Dual exosome power for skin rejuvenation

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ABSTRACT

The potential of exosomes - a type of extracellular vesicles enabling cell-cell communication - for the cosmetic field has become increasingly clear over the past years. Mesenchymal stem cells in the skin, for instance, can improve collagen production and regenerate the skin by exosome-based communication with fibroblasts. Interestingly, plant-derived exosomes can also be taken up by and communicate with human cells, providing a great potential for cosmetic applications. This study investigated the effect of a goji stem cell active, naturally containing plant exosomes, on the skin. The active delivers plant stem cell-derived exosomes from outside into the skin directly acting on the epidermis to strengthen the skin barrier, as shown by the upregulation of genes important for the skin barrier function in keratinocytes treated with the active ingredient. In addition, it was shown that the goji stem cell active can boost the exosome secretion by mesenchymal stem cells and thereby improves communication with fibroblasts resulting in the improved production of extracellular matrix components such as collagen and elastin. This dual function based on exosomes – delivery of plant exosomes and boosting of the endogenous exosome production – leads to skin protection and rejuvenation. The effect was confirmed in three clinical studies demonstrating improved skin density and wrinkle depth as well as reduced skin sagging which led to a more oval shaped face and lifted breasts.

Exosomes have been increasingly discussed in the past few years due to their potential for the diagnosis and therapy of diseases, such as cancer and neurodegenerative diseases. Considering the effect of exosomes on wound healing, skin pigmentation, and hair loss, it is no surprise that they are applied in cutaneous medical aesthetics and in the meantime have also evolved into a hot topic in the world of cosmetics.

Exosomes – vesicles for cell-cell communication

Exosomes, a type of extracellular vesicles with a double-layer membrane and a size of 30-200 nm, are released by eukaryotic cells and contain lipids, RNAs, proteins, and metabolites (Figure 1A). They are generated from larger intracellular vesicles called multivesicular bodies, which in turn are formed by the budding of the cell membrane and are released from a cell when multivesicular bodies fuse with the cell membrane in a process known as exocytosis (Figure 1B).

Therefore, exosomes can be detected in biological fluids and in the supernatant of cultured cells. While initially being discussed as a delivery vehicle for cellular waste, it is nowadays acknowledged that exosomes are an important component of cellular communication systems.

Exosomes released by one cell type can initiate a response in other cell types via different mechanisms. They can either activate surface receptors of recipient cells be direct binding, or deliver functional proteins, lipids and RNA to the recipient cell via membrane fusion or be completely taken up into the recipient cell via endocytosis.

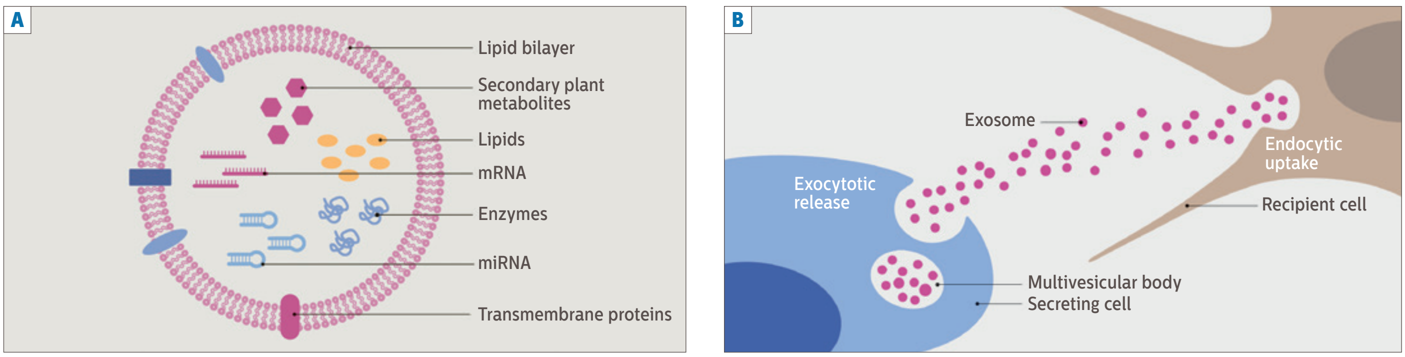

Figure 1: Structure and function of exosomes. A: Exosomes are vesicles composed of a double-layer membrane and contain lipids, RNAs, proteins and metabolites. B: Exosomes are secreted from one cell to communicate with another cell

Signaling via exosomes plays a major role in many cellular processes, such as signal transduction and immune response. In the skin, for instance, mesenchymal stem cells, precursors of different cell types, are essential for regeneration processes, such as wound healing. Interestingly, mesenchymal stem cells themselves are not needed for this process, but rather the exosomes that they secrete to communicate with other cells such as fibroblasts.

The potential of plant-derived exosomes

As is the case with mammalian cells, plant cells use extracellular vesicles to transfer RNAs, proteins, lipids, and secondary metabolites. Such plant-derived exosome-like nanovesicles ('plant exosomes') function in intercellular communication, the plant's defence system or cell wall organization. Interestingly, plant-derived exosomes can be taken up by mammalian cells and have a beneficial effect on human health through cross-kingdom cell communication.

Exosomes from different plants, such as apples, grapes, aloe vera, lemon, and ginger, showed anti-inflammatory, antioxidant, anti-tumour, or wound healing potential.[1-3] Considering these effects and their high stability and safety, plant-derived exosomes are being investigated for medical and cosmetic use.

Due to their size and composition, plant exosomes, similar to mammalian exosomes, can be taken up by the skin where they can unleash their full potential. As the use of human-derived or animal-derived exosomes in cosmetics is not permitted in most countries, biomimetic or plant-derived exosomes are a promising alternative. Despite the advantages of plant-derived exosomes and the intensive research that is currently being performed, it remains difficult to extract pure plant-derived exosomes due to the intricate structure and cell walls of plant cells. Complex extraction and purification protocols, as well as impurities, are the challenges faced.

Plant stem cells as optimal source for plant exosomes

To benefit from the great potential of plant exosomes but circumvent the challenges of isolation, cultivated plant stem cells can be used as source of exosomes. The PhytoCellTec technology developed by Mibelle Biochemistry relies on the wound healing mechanism of a plant, whereupon part of a plant is wounded to induce the formation of callus cells.

This healing tissue consists of dedifferentiated cells, which have stem cell properties. Such plant stem cells do not have rigid cell walls, contain no chlorophyll, and have a high number of exosomes inside and around the cells.

In this study, an extract from a plant stem cell culture originally derived from a goji seedling naturally containing exosomes was analyzed for its beneficial effect on the skin. Goji (*Lycium barbarum*) plants are native to Southeast Europe and Asia and their fruits, the goji berries, are one of the most famous superfruits. Recent studies specifically examining the exosomes derived from goji plants demonstrated that these exosomes provide health benefits in the context of muscle atrophy and spinal cord injuries.[4,5]

Materials and methods

Characterization of plant stem cell-derived exosomes

Goji stem cells were cultivated at 25°C in the dark on a wave reactor. For sample preparation, the cell culture was homogenized using a microfluidizer (Microfluidics Corporation Newton) and afterwards centrifuged.

The supernatant was lyophilized and filtered in several steps. The particle size of the resulting sample was determined using a particle size analyzer (Malvern Panalytical Ltd.).

For freeze-fracture transmission electron microcopy, a sample was placed onto a gold sample holder between copper plates before jet-freezing with liquid propane as cryogen. Samples were fractured and freeze-etched (Leica Microsystems) and replicas were made by platinum-carbon shadowing, cleaned and dried before being examined by a transmission electron microscope at 80 kV (TEM CM 10, Philips). Images were taken with a CCD camera.

Gene expression in keratinocytes

Normal human epidermal keratinocytes (NHEK) were seeded in 24-well plates and grown for 24 hours before treatment with 0.1% goji stem cell extract for 24 hours. Cells were harvested and washed in phosphate buffered saline (PBS), replicates were pooled and total RNA was extracted from cells of each condition.

RNA was reverse transcribed and qRT-PCR analysis was performed on genes selected for their importance in skin physiology. Normalization was performed using house-keeping genes and the resulting gene expression data was compared to the control.

Quantification of exosome production by mesenchymal stem cells

Dermal derived human mesenchymal stem cells (MSCs) were either treated or not (control) with 0.1% goji stem cell extract for a period of 24 hours. The exosomes that were released from the cells were isolated using EXOPrep kit (Hansabiomed) and quantified in two different ways: quantification of total protein amount and quantification of the activity of acetylcholinesterase, which is a known exosomal protein using the Pierce BCA Assay kit (Thermo Fisher Scientific) and Fluorocet exosome quantitation assay kit (System Bioscienes), respectively.

Gene expression in fibroblasts after treatment with MSC-conditioned medium

Human mesenchymal stem cells (MSCs) were seeded in 6-well plates and cultured for 24 hours. The medium was then removed and replaced with culture medium containing or not (control conditioned medium) 1% goji stem cell extract and cells were incubated for 72 hours. All experimental conditions were performed in n=2.

At the end of incubation, the supernatants were collected, and the replicates of each condition were pooled to be used for the treatment of fibroblasts (conditioned medium).

Fibroblasts were previously seeded in 12-well plates and cultured in culture medium for 72 hours and then in assay medium for further four hours. Afterwards, for one series, medium was removed and replaced with assay medium containing or not (control) goji stem cell extract. For another series, medium was removed and replaced with the conditioned medium from MSCs (control or goji stem cell extract treated MSCs). Cells were then incubated for 24 hours. All experimental conditions were performed in n=3.

At the end of incubation, cells were washed

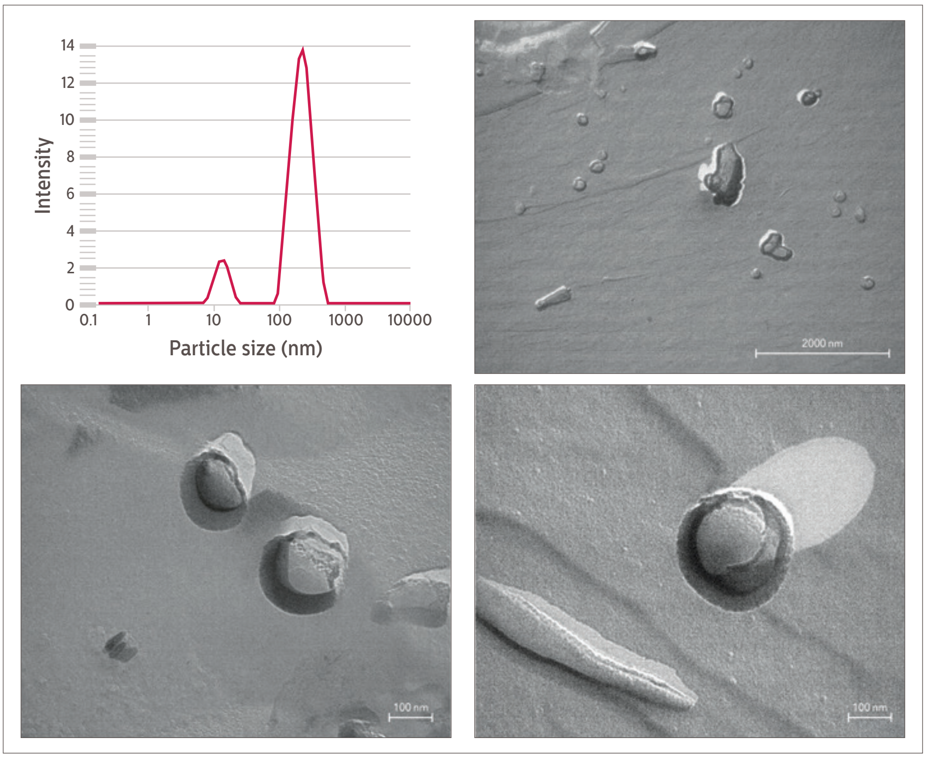

Figure 2: Structure and size of plant exosomes in the goji stem cell extract

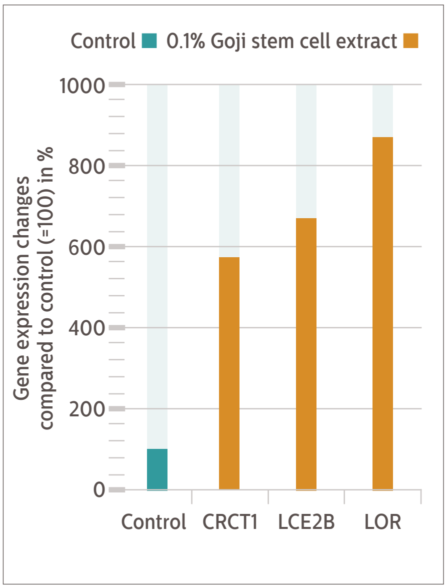

Figure 3: Increased expression of genes important for skin barrier formation

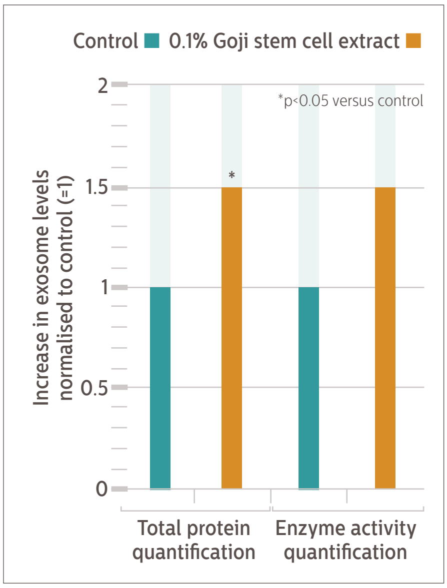

Figure 4: Increased exosome production in mesenchymal stem cells

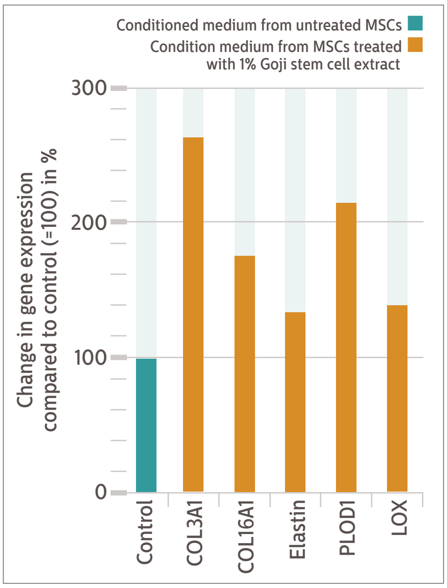

Figure 5: Improved cell-cell communication assessed by gene expression analysis in fibroblasts treated with conditioned medium from mesenchymal stem cells (MSCs)

in phosphate buffered saline (PBS) solution and immediately frozen at -80°C. Total RNA was extracted from the cells and the expression of extracellular matrix genes was then assessed with RT-qPCR using the LightCycler System (Roche).

Clinical anti-aging study

In a randomized, placebo-controlled clinical study, 23 Caucasian women aged between 41 and 69 years (mean age: 56 years) who displayed signs of photoaging applied either a cream with 0.4% goji stem cell active (INCI: Lycium Barbarum Callus Culture Extract (and) Isomalt (and) Lecithin (and) Aqua / Water) on one half of the face and one forearm and the corresponding placebo on the other half of the face and the other forearm, twice daily for a period of 56 days.

The wrinkle depth was measured using PRIMOS lite (Canfield). The density of the skin (epidermis + dermis) was determined by ultrasonic measurements in triplicates using DermaScan C (Cortex) which counts the number of low-echogenic pixels corresponding to low-density areas on a 2-D portion of epidermis and dermis. The lower the number of dark pixels the higher the skin density.

Clinical face contour study

In a randomized, placebo-controlled clinical study, sixty-seven Caucasian women aged between 39 and 70 years (average age 57 years) with sagging facial skin were split into two groups. One group applied a cream with 0.4% goji stem cell active and the other group applied the corresponding placebo cream on the entire face and neck twice daily for 28 days.

The facial sagging and oval face shape was determined by measuring the size of the neck/submandibular triangle area by image analysis of the pictures of the volunteers' faces that were taken with Visioface 1000 D (Courage + Khazaka). For the image analysis, a straight line was drawn vertically through the middle of the chin.

On either side of the face, vertical parallel lines were drawn to intersect at the start of the jawline/sagging skin. The points of the jawline intersection were then connected with the middle of the chin. The area below the drawn V lines was determined. A reduction of this area would suggest a reduction in facial sagging and an improved V-shape of the face.

Clinical breast lifting study

The effect of goji stem cell active on breast sagging was tested in a randomized, placebo-controlled clinical study with 44 women aged between 18 and 44 years (mean age: 29.4 years) with breast sizes 80B, 85A, 90A, and 90B. The women were split into two groups of 22

Figure 6: Improved skin density and wrinkle appearance. A: Subepidermal low-echogenic bands (SLEB) were reduced after 28 days of treatment with goji stem cell active. B: Wrinkles appearance in the crow's feet area was visibly improved after 56 days of treatment with goji stem cell active

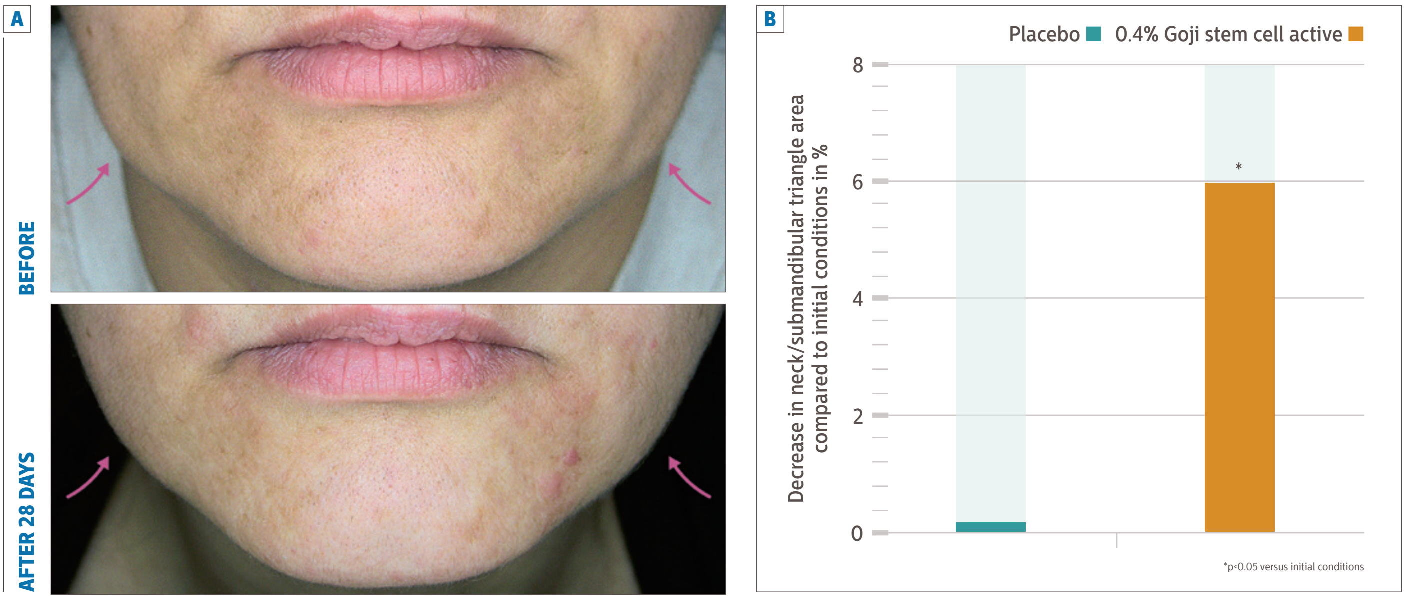

Figure 7: Improved oval shape of the face after 28 days. A: Visible effect on the V-shape in the jawline region. B: Decrease in the neck/submandibular triangle area

volunteers, with each group applying either a cream with 0.4% goji stem cell active or the corresponding placebo on the breast area twice daily for a period of 56 days.

The bust distances between the nipple and the sternum as well as the nipple and the inframammary fold were measured by 3D Breast Imaging using VECTRA-XT (Canfield Scientific).

A lifting of the breasts would result in a reduced nipple-sternum distance while the nipple- inframammary fold distance would increase. The overall breast condition of the volunteers was determined by an investigator using the Regnault ptosis classification (score 0 = normal, score 1 = mild sagging, score 2 = moderate sagging, score 3 = severe sagging, score 4 = pseudoptosis). The elasticity of the skin was determined on the upper part of the breasts using the Cutometer Dual MPA 580 (Courage + Khazaka).

Results and discussion

Plant exosome-containing stem cell extract

The goji stem cell extract used for this study naturally contains exosomes derived from the cultivated goji plant stem cells. The particle size analysis of samples of the stem cell culture revealed the presence of exosomes with a size of approximately 200 nm determined by dynamic light scattering (Figure 2).

Images taken by transmission electron microscopy confirmed the presence and the size of these exosomes. In addition, the structure of intact exosomes enclosed by a double-layer membrane was visible in these images (Figure 2).

Plant exosomes to protect the skin

The effect of the plant exosomes on the skin was assessed in keratinocytes treated with 0.1% goji stem cell extract for 24 hours. The treatment increased the expression of genes involved in the late differentiation and cornification process (CRCT1, LOR, LCE2B, LCE3D, FLG, CRNN) (Figure 3), whereas genes linked to early differentiation were downregulated (IVL, KRT2, KRT1, CALML5) (data not shown).

In aged skin, the barrier function can be impaired and shows a reduced ability to recover. This leads to decreased protection of the skin and body from external aggressors, such as UV radiation, pollution or pathogens, and to difficulties in maintaining skin hydration. Thus, by supporting the formation of the stratum corneum to improve the function of the skin barrier, goji stem cell extract protects the skin from water loss and external aggressors.

Additionally, in cells treated with goji stem cell extract the expression of the two growth factors EGF (epidermal growth factor) and FGF7 (fibroblast growth factor 7) was significantly upregulated by 108 % and 161 %, respectively, and the gene expression of heat shock protein HSPA6 and antioxidant gene SOD3 was upregulated by 407 % and 64 %, respectively. Therefore, goji stem cell active may also stimulate dermal regeneration and protection by acting on keratinocytes.

Increased exosome release from mesenchymal stem cells in the skin

To assess the effect of the goji stem cell extract on the exosome production inside the skin, human MSCs were either treated or not (control) with 0.1% goji stem cell extract for a period of 24 hours.

The exosomes that were released from the cells were quantified in two ways, either measuring the total protein or enzymatic activity of a known exosomal protein. Both quantification methods revealed that treatment with goji stem cell extract leads to an increase in exosome production by MSCs (Figure 4).

Stimulation of extracellular matrix genes through cell-cell communication

After demonstrating the effect on exosome production in MSCs, it was investigated as to whether MSCs treated with goji stem cell extract can communicate with fibroblasts to produce more extracellular matrix (ECM) proteins.

For this purpose, human MSCs were treated with 1% goji stem cell extract. The supernatant, which includes soluble factors as well as exosomes ("conditioned medium"), was added to fibroblasts for a period of 24 hours. Fibroblasts treated with medium from untreated MSCs served as the control.

Results showed that the conditioned medium from goji stem cell extract-treated MSCs increased the gene expression of several ECM factors such as collagen 3 and 16 and elastin (Figure 5). PLOD1 is crucial for collagen production, while LOX encodes lysyl oxidase which connects collagen and elastin to ensure more stability and elasticity. The direct treatment of the fibroblasts did not have the same effect. Thus, the goji stem cell active improves cell-cell communication between MSCs and fibroblasts and thereby intensifies its plant exosome-based anti-aging effect.

Improvement of wrinkle depth and skin density

In a clinical study, 23 Caucasian women aged between 41 and 69 years who displayed signs of photoaging, applied a cream containing 0.4% goji stem cell active and the corresponding placebo cream for two months. After 28 days, a significant improvement of skin density could be observed via ultrasonic measurements for the treatment with the goji stem cell active (data not shown).

The collagen and elastic fibre structure of an intact dermis yields many reflections from the ultrasonic wave that are visible as bright colours in the ultrasonographic image. However, disruption to this regular architecture leads to weaker reflections and dark patches, as can be seen in the ultrasonographic image at day 0. These so-called subepidermal low-echogenic bands (SLEB) are commonly found in aged and photodamaged skin. The SLEB could be reduced after 28 days treatment with 0.4% goji stem cell active (Figure 6A).

Furthermore, after 56 days, a significant decrease in wrinkle depth in the crow's feet area by 11% compared to placebo could be measured (Figure 6B). Not only fine wrinkles below the eye appeared smoother, but deeper lines were also visibly improved (Figure 6B).

Improvement of the oval face shape

In a second placebo-controlled clinical study, 67 Caucasian women aged between 39 and 70 years with sagging facial skin were split into two groups and used either a cream with 0.4% goji stem cell active or the corresponding placebo cream on the entire face and neck twice daily for 28 days.

The facial sagging and oval face shape was determined by measuring the size of the neck / submandibular triangle area by image analysis of pictures of the volunteers' faces. Results showed that treatment for four weeks with 0.4% goji stem cell active significantly improved the oval face shape by 6% compared to initial conditions.

The improved V-shape of the face was also visible in the pictures of the jawline (Figure 7). This demonstrates that goji stem cell active is indeed able to reduce facial sagging and improve the oval shape of the face.

Breast lifting effect

In addition, the effect of goji stem cell active on breast sagging was tested in a placebo-controlled clinical study with 44 women aged between 18 and 44 years. The women were split into two groups, with each group applying either a cream with 0.4% goji stem cell active or the corresponding placebo on the breast area twice daily for a period of 56 days.

The bust distances between the nipple and the sternum as well as the nipple and the inframammary fold were measured by 3D Breast Imaging. Treatment with goji stem cell active for 56 days led to a significant breast lifting effect compared to initial conditions.

On average, the distance from the nipple to the sternum was decreased by 0.9% whereas the distance from the nipple to the inframammary fold was increased by 1.7% (Figure 8A). In addition, the overall breast condition of the volunteers was determined by an investigator using the Regnault ptosis classification.

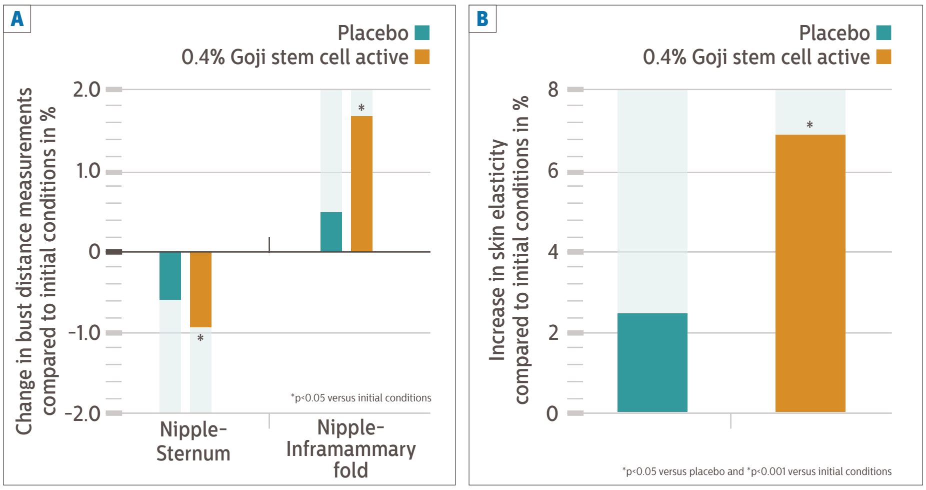

Figure 8: Breast lifting effect and improved skin elasticity after 56 days. A: The breast lifting effect is indicated by a reduced nipple-sternum distance and increased nipple-inframammary fold distance. B: Increased skin elasticity measured in the breast area

According to this clinical scoring system, the application of goji stem cell active significantly improved the breast condition by 33.3% after two months. An improvement in the breast condition was measured in 54.5% of the volunteers, with the best effect in those initially classified in the Regnault ptosis score 1.

This positive outcome confirms that goji stem cell active can reduce breast sagging through a breast lifting effect. In addition to the investigation of the breast condition and lifting, treatment with goji stem cell active for two months significantly improved the skin elasticity compared to the placebo (Figure 8B), supporting the anti-gravity effect observed in this clinical study.

Conclusion

The goji stem cell active contains plant stem cell-derived exosomes, which can be applied to the skin from the outside to directly act on the epidermis. By upregulating genes important for the skin barrier function in keratinocytes, goji stem cell active can protect and rejuvenate the skin.

In addition, goji stem cell active boosts the exosomal secretion from mesenchymal stem cells inside the skin to improve communication with fibroblasts resulting in the increased production of extracellular matrix components such as collagen and elastin. Based on this dual exosome-based mechanism (Figure 9), goji stem cell active rejuvenates the skin leading to reduced sagging and thus an improved V-shaped face and lifted breasts. **PC**

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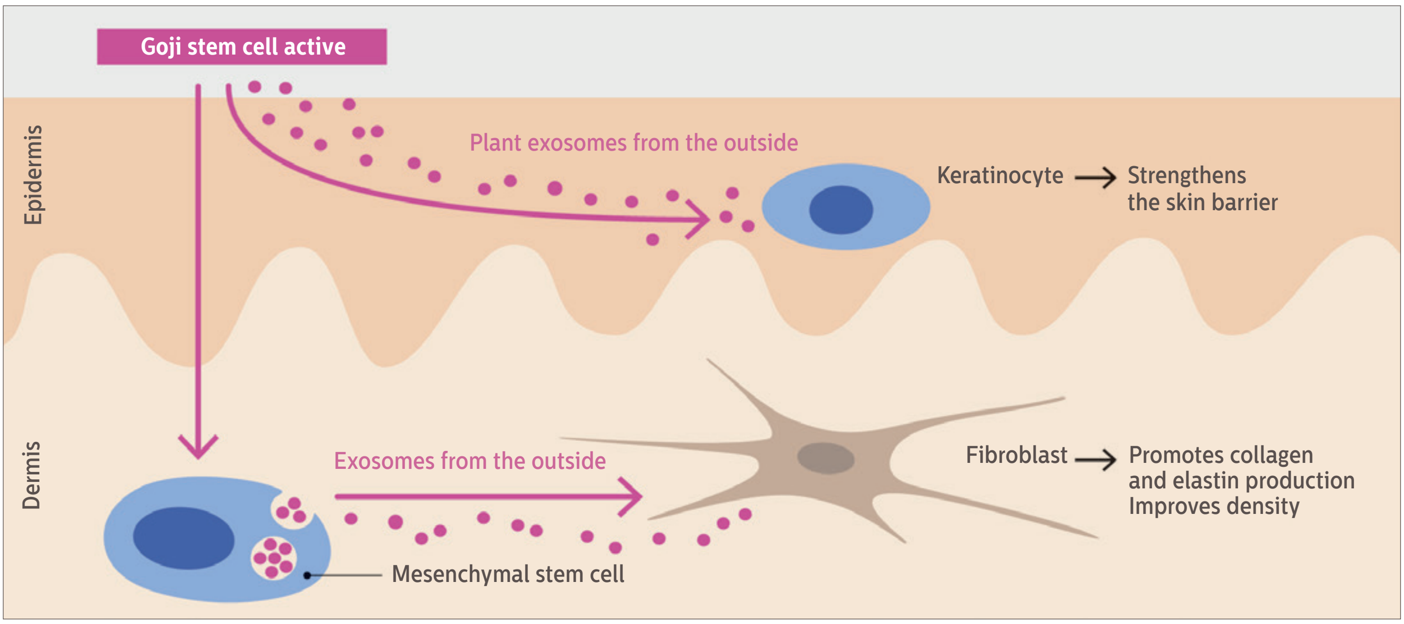

Figure 9: Dual approach of the goji stem cell extract to rejuvenate the skin