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Acidification of blue photosensitizers for antimicrobial photodynamic therapy impairs photodynamic reaction

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Abstract

Introduction: Antimicrobial photodynamic therapy (aPDT) is an adjuvant treatment for periodontal disease. Methylene blue (MB) and toluidine blue O (TBO) are commonly used as photosensitizers in aPDT. However, the pH of blue dyes is a rarely discussed topic.

Objectives: The aim of this study was to evaluate dentin demineralization and optical properties of acidified (pH1) MB and TBO. A secondary objective of this paper was to discuss the regular pH of blue photosensitizers and their clinical applications.

Materials and Methods: Acid TBO and MB (pH1) were compared to conventional dyes and/or treatments. Dentin demineralization was measured by profilometry and microhardness test. Optical properties were evaluated by absorbance measurements and photodegradation rate. Statistical analyses were performed with Kruskal-Wallis test ($p<0.05$).

Results: Acid TBO promoted dentin demineralization like positive control (citric acid/tetracycline) ($p>0.05$). Acidification of MB and TBO promoted no photodegradation and change of absorption band, respectively. Acid MB and TBO promoted similar demineralization of dentin surfaces as citric acid/tetracycline.

Conclusion: Acidification impaired a photodynamic reaction, impeding the use of acidified photosensitizers in aPDT procedures.

Keywords: Photodynamic therapy. Photosensitizers. pH. Methylene blue. Toluidines.

Introduction

Antimicrobial photodynamic therapy (aPDT) consists of an association of a light source and a photosensitizer, to produce a photochemical reaction, aiming bactericidal or bacteriostatic effects (Passanezi *et al*., 2015; Damante *et al*., 2016).

This therapy is successfully associated to nonsurgical and surgical periodontal therapy to modulate extracellular matrix and bone remodeling (Andrade *et al*., 2013), to reduce periodontal pockets and reestablish periodontal health in a short-term period (Sgolastra *et al*., 2013). A best evidence consensus from the American Academy of Periodontology stated that aPDT is a promising therapy for periodontitis treatment (Mills *et al*., 2018). Meta-analysis showed statistically significant benefits on improvement of periodontal health in non-surgical treatment of different periodontitis stages and grades, although a clinical recommendation cannot be established (Chambrone *et al*., 2018).

The most common photosensitizers for aPDT are blue dyes, as methylene blue (MB) and toluidine blue O (TBO). The resonance of a photosensitizer occurs when the absorption band is in the same wavelength range as the light source (Damante, 2021). This concept is based on the Grotthus-Draper law of physics — the principle of photochemical activation. Thus, the recommended wavelength for TBO is 635nm and for MB is 660nm.

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Although the antimicrobial potential of blue photosensitizers in aPDT is widely cited in literature, the discussion about photosensitizers with different pHs is rarely seen. When diluted in deionized water, methylene blue has a neutral pH, around 6, while toluidine blue O presents acid pH, around 4.

The pH of MB was discussed by Morgan and Baumgartner (1997) in a study aiming smear layer removal from resected root-ends. A 2% methylene blue solution dissolved in double distilled water and deionized water presented pH5 and pH3.7, respectively. In addition, these authors were the first to mention an experimental solution of 1% methylene blue in 50% citric acid with pH1.4. Their conclusion was that smear layer removal is pH-dependent.

Antimicrobial photodynamic therapy using TBO with acid pH (4.31) causes enamel demineralization (Pessoa *et al*., 2015), loss of Knoop microhardness and wear on dentin surfaces (Damante *et al*., 2016). These studies suggested that those effects could be promising for periodontal surgery. In surgical periodontal treatment, citric acid alone or mixed with tetracycline in gel with pH1 is used for decontamination and superficial demineralization of root surfaces, exposing collagen fibers. The treated surface promotes a reinsertion of fibers from the flap, and better wound healing results (Register, 1973; Register and Burdik, 1975; Damante *et al.*, 2019; Caba-Paulino *et al*., 2020). It was then proposed, based on these studies, that an acid photosensitizer as TBO (pH4) could be employed with the objective of root surface biomodification/demineralization (Damante *et al*., 2016; Damante *et al*., 2019). It would be an off-label indication, in addition to its antimicrobial effects.

Previous *in vitro* studies demonstrated that demineralization/biomodification of root surfaces by aPDT with TBO (pH4) did not impair gingival fibroblasts growth (Damante *et al*., 2016). It augmented the proliferation of gingival fibroblasts (Damante *et al*., 2016; Karam *et al*., 2017) and the viability of osteoblasts (Ferreira *et al*., 2018). In addition, a clinical trial showed a higher percentage of root coverage with subepithelial connective graft and root treatment with TBO pH4 in a aPDT procedure (Karam *et al*., 2017). The percentage of root coverage for the TBO group (82.1%) was similar to citric acid plus tetracycline (81.6%) (Damante *et al*., 2019).

The authors of the present article were among the first to develop and test both toluidine blue O and methylene blue dyes with modified pH1, aiming their use in periodontal surgery for root biomodification/demineralization (Damante, 2015) (University of São Paulo innovation agency protocol #13.1.1130.25.2 – year 2014).

The biocompatibility of an acid methylene blue dye (pH1) was then tested in rat subcutaneous tissue (Gusman *et al*., 2018). Conventional and acid methylene blue dyes (100μg/ml) were inserted in subcutaneous tissues in tubes containing a fibrin sponge. Histological sections showed that acid dye had similar inflammatory reaction to control group.

There is no paper in literature addressing the topic of blue photosensitizers pH and their clinical implications when applied in aPDT procedures for treatment of periodontal disease. Thus, the aim of this study was to evaluate if dentin demineralization is similar to citric acid/tetracycline gel, and the possible changes in optical properties of experimental acidified blue dyes (TBO and MB) with an adjusted pH1. A secondary purpose of this study was to discuss the pH of aPDT blue dyes and their clinical applications.

Materials and Methods

Acid dyes were prepared by diluting toluidine blue O and methylene blue in deionized water in a concentration of 100μg/ml. The pH was adjusted to 1 in a pH-meter with 10M HCl. Conventional dyes were diluted the same way and pH was not adjusted. The pH was monitored and maintained stable for all experimental periods.

Demineralization test

Only toluidine blue O was used in demineralization tests, in comparison to citric acid plus tetracycline gel, in different times of contact to dentin surface. The main outcome variable of this test was measuring demineralization by means of tooth wear and surface hardness loss.

Thirty bovine dentin blocks were prepared as previously described (Damante *et al*., 2016). Bovine teeth are more suitable for the methodology because they are thicker and more resistant for polishing procedure. Fifteen of the thirty blocks were used for profilometry, and fifteen, for microhardness test. The groups were divided as follows: acid TBO (pH1) in contact for 60s (ATB60), 90s (ATB90), 180s (ATB180). A combination of citric acid (50%) plus tetracycline (10%) gel for 180s was used as a positive control (CA180).

For profilometry, central sites of fragments were treated with TBO dyes using a microbrush, as previously described (Damante *et al*., 2016). Then, the specimens were washed with deionized water. In CA180 group, gel was applied for 180s and then washed with saline solution. The diamond stylus was moved from the first control to the exposed area, and then to the other control area (2.0-mm long and 1.0-mm wide, contact profilometer). Five profile measurements were performed at intervals of 0.25 mm. Lines were defined as tooth wear using the software Mahr Surf XT20, version 2009 (Mahr Ltda, Göttingen, Germany).

Baseline and final surface hardness (SH) determination was performed by the measurement of five indentations with 100-μm distance from each other (Knoop diamond, 25g/10s for enamel and 10g/15s for dentin, HMV- 2; Shimadzu Corporation, Tokyo, Japan).

The percentage of surface hardness loss (SHL) was calculated as follows: %SHL = 100 x (final SH – baseline SH)/ baseline SH. Each fragment was its own control, thus, there was no control group for this test. Statistical analysis was performed with Kruskal-Wallis test complemented by Dunn's multiple comparisons test ($p<0.05$).

Optical properties of acid blue dyes

This experiment was performed with both TBO and MB with acid pH1. The main outcome of this test was the possible change in absorption band of the photosensitizer after lowering the photosensitizer pH. Absorbance measurements were performed in a spectrophotometer (Variant Cary 50 Bio – UV – VIS, USA). Samples were positioned in a 2mm-optical path cuvette. Toluidine blue was analyzed with a concentration of 100μg/ml. Methylene blue saturated the readings, and it was diluted to a concentration of 50 μg/ml. Photodegradation was performed with a light font with 660nm for MB and 585.9 for TBO. A quartz cuvette was filled with 400 μl of solution and optical path of 2 mm. The optic fiber was positioned 45mm distant from sample. Irradiance was 60mW/cm^2; illumination times were 0, 5, 10, 20, 25 and 40 minutes. The absorbance spectra was measured in a spectrophotometer.

Results

Demineralization test

All treatment groups presented demineralization, by means of surface hardness loss, and it was similar to citric acid/tetracycline gel ($p>0.05$) (Table 1).

Table 2 shows that all treatments promoted dentin wear (Table 2). Acid TBO demineralization for 180s was similar to citric acid/ tetracycline gel ($p>0.05$) (Figure 1).

Table 1. Percentage of surface hardness loss (n = 15 bovine dental fragments).

	ATB60	ATB90	ATB180	CA180
Mean	93.721^{d}	86.385^{ac}	89.713^{ad}	90.359^{ad}
SD	2.335	8.648	2.789	4.138

Different letters = $p<0.05$. SD = standard deviation. Acid toluidine blue (pH1) for 60s (ATB60), 90s (ATB90), 180s (ATB180), citric acid/tetracycline gel (pH1) (CA180) 180s.

Table 2. Wear (μm) obtained by profilometry (n = 15 bovine dental fragments).

	ATB60	ATB90	ATB180	CA180
Mean	1.579^{ac}	1.365^{ab}	2.271^{cd}	7.335^{d}
SD	0.542	0.417	0.426	1.653

Different letters = $p<0.05$. SD = standard deviation. Each fragment was its own control. Acid toluidine blue (pH1) for 60s (ATB60), 90s (ATB90), 180s (ATB180), citric acid/tetracycline gel (pH1) (CA180) 180s.

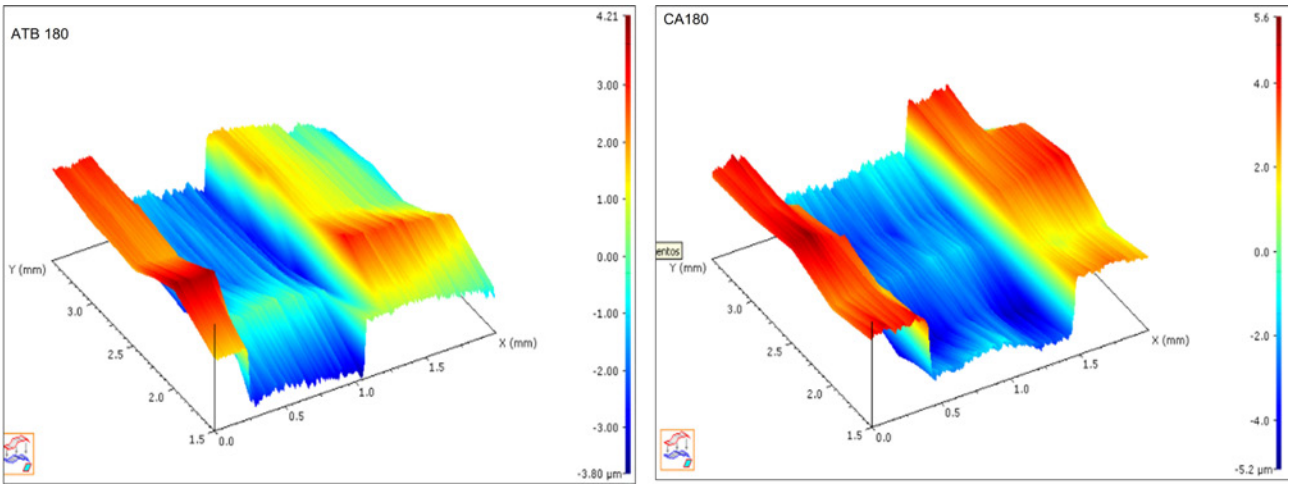

Figure 1. Image of profile measurement at the central part of tooth fragment. Acid Toluidine Blue (ATB180) promoted a similar demineralization to citric acid plus tetracycline gel (CA180).

Optical properties of acid blue dyes

Conventional TBO presented an absorbance band of 585 nm, and a minor peak at 630 nm. Acid TBO showed a migration of absorbance band to 575 nm (-10nm) (Figure 2). The change in absorbance band impairs a photodynamic reaction, once the common market lasers have wavelengths of 635 and 660 nm.

Based on the above-mentioned results, photodegradation rate experiment was conducted only for regular TBO with 630-nm and 660-nm laser. A higher rate of photodegradation was observed with the 630-nm laser (Figure 3). Regardless of a minor rate, some photodegradation was still observed when using regular TBO combined with 660-nm laser.

Conventional and acid MB dyes (50µg/ml) presented an absorbance peak at 660 nm (Figure 4), showing that acidification did not change the absorbance band. Nonetheless, photodegradation rate for acid MB did not occur (Figure 5). Regular MB with pH5.5 – 50 µg/ml presented a very slow degradation rate, and the adjusted curve showed a degradation rate of 0.0059±0.0001pM/second.

The acidification of TBO or MB impairs photodynamic reaction by changing absorption band of the photosensitizer or slowing the photodegradation rate.

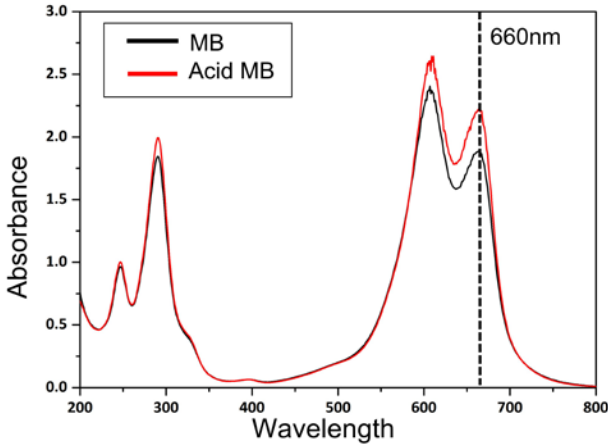

Figure 2. Peak of absorption for conventional (black line) and acid (red line) methylene blue.

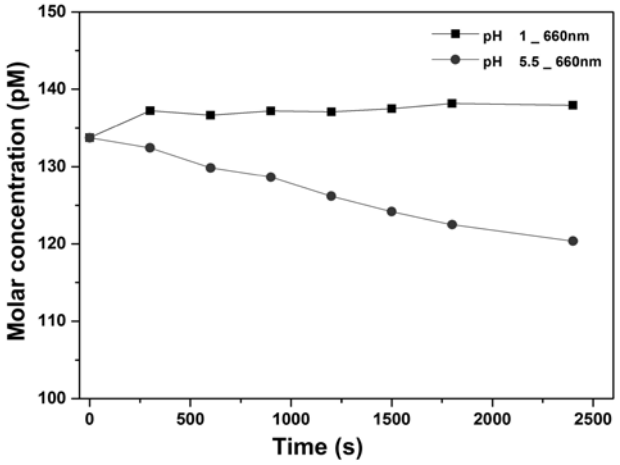

Figure 3. Photodegradation for conventional (pH5.5) and acid (pH1) methylene blue. Observe an absence of photodegradation for acid MB.

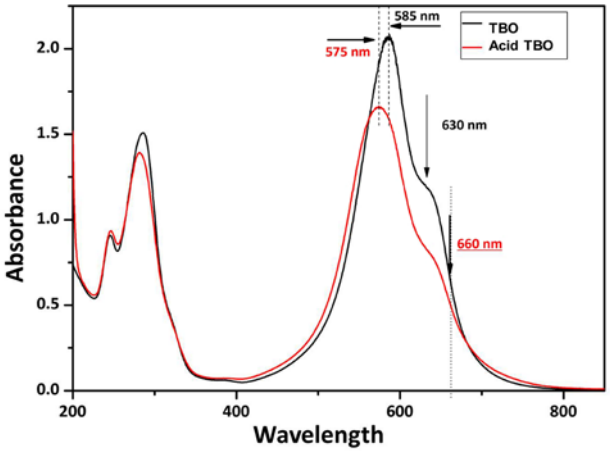

Figure 4. Peak of absorption for conventional (black line) and acid (red line) toluidine blue O. Observe the change on absorption peak for acid TBO for 575 nm.

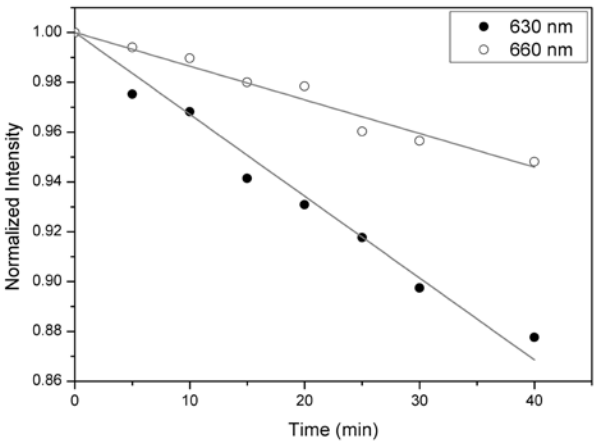

Figure 5. Photodegradation for conventional TBO. A higher photodegradation occurred with the 635-nm laser. Even with the 660-nm laser, some degradation was still present.

Discussion

The present study evaluated dentin demineralization and optical properties of blue photosensitizers (MB and TBO) with pH1. Results showed that acid TBO is capable of demineralizing bovine dentin surfaces with similar values of surface hardness loss and wear to the citric acid/tetracycline gel. Optical properties of MB and TBO were jeopardized with acidification of photosensitizers. Acidification of TBO changed its absorption band from 585 nm to 575 nm, without absorption peak for 635-nm and 660-nm lasers. MB peak of absorption was maintained in 660 nm even with acidification, but the photodynamic reaction did not occur. Although biocompatibility of acid blue dyes was not tested, a previous histological study showed that MB at pH1 provides better biocompatibility in rat soft tissues than pH7 (Gusman *et al*., 2018).

Acidification of a blue photosensitizer was first mentioned in literature in a study of smear layer removal from resected root ends, a treatment used in endodontics. The authors described an experimental solution of MB with citric acid to obtain an acid pH1.4 (Morgan and Baumgartner, 1997). In the present study, the acid MB and TBO were prepared with hydrochloric acid. By using a strong acid, just a few microliters were enough to reach pH1 without changing dye concentration.

Tardivo *et al*. (2005) explained why an acid pH may impair the photodynamic reaction with MB. In acid pH, MB triplet states are observed. Triplets have a higher energy level and react to oxygen in a smaller rate, meaning that less singlet oxygen is produced. As the pH is capable of diminishing the photosensitization mechanisms, there was an alert for treating target tissues with inflammation or low pH (Tardivo *et al*., 2005). The present optical test results showed that acidification of TBO or MB impaired photodynamic reaction by changing absorption band of the photosensitizer or slowing the photodegradation rate. If the photosensitizer absorption peak is not the same wavelength of laser, a photodynamic reaction does not occur (Damante, 2021). Moreover, a slow photodegradation rate may be due to a higher production of triplet states at the photosensitizer.

The research group of the present article has been also investigating blue photosensitizers with their usual pH when diluted in deionized water. Although a pH of a solution depends on concentration and type of solvent, it can be inferred that methylene blue has a neutral pH and toluidine blue O has an acid pH, on regular concentrations used in aPDT. The present measurements showed pH 5.91 and pH 6.42 for methylene blue (Sigma-Aldrich - USA) diluted in deionized water with concentrations of 10mg/ml and 100 μg/ml, respectively. For toluidine blue O (Sigma-Aldrich, USA), a mean pH3.5 was obtained for a dilution of 10mg/ml and pH4.15, for 100 μg/ml. Information from the manufacturer (Sigma Aldrich, USA) showed even a lower acid pH for toluidine blue O: pH2.8 at a concentration of 30g/l – 25°C.

Based on this information, it was investigated if TBO (100 μg/ml) at pH4.31 was capable of demineralizing root surfaces, to propose an off-label application for periodontal surgery purposes (Damante *et al*., 2016). In periodontal surgery, citric acid at pH1 is used for demineralization and detoxification of root surface contaminated by dental biofilm. Toluidine blue O at pH4.31 promoted surface hardness loss (71.5%) in bovine dentin blocks similar to citric acid (76.1%). It also promoted similar wear measurements (TBO - 12.58 μm / citric acid - 11.74 μm). Concerned with the product safety, a cell culture study found that the demineralization in dentin blocks promoted a biocompatible surface for human gingival fibroblast adhesion and proliferation (Damante *et al*., 2016). Finally, the effects of aPDT with TBO on root demineralization in root coverage procedures was evaluated in a clinical trial. The percentage of root coverage in exposed roots treated by aPDT was higher (82.1%) than control (57.7%) (treatment with scaling and root planning) and similar to citric acid plus tetracycline (81.6%) (Damante *et al*., 2019).

The present study showed that lowering the pH of TBO and MB may impair photodynamic reaction. It can be suggested that demineralization occurred due to acid pH of the solution. If the observed effects were due only to an acid pH, any substance could be used with that purpose. Indeed, it is well established, in literature related to periodontal surgery, the application of acid agents (citric acid/tetracycline) to obtain root biomodification and detoxification (Register, 1973; Register and Burdik, 1975; Damante *et al*., 2019). It is noticeable, on the other hand, that TBO and MB, with their natural pH (dilution with deionized water), maintain optical properties favorable to photodynamic reaction. The present study showed that even with 660-nm lasers, there is a photodegradation of conventional TBO. Using that information, it is suggested a decision tree for when to use TBO and when to use MB for aPDT in clinical practice. For surgical procedures, when the objective is demineralization and detoxification of root surfaces, TBO (pH4) is indicated, since it can demineralize root surfaces and it is biocompatible (Damante *et al*., 2016), without losing its optical properties. When aPDT is used as an adjuvant treatment to scaling and root planning (no surgical procedure), MB is indicated for irrigation of periodontal pockets without harming surrounding tissues, due to its neutral pH.

The clinical relevance of the present study is to clarify to other researchers and periodontists the properties of photosensitizers, regarding their pH and the possible effects on demineralization of dental surfaces. The information that toluidine blue with slightly acid pH is capable of demineralization is important to mention and to recommend avoiding its use on enamel surfaces. Modification of usual photosensitizers or research focusing new photosensitizers is a reality. Therefore, the information of the present paper is paramount to clarify that lowering the pH of photosensitizers to pH1-3 is not suitable to promote a

photodynamic reaction. The aim of this study was to alert other researchers not to conduct future studies with acidification of a photosensitizer.

Besides similar results between acid MB and citric acid/tetracycline on dentin demineralization, the acidification of photosensitizers to pH1 impaired the photodynamic reaction. This result, by itself, precludes a clinical recommendation of their use in an antimicrobial photodynamic therapy procedure.

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