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ORIGINAL ARTICLE

pH influences the biocompatibility of methylene blue solutions

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Abstract
Objective The aim of this study was to investigate the biocompatibility of methylene blue at different pH levels through the method of implantation in subcutaneous tissue.
Materials and methods Eighty-four sterilized polyethylene tubes were allocated in the subcutaneous tissue of 28 rats, each one receiving four tubes, set into four groups: group tube (G-T)—empty tube, fibrin group (G-F)—tube filled with fibrin sponge, group methylene blue pH 7 (G-MB/pH 7)—tube filled with fibrin sponge soaked by methylene blue (100 μg/ml) at pH 7.0, and group methylene blue pH 1 (G-MB/pH 1)—tube filled with fibrin sponge and soaked by methylene blue (100 μg/ml) at pH 1.0. After 7, 15, and 30 days, seven animals from each group were euthanized, and the tubes involved by the surrounding tissue were removed and fixed with 4% buffered formaldehyde solution. The collected pieces were processed and histological sections (4 μm) were stained with hematoxylin and eosin and analyzed by light microscopy. Scores were assigned to analysis of histopathologic parameters. The results were statistically analyzed by the Kruskal–Wallis test ($p \leq 0.05$).
Results At 7 and 30 days, the G-MB/pH 1 group showed no significant difference in the G-T control group, while G-MB/pH 7 had a significant increase on tissue reaction, also when compared to G-T. At 15 days, there was no statistical difference between the groups.
Conclusion Within the limits of this study, it is concluded that methylene blue at pH 1.0 provides better biocompatibility than at pH 7.0.

Keywords Biocompatibility testing · Subcutaneous tissue · Hydrogen-ion concentration · Methylene blue

Introduction

The capacity of a foreign material to reside in an organism by making contact with an organism's living matter (tissue or organ) is called biocompatibility [1, 2]. Foreign material needs to be accepted by tissues within physiological limits, and non-biocompatible dental materials that negatively affect patients have become an increasing public concern [1, 3].

One of the main substances used in infections treatment is methylene blue, mostly combined with antimicrobial photodynamic therapy (aPDT), since it has a high degree of selectivity for damaging gram-positive and gram-negative bacteria [3].

Methylene blue is a photosensitizing agent, activated by light at a specific wavelength (660 nm) that corresponds to maximum absorbance. The combination of visible light irradiation and photoactivated substances triggers the photodynamic process [4–8]. This process produces free radicals, singlet oxygen, and other reactive oxygen species, leading to photooxidation of organic matter [9, 10]. The lethal photosensitization of microorganisms must involve changes mediated by singlet oxygen in the membranes and/or plasma membrane proteins and DNA. [11–16].

Thereby, aPDT serves as a noninvasive therapy for infection control and has been used as adjuvant in the treatment of

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chronic [17] and aggressive [18] periodontitis, periimplantitis [19], apical periodontitis [20], after impacted third molar removal [21], endodontic treatment [20, 22] and retreatment [23], endodontic surgery [24], and it may be considered promising for treatment of dental caries lesions [25].

For chronic and aggressive periodontitis, scaling and root planning (SRP) is considered the standard treatment, providing clinical improvements by disrupting subgingival biofilm, decreasing amount of bacteria, and delaying the repopulation of microorganisms [26, 27]. However, difficulties in accessing furcation areas and root concavities and difficulty in removing bacteria that has penetrated into dentin tubules [17, 28, 29] demonstrate that SRP may not be completely effective, and for these reasons, aPDT has been recommended [17].

On the other hand, acidic substances such as citric acid (pH 1), phosphoric acid (pH 1.02), boric acid (pH 4.9), ethylenediaminetetraacetic acid (EDTA) (pH 7.0–7.4), and tetracycline (pH 2.2) are commonly used for chemical conditioning of root canal walls and the outer root surface [30–32]. The acidic root surface modifier substances are intended to detoxify the root surface, removing the smear layer and promoting demineralization, increasing the adhesion of fibrin networks and fibroblasts cells to the root surfaces, and leading to exposure of the collagen matrix, favoring periodontal tissue repair and regeneration [20]. Moreover, in recent years, various studies have proposed the use of aPDT in different treatments associated with various photosensitizing agents, but all with substances with alkaline and neutral pH.

Considering all these points, it is important to evaluate the biocompatibility of solutions used in combination with aPDT, especially the influence of the pH levels of these solutions. Since the hypothesis is that the tissue behavior against acidic methylene blue could be as healthy as against neutral methylene blue, the aim of this study was to investigate the biocompatibility of methylene blue with different pH levels through the method of implantation in subcutaneous tissue (ISO 10993-6/2007).

Materials and methods

Animals

Twenty-eight, 4-month-old, male rats (*Rattus norvegicus* Albinus, Wistar), weighing 250–300 g (UNESP, Dental School of Aracatuba, Animal Care Unit) were kept in plastic cages with access to food and water ad libitum in a room with 12-h light/12-h dark cycles and temperature between 22 and 24 °C. The research protocol was approved by the Ethics Committee in Animal Experimentation (00310-2016) at Universidade Estadual Paulista - Julio de Mesquita Filho - Dental School of Araçatuba.

Arrangement of the tubes

Polyethylene sterilized tubes (CPL Medical's, Sao Paulo, SP, Brazil) with 1.6 mm internal diameter, 2.0 mm external diameter, and 10.0 mm length were used. The materials that filled the tubes were fibrin sponge, methylene blue pH 7.0 (Azul de metileno—pH 7.0, 100 μg/ml—Apothicário, Araçatuba, São Paulo, Brazil), and methylene blue pH 1.0 (Azul de metileno—pH 1.0, 100 μg/ml—Apothicário, Araçatuba, São Paulo, Brazil). Citric acid (60 μg/ml) was used to reach pH 1 in the acidic methylene blue solution. Both methylene blue solutions were 98.5% pure.

Experimental groups

The tubes were separated into four experimental groups ($n = 7$): tube without any filling as control group (G-T); group filled with fibrin sponge (G-F); group filled with fibrin sponge and soaked by methylene blue pH 7.0 (G-MB/pH 7); and group filled with fibrin sponge soaked by methylene blue pH 1.0 (G-MB/pH 1).

Subcutaneous implantation

The animals were anesthetized by intramuscular injection of ketamine (70 mg/kg—Francotar—Virbac do Brasil Ind. e Com. Ltda, Roseira, Brazil) and xylazine (6 mg/kg—Rompum—Bayer S. A., São Paulo, Brazil). The surgical procedure was similar to other studies [33, 34]. The dorsal side of the rats was trichotomized and disinfected with 0.2% chlorhexidine digluconate (Colgate-Palmolive, New York, NY). A 2-cm incision was made in the head-to-tail direction with a 15 Bard-Parker blade (Franklin Lakes, NJ). Two pockets on each side were created in the cranial portion (housing two tubes) and in the caudal portion (housing two tubes) by blunt dissection, and the tubes were implanted in the subcutaneous tissue and the skin was sutured with black silk wire 4–0 (Johnson & Johnson/Ethicon).

Experimental periods and laboratory procedures

The animals were euthanized at 7-, 15-, and 30-day intervals by thiopental (150 mg/kg—Thiopentax—Cristália—Produtos Quím. Farm. Ltda, São Paulo, Brazil) and lidocaine (6 mg/kg—Lidovet—Bravet. Ltda, Rio de Janeiro, Brazil). The tubes and surrounding tissue were removed in blocks and fixed in 4% buffered formaldehyde solution. The specimens were processed in paraffin in order to obtain 4-μm thick sections of tissue prepared longitudinally through the midline of the tubes and stained with hematoxylin-eosin.

Histological analysis

An average value for each animal was obtained from the total cells counted in ten different areas under ×400 magnification. The analysis was performed by a blind examiner using a light microscope (Olympus BX 50 F4, Olympus Optical Co., Ltd., Tokyo, Japan). The images were recorded using a digital camera (Olympus DP 10, Olympus Optical Co. Ltd., Tokyo, Japan) connected to the microscope.

Reactions in the edges of tissues in contact with the materials on the opening of the tubes were analyzed and scored in accordance with a system reported in previous studies [33–35]. The inflammatory reactions were categorized as:

- Score 0, without inflammatory cells
- Score 1, with mild inflammation (cells <25)
- Score 2, with moderate inflammation (cells = 25–125)
- Score 3, with severe inflammation (>125 cells)

The thickness of fibrous capsules when analyzed was considered to be:

- Thin when <150 μm
- Thick at >150 μm

Necrosis was recorded as present or absent and a mean of the number of cells for each group was obtained from ten separate areas and ranked according to the scores 0–3.

Statistical analysis

The measurements were repeated twice to ensure reproducibility, and median and range of the grades were calculated. Results were statistically analyzed by Kruskal–Wallis tests ($p < 0.05$).

Results

Control groups (G-T)

At 7 days, moderate inflammatory cell infiltration consisting of lymphocytes and macrophages was present in the fibrous capsule (Fig. 1a). At days 15 and 30, the connective tissue was well organized, and infiltration of a few chronic inflammatory cells was observed (Fig. 1b, c). The fibrous capsule surrounding the tube was thin, with few chronic inflammatory cells at 15 and 30 days (Fig. 1b, c).

Control groups (G-F)

At 7 days, severe inflammation cell infiltration consisting of lymphocytes and macrophages was present in the fibrous capsule (Fig. 1d). The observed intensity of inflammation was reduced at 15 and 30 days (Fig. 1e, f), becoming moderate and mild, respectively. The fibrous capsule near the tube was comparatively thin at 30 days (Fig. 1f).

Test group (G-MB/pH 7)

At 7 days, severe inflammation cell infiltration consisting of lymphocytes and macrophages was present in the fibrous capsule (Fig. 1g). However, at 15 and 30 days, moderate inflammatory cell infiltration consisting of lymphocytes and macrophages was present in the fibrous capsule (Fig. 1h, i), which was considered thin at 30 days (Fig. 1i).

Test group (G-MB/pH 1)

At 7 and 15 days (Fig. 1j, k), moderate inflammation cell infiltration consisting of lymphocytes and macrophages was present in fibrous capsule. At 30 days (Fig. 1l), mild inflammation was observed. The intensity of the inflammation and the thickness of the fibrous capsule were reduced by day 30 (Fig. 1l), with fewer chronic inflammatory cells consisting of lymphocytes and macrophages present in the thin fibrous capsule.

Comparison between the groups

The data for each time point were compared, as shown in Table 1. At days 7 and 30, there were statistically significant differences between inflammatory cell numbers in the control group and the G-MB/pH 7 group ($p = 0.0014$ and 0.0010, respectively). At 7 days, G-MB/pH 1 (median score 2) had no significant difference with G-T group (median score 1), and the G-MB/pH 7 (median score 3) group was statistically significant, showing more tissue reaction. At 15 days, no significant difference between the groups was observed ($p = 0.0051$). At 30 days, G-MB/pH 1 (median score 1) had no significant difference with G-T (median score 1), and G-MB/pH 7 (median score 2) group was statistically significant, showing more tissue reaction.

Discussion

Nowadays, the development of biomaterials must consider the aesthetics, strength, functional aspects of the material, and also its biocompatibility with connective tissue [1]. Implantation of sterilized polyethylene tubes in the subcutaneous connective tissues of rats has been widely used to evaluate biocompatibility materials according to ISO 10993-6/2007 standards [33, 36]. The aim of this study was to investigate the biocompatibility of methylene blue at different pH levels.

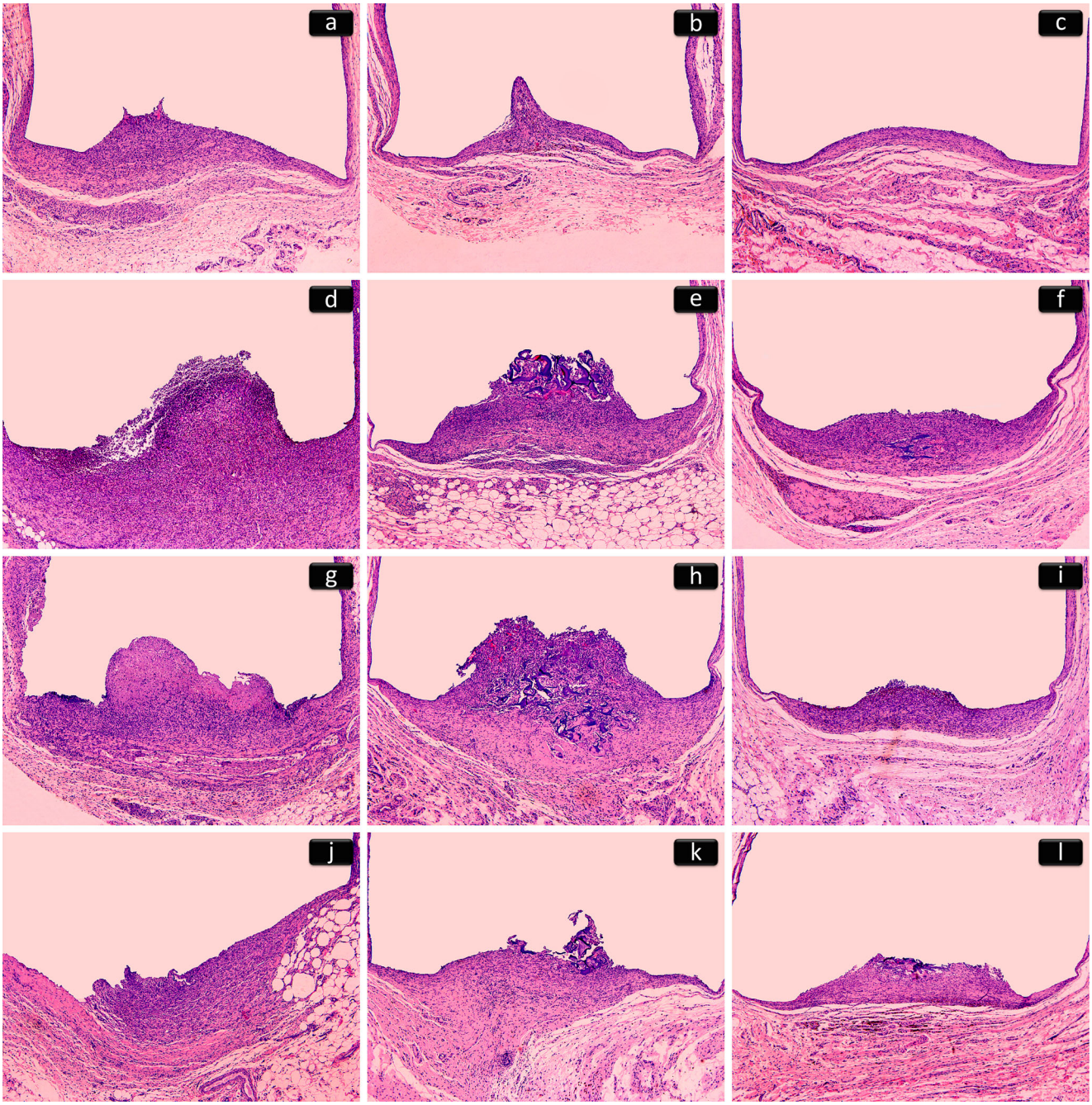

Fig. 1 G-T. **a** Thick fibrous capsule and moderate inflammatory cell infiltration consisting of lymphocytes and macrophages (7 days; hematoxylin-eosin; original magnification, ×50). **b**, **c** Thin fibrous capsule and infiltration of a few chronic inflammatory cells (15 and 30 days; hematoxylin-eosin, ×50). G-F **d** Thick fibrous capsule and severe inflammatory cell infiltration consisting of lymphocytes and macrophages (7 days; hematoxylin-eosin; original magnification, ×50). **e** Thick fibrous capsule and moderate inflammatory cell infiltration (15 days; hematoxylin-eosin; original magnification, ×50). **f** Thin fibrous capsule and mild inflammatory cell infiltration (30 days; hematoxylin-eosin; original magnification, ×50). G-MB/pH 7. **g** Thick fibrous capsule and severe inflammatory cell infiltration consisting of lymphocytes and macrophages (7 days; hematoxylin-eosin; original magnification, ×50). **h** Thick fibrous capsule and moderate inflammatory cell infiltration (15 days; hematoxylin-eosin; original magnification, ×50). **i** Thin fibrous capsule and moderate inflammatory cell infiltration (30 days; hematoxylin-eosin; original magnification, ×50). G-MB/pH 1. **j**, **k** Thick fibrous capsule and moderate inflammatory cell infiltration consisting of lymphocytes and macrophages (7 and 15 days; hematoxylin-eosin; original magnification, ×50). **l** Thin fibrous capsule and mild inflammatory cell infiltration (30 days; hematoxylin-eosin; original magnification, ×50)

Methylene blue is used in aPDT as a photosensitizer for root decontamination [37] and could interact with connective tissue in different ways. In subgingival SRP, the curettes reach the connective tissue and facilitate the input of methylene blue

Table 1 Inflammatory scores and size of the fibrous capsule at different observation times

Time/*p* value	Material	Scores				Median	Capsule		*n*
		0	1	2	3		Thin	Thick	
7 days $p = 0.0014$	G-T	0	4	3	0	1[a]	4	3	7
	G-F	0	0	3	4	3[b]	2	5	
	G-MB/pH 7	0	0	3	4	3[b]	2	5	
	G-MB/pH 1	0	1	3	3	2[a]	2	5	
15 days $p = 0.0051$	G-T	0	5	2	0	1[a]	5	2	7
	G-F	0	1	5	1	2[a]	2	5	
	G-MB/pH 7	0	1	4	2	2[a]	2	5	
	G-MB/pH 1	0	1	5	1	2[a]	2	5	
30 days $p = 0.0010$	G-T	4	3	0	0	0[a]	6	1	7
	G-F	0	4	3	0	1[a]	4	3	
	G-MB/pH 7	0	3	4	0	2[b]	4	3	
	G-MB/pH 1	0	4	3	0	1[a]	5	2	

Different superscript letters indicate statistical difference among the groups ($p \leq 0.05$)

into it [38]. Other ways methylene blue could interact with connective tissue are after periodontal surgery, such as surgical SRP [39] and subepithelial connective tissue graft [40].

Its use at an acidic pH level could increase the effectiveness of these therapies, since the acidic substances could improve healing and periodontal regeneration after an SRP procedure, as shown by Nanda et al.'s [32] study in vitro; however, the clinical benefits are discussed in the literature.

In a systematic review by Mariotti [41], it was concluded that the use of citric acid, tetracycline, or EDTA to modify the root surface provides no clinically significant benefit for regeneration in patients with chronic periodontitis. However, a meta-analysis found 16 studies that applied tetracycline as an adjunct to SRP, finding benefits in reducing probing depth after statistical analysis [42].

Clinical results without benefits can be explained by Lan et al. [43] because low pH substances such as citric acid exhibit a potentially necrotizing effect, inducing cell death and cell structural changes within 30-s intervals, generating superficial necrosis. However, the present study did not find a necrotizing effect of the methylene blue solution at a pH level of 1.0. Cellular and tissue necrosis caused by exposure to acidic substances may not be uniquely explained due to its low pH, since several different characteristics, such as volume, acid strength, concentration, and origin (organic or inorganic), can positively or negatively affect this process.

Thus, aPDT benefits through increasing cell proliferation on infected wounds and stimulates an angiogenic response [44, 45], reduces the amount of pro-inflammatory interleukins (IL-1α, IL-1β, IL-2), induces proliferation of marked fibroblasts [46, 47], and increases FGF2 expression enhancing the repair process. These benefits could be increased by methylene blue at an acidic pH level once it is exposed to the collagen matrix [20, 48]. However, other studies must be performed applying methods to evaluate their photochemical interactions.

G-MB/pH 1 group did not show statistical difference in any experimental period compared to the positive control group. On the other hand, the G-MB/pH 7 group showed greater presence of inflammatory cell infiltration, mainly lymphocytes and macrophages, present in a fibrous capsule at 7 and 30 days. All groups showed an improvement in the inflammatory process over long time, but the G-MB/pH 7 group presented more concentration of the inflammatory infiltrate.

Previous studies indicate that alkaline and acidic conditions influence negatively inflammatory cells; however, in this study, an acidic substance had better results [49–51]. Saghiri et al. 2013 analyzed the inflammatory response of Geristore (resin glass ionomer) in subcutaneous tissue of rats and reported that the fact of it being a substance of low pH may explain the induction of significantly more inflammation [49]. However, as mentioned by the authors [49], Geristore releases five monomers of Bis-GMA, Bis-DMA, TEGDMA, UDMA, and Bisphenol A. As resin monomers are reported to show cytotoxic effects [52, 53] and might be capable of tumor initiation at relatively low concentrations [54], this may be listed as another plausible explanation for the exacerbated inflammatory response.

Citric acid was the substance used to reach pH 1 in the methylene blue solution. Citric acid was the less toxic and the most effective among all acidic substances tested by Register and Burdick 1975 [55]. Moreover, Prasad et al. 2012 [56] concluded that citric acid is more effective in removing the smear layer, exposing the root collagen and causes greater degree of morphological alterations (mean diameter, mean total surface area occupied by dentinal tubules). These characteristics may allow cementum to form within the tubules. Also, the exposed root collagen fibers can splice with the collagen fibrils of a soft tissue graft or flap (collagen splicing) resulting in collagen adhesion.

These different results of acidic substances may also be explained by acid strength, which is the ability of the acids to lose a proton (H^+) [57, 58]. The acid strength could be adjusted by different factors, for instance, form of physical state, topology structure and morphology, and crystallinity in addition to the chemical compositions [57]. However, other animal studies and subsequent clinical studies are necessary to evaluate the use of methylene blue at pH 1.0.

Conclusion

Within the limits of this study, it is concluded that methylene blue at pH 1.0 provides better biocompatibility than at pH 7.0.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding The work has no source of funding.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent For this type of study, formal consent is not required.

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