In vitro inactivation of medically relevant bacteria following exposure to Biovitae ® light

Rif. Prestazione 137/2019/PE

Responsabile delle prove: Ing. Martina Roso

Padova, 10th February 2020

Object: **Evaluation of *in vitro* inactivation of medically relevant bacteria following exposure to Biovitae ® light.**

MATERIALS

Two Biovitae® Lamps have been supplied by Rosa Group in order to evaluate their antimicrobial activity.

Two different grades of titanium dioxide, that is Tronox® 8400, here TiO_2-T, and Titanium(IV) oxide rutile (Product number 637262 Sigma-Aldrich, Merck: rutile nanopowder, <100 nm particle size, 99.5% trace metals basis), here TiO_2-R, were supplied by Rosa Group.

Polymethylmethacrylate was (PMMA) purchased from Sigma-Aldrich-Merck with a Mw=34000 Da

N,N-Dimethylformamide (Sigma Aldrich-Merck) and acetone (reagent grade) were used as solvents without any further purification.

METHODS

1. *Titanium oxide films production*

Phase separation was used for titanium oxide-film preparation. A 25% w/w solution of PMMA in N,N-Dimethylformamide : Acetone equal to 1:1 was prepared, and a proper amount of titanium oxide (both TiO_2-T and TiO_2-R) was added to obtain a final solid ratio polymer :TiO_2 equal to 30:70. This suspension was kept stirred overnight. Accurate precision wet film applicator lay down a uniform thickness (10 mils) of film on a glass plate. After 2 mins, the glass plate was immersed in water and the final film was peeled off from the support, dried overnight at room temperature and kept under vacuum into a drier.

The films were cut and weighed before use in order to get square-shaped pieces to fit culture wells.

2. *Microbiological assay*

All the experiments were carried out at the Department of Molecular Medicine – Microbiological section and the obtained results are here reported.

In this study, two clinically important bacterial strains, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*, were grown at 37°C in nutrient broth under standard conditions and $1x10^9$ CFU (colony-forming unit)/mL were seeded onto 12 wells culture

plates, final volume 2 mL. Samples of TiO_2-R or TiO_2-T were place on the bottom of the culture wells, as indicated. Bacterial cultures were exposed to visible light for 480 minutes at room temperature and with a distance plate-lamp equal to 4 cm. These factors were maintained constant for all the experiments. Control bacterial cultures were prepared in the same conditions with no exposure to light.

At the end of incubation, samples were collected, properly diluted, and seeded on nutrient agar plates. Agar plates were incubated at 37°C and check for bacterial colonies appearance up to 72 hours.

Bacterial colonies were enumerated and data are reported as geometric mean ± standard error of bacterial CFU obtained from 3 different experiments. Statistical analysis was performed using ONE-way ANOVA, followed by Bonferroni's multiple comparisons test. Data were considered significantly different when $P \leq 0.02$..

RESULTS

As reported in Figure 1, exposure to visible light for 480 minutes significantly reduced bacterial count in MRSA cultures. Indeed, we did not recover bacterial cells from MRSA cultures exposed to visible light whereas we registered 3.68×10^9 CFU in cultures of MRSA not exposed to light. Cultures of MRSA seeded on TiO_2-R or TiO_2-T surfaces for 480 minutes but not exposed to visible light reported a bacterial count comparable to not treated MRSA. Following exposure to visible light for 480 minutes, cultures of MRSA seeded on TiO_2-T were completely inactivated. On the contrary, MRSA seeded on TiO_2-R survived following exposure to visible light even if we registered 9×10^5 CFU (P=0.01 *vs* MRSA seeded on TiO_2-R but not exposed to light).

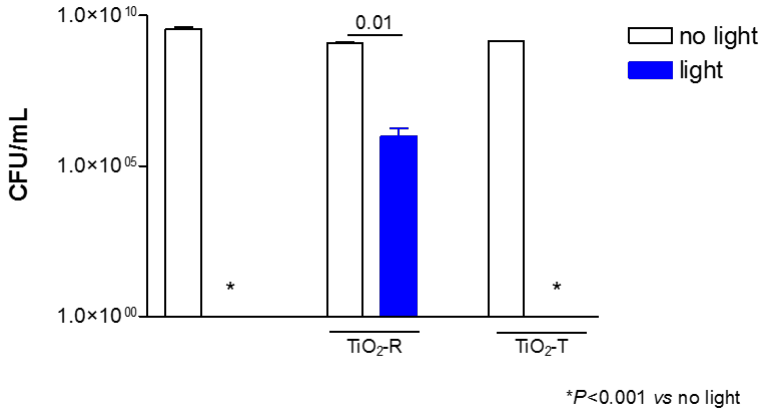


Figure 1. MRSA inactivation following exposure (480 minutes) to visible light seeded or not on TiO_2-R or TiO_2-T surfaces; data are reported as count of bacterial colonies (CFU/mL).

Cultures of *E. coli* exposed to visible light reported a significant CFU reduction ($4.85x10^3$ CFU) as compared with *E. coli* not exposed to light ($8.73x10^9$ CFU; *P*=0.01). As reported for MRSA, cultures of *E. coli* did not report a significant reduction when seeded for 480 minutes on TiO_2-R or TiO_2-T. Following exposure to light, we registered a reduction in CFU of *E. coli* seeded on titanium surfaces comparable to CFU reduction reported for cultures of *E. coli* exposed to visible light and not seeded on TiO_2-R or TiO_2-T (Figure 2).

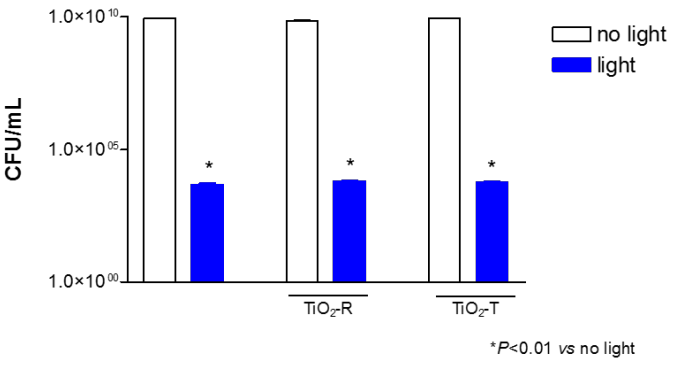


Figure 2. *E. coli* inactivation following exposure (480 minutes) to visible light seeded or not on TiO2-R or TiO2-T surfaces; data are reported as count of bacterial colonies (CFU/mL).

Table 1 reported log_{10} reduction registered in cultures of MRSA and *E. coli* under the described experimental conditions.

bacterial strain	experimental conditions	log_{10} reduction
MRSA	no treatment	0
	TiO_2-R	0.47
	TiO_2-T	0.42
	visible light	9
	visible light+TiO_2-R	3.1
	visible light+TiO_2-T	9
E. coli	no treatment	0
	TiO_2-R	0.07
	TiO_2-T	0.006
	visible light	6.25
	visible light+TiO_2-R	6.04
	visible light+TiO_2-T	6.15

Table 1. Bacterial inactivation following exposure (480 minutes) to visible light seeded or not on TiO2-R or TiO2-T surfaces; data are reported as log_{10} reduction.

FINAL CONSIDERATIONS

Since the emergence of multi-drug resistant bacterial strains is becoming a major and alarming threat to public health, innovative non-antibiotic (such as exposure to visible light) are attractive and promising approaches for the prevention of infectious diseases.

To strengthen clinical applications, additional investigations are mandatory:

- Investigate the antimicrobial effect of visible light on a broader range of clinically relevant bacteria, viruses and fungi;
- Investigate the maintenance of the antimicrobial effects of visible light under more reliable conditions (i.e. gaseous phase, reduced time of exposure, increased distance between light and samples);
- Assess the development of resistance to visible light by microbes under repeated exposure to visible light;
- Check the synergism of visible light with other antimicrobial agents

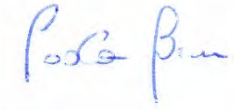
In these experiments, it was not possible to discriminate among the antimicrobial efficacy of visible light and the potential additional effects of the titanium surfaces.

Possible approaches to better elucidate this point:

- Reduce the time of exposure to light and increase the distance between the visible light and the microbial samples in the aim to reduce the bactericidal effect of the light and bring out the possible light-induced antibacterial effect of the titanium surfaces.
- Set a Kirby Bauer-like method using nutrient agar plate and titanium surfaces with the evaluation of zone of bacterial growth inhibition following exposure to visible light.

Microbiology Supervisor

Dr. Paola Brun



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Project:

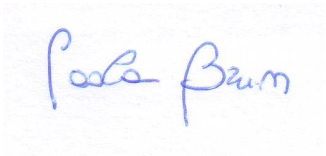
Bacterial Inactivation by UV light exposure – 2nd step

Experimental plan description:

During the second step of the protocol, experiments will be divided into two different points:

1) Methicillin-resistant *Staphylococcus aureus* (MRSA) will be placed into 12 wells plates in liquid culture at final volume of 2 mL ($1x10^9$ CFU/mL) and exposed to light. Bacterial samples will be exposed to light for 10 min, 30 min, and 60 min. The distance between the bacterial samples and the light will be better set before experimental procedures in order to obtain 1 log reduction. Bacteria not exposed to light will serve as control. Bacteria will be properly diluted and plated on agar medium for colonies development. Inactivation of bacteria will be evaluated by colony enumeration. Experiments will be performed in triplicate. (experimental samples, n= 18)

2) To evaluate the role of titanium surfaces on bacterial inactivation with and without exposure to light, a Kirby-Bauer protocol will be set up. Briefly, TiO_2-R, TiO_2-T, and PMMA as control will be placed on Petri dish and covered with a thin layer of solid culture medium (LB agar medium). MRSA will be uniformly distributed on Petri dish and exposed to light under the conditions determined at point 1). Petri dishes treated in the same manner but not exposed to light will serve as control. After light exposure, Petri dishes will be incubated at 37°C. Inactivation of bacteria will be evaluated by colony enumeration. Experiments will be performed in triplicate. (experimental samples, n= 6)



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