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Methylene blue-mediated antimicrobial photodynamic therapy on chicken semen

GA Novaes [a,b], MH Blank [a], TM Yoshimura [c], MS Ribeiro [c], RJG Pereira [a,*]

[a] Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP, SP 05508-900, Brazil
[b] Cobb-Vantress Brasil Ltda., Guapiaçu, SP, Brazil
[c] Center for Lasers and Applications, Nuclear, and Energy Research Institute (IPEN-CNEN), São Paulo, SP, Brazil.

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ABSTRACT

Background: Artificial insemination is widely employed in poultry, but high degrees of bacterial contamination are often observed in semen because of its passage through the cloaca. Consequently, most semen extenders for birds have antibiotics that could aggravate bacterial resistance.
Methods: We evaluated the potential of antimicrobial photodynamic therapy (PDT) as an alternative to the use of antibiotics, and assessed whether changes in concentration and incubation time with methylene blue (MB), radiant exposure, and irradiance of light affect spermatozoa activity and bacteria in chicken semen.
Results: Incubation with MB (< 25 μM) did not alter sperm motility, regardless of the pre-irradiation time (PIT, 1 or 5 min). Following 1 min of PIT with MB at 10 μM, samples were irradiated for 30, 60, 120, and 180 s at irradiances of 44, 29, and 17 mW/ cm^2 (660 nm LedBox). MB and light alone did not interfere with the analyzed parameters. However, when both factors were associated, increases in light dose led to greater reductions in sperm parameters, regardless of the irradiance used. Besides, PDT conditions that were less harmful to spermatozoa were not able to significantly reduce bacterial colonies in chicken semen.
Conclusions: A failure in MB selectivity could explain unsuccessful bacterial reduction following PDT. Further research involving other photosensitizers or conjugating molecules to MB to target microbial cells is needed for PDT application in poultry breeders.

1. Introduction

The use of reproductive biotechnologies in livestock represents a major milestone for world agriculture since they have enabled greater food production in a smaller demographic and temporal space [1,2]. In this regard, artificial insemination (AI) offers many advantages such as genetic improvement intensification, disease control, application of cross-breeding schemes, rationalization of reproductive management, and the possibility of using material from genetically superior animals even after death, through semen freezing. Given these benefits, all commercial meat turkeys are bred artificially due to extremely heavy males that are ineffective for natural copulation [3–5]. However, although AI provides good outcomes for turkey breeders, the passage of the ejaculates through the cloaca during semen collection as well as the practice of pooling semen from several males make the transmission of intestinal microorganisms among individuals an inherent risk of the technique [4,6]. This issue is particularly relevant considering the epidemiological and economic impact of some sexually transmitted pathogens such as *Salmonella, Campylobacter* e *Mycoplasma* on the poultry industry [7–11]. In addition, it has been shown that certain intestinal bacteria in seminal plasma can significantly reduce sperm motility in poultry [6].

In an attempt to mitigate the risks of contamination, several commercial extenders for poultry have antibiotics in their composition such as gentamicin and tylosin [12]. Yet, the extensive use of antibiotics poses a major threat to public health since it contributes to the selection and propagation of multidrug-resistant bacteria [13]. This underlines the need for alternative methods to control microorganisms in poultry semen so that animal production goals align with those of food safety. In this regard, one approach that has attracted great interest among researchers is antimicrobial photodynamic therapy (PDT), which consists of combining a photosensitizing substance, a light source, and oxygen to

* Corresponding author.
E-mail address: ricpereira@usp.br (R. Pereira).

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form reactive oxygen species (ROS), leading target cells to death by oxidative stress [14]. As a therapeutic resource, PDT offers dual selectivity, i.e. light and preferential location of photosensitizers (PSs) in microorganisms, works on multi-resistant microorganisms, and does not induce resistance due to its mechanism of action [14,15]. It has been proven that once suitable conditions are established (e.g. PS class and concentration, pre-irradiation time (PIT), and light parameters), PDT can be effective to reduce Gram-positive and Gram-negative bacteria, protozoa, viruses, and fungi [14].

Although PDT has been largely investigated for microbial control *in vitro*, its impact on seminal samples is poorly explored. Herein, we assessed the impact of some PDT protocols using different conditions for methylene blue (MB, e.g. incubation time and concentration) and light parameters (e.g. irradiance and radiant exposure, i.e., light dose) on chicken spermatozoa and bacteria found in ejaculates. Our choice for MB was based on its large use to fight local infections in Veterinary Medicine [16].

2. Material and methods

All procedures performed on animals were approved by the Ethics Committee of the College of Veterinary Medicine and Animal Sciences – São Paulo University (protocol No 3579040418).

2.1. Experimental design

This study was divided into three major phases: (1) MB dark toxicity; (2) light dosimetry; and (3) microbiological analyses. In phase 1, we first established the degree of toxicity of the PS on chicken spermatozoa through the incubation of semen pools in the dark (n = 8) with different concentrations of MB. Total and progressive motilities were used in this assay as indicators of sperm viability. After defining the least toxic concentration of MB, we assessed the effects of MB on sperm kinetics for 1 and 5 min to find an ideal pre-irradiation time (PIT, i.e., the period between the PS application and the beginning of the irradiations). Upon completion of this phase, different light parameters regarding irradiance and light dose were tested to determine the least impacting PDT conditions for sperm physiology. To this end, 8 semen pools were divided into four groups: CON, MB, LIGHT, and PDT. In this phase, LIGHT and PDT groups were irradiated using a LedBox 660 nm (Biolambda, SP, Brazil) for 30, 60, 120, and 180 s under irradiances of 17, 29, and 44 mW/cm^2. Additionally, we evaluated if PDT had a residual and irreversible effect on spermatozoa (i.e., if these cells could restore their motion parameters over time). Therefore, irradiances of 17 and 29 mW/cm^2 were used for 0 (control), 60, 120, 180, and 240 s (6 semen pools for each irradiance). Finally, once the best PIT and light dosimetry were set, we examined the impact of this protocol on microorganisms present in chicken semen through a microbial culture of the samples in Mueller-Hinton agar and Macckonkey agar (for total coliforms and Gram-negative bacteria, respectively) before and after PDT (6 semen pools in triplicate, totaling 18 replicates in this third phase).

2.2. Animals

Twenty Dekalb White roosters (33-week-old) donated by Hendrix Genetics Ltda – Brazil were housed in cages under standard management practices, which included individual cages (25×45×44 cm), 14L: 10D photoperiod, water *ad libitum*, and twice-a-day feeding regimen (120 g/day). A standard commercial diet was fed to the animals throughout the study (2750 kcal ME/Kg, 17% CP, and 3% Ca–Presence Postura 17, Presence Nutrição Animal, Brazil). Semen collection was performed twice a week, starting one month before the experiment (conditioning period). Afterward only males that responded well to dorso-abdominal massage (i.e., high volume and sperm concentration and low contamination with urine and feces) were maintained throughout the study.

2.3. Semen collection and processing

After collection, clean ejaculates (i.e., without visible feces or urine contamination) from 2-3 males were pooled, and sperm concentration was immediately ascertained by spectrophotometry (Accuread rooster and turkey photometer, IMV, France). A final concentration of 1 billion spermatozoa per mL was achieved by diluting these pools with the Lake extender. During the pre-irradiation phase, 8 semen pools were divided into 10 aliquots with different MB concentrations (0, 5, 10, 15, 20, 25, 50, 75, 100 e 150 μM – Sigma Aldrich, Missouri, USA). Following 5 min of incubation in the dark, all aliquots were assessed for sperm motility parameters by a computer-assisted sperm analysis (CASA) [17]. Later on, another 8 pools were incubated in the dark for 1 and 5 min (using the concentration earlier defined), and sperm kinetics was again checked to establish the best PIT.

As aforementioned, pools in the light dosimetry phase were divided into 4 groups: (CON: untreated control, MB: MB only, LIGHT: light only, and PDT: MB+light). Immediately after irradiation, samples from all groups were examined for sperm motility parameters, plasma and acrosomal integrity, and mitochondrial activity. To verify whether or not possible deleterious effects of PDT could be mitigated by subsequent storage at lower temperatures [18], an additional experiment was performed where semen pools were divided into 5 aliquots (control and PDT for 60, 120, 180 e 240 s), irradiated and kept at 4-5°C. Sperm motility parameters were assessed before and after their storage in the refrigerator (30 and 60 min). In the final phase, we conducted bacterial cultures of seminal aliquots in addition to the evaluation of sperm motion parameters.

2.4. Irradiation of semen samples

Pooled semen samples were irradiated using a LedBox 660 nm (Biolambda – São Paulo – Brasil) with irradiances of 17, 29, and 44 mW/cm^2.

2.5. Semen analysis

Sperm motility parameters were conducted using the Hamilton Thorne equipment (CASA; HTM-IVOS-Ultimate 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA) as depicted by Blank et al. [18]. In short, 6 μl of diluted semen samples (30×10^6 sperm cells/ mL) were loaded in a prewarmed glass slide with a coverslip, where at least 1000 spermatozoa were examined per sample using standard settings (10 frames acquired at a frame rate of 60 Hz and a temperature of 39ºC). Samples were analyzed for total and progressive motility (TM and PM, respectively), rapid spermatozoa (RAPID), curvilinear velocity (VCL), straight-line velocity (VSL), average pathway velocity (VAP), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF). Total motility refers to the percentage of sperm making any sort of movement (this movement can include non-progressive movement) whereas progressive motility refers to cells moving forward.

Acrosomal integrity was assessed using a modified method with a single-stain solution containing 1% (w/v) rose Bengal, 1% (w/v) fast green FCF, and 40% ethanol in McIlvaine's citrate phosphate buffer, which was previously validated for chicken spermatozoa by our lab [19]. Five-μl of stain solution were added to 5 μl of diluted semen on a pre-warmed slide (37°C), and after 70 s the mixture was smeared with another slide. Next, smears were air-dried and at least 100 spermatozoa were examined with bright field microscopy (1000x magnification). Intact acrosomes stained purplish-blue and exhibited a conical shape, whereas absent or reacted acrosomes appeared as blunt and colorless edges.

Plasma membrane integrity was performed with an eosin-nigrosin stain by the protocol for chicken spermatozoa used by Rui et al. [20]. A drop of 5 μl of semen was mixed with 5 μl of stain solution (0.67%

eosin and 10% nigrosin) and after 30 s, samples were smeared on glass slides. After drying, 200 cells were counted under light microscopy (Nikon® E200, Nikon, Tokyo, Japan) at 1000x magnification. Cells with the more permeable plasma membrane allow the dye to enter, being observed under the microscope with a pink stain, while in less permeable plasma membrane cells remain unstained.

To ascertain sperm mitochondrial activity, samples were analyzed using the 3'3 diaminobenzidine (DAB) assay. In this assay, DAB is oxidized by the cytochrome c complex (including cytochrome c oxidase) in a chain reaction in which it is polymerized and deposited as granules in the mitochondria [21]. Briefly, semen was diluted (1:1) in a 1mg/mL solution of DAB in phosphate-buffered saline (PBS) and incubated in a water bath at 37°C for 1 h in the dark. Smears (10 μl) were then prepared on microscope slides and air-dried. The slides were fixed in 10% formaldehyde for 10 min, washed, and air dried again. At least 100 spermatozoa were counted using a phase contrast optical microscope (1000x magnification), and cells were classified into four categories: all mitochondria active (100% of the midpiece was stained – DAB I), most mitochondria active (more than 50% of the midpiece was stained – DAB II), most mitochondria inactive (less than 50% of the midpiece was stained – DAB III) and all mitochondria inactive (absence of staining in the midpiece – DAB IV). These results were expressed as Mitochondrial Activity Index (MAI) according to the equation given by Hrudka [22] ($MAI = \{(\%\ DAB\ I * 1) + (\%\ DAB\ II * 0.5) + (\%\ DAB\ III * 0.25) + (\%\ DAB\ IV * 0)\}$).

2.6. Microbiological analysis

Aliquots (~90 μL) were serially diluted in PBS (proportions of 1:10, 1:100, 1:1000, and 1:10000). Then, 10 μL aliquots and from each dilution were seeded in Petri dishes (all in triplicate) according to the method described by Jett et al. [23]. Mueller-Hinton and Macconkey agar were used to grow total coliforms and Gram-negative bacteria, respectively. Later on, plates were incubated at 37°C. After 18-24 h of incubation, colony-forming units (CFU) were counted and results were multiplied by dilution factors.

2.7. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System 9.3 software (SAS Institute, Cary, NC, USA). Variables were tested to determine variance homogeneity and data normality (Guided Data Analysis – SAS System). ANOVA and ANOVA with repeated measures were used depending on the assay. We also used Dunnet's test to compare test groups with the control when statistically significant differences were detected. All data are expressed as mean ± SEM with statistical significance set at $p < 0.05$.

3. Results

3.1. MB dark toxicity

Our results regarding total and progressive motilities indicated that MB concentrations up to 25 μM had no detrimental effects on total sperm motility, but above this concentration, MB was harmful to progressive motility (Fig. 1A). Therefore, we decided to assess the influence of MB concentrations below 25 μM at two different PITs (1 and 5 min). No statistically significant differences were detected for the total and progressive sperm motility among MB concentrations regardless of the PIT (Figs. 1B and 1C).

3.2. Light dosimetry

MB (10 μM) and light were individually ineffective on sperm characteristics regardless of the exposure time employed (Figs. 2–4, Tables 1–3). In contrast, the combination of these factors during PDT

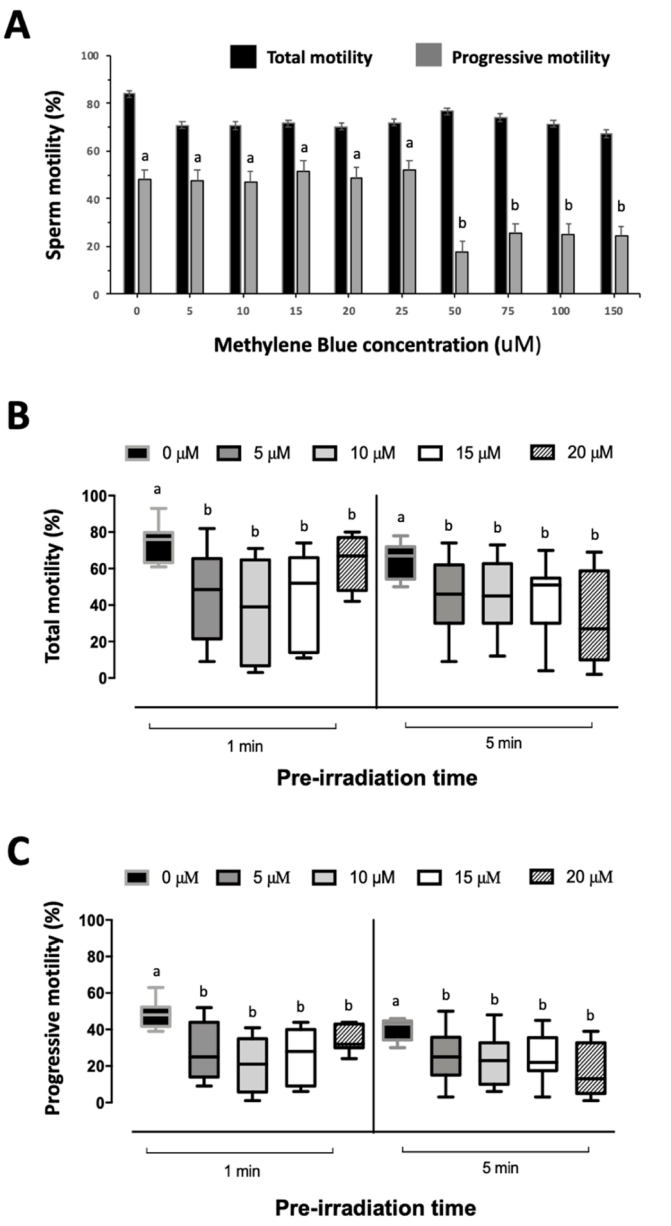

Fig. 1. – Effects of methylene blue (MB) concentration and pre-irradiation time on chicken sperm motility during the MB dark toxicity phase. (A) Total and progressive motilities of chicken spermatozoa incubated for 5 min with several concentrations of MB. (B) and (C) Total and progressive motilities of chicken spermatozoa incubated with MB concentrations below 25 μM for 1 and 5 min. Different lowercase letters denote statistically significant differences among MB concentrations.

affected not only several sperm kinetic parameters in chicken spermatozoa but also plasma membrane integrity and mitochondrial activity. The highest irradiance (44 mW/cm^2) induced a statistically significant decrease in total and progressive motilities within 30 s of light exposure (1.3 J/cm^2) and a large part of the sperm kinetic parameters (Fig. 2, Table 1). Likewise, we noticed a decline in plasma membrane integrity and mitochondrial activity after 180 s (7.9 J/cm^2). The only sperm trait that remained unaffected by PDT at this irradiance was acrosomal integrity.

When 29 mW/cm^2 was applied, total sperm motility only dropped at 120 s of light exposure (around 3.5 J/cm^2), whereas progressive motility fell at 30 s (0.9 J/cm^2) (Fig. 3 and Table 2). Deterioration of the plasma membrane and mitochondrial activity were also detected at this irradiance but from 60 s onwards. Once again, acrosomal integrity was not

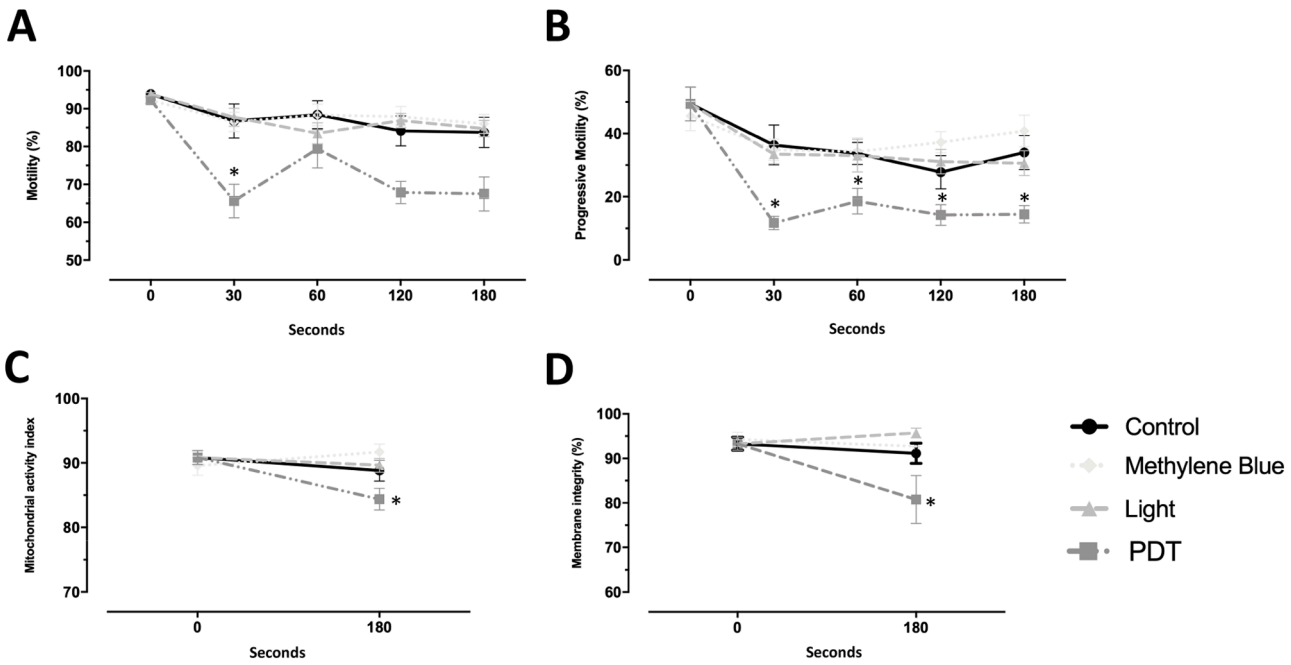

Fig. 2. – Means (± SE) of the (A, B) total and progressive motilities, (C) mitochondrial activity index, and (D) plasma membrane integrity of chicken seminal samples incubated with 10 μM of methylene blue (MB) and irradiated using a LedBox 660 nm under an irradiance of 44 mW/cm^2. Seconds refer to irradiation time. Asterisks indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

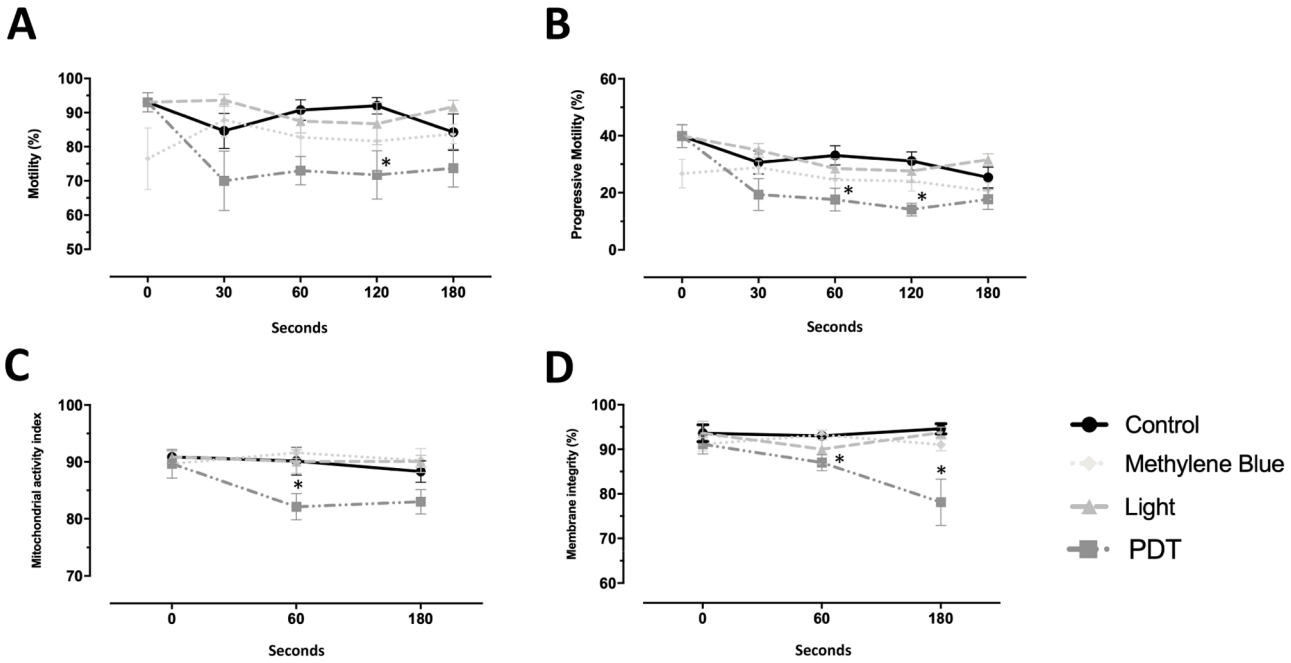

Fig. 3. – Means (± SE) of the (A, B) total and progressive motilities, (C) mitochondrial activity index, and (D) plasma membrane integrity of chicken seminal samples incubated with 10 μM of methylene blue (MB) and irradiated using a LedBox 660 nm under an irradiance of 29 mW/cm^2. Seconds refer to irradiation time. Asterisks indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

altered.

The lowest irradiance (17 mW/cm^2), in turn, exhibited slightly milder adverse effects on total and progressive motilities at 30 s of light exposure (0.51 J/cm^2) when compared to the other two irradiances (Fig. 4 and Table 3). Nevertheless, some sperm kinetic parameters started to decline in the PDT group with 60 s of irradiation (1 J/cm^2). Plasma membrane integrity and mitochondrial activity dropped at 180 s of light exposure (3.1 J/cm^2) but acrosome integrity remained unchanged.

To test whether PDT has an irreversible impact or whether sperm cells could restore their motion parameters over time, we also assessed sperm motion parameters of seminal samples kept at 4°C for 0, 30, and 60 min following irradiations with 17 and 29 mW/cm^2 for 0, 60, 120, 180 and 240 s. No statistically significant differences were detected among PDT groups regardless of the irradiance and storage time (data not shown). This means that PDT damages to the sperm kinetics are

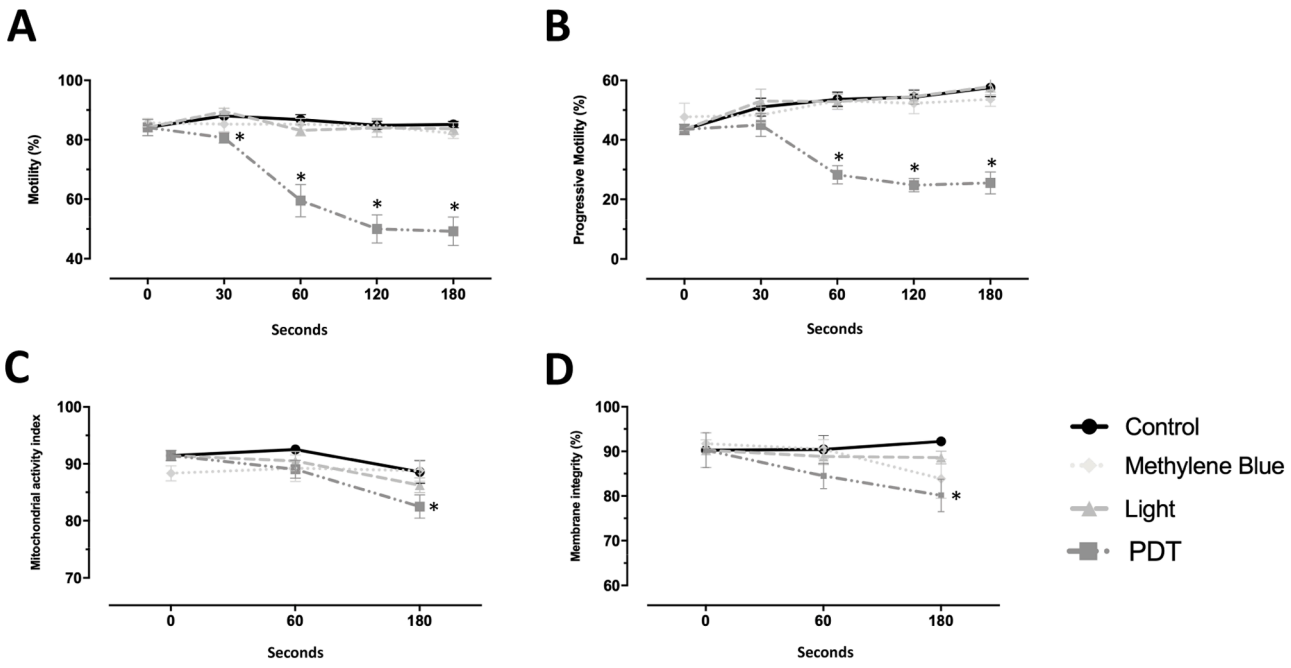

Fig. 4. – Means (± SE) of the (A, B) total and progressive motilities, (C) mitochondrial activity index, and (D) plasma membrane integrity of chicken seminal samples incubated with 10 μM of methylene blue (MB) and irradiated using a LedBox 660 nm under an irradiance of 17 mW/cm^2. Seconds refer to irradiation time. Asterisks indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

irreversible.

3.3. Microbiological analyses

To evaluate the impact of PDT on bacterial killing, we applied 2 exposure times (30 and 60 s) using 3 irradiances (17, 29 e 44 mW/cm^2) with a PIT of 1 min and MB at a concentration of 10 μM. Fig. 5 shows that PDT was not able to significantly decrease the bacterial load compared to control, MB, and light groups, regardless of the irradiance or exposure time used. Besides, some sperm parameters were significantly affected following PDT at 30 or 60 s, regardless of the irradiance (Table 4).

4. Discussion

According to the literature, the PIT is necessary for the PS uptake by the cells, and PDT efficiency depends directly on this component [24]. Here, MB in the absence of light proved to be toxic to chicken spermatozoa in concentrations greater than 25 μM, impacting severely progressive motility. Although MB has been used for decades as a drug for the treatment of methemoglobinemia, its concentration as a PS is significantly lower than those needed to damage somatic cells like fibroblasts, keratinocytes, or neutrophils [25]. An earlier study confirmed MB's ability to easily cross the plasma membrane and accumulate in the mitochondria, inducing their structural swelling and reducing oxidative phosphorylation [26]. Chicken spermatozoa carry 25-30 mitochondria in their middle piece to generate energy for motility, and previous researches reveal that any mitochondrial abnormalities cause decreases in sperm oxygen consumption, motility, and straight-line velocity [27]. Another assumption involves a dose-dependent reduction in the production of nitric oxide (NO) promoted by MB, which in human spermatozoa led to an inhibition of progressive motility and a reduction in the velocity patterns [28]. Thus, given the detrimental effects of higher MB concentrations on mitochondria and sperm motility, we decided to employ an intermediate concentration of this PS (10 μM), in an attempt to maintain a balance between microbial inactivation and sperm survival. Furthermore, our results indicated that PITs (1 and 5 min) did not impair the total and progressive motilities of spermatozoa incubated at MB concentrations lower than 25 μM. Then, we adopted the shortest PIT (1 min) to optimize the experimental procedure since it seems that PS uptake does not depend on the incubation time [29].

PDT dosimetry as a whole is a complex phenomenon involving dynamic interactions among light, PS, and oxygen, which also vary depending on the target tissue or cell [14]. In this context, although there is a plethora of PDT studies covering a variety of areas and cell types, to date only one study addressed the effects of this approach on spermatozoa [30]. The authors reported no changes in the evaluated variables when bovine ejaculates were only irradiated with either He-Ne laser or yellow-green light (wavelength 632.8 and 580 nm, respectively). Nevertheless, meaningful decreases in progressive motility and acrosome integrity occurred whenever bovine spermatozoa were incubated with thiopyronine and irradiated with yellow-green light (light doses of 0.054, 0.135 and 0.27 J/cm^2) [30]. At the same time, only acrosome integrity was impacted after incubation with hematoporphyrin and irradiation with a He-Ne laser (light dose of 0.068 J/cm^2) [30]. Interestingly, we noticed that MB-mediated PDT was able to preserve acrosome integrity regardless of light parameters. Besides, the dose of 0.52 J/cm^2 (PDT protocol with 17 mW/cm^2 for 30 s) was not considered so harmful for the sperm kinetic parameters. Indeed, plasma membrane and mitochondrial activity fell slightly with the increase in light exposure times regardless of the irradiance used, and even after 180 s of light exposure, values for these variables remained equal to or greater than 80%.

It is acknowledged that during their maturation process, spermatozoa extrude cytoplasm, which is the major source of antioxidants. Thus, the lack of cytoplasm results in decreased antioxidant defense making them less resistant to oxidative damage [31]. Apart from that, avian spermatozoa are known to be more susceptible to ROS than mammalian spermatozoa given their higher amount of polyunsaturated fatty acids in the plasma membrane, and their lower capacity to carry antioxidants due to their smaller cytoplasm [32]. Hence, we assume that the negative impact during PDT may be related to the unintentional uptake of MB by sperm mitochondria, which in turn could have culminated in excessive production of ROS. Such production can lead to lipid peroxidation of the mitochondrial membranes that reduce sperm motility and viability due

Table 1
–Means ± SE of the sperm kinetic parameters and acrosomal integrity of chicken seminal samples incubated with 10 μM of methylene blue (MB) and irradiated using a LedBox 660 nm under an irradiance of 44 mW/cm^2.

Exposure time	0 s			30 s					60 s					120 s					180 s				
Group	CON	MB	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P
Average path velocity (VAP)	82.27 ± 5.87	76.82 ± 4.86	0.4862	71.70 ± 3.52	71.97 ± 4.07	72.69 ± 3.35	49.74 ± 1.99*	<0.001	72.04 ± 2.68	73.44 ± 3.55	78.62 ± 2.79	59.10 ± 4.30*	0.0029	63.00 ± 4.16	72.61 ± 1.97	68.80 ± 3.59	55.09 ± 2.96	0.1149	72.45 ± 4.65	70.07 ± 4.96	70.37 ± 4.52	53.36 ± 3.08*	0.0152
Straight-line velocity (VSL)	69.39 ± 5.25	61.90 ± 4.62	0.3024	57.17 ± 3.83	56.42 ± 2.52	56.50 ± 2.12	41.01 ± 2.37*	0.0006	55.97 ± 2.14	56.30 ± 3.19	60.75 ± 3.25	47.11 ± 3.41*	0.0287	52.32 ± 3.53	57.06 ± 2.38	55.82 ± 2.28	44.70 ± 2.39	0.0676	58.77 ± 3.78	57.54 ± 4.54	56.39 ± 3.95	43.74 ± 2.08*	0.0268
Curvilinear velocity (VCL)	119.14 ± 5.69	115.87 ± 3.78	0.6404	116.97 ± 3.16	113.71 ± 5.14	115.35 ± 4.43	84.12 ± 2.85*	<0.001	114.51 ± 5.30	117.95 ± 4.08	122.29 ± 2.32	99.35 ± 5.92*	0.0062	102.64 ± 5.19	115.72 ± 1.45	110.95 ± 5.58	90.51 ± 4.06	0.0732	111.10 ± 5.96	111.50 ± 5.63	109.05 ± 6.15	87.09 ± 5.29*	0.0146
Amplitude of lateral head displac. (ALH)	4.84 ± 0.17	4.95 ± 0.16	0.6366	5.04 ± 0.13	4.86 ± 0.20	4.86 ± 0.14	4.49 ± 0.25	0.2283	4.93 ± 0.21	4.90 ± 0.07	5.02 ± 0.11	4.82 ± 0.15	0.7824	4.77 ± 0.07	5.05 ± 0.13	4.95 ± 0.15	4.60 ± 0.18	0.1268	4.76 ± 0.16	4.93 ± 0.14	4.80 ± 0.14	4.54 ± 0.09	0.3112
Beat cross frequency (BCF)	32.14 ± 0.69	31.50 ± 0.43	0.4673	32.94 ± 0.41	33.46 ± 0.59	33.45 ± 0.80	30.30 ± 0.80*	0.0062	32.39 ± 0.88	33.80 ± 0.78	32.52 ± 0.68	31.61 ± 0.95	0.3167	33.77 ± 0.80	33.15 ± 0.73	33.14 ± 0.63	31.04 ± 1.05*	0.0326	33.72 ± 0.16	32.31 ± 0.73	33.24 ± 0.53	31.74 ± 0.67*	0.0258
Straightness	83.00 ± 1.32	79.37 ± 1.81	0.1285	78.50 ± 1.70	78.50 ± 2.13	77.62 ± 2.49	82.25 ± 1.85	0.4094	77.29 ± 1.81	76.37 ± 2.09	76.37 ± 2.44	79.75 ± 1.99	0.6301	82.75 ± 1.58	77.75 ± 1.64	80.75 ± 2.19	81.37 ± 1.70	0.2692	81.12 ± 2.36	81.37 ± 2.23	80.00 ± 3.18	82.37 ± 1.74	0.9219
Linearity	57.50 ± 2.51	52.87 ± 2.63	0.2243	48.75 ± 2.26	49.75 ± 1.91	49.75 ± 2.06	50.37 ± 3.02	0.9694	49.57 ± 2.06	48.37 ± 2.03	49.87 ± 2.38	48.00 ± 1.87	0.9024	50.87 ± 1.73	49.37 ± 1.37	50.50 ± 1.85	50.62 ± 2.03	0.9337	53.75 ± 1.89	51.50 ± 2.16	52.37 ± 2.82	52.00 ± 2.04	0.9072
Rapid cells (%)	61.00 ± 6.20	56.75 ± 6.60	0.6462	48.75 ± 6.79	46.00 ± 5.66	49.87 ± 4.67	14.37 ± 2.18*	<0.001	50.43 ± 5.10	48.00 ± 5.84	50.25 ± 5.02	25.75 ± 5.58*	0.0073	35.75 ± 6.44	52.87 ± 3.04	41.62 ± 5.51	18.50 ± 4.03*	0.0004	46.12 ± 7.12	40.00 ± 8.87	43.37 ± 4.72	18.75 ± 3.72*	0.0220
Medium cells (%)	13.25 ± 3.11	14.50 ± 2.65	0.5998	13.50 ± 1.16	12.14 ± 1.53	14.12 ± 2.37	18.87 ± 1.59	0.0563	14.14 ± 1.64	11.37 ± 1.51	9.00 ± 1.25*	15.50 ± 1.38	0.0158	19.75 ± 1.98	14.62 ± 1.46	15.14 ± 2.27	18.12 ± 2.71	0.2951	16.00 ± 1.83	12.50 ± 2.03	14.57 ± 2.75	18.00 ± 1.58	0.2885
Slow cells (%)	19.75 ± 4.85	21.00 ± 4.74	0.7590	24.62 ± 3.60	26.12 ± 3.52	23.87 ± 1.98	32.62 ± 4.31	0.2853	23.57 ± 1.21	28.87 ± 2.33	24.50 ± 3.47	30.14 ± 2.27	0.2209	28.75 ± 3.36	20.25 ± 1.67*	27.12 ± 2.68	31.25 ± 3.54	0.0482	22.00 ± 2.50	21.00 ± 4.02	23.62 ± 1.56	30.62 ± 2.64	0.0762
Static cells (%)	6.12 ± 0.39	7.75 ± 0.86	0.1087	13.25 ± 4.51	13.50 ± 2.56	12.25 ± 2.39	34.37 ± 4.44*	0.0021	11.57 ± 3.93	11.62 ± 2.83	16.50 ± 2.77	20.57 ± 1.31	0.1106	15.87 ± 3.97	12.12 ± 2.70	13.12 ± 1.88	32.12 ± 2.94*	0.0006	16.25 ± 3.99	14.00 ± 2.72	15.25 ± 2.14	32.50 ± 4.48*	0.0105
Acrosomal integrity (%)	96.57 ± 0.84	97.57 ± 0.81	0.4092	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	97.14 ± 0.70	97.71 ± 0.36	96.29 ± 0.47	96.50 ± 0.71	0.3395

[1]CON (Control – without photosensitizer or light); [2]MB (10 μM of MB and no light); [3]LIGHT (light without MB); [4]PDT (with 10 μM of MB and light). Asterisks in the same line indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

Table 2
–Means ± SE of the sperm kinetic parameters and acrosomal integrity of chicken seminal samples incubated with 10 μM of methylene blue (MB) and irradiated using a LedBox 660 nm under an irradiance of 29 mW/cm^2.

Exposure time	0 s			30 s					60 s					120 s					180 s				
Group	CON	MB	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P
Average path velocity (VAP)	75.95 ± 4.62	67.81 ± 4.19	0.2129	66.41 ± 3.00	66.77 ± 2.04	69.80 ± 3.32	54.24 ± 5.17*	0.0227	67.65 ± 3.28	63.01 ± 2.72	64.32 ± 2.49	55.81 ± 3.05*	0.0494	66.42 ± 2.56	63.49 ± 2.18	64.82 ± 3.69	54.31 ± 2.38*	0.0197	63.36 ± 2.67	59.94 ± 2.33	68.34 ± 2.41	58.23 ± 3.99	0.1037
Straight-line velocity (VSL)	58.41 ± 2.98	52.04 ± 2.53	0.1254	52.45 ± 2.89	51.70 ± 2.40	53.46 ± 2.03	43.57 ± 4.32	0.1068	52.77 ± 2.47	49.17 ± 2.03	50.06 ± 1.77	44.76 ± 2.73*	0.0251	51.87 ± 2.08	49.95 ± 1.69	50.17 ± 2.13	43.04 ± 2.58*	0.0342	49.92 ± 2.16	46.85 ± 1.97	53.13 ± 1.94	46.93 ± 3.92	0.2953
Curvilinear velocity (VCL)	116.22 ± 6.69	104.24 ± 5.86	0.1992	102.49 ± 3.49	105.44 ± 2.90	107.02 ± 5.19	87.57 ± 7.75	0.0636	109.40 ± 4.28	104.31 ± 3.03	104.29 ± 4.11	90.07 ± 4.12*	0.0106	109.55 ± 3.17	102.67 ± 3.06	104.67 ± 5.73	91.35 ± 3.57*	0.0154	103.76 ± 4.17	100.91 ± 3.06	110.07 ± 3.23	91.69 ± 3.63*	0.0139
Amplitude of lateral head displac. (ALH)	5.07 ± 0.19	4.66 ± 0.15	0.1101	4.75 ± 0.17	4.67 ± 0.18	4.70 ± 0.12	4.40 ± 0.17	0.4483	4.96 ± 0.12	4.70 ± 0.11	4.74 ± 0.11	4.51 ± 0.12*	0.0159	4.97 ± 0.18	4.79 ± 0.16	4.89 ± 0.17	4.70 ± 0.24	0.7072	4.90 ± 0.19	4.77 ± 0.09	4.99 ± 0.06	4.56 ± 0.19	0.2538
Beat cross frequency (BCF)	30.34 ± 0.73	30.22 ± 0.83	0.9205	31.04 ± 0.65	31.62 ± 0.79	31.41 ± 0.76	32.09 ± 0.97	0.8269	32.61 ± 0.63	32.74 ± 0.71	32.19 ± 0.53	29.06 ± 1.12*	0.0069	32.97 ± 0.25	32.20 ± 0.37	31.57 ± 0.63	30.07 ± 0.67*	0.0035	31.66 ± 0.89	32.16 ± 0.77	32.14 ± 0.95	29.60 ± 0.69	0.1364
Straightness	76.75 ± 1.49	76.62 ± 1.76	0.9576	78.50 ± 1.52	77.00 ± 1.73	76.62 ± 1.82	80.00 ± 1.67	0.4872	77.75 ± 1.31	78.00 ± 1.56	77.87 ± 1.56	80.00 ± 1.53	0.6786	77.75 ± 0.99	78.37 ± 1.75	77.62 ± 1.73	79.25 ± 1.31	0.8592	78.25 ± 1.05	78.37 ± 1.88	77.86 ± 1.08	79.86 ± 1.35	0.7779
Linearity	51.12 ± 1.20	50.75 ± 1.29	0.8348	51.75 ± 2.16	49.75 ± 2.18	50.87 ± 1.88	50.12 ± 2.28	0.8977	48.75 ± 0.99	46.14 ± 0.96	48.75 ± 1.48	50.25 ± 2.10	0.3132	48.37 ± 1.87	49.25 ± 1.81	48.75 ± 1.62	48.62 ± 2.82	0.9924	48.62 ± 1.45	47.37 ± 1.75	49.29 ± 1.37	52.14 ± 2.91	0.3780
Rapid cells (%)	60.75 ± 6.94	42.12 ± 8.79	0.1185	43.00 ± 5.11	43.75 ± 3.63	54.00 ± 5.05	26.62 ± 7.97*	0.0174	47.50 ± 5.07	35.12 ± 4.68	41.25 ± 5.58	23.37 ± 5.47*	0.0191	45.50 ± 4.89	35.00 ± 5.53	41.50 ± 7.12	19.37 ± 2.94*	0.0093	35.12 ± 5.49	29.37 ± 3.66	47.29 ± 3.76	23.71 ± 4.72*	0.0094
Medium cells (%)	12.62 ± 2.51	12.25 ± 2.22	0.9124	16.62 ± 2.55	17.75 ± 2.05	16.75 ± 2.05	14.57 ± 2.53	0.8115	18.25 ± 2.58	16.75 ± 2.11	17.87 ± 1.66	18.00 ± 2.69	0.9685	17.87 ± 1.81	19.62 ± 2.94	18.62 ± 2.69	18.62 ± 2.69	0.9714	18.37 ± 2.24	19.12 ± 1.39	17.86 ± 1.32	18.29 ± 1.19	0.9562
Slow cells (%)	19.37 ± 2.71	22.12 ± 2.91	0.5003	25.12 ± 1.65	26.37 ± 0.86	22.50 ± 2.46	25.75 ± 4.59	0.7688	25.12 ± 2.21	30.62 ± 3.81	28.37 ± 2.76	31.25 ± 3.45	0.5103	28.62 ± 2.77	26.87 ± 2.99	27.00 ± 2.87	33.62 ± 4.13	0.4275	30.75 ± 3.56	35.12 ± 2.98	26.71 ± 2.83	31.57 ± 3.07	0.2294
Static cells (%)	7.00 ± 2.79	10.50 ± 2.53	0.2696	15.37 ± 5.16	12.12 ± 5.02	6.37 ± 1.75	21.00 ± 7.22	0.5070	9.25 ± 2.99	17.25 ± 5.61	12.50 ± 3.46	20.14 ± 5.41	0.3458	8.00 ± 2.43	9.71 ± 2.83	7.57 ± 2.79	28.25 ± 6.61*	0.0304	15.75 ± 5.33	16.25 ± 2.83	8.29 ± 2.02	26.29 ± 3.94	0.9994
Acrosomal integrity (%)	94.87 ± 1.33	95.75 ± 1.45	0.6630	—	—	—	—	—	97.00 ± 1.34	94.75 ± 1.80	96.87 ± 1.08	96.37 ± 1.12	0.6347	—	—	—	—	—	96.00 ± 1.27	96.37 ± 1.32	97.00 ± 1.10	96.75 ± 0.98	0.9361

[1]CON (Control – without photosensitizer or light); [2]MB (10 μM of MB and no light); [3]LIGHT (light without MB); [4]PDT (with 10 μM of MB and light). Asterisks in the same line indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

Table 3
–Means ± SE of the sperm kinetic parameters and acrosomal integrity of chicken seminal samples incubated with 10 μM of methylene blue (MB) and irradiated using a LedBox 660 nm under an irradiance of 17 mW/cm^2.

Exposure time	0 s			30 s					60 s					120 s					180 s				
Group	CON	MB	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P	CON	MB	LIGHT	PDT	P
Average path velocity (VAP)	108.04 ± 4.63	114.79 ± 6.10	0.3876	109.05 ± 6.14	107.41 ± 6.35	122.45 ± 5.18	108.56 ± 4.99	0.2240	107.69 ± 4.57	108.07 ± 5.59	112.11 ± 4.15	85.95 ± 2.37*	0.0003	114.56 ± 5.23	107.44 ± 4.61	111.96 ± 3.90	92.39 ± 4.57*	0.0093	107.69 ± 4.57	108.07 ± 5.59	112.11 ± 4.15	85.95 ± 2.37*	<.0001
Straight-line velocity (VSL)	81.80 ± 4.14	89.03 ± 7.82	0.4119	87.56 ± 4.79	84.66 ± 3.96	95.14 ± 6.04	85.26 ± 5.79	0.4759	88.84 ± 4.30	88.45 ± 4.53	94.01 ± 4.26	70.35 ± 2.77*	0.0016	95.09 ± 5.34	87.79 ± 4.99	93.60 ± 4.23	77.07 ± 4.74*	0.0180	88.84 ± 4.30	88.45 ± 4.53	94.01 ± 4.26	70.35 ± 2.77*	<.0001
Curvilinear velocity (VCL)	156.07 ± 5.17	158.09 ± 4.18	0.7716	154.86 ± 5.38	151.61 ± 6.25	167.11 ± 3.76	152.09 ± 3.89	0.1130	151.85 ± 3.25	149.39 ± 3.96	154.44 ± 2.29	129.99 ± 1.86*	<.0001	159.06 ± 3.85	152.64 ± 2.77	154.17 ± 3.24	133.42 ± 3.46*	<.0001	151.85 ± 3.25	149.39 ± 3.96	154.44 ± 2.29	129.99 ± 1.86*	<.0001
Amplitude of lateral head displac. (ALH)	6.32 ± 0.11	6.11 ± 0.14	0.2585	6.27 ± 0.12	6.37 ± 0.15	6.37 ± 0.06	6.10 ± 0.06	0.2076	6.22 ± 0.09	6.20 ± 0.16	6.14 ± 0.12	5.79 ± 0.08*	0.0191	6.35 ± 0.09	6.15 ± 0.08	6.27 ± 0.07	5.62 ± 0.11*	<.0001	6.22 ± 0.09	6.20 ± 0.16	6.14 ± 0.12*	5.79 ± 0.08*	0.0221
Beat cross frequency (BCF)	25.35 ± 1.04	25.06 ± 0.91	0.8380	24.97 ± 1.04	25.21 ± 1.06	23.09 ± 0.91	26.61 ± 0.53	0.0773	25.21 ± 0.66	24.91 ± 0.68	26.34 ± 0.58	28.37 ± 0.72	0.9796	25.81 ± 1.02	25.86 ± 0.76	25.45 ± 1.26	28.52 ± 1.02	0.1533	25.21 ± 0.66	24.91 ± 0.68	26.34 ± 0.58	28.37 ± 0.72	0.9995
Straightness	74.12 ± 2.14	75.57 ± 2.66	0.6757	78.12 ± 1.19	77.50 ± 1.28	75.87 ± 2.34	76.62 ± 2.12	0.8261	80.25 ± 1.03	79.62 ± 1.21	81.37 ± 0.90	80.00 ± 1.19	0.7016	80.25 ± 1.38	79.12 ± 1.79	81.12 ± 1.96	81.12 ± 1.94	0.8372	80.25 ± 1.03	79.62 ± 1.21	81.37 ± 0.90	80.00 ± 1.19	0.2671
Linearity	52.00 ± 2.27	55.43 ± 3.52	0.4170	55.37 ± 1.55	54.75 ± 0.70	56.37 ± 2.81	54.87 ± 2.32	0.9384	57.50 ± 1.84	56.62 ± 1.32	59.25 ± 1.96	53.00 ± 1.50	0.0828	58.37 ± 2.03	56.50 ± 2.33	59.62 ± 2.37	55.75 ± 2.90	0.6673	57.50 ± 1.84	56.62 ± 1.32	59.25 ± 1.96	53.00 ± 1.50*	0.0101
Rapid cells (%)	72.57 ± 1.70	72.00 ± 2.53	0.8543	71.00 ± 3.82	68.25 ± 4.78	78.62 ± 1.68	64.75 ± 2.53*	0.0275	70.37 ± 2.64	71.25 ± 2.43	67.37 ± 3.31	37.25 ± 4.41*	<.0001	71.00 ± 2.17	70.37 ± 1.95	71.25 ± 3.22	31.75 ± 3.31*	<.0001	70.37 ± 2.64	71.25 ± 2.43	67.37 ± 3.31	37.25 ± 4.41*	<.0001
Medium cells (%)	3.43 ± 0.61	3.86 ± 0.59	0.6378	5.62 ± 0.98	4.57 ± 0.97	2.50 ± 0.42*	5.25 ± 0.67	0.0099	5.62 ± 1.07	4.75 ± 0.86	5.37 ± 0.82	7.75 ± 0.79	0.1192	5.00 ± 0.94	4.37 ± 0.53	4.12 ± 0.81	6.00 ± 0.91	0.4020	5.62 ± 1.07	4.75 ± 0.86	5.37 ± 0.82	7.75 ± 0.79*	0.0199
Slow cells (%)	10.62 ± 1.08	9.57 ± 1.32	0.5452	9.71 ± 1.06	9.29 ± 0.81	8.25 ± 1.03	10.75 ± 0.99	0.3400	10.87 ± 1.35	9.25 ± 1.16	8.43 ± 0.87	14.62 ± 1.25	0.9991	9.12 ± 1.04	9.62 ± 0.70	8.37 ± 1.10	11.87 ± 1.63	0.2209	10.87 ± 1.35	9.25 ± 1.16	8.43 ± 0.87	14.62 ± 1.25	0.1838
Static cells (%)	13.57 ± 1.74	14.29 ± 1.44	0.7577	12.00 ± 1.43	12.43 ± 1.11	10.50 ± 1.10	19.37 ± 1.69	0.4194	13.25 ± 1.72	14.75 ± 1.87	16.87 ± 1.84	40.50 ± 5.40*	<.0001	15.12 ± 1.35	15.37 ± 2.20	16.00 ± 3.09	50.00 ± 4.70*	<.0001	13.25 ± 1.72	14.75 ± 1.87	16.87 ± 1.84	40.50 ± 5.40*	<.0001
Acrosomal integrity (%)	98.62 ± 0.59	97.87 ± 0.69	0.4255	—	—	—	—	—	99.37 ± 0.26	98.87 ± 0.48	97.75 ± 0.73	98.57 ± 0.37	0.1488	—	—	—	—	—	99.37 ± 0.26	98.87 ± 0.48	97.75 ± 0.72	98.57 ± 0.37	0.3914

[1]CON (Control – without photosensitizer or light); [2]MB (10 μM of MB and no light); [3]LIGHT (light without MB); [4]PDT (with 10 uM of MB and light). Asterisks in the same line indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

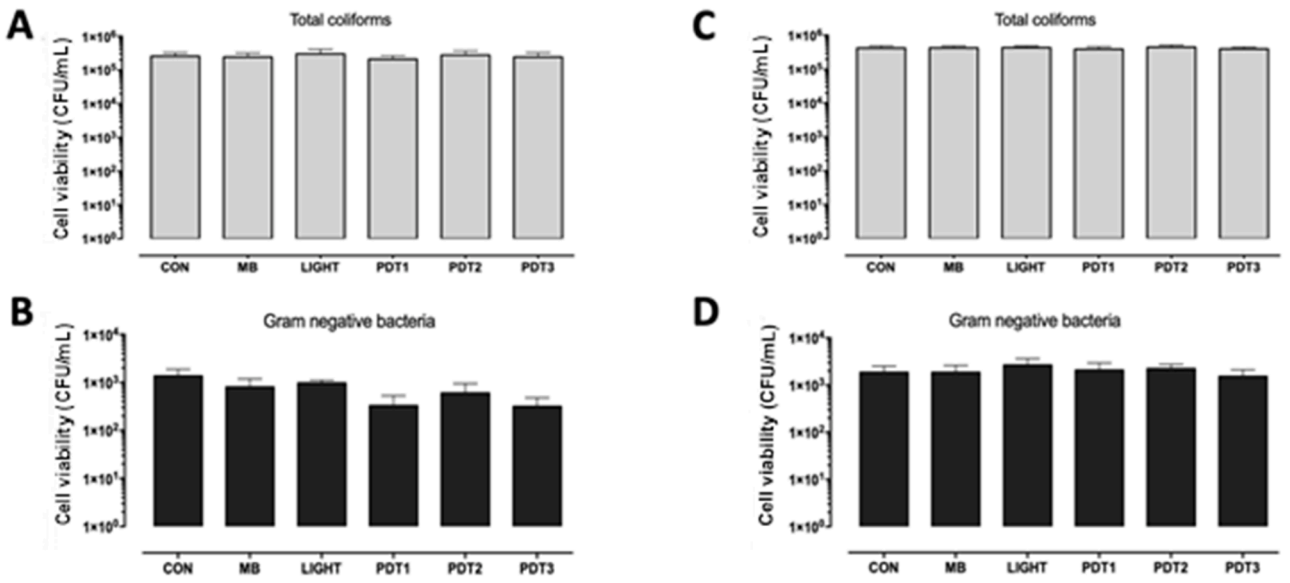

Fig. 5. – Mean (± SE) of total coliforms and Gram-negative bacteria reduction after treatments following 30 (A, B) and 60 s (C, D) of exposure to light. CON (Control – without photosensitizer or light); MB (10 μM of methylene blue and no light); LIGHT (light without MB); PDT1(10 μM of MB and 17 mW/cm^2); PDT2 (10 μM of MB and 29 mW/cm^2); and PDT3 (10 μM of MB and 44 mW/cm^2).

to decreased mitochondrial membrane potential and the formation of pro-apoptotic factors [33,34]. Rui et al. verified that hydrogen peroxide and hydroxyl radicals severely impaired chicken sperm function but their mechanisms of action seemingly comprise different pathways [20]. Those authors suggest that hydrogen peroxide compromises sperm movement by damaging axoneme through its action on ATP synthesis/utilization or on the contractile apparatus of the flagellum, an assumption that is supported by former research with human and boar spermatozoa [35,36]. However, further analysis is required considering that our tests were unable to detect significant changes in the functionality of mitochondria.

Over the last three decades, several studies examined PDT antimicrobial potential using a range of chemical classes and light sources. Although most of them demonstrated the efficacy of PDT against several classes of microorganisms, many determined the inactivation of planktonic cultures *in vitro*. Our study, on the other hand, investigated the inactivation of total coliforms and Gram-negative bacteria in chicken semen, a complex biological sample consisting of different biomolecules (e.g., proteins, lipids, vitamins, etc.). Together, these compounds confer the semen particularities to the PS uptake and distinct interactions with light. Particularly, Eaglesome et al. noticed a clear difference in PDT efficiency when treating pathogens kept in isolated cultures or associated with bovine ejaculates [30]. In isolated cultures, PDT successfully inactivated bovine herpes virus-1, *Mycoplasma bovigenitalium, M. canadense* and *Ureaplasma diversum*, but when suspended in seminal samples it was only effective against bovine herpes virus-1. Here, we chose MB as a PS because of its low cost, easy acquisition, and proven *in vitro* efficiency against a wide range of microorganisms [25,37]. Besides, it is largely applied in Veterinary Medicine to fight infectious diseases [16].

Unfortunately, all PDT protocols that we judged less damaging to the spermatozoa were ineffective against total coliforms and Gram-negative bacteria in chicken semen, since contamination patterns among experimental groups were alike. Shi et al. stated that PDT efficiency depends not only on the amount of ROS produced but also on the selectivity of the PS molecules concerning target cells [38]. Yet, the choice of the PS class to be used is one of the most critical elements in PDT treatment. Our data in phases 2 and 3 strongly suggest a low selectivity of MB to coliforms present in chicken semen, which could be related to high PS uptake by spermatozoa or to its expulsion by efflux pumps in the bacterial cell wall [39], thus compromising the inactivation results. For this reason, we assume that a way to optimize the antimicrobial efficiency of PDT consists of either researching PSs that are more specific for those pathogens found in chicken semen or conjugating MB molecules to nanoparticles, polypeptides, or proteins that target receptors absent in spermatozoa and present in bacterial cells [38]. A previous study has shown that the combination of MB with antimicrobial peptides (AMPs) conferred a higher affinity of the molecule for bacteria than for somatic cells [40]. Another approach that has recently gained ground in PDT is nanotechnology since its effectiveness against bacteria can be enhanced when the PS is associated with nanoparticles such as metallic or polymeric nanoparticles [41]. Indeed, PSs can accompany, be encapsulated, or be bounded to the surface of nanoparticles to promote an improvement in the effectiveness of PDT [42]. Thus, despite the antimicrobial inefficacy observed with the protocols proposed here, we showed that it is possible to use PDT in seminal samples without compromising sperm quality. Although additional studies are required to understand the impact of PDT on *in vivo* fertility, our outcomes will serve as a starting point for future research seeking to use PDT in the microbial control of seminal samples from birds and mammals.

Conclusions

MB concentrations higher than 25 μM are detrimental to chicken spermatozoa even in the absence of light, whereas lower concentrations caused some decrease in motility but overall do not compromise semen quality. Although the association of 10 μM of MB with red light (660 nm) at irradiances of 17, 29, or 44 mW/cm^2 for 30 or 60 s of exposure was able to maintain sperm quality at acceptable levels, it was not effective in significantly decreasing the bacteria present in the chicken semen.

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Data availability statement

The data that support the findings of this study are available from the

Table 4
– Means ± SE of the colony-forming units (CFU) and sperm kinetic parameters of chicken seminal samples incubated with 10 μM of methylene blue (MB), irradiated using a LedBox 660 nm under irradiances of 17, 29, and 44 mW/cm^2 (PDT 1, PDT 2 and PDT 3, respectively) for 30 and 60 s.

Exposure Time	30 s							60 s						
Group	[1]CON	[2]MB	[3]LIGHT	PDT 1	PDT 2	PDT 3	P	CON	MB	LIGHT	PDT 1	PDT 2	PDT 3	P
Total bacteria (x 10^3 cfu/ mL)	256.11 ± 71.1	241.55 ± 80.4	301.67 ± 112.6	209.44 ± 58.7	277.44 ± 94.1	241.83 ± 84.43	0.999	420.00 ± 67.25	423.33 ± 58.04	431.33 ± 51.50	388.33 ± 72.07	445.28 ± 69.10	398.00 ± 53.49	0.993
Gram-negatives (x 10^3 CFU/mL)	1.36 ± 0.52	0.80 ± 0.38	0.97 ± 0.14	0.33 ± 0.20	0.60 ± 0.34	0.31 ± 0.17	0.207	1.86 ± 0.64	1.87 ± 0.73	2.65 ± 0.99	2.07 ± 0.87	2.21 ± 0.54	1.53 ± 0.58	0.881
Total motility (%)	89.00 ± 2.07	85.50 ± 8.50	85.00 ± 2.83	65.00 ± 5.72*	71.00 ± 10.38	60.40 ± 11.65*	0.030	87.67 ± 1.94	80.67 ± 3.23	86.50 ± 3.65	70.17 ± 4.36*	68.50 ± 4.69*	65.67 ± 6.04*	<0.001
Progressive motility (%)	55.80 ± 1.39	36.50 ± 1.50	37.00 ± 5.66	24.40 ± 8.27*	30.40 ± 9.22*	22.60 ± 7.64*	0.030	42.00 ± 2.91	40.00 ± 4.48	39.00 ± 5.16	28.67 ± 3.66	21.17 ± 5.69*	17.50 ± 3.87*	<0.001
Average path velocity (VAP)	95.18 ± 4.55	73.50 ± 0.10	81.20 ± 3.68	69.48 ± 9.48	77.82 ± 8.94	72.38 ± 8.05	0.257	77.05 ± 2.48	76.10 ± 3.08	74.80 ± 3.94	65.13 ± 2.93*	59.43 ± 4.11*	57.52 ± 2.17*	<0.001
Straight-line velocity (VSL)	80.32 ± 5.56	53.35 ± 2.05	59.15 ± 1.24	55.90 ± 10.13	61.74 ± 8.08	55.42 ± 6.69	0.191	56.93 ± 2.35	59.13 ± 4.70	55.40 ± 3.67	49.78 ± 2.64	45.35 ± 3.62*	42.62 ± 1.58*	0.002
Curvilinear velocity (VCL)	138.14 ± 5.47	121.10 ± 6.00	131.00 ± 5.51	118.66 ± 8.98	120.72 ± 10.1	118.28 ± 9.54	0.560	125.00 ± 3.21	121.92 ± 4.46	125.00 ± 4.47	107.03 ± 3.06	95.94 ± 4.25	102.72 ± 4.64	<.001
Amplitude of lateral head displac. (ALH)	5.32 ± 0.12	5.25 ± 0.05	5.50 ± 0.00	5.44 ± 0.14	5.38 ± 0.16	5.42 ± 0.16	0.948	5.55 ± 0.04	5.52 ± 0.15	5.55 ± 0.08	5.17 ± 0.11*	5.27 ± 0.14	5.42 ± 0.12	0.045
Beat cross frequency (BCF)	31.54 ± 1.09	31.00 ± 1.70	31.50 ± 0.64	29.26 ± 2.13	28.50 ± 1.49	29.90 ± 1.64	0.793	30.30 ± 0.99	28.68 ± 1.32	30.55 ± 0.59	27.13 ± 1.26	26.65 ± 1.53	27.62 ± 0.86	0.093
Straightness	83.00 ± 2.24	72.50 ± 2.50	72.50 ± 1.77	77.60 ± 3.19	78.00 ± 2.79	75.40 ± 1.29	0.189	73.33 ± 1.11	71.75 ± 0.85	73.50 ± 1.18	76.00 ± 1.00	75.83 ± 1.58	74.00 ± 0.45	0.127
Linearity	57.20 ± 3.12	44.50 ± 3.50	45.50 ± 1.06	45.60 ± 4.48*	50.20 ± 3.51	46.60 ± 2.38	0.045	46.00 ± 1.65	48.83 ± 4.03	44.50 ± 1.76	46.83 ± 1.72	44.67 ± 2.35	42.17 ± 0.54	0.468
Rapid cells (%)	69.60 ± 2.84	62.00 ± 9.00	68.00 ± 4.95	30.60 ± 8.48*	40.40 ± 11.92	31.80 ± 10.75*	0.023	67.83 ± 4.32	58.83 ± 3.47	61.67 ± 6.71	41.17 ± 5.15*	30.67 ± 7.71*	27.17 ± 5.92*	<.0001
Medium cells (%)	8.40 ± 1.03	9.00 ± 1.00	8.00 ± 0.71	13.60 ± 1.69	10.80 ± 2.15	8.40 ± 1.83	0.251	8.50 ± 1.09	10.83 ± 1.25	10.83 ± 1.74	14.17 ± 1.40*	18.00 ± 1.57*	17.50 ± 1.61*	<0.001
Slow cells (%)	11.20 ± 0.58	14.50 ± 1.50	16.00 ± 3.53	20.60 ± 2.80*	22.25 ± 1.11*	20.20 ± 2.08*	<0.001	11.50 ± 1.76	11.33 ± 0.95	13.67 ± 2.25	15.00 ± 1.53	19.67 ± 2.22*	21.17 ± 1.72*	<0.001
Static cells (%)	11.00 ± 2.07	14.50 ± 8.50	15.00 ± 2.83	35.00 ± 5.72*	29.00 ± 10.3*	39.60 ± 11.65*	0.034	12.33 ± 1.94	19.33 ± 3.23	13.50 ± 3.65	29.83 ± 4.36*	31.50 ± 4.69*	34.33 ± 6.04*	<0.001

[1] CON (Control – without photosensitizer or light);
[2] MB (10 μM of MB and no light);
[3] LIGHT (light without MB). Asterisks in the same line indicate significant differences compared to CON (Dunnett test – $P < 0.05$).

corresponding author, [RJGP], upon reasonable request.

Ethics statement

Does not apply.

CRediT authorship contribution statement

GA Novaes: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **MH Blank:** Validation, Methodology, Formal analysis, Data curation. **TM Yoshimura:** Writing – review & editing, Methodology, Formal analysis, Data curation. **MS Ribeiro:** Writing – review & editing, Validation, Project administration, Funding acquisition, Formal analysis, Conceptualization. **RJG Pereira:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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