

Article

Effect of Silver Nanoparticles on Blue Light Phototoxicity against *Fusobacterium nucleatum*

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Abstract: A previous study showed that sub-lethal exposure of blue light caused cell membrane damage in *Fusobacterium nucleatum* (Fn). The aim of the present study was to test the combined effect of blue light and silver nanoparticles against Fn. Bacterial suspensions were exposed to blue light (400–500 nm) with or without silver nanoparticles (10 nm). Exposed and non-exposed samples were studied for malodor production (Odor judge scores), VSC levels (Halimeter), reactive oxygen species (ROS) production (fluorimeter), and bacterial cell membrane damage (fluorescence microscopy). The results showed that combining blue light exposure and silver nanoparticles significantly reduced malodor and VSC production by Fn concomitant with increased ROS levels and bacterial cell membrane damage. These results suggest that silver nanoparticles may increase blue light phototoxicity against Fn.

Keywords: *Fusobacterium nucleatum*; blue light; silver nanoparticles



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1. Introduction

The proteolytic activity of anaerobic Gram-negative oral bacteria such as *Fusobacterium nucleatum* has been associated with oral malodor and gum disease [1]. These bacteria break down oral proteins and glycoproteins into their amino acid building blocks. These are further metabolized, yielding malodorous compounds such as the volatile sulfide compounds (VSC) [2] that are felt during exhalation and speech.

Silver nanoparticles are considered a promising antimicrobial agent due to the multi-target action of silver [3]. Due to their antimicrobial activity [4], low cytotoxicity, and low immunogenicity [5] silver nanoparticles have multiple biomedical applications. Although their antibacterial mechanism is not yet clear, several mechanisms have been proposed. These include the release of silver ions [6], cell wall and cytoplasmic membrane damage [7], and reactive oxygen species (ROS) induction [8]. The antimicrobial effect of silver nanoparticles combined with blue light against antibiotic resistant *Pseudomonas aeruginosa* was also shown [9]. However, the mechanism for this effect is still unclear.

Previous studies have demonstrated the phototoxic effect of blue light against malodor producing bacteria [10] with or without the added effect of photosensitizers [11] or photoactivators [12]. Furthermore, the main antibacterial mechanism of blue light against *Fusobacterium nucleatum* was shown to be membrane damage [13] that could be leveraged to increase the efficacy of other antibacterial agents [14]. The aim of the present study was to test the added effect of silver nanoparticles on blue light phototoxicity against *Fusobacterium nucleatum*.

2. Materials and Methods

2.1. Light Source

A high intensity non-coherent visible light, known in dentistry as the plasma-arc, i.e., a xenon light source supplemented with a filter (wavelengths, 400–500 nm) (Sapphire[®] Supreme, Den-Mat[®], Lompoc, CA, USA) fitted with a 9 mm diameter light guiding tip

was applied. The average light power (1500 mW/cm^2 , 'SC' mode) was measured with the unit's built in power meter prior to each experiment.

2.2. Bacterial Strain and Growth Conditions

Fusobacterium nucleatum (PK1594) was cultured in brain heart infusion (BHI) broth at 37°C for 72 h under anaerobic conditions using an anaerobic jar and kit (GasPack[®] EZ, BD, Oxford, UK).

2.3. Experimental Protocol

Bacterial suspensions were spun down ($5000 \times g$, 5 min), the supernatant was discarded and replaced with $200 \mu\text{L}$ of phosphate-buffered saline (PBS) with or without silver nanoparticles ($0.02\% w/v$, 10 nm, Sigma), and samples were placed in 48-well plate (Nunc) with a two well gap between samples and exposed to intermittent blue light (5 s) from a constant distance (5 mm) for a total exposure time of 60 s equivalent to light fluence of 82 J/cm^2 as indicated. Following light exposure, the samples were studied for cell membrane damage, ROS levels, and malodor and VSC production abilities, as described below.

2.4. Malodor Scores

Test tubes containing 2 mL BHI medium were supplemented with $100 \mu\text{L}$ of the above treated bacterial suspensions and incubated at 37°C for 48 h under anaerobic conditions. Following incubation, the test tubes were randomized, and malodor production levels were blindly scored by a trained odor judge. Malodor levels were measured organoleptically immediately after opening each test tube. The scores were recorded according to a semi-integer scale of 0 to 5, as follows: 0, no appreciable odor; 1, barely noticeable odor; 2, slight, but clearly noticeable odor; 3, moderate odor; 4, strong odor; and 5, extremely strong odor.

2.5. VSC Levels

Volatile sulfide compounds (VSC) levels were measured in the test tubes' headspace using a sulfide monitor (HalimeterTM, Interscan Corp., Simi Valley, CA, USA). The monitor was zeroed on ambient air, and a one quarter inch diameter disposable plastic straw was attached to the air inlet of the monitor. VSC levels were measured by inserting the other end of the straw 2 cm into the gas headspace of each test tube immediately after removing the cap and recording the maximal reading in ppb sulfide equivalents.

2.6. Reactive Oxygen Species (ROS) Levels

ROS levels were quantified using 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescent probe [15]. Aliquots of $50 \mu\text{L}$ treated bacterial samples, and controls were added to $150 \mu\text{L}$ phosphate-buffered saline (PBS) in a 96-well dark plate. Then, $5 \mu\text{L}$ of 1 mM H2DCF-DA were added to each well and incubated for 1 h in the dark, at 37°C . The fluorescence density was measured using a fluorescence microplate reader (Infinite F-200, TECAN, Männedorf, Switzerland) with excitation at 480 nm and emission at 530 nm.

2.7. Membrane Integrity

Membrane damage was determined [16] using a Bacteria Live/Dead Staining Kit[®] (Promokine, Heidelberg, Germany), which comprised of DMAO, a green-fluorescent dye that binds nucleic acids of both membrane intact and membrane damaged bacterial cells, and Ethidium homodimer-III (EthD-III), a red-fluorescent dye that binds only to nucleic acids of membrane damaged bacterial cells. Bacterial samples ($100 \mu\text{L}$) were added with $1 \mu\text{L}$ of the dye mixture and incubated in the dark for 15 min at room temperature. The samples ($10 \mu\text{L}$) were wet mounted on slides, covered with cover slips, and studied under a fluorescent microscope ($\times 1000$, L3201LED, MRC), using an excitation light of 460–470 nm and a blue LED filter with cutoff of 500 nm. Digital images of six random fields were taken using a mounted camera (AM7023, DinoEye[®], Anmo, Taiwan). Results were recorded as percentage of red fluorescent dyed bacteria of total counts.

2.8. Statistical Analysis

To compare the effect of the various treatments on measured parameters, ANOVA was applied with post hoc pairwise comparisons, according to Dunnett and Scheffe. The tests applied were two-tailed, and $p \leq 0.05$ was considered statistically significant. Experiments were conducted in six replicates.

3. Results

3.1. Malodor and VSC Levels

The effect of the different treatments and controls on malodor scores and VSC production are presented in Figures 1 and 2. Although silver nanoparticles did show a statistically significant effect on the malodor related parameters ($p < 0.001$), only the combined treatment of blue light and silver nanoparticles was effective in reducing malodor and VSC levels below detection threshold (OJ scores < 2 and VSC < 110 ppb).

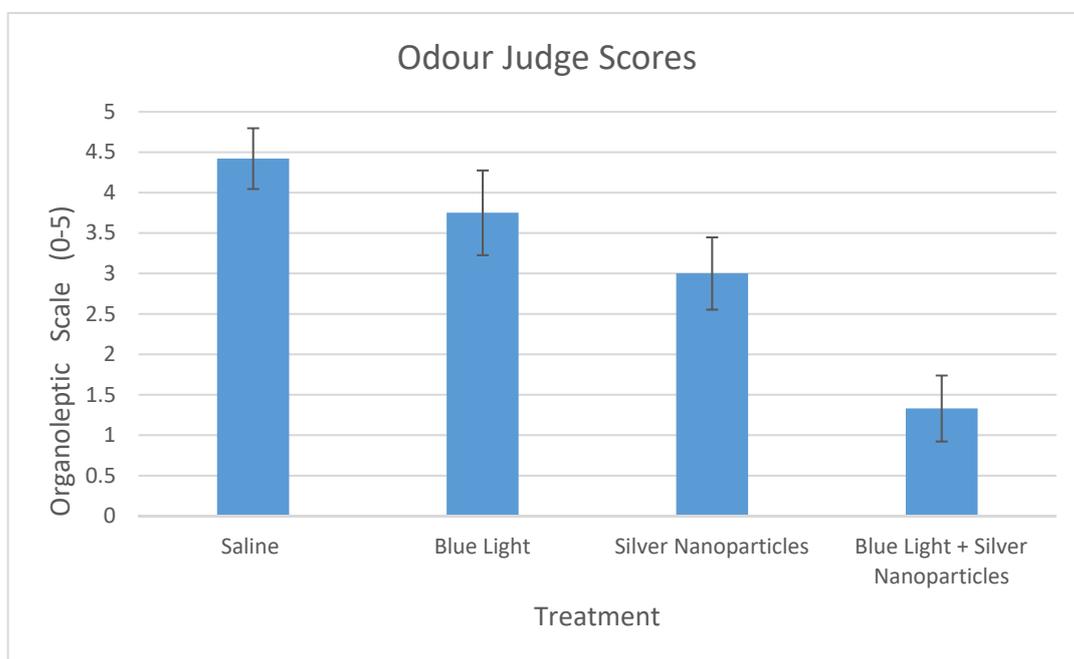


Figure 1. The mean results (\pm SD) of the odor judge scores given using an organoleptic scale of 0–5 for the various treatments, as indicated.

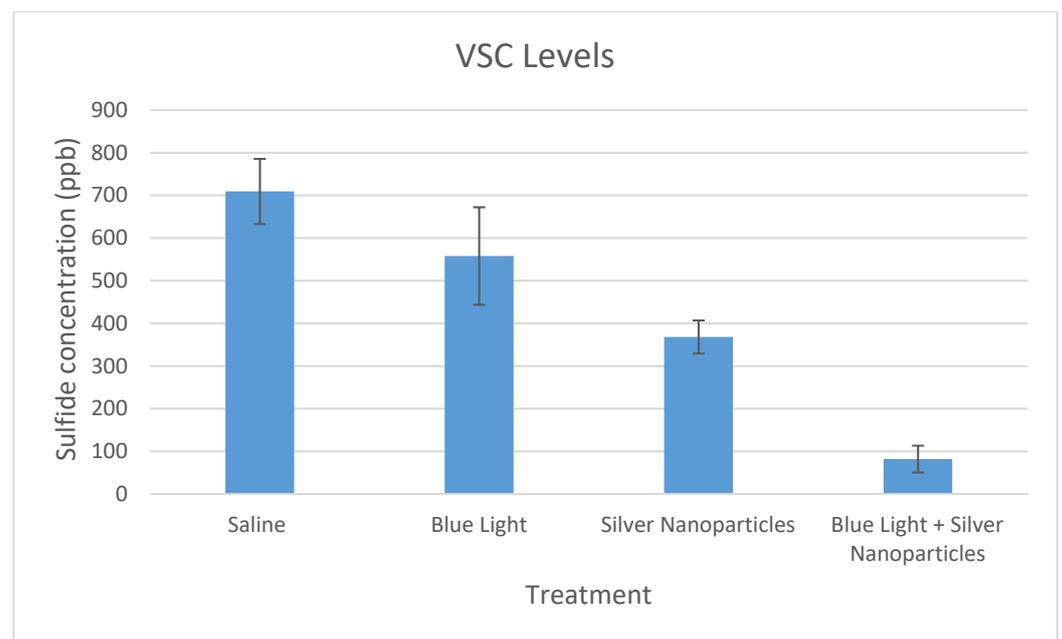


Figure 2. The mean results (\pm SD) of the volatile sulfide compounds (VSC) levels measured using a sulfide monitor (Halimeter) for the various treatments, as indicated.

3.2. Reactive Oxygen Species (ROS) Levels

The effects of the different treatments on reactive oxygen species (ROS) production are presented in Figure 3. Although blue light showed slight elevation in ROS levels, only the combined effect of blue light and silver nanoparticles resulted in a significant increase in ROS levels ($p < 0.001$).

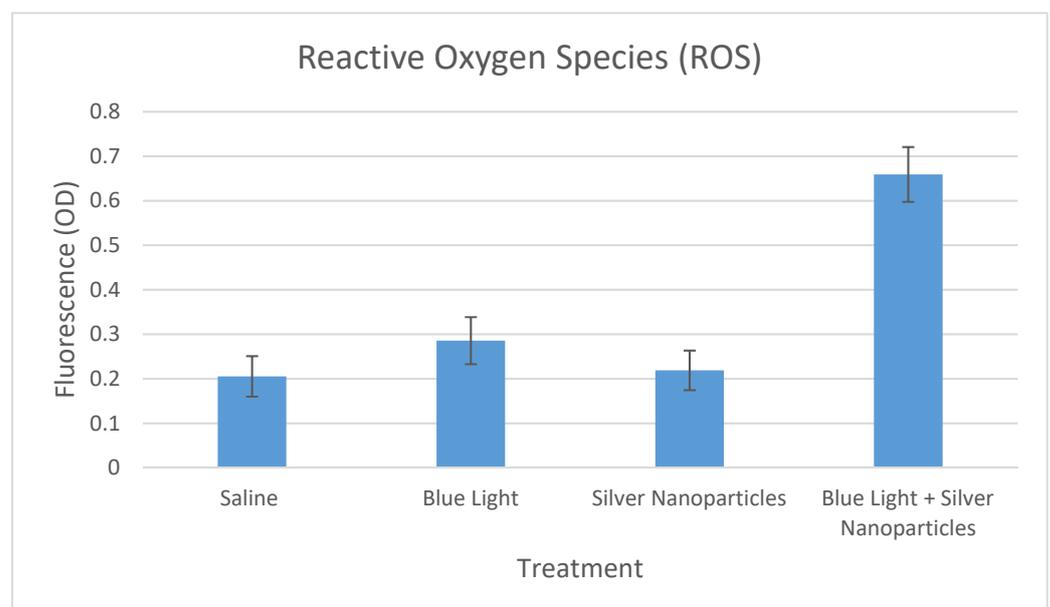


Figure 3. The mean results (\pm SD) of the reactive oxygen species (ROS) levels measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) for the various treatments, as indicated.

3.3. Membrane Integrity

The effects of the different treatments on cell membrane damage are presented in Figures 4 and 5. The combined treatment of blue light and silver nanoparticles was most

effective, resulting in approximately 60% of bacterial cells with damaged cell membrane. This effect was statistically significant ($p < 0.001$) as compared with the individual treatments and control.

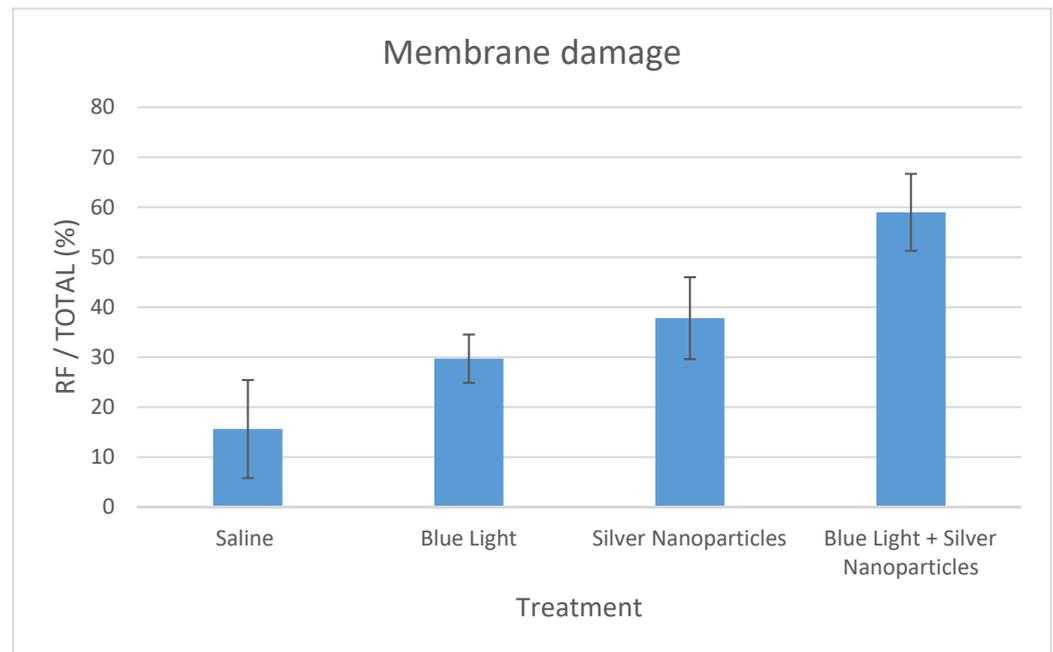


Figure 4. The mean results (\pm SD) of the percentage of membrane damaged bacterial cells measured using digital image analysis from fluorescent microscopy for the various treatments, as indicated. Membrane damaged bacterial cells are stained with ethidium homodimer-III (red fluorescence) and non-damaged ones are stained with DMAO (green fluorescence).

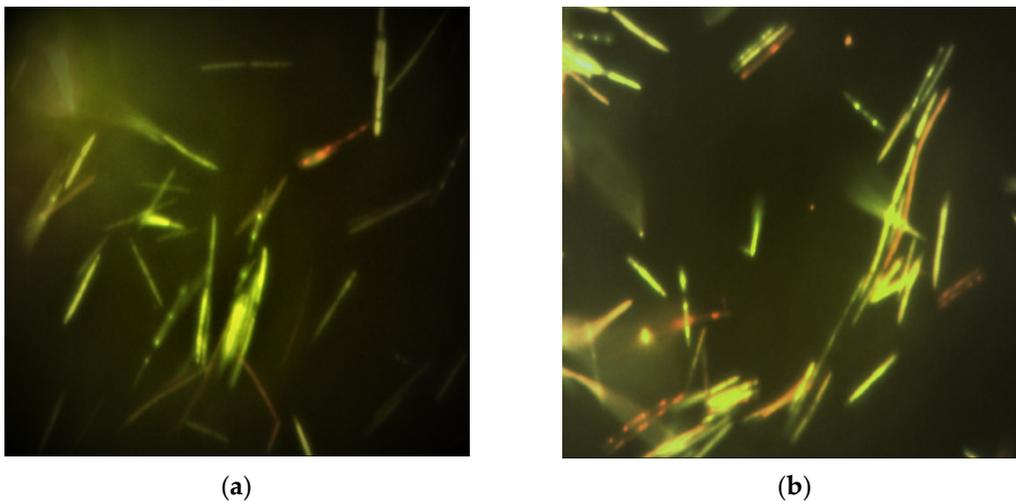


Figure 5. Cont.

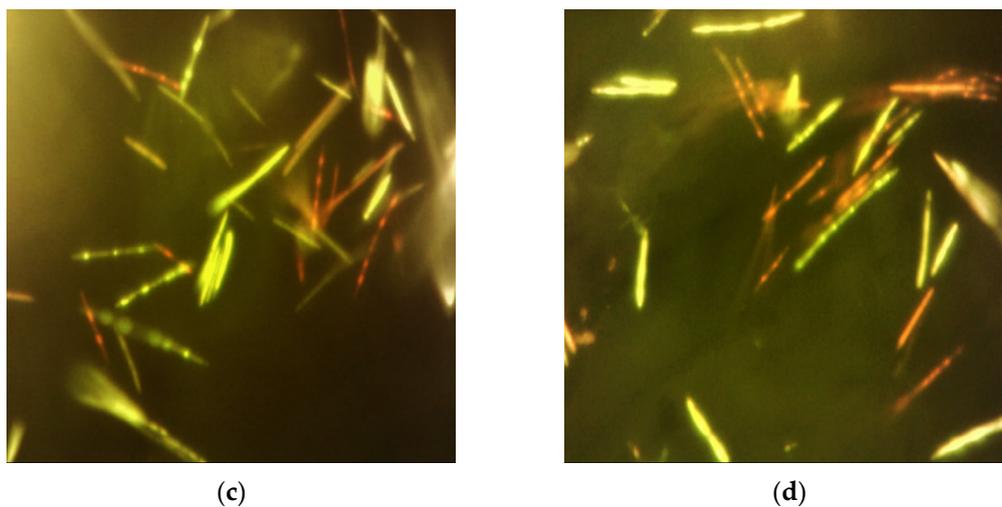


Figure 5. Fluorescent microscopy (FM) images (magnification $\times 1000$) showing membrane damaged (stained red) and undamaged (stained green) bacteria for the various treatments: (a) saline (negative) control; (b) blue light; (c) silver nanoparticles; and (d) blue light combined with silver nanoparticles.

4. Discussion

Previous studies showed that sub-lethal dosage of blue light can increase cell membrane permeability in *Fusobacterium nucleatum* [13,14] and suggested that this effect was mediated through the production of reactive oxygen species [11]. In the present study, we tested the combined effect of sub-lethal blue light and silver nanoparticles on the ability of *Fusobacterium nucleatum* to produce malodor, its cell membrane integrity, and measured the levels of ROS produced following this treatment in order to understand the mechanism for the combined phototoxic effect. The results showed that the combined treatment caused a significant reduction in malodor related parameters concomitant with a rise in ROS levels and cell membrane damage as compared with the individual treatments and control.

Other researchers have also shown that ROS induced sub-lethal damage can be induced in bacteria using a low dosage of blue light exposure [17], resulting in increased membrane permeability. Silver nanoparticles, on the other hand, were reported to have several potential antibacterial mechanisms that include direct membrane damage [7] and ROS production [8]. The results of the present study show that treating *Fusobacterium nucleatum* with silver nanoparticle alone did cause increased cell membrane damage but did not affect ROS levels, whereas combining it with sub-lethal blue light resulted in increased membrane damage concomitant with significant increase in ROS levels, suggesting that silver nanoparticles may also serve as a potent photosensitizer.

The rising problem of bacterial resistant strains to antimicrobial therapy, as well as the risk of floral shift that may give rise to opportunistic infections, has stressed the importance of developing new approaches towards treating bacterial infections. For example, crippling strategies known as anti-virulence treatments [18] that target the bacteria's virulence factors rather than their vitality and growth may eliminate the selective stress that promotes bacterial resistance while decreasing their pathogenicity.

Volatile sulfide compounds (VSC) are considered a key element in oral malodor production as well as an important virulence factor for bacteria, such as *Fusobacterium nucleatum* [19]. VSCs have been shown to induce the progression of periodontal disease and to promote DNA damage and gingival cells death [20].

Within the limitations of an in vitro study, the results of the present study suggest that using sub-lethal blue light exposure combined with silver nanoparticles may increase the efficacy of the antibacterial phototoxic effect against *Fusobacterium nucleatum*, even at a sub-lethal dosage. However, in addition to its beneficial antibacterial phototoxic effect, blue light may also have disruptive effects on mammalian cells [21], possibly mediated by

light-induced ROS. Therefore, in any prospective clinical trial exposure, time should be minimized to reduce any risk to the oral soft tissues. Furthermore, the size and concentration of silver nanoparticles is a complex and controversial issue, since smaller size particles are most effective but show greater risk for mammalian cells toxicity [22]. These points merit further study.

5. Conclusions

The results of the present study suggest that the antibacterial phototoxic effect of blue light against *Fusobacterium nucleatum* may be enhanced by silver nanoparticles.

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