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Plant Cell Cultures Instead of Wild Plant Harvesting

Plants that survive in high altitude habitats of the Alps, medicinal herbs from Tibet or orchids from the Amazonian area are examples of attractive raw materials for cosmetic ingredients. These are all rare plants or plant species that are protected by CITES, the Convention on International Trade in Endangered Species of Wild Fauna and Flora. Harvesting of wild plants is forbidden or not done because of sustainability reasons. Cultivation of these plants in fields is in many cases not successful. Use of the plant cell culture technology is the only way to obtain vegetal raw materials from these plants. The technology is mainly applied for *in vitro* propagation of plants or for the production of complex plant compounds used as pharmaceuticals such as the anticancer drug taxol. This diterpenoid can be isolated from the bark of the yew *Taxus brevifolia*, but 9 tons of bark are needed to obtain 1 kg taxol. It is therefore mainly produced via plant cell cultures. Several years ago plant cell culture technology started being used in the cosmetic industry. Besides sustainability there are also other advantages compared to traditional plant raw materials. The use of plant cell cultures allows a controlled production independent of the season and weather influences and there are no risks of contamination from pesticides.

The Plant Cell Culture Technology

The technology relies on the concept of totipotency. It is the capacity of differentiated plant cells (e.g. leaf cells, fruit cells) to undergo de-differentiation and, under the right stimuli, to regenerate a whole plant. Because of their sessile nature, plants had to adopt this plasticity in order to adapt to environmental conditions. In this way, plants can survive dormancy periods and regenerate when the conditions are again optimal. Totipotency can be used for *in vitro* propagation of plants or for the cultivation of undifferentiated plant cells. Plant cell cultures can be initiated from nearly all plant tissues. The tissue material which is obtained from the plant to culture is called an explant. As a kind of wound reaction, new cells are formed on the cut surfaces of the explant. The cells slowly divide to form a colorless cell mass which is called callus **(Fig. 1)**. These cells have de-differentiated into cells that lack the distinctive features of normal plant cells.

Abstract

There is an ongoing trend to use attractive exotic or rare plants to develop new cosmetic ingredients. On the other hand, there is an increased awareness for sustainable development. The plant cell culture technology makes it possible to obtain vegetal raw material from endangered species in a sustainable way. An old apple variety and the UNESCO protected argan tree were used as source materials to develop plant cell cultures. Extracts of the cultured apple and argan tree cells were found to positively act on isolated adult stem cells of the epidermis and of the dermis respectively. The results of the cell culture assays could be reproduced in clinical studies.

Fig. 1 Callus formed by undifferentiated plant cells

Callus cells represent stem cells of the plant. For high yield production, callus cells can be cultured as individual cells or small cell clusters in a liquid culture. Mibelle Group Biochemistry uses a flexible bioreactor-system (Wave-Biotech AG, Tagelswangen, Switzerland) for biomass production. The cells are cultured in plastic bags of 25 liters volume that are placed on tiltable dishes **(Fig. 2)**. The cells

are cultured in the dark which is why they do not produce chlorophyll pigments. Once the sugar in the culture medium is completely metabolized, the cells are harvested and disrupted by high-pressure homogenization.

Plant Cell Extracts to Protect the Skin's Stem Cells

Declining regenerative potential at the tissue level is a major contributor to the aging process. Since regeneration depends centrally on adult stem cells to supply the new cells required for tissue repair and replacement, any decline in stem cell activity will accelerate the aging process. Adult stem cells are undifferentiated cells with a capacity for self-renewal and the potential to develop into the different cell types within the tissue in which they are found. The skin consists of two layers that derive from different germ layers. The epidermis is of ectoderm origin and the subjacent dermis originates from the mesoderm. Both layers have their own adult stem cells. In the epidermis, the stem cells reside in the basal layer. Undifferentiated cells in the epidermis can be identified *in vivo* via label-retention studies enabling detection of slow-cycling cells, and by the expression of specific marker proteins such as alpha6-integrin and CD34 (1). Following asymmetrical division, epidermal stem cells reproduce more rapidly dividing transient amplifying cells, which after a limited number of divisions enter terminal differentiation. Recently, the dermal papilla was identified as a niche for dermal stem cells (2). Cells of the dermal papilla were shown to express the stem cell marker gene Sox2, to self-renew and to migrate into the inter-follicular dermis where they proliferated and differentiated to fibroblast cells, able to regenerate the extracellular matrix **(Fig. 3)**. These dermal stem cells do not grow adherent like normal fibroblast cells but grow by forming non-adherent cell aggregates (3D spheres).
Could extracts of plant cells from selected species be used to stimulate the activity of the adult stem cells in the skin? Both, plant cells cultured *in vitro* by the technique described above and human

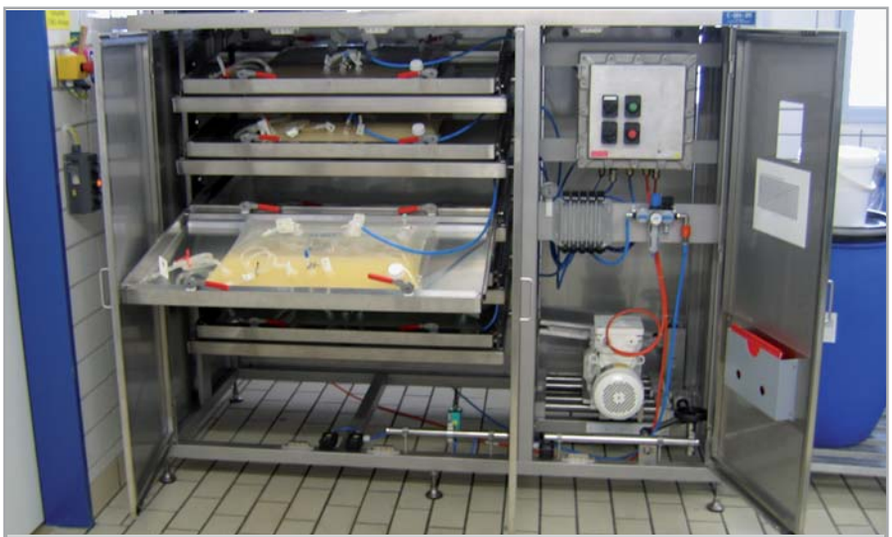

Fig. 2 Flexible bioreactor system for production of plant cells

adult stem cells are in an un-differentiated state. The degree of cellular differentiation is regulated by altered gene expression. Modifications of the DNA and the histone proteins by methylation and acetylation, known as epigenetics, are used to modulate gene expression. Epigenetic regulation of gene expression depends therefore on the activities of the modifying enzymes such as methyltransferases, acetyltransferases and deacetylases. The activity of these enzymes can be regulated by small molecule modulators (activators and inhibitors) (3). It is

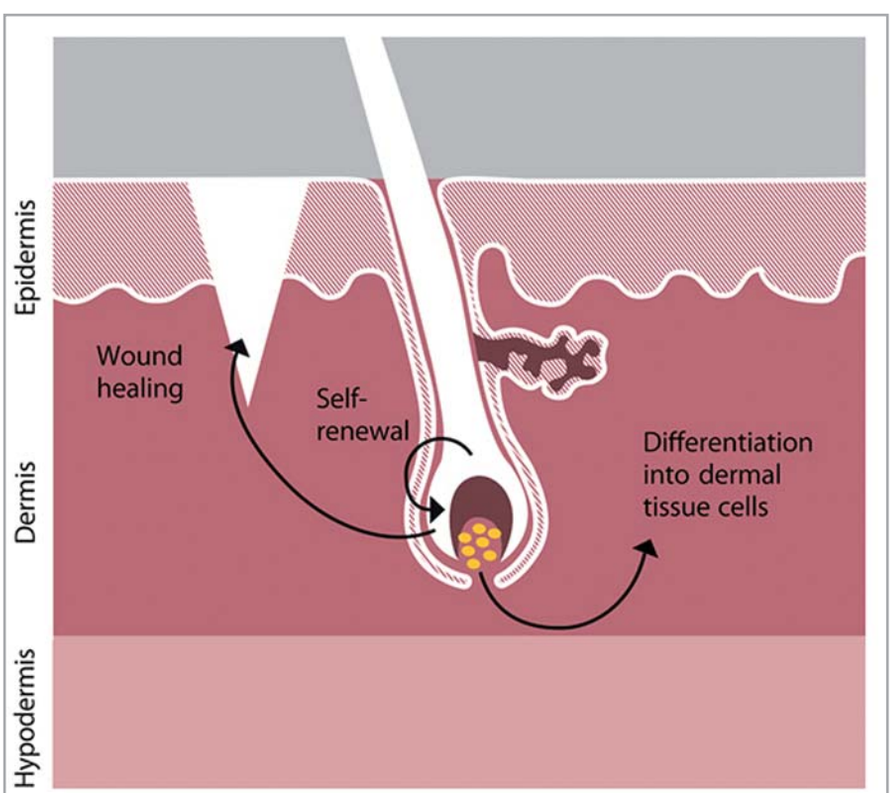

Fig. 3 The dermal papilla as niche of dermal stem cells

possible that un-differentiated plant cells have per se a favorable set of small molecule modulators for adult skin stem cells and that secondary metabolites of selected plant species additionally contribute to the positive effect.
Two active plant cell ingredients were developed using the described culture technology and tested for beneficial effects on isolated epidermal stem cells and dermal stem cells respectively.

Effect of a Plant Stem Cell Ingredient on Epidermal Stem Cells

Epidermal stem cells were isolated from a skin sample using a new technique known as Progenitor Cell Targeting (developed by CELLnTEC Advanced Cell Systems). This technique uses specialized culture media to specifically mimic the environment of the stem cell niche. FACS analysis has demonstrated that this method rapidly selects and enriches the valuable undifferentiated cells found in primary keratinocyte cultures. Using these enriched cultures, the ability of cosmetic ingredients to maintain epidermal stem cell potential was evaluated. The enriched cell population was cultured for different time periods in a medium containing the ingredients at various concentrations. The epidermal stem cell potential was then evaluated by assessing colony forming efficiency (CFE) and/or the ability to form a pluristratified epidermis (organogenic potential). For analysis of CFE, cells are seeded at low density, and then allowed to form colonies. Colonies are then counted, and the percentage of cells seeded that formed colonies (CFE) calculated. This percentage gives a direct indication of the number of cells with stem cell potential, as only these undifferentiated cell types retain the ability to form colonies. To determine the organogenic potential, cells were seeded onto a permeable substrate, then cultured at the air-liquid interface to encourage the establishment of a three dimensional (multi-layered) epidermal structure.

An old apple variety (Uttwiler Spätlauber) was chosen to prepare plant stem cells for an epidermal stem cell ingredient. The Spätlauber apple derives from a seedling planted in the middle of the 18th century. It was especially prized in southern Germany and Switzerland for its excellent longevity during storage. The flesh from an Uttwiler Spätlauber apple was used to start the plant cell culture. Treatment of the enriched epidermal stem cells with an extract from the apple stem cell culture (INCI: Malus Domestica Fruit Cell Culture Extract) was found to stimulate both colony forming efficiency (CFE) and organogenic potential. Compared with a control culture, CFE was increased by up to 100% in the presence of 0.04% apple stem cell extract. Similarly, treatment with the apple stem cell extract massively extended the age at which epidermal cells were able to form a 3-dimensional epidermal structure *in vitro* (organogenic potential). A young culture of epidermal stem cells (passage 5) was found to reliably generate a 3D epidermis both in the presence and ab-

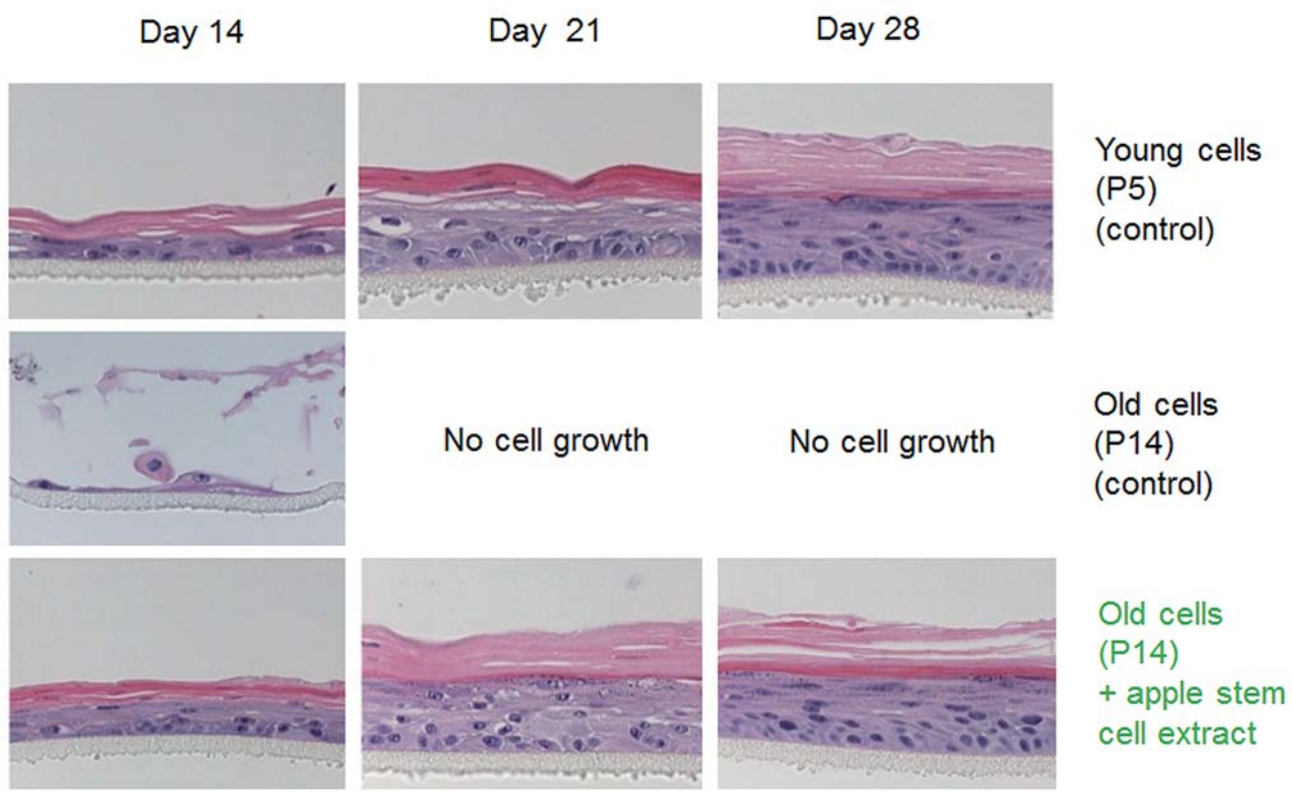

Fig. 4 Plant stem cell extract maintains the capacity to form a stratified epidermis

sence of the apple stem cell extract **(Fig. 4)**. In contrast, old cells (passage 14) were completely unable to form the 3D epidermal structure. However old cells that had been maintained in the presence of the apple stem cell extract exhibited no loss of function, and were able to establish a perfectly normal 3D epidermal structure even at this advanced age (Figure 4; old cells + apple stem cell extract). This clearly shows that the apple stem cell extract improves the maintenance of epidermal stem cell characteristics.

Effect of a Plant Stem Cell Ingredient on Dermal Stem Cells

Dermal stem cells were isolated from the dermal papilla of excised human hair follicles. These cells were maintained as a monolayer culture for at least 11 passages. At both passage 3 and passage 11 cells transferred into hanging drops formed 3D spheres, demonstrating that this important characteristic of progenitor cells was retained even after longer-term cultivation (4). In addition, immunofluorescent labeling of whole mount spheres showed positive staining for the Sox2, a proposed dermal stem cell marker. When cells dissociated from primary spheres were seeded back into classical cell culture dishes used for routine monolayer culture, numerous secondary spheres were spontaneously formed. This indicates that once cells have formed primary spheres, they seem to retain a memory of the 3D progenitor phenotype, and preferentially re-form spheres where normally monolayer cultures would be expected.

The argan tree (*Argania spinosa*) was chosen to prepare plant stem cells for a dermal stem cell ingredient. The argan tree belongs to the oldest tree species of the world and grows only in the south-western part of Morocco. Nowadays, argan trees are an endangered species and as such they cannot be used as a raw material for a cosmetic ingredient. As an alternative, the plant cell culture technique was used to produce vegetal raw material from argan. A piece of an argan shoot was used to start a plant cell culture. In order to evaluate an extract of argan stem cells (INCI: Argania Spinosa Sprout Cell Extract) for its potential to vitalize dermal stem cells, the intensity and uniformity of Sox2-labelling in primary spheres and the number of secondary spheres formed were used as parameters. Compared to control cultures, the immunofluorescence pictures showed clearly an enhanced expression of Sox2 in spheres formed by dermal papilla cells cultured in the presence of 0.1% of the argan stem cell extract (results not shown). For the formation of secondary spheres, primary spheres in hanging drops were digested with TrypLE for 30 minutes at 35 °C. The cells were counted and seeded at 10'000 cells per well into 24 well plates. After 3 weeks, the number of secondary spheres formed was determined. Incubation with the argan stem cell extract was found to significantly stimulate the vitality of dermal stem cells **(Fig. 5)**.

Clinical Studies with the Dermal Stem Cell Ingredient

An ingredient based on an extract of argan stem cells (INCI: Argania Spinosa Sprout Cell Extract, Isomalt, Lecithin, Sodium Benzoate, Aqua), formulated at 0.4% into an o/w emulsion was tested for anti-aging and anti-cellulite effects. The anti-aging study was carried out over 56 days with 21 women aged from 39 to 61 having sun-damaged skin (Phototype I-III). The test products were applied twice per day on the crow's feet area (wrinkle depth) for a vehicle-controlled half side comparison and on the inner side of the forearms (density). Wrinkle depth was determined by PRIMOS pico and skin density by ultrasonography by DermScan C at days 28 and 56. In the ultrasonography of young skin collagen bundles are homogenous and generate therefore high-echogenic signals in the dermal region. Aged and photo-aged skin shows subepidermal low-echogenic bands (SLEB). The thickness of SLEB increases with age progressively and is greater on sun-exposed skin. Collagen bundles are replaced by a deposit of a greater amount of hydrated proteoglycans, glycosaminoglycans and unbound water.

Results showed that the treatment with argan stem cell ingredient led to an increase in skin density of 12.7% compared

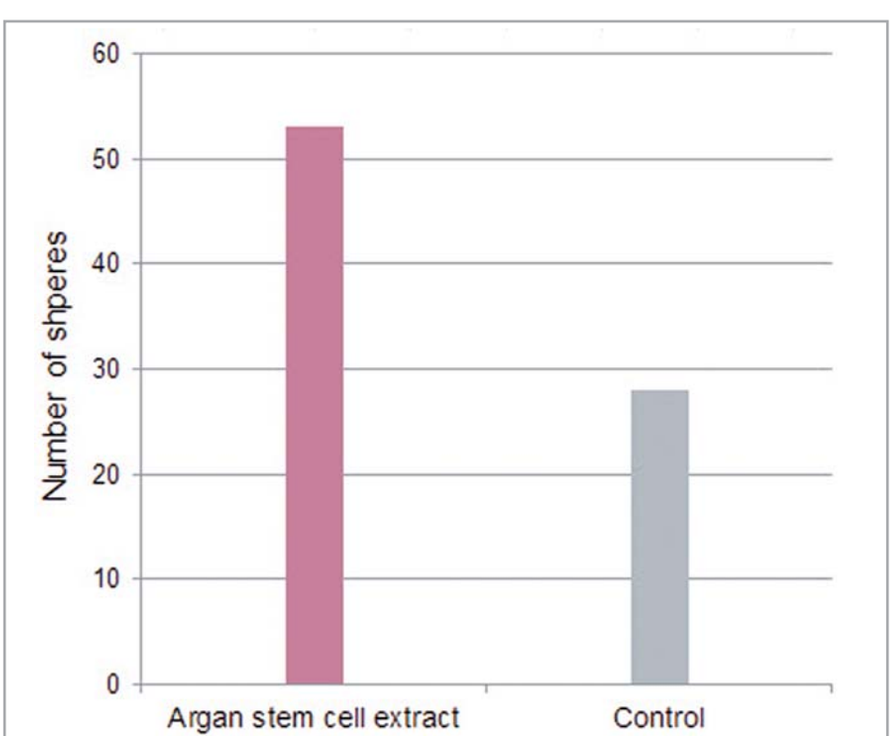

Fig. 5 Argan stem cell extract stimulates the formation of secondary spheres

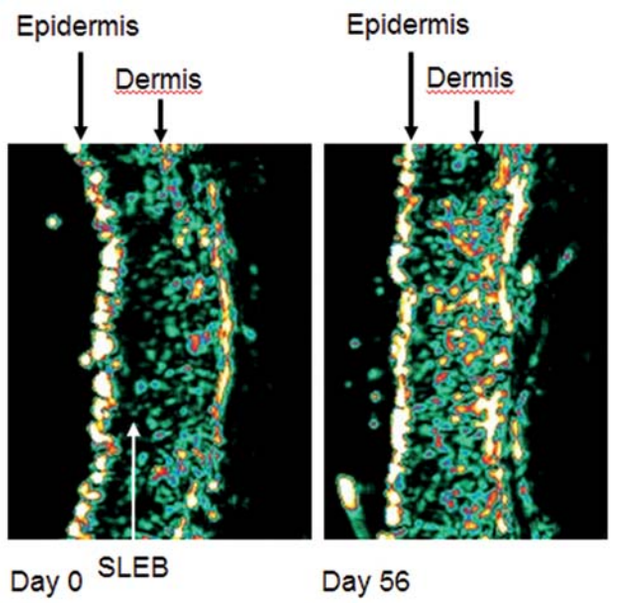

Fig. 6 a Ultrasonographic images of forearm skin at the begin (left) and at the end of the treatment (right) with the argan stem cell ingredient

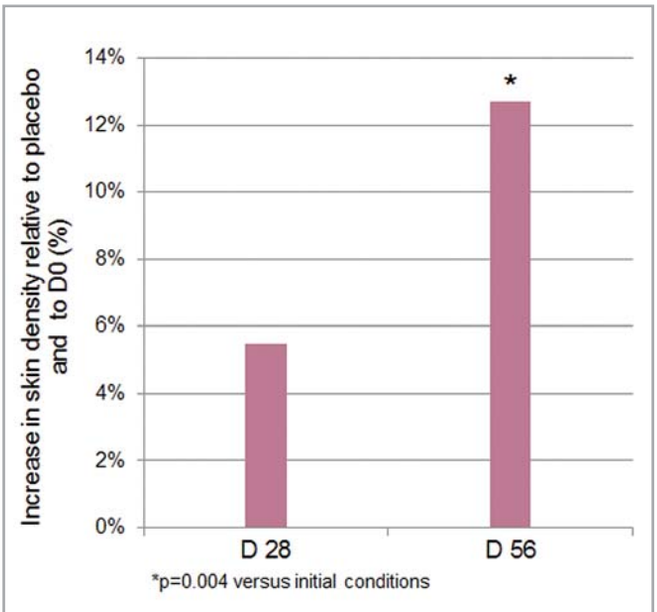

Fig. 6 b Increase of skin density compared to the placebo and to initial conditions

to the placebo and after 8 weeks treatment **(Fig. 6 a, b)**. It was also observed that the ingredient successfully reduced the wrinkle depth in crow's feet area by 19 % after 28 days of treatment and by 26 % after 56 days of treatment **(Fig. 7)**. The anti-cellulite trial was conducted on 22 women aged between 22 and 53 with cellulite grade II-III on thighs. The cream with the argan stem cell ingredient was applied twice daily for 56 days to one thigh. The other thigh was treated with the placebo cream. The dermis-hypodermis junction area (DHJA) was determined by ultrasonography by DermScan C. Ultrasonography is a technique for measuring skin density/tightening where pulses of ultrasound waves are emitted into the skin. Reflections which occur from transitions between tissue layers with different acoustic impedance, are detected and visualized. In young and tight skin, the dermis-hypodermis area appears as a straight transition, visibly in ultrasonographs as a linear junction between the high echogenic dermis and the low echogenic hypodermis. In the case of cellulite, however, the dermis-hypodermis junction appears as an irregular surface and the dermis-hypodermis junction area is increased.

After 56 days of use, and compared to the placebo product, the cream with the argan stem cell ingredient induced a decrease in the dermis-hypodermis junction area of 11% **(Fig. 8 a, b)**. The skin was tighter and the smoothness was improved.

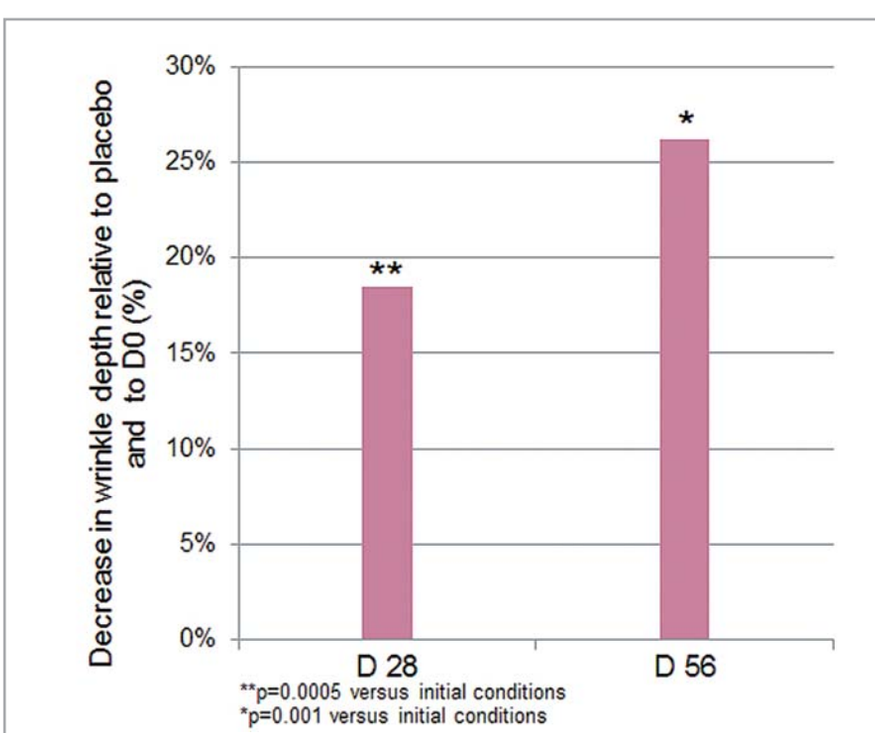

Fig. 7 Analysis of wrinkle depth after 28 and 56 days of treatment

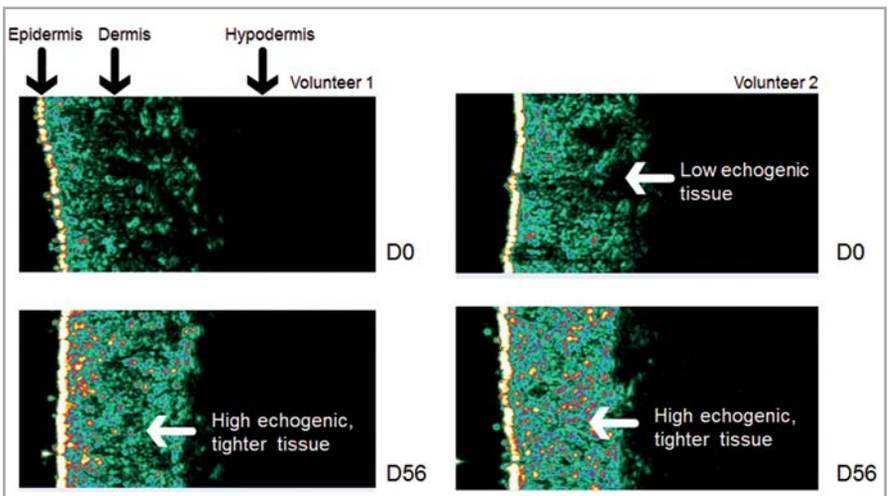

Fig. 8 a Ultrasonsographic images of woman's thigh skin at the beginning and at the end of the treatment with the argan stem cell ingredient

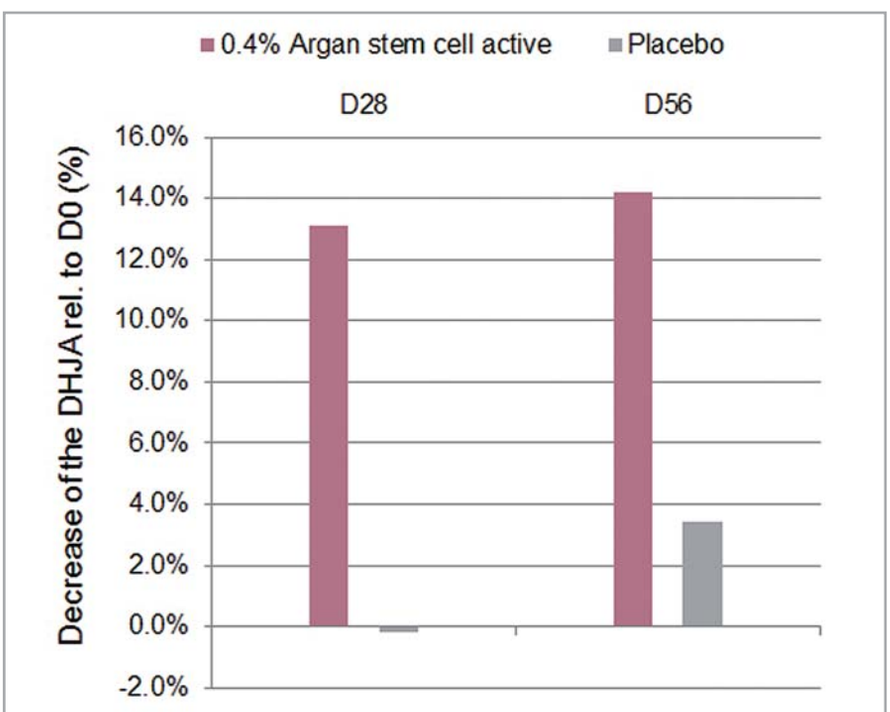

Fig. 8 b Decrease of the dermis-hypodermis junction area after 28 and 56 days of treatment

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