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Antimicrobial effect of a local release system containing metronidazole against a *Porphyromonas gingivalis* biofilm

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The aim of this study was to evaluate a semi-solid system containing metronidazole (MDZ) in presence of challenging conditions for drug release, as well its antimicrobial effect against *Porphyromonas gingivalis* biofilm. Biofilms grown in culture medium were exposed to a formulation containing MDZ or its vehicle. After 24, 48, and 72 h, biofilm viability were analyzed while MDZ was quantified in culture medium and buffer solution (control). MDZ formulation reduced bacterial viability when compared to control groups. The vehicle formulation also affected bacterial viability in relation to control at all periods. Culture medium impaired MDZ release compared to buffer solution at 24 h. The semi-solid system reported herein is able to release MDZ and maintain its levels at concentrations that control viability of *P. gingivalis* in 1- to 3-day-old biofilms.

1. Introduction

Periodontitis is a gingival inflammation characterized by destruction of tooth supporting structures (Schwach-Abdellaoui et al. 2000). In designing a delivery system for periodontology, it is important to consider its antimicrobial effect, as well the main challenges for drug release in an environment containing bacteria, enzymes, and other conditions that are unique to the buccal cavity. Although considered as a good choice to treat periodontitis, the efficacy of metronidazole (MDZ) has often been questioned when formulated into a local drug delivery system (Da Rocha et al. 2016). Probably, drug release tests using only the standardized pharmacopoeial conditions are not enough to guarantee good drug release in the challenging conditions presented by the buccal cavity. Thus, the aim of the study was to evaluate a semi-solid system containing MDZ in presence of challenging conditions for drug release and its antimicrobial effect against *Porphyromonas gingivalis* biofilms, which is one of the most representative bacteria in periodontitis.

2. Investigations, results and discussion

In the presence of *P. gingivalis* biofilm, the formulation containing MDZ released about 19 % of antibiotic at the first 24 h and 5 % of the drug during the remaining time (Table). According bacterial viability results (Fig.), those amounts impaired biofilm growth for 3 days (72 h) which could represent a good therapeutic strategy to avoid rapid colonization of the treated sites.

The vehicle formulation control (VC), composed of monoglycerides and sorbitan monostearate, also affected bacterial viability in relation to negative control group at all periods ($p<0.05$). Although both components are used to control the drug release rate (Uvanesh et al. 2016), their antimicrobial effects were not described in literature. Sorbitan is a mixture derived from sorbitol and is established that this sugar alcohol decreases *P. gingivalis* biofilm density (Hashino et al. 2013). Probably this can explain, at least in part, why the vehicle of our formulation affects bacterial viability. The bioactivity of the formulation vehicle can provide additional therapeutic benefits but future studies should investigate the mechanism of this property.

Table: Metronidazole (MDZ) quantified (w/v) into buffer solution and culture medium from *P. gingivalis* biofilms growth at 24, 48 and 72 hours

Time (hours)	Buffer solution		Culture medium	
	[MDZ] (μg/mL)	MDZ (% Quantified)	[MDZ] (μg/mL)	MDZ (% Quantified)
24	274.8 ± 18.6^{A}	22.7 ± 1.5^{A}	250.9 ± 1.6^{C}	18.7 ± 0.1^{C}
48	27.7 ± 6.0^{B}	2.2 ± 0.5^{B}	67.1 ± 0.8^{D}	5.3 ± 0.1^{D}
72	28.5 ± 3.2^{B}	2.3 ± 0.3^{B}	60.5 ± 0.7^{D}	5.1 ± 0.1^{D}

MDZ: metronidazole. Data were analyzed intra-group and expressed as mean ± standard deviation (n = 3). Values that do not share the same letter (A to D) are significantly differ from each other; Shapiro-Wilk test followed by the *post-hoc* Tukey-Kramer test, $p<0.05$.

In relation to drug release quantification, although a later release of MDZ from formulation in culture medium compared to the same system in buffer solution at 24 h ($p<0.05$), higher amounts of MDZ were released into culture medium than buffer solution at the remaining time (48 and 72 h, $p>0.05$). Probably, enzymatic degradation processes of the formulation matrix at these points contributed to this result since that biofilm culture medium is a highly enzymatic environment. Thus, considering that the formulation containing MDZ was exposed to bacterial enzymes, electrolytes, minerals, buffers, and proteins, the drug release profile evaluated in this condition could be more adequate than the same evaluation into buffer solution (Patel et al. 2012).

The simple anaerobic biofilm model that we used, while surely an imperfect representation of a real biofilm on buccal cavity, does capture the drug release profile in a system containing a hostile environmental variables that are absent in ideal conditions for drug release studies. Thus, this tool could offer new opportunities of formulation design improvements before more advanced biological tests involving *in vivo* studies. The semi-solid system reported herein is able to release MDZ and maintain its levels at concentrations that control viability of *P. gingivalis* in 1- to 3-day-old biofilms.

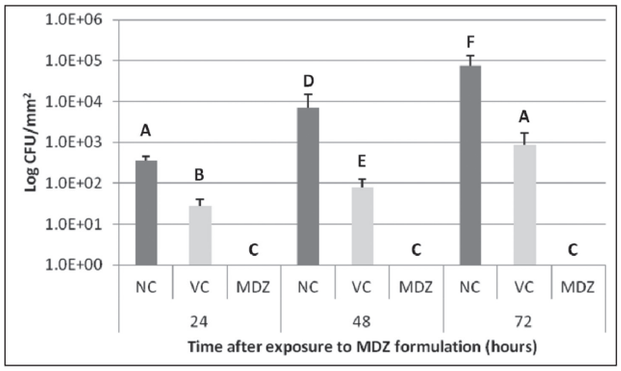

Fig.: Bacterial viability of *P. gingivalis* biofilm after treatment with metronidazole formulation. MDZ= formulation with metronidazole; VC= vehicle control (formulation without MDZ); NC= negative control (untreated biofilm). Data are expressed as mean ± standard deviation (n = 6). Values not sharing the same letter significantly differ from each other; Shapiro-Wilk test followed by the *post-hoc* Tukey-Kramer test, $p<0.05$.

3. Experimental

3.1. Preparation of formulation

A semi-solid local release system with a mixture of monoglycerides (Myverol 18-92k®, Kerry®, Munster, Ireland), sorbitan monostearate (Span 60®, Sigma Aldrich, Missouri, USA), and 25 % metronidazole (MDZ) (32.2 % of MDZ benzoate, equivalent to 20 % of MDZ, plus 5 % of MDZ base; Fagron, Rotterdam, Netherlands) was prepared according to Ré et al. (2016). Briefly, monoglycerides and sorbitan monostearate was prepared by melting at 42 °C. MDZ was incorporated under agitation at room temperature and the system was maintained at room temperature for 24 h for equilibration. After sterilized by X-ray radiation (130 Gy; average proton energy of 160 kV, 25 mA, 0.3 mm Cu filter) for 30 min (Rad Source's RS 2000 Biological Research Irradiator, Shanghai Medicilon, Shanghai, China), formulation was stored at 4 °C.

3.2. Biofilm analysis

In this study, we used a 3-days *P. gingivalis* biofilm model developed by Papaioannou et al. (2012), but using a formulation exposed to the biofilms. This study was approved by the Research and Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto (protocol nº 375). Briefly, *P. gingivalis* W83 inoculum (10^8 bacteria/mL) was transferred to 50-mL tube containing a vertical thin strip of one of the following formulations (241.5±1.9 mg; n=3): i) formulation containing MDZ; ii) formulation without MDZ (vehicle control, VC). After addition of culture medium (Brain Heart Infusion, BD Diagnostics, Sparks, MD, USA) supplemented with 5 µg/mL hemin and 1 µg/mL menadione, glass slide coated with human salivary pellicle was added to allow biofilm formation. Untreated biofilms (negative control, NC) were used as controls. The tubes were incubated in anaerobic jars using a gas generating kit (BD Diagnostics, Sparks, MD, USA) and the culture medium was replaced daily. After 24, 48, and 72 h the biofilms were collected and culture medium was stored at -20 ºC until drug quantification. Biofilms were transferred into tubes containing 1 mL of 0.9 % NaCl and sonicated (Aires et al. 2008). A 100-µL aliquot was diluted in saline solution and serial decimal dilutions were inoculated in duplicate (Herigstand et al. 2001) in blood agar (supplemented with hemin and menadione) and incubated under anaerobiosis at 37 ºC for 7 days. The results were expressed in CFU/mm^2. Two independent experiments were performed in triplicate (n = 6).

3.3. Drug quantification

Bacterial culture medium was prepared for MDZ quantification as reported previously (Ré et al. 2016). Briefly, the culture medium was defrosted and centrifuged, and the resulting supernatant was filtered through a 0.22-µm membrane. After methanol addition followed by freeze and thaw cycles, the filtrate was centrifuged and filtered through a 0.45-µm membrane. The filtrates were analyzed by HPLC on a chromatograph (Prominence; Shimadzu, Kyoto, Japan), with the diode array detector (DAD-UV, SPDM20A model) set at 320 nm and a C-18 column (250 x 4.6 mm; 5.0 µm particle size, Shim-pack VP-ODS) using methanol/ultrapure water 50:50 (v/v) as the mobile phase. The flow rate was 0.8 mL min^{-1}, and the column temperature was 40 °C (n = 3). As control, drug dissolution was performed using sodium phosphate buffer (30 mM, pH 6.8, at 37 °C) under the same conditions designed by culture medium samples except by withdrawn aliquots and filtered through a 0.22-µm membrane for MDZ quantification. The results were calculated by comparing the initial amount of the MDZ in the formulation vs. the amount of MDZ quantified in each dissolution medium (buffer or culture medium from biofilm growth).

3.4. Statistical analysis

Experimental data were analyzed using the SAS System (release 9.3; SAS Institute Inc., Cary, NC, USA, 2012). The analysis of variance was adjusted and the adherence of the residuals to the Gaussian distribution was evaluated by the Shapiro-Wilk test, coefficient of skewness, kurtosis, and graphical analysis. Groups were compared using the *post-hoc* Tukey-Kramer test, at a significance level of 5%.

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Conflict of interest: None declared.

References

Aires CP, Del Bel Cury AA, Tenuta LMA, Klein MI, Koo H, Duarte S, Cury JA (2008) Effect of starch and sucrose on dental biofilm formation and on root dentine demineralization. Caries Res 42: 380–386.

Da Rocha HA, Silva CF, Santiago FL, Martins LG, Dias PC, De Magalhães D (2014) Local drug delivery systems in the treatment of periodontitis: a literature review. J Int Acad Periodontol 17: 82–90.

Hashino E, Kuboniwa M, Alghamdi AS, Yamaguchi M, Yamamoto R, Cho H, Amano A (2013) Erythritol alters microstructure and metabolomic profiles of biofilm composed of *Streptococcus gordonii* and *Porphyromonas gingivalis*. Mol Oral Microbiol 28: 435–451.

Herigstad B, Hamilton M, Heersink J (2001) How to optimize the drop plate method for enumerating bacteria. J. Microbiol. Methods. 44: 121–129.

Papaioannou W, Panagopoulos A, Koletsi-Kounari H, Kontou E, Makou M (2012) Adhesion of *Porphyromonas gingivalis* and biofilm formation on different types of orthodontic brackets. Int J Dent 2012: 471380.

Patel VF, Liu F, Brown MB (2012) Modeling the oral cavity: in vitro and in vivo evaluations of buccal drug delivery systems J. Control. Release 161: 746–756.

Ré ACS, Ferreira MP, Freitas O, Aires CP (2016) Local antibiotic delivery in periodontitis: drug release and its effect on supragingival biofilms. Biofouling 32: 1061–1066.

Schwach-Abdellaoui K, Vivien-Castioni N, Gurny R (2000) Local delivery of antimicrobial agents for the treatment of periodontal diseases. Eur J Pharm Biopharm 50: 83–99.

Uvanesh K, Sagiri SS, Senthlguru K, et al. Effect of Span 60 on the microstructure, crystallization kinetics, and mechanical properties of stearic acid oleogels: An in-depth analysis J. Food Sci 2016; 1: E380-7.