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Contents

Editorial

Can the pandemic by COVID-19 (SARS-CoV-2 infection) increase the number of adverse effects after the use of dermal fillers?

On January 7th, 2020, the appearance of a new coronavirus (CoV) was officially reported in Wuhan (China). At the time, no one could have suspected the chain of events that, with unusual speed, would lead to the greatest pandemic affecting the world population today.

Medical literature on COVID-19 is currently overwhelming, with new findings being published every day. However, we are far from knowing all the intricate details about its mechanism of action, its physiopathology, the response it elicits on different subjects or even its symptoms. Questions are accumulating and we still have a lot of work ahead of us to learn about and fight this virus.

We do agree that it spreads easily, that it is not just a lung disease, and that it causes significant changes in the immune system.

We also know that there are many asymptomatic carriers and people who have suffered mild, and even moderate, forms of COVID-19, whose diagnosis could not be confirmed by the different tests available.

In Spain, as well as in other countries, the severity of the pandemic called for a mandatory confinement of the general population and the declaration of a state of emergency, which entailed the closure of Aesthetic Medicine clinics from mid-March until mid-May.

In general, those measures intended to ensure the safety of the medical body and patients have been recorded in a protocol.

However, we are solely responsible for the reevaluation of our actions with regard to potential risks that some of our therapies may entail.

In recent days, we have witnessed the increasingly number of warnings issued by several sources about possible complications that we will have to deal with in our daily practice. Despite that an analysis of said sources exceeds the aim of this brief text, a few are mentioned below:

- The *Joint Council of Cosmetic Practitioners* (JCCP) from the United Kingdom has proposed a recommendation guideline that states the following: "There is increasing evidence that dermal fillers given in the presence of any viral infection can increase the risk of delayed hypersensitivity reactions."
- The publication of a review in *J Cosmet Dermatol* (2020; 00:1-4): Aesthetic Dermatology Procedures in Coronavirus Days highlights the following: i) permanent filler materials and some resorbable ones may cause chronic inflammation; ii) in comparison, reactions are minor (in principle) when the material used is hyaluronic acid; iii) there are also late hypersensitivity reactions to hyaluronic acid; iv) viruses may activate cytokines and T cells, and promote a proinflammatory state, therefore they recommend: v) to return to antibiotic empirical treatment (macrolides or tetracyclines); vi) use needles with less caliber; and vii) avoid high-risk areas.

In order for this to be reflected in patients' medical history, their documentation must be duly adjusted and this new information must be included in the informed consent provided to the patient. The professional, for his/her part, must consider this possibility in terms of the care that patients may require after the performed procedure.

Reflections by Paloma Tejero, MD, PhD

In my personal experience, because of my doctoral thesis on adverse effects of filler materials in 2013 and because I was part of the SEME committee of adverse effects, my colleagues usually referred patients to me or consult with me on different issues regarding filling materials. From May to July 2020, I have received several reports of exacerbated inflammatory responses after implant placement. Some have called my attention, particularly: i) non-permanent fillers in the perioral area; and ii) two patients with granulomatous abscess of permanent fillers that had been inactive for 14 and 7 years, respectively. All patients had negative serology results, although they reported having been near patients with COVID. However, there are also studies that support the disappearance of antibodies after two or three months of exposure.

In the nearby future, we will be able to weigh on the usefulness of these events considering several factors, which will prevent biased observations: i) accept that AE reporting has been very low (or non-existent) during the months of confinement, and measure it; ii) assess and compare data against reported AEs between March and July of 2019; and iii) assess the frequency with which permanent fillers have AEs within five years of their implantation.

In a word: we currently have more questions than conclusions, but I believe it is important to be alert, try to minimize risks and conduct prospective studies that allow us to learn about the possible interaction between COVID-19 and our practice.

Reflections by Hernán Pinto, MD, PhD

As it happens with any other topic that becomes fashionable, words fly. However, this is a fashion that has been imposed by the circumstances we are living in. And each one of us must do whatever we can to improve our own lives and safety, as well as those of our families, friends, professional colleagues and patients.

Every day we learn more about this virus and COVID-19. But, as usual, a serious search for knowledge gets us answers that, in turn, raise more questions. And, unfortunately, the relationship is not linear: for each answer we get, several new questions emerge. That is, each day we know more than the day before but, at the same time, what we have left to learn also increases. The more we study, the more we have left to study. It is normal.

The protocolization of Aesthetic Medicine practices surrounding all the implications that this virus may have is now a necessity. However, the evidence we have is dissimilar and contradictory, both in terms of quality and conclusions. In a word, we don't know what it is going on. The creation of evidence from and for our collective has become fundamental because dermal fillers represent a high percentage of aesthetic-medical practice. That is why the Spanish Society of Aesthetic Medicine (SEME) will create a commission to study the relationship between Aesthetic Medicine

and the coronavirus (COVID-19), which will allow us to combine our efforts and, among other things, sponsor scientific evidence-based studies nationwide in order to ensure patients' safety.

Hernán Pinto
Main Handling Editor

Paloma Tejero
Spanish Society of Aesthetic Medicine

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Newspaper article - online	Pollack A. FDA approves new cystic fibrosis drug. *New York Times.* January 31, 2012. http://www.nytimes.com/2012/02/01/business/fda-approves-cystic-fibrosis-drug.html?ref=health Accessed February 1, 2012.
Websites	Outbreak notice: Cholera in Haiti. Centers for Disease Control and Prevention Web site. https://www.cdc.gov Published October 22, 2010. Updated January 9, 2012. Accessed February 1, 2012.
Entire book - in print	Modlin J, Jenkins P. *Decision Analysis in Planning for a Polio Outbreak in the United States.* San Francisco, CA: Pediatric Academic Societies; 2004.
Book chapter - in print	Solensky R. Drug allergy: desensitization and treatment of reactions to antibiotics and aspirin. In: Lockey P, ed. *Allergens and Allergen Immunotherapy.* 3rd ed. New York, NY: Marcel Dekker; 2004:585-606.

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Example Article

1. Zoellner J, Krzeski E, Harden S, Cook E, Allen K, Estabrooks PA. Qualitative application of the theory of planned behavior to understand beverage consumption behaviors among adults. J Acad Nutr Diet. 2012;112(11):1774-1784. doi: 10.1016/j.jand.2012.06.368.

In-Text Citation Example

LARGE INCREASES IN AMERICANS' CONSUMPTION OF sugar-sweetened beverages (SSB) have been a topic of concern. Between 1977 and 2002, the intake of "caloric" beverages doubled in the United States, with most recent data showing that children and adults in the United States consume about 172 and 175 kcal daily, respectively, from SSB,[1] it is estimated that SSB account for about 10% of total energy intake in adults[2,3]. High intake of SSB has....

References Section Example

References

1. Duffey KJ. Popkin BM. Shifts in patterns and consumptions of beverages between 1965 and 2002. *Obesity.* 2007:15(11):2739-2747.
2. Nielsen SJ. Popkin BM. Changes in beverage intake between 1977 and 2001. *Am J Prev Med.* 2004;27(3):205-210.
3. Drewnowski A. Bellisle F. Liquid calories, sugar, and body weight. *Am J Clin Nutr.* 2007;85(3):651-661.

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aesthetic medicine

Original Article

Observational, prospective, randomized and controlled in-subject study on the safety and efficacy of Evryàl Strong injectable gel compared to Teosyal Global action injectable gel for the correction of moderate to severe nasolabial wrinkles

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Abstract

Introduction: this single center, double-blind randomized controlled study compares two hyaluronic acid fillers, Evryàl Strong and Teosyal Global Action in the treatment of nasolabial wrinkles.
Materials and Methods: each subject, with moderate or severe bilateral nasolabial wrinkles, was treated with both products, one was applied on the right side and the other on the left side. The quantity of product injected and any general or local reactions (erythema, edema or pain) were recorded and reassessed at 1, 3 and 6 months and then monthly until complete products absorption. The Wrinkle Severity Rating Scale (WSRS) and the Global Aesthetic Improvement Scale (GAIS) were used for assessment, as well as an ultrasound (US) measurement of the skin thickness.
Results: complete data were available for 15 subjects. At 1, 3 and 6 months both products showed improvement in the WSRS and GAIS score in the areas treated compared to pre-treatment assessments, although no significant differences were observed between them. No significant differences were observed on skin thickness and persistence among the two products. 2 subjects showed permanence of both products for longer than six months of the expected follow-up (for 7 and 8 months respectively). No significant differences were observed with regards to the subject's satisfaction and adverse events.
Conclusions: both products achieve long-term and similar permanence in correction of moderate and severe wrinkles, and showed a high safety profile and a high degree of subject and physician satisfaction.

Keywords

Hyaluronic dermal fillers, nasolabial wrinkles

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Introduction

Dermal fillers are very useful for wrinkle correction, including Naso Labial Folds (NLFs). Dermal filler treatments provide good aesthetic results with minimal invasiveness. Hyaluronic Acid (HA) dermal fillers are easy to administer, offer predictable effectiveness, a good safety profile, and quick recovery[1-4].
HA is a glycosaminoglycan, a large hydrophilic molecule that ensures skin hydration, turgor and flexibility[5]. It is degraded by a family of enzymes, named hyaluronidases. Various crosslinking techniques have been developed to prevent its rapid degradation and provide long-term treatment effects[6,7].
Crosslinking technology, HA concentration, the uniformity and size of particles affect the viscoelastic properties of filler and its clinical effect[8,9].
Hyaluronic acid soft tissue fillers include a wide range of products with unique characteristics[10].
This clinical investigation was undertaken to assess the safety and efficacy of Evryàl Strong injectable gel compared to Teosyal Global Action injectable gel in the correction of moderate to severe nasolabial wrinkles.

Materials and Methods

Commercially available Evryal Strong (Apharm s.r.l. Italy) and Teosyal Global Action (Teoxane SA) were used for this study.

Subjects and Clinical Investigation

This prospective observational, randomized, controlled in-subject and post-market study was conducted between 12th February 2019 and 12th November 2019, in accordance with principles set forth in the "Guidelines on clinical investigation: a guide for manufacturers and notified bodies - guidelines on medical devices" of the European Commission[11].
Both male and female subjects aged between 18 and 75 years, with 2 visible and approximately symmetrical NLFs and a score of 3 or 4 on the Wrinkle Severity Rating Scale (WSRS: 1 = absent, 2 = minor, 3 = moderate, 4 = severe, 5 = extreme)[12,13] were enrolled and agreed to return for periodic checks. Subjects were free of diseases and willing to abstain from any aesthetic or surgical procedures in the treatment area for the duration of the clinical investigation.
Subjects were excluded from participation for any of the following reasons: pregnancy, lactation, planned pregnancy or unwillingness to use contraception at any time during the investigation (for women of childbearing potential only); allergic reaction or hypersensitivity to HA and/or lidocaine; any corrective procedures performed or planned in the nasolabial region (e.g., silicone implants, permanent fillers); infectious, inflammatory lesions in the nasolabial region; cutaneous lesions in the treatment area; human immune deficiency virus-positive; recurrent herpes simplex virus 1; tendency to hypertrophic scars, and/or pigmentation disorders; any autoimmune or connective tissue disease, or current treatment with immune therapy; diabetes mellitus or uncontrolled systemic diseases and the use of anticoagulant, antiplatelet or thrombolytic medication. Written informed consent was obtained before the administration of treatment and subjects were medically examined, as was documentation of their medical history and current medication records. Facial frontal view, left oblique and right oblique photographs were taken on day 0 (before treatment) and at all post-treatment visits.

Treatments

NLFs were treated into the mid to deep dermis using a prefilled syringe and a 27G½″ disposable needle with both products; one was administered on the right side and the other on the left side. The injection technique (retrograde) and the injected volume were chosen at the investigator's discretion, according to characteristics of the defect under correction. Subjects received the initial treatment for both NLFs on day 0, with an optional touch-up treatment on day 30 if the investigator was not satisfied with the result of the first treatment. Subjects were followed up for 6 months or until complete product reabsorption, evaluated by ultrasound.

Assessments

Immediately after injection and 15 minutes later on day 0, subjects were asked to quantify the pain associated with the procedure, on a semi-quantitative numeric rating scale ranging from 0 (no pain) to 10 (worst imaginable pain).
The severity of NLFs was graded on the WSRS by a blinded investigator on day 0 (before treatment), and at every follow-up visit. The WSRS ranges from none/minimal (Grade 0) to extreme (Grade 4).
The aesthetic improvement of the subject's appearance after NLF correction was assessed by the blinded investigator and by self evaluation at 1 month, 3 months and 6 months, comparing the result for each NLF with photographs of pre-treatment appearance using the 5-point Global Aesthetic Improvement Scale (GAIS). The GAIS ranges from "very much improved" (score = 1) to "worse" (score = 5). At each visit the investigator carried out an aesthetic evaluation and subjects rated their satisfaction with treatment on a 5-point scale ranging from "very unsatisfied" to "very satisfied". The safety and tolerability of the treatment was assessed based on the spontaneous reporting of adverse events (AEs) by the subjects, clinical examinations, the asking of non-leading verbal questions about the subjects' general well-being by the investigators, who also examined the treatment area for injection site reactions, at each scheduled visit. The ultrasound examination of treated areas (nasolabial folds) was performed by a blinded investigator using a Merlin 1101 ultrasound scanner (BKi Medical Corporation, Tokyo, Japan), with a high-resolution probe for small parts and the interposition of an ultrasound gel (Aquasonic gel 100, Parker Laboratory, Fairfield, NJ, USA). Before the first filler treatment and at each follow- up visit, the probe was directed bilaterally to each nasolabial fold, to measure upper mid and deep-dermal thickness. The increase in skin measurement thickness after filler infiltration, corresponding to wrinkle flattening, was evaluated during the examination. All scan images were acquired and saved. The sonoCT software program was used to take measurements of the

filler and soft tissues.

Measures and Endpoints

The primary efficacy endpoints were average change compared to the baseline (day 0) in the WSRS of NLFs at 3 months, as evaluated by the blinded investigator, and the proportion of subjects with a WSRS score reduced by ≥1 point compared to the baseline at 3 months. The secondary endpoints included the average change in WSRS grade and the proportion of subjects with improvement at all visits, as evaluated by a blinded investigator and by means of self-evaluation; improvement was measured according to GAIS score, subject satisfaction and pain rating.

Statistical Analysis

All primary and secondary endpoints were analyzed descriptively. For the change in the average grade of NLF severity based on the WSRS from baseline to 3 months, 95% confidence intervals of the mean were calculated. Responder analyses based on the proportion of subjects achieving a ≥1 grade or 2 grade improvement from baseline were performed. 15 subjects received treatment and were included in the safety analyses. 14 subjects completed all visits until the end of the investigation at week 36 and were included in all efficacy analyses.

Results

15 subjects were enrolled and each received the injection with both products, one on the right side and the other on the left side to both NLFs. All subjects had NLF severity of 3 (moderate) to 4 (severe) according to the WSRS, at baseline. 15 women were enrolled. All subjects were 39 to 63 years old with a median age of 53.9 years.1 subject was lost in follow up, the remaining 14 subjects completed all visits until the end of the investigation at week 36.

Dosing and Administration

At baseline (day 0), 15 subjects were injected. The median-injected volume was 0.75 mL to the right and 0.75 mL to the left NLF, so the amount of hyaluronic acid injected in NLFs was the same. The retrograde injection technique was used in 100% of subjects. At month 1, the investigator judged the initial treatment to be complete for all subjects.

Efficacy

NLF Severity Based On the WSRS

A blinded investigator assessed the proportion of subjects with an improvement of at least 1 point on the WSRS. At 1 month, all 15 evaluated subjects showed a bilateral improvement in NLF severity of at least 1 point.

Aesthetic Improvement Assessed By GAIS

All 15 evaluated subjects showed a bilateral aesthetic improvement in appearance at 1 month, 3 and 6 months. At 1 month, 14 subjects (93.3%) were “very satisfied” or “satisfied” with the treatment, 1 (6.6%) subject was “very unsatisfied”. This high degree of satisfaction with the treatment outcome was noted at 1 month and consistently maintained until the end of the investigation.

Safety

The treatment was generally safe and well tolerated. In total 5 Adverse Effects (AEs) were reported by 15 subjects. All of these AEs were related to the procedure and classified as adverse device effects (ADEs), with no event being related to the investigational medical device. All ADEs were mild or moderate temporary effects that were localized to the injection site (*Table 1*). Nearly all ADEs were resolved within 14 days post-onset. 1 subject experienced serious AEs, a metastatic ovarian cancer, which was not related to the investigational medical device or the procedure.

ADEs	N (%)	n
Hematoma	2 (13%)	2
Pain	2 (13%)	2
Swelling	1 (6.6%)	1

Table 1 *- Incidence of ADEs Reported During the Investigation (N, number of subject; n, number of events).*

Discussion

In this investigation, 15 white subjects (female; N=15), with a median age of 53.9 years were enrolled. Of these, 15 subjects were evaluated at 1 month, 3 months, and 6 months and until complete product reabsorption; 1 subject was lost in follow-up after 1 month.

The aim of this investigation were to evaluate the safety and efficacy of Evryàl Strong injectable gel compared to the Teosyal Global Action injectable gel for the correction of moderate to severe nasolabial wrinkles. In all but one evaluated subject (96.7%), after 3 months the severity of NLFs improved by at least 1 NLF-SRS point, as assessed by the investigator. The efficacy of nasolabial folds treatments was previously assessed by the investigators, using other comparable devices (Ial System Duo and Belotero Basic/Balance)[14]. Dong et al reported the Nasolabial Fold Severity Score (NLFSS) responder rates for Juvéderm Ultra Plus were 90.8%, compared to 89.9% for Restylane[15]. Lupo et al reported that 96% of subjects treated with Juvéderm Ultra Plus maintained clinically significant improvement[16].

In a study performed by Goodman et al Juvéderm, Ultra Plus was reported to have generated a 90% compared to 65% for Perlane®[17]. Juvedérm Vollure XC (VYC-17.5L) revealed a NLFSS responder rate of 93.2%, as reported by Monheit et al[18]. In all instances, efficacy or clinically significant improvement was defined as at least a 1 point improvement on a nasolabial folds severity scale. Based on this data, it can be concluded that the efficacy results for Evryàl Strong are similar to other benchmarked devices. NLF improvement was apparent at 1 month and was maintained throughout the investigation until 3 months, confirming a tissue residency for Evryàl Strong of at least 6 months after administration. Therefore the median injected volume

was comparable or even slightly lower than the volume used with other HA fillers[18,19]. Treatment with Evryàl Strong also improved the subject's aesthetic appearance: as indicated by improved GAIS scores, from 1 month all evaluated subjects 96.6% reported "very much improved"/"improved" and until 6 months 96.7% reported "very much improved"/"improved".
The subject satisfaction evaluation matched the independent reviewer's assessments, with over 90% of subjects being satisfied to very satisfied upon the completion of the investigation. The subjective treatment outcome of Evryàl Strong injections matched the independent reviewer's assessments, with over 90% of subjects being satisfied to very satisfied upon the completion of the investigation.
Moreover, the acceptance of pain was satisfactory throughout the study and for those subjects experiencing pain after injections, there was no residual pain sensation at 15 minutes post-treatment.
All ADEs reported in this investigation (i.e. injection site hematoma, pain and swelling) were mild or moderate, transient, and are commonly reported following treatment with dermal fillers.

Conclusion

Treatment with Evryàl Strong is a safe, well tolerated and effective method for reducing the severity of NLFs; >96% of subjects showed an NLF severity improvement of at least 1 point after 1 month compared to their baseline status and this improvement was maintained for 6 months; 93.3% of the subjects were "very satisfied" or "satisfied" with the treatment outcome.

Disclosure

The authors report no other conflicts of interest in this work.

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aesthetic medicine

Original Article

Patient-Evaluation of the efficacy and tolerability of the hyaluronic acid-based gel Viscoderm® Hydrobooster for the treatment of facial wrinkles

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Short title: Viscoderm® Hydrobooster in real life setting

Abstract

Background: Viscoderm® Hydrobooster, an injectable formulation of Hyaluronic acid, has been shown in registration studies to be effective in the treatment of facial wrinkles.
Aim: in this study we aimed to evaluate the efficacy of this formulation in a real-life setting. Methods: Viscoderm® Hydrobooster was tested on 29 subjects in four different Austrian centers for its effectiveness in the treatment of wrinkles. Subjects were administered two injections of the studied product two months apart and were required to self-evaluate efficacy and tolerability after each injection and at a final visit, 4 months after the first injection.
Results: the vast majority of subjects reported an improvement after either the first or second treatment, as well as an overall amelioration of well-being. Such positive effects of the treatment were paralleled by very good tolerability and a minor level of pain at injection sites.
Conclusions: overall, as a measure of satisfaction, almost all subjects recommended the treatment to other people.

Keywords

Hyaluronic acid, self-evaluation, superficial wrinkles, hydration

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Introduction

Hyaluronic acid (HA) is one of the major components of fillers and creams used for aesthetic purposes[1-6]. The widespread use of HA is justified by its unique characteristics. It is present in the human body in high amounts, playing a pivotal role in dermis hydration, in maintaining the structural integrity of the skin and repairing skin injuries[7-9]. HA is also very well tolerated and is not immunogenic, a property that is essential for the safe application of HA-based products[2].
Aesthetic medicine is gaining increasing importance not only due to injury restoration, but also skin appearance improvement applications. In ageing, skin gradually loses HA, resulting in the formation of wrinkles and decreased skin elasticity[10-12]. Improving skin condition, particularly in ageing people, is not only important for well-being, but also generates a positive psychological impact. HA has particularly favorable properties and low or no adverse effects, which has resulted in a sharp increase in the number of formulations over the last few decades. HA can be present in different formulations with different molecular weights, in injectable fillers or in creams, either as a single agent or in combination with other substances of proven aesthetic performance[3,5,13-15].
Viscoderm® Hydrobooster (HB) is a special HA formulation whose characteristics are high deformability and low stiffness. These properties enable deep down dermal hydration and wrinkle repair.
The product has been reported to be effective in the treatment of both static and dynamic wrinkles in female subjects with moderate skin ageing[16]. In a larger real life study involving a hundred subjects requiring skin hydration, the application of HB resulted in high levels of satisfaction not only for physicians, but also for subjects, who were required to self-evaluate the treatment[17]. In both studies HB was extremely well tolerated and generated none, or very slight, unpleasant effects[16,17]. This report adds new information on the product's effectiveness and includes patient self-evaluations regarding the effectiveness and tolerability of two HB injections in human subjects.

Patients and methods

Subjects and treatments

The study was performed in four different centers in Austria (under the guidance of dermatologists, aesthetic doctors or plastic surgeons), in accordance with ethical guidelines and principles for medical research (Ethical Principles for Medical Research Involving Human Subjects, Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and its amendment). The general aim of the study was to confirm the efficacy of a product positively tested in the authorization study, in real life[16]. In order to minimize invasiveness, effectiveness was evaluated by means of a simple and rapid questionnaire.
Subjects participating in the study were required to sign an informed consent form before enrollment. Volunteers were excluded if they had undergone any treatment in the same area in the last six months, if they had autoimmune diseases, if they were pregnant, lactating and if they failed to comply with the protocol and post treatment recommendations.
HB was injected twice; the second treatment was administered two months after the first one. Treatment was recommended for superficial wrinkles including forehead lines, glabella, crow's feet and in the perioral area. The subjects were required to fill out a questionnaire five times: before the first treatment, immediately after the first treatment, two months after the first injection just before the second treatment, right after the second treatment and lastly, at the final follow-up visit (4 months after the first injection).
Viscoderm® Hydrobooster was injected (volume of 10 microliters) as microdroplets for the treatment of low severity wrinkles. A combination (10 microliters volume each) of microlinear retrograde technique followed by injection of microdroplets was used for more prominent wrinkles[16]. The area of treatment was based on specific requirements of the subjects. Twenty out of twenty-nine subjects selected the perioral area, while the glabella, forehead lines and crow's feet were chosen by the remaining 9 subjects. *Figure 1* contains a summary chart of the study.

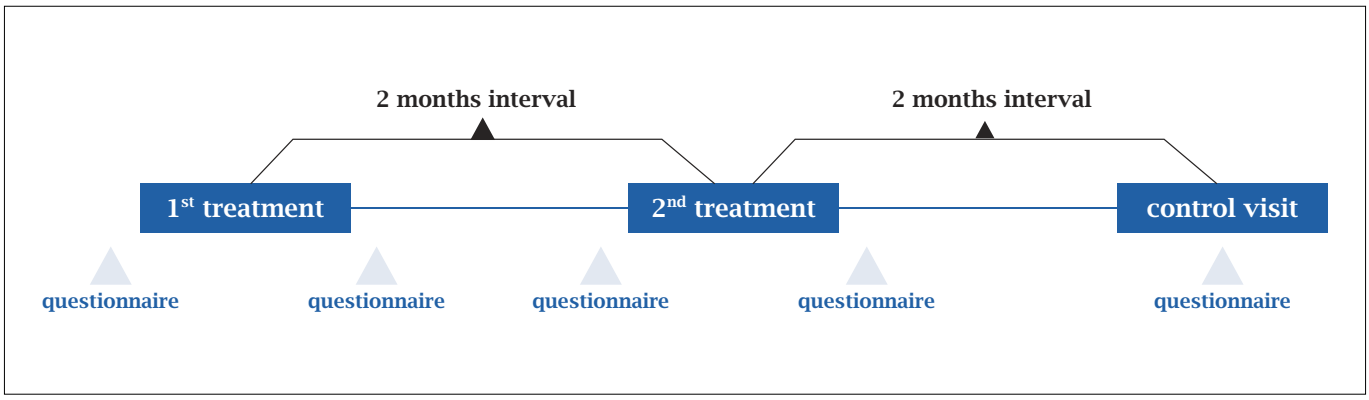

Figure 1 *- Summary chart of the protocol used for this study.*

Results

Twenty-nine healthy volunteer (28 women and 1 man) participated in the study. Their age ranged from 29 to 69 years, with a mean age of 55.3 years.
Before participating in the study, they were required to answer questions regarding their past experiences. Specifically, 20 subjects out of 29 had received previous injection treatments, consisting of HA in 18 cases and botulinum toxin in 14 cases (some subjects had had both treatments). Subjects were generally satisfied by previous treatments, 16/20 were very satisfied and the remaining 4 satisfied. Previous treatments were performed on forehead lines (17 cases), glabella (16 cases) the perioral region (13 cases) and crow's feet, in 12 cases.

The volunteers were asked which area they would like to receive treatment for and the majority (20 subjects) selected the perioral area, while the remaining 9 requested treatment for the glabella, forehead lines and crow's feet. The overall results of treatment are reported in *Figure 2*. The subjects were required to provide an overall evaluation after the first treatment, just before the second treatment and right after the second treatment.
The figure shows that the majority of subjects evaluated treatments very positively, with a 99 to 100% satisfaction rate. The same can be said for results achieved in the treated area (*Figure 3*). Indeed the majority of subjects reported a satisfaction rate which remained constant over a period of 4 months after the first application (i.e. at the time of their last visit).

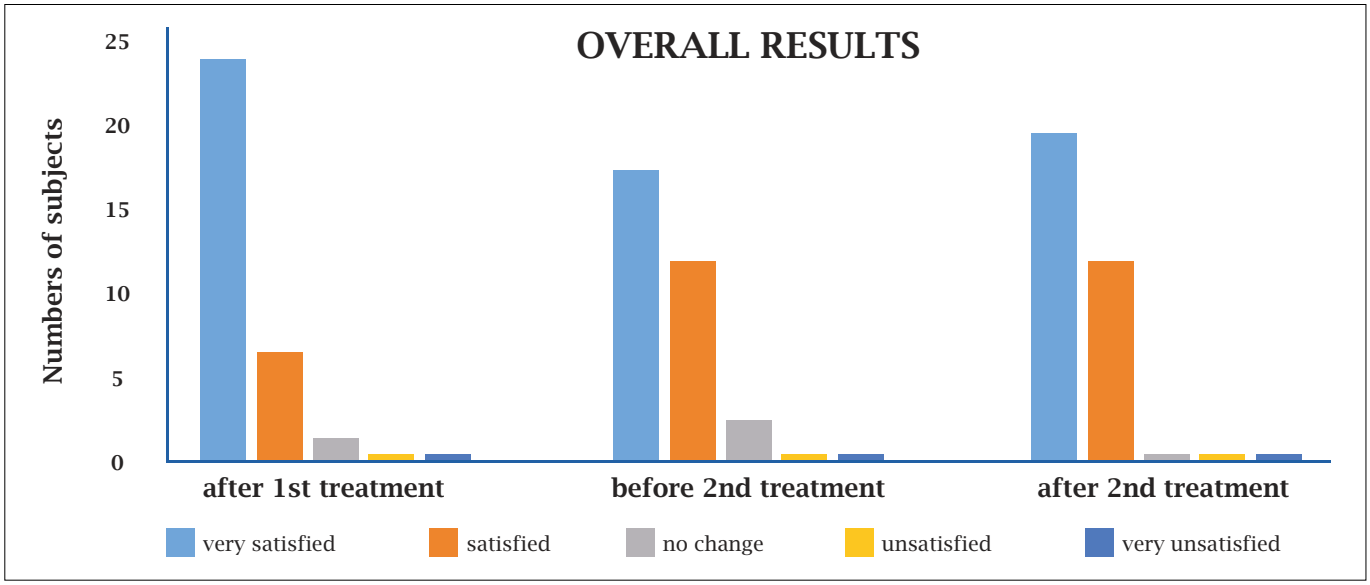

Figure 2 *- Subjects' overall self-evaluation of the efficacy of HB. The ordinate shows the number of subjects responding for each category of answer (plotted on the x axis).*

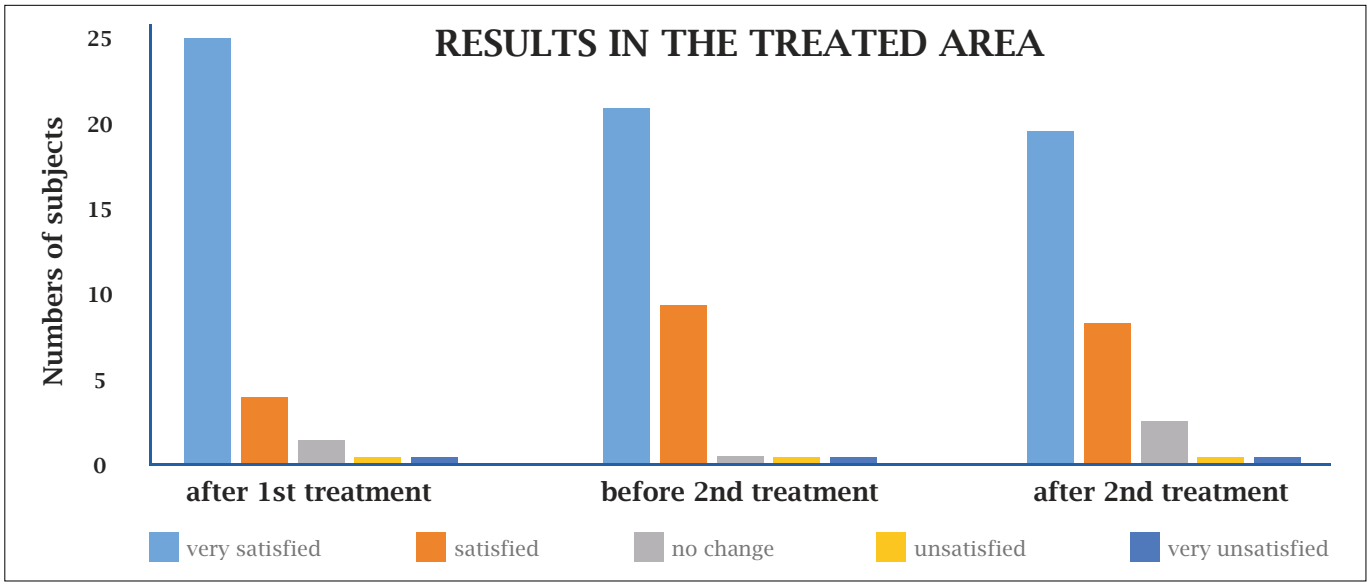

Figure 3 *- Results of subjects' answers to product evaluation for treatment area. For each category of answer (reported in the abscissa), the number of responding subjects is reported in the ordinate.*

The positive judgment of the two injections was paralleled by good product tolerability. In response to the question "have you experienced injection-related side effects?" 19 out of 29 answered no after the first injection and this number increased to 23 after the second injection. Reported side effects were hematomas (5 out of 29 both after the first and second injection) redness (4 and 1 after the first and second treatment, respectively) and 1 case of swelling either after the first or second injection. Only one subject reported other side effects (white spots), after the first injection only, and none after the second treatment. This effect was likely due to the injection of a volume of product that was too much for this specific patient.
As regards the painfulness of treatment, subjects were required to report according to a scale ranging from 0 (no pain) to 10 (most painful). After the first treatment, 27 out of 29 reported low-scale pain (0-4) with one subject reporting grade 5 and one grade 6. Similar results were reported after the second treatment, with 25 out of 29 subjects reporting low-scale pain (0-4) and the remaining four with values of 5 (2 subjects), 6 (1 subject) and 7 (1 subject).
Interestingly, all the subjects recommended the same treatments to others, either right after the first or after the second treatment and more importantly, all the subjects wanted the second treatment.

At the last visit (4 months after the first injection), the majority of subjects (27 out of 29) still recommended the treatment to others. Overall, the majority of subjects reported a rise in their quality of life (*Figure 4*).

Conclusion

Treatment with Viscoderm Hydrobooster® was positively assessed in terms of efficacy and tolerability by 29 subjects independently enrolled in this study which was carried out at four different centers. Almost all patients recommended the treatments to other subjects and the majority were satisfied, reporting an increased quality of life. The pain associated with the injection was modest.

Acknowledgment/Conflict of interest

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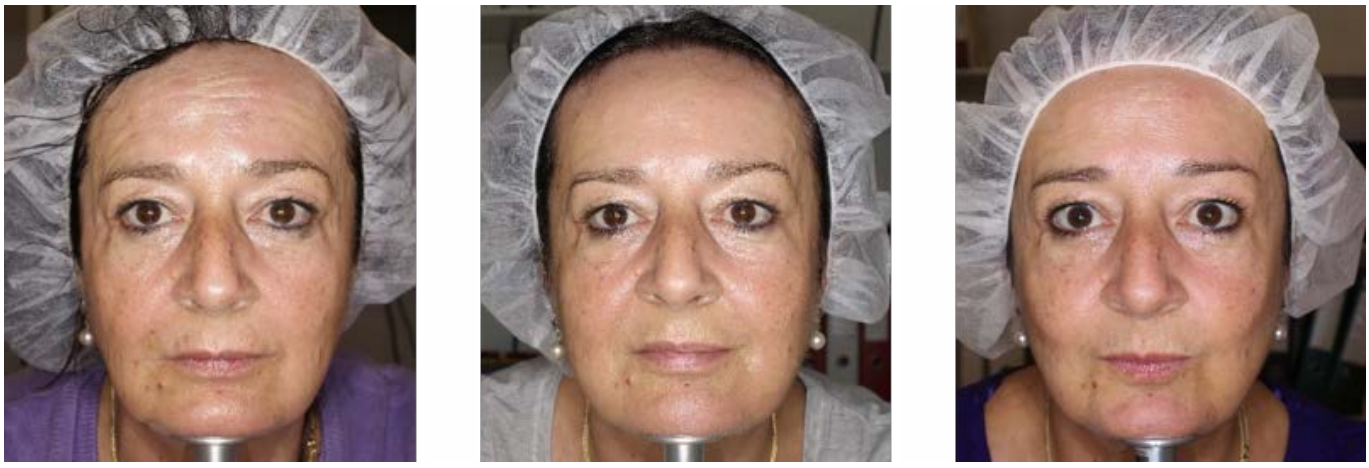

Figure 4 *- Clinical evaluation of treatment at different time points.*

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aesthetic medicine

Original Article

Use of intravaginal device based on photobiomodulation for the treatment of vaginal dryness: a pilot study

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Running head: Photobiomodulation to Treat Vaginal Dryness

Abstract

Purpose: this study aimed to evaluate the use of a treatment based on photobiomodulation, for the improvement of vaginal dryness associated with or without menopause.
Methods: this prospective non-placebo-controlled study included women who had reported vaginal dryness associated with or without menopause and had not received any physical or chemical aesthetic treatments. All patients underwent several treatment sessions with the MILTAPLUS intravaginal probe, a therapeutic device for genital restoration, based on non-invasive photobiomodulation and magnetic field techniques, and followed up 1 and 12 months after the last session. Vaginal tissue revascularization, the improvement of dryness symptoms, tissue characteristics of secretion/fluid and lubrication, the percentage of lubrication and pain variation and mean value of the patient's overall amelioration level of symptoms were assessed.
Results: twenty women with a median age of 44.8 (SD 7.4) years were included. Efficacy outcomes were: (1) vaginal tissue revascularization (34.6%); (2) reduction of dryness, stinging and dyspareunia measured using MBS (50%, 100%, and 50% respectively); (3) improvement of tissue characteristics using VHIS (16.1%); (4) mean amelioration of lubrication (94.6% [SD 8.7]) and pain (79.5% [SD 16.8]) one month after treatment using FSFI. The mean value of patients' overall amelioration level of symptoms was 7.5 (SD 1.1). The treatment was safe and no adverse effects were reported.
Conclusion: the use of photobiomodulation for the treatment of vaginal dryness provided excellent results, with the improvement of most symptoms of this condition. However, more research is required to determine the most suitable protocol for maintaining these outcomes.

Keywords

Vaginal dryness, photobiomodulation light-emitting diode, magnetic field, vaginal restoration, low-level laser therapy

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Introduction

Oestrogen deprivation has a significant impact on vaginal wall structure and function, affecting connective tissue components, including collagen, elastin, and smooth muscle, resulting in the degeneration of these structures and leading to vaginal atrophy (VA)[1].
The following major changes occur: (1) the vaginal epithelium becomes less cellular and thinner; (2) glycogen production - responsible for vaginal secretion - gradually declines and stops completely; (3) blood flow to the vagina is also reduced, which is associated with decreased fluid secretion during sexual arousal. These changes cause a variety of symptoms and vaginal dryness in particular, as a result of decreased natural lubrication[2-5]. Other symptoms include a burning sensation, irritation, discomfort or pain, and dysuria. VA symptomatology may vary from bothersome to debilitating, thus making treatment essential. VA makes vaginal dryness a common condition, particularly during menopause, which in turn often leads to dyspareunia (pain during intercourse)[6].
Vaginal dryness can affect patients' quality of life and sexual relationships[7]. The choice of therapy depends on the severity of symptoms, effectiveness and safety, always in accordance with patient preferences. All treatments are addressed to improve genital symptoms and restore the vaginal environment to a healthy condition[8]. The introduction of new medical devices for non-pharmacological therapies offers the possibility to improve treatments, for better results and greater patient satisfaction. Light in the visible-to-far-infrared spectrum has been applied to the female genital tract for nearly 50 years. Photobiomodulation (PBM) therapy is tissue exposure to visible and near infrared light sources (laser, LED, etc.), based on non-thermal and non-cytotoxic biological effects.
PBM therapy has been proposed as an alternative for use in managing the genitourinary syndrome of menopause (GSM) and stress urinary incontinence (SUI)[8].
The main biological effects are reduced tissue repair, greater inflammation, infection, and pain[9]. Some studies have reported the use of Er:YAG or fractional CO2 thermal laser for vaginal atrophy[10-12], for the reduction of pain, edema, and inflammation. Other studies have used devices based on light-emitting diodes (LEDs)[13], as well as therapy combining LED phototherapy with thermal laser procedures[14,15], to reduce thermal laser side effects, such as pain and edema. The rationale behind LEDs is based on their reported efficacy at a cellular and subcellular level, particularly for 660-nm and 850-nm wavelengths. Phototherapy with LEDs has been proven to reduce the production of pro-inflammatory cytokines, allogenic factors, and increases the production of procollagen and collagen[16]. It may also reduce collagen degradation, due to the enhanced trophicity of subcutaneous and submucosal muscle tissue[8].
Other observed benefits of phototherapy with LEDs are improved blood flow and neovascularization, as well as the inhibition of apoptosis[17]. LED treatment also reduces pain, including postoperative pain and edema, along with many types of inflammation[14,18]. The study aimed to assess the effectiveness and safety of a new photobiomodulation- based device in the improvement of vaginal dryness symptoms in women with or without menopause.

Methods

Study Design

This was a prospective non-placebo-controlled pilot study carried out at Clinica Elite Laser, (Madrid, Spain). Study participants were women (n=20) with symptoms of dryness associated with VA, with or without menopause. The complete treatment course included 12 sessions of photobiomodulation (one session of 5 minutes per week for 12 weeks). Patients were followed up at one and twelve months after the last treatment session.
The study was conducted in accordance with principles set forth in the current revised version of the Declaration of Helsinki, Good Clinical Practice (GCP), and in compliance with all applicable laws and regulatory requirements for medical device use in Spain. All patients signed an informed consent form in order to participate in the study, before undergoing any procedures.

Subjects

Consecutive patients were invited to participate in the study and their need for treatment was confirmed. Participants were women >18 years old with vaginal dryness associated or not associated with menopause. Exclusion criteria were: previous hormonal or other treatments for VA in the last six months; women with an active sexually transmitted disease or infection; neurological disorders; morbid obesity; current or attempted pregnancy; diabetes; breastfeeding or lactating; previous vaginal surgery or toning therapy; a history of cancer, chemotherapy or radiation therapy; vesicoureteral reflux; bladder calculi or tumor.

Variables Assessed

Objective Assessments: Number of microvessels per mm^2 of the vaginal tissue: this variable was measured using a transvaginal Power Angio-Doppler with a 3.5-5-MHz convex probe(Mindray® Bio-Medical Electronics Co Ltd., Shenzhen, China), at baseline and immediately after the treatment session.
Subjective Assessments: Symptoms were assessed according to the most bothersome symptom (MBS), including dryness, stinging, pain, dysuria, dyspareunia, bleeding during sexual intercourse; the values for each one were described as: none (N), low (L), moderate (M), and severe (S). Tissue characteristics were assessed using the Vaginal Health Index Scale (VHIS) score, consisting of five vaginal parameters: Elasticity, Secretion/Fluid Volume, Vaginal pH, Integrity of the Epithelium, and self-reported Lubrication/Moisture of Vaginal Tissue with a 5-point Likert scale, where 1 indicates "None," 2 is "Low," 3 is "Minimum," 4 is "Moderate" and 5 is "Normal." The sum of the five components can provide a maximum score of 25 and a minimum of 5. A score of ≤15 defines the presence of vaginal atrophy. Pain and lubrication were assessed using the Female Sexual Function Index (FSFI), a 19-item questionnaire with self-reported measures of sexual functioning in women, with a specific focus on six domains of sexual arousal, orgasm, satisfaction, and pain,

as well as a total score. The patient's overall amelioration level of symptoms with the treatment procedure was assessed using a 10-point Likert scale, where 1 indicates "Very Dissatisfied," and 10 is "Very Satisfied."

Procedures

Basal assessments: Before treatment, variables self-assessed by patients were: MBS, VHIS and FSFI; investigators carried out an Angio-Doppler to measure the number of microvessels per mm^2.

Treatment procedure: All patients underwent complete treatment (one 5 minute session per week, for 12 weeks) with the MILTAPLUS intravaginal probe (Physioquanta, Mudaison, France), a non-invasive therapeutic device combining technologies based on photobiomodulation and magnetic fields for vaginal tissue restoration (*Figure 1*), the technical characteristics of which are detailed in *Table 1*. The procedure did not require any anesthesia. Each program, LED and laser, lasted five minutes, therefore a complete session lasted 5 minutes. Immediately after the 12-session treatment: A transvaginal Angio-Doppler was performed to assess the number of microvessels per mm^2 and evaluate tissue neovascularization. One and twelve months after session 12 (end of treatment): MBS, VHIS and FSFI variables were assessed. All patients were asked about the amelioration of their symptoms and pain during the procedure.

***Figure 1** - Miltagynea® (Milta Technologie, Mudaison, France) intravaginal probe.*

Efficacy Outcomes

Efficacy outcomes were: (1) vaginal tissue revascularization, evaluated by a transvaginal Angio-Doppler, using the percentage increase in the number of microvessels per mm^2 from baseline to immediately after the treatment; (2) percentage of improvement of symptoms of dryness, stinging and dyspareunia, measured using MBS; (3) the improvement of tissue characteristics of secretion/fluid and lubrication, assessed using VHIS; (4) percentage of lubrication and pain variation using FSFI; and (5) mean value of overall amelioration level of patients' symptoms.

Safety Data

Treatment safety was assessed by recording all procedure complications and any adverse events that may have occurred during treatment right up until the follow-up visit, as well as by patients' self-perception of pain during treatment.

Statistical Analysis

Quantitative variables were described as the mean, standard deviation (SD) and range, whereas categorical variables were described as percentages.

Efficacy outcomes were assessed as the change of the corresponding variable from baseline to one month after treatment.

For this pilot study, the sample size was set at 20 patients. The statistical analysis also included suitable measures for statistical significance (Student's paired two-sample t-test) using the standard cut-off for significance of $p<0.05$.

Results

Subject Characteristics

A total of 20 women with a median age of 44.8 (SD 7.4; range of 29-53) years, eight with menopause (40%) and 12 without menopause (60%), were enrolled in the study and all of them completed it.

Source	Output Power Density	Output Wavelength	Dimension of LED Active Area
LEDs			
VIOLET (12 LEDs)	2160 mW, or 42 mW/cm^2	415 +/- 5 nm	50.9 cm^2
RED (12 LEDs)	900 mW, or 18 mW/cm^2	660 +/- 6 nm	50.9 cm^2
IR (12 LEDs)	540 mW, or 11 mW/cm^2	850 +/- 15 nm	50.9 cm^2
Laser			
Laser IR (12 diodes) Pulse frequency 10 kHz	120 W peak maximum Order of magnitude of the pulse: 100 ns	850 nm	50.9 cm^2
Constant Magnetic Field	70 mT		

Abbreviations: LEDs, light-emitting diodes; IR, infrared; mW, milliwatt; nm, nanometers; cm^2, square centimeter; mT, milliTesla.

***Table 1** - Technical characteristics of Milta emissions.*

Objective Variables: At baseline, global vaginal tissue vascularization had a median of three microvessels per mm^2 (range of 1-4); after one month of treatment, the median value was four microvessels per mm^2 (range of 1-5); twelve months after the last treatment, the median value was four microvessels per mm^2 (range 1-5).
The number of patients with five microvessels per mm^2 at baseline was 0 (0%), at one month after treatment, five (25%) women had five microvessels per mm^2 (*Figure 2*); 100% of said patients were not in menopause. At twelve months after the last treatment, three (15%) women had five microvessels per mm^2; 100% of these were not in menopause.
Subjective Variables: the most prevalent MBS at baseline with a high severe score percentage was pain (65%) followed by stinging (20%) and dyspareunia (20%), dryness (10%), and dyspareunia (10%); no patients reported bleeding during sexual intercourse. After one month of treatment, symptoms with a high severe score percentage were dryness (5%) and dyspareunia (5%) (50% decrease). After twelve months of treatment, symptoms with a high severe score percentage were dryness (5%) and dyspareunia (5%) (50% decrease).
No severe score was reported for any other symptoms.

Table 2 (click here) shows global results obtained with MBS throughout the study and according to menopause condition: Dryness, stinging, pain, dysuria, dyspareunia, and bleeding during sexual intercourse. VHIS at baseline had a mean value of 19.9 (SD 2.4; range of 16-23); one month after treatment, the mean value was 23.0 (SD 2.0; range of 19-25); twelve months after treatment, the mean value was 22.3 (SD 2.6; range of 17-25).
Table 3 (click here) shows the global results of VHIS and FSFI, variables assessed and their results throughout the study; *Table 4 (click here)* shows the results of VHIS and FSFI for patients with or without menopause.

Efficacy Outcomes

For vaginal tissue revascularization, the number of microvessels per mm^2 from baseline and one month after treatment increased by 34.6% (SD 23.5); differences were statistically significant (p=0.0008). At 12 months after the last treatment, the number of microvessels per mm^2 from baseline increased by 23.8% (SD 23.6); differences were statistically significant (p=0.0144). Symptoms of dryness, stinging, and dyspareunia, measured using MBS, improved by 50%, 100%, and 100% respectively, at one month after treatment; the same results were obtained 12 months after the last treatment.

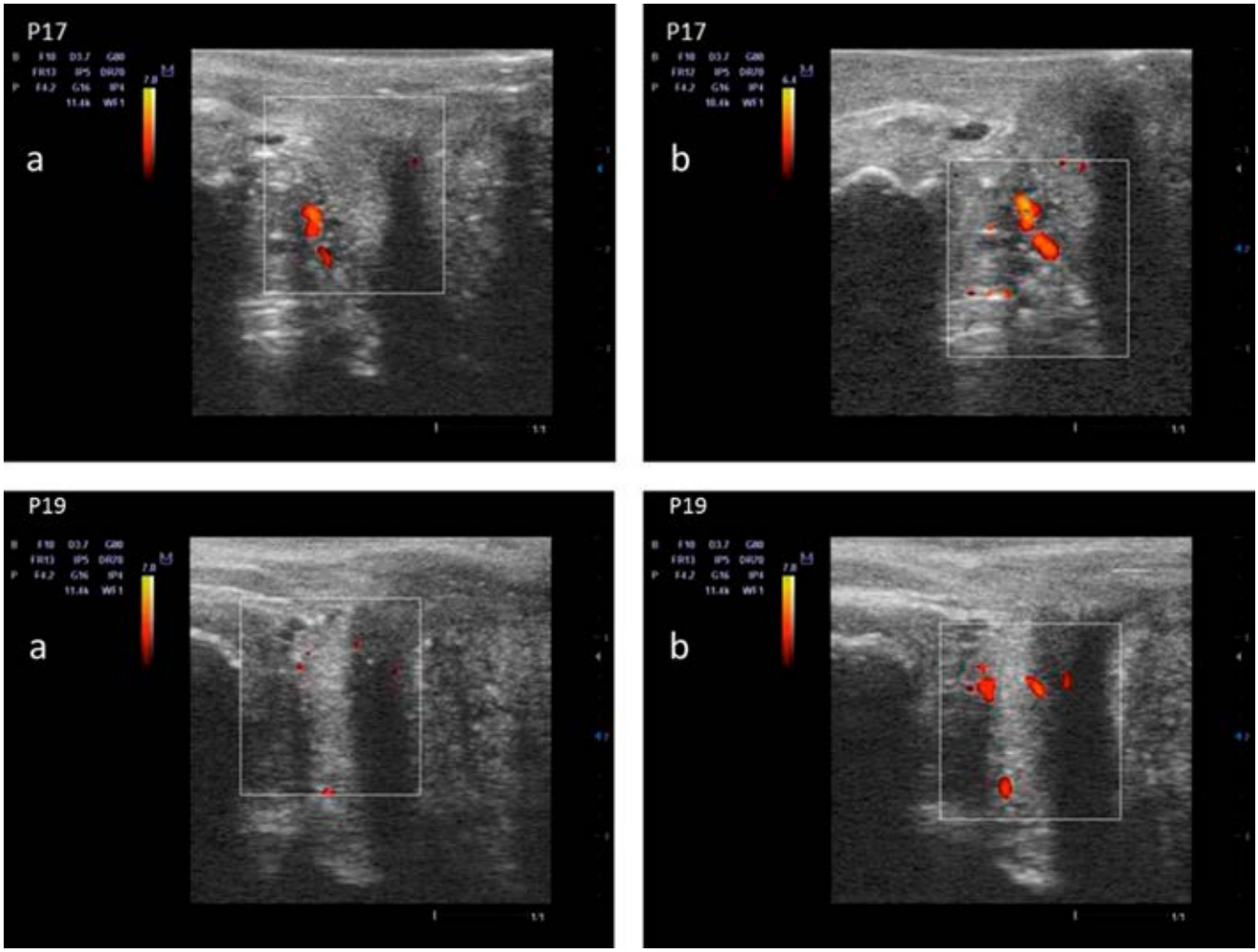

Figure 2 *- Example of a patient's Power Doppler at baseline and at one month after the last treatment session. a: Pre-treatment; b: At one month after the treatment.*

Table 2 shows all differences obtained for each MBS measurement from baseline to one month and 12 months after treatment for all patients and according to menopause condition. The improvement of tissue characteristics at one month after the last treatment, assessed using VHIS, was 16.1%. After one month of treatment, this improvement was significant (p=0.0003). *Figure 3* shows the mean of tissue characteristics at baseline and one month after the last treatment session, for each variable. 12 months after the last treatment session, a 12.5% tissue improvement was recorded, which was significant (p=0.0062).

At one month after treatment, the mean percentage of variation and the SD of FSFI lubrication and pain of 94.6% (SD 8.7) and 79.5% (SD 16.8), respectively. Improvement at one month was significant in both domains (*Table 2*). The mean value of overall amelioration level of patients' symptoms was 7.5 (SD 1.1). *Figure 4* shows the mean value at baseline and one month after the last treatment session, in each domain. At 12 months after treatment, the mean percentage of variation and SD of FSFI lubrication and pain was 71.7% (SD 8.2) and 63.3% (SD 14.7), respectively. Improvement at 12 months was significant in both domains (*Table 2*). The mean value of overall amelioration level of patients' symptoms was 7.0 (SD 1.0).

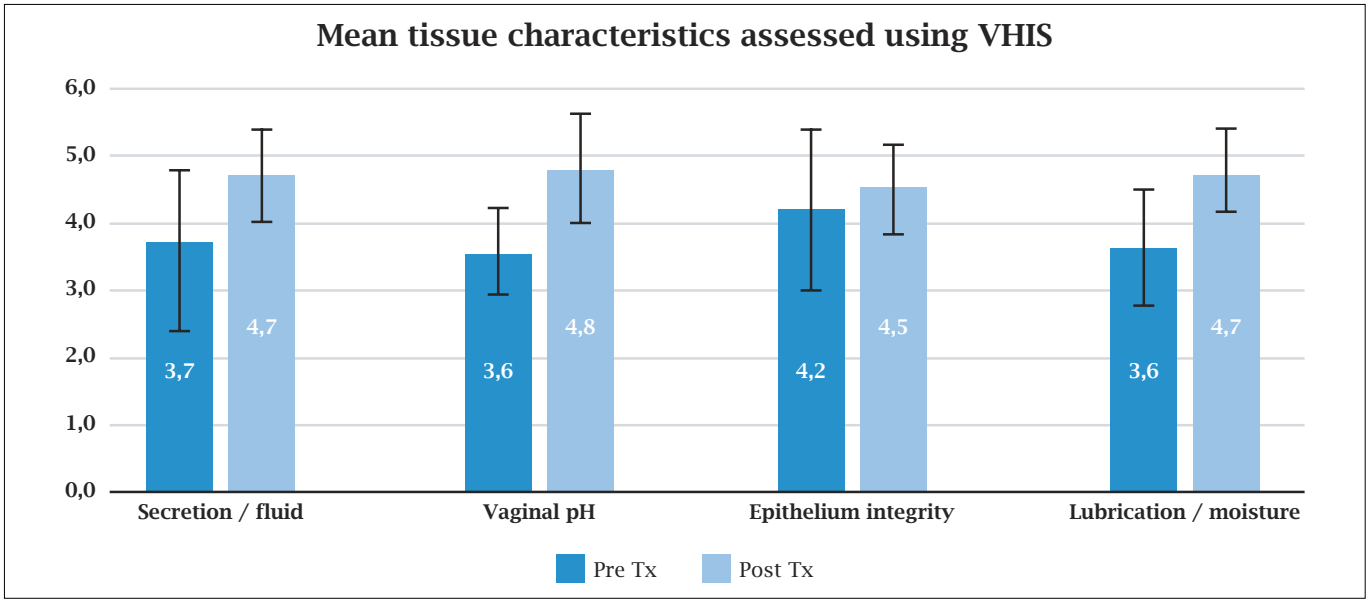

Figure 3 *- Mean of tissue characteristics at baseline and one month after last treatment session assessed using VHIS. Abbreviations: VHIS: Vaginal Health Index Scale; FSFI: Female Sexual Function Index; Tx: treatment.*

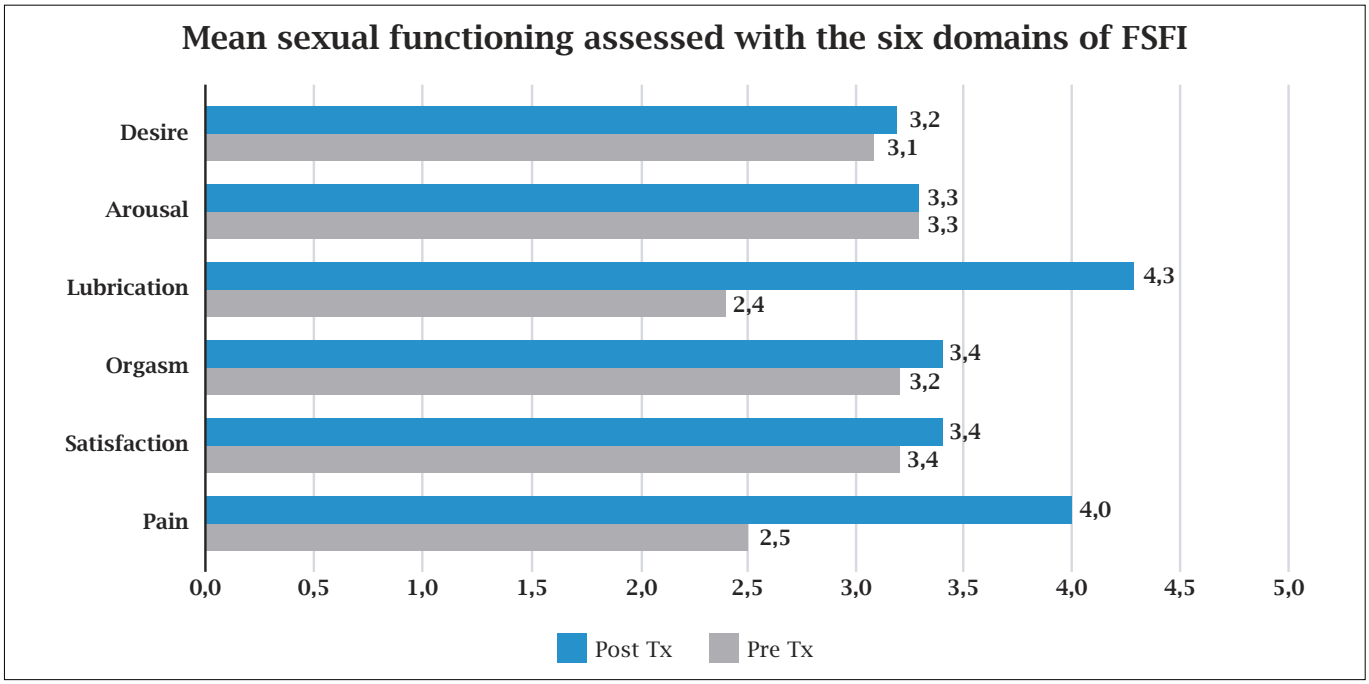

Figure 4 *- Mean patients' sexual functioning at baseline and one month after the last treatment session assessed using the six domains of FSFI. Abbreviations: FSFI: Female Sexual Function Index; Tx: Treatment.*

Safety Outcomes

The procedure did not require analgesia/anesthesia. No pain, complications or side effects were reported during treatment.

Discussion

Study results showed that the combination of LED, soft laser and magnetic field treatment was associated with improved vaginal health and VA symptoms, resulting in better vaginal tissue vascularization immediately after the treatment protocol (34.6%, SD 23.5; p=0.0008) (*Figure 2*) and after 12 months (23.8%, SD 23.6; p=0.0144). Patients' vaginal and sexual symptoms also improved, with decreased severity at all follow-ups. MBS symptoms with a sharp decrease (100%) in severity were: stinging, pain and dysuria at one month of treatment, and stinging and dysuria at twelve months after the last treatment session (see *Table 2*). A significant improvement of the following VHIS symptoms was reported: secretion and lubrication, both with a significant increase (p<0.0001) (*Table 3*). The VHIS score at one month and 12 month after the last treatment, showed a significant improvement compared to baseline (p=0.0003 and p=0.0062 respectively). An improvement in all FSFI components was observed at all follow-up visits. The "lubrication" and "pain" domains showed a significant improvement (*Table 3*). The overall value of patients' self-perceived amelioration of symptoms at one month was 7.5 (SD 1.1), and 7.0 at 12 months after the last treatment session (SD 1.0). All patients resumed usual activities after the treatment session.

Patients without menopause had better outcomes than patients with menopause, however improved conditions were observed in both groups. VHIS score variables remained, with amelioration at twelve months after treatment, in both groups (*Table 4*).

Since no similar studies can be found in literature, results must be compared with previous studies conducted by us. In 2018 we carried out a study with an intravaginal device using only LED technology, for the treatment of vaginal atrophy; increased vaginal tissue revascularization, measured by a transvaginal Angio-Doppler; results were not significant however (p=0.3369); regarding VHIS, the FSFI domains of "lubrication" and "pain" improved, and results were statistically significant[13]. In another study with Erbium YAG (Er:YAG) or carbon dioxide (CO2) lasers and LEDs, the number of microvessels was higher immediately after treatment, and results were statistically significant (p<0.0001)[15]. On the basis of our experience with a combination of photobiomodulation-based technologies, the perception is that this synergy (magnetic field, LED, soft laser) primarily enables greater depth of photon penetration in soft tissues, acting directly on the target area and obtaining better benefits from these technologies, promoting optimal tissue regeneration without side effects. In this study, the added benefit is that the device includes all three technologies in one device, facilitating the professional's handling of the procedure and providing more patient comfort.

Despite considerable improvement in all variables assessed in this study, our results should be considered in the context of the limitations of the study design, i.e. a low number of participants. Thus, unlike other studies previously mentioned, ours was not randomized and did not compare the efficacy of the investigation device with that of a sham device. The study aimed to relieve symptoms that are experienced subjectively by individual patients and thus patient self-assessment was deemed a good representation of treatment effectiveness.

In short, the combined use of these three technologies (LEDs, soft laser, and magnetic field) for the treatment of vaginal dryness provided excellent results for tissue regeneration and symptom amelioration. However, future randomized, double-blind studies with sham devices and a more significant number of patients will be necessary to confirm these results.

Conflicts of Interest and Disclosure

The authors have no conflicts of interest or financial ties to disclose.

Role of funding source

This study did not receive any funding.

Ethical Approval

The study was conducted in accordance with the principles set forth in the current revised version of the Declaration of Helsinki, Good Clinical Practice (GCP), and in compliance with all applicable laws and regulatory requirements for the use of medical devices in Spain. The authors do not have a formal ethics review committee.

Informed Consent

Before undergoing any procedures, patients signed an informed consent form in order to participate in the study.

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aesthetic medicine

Research

Preliminary clinical validation of cryoadipolysis treatment under effects of ischemia simulated by COMSOL Multiphysics® software

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Running head: Ischemia in cryoadipolysis

Abstract
Cryoadipolysis is a non-invasive technique for subcutaneous fat reduction, which is used to circumvent problems associated with surgical methods like liposuction or abdominoplasty. During cryoadipolysis, local ischemia is caused due to the suction process and blood flow is reduced. This leads to a reduction of the biological heat within the suctioned tissue, which can increase the cooling capacity of cryoadipolysis applicators. In this study we performed a preliminary clinical investigation to validate a mathematical model designed to determine the percentage of bloodstream that flows through treated tissue, depending on the geometric characteristics of the cryoadipolysis applicators used. We observed a very strong correlation (R2 > 97%) between experimental and simulation data. The use of numerical simulations and accurate models that reproduce the thermal behavior of biological tissues can be used to better understand the cryoadipolysis process, estimate the efficacy and safety of cryoadipolysis applicators, and develop better and safer devices.

Keywords
Ischemia, Cryoadipolysis, Simulation, Clinical study, Cooltech Define, COMSOL

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Introduction

Fat accumulation is a serious aesthetic and health concern for modern society.
Cryoadipolysis is a non-invasive technique for fat reduction that is used as an alternative technique to circumvent general problems associated with analogous surgical methods (i.e. liposuction)[1].
During cryoadipolysis, adipocytes are selectively lysed after local cold induction and no harm is caused to adjacent cells[1]. Since the approval of the first cryoadipolysis device for the reduction of undesired subcutaneous fat by the FDA in 2010 (K080521), many studies have confirmed the efficacy and safety of this non-invasive technique, both *in vitro and in vivo*[2-4].
Moreover, various clinical trials have confirmed that it is a safe technology which causes minimal pain and guarantees a quick recovery for patients[1].
Cooltech® and Cooltech Define are among the world's leading cryoadipolysis platforms in this regard.
In previous studies, we have recreated the Cooltech procedure using numerical simulation with COMSOL Multiphysics®; simulations have proved to be an excellent tool for improving understanding of the cryoadipolysis process and for optimizing applicator design for better efficacy and safety[5,6].
These simulations have previously been validated using in-vitro experiments[7].
However, there is one aspect of cryoadipolysis that cannot be observed through in-vitro experimentation and therefore has barely been studied: ischemia[4]. Local ischemia (decreased blood flow inside suctioned tissue) is caused by the vacuum suction of the applicators when cryoadipolysis is performed.
Since even a small decrease in the percentage of blood flow produces important changes in the thermal behavior of tissue, ischemia during cryoadipolysis may have significant consequences on the efficacy of treatment. We developed a mathematical model of ischemia based on the applicator geometry and suction pressure[6].
Here, we performed a preliminary clinical validation of our *in-silico* model of the cryoadipolysis method that includes a model for ischemia.
A thermocouple probe was introduced into the fatty tissue during a cryoadipolysis procedure, to monitor internal temperature. Subsequent experimental data were compared with the results of simulations.
A more accurate *in-silico* model that includes blood perfusion will serve to improve the design of the Cooltech cryoadipolysis procedure, in order to provide maximal efficacy and safety for subjects.

Materials and methods

In-silico model

The *in-silico* model simulating the physics of the heat exchange between the biological tissues and the Cooltech® device was created with the COMSOL Multiphysics® software using the finite elements method[5-7].
The meshed geometrical domains consisted of the biological tissues involved (skin and fat) and the cryoadipolysis applicators (*Figure 1A*). Applicators were designed using SolidWorks® software; designs were then imported to the COMSOL Multiphysics® software (*Figure 1B*). The simulation was conducted by solving the equation of heat transfer for biomaterials (*Eq 1*):

A. $$\rho C_p \frac{\partial T}{\partial t} + \nabla \cdot q = Q + Q_{bio}$$

B. $$q = -k \nabla T$$

C. $$Q_{bio} = \rho_b C_{pb} \omega_b (T_b - T) + Q_{met}$$

Equation 1 *- A: Equation of heat transfer for biomaterials; B: Heat flux by conduction in the tissue and C: Biological heat. Parameters and variables of the equations: ρ: density of the tissue; C_p: specific heat at constant pressure of the tissue; ∂T/∂t: partial derivative of the temperature (T) with respect to the time (t); ∇: mathematical operator Nabla (operator that applies partial derivatives in space to a magnitude); q: heat flux by conduction in the tissue; Q: heat source; Q_{bio}: biological heat; k: coefficient of heat conductivity; ρ_b: blood density; C_{pb}: blood specific heat at constant pressure; ω_b: blood perfusion rate; T_b: arterial blood temperature; Q_{met}: metabolic heat source.*

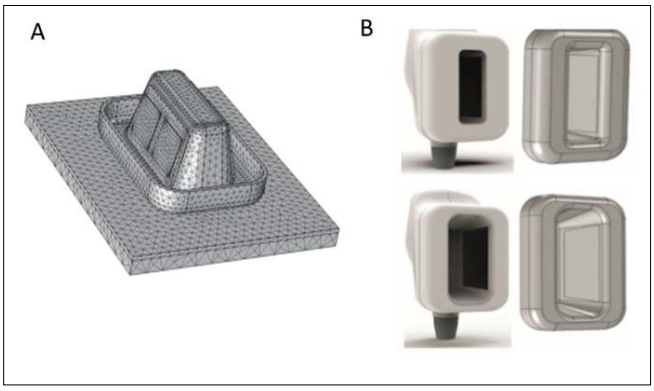

Figure 1 *- (A) Mesh used for the heat transfer equation resolution by finite domains method (Straight HP case). (B) Tight design (top) and Straight design (bottom) of Cooltech applicators.*

For each material used in the simulation, three basic parameters were required: (1) specific heat (C_p), (2) thermal conductivity (k), and (3) density (ρ).
The materials used in the simulations were fat, skin, the polypropylene of the applicator's outer body, the anodized aluminum of the cooling plates' surface and the anodized aluminum of the cooling plates [8-11] (*Table 1*). Four parameters were required to simulate blood perfusion: (i) blood temperature (T_b), (ii) blood-specific heat capacity at constant pressure (C_{pb}), (iii) blood density (ρ_b), and (iv) blood perfusion rate (ω_b) in fat and skin[12,13] (*Table 2*).

Material	Cp (J/kg·K)	k (W/m·K)	ρ (kg/m³)
Fat [8] *T in oC	1984.2 + 1.4733T – 4.8008·10^{-3}T^2	0.18071 – 2.7604T·10^{-4} – 1.7749·10^{-7} T^2	925.59 – 0.41757T
Skin [9]	3391	0.37	1109
Plastic (Polypropylene) [10]	1800	0.16	1040
Anodized Aluminum [11]	880	18	2700
Aluminum [9]	900	238	2700

Table 1 *- Required physical parameters to solve the heat transfer equation.*

Blood Temperature	36 (ºC)
C_p blood[12]	3220 (J/kg·K)
ρ blood [12]	900 (kg/m³)
ω in fat [13]	4.2·10^{-4} (1/s)
ω in the skin [13]	0.0018 (1/s)

Table 2 *- Required physical parameters to simulate blood perfusion.*

The different stages of a cryoadipolysis treatment were recreated with the simulations, including the cooling of tissues through contact with cooled aluminum plates, the use of cryoprotectant on the skin and the ischemia produced by the suction of the tissues into the applicators. Regarding the original conditions of the simulations, the initial temperature was 36ºC for biological tissue (simulating body temperature) and 20ºC (room temperature) for all other materials.
As a first boundary condition, an isolated system was considered, which means that there was no heat flow in its contours (*Figures 2A and 2B*).
As a second boundary condition to simulate the cooling process, a time-dependent temperature was applied to the aluminum plates.
Aluminum plates, which in a real-life situation are cooled by Peltier cells, do not achieve a cold temperature instantaneously and require some time to reach the set temperature (-8ºC with the Cooltech® device). We incorporated the temperature drop function of the Cooltech applicators in the simulation (*Figure 2C*).

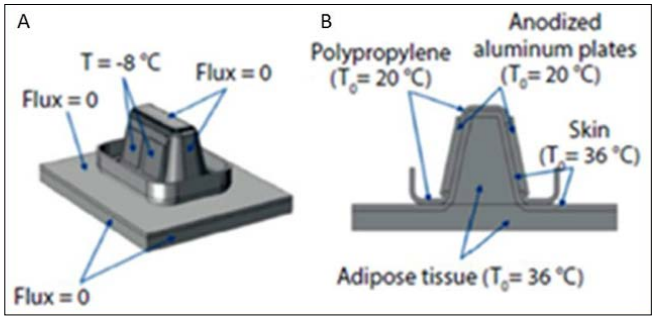

Figure 2 *- (A) Boundary conditions and (B) initial conditions used during simulations.*

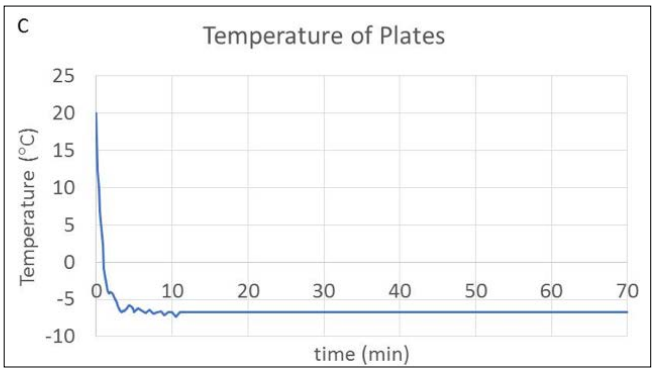

Figure 2 *- (C) Temperature curve of aluminum cooling plates as a function of time. Abbreviations: T, temperature; oC, degrees Celsius.*

In order to simulate the suction effect of the applicator on the tissue to be cooled, the treated biological tissue was divided into two parts: (i) the suctioned tissue, consisting of skin and fat inside the applicator, and (ii) the non-suctioned tissue, consisting of the skin and fat adjacent to the suctioned tissue. Assuming partial ischemia, a model was proposed to estimate a blood perfusion factor (BPF) [6] (Eq 2).

$$BPF = 1 - e^{-\left(k \times \frac{Width\ of\ the\ applicator\ cavity}{Suction\ pressure \times Angle\ of\ cooling\ surfaces \times Depth}\right)}$$

Equation 2 *- Equation for the blood perfusion factor.*

where *e* is a mathematical constant with an approximate value of 2.71828 and is the base of the natural logarithm, and k is a constant to determine the perfusion factor for each applicator, obtained by applying the model to a reference value[6]. The following premises were also considered in order to develop the model:
a) BPF of between 0 and 1, multiplying the blood perfusion frequency (ω) of the biological tissue in its natural state. Without ischemia, the factor is 1 and there is natural biological heat; with 100% ischemia, the factor is 0 and biological heat is null.
b) BPF increases with the blood perfusion surface (surface of the suctioned tissue through which blood flows).
c) BPF decreases with the depth and the inclination angle of the lateral surfaces inside the applicator, and with suction pressure.
d) For high BPF values (low ischemia), changes in the

design parameters do not significantly affect ischemia. On the other hand, for low BPF values (high ischemia), small design changes can significantly affect the resulting ischemia.
For the Tight and Straight Cooltech applicators, the perfusion factor was estimated at 0.31 and 0.42, respectively[6].

Clinical procedure

In order to compare simulated results with clinical data, two subjects with localized adipose tissue in the abdominal area were recruited. The study was conducted in compliance with the principles set forth in the current version of the Declaration of Helsinki, Good Clinical Practice, and the laws and regulatory requirements for the use of medical devices in Spain. Both subjects were consulted, provided their consent and clearly understood the procedure before the study. All procedures fulfilled Organic Law 15/1999 on the Protection of Personal Data and Regulation (EU) 2016/679 of the European Parliament and the Council of April 27 2016, concerning the protection of natural persons with regard to the processing of personal data and the free circulation of said data. Both subjects were treated with the Cooltech device, one with a Straight applicator (Subject 1) and the other with a Tight applicator (Subject 2) (*Figure 3A*).
Prior to the treatment, a thermocouple probe (generic K-type thermocouple) was introduced through a cannula reaching the dermal fat layer through a dermal incision. The probe was connected to a GM1312 Dual-Channel LCD Digital Thermometer to track the temperature during cryoadipolysis treatment (*Figure 3B*).
The cryoadipolysis treatment began with a suction test to ensure the suitability of the applicator for the fatty tissue to be treated. A cryoprotectant CGP (Cool Gel Pad) membrane was then placed on the area to protect the epidermis and dermis. The applicator was positioned over the CGP and then activated. Suction was set at 240 mbar and the temperature at -8ºC.
After 70 minutes of treatment, the applicator was removed along with the CGP membrane and a massage was performed on the treated area.
After the treatment, the depth and position of the probe was verified by palpation and by ultrasound with a Mindray M5 ultrasound system (*Figure 3C*).
The simulation was performed at the same x, y and z coordinates of the simulated tissue.

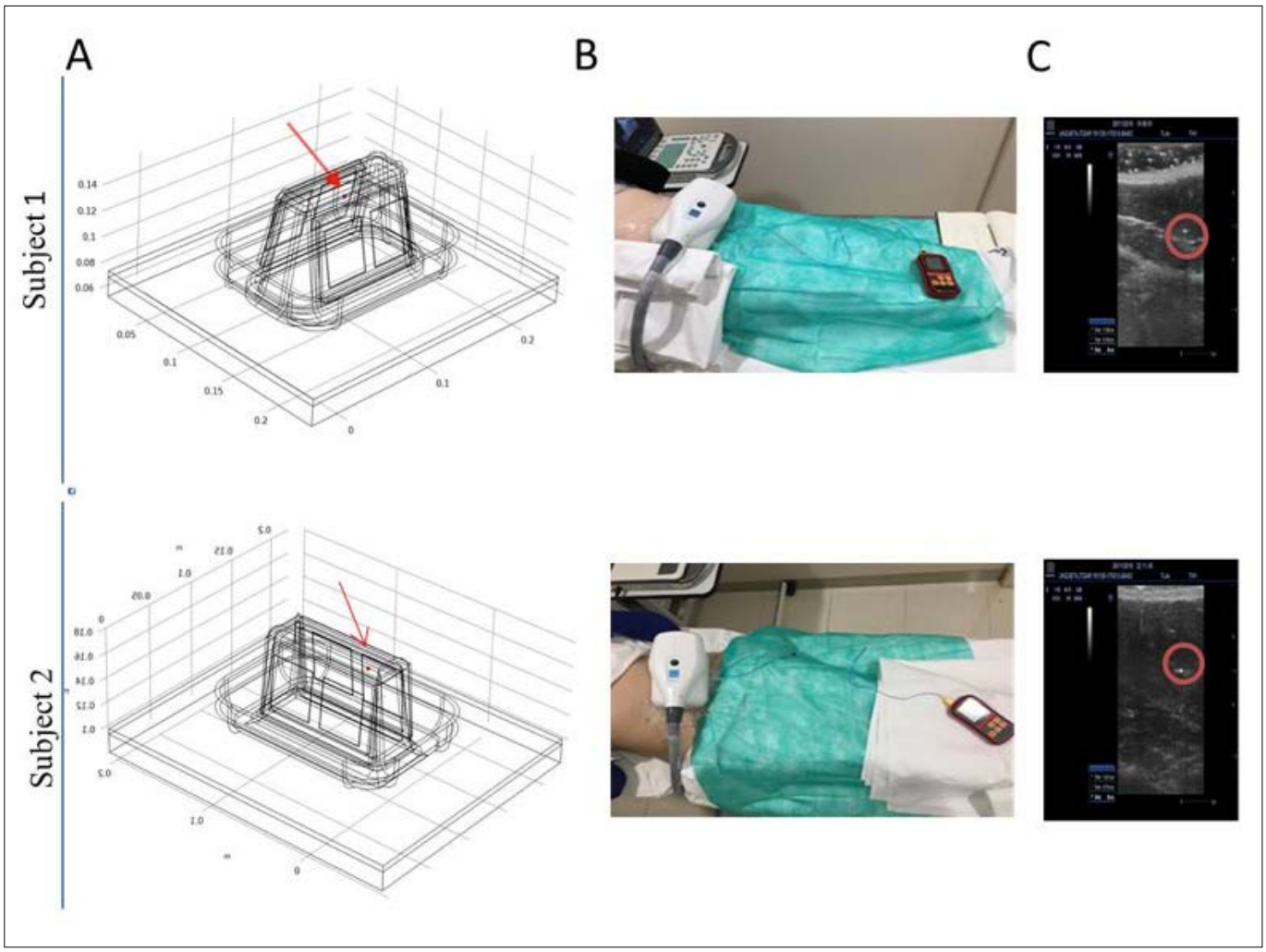

Figure 3 *- (A) Point evaluated in the simulation. (B) Treatment images with thermocouple probe attached for Straight applicator (Subject 1) and Tight applicator (Subject 2). (C) Ultrasound images of the treated tissues. Probe position inside the tissue is marked in red.*

Statistics

In order to evaluate the correlation between simulated and observed results, the coefficient of determination (R2) has been computed. The variance of the difference between both data sets and the maximum value of the difference is also shown.

Results

We described the temperature obtained from the clinical observations during cryoadipolysis as a function of time, and we compared the obtained results with the outcome of the simulations, which took into account ischemic processes. In the case of Subject 1 (treated with a Straight applicator), the experimental data closely matches the data obtained from the simulation. In this case, the greatest discrepancy between the experimental data and the data obtained from the simulation was - 2.21ºC at 10 min. The variance of the difference was 1.01ºC and R2 was 97.87% (*Figure 4A*).
Similarly, in the case of Subject 2 (treated with a Tight applicator), the experimental data matches the data obtained from the simulation very well, with a maximum difference of -2.92ºC at 20 min. The variance of the difference is 1.09ºC and the coefficient of determination (R2) is 99.08% (*Figure 4B*).

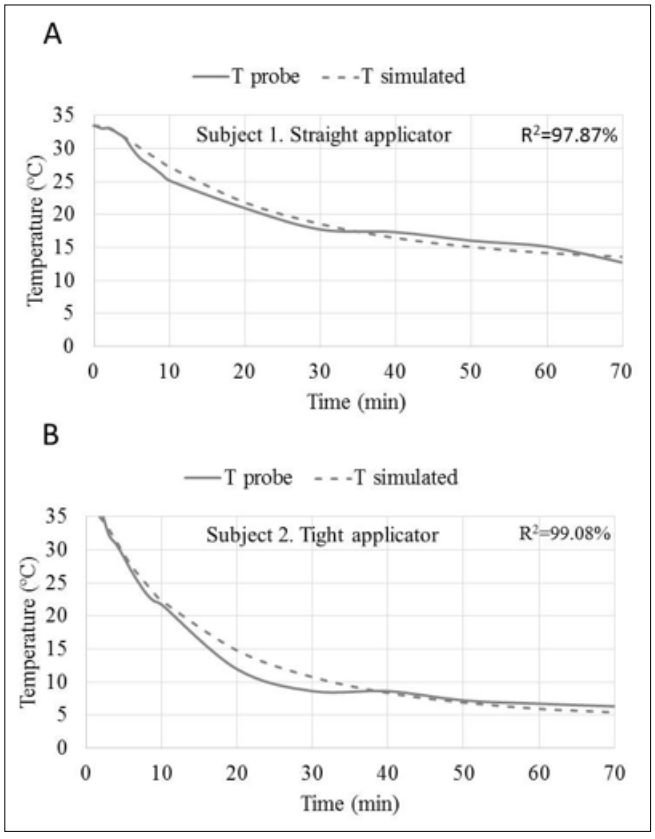

***Figure 4** - Experimental Temperature versus Simulation data for Subject 1 (A) and Subject 2 (B).*

Discussion and conclusions

During a cryoadipolysis procedure, ischemia (reduced blood perfusion) is caused in the treated area due to it being sucked into the applicator[4]. Decreased blood flow in the suctioned tissue leads to a reduction of biological heat (Qbio), which does indeed have a great influence on the cooling dynamic of the cryoadipolysis procedure. In contrast, the metabolic heat (Qmet) of each tissue (Eq 1C) is considered to be negligible during cryoadipolysis[12,13]. Therefore, the 3D heat transfer *in-silico* model used in this study considers ischemia as the only parameter for determining biological heat.
Accordingly, the efficacy of cryoadipolysis does not depend solely on tunable parameters (i.e. temperature, pressure) but is also affected by the geometry of the selected applicator. The shape of the applicator determines the contact surface and the amount of tissue to be treated along with the induced ischemia, thus directly influencing bodily thermal response.
We have validated the described 3D *in-silico* model using two applicators that differ in shape. The correlation between the theoretical data, obtained by means of simulation, and the clinical data obtained during cryoadipolysis administered to two subjects with the Straight and Tight applicators, was 97.87% and 99.08%, respectively. The maximal reported difference was less than 3ºC. Notably, we can confirm that the Tight applicator provides better treatment efficacy. This is due to its long and narrow design, which provides an elevated contact area with the skin and therefore better cooling of the fat volume lodged inside[5].
In conclusion, this experimental study has proven that it is possible to generate mathematical models enabling the simulation of the cooling dynamics of Cooltech® applicators. We have created a realistic model by determining the relationship between the ischemia produced in the suctioned tissue within the applicator and the geometry of said applicator. This information has no precedents and contributes great value to existing clinical experience.
More studies should be performed, considering a variety of different applicators; the number of subjects should also be increased. Confirmation of results would result in a fully validated method for determining the extent of cooling generated by cryoadipolysis applicators by means of numerical simulations that are only dependent on their geometric parameters. This may lead to the design of more effective cryoadipolysis procedures, primarily in terms of patient safety.
A validated *in-silico* model may also substantially reduce the need for clinical subjects to validate new device designs, thus clearly providing a significant added value.

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Conflict of interest

The authors of this publication conduct research in Cocoon Medical S.L.U., a company which is developing products related to the reported study. However, this publication strictly adheres to objectivity and ethical principles for independent research.

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aesthetic medicine

Review

Current understanding of the pharmacology of botulinum toxin type A: penetration, distribution, triple receptor binding, the mechanism of action and factors affecting it

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Abstract

The aim of this review is to structure current information clarifying the most disputable issues of botulinum neurotoxin type A (BoNT/A) pharmacology after systemic (botulism) impact and local medical application. The evaluation of botulinum neurotoxin (BoNT) pharmacological features requires the study of factors affecting its biological activity to extend/shorten the duration of its effect and to increase/decrease BoNT sensitivity in specific patient populations.

This review presents unique molecular mechanisms underlying BoNT/A pharmacokinetics and pharmacodynamics: entry into the body, distribution, receptor binding, translocation, mediator release suppression, zinc metabolism as well as factors affecting body sensitivity to BoNT at each of those stages.

Botulinum neurotoxin pharmacokinetics and pharmacodynamics features discussed herein are of significant clinical relevance since they determine botulinum treatment safety and effectiveness. They also open the way for developing both BoNT-based therapies and anti-botulinic agents.

Keywords

Botulinum neurotoxin, Ganglioside, Synaptic vesicle protein, Fibroblast Growth Factor Receptor, SNARE proteins, translocation

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Introduction

Botulinum neurotoxins (BoNTs) are the most potent protein toxins among bacterial, animal, plant and chemical toxic compounds and are the cause of botulism[1]. BoNT-based therapeutics are widely used in the treatment of various diseases and esthetic disorders. Various highly promising features of BoNT pharmacology in the development of both BoNT-based therapeutics and anti-botulinic agents are currently under study. Unique molecular mechanisms underlying various stages of botulinum neurotoxin type A pharmacological activity as well as potential factors affecting body sensitivity to BoNT are described in this paper.

Neurotoxin complex and BoNT molecule structure

Botulinum neurotoxin is a protein dimer with a molecular weight of 150 kDa and the chemical formula C6760H10447N1743O2010S32, consisting of two chains: light and heavy. The light chain constitutes approximately one third of toxin molecular weight and is bound to the heavy one with a disulfide link[2].

The light chain (L-chain) is a protease blocking synaptic release. It forms the BoNT molecule catalytic domain. The heavy chain (H-chain) consists of 2 domains: the binding domain bounds to target cell surface receptors; the translocation domain is involved in light chain translocation, creating the cell membrane channel. The BoNT molecule is a dipole with an electric charge attenuating from the binding domain to the catalytic one[3]. It is of importance when the molecule is directed to cell membranes as it facilitates receptor binding.

In natural settings BoNTs are synthesized by bacteria as a complex with several proteins: one non-toxin non-hemagglutin (NTNHA) and several hemagglutinins[1].

NTNHA has a molecular weight of 130 kDa and its amino acid sequences are extremely similar to BoNT but without protease motif. “Hand in glove”-type interaction with the BoNT molecule protects from the aggressive effects of environmental factors, including GIT proteolytic enzymes[4].

There are three classes of hemagglutinins with molecular weight of 33-35, 15-18 and 70 kDa[5]. They do not directly come into contact with the BoNT molecule, but rather with NTNHA, working as an adhesin molecule when said toxin complex is absorbed.

Non-toxic hemagglutinin and hemagglutinin proteins can form various multimeric complexes with BoNT called botulinum neurotoxin complexes. Each contains only one BoNT molecule, which is released from the complex if medium pH changes[2].

BoNT absorption and distribution

BoNTs can enter the human body via both injured and intact tissues. Therapeutically, botulinum neurotoxin type-A (BoNT/A) based agents are mainly injected as close as possible to their target cells. However, BoNT/A forms that can be applied without damaging skin are already under development, yet to reach Phase III clinical studies[6,7].

In natural settings, BoNTs show systemic action causing botulism and enter the body mainly through intact membranes.

Depending on the mode of toxin entry, botulism forms can be classified as follows: food botulism (ingestion of BoNT-contaminated food), infant (ingestion of food with bacteria spores), inhalation (breathing-in BoNT-containing aerosols), wound (in majority of cases related to injectable drug use), iatrogenic[8].

In natural settings, the botulinum neurotoxin must cross epithelial barriers and reach general circulation in order to hit its target cells. This process is called absorption.

There are two modes of BoNT intestinal or pulmonary epithelium penetration: intracellular route and intercellular junction-related.

In the case of transcytosis (penetration through epithelial cells), BoNT binds to ganglioside receptors at the epithelial cell surface and undergoes endocytosis (captured in a vesicle). Transport vesicles transfer the toxin to the entire cell and release it into general circulation. Toxin structure is not altered, nor is it released in cell cytosol during transcytosis, which differentiates BoNT binding with epithelial cell from binding with neuronal ones[9,10].

The paracellular route (through intercellular junctions) may or may not involve complexing proteins. Complexin hemagglutinins can bind to E-cadherin in epithelial intercellular junctions and disrupt the latter, releasing BoNT into general circulation[4]. BoNT molecules are also able to break epithelial barriers without complexing proteins. Studies by Maksymowych et al., 1999; Al-Saleem et al., 2012[11,12] showed that the introduction of equimolar amounts of free BoNT/A and BoNT/A complexes resulted in equivalent BoNT titers in general circulation with similar toxicity and effectiveness. However, hemagglutinins are assumed to boost BoNT transportation through the epithelium.

When transported through the intestinal wall, BoNT may bind to cholinergic and serotoninergic neurons of the enteral (intestinal) nervous system located in the intestinal submucosa, blocking gut motor and secretory activity. This explains impaired bowel movement (constipation) as one of the early signs of alimentary and infant botulism[13].

BoNT penetrates the epithelial barrier into general circulation and is distributed in all extracellular fluid compartments in the body except for in the central nervous system.

Eisele et al. (2011) conducted a series of experiments[14] demonstrating that with pH values close to neutral (arterial blood pH of 7.37-7.43[15,16] botulinum neurotoxin complex dissociates on active BoNT and complexing proteins with a half-life of under one minute. Once the toxin complex dissociates, complexing proteins are no longer of any significance in the occurrence of the clinical effect of BoNT. Studies by Al-Saleem et al. (2008)[17] proved that the toxin reaches general circulation without any evident structural or biological activity changes. General circulation acts as a toxin storage compartment until BoNT reaches its target cells. While in general circulation BoNT undergoes slight biotransformation, it is not accumulated in blood cells and mostly remains in its free active form. This concept of “general circulation - botulinum neurotoxin storage compartment” has been confirmed by many researchers. Fagan et al., 2009[18] described active BoNT/A presence in human blood serum 11 days after contaminated food ingestion, Sheth et al.,2008[19] - 25 days after disease onset, Delbrassinne

et al., 2018[20] - 29 days after the consumption of contaminated food. From the intravascular fluid compartment, the botulinum neurotoxin enters the extravascular compartment and then intercellular fluid.
When locally injected for therapeutic purposes, the botulinum neurotoxin is directly introduced into the extravascular compartment (or intravascular one if in a blood vessel) adjacent to target cells, thus bypassing the absorption stage. From the intercellular compartment, the botulinum neurotoxin should reach its target, peripheral cholinergic nerve endings, and bind to receptors there.
In order to better understand the binding mechanism of botulinum neurotoxin with receptors, a knowledge of normal neurotransmission in synapses is required.

Normal synapse neurotransmission

Neuromediators are synthesized in neuron cytosol and then stored in pre-synaptic nerve endings in synaptic vesicles. The Synaptic vesicle membrane contains a proton pump (vesicular ATPase), which increases intravesicular proton concentration when activated[8]. The electrochemical proton gradient ensures mediator influx from cytosol and its accumulation in such vesicles. The uptake of mediators within the synaptic vesicles is also regulated by receptors on the neuronal membrane, not only by the proton gradient. Mediator-containing vesicles are located in neuron cytoplasm and are bound to specific presynaptic membrane regions (active zones[21]) during so- called docking[22]. Vesicles are docked with cell membranes in active zones only and docking is controlled by numerous transport proteins[23].
When a nerve impulse arrives, the axonal presynaptic membrane is depolarized, calcium channels open and Ca^{2+} ions flow into the axon[24]. In response to the Ca^{2+} influx, the mediator- containing vesicle fuses with the presynaptic membrane in the active zone. This stage is called priming. It is regulated by two integral membrane synaptic vesicle proteins (synaptobrevin and synaptotagmin) as well as two presynaptic membrane proteins (SNAP25 and syntaxin), and cytosol proteins including complexin[25-28].
Rapid vesicle conformation changes caused by regulatory proteins result in full synaptic vesicle fusion with presynaptic membrane and pore formation, enabling neuromediator release into a synaptic cleft[29].
The neuromediator diffuses from its nerve terminal and binds to post-synaptic receptors that trigger post-synaptic cell signaling. In neuromuscular junctions, acetyl choline binds with its receptor on myocyte plasmalemma, resulting in muscle cell membrane depolarization. Membrane depolarization kicks off Ca^{2+} influx in myocyte and muscle contraction.
During neuromediator release the synaptic vesicle lumen opens temporarily into a synaptic cleft; later it internalizes in the nerve terminal during endocytosis. After endocytosis, the vesicle is filled once more with the neuromediator; this is followed by the start of the neurotransmission cycle[30].

BoNT/A binding with target cells

Active BoNT/A molecules bind with target cells via their receptors on cell surface[31]. In order to bind with the neuronal membrane, BoNT/A molecule must interact with a set of high and low affinity receptors[32]. Currently 3 receptors (polysyaloganglioside GT1b, fibroblast growth factor receptor 3, transmembrane vesicular receptor SV2) and several co-receptors have been described in such combination. Active neurotoxin molecule endocytosis and further changes are possible only when it binds with the entire receptor combination on the axonal surface[5]. Binding to one of the receptors without interaction with others does not induce toxin internalization.
This multistage process for BoNT/A binding with receptors accounts for a low BoNT/A concentration in circulating fluids, a high rate of extracellular flow around cells and a small axonal surface area.

First receptor - polysyaloganglioside

The first BoNT/A receptor on the neuronal surface is polysyaloganglioside GT1b (PSG). Gangliosides are glycosylated lipids contained in cell membranes. Though gangliosides are present in all tissues of vertebrates, they are more prevalent in neuronal membranes[33] where they are involved in optimal myelin production, axon-myelin interactions, peripheral and central axon stability[32].
PSG density on presynaptic membrane is high. PSGs are grouped as microdomains next to presynaptic membrane active zones[34]. The presence of PSG receptors in these zones is important for binding processes of botulinum neurotoxin with other receptors.
Oligosaccharide (BoNT-binding part) PSG projects quite far outside the membrane surface into a synaptic gap and is negatively charged[8]. The BoNT/A molecule is a dipole with a positively charged binding domain[3].
Such a difference in electric charge of the BoNT/A binding domain and PSG receptors (and other anion lipids on the axonal membrane) makes it possible to redirect BoNT/A molecule to the cell membrane, thus enhancing chances of receptor binding. Currently polysyaloganglioside are considered as initial binding regions, drawing the toxin from a relatively vast 3D extracellular fluid space into a 2D membrane surface one[5]. Therefore toxin binding must follow receptors. On the one hand, binding to PSG is irreversible since BoNT/A is extracted from ground substance and is fixed onto the axonal membrane. On the other hand, at this stage the toxin can still be affected and neutralizing antibodies can still reach it.
However, polysyalogangliosides are membrane receptors for both botulinum neurotoxin and human neuropathy-associated antiganglioside autoantibodies. Anti-PSG autoantibody production in neuropathic patients may induce diminished botulinum neurotoxin sensitivity and resistance development[35].

Second receptor - fibroblast growth factor receptor 3

The HC subdomain structure of botulinum neurotoxin type A is similar to basic fibroblast growth factor (FGF)[36]. This similarity enables BoNT/A high-affinity binding with protein fibroblast growth factor receptor 3 (FGFR3b) on a neuronal surface[37].
However, FGFR3b receptors bear an affinity not only to BoNT/A but also to multiple fibroblast growth factors. Moreover, this receptor affinity to growth factors exceeds the one to the botulinum neurotoxin. Native FGFR3 ligands - growth factors FGF1, FGF2 and FGF9 - compete for binding with FGFR3 and occupying receptors are able to jam BoNT/A absorption by cells[8].
FGFR3b receptor activity is regulated by several low-

affinity cofactors including heparansulfate, neuropilin-1, anosmin, etc[38]. Non-specificity and competitive binding of FGFR3 receptors with BoNT/A and fibroblast growth factors cofactor impact on receptor activity, which may explain the fragility of this receptor mechanism and, therefore, variable sensitivity to botulinum neurotoxin. Moreover, some FGFR3 mutation-related conditions (skeletal dysplasias, epidermal nevus, seborrheic keratosis, hyperinsulinemia) might demonstrate defective FGFR3 expression[39-43]. FGFR3 mutation influence on botulinum neurotoxin sensitivity is yet to be studied.

Third receptor - transmembrane vesicular receptor SV2

SV2 is a protein receptor located on the vesicular membrane[44] of all peripheral and central neurons as well as on the secretory granule membrane of endocrine cells[45]. SV2 is expressed on vesicular membranes in cells accumulating not only acetyl choline but also GABA, dopamine, glutamate, substance P and several other mediators[46].

Unlike polysyaloganglioside receptors expressed into a synaptic gap, the SV2-receptor BoNT/A- binding site is projected into synaptic vesicle lumen and is not approachable for the neurotoxin while such vesicle is in axonal cytosol[47] SV2 becomes reachable for BoNT/A at the time of vesicle fusion with presynaptic membrane and acetyl choline exocytosis[48].

Thus, BoNT/A binding with the entire receptor combination occurs in active zones only after synaptic vesicle fusion with the presynaptic membrane and the opening of vesicular lumen into the synaptic cleft, facilitating further BoNT/A endocytosis. After binding with the receptor combination and endocytosis, the botulinum neurotoxin can no longer be reached by neutralizing antibodies.

Endocytosis

BoNT/A molecule binding with receptors results in receptor-mediated endocytosis of both receptors and toxin[49]. The vesicular lumen has a neutral pH immediately after endocytosis. The vesicular ATPase proton pump controls mediator re-uptake[50] and injects protons into the synaptic vesicle, thus gradually decreasing vesicular lumen pH[51].

Light chain translocation

Vesicular medium acidification results in irreversible conformation changes to both heavy and light BoNT/A chains. With these changes, via receptors the heavy chain links to the vesicular membrane to form a transmembrane H-channel[52,53]. The conformation-altered light chain leaves the vesicle for cytosol[54] through the channel, resulting in the break-up of the chain-binding disulfide link. L-chain translocation occurs with a pH of between 4.5 and 6[55]. A pH decrease results in the protonation of carboxylated amino acid residues present in BoNT/A heavy and light chains. Carboxylated residues are located on one side of the toxin molecule and their protonation results in significant changes in molecular shape[55]. With its positively charged surface, the BoNT/A molecule interacts with the anion vesicular membrane surface to form a protein and lipid complex[56]. The L-chain is assumed to turn into a “molten protein globule”, thus assuming hydrophobic features[8]. On the one hand, L-chain hydrophobicity ensures its translocation via the H-chain-formed membrane channel. On the other, a lower pH molecular surface where the disulfide bond is located results in increased hydrophobicity. This ensures disulfide bond integrity until complete L-chain translocation. In order to cross the vesicular membrane, L-chain should maintain a disulfide bond with the H-chain throughout the entire translocation sequence[55]. Premature disulfide bond breakage at any stage before exit into the cytosol interrupts L-chain translocation[57].

At the end of translocation process, the disulfide bond is destroyed by the thioredoxin reductase- thioredoxin system, releasing a light chain to express its catalytic activity in cytosol[58]. The Thioredoxin reductase (TrxR) - thioredoxin (Trx) system is a main cellular redox system. TrxR and Trx are cytosol side proteins of the vesicular membrane and their inhibition may block BoNT/A action in stages in which the neurotoxin is beyond the reach of neutralizing antibodies[59]. In vitro experiments by Zanetti et al., 2015 showed that inhibitors for the TrxR-Trx enzymatic couple hamper L-chain protease activity for all known botulinum neurotoxin serotypes in cultured neurons. In vivo, they prevent toxin-induced paralysis in mice irrespective of botulinum neurotoxin serotype[60].

In life cycle model terms, disulfide bond reduction is the end of intracellular existence for the intact active BoNT/A molecule (holotoxin). Even if a light or heavy chain were to be exported beyond the cell, neither would be able to disrupt cell functioning. Only holotoxin can undergo multiple stages that result in conduction block[61]. On the other hand, conformation changes related to pH-induced L-chain translocation in cytosol create “a trap”, preventing both retrotranslocation into the endosome and active toxin molecule return in the extracellular environment[5].

L-chain cleaves transport proteins

The modified L-chain enters the neuron cytosol through the H-channel, where it behaves as a metalloprotease. It catalytically cleaves nine amino acids from the C-terminal of soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) for SNAP25 protein (SNAP25206) forming SNAP25197[62,63] Intact SNAP25 is required for mediator-containing vesicle attachment with further neurotransmitter release and it is also involved directly in Ca- channels activity regulation in the presynaptic membrane[64]. SNAP25 cleavage impairs mediator exocytosis, causing nerve impulse conduction block and muscle paralysis[65].

Synaptic activity is highly sensitive to the cleavage of minimal SNAP25 amounts. It was hypothesized that SNAP25 in neuron cytosol exists as various pools and that only small amounts of SNAP25 are actively involved in exocytosis and reachable for L-protease effects[66]. It was confirmed experimentally by showing that a cleavage of 10-15% of the total intracellular SNAP25 pool is sufficient for complete neuromediator release block[67-69]. L-protease cleavage of as little as 2-3% of SNAP25 pool results in the blockage of miniature post-synaptic cell potentials (weak depolarization of post-synaptic membranes at neuromuscular rest)[70]. Along with the SNAP25 proteolysis product, the SNAP25197 protein inhibits exocytosis on its own[71]. Meunier et al.[72] reported how SNAP25197 is able to persist for a long time while

in cytosol, as a component of the non-productive SNARE complex, thus prolongating BoNT/A effects. Conversely, the removal of several amino acids from SNAP25197 results in rapid exocytosis restoration.

Zinc metabolism and translocation

Zinc is necessary for light chain catalytic activity. One botulinum holotoxin molecule contains 1 zinc atom retained by the L-chain zinc-binding amino acid sequence and such binding is reversible[73].

Vesicle acidification causes the protonation of zinc-binding sections in the BoNT molecule. Translocation causes light chain denaturation, obliterating chelate site integrity. As a result, bound zinc dissociates and enters the cytosol zinc pool.

In their in vitro studies, Simpson et al.[74] demonstrated that zinc removal from the active botulinum neurotoxin molecule caused L-chain catalytic activity loss in cell-free samples. Activity in intact neuromuscular junctions is retained due to internalized toxin bound cytosol zinc. Thus, zinc retained by holotoxin (intact active molecule) is not the same zinc that is bound with the catalytically active light chain. Light chain binds cytosol zinc.

Mediator release block

The main BoNT/A target is peripheral neurons, where the botulinum neurotoxin inhibits acetylcholine release[75].

Many cell-based studies have shown that BoNT/A not only blocks acetylcholine release but also prevents the release of numerous other neuromediators if they are accumulated and stored in vesicles[32]. These neuromediators are as follows: epinephrine, norepinephrine, dopamine[76,77], glutamate[78], glycine[79], serotonin[80], substance P[81], etc. Therefore, the botulinum neurotoxin is to be considered not as a specific acetylcholine release inhibitor, but rather as an exocytosis blocker for various mediators that offers tremendous promise for the treatment and prevention of various disorders.

Conclusion

Further studies of the pharmacological mechanisms unique to the botulinum neurotoxin are quite promising in the search for ways to influence its effects: to extend/shorten the duration of its action, to increase/decrease BoNT sensitivity in specific patient populations. This will also help to develop protocols for optimal combinations of botulinum neurotoxin with esthetic medicine procedures of all kinds. Better insights into multiple aspects, not only of BoNT neuronal selectivity but also of BoNT/A interaction with non-neuronal cells, will lead to the development of new therapeutic applications of botulinum neurotoxin-based agents in various areas of medicine.

Conflict of interest

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aesthetic medicine

Narrative Review

Introduction to Polynucleotides Highly Purified Technology

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Polynucleotides, as ubiquitous molecules, are physiologically distributed in all tissues. Endogenous polynucleotide-like derivatives are physiologically released in the extracellular space by damaged or dying cells and in conditions of hypoxia[1-3].

Exogenous polynucleotides are extracted from the gonad DNA of trouts bred for human consumption, and purified with high-temperature sterilizing procedures to obtain a pure ingredient without pharmacologically and allergically active protein contaminants[1].

Thanks to the advanced procedures adopted, the highly purified polynucleotides discussed in the chapters of this document are referred to using the acronym PN-HPT™ (Polynucleotides Highly Purified Technology).

An Italian company, Mastelli Srl, patented PN-HPT™ technologies and introduced the first medical devices based on PN-HPT™ from trout gonad DNA in Italy in 2004. PN-HPT™-based medical devices based on Mastelli's top-standard biotechnologies, refined over 60 years, are nowadays distributed in over 30 countries all over the world. High-tech PN-HPT™ purification procedures eliminate all risks from protein contaminants. Mastelli is the first company to control the entire production chain, in compliance with world-class GMP and QA standards, from trout breeding and PN-HPT™ purification to shelf-ready PN-HPT™-based medical devices. The evolution of PN-HPT™ devices has been steady over the years, up to the Newest® (patent EP 2 407 147 B1 - Composition with bio-regenerative, restorative and eutrophying activities). In the short term, strongly hydrophilic PN-HPT™ have both tissue hydrating and viscoelastic effects that are similar to those of hyaluronic acid (HA); in the long term, PN-HPT™ primes wounded, atrophic or wrinkled tissues to improve the production of new collagen and elastin fibers and new dermal matrix glycosaminoglycans. Their overall effect is to promote the viability of cells like human fibroblasts and other mesenchymal derived cells, including chondrocytes in cartilage, among others[4,5].

The most recent studies steadily support the favorable effect of PN-HPT™ as an enhancer of fibroblast proliferation and viability (*Figure 1*)[6].

New investigations also continue to confirm the ability of PN-HPT™ to rapidly enhance the production of collagen fibers (*Figure 2*)[6].

By binding large amounts of water and the re-orientation and coordination of water molecules, PN-HPT™ reorganizes its structure and forms a 3-D gel. The concentration in this 3-D gel of PN-HPT™ chains and their high molecular weight are key factors, leading to high viscosity. After tissue infiltration, these natural-origin polymers have a filling effect and strongly moisturize tissues. Their physiological degradation leads to the progressive release of water molecules and smaller-sized oligonucleotides that retain the moisturizing and viscoelastic properties of PN-HPT™ progenitors, prolonging PN-HPT™ effects

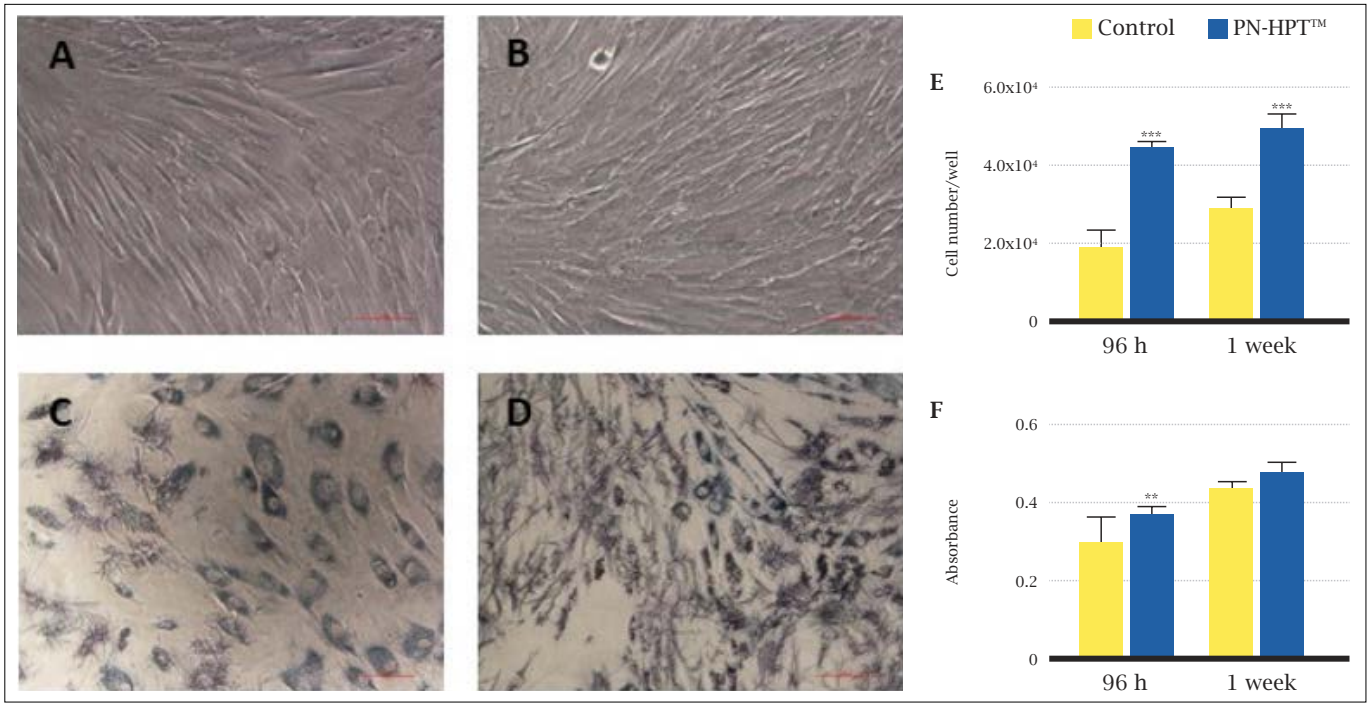

Figure 1 *- Phase-contrast microphotographs (magnification, 20X) of primary human fibroblasts cultured in-vitro for one week without (B) or after exposure to PN-HPT™ (100 µg/mL) (A). After the addition of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] reagents, the density of viable fibroblasts, revealed by formazan blue needle-shape precipitates (enzymatically reduced MTT), appears higher after PN-HPT™ exposure (D) compared to controls unexposed to PN-HPT™ (C). Viable cultured fibroblasts, assessed by Coulter Counter, after 96 h and 1 week (E): viable fibroblasts exposed to PN-HPT™ (blue column) are significantly more numerous compared to unexposed control fibroblasts (yellow column) (***, p< 0.001). Formazan deposits (absorptiometry) after 96 h and 1 week (F): fibroblasts viability significantly higher for PN-HPT™- exposed cells after 96 h (blue columns) compared to unexposed controls (yellow column) (**, p< 0.01) [6].*

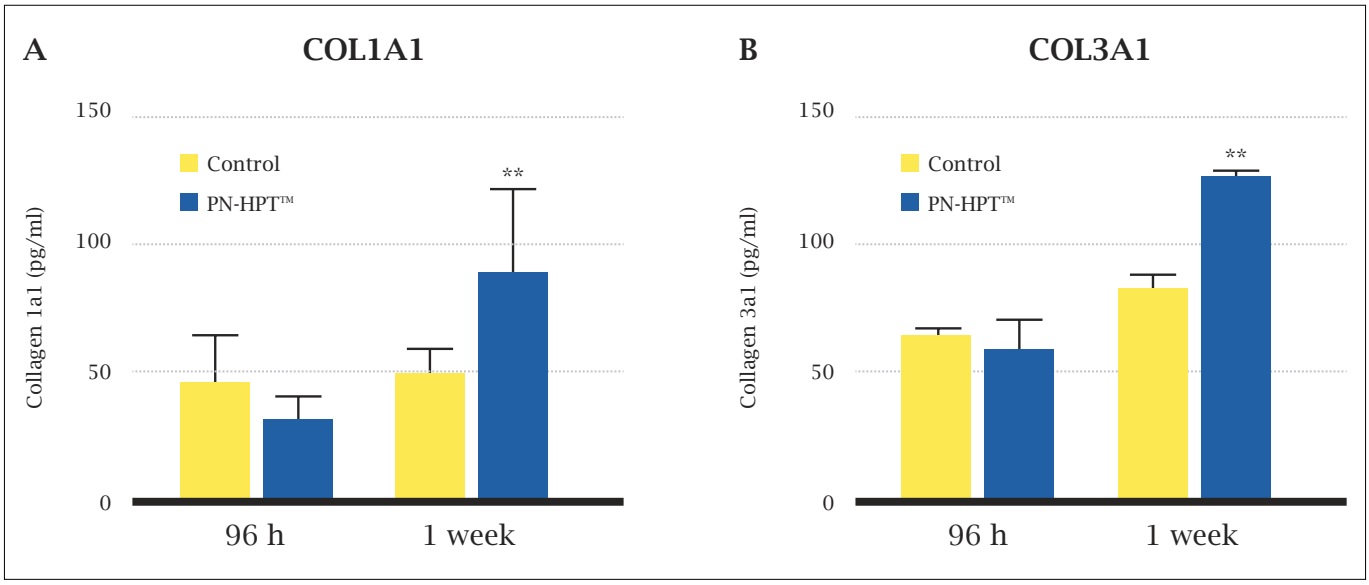

Figure 2 *- Expression of collagen 1a1 mRNA and collagen 3a1 mRNA in the supernatant of cultured primary human fibroblasts exposed or unexposed to PN-HPT™ (100 µg/mL) in the culture medium; assessments after 96 h and 1 week (**, p< 0.01) [6].*

over time[7,8]. All these properties are associated with a lack of clinically significant side effects and unpleasant sequelae, overall high safety and high compliance in treated subjects. This is well shown by the experience in hundreds of thousands of individuals so far treated all over the world with PN-HPT™ devices - with the exclusion, as always advisable, of any other infiltrative treatment in the same session - and no more than occasional transient erythema and wheals[9].

Hyaluronic acid (hyaluronan, HA) derivatives may act as a suitable pro-trophic benchmark for comparisons, including in severe conditions such as burns and non-healing wounds. Like PN-HPT™, HA derivatives can also promote healing in burns and chronic wounds by increasing the deposition of new collagen and elastin fibers; collaterally, HA enhances the hydration of the extra-cellular matrix, a key prerequisite for fibroblast trophism[10].

A crucial point, with the aim in mind of devising an ideal pro-trophic strategy, is the demonstrated interaction between PN-HPT™ and HA. In-vitro studies show that concomitant exposure to PN-HPT™ and HA is associated with a dose-dependent increase of PN-HPT™ activity of up to 20%[1]. The accelerated growth of dermal fibroblasts after exposure to 320 µg per mL of PN-HPT™ alone is indeed similar to the accelerated cell growth induced by PN-HPT™ at the much lower dose of 64 µg/mL with the addition of HA (1,000 µg/mL) (*Figure 3*)[1].

The clinically significant point is the weaker pro-trophic effect of HA compared to PN-HPT™. The maximal increase of fibroblast proliferation on dermal fibroblasts (+14% vs. controls) is attained only at very high HA doses (1,600 µg/mL); the same acceleration of fibroblast growth is achieved with the more powerful PN-HPT™, at a much lower range of doses (64 to 320 µg/mL)[1]. Because of this differential pro-trophic activity with HA, PN- HPT™ might indeed belong to a different pro-trophic class compared to HA. This may be true not only for critical situations like chronic wound care, but it is also likely to have a significant impact in all situations where a strong pro-trophic effect is useful and actively sought, including

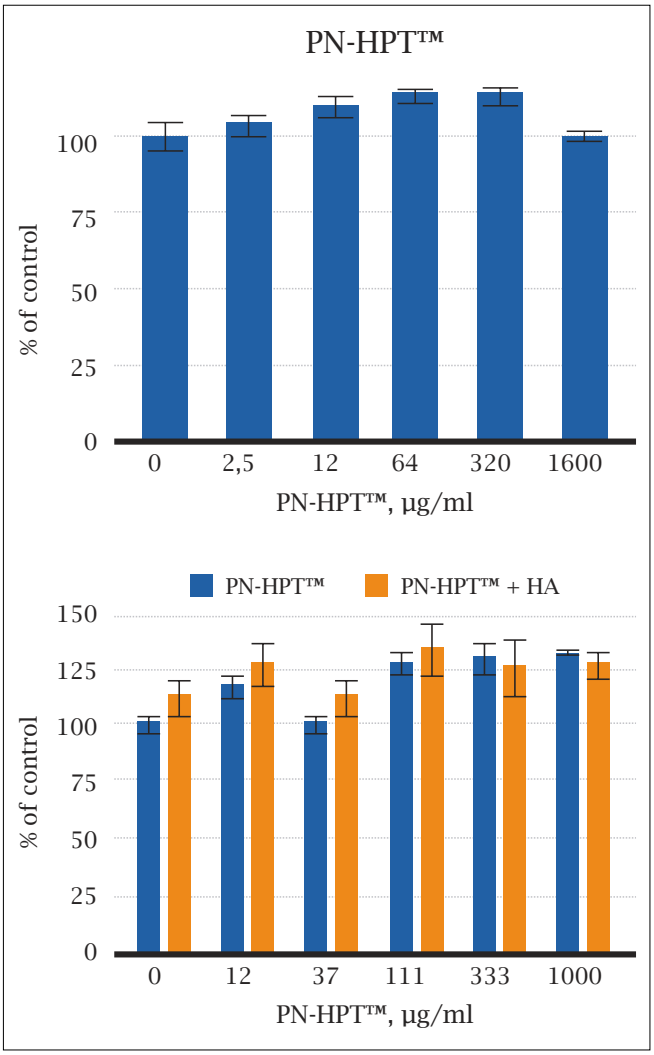

Figure 3 *- Dose-response effect of PN-HPT™ doses increasing between 0 and 1600 µg/mL on proliferation and viability of cultured dermal fibroblasts; MTT assay for cell viability and proliferation performed after 3 days of exposure to PN-HPT™:*
- Upper graph: PN-HPT™ alone.
- Lower graph: with or without co-exposure to fixed-dose HA [1].

many conditions in esthetic medicine and surgery[1]. The observation that PN-HPT™ might belong to a different pro-trophic class compared to HA gives a rationale for the clinical "PN-HPT™ priming" concept, well documented in the "Polynucleotides Highly Purified Technology in the biorevitalization of postmenopausal labia major" section and supported by an expanding body of clinical evidence, as shown in the following sections. The text box at the end of this section summarizes the concept.

Polynucleotides and the "PN-HPT™ priming" concept

Based on previous considerations, the key of the "PN-HPT™ priming" concept is letting the main pro-trophic biorevitalization effort to fall on the more effective PN-HPT™, which have been preliminarily infiltrated in non-healing wound care and other indications in hundreds of thousands of individuals. PN-HPT™ should be administered earlier than other agents or instrumental treatments, to leverage their strong "tissue priming" effect and reactivate the metabolism of fibroblasts and other cells derived from primitive mesenchyme. This sort of preliminary metabolic plowing of the extracellular matrix, which re-activates the synthesis of the ground substance and the collagen, reticular and elastic fibers of the sub-epithelial and sub-mucosal connectives, aims to help the second-step efficacy of other agents like HA and other treatments. With specific reference to HA as an ideal pro- trophic benchmark due to its biological activities, HA synergizes and enhances the PN-HPT™ effect. At the same time, during the second-step of the PN-HPT™ / HA combination treatment, HA enhances the hydration of the extra-cellular matrix, a crucial condition for fibroblast trophism[1,10].

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aesthetic medicine

Narrative Review

Polynucleotides Highly Purified Technology and nucleotides for the acceleration and regulation of normal wound healing

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Cutaneous wound healing is a physiological process involving the collaboration of numerous cell strains and cell products[1,2]. Inflammation and cell proliferation, the two first stages of wound repair, are followed by the synthesis of elements making up the extracellular matrix. The final step is remodeling, although it should be noted that all these stages are not mutually exclusive and may overlap over time[3].

Wound bed preparation, defined as "the management of a wound in order to accelerate endogenous healing or to facilitate the effectiveness of other therapeutic measures", is the gold standard, enabling clinicians to identify and break local barriers to wound healing[4,5]. Constantly assessing wound bed evolution should drive the selection of treatments and dressings at each step.

This consideration led a group of wound care experts to develop the "TIME" protocol, a guide to wound bed preparation[6,7].

The concept summarized in the "TIME" acronym (tissue debridement, infection and/or inflammation, moisture balance, edge effect) was conceived in the early 2000s and has since been considered the ideal tool for effective wound bed preparation[6,7]:

T (Tissue debridement) - If there is non-viable tissue, necrosis, slough or eschar, identify the best type of debridement that would be best suited for the patient (enzymatic, autolytic, surgical, mechanical, etc.) in order to isolate the any tissue that is still viable.

I (Infection and/or inflammation) - creates a barrier to healing. In the event of infections, antibiogram-guided systemic antibiotics will be crucial, and local antimicrobials will assist at the local level.

M (Moisture balance) - Essential for positive outcomes in wound healing and translating into the practice of "moist wound healing", adding moisture if the wound is too dry or desiccated. Conversely, if the wound is too moist or macerated, with maceration at wound edges and around the wound, the wound dressing should facilitate drainage, moderate to heavy.

E (Edge effect) - When healthy as a marker of sound wound management, wound edges appear attached, open, and migrating or contracting.

Granulation tissue on the wound bed, the expression of a dense network of blood vessels and new capillaries looping together on the wound surface, is the marker of sound wound healing.

Newly formed epithelializing cells, which slowly migrate from the wound edges over the granulation tissue until the wound is closed, provide a natural barrier and marks the beginning of the final stages of the wound healing process[1-3].

Polynucleotides Highly Purified Technology in wound healing

Highly purified, natural-origin polynucleotides (PN-HPT™, Polynucleotides Highly Purified Technology) associate lenitive and hydrating properties with a strong trophic activity on dermal fibroblasts, based on the replenishment of tissue reservoirs of purines, pyrimidines, nucleotides and nucleosides (see also section "Introduction to Polynucleotides Highly Purified Technology"). Physiologically, such derivatives are released in the wound bed, where they promote fibroblast activation and healing, due to local hypoxia and damaged or dying cells[8,9]. Hyaluronic acid (hyaluronan, HA) derivatives also accelerate healing in burns and chronic wounds by increasing the deposition of new collagen and elastin fibers. Collaterally, HA enhances the hydration of the extra-cellular matrix, a key prerequisite for fibroblast trophism[10,11].

PN-HPT™ and HA are the two most active natural-origin agents activating wound healing, for the first time patented and associated in the same sterile medical device to facilitate the uncomplicated healing of skin wounds and ulcers. PN-HPT™ from trout gonads and hyaluronic acid, both highly concentrated, are commercially available as a sterile, viscoelastic, isotonic gel formulation for topical application in prefilled single-use glass syringes (Nucliaskin S®, Class III CE 0373 Medical Device, Mastelli Srl, Sanremo, Italy; 2 mL - patent EP 2 407 147 B1 - Composition with bio- regenerative, restorative and eutrophying activity).

The therapeutic scope of such innovative combination is extensive, including the uncomplicated healing of burns, diabetic foot and ulcers, as well as skin lesions due to venous insufficiency, trauma and neuropathic disorders. PN-HPT™ can also be used to facilitate the adhesion of biological membranes, skin substitutes and autografts. Several studies suggest that the combined PN-HPT™ / HA strategy enables more rapid, efficient, and complete wound repair, free from side effects, compared to the topical application of hyaluronic acid alone. The text box illustrates two recent examples of such studies.

Two-center study in 39 patients above the age of 20 years with lower limb venous ulcers, randomized (after surgical debridement) to topical application of either the proprietary PN-HPT™ / HA combination gel (n=20) or HA gel (n=19) twice weekly for 6 weeks. Endpoints: number of healed ulcers and variation in total area of ulceration after 45 days (*Figure 1*)[12].

- Complete ulcer healing: 60% and 22% of patients respectively,
- Average area reduction: 67% vs. 34% respectively.

Prospective cohort study in 50 patients with unhealed diabetic ulcers of feet and lower limbs (lesions for more than 6 months), treated with topical application of the proprietary PN-HPT™/ HA combination gel once or twice weekly (according to the patient's clinical needs) after surgical debridement. Endpoints: number of healed ulcers, time to complete healing, variation in total area of ulceration) (*Figure 2*)[13]:

- Complete ulcer healing: 71% of patients,
- Average area reduction: 77%,
- Mean time to healing: 6.6±1.8 weeks.

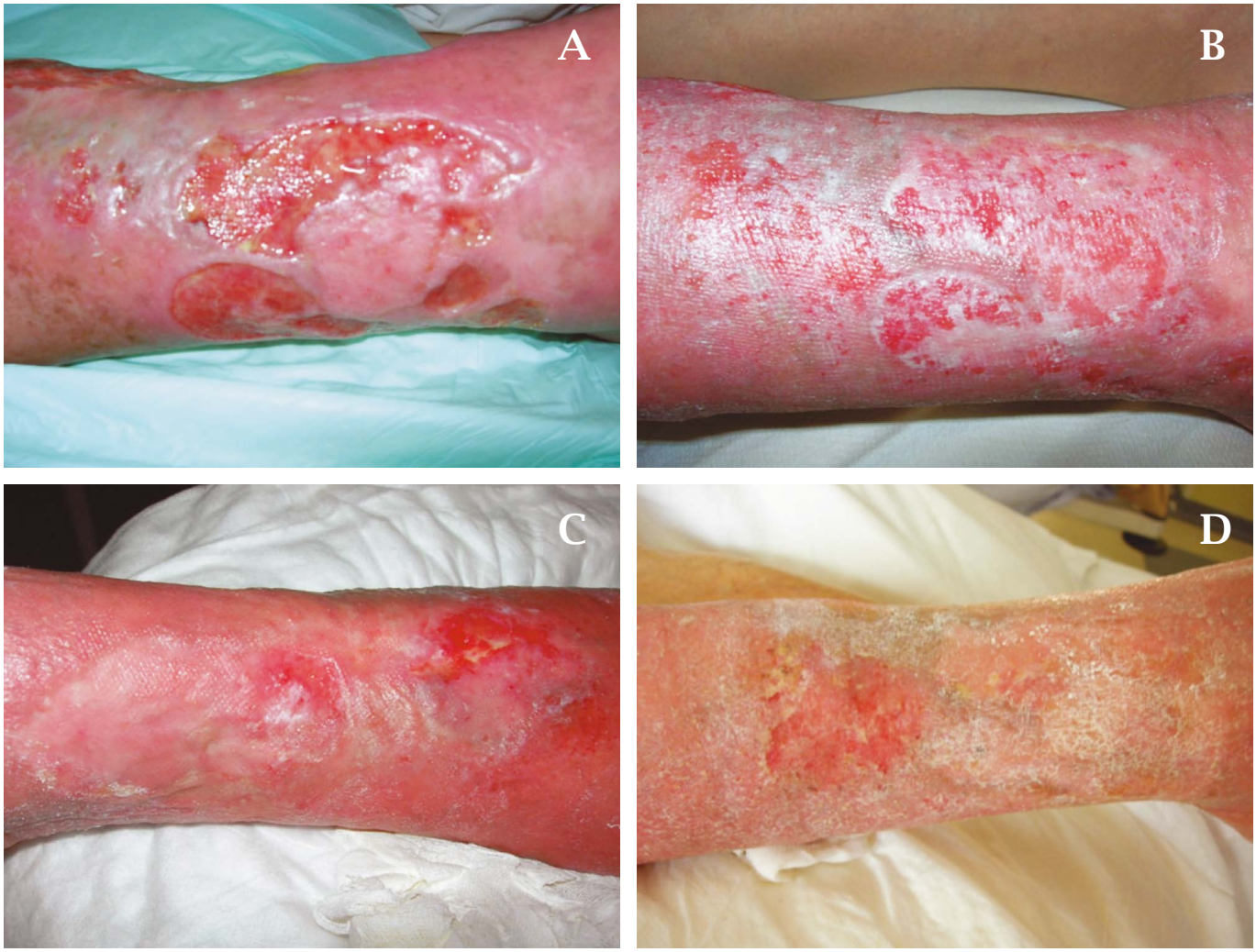

Figure 1 *- Class-6 (CEAP classification) venous insufficiency leg ulcers associated with venous reflux, ankle brachial pressure index >0.9, and more than 50% granulation tissue on the wound bed. Procedure at each treatment session (twice weekly for six consecutive weeks): cleaning with saline solution followed by topical application of the PN-HPT™ / HA combination (Nucliaskin® S viscoelastic gel formulation) as adjuvant treatment; lastly, standard sterile dressing of the wound - (a) Clinical picture of leg ulcerations at baseline; (b) evolution after 15 days; (c) after 30 days; (d) after 45 days. Reproduced with permission from the authors; CEAP: Clinical-Etiological-Anatomical-Pathophysiological[12].*

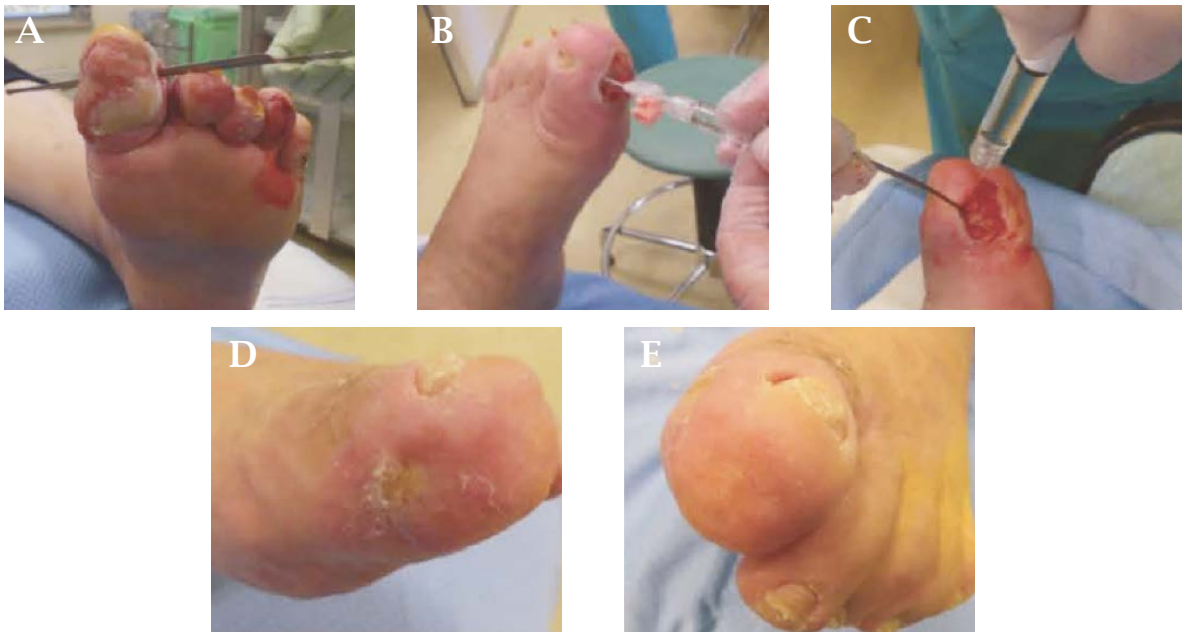

Figure 2 *- Application of the Nucliaskin® S proprietary PN-HPT™ / HA combination in a patient with severe diabetic foot - (a) Baseline clinical picture; (b) and (c) application technique; (d) and (e) final clinical assessment. Reproduced with permission from the authors[13].*

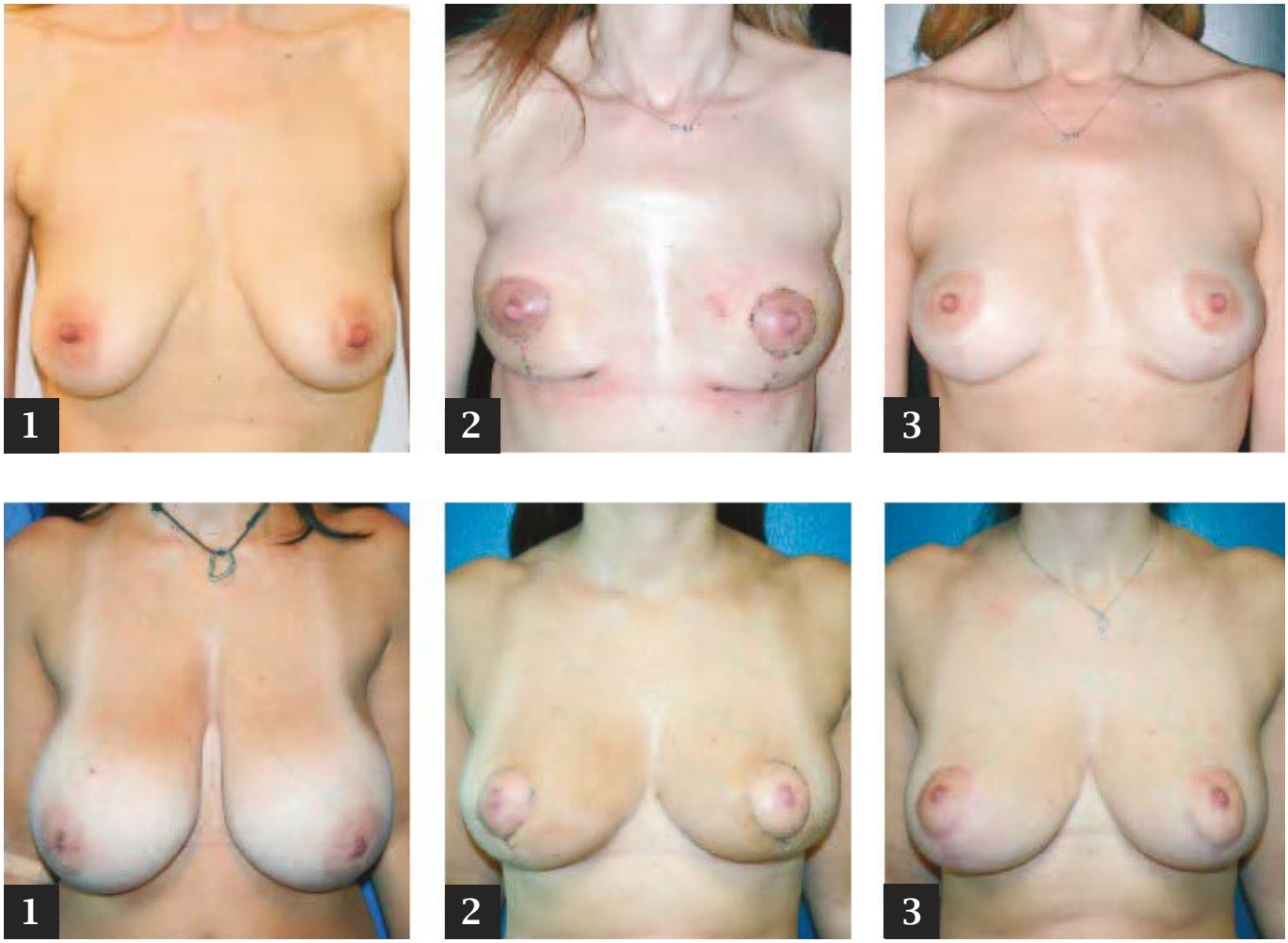

Figure 3 *- Mastopexy (upper three photographs) and breast reduction surgery (lower three photographs) - (1) before surgery, (2) immediately after removal of the non-suture closure of wounds 21 days after surgery, (3) after 3-month application of the commercial Makeskin® nucleotide-based proprietary cream twice daily for 3 months. Reproduced with permission from the authors[20].*

Natural-origin nucleotides as regulators of wound healing with lack of scar hypertrophy and keloids

The strong lenitive properties of nucleotides also act as a brake on inflammation in the development of hypertrophic scars and keloids. Once again, some hyaluronic acid may usefully support nucleotides thanks to its proven anti-inflammatory properties[14]. Other agents known to interfere with scar overgrowth can also be associated with nucleotides. In blinded studies, onion (Allium cepa) extract, for instance, has been repeatedly shown to improve scar softness, redness and texture, both by objective (Vancouver Scar Scale, Image Panel Scale) and subjective (Body Image Scale, Cosmetic Scale) scar assessment[15-17].
For the control of hypertrophic scars and keloids, nucleotides are commercially available as a cream, together with natural hyaluronic acid (1%), onion extract (10%) and other supportive lenitive agents such as osmoprotectant and methyl group donor betaine [18] and antioxidant vitamin E (1%)[19].
Clinical experience repeatedly confirmed the efficacy of nucleotides in the control of post-surgical scars, for instance in a study in 70 women (mean age, 36.4 years) who underwent mastopexy or breast reduction surgery[20]. The commercial nucleotide-based proprietary cream was applied twice daily for 3 months at the site of surgical incisions, in combination with massotherapy, after removal of the non-suture closure of wounds. *Figure 3* illustrates two examples of the good final esthetic outcome, with softness and lack of surgical scar pain[20].

Suggested treatment procedures with PN-HPT™ (Nucliaskin® S) and nucleotides (Makeskin®)

Open wounds and ulcers: PN-HPT™ / HA-based control of appropriate skin healing (sterile viscoelastic gel)

At each session, when the ulcer is in the final "E" phase of the TIME protocol, ulcer cleaning with saline solution, followed by topical gel application and standard sterile dressing.
The length of treatment is variable according to the clinical situation. Based on available clinical data, the suggestion is for 2 weekly topical applications for at least 6 weeks.

Closed wound: topical nucleotide-based cream prevention of scar overgrowth during the remodeling phase of wound healing

Twice daily applications with light massage until complete absorption.

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