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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 consumption 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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References

Citing AMA guide website. http://libguides.stkate.edu/content.php?pid=99799&sid=749106. Updated April 2011. Accessed October 24, 2012.

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EDITORIAL

In modern years, aesthetics has become quite important in every aspect of everyday life: following the hundreds of journals, magazines, blogs and websites pointing their attention towards this interesting and fascinating topic, the request for aesthetic medicine has increased manifolds.

Aesthetic Medicine is a new field of medicine, in which different specialists share the aim of constructing and reconstructing the physical equilibrium of the individual. Treatment of physical aesthetic alterations and unaesthetic sequel of illnesses or injuries, together with the prevention of aging, are perhaps two of the most iconic areas of intervention for Aesthetic Medicine. However, in order to prevent frailty in the elderly, a program of education is similarly important. Furthermore, the line between health and beauty is extremely thin: psychosomatic disorders resulting from low self-esteem due to aesthetic reasons are frequent and cannot be ignored by a clinician.

It is therefore clear that there is no figure in the field of medicine which is not involved in Aesthetic Medicine: endocrinologists, gynecologists, angiologists, psychologists and psychiatrists, plastic surgeons, dermatologists, dieticians, physiotherapists, orthopedists, physical education instructors, massophysiotherapists, podologists, and rehabilitation therapists are just some of the specialists who are sooner or later going to have to answer their patients' needs for aesthetic interventions. The involvement of all these specialists fits the description of health as defined by the WHO: "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" for which, undeniably, a team of different physicians is required.

The number of patients requiring medical consultation for esthetic reasons is rapidly increasing: in order to be able to provide adequate feedback, medical and paramedical specialists should be trained and, more importantly, should be taught how to work together. Existing Societies of Aesthetic Medicine from different countries share the aim of creating such teams and provide constant updates to the literature: the creation of an international network of specialists from all around the world under the flag of Aesthetic Medicine represents a challenge, but at the same time it is the proof of the widespread interest in this topic.

The first issue of this Journal represents the results of the efforts of the many national Societies and of the *Union Internationale de Médecine Esthétique,* now together as one; it is our hope that in years to come this Journal might improve our knowledge in this field, and provide adequate scientific advancement in the field of Aesthetic Medicine.

Francesco Romanelli, MD
Editor-in-chief
Associate Professor at "Sapienza"
University of Rome

EDITORS' NOTES

Aesthetic Medicine, the booming medical activity

Aesthetic Medicine was born in France 40 years ago. The French Society of Aesthetic Medicine was the first of its kind in the world, followed by Italy, Belgium and Spain. Starts were rather difficult as aesthetic procedures in those early years were only surgical. At that time aesthetic doctors and cosmetic dermatologists had very few real medical procedures to offer to their patients for treating aesthetic problems on face and body.

At the beginning of the '80s, viable medical procedures started to emerge in Europe for aesthetic and cosmetic purposes. Mostly, at that time, they were imported from the United States: those included collagen injections for wrinkles (Zyderm by Dr. Stegman), and chemical peels (phenol by Dr. Baker, TCA by Dr. Obagi). But, subsequently, European research on Aesthetic Medicine gained momentum. Hyaluronic acid appeared on the market, as it was discovered that it could be used as a dermal filler for wrinkles.

During the '90s, the use of lasers offered aesthetic doctors and cosmetic dermatologists new possibilities. The "beam revolution" started with CO2 laser for facial resurfacing. Today, CO2 resurfacing is not used as much anymore, because of the long and difficult post-op. CO2 laser was replaced with the gentler Nd-YAG and Erbium lasers and more recently with non-invasive photonic devices for facial rejuvenation, including IPL, US and radiofrequency. These new technologies allow today's aesthetic doctors and cosmetic dermatologists to offer their patients procedures with low risk of post-op complications.

Then, Botulinum Toxin has "invaded" both sides of the Atlantic Ocean. Today, Botox injections are the most popular treatment for facial expressive wrinkles. Botox injections are now so common everywhere that many cosmetic surgeons have given up their bistouries for syringes.

Last but not least, development in Aesthetic Medicine is shown by mesotherapy and adipolipolysis. About lipolysis, new data and recent publications have explained that radiofrequency, ultrasounds and cryolyse could have positive action to dissolve fat and to improve some unaesthetic disorders like cellulite. These non invasive procedures intend to replace the surgical liposculpture with success.

Nowadays, Aesthetic Medicine has the necessary tools to address all major disorders within the aesthetic field.

After 40 years, Aesthetic Medicine is now active in 27 countries in the world (France, Italy, Spain, Belgium, Morocco, Poland, Russia, Switzerland, Romania, Kazakhstan, Algeria, Brazil, Argentina, Uruguay, Venezuela, Colombia, Chile, Mexico, U.S.A, Canada, South Korea, and recently Ecuador, China, South Africa, Turkey, Ukraine and Georgia). All 27 national Societies are members of the *Union Internationale de Médecine Esthétique* (U.I.M.E.).

Aesthetic Medicine is taught in 8 countries (France, Italy, Spain, Brazil, Argentina, Mexico, Venezuela, Kazakhstan) in universities that deliver UIME's diplomas after 3 to 4 years of studies.

What is the future of Aesthetic Medicine?

In the last few decades, patients' desires to look and feel younge, have fueled Aesthetic Medicine and Cosmetic Dermatology: many different procedures have been developed to satisfy the demands.

As life-span have increased, patients today are not only asking about aesthetic procedures, they are also asking for a way to stay in good physical conditions in the last decades of their lives.

As a direct result, Anti-Aging Medicine, which covers skin aging and general aging, has recently emerged and expanded very quickly.

Anti-Aging Medicine can offer senior patients better nutrition, dietary supplementation with vitamins, minerals, antioxidants, and eventually hormone replacement therapy, but only when needed.

Today, and in the near future, both Aesthetic Medicine and Anti-Aging Medicine will offer to our patients, who now live longer, better wellness with aesthetic treatments for skin aging and anti-aging treatments for general aging.

Aesthetic Medicine is booming, but all medical practitioners should be correctly trained, so its future will be bright.

Jean-Jacques Legrand, MD
General Secretary of UIME

Aesthetic Medicine: a bioethic act

When in 1977 the Italian Society of Aesthetic Medicine published the first issue of the magazine "La Medicina Estetica" Carlo Alberto Bartoletti, the Founder, wrote an editorial in which traced the pathway of the discipline and of the Scientific Society, still valid and projected into the future.

Today from that Editorial Board arise an International Journal, which wants to be indexed, in order to give to the doctors practicing Aestehetic Medicine all around the world a solid basis of shared knowledge.

In the late '60s, what was called in Italy Aesthetic Medicine, moved its first steps thanks to "remise en forme and anti aging projects" imported from the experience the "Institutul de geriatrie Bucuresti", directed by Dr. Ana Aslan.

For this reason,there is the bioethical imperative that the Discipline should be first prevention, then return to physiology and finally correction.

The worldwide diffusion and the efforts of Industries born on the wave of the phenomenon have often led to choose the fastest route to achieve and maintain the physical aspect in the myth of beauty at all costs, without considering that aesthetic is not synonymous of beauty, but it is a balance between body and mind, and the role of the doctor is to take care of the Person globally and not only focusing on the correction of "a badly accepted blemish".

Faithful to the teaching of my Master had almost 50 years ago, this new journal will have the task of elevating the human resources, aligning and validating methodologies, but above all affirming the *humanitas* of the medical art in its purest sense to pursue the good and the graceful for the person who relies on it.

Fulvio Tomaselli, MD
Honorary President of the Italian
Society of Aesthetic Medicine

Aesthetic Medicine needs science. All over the world.

All Aesthetic Doctors know that science is the basis for safety. Safety is the most important issue in our discipline.

Unfortunately, Aesthetic Medicine is more often surrounded by marketing than by science, despite the hard work done by Scientific Societies all over the World. And, too often doctors working in this field are dealing with sellers that promote products with insufficient scientific studies. However, they sell it anyway. I think that doctors must learn that the first thing to ask about a medical device is the scientific background regarding that product: patients treated, follow up period, adverse events and, most of all, publications.

With this new International Journal completely dedicated to Aesthetic Medicine, proposed by the Italian Society of Aesthetic Medicine, endorsed by UIME and shared by all the National Societies of Aesthetic Medicine belonging to UIME, World Aesthetic Medicine wants to stimulate scientific production in this discipline to increase safety and quality in aesthetic medical procedures.

Another important goal of the Journal is to catalyze the proposal of new protocols and guidelines in Aesthetic Medicine, with the consensus of the entire Aesthetic Medicine Scientific Community.

What this Journal should achieve in the near future is to improve the number and quality of scientific production in Aesthetic Medicine, in order to allow this discipline to grow in the field of evidence based medicine, not only in the rationale field.

I hope this can be the start of a new era for Aesthetic Medicine, with the commitment of all Scientific Societies all over the world.

Emanuele Bartoletti, MD
Managing Editor
President of the Italian
Society of Aesthetic Medicine

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In vivo HPLC tissue concentration study of three drugs (caffeine, biotin and procaine) administered by microneedling, electroporation and both techniques combined

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ABSTRACT

Topical application of substances for therapeutic or aesthetic goals has been used since the dawn of time. In beauty treatments, skin is the final organ for product action; transcutaneous absorption of the active ingredient is consequently unnecessary. However, to achieve general therapeutic effects, topically-applied substances have to overcome a series of barriers that make their passage to the blood flow difficult. To increase transcutaneous penetration of active ingredients without causing skin damage, we can use physical permeation systems, such as microneedling (MN), electroporation (EP) and the combination of the two methods (MN+EP).

In this study carried out on pigs, high pressure liquid chromatography (HPLC) was used to analyse the skin concentrations of 3 standard medicines (caffeine, procaine and biotin) at various post-administration points.

The results showed concentrations of these substances as late as 7 and 14 days after their administration. Whether these concentrations reach therapeutic levels are yet to be determined.

Keywords
microneedling, electroporation, HPLC

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Introduction

Skin is a heterogeneous membrane having lipophilic surface properties and hydrophilic properties in the deep layers. The stratum corneum functions as a barrier, given that its structure helps to stop water loss from the underneath and protect against aggressions produced from the outside. It is composed of some 15-20 layers of dead cells joined together by a continuous interstitial lipid phase. This stratum is lipophilic, with a water content of approximately 13%. Viable epidermis, represented by (from the most superficial to the deepest layer) the granulosumn, spinosum and basale strata, has significantly higher hydrophilic properties and a water content of more than 50%; in the dermis, the water content exceeds 70%, which facilitates the passage of hydrophilic substances. According to it, highly lipophilic substances accumulate in the stratum corneum, highly hydrophilic ones remain on the skin surface and amphiphilic structures (micelle-type) can pass through the epidermis, depending on their molecular weight and the volume of the administered active agent.

Passive diffusion of any topically-applied substance on the skin can follow various pathways. The transcellular route makes multiple cell entrances and exits necessary, penetrating cell membranes, for which the substance has to diffuse through inter- and intracellular stages; the intercellular route avoids the transmembrane step, following through the lipid stage; while a third pathway, the transappendageal or shut route (through the pores of sweat and sebaceous glands) is of little significance[1]. The route through hair follicles is the most important electro-osmotic route[2]. The relative importance of each penetration route depends on factors such as the molecular weight, solubility and logarithmic acid dissociation constant (pKa), among others.

There are two different components in the stratum corneum: one rich in proteins, constituted by dead cellular elements (keratin); and another, the intercellular component, rich in lipids. They are set up forming a continuous laminar structure of lipids and watery areas, which makes diffusion of polar and non-polar substances more difficult.

There are also poorly hydrophilic compounds, with mainly hydrophobic fractions, in the stratum corneum[1]. The proportions of both stages and the stratum corneum thickness determine resistance to the transdermal passage of topically-applied substances over the skin surface. This causes important differences in absorption depending on, for example, the skin area where the product is applied. Skin penetration criteria are consequently established in a descending order: genitals > head > trunk > limbs.

The physical and chemical properties of the active ingredient that determining its skin penetration are its molecular mass, its size, the hydrophilic-lipophilic balance, the distribution of polar and non-polar parts within the molecule and the ionisation state[3]. These parameters greatly influence solubility and partition coefficient: the molecules with an intermediate partition coefficient (with log P values between 1 and 3) are sufficiently soluble in the stratum corneum lipids to permit their passage, while being sufficiently hydrophilic to reach the deepest layers of the epidermis. Molecular mass and molecule size also affect the diffusion coefficient and melting point (and consequently solubility); optimum permeability is reached using low molecular mass molecules (ideally less than 500 Da), which especially limits the use of proteins and peptides[4].

Various procedures have been proposed to increase skin permeability. Noteworthy among them are permeation-enhancing agents, electric currents in iontophoresis or electroporation, micropore formation using needle matrices in hand units or patches and pressure waves generated by ultrasound, radiofrequency or thermal ablation from different types of laser. All of these techniques have the goal of increasing the passage of molecules, especially drugs, through the skin to act directly at lower dosages than when other pathways (oral, intramuscular, intravascular, etc.) are used. This avoids the prior step of enterohepatic circulation that metabolises a great part of the dose administered; passing through the enterohepatic stage, it involves, using higher dosages, greater risk of iatrogenicity and secondary effects that sometimes make it impossible to continue treatment or increase its cost because it becomes necessary to use other drugs concomitantly to counteract these collateral effects. However, in spite of all this, *in vivo* experimental and clinical studies are needed to establish the potential applications of skin diffusion[5].

Current view of the skin as a drug delivery pathway: transdermal routes

Skin is the largest organ in the human body, with a surface of some 2 square meters[3]. Its role is to protect against physical, chemical and biological aggressions from the surroundings. However, it is also an important source of external sensory stimuli that contribute to the "socialisation" of the human species. As it is extremely accessible, it has been used from time immemorial to apply therapeutic remedies topically, using various pharmaceutical presentations that suit, to a greater or lesser degree, the active ingredients whose action is sought. Its barrier ability basically depends on its histological structure.

Certain poisons are lethal if administered orally but not when applied over intact skin. This led to the belief that skin was completely impermeable to any type of substance applied to it. However, it is now known that skin does possess a certain degree of permeability. Various

authors have studied the circumstances that determine transdermal substance delivery, leading to the development of patches for active ingredients such as scopolamine, nitroglycerin and nicotine. Such patches ensure precise dosage and absence of irritation in the skin area where they are applied[6].

Skin is now considered a membrane with selective permeability for chemical elements, offering potential advantages over other more commonly used pathways. These advantages include avoiding the initial hepatic metabolic step, not having the restrictions associated with the oral route, being easily used and simplifying patient treatment monitoring. However, only strong small-sized lipophilic drugs reach therapeutic levels administered by passive diffusion through the stratum corneum[7]. Cutaneous permeation is also a multifactorial phenomenon involving biological, physical and chemical factors and it is practically impossible to develop mathematical models able to predict the amount of medicine absorbed[3]. Apart from molecule-specific factors, the skin itself conditions permeability. Besides individual factors such as age, sex and anatomical area, the main biological determinants to consider in penetration speed are the state of the skin and its hydration. It is a well-known fact that drugs penetrate desquamated skin much more rapidly due to the lack or thinness of the stratum corneum.

The physical and chemical properties of the molecules (some depending on the drugs in question and others on the excipients) have to be considered as well. Among these are the *molecular weight* (drug size conditions its diffusion in the stratum corneum and the dermis), *lipophilia or partition coefficient* (permeability in the stratum corneum rises with lipophilia, but permeability is better in the dermis for low-lipophilic compounds and tends to stabilise as lipophilia increases), *excipients* (which are not merely a support for the drug; they constitute a release system that directs the drug towards its biophase or action site) and *penetration capability* (traditional vehicles are generally incapable of penetrating the stratum corneum by themselves; what they do is release the drug to the skin surface and, once the drug dissolves, its penetration depends on the stratum corneum/surface water partition coefficient).

Keratin makes it difficult for all type of molecules, whether hydro- or liposoluble, and the intercellular substance formed by hydrophobic lipids is impermeable to hydrophilic substances. The viable epidermal layers (50 to 1500 µm thick and avascular) also help to determine cutaneous permeability[8].

Passive transdermal passage of a substance is limited by: water solubility greater than 1 mg per ml, molecular weight lower than 500 Da, melting point less than 200°C, a pH of 5-9 and a relatively high, but not excessive, lipophilia so that the substance is not trapped between the layers of the stratum corneum[9,10]. The chemical and physical properties of the molecules studied are presented in Table 1.

Substance	Biotin	Procaine	Caffeine
Molecular formula	$C_{10}H_{16}N_2O_3S$	$C_{13}H_{20}N_2O_2$	$C_8H10N4O2$
Molecular weight	244.31 g/ml	272.8 g/mol	194.2 g/mol
pK_a	4.51	8.05	14
Water solubility	220 mg/L	9450 mg/L	21600 mg/L
Melting point	232°C	61°C	238°C
Log P	0.5	1.92	-0.07
Charge	Negative	Positive	Positive

Table 1 - Physical and chemical properties of the substances studied

The transdermal pathway is potentially an alternative route for oral or injected drug administration[11]. It has notable advantages over them, above all in avoiding the initial metabolic step (associated with oral administration) and preventing pain (associated with injection techniques)[12]. Circumventing the epidermis to reach the dermis directly makes it possible to use substances with molecular weights greater than 500 Da[13]. However, not all epidermal permeabilization methods are appropriate for all applications. Detailed knowledge of each of them and of the physical and chemical characteristics of the active principles to be used is required to guarantee skin integrity[14].

Given that the success of a therapeutic transdermal system depends on the capacity of the substance to spread through the skin in amounts sufficient to achieve the desired therapeutic effect, various options to increase skin permeability have been examined[15]. These can be of chemical or physical nature. Chemical options include what are generically called the permeation-enhancing agents, compounds that can penetrate the skin and lower its resistance to diffusion and facilitate delivery to more internal layers where the molecule applied is expected to act.

Two physical manipulations are useful in encouraging the permeation of active ingredients: electroporation (which strengthens and controls the permeation of ionic active ingredients by the mechanisms of electro-repulsion, electro-osmosis and the physical perturbation of the skin barrier to the passage of current) and microperforation or microneedling (which causes small transcutaneous openings through which various active ingredients can be introduced).

Electroporation (EP) causes small nanometric micro-openings in the stratum corneum that allow the passage of substances applied under an electric field, conditioned by factors such as the size of the molecule to be introduced or its electrical charge16. Electrophoresis (EF) is based on the principle of repulsion of charges of the same sign to induce the

passage of an electrically-charged substance can penetrate the stratum corneum.

Using microneedles in the skin area before applying electroporation or electrophoresis seems to be useful for greatly increasing the capacity of active substances to be absorbed transcutaneously without causing any type of pain or with minimal discomfort[16].

In 1848 Baunscheit patented a multiple needle unit that he called "Lebenswecker" or "resuscitator". It was equipped with a system for calibrating the needle penetration depth in the skin and made it possible to apply a revulsive oil that mechanically replicated an insect bite. Based on medieval medical knowledge, he attempted to extract the bad humours responsible for inflammatory illnesses through the micropores created with the device.

Baunscheit even glimpsed a hypothetical electrical theory on the effect of the needle pricks and the use of revulsive oil applied on the microperforated skin. As he indicated in his writings (cited by Dirk[17]), this oil would serve to maintain the galvanic connection between the needles, an antecedent of the "electrical theory" that Liebl and Kloth (2012[18]) recently postulated to explain the mechanism of microneedle action.

This type of device had been patented in 1976 (cited by Prausnitz[16]), 1996 and 1997 (cited by Henry[19]), although varying in size, form, material used, etc. The skin perforation technique using microneedles was rediscovered by Henry[19] in 1998. These researchers used systems equipped with solid microneedles to make small openings in the skin surface before applying a substance (especially those of macromolecules) topically, to facilitate its passage through the stratum corneum effractions[20]. Combining this mechanical method with the electric method described earlier makes it possible to use larger molecules[12]. In addition, a direct relationship between the number of openings performed and the increase in skin permeability has been seen[21].

Using swine skin, there has been in vitro demonstration of the advantage of using various physical permeation methods together to facilitate transdermal passage of hydrophilic macromolecules without causing skin damage[22].

Electroporation (EP), together with the technique of perforation with microneedles (microneedling [MN]), constitute part of what has become known as "*third-generation transdermal systems*"[11]. They increase stratum corneum permeability, making the transcutaneous drug release systems more effective, enable the use of macromolecules up to 40 kDa[20] and minimise the importance of the electrical charge with fewer irritating effects than those associated with the use of specific permeation-enhancing agents.

It has been demonstrated that prior MN allowed greater control of the drug flow by modulating the current applied, as well as decreasing the time required for skin penetration. Known as "*in-skin electroporation*", this technique combines the advantage of MN with those of EP[23]: it facilitates the passage of high molecular weight hydrophilic molecules to deep skin layers and increases permeability capacity. It also makes it possible to use lower voltages and shorter pulse widths than when only EP is used. The openings formed with the combination of both techniques are wider and deeper than those found using EP alone.

Objective

The goal of this study was to demonstrate that skin can be used as an effective drug delivery route when its permeability is increased using permeability-enhancing systems such as EP or MN. The effectiveness of EP and the combination of MN plus EP (MN+EP) in transdermal absorption of a series of molecules (procaine, caffeine and biotin) were explored in an animal model. To do so, high pressure liquid chromatography (HPLC) was used to calculate the concentrations of the substances administered according to the transcutaneous permeation methods mentioned and their chronological evolution during the first hour and at the end of 7 and 14 days following their administration.

The animal used, the domestic pig *(Sus scrofa, domestica)*, is widely validated as an experimental model.

Materials and methods

Experimental animal

The best way to study the degree of penetration or percutaneous absorption of chemical substances is *in vivo* study in human beings. However, this poses a series of ethical problems that make this possibility unviable. In our study, we used the pig because it is considered a good experimental model due to the characteristics of its skin[24,25]. This animal has a series of advantages over other mammals: its skin can be easily obtained, the skin extension allows carrying out the experimental design with fewer animals than with other species and, in third place, it is an animal that is manageable and adapted to the Vivarium installations at the University of Oviedo. In addition, swine skin has many similarities with human skin, which are summarised in Table 2.

To carry out this thesis project, we used 6 crossed-race Pietrain x Duroc pigs (4 females and 2 castrated males), weighing from 35 to 38 kg. Animal handling was performed fulfilling all the procedures recommended by the Department of Biomedical Science and Ethics at the University of Birmingham[26]. The experiment protocol was approved by the Department of Agriculture and Indigenous Resources of the Government of the Principality of Asturias, Spain

Clear differentiation between the epidermis and the dermis
Very similar follicle structure
Very similar replacement time and keratin
The stratum corneum contains practically the same proteins
The dermal-epidermal junction also shows great similarities
Swine dermis is well structured and rich in elastic tissue
The number, size, distribution and communications of the dermal vessels are similar in both species
The architecture, disposition and thickness of the dermal collagen fibres are also similar
They share great similarities from the immunohistochemical and biochemical standpoints
The thickness of the skin layers is practically the same in swine skin and human skin

Table 2 - Similarities between human skin and pig skin

(Authorisation no. PROAE 09/2014).

Before performing the experimental procedures described below, the animals were premedicated with Azaperone (2 mg/kg, IM) and ketamine (10 mg/kg) + xylazine (0.08 mg/kg), IM. After sedation, the animals were taken to the presurgery area, where a 20 G catheter was placed in the auricular vein and 0.01 mg/kg Buprenorphine was applied subcutaneously.

The skin area on which the experiments were to be performed was washed with a soapy antiseptic solution, which was then dried.

The hair was shaved on both sides of the animal loins with an electric shaver, ensuring that no mechanical lesions were produced in the epidermis. Next, the skin was sterilised again, using an iodised povidone solution and then drying carefully with sterile gauze.

On each side of the animals on the shaved skin surface, 2 10x10 cm squares were drawn, subdividing them into 4 5x5 cm^2 quadrants each with a sterile disposable surgical marker (Devon® Skin Marker). The sample taking times were identified as T0, T15, T30 and T60 (minutes) on the small squares. On the right side, these times were referenced in counterclockwise direction, while on the left side they were clockwise; the square corresponding to T0 was always the closest to the head of the animal (Figure 1).

Immediately afterwards, the different products were applied with the various techniques on the pre-established squares. Time 0 (T0) was considered the moment of ending the technique and starting the tissue sample taking. Biopsy tissue was taken again at 15 (T15), 30 (T30) and 60 (T60) min.

The samples were taken with punches of 8 mm diameter. Two samples were taken from each square; one was fixed in formalin for use in structural and immunohistochemical studies, while the other was

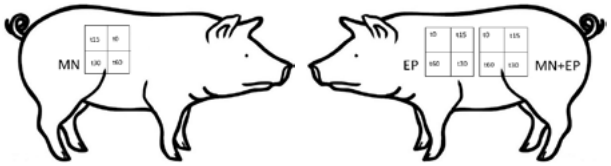


Figure 1

Figure 1 - Schematic design of the study protocol in experimental animals

conserved at -40ºC until used in the HPLC studies. At the end of the experimental process, the wounds were sutured with 00 silk, they were washed with an antiseptic solution and 3 superimposed layers of a semi-permeable acrylic film (Nobecutan®) were applied to ensure that the area studied would not be exposed to environmental contamination and prevent wound maceration as it was not an occlusive dressing.

Immediately afterwards, the animals were returned to the originating farm, where they were strictly monitored to rule out signs of toxicity and/or skin sensitisation, as recommended by the American Association for Laboratory Animal Science[27,28]. As a prophylactic measure, 20,000 IU/kg/IM/day penicillin-streptomycin was administered for 3 consecutive days. The animals were returned to the University of Oviedo Vivarium at 7 (D7) and 14 (D14) days to take new biopsy samples after sedation with ketamine/xylazine. On Day 14, after obtaining the corresponding samples, the animals were euthanized with iv 100 mg/kg sodium pentobarbital.

Materials

TMT System®

The transcutaneous mesodermic therapy (TMT) system® (Mesoestetic Medical Devices, Viladecans, Barcelona, Spain) for electroporation consists of a main module that contains the energy feed, the central processing unit (CPU) and the circuit needed to generate the electrical pulse.

There is a main module containing the energy feed, central processing unit and the circuitry needed to generate electric pulses.

This consists of a pulsed rectified wave generator, modulated by a 3-second wrapped triangular signal having a 3-second period, fed by 12 V stabilised direct current, with a 230 V grid connection (Figures 2a and 2b).

The device has a liquid-crystal display (LCD) graphic screen that makes it possible to see the options for the various treatment programmes.

The range of data is shown in Table 3.

It is programmed to generate mid-frequency alternating current. A rectified sinusoidal wave with variable pulse and frequency is superimposed on the

current pulse, and both can be controlled through the touch-screen and keyboard.

Figure 2a

Figure 2b

Figure 2a-2b - Image of the system for electroporation used in the study with roll-on detail

Pulse (P): 150-1280 usec.
Time (T): 0.56-5 msec.
Frequency (F): 625-1780 Hz.
Amplitude (pv; peak values - in vacuum): 40-50 V.
Direct current component: 10-15 v
Alternating current component: 30-35 v

Table 3 - Data range for the TMT System® device

The CPU stores the programmed data and regulates energy delivery to the application device.

The application time (15 min) is set, along with the polarity of the molecule to be investigated and the programme to be used.

The molecule to be studied is placed in a plastic container that fits into the roll-on stainless steel application device (Figures 2a - 2b) connected to the CPU by conducting cables.

The circuit is closed via a mass that was attached, in this study, to one of the hind legs of the experimental animals an elastic bandage.

The electrical activity of the electroporator in "on" is programmed with a chain of 4 regular pulses of 30-35 V at a time period between 1 and 10 ms , with an interval of 0.5 ms between each 3 pulses during the 15-min application.

Revive MN®

This is a multi-puncture device (MT.DERM GmbH, Berlin) composed of a micromotor that can be programmed for different speeds and that sets in motion a head with 6 microneedles with a maximum length of 1.5 mm and a maximum diameter of 0.2 mm.

Each needle is separated by 1.5 mm; there is a head with swinging needles that is adapted to the surface of the area of the skin treated. It allows making openings perpendicular to the skin barrier without producing tears or lesions in the skin surface (Figure 3).

Figure 3

Figure 3 - Microneedles

In this study, it was applied perpendicularly to the skin of the animal at a depth of 0.8 mm at 150 revolutions per minute (rpm) for 10 min.

Substances studied

We studied the behaviour of 3 drugs (caffeine, biotin and procaine) authorised by the Spanish Agency for Medicines and Health Products for parenteral human use (biotin [Medebiotin fuerte®] and procaine [Procaína Serra®]).

Caffeine was prepared by the Mesoestetic laboratory (Viladecans, Spain) following the guidelines established for sterile non-pyrogenic solutions.

The gels of the products used with the electroporation technique were prepared by the Mesoestetic laboratory.Their formulas are shown in Table 4.

Drug application techniques

Microneedles

During the microneedling process, the 10x10 cm^2 surface was irrigated with 5 ml of the solution to be studied, attempting to make the micropores uniform over the entire surface. The appearance of erythema and small blood suffusions was noted during the process.

Biotin gel	
Ingredient	% w/w
Biotin	0.50
Methocel E4M	1.60
Kathon CG	0.10
Water q.s. 100	97.80
Procaine gel	
Ingredient	% w/w
Procaine hydrochloride	2.00
Methocel E4M	1.60
Kathon CG	0.10
Water q.s. 100	96.30
Caffeine gel	
Ingredient	% w/w
Anhydrous caffeine	3.00
Sodium benzoate	3.00
Methocel E4M	1.60
Kathon CG	0,10
Water q.s. 100	92.30

Table 4 - Composition of the study gels

Electroporation

The substances to be studied were prepared by the Mesoestetic laboratory in the form of a gel, whose composition is shown in Table 3. The container connected to the roll-on applicator of the equipment made it possible to apply each product over the skin, while the mass closing the circuit was fixed on one of the hand limbs of the animal to ensure optimum contact.

The polarity of each of the molecules was respected to facilitate the electro-repulsion phenomenon and the unit was programmed to work for 15 min. In this time period, circular movements were made on the square of skin corresponding to the study substance to facilitate the functioning of the roll-on device. The equipment shut off automatically at the end of the pre-set time.

Tissue concentration study technique: HPLC (high pressure liquid chromatography)

The skin samples were homogenised in 4 cc of normal saline with an Ultra-Turrax T8 unit (IKA Labortechnik, Staufen, Germany) for 1 min at 20,000 rpm, until achieving a uniform mixture.

The study was carried out in the Technical Service installations at the University of Oviedo. The equipment used for the analyses was the HPLC Dionex UltiMate 3000 (Thermo Scientific) connected to an Impact II (Bruker) quadruple mass spectrometry with flight time, with an electrospray source. Detection was performed between 100 and 800 Da in positive mode for biotin, procaine and caffeine.

To extract biotin, 50 µl of sample were taken, diluting to 1 mL with water. Twenty µl of 33% ammoniac were added, then 300 µl of dichloromethane and shaken in vortex for 1 min. Next, it was centrifuged at 10000 rpm for 5 min and the resulting watery extract was diluted 1:10 and injected into the unit.

As for procaine, 50 µl of sample were taken, diluting to 1 mL with water acidified to pH 2.5 with formic acid. Next, 300 µl of dichloromethane were added and shaken in vortex for 1 min. The sample was centrifuged at 10000 rpm for 5 min and the resulting watery extract was injected directly into the unit without dilution.

As for caffeine, 950 µl of water/methanol were added in a 1:1 ratio to 50 µl of sample. The dissolution was shaken in vortex for 1 min and then centrifuged at 10000 rpm for 5 min. The supernatant was diluted 1:20 in water and injected into the unit.

The standard calibration curves were prepared in the following calibration intervals: procaine: 5-1000 ng/ml; caffeine: 1-1000 ng/ml; and biotin: 5-2000 ng/ml.

Results: skin concentration of the molecules studied based on technique used and time elapsed (HPLC)

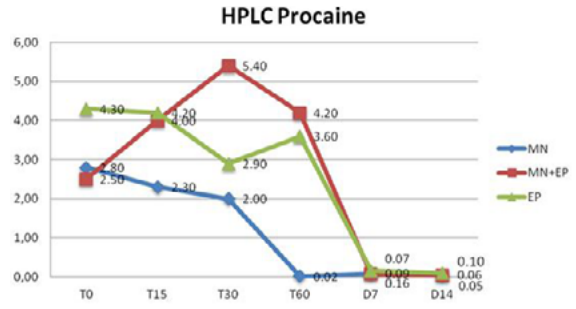
Procaine

The results from the skin samples according to the times and administration methods are shown in Table 5 and Graph 1 (See quatitification in Annex 1).

The individual values for each sample are presented in Annex 1. The highest tissue values were found in the first 60 min; the highest values were reached using the MN+EP technique at 30 min. At 1 week and at 2 weeks after terminating the experiments, the molecules were still detectable in the skin but at residual levels.

	MN	**MN+EP**	**EP**
T0 min	2.8	2.50	4.3
T15 min	2.3	4	4.2
T30 min	2.0	5.4	2.9
T60 min	0.02	4.2	3.6
D7 days	0.09	0.07	0.16
D14 days	0.06	0.05	0.10

Table 5 - Mean cutaneous concentrations of procaine (expressed en µg/ml) based on technique and post-administration time.
EP: electroporation; MN: microneeding; MN+EP: both techniques together



Graph 1 - Procaine concentrations at the different study times with various forms of administration

We can confirm that there is no significant difference among the techniques used in the study according to the time. The higher concentration is shown at T30 by the combined use of both techniques of transcutaneous perfusion.

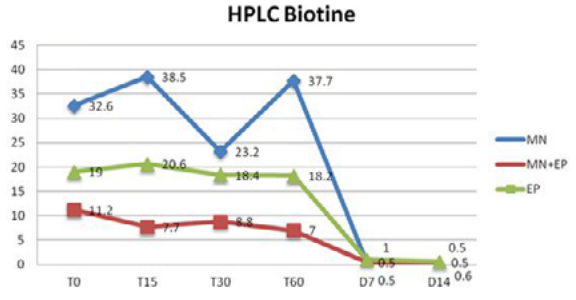
Biotin

The results from the skin samples according to the times and administration methods are shown in Table 6 and Graph 2.

We obtained higher concentrations using MN technique 15 minutes from administration. In that time wee obtained higher concentrations using EP techinique too. We obtained the lowest concentrations by the combined use of electroporation with micro-needles in every time studied.

	MN	MN+EP	EP
T0 min	32.6	11.2	19.00
T15 min	38.5	7.7	20.6
T30 min	23.2	8.8	18.4
T60 min	37.7	7.00	18.2
D7 days	0.5	0.5	1
D14 days	0.5	0.5	0.6

Table 6 - Mean cutaneous concentrations of biotin (expressed en µg/ml) based on technique and post-administration time.
EP: electroporation; MN: microneeding; MN+EP: both techniques together



Graph 2 - Biotin concentrations at different study periods with various forms of administration

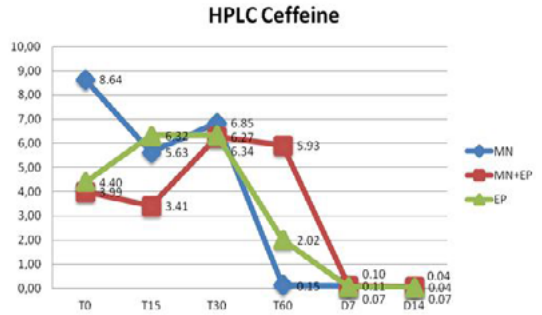
Caffeine

The results from the skin samples according to times and administration methods are shown in Table 7 and Graph 3.

We obtained highest concentrations using MN technique at the time of administration of molecule (T0) followed by EP at the same time; however the combined use of MP+EP allows to keep for long time tissue concentration higher than it was when the two techinques were used individually.

	MN	MN+EP	EP
T0 min	8.64	3.99	4.40
T15 min	5.63	3.41	6.32
T30 min	6.85	6.27	6.34
T60 min	0.15	5.93	2.02
D7 days	0.11	0.10	0.07
D14 days	0.04	0.07	0.04

Table 7 - Mean cutaneous concentrations of caffeine (expressed en µg/ml) based on technique and post-administration time.
EP: electroporation; MN: microneeding; MN+EP: both techniques together



Graph 3 - Cutaneous caffeine concentrations according to study period and form of administration

Annex 1

Quantification of Procaine

Analysis	Sample Type	Area	Height	RT [min]	Concentration	Dilution	Concentration exp.	Unit
151029_5ppb_RA1_01_274	CALIBRANT	81673	16192	3,44	5,0		5,0	ng/ml
151029_20ppb_RA2_01_275	CALIBRANT	372538	68274	3,38	19,9		20,0	ng/ml
151029_100ppb_RA3_01_276	CALIBRANT	1901450	363153	3,37	98,3		100,0	ng/ml
151029_200ppb_RA4_01_277	CALIBRANT	4436409	871830	3,37	228,3		200,0	ng/ml
151029_500ppb_RA5_01_278	CALIBRANT	10154187	2011221	3,37	521,5		500,0	ng/ml
151029_1000ppb_RA6_01_279	CALIBRANT	19101312	3704008	3,36	980,3		1000,0	ng/ml
Analysis	Sample Type	Area	Height	RT [min]	Concentration	Dilution	Concentration exp.	Unit
151029_99_RB1_01_282	SAMPLE	7197	1193	3,34	1,2	21,0	0,02	ug/ml
151029_146_RB8_01_289	SAMPLE	24940	5221	3,36	2,1	21,0	0,04	ug/ml
151029_101_RC1_01_290	SAMPLE	32479	7661	3,37	2,5	21,0	0,05	ug/ml
151029_149_GA1_01_314	SAMPLE	32554	7690	3,39	2,5	21,0	0,05	ug/ml
151029_147_RC8_01_297	SAMPLE	39642	9599	3,37	2,8	21,0	0,06	ug/ml
151029_107_RE1_01_306	SAMPLE	46890	11233	3,38	3,2	21,0	0,07	ug/ml
151029_109-2_RA8_01_281	SAMPLE	51470	11248	3,35	3,4	21,0	0,07	ug/ml
151029_109_RA7_01_280	SAMPLE	53887	13216	3,39	3,6	21,0	0,07	ug/ml
151029_108_GA2_01_315	SAMPLE	68920	16129	3,40	4,3	21,0	0,09	ug/ml
151029_148_RD8_01_305	SAMPLE	76781	18237	3,39	4,7	21,0	0,10	ug/ml
151029_106_RD1_01_298	SAMPLE	129355	29590	3,37	7,4	21,0	0,16	ug/ml
151029_51_RD5_01_302	SAMPLE	405096	98476	3,38	21,6	21,0	0,45	ug/ml
151029_53_GA6_01_319	SAMPLE	468180	110440	3,38	24,8	21,0	0,52	ug/ml
151029_52_RE6_01_311	SAMPLE	1107813	277813	3,38	57,6	21,0	1,2	ug/ml
151029_54_RB4_01_285	SAMPLE	1332667	297919	3,35	69,1	21,0	1,5	ug/ml
151029_11_GA7_01_320	SAMPLE	1657316	397126	3,39	85,8	21,0	1,8	ug/ml
151029_94_RE2_01_307	SAMPLE	1885867	450718	3,38	97,5	21,0	2,0	ug/ml
151029_6_GA8_01_321	SAMPLE	1917231	464940	3,38	99,1	21,0	2,1	ug/ml
151029_88_RB2_01_283	SAMPLE	2046715	462199	3,36	105,8	21,0	2,2	ug/ml
151029_90_RC2_01_291	SAMPLE	2127152	488794	3,36	109,9	21,0	2,3	ug/ml
151029_74-2_RE5_01_310	SAMPLE	2221501	559104	3,38	114,7	21,0	2,4	ug/ml
151029_96_GA3_01_316	SAMPLE	2283487	577427	3,40	117,9	21,0	2,5	ug/ml
151029_7_RB6_01_287	SAMPLE	2285742	507853	3,35	118,0	21,0	2,5	ug/ml
151029_74_RE4_01_309	SAMPLE	2405971	575443	3,38	124,2	21,0	2,6	ug/ml
151029_9_RD6_01_303	SAMPLE	2408169	583615	3,38	124,3	21,0	2,6	ug/ml
151029_86_GA4_01_317	SAMPLE	2573699	618562	3,38	132,8	21,0	2,8	ug/ml
151029_12_RB5_01_286	SAMPLE	2598303	586262	3,35	134,0	21,0	2,8	ug/ml
151029_76_GA5_01_318	SAMPLE	2679447	646380	3,39	138,2	21,0	2,9	ug/ml
151029_82_RD3_01_300	SAMPLE	3334731	798515	3,38	171,8	21,0	3,6	ug/ml
151029_13_RC5_01_294	SAMPLE	3454469	772893	3,35	177,9	21,0	3,7	ug/ml
151029_78_RB3_01_284	SAMPLE	3661600	826348	3,36	188,6	21,0	4,0	ug/ml
151029_8_RC6_01_295	SAMPLE	3740943	850929	3,37	192,6	21,0	4,0	ug/ml
151029_84_RE3_01_308	SAMPLE	3861443	916743	3,38	198,8	21,0	4,2	ug/ml
151029_72_RD4_01_301	SAMPLE	3923418	932840	3,37	202,0	21,0	4,2	ug/ml
151029_70_RC4_01_293	SAMPLE	3961393	910942	3,35	203,9	21,0	4,3	ug/ml
151029_92_RD2_01_299	SAMPLE	4395806	1005666	3,37	226,2	21,0	4,8	ug/ml
151029_5_RE8_01_313	SAMPLE	4914983	1218722	3,40	252,8	21,0	5,3	ug/ml
151029_80_RC3_01_292	SAMPLE	4992932	1144392	3,36	256,8	21,0	5,4	ug/ml
151029_4_RD7_01_304	SAMPLE	5102086	1223337	3,39	262,4	21,0	5,5	ug/ml
151029_2_RB7_01_288	SAMPLE	12499875	2719484	3,36	641,8	21,0	13,5	ug/ml
151029_10_RE7_01_312	SAMPLE	14197672	3364187	3,37	728,8	21,0	15,3	ug/ml
151029_3_RC7_01_296	SAMPLE	15903694	3513789	3,37	816,3	21,0	17,1	ug/ml

Quantification of Biotine

Analysis	Sample Type	Area	Height	RT [min]	Concentration	Dilution	Concentration exp.	Unit
151106_20ppb_RA1_02_509	CALIBRANT	9758,5684	2481,209	4,27711	26,88958	20	134,448	ng/ml
151105_100ppb_RA2_01_510	CALIBRANT	37897,52	9784,197	4,2771	89,72719	100	89,7272	ng/ml
151105_500ppb_RA3_01_511	CALIBRANT	188189,47	50455,12	4,2803	425,3469	500	85,0694	ng/ml
151105_1000ppb_RA4_01_512	CALIBRANT	385038,97	102939,1	4,28519	864,9351	1000	86,4935	ng/ml
151105_2000ppb_RA5_01_513	CALIBRANT	765185,94	209130	4,29552	1713,848	2000	85,6924	ng/ml
151105_5000ppb_RA6_01_514	CALIBRANT	2332166,5	649101,6	4,28151	5213,101	5000	104,262	ng/ml
Analysis	**Sample Type**	**Area**	**Height**	**RT [min]**	**Concentration**	**Dilution**	**Concentration exp.**	**Unit**
151105_46_RC1_01_524	SAMPLE	3514,5557	762,2807	4,27254	12,94596	20	0,25892	ug/ml
151105_157_GA5_01_552	SAMPLE	7333,7959	1683,531	4,27108	21,47478	20	0,4295	ug/ml
151105_145_GA1_01_548	SAMPLE	7775,3555	1536,618	4,27496	22,46083	20	0,44922	ug/ml
151105_156_GA4_01_551	SAMPLE	7883,1045	1596,574	4,25888	22,70145	20	0,45403	ug/ml
151105_144_RE8_01_547	SAMPLE	8717,834	1987,081	4,27358	24,5655	20	0,49131	ug/ml
151105_155_GA3_01_550	SAMPLE	9200,042	1994,641	4,2655	25,64232	20	0,51285	ug/ml
151105_143_RE7_01_546	SAMPLE	9927,9336	2601,642	4,27629	27,26779	20	0,54536	ug/ml
151105_154_GA2_01_549	SAMPLE	10930,479	2645,335	4,28098	29,50659	20	0,59013	ug/ml
151105_26_RA8_01_515	SAMPLE	13800,951	3375,04	4,27666	35,9167	20	0,71833	ug/ml
151105_39_RB6_01_521	SAMPLE	14133,145	3315,052	4,25234	36,65853	20	0,73317	ug/ml
151105_142_RE6_01_545	SAMPLE	19526,635	4830,923	4,27375	48,70283	20	0,97406	ug/ml
151105_59_RC4_01_527	SAMPLE	19758,105	4755,105	4,27687	49,21973	20	0,98439	ug/ml
151105_36_RB4_01_519	SAMPLE	22466,459	5860,402	4,25852	55,2678	20	1,10536	ug/ml
151105_44_RB8_01_523	SAMPLE	35754,781	9150,688	4,27505	84,9422	20	1,69884	ug/ml
151105_124_RD5_01_536	SAMPLE	153334,94	39558,9	4,28529	347,5126	20	6,95025	ug/ml
151105_118_RD2_01_533	SAMPLE	169797,3	42435,52	4,263	384,275	20	7,6855	ug/ml
151105_122_RD4_01_535	SAMPLE	194554,36	49020,23	4,27251	439,5605	20	8,79121	ug/ml
151105_114_RC8_01_531	SAMPLE	248642	64381,88	4,25991	560,3445	20	11,2069	ug/ml
151105_120_RD3_01_534	SAMPLE	404786,59	105381,4	4,2795	909,0339	20	18,1807	ug/ml
151105_116_RD1_01_532	SAMPLE	409324,47	106156,1	4,27685	919,1675	20	18,3833	ug/ml
151105_110_RC6_01_529	SAMPLE	423851,09	108092	4,25986	951,6072	20	19,0321	ug/ml
151105_112_RC7_01_530	SAMPLE	458256,5	119181,4	4,2595	1028,438	20	20,5688	ug/ml
151105_134_RE2_01_541	SAMPLE	517779,13	133647,5	4,26084	1161,36	20	23,2272	ug/ml
151105_37_RB5_01_520	SAMPLE	520123,78	137310,7	4,26177	1166,595	20	23,3319	ug/ml
151105_49_RC3_01_526	SAMPLE	548278,94	140696,6	4,2681	1229,469	20	24,5894	ug/ml
151105_140_RE5_01_544	SAMPLE	683207,63	179406	4,27549	1530,781	20	30,6156	ug/ml
151105_28_RB1_01_516	SAMPLE	713360,13	180248	4,27265	1598,115	20	31,9623	ug/ml
151105_126_RD6_01_537	SAMPLE	728506,5	188819,4	4,26605	1631,939	20	32,6388	ug/ml
151105_32_RB3_01_518	SAMPLE	744227,31	195853,7	4,26318	1667,045	20	33,3409	ug/ml
151105_61_RC5_01_528	SAMPLE	771114,13	201461,4	4,25783	1727,087	20	34,5417	ug/ml
151105_138_RE4_01_543	SAMPLE	840771,19	219379,1	4,2711	1882,639	20	37,6528	ug/ml
151105_130_RD8_01_539	SAMPLE	859830,63	226636,4	4,26006	1925,201	20	38,504	ug/ml
151105_136_RE3_01_555	SAMPLE	1040395,6	286017,2	4,29393	2328,424	100	232,842	ug/ml
151105_29_RB2_01_517	SAMPLE	1458912,8	386157,3	4,25712	3263,022	20	65,2604	ug/ml
151105_128_RD7_01_553	SAMPLE	1485273,6	404745,6	4,28091	3321,889	45	149,485	ug/ml
151105_132_RE1_01_554	SAMPLE	1700113,8	463428,2	4,286	3801,653	100	380,165	ug/ml
151105_42_RB7_01_522	SAMPLE	2242743,3	590912,5	4,25156	5013,409	20	100,268	ug/ml
151105_47_RC2_01_525	SAMPLE	5504520	1454640	4,2794	12297,34	20	245,947	ug/ml

Quantification of Biotine

Analysis	Sample Type	Area	Height	RT [min]	Concentration	Dilution	Concentration exp.	Unit
151103_1_RA2_01_337	CALIBRANT	31891,865	7446,851	3,9025	1,081401	1	108,14	ng/ml
151103_5_RA3_01_338	CALIBRANT	153704,28	39144,36	3,90547	5,578274	5	111,565	ng/ml
151103_20_RA4_01_339	CALIBRANT	506816,78	133737,9	3,90683	18,61391	20	93,0695	ng/ml
151103_100_RA5_01_340	CALIBRANT	2358794	634809,1	3,92827	86,98219	100	86,9822	ng/ml
151103_500_RA6_01_341	CALIBRANT	13240163	3549568	3,90941	488,6829	500	97,7366	ng/ml
151103_1000_RA7_01_342	CALIBRANT	27769718	7269189	3,90692	1025,061	1000	102,506	ng/ml
Analysis	Sample Type	Area	Height	RT [min]	Concentration	Dilution	Concentration exp.	Unit
151103_105_GA3_01_378	SAMPLE	10479,532	2569,01	3,91696	0,290935	80	0,02327	ug/ml
151103_153_GA7_01_382	SAMPLE	11291,671	1848,266	3,93149	0,320916	80	0,02567	ug/ml
151103_152_GA6_01_381	SAMPLE	14766,257	3736,948	3,91485	0,449186	80	0,03593	ug/ml
151103_151_GA5_01_380	SAMPLE	15658,706	4061,127	3,91957	0,482132	80	0,03857	ug/ml
151103_100_RE7_01_374	SAMPLE	25040,238	5558,689	3,93617	0,828464	80	0,06628	ug/ml
151103_103_GA1_01_376	SAMPLE	25060,965	5790,83	3,92439	0,829229	80	0,06634	ug/ml
151103_56_RC5_01_356	SAMPLE	25486,996	6068,388	3,91299	0,844956	80	0,0676	ug/ml
151103_150_GA4_01_379	SAMPLE	25885,057	4493,187	3,92897	0,859651	80	0,06877	ug/ml
151103_102_RE8_01_375	SAMPLE	36331,926	8636,536	3,9145	1,245312	80	0,09962	ug/ml
151103_104_GA2_01_377	SAMPLE	41126,199	8405,876	3,92878	1,422299	80	0,11378	ug/ml
151103_98_RE6_01_373	SAMPLE	54957,32	12833,72	3,91055	1,932894	80	0,15463	ug/ml
151103_55_RC4_01_355	SAMPLE	57898,258	11113,12	3,91352	2,041463	80	0,16332	ug/ml
151103_57_RC6_01_357	SAMPLE	147404,58	37839,7	3,91812	5,345711	80	0,42766	ug/ml
151103_58_RC7_01_358	SAMPLE	190889,3	49665,65	3,91564	6,95101	80	0,55608	ug/ml
151103_24_RC2_01_353	SAMPLE	312038,94	83584,46	3,89978	11,42342	80	0,91387	ug/ml
151103_22_RB8_01_351	SAMPLE	318509,28	83091,66	3,90082	11,66228	80	0,93298	ug/ml
151103_25_RC3_01_354	SAMPLE	401364,13	105597,2	3,91907	14,72098	80	1,17768	ug/ml
151103_23_RC1_01_352	SAMPLE	450460,25	117821	3,89627	16,53343	80	1,32267	ug/ml
151103_21_RB7_01_350	SAMPLE	509187,41	134465,2	3,90386	18,70142	80	1,49611	ug/ml
151103_14_RA8_01_343	SAMPLE	608114,19	163251,1	3,9372	22,35344	80	1,78828	ug/ml
151103_20_RB6_01_349	SAMPLE	681494,5	181880,6	3,90174	25,06237	80	2,00499	ug/ml
151103_83_RD6_01_365	SAMPLE	687287,06	182049	3,91171	25,27621	80	2,0221	ug/ml
151103_17_RB3_01_346	SAMPLE	1049222,3	280593,8	3,91867	38,63755	80	3,091	ug/ml
151103_79_RD4_01_363	SAMPLE	1156305	303748,2	3,91049	42,59066	80	3,40725	ug/ml
151103_97_RE5_01_372	SAMPLE	1326256,5	341956,9	3,89806	48,86465	80	3,90917	ug/ml
151103_75_RD2_01_361	SAMPLE	1352125,9	354748,2	3,90672	49,81965	80	3,98557	ug/ml
151103_16_RB2_01_345	SAMPLE	1359658,9	361792,8	3,91323	50,09774	80	4,00782	ug/ml
151103_93_RE3_01_370	SAMPLE	1390618	363026,6	3,91721	51,24064	80	4,09925	ug/ml
151103_71_RC8_01_359	SAMPLE	1491850	396244,3	3,89919	54,97776	80	4,39822	ug/ml
151103_15_RB1_01_344	SAMPLE	1743974,5	462344,4	3,91955	64,28529	80	5,14282	ug/ml
151103_91_RE2_01_369	SAMPLE	1907970,3	497055,8	3,91978	70,33941	80	5,62715	ug/ml
151103_85_RD7_01_366	SAMPLE	2011531,9	531210,9	3,90008	74,16253	80	5,933	ug/ml
151103_81_RD5_01_364	SAMPLE	2125040,3	556895,5	3,91702	78,35285	80	6,26823	ug/ml
151103_73_RD1_01_360	SAMPLE	2141804,5	560301,9	3,90255	78,97173	80	6,31774	ug/ml
151103_77_RD3_01_362	SAMPLE	2149168,5	560888,8	3,90768	79,24358	80	6,33949	ug/ml
151103_18_RB4_01_347	SAMPLE	2307726,3	620214,6	3,91663	85,09695	80	6,80776	ug/ml
151103_95_RE4_01_371	SAMPLE	2322275,3	596731,6	3,91117	85,63405	80	6,85072	ug/ml
151103_19_RB5_01_348	SAMPLE	2518676,8	666361,3	3,92784	92,88448	80	7,43076	ug/ml
151103_87_RD8_01_367	SAMPLE	2928303,3	754541,7	3,92582	108,0064	80	8,64051	ug/ml
151103_89_RE1_01_368	SAMPLE	6461499	1619293	3,92302	238,4392	80	19,0751	ug/ml

Discussion

Skin constitutes an almost impossible barrier for chemical elements (especially water soluble factors) to pass. Nevertheless, it presents advantages as a drug administration route because it causes no gastric damage and avoids the first hepatic step that partially or totally destroys such elements. For those reasons, various methods to make skin permeable have been developed.

Data from HPLC skin sample analyses show differences in maximum concentrations reached by the substances studied according to the permeation technique used to administer them. In the case of procaine and caffeine, the highest concentrations are from the EP and MN+EP techniques at T30 and T60. With biotin, microneedling seems to be the mostly effective at T60. Briefly, if the effectiveness of a technique is defined by the permanence of the greatest concentration of the administered product, at T60 the most effective techniques are electroporation, microneedling and the combination of both. All the previous comments refer to the first 60 min after drug administration, given that only residual levels of the molecules are detected in the animal skin in the other time periods studied (at 1 and 2 weeks). However, it is not known whether such low levels are capable of producing any biological effect or not.

If the efficacy of a drug is related to greater tissue permanence, based on the results it can be stated that the most efficacious administration technique at T60 is the combination of microneedling plus electroporation.

The fact that procaine and caffeine share physical and chemical properties (positive charge, water solubility and high pKa levels) and that these are notably different from those of biotin (lipid solubility, negative charges and low pKa levels) seems to be relevant in regard to facilitating or obstructing transdermal passage, even once the stratum corneum has been transcended.

The advantage in using this type of transcutaneous administration is the increase of therapeutic action over time, demonstrated by the presence of residual amounts of all the products at D7 and D14. In addition, the amount of administered drug does not decrease when washing the skin area where it was applied, as it does indeed occur with topical administration or using patches[29].

Combining physical techniques, as it has been done in this study, is known to contribute to increasing drug release through the skin. However, there are no references about its application *in vivo*, as has been developed in this study. If microneedling (alone or associated with electroporation) and electroporation are added to the normal physical strategies to increase transdermal delivery of active substances, the amount of actual intradermal drug increases and also remains for a longer time. In short, the bioavailability of the product is strengthened. Microneedling is a simple, minimally invasive way to increase the absorption of substances through the percutaneous pathway and represents a potentially useful method for other applications and other active ingredients[30]. The depth of the micropores formed with our protocol is limited by the transition from the epidermis to the dermis (Figure 4); the painful sensation that can be produced is minimised, and the technique is well tolerated by individuals with needle phobia.

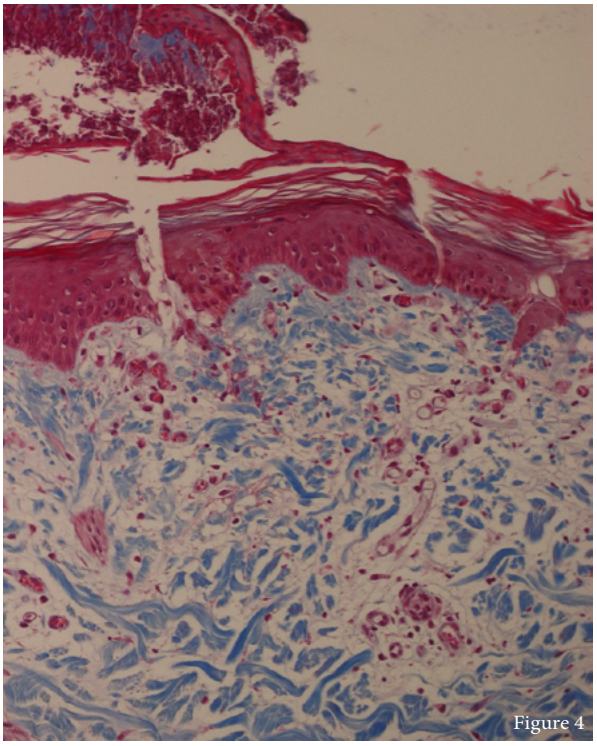


Figure 4 - MN impacts. Microscopic injuries and micro-haemorrhages entering into dermoepidermal junction. Masson's trichrome x20

Conclusions

After analysing the results, the following conclusions have been reached:

- Skin is an absorption pathway for molecules with different sensory properties (procaine, biotin and caffeine) and their local action takes place mainly in the first 7 days after administration.
- The cutaneous concentrations of the analysed molecules are maintained over time (for the first 60 min after administration) with the techniques of microneedling, electroporation and the combination of both.
- Microneedling is a simple, minimally invasive way to increase substance absorption through the percutaneous pathway and it represents a potentially

useful method for administering procaine, biotin and caffeine. Associating it with electroporation strengthens microneedling effectiveness.

- Procaine: The maximum concentration was obtained with the combination of microneedling plus electroporation, while electroporation alone was second. The technique showing the lowest concentrations was microneedling.
- Biotin: Microneedling provided the highest concentrations, which were reached most rapidly and in the most sustained form. The combination of microneedling plus electroporation yielded the lowest concentrations.
- Caffeine: Microneedling showed the highest concentrations at the initial analysis (T0), while at 30 min the 3 techniques behaved similarly. However, at 60 min the combination of microneedling plus electroporation revealed the highest concentrations.
- Caffeine and procaine: These share high water solubility; consequently, this factor (which limits penetration through intact skin) seems to improve transdermal passage when microneedling plus electroporation are used as permeation-enhancing factors.

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Aesthetic Medicine
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The use of Hyaluronidases in Aesthetic Medicine: indications, technique and complications

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ABSTRACT

Hyaluronidases are enzymes that regulate the homeostasis of extracellular matrix and cell growth through the process of hylauronic acid degradation. In nature, these proteins are found in mammalian spermatozoa and lysosomes, as well as in snake and reptile venom in hymenoptera, microbes and hookworms.

Their use in medicine is widespread by virtue of their optimal diffusion properties. The increasing popularity of hyaluronic acid fillers has recently seen a new indication for these enzymes. Hyaluronidases can correct minor imperfections and nodules can completely reverse the volumizing effect of hyaluronic acid fillers and treat cutaneous ischaemic complications. When treating a patient with such enzymes, it is essential for the aesthetic practitioner to be familiar with indications, dosage an potential complications

Keywords
hyaluronidase, hyaluronic acid, filler, necrosis, complication

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Introduction

Hyaluronidases are enzymes synthesized in nature by mammals, microbes and hookworms. These proteins regulate homeostasis of the extracellular matrix thus affecting the cell growth. Each class has a different mechanism of action. They primarily and evenly degrade hyaluronic acid (HA) and secondarily degrade vchondroitin and chondroitin sulfates (ChS)[1].

Mammalian hyaluronidase can be found in mammalian spermatozoa and lysosomes, as well as in snake and reptile venom and in hymenoptera.

Hookworm hyaluronidases are found in the salivary glands of these nematodes. Specifically, they are endo-β-D-glucuronidases and can degrade only HA[2].

Microbial hyaluronidases are produced by several micro-organisms such as Clostridium, Micrococcus, Streptococcus, and Streptomyces[1].

Such enzymes have gained widespread use in medicine mainly as a diffusing factor (hypodermoclysis) due to the property of increasing both absorption and diffusion of an injected drug, such as a contrast medium, anaesthetics (e.g. for obstetric block of pudendal and ileoinguinal nerves or in opthalmology for retrobulbar and peribulbar blocks) and ergometrine to prevent post-partum bleeding.

Interestingly hyaluronidases are administered to reduce post-operative oedema (especially in transplant surgery by reducing the risk of interstitial oedema and rejection) and to lower the risk of local damage after extravasation of toxic substances. In cardiology they are thought to reduce the ischaemic damage after coronary occlusion[3].

The use of hyaluronidases in soft tissues has raised safety concerns for their potential toxicity. A study by Cavallini et al.[4] demonstrated that hyaluronidases do not affect fibroblast viability nor skin viability.

Aesthetic Medicine and hyaluronidases

The use of hyaluronidases in plastic surgery, dermatology and aesthetic medicine usually aims to improve the absorption of a local anaesthetic and mainly to treat over-injection or complications by HA dermal fillers. Nevertheless their use has also been reported in septorhinoplasty[5] in order to reduce tissue distortion due to local anaesthetics.

The most common application in the field of aesthetic medicine is the correction of HA infiltration, of nodules and of excessive infiltration or superficial applications of HA.

Products and regulations

In the United States of America, the use of hyaluronidases is regulated by the Food and Drug Administration (FDA). Many subtypes are approved for clinical purposes, such as Vitrase® (200 i.u./ml) or Hylenex® (150 i.u./ml). The use of these hyaluronidases is off-label for treating complications of HA fillers. In fact these enzymes are authorized for selected indications: urography (to increase resorption of radiopaque contrast), hypodermoclysis and as diffusion adjuvant. Off-label use is possible if considered safe and effective by the practitioner[6] although marketing is not allowed.

In the European Union, promotion for off-label use is not allowed (article 87 of Directive 2001/83/EC) although the off-label use is possible after quality, efficacy and safety have been tested according to centralized or national procedures. The EU has approved the use of hyaluronidases to promote absorption of a contrast medium in urology, to promote subcutaneous reabsorption of hematomas and to increase the penetration of local anaesthetics.

Hyaluronidases for medical purposes are available in powder or liquid form. The former is prepared as a lyophilized protein and needs to be reconstituted with 2 ml of saline and (optional) lidocaine.

Hyaluronidases in powder form are not available in Italy or in most European countries. The use of these enzymes is considered off-label. They are available as galenic formulation by medical prescription. The enzyme can be prepared in different forms such as liquid, gel or topical formulation. More commonly, hyaluronidases come in sterile vials containing each 300-400 Units.

Technique and dosage

In our experience, a dilution of 100 units per one milliliter is appropriate. The infiltration is performed with 1ml syringe and 30-Gauge needle. The amount of enzyme units depends on the anatomical site, the quantity of injected filler, its concentration and cross-linking. 10-20 Units are injected in the peri-orbital region, and 50 to 150 units in the lip and peri-oral area. A similar amount is required to treat the middle-third of the face[7] (Table 1).

However the dosage should be carefully adjusted for each patient and balanced with the volume of injected filler. Non-painful nodules observed immediately after injection commonly disappear in two weeks. In this case massage of the interested area is the first treatment option. If the practitioner aims for a slight correction of an excessive augmentation without completely revering the effect of the HA filler, even a small amount of hyaluronidase could be effective (e.g. less than 3 units for excessive lip augmentation)[8,9].

In case of tissue necrosis induced by hyaluronic-acid (HA) fillers, up to 1500 Units of hyaluronidases may be administered. Cohen et al. recommend immediate treatment with a high dose of hyaluronidase (at least 200 Units)[10].

The symptoms are pale or purple skin. This complication is caused by direct intra-arterial infiltration or compression of subcutaneous tissue. The most commonly affected areas are the nasal ala and the glabellar region[11,12]. The infiltration should be performed in the first 6 hours after a filler injection[12,13]. Later treatments could however improve tissue healing and limit ischaemic damage.

In such cases hyaluronidase administration should be prescribed along with topical nitroglycerine, a warm dressing and antibiotic therapy[14]. Oral aspirin should be prescribed to avoid further vascular compromise[10].

The practitioner should only treat the areas previously injected with HA with due consideration for the enzyme capability to spread in soft tissues. The needle should be introduced perpendicularly to the skin layer; the infiltration depth should be the same as for HA, usually one-third or half the length of the needle. The medication must be injected very slowly.

Author	Ref	Units
Cox	[15]	10-30 U per area
Hirsch	[13]	30-75 U
Sclafani	[14]	10-75 U
Van Dyke	[15]	100-150 naso-labial, 10-15 peri-orbital
Personal experience		15 U palpebral, 50-100 other areas

Table 1 - Hyaluronidase dosage

Special recommendations for inflammatory nodules

Empiric antibiotic therapy should be administered first in case of inflammatory nodules (e.g. clarithromycin 500 mg or minocycline 100 mg twice per day). Treatment duration depends on the severity of the infection. Hyaluronidases are able to dissolve HA and promote the diffusion of antibiotics in the target site[8]. Incision and drainage are only required in severe cases and when HA is superficial and abundant. According to current literature the dosage can range from 3 to 75[9].

Steroid injections should be limited to cases where inflammatory nodules are resistant to any other treatment and always administered in conjunction with antibiotics [8,9,15].

Interactions and complications

A detalied medical history should be acquired before the administration of hyaluronidase. In fact these enzymes are incompatible with benzodiazepines, furosemide, phenytoin, dopamine or alpha adrenergic agonists[16].

Several drugs inhibit hyaluronidases activity: ascorbic acid, anti inflammatory agents (e.g. salicylates, dexamethasone, indomethacin), heparin, antihistamines, several plant-based drugs (e.g. antioxidants, flavonoids), dicumarene and iodinated radiocontrast media. Patients under treatment with any of these drugs should be considered for a higher dosages of hyaluronidases.

Conversely, activator agents such as dopamine, histamine, adrenaline and 0,9% NaCl saline[16,17] should be carefully considered before hylauronidase administration.

The main complication of hyaluronidase injection is an allergic reaction. Symptoms are both local (e.g. local oedema, mild pain) and systemic (rash, urticaria, itching sensation)[18,19]. The severity of symptoms increases with the enzyme[20] dosage and/or in the presence of the above mentioned activators (e.g. in case of retrobulbar anaesthesia in association with adrenaline)[21].

No cases of similar reactions have been described after hyaluronidase injection to reverse the effects of HA dermal fillers. Due to homology of the hyaluronidases molecule between species it is however prudent to investigate[22] known allergies to insects stings or bites.

Conclusion

The use of Hyaluronic acid fillers is a widespread trend and indications are on the increase. Hyaluronidases are a fundamental aid to the aesthetic practitioner to correct excessive infiltrations, to completely reverse the volume augmentation or to treat severe ischaemic complications.

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Tattoos from a medical-toxicological point of view

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ABSTRACT

Tattoos have left their maverick image behind and become "mainstream", particularly among young people.

Consequently, their increasing popularity has led to an enormous growth of tattooed people as ever before.

However, currently, little is known and studied about the complex biochemical interactions taking place when tattoo inks are intradermally inoculated.

In the present study we have tried to extensively analyze numerous tattoo-related issues, such as the current regulations in our country, the complex chemical composition of tattoos and the histological aspects connected to the intradermal presence of pigments.

We have also carefully considered the medical-toxicological implications and the main complications due to the persistence of such pigments inside the human body.

Keywords
tattoo pigment, tattoo toxicity

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Introduction

Historically, tattoo-related health and safety regulations have focused on rules of hygiene and prevention of infections. However, little is known about the toxicological risks of the ingredients used.

In the present study, through a systematic review of the most recent literature data, specific attention has been put on the potential health-damaging effects of said ingredients, with a highlight on their toxicological aspects, their biokinetics and the biochemical modifications experienced by tattoo pigments, once they are intradermally inoculated.

Physical content of tattoo inks

Tattoo ink is a suspension of insoluble pigment particles, with a size scale varying from 10^{-6} m (micro-particles) to 10^{-9} m (nano-particles), carried by a solvent, which distributes pigments uniformly in the fluid matrix, prevents agglomeration and helps the injection into the skin.

Pigments are generally resistant to decomposition due to physical, chemical and biological agents and as such, once injected into the skin, they tend to stay permanently.

The pigment content in the product varies a lot and is often within an interval of 10-30%; according to some scientific studies, however, it seems that there are inks containing pigments up to 50%[1].

Pigment classification

Based on their origin, pigments can be divided into three main groups:

- vegetal pigments
- inorganic pigments
- organic pigments

Vegetal pigments

Vegetal pigments are not numerous, however, the most commonly used black ink is made of carbon black, deriving from incomplete combustion of heavy oil products, such as fossil coal tar or from pyrolysis of fat or vegetal oil.

Inorganic pigments

Inorganic pigments are mainly made of minerals or their oxides.

The most commonly used pigments are graphite (associated with silver-grey and lead-grey), iron oxides (associated with brown-red and dark-grey), titanium dioxide and barium sulphate, which are used for white colours or to brighten darker shades.

Organic pigments

Organic pigments are produced synthetically and they account for 80% of all pigments used in the tattoo industry, main reasons being their strong colouring power, their light-resistance, their enzymatic resistance, low dispersion and relatively low production costs.

They are mainly synthetic organic molecules, azo pigments, polycyclic pigments with a very wide range of colours. Based on functional groups and chromophore groups in their chemical structure, there are different classes of synthetic organic pigments.

The most widely used pigments belong to the group of the mono-azo and di-azo dyes (commonly known as azo dyes); they are synthetic compounds with the typical azo group and two central nitrogen atoms in the molecular structure; they are generally synthesized starting from primary aromatic amines that are diazotisated and coupled with phenols or secondary aromatic amines. In fact, the main chromophore group of the azo compounds is the diarylazo group (Ar-N = N-Ar), which is derived from simpler molecules (organic compounds of nitrogen, where the atom N is connected to one or more organic, alkyl or arylic radicals).

If one of the radicals is an aromatic ring, it can be named aromatic amine.

According to some studies[2,3], from the cleavage in the dermis by human enzymes of said azo dyes, in different time, aromatic amines may be released.

Figure 1 shows how pigment red 5 could be split into three aromatic amines, two of which, p-cresidine and o-toluidine, are carcinogenic.

Figure 1

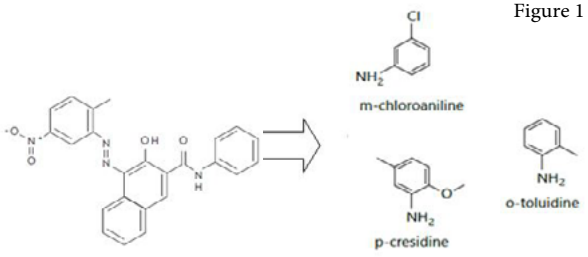

Figure 1 - Cleavage and reduction of pigment red 5
Source: Agnello M, Fontana M. Survey on European Studies of the Chemical Characterisation of Tattoo Ink Products and the Measurement of Potentially Harmful Ingredients in Serup J, Kluger N, Bäumler W, et al, *Tattooed Skin and Health. Curr Probl Dermatol.* Basel, Karger. 2015; 48:142-151.

Chemical impurities

Tattoo inks may contain chemical impurities, which may come from raw materials or from the manufacturing

of tattoo colours.

The most frequent chemical impurities are PAHs (polycyclic aromatic hydrocarbons) and PAAs (primary aromatic amines).

PAH is a generic term for polyaromatic combinations, originated from the combustion of organic material, for example, fossil oil, coal tar and wood; it is also found in car exhaust fumes and tobacco smoke.

Carbon black, the most commonly used pigment in tattooing, derives from the incomplete combustion of fossil coal tar or by cracking fat and vegetal oil and may, therefore, include PAH as a chemical impurity.

There are over 100 different PAHs and IARC (International Agency for Research on Cancer) has classified the most dangerous and the most health-damaging ones.

It is proven that a repeated exposure to certain kinds of PAHs can considerably increase the occurrence of cancer.

Others impurities are the PAAs: they may be present in tattoo inks already in the manufacturing phase or released by azo colorants through chemical or enzymatic decomposition.

Other impurities may be represented by heavy metals, such as lead, mercury, cadmium and chromium, whose toxicity is widely known.

Manufacturing and sale of tattoo ink

There are no internationally or nationally recognized regulations or specific standards for the manufacturing of tattoo ink, that is there is no GMP, good manufacturing practice.

Being injected into the skin, that is for internal use, tattoo ink cannot be regarded as a normal industrial product, but it should undergo strict controls.

The major problem to this regard concerns the safety in the production and selling process and the lack of or incorrect information labeling of tattoo ink packaging.

Høgsberg et al., in a recent study about the 58 most commonly used tattoo ink supplied by established manufacturers, have found that the labels were extremely inadequate[4].

Similar studies have been made in several other European countries, such as Italy, Denmark and Sweden, with similar results[5,6,7].

The study of Høgsberg et al. has highlighted inaccuracies with regard to the indication of the main pigment and its CI number, other ingredients (including preservatives) and the indication of the dates of manufacturing and expiry. In 10% of the ink sample, which according to the label was sterile, a proliferation of human pathogenic bacteria was found and in 28% of the sample there were problems with the packaging, where the physical sealing of the content was defective.

After requesting the suppliers and manufacturers to send product data sheets, it turned out that they were inadequate or full of omissions; in addition none of the manufacturers could explain the ink chemical content in detail.

Chemical analysis

The focus of chemical analyses of tattoo ink has, as a starting point, been on the health-damaging effects of substances based on toxicological studies of chemically pure individual substances (studies on cell cultures, animals or other forms of experimental tests) combined with human data of every available kind, especially commercial exposure of industrial workers to substances that have caused illness.

Analysis have primarily concentrated on chemical impurities, that is PAAs, PAHs and metals (especially heavy metals) studied in accordance with Council of Europe Resolution ResAP (2008)[8].

Tattoo-related risks and complications

It has been documented that there is the possibility that tattoo ink, mainly made of organic pigments, may contain chemical impurities, such as PAAs and PAHs, as well as several heavy metals, such as aluminum, barium, cadmium, cobalt, chromium, copper, nickel, titanium, whose salts cover a wide range of colours.

The average tattooed individual has 100-300 cm^2 of tattooed skin surface with a pigment concentration ranging from 0.60 to 9.42 mg/cm^2 (mean value 2.53 mg/cm2).

This concentration is rather high, considering that tattoo inks are toxicologically undefined mixtures with pigment purities lower than 80%[9].

Nanoparticles of pigments can reach the lymphatic system and hence accumulate in the loco-regional lymph nodes; such particles or products coming from the metabolic transformation of some pigments may reach the bloodstream and be carried to the vital organs[10].

As of today no diagnostic classification system that categorizes and specifies tattoo-related complications has been developed, due to the lack of the necessary epidemiologic tools for systematic gathering and analysis of data.

In 2010 Klügl et al.[11] reported health problems associated with tattooed skin based on 3.411 spontaneous reports collected over the internet in German-speaking countries after an open public invitation. About 68% of respondents reported complications particularly connected to coloured tattoos; 7% were systemic and 6% were persistent.

Complications

In the medical literature, tattoo-related complications are generally divided in two big categories: infectious complications and non-infectious complications.

To this regard, we have found very significant the experience of the 'Tattoo Clinic' of Bispebjerg University Hospital[12], recently quoted in the medical literature and presented at several international congresses, such as the ECTP (European Congress on Tattoo and Pigment Research) held in Belgium in 2015.

The authors have tried to shed a light on the situation and propose an etio-pathogenetic and clinic classification.

Infectious complications

Because of an increased awareness of hygienic regulations, infections are now caused mainly by opportunistic pathogens and commensal skin microorganisms[13].

Another source of infection may be the inks; several reports have showed that up to 20% of sampled inks are contaminated, with bacterial counts as high as 10^8 CFU per mL, including inks labelled as sterile. Contaminations can also originate from poor manufacturing practice or are the result of the use of tap water as unsterile diluent[10].

A recent Danish study[4] has analysed 58 different tattoo inks supplied by 13 different manufacturers and has found human pathogenic bacteria in 7 inks from 7 different manufacturers.

Italian authorities have also found, both in sealed ink samples and in ink samples being used in tattoo studios, a bacterial contamination in up to 86% of the analyzed ink samples[14].

Non-infectious complications

Non-infectious complications can be divided, according to clinical signs, symptoms and pathophysiology, into allergic and non-allergic reaction types, which is a distinction of great significance for the choice of treatment, because allergies may require a radical removal of the pigment from the skin.

A recent Danish study[12] has shown that chronic adverse events after tattooing are mainly dominated by allergic reactions causing remarkably strong itching and, sometimes, pain.

Reactions can appear months or years after the tattoo was done. This is a remarkably long period of sensitization induction and, although the exact reasons have not yet been elucidated, this delayed complication is part of a wider issue: intradermal deposit of tattoo pigments results in lifelong exposure.

The Tattoo Clinic of the Bispebjerg University Hospital has tested a sample of 90 patients, who had previously been tested positive for chronic allergic tattoo reactions, with patch tests using a set of 43 standard allergens, 32 textile dyes and a battery of 8 tattoo inks considered dangerous.

Individual patch tests against specific culprit dyes were mostly negative.

The study has concluded that allergic reactions develop slowly and are unlikely to be caused by an allergen directly present in the tattoo ink, but they are rather due to haptens being formed inside the skin over months or years[15].

Red pigments, the most frequently used in tattoos, show a highly relevant recorded allergenic potential; about the reasons causing such reactions there are only hypothesis, because of the lack of reliable clinical tests.

The most plausible hypothesis is that the allergen is formed inside the skin, probably through metabolism, haptenation or both. The epitope is unlikely to be a defined PAA or of azo dye origin, as indicated by the negative outcome of PAA patch testing of patients with textile dye allergies.

The absence of reliable allergy tests for tattoo colours remains a pressing problem, more so because these allergies cause not only severe complications, but also sensitization against textile dyes.

A very important role is also played by metals, given that their salts, recognized as allergenic, such as cobalt (Co), chromium (Cr) and nickel (Ni) can be the components of colours like green, blue and red. In addition to that, metals can combine with organic pigments to obtain different nuances, shades or brightness.

Among the European population, the prevalence of skin reactions to Co, Cr and Ni is extremely high (respectively 7%, 4% and 20%) with an average time of sensitization getting lower and lower (10-20 years).

It is also important to underline that sensitization to a specific metal can imply a cross-reaction to other metals, that can affect the patient in many ways, i.e. through jewellery, cosmetics, fabrics, accessories, diet, etc.

Carcinogenetic risk in connection with tattoos

As of today no cases of cancer on the lymph nodes connected with tattooed skin areas have been found, even though the dark-coloured lymph nodes, which were found to contain tattoo pigment are often routinely removed and microscoped in connection with sentinel node surgery of malignant melanomas in the skin[16,17].

A single case of pseudolymphoma in a red tattoo has been described. The change apparently healed spontaneously and was, therefore, not malignant[18].

There are no reports in medical literature about lymphomatous cancer, that is, malignant lymphoma and leukaemia caused by tattoos. This is notable, because the lymph node is the most important first

pass organ for tattoo pigments, that is, the organ to which the substances in the tattoo pigments arrive first and in the most concentrated form, and in contrast to the dermis, contain many proliferating cells, which may potentially be exposed to a carcinogen, that is, a cancer-causing substance from the tattoo ink.

A considerable risk is connected with PAAs and PAHs, which, as previously mentioned, resulted to be carcinogenetic in laboratory animals as well as genotoxic in experimental systems both in-vivo and in-vitro.

In addition, some pigments can experience a photodecomposition or a metabolism through cellular enzymes, with the formation of metabolic cleavage compounds, suspected or recognized as human carcinogens.

The most significant articles to this regard are the ones published by Lancet Oncology in July 2015[10] and by the Danish Environmental Protection Agency.

According to the authors, tattoo pigments, after their intradermal deposition, can react with the surrounding tissue and be subject to intracellular uptake[10].

However, the issue of metabolism is disputed: some have argued that low solubility renders the respective pigments to be biologically unavailable[10], making them basically inert. Indeed, the persistence of tattoo colouring indicates that metabolic processes are slow. Yet, low solubility is not a feature of all colourants and ink components and, with lifelong deposit, even slow metabolism is relevant.

Other human P450 cytochrome members including CYP2B6, 2C8, 2C9, 2C18, 2C19 and 2D6 are known to play a relatively minor role in the metabolic activation of procarcinogens and protomutagens.

With regard to pigments, a group of American researchers from FDA have studied the pigment yellow 74, a nitro-aromatic compound containing an azo group, which is metabolized in-vitro by human P450 cytochromes, producing two phase 1 metabolites.

There are no scientific reports about mutagenicity, carcinogenicity or potential photo-carcinogenicity of yellow 74, however, one of the metabolites, enzymically activated in-vitro, can bind to DNA and it is proven to be carcinogenic in the urinary bladder of mice and rats[19].

Other authors (Cui et al. 2004, Vasold et al. 2004) have showed that sun and laser light, upon tattoo removal, can produce a photochemical cleavage of the azo group of said pigment and generate aromatic amines.

Other risk factors are the nanoparticles, which, depending on their shape, size and superficial charge, once intradermally inoculated, can reach the lymphatic vases, carried by macrophages and dendritical cells. From lymphatic circulation they are supposed to reach the bloodstream and be distributed to the whole body.

In black inks, the most frequently used by tattooists, the presence of carbon nanoparticles (carbon black is classified by IARC as a potential human carcinogen) is documented, as well as the presence of Ti=2 nanoparticles in white inks.

As a consequence, metals and nanoparticles, resulting carcinogenetic, mutagenetic and reprotoxic (classified in categories 1A, 1B or 2 in Regulation 1272/2008) should not be used in tattoo ink manufacturing.

Nevertheless, as of today there are no specific regulations about nanoparticles in the tattoo industry.

So far, however, any association between tattoo inks and induction of malignant neoformations is not proven by medical literature.

The occurrence of cancer in tattoos can happen both in the directly tattooed skin and in the areas of the tattoo that are not tattooed and have no tattoo pigment.

The common forms of skin cancer are basocellular carcinomas, spinocellular carcinomas (both of which are skin cancers primarily caused by sun exposure) and malignant melanomas, as well as keratoacanthomas, the latter of which is, however, clinically benign and self-limiting.

There is no scientific evidence of an association between tattoos and said tumours.

In a recent review of the medical literature of the past 40 years published on Lancet Oncology in 2012, a wide assessment of clinical data related to malignant tumours potentially arising from tattoos has been made.

The authors have only found 50 studies reporting malignant skin cancers in tattooed skin; particularly: 23 cases of squamous-cell carcinoma and keratoacanthoma, 16 cases of melanoma and 11 cases of basalioma and have concluded that said cases may be coincidental, because skin cancer occurs extremely frequently in the population[20].

Based on the clinical data in our possession, we can conclude that the vast majority of skin cancer cases are primarily induced by UV radiation and not by the hypothetical carcinogenic potential of chemical constituents of tattoo inks.

A few counter-trend authors have described the possibility that dark tattoos, because of the absorption of sunlight, may work as a physical sun filter; the pigment would then absorb sunlight, which could not be backscattered from the dermis, would provide protection against skin cancer[13].

This has recently been studied in a photocarcinogenicity study with mice[21].

As for tumours in other sites where pigment particles or derivatives from enzymatic and photolysis cleavage may arise, there are no reports of cancer occurring in the regional lymph nodes, even though, as first pass organs, they are frequently exposed to tattoo pigment (tissue concentrations up to 11,8 µg/g are reported)[21].

Neither are there any reports of cancer of the inner organs related to tattoos. Finally, there are no reports of tattooed people, who have large areas of tattooed skin, who have contracted skin or inner organ diseases,

including cancer, which could be related to said tattoos.

The available epidemiology data are scarce and have many confounding social and environmental factors.

Another limitation is the fact that the available toxicological studies refer to a very limited induction time, whereas cancer development time would require a much wider time frame.

Conclusions

In western countries it is reported that tattoo practice has gradually increased over the past decade; it seems that in Europe 100 million people have one or more tattoos[13].

The mostly involved social class seems to be the young population, among which tattoos have become "mainstream", a big trend, also broadcast by mass media as a fashion phenomenon.

It is documented that tattoo ink contains substances classified as carcinogenic, some of which belong to class I of IARC, that is proven human carcinogens.

The most problematic substances to this regard are the PAHs for black colouring, which are present in most cases as contaminants deriving from the manufacturing of carbon black and from aromatic amines released by azo dyes and mainly present in coloured inks (blue, green, red, yellow and orange)[22].

In the current European regulation, aromatic amines are forbidden, whereas PAHs are permitted in restricted values.

Behind the glamour surrounding tattoos, there are reasonable suspects that some substances present in tattoo inks contain toxic elements that somehow may alter the normal cellular process and create clinical skin and systemic complications.

Medical literature seems to exclude a direct association between the pigments in the dermis and the increased occurrence of cancer; as such this association is considered coincidental.

It looks like there is an apparent paradox due to the fact that tattoo inks contain proven carcinogenic substances, but at the same time there is no clinical evidence given by an increased occurrence of cancer, particularly skin cancer.

We have tried to outline the possible explanations, sometimes conflicting, that scientific research has come upon. The unresolved queries are still numerous and we believe that some time is still needed to explain the complex biochemical interactions between pigments and their final host.

However, considering that many millions of people have been tattooed with ink and colours containing potentially carcinogenic substances, based on current knowledge, there is no clinical evidence of significant risk of cancer caused by tattoo pigment and tattoos.

Given the wide exposure of the population and the seriousness of the pathology, we need to be cautious and do not underestimate these hypothetical risks, also because the long latency of cancer would require a sufficiently big cohort to detect development.

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Enhancement of Eyelash growth using bimatoprost. A clinical trial

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ABSTRACT

Hypertrichosis of eyelashes, characterized by excessive eyelash growth, is a regular phenomenon associated with ophthalmic prostaglandin and prostamide analogues for the treatment of ocular hypertension. Prostamide bimatoprost (Lumigan) has been originally approved for glaucoma treatment. In December 2008 the FDA approved Latisse® (bimatoprost 0.03% solution) which is identical to the ophthalmic solution for glaucoma treatment, with a further indication, that of promoting increased eyelash length, thickness and darkness in patients with hypotrichosis of the eyelashes. When prostaglandin and prostamide analogues interact with the prostanoid receptors in the hair follicle, stimulation of resting follicles (telogen phase) to growing follicles (anagen phase) most likely occurs. Prostaglandin and prostamide analogues may also prolong the anagen phase of eyelashes leading to an increase in eyelash length. Bimatoprost-induced stimulation of melanogenesis appears to result in darker lashes and, at the same time, appears to increase the size of dermal papilla and hair bulb. Dermally applied bimatoprost appears to be associated with a lower incidence of adverse events than its administration as an eye drop.

This clinical trial has been performed on fifteen healthy female patients who applied bimatoprost 0.03% on the upper eyelid daily for six weeks. The results were evaluated with GEA scale. All patients saw improvement of at least one grade.

Keywords
bimatoprost, eyelash growth

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Introduction

The ideal of beauty has varied greatly across centuries, fashions trends and cultures.

Eyes have always played a significant role in face aesthetics. All women have always wished for long, thick, dark eyelashes. Prominent eyelashes are generally recognized as a beauty enhancing feature. Beautiful eyes are associated with social advantage. Eyelashes that are long and thick are considered a sign of beauty and often have a positive psychological effect on women[1].

Until recently, the available options for increasing the prominence of eyelashes were limited to makeup, over-the-counter products, artificial eyelashes, and eyelash transplantation. Originally approved for the treatment of ocular hypertension, prostamide bimatoprost is now approved for treatment of hypotrichosis of the eyelashes. Bimatoprost ophthalmic solution 0.03%, applied once daily to the skin of the upper eyelid margin, increases eyelash growth, length, thickness, and darkness. The efficacy of bimatoprost in eyelash growth has been demonstrated by clinician ratings, digital image analysis, and patient-reported degrees of satisfaction.

The effects of bimatoprost treatment on eyelash length, thickness, and darkness are believed to stem, respectively, from a longer anagen phase duration, increased hair bulb thickness, and increased melanogenesis. When dermally applied, bimatoprost appears to be associated with a lower incidence of adverse events than when administered as eyedrop formulation.

This more favorable safety and tolerability profile is likely mediated by decreased exposure of ocular tissues to dermally applied bimatoprost. The set of available data suggests that cutaneous application of bimatoprost ophthalmic solution 0.03% safely and effectively enhances upper eyelash growth[2].

Anatomy and physiology of eyelashes

On the basis of their length, medullation and dark pigmentation, eyelashes are properly classified as terminal hair, in contrast with vellus, or intermediate hair. In fact, eyelashes represent the thickest hair on the body[2]. Humans have 90 to 160 eyelashes on the upper eyelid and 75 to 80 on the lower eyelid, with great variation of length.

The lashes typically originate from the anterior lamella of the eyelid where they lie on the tarsal plate. They grow in imperfect rows of 5 to 6 on the upper lid and 3 to 4 on the lower lid in the caucasian ethnic group. An eyelash is a terminal hair and as such it is coarse, medullated, long and pigmented. In comparison vellus hair, which is often found on a woman's face, is soft, unmedullated, short and non-pigmented. Eyelashes have the widest diameter of any body hair and are the most pigmented of terminal hair.

Hair follicles have a unique cyclical behaviour pattern and the entire cycle varies in duration depending on body location. Each human eyelash lives an average of 3 to 6 months.

The growth phase (anagen) of the eyelash follicle lasts approximately 30 days[3]. During the anagen phase, in addition to growth, melanogenesis and the subsequent transfer of pigment to the hair shaft also occur. The duration of the anagen phase crucially impacts hair length. Anagen is a period of rapid cell proliferation and differentiation. At the end of the eyelash growth period a brief transition stage (catagen) of 15 days follows when shirinking of the hair follicle occurs[3].

At this time epithelial elements of the follicle undergo apoptosis or programmed cell death[1]. Finally the follicle enters a resting phase (Telogen) of about 100 days, leading to the detachment of the eyelash[3]. Throughout telogen, no significant cell differentiation, proliferation or apoptosis occurs. Human hair growth is asynchronous so that at any given time some follicles are undergoing anagen while others are in the catagen or telogen phase and therefore the proportion of follicles in one phase of the hair cycle is indicative of the relative duration of that phase[2]. The proportion of follicles in telogen is normally higher in eyelashes than scalp hair.

One study estimated that 59% to 85% of eyelash follicles are in telogen phase, depending on whether they are on the upper or lower lid[3]. In contrast with eyelashes, scalp follicles have a much longer cycle lasting several years. Relative differences in the duration of hair cycle phases for eyelash and scalp hair result in approximately 50% of upper eyelash follicles being in telogen at any given time compared with only 5% to 15% of scalp follicles[1].

The length of an eyelash can vary greatly, from 8 to 12mm on the upper lid and from 6 to 8mm on the lower lid: it also depends on ethnic origin. Eyelash growth rate is also quite variable, with an average of 0.12 to 0.14mm daily. All eyelashes are characterized by a tendency to curve from the bulb to the top of the shaft. The degree of curvature depends on ethnic origin. Interestingly eyelashes do not turn grey with aging, or only at a very late stage. Several factors are involved in hair follicle growth and cycles but their effect on eyelash growth is unclear. Androgens are the principal hormones that control sexual hair growth through receptors located in the dermal papilla but eyelashes do not seem to be as responsive[3]. Unlike other types of hair eyelashes are devoid of arrectores pilorum muscles[4].

Beyond their aesthetic and social functions, eyelashes have a protective function. They screen the eye against debris and trigger the blink reflex[1]. Eyelash hypotrichosis is characterized by reduced eyelash

growth. Although its aetiology is often unknown, eyelash hypotrichosis or complete eyelash loss may develop as an adverse effect of cytotoxic chemotherapy.

For many patients hair loss, including eyelash loss, can negatively affect self-image and can lead to impairments in psychosocial functioning[5]. The number of follicles cannot be increased after birth because all follicles develop during embryogenesis[2].

Bimatoprost

Bimatoprost ophthalmic solution 0.03% (Lumigan®; Allergan, Inc.) was initially developed as an ocular antihypertensive and in 2001 was approved for the reduction of elevated intraocular pressure (IOP) in patients with open-angle glaucoma or ocular hypertension. As a glaucoma treatment, bimatoprost ophthalmic solution 0.03% is administered once daily as a drop into the affected eye[2]. The mechanism of IOP reduction involves secretion of metalloproteinases by ciliary smooth muscle cells and remodelling of the extracellular matrix leading to the widening of intermuscular space, and ultimately to an increase of uveoscleral outflow of aqueous humor[3].

Bimatoprost is a synthetic prostamide F2α analogue. Prostamides and their structural analogues are structurally, pharmacologically and functionally distinct from prostaglandins and prostaglandin analogues (Figure 1).

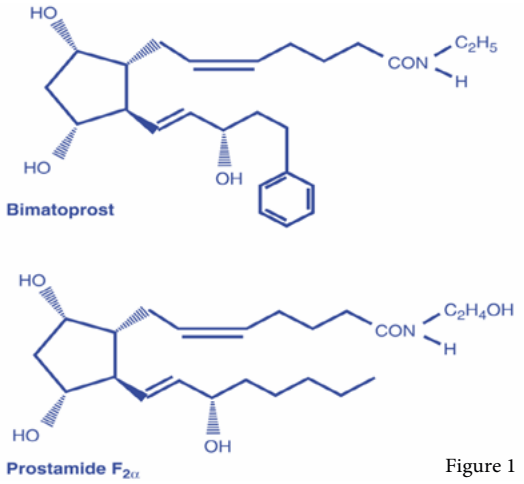

Figure 1

Figure 1 - Bimatoprost and Prostamide F2α structures

Prostamides have recently been identified as a unique class of compounds that are formed from anandamide catalyzed by cyclooxygenase-2 (COX-2). Anandamide is one of the naturally occurring mammalian endogenous cannabis-like ligands (endocannabinoids) derived from arachidonic acid[3].

The capability of bimatoprost ophthalmic solution 0.03% to influence eyelash growth was a fortuitous discovery based on the observation of patients receiving the drug during trials for the assessment of the drug's antihypertensive properties[2].

Although the precise mechanisms by which bimatoprost ophthalmic solution 0.03% increases eyelash growth have not been fully clarified, this medication is believed capable of altering the eyelash

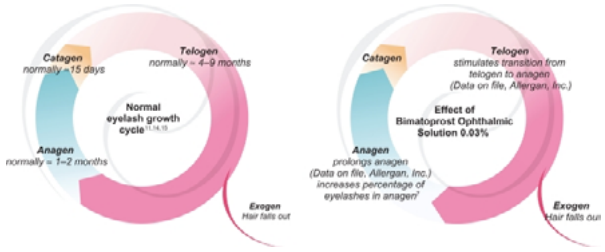

Figure 2

Figure 2 – Normal eyelash growth cycle vs. potential effect of Bimatoprost

hair cycle[2] (Figure 2).

The findings suggest that bimatoprost ophthalmic solution 0.03% stimulates the transition from telogen to anagen phase and prolongs the duration of anagen thereby contributing to increase the length of eyelashes.

Animal studies also demonstrate that treatment with bimatoprost ophthalmic solution 0.03% results in increased diameter of both dermal papilla and hair bulb of follicles in early anagen phase. Increased eyelash darkening results from increased melanin production.

A bimatoprost-induced increase in melanogenesis does not appear to be associated with inflammatory reactions, melanocyte proliferation or melanocyte atypia.

Treatment with bimatoprost does not increase the original number of eyelash follicles. While the number of follicles remains constant, the type of hair they produce can change since bimatoprost may cause previously undetectable or less visible vellus hair to become pigmented and thicker terminal hair[2].

Bimatoprost eyelash gel appears promising for chemotherapy-induced madarosis. Patients may find the results both restorative and cosmetically pleasing[6].

In December 2008 Allergan released Latisse, the first FDA-approved treatment for hypotrichosis of the eyelashes[7] which was clinically tested in a Phase III study to assess safety and efficacy.

As detailed in the Latisse prescribing insert, Latisse contains the active ingredient bimatoprost 0.3 mg/mL, which is the same active ingredient as in Lumigan. Inactive ingredients in Latisse include the preservative benzalkonium chloride 0.05 mg/mL, sodium chloride sodium phosphate, dibasic, citric acid, purified water and sodium hydroxide and/or hydrochloric acid may be added to adjust the pH[7].

Bimatoprost side effects and safety in hypotrichosis

Prostaglandin and prostamide analogues have been associated with similar systemic and local side effects when administered topically on the surface of the eye as an ocular hypotensive agent for glaucoma[3]. Since it has a long history as a glaucoma treatment, safety data for bimatoprost ophthalmic solution 0.03% as an eyedrop are understandably more extensive than for dermal administration[2]. When bimatoprost is applied to the eyelashes and lid margins in very close proximity to the surface of the eye to promote eyelash growth, potential side effects may occur if the medication is inappropriately applied to the surface of the eye[3].

The most common adverse effects are eye pruritus, conjunctival hyperemia, skin hyperpigmentation, ocular irritation, dry eye symptoms, and erythema of the eyelid[2].

As an ocular antihypertensive, the incidence of increased iris pigmentation, which is believed to be a permanent change, associated with once-daily bimatoprost is estimated to be 1.5%, with all recorded events occurring within the first year of treatment[2].

Several factors may contribute to the improved safety profile observed when bimatoprost ophthalmic solution 0.03% is dermally applied. Skin hyperpigmentation, an adverse effect reversible upon discontinuation of bimatoprost, appears to be the result of drop-skin contact[2]. The risk of such pigmentary changes can be minimized by reducing contact of medication with the skin.

Detailed instructions on safe and effective use have become commonplace in practitioners' offices who prescribe and/or dispense bimatoprost ophthalmic solution 0.03%. The proper application for eyelash growth with the aid of the accompanying applicator would seemingly minimize the chance of unintended skin-bimatoprost contact.

As demonstrated in an ocular splash test conducted using lissamine green dye, dermal application of bimatoprost ophthalmic solution 0.03% appears to deliver less medication to the ocular tissues than a drop instilled into the eye. Additional testing has demonstrated that, based on weight, approximately 5% of the dose of bimatoprost ophthalmic solution 0.03% on the applicator (i.e. one drop) is delivered to the patient (data on file; Allergan, Inc.). Exposure of ocular tissues to dermally applied bimatoprost ophthalmic solution 0.03% may be further decreased as a result of the skin's barrier function (data on file; Allergan, Inc.)[2].

Other safety assessments

No clinically meaningful changes were reported in best-corrected visual acuity, vital signs, or physical examination[5]. There were no clinically meaningful changes in mean IOP during bimatoprost treatment[5].

Topically applied prostaglandin F2α analogues (bimatoprost, latanoprost, travoprost and unoprostone) all have similar side-effect profiles, which include both side effects that occur frequently (e.g. conjunctiva hyperaemia, increase of iris pigmentation and eyelash changes) and rare adverse reactions (e.g. periocular pigmentation, damage to the blood–aqueous barrier and cystoid macular oedema). Conjunctiva hyperaemia, eyelash changes and cystoid macular oedema are reversible but certain other side effects, such as increased iris pigmentation, are not.

The systemic side-effect profile is however, favourable for all the prostaglandin analogues and some of the local side effects are only of cosmetic significance. Numerous clinical studies suggest that discontinuing treatment with prostaglandin analogues on account of their side effects is rare in clinical practice[8]. A study by Sherwood et al. reported the most common side effects of topical ocular application of prostaglandin analogues. These included mild hyperemia of the eye in approximately 31% of patients, ocular pruritus in about 14% of patients, and ocular dryness in 7% of patients. Less common side effects (1% of patients) included hyperpigmentation, swelling of periorbital skin and heterochromia, a permanent darkening of the iris more commonly seen in blue/brown or green/brown irises[7].

All cases of conjunctival hyperemia in the bimatoprost-treated patients were transient and resolved before the end of the study despite continued application of the product[4].

Other adverse effects include lowering of IOP, deepening of the eyelid sulcus, ocular irritation, and macular edema. These reactions occurred in less than 4% of patients involved in clinical trials.

In summary, the current studies demonstrated that bimatoprost 0.03% applied topically to the upper eyelid margin is effective in producing longer, darker, thicker and more prominent eyelashes with a favourable safety profile supported by ophthalmic examination[9].

It is also important to notice that Bimatoprost topical dermal application to the eyelid margin, once daily provides a clinically meaningful benefit of rapid eyelash recovery in patients with chemotherapy-induced eyelash loss or hypotrichosis. The treatment was well-tolerated with minimal adverse events[5].

In two multicenter, double-masked, randomized, parallel-group studies on Japanese subjects, ophthalmic examination showed slightly greater mean reductions in intraocular pressure (IOP) with bimatoprost than with vehicle and the reductions were within the normal range for daily IOP fluctuations[9].

Materials and methods

This clinical trial has been performed on 15 healthy caucasian women (age 41-81). Participants received treatment with bimatoprost ophthalmic solution 0.03%

once a day for at least 6 weeks on a regular basis after their evening routine.

All fifteen subjects enrolled in this study completed the study. Most of them continue to use bimatoprost after the conclusion of this study.

GEA Scale (Global Eyelash Assessment Scale)

The primary efficacy measure was the four-point Global Eyelash Assessment (GEA) Scale. The GEA Scale is a validated 4-point scale with photonumeric guide (1=none or minimal eyelash prominence; 2=moderate eyelash prominence; 3=marked eyelash prominence; 4=very marked eyelash prominence)[5], a proven, reliable and reproducible instrument. It asks raters (in this case the patients) to evaluate their overall upper eyelash prominence, focusing on the qualities of length, fullness, and darkness.

GEA scores of 1, 2, 3, and 4 indicate minimal, moderate, marked, and very marked eyelash prominence respectively and it includes a photonumeric guide (Figure 3) to assist raters. Response was defined at 1-grade increase in GEA score. It corresponds to frontal and upper eyelash view. The patients expressed their evaluation at the end of the trial (six weeks).

Figure 3

Figure 3 – GEA Scale Photonumeruc guide

All patients received both a specific informed consent and a specific instructions sheet for the safe use of the Lumigan solution. Lumigan ophthalmic solution has the same composition as Latisse.

Adverse Effects survey

All the patients enrolled in this study received a specific warnings' sheet about bimatoprost side effects.

The most common adverse events associated with bimatoprost use were eye pruritus, conjunctival hyperemia, skin hyperpigmentation, ocular irritation, dry eye symptoms, and erythema of the eyelid, all occurring in fewer than 4% of patients (Latisse package insert, Allergan, Inc., 2008).

Conjunctival hyperemia was the only specific event reported at a significantly higher rate by subjects receiving bimatoprost (3.6%) than those receiving vehicle (0%) ($P = 0.03$)[1]. To evaluate the most common bimatoprost side effects on palpebral application all the subjects enrolled in this study filled in a short questionnaire (Table 1).

Adverse effects Survey			
Discomfort or adverse effects using bimatoprost?	Often ×	Sometimes ×	Rarely ×
Which ones and how often?			
Eye redness	Often ×	Sometimes ×	Rarely ×
Itch	Often ×	Sometimes ×	Rarely ×
Eye dryness	Often ×	Sometimes ×	Rarely ×
Eyelid pigmentation	Yes	A little ×	No ×
Iris Pigmentation	Yes ×	A little ×	No ×
Other (Specify)			

Table 1 - Adverse effects survey form

How to apply Bimatoprost

To help the patient with a correct application of the drug and to minimize complications we provided an instructions sheet.

Instructions

Before applying Bimatoprost each night, ensure that your face is clean, makeup and contact lenses are removed and that any facial care product has already been applied. Hold the small applicator brush and place half a drop of Bimatoprost on the brush without the container touching it. Move the brush carefully along the skin at the base of the upper eyelashes (where they meet with the skin) starting at the nose side of your lash line and continuing outwarads. Do not apply on the lower lid because excess hair can grow outside the treatment area. Blot any excess solution beyond the eyelid margin with a tissue. Repeat for opposite eyelid. Be patient. It will take some time to achieve a satisfactory result, at least six weeks! Take a picture of your eyelashes before starting, you will be pleasantly surprised at the results.

Contact lenses may be worn after 15 minutes. If you stop using Bimatoprost your lashes will gradually return to their previous appearance.

Informed consent

Bimatoprost 0.03% is an ophthalmic solution used to reduce Intraocular pressure. One of its side effects is eyelashes growth. Its use for this purpose is not authorized in Italy. This is an off-label use. In 2008 the FDA approved Bimatoprost 0.03% (Latisse®) for increasing eyelash length, darkness and thickness. Bimatoprost can cause increased brown pigmentation of the iris which is likely to be permanent. Eyelid skin darkening may occur but may be reversible. If you are using or have used prescription products for any eye pressure problems, only use bimatoprost under close medical supervision. If you develop or experience any eye problems or need

eye surgery, consult your doctor immediately. The most common side effects after using bimatoprost solution are itchy eyes and/or eye redness[10].

Results

In our little clinical trial Bimatoprost Ophthalmic solution 0.03% was generally well tolerated.

Adverse events most frequently reported were: itchiness, eye redness, eye dryness and two cases of eyelid pigmentation. Itchiness has been mild and well tolerated, so has the eye redness (Figure 4).

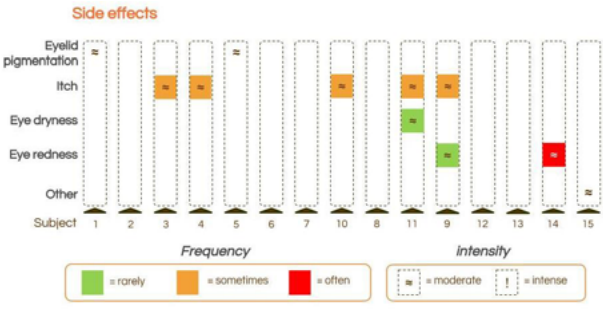

Figure 4

Figure 4 – Graphic display of symptoms reported by patients

No patient has stopped treatment due to these AE. Skin darkening in one of two cases disappeared as the treatment ended. In the other case it has been aptly transformed into semi-permanent makeup. No AE resulted in study discontinuation. No serious or unexpected AE were reported. We have no data on ophthalmic examination (IOP measurement, visual acuity, biomicroscopy and iris colour evaluation).

On the GEA scale all patients reported at least on grade of improvement and three patients reported up to two grades (Figure 5).

Comparisons are also reported through figures 6 to 12, where it is clearly shown that every subject got at least one degree enhancement after a 6 weeks treatment.

Discussion

Although we had no adverse events in our small study, given the properties of prostaglandin analogues and the potential for side effects, we recommend evaluation, prescription and monitoring by an aesthetic doctor/ophthalmologist if use of such a product is going to be considered, especially in the growing cosmetic industry where many non-qualified physicians may be eager to recommend such treatment[7].

The mechanism by which bimatoprost enhances eyelash growth has not been fully clarified. It appears to increase both the duration of the anagen phase and the

Figure 5

Figure 5 – Efficacy of Bimatoprost as assessed by GEA scale. All subjects had at least one grade increase from baseline

Figure 6 – Eyelash enhancement achieved (6th week of treatment - Subject#14)

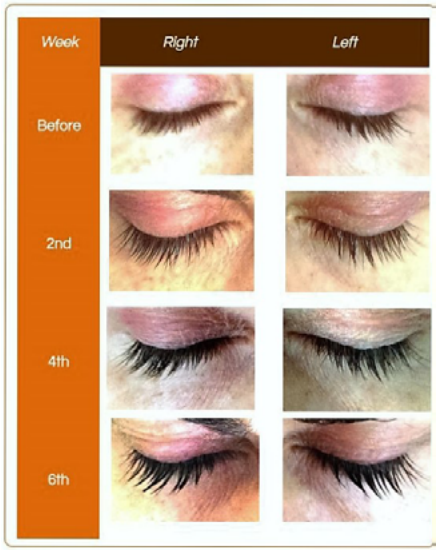

Figure 7

Figure 7 – Eyelash enhancement achieved (6th week of treatment - Subject#11)

Figure 8

Figure 8 – Eyelash enhancement achieved (6th week of treatment - Subject#12)

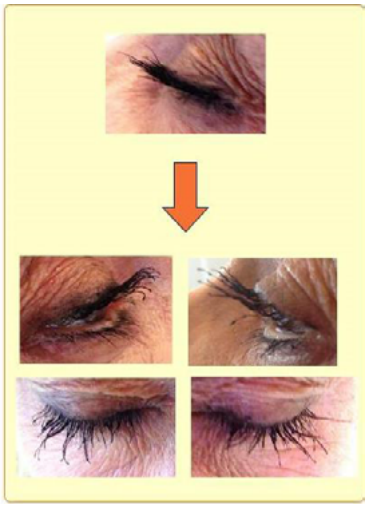

Figure 9

Figure 9 – Eyelash enhancement achieved (6th week of treatment - Subject#15)

Figure 10

Figure 10 – Eyelash enhancement achieved (6th week of treatment-Subject#9)

Figure 11

Figure 11 – Eyelash enhancement achieved (6th week of treatment-Subject#5)

Figure 12

Figure 12 – Eyelash enhancement achieved (6th week of treatment-Subject#1)

percentage of hair follicles in this phase (Latisse package insert, Allergan, Inc., 2008). Bimatoprost also appears capable of stimulating melanogenesis which likely explains the changes in eyelash pigmentation observed during its use[1].

Patients should be advised of the small potential for increased brown iris pigmentation which is likely to be permanent[1].

Conclusion

Bimatoprost ophthalmic solution 0.03% is an effective and safe treatment in a host of applications and another treatment that appears to improve the quality of life for those patients seeking aesthetic improvement with eyelash growth and prominence.

Compared to vehicle-treated subjects, Bimatoprost-

treated subjects reported statistically significant levels of satisfaction with their eyelashes in terms of the lashes' physical and subjective attributes (satisfaction with eyelashes as they relate to feelings of confidence, professionalism, and attractiveness) and the daily routine of making their eyelashes presentable.

Current evidence suggests that topical cutaneous application of bimatoprost 0.03% to the upper eyelid margin is a safe and effective way of enhancing eyelash growth[4].

Bimatoprost ophthalmic solution 0.03% increases growth of natural eyelashes and increased growth is correlated with increased patient satisfaction.

We can conclude that Bimatoprost is an excellent way to increase eyelash prominence thanks to:

- A greater amount of anagen follicle
- A reduction of telogen follicle
- An increase in eyelash length
- An increase in eyelash pigmentation
- An increase in eyelash thickness
- A more acute exit angle from the eyelid
- Vellus hair changes into medullated hair (thicker and darker)
- Eyelash growth also in cantal zone
- Generally well tolerated
- Minimal AE
- Less time for daily routine
- Great patient satisfaction

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Courses and Congresses

2016

16-17 September – Paris (France)
37th National Congress of Aesthetic Medicine and Dermatologic Surgery
French Society of Aesthetic Medicine
French Association of Morpho-Aesthetic and Anti-Aging Medicine
National Institute of education in aging prevention
Venue: Palais de Congres
www.sfme.info
congress@sfme.info

9-10 December – Lisboa (Portugal)
1st National Meeting of Aesthetic Medicine
Portuguese Society of Aesthetic and Anti-Aging Medicine
President: Joao Pedro Vale
Venue: Sana Maloha Hotel
www.spme2016.com
secretariado@spme.pt

2017

16- 18 February - Malaga (Spain)
32th Congress of the Spanish Society of Aesthetic Medicine
President: Petra Vega
Venue: Palacio de Congresos y Ferias de Málaga
www.seme2017.org
seme2017@pacifico-meetings.com

17 March - Opatija (Croatia)
Photo and Chrono skin aging
Venue: Grand Hotel Adriatic
Croatian Society of Aesthetic Medicine

21 April - Opatija (Croatia)
Insufficiency of oligoelements and presence of heavy metal in correlation with degenerative deseases in Primorsko Goranska region
Venue: Grand Hotel Adriatic
Croatian Society of Aesthetic Medicine

12-14 May – Rome (Italy)
38th National Congress of the Italian Society of Aesthetic Medicine
12th National Congress of the Italian Academy of Aesthetic Medicine
Venue: Congress Centre Rome Cavalieri
President: Emanuele Bartoletti
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congresso@lamedicinaestetica.it
www.lamedicinaestetica.it

8-9 September - Paris (France)
38th National Congress of Aesthetic Medicine and Dermatologic Surgery
French Society of Aesthetic Medicine
French Association of Morpho-Aesthetic and Anti-Aging Medicine
National Institute of education in aging prevention
President: J.J. Legrand
www.sfme.info

22-24 September - Almaty (Kazakhstan)
9th National Congress of Aesthetic Medicine and Plastic Surgery
Kazakhstan Association of Aesthetic Medicine and Plastic Surgery
President: G. Zhumatova
info@estetic.kz
www.estetic.kz

27-29 October - Istanbul (Turkey)
21th World Congress of Aesthetic Medicine
Turkish Society of Aesthetic Medicine
President: Hasan Subasi
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Contents