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Category	Title	Author
Newsletter	**Oligopeptides from *Hibiscus esculentus* seeds to smooth expression lines**	Isabelle Benoit, Louis Danoux, Véronique Gillon, Philippe Moussou, Gilles Pauly

Abstract

In its search for innovative approaches to reverse the visible signs of aging, the cosmetic dermatology industry has embraced a powerful molecule, capable of temporarily relieving facial expression lines: botulic toxin (Botox®* Cosmetic or Botulinum Toxin Type A). This neurotoxin inactivates the neuromuscular junction, thereby locally paralyzing muscle contraction. When injected at appropriate locations on the face, this toxin inhibits contraction of facial muscles, preventing the appearance and imprinting of "dynamic wrinkles" or "expression lines". This treatment offers an effective and immediate anti-wrinkle solution, yet hardly falls within the definition of a cosmetic product. Such a drastic procedure is at odds with trends in modern society, wherein consumers increasingly favor the use of natural, plant-derived ingredients. Furthermore, many consumers are squeamish about the prospect of having any substance, let alone a neurotoxin, injected in their faces. This apprehension is heightened by potential side effects; when applied at inappropriate levels or locations, botulic toxin injections can temporarily cause sagging eyelid muscles (ptosis). Moreover, the long-term impact of continued treatments with botulic toxin injections is not known.

Responding to market demand for mild, natural alternatives to botulic toxin, Laboratoires Sérobiologiques have developed Myoxinol® LS 9736**, a patented active ingredient for topical application, displaying anti-aging activity.

As its activity is not limited to relieving the mechanical formation of wrinkles, Myoxinol® offers more cosmetic anti-aging benefits than botulic toxin injections. The anti-free radical/antioxidant activity of Myoxinol® also slows extrinsic biological aging of cells and dermal macro-molecules, preserving skin's elasticity and delivering long term anti-aging benefits.

Introduction

There are two main categories of wrinkle formation mechanisms in skin: biological (aging of cells, oxidation or glycation of macromolecules and mechanical (i.e. when face muscles are involved). The cosmetics industry has been addressing biological parameters for a long time: antioxidant systems, cell metabolism boosters, anti-glycation actives - all aiming at preserving skin's elasticity, and protecting it

from chronological and environmental aging. The mechanical stresses involved in skin aging, e.g. facial muscle contraction, used to be treated in beauty salons via specific massage techniques. They only lately became the focus of more general attention from customers and the cosmetic dermatology industry. Facial muscles are responsible for the formation of dynamic wrinkles: horizontal and vertical frown lines across the forehead, crow's foot around the eye and naso-labial lines around the mouth.

As long as the biological aging mechanisms have not degraded the skin's elasticity, these lines are reversible and the skin recovers its smooth appearance when facial muscles relax. But when biological and mechanical factors work in concert, the skin loses its elasticity and its ability to return to its initial state after muscle contraction. The dynamic wrinkles become permanent, generating "expression" lines.

Botulic toxin injections offer a solution to diminish these mechanical stresses. The active substance in Botox®* Cosmetic injections is Botulinum Toxin Type A, a protein complex (MW ~ 150 kDa) produced by a strain of Clostridium botulinum, the Gram-positive bacteria responsible for botulism. Injected neurotoxin prevents facial muscle contraction through inhibition of acetylcholine release from the stimulated nerves at the neuro-muscular junction.

These injections require appropriate expertise during treatment and are perceived by many as risky because of the toxicity of the molecule. Thus, their routine administration for cosmetic purposes is not authorized by all local regulations. Moreover, even where the procedure is approved some customers remain deterred by the severity of the procedure and its potential side effects.

For these reasons, there is growing market interest in alternatives to botulic toxin injections. Not mentioning the needle-shy ones, many consumers seek a topical cosmetic alternative, preferably one offering a mechanism of action that addresses wrinkles formed by facial muscles. Additionally, among those who undergo botulic toxin treatments, many seek cosmetic products that will extend the time between treatments.

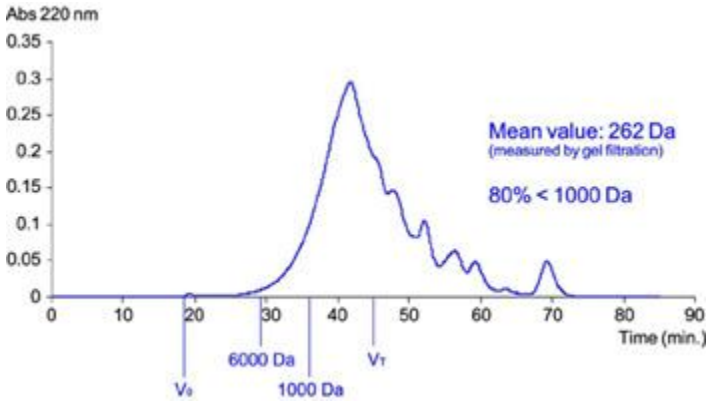
Myoxinol®, a natural complex of oligopeptides obtained from the seeds of Hibiscus esculentus, offers more than just wrinkle reduction. In addition to inhibition of muscle cell contraction, demonstrated with an innovative in vitro model, Myoxinol® also protects cells and dermal macromolecules from oxidative stress. Myoxinol® is a comprehensive, patented anti-aging active, suitable for a gentle topical treatment, effective against both mechanical and biological modes of wrinkle formation.

Material and Methods

Hibiscus esculentus (okra) is a tropical plant native to Central Africa, India, Malaysia and the Philippines. A member of the mallow family, this annual plant has been cultivated as a food source for centuries. Its long, green, mucilaginous seedpods are commonly used in traditional recipes. The high nutritional value of Hibiscus seeds has recently been confirmed scientifically. Flour and milk prepared from these seeds contain lipids and proteins having a composition close to that of the casein fraction of milk. Hibiscus seeds are hence recommended as food supplement in Africa.

Myoxinol® is obtained by biotransformation of native proteins from Hibiscus esculentus seeds. This complex is predominantly composed of low molecular weight oligopeptides (Figure 1), (data obtained by gel filtration chromatography: average molecular weight 262 Da; 80% of oligopeptides with molecular weight < 1 000 Da), allowing optimal bioavailability.

Figure 1: Molecular weight distribution of Myoxinol® (Gel filtration on Superdex Peptid Column).



Myoxinol® was recently found to inhibit muscle contraction in vitro. This has been demonstrated by an innovative test performed with cultured cells mimicking the hyperactivity of facial muscles. The system comprises a co-culture of muscle cells with neurons that spontaneously displays rhythmic contractions. The inhibitory effect of Myoxinol® was evaluated as a decrease in the frequency of contractions of the co-culture matrix.

This confirmation of a muscle-relaxing mechanism was complemented by an evaluation of the anti-free radical capacity of Myoxinol® in a battery of in vitro and in tubo tests, covering primary free radicals and secondary reactive oxygen species (ROS). The anti-wrinkle activity of topically-applied Myoxinol® was confirmed in a clinical study.

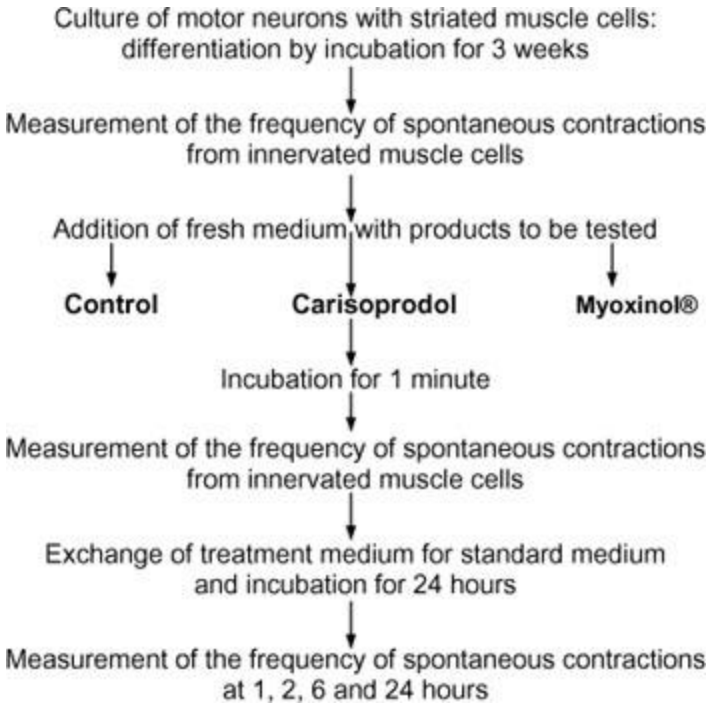
Protocols and Results

1. Efficacy test on contraction of innervated muscle cells

1.1 Protocol (Figure 2)

Motor neurons were seeded on striated muscle cells in growth medium and incubated for approximately 3 weeks at 37°C in a 5% CO_2 atmosphere. The frequency of spontaneous contractions was recorded during a 30 s interval immediately before each treatment. The co-culture was treated by changing to fresh medium containing a range of concentrations of products to be tested. After 1 minute of contact with each test medium, the frequency of contractions was measured during a 30 s interval. Then the test medium was removed and the co-cultured cells were rinsed and replenished with standard medium for an incubation period of 24 h. The frequency of contractions was evaluated at 1, 2, 6 and 24 h after the initial treatment. The benchmark compound used in this study was Carisoprodol (N-isopropyl-2-methyl-2-propyl-1,3-propanediol dicarbamate), a prescription drug that temporarily inhibits contraction of striated muscle induced by a nervous stimulus.

Figure 2: Protocol of the test on striated muscle cells innervated by motor neurons.



1.2 Mechanism of action

Carisoprodol (1) is a commonly used muscle relaxant and analgesic agent which is metabolized in cells into meprobamate. The pharmacological mechanism of Carisoprodol and its metabolite meprobamate are not fully understood, but it is believed they work by blocking nerve impulses. In the central nervous system, meprobamate potentiates GABA (µ-amino-butyric acid) response and therefore meprobamate has been used as a strong sedative tranquilizing agent, but with addictive properties.

1.3 Results

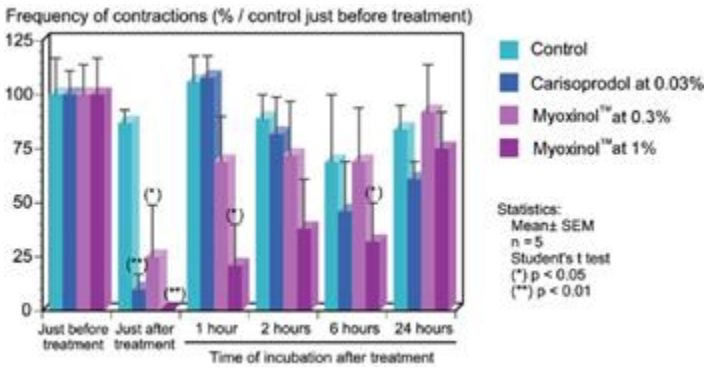
Test of cell viability

As a prelude to in vitro studies of muscle relaxation by Myoxinol®, the potential for cytotoxicity was examined. Tested concentrations of Carisoprodol and Myoxinol® have not shown any significant cytotoxic effects on cultured human fibroblasts after an incubation for 3 days.

Figure 3a: Contraction frequency measurements

Number of contractions (% / control just before treatment)	Dose of use (%) (w/v)	Time of incubation after treatment					
		Before Treatment	0 minute	1 hour	2 hours	6 hours	24 hours
Control	/	100 ± 17	87 ± 6	106 ± 12	89 ± 11	69 ± 31	84 ± 11
Carisoprodol	0.03	100 ± 11	10 ± 7 (**)	108 ± 10	82 ± 17	46 ± 23	61 ± 18
Myoxinol®	0.3	100 ± 14	25 ± 24 (*)	69 ± 21	72 ± 25	69 ± 25	92 ± 22
	1	100 ± 17	0 ± 0 (**)	21 ± 19 (*)	38 ± 23	32 ± 18 (*)	75 ± 17

Figure 3b: Effect on the frequency of contraction of the innervated muscle cells as a function of the incubation time.



Innervated muscle cells display a frequency of around 130 contractions per minute. Carisoprodol at 0.03% has shown a strong immediate inhibition which is reversed within 1 hour. Myoxinol® at 0.3% and 1% has shown a significant and dose-dependent inhibition which is completely reversed at 24 h, providing evidence of the harmlessness of Myoxinol® (further guaranteed by complete toxicological file).

1.4 Conclusion

Myoxinol® at 0.3% and 1% has shown a good, reversible potential to inhibit muscle contraction in vitro. This efficacy has been demonstrated through an innovative model relying on genuine muscle cells. Muscle cells have fully recovered their natural functioning 24 hours after incubation. This eases concerns about possible harmful side effects of topical treatment with Myoxinol®. It can hence be concluded that Myoxinol® will counteract the mechanical parameters responsible for the apparition of expression lines.

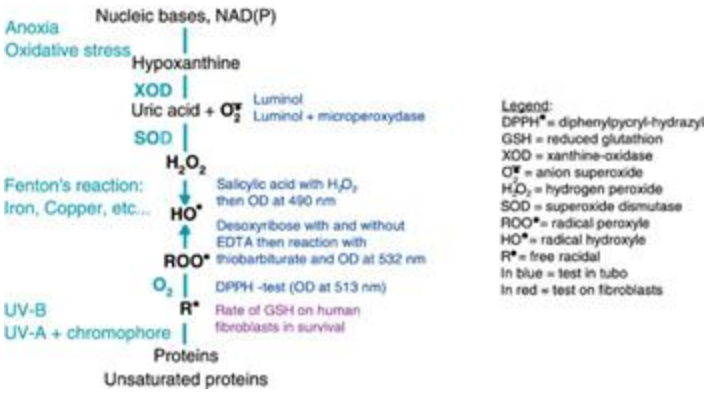
2. Cell protection against free radicals

2.1 Aim

Free radicals (FR) are reactive molecular species possessing one or more unpaired electrons. They are formed in the atmosphere by UV-induced cleavage of chemical bonds in otherwise stable molecules. Free radical concentrations are especially high in severely polluted environments, e.g. photochemical smog. Free radical toxicity, exacerbated by oxygen through the formation of reactive oxygen species (ROS), contributes significantly to extrinsic aging. Free radicals and ROS are especially detrimental to the functionality of macromolecules responsible for the skin's elastic properties. When these macromolecule can no longer perform their role in supporting skin tissue, the skin loses its resiliency. Anti-free radical (AFR) activity is evaluated by *in tubo* chemical and biochemical tests. The chemical tests allow the quantification of free radicals (e.g. DPPH•), including a very toxic form of ROS, the hydroxyl radical (HO•). The biochemical tests permit quantification of another form of ROS, the superoxide anion ($O_2^{-\bullet}$). An in vitro test is used to quantify a product's ability to boost the natural defense of cells against FR by preservation of reduced glutathione (GSH).

2.2 Protocol (Figure 4)

Figure 4: Protocol of in vitro test on cell protection against free radicals.



2.2.1 Chemical tests (Figure 5)

Anti DPPH• test:

DPPH• (diphenylpicryl-hydrazyl) is a stable free radical that forms a purple solution which becomes transparent when a FR scavenger is added. The extent of FR scavenging is evaluated by recording the optical density (OD) at 513 nm. Ascorbic acid was tested as benchmark (2).

Anti HO• test

With Salicylic acid: Fe2+ in presence of EDTA and H_2O_2 forms HO•, that reacts with salicylic acid to form a red solution. Anti-free radical substances scavenge HO• radicals, reducing the formation of this red compound (3).

Fenton Reaction: method with and without EDTA. HO• , formed by H_2O_2 in presence of Fe2+ and EDTA, oxidizes desoxyribose (a component of DNA), then a pink compound is formed by condensation of thiobarbiturate with oxidized form of desoxyribose. The optical density at 532 nm corresponds to the level of oxidized desoxyribose. An anti-free radical substance reacts with these HO• radicals and reduces the formation of this pink compound. O-phenantroline was tested as reference compound.

2.2.2 Biochemical test

Anti O_2–• activity:

Luminol method: Xanthine oxidase (XOD) was incubated with its substrate hypoxanthine and the ingredient to be tested. After addition of luminol, the rate of released O_2–• can be revealed by recording the luminescence. Anti-free radical substances react with these O_2–• radicals, reducing luminescence intensity (4).

Luminol + microperoxidase method: H_2O_2 and O_2–• react with microperoxidase to form singlet oxygen (O21), a ROS which degrades luminol. Anti-free radical substances react with O_2–•, H_2O_2 or $O_2$1, reducing the luminescence intensity (5).

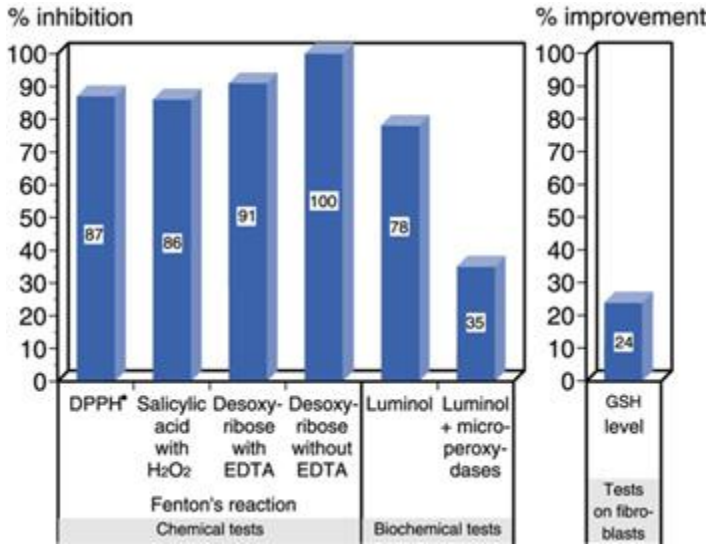
2.2.3 GSH test, on human MRC5 fibroblasts in survival

Glutathione is an endogenous tripeptide (glutamate-L-cysteine-glycine) that, in its reduced state (GSH), protects cell membranes and the stratum corneum, particularly via anti-peroxide potential (H_2O_2 and lipoperoxides). A substance which reserves glutathione in its reduced state will thereby support the cell's natural defense system versus oxidative stress (6).

In the GSH assay, MRC5 fibroblasts were seeded in growth medium (3 days) and grown to saturation. The culture was then washed with PBS and subsequently treated by addition of a test product for 3 days at 37°C. The culture was then washed and incubated with orthophtaldehyde for 15 minutes and its fluorescence intensity measured (7).

2.3 Results (Figure 5)

Figure 5: Results on the anti-free radical screening: chemical, biochemical and on in vitro human MRC5 fibroblasts.



2.4 Conclusion

Myoxinol® presents a spectrum of anti-FR activity, covering the initial radical forms as well as the induced reactive forms of oxygen. Myoxinol® has also supported natural defenses versus peroxides by increasing the concentration of reduced glutathione in the human fibroblasts. Therefore, Myoxinol® protects the skin versus biological oxidative aging. This activity strengthens Myoxinol's® positioning as a comprehensive anti-aging active, efficient on both mechanical and biological parameters of wrinkle formation.

3. Anti-wrinkle activity (clinical)

3.1 Protocol (Figure 6)

The anti-wrinkle efficacy of Myoxinol® was quantified in a double-blind clinical study, with placebo control and randomization, on 12 volunteers presenting wrinkles, especially expression lines, at the crow's foot area. Half-face treatments were performed twice daily, morning and evening. Use of other skin care and UV protection products was discontinued 8 days before the study. The visibility of wrinkles was determined quantitatively by numerical image analysis of photographs of illuminated negative print realized individually on the two crow's foot before and after 3 weeks of treatment. The wrinkle's depth was correlated with the average surface area of the shadow generated on the negative print and measured by a standardized optical system.

Figure 6: Protocol of the clinical test on human crow's foot.

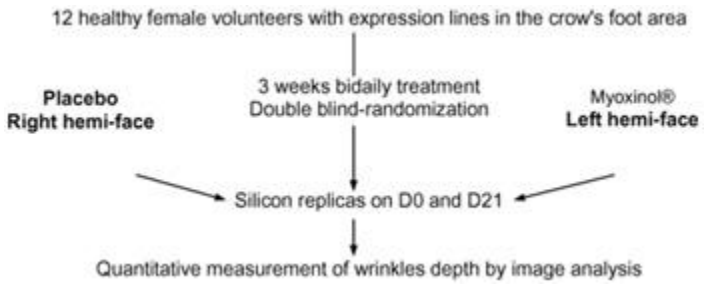
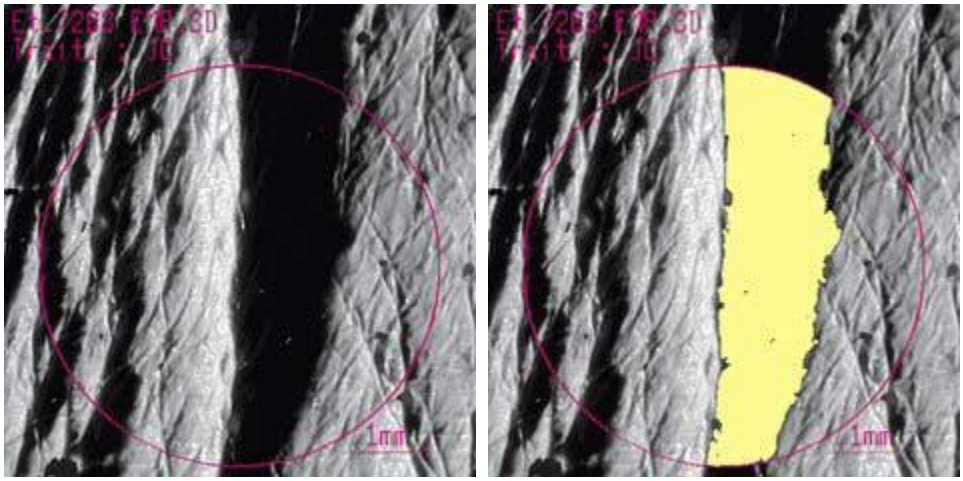


Figure 7: Anti-wrinkle activity of Myoxinol®, measured by image analysis.

Before treatment



After 3 weeks of treatment

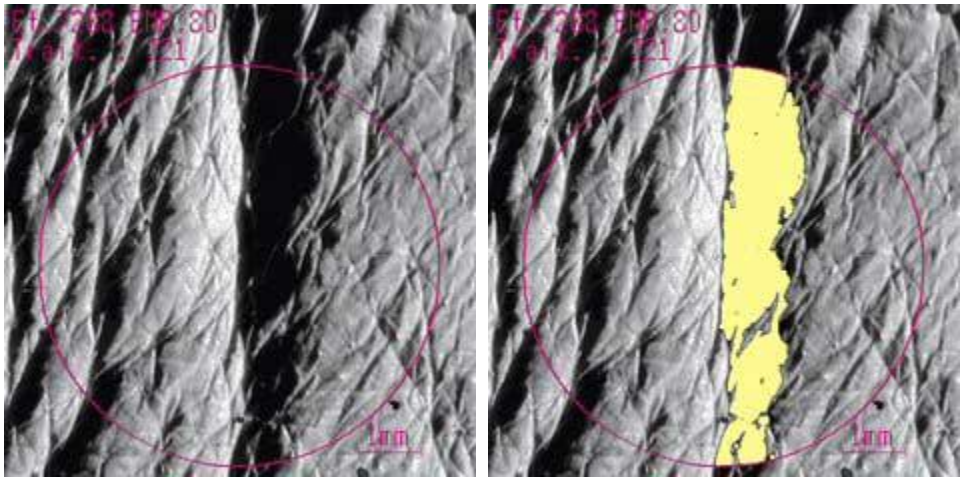
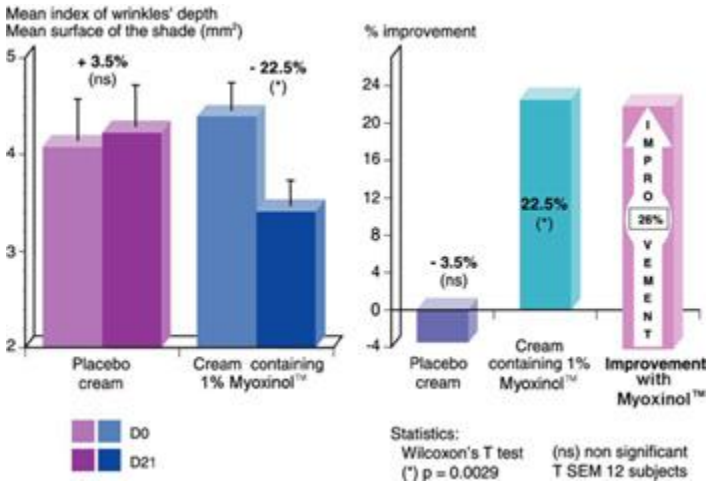


Figure 8: Anti-wrinkle activity of Myoxinol®.



4. Conclusion

The anti-wrinkle activity of Myoxinol® is demonstrated by these clinical results: a 26% reduction of wrinkle depth (mean value versus placebo) after a 3-week treatment by topical applications of an emulsion containing 1% Myoxinol®. This further confirms the bioavailabilty of Myoxinol®, which was already suggested by its molecular weight distribution (predominance of low molecular weight oligopeptides).

Discussion

Myoxinol® can be used to support a ”Botox®-like” marketing story for topical products, as its ability to reduce muscle contraction has been demonstrated in vitro. It should be emphasized that the test has been performed on genuine muscle and neuronal cells, substantiating the “muscle-relaxing” claim.

The activity of Myoxinol® actually surpasses a “Botox®-like” positioning, as its efficacy has also been demonstrated in more “traditional”, yet effective, anti-aging mechanisms of action. The two modes of activity - mechanical and biological - complement each other. Indeed, protection of skin’s elasticity against oxidative aging is a key factor when one wants to maintain the skin’s ability to recover its initial stage upon muscle relaxation.

The combination of both mechanisms of action makes of Myoxinol® a pioneer, patented anti-wrinkle active. Myoxinol®’s ability to smooth expression lines has been demonstrated by an in vivo test after only 3 weeks of treatment.

Conclusions

Modern society seems torn between two paradoxical trends: the relentless drive toward sophisticated technological solutions to every problem and an abiding preference for natural products. This dilemma is clearly illustrated in cosmetics: women remain trapped between the desire to delay as long as possible the visible signs of aging, and the apprehension generated by drastic cosmetic or surgical solutions.

The “Botox® phenomenon” is a good example of this evolution; although considered a mild alternative to cosmetic surgery, and widely appreciated for its immediate effects, its use and potential side effects generate fears.

Myoxinol® (INCI name: Hydrolyzed *Hibiscus Esculentus* Extract (and) Dextrin) escapes this paradox, offering an advanced, muscle-relaxing mechanism of action, while maintaining the safety and positive marketing image of a vegetable-derived active ingredient. Beyond this anti-wrinkle activity, Myoxinol®

helps protect the skin against environmental factors that accelerate aging. With its broad spectrum of anti-aging activity, Myoxinol® offers a gentle, natural alternative to invasive cosmetic dermatological procedures.

* Botox® is a registered trademark of Allergan Inc.
** Myoxinol® LS 9736 - INCI Name: Hydrolyzed Hibiscus Esculentus Extract (and) Dextrin is a registered trademark of Laboratoires Sérobiologiques-Cognis, France.

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