Prevention of UV-induced Skin Damage by Activation of Tumor Suppressor Genes p53 and $p14^{ARF}$

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Summary

UV radiation is the most dangerous stress factor among permanent environmental impacts on human skin. Consequences of UV exposure are aberrant tissue architecture, alterations in skin cells including functional changes. Nowadays new kinds of outdoor leisure-time activities and changing environmental conditions make the question of sun protection more important than ever. It is necessary to recognize that self-confident consumers do not consider to change their way of life, they demand modern solutions on the basis of new scientific developments. In the past one fundamental principle of cosmetics was the use of physical and organic filter systems against damaging UV-rays. Today new research results demonstrate that natural protecting cell mechanisms can be activated. Suitable biological actives strongly support the protection function not from the surface but from the inside of the cell.

A soy seed preparation (SSP) was proven to stimulate natural skin protective functions. The major functions are an increased energy level and the prevention of DNA damage. These functions can be defined as biological UV protection.

The tumor suppressor protein p53 plays a key role in the regulation of DNA repair. p53 must be transferred into the phosphorylated form to work as transcription factor for genes which are regulating the cell cycle or organizing DNA repair.

A pretreatment with SSP increases the phosphorylation rate of p53 of chronically UV-irradiated human keratinocytes significantly. According to the same test procedure SSP induces a dramatic increase in the expression of the tumor suppressor protein $p14^{ARF}$ that is supporting the p53 activity by blocking the antagonist of p53, the oncoprotein Mdm2. Mdm2, a ubiquitin E3-ligase, downregulates p53 and at the same time it prevents phosphorylation of p53.

The positive influence of the tumor suppressor proteins explains the stimulation of DNA repair and prevention of sunburn cell formation by SSP, which was proven in cell culture experiments.

In vivo the increased skin tolerance against UV irradiation by SSP could be confirmed too.

We have assumed, that an increased repair potential provides full cell functionality.

Introduction

The tumor suppressor gene p53 is described as a "guardian of the genome" (1). It is part of the protective mechanisms, that ensures progress in cell cycle only if the genome is fully intact. The special role of p53 results in the initiation of both: apoptosis and cell cycle arrest.

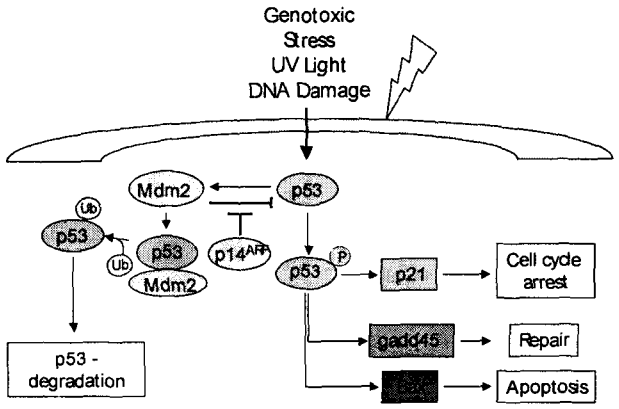

Fig. 1 p53 pathway: The tumor-suppressor protein p53 induces cell cycle arrest, DNA repair and apoptosis in response to DNA damage. The p53-dependent pathways help to maintain genomic stability by eliminating damaged cells. (2) (3) (4)

The p53 gene encodes a 53 kDa protein. The phosphorylated p53 activates distinct genes, e.g. p21 and GADD45. p21 is organizing cell cycle arrest at the G1 checkpoint (5) (6) if DNA damage occurs prior to DNA synthesis. During interruption of the mitosis procedure GADD45 protein is organizing DNA repair (7). At a second G2 checkpoint the cell cycle can be arrested, which is important for those cells with finished S-phase where the DNA replication already is finished. The induction of apoptosis is preventing mitosis of cells with damaged DNA (8). An interaction of the tumor suppressor protein $p14^{ARF}$ with the p53 pathway was recognized recently (9) (10). $p14^{ARF}$ degrades the oncoprotein Mdm2, an antagonist of p53 , thus modulates the p53 activity, including cell cycle arrest (11).

The protein $p14^{ARF}$ can be considered as a second defense line in cases of hyperproliferation with the existing risk of DNA damage. $p14^{ARF}$ responds to proliferation signals by activation of a $p14^{ARF}$ dependent checkpoint, if these signals exceed a critical value. In this case the oncoprotein Mdm2 is inhibited and p53 stabilized without activation of the retinoblastom protein pRb. Thus two pathways are linked and the repairing process is rendered secure. Loss or mutation of p53 disrupts the tumor suppressive functions of the protein. Cell cycle arrest in cases of DNA damage is no longer organized. Mutation and cancer could be the consequences.

The interaction of a soy seed preparation (SSP) on the endogenous protective mechanisms after chronic and single UV irradiation has been investigated. It has been shown that a stimulation and stabilization of p53 by $p14^{ARF}$ can explain the increase of DNA repair effectiveness and the reduction of non-repairable cell formation (apoptotic cells).

We hypothized that the induction of natural cellular protective functions could lead to a higher UV tolerance of the skin and to counteract premature skin aging. Specifically designed tests on humans were performed to prove this correlation.

Materials and Methods

Preparation of the active substance SSP (Soy Seed Preparation)

Minced soy seeds were extracted under non-hydrolytic conditions.
The main compounds in the received fraction are proteins, glycoproteins and carbohydrates.
The aqueous solution of SSP needs preservation. Before SSP could be used for cell culture tests it was dialyzed by a membrane with cut-off 1000 Dalton.

Cell culture

Primary normal human epidermal keratinocytes from female donors, aged between 29 and 51 years, were grown in serum-free culture medium (Cell Systems; GmbH) at a 0.15 mM calcium concentration supplemented with insulin, hydrocortisone, epidermal growth factor (EGF), bovine pituitary extract (BPE), epinephrine and gentamicin / amphotericin B. The culture medium was changed every 2 - 3 days. After trypsinization in a non-stationary growth phase, cells were seeded on a 24-well plate with 1 $x10^5$ cells/well. 24 hrs. before starting UV irradiation, SSP was applied on cells.

Human epidermal keratinocytes (a gift of Prof. Fusenig; DKFZ Heidelberg, Germany) were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 5% FCS. After trypsinization in a stationary growth phase, cells were seeded on a 96-well plate with 3 $x10^4$ cells/well. 72 hrs. before starting UV irradiation, SSP was applied on cells.

UV irradiation

After several pre-incubation periods with SSP, the supernatant was removed and the wells were washed with phoshate buffer solution (PBS). During the variable dosages of irradiations, the cells were incubated with PBS. The UV irradiation was carried out with the UV lamp SOL 500 (Dr. Hönle; Munich, Germany). Afterwards PBS was removed again and the cells were incubated in the appropriate medium. Depending on the tests performed, SSP was added once again to the cells.

ATP assay

Sample dilution (0.1% SSP) was filled into proper wells together with 5% FCS. Cells with 5% FCS only were used as control. The plates were incubated at 37 °C, 5% CO_2 for 72 hrs. Then the supernatant was removed and each well was washed with phoshate buffer solution (PBS). The plates were irradiated with an UV lamp. The supernatant was removed again and a FCS-free medium was added followed by incubation for 18 hrs. The ATP level was detected by the chemiluminescent detection assay ATPLite-M (Packard Instruments Co.). The supernatant was removed and the cells washed with PBS. The lysed cells were incubated with the substrate solution for 10 minutes. The luminescence was measured in a luminescence reader (Fluoroscan Ascent FL; Labsystems Ltd.).

BrdU assay (DNA Repair)

Sample dilution (0.1% SSP) was filled into proper wells together with 5% FCS. Cells with 5% FCS only were used as control. The plate was incubated at 37 °C, 5% CO_2 for 72 hrs. Then the supernatant was removed and each well washed with phoshate buffer solution (PBS). The plate was irradiated with an UV lamp. Supernatant was removed and a FCS-free medium with BrdU labeling solution was added followed by 18 hrs. incubation.The BrdU content was measured using the Cytotoxicity Detection Cell Proliferation ELISA (Roche Molecular Biochemicals). After UV irradiation, cells were labeled with BrdU for 18 hours. The antibody-substrate-complex was incubated for 15 minutes. The absorbance was taken in a microplate reader (MRX; Dynex Technologies, Inc.) at 405 nm, reference 490 nm.

Detection of non-repairable (apoptotic) cells

Sample dilutions (0.1%, 0.5% SSP) were filled into proper wells together with 5% FCS. 5% FCS was used as control. The plates were incubated at 37 °C, 5% CO_2 for 72 hrs. Then the supernatant was removed and each well was washed with phoshate buffer solution (PBS). The plates were irradiated with 3 J/cm² UVA + 0.3 J/cm² UVB. Supernatant was removed again and a FCS-free medium was added followed by 15 hrs. incubation. The number of apoptotic cells was identified by using Cell Death Detection ELISA^{PLUS} (Roche Molecular Biochemicals). The measurement of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) was carried out by using ELISA technique. The absorbance was taken in a microplate reader (MRX; Dynex Technologies, Inc.) at 405 nm, reference 490 nm.

p53 phosphorylation assay

Sample dilution (1.0% SSP) was filled into proper wells. Wells without sample were used as control. The plates were incubated at 37 °C, 5% CO_2, for 48 hrs. Then, the supernatant was removed and each well washed with PBS. The plates were irradiated with 0,5 J/cm^2 UVA + 0.05 J/cm^2 UVB. The supernatant was removed and the defined medium with and without sample were added to the

wells followed by 24 hrs. incubation. This irradiation cycle was repeated every 24 hours for 7 days. The phosphorylation rate of p53 was detected by using ELISA technique. The anti-phospho-p53 (Ser15) antibody (Oncogene Research Products [ORP]; EMD Biosciences, Inc.) was diluted 1:2000 and detected with the anti-rabbit-IgG-biotin antibody (Sigma). The streptavidin-POD complex (Sigma-Aldrich Co.) was incubated for 1 hour. The resulting OD values were read at 450 nm in a microplate reader (MRX; Dynex Technologies, Inc.).

$p14^{ARF}$ assay

Sample dilution was filled into proper wells. Wells without sample were used as control. The plate was incubated at 37 °C, 5% CO_2, for 72 hrs. Then, the supernatant was removed and each well washed with PBS. The plate was irradiated with 0,5 J/cm^2 UVA + 0.05 J/cm^2 UVB.The supernatant was removed and the defined medium with and without sample were added to the wells followed by 6 hrs. incubation.

$p14^{ARF}$ was detected by using ELISA technique. The anti-$p14^{ARF}$ antibody (Novus Biologicals, Inc) was diluted 1:2000 and detected with the anti-rabbit-IgG-biotin antibody (Sigma-Aldrich Co.). The streptavidin-POD complex (Sigma-Aldrich Co.) was incubated for 1 hour. The resulting OD values were read at 450 nm in a microplate reader (MRX; Dynex Technologies, Inc.).

***In vivo*-tests**

Elastase assay

In the beginning of the study the Minimal Erythema Dose (MED) was determined for each volunteer. A formulation containing 5% of SSP as well as a formulation without SSP (placebo) were applied to 12 test persons twice daily on the inner forearm over a period of one week. After one week the test areas of the volunteers were irradiated with UV-rays and elastase activities were determined. Measurements were done according to a method described by Hornebeck et al (12).

Influence of SSP on skin redness

In the beginning of the study the Minimal Erythema Dose (MED) was determined for each volunteer. A formulation containing 5% of SSP as well as a formulation without SSP (placebo) was applied to 5 test persons twice daily on the inner forearm over a period of one week. After one week of skin treatment the test areas were irradiated with UV-rays (1.75 x MED) and the skin color was measured 24 hrs. after irradiation by using a chromameter (a*).

Determination of Sun Protection Factor (SPF)

The determination of the influence of a formulation with 5% SSP on SPF was done according to a modified Colipa-Method [Sun Protection Measurement (Colipa), July 1995].

12 volunteers were pretreated with a SSP containing cream against placebo on the back on two test areas twice a day for a period of one week.

Before UV exposure a sun milk with a known SPF of 4 was applied to the SSP and placebo treated test areas.

After calculation a difference between the test areas could be expressed in SPF units.

Results and Discussion

Effect of SSP on the ATP level

The energy level of cells expressed by the amount of ATP is essential for any cell activity including the protective functions. The generation of ATP by oxidative phosphorylation takes place in the mitochondria of cells. ATP depletion is observed, as a consequence of any type of stress given to the cells.

The influence of SSP on the ATP levels of non-irradiated and UV-irradiated cells has been studied. In a dose dependent manner SSP acts against ATP depletion depending on the UV intensity. The stimulation of ATP by SSP in non-irradiated cells was insignificant. The incubation of the cells in a medium containing 0.1% SSP for 24 hrs. before UV irradiation was important for prevention of ATP decrease. The active SSP induces a higher stress resistance of cells which is also important for other protective functions.

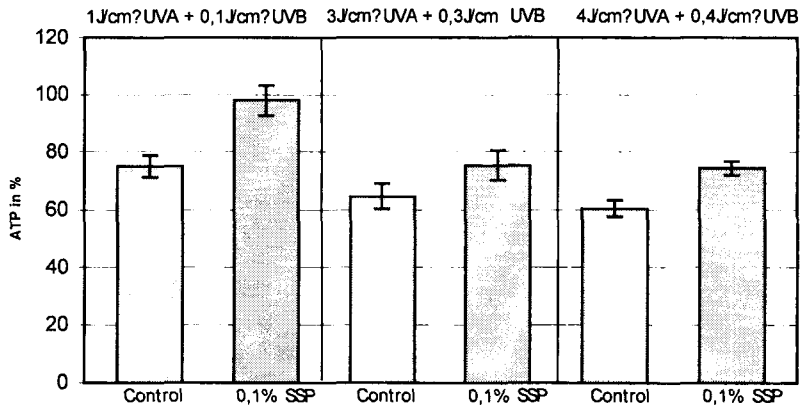

Fig. 2 Difference in ATP depletion of UVA/UVB-irradiated cells related to non-irradiated cells (100%)

Influence on DNA repair

80% of the UV-induced DNA damage are results of thymidine dimerization. The enzyme based excision repair system excises the segment containing the damage and fills the gap with a newly synthesized nucleotide segment using the correct complementary DNA-strand as a template. The repair system of aged skin loses efficacy with an increasing risk of survival of DNA damaged cells. A contact of the cells with SSP before UV irradiation was shown to increase the repair capacity and

to avoid the division of cells with non-repaired DNA. Influence of SSP on DNA repair was only observed when the cells were damaged with UV.

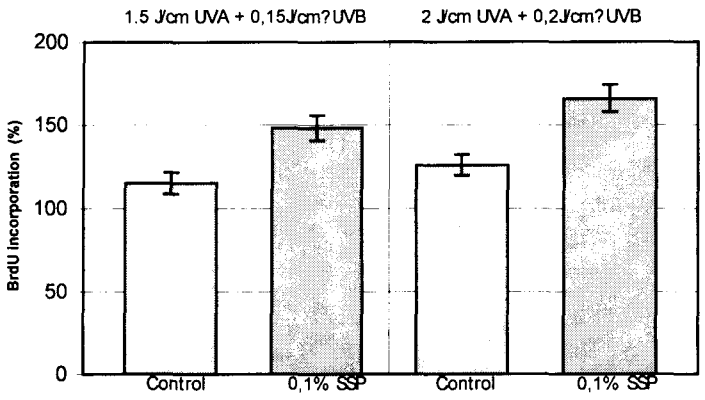

Fig. 3 BrdU incorporation expresses the DNA repair rate compared to the cells own capacity (control). The rate of non-irradiated cells was standardized as 100%.

Prevention of non-repairable cell formation after UV irradiation

Cells with DNA double strand breaks are non-repairable apoptotic cells. Distinct cytokines e.g. TNF-α or tumor suppressors e.g. p53 induce the programmed cell death, the apoptosis. The formation of apoptotic cells after UV irradiation is influenced by DNA repair rate. If the DNA repair process works sufficiently, reactions from a single to a double strand break could be avoided.

Apoptotic cells were detected by quantifying the amount of nucleosomes as DNA fragments. Reduction of apoptotic cell formation after UV irradiation by SSP in different concentrations reflects the increase of the DNA repair rate.

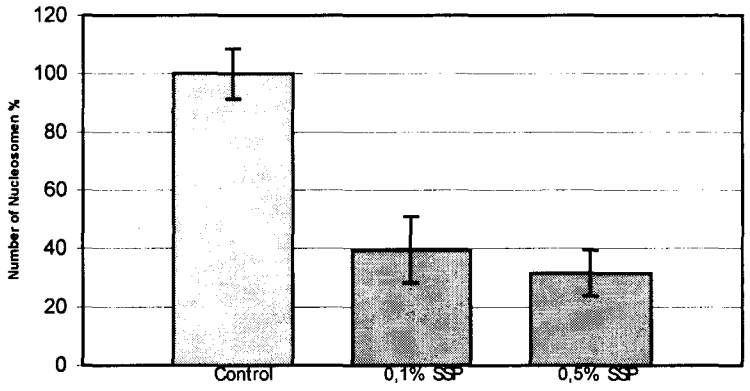

Fig. 4 Preincubation of cells in different SSP dilutions acts against formation of apoptotic cells expressed in nucleosome concentration. Control measures are irradiated cells without additives.

Activation of p53 by phosphorylation

p53 is the dominating tumor suppressor gene for the protection against mutagenesis. After chronic UV exposure mutations in the p53 gene are the most common gene mutations identified in cancer cells. In intact cells the amount of p53 protein (wt-p53) is low. In cell culture it was found that DNA repair induced by UV irradiation with an energy of < 20 mJ/cm² does not involve p53 regulation. Higher UV energy or chronic UV irradiation with lower energy levels will increase the p53 protein level. The amount of p53 correlates with the extent of damage. Severe DNA damage needs a coordination of the distinct protective pathways by p53. Phosphorylation of p53 protein is the essential activation step for transcription of further genes involved in cell cycle control (p21) and DNA repair (GADD45).

The phosphorylation rate of p53 by using anti-phosphorylated p53 (Ser 15) antibody was detected in lab experiments. The phosphorylation rate was increased up to 150% compared to irradiated control cells. This strong activation of p53 explains the influence of SSP on DNA repair and the regulation of apoptosis.

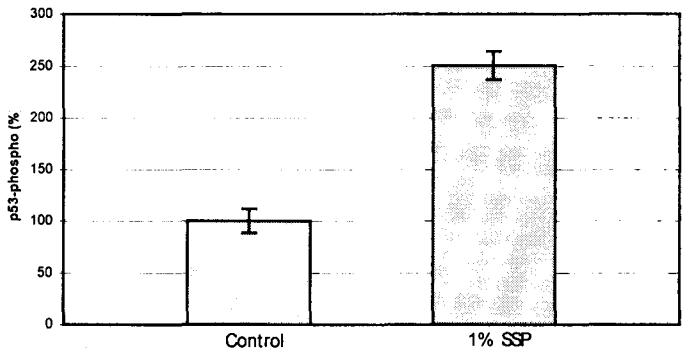

Fig. 5 Influence of SSP on the phosphorylation of p53 after chronic UV irradiation.

In case of a single UV irradiation of cells with a low dosage, it can be assumed that the p53 pathway will not be activated. An increased dosage of cell damage leads to a rise of p53. If the cell damage exceeds 4 mJ/cm^2 (UV-B irradiation), the Mdm2 activity increases and the p53 level is downregulated. SSP was found to counteract the p53 downregulation. The threshold for UV induced damage is shifted and the cells show a significant p53 phosphorylation rate at high UV-ray dosages. This finding is tantamount to an improved repair potential of the cells.

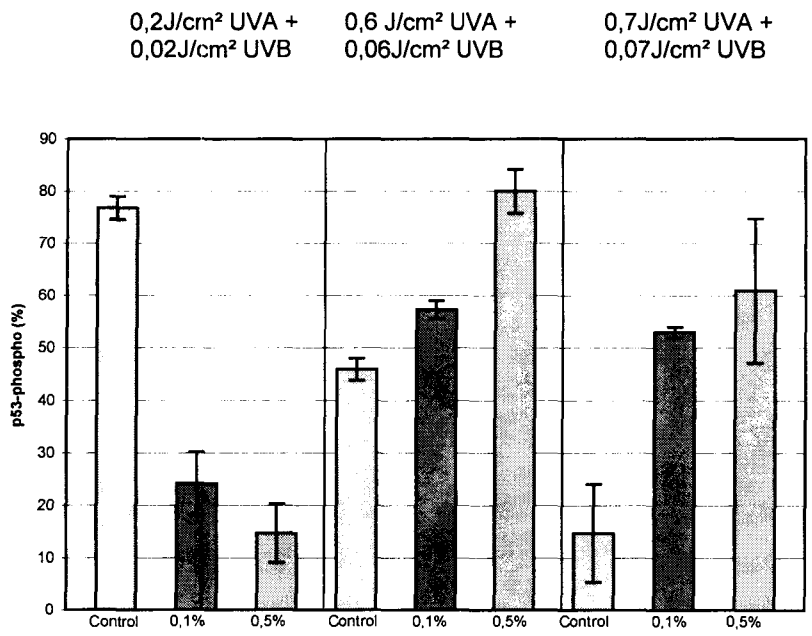

Fig. 6 Influence of SSP on the phosphorylation of p53 after single UV irradiation.

Activation of tumor suppressor p14^{ARF}

p14^{ARF} protein functions as tumor suppressor via two pathways:

- the retinoblastoma (pRb) pathway (13) (14)
- the p53 pathway (9)

The retinoblastoma is a tumor suppressor that is involved in the regulation of transcription factors which control DNA synthesis and cell cycle progression. In case of a pRb hyperphosphorylation a hyperproliferation of new cells can be induced (15). However, p14^{ARF} will directly be activated to inhibit Mdm2 and activates or stabilizes p53 which leads to a cell cycle arrest. p14^{ARF} is the connecting element between the pRb and the p53 pathway.

The proven activation of p14^{ARF} is a perfect correlation with the p53 results. SSP is able to activate both tumor suppressor genes p14^{ARF} and p53 after UV exposure.

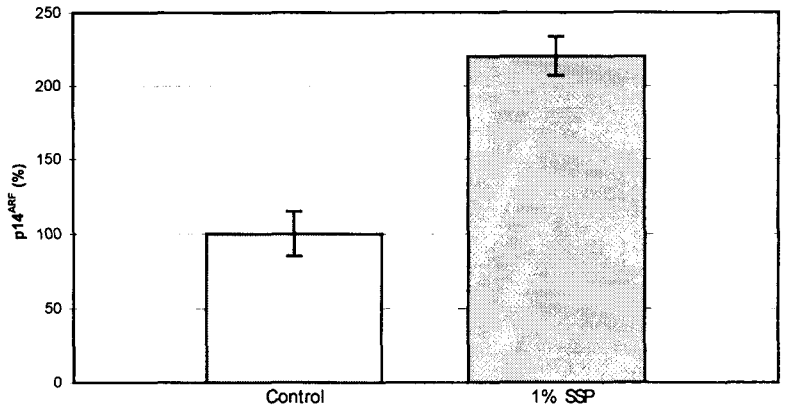

Fig. 7 Influence of SSP on activation of the tumor suppressor $p14^{ARF}$

In vivo test on humans: Influence on the sun protection factor0

The test design was developed to support the hypothesis that stimulation of cellular protective functions must show a measurable increase of UV protection *in vivo*.

The modified Colipa Method was used to demonstrate the influence of SSP on the total SPF of a sunscreen formulation. Skin areas treated with an SSP containing formulation of SPF 4 were shown to increase protection to SPF 7. A pretreatment of the skin with an SSP containing skin care formulation demonstrated to strengthen the cellular protective function with an enhanced UV protection.

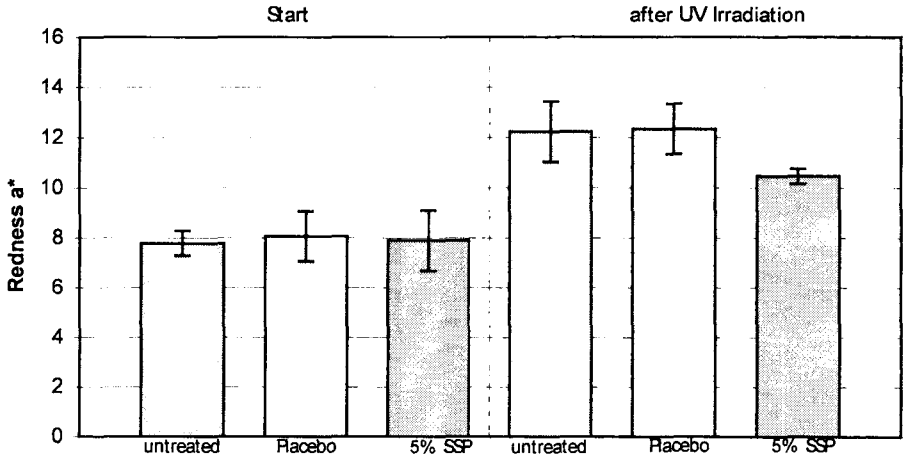

Fig. 8 Skin redness after UV irradiation

Product	SPF (mean)	SD
Standard Cream of SPF 4 with 5% SSP	7,0	0,9
Standard Cream of SPF 4 without 5% SSP	4,2	0,7

Fig. 9 Increase of Sun Protection Factor with SSP

***In vivo* test on humans; Influence on UV-induced elastase release**

Wiedow et al. (16) described that in case of inflammatory skin diseases immunorelevant cells release elastase. Elastase can be detected on the skin surface after irritation.

The pretreatment with a formulation containing 5% SSP for one week reduced the elastase release after UV irradiation by more than 30%.

Prevention of elastase release maintains intactness of the extracellular matrix (ECM).

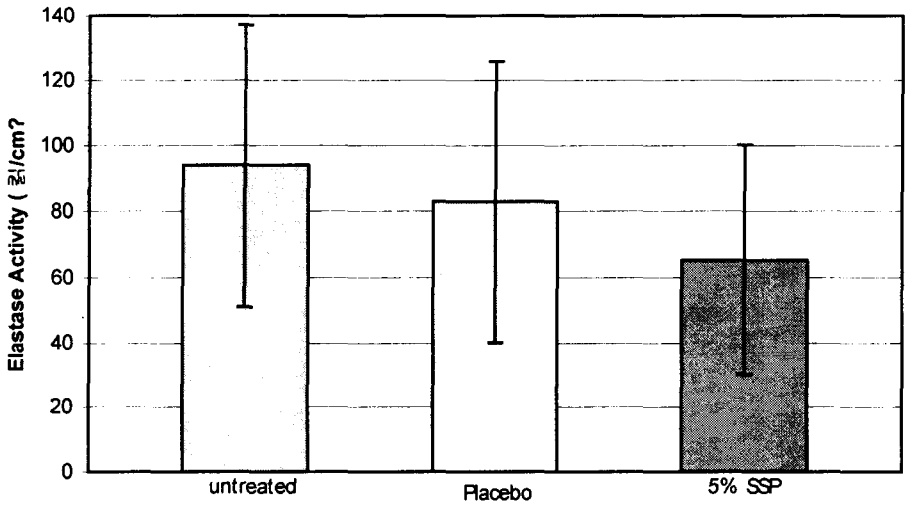

Fig. 10 Reduction of UV-induced elastase release

Conclusion

Beginning with the day of birth we are doomed to age. A fact people like to ignore. A young looking appearance with a perfect skin is partly considered to personal welfare, but also to social acceptance and success.

UV-induced premature skin aging is one of the biggest challenges for the cosmetic industry. Much research was done to better understand the complex regulatory interactions on the cellular level

and the modifications under the influence of environmental stress. Major progress to understand the working principles of cosmetic actives was achieved using new or more sensitive bioassays in conjunction with cell cultures.

A cosmetic active to stimulate natural skin protection functions against UV irradiation can be defined as a biological UV protector. This important way of UV protection could be expressed in well known SPF units. The biological UV protector, like topically applied UV filters, is able to prevent severe skin damage. While UV filters are working on the surface, the biological UV protector is actively supporting the skin functions from the inside. The increased effectiveness of the protective functions strongly supports the skin against UV-induced accelerated aging. *In vitro* tests on cell cultures showed measurable effects. *In vivo* tests on human volunteers confirmed the *in vitro* findings with an improved SPF factor and the reduction of elastase release. Both test methods, *in vitro* and *in vivo*, lead to the conclusion that screening results based on cell cultures not only provide more detailed knowledge of the complex biological reactions inside the skin but also allow to transfer the knowledge into measurable and reliable results on human volunteers. The biological assays used allow to get new and much deeper insights into biochemical reactions in skin to develop effective cosmetic products with truly proven claims.

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