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Methylene blue-mediated antimicrobial photodynamic therapy can be a novel non-antibiotic platform for bovine digital dermatitis

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ABSTRACT

Background: Bovine digital dermatitis (BDD) is one of the most important diseases that effect dairy cows. Methylene blue-mediated antimicrobial photodynamic therapy (MB-APDT) emerges as a promising technique to treat superficial infections in bovines.
Methods: Twenty BDD lesions located at the skin horn transition of the claw of pelvic limbs of 16 cows were treated by MB-APDT, using a red LED cluster ($\lambda = 660$ nm, irradiance $=60$ mW/cm^2, exposure time $= 40$ s) combined with topical application of MB at 0.01 %; or by topical application of OXY (500 mg in 20 % solution). Each lesion was treated twice with an interval of 14 days. Lesions were weekly evaluated until day 28 by clinical analysis and by histological examination on days 0 and 28.
Results: Both treatments led to a similar reduction of lesions area. At day 28, three lesions treated by OXY did not present completely recovery, whereas no lesions were observed in MB-APDT group. OXY resulted in a slight increase in type I and III collagen levels, while MB-APDT led to a significant increase in the total area of both collagen types. An abundant number of spirochetes were histologically observed in all lesions before treatments. On the 28th day, five lesions treated by OXY still presented a slight number of spirochetes, whereas in MB-APDT group no spirochetes were evidenced.
Conclusion: Our findings suggest that MB-APDT is more effective than OXY and could be used in Veterinary practice to fight BDD.

1. Introduction

Bovine digital dermatitis (BDD) is the major infectious disease that causes lameness in cattle worldwide [1–3]. It is characterized by the presence of ulcerative or proliferative skin lesions on the bulbs of the heel or in the interdigital cleft, being frequently associated with pain [1,4,5]. Together with lameness, BDD could lead to nonspecific symptoms such as weight loss, reduction of milk production and reproductive problems, increasing animal production costs related to its prevention and treatment [6–8].

Over the past decades, even though different microorganisms have been identified in BDD lesions, there is a global consensus indicating that *Treponema* species are the most commonly associated pathogens [1,2,9]. Considering the infectious nature of BDD, antibiotic therapy has been adopted as the gold standard option [1,10]. In this regard, treatments with antibiotics such as tetracyclines and lincosamides have been the most performed in dairy farms [10–13].

Oxytetracycline (OXY) is a broad-spectrum tetracycline antibiotic,

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indicated for the treatment of infections caused by a wide range of Gram-positive and Gram-negative microorganisms [14]. Specifically for dairy cattle, OXY has been commonly employed to treat various infectious diseases and is considered a first-choice antibiotic for BDD in many countries [15,16]. However, even though tetracyclines have been topically applied, contamination of milk by antibiotic residues may occur [17].

Antimicrobial photodynamic therapy (APDT) has been broadly investigated as a non-antibiotic alternative to treat superficial infections in Medical Sciences [18,19]. More recently, APDT also triggered special interest of veterinarians for the treatment of infectious diseases of livestock animals [20–24]. APDT combines the use of a photosensitizer (PS) agent that is activated by light to inactivate microbial cells [25,26]. Upon light activation, the PS reacts with molecular oxygen or biomolecules yielding large amounts of reactive oxygen species (ROS) that kill microbial cells by oxidative stress [25,26]. Since ROS do not present target specificity, the selection of APDT-resistant microorganisms seems unlikely [25–27]. Furthermore, broad-spectrum antimicrobial activity (*i.e.,* bacteria, fungus, protozoa, virus, prions and pathogenic algae) can be achieved without harmfulness for the host cells [26,28]. Methylene blue (MB) is a hydrophilic cationic dye that has been widely used as a safe and effective PS against a broad range of pathogenic microorganisms [26,29–33].

In this study, we investigated the use of MB-APDT as a novel non-antibiotic strategy for the treatment of BDD. Combining clinical and histological analysis, we compared the outcome of MB-APDT and OXY by evaluating the presence of spirochetes and as well as collagen type I and III.

2. Materials and methods

2.1. Animals

This study was approved by the local animal care and use committee of the School of Veterinary Medicine and Animal Science from University of São Paulo (São Paulo, Brazil, reference number 3633030214).

Fifty-eight multiparous Holstein cows presenting lameness were screened for BDD. The animals were from two different dairy farms (-22.943341, -46.600468 and -23.4339573326, -45.6925229052) located in Sao Paulo state, Brazil. The animals were kept in free stall systems with sand bedding under semi-intensive management. Locomotion score was determined as proposed by Sprecher et al. [34] as follows:

- score 1 (normal): The cow stands and walks with a level-back posture. Her gait is normal.
- score 2 (mildly lame): The cow stands with a level-back posture but develops an arched-back posture while walking. Her gait remains normal.
- score 3: (moderately lame): An arched-back posture is evident both while standing and walking. Her gait is affected and is best described as short-striding with one or more limbs.
- score 4: (lame): An arched-back posture is always evident and gait is best described as one deliberate step at a time. The cow favors one or more limbs/feet.
- score 5: (severely lame): The cow additionally demonstrates an inability or extreme reluctance to bear weight on one or more of her limbs/feet.

Twenty BBD lesions located at the skin horn transition of the claw of pelvic limbs of 16 cows were randomly distributed in two treatment groups (only one type of treatment per animal). Forty-two animals presenting systemic comorbidities or other hoof lesions were excluded to avoid influences on healing process. Additionally, a control group, with ten healthy (lesion free) and non-claudicating cows was used for a comparative histological analysis with the treated groups. Claw trimming was performed in all animals at the end of follow-up period.

2.2. MB-APDT

Ten lesions were covered with MB (Sigma-Aldrich, USA; 0.01 %) and, after 5 min of pre-irradiation time, lesions were irradiated by a red LED cluster (Vetlight, DMC®, Brazil; $\lambda = 660 \pm 10$ nm, output power- = 2.1 W, irradiance =60 mW/cm^2, cluster area = 13.2 cm^2, Fluence = 6.4 J/cm^2, energy = 84 J, exposure time = 40 s). Each lesion was treated twice with an interval of 14 days. Lesions were covered by bandages and changed once a week to reduce environmental contamination.

2.3. OXY treatment

The topical OXY treatments were performed in the same schedule as in MB-APDT group, *i.e.*, twice with an interval of 14 days. Lesions received 500 mg doses of OXY (Tetrabac 20 %, Bayer, São Paulo, Brazil) that were delivered in gauze bandages directly over the lesions. Similar to the MB-APDT group, lesions were covered by bandages that were changed once a week.

2.4. Follow-up period

Complete cure of the lesions was accomplished when the following was observed: absence of clinical lesions; no histological changes in the epidermis (ulcer and/or hyperplasia), and absence of spirochetes.

2.5. Clinical evaluation of bovine digital dermatitis lesions

Cows and their lesions were weekly evaluated until the 28th day. Macroscopic classification of the lesions was performed at day 0 according to the lesion score proposed by Döpfer et al. [4]. Briefly, this clinical classification is presented as follows: M1) early stage of digital dermatitis with a circumscribed granulomatous area; M2) classical ulceration, which is an area close to the coronary band affecting skin or horn; M3) classical ulceration in the process of healing; M4) cutaneous lesions are hyperkeratotic and can present themselves with a proliferative aspect.

Digital photographs were taken in days 0, 7, 14, 21 and 28 using a digital camera (Nikon Coolpix P500). The camera was positioned in standard orthogonal angle at approximately 30 cm of distance from the lesions. We used Image J software (NIH, USA) to quantify areas of lesions (cm^2) based on a 1 × 1 cm reference object (black graph paper) placed outside the border of each lesion.

2.6. Histological evaluation of the lesions

Prior to the biopsies, trichotomy, antisepsis (2% polyvinylpyrrolidone-iodine) and intravenous regional anesthesia were performed. For the anesthetic procedure, 20 mL of lidocaine hydrochloride without vasoconstrictor (2%, w/v) was administered in the lateral plantar digital vein. Samples were collected from different peripheric points by punch biopsy (6 mm) at days 0 and 28.

The collected fragments were fixed in neutral buffered formalin (10 %), dehydrated and embedded in paraffin at 58 C°, and then sectioned into consecutive 5 μm-thick slices with semi-automatic microtome (Leica, Wetzlar, Germany). Hematoxylin and eosin (H&E), Warthin-Starry, and Picrosirius red stains were used to evaluate the healing process and identify spirochetes. Histological slides were evaluated by a veterinary pathologist in a single-blinded manner.

H&E analysis was proposed to qualitatively analyze the superficial and deep dermis for presence of ulcer, hyperplasia, and to determine the type of lining epithelium. Warthin-Starry staining was performed to detect the presence/absence of spirochetes. In addition, the amount of spirochetes on slides was scored from 0 to 3 (absent, slight, moderate and severe).

The methodology of Picrosirius red staining was performed

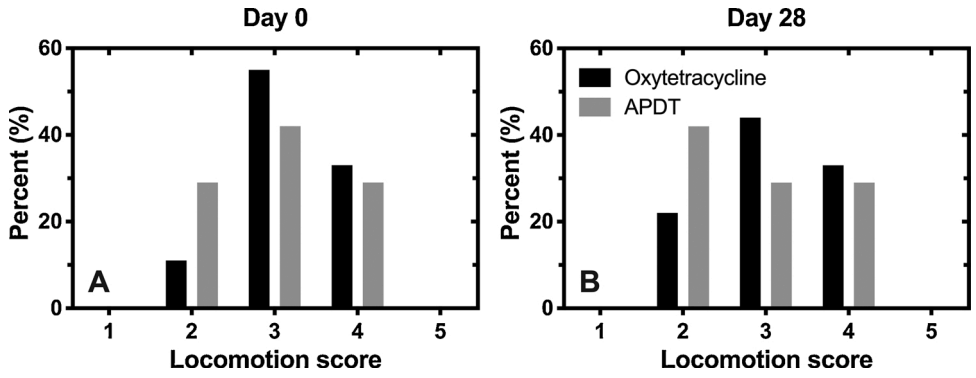

Fig. 1. Distribution of the locomotion score of Holstein cows (N = 16) affected by bovine digital dermatitis at days 0 (before treatments) and 28 (after treatments).

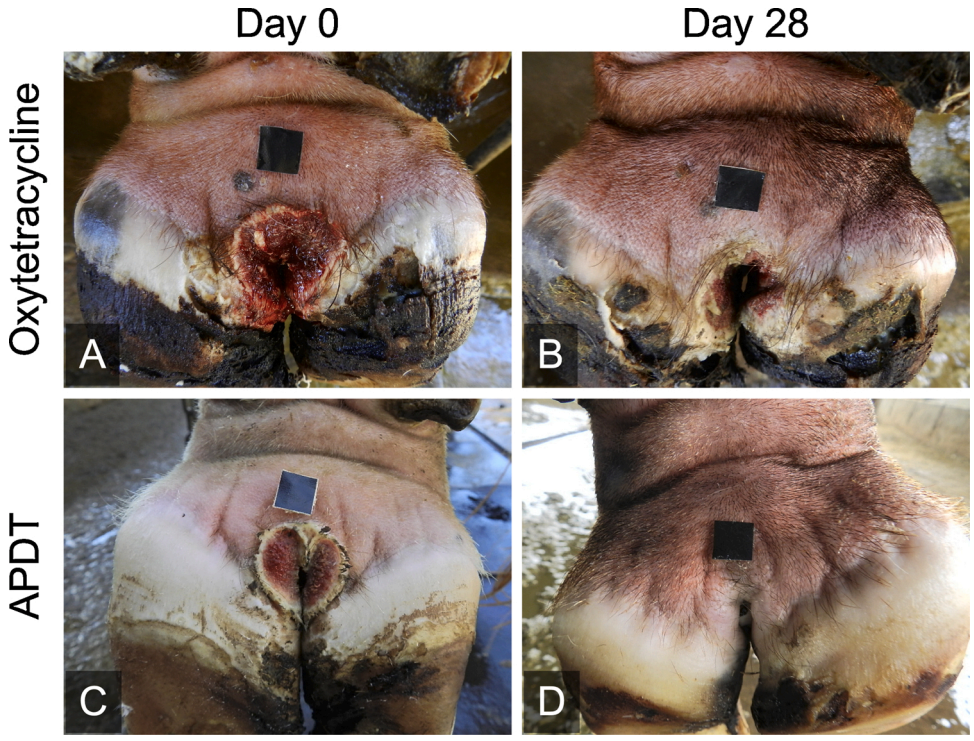

Fig. 2. Representative images of BDD lesions treated by OXY or MB-APDT. Lesion images from day 0 are presented in **A** and **C**; Lesion images from day 28 are presented in **B** and **D**.

according to Lattouf et al. [35]. A polarized light microscope was used to classify type I collagen – characterized as more mature, thicker and strongly birefringent fibers – and type III collagen – characterized as undeveloped, thinner non-refringent fiber. Ten fields in the deep dermis were randomly selected for each slice under 400x magnification. A microscope (Eclipse Nu, Nikon) coupled to a Nikon camera (DSU3) was used to evaluate the images projected then onto a monitor *via* software (NIS Elements, Nikon) for image acquisition. The photomicrographs were evaluated by Image Pro Plus® software (version 4.5, Media Cybernetcs Inc., Silver Spring, USA), previously calibrated for the 400x magnification in order to determine the correlation between pixels and μm.

2.7. Statistical analysis

Quantitative data were analyzed by statistical software SPSS 24 (IBM Corporation, Armonk, NY - USA) and for statistical inferences a significance level of 5% was adopted. We used non-parametric tests because the Shapiro-Wilk test indicated that variables did not present normal distributions. We adopted median and 95 % confidence interval to present all data. Mann-Whitney's test was performed to evaluate effect of treatment. Friedman's test was used to evaluate the difference between times, followed by Wilcoxon's test. To control type I error we proceed a Bonferroni adjustment. Finally, to compare total and relative area of type I and type III collagens between treatment before and after was applied Kruskal-Wallis test, followed by Mann-Whitney *U* test as *post hoc*, whereas Wilcoxon signed rank test evaluated differences between times for the same treatment.

3. Results

Regarding the locomotion score, at day 0, one animal presented score 1, three score 4 and five score 5, in the OXY-treated group; in MB-APDT group, two animals presented score 2, three score 3, and two score 4. At day 28, only two animals (one animal from each group) improved locomotion score, both from score 3 to score 2 (Fig. 1).

In both treatments it was possible to observe the reduction of lesion areas, however, there was no significant difference between the

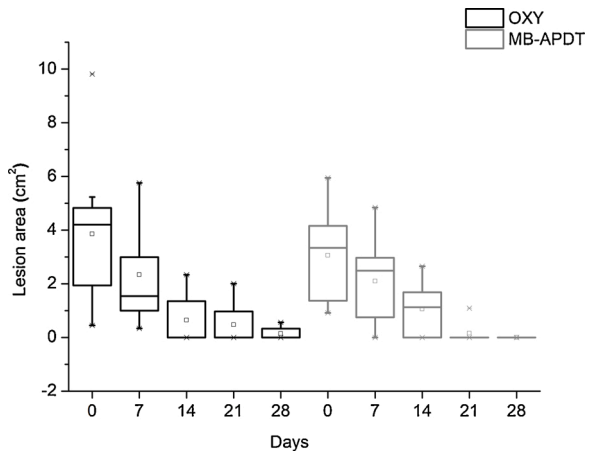

Fig. 3. Bovine digital dermatitis lesion areas treated by OXY or MB-APDT over a 28 days follow-up. Squares represent mean values; boxes represent 25 and 75 percentile; bars indicate 95 % CI; X denotes minimum and maximum values; the horizontal lines inside the boxes correspond to the medians. N = 10/group.

treatments ($p > 0.05$). After 28 days, all lesions have significantly regressed for both treatments ($p < 0.05$). However, three animals treated with OXY remained with small lesions while none of the MB-APDT-treated animals presented any lesions at day 28 (Figs. 2 and 3).

In the OXY group, we observed two M2 (20 %), six M3 (60 %) and two M4 lesions (20 %), while in the MB-APDT group, three M2 (30 %), two M3 (20 %) and five M4 lesions (50 %) were observed. No early-stage lesions (M1) were identified in either group.

Using H&E staining we compared the clinical classification proposed by Döpfer et al. [4] with histological observations at day 0. In this sense, it was possible to see that the H&E stained samples presented similar characteristics with clinical classification. At day 28, all APDT-treated lesions (100 %) and seven (70 %) of OXY-treated lesions were considered cured under clinical and histological points of view (Fig. 2 and 4).

Picrosirius red staining revealed important information regarding the amount of collagen before and after treatments. After MB-APDT there was an increased deposition of type I and type III collagen ($p = 0.007$) (Figs. 5 and 6). Contrarily, lesions treated with OXY presented a similar occupied area by type I collagen at days 0 and 28 (Figs. 5 and 6). At day 28, the collagen type I and III area was also significantly higher in MB-APDT-treated lesions compared to OXY treatment ($p = 0.009$). There was no significant increase in the area occupied by type III collagen in each group comparing days 0 and 28. However, the total area of type III collagen was significantly higher in MB-APDT-treated group at day 28 when it was compared to OXY group (Fig. 6).

Warthin Starry staining revealed spirochetes in all lesions at day 0, suggesting that *Treponema* spp. were the actual predominant infectious agents. Additionally, spirochetes were not detected in healthy animals (score 0), which contribute to the validation of Warthin Starry staining for this purpose (data not shown). Semi-quantitative analyses revealed that all lesions (from both groups) presented score 5, indicating an abundant number of spirochetes before treatments (day 0). After the treatments, it was possible to notice a lower number of spirochetes for both groups (Fig. 7) as well as a reduction of the lesions' area (Fig. 3). Thus, we can assume that both treatments presented antimicrobial activity. Interestingly, on day 28, seven lesions (70 %) treated with OXY presented total regression of lesion area, however, in five (50 %) it was still possible to visualize a slight number of spirochetes (score 1). On the other hand, all lesions treated with MB-APDT showed complete healing and absence of spirochetes (score 0) during this same period (Figs. 3 and 7).

4. Discussion

In this study, we compared two treatments for BDD by clinical and histological analysis. All lesions presented spirochetes, whereas such bacteria were not evidenced in the skin of healthy animals. Our findings show that spirochetes are frequently involved in the pathogenesis of BDD, corroborating with studies presented by Döpfer et al. [4] and Evans et al. [36].

To date, there is no consensus on the best treatment for BDD. Studies

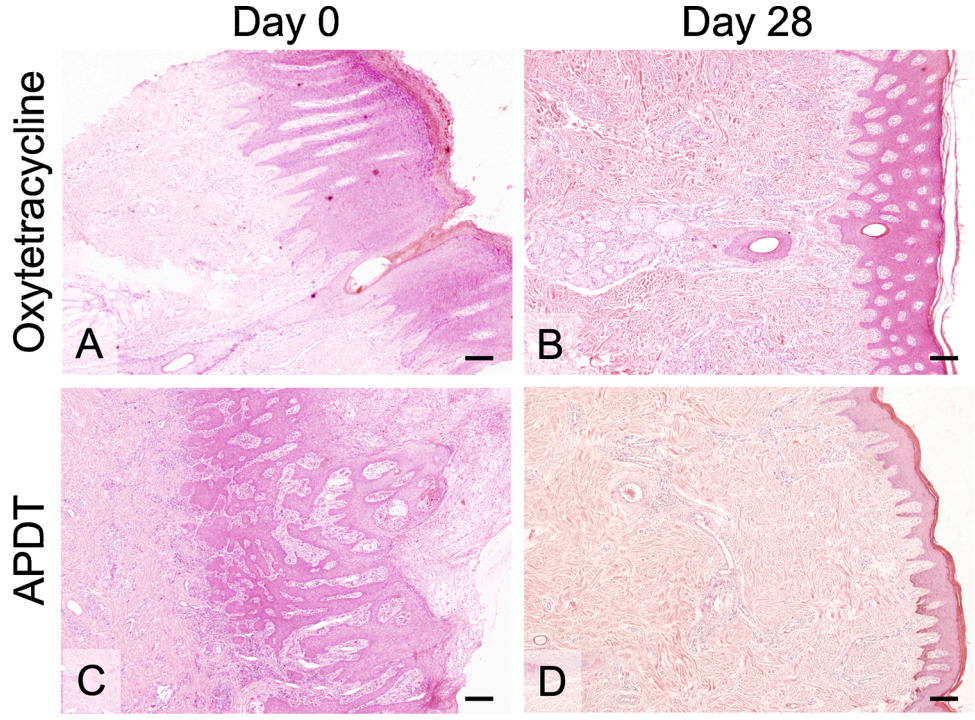

Fig. 4. Representative photomicrographs of BDD lesions treated by OXY or MB-APDT stained by H&E. Histopathological features of digital dermatitis skin biopsy at day 0 (**A** and **C**) and day 28 (**B** and **D**). Scale bar corresponds to 200 μm.

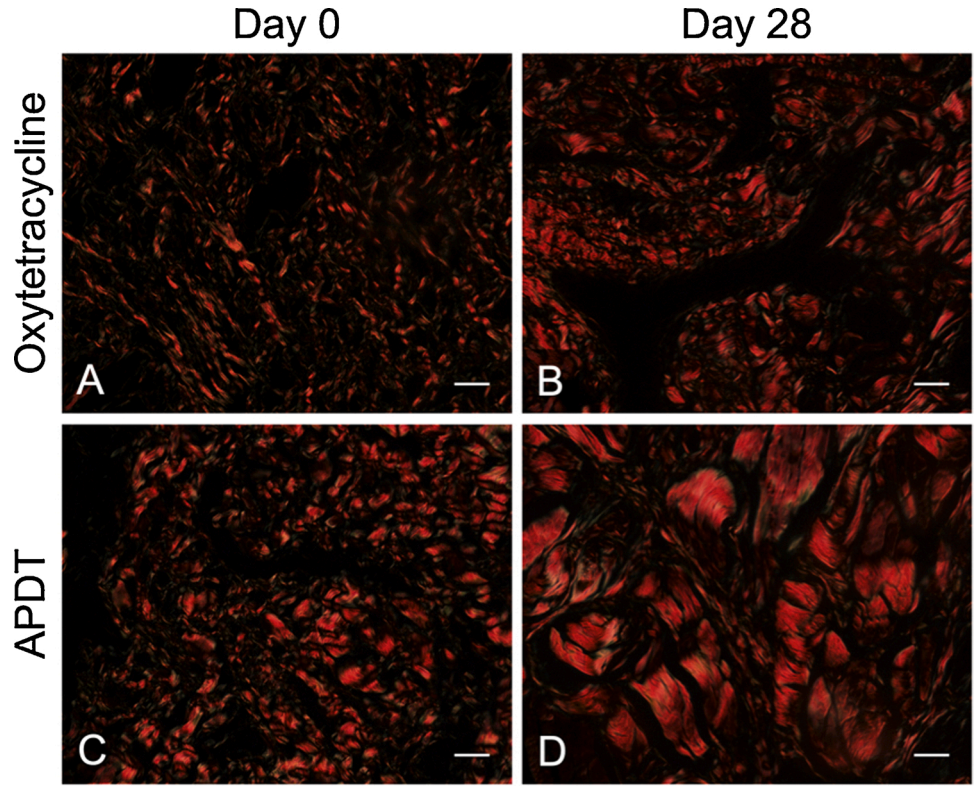

Fig. 5. Representative picrosirius red staining tissue photomicrographs of collagen type I. Oxytetracycline-treated lesions at days 0 (**A**) and 28 (**B**) present a slight decrease in type I collagen, while APDT-treated lesions showed significant increase of collagen type I from day 0 (**C**) to day 28 (**D**). Scale bars correspond to 50 μm.

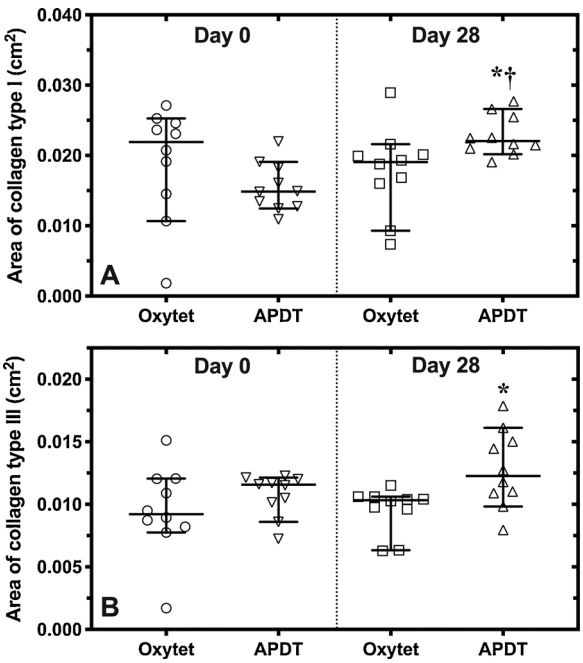

Fig. 6. Comparison between total areas of collagens type I (**A**) and type III (**B**) before and after treatments. Data points are represented by medians and 95 % CI. Asterisks (*) indicate significant statistical difference between same moment of different treatments (Mann-Whitney Test) and dagger (†) indicates significant difference between different moments of the same treatment (*i.e.*, day 0 and 28, Wilcoxon test).

conducted over the past decade have suggested that cure rates of topical antibiotics may range between 60 and 70 % [13]. According to Berry et al. [12] treatments with lincomycin and OXY resulted in a cure rate of 73 and 68 %, respectively. Although these treatments were considered effective, these numbers still are low when compared to infections caused by *Treponema* spp. in humans [1]. In our study, both treatments led to reduction of lesion area. However, regarding the reduction of spirochetes infection burden, MB-APDT showed more pronounced results. In fact, at day 28, a slight number of spirochetes (score 1) was identified in five (50 %) lesions treated with OXY whereas no spirochetes were observed in any of the MB-APDT-treated lesions. These findings are remarkably important because it may suggest that treatments with OXY do not guarantee complete inactivation of spirochetes.

In line with it, *in vitro* studies have demonstrated that *Treponema* spp. isolated from BDD lesions (mostly M4 presentation) can be kept in a dormancy/dormant form during antibiotic treatments [37,38]. The absence of spirochetes in the deep epidermis is required for a full recovery because lesions could present clinical improvement, but if these etiological agents persist complete cure may not occur. Thus, persistence of spirochetes could lead to the recurrence of lesions and could also contribute for the silent dissemination of these bacteria in the herds by asymptomatic carriers. Therefore, clinical and histopathological evaluations should be analyzed together to strengthen the real prognosis of a successful treatment.

Another important point refers to the abundance and composition of collagen fibers. Interestingly, treatment with OXY did not result in any changes on abundance of collagen type I and III, while APDT showed a significant increase in the total area of both collagen types. In this respect, other studies have pointed out that treatment with tetracyclines may present different results on BDD healing time, and in some circumstances, with lower healing rates when compared to other drugs [39]. Unfortunately, the lack of methodological conformity of most of the studies that investigated antibiotic treatments for BDD, including tetracycline's concentration and administration interval, makes

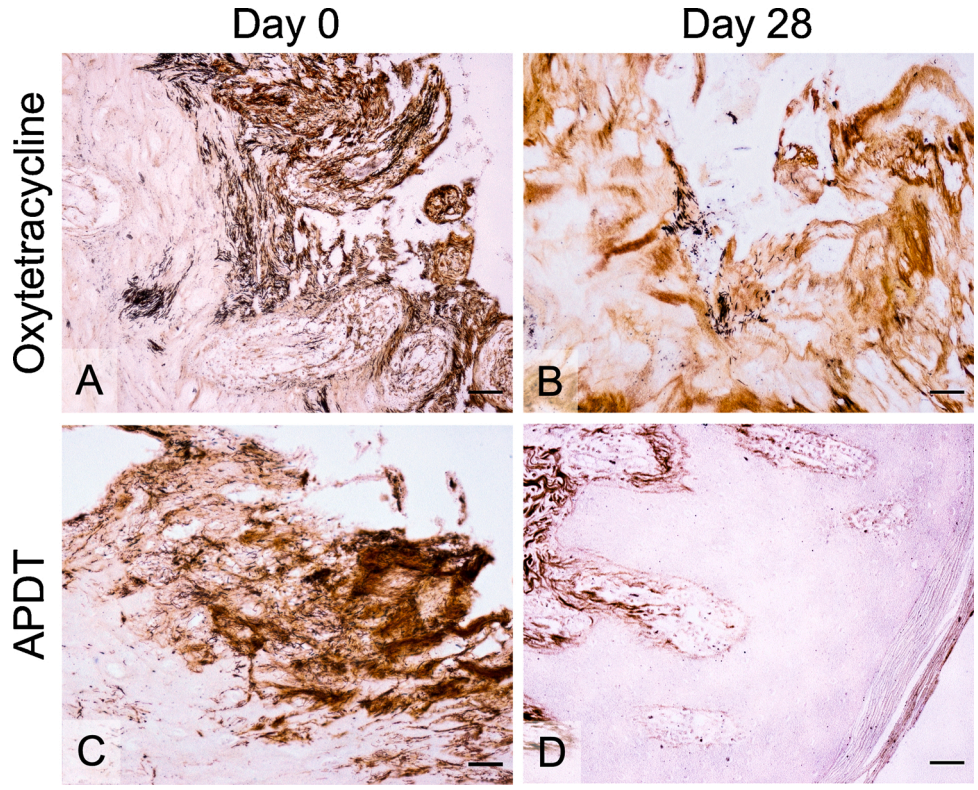

Fig. 7. Representative photomicrographs from BDD skin biopsy stained with Warthin-Starry method to evidence spirochetes in dark brown color. In **A-C**, it is possible to observe long spiral-shaped features that are compatible with spirochete morphology. In **D**, no spirochetes are observed. Scale bars correspond to 25 μm.

comparisons imprecise [1,39–41].

In our study, both treatments were administered twice with an interval of 14 days. At the end of this period, APDT promoted better healing of the lesions. Substantial evidences are reported that MB-mediated APDT doses are sufficient to inactivate more than 99.9 % of clinically relevant pathogens with safety for host cells, including neutrophils, keratinocytes and fibroblasts [28,42–44]. Therefore, MB- APDT may represent an effective and low-risk antimicrobial approach for the treatment of localized, superficial infections in veterinary medicine.

Other studies have investigated therapeutic alternatives for BDD, such as salicylic acid and solutions containing copper sulphate, highlighting the urgent need for the development of novel non-antibiotic approaches [39,41,45]. However, despite the efficacy of copper sulphate, it could also lead to environmental damages. Hence, the topical use of antibiotics still remains as the treatment of choice for large animal clinicians [1].

The risk of contamination of milk and meat by antibiotic residues and the increasing rates of antibiotic-resistant bacteria in cattle have been raised serious public health concerns worldwide [46]. Although, these drugs have demonstrated contradictory *in vitro* efficacy against BDD-associated *Treponema* spp. [40], the topical administration of tetracyclines has been extensively used to treat BDD [1]. In addition, a recent study demonstrated that weekly treatment with tetracycline was no more effective than saline and discouraged the use of this antibiotic for BDD [47]. More worryingly, a recent study demonstrated that extra-label administration of topical tetracycline for BDD could result in the contamination of milk by tetracycline residues [17].

Facing these concerns, MB-APDT is a promising therapeutic option to treat superficial infections in dairy cattle, mostly because antibiotic-resistant bacteria are equally susceptible as their non-resistant counterparts [25,26,32,33,48]. Moreover, *in vitro* studies have already demonstrated that *Treponema* spp. isolated from periodontitis and peri-implantitis are susceptible to MB-APDT [49,50].

The major limitations of this study are that we treated different stages of BDD and we did not identify the genera/species of spirochetes. Another point refers to a longer follow-up period after treatments, which could elucidate possible recurrences of lesions. Also, difficulties in implementing MB-APDT for BDD can be related to operator training. However, we particularly believe that the cost and effectiveness of this procedure may also encourage its adoption for mainstream use. Additionally, MB-APDT paves the way to the sustainable production of antimicrobial-free food.

5. Conclusion

In summary, our study demonstrates that MB-APDT offers an effective therapeutic approach to treat BDD. Our protocol presented better results than OXY for the elimination of spirochetes, production of collagen, and wound healing. This therapeutic platform can significantly reduce the use of antimicrobial drugs in localized infections of cattle. We hope to motivate further studies to develop optimized protocols of MB-APDT for BDD.

Ethics approval

The project was granted ethical approval by the local animal care and use committee of the School of Veterinary Medicine and Animal Science from University of São Paulo (São Paulo, Brazil, reference number 3633030214).

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Declaration of Competing Interest

The authors report no declarations of interest.

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