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Updates from Medicine

Cold atmospheric pressure (physical) plasma in dermatology: where are we today?

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Abstract

Cold atmospheric pressure plasma is physical plasma (essentially ionized gas) created at room temperature and atmospheric pressure, and it has complex effects on cells, tissues, and living organisms. These effects are studied extensively for medical and dermatological use. This article reviews current achievements and new trends in clinical dermatological cold plasma research, discusses the basics of plasma physics and plasma engineering, and describes the most important areas of laboratory plasma research to provide a well-rounded understanding of the nature, present applications, and future promise of this exciting, emerging technology.

Introduction

Over the course of the past several years, clinical trials and small case series appeared in the dermatological literature describing the use of cold plasma in skin conditions, such as chronic wounds, onychomycoses, actinic keratoses, and warts.[1-4] If someone decides to look deeper, it is easy to find the vast body of literature describing the effects of cold plasma on living tissues from laboratories all over the world. Countless publications exist showing the ability of cold plasma to kill bacteria, selectively inhibit the growth of cancer cells, and induce stem cell differentiation, just to name the most important ones.

Compared to the large amount of accumulated *in vitro* and animal data, clinical studies may seem to be just trickling drops, but the opening of the flood gates is inevitable: we are likely to witness a cold plasma boom in medicine and especially in dermatology very soon.

In this article, we will review the highlights of cold plasma physics and experimental data with a focus on the most recent clinical developments relevant to dermatology.

Physics of cold physical plasma

In medicine, plasma means blood plasma, but there is another use of the term in physics, where the entity "plasma" is referred to as the fourth state of matter or as an ionized gas. This is what can be distinguished as physical plasma. Compared to "regular" gas, where entire molecules or atoms move freely, plasma is in a higher energy state where atoms lose their electrons – become ionized – and the resulting electrons and ions move freely and independently from each other. If all particles (electrons, ions) are in energy equilibrium, meaning that they have the same very high temperature, we get hot plasma: the corona of the sun, the plasma in a fusion reactor, or a discharge arc such as in welding or arc lamps. There are examples of hot plasma use in medicine, mostly as a surgical tool, but this review is limited to cold plasmas. If only the electrons are hot, but the rest of the particles, representing the main mass of the gas, are at a lower temperature – the plasma is in energy non-equilibrium – we get cold plasma, which can be cool enough to be safe on the skin surface. Of cold plasmas, those created under atmospheric pressure conditions (i.e. not in a vacuum) are the most feasible for medical applications. Various shorter or longer designations have been used to identify this type of plasma based on the linguistic tastes of different research groups, but lately consensus seems to be forming around the designation "Cold Atmospheric Plasma" or CAP. CAP is a complex entity, and its composition is not uniform: it depends on the gas in which it was created and on the way it was created. The main active components of plasma are reactive oxygen and nitrogen species, but other reactive species, charged particles, its electric field, and partly even the UV photons and ozone generated during plasma production may play a role in its complex effects on living tissues.

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Just as plasma composition is complex, so are its interactions with biological targets. For example, plasma's inhibitory effect on bacteria is believed to be related both to direct oxidative stress caused by reactive species, and damage to the cell wall and cell membrane caused by charged particles contained in plasma.[5] Some plasma-induced changes in eukaryotic cells are attributed to reactive oxygen or nitrogen species acting directly as signaling molecules[6] or via inducing oxidative stress-related triggering of various pathways,[7,8] or by other mechanisms, such as changing intracellular ion concentrations.[9,10] Likely it is the differences between different cell lines in their status of cell proliferation and cell death-related pathways that can explain plasma's intriguing selectivity when it comes to malignant and nonmalignant cells.[11] The effect of plasma as an electric entity, unrelated to reactive species, is also a consideration. Studies using not plasma but nanosecond electric fields on cell cultures demonstrated increased intracellular calcium influx, and effects on intracellular signaling just as it is seen with CAP application.[12,13] On the other hand, plasma effects are observed even when using experimental designs that eliminate the presence of an electric field, underscoring the complex nature of these interactions.[14,15] It seems that not just one but multiple plasma components together shape the effect of CAP, and that effect also varies based on the targeted biological structures.

Another important question is the depth of penetration of plasma. It seems it can be answered only in terms of penetration depth of specific components of CAP as it greatly differs. A study measuring CAP penetration through tissue using slices of pig muscle of various thicknesses found that while only 5% of reactive oxygen species penetrated 0.5 mm of tissue, the penetration rate of reactive nitrogen species was 80%. The authors also detected some reactive species penetration through 1.25 mm tissue slice.[16] In animal experiments, plasma was shown to induce apoptosis throughout a 2.8 mm thick tumor in a mouse. The same study also demonstrated penetration of plasma-induced reactive species through a 1 mm thick layer of pig skin and multiple millimeters of agarose gel and noted differences in the penetration depth of various species and with different plasma settings.[17] Plasma penetration depth was also examined in human mucosa: a Raman microspectroscopy study detected CAP tissue penetration to about 270 μm of the human cervix mucosa.[18] Biological liquids present on the target surfaces also influence not only the depth of plasma and reactive species penetration but also the composition of plasma components reaching deeper layers of the targeted tissue.[19,20] The concepts and challenges of tracking penetration of plasma and reactive species into tissues, the various surface and deep interactions, and the challenges represented by half life differences between different components of plasma were reviewed and discussed in detail by Szili et al.[21]

In any medical treatment, the amount of "medicine" given is of great importance. Just as plasma penetration is complex, so is the dosing of plasma. For example, in experiments showing the apoptosis-inducing effect of CAP-treated medium on melanoma cells, the results depended on the exposure length.[22] Exposure time is only useful as a valid measure of plasma dose when used in the context of a single plasma source but not when comparing different plasma devices as the composition of the plasma generated by them can differ greatly. For example, using the very same jet plasma device but different feeding gases changes the biological effect exerted on bacterial growth.[23] A detailed study using jet plasma and a 3D skin model showed that interleukin expression induced by CAP can be modified by changing not only the length of exposure but also energy input, carrier gas.[24] One possible approach is to characterize every system for every application individually. The more comprehensive way would be to develop core markers of plasma effect to enable comparison between different devices. One such attempt was based on the significance of reactive species in CAP effect: in a complex study the utility of total oxidation potential as a plasma dose measure was proposed.[25] The authors suggest that total oxidation potential quantifies the effects of generated reactive species, the main driver of – for example – CAP-induced decontamination, while also acknowledging that other factors that have no influence on total oxidation potential may also be important in different CAP functions. Dose measurement and dose limit setting ties into plasma safety as well. From that perspective, another approach is under development to use biological assays to measure the effects of CAP devices, which ultimately may be the most meaningful way to assess not just safety limits but also effective doses.[26]

CAP parameters, concentrations of species, and their energies are very much controllable, which permits for any specific treatment to find a niche where efficacy of treatment can be achieved without short and long-term skin damage.

Plasma device designs

CAP is primarily created by high frequency electric discharge, using a pulse generator. Settings of the pulse generator, such as frequency, power, wave form, and also the composition of gas in which the plasma is generated and the length of application, have a significant impact on the biological effects of CAP. Clinical trials have used three main types of CAP device designs: jet, dielectric barrier discharge (DBD), and surface microdischarge plasmas. These devices have very important practical differences, which determine how and for what purpose they can be used best. The physics, engineering, and characteristics of the different CAP devices have been described in great detail.[27-34] The following is a concise summary of their main features. The plasma jet has an electrode away from the skin, and the plasma generated around the electrode is literally blown onto the skin using gas flow, typically a noble gas, such as argon or helium (Fig. 1). This design reduces potential issues related to electric contact with the skin,

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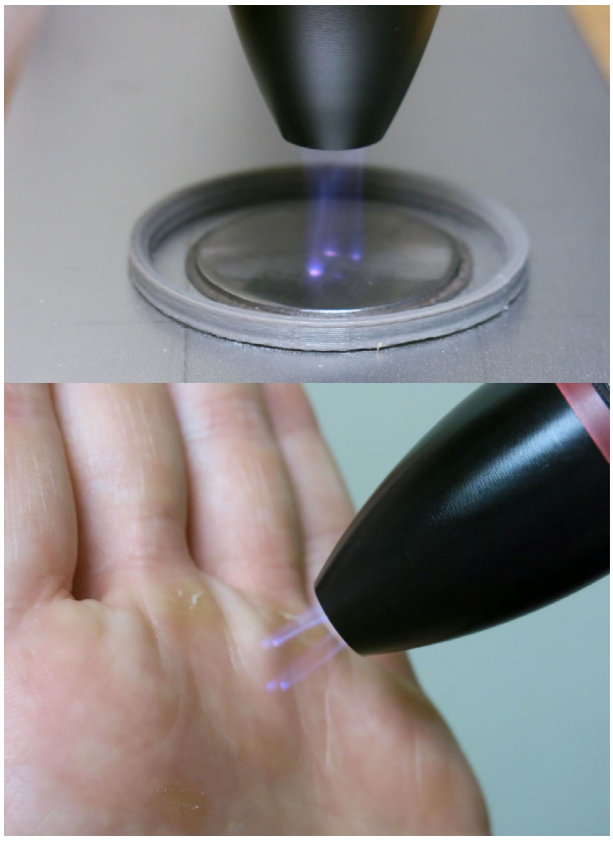


Figure 1 Helium plasma jet. Handpiece of a plasma jet device with outflow of helium plasma showing the characteristic "plasma glow". In this particular device, plasma is generated by a rectangular electrode inside the hand piece. Most of the plasma develops at the corners of the electrode, resulting in the brighter, more "plasma-rich" streams in the plasma flow. The device pictured is a Piezobrush, manufactured by Relyon Plasma GmbH (formerly Reinhausen Plasma GmbH)

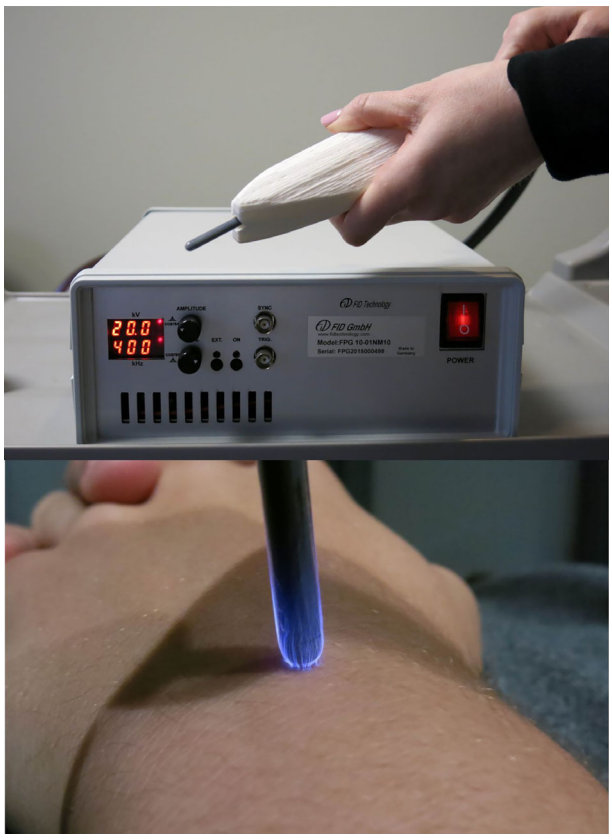


Figure 2 Floating-electrode DBD plasma. Top panel: pulse generator and hand-held electrode. Bottom panel: Plasma is being generated using the electrode tip. The electrode connected to the pulse generator must be kept near the treated surface. Either the tip or the side of the electrode can be used, but plasma will only form where the gap between the electrode and the target surface does not exceed 1 mm. The device pictured is an experimental prototype described previously by Friedman et al.[3]

device contamination problems, and need for sterilizing electrodes. It also facilitates the treatment of uneven, incongruous surfaces but is only applicable for larger target areas when wider, multi-jet torches are used, or by moving the jet or torch over the treated surface. The plasma energy delivered to the surface is limited, and most jet plasmas need a continuous source of gas requiring considerable installation reducing, but not entirely eliminating, portability. DBD plasma devices with a so-called floating electrode create CAP directly on the surface of the skin by generating electric discharges between an electrode connected to the pulse generator and the skin, which serves as the other electrode (Fig. 2). This plasma can have substantially higher energy compared to jet plasmas. Because of the need for the electrode to cover the target area with a very narrow but steady gap, uneven, large, or nonflat surfaces represent a challenge for floating-electrode DBD plasma. Engineering variously shaped, larger, possibly flexible electrodes may solve this issue. Alternatively, moving an electrode over the surface can also result in sufficient cumulative plasma delivery over time even for a larger target area. Because of the close proximity to the skin, contact is practically unavoidable. This necessitates electrode sterilization or single use parts to avoid contamination. The safety of electricity affecting the target surface must also be addressed. A variation of DBD principles called surface microdischarge uses a powered plane electrode and a grounded wire-mesh electrode near the skin. This setup eliminates the electric current going through the skin and may cover a larger area, helping to overcome some of the limitations of floating-electrode DBD plasma designs, while other issues, such as contact contamination concerns, remain.

A fundamentally different type of plasma delivery yet to be seen in published clinical trials in dermatology but used extensively in cell culture based plasma experiments is indirect plasma: application of plasma to a liquid, such as cell culture

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medium or saline solution, which is then used to create the effects of plasma treatment on the desired target without the presence of the original plasma source. It works because at least some of the components of plasma that are responsible for its effect on living tissues, such as free oxygen and nitrogen radicals, can survive in solutions and may have the same effect as applying plasma itself. Indirect plasma, however, is more complex than mixing plasma and a liquid. Plasma generation in the vicinity of a liquid alters the physics of the process. Plasma-liquid interactions can be created using different settings, such as direct discharge in liquid, gas phase discharges, and multiphase discharges. The generated reactive species differ in the liquid and gas phase and vary according to the creation method, liquid, and plasma parameters used.[35,36] Chemical and biochemical reactions induced by CAP are also important parts of the equation.[37] Indirect plasma can be an appealing CAP delivery method for a number of fields in medicine.[38] Adjusting the plasma "dose" to obtain a predetermined composition of reactive species based on the needs of a particular application requires the careful selection of plasma sources, delivery parameters, and treated liquid.[39]

If in fact proven to be equivalent to actual plasma delivery, indirect plasma could eliminate many practical issues of plasma treatments, as the treated intermediary liquid can be stored, shipped, and may also be used over very large areas of skin.

As indirect plasma removes plasma generation from the treated area, some plasma components are not transferred. Two of these are UV photons and ozone. Eliminating UV and ozone exposure of plasma-treated skin can easily be seen as a benefit, as it voids patient safety concerns related to these factors. Table 1 summarizes the most important features of various plasma device designs.

In vitro plasma research

CAP has numerous, well-investigated effects on biological targets, but their detailed analysis is beyond the scope of this review. Here we will only discuss the two research areas most interesting from a dermatological perspective, while others will be referenced as needed when reviewing corresponding clinical plasma applications.

For general reference purposes, one of the earliest most comprehensive experimental studies was published by Dobrynin et al.[40] The authors presented extensive experimental findings describing the biological effects of plasma on different types of living cells, analyzing and discussing in detail the physics of the interaction and the molecular and biological changes in the target cells. The expansion of plasma science resulted in many reviews of either the entirety of *in vitro* plasma research,[41,42] or specific subtopics, such as cancer CAP studies,[43,44] antimicrobial effects,[45,46] and biological safety,[47] for example.

Selective cell death of malignancies

A literature review from 2018 found 190 original research articles published up to and including 2017 describing CAP use in oncology, which included *in vitro* experiments, *in vivo* animal studies, and clinical trials (of which they have found only three).[43] There was an exponential increase of the number of publications annually during the period of 2005 and 2017. Eighty-seven percent of these works studied human cancer cell lines, and 11% of those were melanomas, but murine melanoma cells (9% of the total publications) were the most studied of all the murine cell lines at 58.6%. The three identified clinical trials described plasma use in nondermatological cancer treatments, showing encouraging results applying CAP as a part of a combination treatment for malignant pleural mesotheliomas[48] and advanced head and neck cancers,[49,50] although in the latter case, it is possible that the clinical improvement had to do with the antibacterial and wound healing effect of plasma, which we will review under clinical applications.

In vitro studies show that CAP can selectively induce cell death in a variety of malignant cell lines. As early as 2007, Fridman et al.[22] showed that low dose plasma treatment induces apoptosis in cultured melanoma cells using a floating-electrode DBD plasma device comparing treated and nontreated cells. Another group went one step further by comparing CAP effect

Table 1 Plasma device types

	High-energy plasma	UV exposure	Easy to use on large areas	Easy to use on uneven surfaces	Fully portable	Independent use by patients is feasible
Plasma Jet	No	Yes	Somewhat	Yes	Somewhat	No
FE-DBD[a]	Yes	Yes	No	No	Yes	No
SMD[b]	No	Yes	Somewhat	Somewhat	Yes	Somewhat
Indirect plasma	No	No	Yes	Yes	Yes	Yes

The table shows the typical features of various plasma generation technologies, based only on devices that have been used in published research. Other device designs may have different characteristics. These features are important when evaluating the best target diseases for each device type.

[a]Floating-electrode dielectric barrier discharge plasma.

[b]Surface microdischarge plasma.

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on melanoma cells and primary human keratinocytes. After using helium jet CAP treatment, they found a substantially higher percentage of cell death in melanoma cells, and the difference was shown to be caused by CAP-induced apoptosis, as demonstrated using TUNEL assay.[51] This selectivity is likely not absolute: adjustments of plasma characteristics, such as plasma emission intensity and power, could be used to optimize the selective ablation and migration inhibition effect of a helium jet plasma on cultured human metastatic MDA-MB-231 breast cancer cells, using bone marrow-derived human mesenchymal stem cells as controls.[52] In another study, comparison of CAP effect on four different head and neck squamous cell carcinoma cell lines and two normal oral cavity epithelial cell lines *in vitro* showed significant but varying inhibitory effect on the malignant cell lines and only minimal deleterious effect on the nonmalignant cells.[53] A comparison of A431 squamous cell carcinoma cells and HaCaT keratinocytes treated with indirect plasma (cell culture medium and phosphate buffered saline previously exposed to CAP) showed a dose-dependent increase in apoptosis of the squamous cell carcinoma cells, which was attributed to the presence of reactive oxygen species.[54] These findings suggest that because of the different plasma sensitivities of various cancer (and nonmalignant) cell lines, adjustment and optimization may be needed not only when targeting malignant cells of different tissue origins but even those of the same cell type. Demonstrating the complexity of CAP treatment, Biscop et al.[55] evaluated the influence of cell type, cancer type, and cell culture medium composition on the efficacy of direct and indirect CAP treatment and found that the selectivity of *in vitro* CAP effect is indeed influenced by those variables, especially when indirect plasma treatment is used. Changes in treatment parameters may influence not just what plasma does but also the way it does it: it was shown using surface microdischarge plasma on cultured melanoma cells, that while higher doses of plasma cause apoptosis, shorter exposure time leads to non-apoptotic cell inactivation via induction of cell senescence.[56] A follow-up study using a similar experimental setup demonstrated that the melanoma cell senescence effect of *in vitro* CAP treatment was triggered by plasma-induced intracellular Calcium influx.[9] The *in vivo* effects are perhaps even more complex: while they are possibly simply the result of CAP-induced apoptosis or cell senescence, it was also suggested that CAP can restore the immunogenicity of malignant cells,[57] or it can cause direct immune stimulation to induce cancer ablation.[58]

Stem cell differentiation and cell proliferation and wound healing

Perhaps the most exciting CAP research topic is the induction of stem cell differentiation and cell proliferation using plasma. One of the earliest studies showed enhanced differentiation of chondrocytes and osteoblasts after CAP treatment, first in cell culture,[59] and later in an organ culture system where plasma-treated mouse limb buds showed superior growth and survival, which the authors attributed to increased Wnt signaling caused by CAP-generated reactive oxygen species.[60] Taken together with another study, which (although without examining stem cells specifically) showed that CAP treatment stimulated angiogenesis in an *ex vivo* experimental setting,[61] it seems that CAP may be a very promising tool to heal wounds and to assist in tissue engineering.

Neural cell differentiation, for example, is also influenced by plasma. A study found that CAPinduced neural differentiation in cultured mouse neuroblastoma cells. The authors demonstrated that this was a result of a complex cascade leading to the activation of the Trk/Ras/ERK signaling pathway by CAP-derived cytosolic and mitochondrial reactive species.[62] Another study using a culture of immortalized murine neural stem cell line C17.2 also found increased neural differentiation when treated with CAP. While no specific signaling pathway was identified, the authors concluded that CAP achieved its effect via reactive nitrogen species.[63] These *in vitro* findings raise the hope of using CAP as a potential treatment for neurodegenerative diseases, although given the complexity of the neural tissue, a lot of work still needs to be done using what one author described as "more realistic models of neurological disease".[64]

Keratinocytes: an *ex vivo* study of CAP-treated skin biopsy specimens maintained in organ culture found no change in select differentiation markers but showed increased basal keratinocyte proliferation and Ki67 expression. This effect was only observed when a specific treatment time was used: longer or shorter CAP exposure failed to achieve the same results.[65] A complex and detailed study using both HaCaT human keratinocyte culture and an *in vivo* mouse model examined the effect of CAP on cell proliferation in the context of wound healing. They found induction of epidermal cell proliferation and increased skin remodeling when compared to untreated controls.[66] The authors also showed CAP-mediated β-catenin activation and translocation to the nucleus in both *in vitro* and *in vivo* models, which seem to support the above discussed implication of Wnt signaling (which is profoundly linked to β-catenin activation) being the effector of CAP treatment in mouse limb bud growth experiments. Another study using argon jet plasma on *in vitro* cell culture studies with human fibroblasts and an *in vivo* mouse skin wound healing model found that plasma treatment induced the expression of IL-6, IL-8, MCP-1, TGF-ß1, and TGF-ß2, and promoted the production of collagen type I and alpha-SMA, which play a role in wound healing.[67] Angiogenesis and tissue macrophage activation induced by CAP are also likely to improve wound healing. Miller et al. demonstrated that microsecond pulsed DBD plasma treatment stimulates the production of vascular endothelial growth factor, matrix metalloproteinase-9, and CXCL 1 that in turn induces angiogenesis in mouse aortic rings *in vitro*.[61] A host of proangiogenesis factors, including growth factors, cytokines are modulated by CAP in an autocrine and paracrine way, providing further insight into at least one of the ways CAP enhances wound healing.[68] Other mechanisms, such as influencing redox-

mediated tissue response, activation of the nuclear E2-related factor signaling, and stabilization of the scaffolding function and actin network in dermal fibroblasts, have also been studied.[69] The role of CAP modulation of the nuclear E2-related factor pathway (along with plasma-induced p53 inhibition) was confirmed by studying plasma effects in a dermal, full-thickness wound model in mice. In the same study, the authors described a complex CAP treatment-related change in the inflammatory response leading to a conclusion that singling out one pathway as the main driver of changes in wound healing was not appropriate as effects of CAP as well as their consequences are complex and many.[70] Another in vitro study using mouse fibroblasts and human keratinocytes detected elevated expression of genes that the authors considered important for maintaining skin function (such as Type I collagen, fibronectin, and VEGF) leading to the suggestion of CAP being potentially beneficial for skin rejuvenation.[71]

Clinical cold plasma applications in dermatology

Wound healing

The cold plasma application most extensively studied in clinical trials is the treatment of wounds. While the pathogenesis of chronic wounds is complex, the presence of bacteria does play a role. Plasma has a well-documented inhibitory effect on bacteria,[72,73] and this is what initially led to the idea of using cold plasma to reduce bacterial load of wounds and thus facilitate healing.[2] The first published studies employed jet-type plasmas using argon as carrier gas.[74] The daily treatments may not be practical for all patients, and the onerous treatment schedule was in fact an identified reason for patient dropout. Another group used a floating-electrode DBD plasma device for chronic wound treatment with three applications a week for 8 weeks, followed by an observation period for 4 weeks. The DBD plasma treatment – similarly to jet plasma – resulted in a reduction of bacterial load, and it also provided some advantage to wound healing as assessed by ulcer size.[1] A more recent trial – also using argon jet plasma – had a once a week treatment schedule. Not only the authors achieved significant decrease of bacterial load in plasma-treated patients, they also used Pressure Ulcer Scale for Healing scores to demonstrate the superiority of CAP treatment of chronic wounds compared to wound care alone.[75] Reducing bacterial load is not the only beneficial effect plasma has on wounds. As we have reviewed above, CAP has been shown to induce stem cell differentiation and cell proliferation in various cell types. A study investigating the effects of argon jet plasma on skin graft donor sites in a non-bacteria-colonized environment demonstrated a positive impact on wound healing, compared to untreated controls in a split-site experimental design.[76] Advances in engineering play an important role in the future of plasma wound treatment: the development of hand-held, portable devices increases access to treatment. Fine tuning plasma settings may also help to optimize delivery to reach maximum inhibition of bacteria and induction of wound healing, while lowering treatment frequency to create an effective and practical solution.

A case report of CAP plasma treating chronic postoperative auditory canal and nasopharynx bacterial infection following tympanoplasty, incus resection, and reconstructive surgery of the auditory canal highlights that plasma can be used for different kinds of chronic infectious and nonhealing wounds.[77] Bacterial load reduction is only part of the way CAP improves healing: a randomized, placebo-controlled clinical trial of 37 patients with herpes zoster showed that CAP treatment was superior to placebo in improving herpes zoster-related pain.[78] A single case report found CAP beneficial even in a case of Hailey-Hailey disease, where the primary etiology of chronic erosions is a genetic mutation, not infection.[79]

Onychomycosis

Argon jet plasma was used successfully to inactivate fungal proliferation of Trichophyton interdigitale, Trichophyton rubrum, Microsporum canis, and Candida albicans isolates *ex vivo*, with different species responding differently: Candida albicans was the most and Microsporum canis the least susceptible to CAP.[80] An *in vitro* study using surface microdischarge plasma achieved fungal growth inhibition in Trichophyton rubrum and Microsporum canis but only when daily 10-minute long treatments were used for 9 days.[81] Another *ex vivo* nail infection model based study used helium jet plasma to successfully inhibit Trichophyton rubrum adhesion, germination, and growth *in vitro*.[82] The first clinical pilot used DBD plasma on toenails. The authors achieved over 50% clinical and about 15% mycological cure in 13 patients who have completed the study.[83] Given the recalcitrant nature of onychomycosis and the limited options to treat it, a nail treatment CAP device could be a useful addition to our tool box.

Actinic keratosis treatment

A recent, landmark, large scale, multicenter study comparing the four most common field-directed treatments of actinic keratosis (AK) found that only 29–75% of patients achieved at least 75% lesion count reduction 12 months after the end of treatment, with an adverse effect percentage in the 90s,[84] indicating that there is certainly room for improvement, and there is an unmet need for highly effective and well-tolerated AK treatments. The extensive *in vitro* research showing the ability of CAP to induce selective cell death in various cancer cell lines paired with evidence of its safety and great tolerability laid the foundation for clinical use in AK, which may also serve as a clinical surrogate for (skin) cancer, the ultimate target of many plasma researchers. The first reported trial – by our group – used a floating-electrode DBD plasma device for lesion-directed treatment of AKs. Seventeen lesions were treated (in five patients) only one time and evaluated one month later. Nine lesions resolved fully, and three improved significantly with a single treatment, which corresponds to a 53% clearance rate and a 70% rate of at least significant improvement or clearance.

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Figure 3 Field-directed actinic keratosis treatment using floating-electrode DBD plasma. Left panel: chest with multiple actinic keratoses before being treated using a 10-cm long quartz covered electrode with multiple passes of slow sideways motion utilizing the side of the electrode to evenly cover the entire target area. Treatment settings were based on our previously reported lesion directed AK treatment study.[3] Middle panel: significant decrease of AK lesion number one month after a single treatment. The patient received four additional treatments at 1-month intervals. Right panel: Maintained improvement five months after completing the last treatment of the series. (Khan A, Fridman G, Fridman A, Friedman PC, unpublished data. The photographs were taken as part of an IRB controlled clinical trial: Western Institutional Review Board, date: 11/10/2016, protocol number: 20130084, ClinicalTrials.gov, registration number: NCT02759900)

The procedure was extremely tolerable with no discomfort during or after treatment.[3] Later, another group used jet plasma for a series of field-directed AK treatments of seven patients. Target areas were treated twice a week, seven times total. The authors presented before and after total lesions counts and counts of improved lesions according to the Olsen clinical AK severity scale. They found that the total lesion count decreased by 23%, and 53% of lesions were given a lower Olsen grading, marking clinical improvement. Patients tolerated the treatments well.[85] While it is encouraging that two different types of plasma devices, using different protocols, were shown to be effective and tolerable for AK treatment, larger trials are needed to confirm these findings and to fine tune our plasma delivery techniques to achieve the best possible outcome. Although the DBD plasma study produced higher clearance and improvement rates with a single application than the jet plasma trial with seven applications, the comparison is not entirely valid because one trial was lesion directed, the other field directed. Modifying electrode design and delivery technique may allow to keep the higher efficacy of DBD plasma even when used for larger target areas and field-directed treatment of AKs (Khan A, Fridman G, Fridman A, Friedman PC, unpublished data) (Fig. 3). It also needs to be investigated what effect, if any, CAP field treatment has on long-term keratinocyte cancer development, as the transformation rate of AK into skin cancer may not be directly linked to the clinical severity of AK lesions.[86]

Treatment of viral warts

A recent disease target of CAP in clinical trials is viral warts. The rationale stems from multiple sources: plasma has been shown to destabilize adenoviruses,[87,88] which are nonenveloped double-stranded DNA viruses just like the human papillomavirus. It has been long established that plasma induces intracellular calcium influx.[40] There is evidence that the well-established anti-wart agent, imiquimod, may act at least in part by inducing intracellular calcium influx.[89] Heat therapy, which was shown to be effective at treating warts,[90] also possibly exerts its effect by triggering calcium influx, based on studies examining the effect of hyperthermia on epithelial tumor cell lines.[91] These findings make it feasible that either by inducing intracellular calcium influx, or possibly by prohibiting viral replication,or both, CAP may be an effective treatment against warts. Our group has published the first case series showing the efficacy of CAP in adult patients with warts.[4] Not only was CAP able to clear warts effectively and without recurrence for at least 5 months, the treatments were entirely painless and there was no discomfort or blistering following the procedure. This tolerability is crucial when treating larger warts and special populations, such as children (Fig. 4). A follow-up study also demonstrated the efficacy and exceptional tolerability of CAP when used to treat warts in children.[92] Both of these proof-of-concept trials were limited by small patient number, and while they are promising, further research must confirm their findings on substantially larger number of patients. Additional studies should also investigate the optimal settings of plasma delivery for different types of warts.

Treatment of hair loss

As discussed above, CAP has been shown to induce stem cell differentiation in various tissues. Minoxidil, one of the most established treatment modalities for androgenetic alopecia (AGA), may induce hair follicle stem cell differentiation and hair growth *via* triggering cellular calcium influx.[93] As CAP has also been shown to induce cellular calcium influx *in vitro*,[40] it can be hypothesized that CAP may be able to improve AGA by inducing hair follicle stem cell differentiation based on its similar effect on other stem cell populations and specifically *via*



Figure 4 Extensive plantar warts treated with floating-electrode DBD plasma. Left panel: plantar warts prior to plasma treatment. Right panel: fully resolved warts 3 months after a single treatment with a floating-electrode DBD plasma device using previously reported treatment settings.[4] (Friedman PC, Fridman G, Fridman A, unpublished data. The photographs were taken as part of an IRB controlled clinical trial: Western Institutional Review Board, date: 11/10/2016, protocol number: 20130084, ClinicalTrials.gov, registration number: NCT02759900)

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triggering cellular calcium influx. A recent rat model based study showed that CAP treatment using a nitrogen-CAP jet-type device increased hair follicle diameter.[94] Using CAP on the human scalp presents challenges, as the large surface area would require long treatment times using currently available devices and the presence of hair may impede plasma delivery. Another concern is the questionable feasibility of frequent, ongoing, office-based treatments for a chronic condition. As discussed above, a plasma-treated liquid medium can exert the effects of plasma and, in this case, it would allow for easy treatment of the entire scalp and the treatment could be self-administered by the patients at home. Our group has begun a clinical trial based on the above principles (clinicaltrials.gov: NCT04379752). Preliminary assessment showed that ongoing indirect plasma treatment for the scalp is well tolerated and preferred by patients (Khan A, Fridman G, Fridman A, Friedman PC, submitted for publication). Large, controlled clinical trials and examination of the actual effects of direct and indirect CAP on human hair follicles are needed to explore this potential application.

Clinical safety of plasma

Plasma is a very complex physical entity, and all plasma components may raise safety concerns. For some aspects of plasma such as UV radiation, ozone, nitrogen oxide, and electrical current, there are already existing safety standards issued by national regulatory authorities. Assuring compliance with such standards is a matter of simply measuring the values for every CAP device. Other secondary aspects may require more CAP-specific analysis and perhaps the development of new safety standards. For example, chromosome damage of cultured brain cancer cells caused by DBD plasma was measured using a micronucleus assay, which can be used as a tool to measure possible genotoxicity in other cells and tissues as well.[95] A more recent study using such assay, along with apoptosis assay and hypoxanthine phosphoribosyl transferase (HPRT) gene mutation assay on plasma-treated liver cells, found time-dependent formation of micronucleus formation after CAP exposure, but no delayed genomic instability like delayed reproductive cell death and micronucleus formation was found in the progeny cells, nor was there an increased HPRT mutation frequency either in the target cells or their progeny.[96] One study concluded that when using a jet-type plasma, UV radiation did not penetrate beyond the stratum corneum and the exposure was one order of magnitude lower than that of sun exposure.[97] Another study presented an *ex vivo* model to examine the safety of any CAP devices. The authors analyzed skin specimens treated with a surface microdischarge plasma using microscopy, electron microscopy, and a DNA double-strand break assay. They found no signs of structural changes, but there was an increase of double-strand DNS breaks at some settings.[98] Overall, the authors found the tested CAP device safe and tolerable, at least within the given exposure parameters. Yet another study examining a jet-type plasma device also concluded that the application was safe but noted that changing parameters, like extending exposure time, may increase risks.[99] The presence of reactive species raises a concern of mutagenicity for CAP. Jet-type and DBD CAP devices were examined for mutagenicity, and they were found safe within the operating parameters studied.[100,101] Another approach, study of skin barrier function and skin moisture, also found CAP tolerable and safe.[102] As mentioned in the context of plasma dosing, another approach is to examine the effects of CAP on the actual biological targets, in our case on the human skin. This approach uses measurements to assess changes in the structure and function of the human skin after being treated with plasma. One such proposed model uses dermatoscopy, confocal laser scanning microscopy, hyperspectral imaging and also quality-of-life questions and visual assessment to determine the safety and tolerability of CAP.[26] All studies addressing safety highlight the device and treatment setting specific nature of their results. Even though CAP seems to be generally safe, likely every single device needs to be tested. To ensure that CAP can be used with confidence, there is a need for a comprehensive approach. International efforts on developing plasma safety standards are coordinated by IOPMS (International Organization on Plasma Medical Device Standardization), which – at the time of writing this article – is co-chaired by Dr. Eun Ha Choi (Kwangwoon University, Seoul, Korea) and Dr. Kai Masur (Leibniz-Institute for Plasma Science and Technology, Greifswald, Germany), with Dr. Alexander Fridman (C. & J. Nyheim Plasma Institute, Drexel University, Philadelphia, PA, USA) representing the United States on its board. IOPMS is considering device safety under the major aspects: (i) biomedical safety, related to optimization of protocols, suppression of side effects, detailed analysis of clinical studies; (ii) physical, technological, and electric safety, related to limitations of generation of ozone, UV radiation, leak currents, electromagnetic interaction with other devices in medical environment; (iii) safety aspects related to device (and treatment protocol), sensitivity to device positioning, dosimetry, environmental conditions. The diversity of different types of plasma devices represents a substantial challenge for IOPMS. The goal is to address all these concerns in a way that is applicable to all medical plasma technologies and to develop safety standards and parameters that can be adopted by regulatory bodies worldwide.

Future trends

As much as it can be predicted, the near future of plasma dermatology will likely be determined by two main factors, both related to the beginning of commercialization: expansion of clinical use areas driven by commercial competition and by the clinical availability of this technology, and intensified basic research because of increased interest and funding stemming from

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mainstream acceptance of CAP technology. Besides different plasma devices now sold for wound treatment in the European Union and the Canady Helios™ Cold Plasma Therapy helium plasma jet currently in clinical trial to augment cancer surgery (www.usmedinnovations.com), there are at least three conditions (onychomycosis, actinic keratosis, warts) with successful proof-of-concept studies awaiting commercialization. As there is an overlap between the abilities of various types of devices from different research teams, there will likely be a competition between respective device manufacturers to provide the "best" plasma treatment for the already established target diseases and also to expand the scope of each device or device family to new skin conditions to capture the largest market share possible. Off-label use is more the rule than the exception in dermatology. Once cold plasma devices will be available, it will not be surprising to see case reports of their innovative use for various skin ailments. Based on the success of actinic keratosis pilot studies, skin cancer clinical trials are imminent, especially for squamous cell carcinomas, which can be seen as part of a continuum that includes actinic keratosis on the other end (Fig. 5). Any condition that can be improved by reducing the number of microorganisms on the skin is also a fair target. Plasma is known to disrupt biofilms,[72,103] which are shown to play a role in itch in patients with atopic dermatitis, for example.[104] While the first trial using CAP to reduce pruritus failed to show benefit,[105] further studies in this field are expected using different settings and different devices. Skin microbiome is gaining more and more recognition, and plasma seems to be well positioned to modify it to our benefit. Based on our constantly evolving understating of CAP causing selective inhibition of malignant cell growth and induction of cell proliferation, many malignant, proliferative, or – in contrast – degenerative skin conditions can be evaluated for plasma treatment, not to mention using plasma to reverse skin aging by inducing stem cell differentiation. Another field not discussed here in detail is transcutaneous drug delivery. Plasma has been shown to induce the penetration of topically applied substances into the deeper layers of the skin, opening the door for enhancement of the efficacy of medications targeting the skin and also enabling topical administration of medications intended for systemic absorption.[106-108]

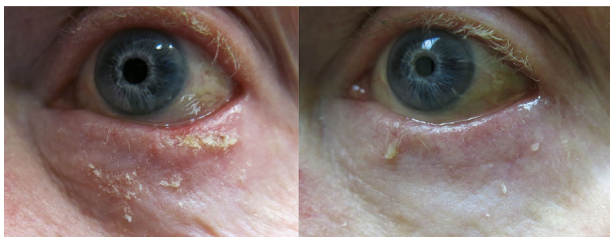


Figure 5 Atypical squamous cell proliferation treated with floating-electrode DBD plasma. Left panel: This extensive, multifocal, ulcerated eyelid lesion was diagnosed to be within the actinic keratosis spectrum, although *in situ* squamous cell carcinoma was part of the differential diagnosis. The entire lesion was treated one time with a floating-electrode DBD plasma device using previously reported treatment settings.[3] Right panel: no sign of recurrent lesion 15 months after a single CAP treatment, although a new lesion is noted medially from the treatment area. (Friedman PC, Fridman G, Fridman A, unpublished data. The photographs were taken as part of an IRB controlled clinical trial: Western Institutional Review Board, date: 11/10/2016, protocol number: 20130084, ClinicalTrials.gov, registration number: NCT02759900)

The cost of plasma devices can vary greatly, but if one takes the German hand-held device plasma care® as an indicator, or even the more sophisticated machines intended for research use, the price can be substantially below even that of the cheapest lasers used so frequently in dermatology offices. Depending on the application, simple, low cost devices may suffice, such as a battery operated hand-held 'flashlight' DBD CAP device described in 2012, which had an estimated cost of around 100 USD.[109] In the United States, some treatments, such as destruction of warts and actinic keratoses, can feasibly be billed to insurance companies using Current Procedural Terminology (CPT) codes, while others, such as onychomycosis treatments, can be offered on a relatively affordable cash-fee basis given the low cost of the devices. Alternatively, a developing plasma dermatology community may lobby for its own CPT codes, just as we saw it happen for reflectance confocal microscopy. The lower price range also means that there will be less of a barrier adapting CAP, in fact it is feasible that one dermatology practice will own more than one type of CAP device, just like it is the case with lasers today.

As we have seen in all plasma research areas, CAP is a complex entity with even more complex effects on cells and tissues, which we are only beginning to understand. A very exciting and rapidly evolving field is the influence of plasma on signaling. For example, CAP effect on intracellular calcium influx is emerging as a putative crucial step in modulating various signaling pathways and mitochondrial and endoplasmic reticulum responses. We hypothesize that it is possibly one of the main mechanisms of most biological plasma effects shown in clinical trials. It has been well demonstrated that modifying delivery parameters can profoundly change the biological response to CAP. It will require extensive and expensive *in vitro*, *in vivo,* and clinical research to explore this field. Fine tuning delivery settings, protocols, and target selection can likely improve efficacy numbers of plasma treatment even for conditions where there are successful proof-of-concept clinical studies, but it requires large and costly clinical trials. Commercialization is hoped to increase available funding for plasma research to better understand how plasma does what it does on the laboratory and clinical level as well, partly because it is in the interest of industry to support at least some research to improve their devices and remain competitive, and partly because government and other funding for academia can also

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be more accessible in a field that is established as a recognized and accepted medical treatment.

If further laboratory and clinical research can lead to a better understanding of how plasma works, and this knowledge is paired with advances in engineering, it is not impossible to envision plasma technology that, for example, can selectively eliminate only certain bacteria from the skin surface to influence diseases via the cutaneous microbiome, or can alter cell fate at will, down to the level of very small groups of cells.

CONCLUSION

Plasma is a physical entity that has a variety of effects on living tissues, many of them potentially important for medical applications. It is easy and relatively inexpensive to create cold plasma at room temperature and under atmospheric pressure so it can be applied to the surface of the skin, with no discomfort, pain, or known harmful effects. Plasma medicine is just taking its first baby steps, but it shows promise for the treatment of various skin diseases. The way plasma acts on living tissues is very complex, but with the expansion of clinical use and research, our understanding of what plasma does and what it can be used for will deepen. Extensive laboratory research and recent proof-of-concept clinical trials raise the hope that plasma may be the answer to some of the most stubborn problems in dermatology by destroying hard to treat bacteria or fungi, causing selective cell death in malignancies, and inducing tissue regeneration.

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Questions (answers provided after references)

1 Among others, which components are implicated in the biological effect of cold plasma?
- a Gas pressure of carrier gas
- b Reactive oxygen and nitrogen species
- c Vacuum used during plasma production
- d Intense visible light accompanying plasma discharges

2 Plasma was shown to be effective treating warts by inducing extreme intralesional heat without direct skin contact.
- a True
- b False

3 Surface microdischarge plasma has intense sterilizing effect, making possible cross-contamination when using the same device on multiple patients a nonissue.
- a True
- b False

4 Which of the following plasma device types are used in current clinical dermatological research?
- a Interpolation electrode plasma
- b Surface microdischarge plasma
- c High-pressure contact plasma
- d Floating-electrode DBD plasma

5 Published clinical studies used cold plasma for which of the following conditions?
- a Psoriasis
- b Bullous pemphigoid
- c Actinic keratosis
- d Pruritus

6 All plasma effects result from electric changes to the cell membrane, without any detectable changes in intracellular signaling pathways.
- a True
- b False

7 Because of their engineering simplicity, cold plasma devices can easily use safety standards already in place for any other electric devices.
- a True
- b False

8 Cold plasma was shown to selectively induce cell death in malignant cell lines but not in nonmalignant controls.
- a True
- b False

9 Cold plasma was shown to induce stem cell differentiation in various types of stem cells.
- a a True
- b b False

10 The extreme high cost of current cold plasma devices is predicted to be the most important barrier of the adoption of this technology.
- a a True
- b b False

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Answers to questions

1 b
2 b
3 b
4 b,d
5 c,d
6 b
7 b
8 a
9 a
10 b

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