinical histological evaluation of a novel human recombinant amelogenin in the regenerative treatment of periodontal defects

Summary of the PhD thesis

by

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PUBLICATIONS PROVIDING THE BASIS OF THE THESIS

1. **Chackartchi T**, Bosshardt DD, Imber JC, Stahli A, Sacks H, Nagy K, et al. Histological evaluation following treatment of recession-type defects with coronally advanced flap and a novel human recombinant amelogenin. *Clin Oral Investig* 2023;27(9):5041-8.

SJR rank: Q1/D1 (Dentistry, miscellaneous)

2. **Chackartchi T**, Imber JC, Stahli A, Bosshardt D, Sacks H, Nagy K, et al. Healing of intrabony defects using a novel human recombinant amelogenin: a preclinical study. *Quintessence Int* 2023;54(8):622-8.

SJR rank: Q2 (Dentistry miscellaneous)

ABBREVIATIONS

aJE	apical termination of the junctional epithelium
aN	apical notch
BC	bone crest
CAF	coronally advanced flap
cC	coronal termination of new cementum
CEJ	cemento-enamel junction
CTG	connective tissue graft
EMD	enamel matrix derivative
GM	gingival margin
hNB	highest point of new bone
hNC	highest point of new cementum
IM	intramuscular
IV	intravenous
JE	junctional epithelium
PM	premolar tooth
SC	subcutaneous

I. INTRODUCTION

A century after the first attempt to use bone graft for this purpose, regeneration of periodontal soft and hard tissues is still a clinical and scientific challenge generating intensive research. The aim of the studies covered in this thesis was to histologically assess the regenerative potential of a new human recombinant amelogenin in the context of gingival recession and intrabony defects.

Gingival recession is defined by the apical displacement of the gingival margin from the cementoenamel junction (CEJ). This condition may manifest as either localized or generalized recession and can be linked to one or multiple surfaces. The displacement of the gingival margin towards the root leads to root exposure in the oral cavity, potentially causing aesthetic concerns, root sensitivity, or even root caries. The goal of treatment is to cover the recession defect. Over recent decades, various surgical techniques and biomaterials have been proposed to reliably address gingival recessions. Systematic reviews evaluating the effectiveness of these surgical methods have indicated that when enamel matrix derivative (EMD) is applied to root surfaces in conjunction with a coronally advanced flap (CAF), it significantly enhances the likelihood of achieving complete root coverage. EMD, a xenogeneic biomaterial derived from the enamel matrix of developing pig teeth, has exhibited favorable outcomes for over two decades and is presently acknowledged as one of the few biomaterials in clinical usage capable of inducing actual periodontal regeneration, as verified histologically.

Periodontal disease can give rise to intrabony defects around teeth. An intrabony defect is defined as a periodontal defect within the bone surrounded by one, two or three bony walls or a combination thereof. Currently, EMD and guided tissue regeneration employing membranes are recognized as the primary treatment modality for deep (≥3 mm) intrabony defects.

EMD is a well-documented material, derived from the enamel matrix of developing pig teeth. This presents evident limitations concerning production quantity and variability in amelogenin content. Additionally, there have been reports of the host producing anti-EMD antibodies. To address these issues, extensive efforts have been dedicated to generating amelogenin through recombinant technology. Studies involving recombinant human amelogenin protein demonstrated its ability to stimulate the regeneration of tissues supporting the tooth following the induction of experimental periodontitis in a canine model. Recently, a novel human recombinant amelogenin (rAmelX) has been synthesized and proposed for use in periodontal regeneration. This thesis describes preclinical histological studies conducted with this material.

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II. OBJECTIVES AND HYPOPTHESES

This thesis summarizes two preclinical studies, both of which involved the histological assessment of rAmelX in a minipig model.

In the first study[1], we aimed to histologically assess how rAmelX impacts periodontal wound healing and regeneration within recession-type defects. These defects were surgically created in the maxilla of three minipigs. The defects were then randomly assigned for treatment with either CAF combined with Recombigain (test group), or CAF combined with Poloxamer 407 only (control group). After three months post-surgery, the animals were euthanized, and histological evaluation was performed.

In the second study[2], our objective was to histologically assess how rAmelX influences periodontal wound healing and regeneration within intrabony defects. These defects were surgically created in the mandible of three minipigs. Twelve defects were randomly allocated for treatment, receiving either Recombigain (test group) or solely Poloxamer 407 (control group). After three months post-surgery, the animals were euthanized, and histological evaluation was performed.

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[1] Chackartchi T, Bosshardt DD, Imber JC, Stahli A, Sacks H, Nagy K, et al. Histological evaluation following treatment of recession-type defects with coronally advanced flap and a novel human recombinant amelogenin. *Clin Oral Investig* 2023;27(9):5041-8.

[2] Chackartchi T, Imber JC, Stahli A, Bosshardt D, Sacks H, Nagy K, et al. Healing of intrabony defects using a novel human recombinant amelogenin: a preclinical study. *Quintessence Int* 2023;54(8):622-8.

In both studies, we hypothesized that rAmelX in the form of Recombigain would be safe to use in the animal model, that is, no adverse reactions to the material would be observable either clinically or in the histological sections. We hypothesized that the application of this novel human recombinant amelogenin would result in the regeneration of the defects, and that at least some histological/histometric parameters in the test groups would be superior to the same parameters of the control groups.

The studies are presented in the above order.

III. METHODS

III.1. The applied material

In our studies presented in this thesis, rAmelX was used in the form of Recombigain, a thermo-sensitive gel-based composition, manufactured by the Israel-based company, Prudentix Ltd., at an authorized, GMP and ISO 13485 certified manufacturing site. Recombigain is delivered in a sterile syringe filled with a gel composed of the active ingredient, rAmelx, a carrier (Poloxamer 407), acetic acid, sodium acetate trihydrate, trehalose dihydrate, glycine, L-methionine, and water. The composition contains no other enamel matrix components.

The active ingredient in Recombigain is rAmelX. This protein is part of a group of endogenous proteins - hydrophobic enamel matrix proteins - , which exist naturally in the body. It is expressed in human cells of various mesenchymal, neural, and other tissues, in developing embryos, neonates and adults. The rAmelX protein itself in this composition is manufactured in an *E.coli* expression system. The recombinant protein is highly pure as opposed to swine extractions.
Poloxamer 407 is a copolymer consisting of a central hydrophobic block of polypropylene glycol flanked by two hydrophilic blocks of polyethylene glycol.

III.2. Animals and general surgical procedures

Three healthy inbred Sinclair miniature pigs were used for the purposes of each study (N=6 altogether). All animals were 18 months old and weighed between 55 to 60 kg. The animals were housed at the Institute of Animal Research in Kibbutz Lahav, Negev, Israel. They were continuously monitored throughout the study duration and were kept under the following standardized environmental conditions: 20°C to 26°C temperature, 30% to 70% relative humidity, and a light/dark cycle of 12 hours. The pigs received a daily diet of standard soft food soaked in water (AMBAR, Feed mill, Granot M.P. Heffer 3881100, Israel), and water was available ad libitum. All procedures were approved by the ethical committee of the Institute of Animal Research (No. IL-20-3-93). The study was conducted following the ARRIVE guidelines for preclinical animal studies and complied with the provisions of the Israeli National Council of Animal Experimentation.

All surgical procedures followed aseptic routines and were conducted by a single experienced surgeon under general and local anesthesia. Postoperative pain management included Buprenorphine (0.05 mg/kg, IM) for seven days and Carprofen (2-4 mg/kg, IM) for the same duration. Antibiotics (Amoxiclav, 1 ml/20 kg, SC) were administered for seven days, once a day. The pre-and postoperative procedures are described in detail in the thesis.

III.4. Creation and treatment of the recession-type defects

Vertical releasing incisions were made on the mesio-buccal aspect of the second premolar (PM 2) and on the disto-buccal aspect of PM 4. Elevating a muco-periosteal full-thickness flap exposed the buccal alveolar bone. Dehiscence defects were created on PM 2 and PM 4, except in cases where accessing PM 4 posed difficulties; then, lesions were created on PM 2 and PM 3. The defects measured 5 mm in depth from the cemento-enamel junction and 3 mm in width. Root surfaces underwent thorough scaling and planing using hand instruments (Gracey curettes) to eliminate the root cementum and periodontal ligament completely. At the bottom of the defect, a notch was prepared on the root surface using a small diamond round bur, while irrigating with sterile saline as a reference for measurements. The maxillary recession defects were randomly allocated to the test group or the control group. Defects in the test group were treated with CAF and rAmelX (Recombigain). Defects in the control group were treated with CAF and poloxamer, the carrier material component of Recombigain. Finally, the flaps were sutured and stabilized using marginal direct sutures to adapt the flap to the neck of the teeth at the interdental papillae, with monofilament 4-0 non-resorbable nylon sutures.

III.5. Creation and treatment of the intrabony defects

The first and third premolars of both mandibular quadrants were extracted, and the extraction sites were left to heal for 4 weeks. After this healing interval, a second surgical procedure was conducted. Bilateral two-wall intrabony defects were meticulously prepared, measuring approximately 4 mm in width and 6 mm in depth. These defects were positioned at the mesial aspect of the mandibular fourth premolars and the mesial aspect of the mandibular second premolars, totaling four defects per animal. Following the elevation of a mucoperiosteal flap, the defects were meticulously created using fissure burs alongside a sterile saline coolant and chisels. Root cementum removal was performed utilizing Gracey curettes and a chisel.

Reference notches were prepared on the root surface at the base of the defects, serving as reference points for the histometric measurements. The 12 bilateral mandibular two-wall intrabony defects randomly assigned to the same treatments as described for the recession-type defects. After the application of the test or control material, the soft tissues were positioned at the pre-surgical level and the wound was closed tension-free by means of nylon (5-0) sutures. After a healing period of 2 weeks, the sutures were removed.

III.6. Histology and histometry

III.6.1. Recession-type defects

Three months post-surgery, the animals were euthanized using Isoflurane inhalation and sedated with Pental (8 ml, IV) and KCl (20 ml, IV) for sacrifice. The maxillae were extracted, and individual bone blocks comprising the implanted biomaterials and surrounding soft and hard tissues were excised, then fixed with 10% neutral-buffered formalin.

Following fixation, the specimens underwent rinsing in tap water, dehydration in increasing ethanol concentrations, infiltration, and embedding in methylmethacrylate. After polymerization, the blocks were trimmed and sectioned along the root axis of each tooth involved, following a bucco-palatal plane. Utilizing a high-precision, slow-speed diamond disk with a coolant, ground sections were created. These sections were mounted onto acrylic glass slides and ground to a final thickness of 100 μm via a custom grinding machine before being superficially stained with toluidine blue/McNeal combined with basic fuchsin. Descriptive and histometric analyses were conducted on the two central-most ground sections per defect, with high-resolution photography performed using a digital camera. Central sections with clearly distinguishable apical and coronal notches, containing the central location within the defect area were chosen for the analysis. Regions of

interest were digitalized with a computer connected to a light microscope.
The following histological landmarks necessary for histometric measurements were identified by two experienced investigators: gingival margin (GM), cemento-enamel junction (CEJ), apical termination of the junctional epithelium (aJE), apical notch (aN), highest (most coronal) point of new cementum (hNC), highest point of new bone (hNB) After identification, the following histometric measurements were performed by a single examiner, blinded to the group allocation:

- defect height: aN - CEJ
- height of JE including sulcus depth: aJE - GM
- connective tissue adhesion (CT): hNC - aJE
- height of new bone: aN - hNB
- height of new cementum: aN – hNC

For the comparative evaluation between the two groups, two slices per defect were analyzed. Between-group differences were assessed utilizing the non-parametric Wilcoxon signed-rank test. Difference was considered statistically significant if $p<0.05$.

Descriptive evaluation included mean and standard deviation, median, minimum and maximum values, along with percentile ranks.

III.6.2. Intrabony defects

Three months after the second surgery (see III.5.), the animals were anesthetized via Isoflurane inhalation and then euthanized using Pental (8 ml, IV) and KCl (20 ml, IV). The mandibles were extracted, and individual bone blocks comprising the implanted biomaterials and adjacent soft and hard tissues were procured, followed by fixation using 10% neutral-buffered formaldehyde.

Post-fixation, the specimens underwent a series of procedures: rinsing in water, dehydration in increasing concentrations of ethanol, infiltration, and embedding in methylmethacrylate. Upon polymerization, the embedded blocks were sectioned along their longitudinal axis into 500 μm-thick mesio-distal ground sections using a high-precision, slow-speed diamond disk with coolant. Following mounting onto acrylic glass slides, the sections were ground down to a final thickness of 100 μm using a custom grinding machine and superficially stained with toluidine blue/McNeal combined with basic fuchsin. High-resolution imaging was conducted using a digital camera linked to a light microscope.

The two most central sections (with clearly distinguishable apical and coronal notches) per defect were chosen for histometric analysis. The same histological landmarks were used as in the case of the recession-type defects. These specific landmarks were identified with the consensus of two investigators. Thereafter, the following

vertical measurements were performed by a single examiner, blinded to the group allocation:

- defect height: aN - CEJ
- height of new cementum: aN - C
- height of new bone: aN - BC
- height of JE including sulcus depth: aJE - GM
- height of connective tissue adhesion: aJE

The defect was selected as the statistical unit for analysis. Averages of two measurements per defect were computed and means along with standard deviations (SDs) for each parameter were calculated across the two treatment groups. Intergroup comparisons were conducted using the Mann-Whitney U test. Difference was considered statistically significant if $p<0.05$.

IV. RESULTS

IV.1. Recession-type defects

The postoperative recovery proceeded without any issues at all surgical sites. There were no indications of compromised systemic health, that is, the behavior of the animals was normal, including their food and water intake. Likewise, no local abnormal occurrences, such as suppuration, abscess formation, or increased tooth mobility were observed at any time during the experimental period. In one of the animals, on one side, only two defects could be generated due to a shallow vestibulum that restricted access, while three defects could be created on the other hemi-maxillas. As a result, a total of 17 defects were treated, comprising 9 in the test group and 8 in the control group.

The test group and the control group exhibited diverse degrees of periodontal regeneration, encompassing the restoration of root cementum, periodontal ligament, and alveolar bone. In the control group, the zone of connective tissue adjacent to the root surface lacking cementum formation appeared wider compared to the test group. The orientation of periodontal ligament fibers varied across locations, with some areas already displaying functional alignment and others not. The newly formed bone in most specimens was predominantly immature, characterized mainly by woven bone.

aN - CEJ measured 4.84 mm in the control group and 5.48 mm in the test group (p=0.219). Statistically, aN-C was significantly higher in the test group (4.38 mm) compared to the control group (3.48 mm, p= 0.047). Conversely, aN - hNB did not exhibit a statistically significant difference when comparing the test (2.15 mm) and the control group (2.24 mm, p=0.938).
aJE-GM was greater in the control group (2.65 mm) compared to the test group (2.24 mm), although this disparity did not reach statistical significance (p=0.297).

IV.2. Intrabony defects

Postoperative clinical healing proceeded without any issues for all animals. At the defect level, there were no observable adverse reactions, such as suppuration, abscess formation, or increased tooth mobility, throughout the entire experiment. Similarly, no signs of compromised systemic health were observed.

All 12 defects were included in the descriptive analysis, with two central sections evaluated for each defect. No pronounced or unusual inflammatory signs were observed in either the test or control group.

New bone and cementum formation were evident in both groups, with more favorable outcomes observed in the test group compared to the control group. The region of connective tissue adjacent to the root surface lacking cementum formation showed similar characteristics in both the test and control groups. The orientation of periodontal ligament fibers varied, either aligned and

directly inserting into the new root cementum or not yet oriented, with no discernible differences between the groups. In both groups, new bone formation extended from the host bone toward the coronal region of the defects.

Histomorphometric analysis was conducted on two sections per defect, resulting in the examination of 24 sections for all 12 defects.

The mean aN-CEJ measured 6.205 ± 0.389 mm in the test group and 5.823 ± 1.019 mm in the control group, with no statistically significant differences between the groups (p=0.309). aN-C was found to be higher in defects treated with rAmelX (4.812 ± 1.167 mm) compared to defects treated with the carrier (4.390 ± 1.708 mm, p=0.937). Similarly, aN - BC reached greater values in the test group (3.508 ± 1.076 mm) compared to the control group (2.965 ± 1.127 mm, p=0.309).

aJE - GM was 2.752 ± 0.648 mm in the test group and 2.323 ± 0.755 mm in the control group, although this difference did not reach statistical significance (p=0.179). aJE was small in both the test group (0.103 ± 0.123 mm) and the control group (0.188 ± 0.133 mm, p=0.225).

V. CONCLUSIONS

This thesis covered studies in which we sought to gain preclinical/histological knowledge about a novel biomaterial intended to treat destructive conditions of the periodontal tissues. The key findings of our studies may be summarized as follows:

- The material was safely applicable in both studied indications; no adverse effects were observed either clinically or histologically.

- In both indications, signs of regeneration were obvious, showing that the treatment was effective.

- In the recession-type defects, the applied material led to the regeneration of cementum, bone, and periodontal ligament. Of these, the regeneration of cementum reached a significant degree.

- In the acute type two-wall intrabony defects, improved formation of root cementum and bone was observed, but the differences from the control group did not reach the level of statistical significance.

- The results indicate that the material is capable of inducing periodontal regeneration, which is histologically evident. However, the results also raise the possibility that the gel carrier itself might contribute to the beneficial effects, which needs to be further explored.

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