405 nm LIGHT EXPOSURE OF OSTEOBLASTS AND INACTIVATION OF BACTERIAL ISOLATES FROM ARTHROPLASTY PATIENTS: POTENTIAL FOR NEW DISINFECTION APPLICATIONS?

R.S. McDonald[1,2,§], S. Gupta[1,2,3,§], M. Maclean[2], P. Ramakrishnan[1,2], J.G. Anderson[2],
S.J. MacGregor[2], R.M.D. Meek[3] and M.H. Grant[1,*]

[1] Department of Biomedical Engineering, University of Strathclyde, Wolfson Centre, Glasgow, Scotland, UK
[2] The Robertson Trust Laboratory for Electronic Sterilisation Technologies, University of Strathclyde, Glasgow, Scotland, UK
[3] Department of Trauma & Orthopaedic Surgery, Southern General Hospital, Glasgow, Scotland, UK

[§]These authors contributed equally to the paper.

Abstract

Infection rates after arthroplasty surgery are between 1-4 %, rising significantly after revision procedures. To reduce the associated costs of treating these infections, and the patients' post-operative discomfort and trauma, a new preventative method is required. High intensity narrow spectrum (HINS) 405 nm light has bactericidal effects on a wide range of medically important bacteria, and it reduced bacterial bioburden when used as an environmental disinfection method in a Medical Burns Unit. To prove its safety for use for environmental disinfection in orthopaedic theatres during surgery, cultured osteoblasts were exposed to HINS-light of intensities up to 15 mW/cm² for 1 h (54 J/cm²). Intensities of up to 5 mW/cm² for 1 h had no effect on cell morphology, activity of alkaline phosphatase, synthesis of collagen or osteocalcin expression, demonstrating that under these conditions this dose is the maximum safe exposure for osteoblasts; after exposure to 15 mW/cm² all parameters of osteoblast function were significantly decreased. Viability (measured by protein content and Crystal Violet staining) of the osteoblasts was not influenced by exposure to 5 mW/cm² for at least 2 h. At 5 mW/cm² HINS-light is an effective bactericide. It killed 98.1 % of *Staphylococcus aureus* and 83.2 % *Staphylococcus epidermis* populations seeded on agar surfaces, and is active against both laboratory strains and clinical isolates from infected hip and knee arthroplasties. HINS-light could have potential for development as a method of disinfection to reduce transmission of bacteria during arthroplasty, with wider applications in diverse surgical procedures involving implantation of a medical device.

Keywords: Healthcare associated infections (HAI); High-intensity narrow-spectrum (HINS) light; disinfection; 405 nm light; osteoblast cell cultures; bactericidal effects; *Staphylococcus*.

*Address for correspondence
M. Helen Grant
University of Strathclyde
Bioengineering Unit, Wolfson Centre,
06 Rottenrow, Glasgow, Strathclyde G4 0NW UK
Telephone Number: 0141 548 3438
FAX Number: 0141 552 6098
E-mail: m.h.grant@strath.ac.uk

Introduction

Healthcare associated infections (HAI), defined as infections which are not present at the time the patient enters hospital, are an ever increasing problem in modern healthcare affecting approximately 1 in 10 patients admitted to UK hospitals (Reilly *et al.*, 2007). Despite current attempts to resolve the problem, including campaigns to improve hygiene in hospitals, particularly hand washing, HAI still causes significant patient mortality. A report published by the House of Commons in 2004 found that HAIs are responsible for over 5,000 deaths in the UK each year, and are a contributory factor in over 1,500 deaths (National Audit Office, 2004). In the USA, deaths associated with HAI in hospitals exceeded the number attributable to several of the top ten leading causes of death. A survey performed in 2002 found 1.7 million patients with an HAI, of which 155,668 died (Klevens *et al.*, 2007). The rise in prevalence of HAI can partly be attributed to the increased use of antibiotics leading to antibiotic resistant strains of many bacteria (McGowan, 1983). It is clear that novel approaches to bacterial inactivation are required.

HAI take many forms. The most common type of infection reported by the Scottish National HAI Prevalence Survey was found to be urinary tract infection (UTI), accounting for 17.9 % of cases (Reilly *et al.*, 2008), with a major risk factor being the use of indwelling catheters. Surgical site infections (SSI) are the next most common, accounting for 16 %. The use of indwelling or implanted medical devices is increasing with technological advances, and so the incidence of device-related infection is increasing (von Eiff *et al.,* 2005). Taking infection acquired during hip replacement surgery as a specific example of HAI, studies have shown incidence rates from 1-5 %, increasing considerably after revision procedures (Ridgeway *et al.*, 2005; Hamilton and Jamieson, 2008; Wilson *et al.*, 2008). During mandatory surveillance of 22,160 hip replacement procedures taking place in England between April 2004 and March 2005, *Staphylococcus aureus* was identified in 64 % of infections, 67 % of which were methicillin-resistant strains (Wilson *et al.*, 2008). *Staphylococcus epidermidis* has also been identified in a large number of cases (Hamilton and Jamieson, 2008). The source of these infections is many-fold. Patient skin will contribute, but general contamination of the operation site and equipment is a

major issue. This is particularly well documented in a paper by Davis and co-workers from Manchester, UK, reporting that 63 % of operations showed contamination of the field of operation (Davis *et al*, 1991). A reduction in the bacterial load will therefore be beneficial. Laminar flow ventilation, by which a continuous flow of highly filtered bacteria-free air is recirculated under positive pressure into the operating field and air contaminants generated during surgery are removed from the site, was introduced to orthopaedic operating theatres in the 1980s. However, even with modern laminar flow ventilation, unacceptably high numbers of bacterial isolates are still reported during orthopaedic surgery (Owers *et al.*, 2004), and at the British Hip Society meeting in 2011 McGovern and colleagues demonstrated how easily laminar air flow can be significantly disrupted by external forces during orthopaedic surgery (McGovern *et al*, 2011). Ultraviolet (UV) light in the operating theatres is more effective than laminar flow ventilation at reducing the number of airborne bacteria, and Ritter *et al.* (2007) reported that UV radiation reduced the risk of infection in joint replacement procedures from 1.77 to 0.57 %. Despite this effect, UV radiation has not been widely used in the UK as the protective clothing required by operating staff is deemed to be too hot, thick and uncomfortable for routine use (Godsen *et al.*, 1998).

High-intensity narrow-spectrum (HINS) light is a new disinfection method that utilises the phototoxic effect of 405 nm visible blue light without the need for additional photosensitiser molecules (Anderson *et al.*, 2006; Anderson *et al.*, 2007). Although not as bactericidal as UV light, 405 nm light has advantages including increased human safety due to its lower photon energy (Blatchley and Peel, 1991). Previous studies have demonstrated that this 405 nm blue light can inactivate various bacteria, including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and *Escherichia coli* (Guffey and Wilborn, 2006; Maclean *et al.*, 2008a; Maclean *et al.*, 2009). The mechanism of bacterial inactivation is thought to be by photostimulation of endogenous intracellular porphyrins, which leads to the generation of reactive oxygen species (ROS) (Orenstein *et al.*, 1997; Papageorgiou *et al.,* 2000; Guffey and Wilborn, 2006; Maclean *et al.*, 2008b). Exposure to 405 nm light at doses that inactivate *S. epidermidis* has been shown to not affect the viability of fibroblasts *in vitro*, or their contractile activity in a wound healing model – the fibroblast populated collagen hydrogel lattice (McDonald *et al.*, 2011).

The use of 405 nm HINS-light for environmental disinfection has undergone clinical evaluation in occupied patient isolation rooms in Glasgow Royal Infirmary, where it was used 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 contamination than was achievable by normal disinfection control methods alone (Maclean *et al.*, 2010). It is proposed that 405 nm HINS-light has considerable potential to reduce the environmental airborne bacterial load in orthopaedic operating theatres, and decrease the risk of post-operative infection following arthroplasty procedures. To assess

the efficacy of 405 nm HINS-light for this application, the susceptibility of bacterial isolates from clinical cases of surgical site infections from arthroplasty procedures to HINS-light was assessed. To ensure in principle that any exposed patient bone tissue would not be detrimentally affected by exposure to lethal doses of 405 nm HINS-light, the effect of exposure of osteoblasts *in vitro* to bactericidal doses of HINS-light was determined in terms of viability, morphology and function. Osteoblast functions were assessed by alkaline phosphatase (ALP) activity, collagen synthesis and osteocalcin expression.

Materials and Methods

Culture and 405 nm light exposure of osteoblasts

The effect of 405 nm HINS-light was determined on immortalised neonatal rat calvarial osteoblasts cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % v/v foetal calf serum, 1 % v/v non-essential amino acids, penicillin (50 units/mL) and streptomycin (50 μg/mL). Cells were routinely cultured as monolayers in 75 cm^2 tissue culture flasks in a humidified atmosphere of 5 % CO_2 in air at 37 °C. For experimental use, the required cell concentration was prepared in complete DMEM and seeded onto the surface of multi-well plates (0.2 mL for 96-well plates; 1 mL for 24-well plates), with the seeding density used (5×10^3 cells/cm^2 or 2×10^4 cells/cm^2) determined by the post-exposure test being performed. After seeding, cells were incubated for a minimum of 4 h to allow attachment, and then the culture medium was replaced with Dulbecco's phosphate buffered saline (PBS) for 405 nm HINS-light exposure.

The HINS-light system consisted of a bank of nine narrow-band LEDs (GE Lumination, Cleveland, OH, USA), with peak output at 405 nm and a 20 nm bandwidth at full-width half-maximum, attached to a heat sink supported by two pillars above a moulded base which centralises the position of the treatment dish. The distance between the sample and the LEDs was 8 cm. This short distance was chosen to optimise chances of seeing any deleterious effects of the HINS-light exposure in the experimental set up. The heat sink ensured that the operating temperatures remained constant for the duration of the light treatments, and no heating of samples occurred. Cells were exposed to 1 h durations of 405 nm light with irradiance levels of 0.5, 1.8, 5 and 15 mW/cm^2, corresponding to doses of 1.8, 6.5, 18 and 54 J/cm^2. Control cells that were not exposed to HINS-light were incubated in PBS for an equivalent time. The effect of prolonged exposure times was investigated for up to 3 h in terms of cell viability. After exposure, PBS was removed and the cells were incubated in fresh media until required for assessment of osteoblast function, with media being refreshed every 2-3 days for the duration of the experiment.

Markers of osteoblast function

Cells were seeded at 2×10^4 cells/cm^2 in 96-well plates for measurement of alkaline phosphatase (ALP) activity, collagen synthesis and osteocalcin expression. ALP activity was measured at 24 and 72 h post-exposure to 405 nm

HINS-light at irradiances between 0.5 and 15 mWcm^2 by the dephosphorylation of p-nitrophenyl phosphate (pNPP; 1 mg/mL). DMEM was removed, wells washed with PBS, and 200 µL of 1 mg/mL pNPP in 0.1 M glycine buffer, pH 10.4, containing 1 mM MgCl$_2$ and 1 mM ZnCl$_2$ added to each well. pNPP is dephosphorylated to p-nitrophenol and phosphate in the presence of ALP, and the resultant release of p-nitrophenol was detected at 405 nm immediately and 15 min after addition and calculated using the extinction coefficient of p-nitrophenol (18.75 mM/cm) (Bowers and McComb, 1966).

Collagen synthesis by the osteoblasts was assessed by the method of Walsh *et al.* (1992). At 24 and 72 h post-exposure to 405 nm HINS-light at irradiances between 0.5 and 15 mWcm^2, the cells were fixed by Bouin's solution, and stained with picrosirius red stain for 1 h. Following a wash with 0.01 M HCl, cells were solubilised in 0.25 M NaOH, and the absorbance quantified at 550 nm using a standard curve obtained with collagen type I. Picrosirius red staining was also used to visualise the cells using the above procedure carried out on cells cultured on 10 mm diameter glass slides in 24-well plates at the same seeding density. Instead of solubilising the cells post-staining, they were visualised with bright field microscopy (Zeiss Axioimager microscope; Zeiss, Oberkochen, Germany).

Osteocalcin secreted into the medium 3, 6 and 10 days after exposure of cells to 405 nm HINS-light at irradiances between 1.8 and 15 mWcm^2 was measured with an ELISA kit (BT-490, Biomedical Technologies, Stoughton, MA, USA) according to the manufacturer's instructions. Production of osteocalcin by the osteoblasts was maximised by treating the cells for 3 days with 10^{-8} M 1,25-dihydroxy vitamin D$_3$ (Martinez *et al.*, 2001).

Scanning electron microscopy was carried out on 2×10^4/cm^2 cells cultured on 10 mm diameter poly-l-lysine coated glass slides. Immediately after exposure to 405 nm HINS-light cells were fixed with 2.5 % glutaraldehyde, then treated with 1 % osmium tetroxide and uranyl acetate, before being critical point dried through a series of alcohols, sputter coated with a gold/palladium mixture and viewed at magnifications ranging from ×250 to ×30000 at 10 kV.

Measurement of viability

The total protein content of the osteoblasts attached to the culture dishes was determined 2 and 3 days after exposure to 5 mW/cm^2 HINS-light for between 1 and 3 h. After seeding at 5×10^3 cells/cm^2 in 24-well plates the total protein was measured by the Lowry assay (Lowry *et al.*, 1951) after solubilising the cells in 0.5 M NaOH overnight. Crystal violet staining of cellular DNA in attached cells was quantified after fixing the cell monolayers with formalin, for 30 min followed by staining with 1 mg/mL Crystal Violet for 20 min. The stain was solubilised with 0.1 % Triton X-100 in PBS for 24 h on a rotary plate before measuring the absorbance at 540 nm.

Culture and 405 nm HINS-light exposure of bacterial pathogens

Culture collection strains of *Staphylococcus aureus* NCTC 4135 and *Staphylococcus epidermidis* NCTC 11964 were obtained from the National Collection of Type Cultures (Collindale, UK). Clinical bacterial isolates used in this study were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Corynebacterium striatum*, *Enterococcus faecalis*, *Micrococcus* sp., *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa*. All clinical isolates were isolated from infected hip and knee arthroplasties, and were obtained from the Southern General Hospital Microbiology Department (Glasgow, UK).

For experimental use, bacterial species were inoculated into 100 mL broth (Oxoid/Thermo Fisher Scientific, Basingstoke, UK) and incubated at 37 °C for 18 h under rotary conditions (120 rpm). All microorganisms were cultured in Nutrient Broth (Oxoid/Thermo Fisher Scientific) with the exception of *C. striatum*, which was inoculated in Brain Heart Infusion Broth (Oxoid/Thermo Fisher Scientific). After incubation, cultures were centrifuged at 3939 × g for 10 min and the microbial cell pellet was then re-suspended in 100 mL PBS. Bacterial suspensions were diluted to a population density of 10^3 colony-forming units per millilitre (CFU/mL). For 405 nm light exposure, 100 µL microbial suspensions were seeded onto agar plates, providing a population of approximately 100-300 CFU/agar plate. Nutrient agar was used for all bacterial species except *C. striatum, which* was seeded onto blood agar (Oxoid/Thermo Fisher Scientific).

For exposure of *S. aureus* NCTC 4135 and *S. epidermidis* NCTC 11964, the 405 nm HINS-light source used was the same as that used for exposure of the osteoblast cells. Microbial samples were exposed to 1 h durations of 405 nm light with an irradiance level of 5 mW/cm^2 (~18 J/cm^2). Post-exposure, plates were incubated at 37 °C for 24 h before enumeration. Results represent the mean of a minimum of triplicate replicates, and are reported as the percentage of surviving CFU/plate, as compared to non-exposed control samples. To determine the biocidal activity of 405 nm HINS-light against the range of clinical infected arthroplasty isolates, a 405 nm LED array with 144 LEDs (Enfis/Photonstar Technology, Romsey, Hampshire, UK) was used which provided an average irradiance of 71 mW/cm^2 across the diameter of the agar surface. This higher power 405 nm light source provided higher irradiance, thereby providing a more rapid and convenient exposure system for determination of the susceptibility of the range of arthroplasty isolates to 405 nm light. Isolates seeded on the agar surfaces were exposed to increasing durations of light treatment (between 2.5 min to 1 h), and non-exposed control plates were set-up for all samples. As with the culture collection isolates, results are reported as the percentage of surviving CFU/plate, as compared to non-exposed control samples.

Statistical analysis

Data were compared by ANOVA followed by Dunnett's *post-hoc* test, unless otherwise stated, and significance was assigned at $p < 0.05$.
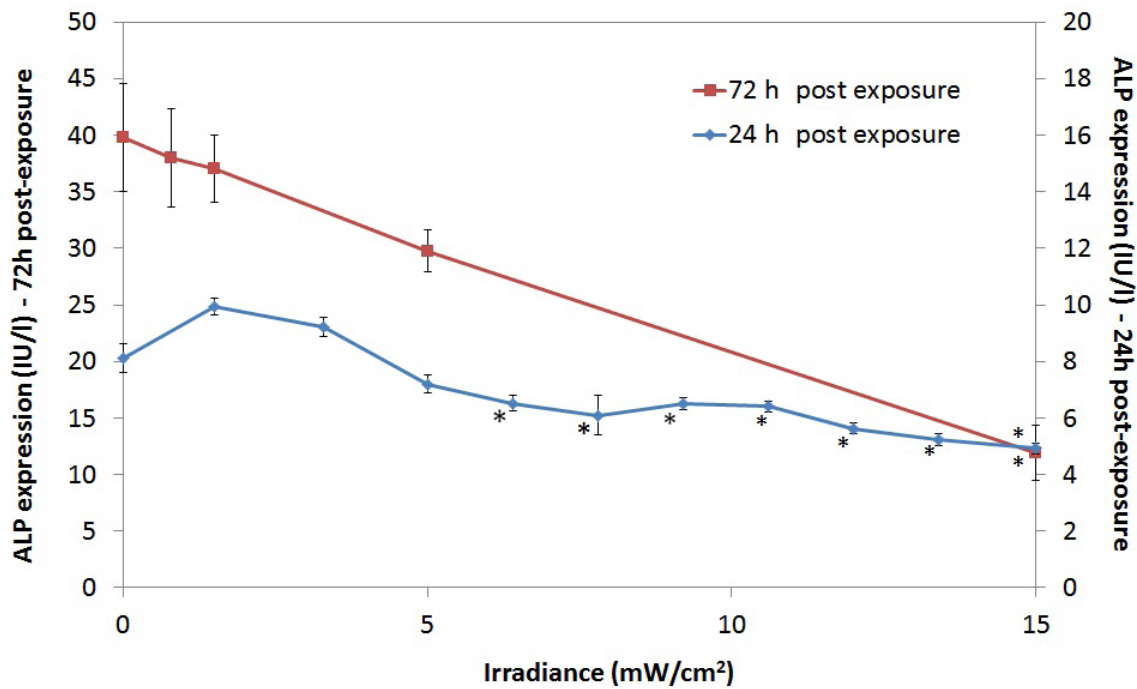
Fig. 1. ALP expression at 24 (♦) and 72 (■) hours post-exposure of osteoblasts treated at between 0.5 and 15 mW/cm². * indicates significant difference from control ($p < 0.05$, ANOVA followed by Dunnett's comparison, $n = 3$ independent experiments ± SEM). Note that the data at 24 h should be read off the y axis on the right of the graph, and those for 72 h from the axis on the left of the graph.

Results

Effect of 405 nm HINS-light exposure on osteoblast function

The function of osteoblasts following exposure to 405 nm HINS-light was monitored by ALP activity and by the ability to synthesise collagen and osteocalcin.

A 1 h exposure to intensities of 405 nm HINS-light at or below 5 mW/cm² (18 J/cm²) caused no significant reduction in activity of ALP at 24 h post-exposure. Intensities above 5 mW/cm² caused a statistically significant decrease in ALP activity. By 72 h post-exposure, osteoblasts treated at 15 mW/cm² showed minimal signs of recovery. Cells exposed to 5 mW/cm² HINS-light and below did not show a significant decrease in ALP activity compared with the control at 72 h post-exposure (Fig.1).

Differential interference contrast (DIC) microscopy images of control cells and cells exposed to 1 h of 15 mW/cm² HINS-light stained with picrosirius red for the presence of collagen are shown in Fig. 2 (A) and (B) respectively. Microscopy was performed at 48 h following exposure. Osteoblasts exposed to 15 mW/cm² retained the elongated shape of healthy osteoblasts and no obviously damaged cells were present in the sample. Collagen synthesis was not affected by exposure to up to 5 mW/cm² HINS-light, at either 24 or 72 h post-exposure. A significant decrease in synthesis was shown in osteoblasts exposed to 15 mW/cm² HINS-light at 24 h post-exposure, and this significant decrease persisted at 72 h post-exposure (Fig. 3) with no sign of recovery.

No decrease was observed in osteocalcin expression by osteoblasts exposed to 5 mW/cm² or lower HINS-light for 1 h at up to 10 days post-exposure. However, expression was significantly suppressed in osteoblasts exposed to HINS-light intensities of 15 mW/cm² for 1 h at 3 and 6 days post-exposure (Fig. 4). By 10 days post-exposure, expression of osteocalcin in cells exposed to 1 h of 15 mW/cm² was not significantly different from the control.

To detect any effects on cell morphology, scanning electron microscopy was performed on cells immediately following a 1 h exposure to 5 and 15 mW/cm² HINS-light. Cells that had not been exposed to HINS-light had classic osteoblast morphology (Fig. 5A). They appear well attached and stretched out, and high magnification images of individual cells showed no remarkable surface features. Osteoblasts exposed to 1 h at 5 mW/cm² appeared to be similar to the controls (Fig. 5B), but after exposure to 15 mW/cm² they had a noticeably different appearance compared to control cells (Fig. 5C). Although the cell density appeared similar, the proportion of cells that were attached and stretched out was less. Higher magnification images of the cell surface showed many small imperfections, like cuts or folds in the membrane, suggesting some sub-lethal effects on the cells (Fig. 5C). A summary of the effects of the maximum dose (15 mW/cm² for 1 h) of HINS-light on the cells is shown in Table 1.

Having determined that exposure to 5 mW/cm² for 1 h was a safe dose of 405 nm HINS-light in terms of the osteoblast functions, cells were exposed to this irradiance level of light for up to 3 h to establish the maximum duration this irradiance of light could be used safely. Viability of the cells in culture was measured 48 and 72 h post-exposure by both total protein content and DNA
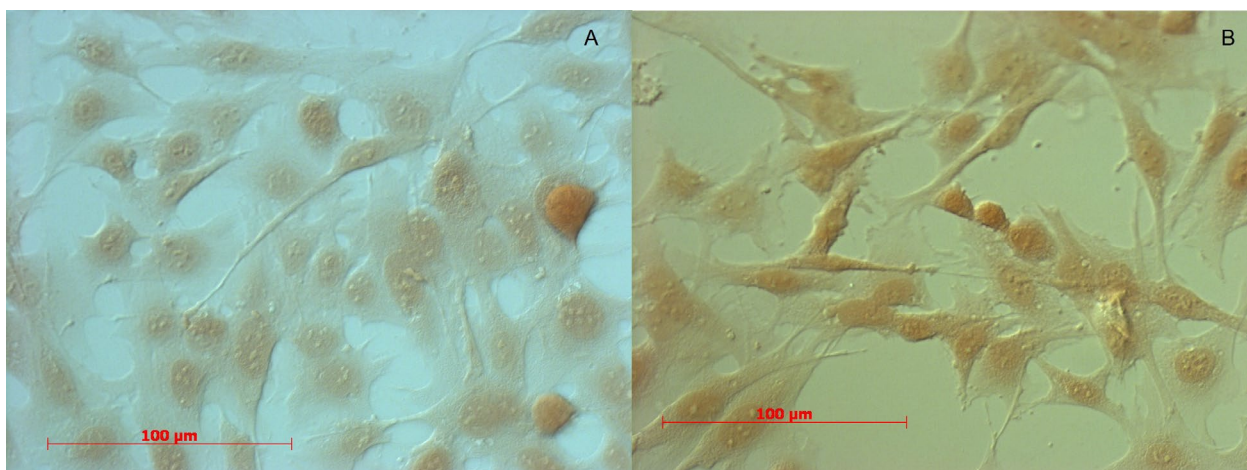
Fig. 2. DIC microscopy images of osteoblasts stained with picrosirius red. Image (**A**) and (**B**) show control cells and those exposed to 15 mW/cm² HINS-light for 1 h, respectively, at 48 h post-exposure.
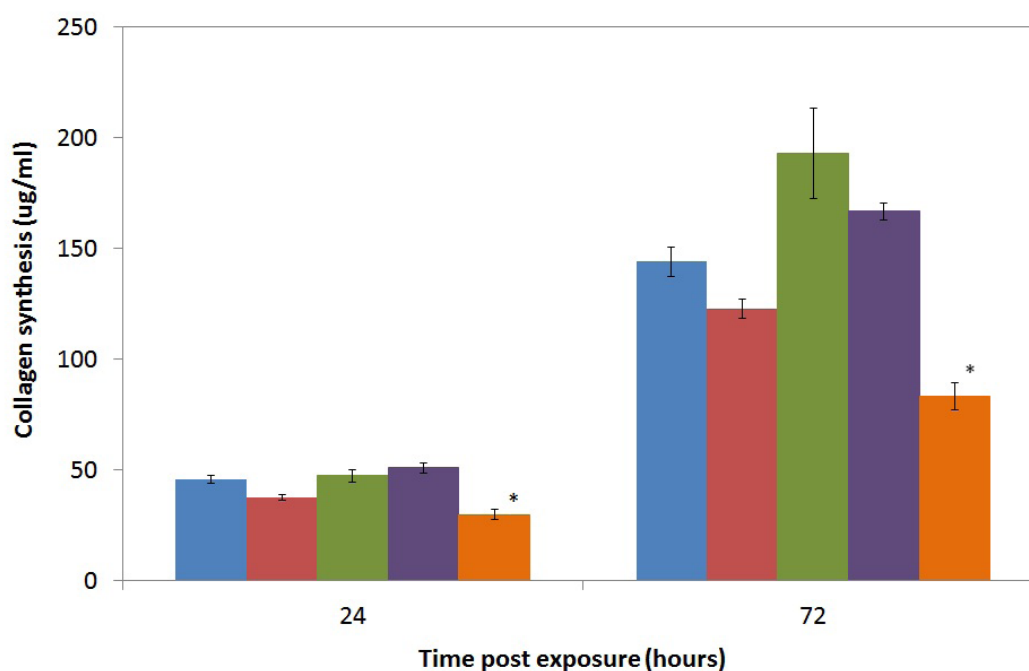

Fig. 3. Collagen synthesis by osteoblasts exposed to HINS-light. Cells were exposed to (from left to right at each time point); control, 0.5 mW/cm², 1.8 mW/cm², 5 mW/cm² and 15 mW/cm² for 1 h. ***** indicates significant difference from control ($p < 0.05$, ANOVA followed by Dunnett's comparison, $n = 3$ independent experiments ± SEM).

staining with Crystal Violet in attached cells. Data in Fig. 6 show that cells tolerated exposure to 5 mW/cm² HINS light for up to 120 min without significant loss of viability, but 3 h exposure resulted in a significant difference in the number of viable attached cells compared with the cells exposed for 60 min.

Effect of 405 nm HINS-light exposure on bacterial pathogens

Osteoblast exposure determined that 1 h exposure to 5 mW/cm² light was non-detrimental to the osteoblast cells. Experiments were carried out to assess whether this level of exposure could induce a bactericidal effect in *S. aureus* and *S. epidermidis*. Results, shown in Table 2, demonstrate that exposure to 5 mW/cm² for 1 h successfully reduced bacterial contamination, with a 98 % and 83 % reduction

in population achieved with *S. aureus* and *S. epidermidis*, respectively.

Exposure of a range of clinical isolates from infected arthroplasties demonstrated that 405 nm HINS-light has a non-selective bactericidal effect, with successful inactivation achieved across a wide range of Gram-positive and Gram-negative species (Fig. 7A and B, respectively). Inactivation of Gram-positive species appeared to occur at a slightly faster rate with >90 % inactivation occurring between 5-30 min, compared to 10-60 min for the Gram-negative species. When comparing the inactivation data for the clinical *S. aureus* and *S. epidermidis* isolates with those of the culture collection isolates it can be seen that use of the higher irradiance of 71 mW/cm² resulted in much faster inactivation than when using the lower 5 mW/cm² irradiance; 90-100 % inactivation in 5-10 min compared
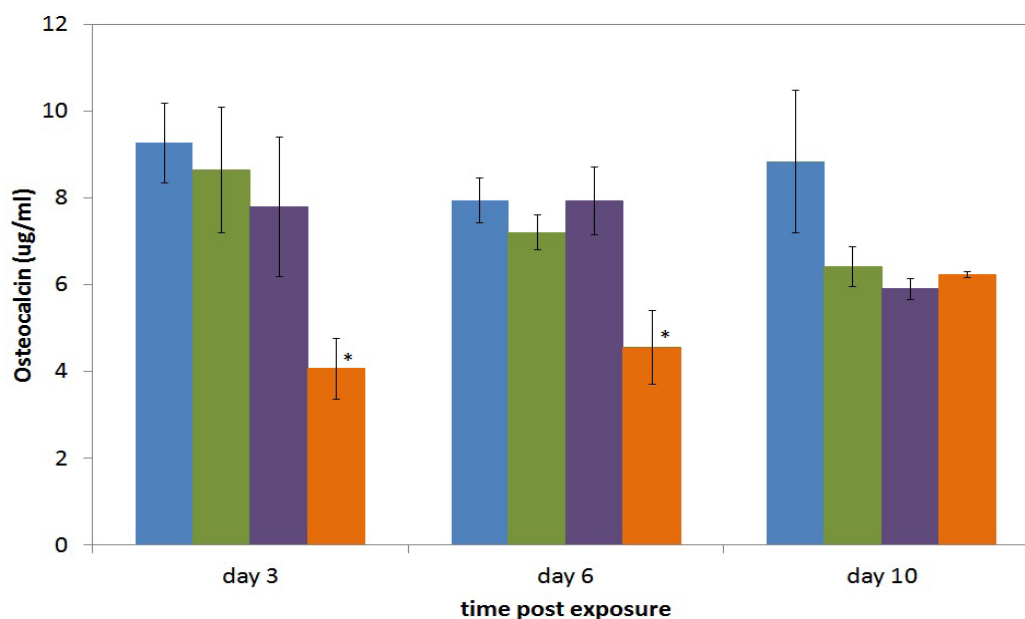
Fig. 4. Osteocalcin expression following 1 h HINS-light exposure. From left to right at each timepoint; control, 1.8 mW/cm^2, 5 mW/cm^2, 15 mW/cm^2. ***** indicates significant difference from control ($p < 0.05$, ANOVA followed by Dunnett's comparison, $n = 3$ independent experiments ± SEM, except for control and 15 mW/cm^2 samples on days 3 and 6 where $n = 6$).

to 80-100 % after 1 h. These results demonstrate that the higher the irradiance of 405 nm light, the faster the rate of bacterial inactivation that can be achieved.

Discussion

The results of this study demonstrate that intensities of HINS-light of 5 mW/cm^2 and below applied over 1 h, do not have a significant effect on osteoblast function in terms of ALP activity, collagen synthesis and osteocalcin secretion. It is important to state that in all experiments carried out on osteoblasts in this study, culture medium was replaced with phosphate buffered saline (PBS) for the duration of HINS-light exposure due to possible generation of ROS in the culture medium. Cell viability has been shown to be significantly reduced by exposure to visible and near-UV radiation when exposed in culture medium compared to PBS (Stoien and Wang, 1974; Smith, 2009). The main component of culture medium responsible for ROS generation has been shown to be riboflavin, with tryptophan, tyrosine, pyridoxine and folic acid all enhancing the effect (Grzelak *et al.*, 2001).

In vitro, ALP activity is well established as a measure of osteoblast ability to synthesise bone (Hoemann *et al.*, 2009), and it is encouraging to find that exposure to 5 mW/cm^2 for 1 h has no inhibitory effect on the activity either immediately, or after 24 or 72 h post-exposure. As the intensity was increased from 5 to 15 mW/cm^2 significant decreases relative to control values were observed at both 24 and 72 h post-exposure. The effect of blue light on ALP activity has not previously been investigated in osteoblasts. Low doses of red laser light have been shown to be without significant effect (Khadra *et al.*, 2005) but inhibition at

high doses (2 J/cm^2) of light at a wavelengths of 670 nm has been reported (Stein *et al.*, 2005).

Collagen synthesis is an essential aspect of bone matrix formation by osteoblasts, and the effects of 405 nm HINS-light exposure on this parameter were found to be similar to the effect on ALP activity. Exposure to intensities of up to 5 mW/cm^{-2} for up to 1 h had no significant effect on the ability of osteoblasts to synthesise collagen, while exposure to higher intensities significantly reduces this function. Although collagen synthesis has been shown to be both stimulated (Stein *et al.*, 2005; Saracino *et al.*, 2009) and inhibited (Marques *et al.*, 2004) by laser irradiation, there are no studies in the literature using an equivalent irradiance of light to that used in the present study. Staining of the collagen with picrosirius red and visualising the stained cells by microscopy proved not to be sensitive enough to detect the inhibition of synthesis in the cells, but revealed the morphology of the cells post-exposure to 405 nm HINS-light very clearly. There was no visible evidence of a change in cell morphology, or the presence of apoptotic cells.

Osteocalcin is the most abundant non-collagenous protein produced by osteoblasts, and studies on knock-out mice suggest that it has osteogenic regulatory functions (Ducy *et al.*, 1996). The ELISA kit used in this study has been shown to be an effective reliable method for determining osteogenic potential (Nakamura *et al.*, 2009). As reported previously, osteoblasts did not secrete measurable amounts of osteocalcin without stimulation with the hormone 1,25-dihydroxy vitamin D$_3$ (Carpenter *et al.*, 1998). Expression of osteocalcin is lower during the log phase of growth of the osteoblasts, and at maximum levels once the cells have reached confluence as reported previously (Owen *et al.*, 1991). Measuring osteocalcin production 3 days after exposure showed that 405 nm
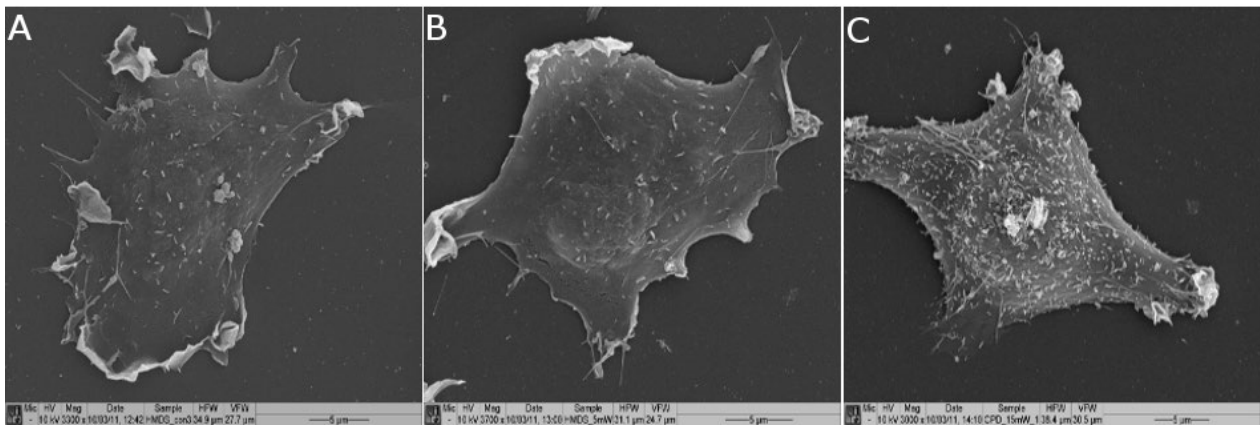
Fig. 5. SEM micrographs showing the effect of a 1 h exposure to 5 and 15 mW/cm^2 (B and C) HINS-light on osteoblast morphology. Control (A). Scale bars are 5 μm.
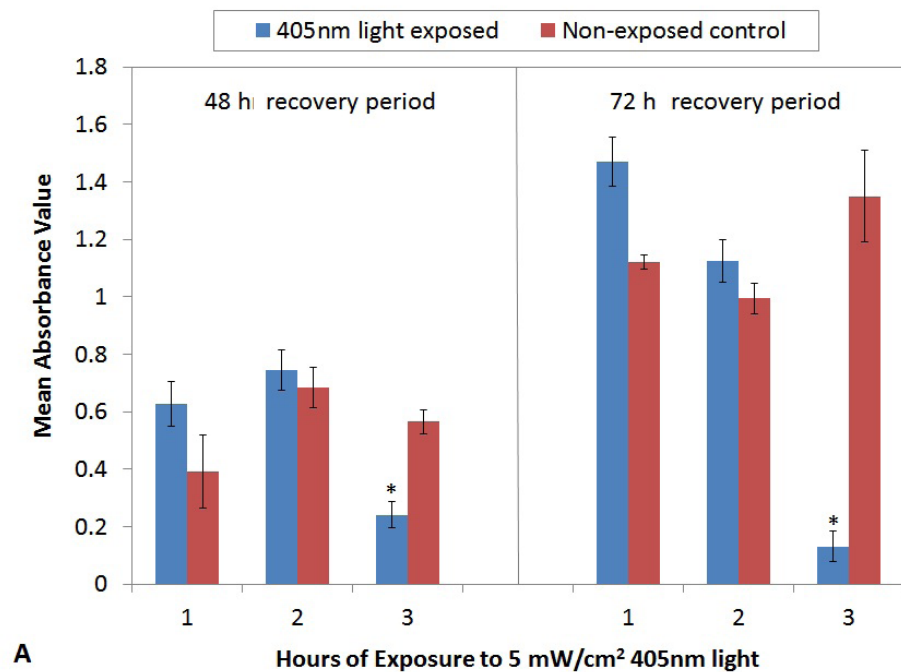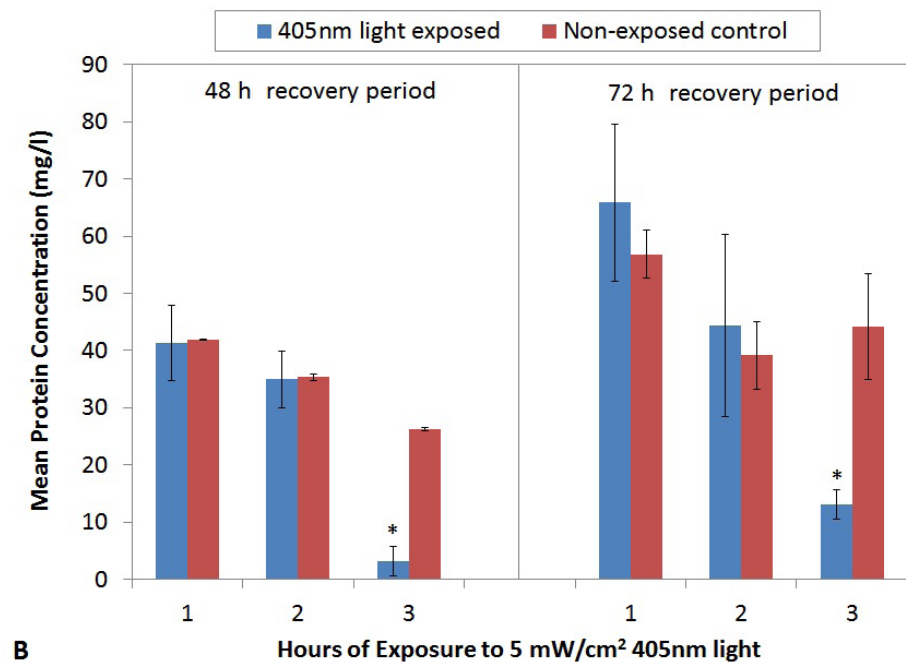

Fig. 6. Viability of osteoblasts following exposure to 5 mW/cm^2 HINS-light for 1, 2 and 3 h. **A** shows the Crystal Violet staining of cultures incubated in the presence of 5 mW/cm^2 HINS-light, and control cultures incubated in the absence of HINS-light. **B** shows the protein content of cultures incubated in the presence of 5 mW/cm^2 HINS-light, and control cultures incubated in the absence of HINS-light. (*$p < 0.05$, unpaired student t-test, comparing the controls and HINS light treated samples after the same exposure time, $n = 4$ independent experiments ± SEM).
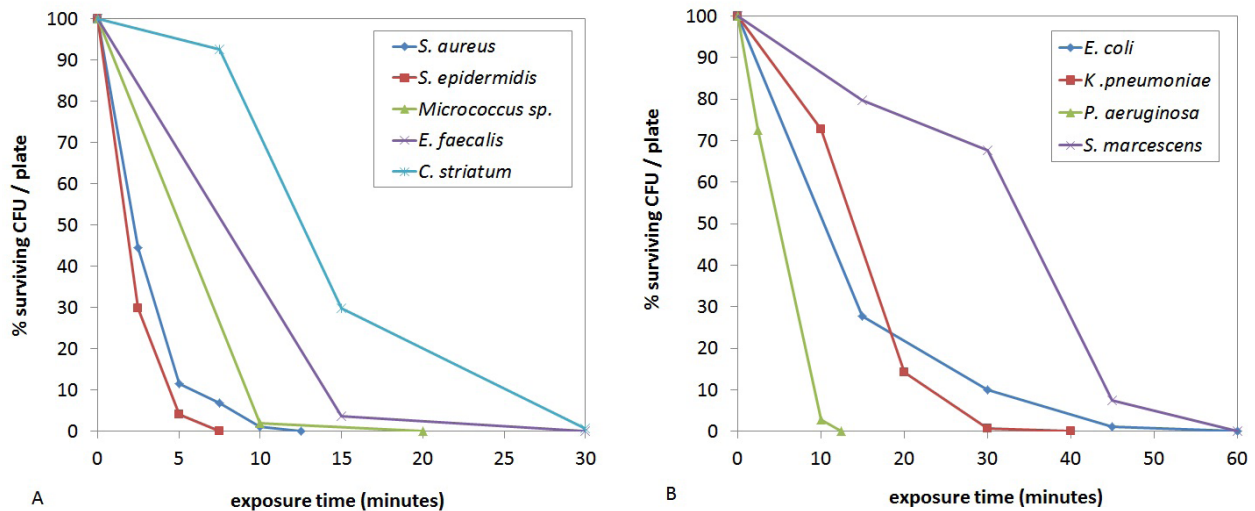
Fig. 7. Inactivation of a range of Gram positive (**A**) and Gram negative (**B**) clinical isolates from infected arthroplasties. Bacterial isolates were exposed on agar plate surfaces using 405 nm HINS-light with an average irradiance of 71 mW/cm².

HINS-light doses of up to 5 mW/cm² did not significantly affect expression, whereas exposure to 15 mW/cm² caused a significant reduction, from which the cells had recovered 10 days after exposure. There are no data in the literature on the effect of blue light on osteocalcin expression in osteoblasts, but near-UV laser irradiation has been shown to stimulate osteocalcin production in human osteoblasts (Khadra *et al.*, 2005).

SEM analyses showed that exposure to 5 mW/cm² did not cause any visible difference to the cell morphology compared to control cells, and the density of the cells was not significantly reduced. However, after the osteoblasts were exposed to 15 mW/cm² for 1 h there was undoubtedly some disturbance caused to the membrane of the cells. The resolution of the images is not high enough to identify the features, but they may represent the initial stage of bleb formation. The decrease in surface area observed after exposure to 15 mW/cm² HINS-light confirms an effect on the cells, and may represent the shrinking associated with apoptosis. A lesser percentage of cells in this group had the stretched morphology and associated large surface seen in the control cells.

The results demonstrate that 405 nm HINS-light has a dose dependent effect on osteoblast function. They imply that irradiances of up to 5 mW/cm² delivered over a period of 2 h should not cause damaging effects to osteoblasts during procedures such as hip replacement, which result in exposure of bone surfaces to the external environment. The time required for implantation of a primary hip arthroplasty is approximately 1 h surgery, and a revision operation is considerably longer; 2 to 2.5 h depending on the complexity of the case. The data shown here illustrate that exposure to HINS-light, under the conditions used for up to 120 min, does not significantly alter osteoblast viability. However, we recognise that the cells used were derived from rat tissue, and there may be differences in the responses of human cells.

Despite the finding that a 1 h exposure to 15 mW/cm² irradiance HINS-light has detrimental effects on

osteoblast function, this may have no significant bearing on bone formation or osseointegration of implants during use of the light in a surgery environment. Light of 405 nm wavelength will not penetrate deeply into bone. Penetration of the visible light spectrum into tissue increases with wavelength, with red light penetrating the skin to 6 mm, and shorter wavelength blue light only penetrating up to 2 mm (Fernandez-Guarino *et al.*, 2007). Macrene (2006) also showed that 50 % of red light photons (wavelength 800 nm) would penetrate 20 mm into soft tissue, compared with 80 μm for photons of 255 nm UV light. Although data on the penetration of blue light are not available, porcine studies have shown that a 4.3 mW/cm² laser of 635 nm light penetrates 1.6 ± 0.4 mm into trabecular bone (Bisland and Burch, 2006). Any damage to osteoblasts in this context would therefore only occur on the bone surface, and would have only limited effects on osseointegration of an implant. Osseointegration is not a surface process and, during total hip replacement, osseointegration of the femoral stem will take place deep within the cavity of the bone, where exposure to 405 nm HINS-light will be minimal. It is therefore unlikely that 405 nm HINS-light at the intensities and duration used in this study would cause any significant delay in osseointegration of implants.

It was important to establish that, whilst being non-detrimental to the osteoblast cells, exposure to 5 mW/cm² 405 nm HINS-light for 1 h was capable of inducing a bactericidal effect on bacterial contamination. *S. aureus* and *S. epidermidis* were selected for use due to their significance as causative pathogens of arthroplasty infections (Hamilton and Jamieson, 2008; Wilson *et al.*, 2008), and at this dose bacterial kill of 98.1 % and 83.2 % were obtained, respectively, demonstrating a potent bactericidal effect against the culture collection isolates. The bactericidal efficacy of HINS-light against clinical isolates from infected hip and knee arthroplasty tissue from the Southern General hospital in Glasgow was convincingly shown for a range of Gram-positive and Gram-negative bacteria. A comparison of the inactivation

Table 1. Analysis of osteoblast SEM images. $n \geq 6$ independent experiments, where each sample contained at least 15 cells. * signifies significant difference from control ($p < 0.05$, ANOVA followed by Dunnett's comparison).

Sample	% viable cells (± SEM)	Surface area (μm^2 ± SEM)
Control (no exposure)	81 ± 0.1	84.7 ± 12.9
5 mW/cm^2 for 1 h	77 ± 2.6	53.0 ± 13.2
15 mW/cm^2 for 1 h	66 ± 3.7 *	31.5 ± 8.5 *

Table 2. Inactivation of *S. aureus* and *S. epidermidis* on agar surfaces following exposure to 5 mW/cm^2 405 nm HINS-light for 1 h (18 J/cm^2). * indicates statistically significant reduction ($p < 0.05$, ANOVA, $n = 6$ independent experiments ± SEM).

Bacteria	Initial Population (mean CFU/plate ± SD)	Final Population (mean CFU/plate ± SD)	% reduction
S. aureus	312.3 (± 22.78)	6.0 (± 3.69)	98.1 % (± 1.1)*
S. epidermidis	70.3 (± 9.07)	11.5 (± 5.82)	83.2 % (± 8.7)*

rates of the Gram-positive and Gram-negative bacteria reveals that, as reported previously (Maclean *et al.*, 2009), Gram-positive bacteria are more susceptible to 405 nm HINS-light. The use of higher intensities of 405 nm HINS-light to kill the clinical isolates proves the principle that clinical isolates as well as type cultures can be killed, and demonstrates that faster cell kill can be achieved with higher intensities of 405 nm HINS-light.

The experiments described in this study were carried out under closely defined laboratory test conditions and have established critical dose levels for safe exposure of osteoblast tissue to 405 nm light. For practical application, this dose level will be dependent on the irradiance from the light source, the distance from the treated tissue and the exposure time. It is anticipated that for the proposed practical application, 405 nm disinfecting light systems would have brightness characteristics similar to typical operating theatre lighting. Further work, involving close interaction with surgical staff, is required to translate these findings into the optimal design of a light-delivery system, which could potentially have application for directed continuous disinfection of the operating environment during real-time arthroplasty surgery.

Conclusions

405 nm HINS-light exposure for 1 h at 5 mW/cm^2 does not significantly alter osteoblast morphology, function or viability. It is an effective bactericide for clinically relevant bacteria and was found to kill 98.1 % of *Staphylococcus aureus* and 83.2 % of *Staphylococcus epidermis* populations at this dose. These findings, along with the limited penetration of the light into tissues, suggest that 405 nm HINS-light at this exposure level could potentially be used for directed environmental disinfection in orthopaedic operating theatres. In fact, osteoblasts exposed to 5 mW/cm^2 for up to 2 h showed no loss of viability. For localised short duration exposure,

such as during high risk surgical procedures, 405 nm HINS-light has potential to be used to reduce the number of airborne and surface bacteria in the area immediately surrounding a surgical site without damaging the exposed tissues, analogous to the results from environmental disinfection procedures already demonstrated in the Burns Unit of Glasgow Royal Infirmary. This visible light-based technology has the potential significantly to reduce contamination, and consequently infection, in arthroplasty, and has applications in many diverse areas of surgery, particularly where medical devices are being introduced into the body.

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Editor's note: All questions and comments by the reviewers were answered by text changes. Hence there is no "Discussion with Reviewers" section.