# Wrinkle Reduction by Stimulation of the Skin's Mechanical Resistance

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**KEY WORDS:** *antiaging, skin mechanical resistance, mechano-receptor, integrin, vinculin, alpha-smooth muscle actin*

**ABSTRACT:** *A rye extract rich in arabinoxylans is shown to stimulate the synthesis of mechano-receptor proteins and alpha-SMA fibers in the skin, improving the skin's mechanical resistance and reducing the appearance of gravity wrinkles.*

Wrinkles are a symptom of structural failure in the dermis. They indicate that the skin is losing its ability to support its own weight, and that fibroblasts in the dermis are losing their capacity to attach to collagen fibers and transmit mechanical information.

This is a report on an arabinoxylans-rich rye extract that stimulates the skin's lifting properties and boosts the mechanical resistance of the dermis.

## Skin Resistance Mechanisms

The dermis is a support connective tissue composed primarily of fibroblasts and a vast microfibril network of collagen, elastin and proteoglycans. This fiber network undergoes a constant but very slow renewal, during which the synthesis of macromolecules and their degradation by matrix metalloproteinases (MMPs) are equilibrated until adulthood. This balance depends on such factors as nutritional supplies, hormonal status, the influence of toxic compounds or the external environment.

In humans at around the age of 30, the skin begins to accumulate a number of changes to cells and their support. In this process called intrinsic aging, the skin acts as if its support structures had lost their intrinsic mechanical properties due to the aging of the structural material or, more likely, due to insufficient maintenance of that material by the cells. In fact, those intrinsic mechanical properties suffer due to the loss of the interaction between fibroblasts and the extracellular matrix (ECM). This interaction normally is ensured by mechano-receptors that are linked to alpha-smooth muscle actin (alpha-SMA) fibers. Mechanical stress is transmitted through the epidermis and then the ECM to reach the mechano-receptors located at the fibroblast plasma membrane. Afterwards, the mechano-receptors transduce the mechanical information into intracellular messages.

> Mechanical forces in tissues and the phenomenon of gravitation play a fundamental role in the appearance of wrinkles.

The clinical manifestations of these deteriorations involve two major types of surface deformations—expression wrinkles and gravity wrinkles. Mechanical forces in tissues and the phenomenon of gravitation play a fundamental role in their appearance.[1] The formation of gravity wrinkles occurs in the skin wherever it has the capacity to stretch under the influence of its own weight. On the face, for example, the upper eyelids and the lower part of the face are the preferred locations.

The dermis also is altered by UV radiation, especially UVA. It leads to an increase in the production of MMPs that are responsible for the degradation of macromolecules that constitute the ECM. The process of extrinsic aging is also characterized by a reduced fibroblast proliferation capacity and by a reduction of their metabolic activity and migration capacity. This reduction in migration is correlated with a decrease of integrins, which are the proteins involved in the attachment of fibroblasts to collagen fibers and in the transmission of mechanical information.[2,3]

The ECM is the adhesion substrate of fibroblasts and the mechanical support of the skin. The ECM transmits mechanical stress all the way to fibroblasts. These cells play the role of a strain gauge, detecting mechanical stresses transmitted by receptors responsible for the junction between the fibroblast and its network of collagen fibers. These mechano-receptors, or shock absorbers, activated by the application of pressure on the surface of the skin, play a central role in the cell mechanics of the skin (**Figure 1**). They integrate the mechanical signal and then act as mechano-effectors by transmitting the shock to adjacent cells by changing their cytoskeleton and activating a variety of intracellular signaling pathways.[4] Fibroblasts respond to these signals by synthesizing components of the dermal matrix and simultaneously inhibiting the production of MMPs and pro-inflammatory cytokines.[4–6] Thus, fibroblasts produce a more resistant support to adapt the resistance of the

skin to the mechanical stress, and the translated mechanical information enables the control of biological homeostasis of the dermis.[7]

## Mechano-receptors and alpha-SMA

The attachment of fibroblasts to collagen fibers, or initial adhesion, is ensured by alpha2beta1 integrins, which are dimeric transmembrane proteins produced in the fibroblasts and specifically involved in activating the cell-signaling pathway.[8] Faced with a mechanical stress perceived as a signal by the cell, initial adhesion is reinforced to form a mechano-receptor (**Figure 2**) composed of a set of structural and signaling proteins, among which are talin, vinculin and paxillin.

Formation of the mechano-receptor is marked by the incorporation of vinculin in initial adhesion. Vinculin is a relevant marker of the mechano-receptor because it is absent in initial adhesions and its accumulation is correlated with the formation of the mechano-receptor[9,10] and with the adhesion force of fibroblasts to the matrix.[11] Stress fibers attached to this mechano-receptor include alpha-SMA, responsible for the generation of retractile forces of fibroblasts.[8]

The mechano-receptors are activated in conditions of stress and translate the mechanical message into an intracellular signal that organizes the alignment of fibroblast stress fibers. The presence of alpha-SMA in fibroblasts ensures the maturation of mechano-receptors, thereby enabling a more efficient transmission of the mechanical signal. The increased tension of the ECM will stimulate the expression of alpha-SMA by fibroblasts.[11] The association of mechano-receptors with alpha-SMA is not only essential for generating tension forces, but also plays a central role in the regulation of dermal mechanical resistance by ensuring a perpetual equilibrium between the states of contraction and relaxation of the skin.

Reinforcement of the initial adhesion into mechano-receptors requires only 10–40 sec. This speed suggests that the formation of mechano-receptors is regulated locally and that it plays a major role in the transmission of forces to the ECM to modulate the mechanical

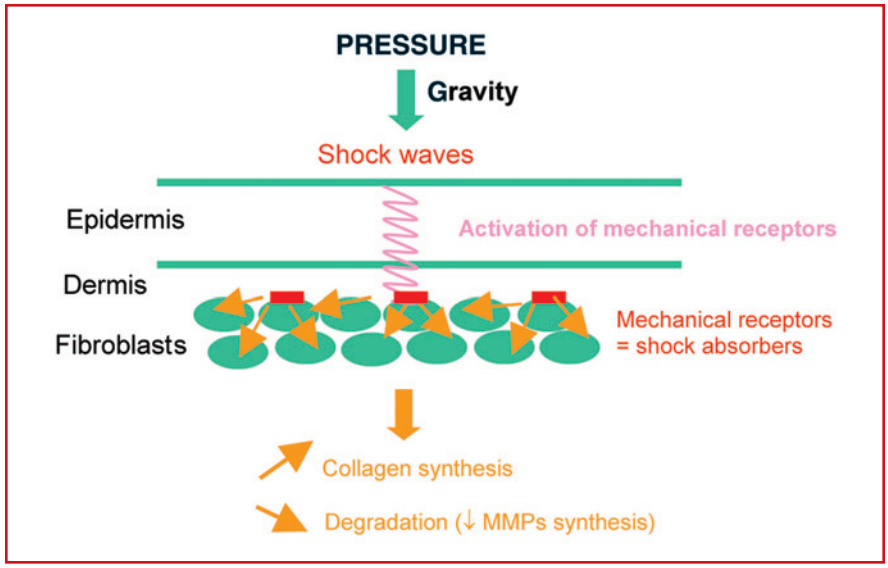


*Figure 1. Mechanisms involved in skin resistance*

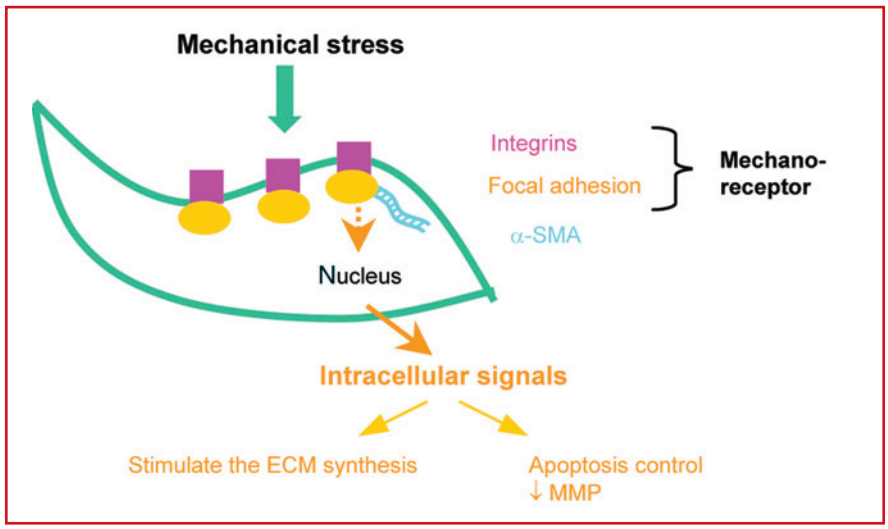


*Figure 2. Role of the mechano-receptor and alpha-SMA in cell mechanics*

resistance of the skin. If the mechanical stress continues, the mechano-receptor continues to develop into a broad and stable focal adhesion that extends along the periphery of the cell. This process requires about 60 min and enables fibroblasts to induce a longer-term elevated mechanical response, because the focal adhesion can exert higher forces than mechano-receptors.[9]

## Age-reduced Interaction Between Fibroblasts and the ECM

As humans age or accumulate exposure to UV, their fibroblasts produce reduced quantities of alpha2beta1 integrins.[2,3] That reduction leads to a loss of their attachment to the ECM, a contact that is indispensable for maintaining the mechanical properties of the skin.

Aged or photoexposed skin is characterized by a degraded ECM composed of fragmented collagen fibers. This deteriorated support no longer ensures optimal adhesion of fibroblasts, leading to a state of mechanical relaxation. In this state, mechano-receptors are dissolved by the breakdown of vinculin,[4,10] leading to a loss in the cell-matrix interaction and thus a reduction in tension of the support. In addition, the expression of alpha-SMA by fibroblasts from old skin is reduced[7] and the organization of these fibers is also altered,[2] causing a reduction in the retractile forces of fibroblasts and in their response to mechanical stresses. The mechanical resistance of

the skin to pressure and gravity shocks is thereby reduced, causing the appearance of wrinkles.[3]

Favoring the interaction of fibroblasts with their support is thus essential for stimulating the adhesion capacities of fibroblasts and for restoring the mechanical properties of the dermis.

## A Rye Extract for Increased Mechanical Resistance of Skin

Researchers at Silab began a search for a new material that would increase the skin's mechanical resistance. Having identified the biological markers—integrins, vinculin and alpha-SMA—involved in the skin's mechanical resistance, the Silab researchers studied the behavioral differences between normal human fibroblasts and aged human fibroblasts in terms of synthesis of the selected biological markers. Using in vitro methods, the researchers screened different plant molecular structures for activity on the three biological markers before finally selecting arabinoxylans. Arabinoxylans, usually extracted from cereals, are polysaccharides composed of a repeated xylan backbone that can be substituted by one or two arabinose.

Published data was searched for a plant that was rich in arabinoxylans and met several additional criteria: low toxicological risk, high potential efficacy, available industrial supply, and free of patent for cosmetic applications. Fifteen plants were screened. They included maritime pine, millet fiber, sugar-apple, cotton, linseed, soy, hemp, nut grass, millet seed, chervil, wheat, apple marc, rye and root of water lily. The results of the screening showed that only the rye (*Secale cereale*) raw material responded to the three biological markers and to the other criteria of raw material selection. Up to now, no other raw material having the same properties as the rye extract has been found. But, now that arabinoxylans has been identified as the active fraction of the rye extract, one can imagine finding another plant rich in arabinoxylans having the same effects as the rye and corresponding to the same concept.

The active ingredient was obtained from the rye seeds after controlled enzymatic hydrolysis. A unique purified rye extract[a] was composed of carbohydrates (92%), mineral ashes (4%) and proteins (3%). Quantification of carbohydrates was carried out by the assay of DuBois et al.[12] The carbohydrate analysis by GC/FID led to identification of glucose and arabinoxylan oligomers. The rye extract with this composition and approximately 10% arabinoxylans is the subject of the study reported here.

The phenolic compound ferrulic acid was used as a marker to study the rye extract in formula and to follow its stability after 6, 12 and 18 months at 42°C. The method used for these studies was HPLC (high performance liquid chromatography) analysis. The rye extract was also tested with in vitro methods in order to check its activity over time.

## Material and Methods

The rye extract was tested in vitro on normal human fibroblasts and aged

[a] *Coheliss (INCI: Water* (aqua) *(and)* Secale cereale *(rye) (seed extract). Coheliss is a registered trademark of Silab.*

human fibroblasts. The syntheses of alpha2beta1 integrins and vinculin were analyzed using quantitative polymerase chain reaction (PCR). The alpha-SMA expression was quantified by spectrofluorimetry and visualized using immunocytology.

***Synthesis of alpha2beta1 integrins and vinculin:*** An in vitro study was carried out by quantitative PCR on a pool (4 donors) of normal human fibroblasts, compared to a model of aged human fibroblasts. The aged human fibroblasts were obtained after 25 successive cell transfers. Cell aging was verified by visualizing a senescence marker[b].

Normal and aged human fibroblasts were inoculated in petri dishes 100 mm in diameter and incubated at 37°C for 48 h in an atmosphere of 5% $CO_2$. The cells were then grown for 24 h in the presence or absence of rye extract at 0.25% (v/v) or TGF-beta1[c] at 1 ng/mL used as reference molecule.

At the end of incubation, the cells were recovered and total RNA was extracted. The RNA was reverse-transcripted and the complementary DNA obtained was analyzed by quantitative PCR. Beta-actin mRNA, the internal standard, also was analyzed in parallel to the mRNA of alpha2beta1 integrins and vinculin. Quantification of fluorescence incorporation (SYBR Green) was measured[d] continuously. The analysis of cycle threshold (relative quantification) was carried out with software[e].

[b] *beta-Galactosidase, available as C-50030 from Sigma, USA*
[c] *T-7039 from Sigma, USA*

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***Expression of alpha-SMA:*** Two in vitro studies were carried out to evaluate the expression of alpha-SMA by normal human fibroblasts and aged human fibroblasts.

[d] *iCycler thermocycler, model MyiQ, Bio-Rad, USA*
[e] *Genex software, Bio-Rad*

In the first study, the expression of alpha-SMA was quantified by spectrofluorimetry. Normal and aged human fibroblasts were inoculated in 100 mm diameter petri dishes and incubated at 37°C for 48 h in an atmosphere of 5% $CO_2$. Then the cells were grown for 24 h in the presence or absence of rye extract at 0.25% and 0.50% (v/v) or TGF-beta1[c] at 1 ng/mL. This treatment was repeated once.

After four days of incubation with the products, fibroblasts were immunolabeled. The cells were recovered, permeabilized and incubated for 45 min at 4°C with an alpha-SMA monoclonal antibody[f] followed by the addition of a second antibody[g]. The level of alpha-SMA was quantified with a plate reader[h] by fluorescence excitation at 488 nm and emission at 530 nm.

The number of cells was quantified by the incorporation of a solution of propidium iodide[k] at 15 µg/mL. The cell count was estimated with a plate reader

[f] *A-2547 from Sigma, USA*
[g] *Murine Alexa Fluor-488-conjugated anti-IgG, A-11017 from Interchim, France*
[h] *Fluorolite 1000, Dynex, USA*
[k] *P-4170, Sigma, USA*

of fluorescence excitation at 365 nm and emission at 605 nm.

In the second study, the expression of alpha-SMA was visualized by immunocytology. Normal and aged human fibroblasts were inoculated on glass slides[m] in complete culture medium for 48 h. Then the cells were treated with the rye extract at 0.10% (v/v) diluted in complete culture medium and incubated at 37°C in an atmosphere of 5% $CO_2$ for 48 h. This treatment was repeated once.

After four days of treatment with the products, alpha-SMA were labeled by immunocytology. The slides were rinsed in phosphate-buffered saline (PBS) and fixed in a 4% (v/v) solution of paraformaldehyde[n] for 15 min. Then the slides were rinsed in PBS, permeabilized in a solution of saponin[p] at 0.50% (w/v) for 5 min and again rinsed in PBS buffer. Finally, the cells were incubated with a murine alpha-SMA monoclonal antibody[f] followed by the addition of a second antibody[q]. Visualization was realized with a microscope[r] coupled to an image analysis system[s].

***Biomechanical properties of the skin:*** An in vivo study of the skin's biomechanical properties was conducted on 20 healthy female volunteers aged 39–70 years (mean age 56 ± 8 years). Measurements of the face were made instrumentally[t] before and after 56 days of twice daily treatment with the rye extract formulated (**Formula 1**) at 4% in an emulsion vs. placebo.

Skin tone and the tensor effect were determined in order to monitor the biomechanical properties of the skin under the effect of the treatments. Skin tone was evaluated with the parameter X and tension was quantified with the parameters Uf and Ue. Uf is the viscoelastic component of the skin; if Uf

**Formula 1. Test emulsion**

| | |
|---|---|
| Isononyl isononanoate (Lanol 99, Seppic) | 5.0% wt/wt |
| Water (*aqua*) (and) *Secale cereale* (rye) seed extract (Coheliss, Silab) | 4.0 |
| Cetearyl alcohol/cetearyl glucoside (Montanov 68, Seppic) | 2.5 |
| Arachidyl alcohol (and) behenyl alcohol (and) arachidyl glucoside (Montanov 202, Seppic) | 2.0 |
| Butylene glycol | 2.0 |
| Methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben (and) phenoxyethanol (Phenonip, Clariant) | 0.7 |
| Sodium polyacrylate (and) C13-14 isoparaffin (and) *Paraffinum liquidum* (mineral) oil (and) polyacrylamide (and) polysorbate 85 (Sepigel 501, Seppic) | 0.3 |
| Water *(aqua)* | qs to 100.0 |

[m] *Labtek, USA*
[n] *F-1635, Sigma, USA*
[p] *S-4521, Sigma, France*
[q] *FITC-coupled murine anti-IgG, Alexa Fluor A488 from Molecular Probes, USA*
[r] *Olympus IX 70 microscope, Japan*
[s] *VisioLab 2000, Biocom, France*
[t] *SEM 575 Cutometer, Courage & Khazaka, Germany. Cutometer is a registered trademark of Courage & Khazaka.*

decreases, the skin is less extensible, thus more taut. Ue is the elastic component of the skin; if Ue decreases, the skin is less flexible, thus more taut. In this study the negative form of the parameter was used to obtain a positive measurement of the tone and tension.

***Antiwrinkle and smoothing properties:*** An in vivo study was conducted on 20 healthy female volunteers aged 39–70 years (mean age 56 ± 8 years). Silicone polymer replicas were made of the crow's-feet and the nasolabial fold before and after 56 days of twice daily treatment with the rye extract formulated (**Formula 1**) at 4% in an emulsion vs. placebo.

The antiwrinkle effect was analyzed by observing the replicas with a profilometer equipped with an image analyzer[u]. Three parameters were studied: the number, total surface and total length of wrinkles. The smoothing effect at the crow's-feet was analyzed by observing replicas with a profilometer equipped with an image analyzer[v]. Two parameters characteristic of skin surface relief were studied: the index of mean roughness (Ra) and that of maximal roughness (Rz).

## Results

***Synthesis of alpha2beta1 integrins and vinculin:*** The expression of mRNA of alpha2beta1 integrins and vinculin by untreated aged fibroblasts was reduced by 16% (**Figure 3**) and 19% (**Figure 4**), respectively, in comparison to untreated normal human fibroblasts. Tested at 0.25%, the rye extract increased the expression of mRNA of alpha2beta1 integrins and vinculin in normal fibroblasts by 20% and 28%, respectively, and also restored their normal levels of expression by aged human fibroblasts. The rye extract boosted the synthesis of alpha2beta1 integrins and vinculin, thereby favoring the formation of mechano-receptors and increasing adhesion between fibroblasts and the ECM, as well as the transmission of mechanical messages.

***Expression of alpha-SMA:*** In cultures of untreated aged human fibroblasts, alpha-SMA levels were reduced by 60% compared to untreated normal human fibroblasts (**Figure 5**). Tested at 0.50%, the rye extract produced a 62% increase in alpha-SMA expression by normal human fibroblasts and exhibited a dose-dependent tendency to restore alpha-SMA expression by aged human fibroblasts.

The expression of alpha-SMA was visualized by immunocytology (**Figure 6**).

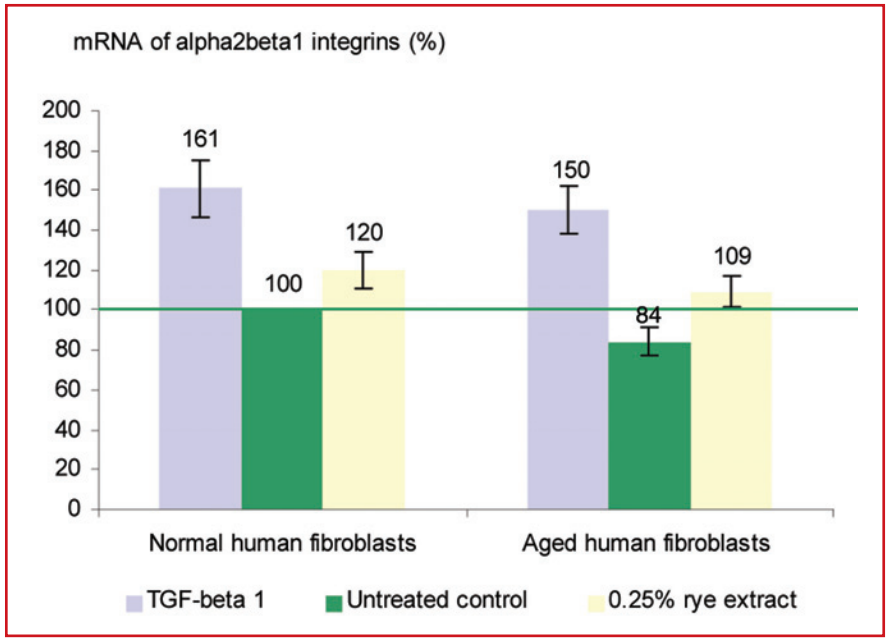


***Figure 3. Effect of the rye extract on the expression of alpha2beta1 integrins mRNA by normal and aged fibroblasts***

[u] *Quantirides 99, Monaderm, Monaco. Quantirides is a registered trademark of Monaderm.*
[v] *Quantilines, Monaderm*

These qualitative results were consistent with those obtained with the spectrofluorimetric assay: the capacity of aged human fibroblasts to express alpha-SMA fibers was reduced, but was stimulated after treatment of cells with the rye extract at 0.10%. The rye extract thus stimulates the contractile properties of fibroblasts that enable the skin to respond to a variety of mechanical stresses to which it is subjected on a daily basis.

***Biomechanical properties of the skin:*** After 56 days of twice daily applications and in comparison to the placebo, the rye extract significantly increased the parameter –X characteristic of skin tone by 19% ($P = 0.0279$). A study of the distribution of the results showed that 72% of the volunteers presented improved skin tone.

In addition, the rye extract also significantly improved parameters –Uf (16%, $P = 0.0136$) and –Ue (19%, $P = 0.0173$), representative of skin tension. This effect was observed in 78% of the volunteers. The rye extract thus renders skin tissue firmer.

***Antiwrinkle and smoothing properties:*** After 56 days of twice daily applications and in comparison to the placebo, the rye extract at 4% in an emulsion presented a significant antiwrinkle effect ($P < 0.05$) at both the crow's-feet and the nasolabial fold, respectively: a 13% and 23% decrease in the number of wrinkles, a 15% and 26% reduction of the total surface of wrinkles, and a 16% and 27% reduction of their length.

The replicas shown in **Figure 7** illustrate the skin surface of the crow's-feet area before and after a volunteer was treated with the rye extract. A study of distribution of the results showed that 67% and 78% of the volunteers presented a decrease in the total wrinkled surface at the crow's-feet and nasolabial fold, respectively. In addition, the rye extract also smoothed skin microrelief by significantly reducing parameters Ra (–7.8%, $P = 0.0071$) and Rz (–5.0%, $P = 0.0247$). The rye extract attenuated roughness of skin microrelief and thereby improved its surface properties.

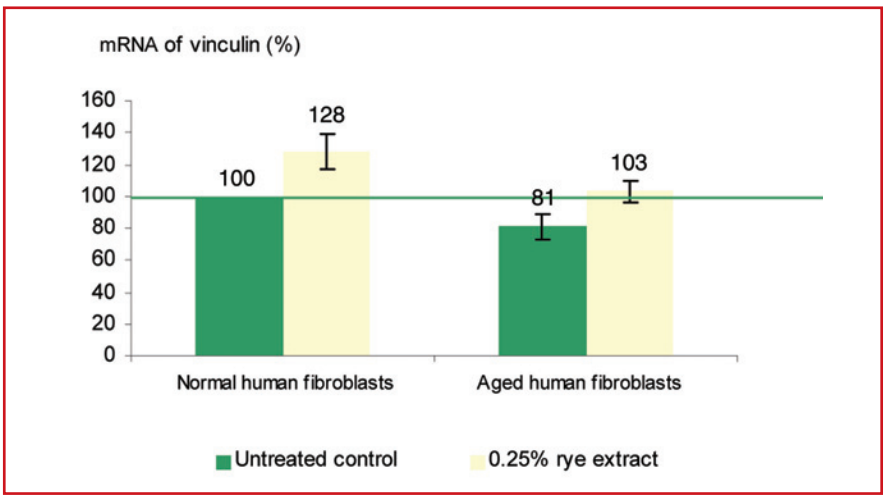


*Figure 4. Effect of the rye extract on the expression of vinculin mRNA by normal and aged fibroblasts*

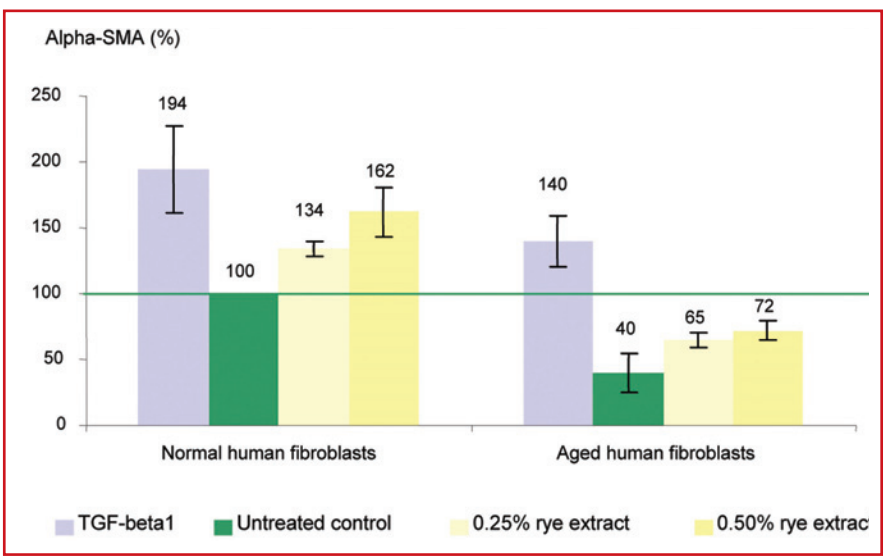


*Figure 5. Effect of the rye extract on the expression of alpha-SMA mRNA by normal and aged fibroblasts*

## Conclusion

The capacity of the skin to adapt to mechanical stresses, or cell mechanics, can be considered as a new approach to the fight against the signs of aging, especially those of chronobiological aging.

An active substance, rich in arabinoxylans and purified from rye seeds, was shown here in several studies to act as an internal tensor agent. The expression of proteins of the mechanical receptor (alpha2beta1 integrins and vinculin) and of alpha-SMA fibers by aged human fibroblasts was reduced in comparison to those of normal human fibroblasts. The biological properties of the rye extract restored the expression of alpha2beta1 integrins and vinculin to normal levels, and had a similar effect on the synthesis of alpha-SMA by aged human fibroblasts.

Tested directly on volunteers, the rye extract formulated at 4% in an emulsion firmed skin tissue as shown by the significant 19% increase in skin tone (-X) and increases of 16% and 19% in two skin tension parameters (–Uf and –Ue, respectively) after 56 days of twice daily applications.

The rye extract also significantly improved the surface properties of the skin. It attenuated wrinkles at the crow's-feet in 72% of the volunteers and at the nasolabial fold in 78% of the volunteers, and it smoothed skin microrelief by reductions of 7.8% and 5.0% in the indexes of mean roughness (Ra) and maximal roughness (Rz), respectively.

The rye extract boosts the natural skin equipment that participates in its mechanical resistance and restores its natural lifting properties.

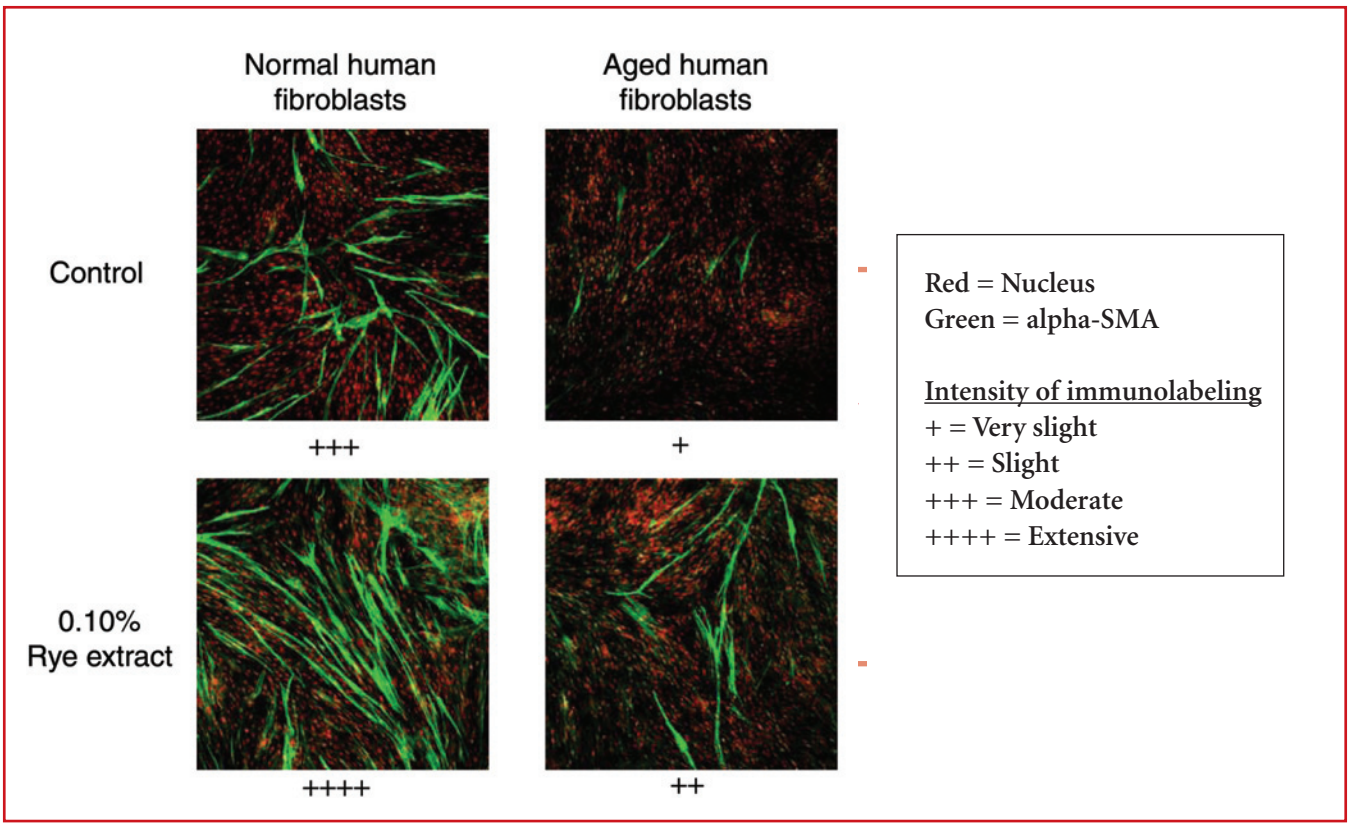


*Figure 6. Visualization of the effect of the rye extract on the expression of alpha-SMA by normal and aged fibroblasts*

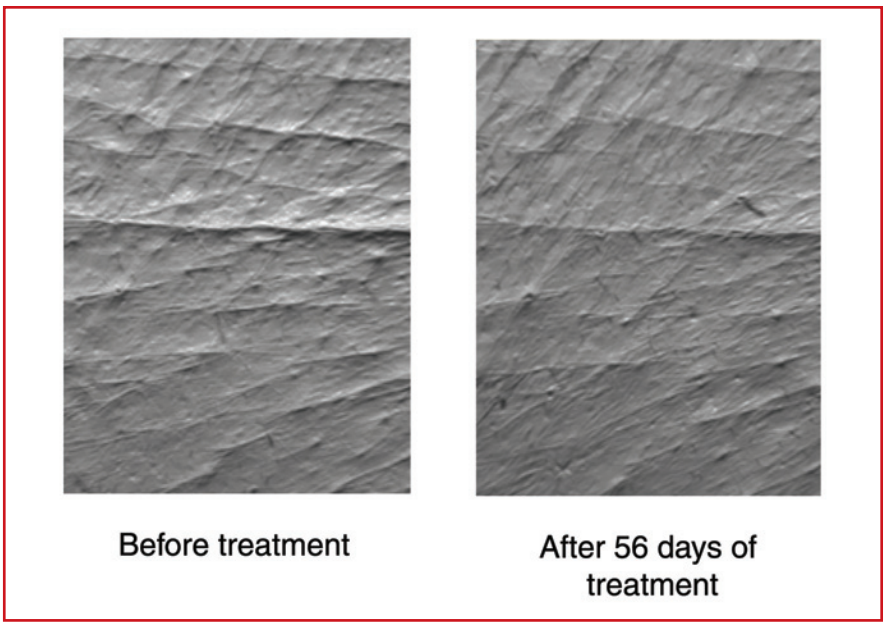


*Figure 7. Visualization of the antiwrinkle effect of the rye extract on the crow's-feet of a volunteer*



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