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**Water-filtered infrared-A radiation (wIRA) is not implicated in cellular
degeneration of human skin**

THESE

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Rapport de synthèse

Introduction: le vieillissement cutané est un processus biologique complexe auquel participe une exposition excessive au rayonnement ultraviolet du soleil. En particulier, les longueurs d'onde des rayons ultraviolets A et B (UV-A et UV-B) peuvent induire une augmentation de la synthèse de protéases, comme la métalloprotéinase matricielle 1 (MMP-1), qui est impliquée dans le processus de vieillissement. La thérapie par infrarouges, dont les longueurs d'onde sont plus longues que celles des UV, est largement utilisée à des fins thérapeutiques ou cosmétiques. Or, il a été démontré que les infrarouges en filtration aqueuse (IRFA) pouvaient induire une augmentation de la production de MMP-1 et par conséquent être nocifs. Il serait donc intéressant d'évaluer les effets des IRFA au niveau cellulaire et moléculaire.

But Expérimental: étudier les effets des lampes à infrarouges en filtration aqueuse utilisées en clinique sur des fibroblastes cutanés humains en culture, afin d'analyser l'expression du gène codant pour la protéine MMP-1.

Méthode: des fibroblastes cutanés humains ont été irradiés d'une part avec approximativement 88% d'IRFA (780-1400 nm) et 12% de lumière rouge (LR, 665-780 nm) avec 380 mW/cm² IRFA(+LR) (333 mW/cm² IRFA) et d'autre part avec des UV-A comme contrôle. Des courbes de survie cellulaire ont été établies après une exposition allant de 15 minutes à 8 heures au IRFA(+LR) (340-10880 J/cm² wIRA(+RL), 300-9600 J/cm² wIRA) ou de 15 à 45 minutes aux UV-A(+BL) (25-75 J/cm² UV-A(+BL)). L'induction de l'ARNm du gène de la MMP-1 a été analysé dans les fibroblastes cutanés humains à deux températures physiologiques (30°C et 37°C) lors d'expositions uniques de 15 à 60 minutes aux IRFA(+LR) (340-1360 J/cm² IRFA(+LR), 300-1200 J/cm² IRFA) ou de 30 minutes aux UV-A(+BL) (50 J/cm² UV-A(+BL)). De plus, nous avons effectué des irradiations répétées, une à chaque passage cellulaire jusqu'au passage 10 de 15 minutes d'IRFA(+LR) 340 J/cm² IRFA(+LR), 300 J/cm² IRFA) .

Résultats: une exposition unique aux UV-A (+BL) entraîne chez des fibroblastes cutanés humains une augmentation de la mort cellulaire, ainsi qu'une forte augmentation de l'expression du gène codant pour la MMP-1. L'augmentation mise en évidence pour cet ARNm varie en fonction de la technique utilisée : elle est de 11 ± 1 fois par RT-PCR classique, de 76 ± 2 fois par RT-PCR quantitative à 30°C, et de 75 ± 1 fois par RT-PCR quantitative à 37°C. Par contre, une exposition unique ou

répétée aux IRFA (+LR) n'induit aucune augmentation de la mort cellulaire, ni de l'expression de l'ARNm de la MMP-1 chez ces fibroblastes.

Conclusions : les résultats de cette étude montrent que, contrairement aux rayons ultraviolets, les IRFA (+LR) ne semblent impliqués ni dans le vieillissement, ni dans la mort cellulaire, même utilisés à des doses très élevées.

Ces résultats sont en accord avec certaines investigations *in vivo* montrant une induction de MMP-1 par des UV et non des infrarouges. Ces dernières études suggèrent d'ailleurs plutôt un rôle protecteur des IRFA (+LR).

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Water-filtered infrared-A radiation (wIRA) is not implicated in cellular degeneration of human skin

Wassergefilterte Infrarot-A-Strahlung (wIRA) ist nicht an der Zelldegeneration menschlicher Haut beteiligt

Abstract

Background: Excessive exposure to solar ultraviolet radiation is involved in the complex biologic process of cutaneous aging. Wavelengths in the ultraviolet-A and -B range (UV-A and UV-B) have been shown to be responsible for the induction of proteases, e. g. the collagenase matrix metalloproteinase 1 (MMP-1), which are related to cell aging. As devices emitting longer wavelengths are widely used in therapeutic and cosmetic interventions and as the induction of MMP-1 by water-filtered infrared-A (wIRA) had been discussed, it was of interest to assess effects of wIRA on the cellular and molecular level known to be possibly involved in cutaneous degeneration.

Objectives: Investigation of the biological implications of widely used water-filtered infrared-A (wIRA) radiators for clinical use on human skin fibroblasts assessed by MMP-1 gene expression (MMP-1 messenger ribonucleic acid (mRNA) expression).

Methods: Human skin fibroblasts were irradiated with approximately 88% wIRA (780-1400 nm) and 12% red light (RL, 665-780 nm) with 380 mW/cm² wIRA(+RL) (333 mW/cm² wIRA) on the one hand and for comparison with UV-A (330-400 nm, mainly UV-A1) and a small amount of blue light (BL, 400-450 nm) with 28 mW/cm² UV-A(+BL) on the other hand. Survival curves were established by colony forming ability after single exposures between 15 minutes and 8 hours to wIRA(+RL) (340-10880 J/cm² wIRA(+RL), 300-9600 J/cm² wIRA) or 15-45 minutes to UV-A(+BL) (25-75 J/cm² UV-A(+BL)). Both conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and quantitative real-time RT-PCR techniques were used to determine the induction of MMP-1 mRNA at two physiologic temperatures for skin fibroblasts (30 °C and 37 °C) in single exposure regimens (15-60 minutes wIRA(+RL), 340-1360 J/cm² wIRA(+RL), 300-1200 J/cm² wIRA; 30 minutes UV-A(+BL), 50 J/cm² UV-A(+BL)) and in addition at 30 °C in a repeated exposure protocol (up to 10 times 15 minutes wIRA(+RL) with 340 J/cm² wIRA(+RL), 300 J/cm² wIRA at each time).

Results: Single exposure of cultured human dermal fibroblasts to UV-A(+BL) radiation yielded a very high increase in MMP-1 mRNA expression (11 ±1 fold expression for RT-PCR and 76 ±2 fold expression for real-time RT-PCR both at 30 °C, 75 ±1 fold expression for real-time RT-PCR at 37 °C) and a dose-dependent decrease in cell survival. In contrast, wIRA(+RL) did not produce cell death and did not induce a systematic increase in MMP-1 mRNA expression (less than twofold expression, within the laboratory range of fluctuation) detectable with the sensitive methods applied. Additionally, repeated exposure of human skin fibroblasts to wIRA(+RL) did not induce MMP-1 mRNA expression systematically (less than twofold expression by up to 10 consecutive wIRA(+RL) exposures and analysis with real-time RT-PCR).

Conclusions: wIRA(+RL) even at the investigated disproportionately high irradiances does not induce cell death or a systematic increase of MMP-1 mRNA expression, both of which can be easily induced by UV-A radiation. Furthermore, these results support previous findings of *in*

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vivo investigations on collagenase induction by UV-A but not wIRA and show that infrared-A with appropriate irradiances does not seem to be involved in MMP-1 mediated photoaging of the skin. As suggested by previously published studies wIRA could even be implicated in a protective manner.

Used abbreviations: BL: blue light; IR-A: infrared-A; MMP-1: matrix metalloproteinase 1; mRNA: messenger ribonucleic acid; PBS: phosphate buffered saline; RL: red light; UV-A, UV-A1, UV-B: ultraviolet-A (315-400 nm), -A1 (340-400 nm), -B (280-315 nm); wIRA: water-filtered infrared-A (780-1400 nm)

Keywords: Matrix metalloproteinase 1 (MMP-1), photoaging, ultraviolet-A (UV-A), ultraviolet-A1 (UV-A1), UV-A radiation, infrared-A radiation (IR-A), water-filtered infrared-A (wIRA)

Zusammenfassung

Hintergrund: Eine übermäßige Exposition gegenüber Ultraviolettstrahlung der Sonne ist mit dem komplexen biologischen Prozess der Hautalterung verbunden. Für Wellenlängen im Ultraviolett A und B (UVA und UVB) wurde gezeigt, dass sie für die mit der Zellalterung verbundene Protease-Induktion, z. B. der Kollagenase Matrix-Metalloproteinase 1 (MMP-1), mitverantwortlich sind. Da längere Wellenlängen häufig für therapeutische und kosmetische Zwecke verwendet werden und die Induktion von MMP-1 durch wassergefiltertes Infrarot A (wIRA) diskutiert worden war, war es von Interesse, Effekte von wIRA, die zur Hautdegeneration führen könnten, auf zellulärem und molekularem Niveau zu untersuchen.

Ziele: Untersuchung der biologischen Wirkungen von klinisch häufig gebrauchten wassergefilterten Infrarot-A-Strahlern auf menschliche Hautfibroblasten anhand der MMP-1-Gen-Expression (Expression der MMP-1-Messenger-Ribonukleinsäure (mRNA)).

Methoden: Menschliche Hautfibroblasten wurden einerseits etwa 88% wIRA (780-1400 nm) und etwa 12% rotem Licht (RL, 665-780 nm) mit 380 mW/cm² wIRA(+RL) (333 mW/cm² wIRA) und andererseits zum Vergleich UVA-Strahlung (330-400 nm, vorwiegend UVA1) und einem kleinen Anteil von blauem Licht (BL, 400-450 nm) mit 28 mW/cm² UVA(+BL) ausgesetzt. Es wurden Überlebenskurven anhand der Koloniebildungsfähigkeit nach Einzelbestrahlungen zwischen 15 Minuten und 8 Stunden mit wIRA(+RL) (340-10880 J/cm² wIRA(+RL), 300-9600 J/cm² wIRA) oder 15-45 Minuten mit UVA(+BL) (25-75 J/cm² UVA(+BL)) erstellt. Sowohl die konventionelle Reverse-Transkriptase-Polymerase-Kettenreaktion (RT-PCR) als auch die quantitative Echtzeit-RT-PCR wurden angewandt, um die Induktion von MMP-1-mRNA bei zwei physiologischen Temperaturen für Hautfibroblasten (30 °C und 37 °C) nach Einzelexposition (15-60 Minuten wIRA(+RL), 340-1360 J/cm² wIRA(+RL), 300-1200 J/cm² wIRA; 30 Minuten UVA(+BL), 50 J/cm² UVA(+BL)) und zusätzlich bei 30 °C nach wiederholter Exposition (bis zu zehnmal 15 Minuten wIRA(+RL) mit bei jedem Mal 340 J/cm² wIRA(+RL), 300 J/cm² wIRA) zu bestimmen.

Ergebnisse: Nach einer einzelnen UVA(+BL)-Exposition von kultivierten menschlichen dermalen Fibroblasten zeigte sich eine sehr starke Zunahme der MMP-1-mRNA-Expression (1.1 ± 1 fache Expression für RT-PCR und 76 ± 2 fache Expression für Echtzeit-RT-PCR jeweils bei 30 °C, 75 ± 1 fache Expression für Echtzeit-RT-PCR bei 37 °C) sowie eine dosisabhängige Minderung des Zellüberlebens. Im Gegensatz hierzu rief wIRA(+RL) kein Zellsterben und keine mit den angewendeten sensitiven Methoden erkennbare systematische Induktion der MMP-1-mRNA-Expression hervor (weniger als zweifache Expression, innerhalb der me-

thodischen Schwankungsbreite). Auch bei wiederholter wIRA(+RL)-Exposition von menschlichen Hautfibroblasten wurde MMP-1-mRNA nicht systematisch induziert (weniger als zweifache Expression bei bis zu 10 aufeinanderfolgenden wIRA(+RL)-Expositionen und Analyse mit Echtzeit-RT-PCR).

Folgerungen: wIRA(+RL) induziert selbst unter den untersuchten unphysiologisch hohen Bestrahlungsstärken im Gegensatz zu UVA-Strahlung weder den Zelltod noch eine systematisch verstärkte Expression von MMP-1-mRNA. Diese Ergebnisse unterstützen Resultate früherer *in vivo*-Untersuchungen zur Kollagenase-Induktion durch UVA, aber nicht durch wIRA, und zeigen, dass Infrarot A bei adäquaten Bestrahlungsstärken nicht in die MMP-1-induzierte Lichtalterung der Haut involviert zu sein scheint. Wie in früher veröffentlichten Studien nahegelegt, könnte wIRA sogar eher mit einer Schutzfunktion verbunden sein.

Schlüsselwörter: Matrix-Metalloproteinase 1 (MMP-1), Lichtalterung, Ultraviolett A (UVA), Ultraviolett A1 (UVA1), UVA-Strahlung, Infrarot-A-Strahlung (IRA), wassergefiltertes Infrarot A (wIRA)

Introduction

Chronic exposure of skin to ultraviolet radiation has been shown to be implicated in photoaging which is characterized by distinct degeneration of the dermal extracellular matrix. Clinical consequences are the appearance of wrinkles, skin fragility and a leathery texture of the skin. It has been clearly shown that ultraviolet-B (UV-B, 280-315 nm) and ultraviolet-A (UV-A, 315-400 nm) are implicated in photoaging of the skin [1]. However, longer wavelengths that are becoming more preponderant in clinical and cosmetic irradiation devices have not been studied in detail. Infrared radiation (IR) [2], [3] and especially water-filtered infrared-A (wIRA) [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15], [16], [17], [18], [19] has been used for therapeutic purposes including promotion of healing in acute and chronic wounds [5], [6], [7], [8], [10], [11], [13], [14], surgery [11], [13], pain therapy [4], [6], [7], [8], [11], treatment of recalcitrant hand and foot warts [4], [12], improvement of penetration of topically applied substances [15], [16], [17], improvement of regeneration after physical activity [18], and improvement of lipolysis [19]. Very little information is available on the induction of genes in human skin after infrared (IR) irradiation [20], although protective effects of infrared have been observed on cultured normal human dermal fibroblasts irradiated with UV-B and UV-A [21]. In contrast to a publication about irradiation of guinea-pigs from 1982 [22], Menezes et al. [21] have shown that a pre-irradiation of human skin fibroblasts with infrared wavelengths from 700-2000 nm at a temperature of 25 °C can protect the cells against subsequent cytotoxicity induced by UV-A and UV-B radiation. The protective effect was found to be accumulative and could last up to 24 hours following the initial irradiation with infrared. The results from this study highlight the cellular effects of infrared radiation irrespective of thermal qualities; the authors conclude that there is an efficient role of infrared

irradiation in the prevention of damage to skin cells by UV radiation. Other scientific teams found as well protective effects of infrared radiation against ultraviolet toxicity [23], [24], [25], and they described the signaling pathway of infrared [24], [25]. In addition, other publications showed cellular (non-thermic) effects of infrared-A like target-oriented growth of cells [26], [27] or changes in cell behavior [28]. Taking the holistic point of view of quantum physics [29] into account, water-filtered infrared-A can be described as flow of photons (quanta) with non-thermic and thermic effects. From the point of view of modern physics [30] – overcoming the deterministic approach, as represented in Newton's laws or Schrödinger's wave equations (with its independence from time) or even quantum mechanics – with the probabilistic approach of modern physics [29], [30], regarding the interaction of elements within a system (with an irreversibility of time and a sequence of events and small influences leading to divergent ways and results (butterfly phenomenon), solving even the quantum paradox), many systems in the world, especially biological systems, are unstable thermodynamic systems, capable to build up and represent complex structures and being far away from a stable (unstructured) point (chaos). Energy delivery to the system can maintain such an unstable thermodynamic system [30]. In this sense an adequate infrared irradiation with appropriate irradiances can help maintain such a desired unstable thermodynamic system: on the macroscopic level predominantly with thermic effects (clinically with increased tissue temperature, perfusion and tissue oxygen partial pressure as energetically important variables [11]) and on the microscopic/molecular level with non-thermic and thermic effects on cells and cell structures.

The receptor for infrared in cells is supposed to be located in the centrosomes [31].

Protective effects of infrared radiation on cultured skin fibroblasts that are exposed to UV-B and UV-A radiation reported above [21] could be related to a direct induction of protective proteins by infrared-A radiation as demon-

strated by recent studies investigating ferritin expression in human skin *in vivo* [32]. Subsequent protection of cultured cells has been shown to be specifically related to ferritin induction following UV-A irradiation [33].

In addition, pulsed low dose infrared-A radiation from lasers has been shown to induce proteins responsible for cell adhesion important in wound healing [34]. This cell adhesion process was dependent on the modulation of the activity of the respiratory chain and free radical and redox processes were also involved in the cell matrix interaction [35], [36].

Within the spectra of infrared-A and water-filtered infrared-A radiation (wIRA) effects especially of the energy-rich wavelengths near to visible light – approximately 780-1000 nm (800-900 nm [26], [31], [37], 800 nm [27], 820 nm [34], [35], [36], 830 nm [38]) – have been described both *in vitro* and *in vivo* and these wavelengths seem to represent the clinically most important part within infrared-A and wIRA.

The combination of broad band UV-A radiation with radiation of visible and infrared wavelengths does not induce the formation of melanocytic nevi in a guinea-pig model for human nevi [39]. We have also seen in human skin *in vivo* that wIRA does not induce direct damage to DNA or oxidative stress proteins or proteases that are involved in carcinogenesis and photoaging of the skin [32], [40]. As wIRA irradiation is used frequently in various routine therapies at present, it is important to investigate the effects of single exposure to wIRA at two physiologic temperatures and of repeated exposure to wIRA on human skin fibroblasts.

Materials and methods

Skin biopsies and cell culture

Skin biopsies from unexposed body sites (i.e. buttocks or behind ears) were obtained from four patients (ranging from 2 to 29 years of age). Biopsies were in accordance with the Hospital Ethical Committee of the University Hospital in Lausanne. Primary skin fibroblast cultures developed in our laboratory from these biopsies were established as described previously [41], and grown in Dulbecco's Modified Eagles Medium (DMEM, Gibco Invitrogen, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, Paisley, Scotland) and glutamine (Gibco Invitrogen, Paisley, Scotland) at 37 °C and 10% CO₂. Primary fibroblast cell lines established in our laboratory used between passages 2 and 10 were grown to 75% confluence and just prior to irradiation medium was removed and the fibroblast monolayer rinsed twice with phosphate buffered saline (PBS: NaCl 6.8 g/L; Na₂HPO₄ 1.48 g/L; KH₂PO₄ 0.43 g/L).

Cell treatment and irradiation

A wIRA irradiation source, a special Hydrosun[®] radiator, was used for irradiation of cultured cells (Labor-Hydro-

sun[®]-Strahler, Hydrosun[®] Medizintechnik, Müllheim, Germany), which emits approximately 88% wIRA (between 780-1400 nm) and approximately 12% visible red light (RL, between 665-780 nm, special red filter RG665, Schott, Mainz, Germany). This radiator was equipped with a water filter (4 mm water cuvette) to eliminate the undesired wavelengths infrared-C and -B and to decrease the absorption bands of water within the infrared-A and thereby the undesired overheating of exposed skin. The spectrum is similar to the spectrum presented in [4] – especially concerning wIRA – with the exception of less visible light (only 665-780 nm instead of 590-780 nm), as filter RG665 was used instead of OG590.

Fibroblasts were plated in 60 mm diameter Falcon culture dishes and were grown to 75% confluence. Just prior to irradiation, growth medium was removed and the fibroblast monolayer was rinsed twice with PBS. Fibroblasts were then covered with 2 ml of PBS and irradiated – covered with plastic tissue culture lids (with a measured spectral transmittance of approximately 90% between 665 and 1400 nm) – at a distance of 40 cm (same for wIRA(+RL) and UV-A(+BL), see below) at 30 °C or 37 °C (two physiologic temperatures for skin fibroblasts) in the culture dishes with a temperature regulated water bath beneath the dishes and in contact with the bottom of the dishes. Irradiation times varied from 15 to 60 minutes, where irradiation doses (radiant exposures) of wIRA(+RL) were 340, 680, 1020 and 1360 J/cm², corresponding to 300, 600, 900 and 1200 J/cm² wIRA, and with a total irradiance (irradiation intensity related to area) of wIRA(+RL) of approximately 380 mW/cm² including the 12% red light, corresponding to approximately 333 mW/cm² wIRA. After irradiation, fresh medium was added to the fibroblasts, and fibroblasts were kept at 37 °C in 10% CO₂ until harvesting. RNA was extracted 24h after irradiation.

Repeated infrared-A irradiation was performed on primary fibroblast cell lines developed in our laboratory by irradiating them at each cellular passage (approximately 75% confluency) with an irradiation dose of wIRA(+RL) of 340 J/cm² over 15 minutes (corresponding to 300 J/cm² wIRA). Cells were passaged until they reached senescence. Irradiation was performed once per week over 3 to 10 weeks as a model for repeated intermittent irradiation over longer periods. After irradiation, fresh medium was added to the fibroblasts, and fibroblasts were kept at 37 °C in 10% CO₂ until harvesting. RNA was extracted 24h after irradiation, at passages 3, 6 and 10.

UV-A irradiation was performed with a high-pressure UV-A radiator Uvasun 3000 (Mutzhas, Munich, Germany) using an (unweighted) irradiation dose of 50 J/cm² over 30 minutes. On average, this dose typically corresponds to one Minimal Erythema Dose (1 MED = 250 J/m² = 0.025 J/cm², erythema weighted irradiation dose) in the unadapted phototype II of human skin [42]. The Uvasun 3000 radiator emitted wavelengths between 330 and 450 nm with an irradiance of approximately 28 mW/cm² at a distance of approximately 40 cm. The spectral output of the radiator was analyzed with a calibrated Optronix

model 742 spectroradiometer (Optronics Laboratories, Pennsylvania, USA) and showed a broad peak between 360 and 410 nm, mainly lying within the range of UV-A1 (defined as 340-400 nm) and a small amount of blue light (BL, 400-450 nm). The Uvasun 3000 radiator was equipped with an infrared filter and with a filter that cuts off sharply all wavelengths below 335 nm. In addition, the plastic tissue culture lids permit no transmission of UV-B or UV-C radiation.

Irradiances were monitored by an International Light Radiometer, IL 1700 (International Light Radiometer, Newburyport, Massachusetts, USA) with UV-A detector head (No. 566 with filter W1327), calibrated by comparison with the spectroradiometer.

Temperature was monitored at the cellular level within the PBS solution with a digital multimeter M-4660 A (Conrad Electronic, Frankfurt/Main, Germany) during all the experiments.

Survival assays

Dishes of fibroblasts (60 mm, approximately 75% confluent) that had received irradiation treatments were trypsinized, diluted, and plated at approximately 5000 fibroblasts per dish (60 mm, three dishes per irradiation treatment). The dishes were incubated at 37 °C for 12 to 14 days, after which they were stained with methylene blue and the colonies (>20 fibroblasts) were counted with the aid of a dissecting microscope. All experiments were carried out with both the laminar flow hood illumination system and room fluorescent lights turned off.

Nucleic acid extraction and reverse transcription

Total RNA was extracted from cells using the NucleoSpin[®], RNA II kit (Marcherey-Nagel, Düren, Germany) as described by the manufacturer. RNA integrity was assessed on a 1% TBE agarose gel (Molecular Biology Grade, Eurobio SA, Chemie Brunschwig, Bale, Switzerland).

One microgram of total RNA was reverse transcribed using 50 units of StrataScript[®] reverse transcriptase enzyme (Stratagene, San Diego, California, USA) in a volume of 50 µl, containing 38 µl (RNA + H₂O) + 3 µl random primers (100 ng/µl) + 1 µl (50 units) StrataScript[®] + 1 µl (40 units) RNasin[®] (Promega, Madison, Wisconsin, USA) + 2 µl dNTP mix 100 mM (Promega, Madison, Wisconsin, USA) + 5 µl (10x) StrataScript[®] first strand buffer (Stratagene, San Diego, California, USA) as described by the manufacturer. The cDNA synthesis was run in a Biometra[®] T-1 thermocycler (Biomedizinische Analytik GmbH, Göttingen, Germany), programmed as follows: 25 °C 10 min, 37 °C 60 min and 90 °C 5 min.

Conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

An MMP-1 cDNA fragment of 234 base pairs (bp) and an actin cDNA fragment of 739 bp were amplified by RT-PCR using the following primers: (MMP-1) forward 5'-ATGCTGAAACCCTGAAGGTG-3' and reverse 5'-CTGCTTGAC CCTCAGAGACC-3', (actin) forward 5'-GTTGCTATCCAGGCT GTG-3' and reverse 5'-CATAGTCCGCCTAGAAGC-3' in a Biometra[®] T-1 thermocycler (Biomedizinische Analytik GmbH, Göttingen, Germany).

After denaturation, 95 °C for 5 minutes, 25 to 35 cycles of amplification were performed as follows: 94 °C for 45 seconds, annealing temperature (MMP-1: 55 °C; actin: 60 °C) for 30 seconds, 72 °C for 30 seconds, and finally an elongation of 72 °C for 15 minutes. PCR products were electrophoresed through a 2% TBE agarose gel (Molecular Biology Grade, Eurobio SA, Chemie Brunschwig, Bale, Switzerland). Semi-quantitative analysis was performed with a Genius[®] Image Analysis System (Syngene, Cambridge, United Kingdom).

Quantitative real-time RT-PCR

The quantitative TaqMan[®] real-time RT-PCR was run in an ABI Prism[®] 7700 (Applied Biosystems, Foster City, California, USA). We measured the gene expression of MMP-1 (Hs00233958_m1) and actin (Hs99999903_m1) using assay-on-demand primers (Applied Biosystems, Foster City, California, USA). Real-time RT-PCR reactions were performed in 25 µl: 11.25 µl of cDNA sample (approximately 160 ng), 12.5 µl TaqMan[®] Universal Master Mix (Applied Biosystems, Foster City, California, USA) and 1.25 µl of 20x assay on demand gene expression assay mix (Applied Biosystems, Foster City, California, USA). Thermal cycle conditions were 50 °C 2 minutes, 95 °C 10 minutes, then 50 cycles at 95 °C 15 seconds, 60 °C 1 minute. Measurements were performed in triplicates. Relative messenger ribonucleic acid (mRNA) expressions were analyzed with the 2^{ΔΔCT} method [43] and normalized to actin mRNA expression levels.

Statistical aspects

It is well known, that non-parametric statistics entail certain advantages like an absence of requirements in reference to the form of distribution [4], [19], however we chose to apply parametric statistics, because in comparable publications especially those who relate to our topic [20], [32], [44] these statistical methods were used. Above all the results are so obvious, that they could be shown in the non-parametric as well as in the parametric form.

Irradiance (irradiation intensity related to area) is presented in the clinically common form "mW/cm²", irradiation dose in "J/cm²" (1 mW/cm² = 10 W/m²; 1 J/cm² = 10 kJ/m²).

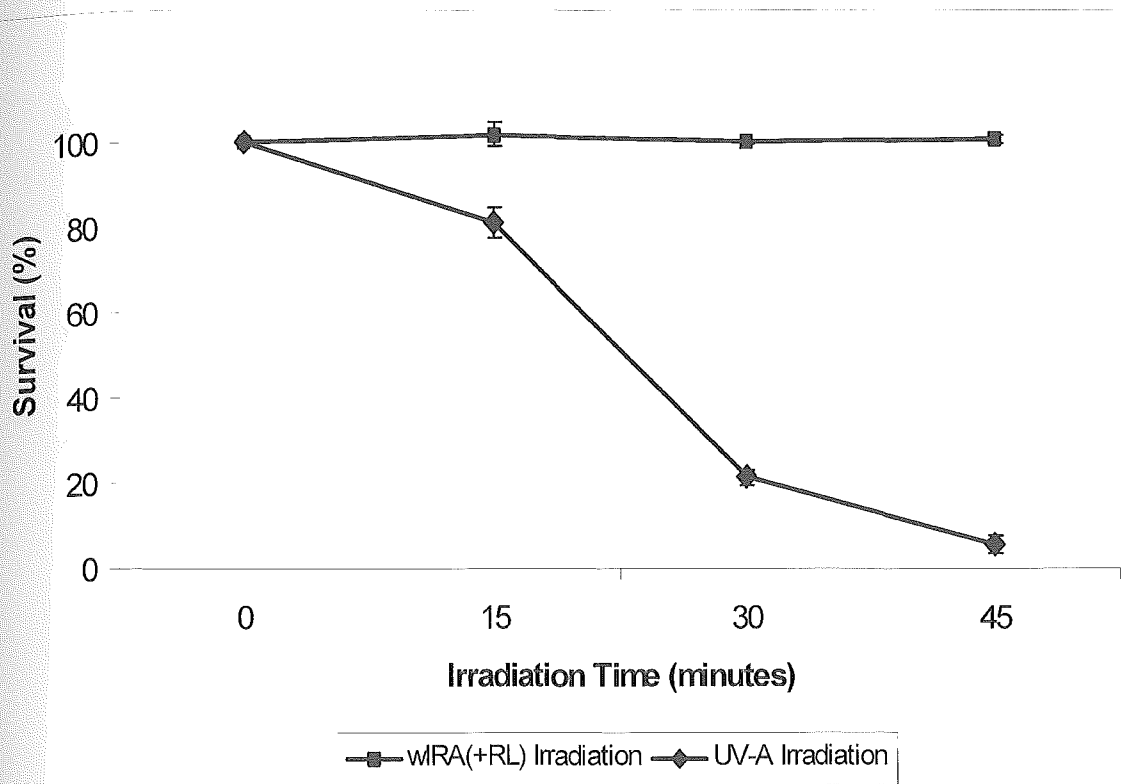


Figure 1: Cell survival after single wIRA(+RL) or UV-A(+BL) irradiation

Percent survival of human skin fibroblasts (average of 3 cell lines from non-sun exposed sites from patients with 2-29 years of age) as a function of increasing irradiation doses of wIRA(+RL) (340, 680 and 1020 J/cm² wIRA(+RL), corresponding to 300, 600 and 900 J/cm² wIRA) or UV-A(+BL) (25, 50 and 75 J/cm²) irradiation (corresponding to 15, 30 and 45 minutes irradiation) at 30°C performed on a 30°C temperature regulated water bath accomplished for each cell line in triplicate.

Results

Cell survival after single wIRA(+RL) or UV-A(+BL) irradiation

Survival curves (expressed as colony forming units) were established for human skin fibroblasts from non-sun exposed skin sites with wIRA(+RL) and UV-A(+BL) irradiation. UV-A irradiation induced cell death in a dose dependent manner with 50% cell death seen at approximately 20 minutes of irradiation with an irradiance of approximately 28 mW/cm², roughly representing 34 J/cm² (see Figure 1). Within the same period of time, no cell death was noted for wIRA(+RL) irradiation treatment (Figure 1). These periods were measured extensively as this corresponds to the normal irradiation time in a therapeutic session with wIRA(+RL) irradiation. When much longer periods were used, corresponding up to 10880 J/cm² over 8 hours, we found that it takes very high irradiation doses of wIRA(+RL) to achieve cell death (see Figure 2). A 10-20% reduction of cell survival was observed only after 6-8 hours of irradiation with wIRA(+RL) with approximately 380 mW/cm² wIRA(+RL), corresponding to approximately 333 mW/cm² wIRA, at 30°C.

In addition, we have verified that dermal fibroblasts from six individuals (22-46 years of age) subjected to

wIRA(+RL) irradiation showed similar resistance to irradiation doses up to 1020 J/cm² wIRA(+RL) irradiation as assessed through staining by trypan blue [20].

Conventional RT-PCR analysis of collagenase MMP-1 mRNA expression after single wIRA(+RL) or UV-A(+BL) irradiation

UV-A(+BL) radiation (50 J/cm² at 30°C) induced MMP-1 mRNA expression and when normalized to actin mRNA expression showed a 11 ± 1 fold expression compared with fibroblasts that were not irradiated (see Figure 3). Fibroblasts exposed to wIRA(+RL) did not display a systematic increase in MMP-1 mRNA expression (less than twofold expression, within the laboratory range of fluctuation) at 30°C (see Figure 3). Similarly, our primary fibroblast cell lines irradiated at 37°C did not show a systematic induction of MMP-1 expression after 30 minutes exposure with wIRA(+RL) (data not shown).

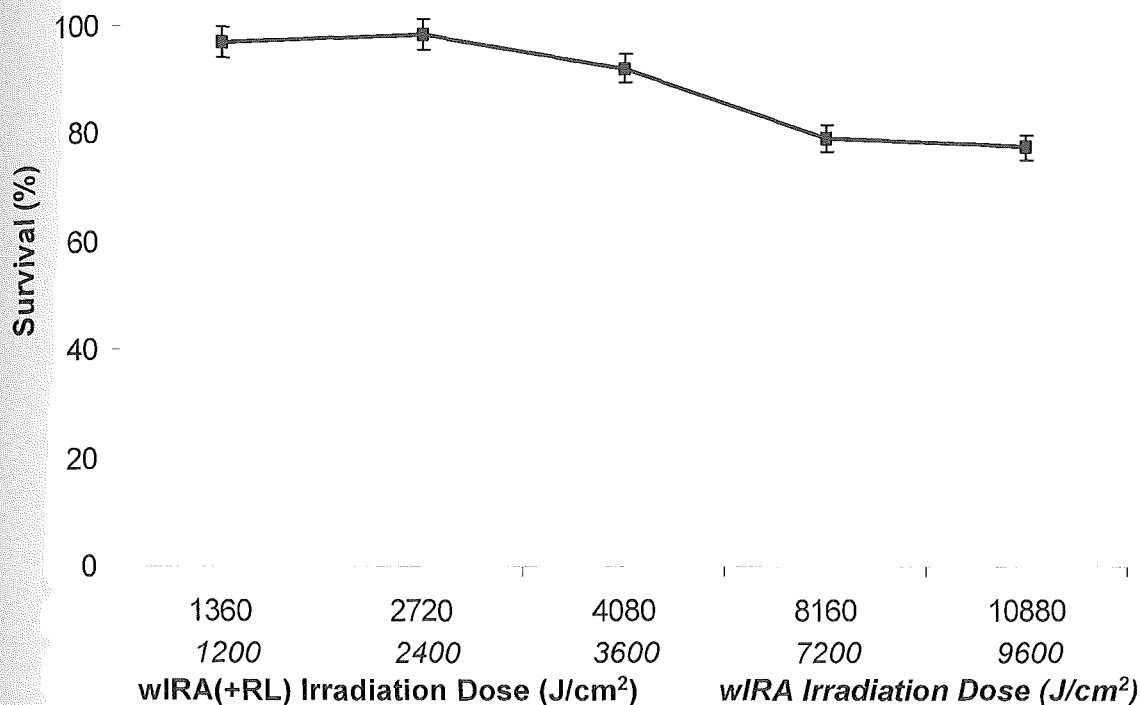


Figure 2: Cell survival after single wIRA(+RL) irradiation

Percent survival of human skin fibroblasts from a donor of 29 years as a function of increasing irradiation doses of wIRA(+RL) at 30 °C performed on a 30 °C temperature regulated water bath (irradiation doses of 1360, 2720, 4080, 8160 and 10880 J/cm² wIRA(+RL), corresponding to 1200, 2400, 3600, 7200 and 9600 J/cm² wIRA and corresponding to 1, 2, 3, 6 and 8 hours of irradiation).

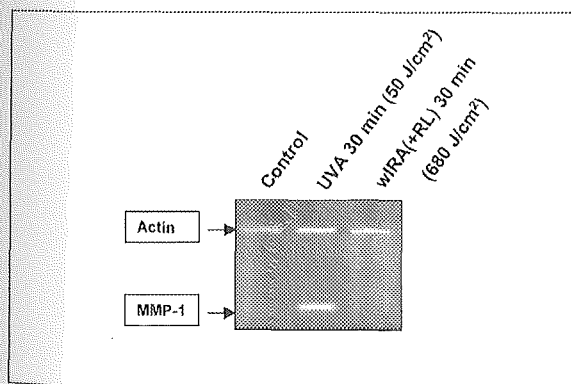


Figure 3: Conventional RT-PCR analysis of MMP-1 mRNA expression after single wIRA(+RL) or UV-A(+BL) irradiation

Human dermal fibroblasts were irradiated over 30 minutes with wIRA(+RL) (680 J/cm² wIRA(+RL), corresponding to 600 J/cm² wIRA) or UV-A(+BL) (50 J/cm²) at 30 °C. MMP-1 cDNA was amplified and x-fold expression of MMP-1 mRNA normalized to actin mRNA was calculated (11 ± 1 fold expression by UV-A(+BL) irradiation, no systematic increase of expression by wIRA(+RL)).

Figure 3 presents an actual sample of a RT-PCR analysis.

Quantitative real-time RT-PCR analysis of collagenase MMP-1 mRNA expression after single wIRA(+RL) or UV-A(+BL) irradiation

wIRA(+RL) irradiation was employed at multiple irradiation doses to dermal fibroblasts at 30 °C and in addition at 37 °C to obtain a potential induction of MMP-1 mRNA expression. At all wIRA(+RL) irradiation doses at 30 °C and at 37 °C no systematic induction of MMP-1 mRNA expression occurred (less than twofold expression) assessed by the sensitive quantitative technique real-time RT-PCR (see Figure 4 and Figure 5). In parallel for each experiment, UV-A(+BL) at an irradiation dose of 50 J/cm² was used as the positive control. There was a clear induction of MMP-1 expression in fibroblasts irradiated with UV-A(+BL), with a 76 ± 2 fold expression (at 30 °C) and a 75 ± 1 fold expression (at 37 °C) related to basal levels normalized to actin mRNA expression (see Figure 4 and Figure 5).

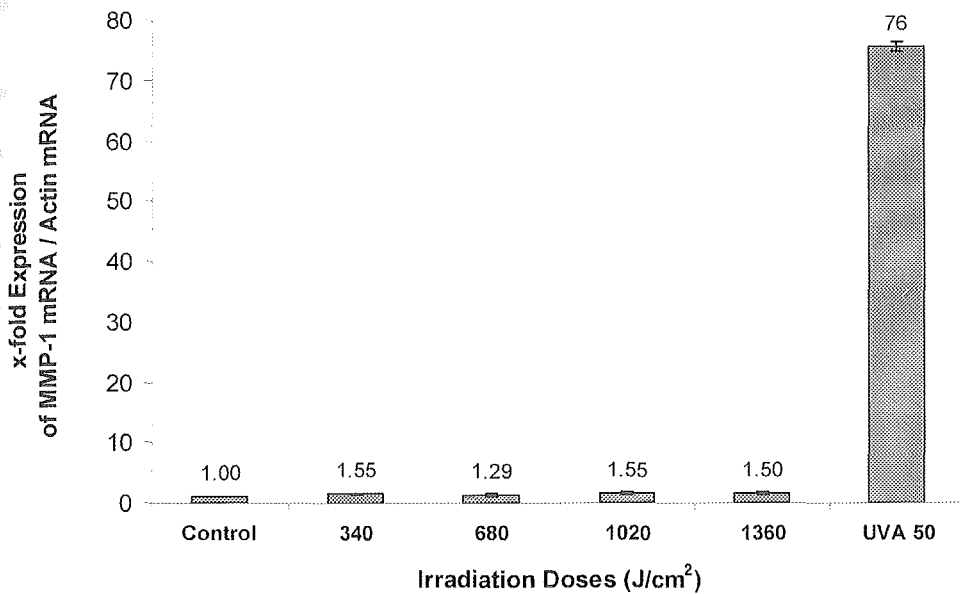


Figure 4: Quantitative real-time RT-PCR analysis of MMP-1 mRNA expression after single wIRA(+RL) or UV-A(+BL) irradiation at 30 °C

An irradiation dose response assessment was performed on human dermal fibroblast cells with wIRA(+RL) irradiation doses of 340, 680, 1020 and 1360 J/cm² respectively (corresponding to 300, 600, 900 and 1200 J/cm² wIRA and corresponding to 15, 30, 45 and 60 minutes irradiation) performed on a 30 °C temperature regulated water bath. Alternatively UV-A(+BL) irradiation (50 J/cm², corresponding to 30 minutes irradiation) was used. Data represent the average of five experiments with the associated standard deviation of the mean. Fibroblasts were harvested 24h after irradiation to assess MMP-1 mRNA expression by quantitative real-time RT-PCR as described in the text. Data were analyzed with the 2^{-ΔΔCT} method [43], results are given as relative MMP-1 mRNA expression normalized to the actin mRNA expression.

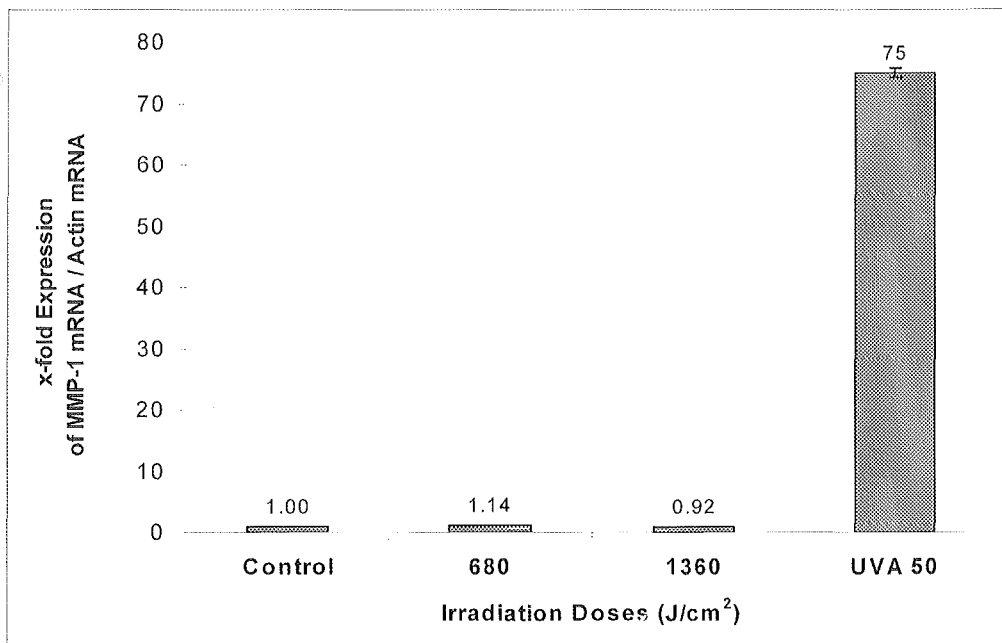


Figure 5: Quantitative real-time RT-PCR analysis of MMP-1 mRNA expression after single wIRA(+RL) or UV-A(+BL) irradiation at 37 °C

Human dermal fibroblast cells were exposed to wIRA(+RL) with an irradiation dose of 680 and 1360 J/cm² (corresponding to 600 and 1200 J/cm² wIRA and corresponding to 30 and 60 minutes irradiation) or UV-A(+BL) irradiation (50 J/cm², corresponding to 30 minutes irradiation) performed on a 37 °C temperature regulated water bath. Data represent the average of three experiments with the associated standard deviation of the mean.

Morphological appearance of fibroblasts

Twenty-four hours after irradiation microscopically the fibroblasts were typically spindle shaped and showed no signs of alteration nor cellular degeneration. Microscopic photos of cultured fibroblasts, representing 6 investigated different conditions of single irradiation (PBS control at 30 °C, 680 J/cm² wIRA(+RL) at 30 °C, 1360 J/cm² wIRA(+RL) at 30 °C; PBS control at 37 °C, 680 J/cm² wIRA(+RL) at 37 °C, 1360 J/cm² wIRA(+RL) at 37 °C), are shown in Figure 6.

Quantitative real-time RT-PCR analysis of collagenase MMP-1 mRNA expression after repeated wIRA(+RL) irradiation

wIRA(+RL) irradiation was employed at repeated irradiation doses to skin fibroblasts at 30 °C with exposure once per week over 3 to 10 weeks. During this period, the fibroblasts had the same growth capacity and showed no morphological alterations (see Figure 7).

Even following repeated exposure during up to 10 passages (with single exposure of 340 J/cm² wIRA(+RL), cumulated exposure 3400 J/cm² wIRA(+RL), corresponding to 3000 J/cm² wIRA), there was no systematic induction (less than twofold expression) of the MMP-1 mRNA as assessed with real-time RT-PCR (see Figure 7). The results of the more sensitive real-time RT-PCR technique are in keeping with those seen with Northern Blot analysis for MMP-1 mRNA expression for the same cells [45].

Discussion

In the present study we showed (i) that single wIRA exposure neither caused cell death nor MMP-1 mRNA up-regulation in skin fibroblasts at two physiologic temperatures (30 °C and 37 °C), and (ii) that the same was true for repeated wIRA exposure at 30 °C, while (iii) UV-A radiation (mainly UV-A1) caused a drastic increase in MMP-1 mRNA expression under all investigated conditions (11 ±1 fold expression for conventional RT-PCR and 76 ±2 fold expression for quantitative real-time RT-PCR both at 30 °C in single exposure, and 75 ±1 fold expression for quantitative real-time RT-PCR at 37 °C in single exposure) and a dose-dependent decrease in cell survival.

Previously we have reported that wIRA, which has low photon energy compared to UV, exerts no effects on DNA, RNA and protein synthesis related to cell proliferation of human cells. Our data are in accordance with our *in vivo* studies in which we could not detect DNA damage (pyrimidine dimers, 8-oxodG, p53) in six individuals following wIRA, however clearly after UV-A irradiation [40], [46].

On the molecular level, a recent publication has indicated that wIRA irradiation could induce MMP-1 gene expression in newborn foreskin dermal fibroblasts using conventional RT-PCR techniques (RT-PCR) [44] (and derived from this

[47], [48], [49]). We were unable to reproduce this induction using both conventional RT-PCR and quantitative real-time RT-PCR at two different physiologic temperatures in young as well as in adult skin fibroblasts employing single and repeated irradiation regimens. As we considered normal skin to be more relevant for the clinical use of wIRA than foreskin of the newborn, we used skin fibroblasts from non-sun exposed skin sites of donors from two to 29 years of age. Within this range of ages no relevant differences concerning fibroblast survival were seen. As we have previously reported that newborn foreskin biologically behaves different compared to other non-sun exposed skin sites [46], differences between the results of the study with newborn foreskin fibroblasts [44] and our results might be related to the different source of skin tissue. A direct monitoring of the temperature within cultured cells is – even considering sophisticated methods such as infrared thermography – not available and is typically substituted by temperature measurements in the culture medium. In a great effort we established a system including a temperature regulated water bath beneath the dishes (with the cultured cells) and being in close contact with the bottom of the dishes while administering wIRA, thus avoiding any temperature alteration of the cells. Even though the mentioned study [44] states that no temperature increase was detectable “immediately” after the irradiation, there seem to be differences between this study and our study regarding the cell culture and irradiation conditions. Of note, MMP-1 has been described to be inducible by heat-shock in a temperature-dependent manner in foreskin cells, mediated via an IL-6-dependent autocrine mechanism [50]. In addition, for quantifications instead of the conventional RT-PCR, as used in the mentioned study [44], the quantitative real-time RT-PCR should be used.

The aforementioned study [44] used approximately 333 mW/cm² wIRA (derived from 200 J/cm² over 10 minutes or 1200 J/cm² over 60 minutes). Because biological effects of irradiation depend not only on irradiation dose, but also on irradiance [51], we used a similar amount of radiance: our total irradiance was approximately 380 mW/cm² wIRA(+RL) (340 J/cm² over 15 minutes or 1360 J/cm² over 60 minutes or 10880 J/cm² over 8 hours) with approximately 88% wIRA and therefore approximately 333 mW/cm² wIRA (approximately 300 J/cm² over 15 minutes or 1200 J/cm² over 60 minutes or 9600 J/cm² over 8 hours).

From a clinical point of view these irradiation intensities and doses are physiologically out of range. In published clinical studies, wIRA(+RL) was applied at total irradiances of approximately 185-250 mW/cm² (185 mW/cm² [10] or 220 mW/cm² [11] or 250 mW/cm² [4]) – corresponding to wIRA irradiances of only 140-190 mW/cm² (140 mW/cm² [10] or 175 mW/cm² [11] or 190 mW/cm² [4]) and wIRA irradiation doses of 210-340 J/cm² over 20-30 minutes (210 J/cm² over 20 minutes [11] or 250 J/cm² over 30 minutes [10] or 340 J/cm² over 30 minutes [4]) per irradiation session or even less when larger irradiation distances than the minimum distance of 25 cm were

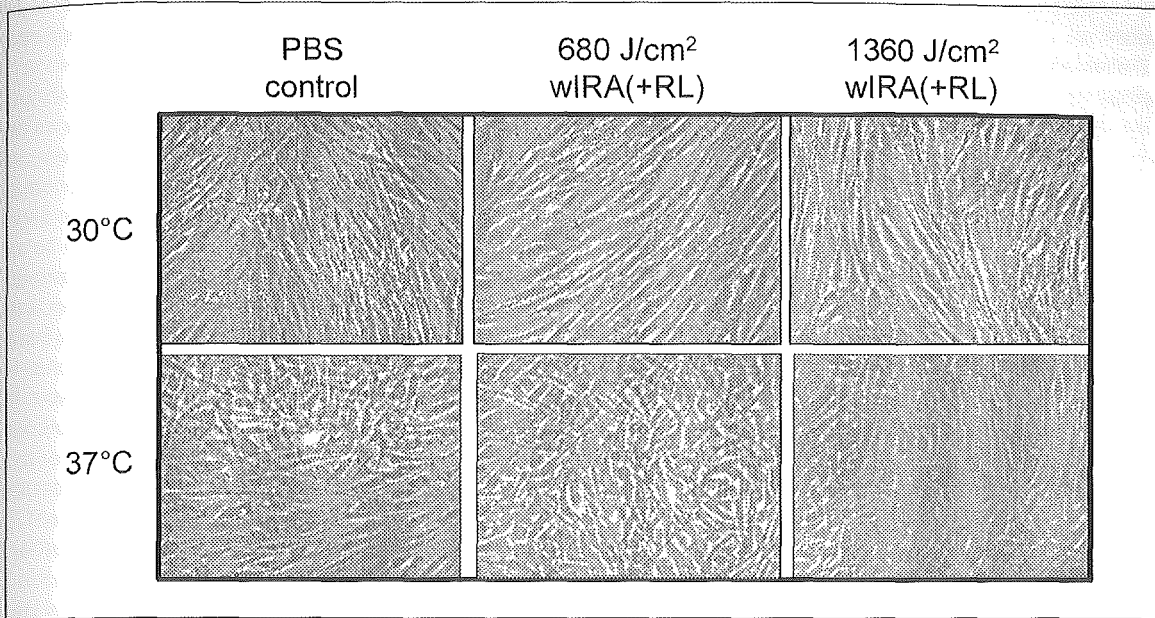


Figure 6: Microscopic photos of cultured fibroblasts 24 hours after irradiation, representing 6 investigated different conditions of single irradiation (PBS control at 30 °C, 680 J/cm² wIRA(+RL) at 30 °C, 1360 J/cm² wIRA(+RL) at 30 °C; PBS control at 37 °C, 680 J/cm² wIRA(+RL) at 37 °C, 1360 J/cm² wIRA(+RL) at 37 °C). The fibroblasts were typically spindle shaped and showed no signs of alteration nor cellular degeneration.

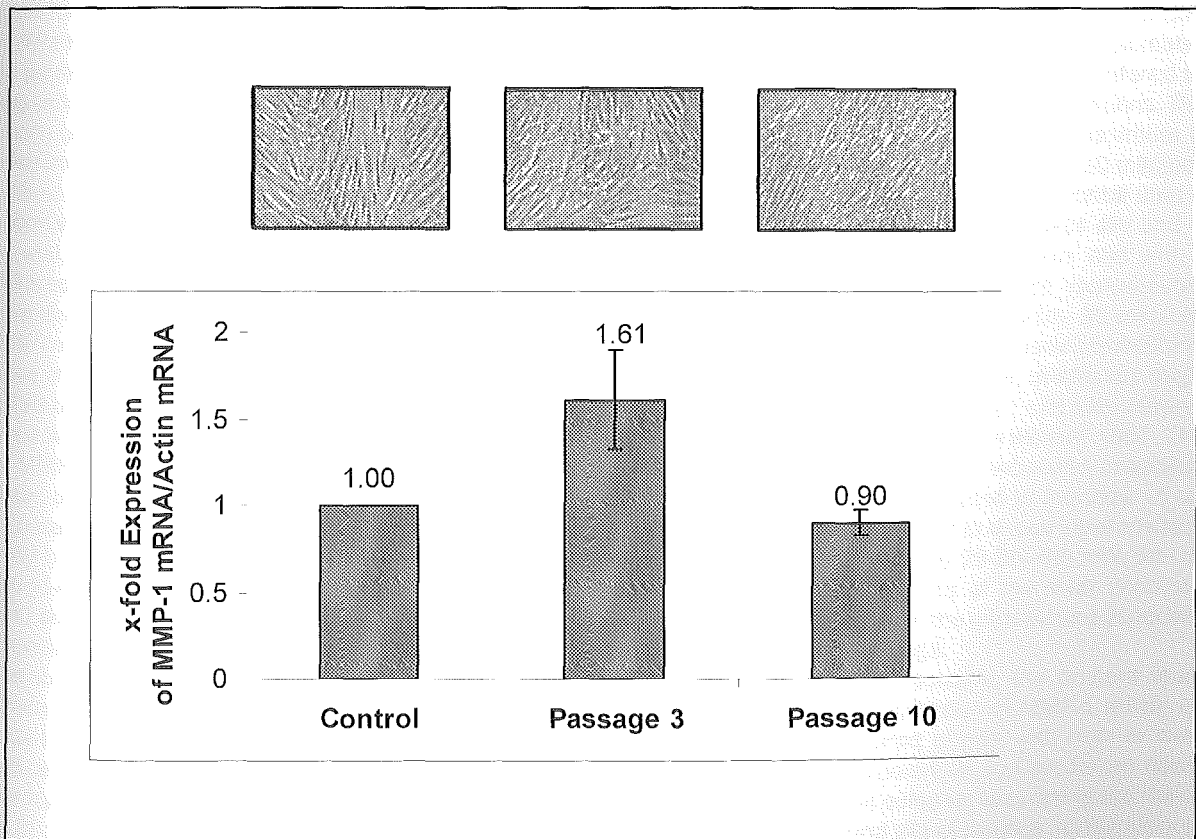


Figure 7: Quantitative real-time RT-PCR analysis of MMP-1 mRNA expression after repeated wIRA(+RL) irradiation
 Human dermal fibroblasts were exposed to radiation at each cellular passage until passage 10 with a single wIRA(+RL) irradiation dose of 340 J/cm² (i.e. cumulated irradiation dose of 3400 J/cm² wIRA(+RL), corresponding to 3000 J/cm² wIRA and corresponding to 150 minutes cumulated irradiation time). Data represent the average of three experiments with the associated standard deviation of the mean.

used. In routine clinical practice with markedly larger irradiation distances than the minimum distance typical total irradiances (wIRA+RL) are approximately 80-160 mW/cm² (depending on size of the irradiated area, on tissue temperature and amount of subcutaneous soft tissues, e.g. lower used irradiances at the tibial border compared to the anterior part of the thigh), corresponding to wIRA irradiances of only 60-120 mW/cm². In addition, the irradiation of cultured cells in dishes (*in vitro*) is different compared to the irradiation of patient skin (*in vivo*) in several respects [51]. Cultured cells in the dish represent a naked monolayer without an epidermis (including horny layer) and there is – with the exception of possible marginal movements of the medium – no circulation, i.e. no blood circulation. In contrast, in patients blood circulation and other effects allow to generate and distribute a field of warmth in tissue up to a depth of several cm [4], [8], [11]. The striking difference between irradiating a material without circulation and a living patient is described and documented, for example, in two infrared thermography video sequences in [10].

Depending upon the assumptions, an irradiance of 100 mW/cm² of human skin (*in vivo*) is equivalent to an irradiance of only approximately 30(-50) mW/cm² of fibroblast cultures in dishes (*in vitro*) [51]. Typical clinical wIRA irradiances of 60-120 mW/cm² (*in vivo*) therefore correspond to irradiances of only approximately 18-36(-60) mW/cm² of fibroblast cultures in dishes (*in vitro*) [51].

All publications which claim to have shown a MMP-1 upregulation by infrared-A used much higher *in vitro* irradiances (approximately three- to tenfold irradiances, 333 mW/cm² [44], [47], [48], 105 mW/cm² [49]) and often with no description that a water-bath to avoid damaging thermal effects was included [44], [47], [48], [49].

As well, publications which showed undesired effects of infrared-A *in vivo* (augmentation of UV-induced skin wrinkling in mice [52]) used extreme irradiances (2020 mW/cm² [52]). Even with an extreme single irradiation dose of 3951 J/cm² (which would mean an irradiance of 1098 mW/cm², if administered during 1 hour; an irradiance is not presented) Kim et al. [53] found no increase of MMP-1 mRNA expression after single infrared irradiation, but only after multiple irradiations.

Publications with appropriate *in vitro* irradiances (45 mW/cm² total irradiance and less than 45 mW/cm² infrared-A [21], [24], [25]) and appropriate water-bath [21], [24] showed positive and protective effects of infrared, especially infrared-A.

The striking difference between appropriate and inappropriate irradiances can be illustrated by the skin temperature at maximum: Mercer [9] showed that a clinically typical irradiation with wIRA increased skin surface temperature (starting from 32.5 °C) by nearly 6 °C to a mean of 38.2 °C (maximum value was 39.1 °C). In accordance with that skin surface temperature (starting with approximately 32 °C) reaches approximately only 38 °C even under Mediterranean conditions in the summer at noon after 30 minutes stay in the sun. These approximately 38(-39) °C are decisively lower than the induced "43 °C

for 90 minutes" with inappropriately high irradiance [53]: above 39.5-40 °C heat-shock proteins can be induced (the heat-shock-induced matrix metalloproteinase-1 expression in human epidermal keratinocytes is mediated by the transient receptor potential vanilloid-1 kation channel [54]), and this is a thermal effect and not a direct radiation effect. So different outcomes of studies can be assigned to thermal effects, when overheating is not avoided.

Interestingly a current publication with emphasis to avoid any overheating [55] from the same institute as the publications [44], [47], [48], [49] showed no damaging effect of water-filtered infrared-A (0, 100, 250, 500, 1000 J/cm²) to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) solution as a model enzyme for environmentally induced protein damage, while UV-A (0, 100, 250, 500, 1000 J/cm²), UV-B (0, 250, 500, 1000, 2000 mJ/cm²), and gamma-irradiation (0, 50, 100, 250, 500 Gy) caused a dose-dependent increase in protein modification (fragmentation and aggregation) and loss of enzyme activity with complete loss of enzyme activity at the highest doses. In addition, when comparing publications different conditions (different spectra, humans/animals/cell cultures/enzyme solutions) have to be taken into account.

Moreover, it is doubtful whether possible negative effects of solar infrared radiation [47] with a reported average of approximately only 75 J/cm² per hour for summertime in Munich (Germany) [47], equivalent to approximately only 20 mW/cm² infrared irradiance, can be inferred for the skin of humans from such *in vitro* observations. This value is in principle accordance with the maximum of solar infrared-A irradiance of approximately 20 mW/cm² spectroradiometrically measured at a horizontal area in Berlin in July at noon time during cloudless sky and of approximately 25 mW/cm² in the tropics and with a maximum of total infrared irradiance of approximately 28 mW/cm² in Berlin and of approximately 35 mW/cm² in the tropics [51], [56]. Even under extreme atmospheric conditions at the equator infrared-A irradiance might reach only approximately 34 mW/cm² and total infrared irradiance only approximately 48 mW/cm² [56]. This means that the maximum of solar infrared-A irradiance at the surface of the earth is limited to the order of approximately 20-25(-34) mW/cm² and total infrared irradiance is limited to approximately 28-35(-48) mW/cm². In addition, the problematic parts of the solar spectrum seem to be located outside of the infrared and especially outside of the infrared-A range.

Taking these aspects into account, the present study evidences no systematic induction of MMP-1 mRNA expression or other undesired effects with high or even disproportionally high irradiances and irradiation doses of wIRA in skin fibroblast cultures, supporting the notion that wIRA does not bring along noxious consequences when clinically administered in an appropriate form. (Beside this, upregulation of MMP-1 takes place physiologically during growth, wound healing, and angiogenesis, and therefore even an upregulation of MMP-1

would not be interpretable automatically as undesired damaging effect.)

This conforms with the clinical experience with wIRA during the last 15 years when wIRA with its profound penetration properties has been used to improve wound healing [5], [6], [7], [8], [10], [11], [12], [13] [14] and in other skin related disorders [4], [9], [12], [15], [16], [17] as well as in neonatology [57], sports medicine [18], [58], [59], rheumatology [60], physiotherapy/orthopedics [61], and improvement of lipolysis [19]. In all these studies, wIRA effectuated no damage when administered appropriately, avoiding overheating.

Additional positive or protective effects of wIRA [21], [24], [25], as shown in experimental studies in which infrared exposure, especially water-filtered infrared-A, induced ferritin and protected against UV induced cell death [20], [32], [62], seem to underlay the clinical experience that wIRA given in non-dermatological indications improves the condition of the skin [8].

This is as well in accordance with the effects of the full spectrum of the sun, which has more positive effects than expected from the UV part alone [63] or the well known negative effects of UV radiation including photoaging [64], [65], [66], [67], as the full spectrum of the sun includes infrared [63], which reaches the surface of the earth in moderate climatic zones water-filtered [68]. It is this water-filtered infrared, which accompanied the evolution of mankind [69] and which served as a model for the technical realisation of wIRA [68], [69] for clinical use [70].

Notes

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Conflicts of interest

None declared.

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