In vitro evaluation of the safety profile of cosmeceuticals containing growth factors and their analogues

Avaliação in vitro do perfil de segurança de cosmecêuticos contendo fatores de crescimento e seus análogos

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ABSTRACT

Introduction: Aging skin is a condition that affects (or will affect) all people, and its treatment is considered a clinical challenge. Growth factors and their analogues are emerging as a promising therapeutic option.
Objectives: To evaluate the safety profile of some dermocosmetic products with formulations based on growth factors – or their analogs intended for that purpose – using in vitro human skin cell culture models.
Methods: Two types of cell cultures were studied, and the effects of the study products on the proliferation of melanoma cells and normal human fibroblasts were evaluated.
Results: No significant morphological alterations were found in the cultured human melanoma, and no significant decrease in the number of healthy cells was verified in the normal fibroblasts culture. In some cases there was even a proliferation of those cells.
Conclusions: These preliminary data demonstrate that cosmeceutical products containing growth factors as an active principle can be considered safe for topical application.
Keywords: intercellular signaling peptides and proteins; sking aging; skin.

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RESUMO

Introdução: Envelhecimento cutâneo é afecção que atinge ou atingirá todas as pessoas, e seu tratamento representa desafio clínico. Os fatores de crescimento e seus análogos surgem como promissora opção terapêutica.
Objetivos: avaliar o perfil de segurança de alguns produtos dermocosméticos à base de fatores de crescimento ou seus análogos destinados a esse fim, utilizando modelos in vitro de cultura de células da pele humana.
Métodos: foram estudados dois tipos de culturas celulares, com a avaliação dos efeitos dos produtos teste sobre a proliferação de células tumorais (células de melanoma) e sobre a proliferação de fibroblastos humanos normais.
Resultados: não foram encontradas alterações morfológicas significativas nas culturas de melanoma humano e não houve alterações significativas no número de células saudáveis, pelo menos na cultura de fibroblastos normais, tendo mesmo havido em alguns deles a proliferação dessas células.
Conclusões: Esses dados preliminares demonstram que os produtos cosmecêuticos que contêm fatores de crescimento como ativos principais podem ser considerados seguros para aplicação tópica.
Palavras-chave: peptídeos e proteínas de sinalização intercelular; envelhecimento da pele; pele.

INTRODUÇÃO

The skin is the human body's largest organ, and plays an important role in protecting it against environmental aggressions and preventing the loss of essential body fluids, the invasion of toxins, microorganisms, and excessive amounts of ultraviolet (UV) radiation. It also protects against electrical currents, mechanical forces, and differences in temperature.[1]

In the last few decades, many authors have claimed that the skin, particularly the epidermis and dermis, presents intense metabolic and endocrinologic activity.[2] Large quantities of hormones and neurotransmitters (including parathyroid hormone, pro-opiomelanocortin-derived peptides, melanocyte stimulating hormone, adrenocorticotropic hormone, beta-endorphins and enkephalins, corticotropins-releasing hormone, urocortin peptides, the neurotransmitters acetylcholine and catecholamines, biogenic amines precursors and growth factors) are also synthesized and released by cells located in the skin. [3-11] All of these substances, produced locally or systemically, act on specific receptors that have been recently described in the skin. [12]

The local and systemic homeostasis regulation mechanisms of the skin's defense play a key role in the pathogenesis and control of a variety of skin disorders, including psoriasis, contact allergic and irritant dermatitis, lichen planus, alopecia areata and vitiligo, and, more recently, cutaneous aging. [13-19]

For years, there has been a constant search for alternatives to halt, reverse, or control the changes that occur in the skin during the aging process. Skin aging is an insidious, multifactorial, and progressive degenerative process of inevitable course, which is virtually irreversible. [20] It is characterized by cellular and molecular alterations, with a progressive decrease of the homeostasis capacity, leading to the programmed cellular senescence and death (apoptosis). [21]

Skin aging is seen as a synergistic result of two processes: intrinsic (or chronological) and extrinsic aging.[22,23] Chronological or intrinsic aging is a genetically programmed process – and is therefore independent of external or environmental factors – that can be triggered or aggravated by neurohormonal factors. Extrinsic aging corresponds to alterations caused in the skin due to lifestyle, and is mainly influenced by UV radiation, and secondarily by chemicals, smoking, heat, and other environmental insults. Both intrinsic and extrinsic factors accelerate the changes experienced by skin tissue during the aging process, directly or indirectly altering the complex skin's neuroimmunoendocrinologic system.[24-25]

Molecular alterations occur over time, triggering organic changes that ultimately lead to aging. An example of a mechanism that results in skin aging is the repair of telomeric DNA at the chromosomes' extremities, which results in their shortening and rupture.[26,27] As the DNA polymerase is unable to transcribe the final sequence of bases found in the DNA strands during replication, telomere length is reduced with every mitosis.[28] This reduction in telomere has been associated with cutaneous aging.[29-32]

Another important alteration is the degradation of oxidized products, a function carried out by proteasome, a multicatalytic protease with activity that appears to decrease throughout life. It is possible to observe the incomplete degradation of oxidized proteins, an increase in protein aggregates, and the acceleration of cellular dysfunction. [33,34]

According to the World Health Organization, the increase in the life expectancy of the population has stimulated both the study of the cutaneous aging process and the search for biochemical, molecular, and cellular mechanism-based tools for its treatment, prevention, and control, which mainly aim to improve the quality of life during the course of this insidious degenerative process. [35]

Many active principles that are claimed to have anti-aging effects are incorporated into so-called cosmeceuticals. This technical denomination is not yet formally recognized by drug regulatory agencies, and therefore comprises a gray area of topical products that are between cosmetics and medicines, based on their mechanisms of action. These products are defined as cosmetic products that provide benefits similar to those of drugs, with safety and efficacy proven using internationally validated methods. Therefore these products may have a useful adjuvant role in medication-based clinical treatment, in the preparation of skin for procedures, and in the maintenance of results. [36]

Many segments of anti-aging active principles have been developed and, depending on the research involved, they may be incorporated into cosmeceuticals with a high potential for clinical benefits. In this setting, growth factors – analog substances – have been emerging as promising substances in the rejuvenation strategies, taking part in that process at several levels. [37-44]

Growth factors are a set of substances, most of a protein nature, that together with hormones, neurotransmitters, and cytokines play an important role in intracellular communication.[11] The main function of growth factors is the external control of the cell cycle, by ending cellular quiescence (G0 phase) and inducing entry in the G1 phase.[38]

Nevertheless, growth factors' functions are not limited to the stimulation of cell proliferation through regulating their cycle and starting mitosis. They are also critical in apoptosis and the maintenance of cellular survival and the stimulation of migration and differentiation.[40] Depending on the type and location of growth factors, they can also promote differentiation and cell maturation. Bone morphogenetic proteins, for instance, stimulate osseous differentiation, while the vascular endothelial growth factor stimulates blood vessel differentiation. [45]

Another aspect of paramount importance is that growth factors function in very low concentrations – mostly in the magnitude of picograms – in bodily fluids or tissues.[45] They act in general as signals between cells, binding to specific cell receptors located on the cell membrane, transmitting the signal from the outside to the inside of the cell by coupling different protein kinases, which phosphorylate and activate a cascade of signals that ends with the activation of one or several genes (signal transduction).[38]

In order to assess the importance of growth factors in the cellular life cycle, studies using in vitro cell culture models have shown that growth factors are transported by serum, and are

produced in a great number of cells. In those studies, it became clear that for the cells to proliferate in the culture medium, the presence of a serum that is rich in growth factors and adhesion molecules is necessary, in addition to other nutritive molecules such as lipoprotein, transferrin, amino acids, and energy-metabolic substrates.[46,47] Furthermore, studies addressing the involvement of growth factors in cutaneous processes have demonstrated their role in wound healing and tissular remodeling. [48,49]

As with lesions, the aging process can be considered an acute and chronic skin injury that triggers a cascade of events including inflammation, new tissue formation, and tissue remodeling, and results in the partial reconstruction of the affected area. This repair process starts immediately after an injury has taken place through the release of several growth factors, cytokines, and serum compounds of low molecular weight resulting from the rupture of blood vessels or from the degranulation of platelets and other cells, including phagocytes, participating in the process. [50]

In addition to the importance of cell–cell and cell–matrix interactions, all stages of the repair process are controlled by a great variety of different growth factors and cytokines. Several studies have demonstrated the beneficial effects of many of those growth factors – e.g. platelet-derived growth factors, fibroblast growth factors (FGF), epidermal growth factor (EGF), granulocytes and macrophage-colony stimulating factor, and transforming growth factor – on the healing process, both in cell cultures (in vitro) and in animals, in addition to patients affected by diverse types of healing disorders. [51-55]

As in the healing of wounds, most of the cell damage caused by free radicals (for example, damage generated by UV radiation), is repaired. Nonetheless, the incremental accumulation of unrepaired damage that occurs over the years results in clinically significant alterations called solar elastosis. [11,56]

Photodamaged skin can therefore be considered a chronic wound that might not completely heal, in part due to the size of the affected area – which is much larger than what is considered acceptable in order for an effective repair to occur – and chronic (direct or indirect) UV exposure. [11]

Due to these factors and the intense involvement of growth factors in the tissue regeneration process, there has been an increasing amount of research and development directed to the subject. Regarding the study of growth factors as agents of anti-aging action, clinical studies have shown positive results in the acceleration of wound healing, therefore calling attention to their importance in products intended to mitigate the damage caused by aging. [56,57]

Its topical application is aimed at stimulating keratinocytes, fibroblasts, and other cells that have had their proliferative capacity reduced due to aging. A clinical and histopathological study that followed up volunteers for six months evidenced the proliferation of extracellular matrix proteins, such as collagen, with neocollagenesis and increased density of fibroblasts as responses, particularly in the papillary dermis. Those events confirmed the previously obtained in vitro observation that growth factors and cytokines participate in the dermal fibroblasts' mitotic activity. [44,58]

This study evaluates the safety profile of a selection of growth factor (or their analogs) based dermocosmetic products designed for that purpose, using in vitro models of human skin cell culture that are validated and recognized by the international scientific community and sectorial regulating bodies in different countries.

METHODS

This study's experimental conditions (the use of human cells in optimal culture conditions) are aligned with the current applied methodologies that are accepted and validated by the international scientific community. The human cell cultures were purchased commercially from qualified and certified international companies. The experiments were conducted with the approval of the Research Ethics Committee of the Faculdade de Ciências Médicas da Universidade Estadual de Campinas (Unicamp), Campinas (SP), Brazil.

The safety assessment described below was carried out for three different dermocosmetic products containing growth factors or their analogs (Caregen Co. Ltd., Seoul, Korea). Those products, as well as their active principles, are described below using their International Nomenclature for Cosmetics Ingredients names and primary use indications (Table 1).

Assessment of the Test Products' Effects on the Proliferation of Tumor (Melanoma Cells

Given that growth factors (and/or their analogs) are substances with well-known biological action, this experiment was designed to evaluate their effects on the proliferation of tumor cells.

The melanoma strain (Cascade Biologics, Portland, Oregon, USA) was seeded in 75 cm^3 bottles, and cultured and expanded in humid incubator at 37°C in the presence of 5% CO_2, using a specific culture medium (10% fetal bovine serum, 1 ml bovine pituitary extract, 50 μg/ml gentamicin, 2 μg/ml insulin, 12.5 μg/ml amphotericin B, and 500 ml DMEM). Upon reaching cellular confluence (approximately 80% of the surface occupied), the cells were trypsinized (0.25% trypsin solution/EDTA) and seeded in six-well plates for subsequent incubation with the three test products (Cosmeceutical 1, Cosmeceutical 2, and Cosmeceutical 3) and evaluation of cell proliferation.

The cells were incubated with non-cytotoxic concentrations of the test products that had been previously determined through MTT trial (MTT assay, Invittox n. 17, Ecvam). Using vital staining MTT (3-(4.5 dimethyl thiazole–2yl)-2.5 diphenyl tetrazolium bromide) to verify viability and cellular cytotoxicity is one of the more commonly used parameters reported in the literature. It is based on the conversion of yellow tetrazolium bromide (MTT) into blue formazan by the action of the mitochondrial enzyme succinate dehydrogenase in viable metabolically active cells. The study of cellular proliferation was sustained for 12 passes, and measurements were taken at six time points:

T1 – 2 DAYS AFTER TREATMENT

T2 – 3 days after treatment

T3 – 6 days after treatment

T4 – 12 days after treatment

T5 – 19 days after treatment

T6 – 26 days after treatment

The cells were kept in contact with the test product, and the medium was changed every 2-3 days. When cellular confluence was achieved in the wells, the plates were trypsinized in order to allow the detachment of cells from the bottles, being subsequently re-attached for the continuity of the evaluation. At each time point, the cells were trypsinized and seeded into 96-well plates, and 24 hours later the MTT was added to the culture, which was then incubated for another four hours. The absorbance of each well was determined at 450 nm through a microplate reader. The rate of cell viability was expressed in percentage terms according to the following formula:

Images were also obtained to record the cells' morphology after each contact period, in order to evaluate a possible alteration in tumor cells.

$$\% \text{ viable cells} = 100 \quad \frac{\text{sample absorbance (treated well)}}{\text{ontrol absorbance}} \quad \times 100$$

Proliferation of Normal Human Fibroblasts

This experiment was conducted to evaluate the effect of products containing growth factors and/or their analogs on the proliferation of normal dermal cells. It aimed to draw conclusions about safety-related criteria, such as their potential action on alterations in cell morphology or possible anomalies. The test was applied for products Cosmeceutical 1, 2, and 3, which (based on their formulations) would have real fibroblast proliferation capacity. For this evaluation, normal human fibroblasts (Clonetics, Cambrex/Lonza Inc., Walkersville, Maryland, USA) were seeded in 75 cm3 bottles, cultured and expanded in humid incubator at 37°C in the presence of 5% CO2, using specific culture medium (10% fetal bovine serum, 50 µg/ml gentamicin, 12.5 µg/ml amphotericin B, 500 ml RPMI). Upon reaching confluence, cells were seeded in 25 cm3 bottles (in triplicate) for later incubation with the test products.

The cells were incubated with non-cytotoxic concentrations of the products Cosmeceutical 1, 2, and 3 (0.006%, 0.006% and 0.012%, respectively), previously determined using the MTT technique. The cellular proliferation study lasted 12 weeks, with measurements taken at 4, 8, and 12 weeks of contact with each product.

The cells were kept in contact with the test products, and the medium was changed every 2-3 days. Upon reaching con-

fluence, the 25 cm3 bottles were trypsinized and re-attached. The cell count was carried out using a Neubauer chamber. Images were also obtained to record the cell morphology after each contact period in order to evaluate possible alterations in normal cells.

RESULTS

For all results the parametric method of analysis of variance (ANOVA) was used to carry out statistical inferences, followed by the Tukey test for multiple comparisons. Statistical significance was defined as $p \leq 0.05$.

Assessment of Proliferation of Human Melanoma Cells

The evaluation of the stimulus for the proliferation of human melanoma cells (Figure 1) was carried out for all products (Cosmeceuticals 1, 2, and 3). No significant morphological alterations were found in human melanoma cultures. Regarding Figure 1A (the untreated control), the volunteer groups that received treatments with Cosmeceutical 1 products (Figure 1B), Cosmeceutical 2 (Figure 1C), and Cosmeceutical 3 (Figure 1D), presented similar behavior for both the number of cells (visual evaluation) and the morphology. Therefore it is possible to conclude that the products evaluated in this study did not promote significant changes when applied to tumor cells – here represented by human melanoma. Table 2 shows the results obtained for each study product; the number of cells after 26 days of treatment was quantified.

Evaluation of the Proliferation of Normal Human Fibroblasts

The normal human fibroblasts cultures' cellularities were assessed after 4, 8, and 12 weeks of treatment with the test products (Cosmeceutical 1, 2, and 3). The results are shown in table 3.

Cosmeceuticals 2 and 3 did not promote significant changes in the number of healthy cells. These results demonstrate that the products are safe, due to the absence of morphological alterations in the cells. In addition, the absence of proliferation does not limit their effectiveness, since the increase in the synthesis of collagen, for instance, does not depend on an increase in the number of cells, but rather on an increase in their metabolism. In turn, Cosmeceutical 1 was able to increase the number of fibroblasts significantly ($p < 0.05$) without altering the cells' morphology, further suggesting its safety.

DISCUSSION

Growth factors are regulating proteins that act as natural biological mediators, influencing repair and regeneration processes, and can be found in various tissues during the healing and/or cellular renewal phase.[48]

According to Fitzpatrick and Rostan, photodamage – the damage inflicted on the skin due to sun exposure – is considered a chronic wound that might not completely healing.[11,56]

In this manner, taking into account the involvement of growth factors in the tissular regeneration and wound healing process, it becomes evident that photoaging is a chronic scarring
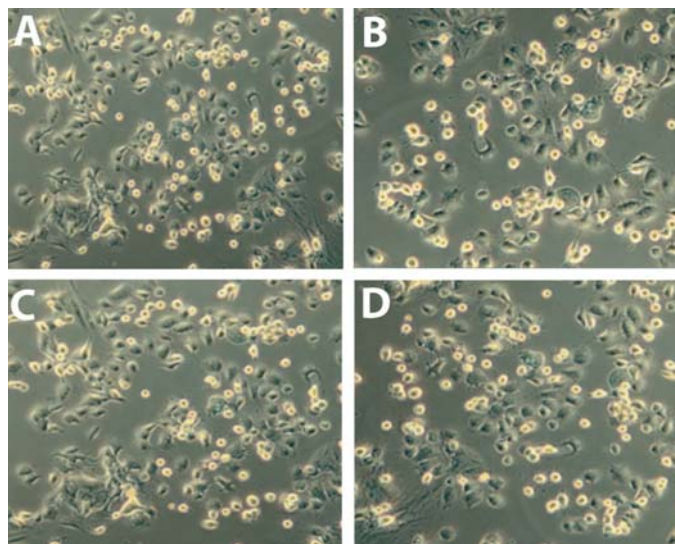
Figure 1 (A, B, C e D): Melanoma cultures kept in 25 cm2 bottles in humid incubator at 37°C. A: untreated control; B: Cosmeceutical 1; C: Cosmeceutical 2; D: Cosmeceutical 3

event, in which the role of growth factors – as well as the involvement of cytokines and neurotransmitters – is of paramount importance for its control and/or treatment.

The action of growth factors has been intensely studied due to the exhaustive search for a tool that can assist in retarding, controlling, or reversing the signs of aging independently of their root causes. This search has stimulated the development of a new generation of cosmeceutical products, the actions of which are based on the effects of endogenous mediators with paracrine and endocrine activity.

This study sought primarily to assess the safety of three cosmeceuticals containing growth factors or their analogs as the main active ingredients in their formulations. The experimental models used complied with internationally recognized and validated protocols and used in vitro cultures of normal and tumor cells of human skin. To evaluate the action of these products on the proliferation of tumor cells (human melanoma cell culture),

each product was kept in contact with the cultures and the measurements were taken at six time points (Days 2, 3, 6, 12, 19, and 26). To measure cellular viability, a colorimetric method was used based on the measurement of the cells' respiratory function, through the conversion of yellow tetrazolium bromide (MTT) into formazan by the mitochondrial enzyme succinate dehydrogenase, in the metabolically active viable cells.

The results demonstrated that the three products tested – Cosmeceuticals 1, 2, and 3 – did not present affect the number of malignant cells, suggesting their inability to promote the progression of potential epidermal cancers. It is worth noting that Cosmeceutical 3 promoted a small, insignificant ($p < 0.05$) reduction in the human melanoma culture's cellularity over time compared to the control group. Despite the absence of more significant differences, the outcomes demonstrate an effect contrary to the induction of proliferation.

In order to complement the analyses carried out with tumor cells, normal human fibroblasts were exposed to the same three products, and the assessment of cell proliferation was performed over a 12-week period. The study evaluated the quantitative change in cell proliferation at three experimental times (4, 8, and 12 weeks after contact with the test products). At the end of the 12-week evaluation, the cultured human fibroblasts maintained their morphological aspect, meaning that the products did not cause cell mutations. Regarding the cell count, there was an increase in the number of normal human fibroblasts in the non-senescent at the three time points in the groups treated with Cosmeceutical 2, with a greater than 30% increase at the eighth week of contact. In turn, in the groups in which Cosmeceuticals 1 and 3 were applied, there was no significant change in the cell count at any time point. These results show that no products evaluated in this study present cytotoxic potential, meaning that they do not cause cell death either in the short or long term, and can be considered safe for daily cosmetic application.

The major growth factors common to the formulations of the three tested products, however at different concentrations, are: Human Oligopeptide-3 (FGFb, basic fibroblast growth fac-

Table 1: Denomination, primary indication of use and active principles* of the study products		
Product description	**Main indication**	**Active principles* (growth factors or their analogues)**
Cosmeceutical 1	Anti-wrinkle cream	Human Oligopeptide-3 (FGFb), Human Oligopeptide-1 (EGF), Human Oligopeptide-13 (FGFa), Human Oligopeptide-2 (IGF-1)
Cosmeceutical 2	Revitalizing facial serum	Oligopeptide-20 (CG-IPD 5), Oligopeptide-24 (CG-EPD 3)
Cosmeceutical 3	Cream for the eye area	Human Oligopeptide-3 (FGFb), Human Oligopeptide-1 (EGF)

* Only growth factors were listed, nevertheless other active principles may be present in the formulations.

Table 2: Evaluation based on the quantitative results of the different treatments, obtained from the cell counts during the treatment course, compared to the untreated control	
Product description	**Effect on human melanoma cells**
Cosmeceutical 1 (0.024%)	Did not promote significant change (p > 0.05) in the cellularity of cultured human melanoma over time, compared to the respective untreated control group
Cosmeceutical 2 (0.024%)	Did not promote significant change (p > 0.05) in the cellularity of cultured human melanoma over time, compared to the respective untreated control group
Cosmeceutical 3 (0.049%)	Promoted small, insignificant reduction (p < 0.05) in the cellularity of cultured human melanoma over time, compared to the respective untreated control group

Table 3: Evaluation based on the quantitative results of the different treatments: cell counts compared to the untreated control at 4, 8, and 12 weeks	
Product description	**Effect on human fibroblasts**
Cosmeceutical 1 (0.024%)	Promoted an increase in cell count in cultures of non-senescent normal human fibroblasts at three time points (4 weeks: 24.37%; 8 weeks: 30.27%; 12 weeks: 18.52%) compared to the untreated control.
Cosmeceutical 2 (0.024%)	Did not promote significant increase in the cell count in cultures of non-senescent normal human fibroblasts, after 4, 8, and 12 weeks of application.
Cosmeceutical 3 (0.049%)	Did not promote significant increase in the cell count in cultures of non-senescent normal human fibroblasts after 4, 8, and 12 weeks of application.

tor), Human Oligopeptide-1 (EGF, epidermal growth factor), Human Oligopeptide-13 (FGFa, acid fibroblast growth factor) and Human Oligopeptide-2 (IGF-1, insulin growth factor).

FGFa and FGFb, Human Oligopeptide-13, and Human Oligopeptide-3, respectively, were considered safe growth factors for topical application in humans (clinical study), in patients with severe burns.[59]

EGF (Human Oligopeptide-1) is one of the most utilized peptides in the cosmetic segment. It has the intrinsic ability to promote cell proliferation and stimulate the metabolism and production of extracellular matrix proteins such as collagen and hyaluronic acid. Studies have shown that although it stimulates cell proliferation, it is safe to use since its specific action on EGF receptors (EGFr) limits its action on the progression of tumors.[60]

IGF-1 (Human Oligopeptide-2) was considered an important, safe, and well-tolerated growth factor with therapeutic action. Its benefits have been proven in two clinical studies of scientific importance – one carried out by Traynor et al., and the other performed by Sorenson et al.[61,62]

The results obtained in the present study corroborate those previous studies, given that all have produced evidence – in different areas of application and with diverse growth factors – that those substances are considerably effective and safe for topical use.

CONCLUSION

Taking into consideration the importance of growth factors in the cosmeceutical industry and the exponential growth in research performed in the area, and based on the results obtained in the present study with the use of *in vitro* models of tumor and healthy human skin cell culture, it is possible to conclude that the preliminary data suggest that cosmeceutical products containing growth factors as active principles can be considered safe and effective for topical application, given that they have not promoted the proliferation of abnormal cells and, in some cases, have stimulated the proliferation of normal fibroblasts – cells of paramount importance for the tissular repair process. ●

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