



Lycoskin Defence

Protect your skin from urban pollution!

Integrating plant biology knowledge and
cell culture expertise into cosmetology

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INTRODUCTION

Heavy Metal Pollution

- Although **heavy metals** are naturally present in the environment, contaminations come from many different sources, mostly industries, agriculture, human waste and road traffic.
- Through food, water or body tissue permeation, heavy metals can accumulate rapidly in the organs and intoxicate them.
- Heavy metals, such as **lead** (Pb), **nickel** (Ni), **cadmium** (Cd), **mercury** (Hg), **copper** (Cu) and **chromium** (Cr), represent the most serious threat for skin health.



Heavy Metal Pollution

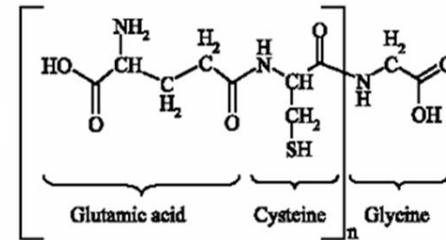
On The Skin

- Skin cells are certainly the most exposed to **heavy metal stress** and thus the most damaged by the devastating effects that these chemicals cause (Jarup, 2003).
- Heavy metals cause **dermatitis, contact allergies, irritation, inflammations and other serious skin diseases** (Thyssen & Mennè, 2009).
- Several studies indicate that about **7-10% of women and 1-3% of men** in the entire human population **are sensitive to nickel**, which is the main constituent of jewellery, coins and other metal objects of common human use (Thyssen et al., 2008).

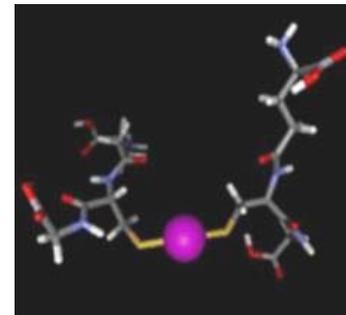


Plants and Heavy Metals

- Plants have evolved sophisticated mechanisms to protect their cells **against heavy metal toxicity**.
- They produce **high amounts of compounds able to bind (chelate) heavy metals**, thus avoiding their dangerous interaction with most of the vital cellular components.
- These compounds include the small **metal-binding peptides** called “**phytochelatins**”.

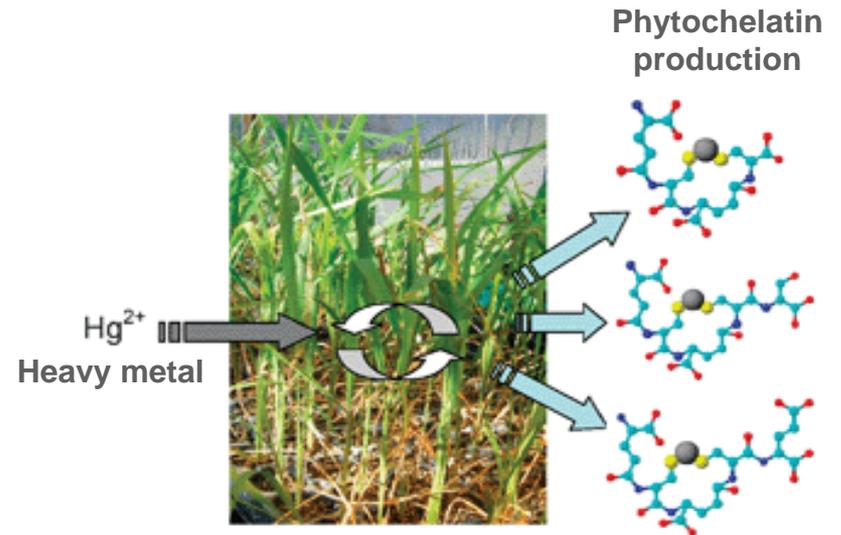


Phytochelatins



Plants and Phytochelatins

- **Phytochelatins** are chemically synthesized by the enzyme **phytochelatin synthase (PCS)** which uses the starting material glutathione.
- The levels of glutathione in the cell are themselves regulated by another enzyme, called GSH2.
- High amounts of both enzymes in the plant cells lead to an increase of **phytochelatin production**, and thus to a better protection of the cells from the toxic effects of heavy metals (Wang et al., 2009).



A close-up photograph of several ripe, red tomatoes, filling the top portion of the slide. The tomatoes are in sharp focus, showing their smooth, glossy skin and some green stems.

Tomato Stem Cells

Thanks to a well established experience in plant stem cell biotechnology, plant signal transduction and plant-derived active compounds, we have developed an **innovative Active Ingredient** to protect skin cells from the devastating effects of **heavy metals**.

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Protect your skin from urban pollution!

the result of integrated knowledge on plant biology, skin cell biology and cosmetology

Tomato Stem Cells

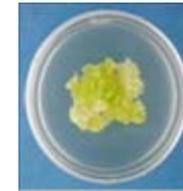
- The solution comes from **tomato stem cells**, very rich in well-known antioxidant compounds. They were used as **biofactories** to produce high amounts of **phytochelatins**.
- After treatment of the tomato stem cells with compounds which stimulate phytochelatin production, we obtained **Lycoskin Defence**, a water soluble extract, rich in **antioxidant compounds** and **phytochelatins**.

Lycoskin Defence ←

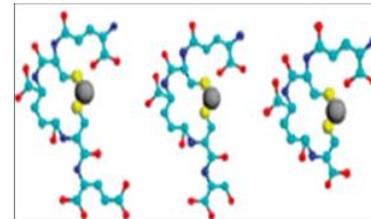
Rich in antioxidants



Tomato cells as bio-factory

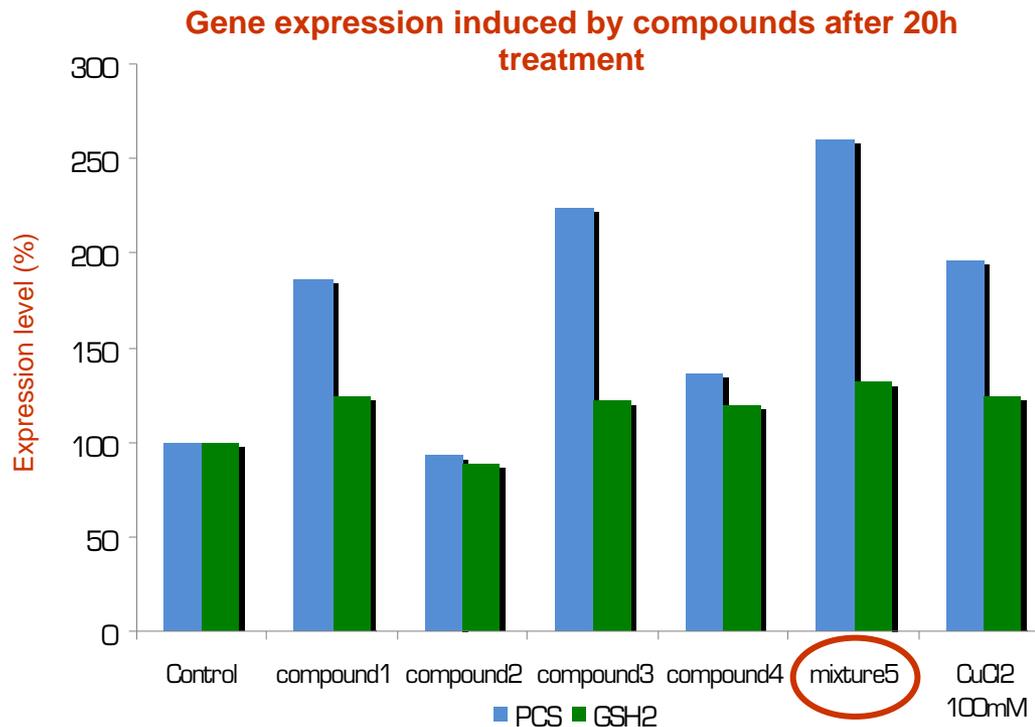


High production of phytochelatin



Phytochelatin Over-Production in Tomato Stem Cells

To boost the production of phytochelatins, tomato stem cells were treated with different mixtures of natural compounds, formulated in our laboratory.



The **mixture 5** is the strongest inducer of **PCS** and **GSH2** expression: the enzymes that are responsible for **phytochelatin** production in plant cells.

A close-up photograph of several ripe, red tomatoes. The tomatoes are the primary focus, with their smooth, glossy skin and vibrant red color. The background is slightly blurred, showing more tomatoes and a hint of a green leaf.

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EFFICACY STUDIES

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Efficacy tests

Metal Chelating Capacity:	Chelating capacity Assay
Cell vitality:	MTT assay in presence of heavy metals
Antioxidant Activity:	TAC Assay
Cell ROS production:	CM-H ₂ DCFDA fluorescent Assay
DNA Protection:	Comet Assay
DNA Repair/longevity:	GADD45 α RT-PCR Sirt-1 RT-PCR
Collagen degradation:	MMP1, MMP3 and MMP9 analysis by RT-PCR
Collagen Induction:	Collagen I & III analysis by ELISA

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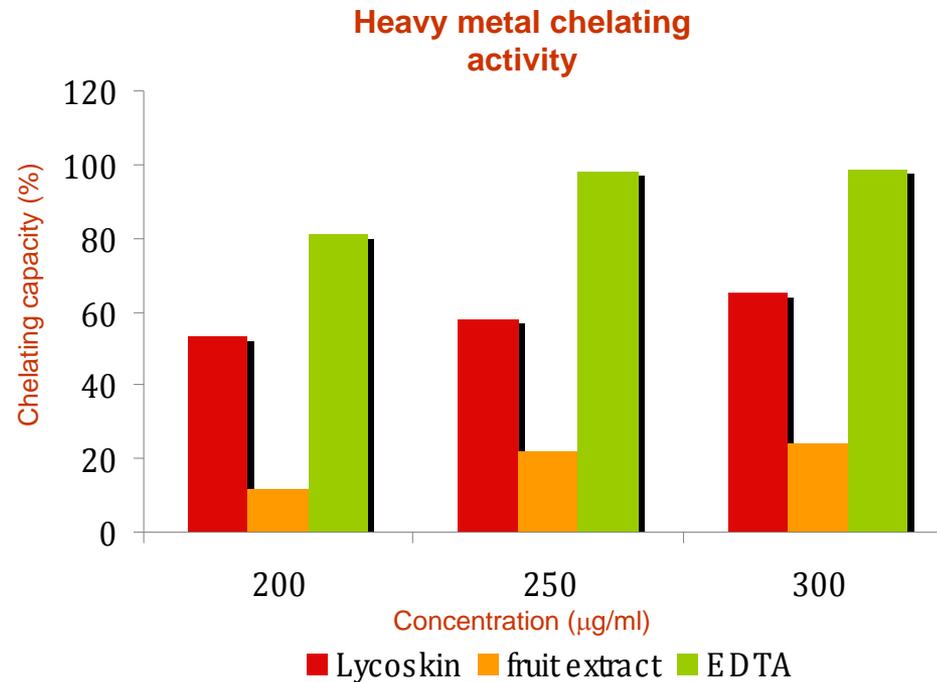
Heavy Metal Chelating Capacity

Principle: the heavy metal chelating capacity assay measures the capacity of a compound or mixture to bind heavy metals.

Protocol: the assay is performed *in vitro* and it measures the changes of colour following the adding of extract or single compounds to the complex ironII-ferrozine. To each reaction tube (1 ml), containing FeCl_2 (0.05 mM) and ferrozine (0.1 mM) in methanol 60%, different amounts of reagents are added and after 10 minutes, the absorbance of the samples is measured at 550 nm by a Multiwell-plate Reader. The reagent EDTA (ethylenediamine tetraacetic acid) is used as positive control in the assay due to its high metal chelating activity. All the values obtained are expressed as percentages of chelating capacity.

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Heavy Metal Chelating Capacity



Thanks to the presence of **phytochelatins**, **Lycoskin Defence** has good **heavy metal chelating (binding) capacity**, significantly higher than that of tomato fruit extract. Thus it is able to chelate metals from the intracellular and extracellular environment, avoiding their dangerous action towards vital cell structures and extracellular matrix components.

Lycoskin Defence

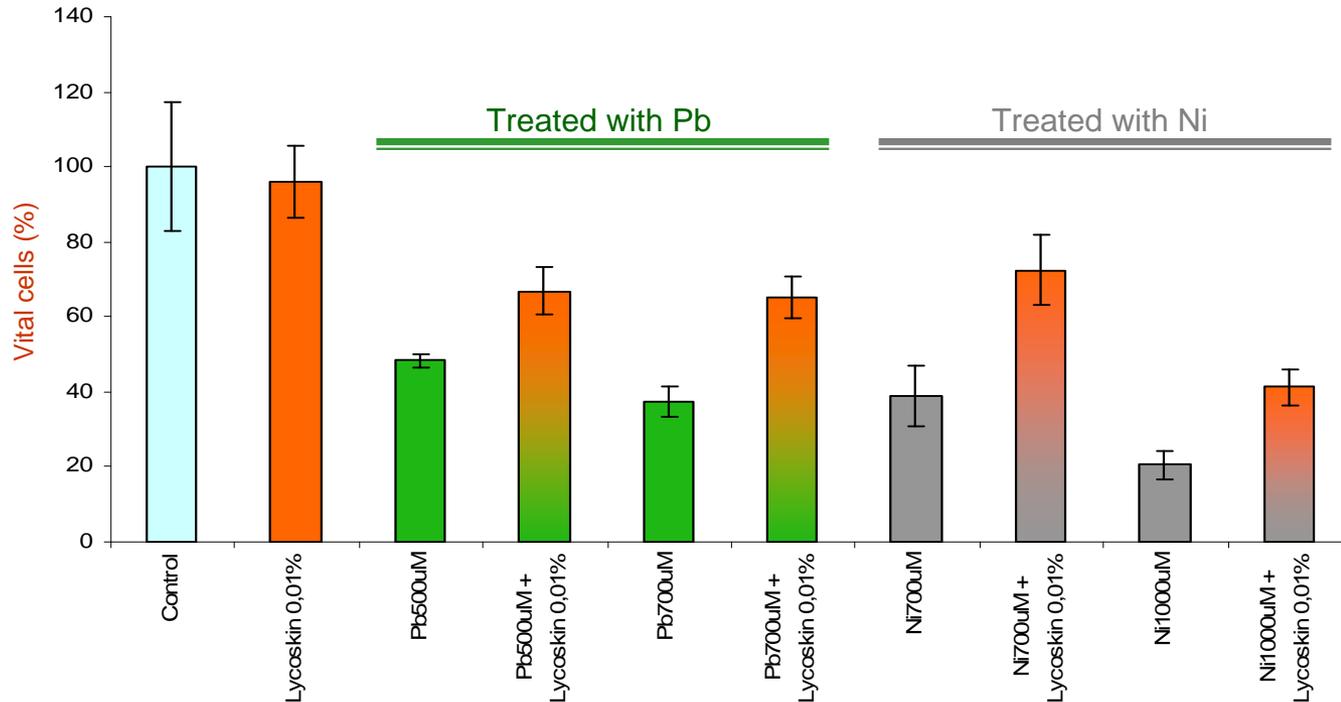
Cell vitality

Principle: the cell vitality assay measures the percentage of vital and healthy cells in the culture after treatment with different compounds or mixtures

Protocol: the assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] and form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals. The number of healthy cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a simple colourimetric assay. The results can be read on a multiwell scanning spectrophotometer (ELISA reader).

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Cell Vitality



Lycoskin Defence has **heavy metal detoxifying capacity**, thanks to the presence of **phytochelatins**.

Thus it is able to protect skin cells from heavy metal toxicity (such as that caused by lead or nickel), as measured by the cell vitality assay.

Lycoskin Defence

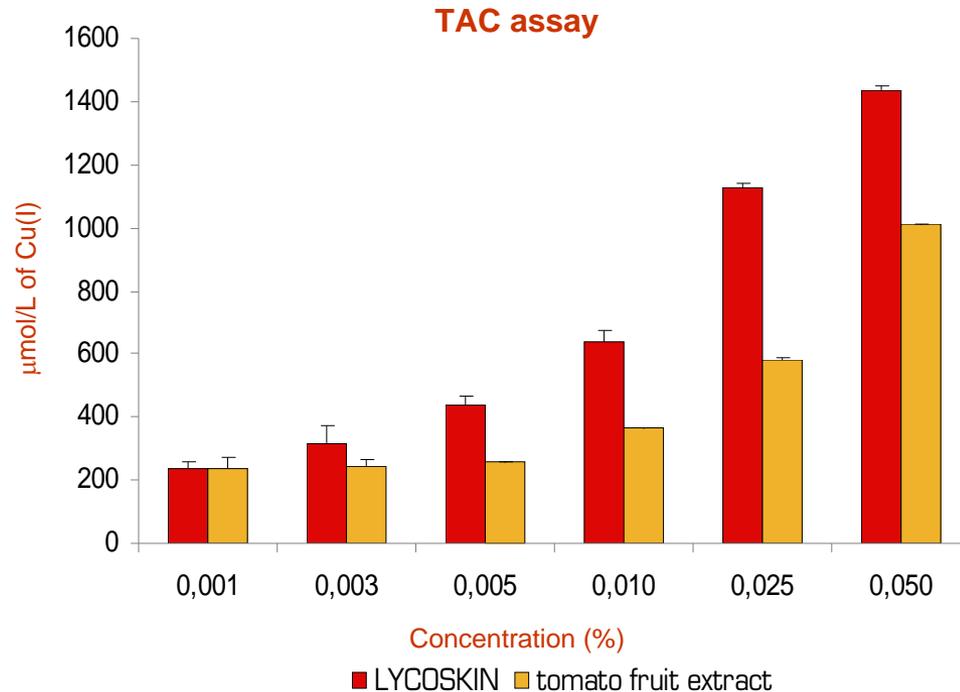
Total antioxidant Capacity (TAC)

Principle: the TAC assay measures the reducing potential of the test material. However, it does not provide any information on the antioxidant induction in living epidermis cells.

Protocol: TAC is an *in vitro* assay and it is based on a redox reaction between a test compound and copper II, Cu(II). If a test compound has got reducing power, the Cu(II) is readily converted into Cu(I), which can be monitored by the addition of a chromophore, bathocuproine (BATO). The absorbance of the samples is then measured at 490 nm by a plate reader. As reference standard, scalar dilutions of CuCl are used, ranging from 10 mM to 0.15 mM.

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Total antioxidant Capacity (TAC)



Lycoskin Defence has an **excellent antioxidant activity**, even better than that of tomato fruit extract, as measured by TAC (Total Antioxidant Capacity) assay.

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Reactive Oxygen Species (ROS) Measurement

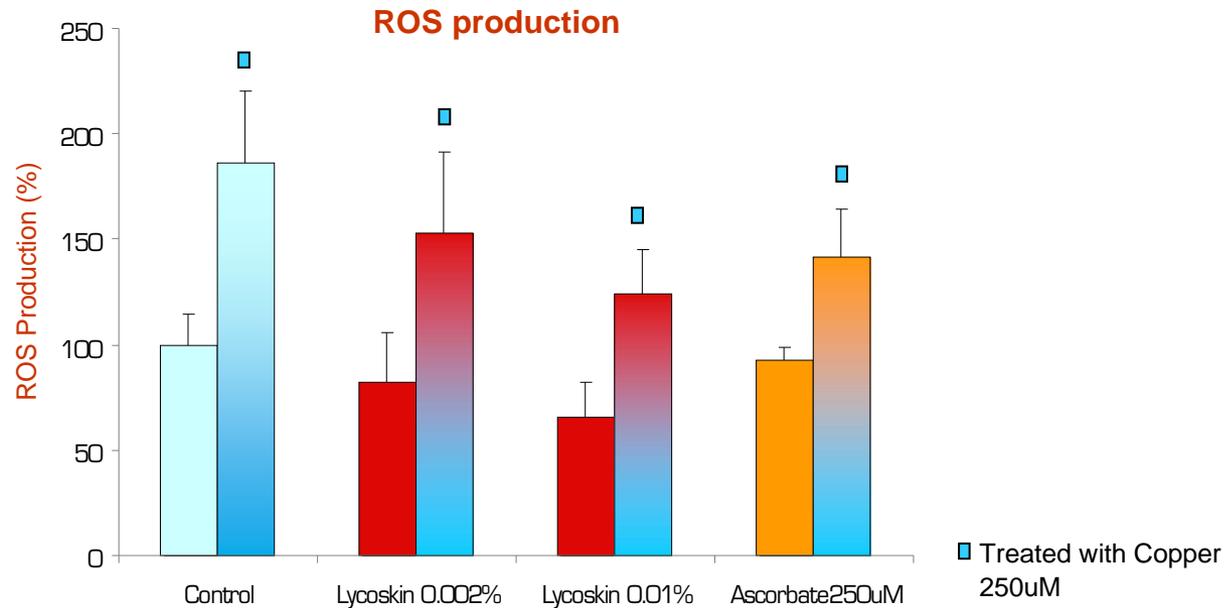
Principle: Unlike the TAC assay, the ROS fluorescent assay measures the amount of Reactive Oxygen Species (ROS) present in living skin cells in the absence and the presence of stress. Stress, caused by heavy metals, UV or H_2O_2 , leads to a substantial increase of intracellular ROS.

Protocol: In the ROS assay, NIH 3T3 cells are pre-treated with test materials and then loaded with CM-H2DCFDA dye that passes through the plasma membrane. Once cleaved by intracellular esterases and trapped in the cell, the dye is oxidized to the fluorescent form depending on the amount of ROS present in the cell. After the loading, cells are stressed by adding $CuCl_2$ 250 μ M for 30 min. The amount of fluorescence emitted by the cells at 535 nm, related to the levels of ROS produced, is measured by a plate fluorimeter.

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Cell ROS Protection

Thanks to its high antioxidant power, **Lycoskin Defence** is also able to protect cells from heavy metal induced oxidative stress. Copper can cause an overproduction of reactive oxidative species (ROS) in the cells, giving a strong oxidative burst (Orhan et al., 2006).



Lycoskin Defence protects cultured skin cells from the toxic effect of metals, **decreasing the amounts of intracellular ROS** induced by metal treatment. Ascorbate was used as positive control, in view of its antioxidants properties.

Lycoskin Defence

Comet Assay

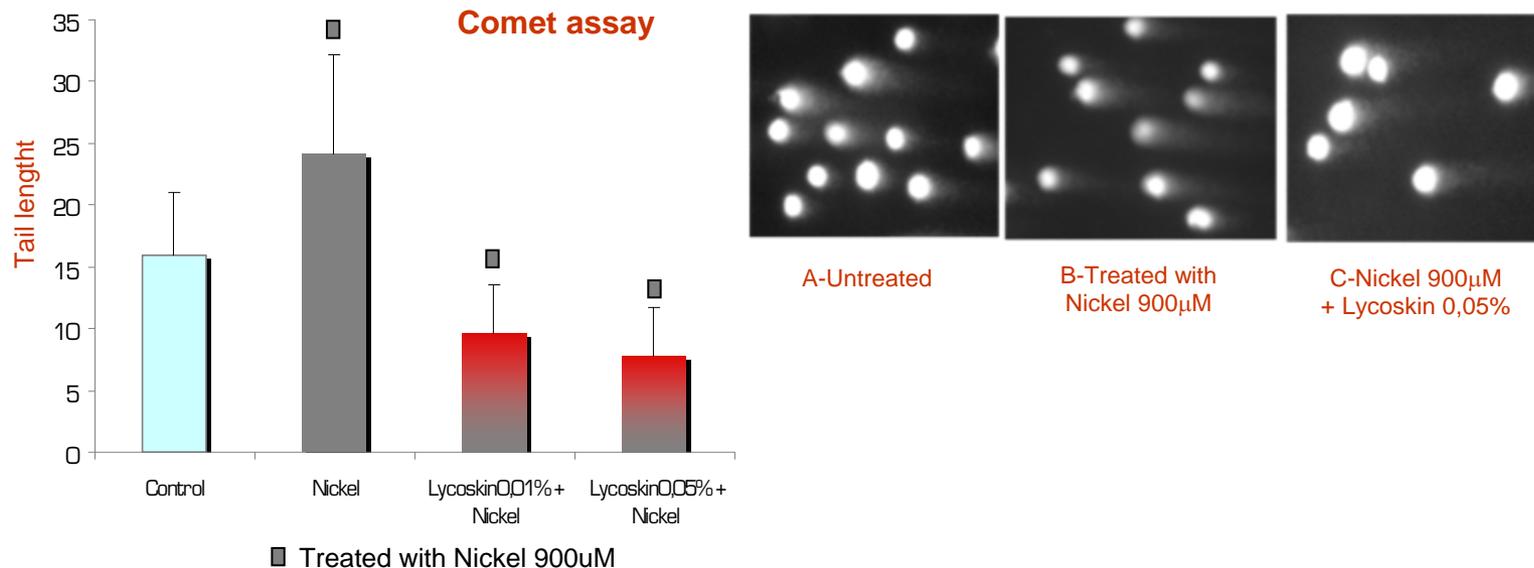
Principle: It is a simple and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. If DNA damage is present, a fluorescent “comet tail” is observed.

Protocol: cultured fibroblasts are incubated for 3 h with different compounds or mixtures and then stressed with Nickel 900 μM for 2.5 h. Cells are then transferred onto agarose pre-coated slides. The slides are placed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base pH 8.5, 1% TritonX100) at 4 °C and then electrophoresed for 20 min. After treatment with cold Neutralization buffer (0.4 M Tris-HCl, pH 7.5) for at least 10 min, the slides are dried and stained with a 10 mg/ml solution of ethidium bromide for fluorescence microscope observation.

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DNA Protection

Besides **ROS** induction, **heavy metals** can cause several **genotoxic effects on skin cells**, which can seriously affect cell gene functionality (Caicedo et al., 2007; Rana, 2008). **The Comet assay** is a simple and sensitive technique for the detection of DNA damage at the level of the individual cell. If DNA damage is present, a long fluorescent “comet tail” is observed.



The length of comet tails induced by metal stress, such as stress from **nickel**, is significantly reduced by **Lycoskin Defence**. This indicates a **good protective effect of nuclear DNA** from metal induced damage.

Lycoskin Defence

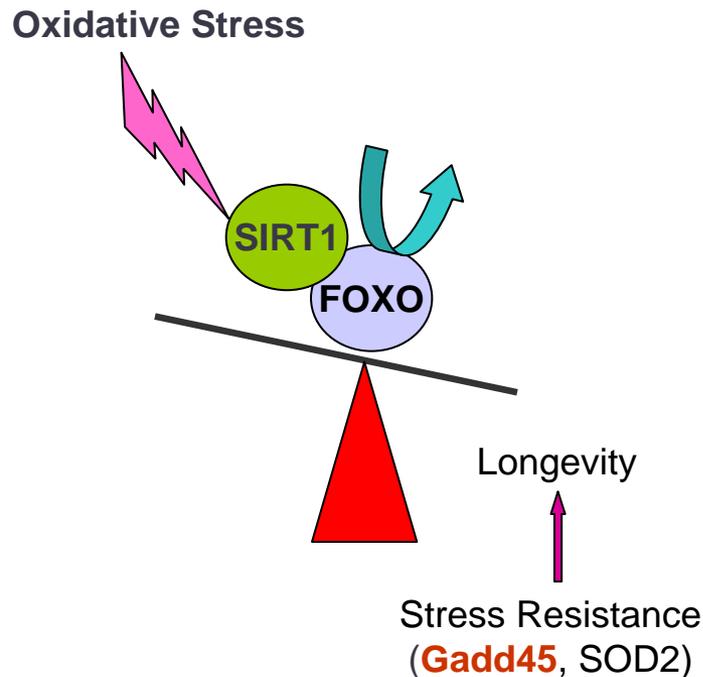
Analysis of GADD45 α , SIRT-1 and MMPs' gene expression

Principle: the expression levels of these genes were analyzed by RT-PCR technique. The expression of GADD45 α , SIRT-1 and MMPs' genes are related, respectively, to DNA repair, longevity and collagen degradation.

Protocol: After treatment of fibroblasts or keratinocytes with compounds or mixtures for 2h, the cells are stressed with nickel 900 μ M or H₂O₂ 150 μ M. At different times, cells are collected and RNA extracted for processing. Starting from RNA, cDNA is synthesized and used as template for PCR amplification using specific primers. The PCR products are loaded on agarose gel, and quantified with the Geliance 200 Imaging system (Perkin Elmer). The amplification band corresponding to the gene analyzed is normalized to the amplification band corresponding to the standard 18S. The values obtained are finally converted into percentage values by setting the measure of the untreated controls as 100%.

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Longevity

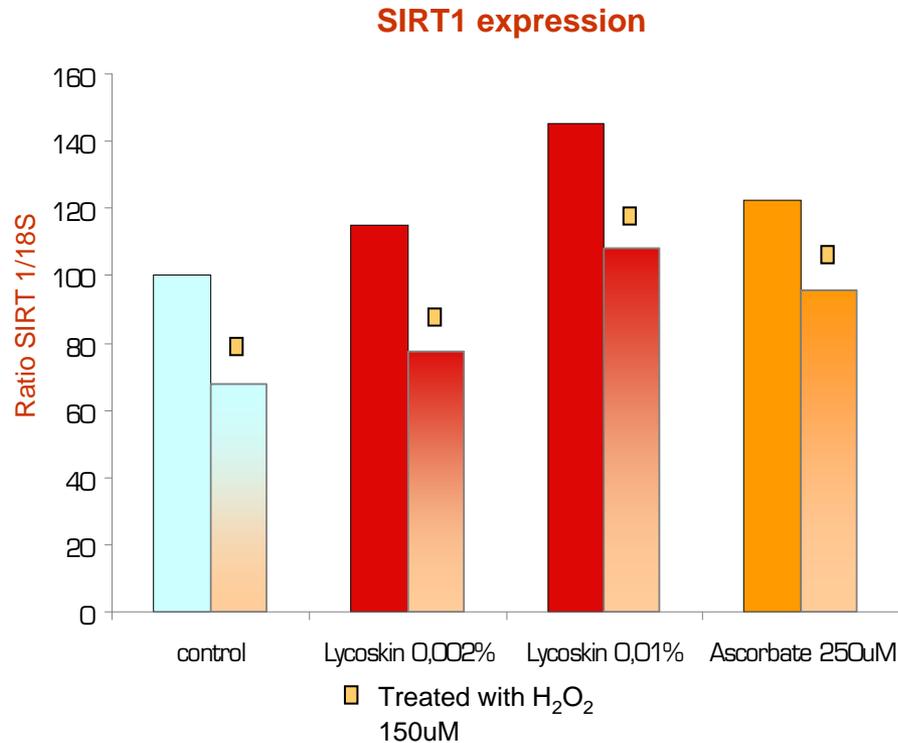


Sirtuins are a family of NAD-dependent protein deacetylases widely distributed in all phyla of life. Accumulating evidence indicates that sirtuins are important regulators of organism longevity. Sirtuins are present in all multicellular organisms and regulate the activity of many transcription factors and DNA binding proteins, acting as nucleus stabilizing factors (Lavu et al., 2008). Recently, some studies demonstrated that SIRT-1, the most characterized member of the protein family, can promote human **cell longevity through an increase of DNA protection** (Zschoernig & Mahlknecht, 2008).

After oxidative stress, **SIRT-1** interacts with the transcription factor FOXO, leading to the activation of antioxidant components and several DNA repairing proteins.

Lycoskin Defence

Longevity

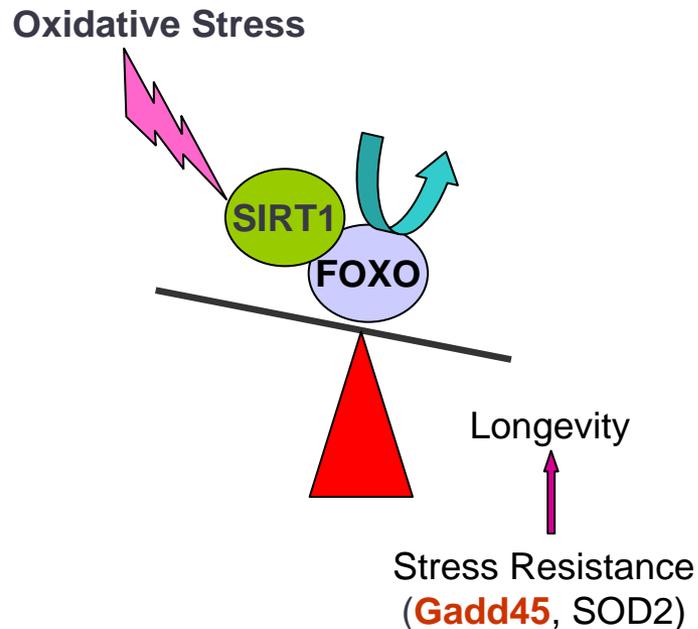


The expression of SIRT-1 is induced by **Lycoskin Defence**, even in the presence of oxidative stress.

At a concentration of 0.01%, **Lycoskin Defence induces SIRT-1** gene expression more than ascorbate, used as positive control for its well-known antioxidant properties.

Lycoskin Defence

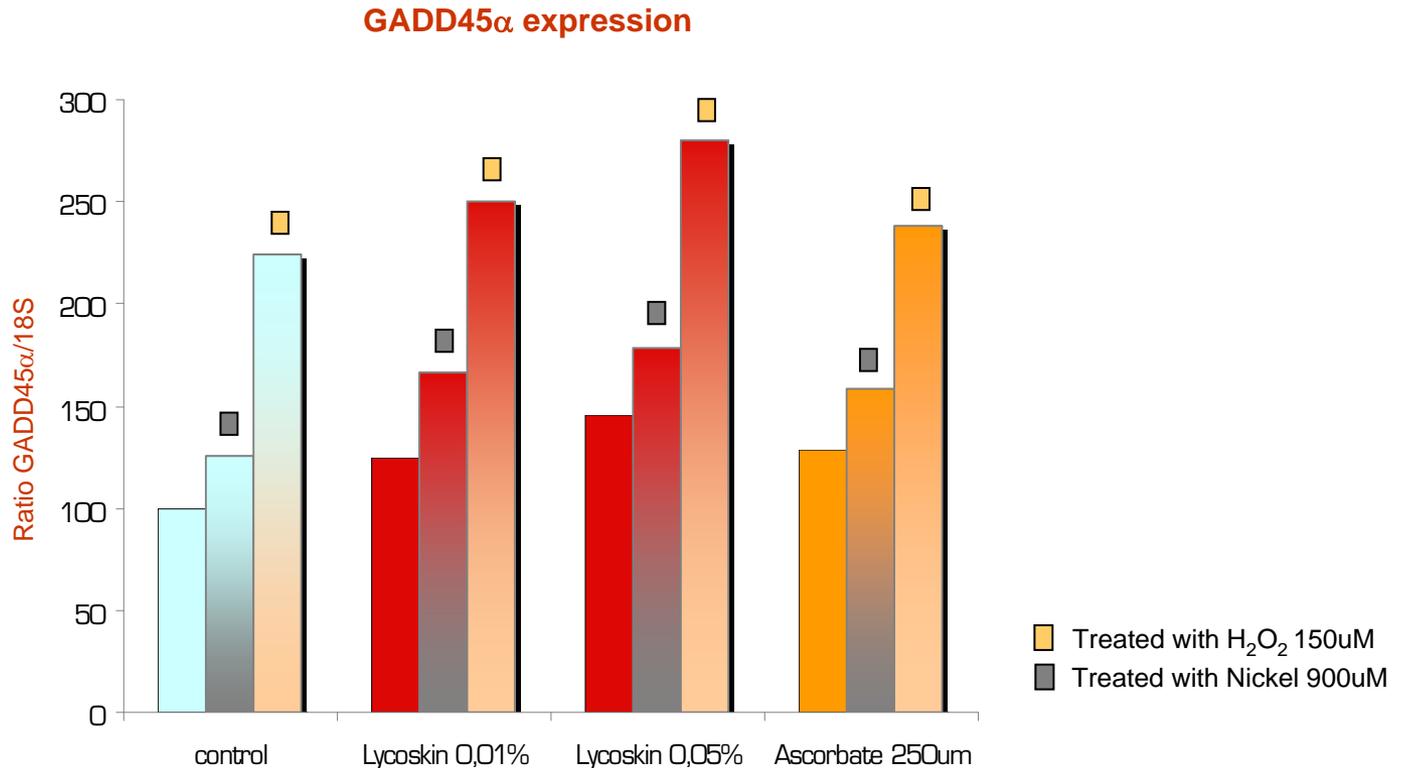
DNA Repair



In the complex scenario that the cell activates to protect its nuclear DNA, there are elements playing pivotal roles in guaranteeing the **integrity of DNA**, and thus the correct functioning of all the cellular processes. GADD45 α is specifically involved in cellular stress response. By the interaction with several other components, **GADD45 α coordinates the transduction mechanisms leading to DNA protection and repair**, cell cycle fulfillment and senescence. High levels of GADD45 α in the cell guarantee good protection from stressing agents which cause nuclear DNA damages (Takekawa et al., 1998).

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DNA Repair

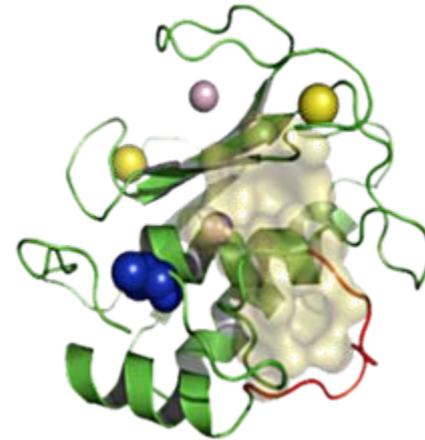


GADD45 α expression level is **significantly higher** in cells treated with **Lycoskin Defence**, whether untreated or in the presence of metal or H₂O₂. This increase is even higher than that produced by ascorbate, used as positive control for its well-known antioxidant properties.

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Matrix metalloproteinases (MMPs) represent a family of zinc-dependent endo-peptidases, capable of digesting extracellular matrix proteins, including collagen, gelatin and stromyelin (Cammarota et al., 2006). The collagenases are capable of degrading triple-helical fibrillar collagens.

A single exposure to **UV irradiation** results in prolonged induction of **collagenase-1** (MMP-1), **stromelysin-1** (MMP-3) and **gelatinase-B** (MMP-9) expression in human skin cells.



Matrix metalloproteinase 1 (interstitial collagenase), also known as MMP1, is a human enzyme which, once secreted, breaks down the interstitial collagens, types I, II and III.

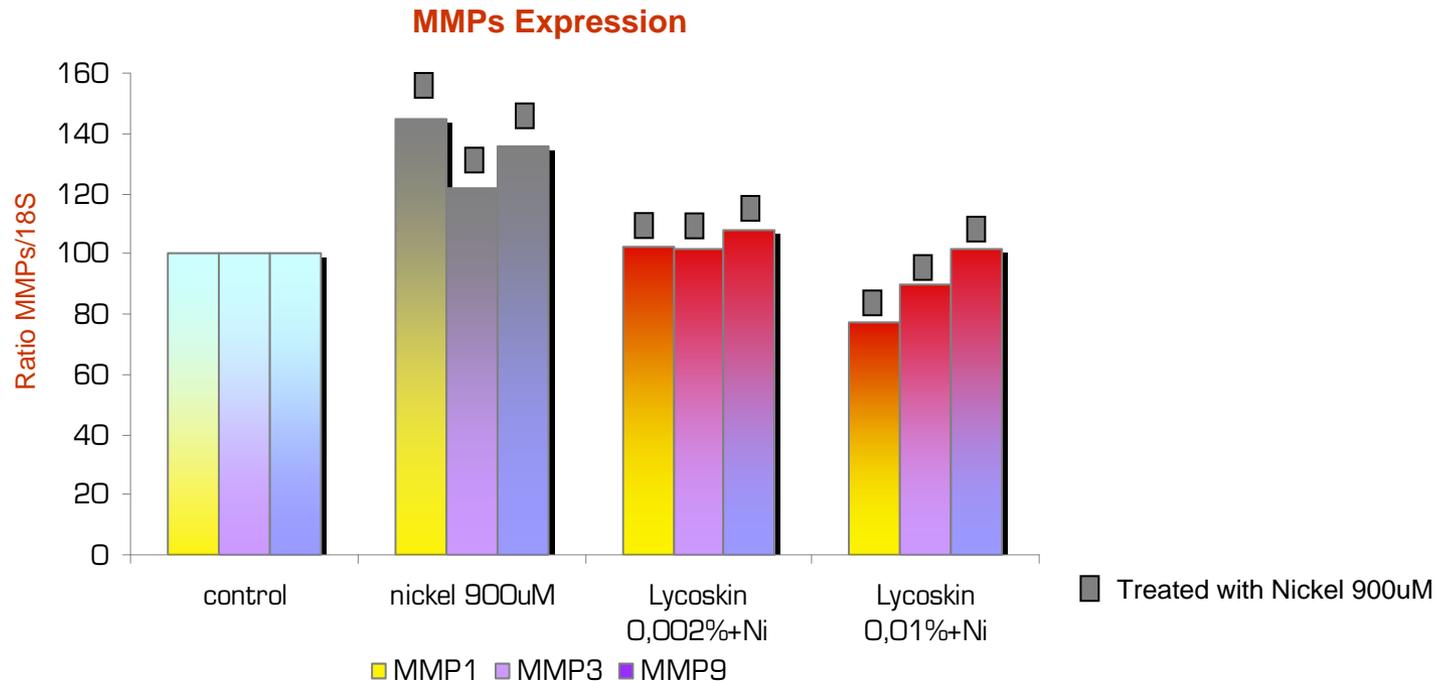
Matrix metalloproteinase 3 (stromelysin 1, progelatinase), also known as MMP3, is a human enzyme which degrades fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans.

Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase), also known as MMP9, is a human enzyme which degrades type IV and V collagens.

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Collagen Degradation

Another dangerous effect caused by metals (in particular nickel) on skin cells is the induction of factors responsible for extracellular matrix degradation, such as **matrix-metalloproteinases (MMP)**.



Lycoskin Defence neutralizes the up-regulation of all three MMP genes caused by nickel.

This indicates that **Lycoskin Defence** can preserve extracellular matrix protein structure, **guaranteeing skin integrity**.

Lycoskin Defence

Collagen Induction assay

Principle: the assay measures the amount of new collagen produced by the cells in response to compounds and mixture treatment. The measure is based on an immunoenzymatic method (ELISA).

Protocol: for collagen expression analysis, fibroblasts are incubated with different concentrations of compounds or mixtures for 20h. Afterwards, the samples are fixed in formaldehyde 4% and incubated with primary goat monoclonal antibodies, raised against collagen type I and type III, dissolved in PBS containing BSA 1%. Two hours later, samples are washed in PBS 3 times, and incubated with anti-goat secondary antibody, HRP labeled. The amount of new collagen produced by the cells is measured by a colourimetric reaction using a 0.5 mg/ml solution of OPD, 0,012 % H₂O₂ in citrate buffer 50 mM.

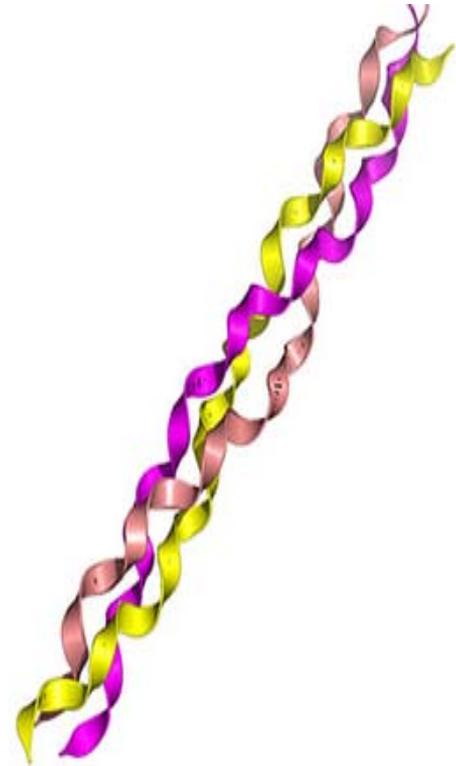
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Collagen

Collagen is the major insoluble fibrous protein in the **extracellular matrix** and in connective tissue. It has great tensile strength, and is responsible for skin elasticity. Its degradation leads to wrinkles that accompany aging.

There are 29 types of collagen, but 80/90% of the collagen in the body consists of **types I, II, and III**. These collagen molecules pack together to form long thin fibrils of similar structure. Collagen is exocytosed in the precursor form (procollagen), which is then cleaved by procollagen proteases to allow extracellular assembly.

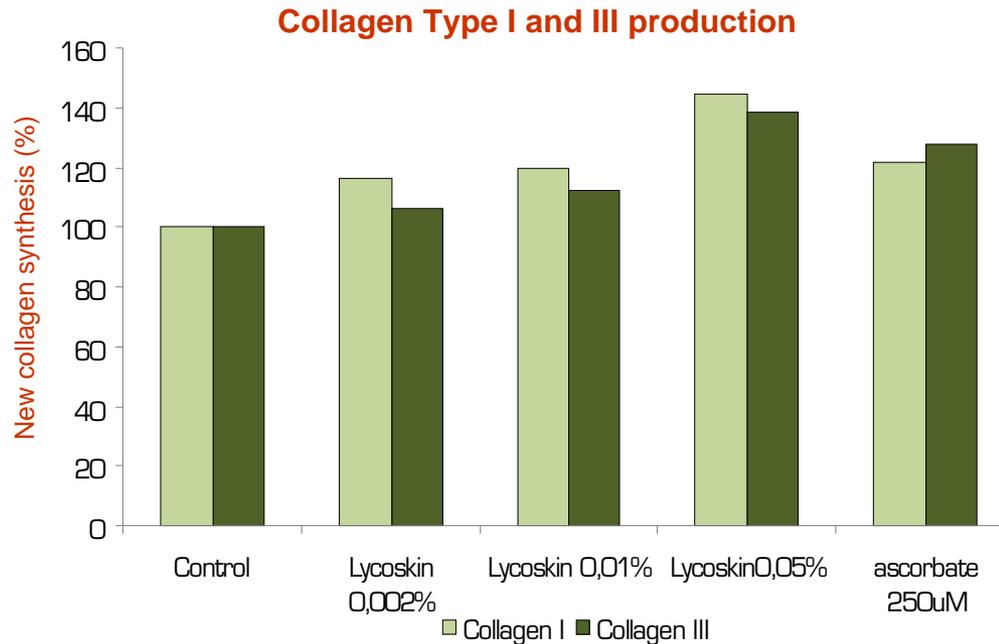
- **Type I collagen** is the **most common** of the collagens in vertebrates. It composes up to 90% of the skeletons of the mammals and is also widespread all over the body: in skin, tendons, ligaments, cornea etc.
- **Type III collagen** is the second most abundant collagen in human tissues and occurs particularly in tissues exhibiting **elastic properties**, such as skin, blood vessels and various internal organs.



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Collagen Induction

Besides its effect on collagen protection and stability, **Lycoskin Defence** also **induces new collagen type I and III** production in cultured fibroblasts.



At a concentration of 0.05%, this induction (**43% for collagen I and 38% for collagen III**) is even higher than that produced by the well-known antioxidant agent, ascorbate.

A close-up photograph of several ripe, red tomatoes with green stems, filling the top portion of the slide.

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CLAIMS and BENEFITS

Lycoskin Defence

Benefits

“In vitro” and “in cell” tests have shown that Lycoskin Defence:

- has heavy metal chelating and detoxifying capacity
- has strong antioxidant power
- has high phytochelatin content
- neutralizes the up-regulation caused by nickel of all three MMP genes
- Decreases the amount of intracellular ROS induced by metal treatment
- Has a protective effect on nuclear DNA towards metal-induced damage
- has DNA repair effect by inducing GADD45 α gene expression, thus guaranteeing good protection from stressing agents, which cause nuclear DNA damages
- induces SIRT-1 gene expression; SIRT-1 is a protein that promotes human cell longevity through an increase in DNA protection
- induces new production of collagen type I and III

Lycoskin Defence

Claim suggestions

- Protects skin from city pollution due to heavy metals
- Protects skin from premature ageing
- Protects skin from damages due to free radicals
- Extends cellular vitality and longevity, also under heavy metal stress
- Helps protect and preserve the skin genome's integrity
- Reinforces the Extra Cellular Matrix

A close-up photograph of several ripe, red tomatoes. The tomatoes are the primary focus, with their smooth, glossy skin and vibrant red color. The background is slightly blurred, showing more tomatoes and some green foliage.

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TOXICOLOGICAL and REGULATORY DATA

Lycoskin Defence

Toxicological profile

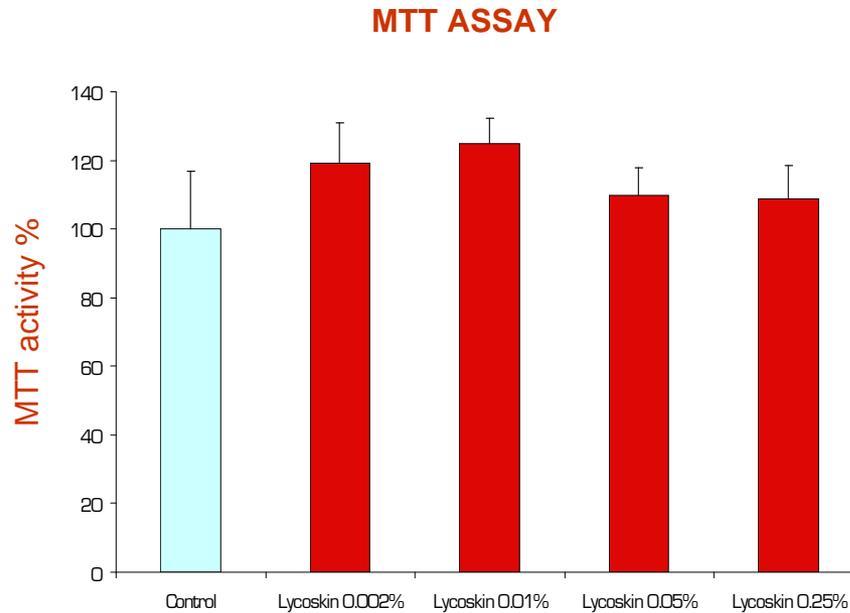
CYTOTOXICITY

MTT ASSAY

MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilised. The number of surviving cells is directly proportional to the level of the formazan crystals created. The colour can then be quantified using a simple colourimetric assay. The results can be read on a multiwell scanning spectrophotometer (ELISA reader).

Lycoskin Defence

Toxicological profile



NIH3T3 cells have been treated with Lycoskin Defence for 12h, before the addition of MTT

Lycoskin Defence has no toxic effect on cells

Lycoskin Defence

Toxicological profile

Cytotoxicity:	MTT Assay
Patch-test:	in humans (20 volunteers)
Evaluations:	Expert Certificate

Lycoskin Defence

Regulatory data

Trade name:	Lycoskin Defence
INCI name:	<i>Lycopersicon esculentum</i> Leaf Cell Culture (and) Glycerin (and) Water
CAS number:	90131-63-8 / 56-81-5/ 7732-18-5
Patent :	MI2009A 002134



Lycoskin Defence

FORMULATION GUIDELINES

Lycoskin Defence

Formulation guidelines

GENERAL RECOMMENDATIONS

Solubility:

Fully soluble in aqueous medium
Soluble in glycerin and ethanol

Stability:

Stable between pH 4.0 and pH 8.0

Incorporation method:

Add at the end of the formulation
Can withstand temperatures up to 70°C

Lycoskin Defence

Formulation guidelines

COSMETIC FORM	COMPATIBILITY
Aqueous Toner	Yes
Hydro-alcoholic Toner	Yes
Transparent Gel	Yes
Opaque Gel	Yes
Transparent Foaming Product	Yes
Non Ionic O/W Emulsion	Yes
Anionic O/W Emulsion	Yes
Cationic O/W Emulsion	Yes
W/O Emulsion	Yes
Oily Fluid	No
Anhydrous Balm	No

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Formulation guidelines

RICH SERUM

INGREDIENTS	DOSE %
I Water	to 100
Disodium EDTA	0.100
Glycerin / Butylene Glycol	6.500
Xanthan Gum	0.200
Carbomer	0.200
Potassium Cetyl Phosphate	0.250
II Glyceryl stearate (and) PEG-100 Stearate	1.500
Cetyl Alcohol	1.000
Vegetable oil	1.000
Coco-Caprylate/Caprates/ Ethylhexyl Palmitate/ Octyldodecanol	10.000
Antioxidant	0.020
Dimethicone	2.000
III Sodium Hydroxide	to pH 6.5
IV Preservatives	Q.S.
V LYCOSKIN DEFENCE	0.500

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Formulation guidelines

HYDRATING CREAM

INGREDIENTS	DOSE %
I Cetearyl Alcohol	2.500
Hydrogenated Olive Oil (and) Olea Europaea Fruit Oil (and) Olea Europaea Oil Unsaponifiables / Shea Butter	4.500
Caprylic/Capric Triglyceride/Isononyl Isononanoate/ Helianthus Annuus (Sunflower) Seed Oil	12.000
Vitamin E Acetate	0.200
Preservatives	Q.S.
II Water	to 100
Butylene Glycol/ Glycerin	6.500
Xanthan Gum	0.250
ROSEFLOR 22 HYP Glyceryl Stearate (and) Cetearyl Alcohol (and) Potassium Behenoyl Hydroxyproline (and) Water (and) Behenic Acid (and) Phenoxyethanol (and) Rosa Centifolia Flower Extract	6.500
Acrylates/C10-30 Alkyl Acrylate Crosspolymer	0.200
III Sodium Hydroxide	to pH 6.0
IV LYCOSKIN DEFENCE	0.500

Most Relevant Publications of the Research Group

Our research group has a well established experience in plant stem cell biotechnology, plant signal transduction and plant-derived active compounds:

- 1) Apone et al. (2009). Mixture of peptides and sugars derived from plant cell walls increases plant defence responses to stress and attenuates ageing-associated molecular changes in cultured skin cells. *Journal of Biotechnology*, Dec 16.
- 2) Lantchheva et al. (2004). Primary asymmetric division and embryo formation in a single cell suspension of embryogenic *Medicago falcata*. *Biotechnol and Biotech Equip*, 18/3, 27-33.
- 3) Apone et al. (2003). The G-Protein-Coupled Receptor GCR1 regulates DNA synthesis through activation of phosphatidylinositol-specific phospholipase C. *Plant Phys*, 133(2):571-9.
- 4) Colucci et al. (2002). GCR1, the putative Arabidopsis G -protein coupled receptor gene is cell cycle regulated, and its overexpression abolishes seed dormancy and shorten time to flowering. *Proc Natl Acad Sci U S A*, 99 (7) 4736-4741.
- 5) Moore et al. (2000). A new lectin in red kidney beans called PvFRIL stimulates proliferation of NIH3T3 cells expressing the Flt3 receptor. *Biochim Biophys Acta* 26;1475(3):216-224..
- 6) Colucci et al. (1996). cDNA cloning of FRIL, a mannose binding lectin from *Dolichos lablab*, that preserves hematopoietic progenitors in suspension culture. *Proc Natl Acad Sci U S A* (2):646-650.
- 7) Apone et al. (2009). A mixture of peptides and sugars derived from plant cell walls increases plant defense responses to stress and attenuates ageing-associated molecular changes in cultured skin cells. *Journal of Biotechnology*, 145: 367-376.

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