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SHORT COMMUNICATION

Evaluation of Emdogain® antimicrobial effectiveness against biofilms containing the keystone pathogen *Porphyromonas gingivalis*

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SUMMARY

This study aimed to evaluate the antimicrobial activity of Emdogain® (EMD) against biofilms containing the periopathogen *Porphyromonas gingivalis*. A brain-Heart infusion broth inoculated with *S. gordonii* and *P. gingivalis* was perfused (7-d, anaerobiosis) through a closed circuit containing two Robbins devices as to form biofilms. The latter were then treated for 2 min with various antimicrobials (Chlorhexidine (CHX) 0.2%, Povidone iodine (PVI) 5%, PVI 10%, essential oils (EO), EO Zero™ or EMD) (n=8) and cell densities were calculated and compared. In the present *in vitro* model, Emdogain® was not statistically effective ($p>0.05$) in killing biofilm bacteria unlike the other tested molecules.

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Periodontitis and peri-implantitis are biofilm-induced diseases leading to the progressive destruction of the teeth/implant surrounding tissues (Darveau, 2010; Berglundh *et al*., 2011). The physiopathogenesis of these conditions involves the disruption of the homeostasis that normally occurs between the oral biofilm and the host (Hajishengallis *et al.,* 2012). The alteration of the normal host/bacteria equilibrium has been associated with the development in the commensal microbiota of specific pathogens, namely keystone pathogens. Even in low quantities these pathogens could influence the metabolic behavior of the whole subgingival community (Hajishengallis *et al.,* 2011) which would then become dysbiotic and virulent (Jiao *et al.,* 2014). Among these, *Porphyromonas gingivalis*, a well-characterized Gram-negative anaerobic bacterium could play a revolt activist role through the manipulation and evasion of the host defense, notably by interaction with the complement system (Darveau *et al.,* 2012; Hajishengallis *et al.,* 2014). This pathogen which usually develops in the subgingival regions could be a target to reach in order to reduce the pathogenicity of the periodontal/peri-implant biofilm (Lamont *et al.,* 1998). Traditionally, periodontal/peri-implant treatment consists in the mechanical dispersion of the subgingival biofilm to dissolve the bacterial communication network and reduce the microbial load. Unfortunately, in some clinical situations, related for instance to the morphology or the depth of the contaminated surface, the clinical outcome is insufficient to control the disease (Adriaens *et al.,* 2004; Toma *et al.,* 2014). Effective antimicrobial molecules could be of interest to complete the mechanical debridement and to achieve a better disinfection of the subgingival pocket. Additionally, if this agent could at the same time promote periodontal or bone regeneration it could potentially improve the prognosis of the diseased tooth or implant. Enamel Matrix Derivative (Emdogain®) is a well-known and documented product that has been shown clinically and histologically to improve periodontal regeneration (Sculean *et al.,* 2001a; 2008; Larsson *et al.*, 2015; Sculean *et al.,* 2015). A plethora of cellular and molecular mechanisms have been attributed *in vitro* to Enamel Matrix Proteins (EMP) and explain, in part, the biological effects encountered in periodontal regeneration (Bosshardt, 2008). This biological mediator in its commercially available form (Emdogain®) have also been shown to exert a certain antibacterial effect *in vitro*. Indeed, Emdogain® was able to reduce the growth of some bacterial species as demonstrated by broth dilution and agar plate diffusion assays (Spahr *et al.,* 2002; Walter *et al.,* 2006). It also presented a bactericidal effect *ex vivo* on 4-day dental plaque biofilms (Sculean *et al.,* 2001b). The following research was thus conducted to investigate the antimicrobial properties of this biological molecule. It addresses the hypothesis that Emdogain® could or could not present an antimicrobial activity against 7-d mature undisturbed biofilms containing the key pathogen *Porphyromonas gingivalis*. The secondary objectives were to compare this potential antimicrobial effect to that obtained with several oral antiseptics and to evaluate if this effect could be influenced by the nature of the surface (hydroxyapatite or titanium).

Dual-species biofilms containing *Streptococcus gordonii* ATCC10558 and *Porphyromonas gingivalis* W83 were grown *in vitro* during a 7-d period in strict anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2). The method used a dynamic model involving two modified Robbins devices (LPMR 12-E; Tyler Research, Edmonton, Alberta, Canada)

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assembled in parallel according to a protocol previously described by Bercy and Lasserre (2007) (*Figure 1A*).
In brief, brain heart infusion (BHI) broths were separately inoculated with each species to achieve a 0.5 McFarland turbidity value for *Streptococcus gordonii* and two McFarland units for *Porphyromonas gingivalis*, respectively. Several other bacterial loads had been previously tested in order to achieve *S. gordonii/P. gingivalis* biofilms with approximately balanced proportions of each bacterial species but the chosen loads were the ones that allowed the most predictable results (data not shown). In parallel, 24 HA (Clarkson chromatography products Inc., South William-sport, PA, USA) and Ti (Southern Implants®, Irene, South Africa) slabs (twelve discs of each type) were soaked during 30 min at 37°C in collected, centrifuged and filtered human saliva to facilitate further early biofilm formation. Thereafter, the 24 discs were attached at the lower surface of the 24 plugs present on the two Robbins devices (twelve discs/plugs per device). The *Streptococcus gordonii* broth was then perfused through the closed circuit via a peristaltic pump (48mL/h) during one hour to initiate biofilm accumulation. It was then replaced by the *Porphyromonas gingivalis* broth which was perfused during seven days.
Once the biofilm formation was achieved, the 24 discs were aseptically removed and independently submitted to one of the following procedures during two minutes:

a) NaCl 0.9%;
b) chlorhexidine (CHX) 0.2% (Corsodyl®, GlaxoSmith-Kline Consumer Healthcare s.a, Wavre, Belgium);
c) povidone iodine (PVI) 5% (Isobetadine® Meda Pharma s.a, Bruxelles, Belgium);
d) PVI 10%;
e) essential oils (EO) (Listerine®, Johnson & Johnson Consumer S.A., Beerse, Belgium);
f) EO Zero™ (Listerine® Zero™);
g) Emdogain® (Institut Straumann AG, Basel, Switzerland).

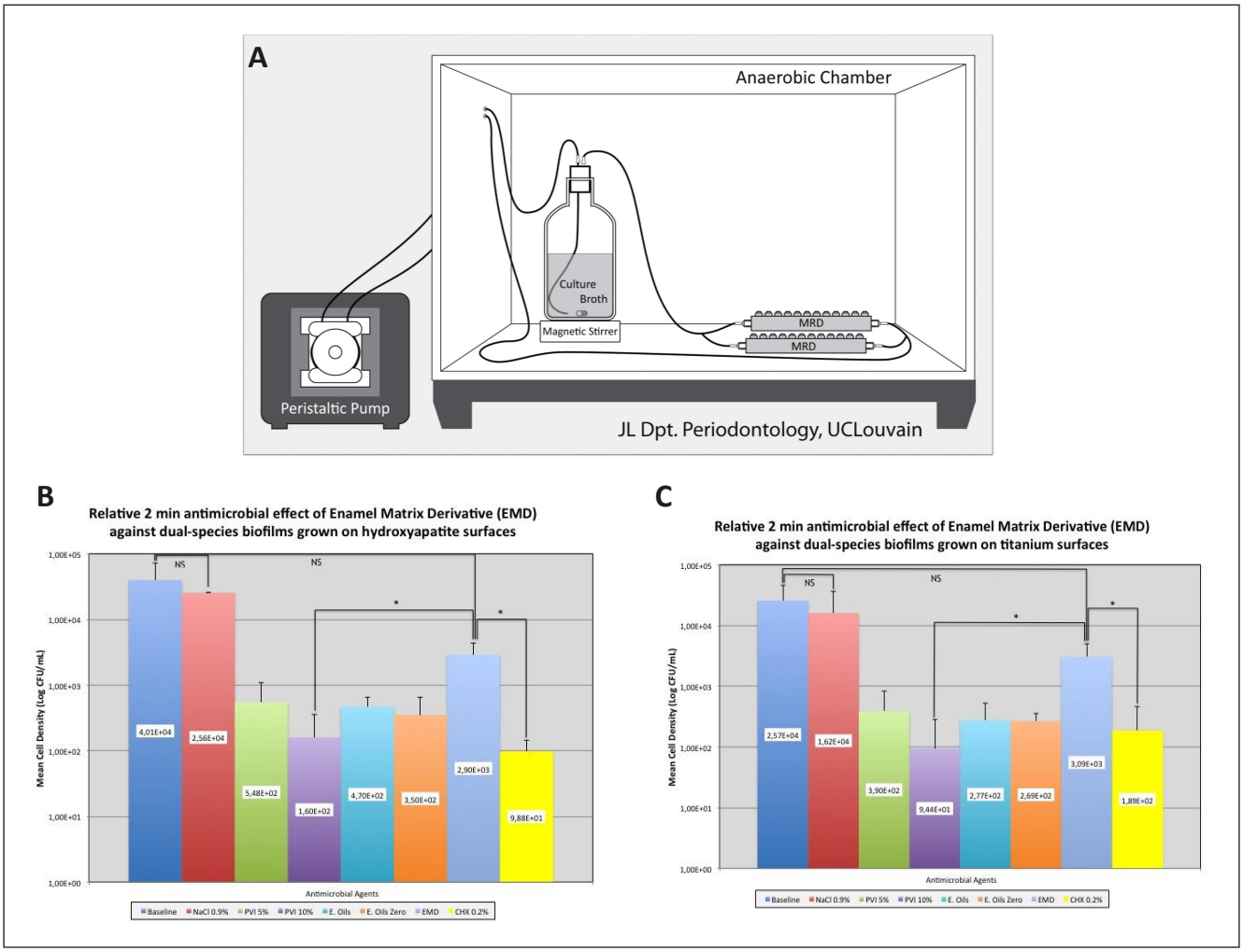

Figure 1 - *A - Diagrammatic representation of the circuit allowing biofilm formation at the bottom of the 12 plugs inserted in each MRD. B: Viability evaluation of dual-species biofilms containing the keystone pathogen Porphyromonas gingivalis after 2-min treatments with EMD or various conventional antiseptics. The columns represent mean values with standard deviations. A Kruskal-Wallis test was performed to compare the observed data between them and to baseline. NS: not significant; *: p<0.05. Biofilms were grown on hydroxyapatite surfaces (5 mm diameter/2 mm width). C: Viability evaluation of dual-species biofilms containing the keystone pathogen Porphyromonas gingivalis after 2-min treatments with EMD or various conventional antiseptics. Results are presented as mean values +/- SD. A Kruskal-Wallis test was used to compare the observed data between them and to baseline. NS: not significant; *: p<0.05. Biofilms were grown on grade 5 machined titanium surfaces (5 mm diameter/2 mm width).*

Non-treated biofilms (one disc per surface: HA and Ti) were used as controls during each experiment.
After treatment, discs were gently rinsed (three times) in PBS and transferred separately in 1mL PBS. They were then vortexed (1min), ultrasonicated (1min) and 100 µL aliquots were cultured anaerobically during ten days on enriched blood agar plates before colony forming unit (CFU) counting using an Acolyte® device. Mean cell densities were calculated in Log_{10} CFU/mL as well as standard deviations (SD). Experiments were repeated to obtain at least eight values per solution and surface.
For each surface and each treatment modality the mean cell densities were compared between them and to untreated controls using a Kruskal-Wallis, Dunn's test. Mann-Whitney tests were also performed to compare the mean cell densities between HA and Ti for each antiseptic molecules.
Results are presented in *Figure 1B* and *1C*. Even though the mean number of CFU was reduced by 1-Log unit after 2-min treatments, the statistical analysis was not able to confirm a significant antimicrobial impact of EMD on bacterial viability. This finding was observed both for hydroxyapatite and titanium surfaces ($p>0.05$). NaCl 0.9% showed no significant effect either from baseline with viability reductions reaching 36% on HA and 37% on Ti ($p>0.05$). On the contrary, all the other molecules were highly significant in killing biofilm bacteria allowing a 2-Log units reduction for both surfaces. CHX 0.2% and PVI 10% displayed the best bactericidal effect with a viability reduction value approaching 2.5-Log units. This represented more than 99% viability decrease compared to baseline ($p<0.001$). Regarding the comparison of the bactericidal effect obtained with the tested antimicrobials, no statistical difference was observed but only between CHX 0.2% or PVI 10% and EMD ($p<0.05$). The antimicrobial effects of the various molecules tended to be similar on the two surfaces considered. This was confirmed when applying a Mann-Whitney test ($p>0.05$).
It has been shown that a subgingival application of Emdogain® was able to reduce gingival inflammation during wound healing after scaling and root planning (Wennstrom *et al.*, 2002). This observation, also reported by some authors after surgical flaps, could be related to a potential antimicrobial effect of EMD (Sculean *et al.*, 2001b). The present study evaluated the bactericidal effectiveness of EMD and other antiseptics on *in vitro* 7-d mature biofilms containing the periodontal pathogen *Porphyromonas gingivalis* W83. A small bactericidal effect was indeed observed for EMD but was not significant at statistical analysis.
Relatively few papers have investigated the antimicrobial effectiveness of Emdogain® gel (Sculean *et al.*, 2001b; Arweiler *et al.*, 2002; Spahr *et al.*, 2002; Newman *et al.*, 2003; Walter *et al.*, 2006). It was first observed by Sculean *et al.* (2001b) who showed that both EMP but especially its vehicle (propylene glycol alginate: PGA) and their mixture (Emdogain® gel) were as efficient as CHX in reducing the viability of 4-d supragingival biofilms in periodontitis patients. In this research, cell viability was analyzed through vital fluorescence microscopy after 2-min treatments with NaCl, CHX 0.2%, EMD, PGA and Emdogain® *ex vivo*. A comparable study was also performed and published one year later by Arweiler *et al.* (2002) with the difference that the antimicrobial treatment was conducted *in vivo*, on 2-d supragingival biofilms and in periodontally healthy subjects. The Emdogain® antibacterial efficacy *in vivo* was less pronounced than that observed by Sculean *et al.* (2001b) and vital fluorescence mean values ranged from 56 to 70% after treatment. These interesting outcomes were nevertheless very significant and it appeared to us of interest to determine if this bactericidal effect could be obtained on biofilms containing key pathogens as is the case in the subgingival plaque. It was also questioned if such endpoints may be found on undisturbed biofilms that had grown anaerobically during a longer 7-d period as to increase biofilm maturation. Indeed, It is currently known that the degree of maturation decreases biofilm susceptibility towards antimicrobial agents (Stojicic *et al.*, 2013). The results obtained in the present study are consistent with this aspect of biofilm maturation since the bactericidal activity observed with Emdogain® was not significant. A moderate antimicrobial activity of this molecule was observed in other works towards bacterial growth which was reduced for various species (Spahr *et al.*, 2002; Newman *et al.*, 2003; Walter *et al.*, 2006). However these works showed notable variations in the significance of the bacterial growth inhibition by Emdogain®. This might be related to differences in the experimental approaches as suggested by Walter *et al.* (2006). If a certain antimicrobial property can be seen with Emdogain®, it could be attributed to its EMP vehicle, namely the PGA as previously demonstrated by some authors (Sculean *et al.*, 2001b; Arweiler *et al.*, 2002).
Another characteristic of Emdogain® that might also contribute to this improved soft tissue healing after EMD application could be its anti-inflammatory properties that have been recently well addressed in a review paper by Miron *et al.* (2014). Such antibacterial/anti-inflammatory activities are important to consider to specify to what extent this biological mediator could be proposed, for instance, as an adjunctive molecule in the treatment of peri-implantitis (Froum *et al.*, 2015).
Dental plaque is a complex biofilm (Socransky *et al.*, 2002). Microbial biofilms show an increased resistance towards antimicrobial agents compared to free-floating bacteria (Gilbert *et al.*, 1997; Wright *et al.*, 2013). This work additionally proposed to investigate the antimicrobial effectiveness of chlorhexidine 0.2%, povidone iodine 5%, 10%, essential oils and essential oils Zero™ against *Porphyromonas gingivalis*-containing dual-species biofilms taking into consideration the biofilm mode of growth as suggested by Scheie and Petersen (2004). In the present model, all the tested molecules presented a highly significant antimicrobial effect. CHX 0.2% and PVI 10% displayed the most efficient antibacterial activity both on HA and Ti surfaces. Within the limitations of the present study, Emdogain® demonstrated a lack of antimicrobial efficacy against 7-d mature biofilms containing *Porphyromonas gingivalis in vitro*. Regarding the activity of other molecules, CHX 0.2% and PVI 10% showed the best antibacterial effectiveness.

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