



Alp Rose stem cells, olive oil squalene and a natural alkyl polyglucoside emulsifier: Are they appropriate ingredients of skin moisturizers – *in vivo* efficacy on normal and sodium lauryl sulfate-irritated skin?

Matične ćelije alpske ruže, skvalen maslinovog ulja i prirodni alkil-poliglukozidni emulgator: da li su odgovarajući sastojci kremova za vlaženje – *in vivo* efikasnost na zdravoj i koži iritiranoj natrijum lauril sulfatom?

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Abstract

Background/Aim. Since skin moisturization may be achieved by both actives and chosen carrier, plant stem cells, squalene and natural alkyl polyglucoside emulsifier may be potential components of contemporary cosmetic products. The aim of the study was *in vivo* evaluation of the skin irritation potential and the efficacy of Alpine Rose stem cells incorporated into liposomes and olive oil squalene as ingredients of moisturizing creams, with respect to the novel emulsifier used for creams' stabilization. **Methods.** With the employment of noninvasive skin biophysical measurements, skin hydration (EC), transepidermal water loss (TEWL), erythema index (EI) and viscoelasticity were measured on 76 healthy volunteers. In the first phase, skin irritation after a 24-hour occlusion and the long-term efficacy of creams (a 21-day study) on healthy skin were evaluated. Phase II of the study focused on the cream efficacy assessment after a 6-day treatment of sodium lauryl sulfate-

irritated skin. **Results.** After a 24-hour occlusion, there were no significant changes in the EI for any tested sample. In the second phase of the study, the EI was not significantly altered for the cream containing squalene, while the application of all active samples resulted in a significant reduction of TEWL. In both phases of the study an EC increase was recorded, especially for the squalene-containing cream. **Conclusion.** Due to the lack of skin irritation and skin barrier impairment along with the marked hydration effect, it could be said that the investigated actives incorporated into alkyl polyglucoside emulsifier-stabilized creams may be safely applied as ingredients for "tailor-made" cosmetic moisturizers intended for normal and dry skin care, whereas olive oil squalene could be used for the treatment of irritated or sensitive skin as well.

Key words:

plant extracts; stem cells; cosmetics; skin; squalene; emulsifying agents; skin irritancy tests.

Apstrakt

Uvod/Cilj. S obzirom na to da vlaženje kože može biti postignuto izborom kako aktivnih supstanci, tako i odgovarajućeg nosača, biljne matične ćelije, skvalen i prirodni alkil poliglukozidni emulgator mogu biti potencijalni sastojci savremenih kozmetičkih proizvoda. Cilj ovog rada bio je *in vivo* procena iritiranog potencijala i efikasnosti matičnih ćelija alpske ruže dodatih u liposome i skvalena maslinovog ulja, kao sastojaka vlažećih kremova, imajući u vidu nov emulgator koji je korišćen za njihovu stabilizaciju. **Metode.** Upotrebom neinvazivnih metoda zasno-

vanih na biofizičkim merenjima na koži, kod 76 zdravih dobrovoljaca mereni su: hidratacija kože (EC), transepidermalni gubitak vlage (TEWL), eritema indeks (EI) i viskoelastičnost kože. U prvoj fazi studije vršena je procena iritacije kože nakon 24-h okluzije, kao i procena efikasnosti krema nakon primene na zdravoj koži dobrovoljaca u trajanju od 21 dana. Druga faza studije bila je bazirana na proceni efikasnosti krema nakon 6-dnevnog tretmana kože prethodno iritirane natrijum-lauril-sulfatom. **Rezultati.** Nakon 24-h okluzije nije bilo značajne promene vrednosti EI ni kod jednog ispitivanog krema. U drugoj fazi studije, krem sa skvalenom nije značajno uticao

na promenu vrednosti EI, dok je primena svih aktivnih krema dovela do značajnog sniženja vrednosti TEWL. U obe faze studije zabeležen je porast EC, naročito nakon primene krema koji sadrži skvalen. **Zaključak.** Uzevši u obzir odsustvo nadraženosti kože i narušavanja kožne barijere, kao i porast hidratacije površinskog sloja kože, može se reći da se ispitivane aktivne supstance ubačene u kreme stabilizovane alkil-poliglukozidnim emulgatorom

moгу bezbedno koristiti kao komponente tzv. „skrojenih“ kozmetičkih ovlaživača namenjenih za negu zdrave i suve kože, dok se skvalen maslinovog ulja može koristiti i za negu iritirane i osetljive kože.

Ključne reči:
ekstrakti, biljni; ćelije, matične; kozmetička sredstva; koža; skvalen; emulzije; koža, iritacija, testovi.

Introduction

Moisturizers play an important role in healthy skin care, as well as in treatment of dermatoses accompanied with dry skin. In addition to moisturizing and preventing dryness, they may also influence the structure and barrier function of both healthy and diseased skin^{1,2}. With long-term daily use, depending on their composition, moisturizers may either improve or deteriorate skin barrier function, and consequently increase penetration of ingredients in/through the skin and cause (further) skin dryness and irritation¹⁻³. Therefore, with the appropriate formulation and ingredient selection it is possible to achieve the preferred effect of topical preparations on the structure and barrier function of healthy or diseased skin in terms of skin hydration, while also repairing the skin barrier and reducing the signs of its irritation.

Plant stem cells, though interesting, are not sufficiently investigated as cosmetic actives. They represent extracts of the whole cell culture containing all the important compounds—epigenetic factors and secondary metabolites significant for protection and maintaining of these factors⁴. According to the manufacturer, stem cells from the Alpine Rose, *Rhododendron ferrugineum*, possess the ability to increase the vitality of epidermal stem cells and protect them against UV-induced stress, due to the content of various polyphenols and proteins called dehydrins. Furthermore, Alpine Rose stem cells (ARSC) are claimed to protect the skin barrier, supposedly by making it more resistant to the combination of strong environmental stress factors (UV, cold, wind) during the winter season. Finally, the anti-wrinkle effect has also been attributed to ARSC⁵.

Squalene is naturally present in the sebum and it is one of the main constituents of skin surface polyunsaturated lipids. The most abundant source of squalene is the shark liver oil, although nowadays, as a cosmetic active, squalene is exclusively obtained from plant-natural sources (eg olive oil). Squalene is used in cosmetics for its emollient, moisturizing and anti-irritant effects, as well as for the repair of skin barrier function. Also, it has been reported that squalene protects skin surface from lipid peroxidation caused by UV light and acts as a highly efficient quencher of singlet oxygen, which explains its antioxidant properties^{6,7}.

Although the proper selection of emollients and active ingredients certainly is the most relevant step towards assuring the desired effect of moisturizing creams, the importance of the used emulsifier system should not be neglected. The applied emulsifier together with the oil phase (emollients) defines the system's structure, and, consequently, the

cream's behaviour during and after the application, as well as its effect on the skin⁸. Additionally, certain safety aspects are based on the final choice of the emulsifier system^{1,2}.

Alkyl polyglucosides (APGs) are non-ionic, polyethylene glycol-free surfactants, derived from natural, renewable sources. APGs show favorable dermatological properties, ie they are mild to the skin and therefore considered as skin friendly. Furthermore, they are highly valued for their biodegradability, and hence frequently labelled as environment friendly emulsifiers. The specific chemical structure, water holding capacity and ability to form certain lamellar structures similar to the *stratum corneum* (SC) structural organization, makes them interesting cosmetic and pharmaceutical raw materials⁸⁻¹⁰. A novel APG-mixed emulsifier, hydroxystearyl alcohol and hydroxystearyl glucoside (INCI), has improved sensory properties and due to the additional diol structure in hydrophilic sugar unit, provides significant, continuous hydration of the upper layers of the skin¹¹. To the best of our knowledge, there is insufficient data available on this natural APG-mixed emulsifier as a potential stabilizer of moisturizing creams.

In accordance with the aforementioned, the aim of this study was to investigate *in vivo* skin irritation as a certain aspect of safety, and *in vivo* skin efficacy of Alp Rose stem cells incorporated into liposomes, olive oil squalene and a novel natural APG-mixed emulsifier, as ingredients of moisturizing creams. Efficacy was evaluated on both normal and experimentally irritated skin.

With that aim, we conducted the two phase study: the first phase was performed so as to assess the irritation potential of the samples after a 24-h occlusion, as well as the long-term effects of the samples' daily use in healthy skin care, while the second phase was carried out to evaluate the efficacy of the samples in the treatment of sodium lauryl sulfate (SLS) – irritated skin.

Methods

Subjects

The study was performed in accordance with the Declaration of Helsinki after obtaining written informed consent from the volunteers and permission from the Ethical Committee of the Faculty of Pharmacy, University of Belgrade, Serbia (the approval number: 1583/1). All measurements in this study were carried out in accordance with the relevant guidelines¹²⁻¹⁵.

A panel of 76 healthy female volunteers without the history or clinical signs of dermatological disease and with

normal to moderate dry skin participated in the study. The type of the skin (normal to moderate dry skin) was evaluated thanks to the basal values of the measured parameters, primarily on the basis of transepidermal water loss (TEWL) values (TEWL values were lower than 12 g/m²h)^{1,16}.

The volunteers were instructed not to use any skin care products on their arms a week before and throughout the study, but were allowed to wash normally during the study.

According to the published guidelines, measurements were carried out under controlled conditions: temperature (21 ± 1°C) and relative humidity (40 ± 5%), after a 30-min acclimatization period of the participants¹⁶.

Materials

A natural emulsifier of APG type, INCI (Simulgreen™ 18-2) was kindly provided by Seppic, France. Olive oil squalene with 96% purity (Olifeel® SQ) was kindly provided by Amedeo Brasca & C. SRL, Italy, and stem cells of Alpine Rose leaves encapsulated in liposomes (PhytoCellTec™ Alp Rose) were kindly provided by Mibelle AG Biochemistry, Switzerland. Olifeel® SQ is clear oily liquid with slight odour, yellow color and high purity which prevents degradation and coloring. According to the producer statement, it can be easily incorporated in all types of cosmetic products. PhytoCellTec™ Alp Rose is powder with specific odor, dissolvable up to 20% in water, which can be incorporated in all advanced “stem cell cosmetic” formulas, face and body care products and every weather formulations⁵.

Other components were: caprylic/capric triglyceride (Levate, Italy), *Prunus amygdalus dulcis* (Sweet Almond) seed oil, isopropyl myristate (A&A Fratelli Parodi, Italy) and mineral oil (R.A.M. Oil S.p.A., Italy). All samples were suitably preserved and also contained tocopheryl acetate (BASF, Germany) as an antioxidant, glycerin (BASF, Germany) as a humectant and Xanthan gum (Gum Technology, Arizona) as an additional stabilizer.

Test samples

In order to determine the optimal formulation, the number of oil in water (O/W) cream samples were prepared containing a multi-component oil phase, fixed emulsifier content (5%) and

different types of the additional stabilizers applied. Also, in order to decrease the number of potential confounding factors, cream samples were formulated as simply as possible. Along with the placebo samples (creams without the active substances), the active samples were prepared accordingly.

Based on the conducted preliminary physical stability evaluation, the placebo sample F1p, the active sample F1a (cream with ARSC incorporated into liposomes, 0.4% w/w, since the recommended use level for this active is 0.4–1% w/w) and the active sample F1s (cream with olive oil squalene, 1% w/w) were singled out and prepared for the subsequent *in vivo* investigation.

The fourth sample (Fc) was a commercial cream from the market with ARSC incorporated into liposomes (the same concentration and the same manufacturer of the active), and the following composition: ingredients (INCI)/Aqua, propylheptyl caprylate, coco caprylate, dicaprylyl carbonate, hydrogenated vegetable glycerides, avocado oil, pentaerythrityl distearate, *Butyrospermum parkii*, ethylhexyl methoxycinnamate, diglycerine, imperata cylindrica root extract, glycerine, polyethylene glycol (PEG)-8, carbomer, tricetareth-4 phosphate, ammonium acryloyldimethyl taurate/VP copolymer, phenoxyethanol, methylparaben, butylparaben, isobutylparaben, ethylparaben, propylparaben, tocopheryl acetate, butyl methoxydibenzoylmethane, parfum, polysorbate-20, *Rhododendron ferrugineum* leaf cell culture extract, isomalt, lecithin, sodium benzoate, lactic acid, tocopherol, ascorbyl palmitate, ascorbic acid, citric acid, disodium ethylenediaminetetraacetic (EDTA), linalool, limonene.

In the second phase of the study two new O/W cream samples were introduced instead of the commercial cream: the cream sample F1a1s containing the combination of ARSC (0.4% w/w) and 1% of olive oil squalene, and the cream sample F1a6s containing the same concentration of ARSC but 6% of squalene. The composition of all the prepared samples is given in Table 1.

Preparation of the formulations

The oil phase was heated in a closed vessel on the heating plate of a magnetic stirrer (IKA Combimag RCH, Germany) to 70°C. The aqueous phase containing glycerin

Table 1

Composition of the investigated samples

Ingredients/INCI	Sample*, % (w/w)				
	F1p	F1a	F1s	F1a1s	F1a6s
A (Oil phase)					
caprylic/capric triglyceride	7.0	7.0	7.0	7.0	5.0
mineral oil	3.5	3.5	3.5	3.5	2.5
isopropyl myristate	4.5	4.5	4.5	4.5	3.25
<i>Prunus amygdalus dulcis</i> (sweet almond) seed oil	3.0	3.0	3.0	3.0	2.25
squalene (Olifeel® SQ)			1.0	1.0	6.0
B (Water phase)					
INCI (Simulgreen™ 18-2)	5.0	5.0	5.0	5.0	5.0
glycerin	2.0	2.0	2.0	2.0	2.0
water	ad 100.0	ad 100.0	ad 100.0	ad 100.0	ad 100.0
C					
xanthan gum	0.5	0.5	0.5	0.5	0.5
tocopheryl acetate	0.3	0.3	0.3	0.3	0.3
<i>Rhododendron ferrugineum</i> leaf cell culture extract (and) Isomalt (and) lecithin (and) sodium benzoate (and) lactic acid (and) aqua (PhytoCellTec™ Alp rose)		0.4		0.4	0.4
Preservatives blend	0.5	0.5	0.5	0.5	0.5

INCI – Hydroxystearyl alcohol and Hydroxystearyl glucoside; Alp – alpine.

*For explanation see Methods (Test samples).

was heated in a closed vessel to 75°C, when the emulsifier was added while stirring (propeller laboratory stirrer, Heidolph Instruments GmbH & Co, Kelheim, Germany) for 1 min at 1,500 rpm. At the same temperature, the oil phase was added to the aqueous phase followed by stirring at 1,500 rpm for 5 min. The cream was being gradually cooled for 3 min at 1,050 rpm, at 725 rpm for 5 min and further at 1,050 rpm to room temperature. Xanthan gum was added in the cooling phase of the cream at the temperature below 60°C, while the preservatives blend and tocopheryl acetate were incorporated at below 40°C. Alp Rose stem cells, previously dispersed in 5 g of water (this was taken into account after having determined the needed amount of the aqueous phase), were carefully added during the cooling phase of the cream (at a temperature below 60°C), after adding of the stabilizer.

The samples containing squalene did not change their appearance (namely color or smell) throughout the temperature stress tests, after having been stored at room temperature or when submitted to the application during the study, which indicated the absence of oxidation products of squalene [eg squalene monohydroperoxide (SQOOH), as a main photo-oxidation product and a primary UV oxidized lipid produced from squalene]⁶. Temperature stress tests were performed by 24-h preservation of samples at three different temperature conditions (4°C, room temperature and 40°C), during 6 cycles (18 days).

Experimental design

The study was randomized, double-blind and organized in two phases.

In the first phase of the study, 52 volunteers were randomly divided into two groups.

The first group of 16 volunteers (mean age 21.8 ± 3.6) participated in the evaluation of irritation potential of cream samples under a 24-h occlusion. Samples were applied on the volar aspects of the forearms using a precisely marked cardboard ruler with three empty rectangular spaces (each measuring 9 cm², 3 cm × 3 cm). Two samples *per* arm were applied, and a rectangle next to the wrist was left as an untreated control (UC) on each forearm. The untreated control on the left arm was occluded (untreated control occluded – UCO). After samples application, the treated sites were immediately covered with Parafilm[®] (Pechiney Plastic Packaging, Inc., Menasha, Wisconsin, USA) and cotton adhesive tape Sensifix[®] (Belgrade, Serbia). In this phase of the study the following parameters were measured: electrical capacitance (EC), TEWL and erythema index (EI), before samples application (baseline values) and 3 h following the occlusion removal.

The second group of 36 volunteers (mean age 20.5 ± 0.5) participated in the evaluation of the efficacy of the same samples during their long-term application on the healthy skin. The samples were applied on the volar aspects of forearms using the same cardboard ruler and in the same, previously described manner. The rectangle closest to the wrist on each forearm served as an untreated control. The samples were distinguished solely by the color of the packaging. The volunteers received clear instructions regarding the sample amount and application manner. They applied sam-

ples to the specific test sites twice a day (in the morning and in the evening). In this part of the study EC, TEWL and skin elasticity were monitored. The measurements were conducted before the application of the samples commenced (baseline values), after a 14-day and subsequently after a 3-week treatment. The volunteers were instructed to skip the application the morning before the measurement.

In the second phase of the study, 24 volunteers (mean age 29.9 ± 8.9) participated in the evaluation of the efficacy of the samples in the treatment of SLS-irritated skin. The experimental irritation with SLS under a 6-h occlusion was performed in accordance with the published guidelines¹⁷ and in the previously reported manner¹⁸. A total of 100 µL of 10% aqueous solution of SLS (purity 99%, Merck, Germany) was placed on each of the six filter papers. The filter papers were subsequently placed on the test sites of the volar aspects of forearms using a cardboard ruler with four rectangular empty spaces in the following manner: three rectangular spaces from the left wrist up and three rectangular spaces from the right elbow down were covered with filter papers. Then, the filter papers were immediately covered with a Parafilm[®] and cotton adhesive tape Sensifix[®]. The test site next to the left wrist represented the induced UCO, and the rectangular empty space next to the right wrist was covered without induced irritation and served as an UC. The volunteers removed Sensifix[®], Parafilm[®] and the filter papers after 6 h. After irritation, a 6-day treatment of the tested sites was conducted. Five samples were applied, two samples to the left and three samples to the right hand, twice a day (in the morning and in the evening). In this phase of the study the following cream samples were investigated: F1p, F1a, F1s, and instead of the commercial cream (Fc), two new samples were introduced (F1a1s and F1a6s). In this part of the study the following parameters were measured: EC, TEWL and EI, before the irritation test ("primary" baseline values), 24 h after the occlusion removal ("secondary" baseline values) and after 6 days of the treatment. The last sample application was performed 12 h prior to measurements.

Corneometer[®] CM825 was used for skin measurement EC as an indicator of skin hydration. TEWL was measured using Tewameter[®] TM210, EI using Mexameter[®] MX18 and the skin elasticity using Cutometer[®] MPA580 (all devices manufactured by Courage + Khazaka Electronic GmbH, Germany).

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Data from different sites (treated with different samples including both controls) at different time points were analyzed using one-way ANOVA, followed by Tukey's *t*-test where appropriate. The differences were accepted as statistically significant at $p < 0.05$. Statistical analysis was performed with commercial statistical software package SPSS for Windows 17.0.

Results

In the first phase, as well as in the second one, all the volunteers completed the study and reported strict compliance with the given instructions.

Phase I

In this phase of the study, the following cream samples were tested: placebo cream (F1p), active cream containing 0.4% of ARSC (F1a), cream with 1% of squalene (F1s) and commercial cream (Fc) containing the same concentration of ARSC as the cream sample F1a.

In vivo evaluation of irritation potential

After the 24-h occlusion, there was no statistically significant change in EI for any tested cream sample, as well as the untreated controls. TEWL was significantly decreased for the cream samples F1p and F1a compared to the baseline. Also, EC was significantly increased for all active creams (F1a, F1s and Fc) compared to the baseline values.

The *in vivo* measured parameters (EC, TEWL and EI)

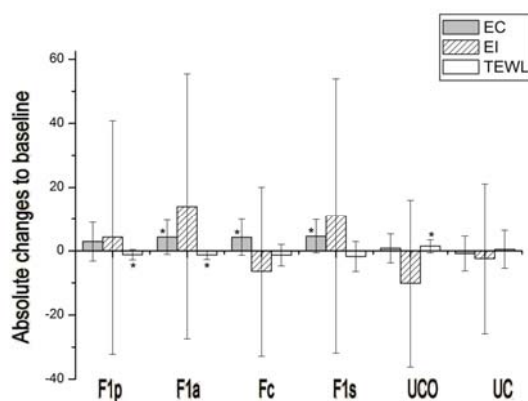


Fig. 1 – *In vivo* irritation potential of the investigated samples† F1p, F1a, Fc, F1s vs the untreated controls – UC: without occlusion (UC) and occluded (UCO).

The data from different sites was analyzed using one-way ANOVA, followed by Tukey's test where appropriate. Electrical capacitance (EC), erythema index (EI) and transepidermal water loss (TEWL) for the same sample at various time points were compared using paired sample *t*-test (significant changes marked with*).

†For explanation see. Methods (Test samples).

were expressed as absolute changes (Δ values) compared to the baseline (Figure 1).

In vivo efficacy on normal skin (the long-term 21-day study)

The results of the long-term 21-day study for EC and TEWL are shown as relative percentage change compared to untreated control (Figures 2 and 3), while Table 2 shows the calculated parameters for viscoelasticity of the skin (R2, R5 and R7) as mean values \pm SD.

In this part of the study, following positive testing for normality, parametric tests were used. Data involving values of the parameters measured in the forearm areas treated by different samples (F1p through F1s) at distinct time points, was analyzed by the one-way within-subjects (repeated measures) ANOVA, followed by Tukey's *t*-test, where appropriate. Differences between the values of the parameters obtained

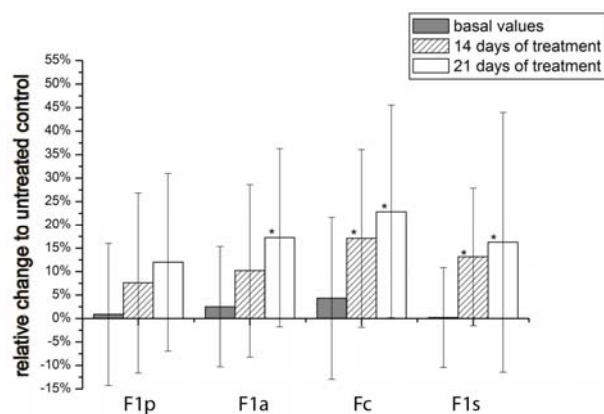


Fig. 2 – The effect of the topical application of the samples† F1p, F1a, Fc and F1s on electrical capacitance (EC), related to the untreated control (percentage change).

Data involving the values of the parameters measured on the forearm skin areas treated by different samples was analyzed by the one-way within-subjects (repeated measures) ANOVA, followed by Tukey's *t*-test, where appropriate; differences between the values of the parameters obtained for the cream-treated skin and the corresponding untreated control were checked by Student's unpaired *t*-test; all significant differences ($p < 0.05$) being marked with (*).

†For explanation see Methods (Test samples).

Table 2

The effect of the investigated samples on selected viscoelastic parameters of the skin (the control values are also given)

Samples*	R2	R5	R7	R2†	R5†	R7†
F1p	0.843 \pm 0.036	0.995 \pm 0.101	0.649 \pm 0.057	0.833 \pm 0.041	0.950 \pm 0.097	0.621 \pm 0.056
F1a	0.828 \pm 0.026	0.921 \pm 0.078	0.606 \pm 0.039	0.847 \pm 0.033	0.955 \pm 0.077	0.632 \pm 0.046
UC1	0.813 \pm 0.041	0.847 \pm 0.081	0.576 \pm 0.051	0.806 \pm 0.061	0.824 \pm 0.094	0.563 \pm 0.072
Fc	0.842 \pm 0.044	0.972 \pm 0.071	0.641 \pm 0.055	0.856 \pm 0.037	0.996 \pm 0.093	0.650 \pm 0.052
F1s	0.828 \pm 0.030	0.924 \pm 0.071	0.616 \pm 0.041	0.820 \pm 0.036	0.909 \pm 0.065	0.599 \pm 0.036
UC	0.808 \pm 0.057	0.846 \pm 0.092	0.601 \pm 0.064	0.812 \pm 0.042	0.831 \pm 0.076	0.611 \pm 0.050

The results are shown as mean values \pm standard errors measured initially (basal values) and after a 21-days application (†).

UC – untreated controls.

*For explanation see Methods (Test samples).

†R2 – the gross – elasticity of the skin including the viscous deformation, and is represented by the ratio of the ability of redeformation of skin to final distension; R5 – the biological elasticity (the portion of elasticity compared to the final distension).

ned for the cream-treated skin and the corresponding untreated control were checked by Student's unpaired *t*-test.

After 14 days of application, although all tested creams exhibited a rising trend, skin hydration (measured as the EC), it was significantly changed only for the creams F1s and Fc compared to the untreated control, whereas after 21 days the increase was significant for all active creams (F1a, F1s and Fc) (Figure 2).

The results for TEWL (Figure 3) showed that after 21 days of treatment, the TEWL values significantly decreased for the cream F1s compared to all the other tested samples. The investigated creams F1a and Fc did not alter TEWL significantly at any time point, whereas the cream F1p significantly increased TEWL after both 14 days and 21 days of application.

Phase II

For the second phase of the study, the following samples were selected for further investigation: placebo cream (F1p), active cream (F1a) with 0.4% of ARSC and the cream with 1% of squalene (F1s). In order to evaluate the joint effects of the investigated actives on previously SLS-irritated skin, as well as the concentration dependent impact of squalene on the tested skin parameters, two new samples were included: active cream with 1% of squalene (F1a1s) and the active cream with 6% of squalene (F1a6s) – both containing 0.4% of ARSC.

In vivo efficacy on SLS-irritated skin

The measured parameters were expressed as absolute changes as compared to baseline (Δ values) and presented in

Figures 4–6. Data from different sites (treated with different samples and the controls) at different time points were analyzed using one-way ANOVA, followed by Tukey's *t*-test where appropriate. The values of the measured parameters after the irritation as well as after six days of the samples' application were compared to both the baseline values and between one another using paired sample *t*-test. The differences were accepted as statistically significant at $p < 0.05$.

After a 6-day treatment of the SLS-irritated skin, a significant decrease in TEWL for all tested creams (F1p, F1a, F1a6s, F1s and F1a1s) was seen (Figure 4) when compared to the values after irritation ("secondary" baseline values), whereas the creams F1a6s and F1s also significantly decreased TEWL related to the "primary" baseline values.

As regards skin hydration, measured as EC, after a 6-day treatment, the creams F1p, F1a6s and F1s significantly increased EC related to the values measured after the irritation ("secondary" baseline values) (Figure 5). A trend of EC increase, though insignificantly, could be noticed for the tested creams F1a and F1a1s (Figure 5).

Furthermore, after 6 days of application, there was no significant change in the EI for the creams F1p and F1s, whereas the active creams F1a, F1a6s and F1a1s significantly increased EI when compared to the "primary" baseline values (Figure 6).

Discussion

In the initial phase of the study, *in vivo* evaluation of the irritation potential and efficacy during the long-term application of the investigated creams was performed on healthy skin with two groups of volunteers.

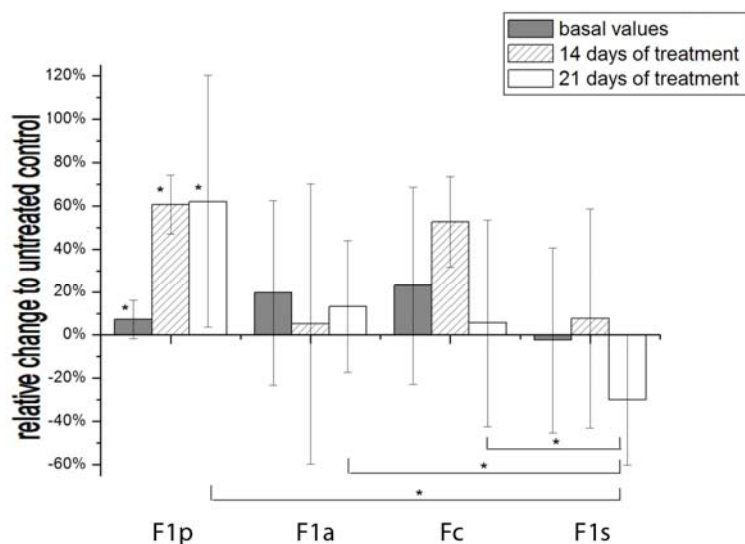


Fig. 3 – The effect of the topical application of the samples[†] F1p, F1a, Fc and F1s on transepidermal water loss (TEWL), related to the untreated control (percentage change). Data involving the values of the parameters measured on the forearm areas treated by different samples was analyzed by the one-way within-subjects (repeated measures) ANOVA, followed by Tukey's *t*-test, where appropriate; differences between the values of the parameters obtained for the cream-treated skin and the corresponding untreated control were checked by Student's unpaired *t*-test; all significant differences ($p < 0.05$) being marked with (*).

[†]For explanation see Methods (*Test samples*).

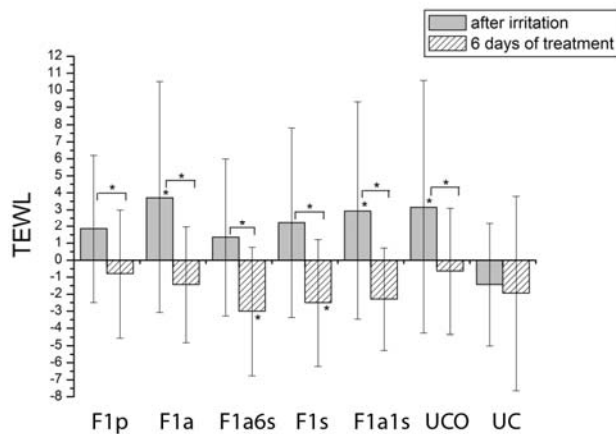


Fig. 4 – The effects of the irritation and subsequent topical application of the samples[†] F1p, F1a, F1a6s, F1s and F1a1s to irritated skin on transepidermal water loss (TEWL) related to the “primary” baseline (absolute changes); both controls are also included occluded (UCO) and without occlusion (UC).

The values of the parameters after the irritation (“secondary” baseline values) as well as after six days of the samples’ application were compared to the “primary” baseline values and between one another using paired sample *t*-test, significant differences ($p < 0.05$) being marked with (*).

[†]For explanation see Methods (Test samples).

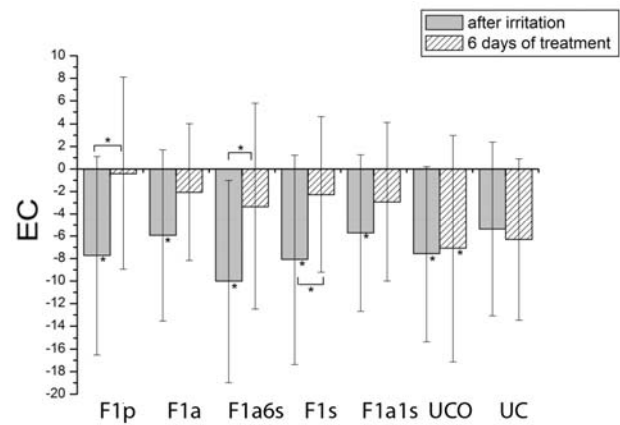


Fig. 5 – The effects of the irritation and subsequent topical application of the samples[†] F1p, F1a, F1a6s, F1s and F1a1s to irritated skin on EC related to the “primary” baseline (absolute changes); both controls are also included occluded (UCO) and without occlusion (UC).

The values of the parameters after the irritation (“secondary” baseline values) as well as after six days of the samples’ application were compared to the “primary” baseline values and between one another using paired sample *t*-test, significant differences ($p < 0.05$) being marked with (*).

[†]For explanation see Methods (Test samples).

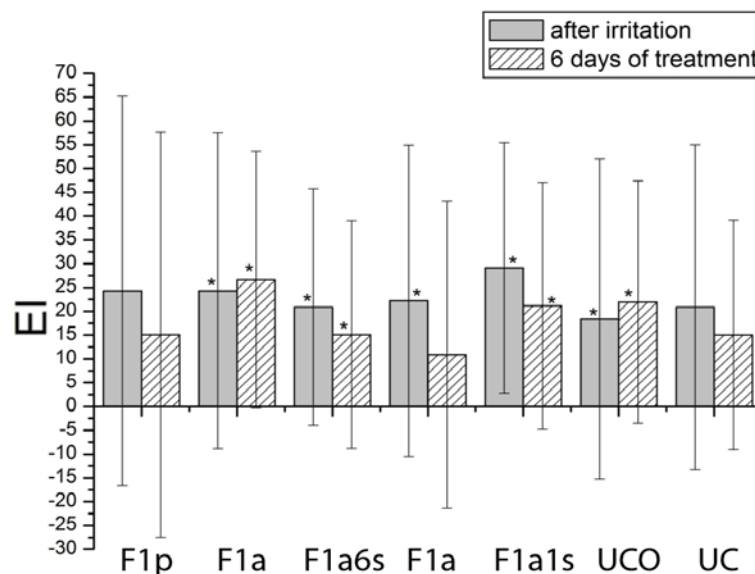


Fig. 6 – The effects of the irritation and subsequent topical application of the samples[†] F1p, F1a, F1a6s, F1s and F1a1s to irritated skin on EI related to the “primary” baseline (absolute changes); both controls are also included occluded (UCO) and without occlusion (UC).

The values of the parameters after the irritation (“secondary” baseline values) as well as after six days of the samples’ application were compared to the “primary” baseline values and between one another using paired sample *t*-test, significant differences ($p < 0.05$) being marked with (*).

[†]For explanation see Methods (Test samples).

The evaluation of the irritation potential, as an important safety aspect of cream samples, was conducted under a 24-h occlusion on the first group of volunteers. In order to investigate the irritation potential, EI was monitored. Additionally, the potential skin barrier impairment was assessed *via* TEWL and EC of the skin.

It is known that occlusion itself induces barrier damage without skin dryness, increases penetration of some ingredients from topically applied products and may cause irritation^{19, 20}. For that reason, to assess the overall irritation potential, as well as the potential skin barrier impairment of the tested samples, it was necessary to eliminate the influence of occlu-

sion. Hence, final measurement of the monitored parameters was performed 3 h after occlusion removal, when the effects of occlusion had subsided¹⁹.

It was shown (Figure 1) that the EI, as an indicator of the skin irritation *via* the measurement of skin redness¹⁴, was not significantly changed compared to the baseline values for any cream sample. Also, for the cream Fc and UCO, an insignificant reduction of the EI related to the baseline could be noticed. The rationale for this is probably a prolonged (24-h) occlusion and maceration of the skin caused by the increased water binding of SC (hyper-hydration of SC)^{21,22}.

As irritants application is often accompanied by the increase of TEWL – a marker of skin barrier function and its structural changes – this measurement is commonly used in safety evaluation studies of cosmetic products^{2,3,20}. Although, in our study, TEWL showed a falling trend, these results may be considered statistically significant only for the placebo cream F1p and the active sample F1a. Since there was no significant change in the TEWL values on the site reserved for UC, and due to the fact that this parameter was significantly increased 3 h after the 24-h occlusion on the site reserved for UCO, it could be assumed that the obtained results are caused by the effect of the investigated active (ARSC incorporated into liposomes). On the other hand, if one compares TEWL values for the placebo F1p and the active creams F1a and Fc, both containing the same active (the same concentration and manufacturer of the active), it could be speculated that these results can be attributed not only to the carrier itself (the cream composition given in Table 1) but also to the used emulsifier. The rationale for the obtained TEWL results is probably the similarity of the liquid crystalline structure of the investigated samples (unpublished data) stabilized with the used emulsifier to the SC structural organization. This assumption is consistent with the already reported results concerning skin mildness and favourable dermatological properties of this type of emulsifiers⁸⁻¹⁰ and the investigated emulsifier, as well¹¹.

As concerns the hydration of the skin (EC), it was increased in all the treated sites, however, only for the placebo F1p without statistical significance. Although occlusion can significantly increase SC hydration by blocking water loss from the skin surface²⁰, the obtained results cannot be attributed to the occlusion. Since there was no significant change in the EC for UCO and the skin hydration on the site reserved for UC was decreased (although without statistical significance), the obtained results can be attributed to the effect of the investigated actives, as well as the carrier and the used emulsifier, which is mainly responsible for the carrier's specific microstructure.

According to these results no alteration of the skin barrier function occurred.

The obtained results from this phase of the study generally indicated that the investigated cream samples had not irritated the skin. The absence of erythema and/or any impairment of the skin barrier function in a 24-h occlusion study may preliminarily imply the satisfying safety profile of the cream samples, as well as of the investigated cosmetic actives and the used emulsifier.

In order to evaluate the efficacy of the investigated samples, a long-term 21-day study was performed. After the initial baseline measurements, the second group of volun-

ters with normal/healthy skin was applying the tested samples twice a day following the written procedure. The first control measurements were performed after a 14-day treatment, and the final measurements were conducted a week afterwards.

As, it is well-known that the short-term application of moisturizers may increase hydration of the skin¹⁻³, EC measurements were conducted to investigate the potential of a long-term (21 days) use of the tested creams to hydrate the skin effectively. After 21 days of application, all tested active creams as well as the placebo sample F1p (although without significance) increased the skin hydration level compared to UC. It should be emphasized that the creams F1s and Fc, effectively changed the EC (a significant increase) as soon as after 14 days of application.

If one compares the active cream F1a and the commercial one Fc, both containing the same concentration of the active (ARSC incorporated into liposomes) but quite a different carrier, it could be assumed that the contribution of the investigated active to the moisturizing effect of the creams is as important as it was expected. Also, a similar assumption could be made by comparing the tested active cream F1a and the corresponding placebo F1p, both containing the same carrier. However, neither the exact mechanism of the moisturizing effect of ARSC leaves encapsulated in liposomes (PhytoCellTec™ Alp Rose) is fully understood, nor their ability to eventually penetrate the SC after the application to healthy skin. Bearing in mind that the investigated active is an extract of the whole cell culture incorporated into liposomes, it could be speculated that the observed hydration effect is probably attributed to some ingredients with water binding capacity which act like humectants, or to phospholipid-based liposomes (physiological lipids) themselves²³. In future research, it would be interesting to investigate the mechanism of the observed hydration effect of ARSC incorporated into liposomes and their penetration ability, as well.

Secondly, according to the obtained results, the cream containing 1% of olive oil squalene, F1s, increased the EC significantly after 14 days, as well as after 21 days of application. Thus, if one considers the EC values for the placebo F1p and the cream F1s, it could be said that 1% of squalene significantly contributes to the cream hydration efficacy. Although, squalene is known as a good moisturizing agent⁶, the obtained results indicate the possible achievement of effective skin hydration even at a low concentration of this active. On the other hand, there existed a rising trend of the EC compared to UC for placebo F1p, although without significance, and the lack of statistically significant difference in the EC values (skin hydration) between the placebo cream F1p and its counterparts – the active samples F1a and F1s. Since these samples are based on the same carrier system, the recorded results may indicate that for effective skin hydration, the carrier itself is beneficial and therefore, probably, the used emulsifier as well. This assumption is consistent with the previously reported results concerning the older generation of APG emulsifiers^{8,10,24,25}, but also the emulsifier used in our study (INCI Simulgreen™ 18-2)¹¹.

It is well-known that due to their specific structure, water binding capacity and the formation of lamellar phases

APG emulsifiers can provide additional skin moisturization^{8, 10, 24, 25}. It has recently been reported that creams stabilized with the used novel APG emulsifier may be attributed with even stronger water binding capacity in comparison with the previous generation of APG emulsifiers. Furthermore, water binding capacity and the formation of liquid crystals around oil droplets in creams stabilized with aforementioned emulsifier, depends on the nature of the used oil; thus the selection of some non-polar oils (eg, vegetable squalane) leads to the visible formation of these structures¹¹. Since squalane is a saturated non-polar derivative of squalene, it could be assumed that squalene may also have certain impact on the specific colloidal structure of the investigated cream F1s. Squalene may contribute to both the formation of the lamellar phase in the tested cream sample and additional skin moisturization. Within the investigated cream samples, especially those containing squalene, anisotropic lamellar structure was recorded by polarization microscopy (unpublished data), performed during the formulation study and the preliminary physical stability investigation of the tested samples.

In the case of the impact of a long-term (21 days) use of the tested cream samples on the skin barrier, the TEWL measurement was conducted, as the most common method of evaluation of the skin barrier function². The obtained values showed that after 21 days of application, the cream F1s significantly decreased TEWL compared to all other tested creams. Having compared the placebo F1p which significantly increased TEWL after 14 and 21 days of application and the cream F1s, it seems reasonable to attribute a favourable effect of the active sample to its cosmetic active substance (squalene). Taking into account that TEWL measurements are generally used for screening and objective, non-invasive perceiving of actives that may have a positive effect on the skin barrier function¹³, it could be assumed that olive oil squalene may even improve the skin barrier function² after long-term application on healthy/normal skin. Furthermore, our findings imply that even a relatively low concentration of squalene (1%) significantly contributes to the moisturizing efficacy of the tested cosmetic cream (F1s).

The remaining tested creams F1a and Fc, both containing the same active (ARSC incorporated into liposomes) but a different carrier, did not alter TEWL significantly at any time point. The obtained results are consistent with the already reported, and indicate that repeated application of moisturizers on normal/healthy skin may increase skin hydration without affecting TEWL¹. Therefore, it could be summarized that a 21-day application of the tested active creams, as well as of the investigated active substances (olive oil squalene and PhytoCellTec™ Alp Rose) have not resulted in the impairment of skin barrier function.

On the other hand, the obtained TEWL values for the placebo sample F1p are somewhat confusing. The TEWL values for this sample were elevated after 14, as well as after 21 days of application, suggesting a possible negative effect of the placebo sample on the skin barrier function during a long-term use. However, considering that TEWL did not increase in the case of samples with the same carrier (F1a and

F1s), and due to the fact that the basal values on the particular site intended for the application of the sample had already been elevated (compared to the UC) with most volunteers, the obtained placebo affected TEWL findings could not be attributed to the carrier itself. Furthermore, it has been reported that TEWL on the dominant forearm might be significantly higher than on the non-dominant one, and that different sites on the same anatomical position might have significantly different TEWL values²⁶. This assumption is consistent with our previously obtained results (the lack of impairment of the skin barrier function) in a 24-h occlusion study for the same sample – placebo F1p.

To evaluate further the impact of the 21-day application of the investigated creams on biomechanical characteristics of the skin, the measurement of skin viscoelastic properties was conducted, as well. It is widely acknowledged that the ageing process alters structural and mechanical properties of the skin through changes of the elastic and collagen fibers. As ageing is accompanied by a decrease of skin elasticity which begins in the early twenties, this measurement could be useful for the study of the influence of the investigated samples on biomechanical characteristics of the skin. Viscoelasticity of the skin was assessed with a calibrated Cutometer® MPA580, suction-based instrument equipped with a 2-mm-diameter probe. Applying the following settings: 400 mbar suction pressure, suction time 2 s, relaxation time 2 s, 3 repetitions, the skin deformation curve (a deformation vs time curve) was obtained. This curve offers two types of parameters: directly measured from the curve so-called *U*-parameters (total deformation recovery *U_a*, total extensibility *U_f*, immediate elastic deformation *U_e* and immediate elastic recovery *U_r*, which are the most commonly used *U*-parameters) and calculated so-called *R* parameters (*R₀* to *R₉* parameters). *R*-parameters were calculated using the Software Cutometer® MPA580, but for the evaluation of viscoelastic properties of the skin only a few were chosen: *R₂* (*U_a*/*U_f*), *R₅* (*U_r*/*U_e*) and *R₇* (*U_r*/*U_f*). The parameter *R₂* (gross elasticity) reflects the overall elasticity of the skin, *R₅* (net elasticity) reflects only the elastic component of the viscoelastic response of the skin (it is affected solely by the elastic fibers of the skin), while the parameter *R₇* represents the elastic recovery ratio and it can be affected by changes of elasticity and viscosity of the skin. These parameters were calculated as those unaffected by skin thickness of the volunteers and the experimental conditions of the study. Moreover, they were the most useful for this type of the study, especially the parameter *R₇* which is most closely related to the skin elasticity and has profound meaning for measuring skin ageing by evaluating its elasticity²⁷. As it could be seen (Table 2) after 21 days of application, there was no significant change in the selected parameters. Hence, the obtained results indicate that the investigated creams do not have an impact on skin elasticity after a 21-day application to the skin of volunteers in their early twenties. This findings may suggest a few possible implications: selection of a proper group of human volunteers is of paramount significance to detect possible effects, if any, of the treatment that could tentatively be define as anti-age skin improvement; claims

indicating anti-age plant stem cells effect have to be supported by a set of adequate objective and subjective techniques/methods of evaluation; proper concentration range and the type of the vehicle/carrier system adjusted for the given active is always the most important task for a formulator attempting to achieve both a satisfying efficacy and an acceptable safety profile of a skin care product.

Overall, upon consideration of the obtained results from the first phase of our study, it was shown that the tested creams stabilized with a novel APG emulsifier have satisfying safety profiles, either with or without the incorporated active substances (1% of olive oil squalene (Olifeel® SQ) and 0.4% of ARSC leaves encapsulated in liposomes (PhytoCellTec™ Alp Rose)). In addition to this, after a long-term use (21 days) on healthy skin, these creams manage to increase SC hydration without compromising the skin barrier function. Finally, this study stage proved that the investigated actives, especially olive oil squalene, are efficient, indicating that they could be appropriate actives for cosmetic moisturizers intended for healthy skin care.

It is widely acknowledged that the application of the effective moisturizers – those with a proper composition and with adequate cosmetic actives in optimal contents, could have a positive impact on the renewal of the (SLS)-irritated skin^{1, 11, 24}, *ie* it may have a general favourable effect on the recovery of irritated skin. So, as to investigate the potential of the tested creams further, in the second phase of the study the samples were evaluated upon their application to the previously irritated skin, again employing non-invasive measurements of the predetermined skin parameters throughout the study. To perform this, an *in vivo* skin irritation test with 10% SLS aqueous solution under a 6-h occlusion was performed in the following manner. Initial basal values were taken before ("primary" basal values) and after ("secondary" basal values) the SLS solution was applied and the occlusion was placed on the investigated skin sites. In order to eliminate the influence of occlusion itself and determine the effects of SLS, the "secondary" basal values were measured 24 h after the irritation test and the occlusion removal¹⁹. TEWL values (in more than 50% of volunteers TEWL greater than 12 g/m²h¹ was recorded), confirmed that skin dryness was successfully induced¹⁶. Final measurement was conducted after 6 days of treatment with the tested creams.

Non-invasive skin biophysical measurements are widely used in the assessment of skin irritation elicited with SLS as a model irritant. In addition to the TEWL measurement, which is a highly sensitive and precise method for the determination of SLS irritation effects on the skin, as well as the most valid measurement for assessment of low irritant skin reactions, it is also useful to monitor hydration of the skin (EC) and EI^{17, 19}.

After the 6-day treatment of the SLS-irritated skin, TEWL was significantly decreased for all investigated creams (F1p, F1a, F1a6s, F1s and F1a1s) related to the "secondary" basal values (values measured upon irritation) (Figure 4). Secondly, for the creams F1a6s (containing ARSC and 6% of squalene) and F1s (containing 1% of

squalene), a significant decrease in TEWL could be noticed when compared to the "primary" baseline (normal skin prior to irritation). Although TEWL was significantly decreased for the induced UCO related to the "secondary" baseline, the results obtained at the treated sites could not be solely attributed to the physiological regeneration of the skin, especially regarding the creams F1a6s and F1s. For the creams F1a6s and F1s, the obtained TEWL values could also be addressed to squalene and the carrier itself. After the application of the aforementioned samples, squalene may remain on the skin surface and due to the skin occlusion it may decrease TEWL or even penetrate the skin and influence its barrier recovery³. On the other hand, stabilized with a novel APG emulsifier, the carrier itself may contribute to the skin barrier recovery after the application on SLS-irritated skin as well, which has already been reported for vehicles based on this type of emulsifiers²⁴. Such results indicate that both the investigated creams and tested actives may have a positive effect on skin barrier integrity. This assumption is consistent with the reported results regarding moisturizers and their impact on the skin barrier recovery after exposure to SLS^{3, 24}.

When it comes to skin hydration, a significant change of the EC compared to the "secondary" basal values was detected (Figure 5) for the placebo sample F1p and the active creams F1a6s and F1s. Namely, the EC value for UCO after 6 days remained significantly lower compared to the "primary" baseline. Thus, the trend of the EC increase, however insignificant, caused by the skin treatment with both the F1a and F1a1s creams, might be interpreted as the contribution in hydration effect of the tested actives. Moreover, these results are in line both with those from the 21-day study and with our initial assumption that the carrier itself contributes to the skin hydration, and presumably together with the used emulsifier as well.

As concerns EI values, after the 6-day application of the tested creams there was no significant change in the EI for the placebo cream F1p or the active cream F1s in relation to the "primary" basal values, although the trend of the EI decrease could be observed compared to the "secondary" baseline (values measured upon irritation) (Figure 6). Secondly, for the cream sample F1a and the samples containing a combination of the actives F1a1s and F1a6s, the EI values were elevated compared to the "primary" baseline, whereas for the samples F1a1s and F1a6s the trend of the EI decrease compared to "secondary" basal values (values measured upon irritation) could be observed as well. These results may imply at least two possible scenarios: adverse effect of the tested active (ARSC incorporated into liposomes) on previously irritated skin; too short period left for skin regeneration even under the treatment. On the other hand, after 6 days of treatment, the EI value remained significantly increased for the induced UC (UCO) compared with the "primary" baseline, and the trend of the EI increase was noticed related to the "secondary" basal values. Regarding the results for UCO and the fact that the reduction of skin irritation without adverse effects has recently been ascribed to ARSC by some researchers²⁸, it can be speculated that the obtained results are somehow a consequence of SLS irritation and occlusion itself.

The results of the second phase of the study generally stand in good agreement with the results of the first phase. The tested creams and the incorporated actives *per se* exerted a positive effect on irritated skin and they proved to have the potential to renew certain skin damages. The fact that hydration of the skin was elevated after the application of all investigated samples (for F1a and F1a1s without any statistical significance) suggests the contribution of the investigated actives, the carrier and its specific colloidal structure, and it consequently implies that the used emulsifier contributed to the observed moisturizing effect. Finally, the tested actives proved to be efficient in the treatment of irritated, dry skin.

Additionally, regarding the creams containing a combination of the actives and different concentrations of squalene (F1a1s and F1a6s, containing 1% and 6% of squalene, respectively), the study shows a concentration-dependent impact of squalene on skin hydration. Although, squalene is considered to be a good emollient and moisturizing agent⁶, the obtained results also imply a certain impact on the specific colloidal structure in the tested creams, probably due to the formation of the lamellar phase, resulting in the additional skin moisturization.

Conclusion

With the use of non-invasive skin biophysical measurements, the performed study confirms that the investigated actives – Alp Rose stem cells incorporated into liposomes and olive oil squalene, as well as the used emulsifier – hydroxystearyl alcohol and hydroxystearyl glucoside, show

no irritation potential, nor any negative influence on the skin barrier function during the application on healthy or irritated skin. In view of these results, it could be said that these ingredients can be safely applied in the formulation of cosmetic moisturizers. The present study also shows a good skin hydration potential of the tested actives used either on the healthy or irritated skin. Furthermore, our results imply that both actives can be used for the improvement of skin barrier function. In addition, considering the lack of impact on skin elasticity after the application of the investigated samples, our study failed to show the potential anti-age skin improvement effect of the tested actives, probably due to the inappropriate selection of young volunteers in their early twenties.

Therefore, Alp Rose stem cells incorporated into liposomes, olive oil squalene and hydroxystearyl alcohol and hydroxystearyl glucoside could be used both alone and combined in moisturizing formulations intended for normal and dry skin care, whereas olive oil squalene could be used for treatment of irritated or sensitive skin as, well.

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