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The contribution of temperature, exposure intensity and visible light to the inhibitory effect of irradiation on acute chlamydial infection

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ABSTRACT

Water-filtered infrared A (wIRA) is radiation with a spectrum ranging from 780 to 1400 nm. *Chlamydiaceae* are obligate intracellular bacteria associated with various diseases in both animals and humans. A recent *in vitro* study demonstrated that wIRA combined with visible light (wIRA/VIS) has potential as a non-chemical method for the treatment of chlamydial infections without adversely affecting the cell viability. The aim of this study was to investigate the influence of various factors on the effect of wIRA/VIS on acute chlamydial infection, namely the impact of temperature, exposure intensity and infectious dose (multiplicity of infection) as well as the efficacy of the visible light component. We demonstrate that non-thermal effects contribute to the inhibition of acute chlamydial infection. Visible light enhances the inhibitory effect of wIRA on extracellular bacteria (elementary bodies or EBs). Moreover, the inhibitory effect of wIRA/VIS following treatment of EBs prior to infection correlated with increased irradiation intensity. The infectivity of mature chlamydial inclusions was significantly reduced upon wIRA/VIS exposure at all irradiation intensities investigated, suggesting the contribution of host cell factors to the anti-chlamydial effect of wIRA/VIS in the late stage of the developmental cycle. The effect of irradiation was not influenced by the infectious dose.

1. Introduction

Water-filtered infrared A (wIRA) is infrared radiation with a spectrum of 780–1400 nm resulting from the light produced by a halogen bulb passing through a water cuvette to exclude wavelengths above 1400 nm and through a black filter to block visible light (VIS) [1]. Various clinical trials have shown that wIRA alone and in combination with visible light (wIRA/VIS) improves acute and chronic wound healing processes [2]. Moreover, two studies showed that wIRA/VIS treatment of abdominal wounds before or after surgery not only improved wound healing and oxygen partial pressure but also significantly reduced the rate of wound infections [3,4]. Surgical site infections are most often caused by the patient's endogenous flora. The common pathogens implicated in wound infections are the Gram-positive *Staphylococcus aureus* and coagulase-negative staphylococci as well as the Gram-negative *Escherichia coli* and *Enterococcus* ssp. [5]. Another study recently demonstrated that wIRA/VIS reduced the inclusion frequency of acute chlamydial infection without adversely affecting the cell viability or inducing cytotoxicity in host cells [6].

The *Chlamydiaceae* are a family of Gram-negative bacteria consisting of one genus designated *Chlamydia* (*C.*) [7]. These obligate intracellular pathogens have a unique developmental cycle comprising an extracellular, infectious but metabolically less active stage (elementary bodies or EBs), which transforms into replicating reticulate bodies (RBs) before differentiating back into EBs [8]. They are responsible for a wide range of diseases, some of which are considered to be a public health concern in both human and veterinary medicine. For example, some strains of *C. trachomatis* are the causative agent of trachoma, the leading cause of infectious preventable blindness of human beings in developing countries, whereas other strains are recognized as the most important cause of bacterial sexually transmitted disease worldwide [9]. *C. pecorum*, a chlamydial species well-described in veterinary medicine, is associated with various medical conditions in pigs and ruminants including abortion, keratoconjunctivitis, pneumonia, polyarthritis and meningoencephalitis [10,11]. Furthermore, *C. pecorum*, together with other chlamydial species normally found in animals, such as *C. psittaci* and *C. suis*, has also been identified in the eye of trachoma patients in Nepal [12]. The treatment of choice for chlamydial infection is antimicrobial therapy; however, in general, the emergence of antibiotic-resistant bacteria raises concerns about the continued efficacy of this approach. Tetracycline-resistant *C. suis* strains, harboring the tetC resistance gene, have been isolated from asymptomatic pigs in the USA [13,14]. Horizontal transfer of this gene to various *Chlamydia* ssp. strains including *C. trachomatis* has been demonstrated *in vitro* [15]. Furthermore, β-lactam antibiotics have often been reported to induce chlamydial persistence *in vitro* and *in vivo* [16]. Persistence is a reversible, viable but non-infectious chlamydial state. Though the importance of

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persistence *in vivo* is largely unknown, it might negatively impact both detection and successful antimicrobial treatment of chlamydial infection [17,18]. Taken together, new therapeutic strategies are needed to overcome current and developing problems with conventional treatment of chlamydial infections.

The aim of this study was to evaluate the impact of increasing irradiation intensity on wIRA/VIS-exposed chlamydial EBs and mature inclusions over a range of infectious doses, to assess the influence of visible light, and to evaluate the effect of irradiation in a temperature-controlled setting using both human (*C. trachomatis* serovar E and HeLa cells) and animal (*C. pecorum* 1710S and Vero cells) infection models.

2. Materials and Methods

2.1. Host Cells and Media

Vero 76 cells (African green monkey kidney cells, CRL 1587 American Type Culture Collection (ATCC), Manassas, VA, USA) and HeLa cells (Homo sapiens cervix adenocarcinoma, CCL-2 ATCC) were cultured at 37 °C with 5% CO_2 in growth culture medium for cell propagation. Vero growth medium consisted of Minimal Essential Medium (MEM) with Earle's salts, 25 mM HEPES, without L-Glutamine (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, BioConcept, Allschwil, Switzerland), 4 mM GlutaMAX-I (200 mM, GIBCO) and 0.2 mg/mL gentamycin (50 mg/mL, GIBCO). HeLa cell culture media were further supplemented with 1% MEM Non-Essential Amino Acids (MEM NEAA, 100×, GIBCO). Medium used for cell propagation intended for infection experiments was without gentamycin. Cells were grown on round glass coverslips (13 mm diameter, Sterilin Limited (Thermo Fisher Scientific), Cambridge, UK) in 24-well plates (Techno Plastic Products AG (TPP), Trasadingen, Switzerland) in 1 mL medium for infection experiments as previously described [6]. HeLa cells were seeded at a density of 3 x 10^5/well, Vero cells at a density of 2 x 10^5/well and infection experiments were performed when cells reached at least 90% confluency.

2.2. Chlamydial Strains

In this study, two different strains of *Chlamydiaceae* were used for *in vitro* infection experiments: *Chlamydia* (*C.*) *pecorum* 1710S (isolate from a swine abortion, kindly provided by Prof. J. Storz, Baton Rouge, LA, USA) and *C. trachomatis* serovar E (kindly provided by Prof. R. V. Schoborg, Johnson City, TN, USA). The isolate of the *C. trachomatis* strain was originally obtained from S. P. Wang and C.-C. Kuo (University of Washington, Seattle, WA, USA). The isolates were both propagated in HeLa cells. In summary, isolates were suspended in infection medium and used to infect HeLa cells. After centrifugation (1000 g, 60 min), infection medium was replaced by growth medium plus gentamicin (0.2 mg/mL, GIBCO) and 1 μg/mL cycloheximide (Sigma-Aldrich, Inc., St. Louis, MO, USA). After an incubation period of 46 h (*C. trachomatis*) or 39 h (*C. pecorum*), monolayers were scraped and processed using sonication and centrifugation (500 g, 10 min to remove cell debris and 10,000 g, 45 min to pellet EBs). EBs were suspended in SPG medium and stored at −80 °C. SPG medium consisted of 218 mM sucrose (Sigma-Aldrich, Inc., St. Louis, MO, USA), 3.76 mM KH2PO4 (Sigma-Aldrich), 7.1 mM K2HPO4 (Merck Eurolab AG, Dietlikon, Switzerland) and 5 nm GlutaMAX-100 (GIBCO).

2.3. Irradiation

Cultures were either exposed to water-filtered infrared A in combination with visible light (wIRA/VIS) or wIRA alone for 20 min using a wIRA radiator (hydrosun 750, Hydrosun GmbH, Müllheim, Germany). The exposure intensity of wIRA/VIS and wIRA alone, respectively, depended on the experiment. The primary radiation spectrum of wIRA/VIS encompasses 400–1400 nm with negligible doses of UVA (400–315 nm; 25–30 W/m^2), UVB (315–280 nm; 15–25 × 10^{-5} W/m^2) and UVC (280–100 nm; 5–21 × 10^{-5} W/m^2 within the range of 250–280 nm) (measurements performed by the manufacturer). wIRA irradiation was attained by the use of a black filter (RG780, Hydrosun GmbH) resulting in a spectrum of 780 up to 1400 nm. The 24-well plates were placed in a thermostat-controlled water bath (SC100, Thermo Fisher Scientific, Newington, CT, USA), which maintained a temperature of 37 °C and was used as a cooling system for the irradiated cultures as previously described [19]. Non-irradiated controls were localized on the same plate and were kept at a suitable distance from the irradiated wells in order to avoid any direct and/or indirect irradiation influence (data not shown). Additionally, the temperature was monitored using a Voltcraft thermometer (Type 2ABAc, Philips, Kassel, Germany) as previously described [6].

2.4. Study Design

2.4.1. Infection Experiments

The experiments were organized in two different treatment groups: i) *Chlamydia*-infected cells with irradiation and ii) *Chlamydia*-infected cells without irradiation. Non-infected and non-irradiated cells were included as negative controls (data not shown). Vero and HeLa cells were infected with either *C. pecorum* or *C. trachomatis* at a multiplicity of infection (MOI) of 1, 0.1 or 0.01 in 1 mL infection medium (growth culture medium without FCS and gentamycin). After centrifugation for 1 h at 1000 g and 25 °C, infection medium was replaced by incubation medium (growth culture medium without gentamycin) containing 1 μg/mL cycloheximide (Sigma-Aldrich) and cultures were incubated at 37 °C as previously described [20].

2.4.2. Irradiation of Chlamydial EBs Prior to Host Cell Infection

Chlamydial EBs at a of MOI 0.01, 0.1 or 1 were irradiated in 1 mL infection medium for 20 min before infection of host cells. Non-irradiated EBs were used as controls. After an incubation period of 43 h, cultures were subjected to i) immunofluorescence microscopy, or ii) infectious titer analysis, as appropriate (Fig. 1A). *C. pecorum* EBs were used to infect Vero and HeLa monolayers. *C. trachomatis* EBs were applied to HeLa cells only.

2.4.3. Single-Dose Irradiation 40 h Post Infection

For single-dose irradiation, HeLa and Vero monolayers were infected with *C. trachomatis* or *C. pecorum*. After an incubation period of 40 h at 37 °C, cultured cells were irradiated for 20 min. Cultures were incubated for another 3 h at 37 °C under standard culture conditions before being subjected to i) immunofluorescence microscopy, or ii) infectious titer analysis, as appropriate (Fig. 1B). Vero monolayers were infected with *C. pecorum* and HeLa cells were infected with *C. trachomatis* (Fig. 1C).

2.5. Immunofluorescence Microscopy

Monolayers were fixed with absolute methanol (−20 °C) for 10 min and immunolabeled as described [20]. Briefly, chlamydial inclusions were detected using a *Chlamydiaceae* family-specific mouse monoclonal antibody directed against the chlamydial lipopolysaccharide (LPS, Clone ACI-P, 1:200; Progen, Heidelberg, Germany) and a 1:500 diluted Alexa Fluor 488-conjugated secondary goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA). Host and chlamydial DNA were labeled using 1:1000 diluted 4′, 6-Diamidin-2′-phenylindoldihydrochlorid (DAPI, 1 μg/mL, Molecular Probes). Coverslips were mounted with FluoreGuard Mounting (Hard Set, ScyTek Laboratories Inc., Logan, UT, USA) on glass slides and assessed using a Leica DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) under oil immersion at 1000-fold magnification with a 100× objective (PL FLUOTAR 100×/1.30, OIL, ∞/0.17/D, Leica Microsystems) and a 10× ocular objective (Leica L-Plan 10×/25 M, Leica Microsystems). In total, 200 cell nuclei