Research Article

Inflamyar™ Possesses Anti-Inflammatory Effect and Induces Growth Factor Expression on Human Dermal Fibroblasts *In Vitro*

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Abstract

Introduction: Wound healing describes a highly complex process that is essential for protecting the human body from the harmful effects of the outside world after skin structure injury. The search for remedies that support wound healing is one of the oldest challenges in medicine. Since ancient times, herbal extracts have been studied for these purposes. Overall, however, the individual effects of active ingredients from plant extracts are very diverse and have so far been described insufficiently due to the enormous diversity of active substances and their processing, application form and dosage. In this study, the effect of Inflamyar™, a commercially available homeopathic spagyric product, on wound healing was evaluated.

Methods: Primary human dermal fibroblasts were pre-incubated with the test substance with/without a subsequent inflammatory stimulus (LPS). Consecutively, the expression of cytokines was assessed.

Results: In the absence of an inflammatory stimulus, an induced cytokine and growth factor expression was found in human dermal fibroblasts. In the presence of an inflammatory stimulus, most of the cytokines showed either no reaction or a reduction, especially at higher concentration levels of the tested substance. An increase in the anti-inflammatory cytokine IL-1ra and the growth factors bFGF, VEGF and GM-CSF were found at higher concentrations of the tested substance.

Conclusions: The test substance enhances normal immune surveillance of human dermal fibroblasts in absence of an inflammatory stimulus. Under inflammatory conditions, the data suggests a shift from an inflammative environment to a tissue regenerative environment after incubation with the test substance.

Keywords: Inflamyar™, Flamyar™, human dermal fibroblasts, Homeopathy, Spagyric

Abbreviations: FGF: Basic fibroblast growth factor; DMEM: Dulbecco´s Modified Eagle Medium; FBS: Fetal bovine serum; G-CSF: Granulocyte-colony stimulating factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; IFN: Interferon; IP-10: Interferon γ -induced protein 10; IL: Interleukin; JAK/STAT: Janus kinase/signal transducer and activator of transcription; LPS: lipopolysaccharide; MIP: Macrophage Inflammatory Protein; MCP-1: Monocyte chemoattractant protein 1; PDGF: Platelet-derived growth factor; RANTES: Regulated on activation, normal T cell expressed and secreted; TNF: Tumor necrosis factor; VEGF: Vascular Endothelial Growth Factor

Introduction

With a surface of 2 m2 and a weight of 10-12 kg, the skin is the largest human organ. In addition to the protection against external mechanical and physical influences, the skin also plays an important role as an active immune organ against the infestation of microorganisms [1]. In the case of a skin structure injury, wound healing and thus the reconstitution of the barrier function is of central importance. Wound healing is a highly complex, dynamic process in which a variety of different cellular and humoral components work

together in different phases. The beginning of wound healing is called the inflammatory or exudation phase. If the blood vessels are damaged, blood coagulation begins and a fibrin net, which serves as a matrix for the newly formed granulation tissue, forms. Neutrophil granulocytes migrate through the capillaries into the tissue, secreting cytokines and protein-degrading proteases. Immigrated macrophages eliminate microorganisms through phagocytosis and secrete growth factors and cytokines. These stimulate the proliferation and migration of fibroblasts and vascular endothelial cells into the wound bed. Capillaries sprout into the wound area and precursors of extracellular matrix are synthesized.

The final closure of the wound area is achieved by contraction of the wound area and by immigration of fibroblasts and keratinocytes into the wound bed [2]. The search for remedies that support wound healing is one of the oldest challenges in medicine. Since ancient times, the use of topically applied extracts from medicinal plants has been widespread throughout the world [3]. Many representatives of the plant kingdom show an enormous potential for the treatment of wounds [4,5]. Their natural active ingredients induce healing and regeneration of the injured tissue through different mechanisms [4]. These components include various groups of substances such as terpenoids, polyphenols, alkaloids and essential oils, polypeptides and lectins and other compounds [6].

Active ingredients of plant origin are not only inexpensive, but also have relatively low side effects compared to many synthetic substances [7,8]. This is not least due to the many years of experience in the use of these substances. Important factors for the effect of these substances are their exact composition, dose, processing and administration form. In particular, the dose of these drugs is an important factor in their effectiveness. This was already expressed in the 16th century by the alchemist and physician Paracelsus ("All things are poison and nothing is without poison; just the dose makes sure that a thing is not poison.") [9]. Thus, different plant compounds show a toxic effect at high concentrations and a healing effect at low concentrations. In fact, the relevant literature does not distinguish between poisonous plants or medicinal plants. Some studies have shown efficacy of plant extracts in different indications even at highly diluted concentrations [10-13]. Overall, the individual effects of active ingredients from plant extracts are very diverse and have so far been described only inadequately [14]. For this reason, further investigations are necessary.

In this study, the effect of Inflamyar™, a commercially available homeopathic spagyric product consisting of plant extracts from Arnica montana, Bryonia cretica, Guajacum, Toxicodendron quercifolium, Bellis perennis, Ledum palustre, Ruta graveolens and Viscum album, on wound healing was investigated *in vitro*.

Material and Methods

Test Substance

The test substance, Flamyar™, is a homeopathic spagyric natural remedy manufactured by PEKANA Naturheilmittel GmbH (Kißlegg, Germany) and distributed in the USA under the name Inflamyar™. The test substance was developed for the treatment of sports injuries, sprains, joint problems, bruises, and muscle strains. Active ingredients are Arnica montana spag. Peka Dil. D12, Bryonia cretica spag. Peka Dil. D4, Guajacum Dil. D4, Toxicodendron quercifolium Dil. D12, Bellis perennis spag. Peka Dil. D8, Ledum palustre Dil. D4, Ruta graveolens spag. Peka Dil. D6, Viscum album spag. Peka Dil. D4.

Cell Culture

Human dermal fibroblasts obtained from Zenbio (Durham, NC, USA) were cultivated in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS) and antibiotics (100ug/mL penicillin/streptomycin, Gibco, Life Technologies, Eugene, OR USA) with a density of 32,000 cells per well in 24-well plates. After 16 hours, cell culture medium was replaced by 900ul fresh medium plus 100ul of each treatment and cells cultivated for another 24 hours. After collection, the supernatants were analyzed for cytokine/growth factor quantitation using Luminex-based arrays. Treatments of cells with the test substance were tested in duplicate. Untreated and LPS alone cultures were tested in triplicate.

Cytokine Expression in Human Dermal Fibroblast Cell Cultures

Supernatants were harvested as described above. The expression levels of the following cytokines were tested: Interleukin (IL)-1β, -1ra, -2, -4, -5, -6, -7, -8, -9, -10, -12 (p70), -13, -15, -17, eotaxin, basic fibroblast growth factor (FGF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), Interferon γ (IFN-γ), interferon γ -induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). The assay was analyzed using magnetic protein multiplex arrays (BioPlex, Bio-Rad Laboratories Inc.) and xMAP technology (Luminex, Austin, TX, USA).

Statistical Analysis

Calculations and statistical analysis was performed using the two-tailed, independent t-test using Microsoft Excel.

Results

Primary human fibroblasts were used as a model for potential immune modulating activities. The direct effect on the one hand and the effect of the preincubation of fibroblasts with the test substance and the subsequent inflammatory stimulus (LPS) on the other hand were tested. The exposure to the test substance without a subsequent inflammatory stimulus (LPS) showed no effect on proinflammatory cytokine expression of IL-1β, IL-5, IL-13, IL-17A, MIP-1β, RANTES and TNF-α. An induction of proinflammatory cytokine expression was seen in IL-6 (27.03 to 36.37%), IL-8 (4.77 to 34.52%), IL-12p70 (43.87 to 80.88%), IP-10 (38.81 to 503.21%), Eotaxin (up to 85.85%), MCP-1 (208.36 to 346.56%) and MIP-1α (211.54 to 279.33%). Variable results were seen in IFNγ (27.05 to -51.03%). An induction of anti-inflammatory cytokines was seen in IL-1ra (6.47 to 20.86%) and, at higher concentrations of the test substance, in IL-10 expression (up to 25.54%).

Regarding expression of cytokines with pro- and anti-inflammatory activity, the treatment of the fibroblasts with the test substance resulted in an increased expression of IL-4 (up to 144.65%), IL-7 (32.52 to 169.92%) and IL-9 (136.11 to 284.32%) and IL-2 (up to 49.66%). No changes were seen in expression of IL-15. Moreover, an induction of the growth factor VEGF was assessed (15.27 to 31.04%), whereas PDGF-BB showed an induction at higher concentrations (up to 65.2%). Expression of bFGF, G-CSF and GM-CSF showed no changes.

The exposure of fibroblasts to the test substance with a subsequent inflammatory stimulus (LPS) led to consistent decreases of proinflammatory IFNγ (-7.31% to 21.92%), IL-1β (up to -12.95%), IL-13 (-15.20 to -39.61%), Eotaxin (-4.82 to 19.31%) and TNF-α (5.15 to -15.31%). No effects were seen on IP-10 or MCP-1 levels, whereas IL-17A (0.98 to 10.10%) and RANTES (15.38 to 34.50%) showed an induction of expression. IL-6 (-19.92 to 2.33%), IL-12p70 (-12.08 to 20.21%), MIP-1α (-21.28 to 11.45%) and MIP-1β (-22.08 to 8.38%) possessed biphasic reactions. Variable effects were measured in the expression of IL-5 (-32.66 to 32.66%) and IL-8 (-21.51 to 8.27%).

A decreased expression of anti-inflammatory cytokine IL-10 (-38.51 to 0.87%) as well as cytokines with pro- and anti-inflammatory activity [IL-2 (up to -35.44%), IL-4 (12.35 to 1.54%), IL-7 (-2.52 to -22.57%), IL-9 (-1.52 to 14.56%), IL-15 (-6.49 to 17.55%)] were also detected. A biphasic expression was seen for IL-1ra (10.52 to 7.81%). Data from growth factor analysis showed no effect on PDGF-BB and biphasic effects on bFGF (-8.45 to 21.92%) and GM-CSF (-48.31 to 23.48%) expression. G-CSF expression was assessed on a decreasing level (-13.69 to -59.03%) whereas VEGF (-13.73 to 16.27%) possessed an induction of expression.

Discussion

The analysis of cytokine expression after treatment of human dermal fibroblasts without a subsequent inflammatory stimulus possessed, except expression of IFN-γ, either no effect or an increased expression of cytokines and growth factors. Increased cytokines of the chemokine group, e.g. IL-8, MCP-1, MIP-1α, IP-10 and Eotaxin, were described to be involved in important aspects of early stages of wound healing [15,16] and re-epithelialization [17]. An up-regulation of anti- and pro- inflammatory cytokines (IL-6, -12p70, -7, -9, -1ra and -10) by topical application of natural products has been suggested to enhance normal immune surveillance of skin tissue and maintenance of homeostasis [18]. Interestingly, the treatment of skin cells with all four concentrations of the test substance resulted in a 20-30% increase in VEGF production. This regenerative growth factor has a role in tissue repair through its function as an angiogenic factor [19] and topical application of VEGF in a diabetic mouse model has been shown to promote wound healing [20]. Moreover, an induction of PDGF-BB was seen after cell stimulation with higher doses of the test substance. PDGF-BB also plays an important role in the late stage of wound healing [21].

In presence of inflammatory stimuli, most of the cytokines tested showed either no reaction or a reduction, especially at higher test substance concentrations. Interestingly, at higher doses of the test substance an increased expression in the anti-inflammatory IL-1ra and the growth factors bFGF, VEGF and GM-CSF were found. The anti-inflammatory IL-10 was detected on a moderately reduced level compared to proinflammatory cytokine expression at higher test substance doses. The most important members of the down-regulated proinflammatory cytokines are key molecules in the induction of other proinflammatory proteins via Mitogen-activated Protein Kinase (MAPK) and Nuclear factor κβ (NFκβ) signaling pathways, promote the immune cells proliferation, activate immune cells and promote binding and immigration of immune cells into the tissue [22-25]. In contrast, the moderately reduced anti-inflammatory IL-10 is a major player in inflammatory processes and inhibits the proinflammatory cytokine expression via interaction with the NFκB and the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway [26]. IL-1ra inhibits the proinflammatory impact of IL1β and adjusts many IL-1 - related inflammatory responses [27].

The growth factors bFGF, VEGF and GM-CSF have an important function and are upregulated in late stages of wound healing like angiogenesis [28], reepithelialization [29], granulation tissue formation [30,31], matrix formation and remodeling [32]. This expression pattern indicates a shift from an inflammative environment to a tissue regenerative environment after incubation

with the test substance under inflammatory conditions. Compared to the direct influences of the test substance under normal culture conditions, the reaction under inflammatory conditions is very different and suggests different mechanisms of action under these two different conditions. Overall, the test substance shows an anti-inflammatory effect on human skin cells under inflammatory conditions and the current data suggest that tissue regeneration is promoted.
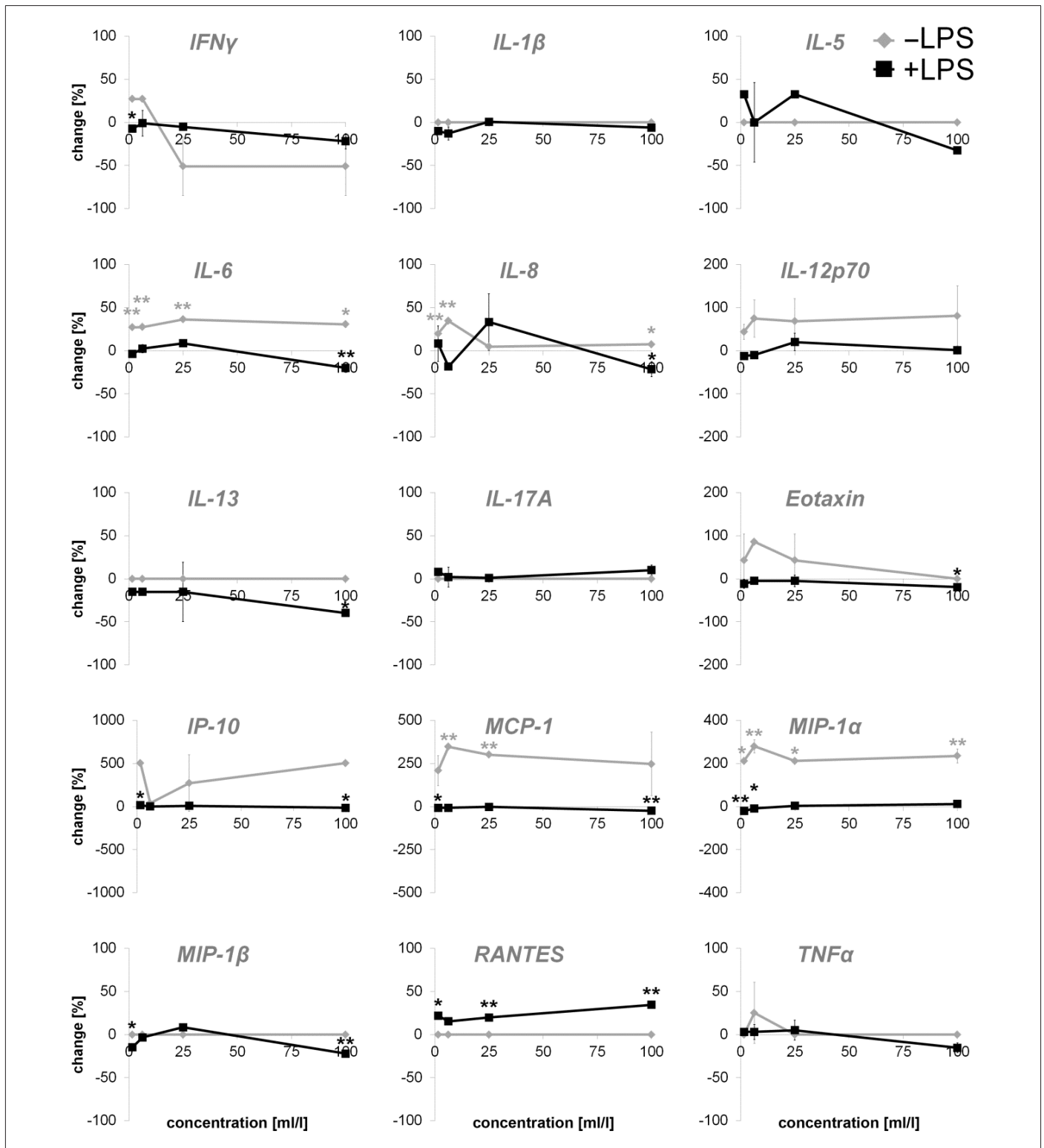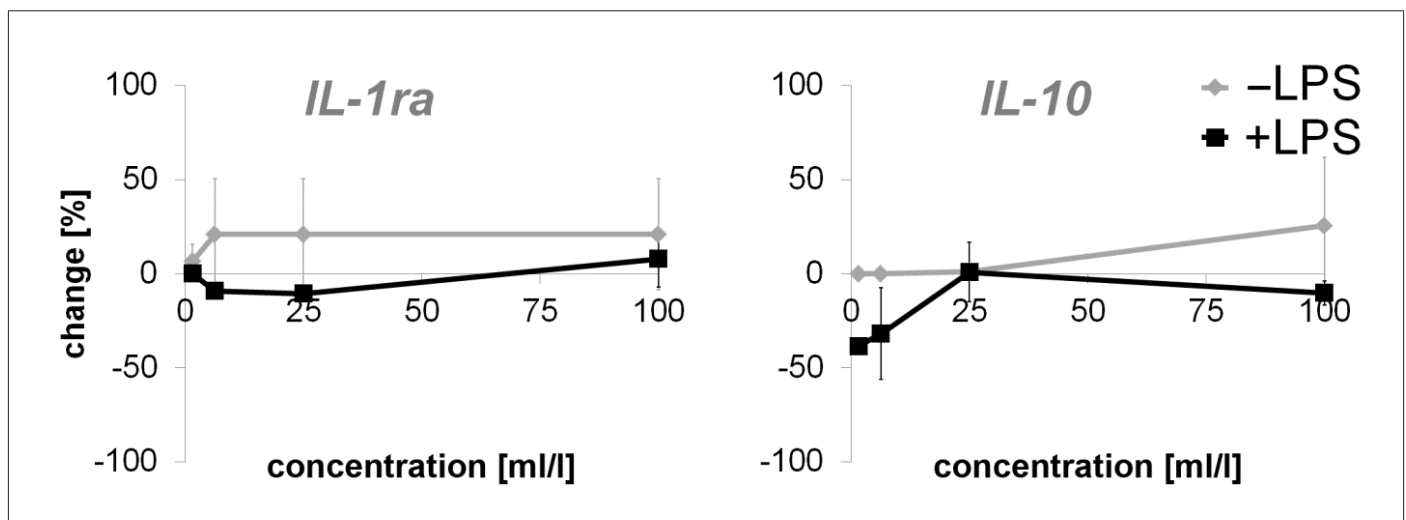

Figure 1: Percent change in proinflammatory cytokine levels in supernatants from dermal fibroblast cultures incubated with serial dilutions of the test product for 24 hours. Effects of the test product alone (-LPS) and effects of a test product pretreatment prior to the addition of the inflammatory compound LPS (+LPS) was observed and displayed in comparison to control cultures without the test substance (* p<0.05; ** p<0.005).

In comparison to previously published work, the data generated in this study is consistent with data from other workgroups. For example, Karow et al. showed reduced wound irritation after treatment of patients with A. montana extracts [33]. Moreover, a stimulation of expression of extracellular matrix genes in a wound-healing phenotype macrophage cell line by A. montana was described by Marzotto et al. [34]. In another study, A. montana showed an inhibition of carragenin-induced rat paw oedema [7]. A. montana extracts have also been reported to promote healing, as some of the modified genes are key factors of tissue remodeling, inflammation, and chemotaxis [35]. In addition, Mahajan et al. show a significant reduction of IL-6, IL-1 and TNFα from human whole blood culture and RAW-264.7 cells after LPS induction and incubation with various homeopathic dilutions of A. montana and Bryonia species *in vitro* [36]. In a study of dos Santos et al. T. quercifolium extracts have anti-inflammatory effects and appear to act on inflammatory processes with prostaglandins, histamine, and other inflammatory mediators [37].

In the circular excision wound model in rats, a wound healing potential of a topically applied ointment made from B. perennis flowers could also be shown [38]. Tolmacheva et al. described an anti-inflammatory effect of L. palustre extracts [39]. Kuonen et al. described a significant and dose dependent promotion of NIH/3T3 fibroblast migration and an enhanced wound closure by V. album extracts [40]. Although plant extracts have been used as medicinal products for several centuries, unfortunately these are only marginally and insufficiently described in scientific studies. However, previous studies in this area reflect the enormous potential of these naturally occurring drugs. For this reason further studies are urgently needed with regard to the applicability and efficacy of these substances (Figure 1 & 2 & 3).

Figure 2: Percent change in anti-inflammatory cytokine levels in supernatants from dermal fibroblast cultures incubated with serial dilutions of the test product for 24 hours. Effects of the test product alone (-LPS) and effects of a test product pretreatment prior to the addition of the inflammatory compound LPS (+LPS) was observed and displayed in comparison to control cultures without the test substance (* p<0.05; ** p<0.005).
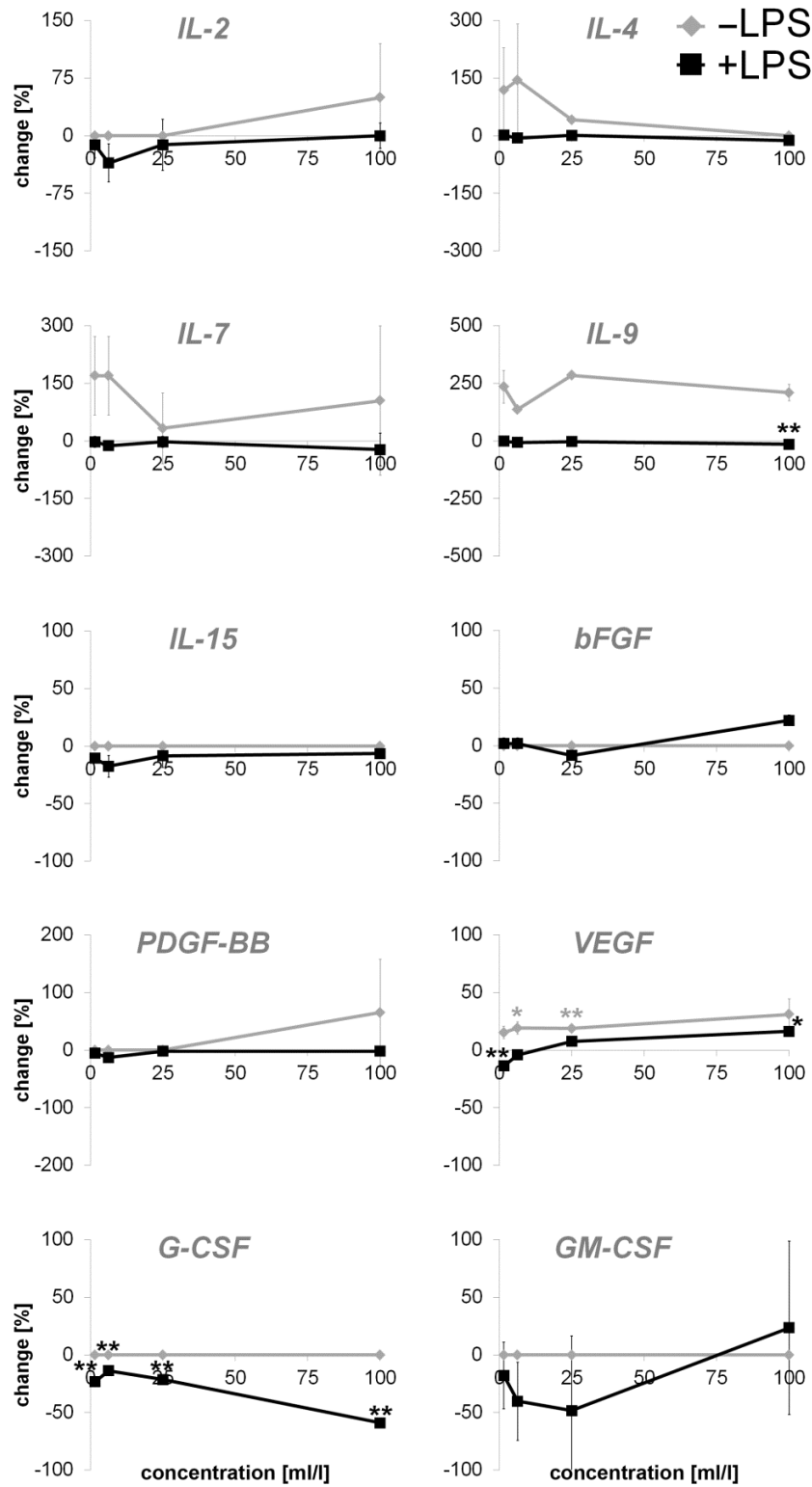
Figure 3: Percent change in levels of cytokines with pro- and anti-inflammatory activity and growth factors in supernatants from dermal fibroblast cultures incubated with serial dilutions of the test product for 24 hours. Effects of the test product alone (-LPS) and effects of a test product pretreatment prior to the addition of the inflammatory compound LPS (+LPS) was observed and displayed in comparison to control cultures without the test substance (* p<0.05; ** p<0.005).

Conclusions

The test substance enhances normal immune surveillance of human dermal fibroblasts in absence of an inflammatory stimulus. Under inflammatory conditions, the data suggests a shift from an inflammative environment to a tissue regenerative environment after incubation with the test substance. In summary, the data of the present study show an anti-inflammatory effect and thus a potential for the test substance for the treatment of acute or chronic inflammatory reactions.

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Declarations

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable.

Availability of Data and Materials

All data described in the manuscript are available from the corresponding author on reasonable request.

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Author's Contributions

All experiments were conducted by NIS Labs, Klamath Falls, USA. MS, VS, MH and LH have performed data analysis and interpretation, MS prepared the manuscript. VS, MH, UK and LH critically reviewed the manuscript and contributed intellectual content. All authors read and approved the final version of the manuscript.

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