UpRegulex® – Sericin Peptides from Silk Protein

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Abstract

Sericin is the short chain molecule that acts as the "glue" that provides silk fibres with their tensile strength and stability. Our research has developed peptide fractions of these molecules that exhibit long term anti-ageing benefits. The beneficial changes in gene expression are presented. Further studies, both *in vitro* and *in vivo* have also been conducted. Together, the data presents a convincing case for the use of these peptides in anti-ageing products as well a potential for use in scar reduction technologies.

Introduction

Silk protein, the structural component of the silkworm cocoon, consists of two major fractions, fibroin and sericin. Fibroin is the large molecular weight fibrous protein consisting of a core of heavy and light chains linked together with a sericin glycoprotein fraction via disulphide bonds. This protein configuration imparts tensile strength to fibroin. This protein is valued in numerous industrial applications for its water absorbency, thermotolerance, insulation, dyeing affinity and lustre. However, with reference to the topical treatment of human skin, the glycoprotein sericin has recently become the more important fraction of silk protein. Found in sutures, wound dressing, cell culture mediums, skin moisturizers, hair products and, more recently, anti-cancer applications, its unique, non-toxic biocompatibility with human skin tissues has prompted its global use in the cosmetic industry [(1)].

Sericin is a family of adhesive glycoprotein consisting of at least four chemically distinct but structurally similar protein moieties that make up approximately thirty percent of the total protein in a silkworm cocoon. They act together to form an occlusive glue-like gel in the silkworm cocoon, sealing the fibroin sac from the outer environment. Sericin glycoprotein is water-soluble as they contain a large percentage of hydrophilic amino acids, in particular serine. Sericin is highly tolerant to heat, chemical modification and hydrolysis due to solubility, amino acid composition and three-dimensional structure (partial *beta* sheet) [(1)].

Previous Biological Studies on Sericin Protein

As it bears a compositional resemblance with natural moisturizing factor (NMF), which is also abundant in serine, the moisturizing benefits of sericin have been previously studied. Results have shown that topical application of sericin leads to a decrease in skin impedance and increased hydration of epidermal cells [(2)]. The photoprotective effect of sericin as an antioxidant protein on UVB-induced acute damage in mouse skin via reduction of oxidative stress and COX-2 activity has also been demonstrated [(3)]. Furthermore, protection against tumour production (in the 7,12-dimethylbenz (alpha) anthracene (DMBA)-initiated and 12-O-tetradecanoylphorbol 13-acetate (TPA)-promoted mouse skin tumourogenesis model) has been postulated and results suggest a suppression of oxidative stress, inflammatory responses and TNF-alpha activity [(4)]. The anti-apoptotic effect of sericin in a UVB (30 mJ/cm2)-irradiated human keratinocyte model was also studied and pre-treatment with sericin was reported to suppress bax expression, up-regulate the expression of bcl-2, and prevent the activation of caspase-3 [(5)]. In an UVB-treated keratinocyte model, both the inhibition of tyrosinase activity and the inhibition of intracellular hydrogen peroxide generation were induced through pre-treatment with sericin, suggesting that sericin probably prevents mitochondrial damage [(6)].

UpRegulex® – Hydrolyzed Sericin in an Enhanced MLV Delivery System

To expand our understanding of the beneficial effects of sericin protein on human skin, a proprietary hydrolyzed fraction of the protein, UpRegulex® (INCI name: Water (and) Butylene Glycol (and) Phospholipids (and) Hydrolyzed Sericin) was developed and further tested. As the hydrophilicity of the peptide fraction leads one to predict inefficient permeation across the *stratum corneum*, the sericin peptides were encapsulated in an enhanced multiplayer vesicle lipid dispersion. It should be noted that this lipid dispersion has been previously shown to possess its own photoprotective benefits to the skin. Therefore, the data presented herein reflect the combination of Sericin – MLV effects and not those of sericin alone. The product is available from the co-authors' company. Initially, the peptide – MLV fraction was evaluated using DNA microarray technology on full thickness epidermal skin substitutes. The results were then analyzed and used to develop other assays to determine if those effects of the sericin – MLV complex predicted from the microarray results were realized.

DNA Microarray Analysis

Using Mattek full thickness epidermal skin tissue substitutes, UpRegulex® was incubated for 48 hours at a final concentration of 0.5%. From the cultures, the resulting mRNA was extracted and analyzed in the usual manner as has been previously reported (protocol available upon request). Using a "norm" of a change in mRNA concentration (increase or decrease) of 30% as a standard measure of significance (that is to say that a change in gene expression of 30% must occur to be a significant effect), 117 genes were upregulated and 28 genes downregulated by the addition of the extract. Analysis of those genes affected was done using DAVID Bioinformatics Resources 2008 [7,8]. A short explanation of the metabolic purpose of each of those genes listed (as provided by in reference by the gene analysis database system) is included for review. As individual references for each of the citations would extend the length of this publication beyond its scope, we ask for the liberty to include the references by citation to the database itself. Individual references are available upon request. Results show that the Sericin – MLV complex had significant effects on three particular biological systems, 1) the cell cycle, 2) eicosanoid metabolism and 3) apoptosis.

Cell Cycle Effects

The most prevalent effect of the extract was on the cell cycle (10 genes upregulated). In particular, the effect was observed to be on the ubiquitination mediated proteolysis pathway as well as important checkpoints in the cell cycle. For the most part, genes involving the G1 to S phase switch in the cell cycle were affected. Results indicate that UpRegulex® acts as a proliferative signal to tissues.

A summary of the activity of each of those genes affected in the cell cycle
The origin recognition complex (ORC) Subunit 1 (inducible) – ORC is a highly conserved six subunit protein complex essential for the initiation of the DNA replication in eukaryotic cells and serves as a platform for the assembly of additional initiation factors to complete the DNA recognition complex essential for DNA replication during S phase. While other ORC subunits are stable throughout the cell cycle, the levels of this protein vary during the cell cycle and thus, the protein encoded by this gene is the regulatory subunit of the ORC complex. Concentration is mediated by ubiquitin-mediated proteolysis after initiation of DNA replication. Activity is selectively inactivated by phosphorylation during M phase. It is also involved in transcription silencing during replication to ensure integrity to the replication process with MYST histone acetyltransferase 2 (MyST2/HBO1).
CDC6 Involved in the initiation of DNA replication, this protein also forms the DNA pre-recognition complex with ORC and activates S phase and DNA replication. It also participates in checkpoint controls that ensure DNA replication is completed before mitosis is initiated and ubiqutin-mediated proteolysis and deactivation of S phase activators occurs.
POLO Kinase 1 This protein is required for cell division. The concentration of this protein accumulates during S phase to a maximum during the G2 and M phases, declines to a nearly undetectable level following mitosis and throughout G1 phase. Induction of this gene occurs only in the presence of growth-stimulating agents.
S Phase Kinase Associated Protein 2 (p45) The substrate recognition component of the SCF (SKP1-CUL1-F- box protein) E3 ubiquitin ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins involved in cell cycle progression, signal transduction and transcription. In particular, this protein is upregulated in order to specifically recognize and initiate degradation of p27, a potent cell cycle inhibitor of the G1 to S phase switch. It allows recognition of the phosphorylated CDKN1B/p27kip complex and causes its removal from the cell to regulate of G1/S transition.
CDC20 A regulatory protein interacting with several other proteins at multiple points in the cell cycle. Synthesis is initiated at G1/S, protein level peaks in M phase and protein is abruptly degraded at M/G1 transition. It localizes in cell nucleus during cell cycle G1, but translocates to the cytoplasm at the start of S phase, a process regulated through its phosphorylation by Cdks, in particular maturation promoting factor (MPF). In G1 to S phase transitions, it is involved in both the initiation of DNA replication as well as checkpoint controls that ensure DNA replication is completed before mitosis is initiated. This protein further acts primarily to activate proteolysis of M phase cyclins and trigger anaphase in a dividing cell. Required for full ubiquitin ligase activity of the anaphase promoting complex/cyclosome (APC/C) and may confer substrate specify upon the complex, thus driving the cell through M phase. It is required for two microtubule-dependent processes, nuclear movement prior to anaphase and chromosome separation.
BUB1 Acts in M phase as a component of the mitotic checkpoint that delays anaphase until all chromosomes are properly attached to the mitotic spindle.

A summary of the activity of each of those genes affected in the cell cycle
Cyclin A2 Functions as a regulator of CDK kinases. It exhibit distinct expression and degradation patterns that contribute to the temporal coordination of each mitotic event. It accumulates steadily during G2 and is abruptly destroyed at mitosis. This cyclin binds and activates CDC2 or CDK2 kinases to form serine/threonine kinase holoenzyme complex imparting substrate specificity to the complex. Thus it is essential for the control of the cell cycle at the G1/S (start) and the G2/M (mitosis) transitions.
Cyclin B2 The protein encoded by this gene also functions as a regulator of CDK kinases. Again, different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. It accumulates steadily during G2 and is abruptly destroyed at mitosis. It is essential for the control of the cell cycle at the G2/M (mitosis) transition. Interacts with the CDC2 protein kinase to form a serine/threonine kinase holoenzyme complex also known as maturation promoting factor (MPF). The cyclin subunit imparts substrate specificity to the complex. Also interacts with transforming growth factor beta RII. Thus, cyclin B2/cdc2 plays a key role in transforming growth factor beta-mediated cell cycle control.
TGF-beta 2 Normally, this cytokine is considered as a growth inhibitor in numerous tissues. In human skin, the production of this cytokine has an anti-proliferative / differentiation promotion effect on keratinocytes. The receptor is present in ample quantities in fibroblasts and interaction of the receptor with the cytokine is beneficial, initiating cell migration, the rate-limiting event in skin wound healing (J Cell Biol. 2006 Mar 27;172(7):1093-105. Epub 2006 Mar 20). Thus, the effect of TGF beta 2 induction is likely terminal differentiation of the *stratum corneum* and / or dermal cell migration (in wound or scar formation events).

Eicosanoid Metabolism

A second system affected by the extract is that of eicosanoid metabolism where two significant genes, both receptors for PGE2 were upregulated. It should be understood that this does imply decrease or increase of inflammatory potential. It has been shown to mediate PGE2 induced expression of early growth response 1 (EGR1), regulate the level and stability of cyclooxygenase-2 mRNA, and lead to the phosphorylation of glycogen synthase kinase-3. Knockout studies in mice suggest that this receptor may be involved in the initiation of skin immune responses. But these activities should be viewed as potentially upgrading the immune system to better deal with an environmental insult. Further, the activity of this membrane bound receptor is mediated by G(s) proteins that stimulate adenylate cyclase and exhibits a relaxing effect on smooth muscle. Only four receptors exist to accept and react with PGE2, lending significance to the effect.

Resistance to Oxidative Stress

A third system affected was that of oxidative stress, alleviated by the up-regulation of MgSOD, a member of the iron/manganese superoxide dismutase family. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. Although this gene has a typical housekeeping gene promoter, it is highly inducible by various physical, chemical, and biological agents.

Reactive oxygen species are required for cell proliferation but can also induce apoptosis. In proliferating cells this paradox is solved by the activation of protein kinase B (PKB; also known as c-Akt), which protects cells from apoptosis. By contrast, in quiescent cells (that lack PKB activity) an alternative mechanism is induced as a consequence of PKB inactivity. This mechanism entails the activation of Forkhead transcription factors, the direct transcriptional activation of MnSOD and the subsequent reduction of reactive oxygen species. It has recently been concluded that the induction of this enzyme decreases with ageing. It is currently thought that increased resistance to oxidative stress is associated with longevity. Although this model originates from the genetic analysis of Caenorhabditis elegans, this concept has been extended to mammalian systems.

TRAIL (TNF-related apoptosis-inducing ligand) is a novel member of TNF superfamily that induces apoptosis in transformed cell lines of diverse origin, was significantly downregulated. Skin expresses a functional form of TRAIL.

Other genes of interest positively affected by the sericin complex are hyaluronidase and MMP8 (downregulated) and cytochrome P450 and collagens XI, XIV and XV (upregulated).

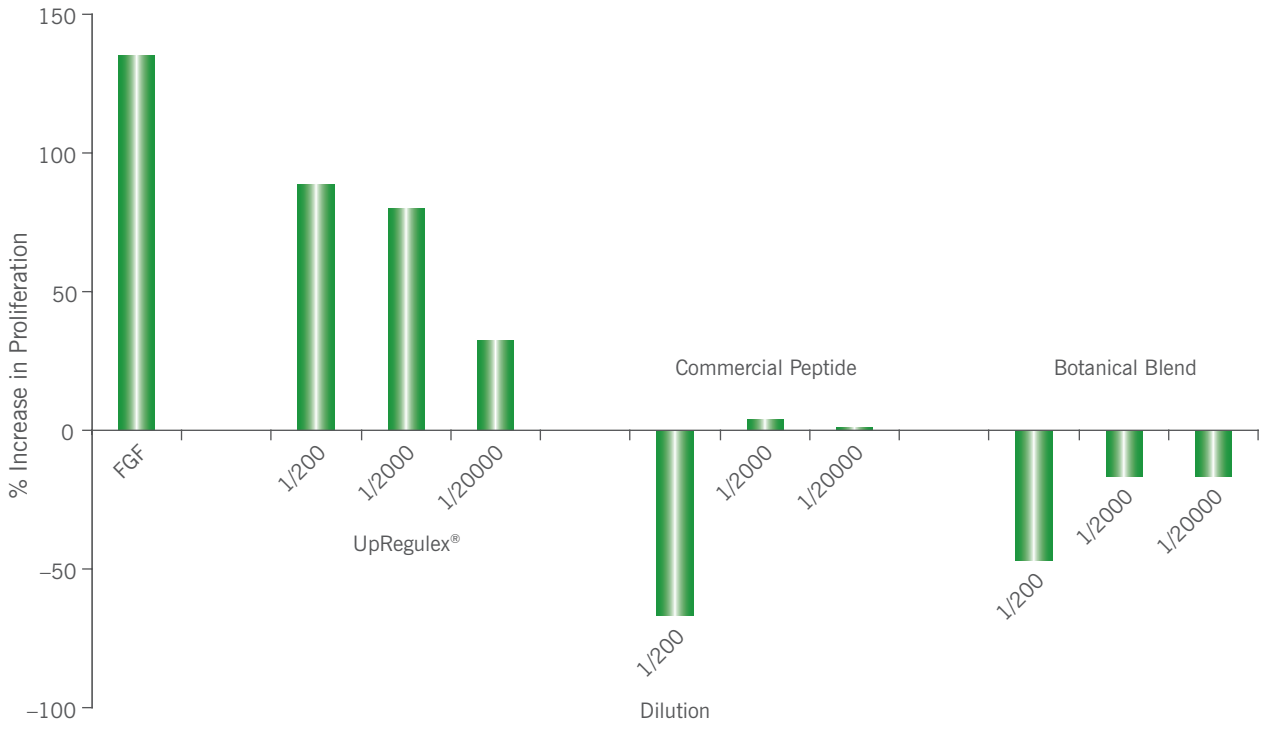
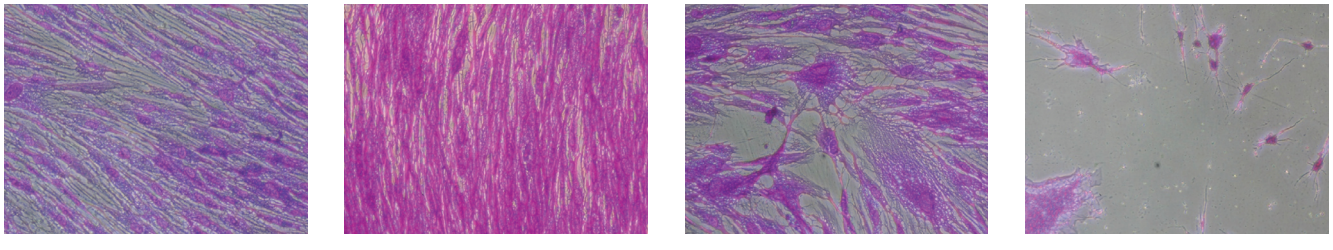


Figure 1 – UpRegulex®: Stimulation of Dermal Fibroblast Proliferation

In vitro Testing

Proliferation – The positive effects on the cell cycle observed in the DNA microarray results indicate that UpRegulex® may act as a stimulatory signal for the proliferation of skin cells. Tests were conducted according to published protocols [9] using human dermal fibroblasts to determine if the sericin – MLV complex stimulated proliferation. At concentrations tested, UpRegulex® not only was found to be non-toxic to the cell cultures but, in fact, stimulated cell proliferation. Interestingly, this effect was not observed using reference collagen 1 stimulators (a leading commercial botanical blend and a leading commercial peptide) (see Figure 1). UpRegulex® also improved cell morphology (sulforhodamine stain of human dermal fibroblast cultures) as shown in Figure 2.

Type 1 collagen Stimulation – Additional tests were conducted according to published ELISA protocols [10, 11] using Collagen 1, a major component of the dermal extracellular matrix, as a marker for dermal tissue generation. As no direct stimulation of collagen 1 by the addition of UpRegulex®was detected in the microarray, increased collagen 1 formation via UpRegulex® application would dictate that dermal growth occurs as a result of the application of the peptide complex. UpRegulex stimulated collagen 1 formation at all concentrations tested and, at some concentrations, was comparable in activity to basic fibroblast



Control | UpRegulex® | Commercial Peptide | Botanical Blend

Figure 2 – UpRegulex®: Improvement in Cell Morphology

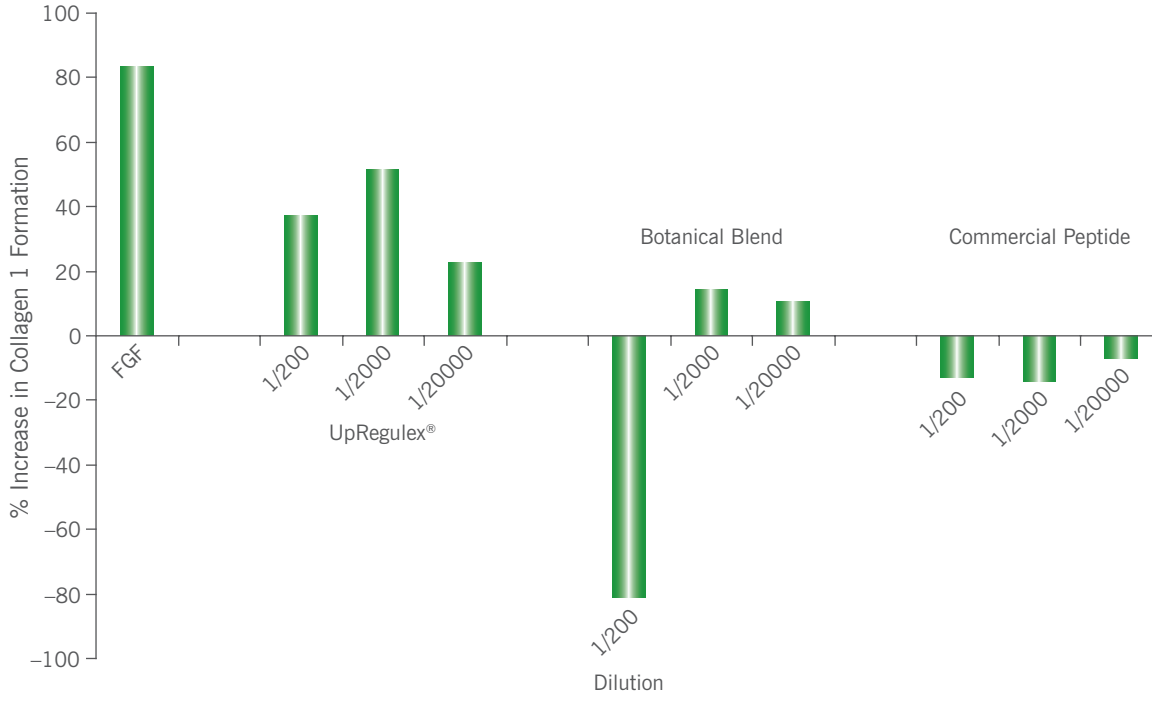


Figure 3 – UpRegulex®: type 1 collagen Stimulation

growth factor (bFGF). In the same test, UpRegulex compared favorably to a leading commercial botanical blend as well as to a leading commercial peptide previously reported to stimulate the production of collagen 1 (see Figure 3).

Hyaluronic Acid – Hyaluronic acid (hyaluronan, HA), is a key component of connective, epithelial and neural tissues. In the skin, this carbohydrate polymer determines the hydration and thickness of the dermal layer. As it production decelerates with age, so does the hydration and thickness of the skin. Results from the DNA microarray showed the downregulation of the gene coding for hyaluronidase, the enzyme that digests hyaluronic acid in the skin. Tests using the measurement of hyaluronic acid concentration were conducted to determine if the UpRegulex® peptide complex can increase the amount of this desirable skin component. Adult human dermal fibroblasts were plated in high glucose DMEM supplemented with 2.5% cosmic serum at 10,000 cells per well. UpRegulex® was tested at a previously determined optimal concentration of 500ug/ml.

For comparative reasons, UpRegulex® was compared to Retinol in the assay. Retinol was first tested for cytotoxicity (maximal DMSO concentration in cell medium: 0.1%). Based on the results from that experiment, two concentrations were selected for testing the effect of retinol on hyaluronic acid concentration: 20ug/ml and 2ug/ml. After 48h, cell culture conditioned media were collected and 100ul samples were used for the HA assay in quadruplicates. HA assay was performed using Hyaluronan Enzyme-Linked Immunosorbent Assay Kit (HA-ELISA). Results are reported as total hyaluronic acid concentration (ug/ml) with respect to a control (H2O added). Results indicate that hyaluronic acid content was increased in cultures where UpRegulex® was applied in comparison to controls (See Figure 3). Retinol was also tested in the same manner and was shown to have no effect on hyaluronic acid concentration.

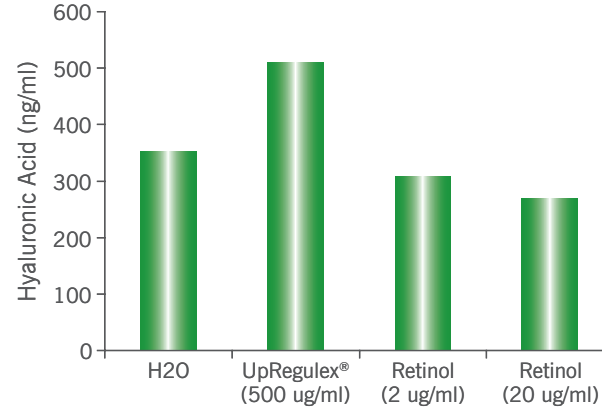


Figure 4 – UpRegulex®: Increased Hyaluronic Acid Concentration in Dermal Fibroblast Cultures

Tyrosinase Inhibition – As stated earlier, sericin protein was found to inhibit tyrosinase activity. Tests were conducted using previously published protocols for a tyrosinase inhibition assay (6). It was determined that hydrolyzed sericin peptides maintained their inhibitory properties (see Figure 4). Arbutin and magnesium ascorbyl-phosphate were also tested in the same manner. Arbutin exhibited only moderate inhibitory activity while no inhibition (and in fact an increase) of tyrosinase activity due to the addition of the acsorbate derivative was observed.

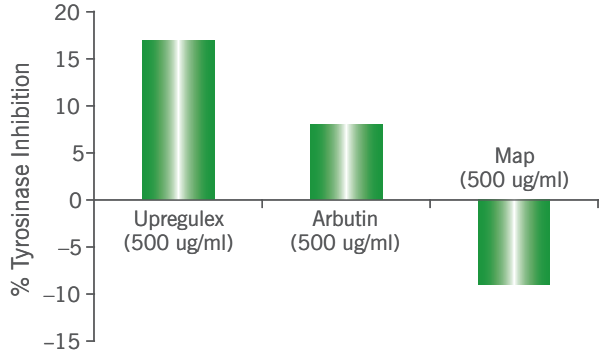


Figure 5 – UpRegulex®: Tyrosinase Inhibition

Conclusion

DNA microarray analysis showed a surprising and positive effect of the hydrolyzed silk peptide sericin preparation UpRegulex® on numerous regulatory genes in the cell cycle. Predictably, the upregulation of those genes would possibly act as a stimulatory signal that may drive the cells through the cell cycle initiating growth while still maintaining DNA integrity. These results were partially confirmed by the stimulation of proliferative growth observed in fibroblast cultures. Subsequent *in vivo* testing showed that the peptide / MLV complex increased levels of collagen 1 and hyaluronic acid. Given these results, UpRegulex® could be a valued addition to products for scar reduction, wound healing and tissue regeneration.

The observed upregulation of the genes involved in eicosanoid metabolism may, at first, seem to be a drawback to product usage with regard to inflammatory reactions. But the particular genes induced in eicosanoid metabolism are more involved in smooth muscle relaxation, which would, predictably, lead to the possibility of wrinkle reduction in topical application. Previous reports on the anti-apoptotic activity of sericin were also confirmed by the upregulation of MgSOD and the downregulation of TRAIL. Both of these genes are involved in the improvement of mitochondrial function and longevity. The inhibition of tyrosinase activity may also improve skin tone and texture. Taken together, UpRegulex® emerges as a unique product with a complementary array of anti-ageing activities.

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Author's Biographies

Glen Gillis, Ph.D. Dr Gillis is the founder and CEO of PhytoChemical Research Associates. Previously, he has directed botanical research, product development and regulatory/safety in the cosmetic industry for over a decade. He now conducts botanical/chemical research in both academic and commercial research laboratories for nutritional, industrial and cosmetic applications. He has authored over 20 patents, posters and scientific papers.

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