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The Combined Effect of Lumenato and Ceramide in the Protection of Collagen Damage Induced by Neutrophils in Normal Human Dermal Fibroblasts

Yulia Solomonov, Rachel Levy*

Immunology and Infectious Diseases Laboratory, Department of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of The Negev, Beer-Sheva, Israel
Email: *ral@bgu.ac.il

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Abstract

Introduction: Collagen is the primary structural protein fibroblasts produce in the skin's extracellular matrix. Infiltration of neutrophils into the epidermis and dermis by exposure to UV causes collagen damage and contributes to photoaging. **Methods**: To study the combined effect of Lumenato and ceramide in preventing collagen-1 damage induced by phagocytes, we used co-cultures of normal human dermal fibroblasts (fibroblasts) and activated human neutrophils. The present study aimed to determine the protective effect of the combination of Lumenato and ceramide on fibroblast collagen-1 damage induced by neutrophils. **Results:** Lumenato (in the range of 6.5 - 208 μg/ml) or ceramide (in the range of 0.1 - 50 μM) inhibited the production of superoxides and MPO by TNF*α*-stimulated neutrophils, as well as the production of NO by LPS-stimulated macrophages in a dose-dependent manner. The combinations of Lumenato and ceramide, in low concentrations, caused synergistic prevention of fibroblasts' collagen-1 damage induced by TNF*α*-activated neutrophils, detected by fluorescence immunostaining and WB analysis. MPO activity in the supernatants of the co-cultures was also synergistically inhibited. Adding Lumenato or ceramide singly or in combinations in these low concentrations to the fibroblast cultures did not affect the expression of collagen-1. The combinations of Lumenato or ceramide in these concentrations also caused a synergistic inhibition of NO production by activated macrophages. **Conclusions**: The results suggest that combining low concentrations of Lumenato and ceramide results in synergistic protection against fibroblasts' collagen-1 damage induced by neutrophils, thus indicating their possible potential for enhanced skin health.

Keywords

Dermal Fibroblasts, Neutrophils, Collagen-1, Lumenato, Ceramide

1. Introduction

Dermal collagen represents the most abundant extracellular matrix (ECM) protein in human skin [1] produced, organized, and maintained by fibroblasts [2]. Collagen-1 is the main constituent of the dermal extracellular matrix, composing approximately 85% - 90% of the total collagen in the skin and playing a significant role in skin elasticity [3]. Its synthesis is reduced in aged and photodamaged skin, contributing to the wrinkled appearance of the skin [4]. It was shown that inflammation and accumulation of *reactive oxygen species induced* aged and photodamaged skin [4] [5]. Several studies have linked the immune system and inflammation genes with photoaging [6]. UV induces different events that can cause inflammation, such as the release of inflammatory cytokines [7], the release of reactive oxygen species (ROS) [8], the production of inflammatory mediators [9], membrane lipids peroxidation [10], and skin cell death [11]. Exposure to natural sunlight of UVB and solar simulating radiation (SSR), infrared radiation, and heat caused infiltration of neutrophils to the skin within the epidermis and dermis [12] [13] [14]. These neutrophils contain potent proteolytic enzymes for the clearance of UV-induced apoptotic cells and for killing skin cells with oxidized surface lipids, but they can also degrade collagen and elastic fibers [15]. Collagen is the only protein susceptible to fragmentation by superoxide anion, as demonstrated by the liberation of small 4-hydroxyproline-containing peptides. Hydroxyl radicals in the presence of oxygen or hypochlorous acid cleave collagen into small peptides, and the cleavage seems to be specific to proline or 4-hydroxyproline residues [16]. In our previous study [17], we have reported that Lumenato protected from fibroblasts' collagen damage induced by activated neutrophils in the co-cultures of fibroblasts and activated neutrophils. The present study aimed to use this co-culture model and to determine whether combinations of Lumenato and ceramide in low concentrations can cause synergistic protection of fibroblasts collagen-1 damage induced by activated neutrophils.

2. Materials and Methods

2.1. Macrophage Isolation and Culture

Peritoneal macrophages were collected from the peritoneal cavity of 6 - 8 week-old male ICR mice (Harlan, Israel) following an intraperitoneal injection of 1.5 ml of thioglycollate broth (4%) 4 days before harvest as previously described [18]. Peritoneal macrophages were washed three times with PBS, and hypotonic lysis of erythrocytes was performed to yield a highly enriched (90% - 95%) macrophage cell population. Peritoneal macrophages (1×10^6 cells/well) were cul-

tured in 96-well plates at 37˚C in 5% CO_2 atmosphere in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine; 100 U/ml penicillin; 100 μg/ml streptomycin (Beit-Haemek, Israel). Cells were stimulated with 200 ng/ml LPS from *Salmonella enterica serotype Typhimurium*. The study was performed following approval by the Ben-Gurion University of the Negev committee for ethical care and use of animals in experiments, Authorization No. IL-33-06-2020 and was conducted according to the Israeli Animal Welfare Act following the Guide for Care and Use of Laboratory Animal (National Research Council, 1996).

2.2. Neutrophil Purification

Thirty ml blood with neutrophil count between 3 - 7 × 10^6/ml was drawn from healthy volunteers with their written contest. Neutrophils at 95% purity were obtained by Ficoll/Histopaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes [19]. Cells were counted, and their viability was determined by trypan blue exclusion. The study was approved by the institutional Human Research Committee of the Soroka University Medical Center (No. 0370-16-SOR). Neutrophils were stimulated with TNFα (Peprotech, Rocky Hill, NJ, USA).

2.3. Ingredients

Lumenato, composed of 0.55% lycopene, 24.06% phytoene, 6.75% phytofluene, 11.27% tocopherols, 0.61% b-carotene, 3.15% phytosterols, 7.48% zeta-carotene, gamma carotene 0.16% and tomato seed oil was supplied by LycoRed Natural Products Industries Ltd. (Beer-Sheva, Israel). C2-Ceramide (d18:1/2:0) was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Working concentrations of phytonutrients were prepared from the stock solution dissolved in DMSO (in a final concentration of 5 μM for ceramide and 200 μg/ml for Lumenato) by adding appropriate volumes to a warmed culture medium.

2.4. Fibroblasts Cell Culture

Normal human dermal fibroblasts (NHDF) of adult donors (Promocell, Heidelberg, Germany) were cultured in Fibroblast Growth Medium-2 (Promocell) supplemented to final concentration in the medium of fetal calf serum 2%, 1 mg/ml basic fibroblast recombinant human growth factor, 5 mg/ml recombinant human insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Beit-Haemek, Israel). The cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. When fibroblasts reached around 80% confluence, the cells were seeded in 24-well plates.

2.5. Cell Survival

Cell viability was assessed by cell count using trypan blue exclusion or the colorimetric MTT ([3-4, 5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium) metabolic activity assay, as done before [17]. The MTT (0.5 mg/ml) was dissolved in a medium and added to each sample in an amount equal to 10% of the culture me-

dium volume. After incubation for 30 min, the formazan crystals were dissolved in 100 mM HCl and 10% Triton X-100, all in isopropanol in an equal volume to the culture medium. The medium only served as a background. Absorbance intensity was measured by a Versamax Microplate Reader (Molecular Devices, Menlo Park, CA, USA) at 570 nm with a reference wavelength of 690 nm.

2.6. NO Production Assay

NO level in cell culture supernatant was determined by detecting nitrite levels using Griess reagent and sodium nitrite as a standard [18].

2.7. Immunofluorescence Analysis

For immunofluorescence detection, fibroblasts or co-cultures of fibroblasts and neutrophils were fixed with methanol at −20˚C for 3 min, followed by a wash in PBS. Fixed cells were incubated with anti-collagen-1 antibodies (Southern Biotech Birmingham, Alabama, USA) 1:500 in 5% BSA/PBS for 90 min at room temperature, as described before [20]. The cells were washed three times in PBS and incubated with Cy3 anti-mouse (1:100 in 5% BSA/PBS; Jackson Immuno Research Laboratories, Inc., PA, USA) for 60 min at room temperature. The cells were washed three times in PBS, and the nuclei were stained with DAPI. Then, a final wash was performed, and the cells were analyzed by fluorescence microscopy (Olympus, BX60, Hamburg, Germany). Fluorescence intensity for collagen I was determined using a CellProfiler Program.

2.8. Total Cell Lysates

Total cell lysates were prepared using 1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 25 mM NaF, 10 mM $ZnCl_2$, 1 mM PMSF, and 100 μM leupeptin as described earlier [18].

2.9. Immunoblot Analysis

Lysate protein (10 mg) was separated by electrophoresis on 7.5% polyacrylamide SDS gels. The resolved proteins were electrophoretically transferred to nitrocellulose and blocked in 5% milk in TBS (10 mM Tris, 135 mM NaCl, pH 7.4). Immunoblot determination was done as described before [18] using primary antibodies against Collagen-1 (Southern Biotech Birmingham, Alabama, USA) and against actin (MP Biomedicals, 9 Goddard Irvine, CA, USA) for overnight incubation at 4˚C and second antibody, peroxidase conjugated goat anti-goat or anti-mouse (Amersham Biosciences, Buckinghamshire, United Kingdom) for 1 hour at room temperature and developed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

2.10. Superoxide Generation

Horseradish peroxidase (HRP)-dependent oxidation of the highly sensitive fluorescent biosensor Amplex Red [17]. The oxidation of Amplex Red occurs outside the cells by HRP, which traps H_2O_2 as soon as it is generated by spontaneous

dismutation of O_2^-, the first product of NADPH oxidase. Resting and activated neutrophils (2×10^5/well) were suspended in KRPG buffer (phosphate buffer, 145 mM NaCl, 4.86 mM KCl, 1.22 mM $MgSO_4$, 5.5 mM D-glucose, 0.54 mM $CaCl_2$, pH 7.35) containing HRP (0.1 unit/ml) and Amplex Red (50 mM). Fluorescence was recorded using a microplate reader with 535 nm-excitation and 595 nm-emission wavelengths. Background fluorescence was measured in the absence of neutrophil cells.

2.11. Myeloperoxidase (MPO) Activity

100 µl of 37˚C O-dianisidine hydrochloride solution (1 mg O-dianisidine, 10 ml phosphate buffer pH 6.0 + 0.0015% H_2O_2) was added to 100 µl supernatant in a 96-well plate immediately before the optical density was followed by the change of absorbance at 450 nm at 2-min intervals on a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA).

2.12. Statistical Analysis

Data are presented as the mean ± SEM. Significant differences from control conditions were determined using either one- or two-way analysis of variance (ANOVA) followed by a posteriori Bonferroni's test for multiple comparisons provided by GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

The dose-response effect of Lumenato or ceramide on NO production assessed as NO secretion levels in culture supernatants of peritoneal macrophages was studied. Lumenato or ceramide was added to peritoneal macrophages 1 h before the addition of 200 ng/ml LPS and cultured for 24 h at 37˚C. The secreted NO was determined in the cell culture supernatants. Either Lumenato (in the range of 6.5 - 208 µg/ml) or ceramide (in the range of 0.01 - 50 µM that is equivalent to 0.0034 - 17 µg/ml) caused a dose-dependent inhibition of NO production (**Figure 1A**, **Figure 1C**), without affecting cell survival detected by MTT (**Figure 1B**, **Figure 1D**).

Similarly, the effect of Lumenato or ceramide in those ranges was studied on the activation of neutrophils. Lumenato or ceramide was added to neutrophils for 15 min at 37˚C.

˚C before activation by TNF*α* overnight, and MPO activity was determined in the supernatant (**Figure 2A**, **Figure 2C**). The effect of Lumenato or Ceramide on the production of short-lived superoxides was determined immediately after activation (**Figure 2B**, **Figure 2D**). As shown in **Figure 2**, adding Lumenato or Ceramide caused a dose-dependent inhibition of MPO release and superoxide production.

To study whether combinations of Lumenato and ceramide result in synergistic inhibition, Lumenato (6.5 or 13 µg/ml) or Ceramide (0.1, 0.5 or 1 µM - that is equivalent to 0.034, 0.17 or 0.34, respectively) were added singly or in combina-

tions to macrophages 1 h before addition of LPS overnight. The secreted NO was determined in the cell culture supernatants. These combinations caused a synergistic inhibition of NO production of about twice the additive effect (Figure 3).

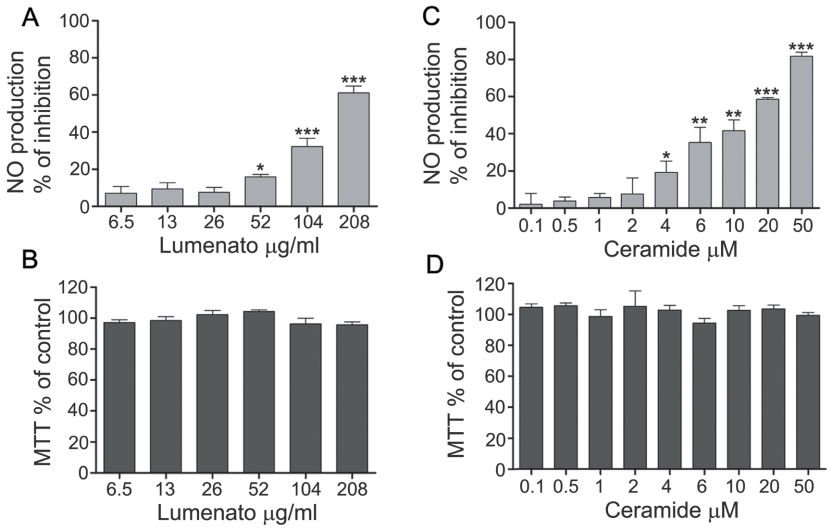

Figure 1. A dose-dependent inhibition of NO production by Lumenato or ceramide in macrophages. Preincubation of macrophages with Lumenato in a range of 6.5 - 208 μg/ml or ceramide in a range of 0.1 - 50 μM for 1 h before the addition of LPS caused a dose-dependent inhibition of NO production determined at 24 h in the cell supernatant. The results are expressed as % of inhibition and are the means ± SEM of 3 independent experiments, each done in triplicates. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, significant increase from the stimulated macrophages.

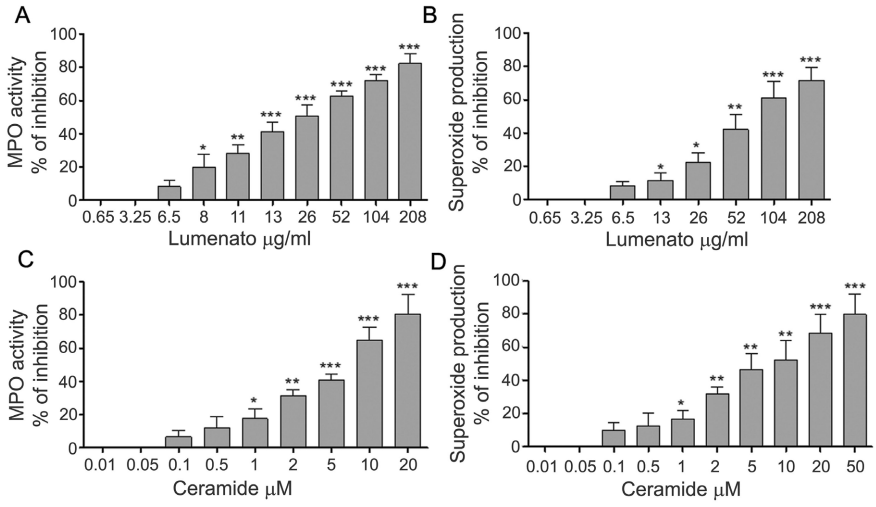

Figure 2. A dose-dependent inhibition of MPO secretion and superoxide production by Lumenato or ceramide in Neutrophils. Neutrophils were pre-incubated with Lumenato in a range of 0.65 - 208 μg/ml or ceramide in a range of 0.01 - 50 μM for 15 min before the addition of 100 ng/ml TNF*α*, and the production of MPO production was determined at 24 h in the cell supernatant (A, C). Superoxide production was measured immediately after the addition of TNF*α* (B, D). The results are expressed as % inhibition and are the means ± SEM of 3 independent experiments each in triplicates. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, significant increase from the stimulated neutrophils.

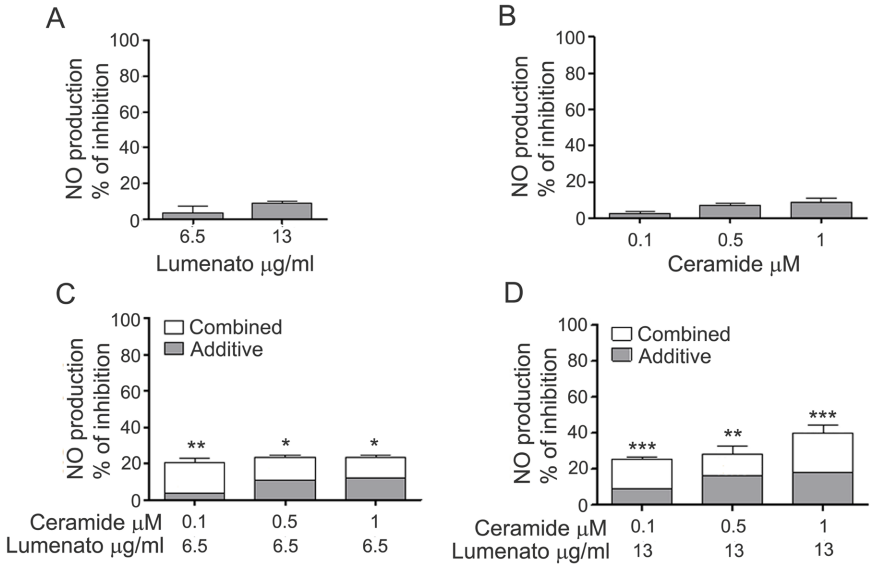

Figure 3. Synergistic inhibition of NO production by combination of Lumenato and ceramide. Macrophages were incubated for 1 h before the addition of LPS with Lumenato or ceramide individually or combined, and NO production was determined at 24 h. The results are expressed as % NO inhibition and are the means ± SEM of 3 independent experiments, each done in triplicate. The horizontal lines represent the calculated additive effect, the effect above this line represents synergistic inhibition. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, significant increase from the additive effect.

To study whether these combinations of Lumenato and ceramide may cause synergistic protection of the fibroblasts' collagen-1 damage induced by TNF*a*-activated neutrophils, we used the optimal conditions of co-cultures of fibroblasts and neutrophils that were determined in our earlier study [17]. Neutrophils were stimulated with TNF*a* since upregulation of this cytokine is an essential early response to sunlight and UVB by keratinocytes in the skin and represents an essential component of the inflammatory cascade in the skin [21]. During overnight, the short-lived neutrophils do not resist and thus do not interfere with collagen staining of the co-culture. For co-cultures, 1×10^5/ml fibroblasts were plated overnight to obtain confluent cultures. Lumenato, ceramide, or their combinations were added for 15 min to 2×10^5 neutrophils/ml before activation with 100 ng/ml TNF*a*. Activated neutrophils with and without the ingredients were added to the fibroblasts overnight. The addition of 2×10^5 activated neutrophils/ml with TNF*a* caused significant ($p < 0.001$) collagen-1 damage, as shown by immunofluorescence staining and densitometry, reducing collagen-1 from 53% ± 3.2% to 38.3% ± 1.9% without affecting fibroblast cell number (**Figure 4A**). The addition of 6.5 μg/ml Lumenato to fibroblasts did not protect from collagen-1 damage induced by activated neutrophils, as shown by immunofluorescence staining (**Figure 4A**). The presence of ceramide (0.1, 0.5, or 1 μM that is 0.034, 0.068 or 0.34 μg/ml, respectively) in the co-cultures caused low and similar protection of collagen-1 damage ($p < 0.05$) as shown by immunofluorescence staining and densitometry (**Figure 4B**). The effect of ceramide was better than that of Lumenato in protecting from collagen-1 damage since much

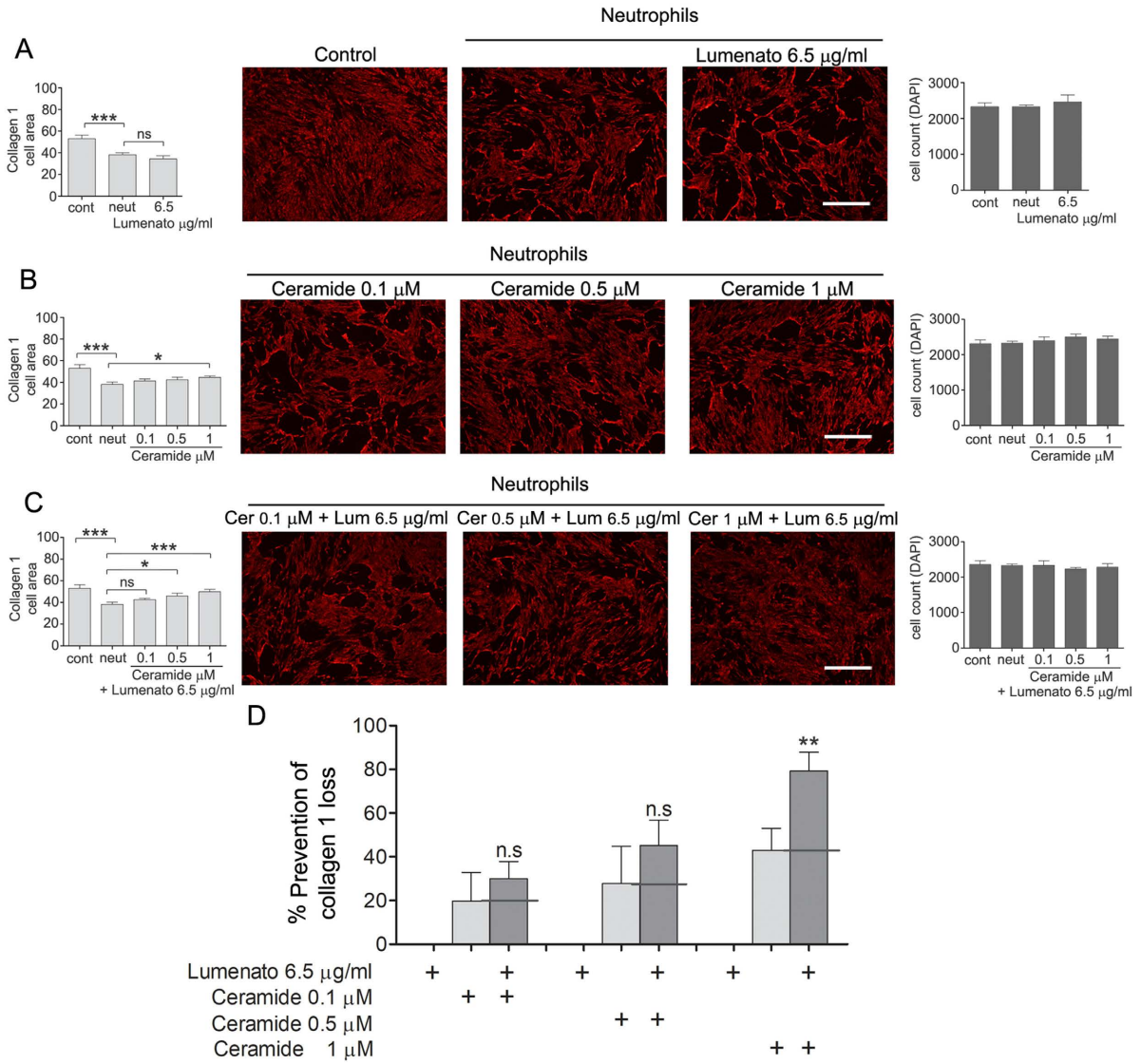

Figure 4. Synergistic prevention of collagen-1 damage by combinations of 6.5 μg/ml Lumenato and ceramide detected by immunofluorescence analysis. 1×10^5/ml fibroblasts were plated for 24 h to obtain confluent cultures before the addition of neutrophils. Neutrophils 5×10^5/ml in growth medium were incubated with 6.5 μg/ml Lumenato or with ceramide 0.1, 0.5, or 1 μM and their combinations for 15 min at 37˚C before activation with 100 ng/ml TNF*α* for 15 min, and added to fibroblast cultures for overnight at 37˚C. Shown are representative immunofluorescence staining of collagen-1 in control, in the co-cultures with neutrophils, and in the co-cultures with neutrophils pre-incubated with Lumenato (A) in the co-cultures with neutrophils preincubated with different concentration of ceramide (B) and in the co-cultures with neutrophils preincubated with combinations of Lumenato and ceramide (C). Magnification X40. The bar graphs on the left present the densitometry of the immunofluorescence staining collagen-1. Shown are the means ± SEM of three different experiments. In each experiment, ten fields of each treatment were scanned. The bar graphs on the right present cell count by DAPI staining in the cultures. Significance * - $p < 0.05$, *** - $p < 0.001$. D. The calculated % of prevention of collagen I damage after scanning the densitometry of the immunofluorescence staining collagen-1. The horizontal lines represent the calculated additive effect, the effect above this line represents synergistic inhibition. ** - $p < 0.01$, a significant increase from the additive effect.

higher concentration of Lumenato (6.8 μg/ml Lumenato versus 0.034 - 0.34 μg/ml ceramide) did not cause any protection. Combinations of Lumenato with each of the ceramide concentrations caused dose-dependent protection of collagen-1

damage as shown by immunofluorescence staining and densitometry (Figure 4C). In all cultures the cell count was not affected (Figure 4). The calculated percent of collagen-1 prevention loss by each ingredient and their combinations are presented in Figure 4D. The best and significant ($p < 0.01$) prevention of collagen-1 damage compared with the additive effect was achieved when 6.5 μg/ml Lumenato was combined with 1 μM Ceramide (79.3% ± 13.2% compared with 43%).

As shown in Figure 5, western blot analysis obtained similar results. The use of the immunoblot analysis technique showed a marked reduction in collagen-1 (of about 50%) by the addition of TNFα-activated neutrophils (Figure 5A). The presence of Lumenato singly caused a low of about 10% protection of collagen damage. The presence of ceramide (0.1, 0.5, or 1 μM that is 0.034, 0.068, and 0.34 μg/ml, respectively) in the co-cultures caused a higher protection of collagen-1 damage (35.9%, 37.8% and 46.6%, respectively). The significant ($p < 0.001$) synergistic calculated prevention of collagen-1 loss compared with the additive effect was obtained with the combination of 6.5 μg/ml Lumenato and 1 μM Ceramide 96% ± 10.8% compared with 46.4% (Figure 5B).

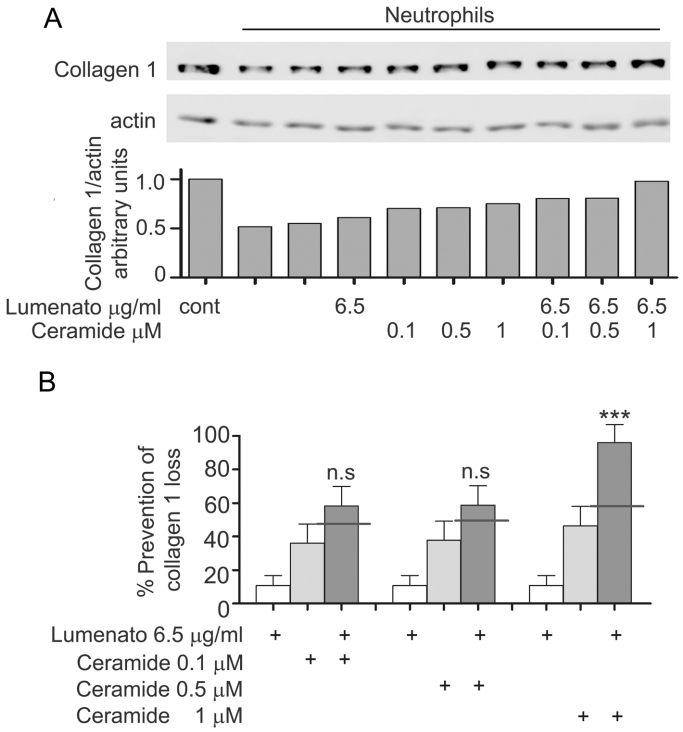

Figure 5. Synergistic prevention of collagen-1 damage by combinations of 6.5 μg/ml Lumenato and ceramide detected by western blot analysis. The experiments were performed as described in Figure 4. (A) Representative western blot analysis of collagen-I and actin in cell lysates under the different treatments at 24 h of incubation. The intensity of each band was divided by the intensity of each actin band after quantitation by densitometry and expressed as arbitrary units in the bar graph. (B) The calculated % of prevention of collagen-I damages. Shown are the mean ± SEM of 3 different experiments. The horizontal lines represent the calculated additive effect, the effect above this line represents synergistic inhibition. *** - $p < 0.001$, a significant increase from the additive effect.

The effect of 13 μg/ml Lumenato was also studied in the same experiments described in Figure 4 and Figure 5. Its addition to the neutrophils before their addition to fibroblast caused a low and non-significant protection of collagen damage (Figure 6A). The presence of the different doses of ceramide caused a

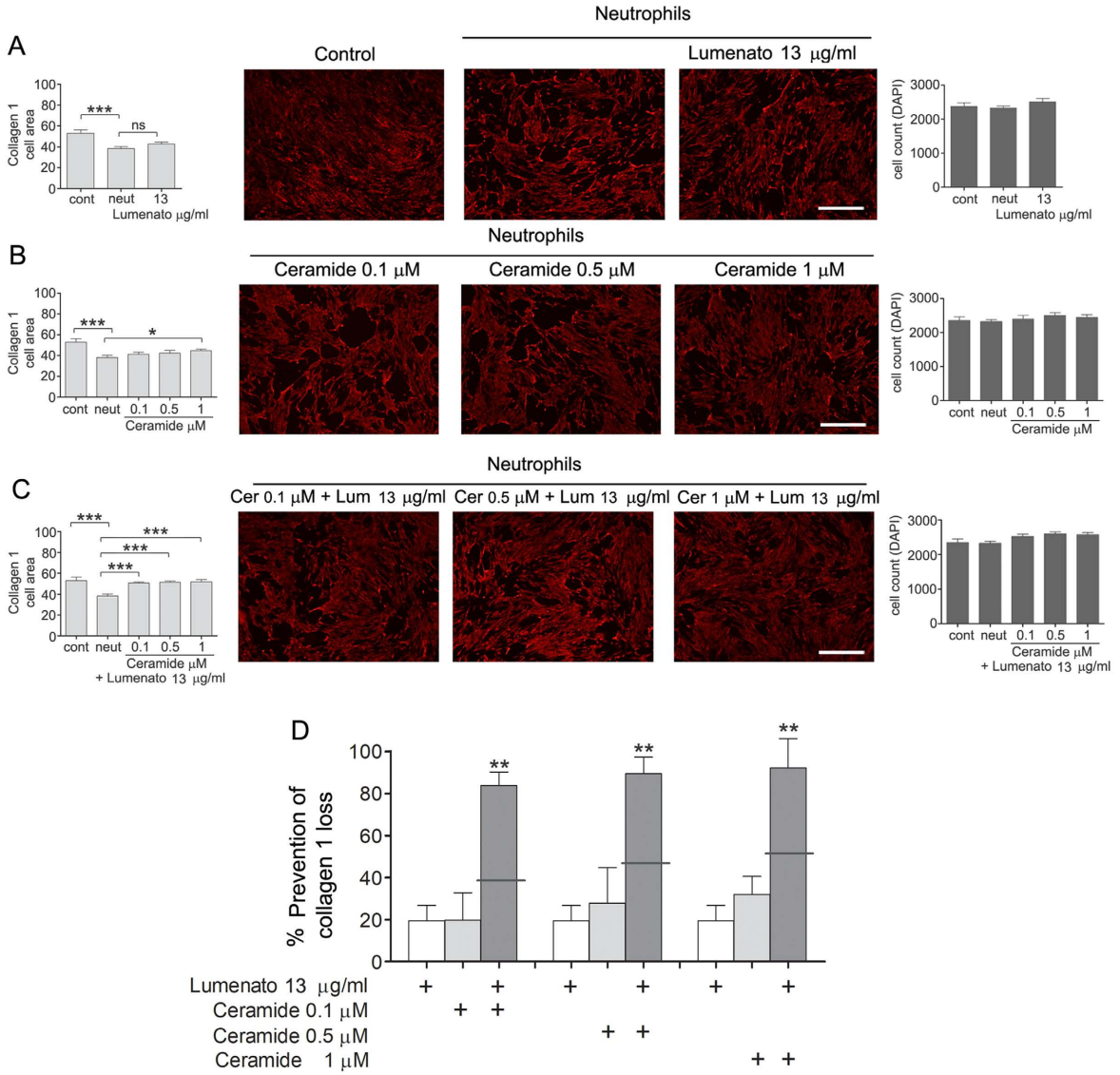

Figure 6. Synergistic prevention of collagen-1 damage by combinations of 13 μg/ml Lumenato and ceramide detected by immunofluorescence analysis. The experiments were performed as described in Figure 4 using 13 μg/ml Lumenato. Shown are representative immunofluorescence staining of collagen-1 in control, in the co-culture with neutrophils, and the co-culture with neutrophils pre-incubated with Lumenato (A) in the co-cultures with neutrophils pre-incubated with different concentrations of ceramide (B) and in the co-cultures with neutrophils preincubated with different with combinations of Lumenato and ceramide (C) Magnification X40. The bar graphs on the left present the densitometry of the immunofluorescence staining collagen-1. In each experiment, ten fields of each treatment were scanned. The bar graphs on the right present cell count by DAPI staining in the cultures. Shown are the means ± SEM of three different experiments. In each experiment, ten fields of each treatment were scanned. * - $p < 0.05$, *** - $p < 0.001$. (D) The calculated % of prevention of collagen-1 damages after scanning the densitometry of the immunofluorescence staining of collagen-1. The horizontal lines represent the calculated additive effect, the effect above this line represents synergistic inhibition ** - $p < 0.01$, a significant increase from the additive effect.

low protection of collagen loss (Figure 6B, same as presented in Figure 4B) that was similar to that caused by Lumenato. Here again, the effect of ceramide is better than that of Lumenato in protecting from collagen-1 damage since a much higher concentration of Lumenato (13.8 μg/ml Lumenato versus 0.034 or 0.064 μg/ml ceramide) caused similar protection to that of ceramide. 0.34 μg/ml ceramide caused better protection than 13.8 μg/ml Lumenato. The combination of 13 μg/ml Lumenato with the different ceramide concentrations caused a significant ($p < 0.001$) protection of collagen-1 loss in the co-cultures (Figure 6C). Cell number was not affected in all cultures. Figure 6D presents the calculated % prevention of collagen-1 loss by the combinations. There was a significant ($p < 0.01$) synergistic protection of the combinations of about twice the additive effect. A combination of 13 μg/ml Lumenato with 0.1 μM ceramide caused a synergistic effect of 83.7% ± 6.4% prevention of collagen-1 loss compared with the additive effect (39%), combination with 0.5 μM ceramide caused 89.4% ± 7.9% protection compared with the additive effect (47.7%) and combination with 1 μM ceramide caused 92.2% ± 13.2% protection compared with the additive effect (52%).

Similar results were obtained using western blot analysis, demonstrating significant synergistic ($p < 0.01$) protection of collagen-1 loss induced by activated neutrophils by the different combinations with ceramide (Figure 7). MPO activity measured in the supernatant of the co-cultures showed that in the combinations of Lumenato and ceramide, there was a synergistic activity in accordance with the synergistic protection of collagen damage induced by activated neutrophils (Figure 8). Addition of Lumenato or ceramide singly or in the combinations to the fibroblast cultures did not affect collagen-1 expression or cell number (Figure 9).

Using combinations of lower concentrations of Lumenato (5 times less and 10 times less: 3.25 μg/ml or 0.65 μg/ml) with 0.1 μM or with 0.01 μM ceramide showed that singly and in combinations had no effect on collagen loss by TNF*α*-activated neutrophils (not shown). Likewise, using a higher concentration of Lumenato (26 μg/ml) with ceramide (0.1 μM, 0.5 μM or 1 μM) resulted in inhibition of collagen loss that was similar or lower than their additive effect (not shown).

4. Discussion

In the present study, we show that collagen-1 damage in fibroblast cultures induced by activated neutrophils could be prevented by Lumenato or ceramide in a dose-dependent manner. Moreover, using combinations of low concentrations of Lumenato and ceramide resulted in significantly synergistic prevention of collagen-1 loss. In our previous study [17], we used 208 μg/ml Lumenato to achieve around 80% prevention of collagen loss induced by neutrophils. In the present study, we used much lower concentrations of Lumenato (6.5 μg/ml or 13 μg/ml) and of ceramide (0.1 - 1 μM that is 0.034 - 0.34 μg/ml), each showing no or low protection of collagen-1 damage, but in combinations reached a synergistic

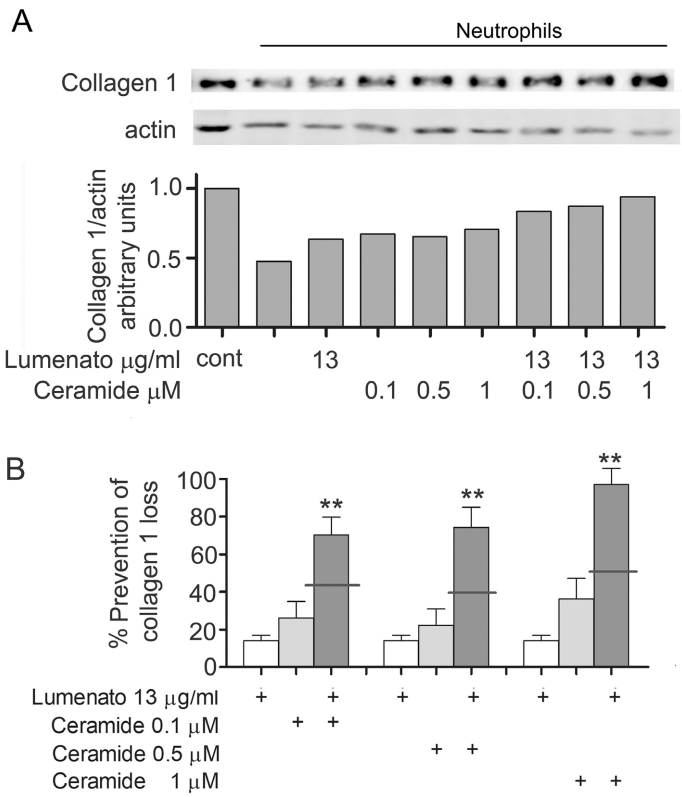

Figure 7. Synergistic prevention of collagen I damage by combinations of 13 μg/ml Lumenato and ceramide detected by western blot analysis. The experiments were performed as described in **Figure 4** using 13 μg/ml Lumenato. (A) A representative western blot analysis of collagen I and actin in cell lysates under the different treatments at 24 h of incubation. The intensity of each band was divided by the intensity of each actin band after quantitation by densitometry and expressed as arbitrary units in the bar graph. (B) The calculated % of protection of collagen I damage. Shown are the mean ± SEM of 3 different experiments. The horizontal lines represent the calculated additive effect, the effect above this line represents synergistic inhibition. ** - $p < 0.01$, a significant increase from the additive effect.

protection against collagen-1 damage that was higher than 80% protection. Ceramide is much more potent than Lumenato in inhibiting phagocyte function and protecting collagen-1 damage induced by activated neutrophils as shown by both immunofluorescence analysis and western blot analysis and thus its tiny amount is sufficient to cause synergistic protection of collagen-1 damage. These results are of high importance since elevated plasma ceramide levels may cause a higher risk of CVD [22]. The effect of Lumenato and ceramide is probably by inhibiting neutrophil activation and not by increasing collagen-1 synthesis, as their addition singly or in combinations to fibroblast cultures did not affect collagen-1 expression or cell count. This suggestion is supported by the dose-dependent inhibition of superoxide production and MPO release by each of these ingredients in stimulated neutrophils and the inhibition of MPO activity detected in the supernatant of the co-cultures. Moreover, in our earlier study [17], we showed that damage to collagen required the presence of neutrophils since

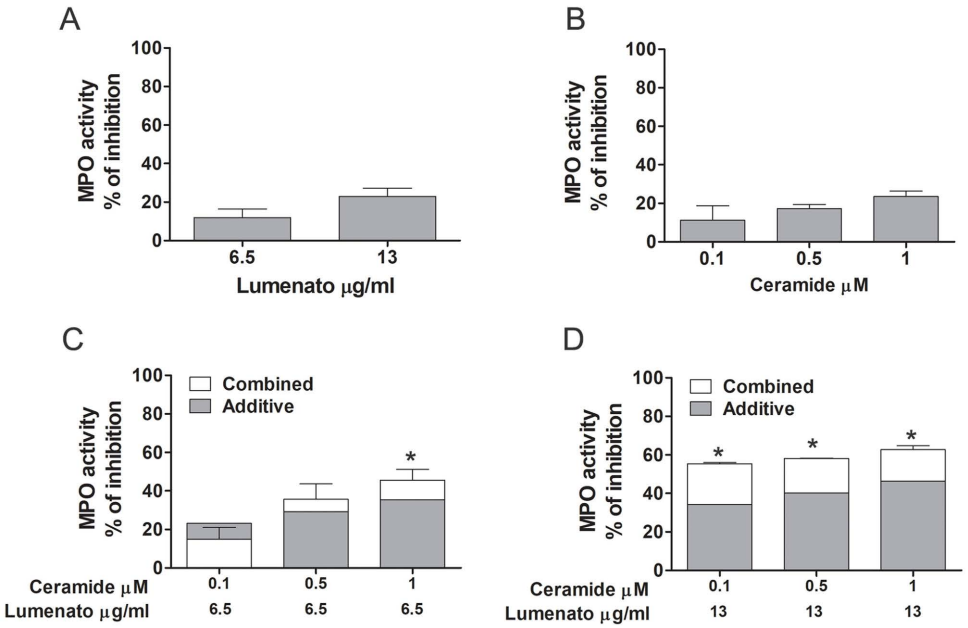

Figure 8. Synergistic inhibition of MPO secretion by combinations of Lumenato and ceramide in the co-cultures. MPO secretion in the supernatant of the co-culture experiments that are described in **Figure 4** and **Figure 6**. Average ± SEM of the experiments. The horizontal lines represent the calculated additive effect, the effect above this line represents synergistic inhibition. Significance: * $p < 0.05$, a significant increase from the additive effect.

their supernatant did not cause any damage, suggesting the crucial role of short-lived ROS in the damage. In accordance with our results, it was reported that C2-ceramides, as used in our study, are potent inhibitors of superoxides release in stimulated neutrophils and their effect is structurally specific [23] [24]. The importance of neutrophils in collagen degradation was reported in *in vivo* studies demonstrating that neutrophil depletion blocked collagen degradation in mouse liver, while the addition of neutrophils to corneal fibroblast did cause collagen degradation [25] [26]. Protection of external collagen damage is critical since collagen is one of the most essential proteins in vertebrates and represents one-third of the human body's total protein. Collagen type 1 is the most critical since it constitutes 90% of the collagen present in our organism and is located primarily in the skin, where it is the main structural component of the extracellular matrix (ECM) of the dermis [27]. With age, there is a progressive decrease in collagen production associated with an increase in its degradation, thus contributing to the wrinkled appearance of the skin [28].

The present study also shows that ceramide or Lumenato inhibited NO production by LPS-stimulated macrophages in a dose-dependent manner, which is in accordance with the literature [29]. We also show herein that the combinations of Lumenato with ceramide, in low concentrations, caused a synergistic inhibition of NO production by stimulated macrophages. Even though it has been demonstrated that neutrophils play a significant role in collagen damage following exposure to natural sunlight or UVB, macrophages present in the skin are also activated under these conditions [30] and thus may contribute to collagen damage.

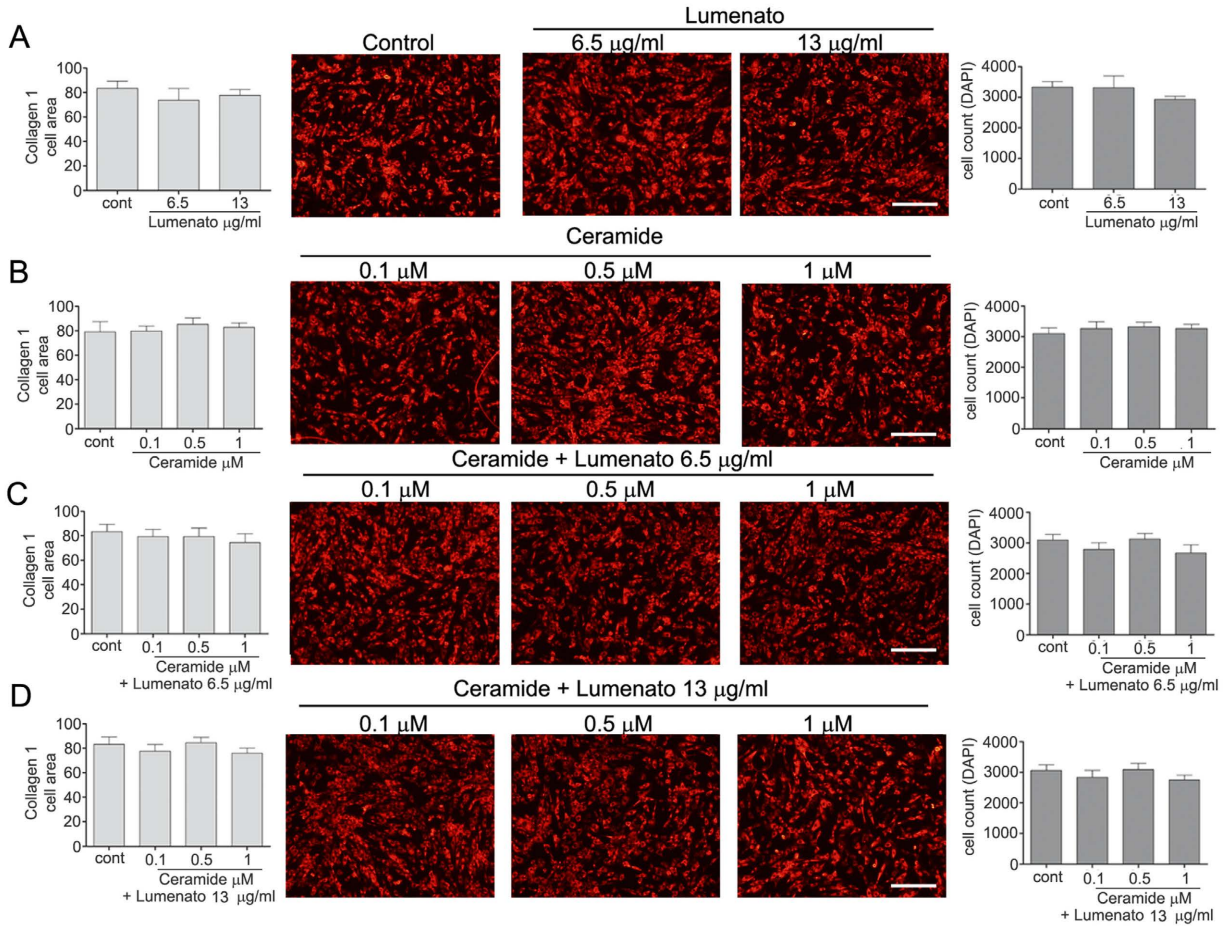

Figure 9. The addition of Lumenato or ceramide and their combinations did not affect collagen-1 expression. Lumenato or ceramide and their combinations were added to fibroblasts plated in concentration of 5×10^4 cells/ml overnight. Shown representative immunofluorescence staining, magnification X40. The bar graphs on the left present the densitometry of the immunofluorescence staining of collagen-1 in the different cultures. In each experiment, ten fields of each treatment were scanned. The bar graphs on the right present cell count in the cultures by DAPI staining. Shown are the means ± SEM of three different experiments.

Ceramides are the main lipid component of lamellar sheets in the intercellular spaces of the stratum corneum. These lamellar sheets provide the barrier property of the epidermis. The intercellular lipid domain is composed of free fatty acids, cholesterol, and ceramides in equimolar concentrations [31], and ceramides play a main role in structuring and maintaining the water permeability barrier function of the skin. It has been shown that with age, there are changes in ceramides and total stratum corneum lipid content, and there is an overall decrease in total stratum corneum lipids of approximately 30% in older people [32]. The short-chain ceramides, such as C2-ceramide used in our study, were reported to act also as intracellular messengers of the sphingomyelin cycle that activates protein kinase C- and stress-activated protein kinases (c-jun N-terminal kinase (JNK) and p38), to induce apoptosis, oxidative stress and epidermal differentiation [33]-[38]. It was further reported that ceramide inhibited the production of IL-1 and IL-6 and prevented cell death induced by TNFα [39]. The

present study shows that C2-ceramide blocks ROS release from neutrophils and prevents collagen-1 damage caused by activated neutrophils. Thus, it may have valuable potential in protecting against collagen damage following exposure to natural sunlight, erythemogenic dose of UVB, or solar stimulating radiation that induces ROS release by infiltrating neutrophils into the skin [12].

The mechanism behind the synergistic protection from collagen-1 damage induced by stimulated neutrophils in the presence of combinations of Lumenato consisting of more than 30% carotenoids (24.06% phytoene and 6.75% phytofluene) with C2-ceramide as shown in the present study, may be attributed to their different physicochemical properties and, or their preferential location in the neutrophil membranes. Carotenoids are found in specific locations and orientations in the membranes and subcellular structures, and their chemical and physical properties are strongly influenced by other molecules in their vicinity, especially proteins and membrane lipids, and in turn, they influence the properties of the membranes and the subcellular structures [40]. Phytoene and phytofluene lacking hydrophilic substituents, probably remain entirely within the inner part of the membrane and affect the motional freedom of lipid polar head groups [40] [41]. The presence of the permeable C2-ceramide probably modifies the membrane structure. Since each Lumenato or C2-ceramide is shown to be an antioxidant, their mutual presence in a different compartment of the membrane may synergistically enhance each compound's scavenging of the immediate superoxide production and may provide shielding effects of MPO secretion, as shown in the present study. It was recently reported [42] that phytoene, phytofluene, and lycopene individually inhibited oxidative injury, ROS generation and proinflammatory cytokines, with higher degrees of inhibition by combined supplementation in a rat model.

5. Conclusion

The fibroblasts' collagen-1 damage caused by activated neutrophils in the co-cultures, could be prevented by either Lumenato or ceramide, and their combinations were highly effective in protecting the damage. Ceramide is much more potent than Lumenato in inhibiting phagocyte function and protecting collagen-1 damage induced by activated neutrophils and thus its use in low levels in the combinations with Lumenato is sufficient. The effect of combining Lumenato and ceramide is probably by inhibiting neutrophil activation and not by increasing collagen-1 synthesis, as their addition singly or in combinations to fibroblast cultures did not affect collagen-1 expression or cell count. The significant synergistic protection of fibroblasts' collagen-1 damage induced by activated neutrophils using combinations of low concentrations of Lumenato and ceramide, indicate their potential for protecting the skin from photoaging.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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List of Abbreviation

C2-ceramide—ceramide

TNFα—Tumor Necrosis Factor-alpha

Fibroblasts—Normal human dermal fibroblasts (NHDF)

HRP—Horseradish peroxidase

MPO—Myeloperoxidase

ROS—Reactive oxygen species

NO—Nitric oxide