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Original Research

Skin Cellular Reprogramming as an Innovative Anti-Aging Strategy for Cosmetic Application: A Clinical Study of Sericoside

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Abstract

Background: While our body ages, skin cells progressively lose their pluripotency and proliferative capacities, as well as remodeling driver role, among other activities. This loss of capacities leads to visible aging signs such as wrinkles, under-eye bags or even aging spots. We studied if the stimulation of cell pluripotency and proliferation by a natural molecule could be an innovative anti-ageing strategy for skin rejuvenation. **Methods**: The activity of sericoside, a compound extracted from the bark of *Terminalia sericea* roots, was evaluated at a concentration of 0.02% *in vitro*. This assessment involved transcriptomic analysis on fibroblasts after 24 hours, as well as proliferation tests on aged fibroblasts after 72 hours. A clinical study was then conducted on 40 volunteers between the ages of 35 and 55. For four weeks, volunteers applied a cream twice daily containing either sericoside or blank emulsion (control group). Skin elasticity was measured by cutometry with R2 parameter. Skin texture and roughness was analyzed by an *in vivo* 3D scanner. **Results**: Transcriptomic analysis showed that sericoside improved the set of gene expressions involved in cell cycle (+85% *MKI67*), cell proliferation (+250% *IGF1*), DNA repair (+56% *OGG1*), pluripotency transcription factors (+36% *NANOG*) and stem cells maintenance (+200% *SOX2*). We substantiated a decrease of proliferation factor with aged cells compared to young cells by 50%, while sericoside increased this proliferation factor by +46%, a similar rate to that of a 22-year-old donor. Clinically, the anti-aging effects of sericoside were evident: the use of sericoside resulted in a 17% increase in skin elasticity and a 10% reduction in skin roughness, underscoring the smoothing effect with sericoside. **Conclusions**: The study highlighted an innovative anti-aging strategy that involves re-activating cells' memory to reprogram cell pluripotency by stimulating the natural tools available in our DNA.

Keywords: cell memory; reprogrammation; proliferation; rejuvenation; sericoside

1. Introduction

The natural aging process affects many biological mechanisms in the body; the large majority of changes begin with modifications in gene control and result in visible signs of aging on the skin [1]. Generally, characteristics of cells differ depending on age. Pluripotent stem cells have the ability to differentiate into different tissues to replace senescent cells. These newly differentiated and functional cells exhibit remarkable vitality and proliferation capacity, which naturally declines with age [2]. As they age, stem cell number and self-renewal capabilities do not necessarily decline; rather, they progressively lose their DNA repair capacities, while their ability to produce new progenitors and differentiated effector cells is affected [3]. As a result, there is a reduction in tissue regenerative potential and an accumulation of non-functional cells throughout life [4].

Stem cells present an important role in different skin layers; their function in the epidermis is well documented in the literature. Indeed, the epidermis is the first part of the body to be exposed to external aggressors, submitting epidermal cells to stress and damage. Accordingly, the multi-layered stratified squamous epithelium requires constant renewal due to its high turnover [5]. The role of stem cells in the dermis, though less described, is equally important: stem cells are present in the dermal tissue and play a role in maintaining homeostasis and regenerating injured skin [6]. Indeed, they have the capacity to differentiate in new functional fibroblasts capable of synthesizing molecules from the extracellular matrix and the elastic network, with a higher proliferation rate than cells becoming senescent.

When skin cells lose their pluripotency and functionality, cell regeneration and repair functions slow down, and their role in driving skin remodeling other activities diminishes. This process leads to the progressive appearance of visible signs of aging such as wrinkles, under-eye bags, and aging spots [7]. Based on this knowledge, we hypothesized that reactivating cell memory to bring cells closer to a pluripotent state could be an effective and innovative strategy for rejuvenating cells.

Recent discoveries in ethnobotany and the traditional use of plants have highlighted the fact that certain botanical compounds have the ability to significantly stimulate genes involved in rejuvenation mechanisms. One such compound is found in the Miombo forest of Tanzania, home to the common *Terminalia sericea* (aka silver tree), which plays a role in maintaining biodiversity. The plant produces sericoside, a pentacyclic terpenoid that accumulates in the bark of its roots, and which is obtained by extraction and purification [INCI: Sericoside]. Sericoside is already known and widely used for its various medicinal properties such as antibacterial, antioxidant or even anti-inflammatory properties [8–11].

In this study, we evaluated the capacities of sericoside to reprogram cells to a younger stage to determine whether it could be an effective candidate for reactivating skin cell memory for a rejuvenation process. We analyzed its effects on stem cells maintenance, DNA repair, cell pluripotency, cell proliferation properties, and broader anti-aging properties on a global scale.

2. Materials and Methods

2.1 Primary Cells Isolation

Experiments were performed on primary fibroblasts obtained from skin surgical residues following plastic surgery. Skin explants were obtained from donors who have sustained an abdominoplasty (Polyclinique Courlancy, Reims) after reading, understanding and signing an Information and No Objection Form for the Use, for Dermocosmetic Research Purposes, of Tissues, Cells, and Products of the Human Body Collected During Surgery (Surgical Residues) aligned with articles L. 1211-2 alinéa 2, and L. 1245-2, Code de la santé publique.

1 hour after surgery, adipose tissue was removed and skin was cleaned by successive bathes of ethanol 70%, PBS and PBS supplemented with 1% antibiotics (Sigma-Aldrich, Saint-Louis, MO, USA) and 1% amphotericin B (Gibco, Life Technologies, Carlsbad, CA, USA). 1 cm^2 pieces of skin were cut and cultured with DMEM culture medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal calf serum (FCS, HyClone Laboratories, Logan, UT, USA), 1% antibiotics (Sigma-Aldrich) and 1% amphotericin B (Gibco). Culture media was renewed every other day until that fibroblasts spread out of skin pieces and reach 90% confluency. Fibroblasts were then detached from the dish with trypsin-EDTA 0.05% (Gibco) and seeded for the next experiments.

Primary fibroblasts used in this study were authenticized in parallel with the experiments by performing an immunostaining of vimentin protein that is a marker of fibroblasts, associated to a counter-immunostaining of cytokeratin-14 and of melan-A to rule out the hypothesis of keratinocytes and melanocytes presence in cell culture.

2.2 Transcriptomic Study

Normal Human Dermal Fibroblasts were stimulated in fetal calf serum (FCS)-free DMEM culture medium (Thermo Fisher Scientific) containing 0.02% sericoside. The *in vitro* testing dose of sericoside was selected following a cell metabolism test on fibroblasts (data not shown). After 24 hours of stimulation, total RNA were extracted by TRIzol method [12]. RNA quality was controlled and a reverse transcription was performed to obtain cDNA using Verso cDNA kit (Thermo Fisher Scientific). RT-qPCR was carried out on specific plates designed to study transcriptomic expression of different genes involved in dermis biology, with 10 ng of cDNA per well and using CFX Touch 96 (BioRad, Hercules, CA, USA) . The results of gene expression obtained with fibroblasts were normalized according to *POLR2A* (RNA polymerase II, subunit A) and *HMBS* (porphobiloinogen deaminase) housekeeping gene. We analyzed the modulation of genes expression relative to the untreated condition.

2.3 Protein Synthesis

Normal Human Dermal Fibroblasts were cultured in fibroblasts growth medium until confluence and were then treated with sericoside for three days versus untreated condition. Treatment was renewed every day and cells were then collected and frozen at –20 °C before being hydrolyzed [13]. Amino acid synthesis dosage was performed with a Beckman 6300 analyzer (Beckman Coulter, Brea, CA, USA) using a column of ninhydrine with a detection at 440 nm.

2.4 Clinical Study

This study has been carried out in compliance with the most recent recommendations of the World Medical Association Declaration of Helsinki-ethical principles for medical research involving human subjects (Helsinki Declaration 64th WMA General Assembly, Fortaleza, Brazil, October 2013) and according to the Colipa Guidelines for the evaluation of the efficacy of cosmetic products. Only after having signed the informed consent the participation in the study was permitted. The original of the informed consent forms were archived at the Abich Clinical study Center. All subjects signed a consent allowing to treat personal data according to the Italian law (privacy. D.Lgs 196/2003).

2.4.1 Panel Description

A placebo-controlled, single blind clinical study was carried out on 40 volunteers (10 men and 30 women, aged 35–55). The subjects were randomly and equally divided in two groups: one of which applied an emulsion containing 0.5% of sericoside (average age: 52) while the other applied a blank emulsion (average age: 47). The clinical testing dose was selected following a full-toxicity evaluation (data not shown). Each group of volunteers was instructed to apply their emulsion twice daily for a duration of four

Table 1. Gene modulation with sericoside relative to the untreated condition.

Pathway	Genes	Fold change	*p* value	Pathway	Genes	Fold change	*p* value
Antioxidant defense	*G6PD*	2.08	0.023	Extracellular matrix	*CD44*	1.48	0.006
	GPX1	1.48	0.031		*CYR61*	1.56	0.031
	GSTT1	1.40	0.056		*FBN1*	1.15	0.004
	HMOX1	1.22	0.061		*HAS2*	1.61	0.097
	MSRA	1.40	0.0001		*TIMP1*	4.03	0.005
	SOD2	1.16	0.011		*SDC1*	1.90	0.0008
Elastic network	*VCAN*	1.79	0.012	DNA repair	*GADD45A*	1.29	0.040
Cell cycle	*MKI67*	1.85	0.0002		*OGG1*	1.56	0.009
Cell proliferation	*FGF7*	1.29	0.001		*XPA*	1.26	0.0002
	IGF1	3.50	0.00005		*XPC*	1.51	0.044
	IGF1R	1.40	0.001	Pluripotency transcription factor	*NANOG*	1.36	0.041
	IGFBP3	1.30	0.0003		*POU5F1*	1.51	0.072
Dermo- epidermal junction	*COL4A1*	1.17	0.056	Retinoic acid receptor	*CRABP2*	1.47	0.026
	COL7A1	1.45	0.044	Signal transduction	*CAV1*	3.86	0.004
Transcription factor	*MYC*	1.39	0.032	Stem cell maintenance	*SOX2*	3.00	0.052

weeks and may not use any other cosmetics product on face for the duration of the clinical study. Several indicators of facial aging were monitored, including skin firmness, skin elasticity, and presence of dark circles and blemishes.

2.4.2 INCI NAME of Formula

AQUA, ISONONYL ISONONATE, ETHYLHEXYL PALMITATE, C20-22 ALKYL PHOSPHATE, C20-C22 ALCOHOLS, ± SERICOSIDE, ARGININE, HYDROXYETHYL ACRYLATE/SODIUM ACRYLOYLDIMETHYL TAURATE COPOLYMER, SQUALANE, POLYSORBATE 60, BENZYL ALCOHOL, DEHYDROACETIC ACID, PARFUM.

2.4.3 Skin Firmness Measurements Using Cutometer®

Skin elasticity was evaluated by Cutometer® MPA 580 (Courage+Khazaka electronic GmbH, Köln, Germany) according to the following parameters:

- R2 parameter (overall skin elasticity, tonicity).

2.4.4 Skin Texture and Roughness Analyzed by DermaTOP-Blue Method

Evaluation by Visio DermaTOP-blue (EOTech, Plymouth, MI, USA) allows contactless measures of skin profilometry and skin texture:

- Skin texture (Sa parameter).

2.4.5 Eye Contour Benefit Measured by Chromameter and DermaTOP-Blue Method

Eye contour benefits in terms of under-eye bags and dark circles were evaluated by Chromameter CR-200 (Konica Minolta, Tokyo, Japan) according to the following parameters (dark circles):

- L parameter (0 = black; 100 = white)
- a (green-red), b (blue-yellow) parameters (+120; –120)

Eye bag volume was evaluated by Visio DermaTOP-blue.

2.5 Statistical Analysis

For *in vitro* and clinical data, data normality was first verified via the Gaussian law using Shapiro Wilk test. According to the results, we used parametric or nonparametric tests to compare the effect of sericoside versus the untreated condition or versus placebo.

3. Results

3.1 Gene Regulation

Transcriptomic analysis was performed by Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) on specific pre-coated plates designed to study transcriptomic expression of different genes involved in dermal function, such as extracellular matrix structure, remodeling, antioxidant enzymes and stress defenses, neurotrophin pathway, cell proliferation, DNA repair and stem cell markers.

We observed a significant upregulation of 30 genes following treatment with sericoside, indicating the strong bioactivity of the active ingredient (Table 1). These genes are involved in a range of processes, including cell proliferation, DNA repair, extracellular matrix, dermo-epidermal junction, antioxidant defense, and regulation of pluripotency transcription factors.

Gene expression was expressed as a fold change after 24 hours stimulation with sericoside at 0.02% in comparison with the untreated control. The significance was calculated by applying a statistical Student *t*-test.

First, we observed the potential benefit of sericoside on cell rejuvenation through the upregulation of genes involved in DNA repair (*XPC, XPA, OGG1,*

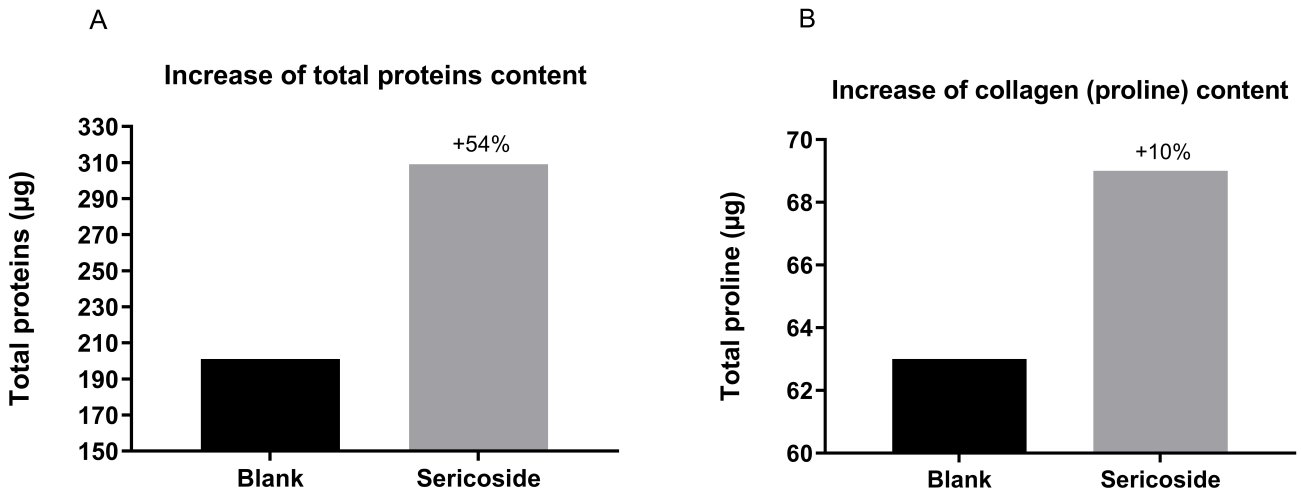

Fig. 1. Impact of sericoside on total protein synthesis (A) and collagen synthesis (B) by fibroblasts. The total amount of synthetized proteins was compared between untreated cells and cells treated with sericoside for 3 days (A). The collagen content was further analyzed in order to evaluate the benefit of sericoside on the extracellular matrix (B).

GADD45A), pluripotency transcription factors (*POU5F1, NANOG*), retinoic acid receptor (*CRABP2*), signal transduction (*CAV1*), stem cell maintenance (*SOX2*) and transcription factor (*MYC*) [14–17].

The transcriptomic analysis on fibroblasts also showed that sericoside significantly upregulated several genes linked to protection and rejuvenation of the extracellular matrix, indicating a potential anti-aging activity. The upregulation of key genes associated with the protection and rejuvenation of the extracellular matrix, including *CD44, CYR61, FBN1, HAS2, TIMP1, SDC1*, and *VCAN*, suggests that potential improvements in skin elasticity, reduction in wrinkles, and increased firmness may be attributed to this mechanism. The upregulation of genes involved in antioxidant defense (*G6PD, GPX1, GSTT1, HMOX1, MSRA*, and *SOD2*) is relevant to the reduction of dark circles, as these genes protect heme from oxidation. Sericoside also upregulated a variety of genes involved in dermoepidermal junction (*COL7A1, COL4A1*), cell proliferation (*IGFBP3, IGF1R, IGF1, FGF7*) and cell cycle (*MKI67*). Taken together, these data suggest that sericoside, through the maintenance of stem cell state and the activation of DNA repair, may contribute to the preservation of cell functionality. The stimulation of these genes can have a positive impact on various biological functions in the dermis, including matrix structure, cell junction, and proliferation.

3.2 Stimulation of Protein Synthesis

Our transcriptomic results showed that we could rejuvenate cells thank to the application of sericoside. The question remained whether biological functions associated with the dermis function, such as collagen production, could be reactivated. Indeed, collagen is the predominant matrix skin protein, and it is known to impart textile strength to skin. However, collagen decreases with aging as well as ultraviolet (UV) exposure and other external challenges.

To assess the impact of sericoside on collagen synthesis, particularly on the production of total protein and proline (one of the crucial amino acids for collagen and elastin production, responsible for providing collagen with its spatial structure), human fibroblasts were subjected to sericoside treatment. The objective was to determine whether there was a potential increase in collagen synthesis compared to the control medium.

We observed a tendency with sericoside to increase total protein synthesis by 54% with a specific reference to proline content which was boosted by 10% (Fig. 1). These data complete the transcriptomic study, showing that reprogramming cells allow restoring the whole functions of cells, from cell proliferation to collagen synthesis.

3.3 Clinical Evaluation

3.3.1 Reversing the Clock of Aging

After showing *in vitro* that cell reprogramming is an efficient strategy to reactivate cell machinery, such as collagen synthesis, we performed a clinical study to offer definitive proof of the anti-aging benefit of sericoside.

First, we evaluated the efficacy of our product on skin elasticity after one month of application. Overall skin elasticity increased by 17% (R2 parameter) (Fig. 2), a significant increase compared to the placebo control. These data showed a significant increase in skin elasticity after only 30 days of usage. This clinical study also demonstrated a tendency to reduce skin fatigue (R9 parameter) by 21% with the cream containing sericoside (data not shown).

We continued to explore the anti-aging property of sericoside by analyzing the skin roughness and texture on periorbital area as a way to see visible reduction of aging

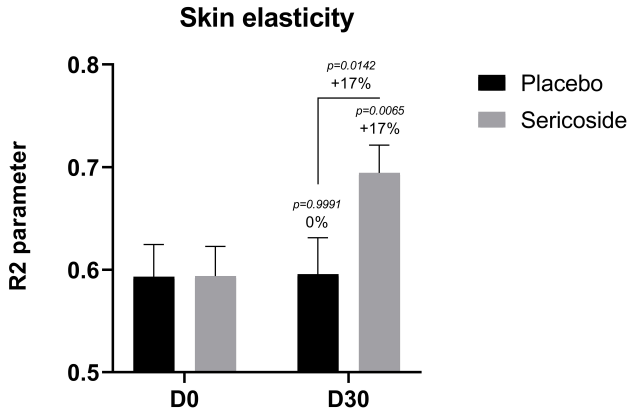

Fig. 2. Impact of sericoside application on skin elasticity measured by Cutometer in comparison with placebo control. Skin elasticity was measured by R2 parameter at day 0 and after 1 month of application of a placebo cream or a cream containing sericoside at 0.5%. The efficacy of Sericoside after 1 month of application was evaluated by a statistical analysis using One-way ANOVA followed by Tukey's multiple comparisons test.

signs on face. A measurement of Rz parameter showed a tendency to skin roughness decrease with sericoside by 16% (data not shown). The skin texture amelioration was perceivable with touch. This effect was confirmed by the visualization of skin texture analyzed by skin surface topography (parameter Sa) which indicates the roughest points (difference between peak and hollowness) in red marks. After the treatment, red marks are notably reduced (Fig. 3).

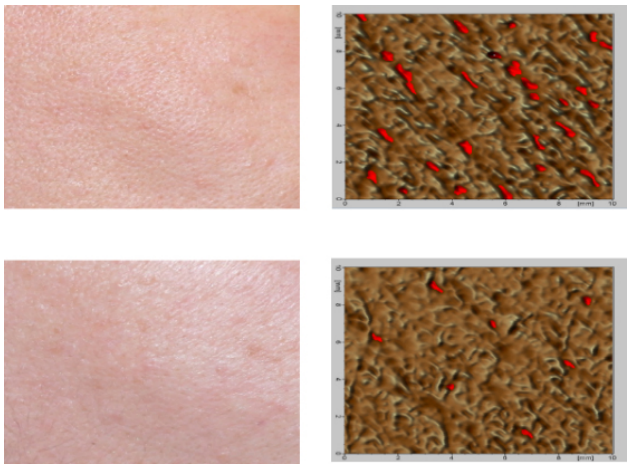

Fig. 3. Impact of sericoside application on skin texture visualization analyzed by DermaTOP-blue method in comparison with placebo control. Surface pictures illustrating skin surface are represented. The upper panel represents the skin surface of a representative volunteer at the beginning of the study before any cream application. The lower panel represents the same area after one month of daily application of a cream containing sericoside at 0.5%. In both panels, skin rugosity is highlighted with red color.

After 30 days of application, a significant reduction of periorbital wrinkles can be observed as a result of controlling the aging clock. Skin wrinkles are represented by 3D imaging and high resolution pictures. Skin wrinkles were visibly less deep after a month of treatment with sericoside (Fig. 4).

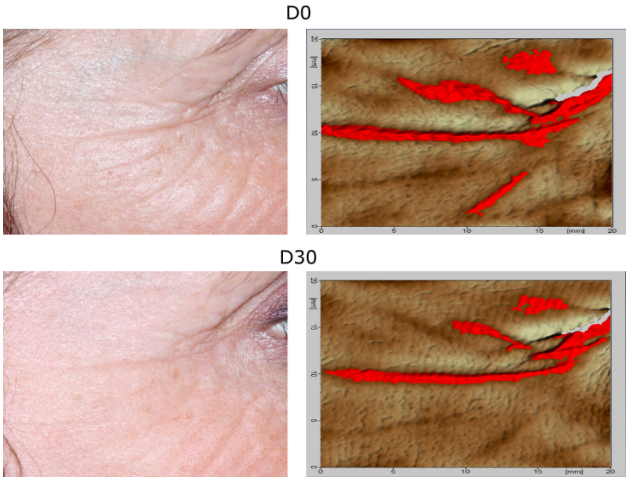

Fig. 4. Impact of sericoside application on skin wrinkles focus on periorbital area visualized by DermaTOP-blue before and after application (D0 versus D30). The upper panel represents "crow's feet" of a representative volunteer at the beginning of the study before any cream application. The lower panel represents the same area after one month of daily application of a cream containing sericoside at 0.5%. In both panels, deep wrinkles are highlighted with red color.

3.3.2 Impact on Dark Circles

We analyzed the impact of sericoside on dark circles after 30 minutes of application using the same panel previously described.

Sericoside resulted in a significant improvement in dark circles for three times as many volunteers compared to the placebo group (Fig. 5). The percentage of volunteers showing a perceivable improvement in the appearance of dark circles reached 45%. The mean positive volume of under-eye bags decreased by 20 mm^3, whereas no improvement was observed in the placebo group.

4. Discussion

We first demonstrated that sericoside is capable of rejuvenating cells by stimulating cell memory, leading to the reprogrammation of cell pluripotency. Indeed, our active ingredient stimulated the expression of genes such as *SOX2*, *NANOG* or *C-MYC* whose expression is decreased while aging, positioning these targets as an efficient cocktail for a natural iPS cell reprogramming [18]. Interestingly, SOX2 has been shown by Narayan *et al.* [19] to work with

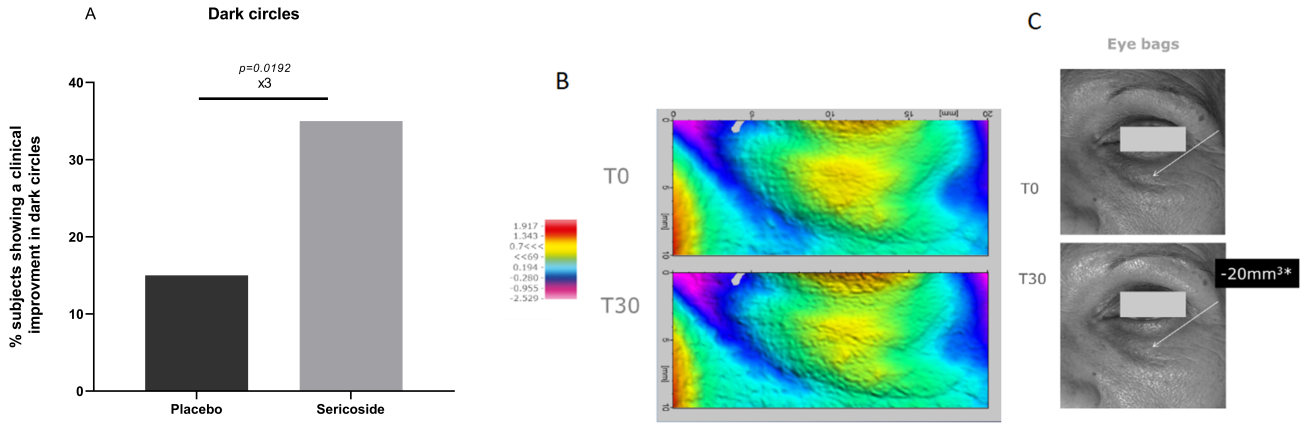

Fig. 5. Impact of sericoside application on dark circles focus on periorbital area visualized d by Chromameter (A) and visualized by DermaTOP-blue (B) and with macroscopic pictures (C) in comparison with placebo control. (A) Number of subjects showing a visible improvement of dark circles after one month of application of a placebo cream or a cream containing sericoside at 0.5%. (B) Illustrative pictures obtained by DermaTOP-blue show an improvement of under-eye bag volume. (C) Macroscopic pictures demonstrate the efficacy of sericoside on periorbital area with a volume reduction. The efficacy of sericoside on dark circles after 1 month of application was evaluated by a statistical analysis using χ^2-test.

POU5F1 as activators during the reprogramming of human fibroblasts to iPS cells. *NANOG* gene is an embryonic stem cell marker involved in the maintenance of undifferentiated state of pluripotent stem cells [20]. In the context of cellular reprogramming, *C-MYC* enhances the conversion of somatic cells into induced pluripotent stem cells [21]. Sericoside also stimulates the expression of *XPC, XPA, OGG1* and *GADD45A*, genes involved in DNA repair, which is a function of stem cells that can be negatively impacted during aging [22]. These transcriptomic results highlighted sericoside as a good candidate to reactivate cell memory to restore them to pluripotency state.

This rejuvenation process induced by sericoside leads to a reactivation of cell functions. Indeed, the active ingredient also stimulated the expression of genes involved in various pathways: reactivation of antioxidant defense with *G6PD, GPX1, GSTT1, HMOX1, MSRA* and *SOD2*; extracellular matrix composition with *CD44, CYR61*, *FBN1, HAS2, TIMP1, SDC1* and *VCAN*; cell proliferation with *IGFBP3, IGF1R, IGF1, FGF7* and *MKI67*; and even dermo-epidermal junction integrity with *COL7A1* and *COL4A1*. These genes are involved in biological pathways that contribute to skin rejuvenation and youthfulness. The role of genes involved in antioxidant defense is to stimulate the skin defenses against oxidative stress induced by various factors from the exposome. Unfortunately, during the chronologic aging process, there is an increase of reactive oxygen species production associated with a decrease of antioxidant defense [23]. Fibroblasts' proliferative capacity, a key factor in skin maintenance and renewal, is impaired during skin aging, leading to an impairment of these capacities. In the case of *IGF1* and *IGF1R*, two genes involved in cell proliferation, recent publications have evidenced the crucial role of *IGF1* that activates *IGF1* receptor to induce an appropriate protective response against UV-induced DNA damages [24,25]. Lewis *et al.* [24] described the loss of *IGF1* expression in ageing skin to be linked to a high occurrence of skin cancers. More, cells' proliferation impairment provokes a reduction of the extracellular matrix component synthesis such as collagen, elastin, hyaluronic acid and chondroitin [26]. The extracellular matrix components are known to be involved in the composition of a tissue framework and cell niche that provides a micro- and macro-architecture that helps drive cell behavior and function [27]. Li *et al.* [27] also showed that the alteration of the dermal extracellular matrix composition and organization is a major driver of human skin aging. The dermis is impacted by skin aging, but not only because the overlying dermoepidermal junction is submitted to skin aging. Langton *et al.* [28] described a reduction of collagen IV and collagen VII expression among other proteins during skin aging, resulting in a flattened dermoepidermal junction. These data, taken together with our findings, confirm that reactivating these pathways that are downregulated by the aging process proves the efficacy of sericoside in promoting cellular rejuvenation. Indeed, the upregulation of these genes in aged skin will rebalance their expression closer to a balanced state, leading to a reactivation of cell metabolism, without being abnormally overexpressed.

Cell rejuvenation was assumed thank to a proliferation study evidencing that aged fibroblasts treated with sericoside showed a strong decrease of their doubling time, underscoring the effectiveness of our active ingredient on cell rejuvenation (data not shown). This rejuvenation of fibroblasts' metabolism was also confirmed with an increase of protein synthesis by 54%, and especially the collagen as observed by the 10% increase of proline content, reinforcing the set of data showing a rejuvenation of cells with serico-

side.

Our clinical studies demonstrate that cell rejuvenation led to a wide anti-aging efficacy with an improvement of skin elasticity and firmness after 30 days of application. This anti-aging effect can be linked to the stimulated expression of genes involved in the protection and production of the extracellular matrix. We further demonstrated a reduction of wrinkles and an improvement of skin texture by reducing skin roughness on the periorbital area and eye bag volume. These clinical benefits go hand in hand with the transcriptomic study which showed an improvement of extracellular matrix composition, cell proliferation and dermo-epidermal junction. Finally, sericoside reduced dark circles, confirming the antioxidant properties of the active ingredient [29].

5. Conclusions

This study demonstrated that stimulating the natural tools available in our DNA to reprogram cells is an innovative method of skin rejuvenation. Sericoside has shown strong efficacy in numerous applications.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

RR, AS, MM and GM designed the research study. MM and MB performed the research. JT provided help for the isolation of human skin cells. MM and AS analyzed the data. MM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This clinical study follows the European law and has been carried out in compliance with the most recent recommendations of the World Medical Association Declaration of Helsinki- ethical principles for medical research involving human subjects (Helsinki Declaration 64th WMA General Assembly, Fortaleza, Brazil, October 2013) and according to the Colipa Guidelines for the evaluation of the efficacy of cosmetic products. Only after having signed the informed consent the participation in the study was permitted. The original of the informed consent forms were archived at the Abich Clinical study Center under the codification number 8693/15-04. All subjects signed a consent allowing to treat personal data according to the Italian law (privacy. D.Lgs 196/2003).

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Conflict of Interest

The authors declare no conflict of interest.

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