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In Vitro and *in Vivo* Anti-Inflammatory Effect of a Biotechnologically Modified Borage Seed Extract: Evidence for Lipid Pro-Resolving Mediators' Implication in the Enhancement of Psoriatic and Atopic Dermatitis Lesions

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Abstract

Aim: Resolvins, maresins and lipoxins are lipid mediators issued from essential polyunsaturated fatty acids which are the first anti-inflammatory and pro-resolving signals identified during the resolution phase of inflammation. As borage oil and/or borage seed extracts have shown beneficial action in treatment of atopic dermatitis or eczema in human and canine, we have modified a borage oil component by using biotechnology in order to get a compound structurally related to a polyunsaturated fatty acid, and we have studied its ability to reduce inflammation mediators production through the generation of resolvins, maresins and/or lipoxins. Additionally, we have demonstrated the potent anti-inflammatory effect of this new compound which consists in borage seed oil aminopropanediol amides, through an *in vivo* study concerning subjects suffering from psoriasis or atopic dermatitis. Study Design/Methods: For the *in vitro* study, inflammation was induced in co-cultures of human dendritic cells and normal keratinocytes by the addition of PMA and the calcium ionophore A23187. Ability of our borage seed oil aminopropanediol amides to increase resolvin D2, maresin 1 and lipoxins A4 and B4 synthesis was then measured. Pro-inflammatory cytokines (IL-1*β*, IL-6, IL-8) and PGE2 productions were also quantified. For the *in vivo* study, 36 subjects suffering from psoriasis or atopic dermatitis have used twice a day during 30

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days, a formulation containing borage seed oil aminopropanediol amides. Before the beginning of the study and after 30 days' treatment, the severity of psoriasis and of atopic dermatitis was evaluated by using the PGA and the SCORAD scoring scales, respectively. Results: Borage seed oil aminopropanediol amides were able to significantly increase the resolvin D2, maresin 1 and lipoxins A4 and B4 synthesis. Concomitantly, they were also able to significantly inhibit the production of IL-1*β*, IL-6, IL-8 and PGE2 induced by the PMA and the calcium ionophore A23187 in the *in vitro* co-culture model used. Introduced in formulation, borage seed oil aminopropanediol amides significantly reduced the clinical manifestations of psoriasis and atopic dermatitis. Conclusion: Our *in vitro* and *in vivo* study clearly showed the anti-inflammatory activity of borage seed oil aminopropanediol amides and emphasized the putative role of pro-resolving lipid mediators in the treatment of atopic dermatitis, psoriasis or other inflammation-induced skin diseases.

Keywords

Human Skin, Biotechnologically Modified Borage Extract, Interleukins, Inflammation, Psoriasis, Atopic Dermatitis, Resolvins, Maresins, Lipoxins

1. Introduction

Resolvins, protectins, maresins and lipoxins are lipid mediators issued from essential polyunsaturated fatty acids (PUFAs) which are the first anti-inflammatory and pro-resolving signals identified during the resolution phase of inflammation (for a review, see [1]). Resolvins derived from both eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), lipoxins (AA metabolites), protectins and maresins (DHA metabolites), all participate to reduce inflammation and were all able to inhibit pro-inflammatory cytokines production in various situations such as obesity [2], asthma [3], chronic airway inflammation diseases [4], cigarette smoke-induced lung inflammation [5] and pulmonary inflammation in general [6], synovial inflammation [7], chronic disorders of the colon and colon cancer [8].

In these conditions, the use of these lipid mediators, or of products able to induce their production, seems to be of a great therapeutic value in the management of human skin inflammatory disorders.

Interestingly, fish or borage oil for example, which contains significant quantities of PUFAs like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has showed potent anti-inflammatory effects in animal [9] and human skin suffering from atopic dermatitis [10]-[13], eczema [14] [15], or psoriasis [16]. Dietary supplementation with borage oil even showed significant efficacy in increasing hydration of human elderly skin [17].

According to various scientific works, generation of lipid pro-resolving mediators from PUFAs contained in fish and borage oils, is the most likely explanation for these anti-inflammatory and curative effects (for a review, see [18]).

Nevertheless, in a very surprising way, nor resolvins, protectins, maresins or lipoxins, nor products able to induce their production, were already tested for their ability to enhance atopic dermatitis or other inflammatory skin diseases after a topical application on the human cutaneous tissue.

Taking advantage of the possibility to use biotechnological process to create a borage oil derivative structurally related to a polyunsaturated fatty acid, we have created borage seed oil aminopropanediol amides (BSOAA) and we have studied their anti-inflammatory effects in an *in vitro* model of human dendritic cells and normal keratinocytes co-cultures. In this first part of the study, the ability of our biotechnological product to induce the lipid pro-resolving mediators' production, and to reduce the pro-inflammatory cytokines and the PGE2 production, was evaluated.

In the second part of this study, we have evaluated the *in vivo* capacity of our borage seed oil aminopropanediol amides to enhance psoriatic and atopic dermatitis lesions of patients suffering from these diseases.

2. Materials and Methods

2.1. Reagents and Materials

Macrophage-SFM and Alamar Blue were purchased from Life Technologies (Saint Aubin, France). Keratino-

cytes growth medium (KGM-2) and Normal Human Epidermal Keratinocyte (NHEK) were purchased from Promocell (Heidelberg, Germany). GM-CSF and IL-4 were purchased from Peprotech (Neuilly-Sur-Seine, France). PMA and A23187 were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). PUFA, DHA and EPA, came from Larodan (Solna, Suède). All plastics for cell culture were produced by Falcon and purchased from D. Dutscher (Brumath, France). Turbocapture mRNA kit for mRNA was purchased from Qiagen (Courtaboeuf, France). Maxima First Strand cDNA Synthesis kit was purchased from Thermo Fisher Scientific (Illkirch, France). The kit used for cytokines multiplex analysis came from Merck Millipore (Saint-Quentin en Yvelines, France). For PCR, SsoFast EvaGreen super mix were purchased from BioRad (Marnes la coquette, France). Oasis HLB 96 wells solid phase extraction were purchased from Waters (Saint Quentin en Yvelines, France).

2.2. Dendritic Cells and Normal Human Epidermal Keratinocytes Co-Culture

Dendritic cells (DCs) were differentiated from human peripheral blood mononuclear cells (PBMCs). PBMCs were obtained from healthy blood donor buffy coats by a standard Ficoll-Hypaque gradient method. Monocytes were isolated from PBMCs by adherence to plastic for 2 hours in serum-free medium (SFM) optimized for macrophage culture, at 37˚C in a humidified atmosphere containing 5% CO_2. Monocytes were then incubated for 7 days with GM-CSF (10 ng/ml) and IL-4 (10 ng/mL) to be differenciated in dendritic cells. In parallel of dendritic cells differenciation, Normal Human Epidermal Keratinocytes (NHEK) were set in culture. At the end of diferenciation step, NHEK were seeded in inserts and were placed with dendritic cells to recreate a skin compartment.

Compounds tested were mixed with the DC/NHEK co-culture 24 hours prior to the inflammatory stimulation with PMA (50 nM) and A23187 (1 µM).

2.3. Test of Viability

Borage seed oil aminopropanediol amides were evaluated on monocyte viability. For that PBMCs were seeded in 96-wells plate at the density of 3.125×10^6 cells/cm^2 for 2 hours. After subsequent washes with PBS, borage seed oil aminopropanediol amides were introduced at 0.005% (v/v) in culture medium for 24 hours. During the last 6 hours, Alamar Blue was introduced in the cell culture and conversion of resazurin to the fluorescent molecule, resorufin, was measured as an indicator of cell death. Measurement was done thanks to the spectrofluorimeter Tecan Infinite F500.

2.4. Specialized-Proresolving Mediators Quantification

The extraction protocol and LC/MS/MS analysis were performed by Ambiotis SAS (France) as described in Le Faouder *et al.*, J. Chrom. B., 2013 adapted from Ambiotis Standard Operating Procedure. Briefly, samples were extracted using oasis HLB 96 wells solid phase extraction (Waters). LC-MS/MS analysis was performed on UHPLC system (Agilent LC1290 Infinity) coupled to Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped with electro-spray ionization operating in negative mode. Reverse-phase UHPLC was performed using ZorBAX SB-C18 column (2.1 mm × 50 mm × 1.8 µm) (Agilent Technologies). We thank Pr Charles Serhan for PD1 standard.

2.5. Genes Expression

mRNAs of DCs were extracted by using specific extraction system, Turbocapture mRNA (Qiagen). Then a reverse transcription was made to obtain cDNA (Fermentas). cDNA have been used in the Applied Biosystems 7500 Fast Real Time PCR system with the SsoFast EvaGreen Supermix (BioRad) and specific primer of gene interest.

2.6. Cytokine Quantification

Cell supernatant was collected after 24 hours of stimulation and analyzed thanks to Millipore kit for expression of IFN-g, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13 and TSLP in accordance with manufacturer instruction.

2.7. *In Vivo* Study

2 groups of 18 subjects (one group suffering from psoriasis, one group suffering from atopic dermatitis), male and female, aged from 3.5 months to 63.1 years, were included in this study. All the subjects have used twice a day, a cosmetic formulation (see below) containing our borage seed oil aminopropanediol amides. Before and after a 30 days' treatment, 5 dermatologists have scored psoriasis severity by using the Physician Global Assessment (PGA) [19] and severity of atopic dermatitis by using the standardized quotation of the SCORAD [20]. Dermatologists also determined for each group, the SRRC Index score (evaluating scaling roughness, redness and cracks) (for a review, see [21]).

Additionally, before and after a 30 days' treatment, volunteers have answered to an auto-evaluation questionnaire regarding the efficacy of the product. Sensations of itching, tightness and discomfort were then evaluated; the Infant's Dermatitis Quality of Life Index [22] and the Dermatology Life Quality Index [23] were also determined.

2.8. Cosmetic Formulation Composition

AQUA (WATER)-URIAGE THERMAL SPRING WATER-BUTYROSPERMUM PARKII (SHEA BUTTER)-CETEARYL ISONONANOATE-ISODECYL NEOPENTANOATE-BUTYLENE GLYCOL–GLYCERIN-HYDROGENATED POLYDECENE-DIMETHICONE-SQUALANE-STEARETH-2-STEARETH-21-CETYL ALCOHOL-PENTAERYTHRITYL DISTEARATE-POLYACRYLATE-13-BRASSICA CAMPESTRIS (RAPESEED) STEROLS-CHLORPHENESIN-PIROCTONE OLAMINE-POLYISOBUTENE-SODIUM DEXTRAN SULFATE-O-CYMEN-5-OL-TOCOPHERYL ACETATE-XANTHAN GUM-CITRIC ACID-RASPBERRY SEED OIL/PALM OIL AMINOPROPANEDIOL ESTERS-POLYSORBATE 20-SORBITAN ISOSTEARATE-ASIATICOSIDE-PHYTOSPHINGOSINE-AMINO-GLYCEROL BORAGO OFFICINALIS FATTY AMIDE.

2.9. Statistics

2.9.1. *In Vitro* Studies

Data are expressed as means ± S.D. of 2 different experiments realized in duplicate (n = 2) and in triplicates (n = 3). The statistical significances were assessed by Student t-tests ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

2.9.2. *In Vivo* Studies

Data are expressed as means of the scores determined for each subject. The statistical significances were assessed by Paired Wilcoxon tests (as indicated).

3. Results and Discussion

As shown in **Figure 1**, in the selected experimental conditions, borage seed oil aminopropanediol amides at 0.005% (v/v) were able to significantly increase the cutaneous cell production of the pro-resolving mediators maresin 7 (R) MaR1 and lipoxins LxA4 and LxB4. We can then reasonably conclude that our BSOAA contain significant amounts of precursors of these mediators. Additionally, we can also logically expect in these conditions, that our BSOAA could afford for efficient anti-inflammatory effects. In order to test this hypothesis, we induced an inflammation in our co-culture model by the addition of phorbol myristate acetate (PMA) and the calcium ionophore A23187, and we evaluate the effect of our BSOAA on pro-inflammatory cytokines and PGE2 productions.

As shown in **Figure 2**, borage seed oil aminopropanediol amides at 0.005% (v/v) were able to significantly counteract the effect of PMA/A23187 in the cutaneous cells co-cultures. The production of the following pro-inflammatory cytokines was significantly reduced: IL-1β, 102.5% this ($p < 0.05$); IL-6, −109.5% ($p < 0.001$); IL-8, −90.3% ($p < 0.001$). PGE2 production, which is induced during inflammatory process, was also inhibited by BSOAA: −110.9% ($p < 0.001$). These results are in line with the work of Miller *et al.* showing that polyunsaturated fatty acids induce epidermal generation of local putative anti-inflammatory metabolites [9]. These results so clearly demonstrate the very potent anti-inflammatory effect of our borage seed oil aminopropanediol amides, which can be merely explained by the ability of BSOAA to induce pro-resolving lipid mediators like

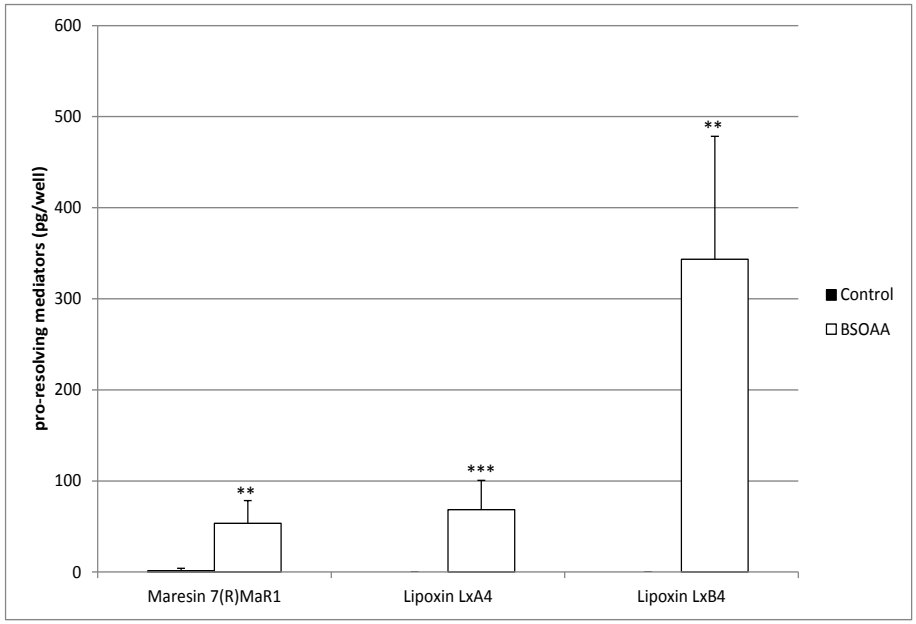

Figure 1. Effect of borage seed oil aminopropanediol amides at 0.005% (v/v) on the cutaneous cell production of resolvin RvD2, maresin 7(R) MaR1 and lipoxins LxA4 and LxB4. **Significantly different from the "control" ($p < 0.01$, Student t-test); ***Significantly different from the "control" ($p < 0.001$, Student t-test); n = 5, from 2 different experiments; NB: histogram for the "control" condition are not visible for the lipoxins measurements because the mean value is equal to 0.

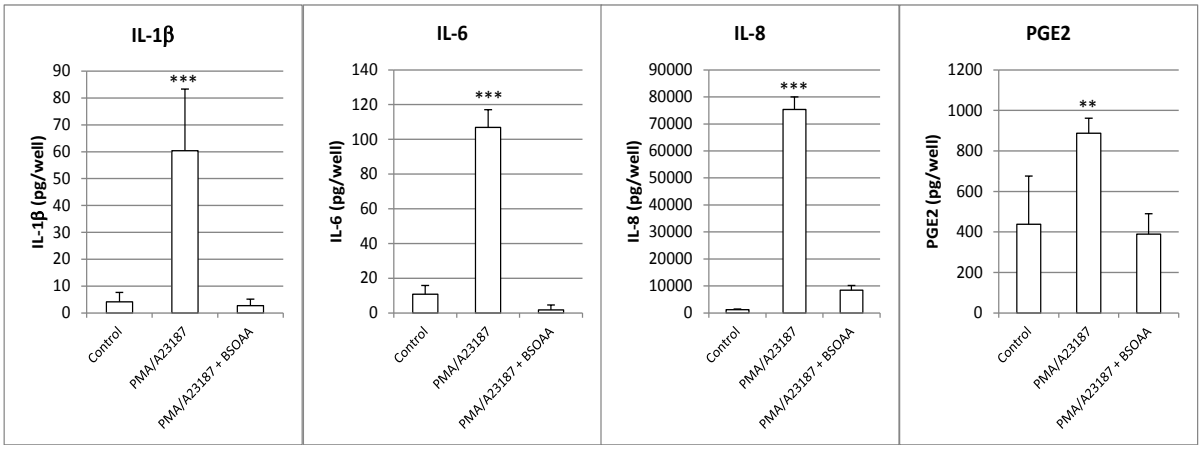

Figure 2. Effect of borage seed oil aminopropanediol amides at 0.005% (v/v) on the cutaneous cell production of IL-1b, IL-6, IL-8 and PGE2. **Significantly different from the "control" ($p < 0.01$, Student t-test). ***Significantly different from the "control" ($p < 0.001$, Student t-test). n = 5, from 2 different experiments.

lipoxins and maresin.

Psoriasis and atopic dermatitis are pathologies notably characterized by high skin levels of pro-inflammatory cytokines such as IL-1 and IL-8. Numerous therapeutic approaches so consider the targeting of the cytokine network as a promising therapeutic approach (for a review, see [24]).

Regarding the *in vitro* efficacy of our borage seed oil aminopropanediol amides, *i.e.* their ability to significantly reduce skin cells pro-inflammatory cytokines production, we were prompted in the second part of this work, to deepen our knowledge of our biotechnologically made product by evaluating its effect through *in vivo* study involving 36 subjects suffering from psoriasis and atopic dermatitis.

As shown in **Figure 3**, topical application of a formulation containing 1% of our borage seed oil aminopro-

panediol amides was able to significantly improve the xerosis of all the subjects' skins (suffering from psoriasis or atopic dermatitis): −67% ($p < 0.01$; visual scoring by dermatologists). As shown in **Figure 4**, the SRRC score of all the volunteers was also significantly improved: −69% ($p < 0.01$). In addition, the auto-evaluation of the product efficacy by the volunteers showed that sensations of itching, tightness and discomfort were also significantly reduced: −60% ($p < 0.01$), −68% ($p < 0.01$) and −74% ($p < 0.01$), respectively (**Figure 5**).

When we consider separately volunteers suffering from psoriasis or from atopic dermatitis, we can note (**Figure 6** and **Figure 7**) that the formulation containing the borage seed oil aminopropanediol amides significantly improve the severity of both psoriasis (PGA scale) and atopic dermatitis (SCORAD index): −13% ($p < 0.01$) and −65% ($p < 0.01$), respectively.

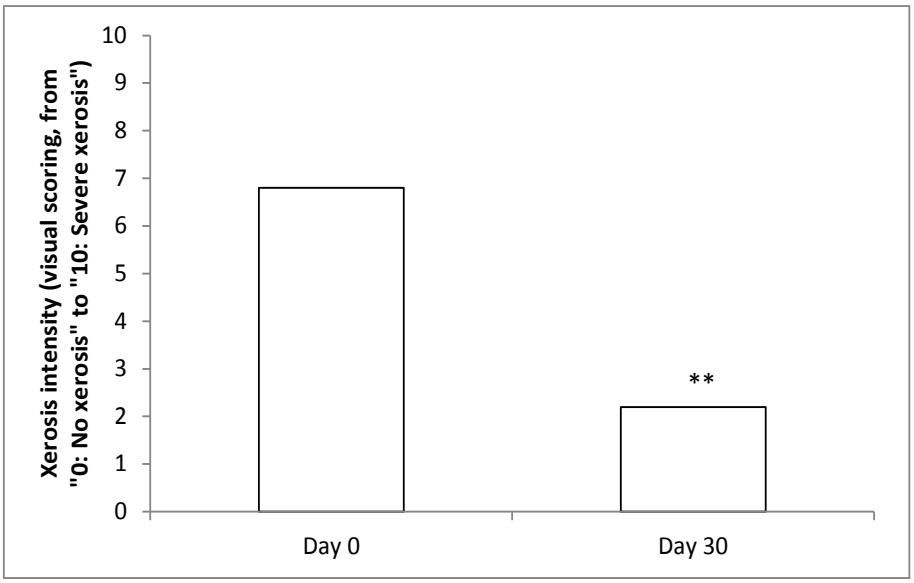

Figure 3. Effect of a formulation containing borage seed oil aminopropanediol amides on skin xerosis. **Significantly different from the "Day 0" ($p < 0.01$, paired Wilcoxon test). n = 36 subjects.

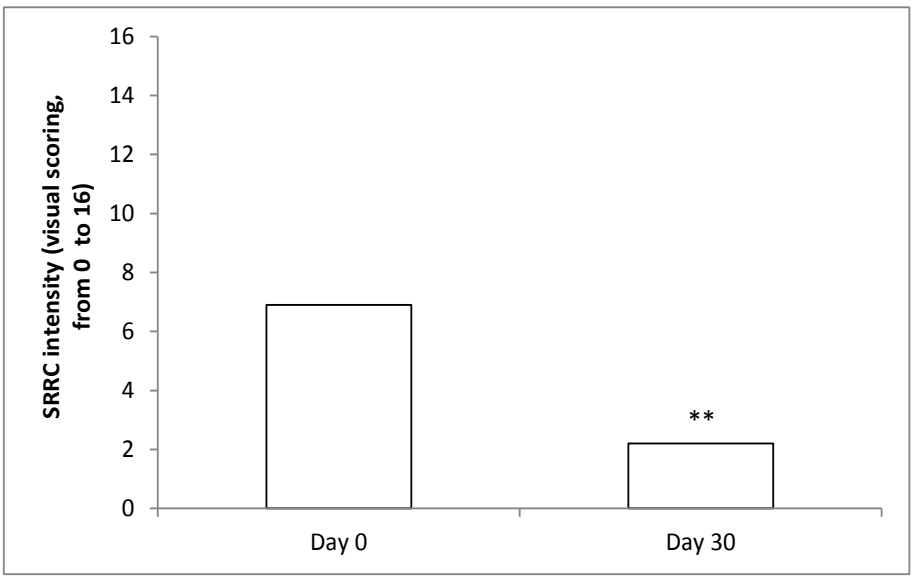

Figure 4. SRRC score after 30 days of use of a formulation containing borage seed oil aminopropanediol amides. **Significantly different from the "Day 0" ($p < 0.01$, paired Wilcoxon test). n = 36 subjects.

At last, as shown in the **Figure 8**, quality of life of the volunteers was significantly improved: the Infant's dermatitis Quality of Life Index (IDQOL) and the Dermatology Life Quality Index (DLQI) were reduced by 65 ($p < 0.01$) and 75% ($p < 0.01$), respectively.

All the results obtained in this *in vivo* study are in line with the works of numerous authors showing that targeting the cytokine network could consist in a very helpful therapeutic approach for the treatment of inflammatory diseases in human skin (for a review, see [24] and [25]).

Our *in vitro* and *in vivo* studies also permit us to demonstrate that pro-resolving lipid mediators, or active compounds able to increase their production in skin, could offer pertinent and efficient tools to improve the skin and the life quality of patients suffering from psoriasis or atopic dermatitis notably.

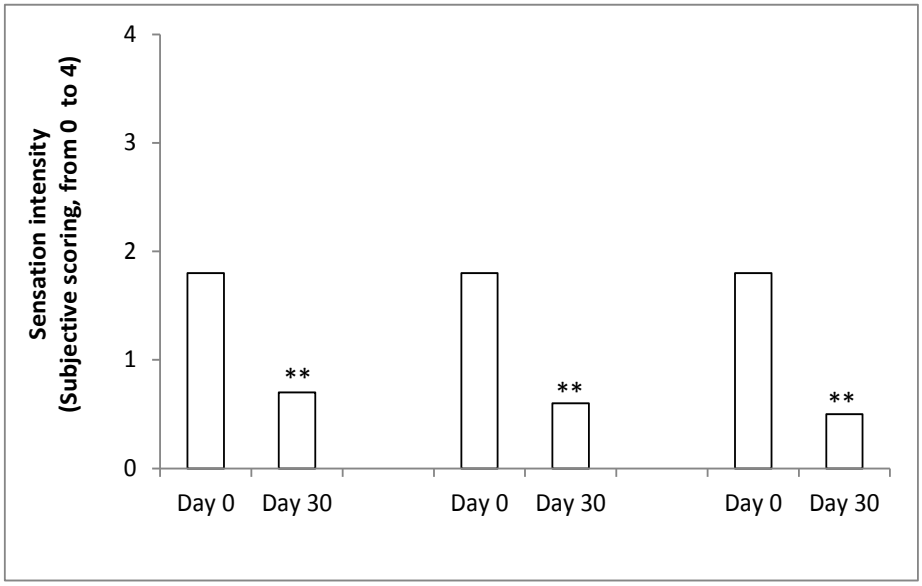

Figure 5. Sensations of tightness, itching and discomfort after 30 days of use of a formulation containing borage seed oil aminopropanediol amides-Auto-evaluation by the volunteers. **Significantly different from the "Day 0" ($p < 0.01$, paired Wilcoxon test). n = 36 subjects.

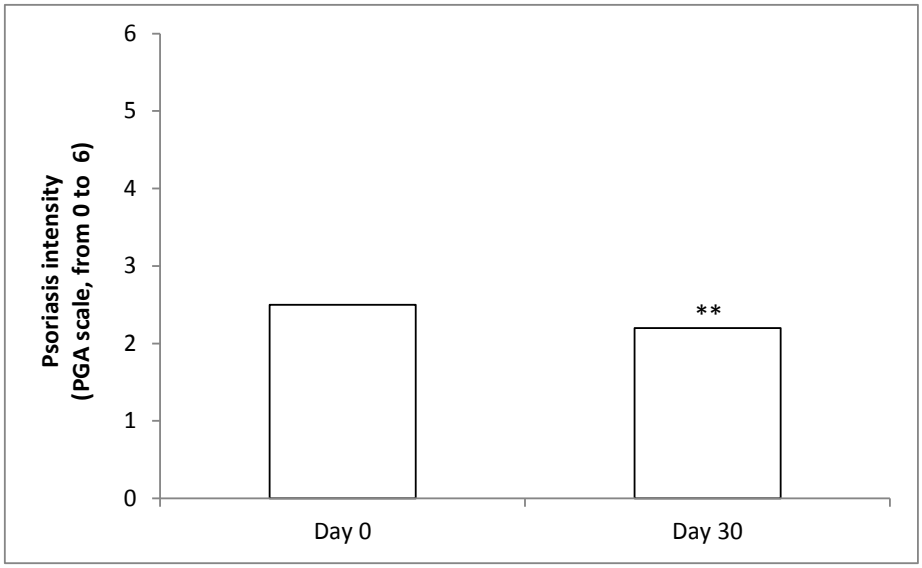

Figure 6. Effect of a formulation containing borage seed oil aminopropanediol amides on psoriasis severity. **Significantly different from the "Day 0" ($p < 0.01$, paired Wilcoxon test). n = 18 subjects.

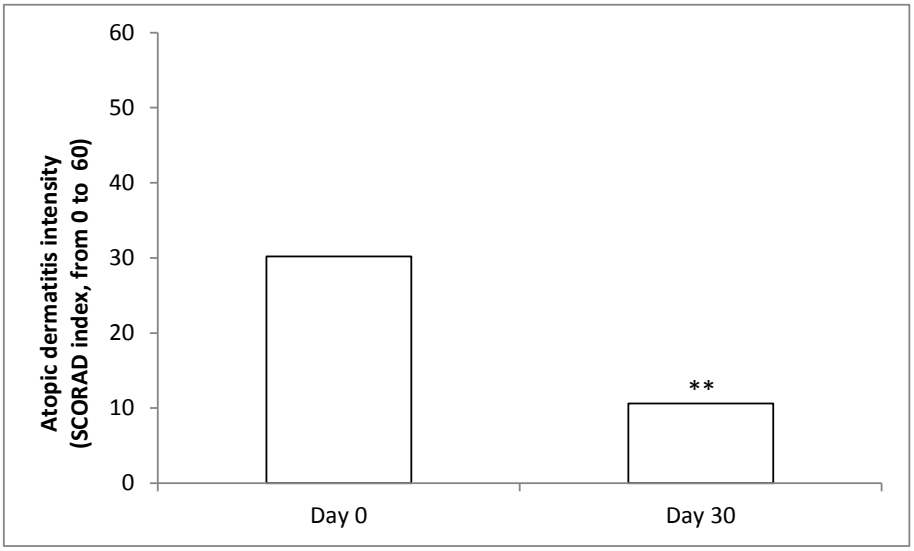

Figure 7. Effect of a formulation containing borage seed oil aminopropanediol amides on atopic dermatitis severity. **Significantly different from the "Day 0" ($p < 0.01$, paired Wilcoxon test) n = 18 subjects.

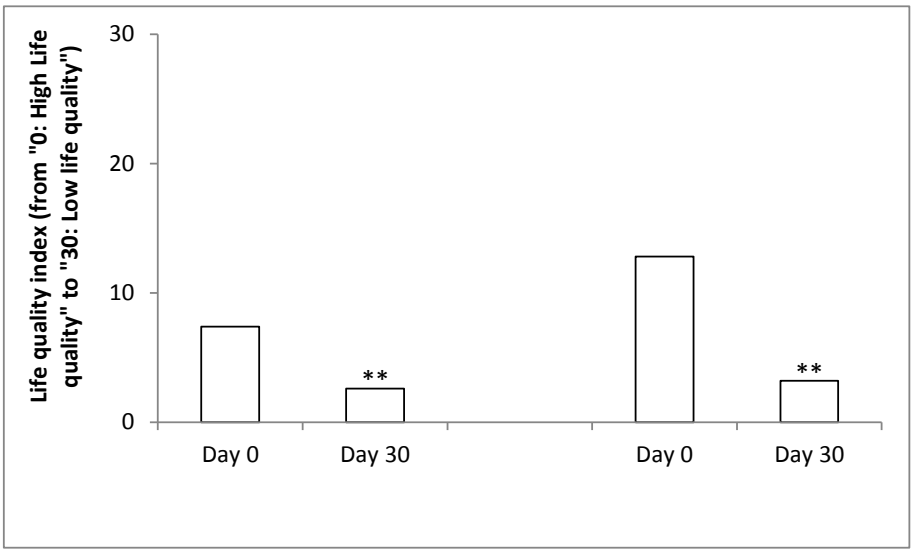

Figure 8. Effect of a formulation containing borage seed oil aminopropanediol amideson the Infant's dermatitis Quality of Life Index (IDQOL) and the Dermatology Life Quality Index (DLQI). **Significantly different from the "Day 0" ($p < 0.01$, paired Wilcoxon test) n = 36 subjects.

4. Conclusion

At last, we show that biotechnologies can be very useful for the generation and/or the amelioration of new therapeutics in human.

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