



Probing the differential effects of infrared light sources IR1072 and IR880 on human lymphocytes: Evidence of selective cytoprotection by IR1072

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Abstract

Light therapy, both laser and LED, have been shown to provide clinical benefit in many therapeutic arenas. The effects of IR1072 and IR880 were investigated, using a range of single and multiple irradiation protocols, for their effect on freshly prepared human lymphocytes stimulated with phytohemagglutinin. Viable cell numbers remained significantly higher after irradiation with IR1072 and were significantly lower after IR880 irradiation compared to untreated controls, following a daily single irradiation over a 5-day period. Cell numbers were significantly higher after pre-treatment with IR1072 and exposure to UVA, compared to cells treated with UVA only. Cells irradiated twice on Day 3 post-harvest with various wavebands confirm on Day 5, an increase in % cell viability after IR1072, and IR1072 alternating with IR1268 irradiation, and a decrease in % cell viability after IR880 irradiation alone. Further, wavebands tested displayed no significant differences compared to the control. Cells were collected after exposure on Days 3 and 5 with IR1072 and IR880 treatments and protein levels were compared using quantitative immunoblotting probed with an anti-iNOS antibody. Following IR1072, but not IR880, treatment there was a 4.9 ± 2.1 -fold higher iNOS protein expression in treated cells compared to the control on Day 5 post-treatment.

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1. Introduction

Sunlight is the most important and universal source of non-ionising radiation essential for life on Earth. Flora and fauna have adapted through evolution to those components of sunlight which reach the surface of the planet having been filtered by the atmosphere.

Comparing the known photobiological effects of light with the transmission spectrum of water shows that all of these are contained within the peak of this spectrum

suggesting that atmospheric or intracellular water may have been influential in determining the course of these evolutionary processes (Fig. 1)

Solar ultraviolet (UV) is short wave high energy radiation known to be damaging to cells and responsible for photoageing and carcinogenesis [1,2], whereas IR is known to be a beneficial therapeutic agent, for example in the treatment of musculo-skeletal disorders and healing of indolent wounds [3,4]. In the laboratory, various photo biological effects of infrared light have been explored, albeit dictated by the random commercial availability of predominantly laser light sources [5–10]. These well-documented experiments have demonstrated unequivocally that selected wavelengths of infrared light

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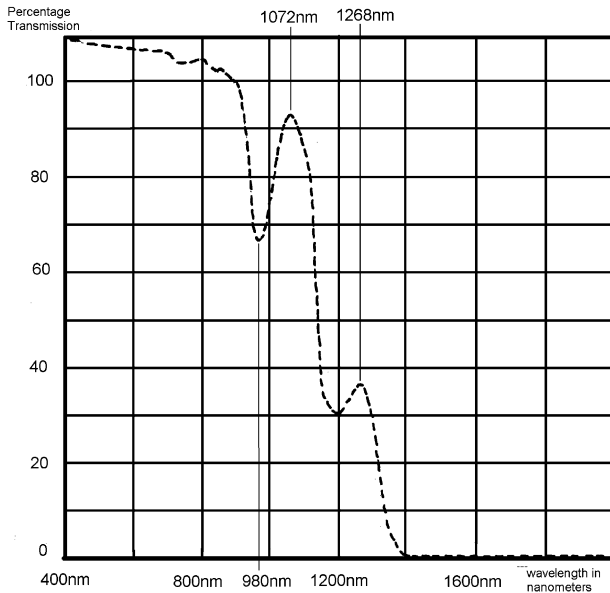


Fig. 1. Transmission spectrum of pure water [9].

have non-thermal photo biological effect. In 1998, Menezes et al. [2] showed that non-thermal quantities of IR light (700–2000 nm) induced a strong cellular defence against solar UV toxicity in normal human fibroblasts. In 2001, Dougal and Kelly [11] demonstrated that one single 5-min application of 1072-nm narrow waveband light was effective in the treatment of *herpes labialis*. 1072-nm light was chosen as it represents a peak in the transmission spectrum of the water molecule. Cold sores (*herpes labialis*) are known to be activated by UV [12], which is known to suppress the immune defence system.

The small, short-lived reactive molecule of nitric oxide (NO) has emerged as a potent inhibitor of apoptosis. Inhibition of apoptosis by NO has been shown in a variety of cells including B-lymphocytes [13], splenocytes [14] and endothelial cells [15]. Nitric oxide (NO) is formed by an enzyme-catalysed reaction between molecular oxygen and L-arginine. NO is an important molecule mediating a wide range of physiological and pathophysiological processes. The amount of NO production [16] may determine its role, as may the type of insult [17]. Three forms of NOS have been described which show approximately 50% identity in amino acid sequence [18]. NO derived from the inducible isoform of nitric oxide synthase (iNOS) is an inflammatory product. iNOS differs from endothelial NOS (eNOS) and neuronal NOS (nNOS), as both eNOS and nNOS require calcium for activity, whereas calmodulin binding to iNOS is so tight that addition of Ca^{2+} is not necessary [19]. iNOS expression can be both upregulated [20–22] or downregulated [20] in a variety of cell types depending on stimulus.

This study was designed to investigate the effect, if any, of a series of narrow wavebands of light on human lymphocytes in an attempt to determine the possible photo biological response of these cells to light within the near infrared spectrum and to consider the effects on iNOS expression after the treatment of infrared light *in vitro*.

2. Materials and methods

2.1. Cell preparation

Heparinised human whole blood was obtained from healthy volunteers (with local ethical approval), and peripheral blood mononuclear cells (PBMC) were separated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway) and centrifuged at 400g for 5 min. The PBMCs were isolated from the interfacial layer, washed twice in RPMI without L-glutamine (Gibco™) and resuspended in RPMIcm (RPMI + 10% v/v fetal calf serum + 1% penicillin/streptomycin + 1% L-glutamine). Cell density was adjusted accordingly to 1×10^6 cells/ml with RPMI. 100 μl PHA ('Lectin', Sigma) was added to the cells to make PHA Blasts. Cells were incubated in 35-mm culture dishes in RPMI media at 37 °C in 5% CO_2 .

2.2. Experimental set-up

A series of multiple exposure protocols all of which have shown therapeutic benefit in cold sore trials (results not shown) were adopted for this study to show the flexibility of the treatments. The five protocols were set-up as follows:

1. PHA Blasts were exposed to infrared light source, IR1072, on Days 3, 4 and 5 post-harvest. Using 35-mm culture dishes, all cells were exposed to a single 3-min treatment of infrared light. Following daily treatments, individual replicate cell samples were analysed for % cell viability on Day 5.
2. PHA Blasts were exposed to IR1072 and IR880 on Days 3 and 5 for 5 \times 3-min treatments and analysed on Day 5. Cell viability and iNOS expression was determined after each treatment on Day 5.
3. PHA Blasts were exposed daily from Day 1 onwards to a single 3-min dose of IR1072 and IR880. After daily irradiation, cells were analysed for % cell viability.
4. PHA Blasts were exposed to IR1072 on Day 3 for 4 \times 3-min treatment and on Day 4 for a single 3-min treatment. Cells were then left for 4 h before exposure to UVA for 40 min and cell viability was then determined.

5. Cells were incubated until Day 3 in tissue culture tubes and exposed to various wavebands for 2×3 min on Day 3. Wavebands included IR660, IR880, IR950, IR1267, IR1072, IR1072 nm alternating with IR1268, IR1072 and IR1267 nm, 1- μ s pulsing of IR1072 nm and 7- μ s pulsing of IR1072 nm. Cells were analysed for % cell viability immediately after irradiation.

Notably for all protocols used, the temperature of all the dishes was maintained at room temperature throughout the IR and control treatments.

2.3. Annexin V apoptosis kit

Cell viabilities were analysed using the Annexin V Apoptosis Detection Kit (Autogen Bioclear, UK). Apoptosis can be detected by the change in position of phosphatidylserine (PS) in the cell membrane. In non-apoptotic cells, most PS molecules are localised at the inner layer of the plasma membrane, but soon after inducing apoptosis, PD redistributes to the outer layer of the membrane. Exposed PS can be easily detected with Annexin V. Cells with bound Annexin V showed green staining in the plasma membrane. Cells that had lost membrane integrity showed red staining (PI) throughout the cytoplasm and a halo of green staining on the cell surface (plasma membrane) [23–26]. Cells at 1×10^5 – 1×10^6 per dish were rinsed and resuspended in Assay Binding Buffer. Five microlitres of Annexin V and 10 μ l of propidium iodide (PI) were added to the cells before incubating at room temperature in the dark for 15–30 min. Cells were observed under a dual filter set for FITC and rhodamine using fluorescence microscopy, and counted blind by at least two observers.

2.4. Western blotting analysis

Thawed cell pellet suspensions were homogenised on ice with a Dounce homogeniser. The protein levels in the cell suspension were determined using the Lowry Assay [27] using bovine serum albumin as a standard. Protein levels were adjusted to 10 μ g protein was loaded in each lane. Standard electrophoresis was performed using a 6% polyacrylamide gel. Following electrophoresis, the protein was transferred to nitrocellulose (NC) membrane for 2.5 h at 50 V. The NC membrane was blocked with 5% non-fat skimmed milk in $1 \times$ Tris buffered saline (TBS) containing 0.2% Tween 20 (Sigma, UK) for 1 h at room temperature. The NC membrane was incubated with primary antibody iNOS (dilution 1:2500) overnight at 4 °C. The NC membrane was washed 4×10 min with wash buffer (2.5% non-fat skimmed milk, 0.2% Tween 20 in TBS) and incubated with anti-rabbit horseradish peroxi-

dase-linked secondary antibody (dilution 1:2000) for 1 h. The NC membrane was washed 4×10 min with wash buffer. The protein bands from the NC were visualised using a substrate of 68 mM luminol, 1.25 mM *p*-couramic acid, 30% hydrogen peroxide. The immunoblot was exposed to Hyperfilm™ for 3 min in a film cassette and were developed and fixed at room temperature. The protein bands were quantified using an ImageQuant® densitometer in the linear range of the film, to determine the relative iNOS expression. Optical density values (standardised with β -actin as in [27]) were compared using a multiple ANOVA with a significance level of $p < 0.05$. Data were obtained from $n = 3$ individual replicate experiments.

2.5. Statistics

Apoptosis was measured using % cell viability, that is,

$$\% \text{ cell viability} = \left[\frac{(\text{No. of viable cells})}{(\text{No. of total cells})} \right] * 100.$$

Data are given as the means \pm standard deviation.

Comparisons between control and treated cells were made by a multiple ANOVA and expressed as mean \pm SD, with a confidence interval of 95%. Statistical analysis was carried out using Prism 3.2.

2.6. Light sources

Both the 880- and 1072-nm light sources emitted multimode light of bandwidth less than 50 nm, continuous mode of optical power 5 mW/cm².

3. Results

Using a range of protocols, IR1072 treatment consistently elicited a significant protective effect upon PHA Blast survival. In contrast, IR880 was consistently cytotoxic compared to control and IR1072 treated cells.

Following irradiation with IR1072, % cell viability significantly increased on Day 5 ($p < 0.05$) compared to the control data following both a single and multiple 5×3 -min treatment protocol on Days 3 and 5 (Fig. 2). In the next protocol, cells irradiated with 5×3 min of IR1072 and IR880, the % cell viability significantly decreased after treatment with IR880 both on Day 5 ($p < 0.01$) compared to cells treated with IR1072 (Fig. 2). The daily treatment protocol elicited a significant decrease in % cell viability for IR880 treated cells over an 8-day period [Day 1 ($p < 0.01$), Day 3 ($p < 0.01$), Day 4 ($p < 0.05$), Day 5 ($p < 0.05$) and Day 8 ($p < 0.05$)], compared to those irradiated with IR1072 (Fig. 3), in paral-

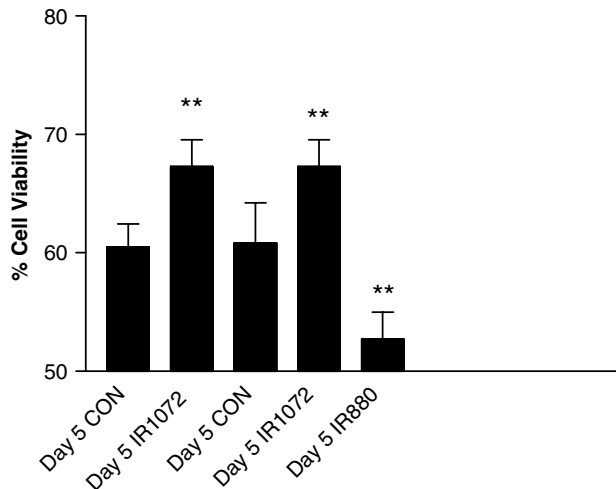


Fig. 2. Columns 1 and 2: The % cell viability of PHA Blasts following a single 3-min treatment of IR1072 on Days 3 and 5 before testing for apoptosis on Day 5. Data were compared to respective controls, and analysed using an ANOVA, where $*p < 0.05$. Columns 3, 4 and 5: The % cell viability of PHA Blasts following multiple 5 \times 3-min treatments of IR1072 and IR880 on Days 3 and 5, before testing for cell viability on Day 5. Data were compared to respective controls on Day 5, and analysed using an ANOVA, where $**p < 0.01$.

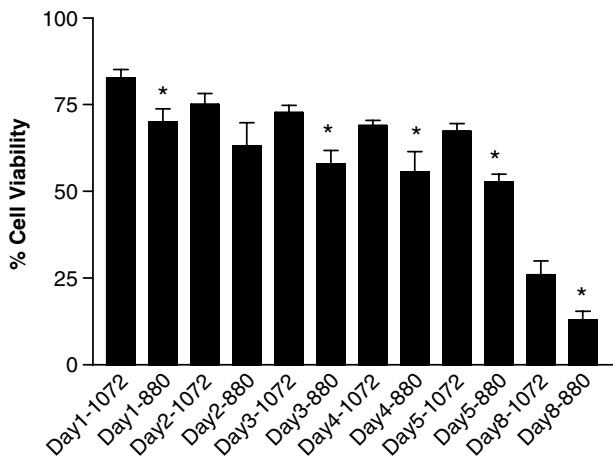


Fig. 3. The % cell viability of PHA Blasts following daily single 3-min treatments irradiated with either IR1072 or IR880. Cell viability was determined using the Annexin V apoptosis kit. IR1072 data were analysed compared to respective IR880 data, using an ANOVA, where significant differences were seen on Days 1, 3, 4 and 5 $*p < 0.01$, and a trend to significance on Day 2.

lel experiments. After pre-treatment with IR1072 and subsequent exposure to UVA, % cell viability remained significantly higher ($p < 0.01$) compared to cells treated only with UVA (Fig. 4). Following irradiation with various wavebands, again cells exposed to IR880 showed significant decrease in % cell viability ($p < 0.01$), whereas the % cell viability was higher following treatment with IR1072 ($p < 0.01$) and alternate IR1072/IR1268 wave-

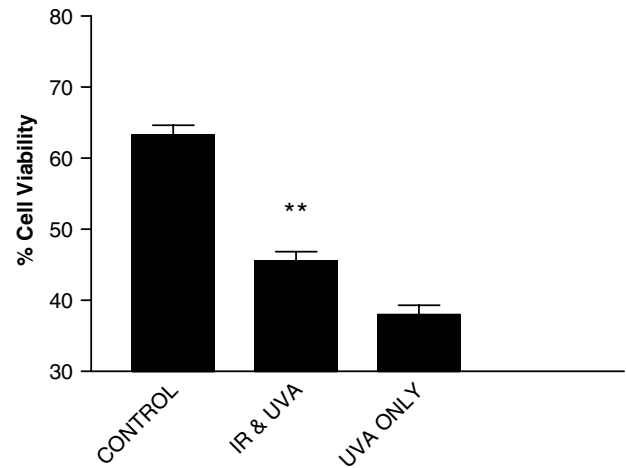


Fig. 4. PHA Blasts were pre-treated 4 \times 3 min on Day 3, and 1 \times 3 min on Day 4 with IR1072, and then cells were incubated for 4 h before UVA exposure for 40 min. Samples were then assayed for cell viability. Data were analysed and compared to UV treated alone using an ANOVA, where $**p < 0.01$.

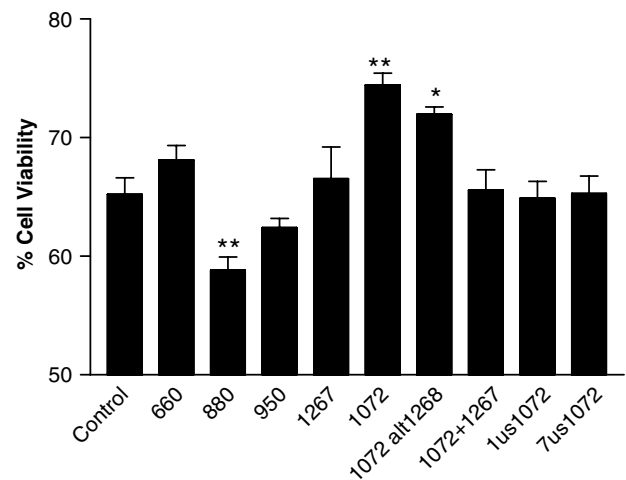


Fig. 5. Effect of various wavebands on PHA Blasts treated on Day 3 for 2 \times 3 min and analysed. Data were analysed and compared to the untreated control using a multiple ANOVA, where $**p < 0.01$.

band light ($p < 0.01$), all compared to untreated controls (Fig. 5). All other wavelengths and conditions tested had no significant effect upon cell viability.

In order to gain a handle on the potential mechanism underlying the observed long-lasting cytoprotection elicited by exposure to IR1072, quantitative immunoblotting was performed probing the expression of iNOS, in comparison to control and IR880 nm. Following pre-treatment with IR1072, a significant increase of 4.9 ± 2.1 -fold ($p < 0.05$) in iNOS immunoreactivity was detected at Day 5, compared to control. In contrast, no significant increase in iNOS was observed with IR880 (2.1 ± 2.2 -fold for Day 5) ($p > 0.05$), performed in parallel studies (Fig. 6).

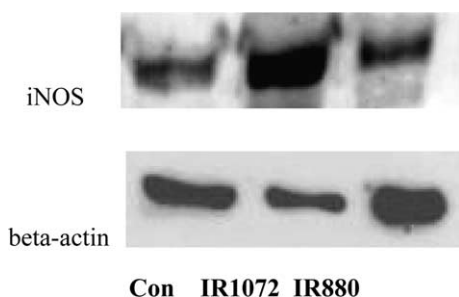


Fig. 6. Effect of IR treatment upon iNOS protein expression levels PHA Blasts were exposed daily to 1×3 -min infrared source, IR1072 or IR880 and assayed on Days 3 and 5, for iNOS protein expression using quantitative immunoblotting with a selective anti-iNOS antibody (Autogen Bioclear, UK). Immunoblots were re-probed and standardised with a β -actin antibody (Sigma, UK). Lane 1, control cells (Day 5); Lane 2, IR1072-treated cells (Day 5); Lane 3, IR880-treated cells (day 5). Data were compared by a multiple ANOVA with a level of significance set at $p < 0.01$.

4. Discussion

This study has identified an *ex vivo* method by which immune cell viability may be improved in the presence of adversity. In this instance, the adverse events were the stress of being cultured outside the human body and, secondly, being exposed to an insult, namely UVA light. Many authors have suggested the concept that a particular range of wavelengths has therapeutic benefit [6–8,10,28–30]. Biostimulation is the commonest means by which therapeutic efficacy is sought. Whilst the wavelengths in the 855–905 nm range may stimulate fibroblast proliferation [9], importantly light in this range also appears to be lymphotoxic as shown by our studies. The cytotoxic and protective effects upon the cells are rapid as the analysis was carried out within 2 h of exposure to the IR light and both effects were long lasting, being observed at least 2 days post-treatments. This study clearly demonstrates that light in the 1050–1100 nm range improves cell viability following both single and multiple treatment protocols. Maintaining lymphocyte viability in the presence of adverse factors is of significance as bacterial endo- and exo-toxins are leucotoxic factors, the effect of which, may be reduced by the irradiation of the inflammatory cells by 1072 ± 25 -nm light. It has long been postulated that IR light has a protective effect against UVA, however, the exact range of wavelengths has been unknown. These present results suggest 1072 ± 25 -nm light is protective against some of the damaging effects of UVA. This concurs with the clinical utility of this wavelength in treating cold sores (e.g. [11] and further unpublished clinical observations). Although significant, the protection is incomplete and therefore requires further optimisation. There are likely to be missing elements, including other cell types and mediators in this *ex vivo* model which are naturally present *in vivo*. Photo-modulation

of the immune response is a potential therapeutic tool yet to be fully evaluated. The protective effect of 1072-nm light against UV damage is an important finding which potentially could reduce the skin damage induced by PUVA in the treatment of psoriasis. Additional therapeutic benefits would be applicable to any pathology which responds to more resilient lymphocytes.

Nitric oxide has been shown to be a potent inhibitor of apoptosis in a variety of cell types [31]. NO diffuses very rapidly both through water and cell membranes, and iNOS is produced more rapidly and efficiently than eNOS and nNOS. iNOS can function without the elevation of intracellular calcium levels and its activity is rapidly inducible in immune cells, for example, primarily activated monocytes and macrophages, following exposure to cytokines and microbial products [32]. Biochemically, these present results show that iNOS has been upregulated in a wavelength-dependent fashion, in comparison to untreated controls. NO is believed to act as an inhibitor of apoptosis by two distinct mechanisms: first through a cGMP-dependent mechanism where NO acts either at the level of caspase-3-like protease activation or upstream of this event to prevent the activation of the protease; second, NO also inhibits the activity of the caspase-3-like protease by *S*-nitrosylation of the enzyme. Suppression of caspase-3-like activity then rescues the cell from programmed cell death [1].

We report the first evidence that IR1072 and IR880 elicit opposing effects upon lymphocyte viability *ex vivo*, the former being protective and the latter wavelength cytotoxic. Furthermore, we provide the first demonstration that IR1072 protects against UV-mediated lymphotoxicity. Preliminary biochemical evidence shows a wavelength-dependent induction of iNOS, which may offer a candidate protective mechanism underlying IR1072-induced long-term preconditioning in immune cells, and warrants further investigation.

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