EPIDERMAL AND DERMAL HISTOLOGICAL CHARACTERISTICS IN RESPONSE TO HYDROPORATION

Epidermale und dermale Hydroporation histologisch charakterisiert

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KEY WORDS: Hydroporation, histology, wound healing, drug delivery, fractional laser

SUMMARY

BACKGROUND: Targeted drug deliver through an intact epidermal barrier is of interest in order to treat various skin conditions and diseases. Among many available methods hydroporation has already been shown to be effective for controlled skin rejuvenation by localized epidermal ablation. Therefore the concept might also be suitable to be used to trans-epidermal scarless deposition of substances, drugs or molecules using recently developed handpieces having nozzles smaller than 120 µm. However systematic studies investigating the tissue effects on those hydroporation systems are lacking.

OBJECTIVES: The aims of this in vitro study were (1) to prove the ability of the hydroporation system to penetrate the epidermal compartment and (2) to be able to deposit liquids (NaCl, vitamin a and c solutions), viscous substances (hyaluronic acid), crystalloid suspensions (triamcinolone, 40 mg/ml), and molecules of higher molecular weight like antibodies (IgG-FITC), PEG's (FITC-PEG's) and sugars (Dextrane-FITC) into the dermis using an skin explant model with and without AFXL pre-treatment using two different types of ablative fractional lasers.

MATERIALS AND METHODS: Skin explants were subjected to hydroporation using alcian blue inked 0.9 % NaCl, unstained 0.9 % NaCl applications for 10 s, 30 s, 1 min, ready to use solutions containing vitamin a and c as well as hyaluronic acid crystalloid suspensions, antibodies, hetero-functionalized polyethylene glycol and sugars to investigate morphological tissue changes and to measure distribution within the epidermis and the dermis. To test the potential synergistic effect of fractional ablative laser pre-treatment in conjunction with hydroporation to apply molecules of higher molecular weights two laser systems have been used.

RESULTS: The hydroporation system has been tested for effective deposition of low molecular weight particles in a homogenous distribution up to a dermal depth of 1436 µm. Furthermore hyaluronic acid of low viscosity and crystalloid suspensions could be placed into the dermis of normal skin. In cases of dense collagen fibers as seen in scars deposition was limited. The transport of high molecular weight substances (2, 70, and 150 kDa) was possible through the nozzle of a standard handpiece, however epidermal penetration was limited. Pre-treatment with either a fractional ablative CO_2- or Er:YAG-laser enabled deep dermal deposition of those molecules.

CONCLUSION: This in large vitro study clearly demonstrated that the hydroporation concept can be applied to human skin in a safe and effective manner not only for controlled ablation but also for scarless dermal application of low molecular weight molecules in liquids of low and medium viscosity. The application of high molecular weight compounds was made possible by pre-treating the skin with fractional ablative lasers.

SCHLÜSSELWÖRTER: Hydroporation, Histologie, Wundheilung, transdermal, fraktionale Laser

ZUSAMMENFASSUNG

HINTERGRUND: Die gezielte intra- und transepidermale Bereitstellung von Therapeutika ist von jeher zur topischen Therapie jedweder Erkrankung von hohem Interesse. Bisher wurden zahlreiche Konzepte entwickelt, unter denen die kontaktfreie Hydroporation bereits zur Hauterneuerung und ablativen Therapie eingesetzt wurde. Das theoretische Potential dieser Technik schließt die narbenfreie trans-epidermale Deposition von Substanzen, Arzneimitteln oder Molekülen bei der Verwendung neuer Applikatoren mit Strahldiametern von nur 120 µm ein. Allerdings fehlen systematische Studien zu Gewebepenetrationscharaktersitka und – interaktionen.

ZIEL: Ziel der Studie war es (1), die Penetrationsfähigkeit von Epidermis und Dermis qualitativ und quantitativ zu erfassen (2) den Depositionseffekt von Fertiglösungen (NaCl, Vitamin A und C), viskösen Substanzen (Hyaluronsäure), kristalloiden Substanzen (Triamcinolon, 40 mg/ml), und Molekülen mit hohem und höherem Molekulargewicht wie Antikörper (IgG-FITC), PEG's (FITC-PEG's) und Zucker (Dextran-FITC) in der Dermis anhand eines Explantatmodels mit und ohne Vorbehandlung mittels zweier faktional ablativer Laser zu untersuchen.

MATERIAL UND METHODEN: Hautexplantate wurden in-vitro mit gefärbter (Alcianblau) und ungefärbter 0,9 % NaCl für 10 s, 30 s und 1 min, Fertiglösungen von Vitamin A und C, Hyaluronsäure, Triamcinolonsuspension, Antikörpern, hetero-funktionalisiertem Polyethylenglycol und Dextran hydroporiert und mikroskopisch die Gewebeinteraktion erfasst. Der Einfluss fraktional ablativer Laser auf das Penetrationsverhalten hydroporierter Moleküle hohen Molekulargewichtes wurde mittels CO_2- und Er:YAG-Laser ermittelt.

ERGEBNISSE: Die Hydroporation niedermolekularer Substanzen konnte bis zu einer Tiefe von 1436 µm nachvollzogen werden. Hyaluronsäure niedriger Viskosität sowie kristalloide Suspensionen konnten ebenfalls nach Hydroporation mikroskopisch dermal visualisiert werden. Im Falle von Narbengewebe war die Deposition jedoch limitziert. Die Applikation hochmolekularer Substanzen war ebenso mit den verwendeten Handstücken möglich, jedoch die Penetrationskapazität ohne Vorbehandlung limitiert. Ein fraktional ablative Vorbehandlung führte zur Verbesserung der Penetrationsleistung.

ZUSAMMENFASSUNG: Die Hydroporation niedrigmolekularer und auch höhermolekularer Substanzen in die Haut kann reproduzierbar und effektiv mit flüssigen und niedrigviskösen Systemen erfolgen. Die Applikation hochmolekularer Substanzen ist per se limitiert kann jedoch mit einer fraktional ablativen Vorbehandlung verbessert werden.

TAB. 1: OVERVIEW OF CURRENT TECHNOLOGIES TO PENETRATE THE EPIDERMAL BARRIER [13].

Designed topicals	Chemical & mechanical penetration	External forces	TOR – Temporarily opened epidermal barrier
Supersaturation Penetration enhancer Encapsulation Nanocarriers	Syringes Suction blisters Dermabrasio Peeling	Iontophoresis Sonopheresis Electroporation Photomechanical waves Hydroproration Laser microjet	Dermaroller Fractional ablative Laser & RF-devices Fractional non-ablative qs Laser
High limitation to size of compound & transport capacity	Limitation to area and depth of penetration & transport capacity	Limitation to size of compound & transport capacity	50 % of skin can be opened without scar formation for 24h Potentially high capacity but inside out pressure gradient

INTRODUCTION

The human skin, the largest organ surface wise has been attractive for topical and transdermal intervention since ever. However, Evolution has designed a very efficient barrier preventing human beings from water loss, penetration of germs, allergens poisons, toxins, radiation and other influences of danger in a very sufficient manner. To overcome the barrier many concepts have been developed (Tab. 1).

Recent insights to the potential of fractional skin treatments have established standard laser procedures to treat aged and sun damaged skin and scars. The biggest potential of it is foreseen with the option of a contact free temporary opening of the epidermal barrier (TOR, German: gate) to promote new and intensified treatment regimen. Fractionated laser therapies are routinely used in the clinic to treat scars and many other conditions [1]. By treating the skin with fractions, a response is initiated that involves the skin replacing itself in up to 50 % of the surface if the individual piece of skin removed is smaller than ~0.3 mm in diameter. These columns of treated skin can reach as deep as the dermal compartment and are called microscopic treatment zones (MTZ or microscopic ablation zones (MAZ)) [2–4]. In recent years, experimental in vitro and in vivo studies have proven that treatment with AFXL enhances the uptake of topically applied small molecules like photosensitizers and facilitates distribution into deep skin layers [5–9].

Among the use of external forces (Table 1) hydroporation has gained a new interest since the development of highly effective devices. It has been effectively used for lymph drainage, gentle massage and also facial rejuvenation [10]. In the latter setting the system was able to ablate of the epidermis in a controlled manner. Interestingly there were no disturbances of wound healing. However, as in traditional laser concepts aiming on full thickness epidermal ablation erythema, herpes virus infection, crusting and emphysema have been reported [10].

Hydroporation however may also ensure a contact free drug delivery approach together with the use target molecules within a jet stream at high speeds to treat large surface areas. This concept avoids epidermal ablation despite the possible application of high volumes or substance concentrations. However systematic studies investigating the tissue effects on those hydroporation systems are lacking. So far it is not known what type of penetration injury takes place and if at all a substance or drug is deposited to the

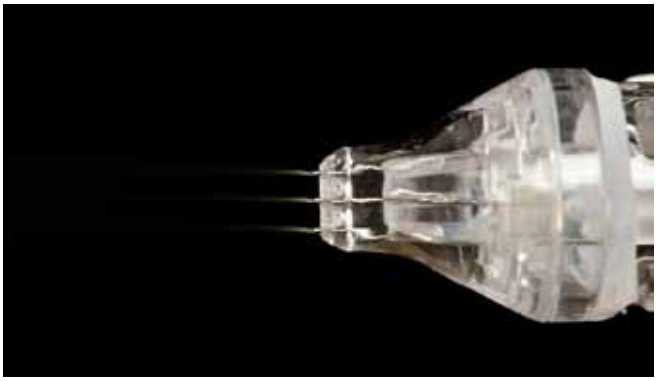

Fig. 1: The triple nose handpiece ensures a larger treatment area while dividing the fluid stream into three.

Fig. 2: The standard single outlet handpiece ensures the most powerful application mode of a stream accelerated up to app. 750 km/h.

Fig. 3: The prototype handpiece with a backpack like opening for standard vials was used to test liquids having a higher viscosity. The system displayed does also have a triple outlet.

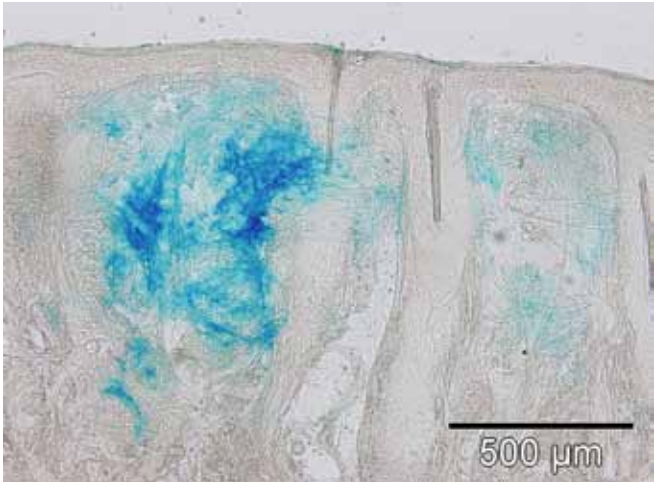

Fig. 4: Visualization of distribution type and depth of ink stained 0.9 % NaCl solution stained with blue ink (patent blue). Without displaying a gross damage on the epidermis the ink is homogenously distributed in an area of 693 µm width and up to 1024 µm depth.

deeper structures of the skin. Of importance is to know penetration depth and delivery capacities in relation to substance viscosity, molecule size and tissue properties.

The aims of this in vitro study were (1) to prove the ability of the hydroporation system to penetrate the epidermal compartment and (2) to be able to deposit liquids (NaCl, vitamin a and c solutions), viscous substances (hyaluronic acid), crystalloid suspensions (triamcinolone, 40 mg/ml), and molecules of higher molecular weight like antibodies (IgG-FITC), PEG's (FITC-PEG's) and sugars (Dextrane-FITC) into the dermis using an skin explant model with and without AFXL pre-treatment using two different types of ablative fractional lasers.

MATERIALS AND METHODS

A prospective, single-center, in vitro study was designed to examine morphological changes visible at microscopically as performed in earlier studies [11, 12]. The study protocol used a previously described model and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Skin samples, obtained at routine skin surgery, were used as skin explants. All subjects consented the use of their skin explants.

Hydroporation

Hydroporation was performed using the system JetPeel™-3 System (TavTec, Israel) in conjunction with two out of the available applicator systems (triples nose, single nose, Figure 1 and 2) and a specific prototype made for application tests (name?). The hydroporation system ensures a fluid stream accelerated up to 720 km/h.

Laser system

AFXL was performed with a 10,600 nm CO_2 laser (Exelo2, former Quantel-Derma now Alma Lasers GmbH) and a 2,940 nm Er:YAG-Laser (Burane FXL, former Quantel-Derma GmbH now Alma Lasers GmbH, optic lens array FX12).

The fractionated CO_2-laser was operated with a scanner, using a spot diameter of 250 µm. The pulse duration (exposure time) was 1 ms, and the pulse energy of 40 mJ was delivered by 1 stack and 1 pass at a density of 250 MAZ/cm^2. The average fluence in each MAZ generated by a scanner was 81.6 J/cm² (spot area 0.049 mm²; 40 mJ / 0.049 mm² = 0.04 J / 0.00049 cm² = 81.6 J/cm²). The average fluence with the treatment area is calculated as 40 mJ * 250 MAZ/cm^2 = 10,000 mJ/cm² = 10 J/cm².

The fractionated Er:YAG-laser was operated with a FX12 optic a lens array, density of 270 MAZ/cm^2, providing a spot diameter of 150 µm. The laser was set to 31.8 mJ pulse energy with a 300 ms pulse duration (exposure time), consisting of 10 stacked subpulses of 3.18 mJ each. The average fluence with the treatment area is calculated as 31.8 mJ * 270 MAZ/cm² = 8,586 mJ/cm² = 8.6 J/cm². Therefore the average fluencies in each treatment areas are comparable, despite differences in absorption characteristics.

Skin explants

Skin explants were subjected to hydroporation (JetPeel™-3 System, TavTec, Israel) using (1) alcian blue inked 0.9 % NaCl, (2) unstained 0.9 % NaCl applications for 10 s, 30 s, 1 min, (3) ready to use solutions containing vitamin a and c as well as hyaluronic acid (4) crystalloid suspensions (triamcinolone), (5) proteins (IgG-FITC, goat anti-mouse IgG-FITC, average molecular weight ~150,000, Santa Cruz Biotechnology, cat# sc-2010), (6) FITC-PEG's (Fluorescein hetero-functionalized polyethylene glycol, MW 2000, cat# PEG4-0002, mPEG-FITC, Nanocs, www.nanocs.com) and (7) sugars (Fluorescein isothiocyanate-dextrane, Dextrane-FITC, average molecular weight 70,000, Sigma cat# 46945) to investigate morphological tissue changes and to measure distribution within the epidermis and the dermis. To test the potential synergistic effect of AFXL in conjunction with hydroporation skin explants were fist subjected to AFXL as described above and then hydroporated using test substances of higher molecular weight (8) IgG-FITC, FITC-PEG's and Dextrane-FITC.

Routine pathology workup

Each skin sample was subjected to 4 % buffered formalin post intervention. Following fixation in formalin, all skin explants were

embedded into paraffin, sectioned into 4 µm to 6 µm thick slices and stained with hematoxyline and eosin and alcian according to in-house routine protocol. Only samples treated with inked NaCl were processed without H&E and alcian staining.

Immunofluorescence

The tissue sections were frozen and sectioned into 5–8 µm thick slices. Slides were the analyzed using the fluorescence microscope using different magnifications (Olympus BX41, Germany, magnification: 1.25, 4, 10, 20, 40, 60, 100x) and documented using a calibrated digital camera system (Olympus DP71, Germany) together with the software evaluation package (Olympus Cell F, Germany). Fluorescence microscopy enabled visualization of FITC-labeled antibodies, PEG's, and antibody distribution in detailed areas of skin the sections before and after hydroporation alone or following ablative fractional laser treatment.

RESULTS

The investigation of the general ability of the Jetpeel hydroporation system to interact with epidermal and dermal human structures was first tested by the application of stained 0.9 % NaCl inked with alcian blue. The test application was performed using the single outlet handpiece with a mean application time of 10 s per area of app. 1 cm^2. The histological slides reveal a homogeneous distribution of the ink which was also visible macroscopically within the upper two thirds of the dermal compartment leaving the epidermis on microscopical level intact. Interestingly only remnants of ink were detectable at the explants surface (Fig. 3).

To further test the safety and efficacy of the hydroporation system over time of application, unstained 0.9 % NaCl has been applied using the triple outlet handpiece for 10 s, 30 s and 60 s in a defined surface area of app. 1 cm^2. As control served massage application in the same area size according to the instructions of the manufacturer. H&E stained slides revealed no epidermal or dermal changes using the so called massage application. In comparison to untreated controls superficial parts of the stratum corneum only have been removed (Fig. 5). When hydroporation was done over a time period of 10 s vacuole

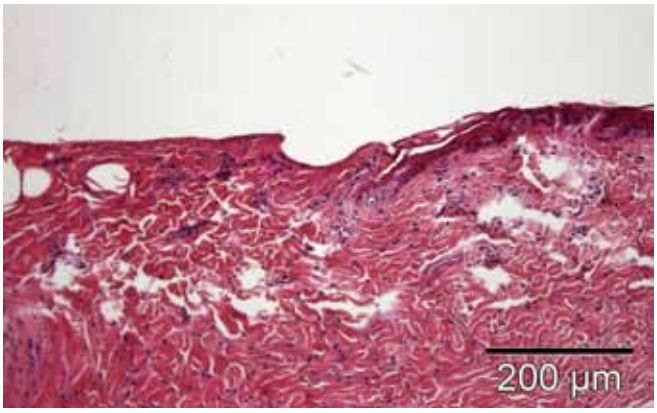

Fig. 7: Hydroporation over 60 s in a defined area led to a complete loss of the epidermis, dermal vacuole formation, and localized separation of dermal fibre bundles.

Fig. 8: Using ready to use solutions containing vitamin a provided by the manufacturer a rather uniform vacuole formation, spreading of dermal fibers within the full depth of dermis up to 1436 µm was measurable. However in some areas there was also a complete destruction of the dermal fiber structure visible.

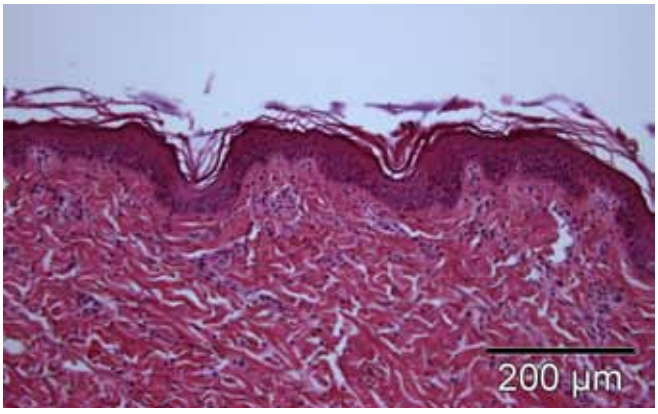

Fig. 5: Hydroporation does not lead to any morphological changes within the epidermis or dermis. In comparison to untreated controls superficial parts of the stratum corneum only have been removed.

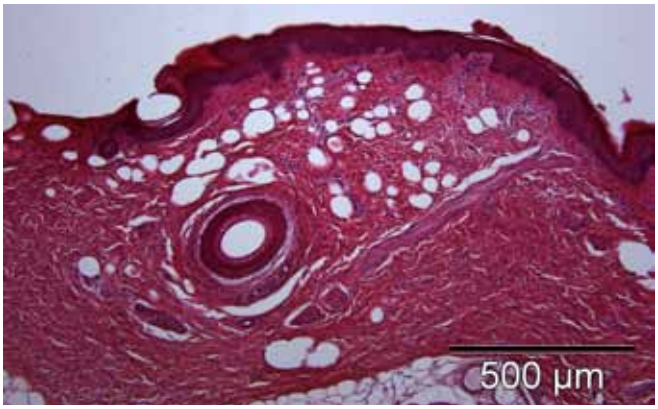

Fig. 6: Hydroporation over 30 s in a defined area led to vacuole formation, removal of the stratum corneum and circumscribed epidermal loss. Localized separation of dermal fibre bundles are visible.

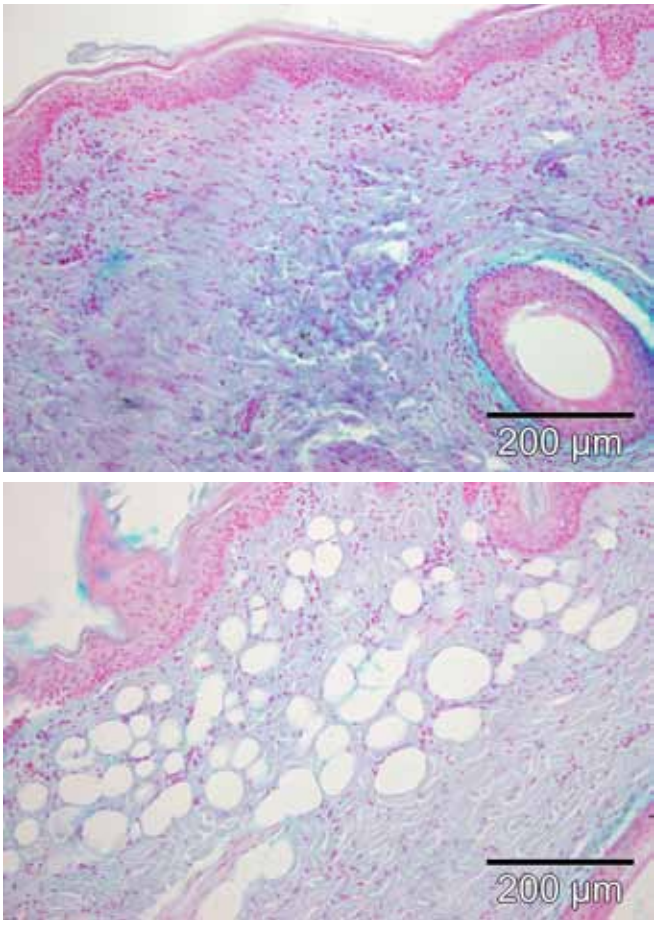

Fig. 9: If the provided hyaluronic acid in low viscosity condition as provided by the manufacturer was applied in the same way equivalent distribution pattern could be achieved as visualized in light blue color by alcian blue staining. Most probably depending on application pattern a predominant vacuole formation or a more in between the fibers deposition pattern was visible.

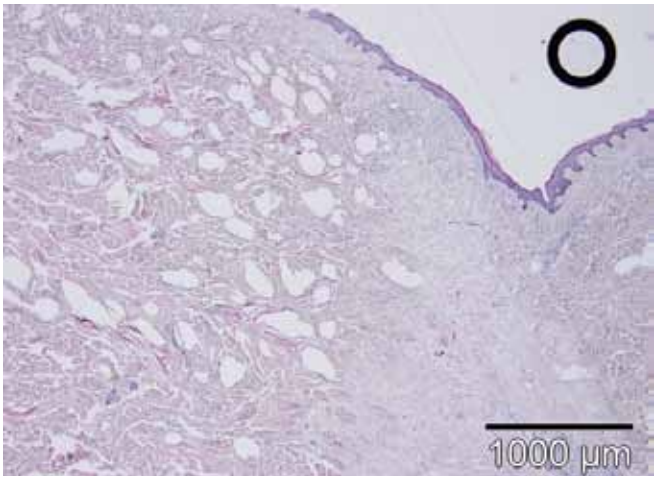

Fig. 10: Microscopy of a scarred tissue explant subjected to triamcinolone hydroporation. The tissue section reveals a homogenous deposition of the drug within the dermis. However denser scar tissue has not been penetrated (H&E). On top of this there was a higher tendency of an epidermal damage.

formation within the upper thirds of the dermal compartment appeared. Extending this time up to 30 s vacuoles increased in size (Fig. 6). Localized separation of dermal fiber bundles was visible. On top of this, in circumscribed areas there was a complete loss of the epidermis. Extending the application time to one minute revealed a total loss of the epidermal compartment (Fig. 7) as described earlier [10].

In a third step of experimentation ready to use solutions containing vitamin a and c as well as hyaluronic acid provided by the manufacturer have been applied to skin explants in order to estimate the distribution of within the dermis and to measure maximum penetration depths. Using those solutions and applying them according to the instructions of the manufacturer in skin explants after standard histology work-up a penetration up to 1436 µm was visible while there was a more or less uniform vacuole formation pattern and spreading of the dermal compartment visible (Fig. 8). There were no major differences visible if vitamin a or vitamin c solution were in use. If the hyaluronic acid of low viscosity condition as provided by the manufacturer was applied in the same way, equivalent distribution pattern could be achieved visualized by alcian staining. Most probably depending on application pattern a predominant vacuole formation or a more in between the fibers deposition pattern was visible (Fig 9 a+b). Interestingly with increasing viscosity there was no more pronounced epidermal damage visible on a microscopic level. As control a classical syringe based filler has been used. There was a clear dermal- and subdermal deposition of an opaque material visible. Volume wise the filler application was much higher than that of the hydroporation system.

To further test the limits of the system and to answer the question if the concept may be also translated into clinics a crystalloid suspension (triamcinolone) as in use for clinical scar treatment has been applied to skin explants bearing scars using the prototype handpiece. Interestingly hence the sizes of the crystals is much smaller that the nozzle size of 120 µm the triamcinolone particle could be transported via stream through the nozzle. Microscopy revealed a homogenous deposition of the drug with the dermis. However, the power of the system was clearly insufficient to penetrate the denser scar tissue (Fig. 10).

To date trans-dermal drug delivery assisted by fractional laser is of extreme importance. Therefore the ability of the system to transport fluorescence labelled molecules like antibodies (IgG-FITC), PEG's (FITC-PEG's) and sugars (Dextrane-FITC) have been tested while using the prototype handpiece. Fluorescence microscopy revealed no significant deposition of the high molecular test substances PEG and the IgG antibody using the hydroporation system only. Only some deposition was visibly applying the sugar (Fig. 11 a–c). Hence the method of AFXL is now widely used to enhance dermal drug delivery in daily clinics, two laser systems were used to facilitate the hydroporation induced uptake of high molecular substances (Fig. 12 a–c). Pre-treatment of both laser systems resulted in an enhanced deposition of all high molecular weight test molecules within the MAZ and also the coagulation zone made by the CO_2-Laser. If an Erbium laser was used the deposition within the dermis

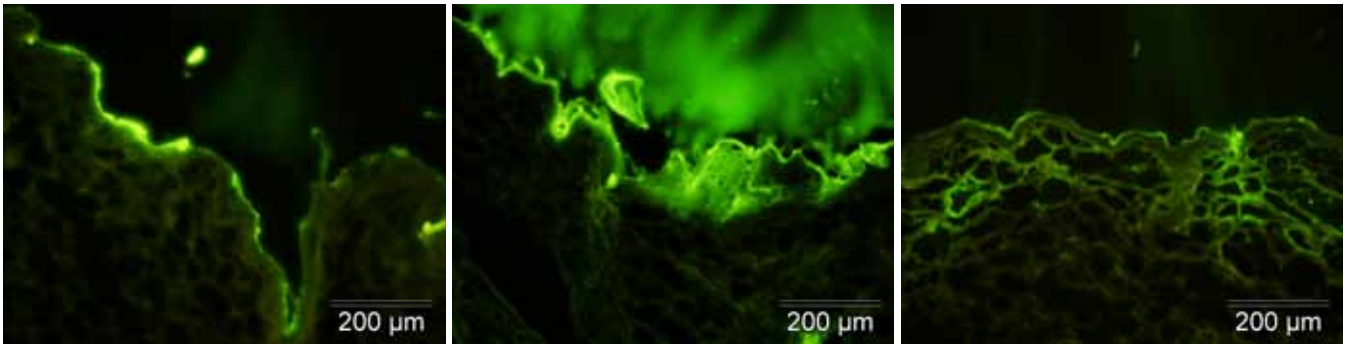

Fig. 11: Immunofluorescence of penetration capacity of hydroporated IgG-FITC (Fig 11a), FITC-PEG's (Fig 11b) and Dextrane-FITC (Fig 11c) without laser pre-treatment.

was less intense around the MAZ most probably due to the fact of a lower residual thermal damage.

DISCUSSION

The investigation of the general ability of the Jetpeel hydroporation to penetrate the stratum corneum and the epidermal compartment in order to deposit liquids and small molecules by using an jet stream of liquids accelerated by pressure up 720 km/h through an very small singular or triple nozzle revealed by using inked 0.9 % NaCl a homogenous localized dermal deposition in unstained tissue slides made of human skin explants.

Hence the amount of liquid applied to a given surface area is strongly operator dependent, the safety of the massage procedure as suggested by the manufacturer has been tested successfully. According to the H&E slides a very superficial removal of the stratum corneum was visible only. However, extended application times resulted in first vacuole formation, lateral spreading of collagen fibers and finally complete loss of the epidermal compartment as known from previous studies [10]. These findings suggest that precise operator skills are necessary for optimal effects and homogenous deposition of substances using the system. The experimental design using skin explants and inked NaCl might provide a cheap an easy training setting hence coloration is visible by the naked eye. Furthermore, states of the art of after care professional assistance of wound healing are required in case of epidermal damage. As known from laser procedures, precautions may be set in place e.g. herpes prophylaxis, sunscreen, down time and post treatment regimen.

The manufacturer does provide a variety of ready to use liquids containing for example vitamin a and c as well as hyaluronic acid of low viscosity for dermal treatments. The question arose if at all and how deep those substances may penetrate and what the distribution pattern looks like. There were no major differences of distribution type and depths visible if vitamin a or vitamin c solution were in use. The total penetration depth was measured as 1,436 µm while preventing a major epidermal collateral damage. In case of the hyaluronic acid which does certainly excels a higher viscosity similar distribution types and patterns were visible on a microscopic level.

In an experimental set-up using the prototype handpiece mounted with a matching vial containing a standard solution of triamcinolone 40 mg/ml as clinically used for scar treatments the hydroporation concept has been tested if crystalloid suspension also may be applied to healthy and scarred tissue. Microscopy revealed a rather homogenous deposition of the drug within physiological tissue while dense scar tissue was almost not penetrated. It is therefore concluded that small insoluble particles in liquid might be transported by the system, however penetration capacity is limited if texture density increases.

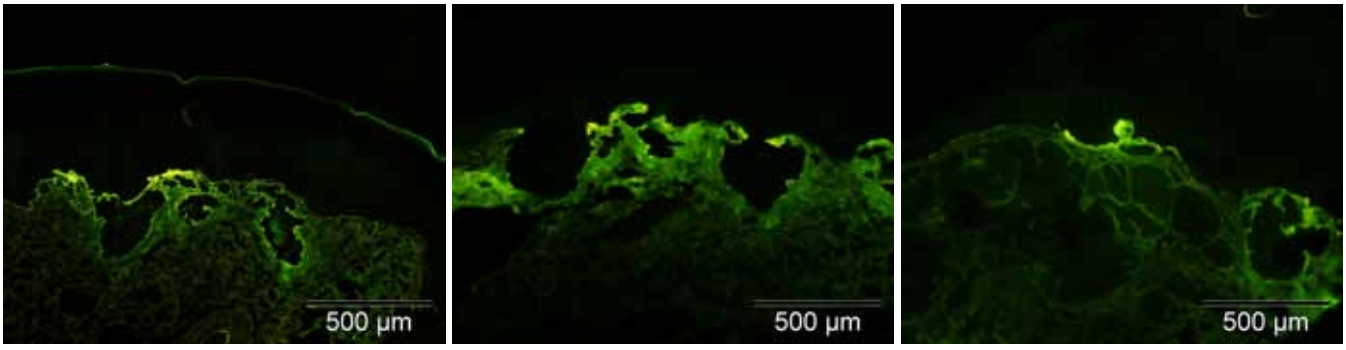

Fig. 12: Immunofluorescence of penetration capacity of hydroporated IgG-FITC (Fig 12a), FITC-PEG's (Fig 12b) and Dextrane-FITC (Fig 12c) using a CO2- ablative fractional laser pre-treatment.

Another potential limitation of the system is assumed if high molecular molecules shall be applied. Hence their application would of clinical interest three test molecules were used to test the transportation capacity and the ability to get those molecules deposited to the dermis. The experimental design was made by using fluorescent labelled antibodies, PEG's and dextrane. Using the hydroporation alone it has to be concluded although transported onto the skin surface there was a limited penetration visible for the dextrane only. Moreover in conjunction with an AFXL pretreatment however this limitation could be overcome clearly.

There are several limitations of the study that warrant attention. First those results gained in-vitro may not directly apply to clinical settings. Especially the experimental design does not allow the exact calculation of how much of the potential drugs can be transported into the skin. Finally it remains open, to test if potentially applied drugs, molecules or substances tolerate this mechanism of transportation without change or loss in function or biological properties. These might be especially the case in large size proteins like antibodies.

CONCLUSION

This in large vitro study clearly demonstrated that the hydroporation concept can be applied to human skin in a safe and effective manner as long as a well-trained operator follows the instructions of the manufacturer. Hence the jet stream is accelerated to enormous speeds liquids containing active substances as well as hyaluronic acid having a higher viscosity can be transported. Even small insoluble particles may be placed into the dermal compartment. However, penetration capacity is limited if texture density increases as it is the case in scars. Also due to the fact of the very high pressure acting on the epidermis inappropriate use of the system might lead to epidermal damage or complete loss of it resulting in wounding. Therefore appropriate after care needs to be provided. Hence hydroporation would be a very nice contact free application method in a wide clinical setting e.g. vaccination, topical biological therapy and many more the transport and deposition capacity of the system has been tested using antibodies, a PEG's and dextrane. Although still being transported via the nozzle the penetration into the dermis was limited. A second approach using two different fractional ablative laser pre-treatment protocols at standard settings revealed again a sufficient dermal deposition.

Taken together this large in vitro study clearly shows the potential and limitation of the concept of hydroporation. Further clinical trials shall be performed to confirm these in-vitro findings.

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