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Developing a Topical Adjunct to Injectable Procedures

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ABSTRACT

Injectable procedures have come to play an enormous part in everyday aesthetic medical practice. Whether its use is directed at volumizing with fillers, decreasing volume using enzymes, skin-tightening using multi-needle approaches, or neuromuscular blockade, the injectable route is the means of delivery in all these cases, making injectable procedures the most common aesthetic procedure performed. As with all procedures, expected and unexpected consequences may follow including bruising, swelling, discomfort, and the possibility of infection. This paper outlines the scientific process and validation of a product designed as an adjunct to injection therapy and the scientific deep dive needed to encompass both symptomatic and adjunctive purposes. On the symptomatic side, bruising, swelling, and pain were considered, while volumetric enhancement, regeneration, and anti-microbial/biofilm effects were desired outcomes from the adjunctive perspective. Utilizing peptides and active agents aimed at reducing excess residual iron and stimulating macrophage absorption of red blood cells, we were able to achieve efficient resolution of bruising. In addition, peptides were included to stimulate collagen, elastin, and hyaluronic acid in synergy with the injectable. Anti-inflammatory, antimicrobial, and antibiofilm agents were added to aid in the safety profile of the injectable. In vivo testing of bruising resolution demonstrated that at day 2/3, participants using the study product (INhance Post-Injection Serum with TriHex Technology®, Alastin Skincare, Inc. Carlsbad, CA) had 73% less bruise color intensity and statistically significant improvement over the bland moisturizer. Overall, 81% of subjects applying the study topical product had less bruising at day 2/3 compared to the bland moisturizer.

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INTRODUCTION

Every invasive surgical procedure from the simplest injection to the more complex surgical procedure creates the potential for bruising and swelling. Even newer techniques such as cannula delivery of injectables still require needle introduction for the portal site, and the cannula itself may still cause a bruise. Bruising is a result of extravasation of red blood cells (RBCs) into the tissue. Once outside the vascular system, RBCs quickly burst, releasing free hemoglobin (Hb) that is prone to oxidation states that have potent pro-inflammatory and pro-oxidant effects.[1] The heme that is released is phagocytized by macrophages. Following internalization by the macrophage, heme is cleaved into biliverdin, carbon monoxide, and iron. This mechanism not only provides effective elimination of Hb, but it also assures iron recycling for new erythropoiesis.[1] However, leaving the byproducts of bleeding around for too long creates the risk of the pro-inflammatory effects mentioned above that can interfere with wound healing, promote pigmentation and (of course) look very unsightly in addition to representing an obvious sign of injection or surgery. In addition, delayed bruising accompanying filler injections has recently been attributed to the hyaluronic acid structure closely resembling that of heparin and behaving in similar ways in certain situations.[2] Sometimes bruising cannot be avoided, and no topical agent currently available can prevent it; nevertheless, there is a way of accelerating bruise resolution by removing by-products of RBC extravasation more efficiently.

A bruise typically appears hours after injury to the tissues just below the skin's surface or even sooner (occasionally instantly) if a blood vessel is breached during a procedure. RBCs seep into the surrounding tissue and macrophages begin to break down the cells that rapidly lose oxygen giving them a bluish hue. The byproducts of hemoglobin breakdown (heme, biliverdin, bilirubin, hemosiderin) transmit the various colors to the skin that slowly resolve once these pigments are absorbed by the macrophages and digested, known as blood product accumulation. This process can vary from days to weeks depending on the degree of accumulation of blood products. The testing that follows was conducted to determine whether lactoferrin, phosphatidylserine, and selected peptides can enhance the process of phagocytosis and improve the functionality of macrophages.

STUDY DESIGN

In Vitro Testing of Macrophage Phagocytosis of Red Blood Cells

First, an in vitro model for hemosiderin-laden macrophages was established and then used to test active agents and peptide activity in hemosiderin clearance. RBCs were labelled with a fluorescent marker (opsonized human RBCs with human RBC antibody); macrophages were added and the number of macrophages that phagocytize RBCs were observed (Figure 1). Non-phagocytized RBCs were then washed out, and the remaining macrophages were lysed, exposing phagocytized RBCs and the absorbance of these labelled RBCs were measured to assess macrophage function. We were able to draw an RBC absorbance curve and validate the model by measuring absorbance of RBCs that came from within the macrophages (Figure 2). Using human RBCs and macrophage cell lines, 24 hours was determined the best time point to measure the phagocytosis in this model. At 24 hours, the peptide-combination treatment described below showed the highest increase in phagocytosis.

In Vivo Testing of Bruising Resolution

A multi-center, randomized, double-blind study was undertaken to assess the efficacy and safety of a topical product formulated to increase the elimination of blood products that manifest as bruises. The study was approved by an institutional review board and all procedures were performed with respect to the ethical principles stated in the Declaration of Helsinki (amended in October 2013). This study investigated the topical study products' ability to improve the appearance of bruises compared to a bland moisturizer. Skin types I–III, male and females aged over 18 were included. Eighteen participants were enrolled to receive bruises. Two were withdrawn due to lack of data leaving 16 participants – 32 bruises. Four additional participants were enrolled and underwent biopsies before and after treatment.

Eligible participants received an induced bruise on both arms via mechanical disruption, venipuncture, of a vessel as in a blood draw (one group) or via removal of blood and re-injection of 0.1 ml subdermally in the proximal inner forearm immediately distal to the antecubital fossa (second group).

Participants were randomized to receive either topical study product or bland moisturizer to use on the designated arm at a minimum of four times daily. One arm was treated with the topical study product on the bruise four times daily and the other arm was treated with the bland moisturizer four times daily. Participants were screened and underwent the procedure at the (day 0, baseline) visit and had follow-up visits at days 1, 2, 3, 6, and 7. Skin colorimetry (SkinColorCatch, Delfin Technologies, Kuopio, Finland) was performed in triplicate at each visit and clinical changes of bruises were documented using photography. Assessed endpoints included global improvement in the appearance of bruises between arms with comparison in SkinColorCatch measurements. Four participants consented to undergo a biopsy on the forearm at Day 0, pre-treatment, and at approximately two weeks post topical application, four times daily.

FIGURE 1. Visual example (higher magnification) of a typical test with macrophage phagocytosis, after RBC washout – what remains are macrophages with and without RBCs in them (identified by hollow circles, white arrows – abundant in comboT as opposed to dark central nuclei without RBCs – more abundant in NT).

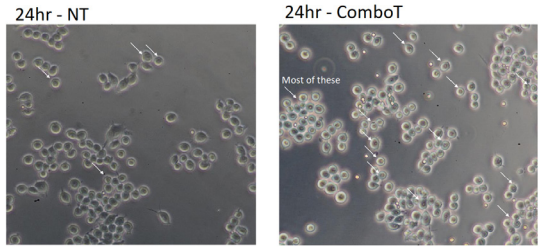
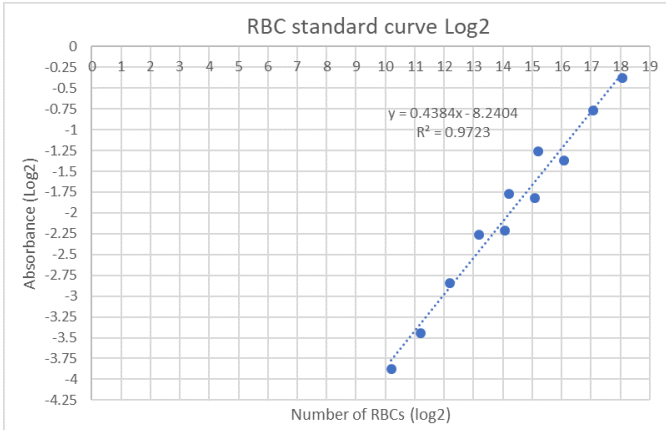


FIGURE 2. An RBC standard curve was plotted to quantitate the number of RBCs that are phagocytized in an assay. From this, we were able to extrapolate the number of RBCs from an absorbance value.

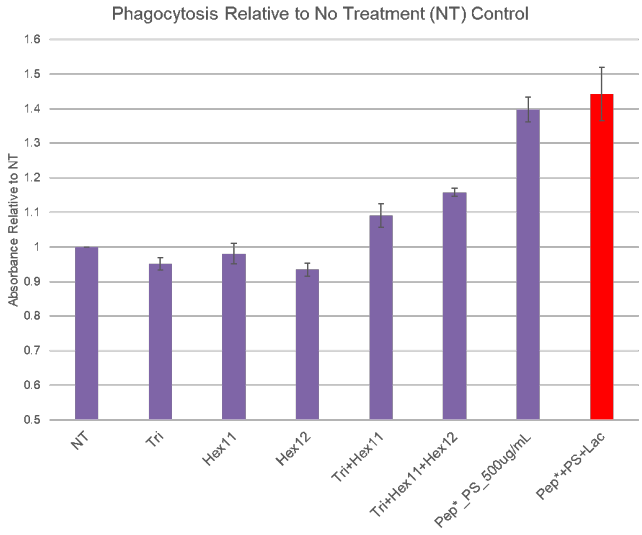


RESULTS

In Vitro

Macrophage phagocytosis of red blood cells: (NT = No treatment; Figure 3). Phagocytosis of RBCs following exposure of peptides and other active (phosphatidylserine-PS-and Lactoferrin-Lf) was assessed: Each 0.1 increment on Y-axis demonstrates a 10% increase in efficacy of macrophage function representing significant increased RBC phagocytosis; the peptide combination (Tri+Hex11+Hex12 - as opposed to individual function) showed an increase in efficacy (15% increase over control); the addition of phosphatidylserine demonstrates a 30% increase over control and thus a substantially increased efficacy over the peptide combination. The addition of lactoferrin gave an increased efficacy of around 47% over control and approximately 30% increased efficiency over peptide combination alone. The

FIGURE 3. Macrophage function and RBC phagocytosis.



results demonstrated here are all represented by triple replicates; the combination of phosphatidylserine and lactoferrin at 500 µg/ml each with peptides tripeptide- 1, hexapeptide-12, and hexapeptide-11 constitute a synergistic combination for increasing macrophage phagocytosis of red blood cells and present exciting possibilities for hastening resolution of bruising.

In Vivo

Objective assessment using colorimetry, SkinColorCatch, was deemed the most accurate means of assessment. Measurement of the intensity of the blue/yellow channel was undertaken at all time points – this score was then compared with the baseline score prior to bruise creation and the delta between blue intensity and original clear skin was compared between the two groups. In effect, this provided information of bruise resolution. In order to ensure equivalence of both groups, the scores immediately post-bruising needed to be comparable – this is illustrated in Figure 4. The two models showed slight variation

FIGURE 4. Bruise color intensity/duration.

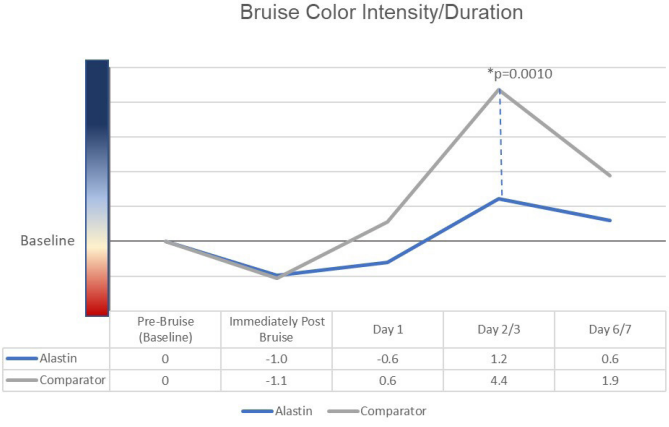
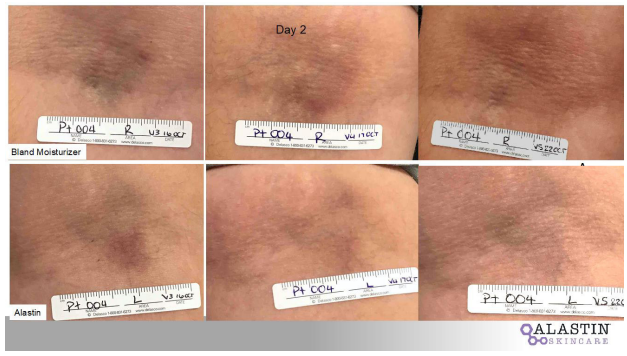


FIGURE 5. Typical examples from Group 2 showing decreased intensity on day 2 heralding hastened resolution of bruising compared to comparator.



– injecting subdermally manifested with bruising slightly later – thus, days 3 and 7 in this group were equivalent to days 2 and 6 in the blood draw group. After analyzing the data, it was immediately apparent at day 2/3 that participants using the study product had 73% less bruise color intensity than the bland moisturizer, a statistically significant improvement over the bland moisturizer. Overall, 81% of subjects applying the study topical product had less bruising at day 2/3 compared to the bland moisturizer. This result suggests that the topical study product hastened resolution of bruising (Figures 5 and 6).

An interesting observation seen in many cases was the transition from blue to red that took place in the experimental group prior to resolution (see day 3, Figure 6 test group 1). Apparently, the blue pigment is absorbed efficiently leaving residual redness that rapidly dissipates. This is different from the often-described phases of bruising from oxygenated Hb (red) to de-oxygenated Hb (blue) then green, yellow, etc. to dissolution. It appears in the cases tested that the product encourages macrophage efficiency (as validated in vitro tests) that then absorbs pigment, leaving residual red pigment that resolves very quickly.

In addition to the bruising resolution, symptomatic relief from inflammation, swelling, and pain is provided by phytoene and phytofluene, colorless carotenoids derived from saltwater microalgae that modulate prostaglandin E-2 (PGE-2) and have the ability to reduce oxygen radicals and absorb potential damaging visible light.[11] In addition, xylitol has been shown to have anti-inflammatory and antibiofilm effects especially when combined with lactoferrin.[4] *Arnica montana* extract contains flavonoids and coumarins, accelerates healing, and reduces inflammation associated with bruising products by stimulating M2 macrophage function.[12] Ledum palustre also known as Marsh Tea or Wild Rosemary has been used homeopathically for insect bites, puncture wounds, and swellings or bruises.[13] It appears to have synergistic activity with arnica.[13] This was demonstrated in split-face testing in patients following radiofrequency (RF) mi-

FIGURE 6. Typical example from Group 1 showing distinct differences at day 3 – blue appears to convert to red as the pigment is absorbed – this is followed by hastened resolution.

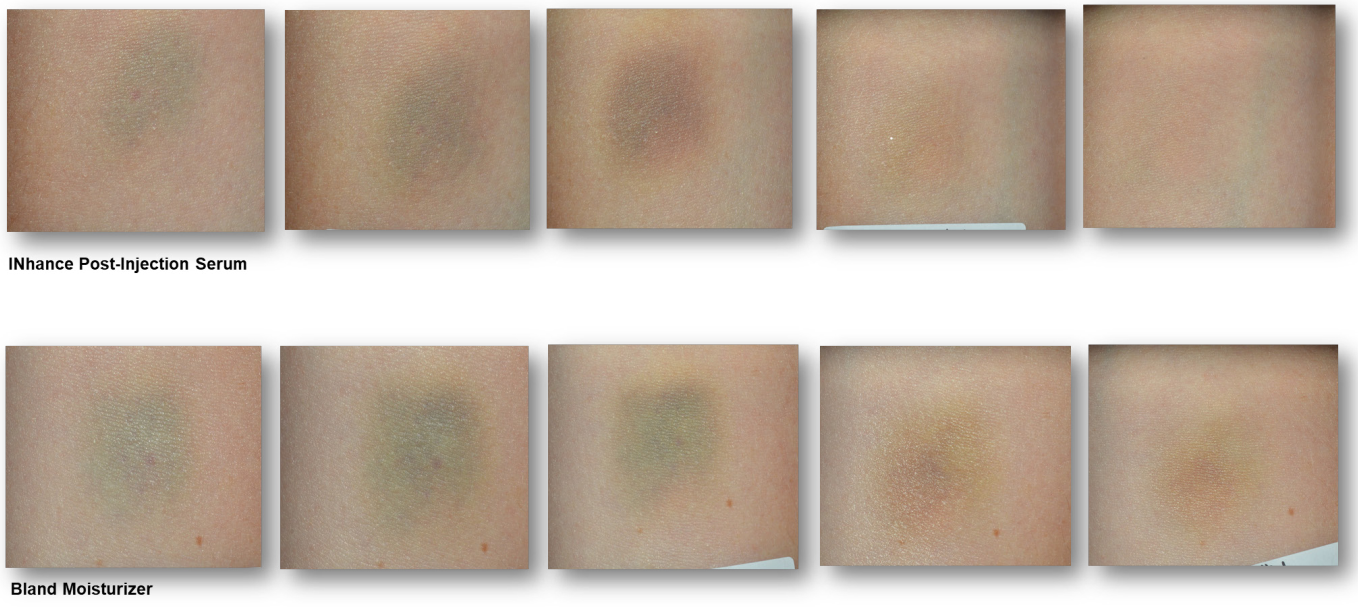
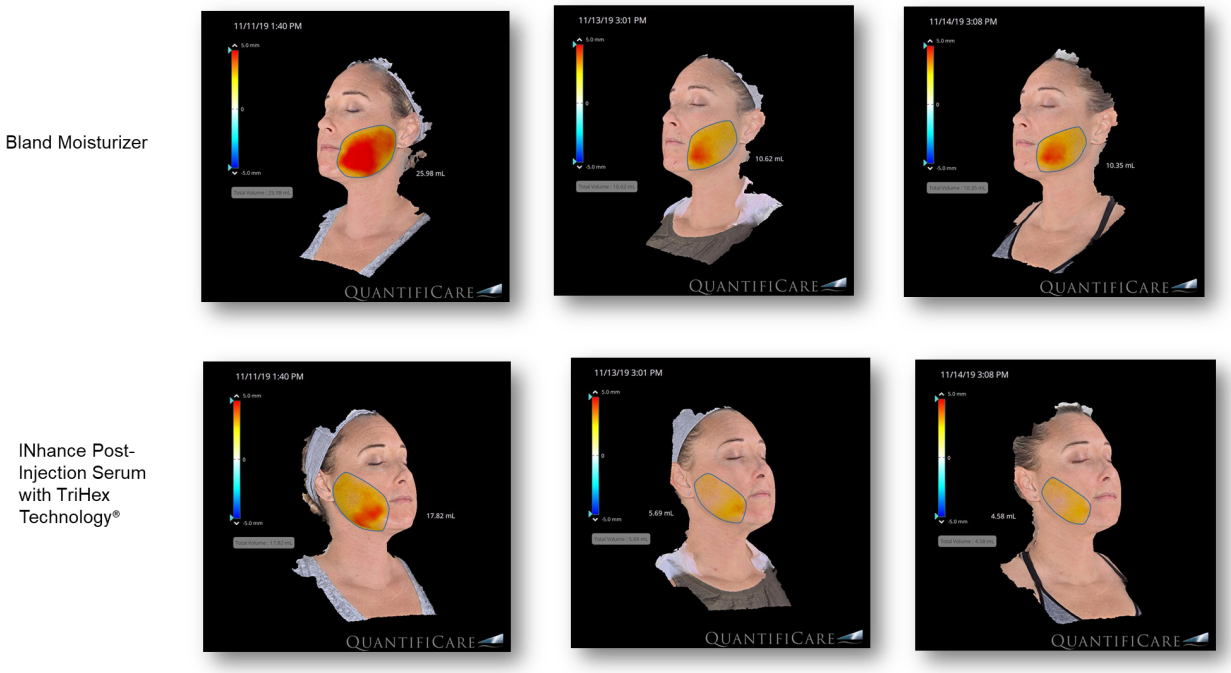


FIGURE 7. Volume/swelling assessment analysis. Red corresponds with increased volume and swelling. Patients underwent radiofrequency microneedling with split face application of topicals following the procedure.



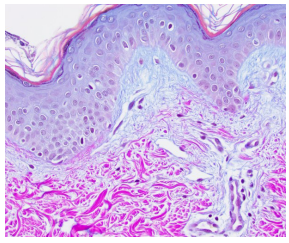
croneedling. Increased volume (red) correlates with swelling on the comparator side versus the test product (Figure 7).

Additional Benefits Related to Active Ingredients in Topical Product: Volumetric Enhancement and Regeneration

Elastin (and collagen) stimulation synergy with fillers (Figure 8 and Figure 9)

Elastin is an assembly of microfibrils and tropoelastin (or soluble elastin). Elastin fibers are formed first by the synthesis of fibrillin microfibers that intertwine and then associate with tropoelastin (TE) protein molecules. TE molecules are bound together and cross-linked together with fibrillin fibers by lysyl oxidases such as enzyme 1 (LOXL1), a key player regulating the assembly of these two elements. This complex is then presented to the fibroblast by Fibulin 5 (FBLN5) that connects the complex to integrins that connect to fibroblasts.[14-16] TriHex (palmitoyl tripeptide-1 and palmitoyl hexapeptide-12) clear the extracellular matrix of aggregated fragmented collagen and elastin and then stimulate increased new collagen and elastin production.[9,17] Acetyl tetrapeptide-2 increases FBLN5 and LOXL1 protein levels, thereby increasing elastin synthesis. It also upregulates genes related to collagen 1 synthesis. In vivo, it has been shown to reduce parameters linked to skin flaccidity and dermal disorganization.[18] *Anethum graveolens* (Dill extract) produces a reinduction of LOXL synthesis.[15] While microfibrils and soluble elastin continue to be synthesized throughout life, LOXL dramatically decreases from the age of 18. In addition, lactoferrin stimulates tropoelastin synthesis.[19,20] As with previous studies, we were able to validate changes taking place at a molecular level involving collagen and elastin in all 4 patients biopsied (Figures 8 and 9). These were early time points (biopsies at 2 weeks after treatment) but even taking that into consideration we were able to demonstrate good early regeneration of collagen and elastin (fibrillin) from topical use of the product. This confirms the advantage of using an adjunctive topical therapy demonstrating synergistic effects on volumization at a molecular level.

FIGURE 8A AND 8B. Herovici stain 40X magnification – this stain demonstrates new mucopolysccharide formation (denoted by blue areas in papillary dermis)- this represents the early step in generation of collagen fibers. 8a – pretreatment; 8b – 2 weeks after use of topical therapy 4X/day. Note good GAG stimulation in papillary dermis.



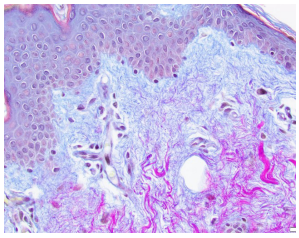
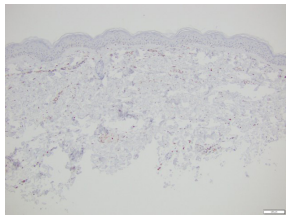
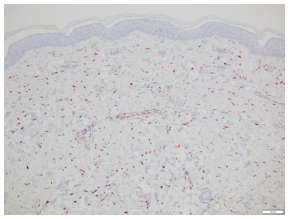


FIGURE 9A AND 9B. Fibrillin stain 10X magnification – stained in brown, fibrillin is one of the first components of elastin to be destroyed by photodamage. Likewise, regeneration of fibrillin is critical to the reversal of solar elastosis and regeneration of new elastin fibers. 9a – pretreatment; 9b low power image showing profuse increase in fibrillin staining 2 weeks after use of topical therapy.





Intrinsic hyaluronic acid (HA) stimulation

The promotion of intrinsic HA stimulation is extremely advantageous over extrinsic addition of HA products. This is important as many topical preparations use combinations of high and low molecular weight (MW) HA to promote skin hydration. However, in the wound healing literature, low MW HA is known to be very pro-inflammatory and should be avoided whenever possible when treating the skin.[21] That leaves high MW HA that is very efficient in treating the skin surface but cannot penetrate the skin depth to the dermis. It seems logical then to combine a high MW HA together with agents that stimulate the fibroblast to produce more HA in the depths of the skin. Hydroxymethoxyphenyl decanone is a potent hyaluronic acid booster, antioxidant, and anti-irritant. It has been demonstrated to stimulate the dermal and epidermal hyaluronic acid level by 259% and 198% versus placebo, respectively, in an ex vivo human skin model.[22] *Tremella fuciformis* extract (from edible white fluffy mushroom) serves as a natural hyaluronic acid providing very high levels of moisture and anti-oxidant properties.[23,24] Lactoferrin has wound healing attributes, promotes proliferation of fibroblasts and increases HA secretion.[25,26] Aside from intrinsic HA stimulation, sodium hyaluronate crosspolymer is a chemically crosslinked hyaluronic acid derived from a non-animal source. It possesses an exceptionally high water-binding capacity resulting in excellent moisturizing abilities.

Adipocyte stimulation – adipogenesis

Newer studies suggest that fillers may stimulate de novo adipogenesis by mechanical stimulation of adipose stem cells possibly in the dermal white adipose tissue layer.[27] This layer sits at a level above the subcutaneous white adipose layer, has direct contact with dermal papilla cells and is an area where follicular stem cells and adipose stem cells appear to engage in cross talk.[27] In addition, acetyl hexapeptide 38 peptide is a PGC1a stimulator (peroxisome proliferator-activated receptor-gamma—PPARγ—coactivator 1 alpha). PGC1a plays a central role in adipogenic activity.[28] PGC1a strongly induces differentiation of preadipocytes into white adipocytes under the influence of PPARγ. The young adipocytes formed under these condi-

tions appear to be small and active, and this size and activity have been seen to be synergistic and in line with good elastin formation.[29] In other words, large, mature adipocytes have been associated with diminished elastin—manifesting as aged sagging skin—whereas younger, smaller, newly synthesized adipocytes are accompanied by increased elastin levels. PGC1a stimulators are thus useful candidates for increasing adipogenesis, providing small active adipocytes through this PPARγ activation pathway.

DISCUSSION/CONCLUSION

Fillers and injection therapy are the most frequently performed aesthetic procedures. Inevitably, a certain amount of discomfort and more significantly occasional bruising is an undesired consequence of medical or cosmetic procedures to the face and body. Additionally, the introduction of synergistic volume replacement of collagen, elastin, hyaluronic acid, and fat, particularly at a more superficial extracellular matrix level serves as an excellent adjunct to the injection therapy. Active agents have been selected that have been demonstrated to improve the efficiency of macrophage phagocytosis of RBCs and heme using a unique in vitro model.

The plasminogen system is essential for dissolution of fibrin clots. Lactoferrin, an iron-binding milk glycoprotein, blocks plasminogen activation on the cell surface by direct binding to human plasminogen, decreasing conversion to plasmin (it is a plasmin inhibitor). It also has anti-microbial activity.[3-5] Lactoferrin effects range from antimicrobial to anti-inflammatory and immune-modulator activities; however, its most striking physicochemical feature is its high iron-binding affinity.[6] Only transferrin has the ability to retain this metal over a wide range of pH values. Thus, it is essential in the process of bruising resolution and in the prevention of post-inflammatory pigmentation.[6] It also induces phagocytosis of cells, microbes and debris.[7] Phosphatidylserine (PS) resides on the inner cell membrane of red blood cells and induces phagocytosis of the RBCs.[8] Tripeptide-1 and hexapeptide-12 in combination have been tested comprehensively in previous in vitro and human studies and have shown important synergistic benefits, particularly in the area of extracellular matrix recycling and neocollagenesis and neoelastogenesis.[9] Hexapeptide-11 is a potent stimulator of autophagy and macrophage clustering.[10]

Volumetric advantages and efficacy of the actives have been previously demonstrated in clinical trials[30] and this combination together with anti-inflammatory, anti-biofilm, bacteriostatic and anti-bruising actives create a comprehensive and unique topical adjunct to injection therapy. Validation of hastened bruising resolution has been achieved using in vitro and in vivo studies.

DISCLOSURES

Alan Widgerow is Chief Medical Office Alastin Skincare Inc. Michaela Bell is Director Clinical Research Alastin Skincare, Inc. John Garruto is a consultant to Alastin Skincare, Inc. Dr. Jacob is a consultant to Alastin Skincare, Inc. Dr. Palm is a consultant and shareholder to Alastin Skincare, Inc.

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