Effect of a Blueberry-Derived Antioxidant Matrix on Infrared-A Induced Gene Expression in Human Dermal Fibroblasts

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ABSTRACT

There is compelling evidence that Infrared A (IRA) from natural sunlight contributes to photoaging of human skin by inducing the expression of matrix metalloproteinase-1 (MMP-1) expression in human dermal fibroblasts. Corresponding mechanistic studies have shown that IRA does so by increasing the production of reactive oxygen species in irradiated cells. In the present study, we therefore asked if treatment of primary human skin fibroblasts with a blueberry-derived antioxidant matrix (Berrimatrix™), which is employed as an active ingredient in commercially available skin care products that are topically applied, can prevent IRA-induced MMP-1 expression in these cells. In this in vitro study, we have found that this antioxidant containing matrix is well tolerated by fibroblast over a broad concentration range and that it efficiently prevents IRA-induced MMP-1 mRNA expression. It may thus be speculated that topical application of this antioxidant containing matrix may be efficient in protecting human skin against IRA-induced wrinkle formation.

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INTRODUCTION

Previous studies indicate that human skin is a target for infrared A radiation (IRA).[1] Infrared A (IRA) irradiation leads to increased expression of matrix metalloproteinase 1 (MMP1), an enzyme involved in wrinkle formation in human dermis after treatment with physiological IRA doses.[1] In addition, IRA irradiation results in a decrease of the skin´s vitamin content.[1]

Mechanistic studies have shown that IRA exerts these effects through the generation of oxidative stress in human skin fibroblasts, indicating the possibility that appropriate antioxidant may be able to prevent IRA-induced gene expression in this cell type. To this end, we studied a water-soluble extract made from blueberry plants grown along the coast of Maine (Berrimatrix™). This antioxidant matrix contains polyphenols, vitamins, and other natural ingredients. In order to test whether this antioxidant matrix is able to prevent IRA-induced damage in human dermal fibroblasts, these cells were pre-incubated (24 hours) and post-incubated (24 hours) in the presence or absence of the extract. IRA-induced expression of MMP-1 has been assessed as read-out.

MATERIALS AND METHODS

Cell Culture

Human dermal fibroblasts (HDFs) prepared from neonatal foreskin were cultured in EMEM (PAA Laboratories GmbH, Kölbe, Germany) supplemented with 5% fetal calf serum (FCS, Invitrogen, Karlsruhe, Germany), 1% L-glutamine, 1% non-essential amino acids (Invitrogen, Karlsruhe, Germany), and 1 % streptomycin/amphotericin B (Invitrogen, Karlsruhe, Germany) in a humidified atmosphere containing 5% CO_2 for 4 days until they reached confluence as described.[1] For all studies, only early passage (<12) fibroblasts have been used to avoid changes in their original phenotype during subculture. Cells were kept in 6 well plates for culture and irradiation.

Viability Testing

In order to test the concentrations to be applied on the human dermal fibroblasts, dose response studies have been performed. Viability of the cells during a period of 48 hours in the presence and absence of the antioxidant matrix has been assessed as read-out.

Cytotoxicity of the antioxidant matrix was evaluated using the MTT colorimetric assay according to Mosmann as described earlier.[2,3] MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), the classical tetrazolium salt, turns bright blue and may form an insoluble precipitate when reduced in a cell either enzymatically or through direct reaction with NADH or NADPH. So, loss of coloring indicates diminished cell metabolism and increased toxicity.

Human dermal fibroblasts were seeded in 96-well plates at 3.000 cells/200 µl in each well. The next day the cells were treated with the substances of interest for 48 hours. After 45 hours the medium was supplemented with 25 µl of MTT (2 mg/ml phosphate-buffered saline) and the mixture was incubated for another 3 hours. Finally, solutions were removed, formazan crystals were dissolved in 200 µl of Me2SO and absorption was measured using a microplate reader (Monochromator

TABLE 1.

Berrimatrix Concentrations Studied in the Infrared Protection Assay	
Vol %	ppm
0.001%	10
0.005%	50
0.01%	100

Infinite 200, Tecan, Crailsheim, Germany) at 540 nm. Viability was calculated as percentage of control cells. Those cells were only exposed to cell culture medium and set to 100%.[4]

In-vitro Irradiation

Primary human dermal fibroblasts were exposed to a dose of 360 J/cm^2 infrared A radiation which previously was found to be optimal to induce gene expression without affecting viability in this cell type.[1] In brief, medium was replaced by PBS, lids were removed, and cells were exposed to infrared A radiation using a Hydrosun 500H IRA device (Hydrosun Medizintechnik GmbH, Müllheim, Germany). The IRA device was water-filtered and equipped with a black filter and emits wavelengths between 760 and 1400nm leading to an irradiance of 360 mW/cm^2 at a distance of 20 cm measured through a Hydrosun HBM1 irradiance measuring device (Hydrosun Medizintechnik GmbH, Müllheim, Germany). The culture dishes were placed on a cooled plate connected to a thermostated bath (Thermo Haake GmbH, Karlsruhe, Germany) to maintain temperatures below 37˚C during irradiation. Control cells were held on a 37°C thermostated plate under similar conditions without irradiation.

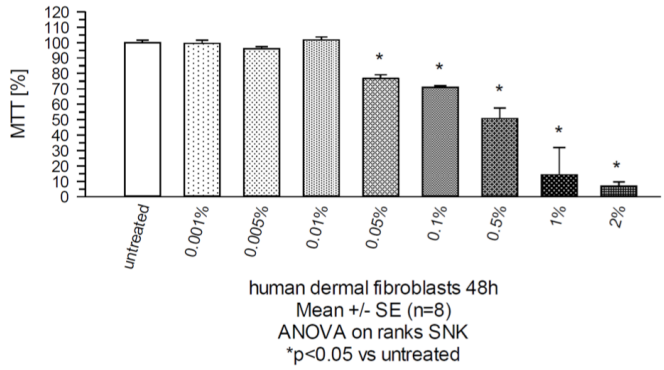
Application of Antioxidant Matrix

Cells were starved for 24 hours prior to irradiation (0% FCS) and the antioxidant matrix was added into the culture medium for 24 hours. Before starvation, before irradiation and before harvest, cells were washed once in PBS. During irradiation, the antioxidant matrix was not present (PBS incubation). After irradiation, the antioxidant matrix was added again by using a pipette to fresh media containing 2% FCS and was present until harvest of cells 24 hours post IRA treatment. The following 3 concentrations (Table 1) of the antioxidant matrix have been assessed based on the observations made during MTT assay (see Figure 1).

RNA Isolation and PCR

Total RNA was performed as described above.[2] For isolation of total RNA, we used RNeasy Total RNA Kits (Qiagen, Hilden; Germany). The RNA concentration was determined via photometric measurement at 260/280 (Biophotometer, Eppendorf, Hamburg, Germany). Aliquots of total RNA (100 ng) were applied for cDNA-Synthesis using SuperscriptIII First-Strand synthesis system for the reverse transcription step with random healers

FIGURE 1. Effect of Berrimatrix on viability of human dermal fibroblasts after 48 hours.



(Invitrogen, Karlsruhe, Germany). A specific primer pair was designed for each gene by Primer Express 2.0 software (Applied Bio systems, Darmstadt, Germany) based on the cDNA sequence published as indicated (Table 2).

Automated pipetting was performed by means of an epMotion 5070 system (Eppendorf, Wesseling-Berzdorf, Germany). The PCR reactions were carried out on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Munich, Germany) using Absolute QPCR SYBR Green Mix (Thermo Fisher Scientific, St. Leon-Rot, Germany). Three samples for each condition were processed with 2 determinations, each employing the universal protocol over 46 cycles, and the mean value of these was calculated. In detail, 15 minutes 94˚ C activation of hot start Taq Polymerase, 20 seconds 95˚ C denaturation, 20 seconds 55˚ C annealing, and 20 seconds 72˚ C extension. For comparison of relative expression in real time, PCR expression observed in untreated samples was arbitrarily set equal to 1 and the corresponding changes were calculated based on the 2 (-ΔΔC(T)) method.[3]

Statistics

The one-way analysis of variance on ranks SNK was used as a non-parametric test for comparison of differences between measurements and *P* values of less than 0.05 were considered significant in case of viability testing. ANOVA SNK was performed, if data passed normality and equal variance check, and *P* values of less than 0.01 were considered statistically significant (SigmaPlot 12.5).

TABLE 2.

Real-time Primers		
Marker	Sequence	Reference
18S rRNA	5´-GCCGCTAGAGGTGAAATTCTTG-3´ 5´-CATTCTTGGCAAATGCTTTCG´-3´	X03205
MMP-1	5´-ATGAAAGGTGGACCAACAATTT-3´ 5´-CCAAGAGAATGGCCGAGTTC-3´	NM_002421.3

TABLE 3.

Inhibition of the IRA Response by Percentage				
Concentration of Berrimatrix	**- IRA Mean±SE**	**+ IRA Mean±SE**	**Remaining MMP1**	**% Inhibition**
0%	1.00±0.00	6.1±0.31	100%	-
0.001%	2.20±0.06	3.30±0.21	45%	55%
0.005%	1.80±0.00	3.67±0.12	52%	48%
0.01%	1.40±0.00	2.63±0.13	32%	68%

RESULTS

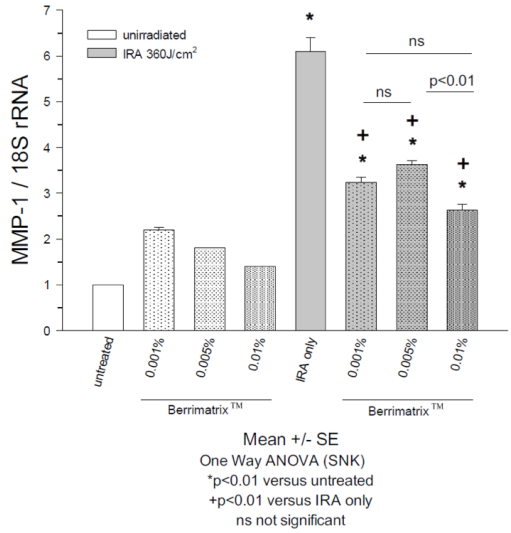
The effect of the antioxidant matrix on viability of human dermal fibroblasts after 48 hours is depicted in Figure 1. Viability of untreated cells has been set equal to 100% and the corresponding effect of treatment with the antioxidant matrix is expressed in relation to that.

As the lowest concentrations assessed eg, 0.001%, 0.005%, and 0.01%, did not affect viability of human dermal fibroblasts after 48 hours below a range of 80%, the named concentrations have been used for testing the ability of Berrimatrix to protect from IRA-induced up-regulation of MMP1.

Next, the ability of the antioxidant matrix to protect from infrared A induced up-regulation of MMP1 was studied (Figure 2). Infrared A irradiation in a dose of 360J/cm^2 significantly induced MMP1 up-regulation by 6.1±0.31 (mean±SE). The application of the antioxidant matrix alone did not significantly affect basal MMP1 expression (ANOVA on ranks SNK, P<0.05). The fact that the basal MMP1 expression of Berrimatrix-treated fibroblast followed an inverse dose-dependency might be explained by the antioxidants included in the blueberry extract itself or the additional compounds included in this antioxidant matrix. Accordingly, antioxidants such as ascorbate, but also flavonoids and many polyphenols, can undergo rapid oxidation in cell culture media resulting in formation of H_2O_2 and other reactive oxygen species.[7] The latter may lead to up-regulation of genes also modulated by reactive oxygen species. The cell culture medium itself does not promote further oxidation because no iron is included.

Pre-incubation with the antioxidant matrix prior to infrared A irradiation (IRA) resulted in a significant decrease of MMP1 expression by all three doses selected. The highest dose of the antioxidant matrix employed (0.01%) inhibited IRA-induced MMP1 up-regulation by 68%. Lower doses such as 0.001% decreased MMP1 up-regulation by 55%. And 0.005% Berrimatrix resulted in an inhibition of 48%.

FIGURE 2. Effect of various concentrations of Berrimatrix on IRA-induced MMP1 expression in human dermal fibroblasts.



CONCLUSION

A dose of 0.01% Berrimatrix was able to inhibit IRA-induced MMP-1 expression by 68%, while lower concentrations resulted in inhibition of 48% for 0.005% and 55% for 0.001%.

DISCLOSURES

This study was funded by Airelle Skincare, LLC. Katherine Wilkens and Kasey D'Amato are employed by Airelle Skincare, LLC. Dr Krutmann has served as a consultant for Airelle Skincare, LLC and Dr Krutmann received honoraria for work on this supplement.

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