Journal of Biomedical Optics

SPIEDigitalLibrary.org/jbo

Effect of 405-nm high-intensity narrow-spectrum light on fibroblast-populated collagen lattices: an *in vitro* model of wound healing

Richard McDonald
Scott J. MacGregor
John G. Anderson
Michelle Maclean
M. Helen Grant

Effect of 405-nm high-intensity narrow-spectrum light on fibroblast-populated collagen lattices: an *in vitro* model of wound healing

Richard McDonald,[a] Scott J. MacGregor,[b] John G. Anderson,[b] Michelle Maclean,[b] and M. Helen Grant[a]
[a]University of Strathclyde, Bioengineering Unit, Wolfson Centre, 106 Rottenrow, Glasgow, Strathclyde G4 0NW United Kingdom
[b]University of Strathclyde, The Robertson Trust Laboratory for Electronic Sterilisation Technologies, 204 George Street, Glasgow, Scotland G1 1XW United Kingdom

Abstract. High-intensity narrow-spectrum (HINS) 405-nm light is a novel technology developed to address the significant problem of health-care associated infection. Its potential for wound-decontamination applications is assessed on mammalian cells and bacteria. The fibroblast-populated collagen lattice (FPCL) is used as an *in vitro* model of wound healing, and the effect of HINS light on contraction is examined. Effects on cell proliferation, morphological changes, and α-smooth muscle actin (α-SMA) expression are investigated. Bactericidal effects are assessed using the bacterium *Staphylococcus epidermidis*. Low doses of HINS light were found to have no significant inhibitory effects on FPCL contraction, cell proliferation, or α-SMA expression. Doses of up to 18 Jcm^{-2} had no significant inhibitory effects on FPCL cell numbers, and this dose was shown to cause almost complete inactivation of bacteria. These results show that HINS light has potential for disinfection applications without adversely influencing wound healing. [DOI: 10.1117/1.3561903]

Keywords: high-intensity narrow-spectrum 405-nm light; contraction of fibroblast populated collagen lattices; *in vitro* model of wound healing; cell viability; *Staphylococcus epidermidis*.

Paper 10609RR received Nov. 16, 2010; revised manuscript received Feb. 11, 2011; accepted for publication Feb. 11, 2011; published online Apr. 4, 2011.

1 Introduction

Health-care associated infections (HAI) were estimated to have cost the NHS $183 million in Scotland in 2007 with a prevalence of 9.5% in patients admitted to hospital.[1] The rise in prevalence of HAI can partly be attributed to the increased use of antibiotics, leading to antibiotic-resistant strains of many bacteria. Infection of wounds can delay wound healing and is the principal cause of death in burn victims. There is an increasing need for a novel decontamination method capable of maintaining wound sterility and decreasing the risk of infection, without hindering the wound healing process.

High-intensity narrow-spectrum (HINS) light is a new disinfection method that utilizes the documented phototoxic effect of 405-nm visible light without the need for additional photosensitiser molecules.[2,3] Previous studies have demonstrated that 405-nm blue light can achieve photoinactivation of various bacteria, including *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and *Escherichia coli*.[4,5] Bacterial inactivation is thought to be the result of photostimulation of endogenous intracellular porphyrins, which leads to the generation of reactive oxygen species, notably singlet δ oxygen ($^1O_2$).

A HINS-light–based decontamination system has recently undergone clinical evaluation at the Glasgow Royal Infirmary, where it was developed as a background lighting system to provide continuous disinfection of air and exposed surfaces in the presence of patients and staff. The results demonstrated a significantly greater reduction in levels of environmental bacterial contamination than was achievable by normal disinfection and control methods alone.[6] The focus of the work reported here is to investigate the potential of exploiting the difference in repair mechanisms between mammalian cells and bacteria[7,8] such that HINS light could be employed to maintain tissue sterility without damaging the wound-healing cells. The effects of HINS light on fibroblasts were assessed in terms of cell motility and morphology, contraction of the lattice, and expression of the contractile protein, α-smooth muscle actin (α-SMA). The bactericidal effects of the maximum dose at which HINS light had no inhibitory effect on fibroblast function was investigated using *Staphylococcus epidermidis*, a bacterium commonly associated with wound and prosthetic implant infections.

2 Methods and Materials

Fibroblast-populated collagen lattices (FPCLs) were formed from type I acid-soluble collagen, which was extracted from rat-tail tendons. Plain collagen gels [0.3% (w/v)] were formed as described previously[9] and seeded with 3T3 mouse fibroblast cells at 2.5×10^4 cells/cm^2. After 24 h, the FPCLs were transferred to a 90-mm dish and free floated in 13 ml Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum, penicillin/streptomycin, and nonessential amino acids.

Address all correspondence to: M. Helen Grant, University of Strathclyde, Bioengineering Unit, Wolfson Centre, 106 Rottenrow, Glasgow, Strathclyde G4 0NW United Kingdom. Tel: 0141 548 3438; Fax: 0141 552 6098; E-mail: m.h.grant@strath.ac.uk

1083-3668/2011/16(4)/048003/4/$25.00 © 2011 SPIE

The HINS-light system consisted of an array of nine narrow-band blue-light LEDs (GE Lumination, Cleveland, OH), with peak output at 405 nm and a 20-nm bandwidth at full width at half maximum, attached to a heat sink supported by two pillars above a molded base, which centralizes the position of the treatment dish. The FPCLs were exposed to HINS light 24 h after seeding. DMEM was removed and replaced with 10 ml phosphate buffered saline (PBS) for the duration of the treatment, with treatments carried out using light intensities of 0.5, 1.8, and 15 mW cm^{-2} for 1 h, giving a total dose of 1.8, 6.5, and 54 J cm^{-2}, respectively. Light intensity was measured using a radiant power meter (model 70260, Oriel Instruments, Irvine, CA) and photodiode detector (model 1Z02413, Ophir, Andover, MA) calibrated at 405 nm.

To measure contractile function of the fibroblasts, dishes containing FPCLs were placed on standard graph paper, illuminated by a light box and an image was captured daily. Image-processing software (ImageJ, National Institute of Health)[10] was used to calculate the area of the gel. To test viability of fibroblast cells after exposure to HINS light, propidium iodide (20 μg/ml) and acridine orange (100 μg/ml) were used to stain FPCLs at 24, 48, and 120 h after exposure and analysis was performed using a Zeiss AxioImager ZI microscope. The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to quantify cell number as described by Ho et al.[9] Immunoblotting was used to establish the effect of HINS light on the expression of α-SMA in fibroblasts. Proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes for immunoblotting (Millipore, Billerica, MA). The membrane was probed with rabbit polyclonal α-SMA antibody at 1 μg/ml (AbCam, Cambridge, UK) and anti-rabbit IgG secondary antibody (Sigma-Aldrich, St Louis, MO). Membranes were developed using an alkaline phosphatase detection system (Bio-Rad, Hemel Hempstead, UK), which were then scanned and the optical density analyzed using ImageJ.

The HINS-light intensity used for bacterial inactivation experiments was defined by the maximum intensity that did not have an inhibitory effect on fibroblast function, as determined by the MTT collagenase assay. *S. epidermidis* NCTC 10956 was inoculated into 100 ml tryptone soya broth (Oxoid, Basingstoke, UK) and cultivated at 37 °C under rotary conditions (125 rpm) for 18 h. The broth was centrifuged at 4300 g for 10 min, and the pellet was resuspended in 100 ml PBS. This suspension was diluted in PBS to give a population density of 10^3 colony-forming units per milliliter (CFU/ml), and 1 ml exposed to 5 mW cm^{-2} HINS light in the central four wells of a 24-well plate. Postexposure, 100 μl of exposed suspension was manually spread onto tryptone soya agar plates, colonies were counted with the aid of a colony counter, and results were converted into viable CFU/ml. Control samples were also set up and subject to identical conditions but not exposed to 405-nm HINS light.

3 Results and Discussion

HINS-light exposure has an intensity-dependent inhibitory effect on fibroblast function. One-hour exposures to low intensities of HINS light (0.8 and 1.8 mW cm^{-2}) have no significant inhibitory effect on FPCL contraction [Fig. 1(a)]. There is also

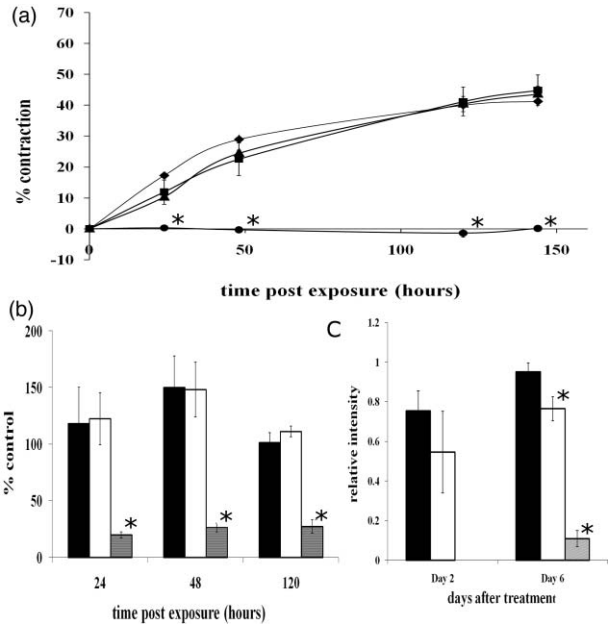

Fig. 1 (a) Contraction curves of FPCL control (♦) and treated for 1 h at 0.5 (■), 1.8 (▲), and 15 mW cm^{-2} (●) on day 1 of contraction. (b) MTT assay results for FPCLs treated for 1 h. (c) Optical density analysis of α-SMA protein bands in western blot. Exposure intensities in (B) and (C) are 0.5 (■), 1.8 (□) and 15 mW cm^{-2} (▮). $n = 3 \pm$ SEM, * *indicates significant difference from control at each time point* ($P < 0.05$, *ANOVA followed by Dunnett's test*).

no significant decrease in cell number as measured by the MTT-collagenase assay [Fig. 1(b)], and expression of the α-SMA protein, a well-established marker of fibroblast contractile activity was unaffected [Fig. 1(c)]. These findings suggest that at these doses, HINS light has little or no inhibitory effect on fibroblast viability or function.

HINS-light doses of 54 Jcm^{-2}, delivered over 1 h at an intensity of 15 mW cm^{-2}, have a noticeable effect on fibroblast function. Contraction of FPCLs halts within 24 h of exposure, and the cells do not appear to recover contractile function significantly within 140 h of the exposure [Fig. 1(a)]. Investigation of the number of cells present on the FPCL following exposure shows that within 24 h, there is an ~80% reduction in cell number and no significant recovery within 120 h [Fig. 1(b)]. Expression of the α-SMA protein is also inhibited by this higher intensity of HINS light [Fig. 1(c)].

Microscopic imaging of the FPCL confirmed that low intensities of HINS light have no obvious effect on fibroblast morphology. Exposure to 15 mW cm^{-2} caused a visible and prolonged decrease in cell number and also showed that cells lose their classic elongated fibroblast morphology following exposure (Fig. 2).

Results of experiments carried out using 1-h exposures at 0.5, 1.8, and 15 mW cm^{-2} indicated that there was a critical point between 1.8 and 15 mW cm^{-2} at which FPCL contraction becomes inhibited by the 405-nm HINS light. In order to determine the maximum intensity at which HINS light did not cause an inhibitory effect, FPCLs were exposed to 3.6–15 mW cm^{-2} for 1 h, giving a dose range of 13–54 Jcm^{-2}, and the subsequent effects on FPCLs were assessed using the MTT-collagenase assay. The maximum intensity at which HINS light did not cause an

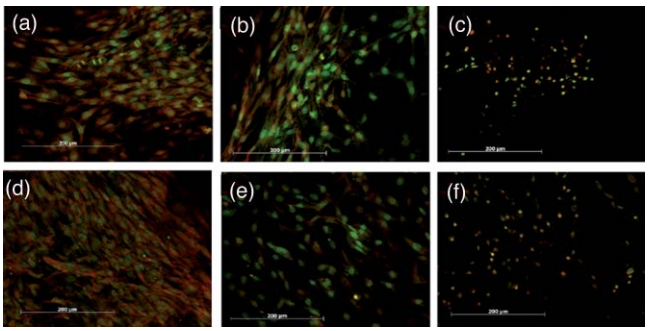

Fig. 2 Microscropy images of HINS-light–treated FPCLs stained with propidium iodide and acridine orange. (a) Control, (b) 1.8, and (c) 15 mW cm^{-2} exposed FPCLs at 24 h postexposure; (d) control, (e) 1.8, and (f) 15 mW cm^{-2} exposed FPCLs at 120 h postexposure. Scale bars are 200 μm. All exposures were for 1 h.

inhibitory effect was established as 5 mW cm^{-2} [Fig. 3(a)], and this intensity was therefore used in the inactivation experiments.

The inactivation results from this study show that almost complete kill of a 3-$\log_{10}$ *S. epidermidis* population is achieved after 1 h exposure to 5 mWcm^{-2} HINS-light [Fig. 3(b)]. Previous data by Maclean et al.[4] demonstrates that complete kill of staphylococcal bacterial species suspended in PBS can be achieved when exposed to high-intensity 405-nm light, and it is likely that this would have been the case if a slightly longer exposure time had been applied; however, the aim of these experiments was to determine the bactericidal effect of HINS light within the parameters of intensity and time that did not inhibit fibroblast function. Although in this case complete bacterial inactivation was not achieved, future work will aim to increase the bactericidal efficacy of doses of HINS light that are nondamaging to mammalian cells.

S. epidermidis, a Gram-positive, coagulase-negative bacterium that is part of the normal skin flora, is a common cause of wound infection.[11] Traditionally, a bacterial count of $>10^5$ CFU/ml is associated with an increased incidence of infection and inhibited wound healing;[12,13] however, as few as 10 colony-forming units have been reported to cause deep infection.[14] In the present study, a population of 10^3 CFU/ml was used in the inactivation experiments, as determined in a recent study by Reinis et al. to be the minimal infective dose of *S. epidermidis* on tested biomaterials.[15] Although a relatively low density, this would be greatly in excess of numbers of staphylococci that could contaminate a wound by environmental transmission. A recent study has, for example, demonstrated that the levels of staphylococcal bacteria contaminating environmental surfaces in a hospital isolation room had a mean value of only 3.5 CFU/cm^2.[6] Consequently, there may be potential for the application of HINS light to maintain the sterility of wounds during periods of wound exposure without having a detrimental effect on the wound-healing process.

Although we have demonstrated that 5 mW cm^{-2} HINS light effectively inactivates *S. epidermidis*, the sensitivity of a range of other clinically important microorganisms to this dose of HINS light needs to be investigated. Other investigations using HINS light have shown significant reductions in populations of bacteria from a variety of genera (*Staphylococcus, Clostridium, Escherichia, Streptococcus, Acinetobacter, Proteus, Pseudomonas, Propionibacterium*, and *Helicobacter*) by various combinations of intensity and dose of blue light.[4,16–18] The mechanisms responsible for the bactericidal effect include the involvement of porphyrins, but there may be other mechanisms involved.

The inactivation of bacteria in environments that more closely replicate the wound bed must be investigated. Lambrechts et al. have shown that the introduction of human blood plasma and human albumin serum reduces the sensitivity of a range of bacteria to photodynamic inactivation (PDI) with exogenous photosensitisers.[19] Spesia et al. experienced a similar reduction in effectiveness of PDI when exposing *E. coli* in the presence of blood plasma.[20] The effect of protein absorption on bacterial inactivation by HINS light is yet to be established. This initial study has been carried out on the 3T3 mouse fibroblast cell line. To establish safety of HINS light in the context of wound disinfection, *in vitro* effects on primary human dermal fibroblasts, skin epithelial cells, and other cell types involved in the wound-healing process should also be investigated.

4 Summary

The results show that exposure of 3T3 fibroblast cells to 1 h of HINS light with intensities of 5 mW cm^2 and below does not have an inhibitory effect on fibroblast function. This result implies that exposing an open wound to these intensities for 1 h would not have a detrimental impact on the wound-healing process. It has also been shown that a 1-h exposure to

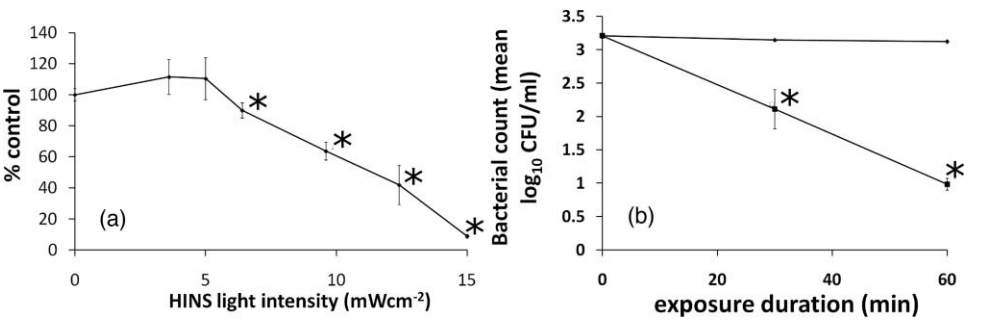

Fig. 3 (a) MTT assay results for FPCLs treated for 1 h from 3.6 to 15 mW cm^{-2}. Exposures of >5 mW cm^{-2} resulted in significant decrease in cell number. * indicates significant decrease in cell number as compared to exposure at 5 mW cm^{-2} ($P < 0.05$, ANOVA followed by Dunnett's comparison, $n = 4 \pm$ SEM) (B) Inactivation of *S. epidermidis* exposed to 1 h of 5 mW cm^{-2} HINS light. ♦ indicates control, ■ indicates exposed bacteria. * indicates significant difference from control ($P < 0.05$, ANOVA followed by Dunnett's comparison, $n = 4 \pm$ SEM).

5 mW cm^{-2} of HINS light causes significant inactivation of the wound pathogen *S. epidermidis*. Although further work is required to extend these investigations, the results demonstrate that HINS-light treatment merits further investigation as a potential tool to help maintain the sterility of a wound or as a technique that could be used to aid wound disinfection.

Acknowledgments

R.M. is supported by the Engineering and Physical Sciences Research Council.

References

1. J. Reilly, S. Stewart, G. Allardice, A. Noone, C. Robertson, A. Walker, and S. Coubrough, "NHS Scotland National HAI Prevalence Survey," Final Report 2007, Health Protection Scotland (2007).
2. M. MacLean, J. G. Anderson, G. A. Woolsey, and S. J. MacGregor, "Inactivation of Gram positive bacteria'' Int. Patent No. WO 2007/012875 A (2007).
3. S. J. MacGregor, J. G. Anderson, M. MacLean, and G. Woolsey, "Light inactivation of problematic micro-organisms (MRSA)," Int. Patent Application No. PCT/GB2006/002841 (2006).
4. M. Maclean, S. J. MacGregor, J. G. Anderson, and G. Woolsey, "Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array," *Appl. Env. Microbiol.* **75**(7), 1932–1937 (2009).
5. J. S. Guffey and J. Wilborn, "*In vitro* bactericidal effects of 405-nm and 470-nm blue light," *Photomed. Laser Surg.* **24**(6), 684–688 (2006).
6. M. Maclean, S. J. MacGregor, J. G. Anderson, G. A. Woolsey, J. E. Coia, K. Hamilton, I. Taggart, S. B. Watson, B. Thakker, and G. Gettinby, "Environmental decontamination of a hospital isolation room using high-intensity narrow-spectrum light," *J. Hosp. Infect.* **76**(3), 247–251 (2010).
7. C. Roehlecke, A. Schaller, L. Knels, and R. H. W. Funk, "The influence of sublethal blue light exposure on human RPE cells," *Mol. Vis.* **15** 1929–1938 (2009).
8. D. L. Croteau and V. A. Bohr, "Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells," *J. Biol. Chem.* **272**(41), 25409–25412 (1997).
9. G. Ho, M. H. Grant, J. C. Barbenel, and C. J. Henderson, "Low-level laser therapy on tissue-engineered skin substitutes: effect on the proliferation rate of 3T3 mouse fibroblast cells," in *Laser Florence 2003: A Window on the Laser Medicine World*, pp. 124–134 (2004).
10. W. S. Rasband, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011.
11. P. G. Bowler, B. I. Duerden, and D. G. Armstrong, "Wound microbiology and associated approaches to wound management," *Clin. Microbiol. Rev.* **14**(2), 244–269 (2001).
12. S. Landis, "Chronic wound infection and antimicrobial use," *Adv. Skin Wound Care* **21**(1), 531–540 (2008).
13. R. H. Bendy, Jr., P. A. Nuccio, E. Wolfe, B. Collins, C. Tamburro, W. Glass, and C. M. Martin, "Relationship of quantitative wound bacterial counts to healing of decubiti: effect of topical gentamicin," *Antimicrob. Agents Chemother.* **10**, 147–155 (1964).
14. P. Gosden, A. MacGowan, and G. Bannister, "Importance of air quality and related factors in the preventation of infection in orthopaedic implant surgery," *J. Hosp. Infect.* **39**(3), 173–180 (1998).
15. A. Reinis, J. Vetra, A. Stunda, L. Berzina-Cimdina, J. Kroica, V. Kuznecova, and D. Rostoka, "*In vitro* and *in vivo* examinations for detection of minimal infective dose for biomaterials," in *Proc. of 6th World Congress of Biomechanics*. August 1–6, 2010 Singapore, R. Magjarevic, Ed., Springer, Berlin, pp. 1204–1207 (2010).
16. L. E. Murdoch, M. Maclean, S. J. MacGregor, and J. G. Anderson, "Inactivation of Campylobacter jejuni by exposure to high-intensity 405-nm visible light," *Foodborne Pathogens Dis.* **7**(10), 1211–1216 (2010).
17. M. R. Hamblin, J. Viveiros, C. Yang, A. Ahmadi, R. A. Ganz, and M. J. Tolkoff, "Helicobacter pylori accumulates photoactive porphyrins and is killed by visible light," *Antimicrob. Agents Chemother.* **49**(7), 2822–2827 (2005).
18. H. Ashkenazi, Z. Malik, Y. Harth, and Y. Nitzan, "Eradication of Propionibacterium acnes by its endogenic porphyrins after illumination with high intensity blue light," *FEMS Immunol. Med. Microbiol.* **35**(1), 17–24 (2003).
19. S. A. G. Lambrechts, M. C. G. Aalders, F. D. Verbraak, J. W. M. Lagerberg, J. B. Dankert, and J. J. Schuitmaker, "Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin," *J. Photochem. Photobiol. B* **79**(1), 51–57 (2005).
20. M. B. Spesia, M. Rovera, and E. N. Durantini, "Photodynamic inactivation of Escherichia coli and Streptococcus mitis by cationic zinc(II) phthalocyanines in media with blood derivatives," *Eur. J. Med. Chem.* **45**(6), 2198–2205 (2010).