IFSCC 2025 full paper (IFSCC2025-969)

"Slowing down skin aging properties of linseed oil concentrate obtained from a regenerative plant sourcing, Linum usitatissimum"

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1. Introduction

Skin aging is a multifaceted process that involves intrinsic and extrinsic mechanisms that lead to various structural and physiological changes in the skin. The epidermis and the dermal-epidermal junction (DEJ) are strongly altered. With age, epidermis capacities of self-renewal, differentiation and stress response decrease. A drop of differentiation markers such as keratin 10 and 14 and DEJ markers (collagen XVII, laminin-332 and integrin alpha-6) had been described [1-3]. These alterations lead to clinical modifications such as skin irregularities, dehydration, skin barrier dysfunction and wrinkles.

We have developed a new patented eco-designed active ingredient from a regenerative culture process of *Linum usitatissimum*. This linseed oil concentrate, obtained by molecular distillation, is enriched in unsaponifiable fraction which contains highly active molecules such as phytosterols. We have investigated the activity of this new active on skin aging by *in vitro* and *in vivo* studies.

2. Materials and Methods

2.1. *In vitro* evaluation:

The efficacy on ceramides was analyzed on a skin explant model cultivated in the open air. Skin explant was topically treated several days (D0, D2, D3, D5) with cream containing 1% of active ingredient (LIN) or placebo. Immunostaining of ceramides was performed at D6. Statistical significance between conditions was assessed using One Way ANOVA followed by Dunnett's test with $p<0.05$ being considered significant.

The anti-aging efficacy was evaluated on a reconstructed aged skin chimeric model and on a UVA stress skin explant model:

- 3D full-thickness skin model was reconstructed with aged dermal fibroblasts (57 years) and young keratinocytes (28 years). LIN (0.01%) or retinol (0.1µM – positive reference) were applied in medium during the equivalent dermis reconstruction, then applied topically (1% vs placebo for LIN or 0.1µM for retinol) on reconstructed epidermis for 8 days. At the end, histologic and immunostaining analyses were conducted. Statistical

significance between conditions was assessed using a two-tailed, unpaired Student t-test, with $p<0.05$ being considered significant.

- Skin explants were pre-treated for 48h by topically application of LIN formulated at 1% or placebo and in parallel by LIN (0.01%) in medium. UVA stress (6J/cm²) was induced on skin explants. Immunostaining of several markers were conducted 6h or 24h after UVA stress. Statistical significance between conditions was assessed using One Way ANOVA followed by Tukey's test with $p<0.05$ being considered significant.

The efficacy on telomeres length was analyzed on human dermal fibroblasts. Cells were pre-treated for 3 days with LIN (0.001%) or positive reference (Astragaloside IV – 30µM). Then, cellular senescence was induced by H_2O_2 stress for 2 days. At D5, the telomere length was measured by fluorescent *in situ* hybridization. Statistical significance between conditions was assessed using a two-tailed, unpaired Student t-test, with $p<0.05$ being considered significant.

2.2. *In vivo* evaluation:

An *in vivo* randomized double-blind placebo-controlled study has been performed on 2 groups of 24 women aged from 45 to 65 years. Subjects presented dry skin and aging signs. Subjects used a cream containing LIN at 1% or a placebo twice a day for 56 days. Assessments were performed at day 0, day 28 and day 56 including:

- EOS ceramides from D-squams taken on the face and analyzed by Gas Chromatography-Mass Spectrometry (GC/MS).
- Trans Epidermal Water Loss (TEWL) measured by Aquaflux.
- Skin micro relief (area close to the eye) and fine wrinkles (global analysis of the face) using non-contact topographic measurement (Aeva3D-HE²).
- Skin irregularities by clinical scoring.
- Self-assessment efficacy of the products by questionnaire.

For each parameter, the comparison of results at each time D28 and D56 compared to D0 is performed using a statistical test. Normality of the data is tested using the Shapiro–Wilk test (level $\alpha>0.01$). Paired t-test (normal data) or Wilcoxon signed rank test (non-normal data) is then used with a significance level $\alpha<0.05$.

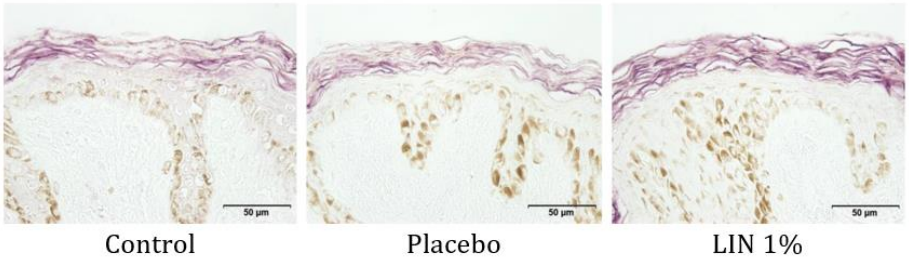
3. Results

3.1. *In vitro* results:

Ceramide quantification:

LIN at 1% significantly increased the quantity of ceramides in *stratum corneum* of +60%$^{\$\$}$ *versus* Control and +123%*** *versus* Placebo (Figure 1).

(a)



(b)

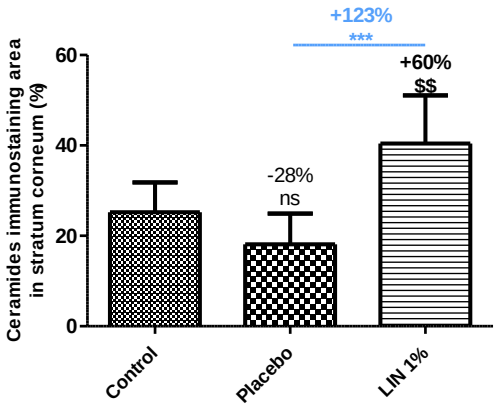


Figure 1: (a) Illustrative photographs of ceramides immunostaining; (b) Quantification of ceramides $$ p<0.01 vs Control; *** p<0.001 vs Placebo (One way ANOVA followed by Dunnett test)

Anti-aging efficacy in reconstructed aged skin chimeric model:

The reconstructed epidermis with keratinocytes cultivated on an aged dermis showed similar alterations to chronological aging such as thinner epidermis and disorganized dermal epidermal junction (Figure 2a). LIN increased the thickness of epidermis and improved its overall structure (Figure 2a). LIN significantly increased the expression of differentiation marker keratin 10 (+18.5%*** vs placebo) (Figure 2b and 2c). It increased also the expression of integrin alpha-6 (+47%*** vs placebo) (Figure 2d and 2e).

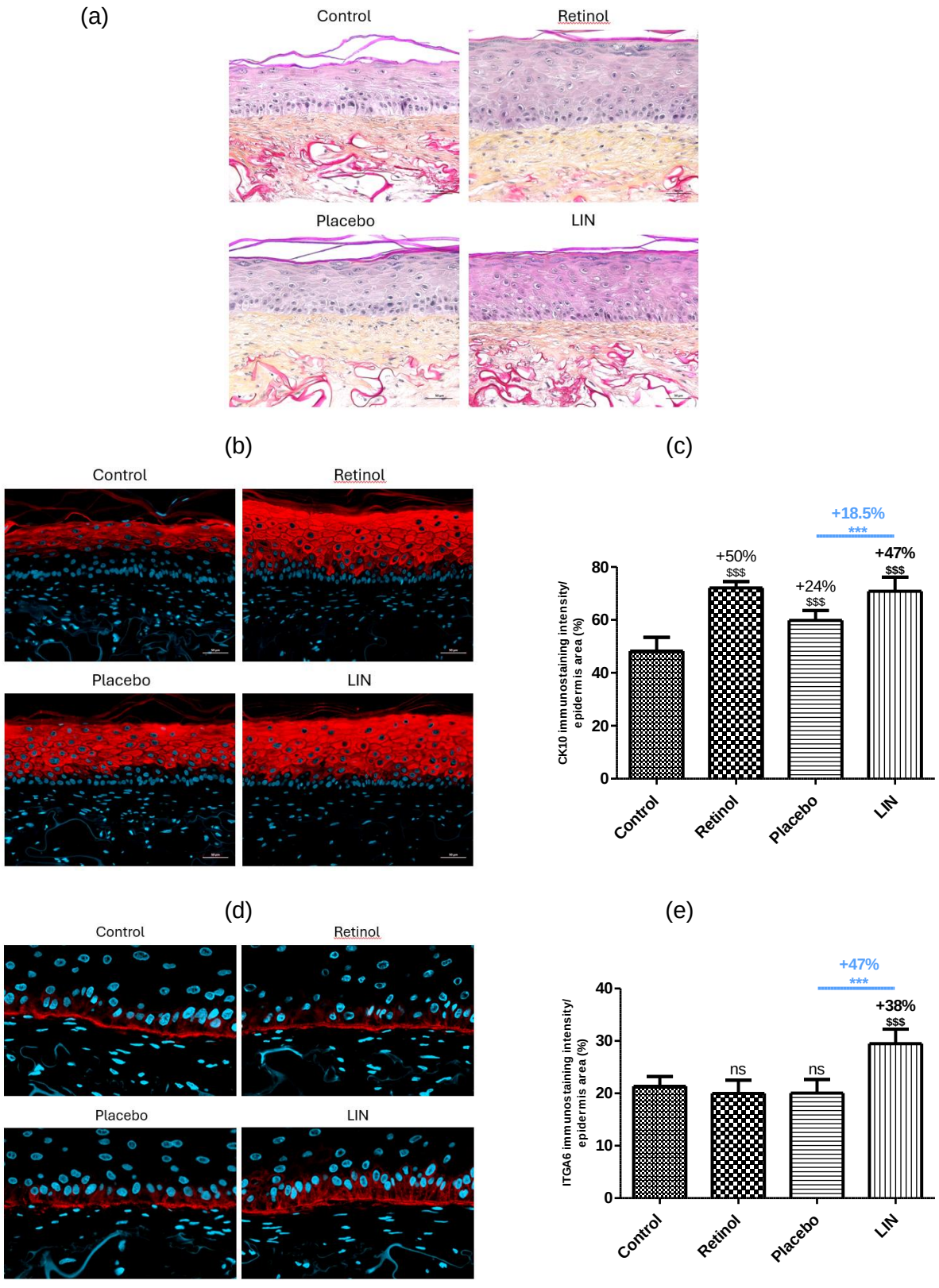


Figure 2: Illustrative photographs of histological coloration (a) and immunostaining of keratin 10 (b); and integrin α6 (d); Quantification of keratin 10 immunostaining (c) and integrin α6 immunostaining (e) $$$ p<0.001 vs Control; *** p<0.001 vs Placebo (Student t test)

Anti-aging efficacy in UVA stress skin explant model:

LIN protected skin against UVA stress thanks to a significant increase in filaggrin (+37%** vs placebo), laminin-332 (+44%** vs placebo) and hyaluronic acid (+41%*** vs placebo) (Figure 3).

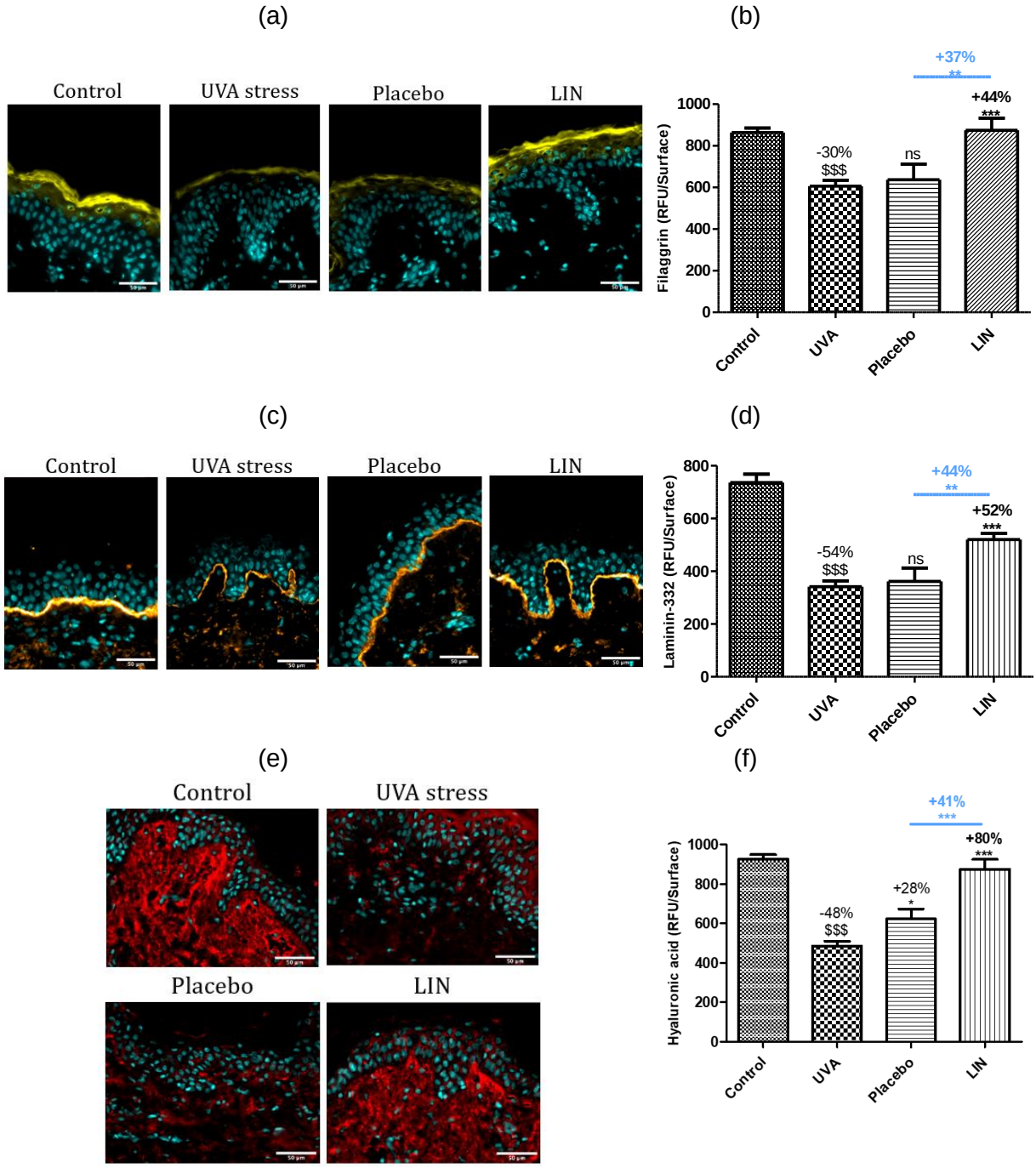
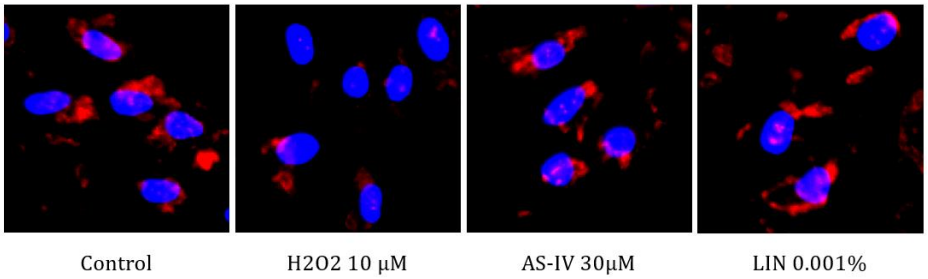


Figure 3: Illustrative photographs of immunostaining of filaggrin (a), laminin-332 (c) and hyaluronic acid (e); Quantification of immunostaining of filaggrin (b), laminin-332 (d) and hyaluronic acid (f) $$$ p<0.001 vs Control; *p<0.5; *** p<0.001 vs UVA; **p<0.01; *** p<0.001 vs Placebo (One way ANOVA followed by Tukey test)

Telomeres length:

Fibroblasts' telomere length decreased with H_2O_2 stress. The treatment by positive reference (Astragaloside IV) and active ingredient (LIN 0.001%) protected the telomere length of H_2O_2 stress (+92%** and +120%*, respectively) (Figure 4).

(a)



(b)

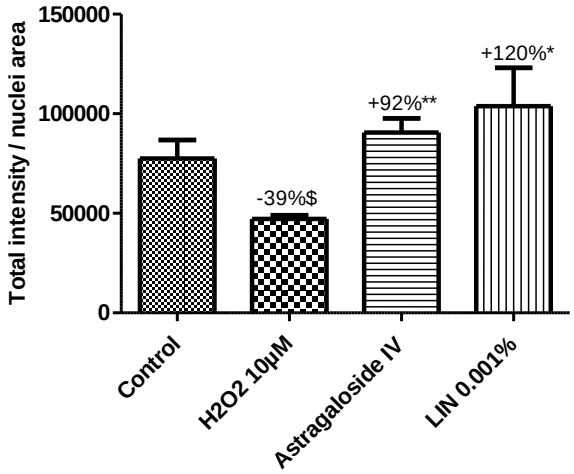


Figure 4: (a) Illustrative photographs of telomere length (Blue: Hoechst, Red in nuclei: Telomeres); (b) Quantification of telomere length.
$ p<0.05 vs Control; * p<0.05; ** p<0.01 vs H_2O_2 (Student t test)

3.2. *In vivo* clinical results:

Clinical results showed a significant improvement of EOS ceramides of the skin for LIN (+12.5%) between day 56 and day 0 and no changes for the Placebo traducing an improvement of the skin barrier (Figure 5).

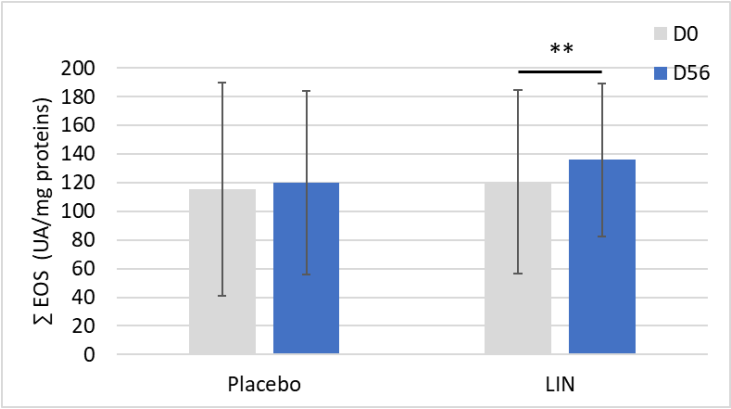


Figure 5: EOS ceramides at D0 and D56 for LIN and Placebo. ** p<0.05 vs D0.

Results of skin microrelief at day 28 showed an improvement of Rz and Rt parameters (Rz: -3,3% and Rt: -3,3%),traducing a smoother skin, with a difference versus Placebo for Rz ($p<0.1$) (Figure 6). At day 56, LIN improved significantly Rz and Rt versus day 0 (-4.7% and -5.9% respectively) (data not shown).

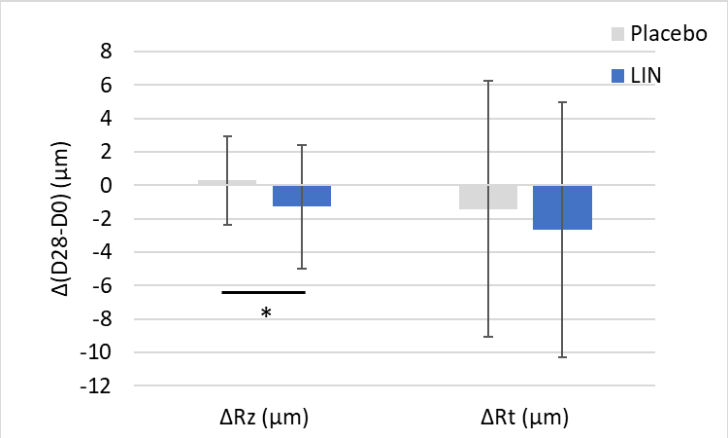


Figure 6: Skin microrelief evolution for Rz and Rt parameters between D28 and D0. * $p<0.1$.

The improvement of the skin surface was confirmed by the clinical scoring of skin irregularities showing a significant improvement at day 28 and day 56 compared to day 0 for LIN with a significant difference with the Placebo at both times (Figure 7).

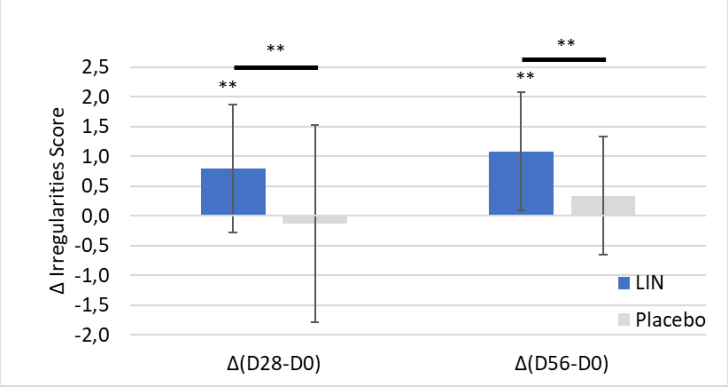


Figure 7: Clinical scoring of skin irregularities at D28 and D56. ** $p<0.05$

4. Discussion

Aging is a biological process that affects most cells, organisms and species. As the largest body organ, skin shows clear signs of aging. Skin aging is a process associated with intrinsic and extrinsic factors that lead to a gradual loss of structural integrity and physiological functions of the skin. With age, keratinocytes partly lose their ability to self-renewal, differentiation and response to stress, leading to an impairment of the structure and function of the epidermis. The epidermis and the dermal-epidermal junction (DEJ) are strongly altered. A drop of differentiation markers such as keratin 10 and 14 and DEJ markers (collagen XVII, laminin-332 and integrin alpha-6) had been described [1-3]. These alterations lead to clinical modifications such as skin irregularities, dehydration, skin barrier dysfunction and wrinkles.

We have developed a new eco-designed active ingredient from a regenerative culture process of *Linum usitatissimum.* This linseed oil concentrate, obtained by molecular distillation, is enriched in unsaponifiable fraction which contains highly active molecules such as phytosterols.

This active ingredient showed anti-aging efficacy on several *in vitro* models and *in vivo* clinical study. In fact, in a reconstructed aged skin chimeric model, LIN countered the effects of aging by improving epidermis differentiation (increase of keratin 10) and reinforcing the dermal-epidermal junction (increase of integrin alpha-6). LIN increased also the expression of another markers of DEJ: collagen XVII (+15.5%**), laminin-332 (+38%***) and fibrillin-1 (+19%***) (data not shown). This effect on DEJ had been observed also on UVA stress skin explant model where LIN preserved the amount of laminin-332, which was altered by UVA stress. In this model, LIN demonstrated its effect on skin barrier and epidermis differentiation by increasing the expression of filaggrin and keratin 14 (+29%*) (data not shown).
The oxidative stress is one of the main mechanisms associated with skin aging. LIN also protected against oxidative stress damages by decreasing carbonylated proteins (-25%*) in the model of UVA stress (data not shown).
Ceramides are important structural components of the epidermis, where they enter in the composition of the lipid matrix of the *stratum corneum.* The role of this matrix is central to the skin barrier function and in particular to protection against the exposome as well as in the regulation of water permeability or epidermal renewal. Ceramide content varies under various conditions, notably it is known to decrease with age [4]. By increasing ceramides expression, LIN could promote skin barrier and contribute to skin moisturization. These different results suggest that LIN can reinforce the skin barrier and DEJ, which are essential for good skin homeostasis.
Telomere length, in particular the abundance of short telomeres, has been proposed as a biomarker for senescence, aging, and overall cell health [5]. Various stress factors (environmental factors, stress, diet, lack of physical activity, etc.) could accelerate the shortening of telomeres and thus aging. LIN significantly increased telomere length decreased by H_2O_2 stress. The active ingredient protected telomeres from H_2O_2 stress inducing premature senescence of fibroblasts. By protecting telomeres, LIN could limit the process of cellular senescence and promote cellular longevity, thus delaying skin aging.

These effects were confirmed by clinical study demonstrating an improvement of skin surface. LIN showed a significant increase of EOS ceramides traducing a reinforcement of skin barrier. This result was confirmed after 28 days with an improvement of the TEWL (-1.7%) observed for LIN with a significant difference with the Placebo (+17.1%) (data not shown). LIN showed an improvement of skin microrelief traducing a smoother skin and an improvement of skin irregularities. We also observed a significant decrease of fine wrinkles (class 20-50 µm) on the whole face between day 56 and day 0 for LIN (-1,1%) (data not shown). Self-assessment questionnaire after 28 days showed that 78% of the subjects observed an improvement in skin moisturization (data not shown).

5. Conclusion

We have demonstrated that linseed oil concentrate is able to reinforce the skin barrier function and the DEJ to promote renewal of the epidermis. By protecting telomeres shortening and preserving cell regeneration capacities, the active could promote longevity to slow down skin aging process. Clinical results confirmed the efficacy of the active on skin barrier and epidermal surface.

References

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