Chapter 7

Cold Atmospheric Pressure Plasmas (CAPs) for Skin Wound Healing

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Additional information is available at the end of the chapter

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Abstract

In the past 20 years, cold atmospheric pressure plasmas (CAPs) have become a new promising way for many biomedical applications, such as disinfection, cancer treatment, root canal treatment, wound healing, and other medical applications. Among these applications, investigations of plasma for skin wound healing has gained huge success both in vitro and in vivo experiments, and also the mechanism behind it has been studied by many groups. In this chapter, we summarize the state-of-the-art progress in wound healing by CAPs. The plasma devices developed for wound healing, the interactions between plasmas and microorganisms/cells/tissues, the in vitro and in vivo treatments, the clinical trials, and biosafety issues are all included.

Keywords: atmospheric pressure plasma, plasma devices, wound healing, disinfection, cell proliferation, clinical trials

1. Introduction

Wound healing is a complex process involved with infection, cell proliferation/migration, and skin remodeling, see **Figure 1** [1]. For normal wounds, generally the first-stage inflammation occurs in 24–48 h after tissue damage. Bacteria, neutrophils, and platelets are abundant with normal skin appendages present outside the wound. The second stage lasts from after 48 h to around 10 days, during which scab would form on the skin and cell migration and proliferation vigorously occurs. New blood vessels populate the wound area. Skin remodeling starts in the following stage and usually lasts a year or even longer. A scar is usually left and the healed area does not contain normal skin appendages. Wounds can typically be categorized as acute and chronic wounds. Acute wounds contain abrasions, scalds, burns, or post-operative incisions; however, chronic wound does not heal in an orderly set of

stages and often remain in the inflammatory stage for too long and associated with systemic illnesses, age, and repeated trauma such as diabetic ulcers, venous ulcers, arterials ulcers, and pressure sores. The number of patients undergoes chronic wounds increases constantly. It has been reported that around 4.5–5 million people in Germany are concerned with chronic non-healing wounds [2]. Traditional treatments of chronic wounds are expensive and time-consuming; patients usually undergo long-term hospitalization with a poor quality of life.

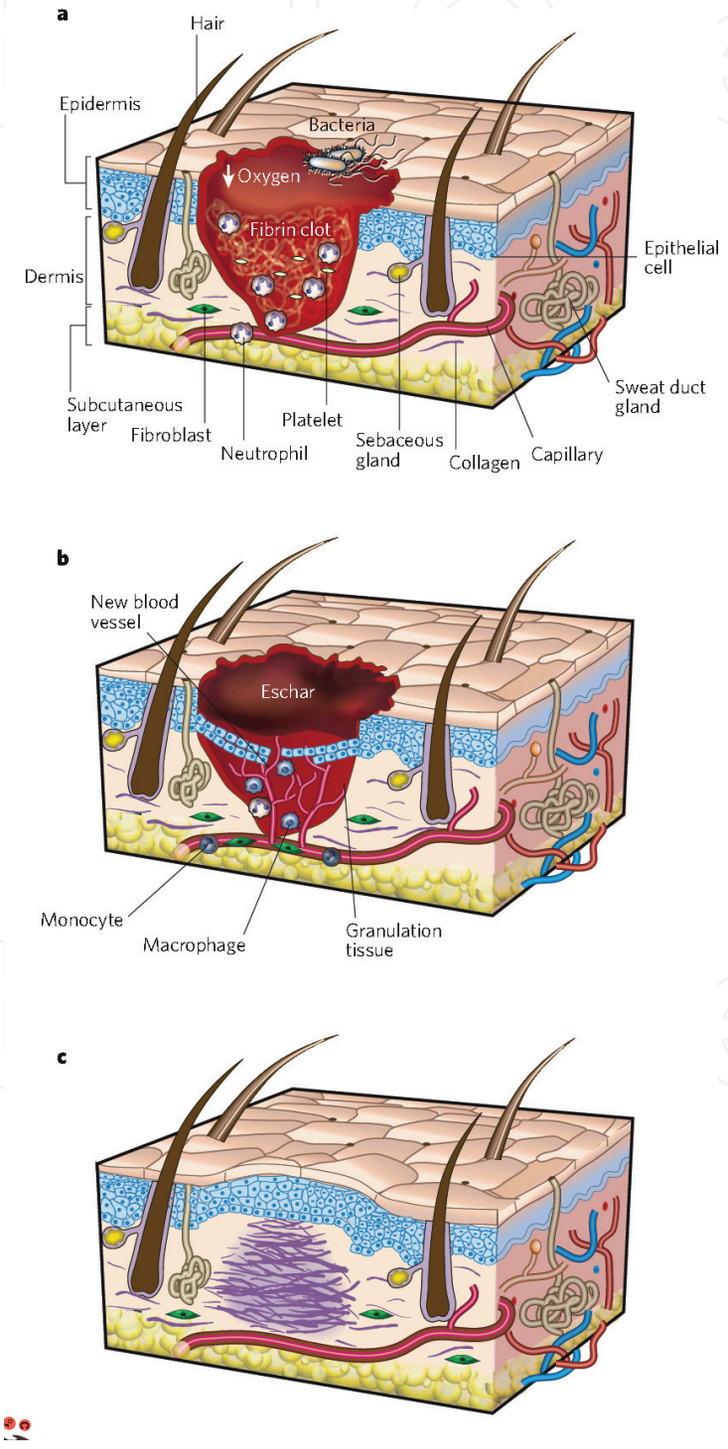

Figure 1. Classic stages of wound repair: (a) inflammation (b) new tissue formation; and (c) remodeling [1].

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The first step of chronic wound treatment begins with bacterial disinfection. However, with involvement of multidrug-resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA), treatment of chronic wound encounters more challenges, because the effect of traditional medical antiseptics is largely restrained. Therefore, there is a huge demand for new methods and strategies for skin disinfection and improving wound healing process. One of the potential candidates is cold atmospheric pressure plasmas (CAPs) [3–6].

Plasma, known as the fourth state of matter, other than plasma from blood, has been studied for centuries. Nevertheless, cold atmospheric pressure plasmas (CAPs) also called nonthermal plasmas have attracted huge attention during last two decades for its unique advantage in a new special field—plasma medicine. In 1996, the first paper of plasma medicine came out, which developed a promising way to solve the problems with traditional medical therapies [7]. Since then, related research groups in the entire world started to follow this new field; they extended the research field from initial decontamination to mechanism study, plasma cell interaction, cancer treatment, skin disinfection, blood coagulation, chronic wound healing, and so on, and since then thousands of peer-reviewed research and review papers have been published. There also have been various CAP devices developed [8–13], based on which, in recent years several commercial plasma devices have started to be used in hospital, for example, RF Argon plasma jet-based products by Leibniz Institute for Plasma Science and Technology (INP Greifswald)—kINPen MED has gained Conformité Européenne (CE) marketing certification in 2013 [14].

We report the latest progress of atmospheric pressure plasma for skin wound healing in this chapter and the sections have been arranged as follows: in Section 2, basic plasma components and typical plasma sources for skin treatment are summarized; in Sections 3 and 4, fundamental studies of plasmas interaction with microorganism and cell/tissue are included; in Sections 5 and 6, in vitro and in vivo studies of CAP treatment of skin wounds on animal model are presented; and in Section 6, clinical trials and a very important issue—plasma biosafety—are presented.

2. Plasma generation and designed devices for skin disinfection

CAPs could be generated in lab using plasma source and driven by power supplies with working gas. Working gases such as noble gases (He, Ar), N_2, O_2, air, or their mixtures could be used to ignite the plasma. Typical constituents of plasma include electrons, ions, neutral particles (background gas molecules), UV radiation (UBA, UVB, UVC), heat, reactive oxygen, nitrogen species (RONS), and so on (see **Figure 2**). All these components within plasma make CAPs highly reactive. The constituents and concentration of species in plasma depends on the plasma source, the power input as well as the working gas. When contacting with microorganisms or cell/tissue, components of plasma will play different roles in the treating processes; however, the mechanism has not been fully understood yet. It is well known that UVB (280–320 nm) and UVC (100–280 nm) could be able to cross the epidermis, and UVA (320–400 nm) can even reach the dermis. UVB and UVA can also trigger skin cancer. However, a lot of reports claimed that UV radiation in plasma is low and does not play a significant role in anti-microorganism process except that from microwave-driven discharge [15–18].

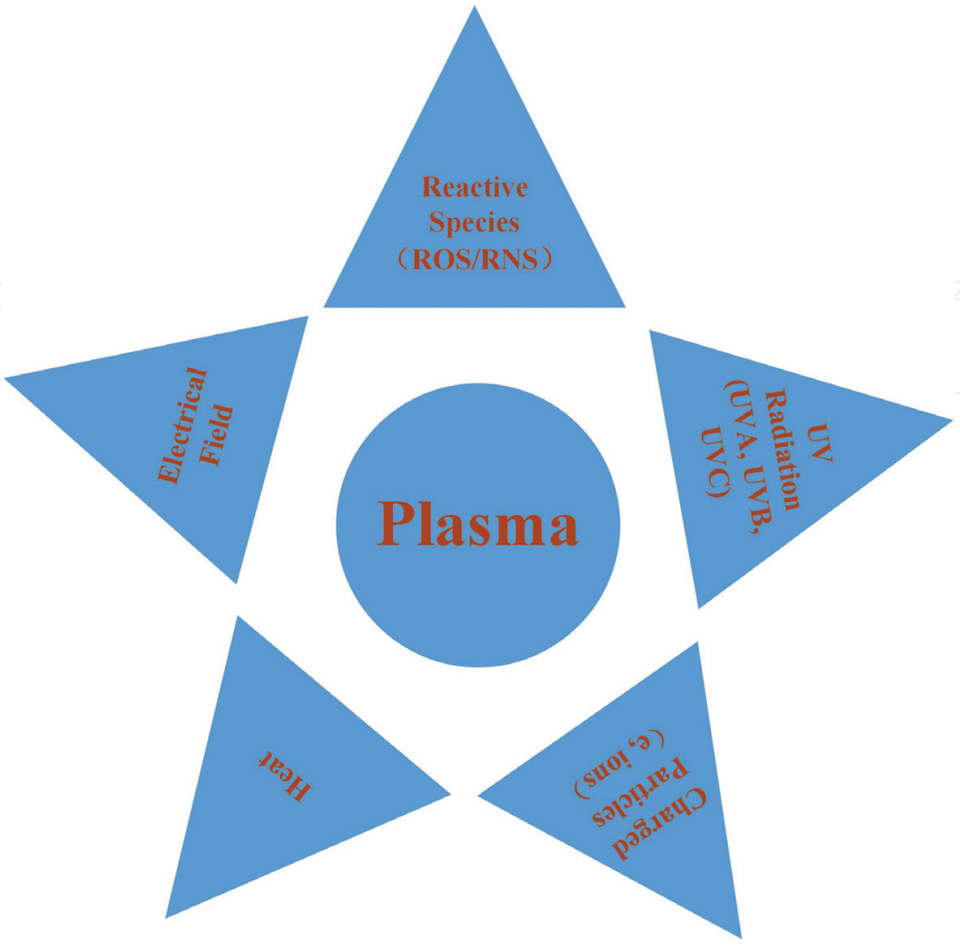

Figure 2. Plasma components.

Charged particles in plasma were concluded to play an essential role in bacterial inactivation by rupturing the outer layer of cell membrane [19, 20]. Mendis et al. reported the electrostatic force caused by charge accumulation on the cell membrane could overcome the tensile strength which leads to rupture [15]. However, other researchers claimed different viewpoints that the anti-bacterial effect of the charged particles is due to the chemical modification of the membrane surface [21]. CAPs are usually designed to operate in near room temperature (less than 40°C); therefore, there would not be substantial thermal effects on microbial cells [22]. The effect of heat can be ignored. The microorganism is under indirect plasma treatment which means that plasma does not directly contact with the target; the electric field was too weak to contribute to the inactivation process. In some cases, when plasma is in direct contact with the samples, the electric field could become high enough to take effect [23]. However, in most of the cases, there is a rare possibility to put the samples within 300 μm away from the electrode to cause damage by the electric field [24]. If the plasma device is using air or O_2-containing mixture or operating in the open air, RONS such as O, O_3, NO, NO_2, OH, and H_2O_2 would present. These highly active species are believed to play an important role in all the plasma treatment processes and have already been reported by many researchers [18, 26–28]. The detailed discussion of the roles of reactive species can be found in [29].

Figure 3 summarizes several typical plasma sources used in different research groups. (a) is called plasma pencil designed by Laroussi group [30]. This device driven by sub-microsecond high-voltage pulse uses He gas flowing through a modified DBD device (with holes in the center of dielectric plates), therefore the plasma created between electrode panels comes out and forms a plasma plume up to 5 cm. Another famous plasma source construct is a floating-electrode DBD device (FE-DBD) which uses the treating object as the second electrode and could generate plasma between the electrode surface and the substrate [31]. FE-DBD could directly use the air inside the short gap as working gas. Safety and stability of FE-DBD largely depends on the power supply, the gas gap, and the electrode shape. Kolb et al. introduced a DC-driven micro-hollow cathode discharge to generate plasma plume outside the tiny hole (c) [32]. It could use air or other gas as working gas. A 51 KΩ ballast resistor is connected to the circuit to restrain the current to 20 mA. The dimension of the plume changes with the gas flow rate as well as the gas temperature. Lu et al. reported a single-electrode plasma jet powered by nanosecond pulse DC [33]. Helium is used as working gas and the length of plasma plume could reach up to 4 cm. The gas temperature is about 300 K and species like O, OH, and N_2^+ are all detected by optical emission spectra (OES) (d). Another structure called plasma needle usually uses bare metal needle as electrode, see (e) and (f) [9, 34]. (g) is a portable DC-driven plasma needle array device called plasma flashlight [35]. This device can directly use a DC battery to power up and create plasma in the open air. (h) is a plasma brush of relatively large area [36]. (i) is also a classical design

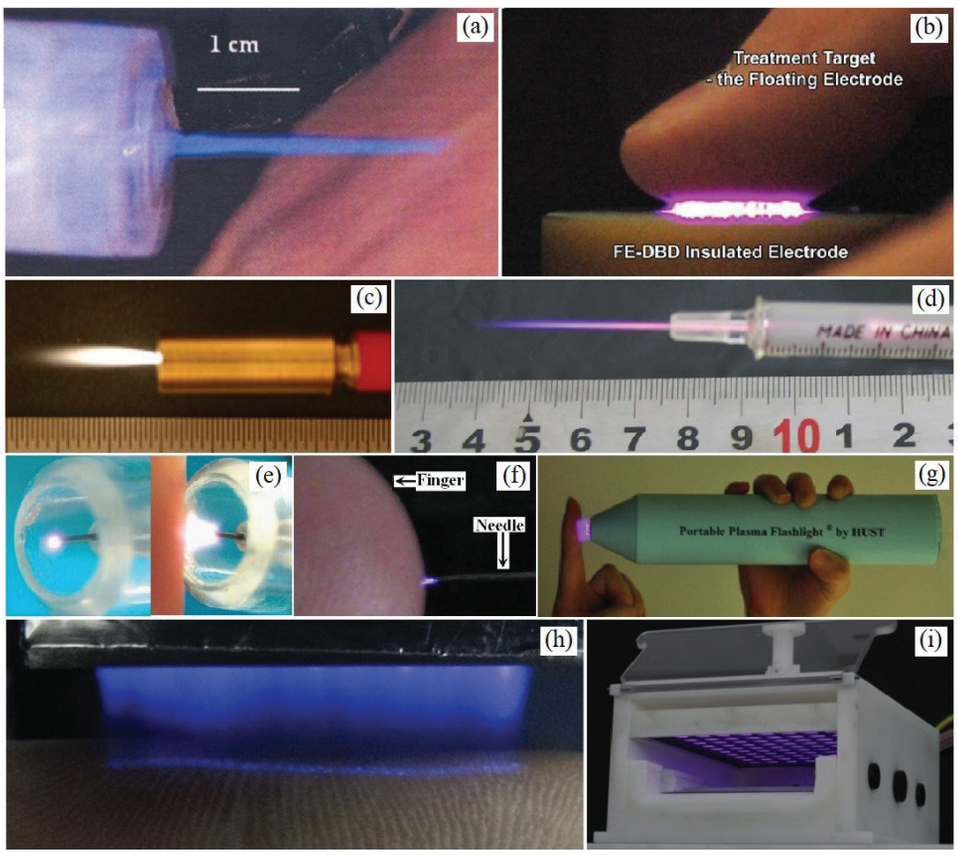

Figure 3. Typical CAP sources.

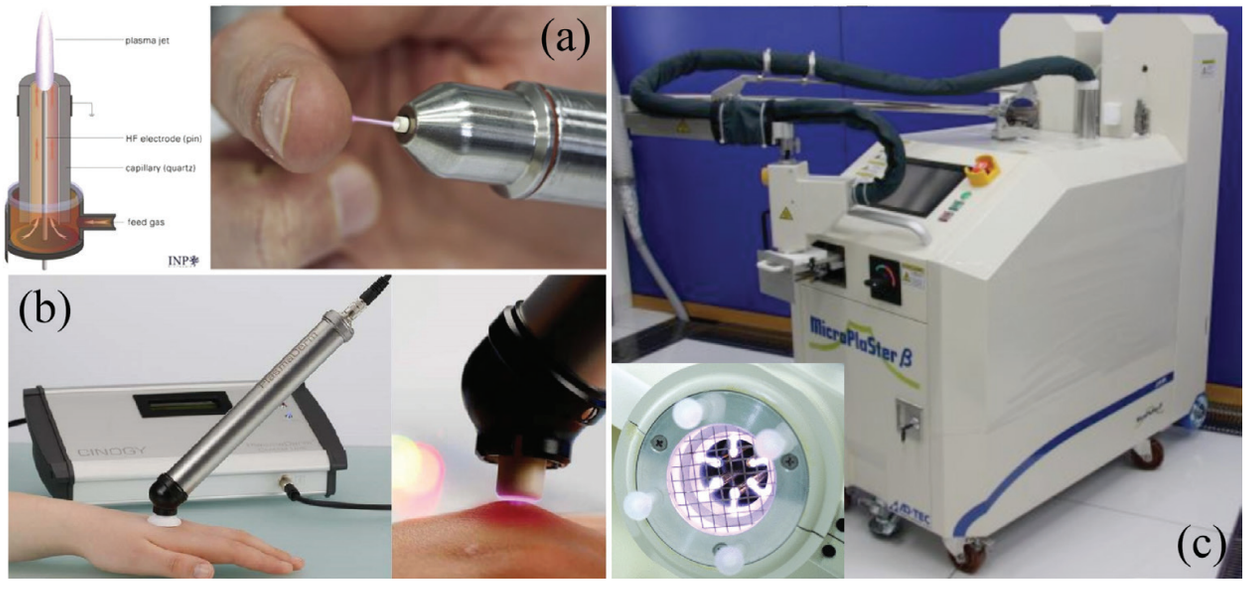

Figure 4. Three commercialized CAPs medical devices.

(surface micro-discharge, SMD) representing 'indirect plasma' [37]. This type of plasma generates on the surface of the electrode, and the plasma does not directly contact with the treatment target. When using air as working gas, SMD could operate in three modes: call ozone mode, transition mode, and NOx mode, respectively. The transition between these modes depends on the input power. More detailed information of the different plasma sources can be found in [38, 39].

With the development of the lab-made plasma sources and fundamental studies, many commercial plasma device products have been delivered into the market. **Figure 4** shows three famous products for skin wound healing based on plasma jet device, FE-DBD and SMD, respectively. **Figure 4(a)** is the world famous kINPen plasma device, which is based on a RF argon plasma jet source and gained CE marketing certification in 2013. (b) is called PlasmaDerm based on FE-DBD. And (c) is MicroPlasma β, which origins from SMD device. The wound healing effect of these three products will be presented in the following sections.

3. Plasma interaction with microorganisms

Plasma sterilization is the first research field of plasma medicine. Since Professor Laroussi published the first paper of plasma sterilization in 1996 on *IEEE Transactions on Plasma Science* [7], there came out thousands of studies on inactivation of bacteria, fungi, and virus using different plasma sources. At the same time, mechanism of plasma interaction with microorganism was studied by using physical, chemical, and biomedical diagnostic methods, such as optical emission spectra (OES), laser-induced fluorescence (LIF), Fourier transform infrared spectroscopy (FTIR), flow cytometry, electrophoresis, ELISA, chemiluminescence assay, and so on. However, the exact mechanism of plasma inactivation microorganisms still

remains unclear. Possible mechanisms proposed by researchers are: (1) electroporation- and oxidation-induced cell wall/membrane dysfunction, which leads to leakage of cellular components; (2) intracellular oxidation and nitrification causing protein damage and gene expression disorder; and (3) direct DNA damage such as causing double-strand breaking.

Bacterial killing effect by CAPs has been investigated for more than 20 years. It is found that CAPs could effectively inactivate different type of bacteria, including gram-positive and gram-negative, anaerobic, aerobic, or facultative anaerobic bacteria [40]. The response of bacteria to CAPs is species-dependent and the Gram-positive bacteria is usually more susceptible to CAPs treatment because of the difference of cell-wall components, which indicates that the CAP-induced damage to the cell membrane and cell wall may be a key factor of antibacterial effect. The most common bacteria found in skin and wound infection are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, and so on, which have been proved to be effectively inactivated by CAPs [41, 42]. Unlike drugs, another advantage of CAPs is that plasma does not show any resistance after multi-treatment against bacteria. Maisch et al. reported significant decolonization of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* without cell damage of a pig skin sample [43]. Alkawareek et al. also found complete inactivation of MRSA [44].

Many fungi are common constituents of skin flora, and under certain conditions, they would cause diseases. The effects of traditional tools such as chemicals, UV radiation, or heat are often unsatisfactory and sometimes accompanied by undesirable side effects. Unlike bacteria, fungus is more resistance to plasma treatment because of the much more complex cell biology. In 2008, Akishev et al. published the first paper of CAP decontamination of *Aspergillus niger* and *Candida lipolytica* on agar surface using $N_2 + O_2$ plasma jet. After 30–60 treatments, inhibition zone of 30–40 mm was observed [45]. Xiong et al. also inactivated *Candida albicans* on agar surface using a $He + O_2$ plasma jet [46]. They compared the antifungal effect of with/without a cap on the petri dish and found that restraining active species inside the chamber largely improved the antifungal effect. Daeschlein et al. used a low-temperature atmospheric pressure plasma jet to treat clinical isolates of *Trichophyton interdigitale*, *Trichophyton rubrum*, *Microsporum canis*, and *Candida albicans*. They found that plasma irradiation could eradicate fungal growth and no isolate exhibited resistance to plasma treatment [47]. In a new research area of plasma treatment of onychomycosis, Xiong et al. used three kinds of CAPs to treat *E. coli* and *Trichophyton rubrum* living on the back side of a nail model and found that bacteria is easier to inactivate than fungus and the inactivation effect also related to the structure of plasma sources [48].

Researchers also use CAPs successfully inactivate various virus and mechanism has been investigated as well [49–51].

It is known that more than 60% of all infections are caused by bacteria in the form of bacteria which could become resistant to treatment and often develop into a chronic state. A biofilm is often formed by a cluster of cells encapsulated by a 3D extracellular matrix (ECM), [52] which forms a good protection barrier for antibiotics and plasma agents. Therefore, cells inside this community have been demonstrated to exhibit higher antibiotic resistance than planktonic cells. However, CAPs have also shown great decontamination effect against biofilms with longer time than treating planktonic cells under same condition [53, 54]. Koban et al. compared the anti-biofilm (*Candida albicans*) effect by dielectric barrier discharge and plasma jet,

and used 0.1% chlorhexidine digluconate (CHX) and 0.6% sodium hypochlorite (NaOCl) as positive control. They found plasma treatment reduced the colony-forming units CFU significantly compared to chemical disinfectants [55]. In a later research, they investigated the synergistic effect of nonthermal plasma and disinfecting agents against single and multispecies dental biofilms. They found that the combination of plasma and agents increases the antimicrobial efficacy of all tested compounds [56]. Xiong et al. firstly used Laser Confocal Scanning Microscopy (LCSM) technology to obtain the depth of biofilm that plasma could penetrate through [57]. A He-O_2 mixture nanosecond pulse DC-driven plasma jet was used to treat a 10-day growth of *Porphyromonas gingivalis* biofilm, and found that 5-min plasma treatment could at least inactivate the bacteria under 15 μm. In their following work, they successfully inactivated a 25.5 μm biofilm using a plasma flashlight [35]. Puligundla and Mok reviewed the potential application of nonthermal plasmas against biofilm-associated microorganism in 2017. For more details about plasma interaction with biofilms, refer [58].

4. Plasma interaction with skin-related cells/tissues in vitro

The interaction between plasma and human cells largely depends on the plasma source, plasma doses as well as cell type. Researches on plasma cells interaction have been studied by several groups [59–61]. For a unique mixed state with electrical field, charged particles, and controllable reactive species, the response of eukaryotic cell to plasma treatment is very different. It is generally accepted that low dose of plasma treatment could stimulate cell viability and enhance proliferation, differentiation, and migration, while high dose induces cell apoptosis/necrosis [62–65]. It has been found that the resistance against plasma treatment is different between cancer cells and normal cells, which makes plasma selectively killing cancer cells while bring less damage to normal cells and become a potential and powerful tool against cancers [66–68].

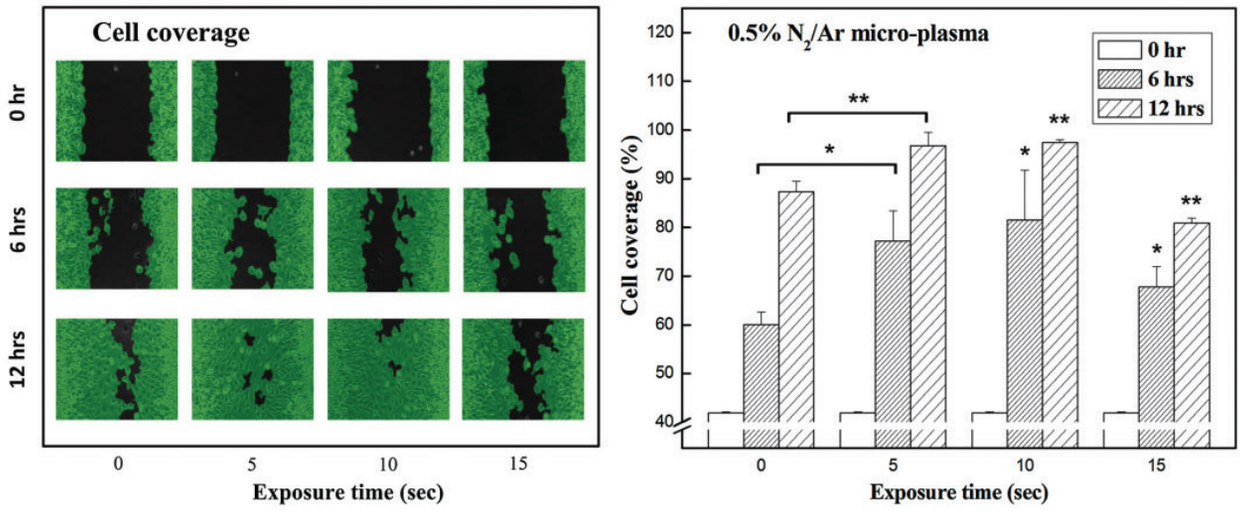

Figure 5. The progression and statistical analyses of cell migration or coverage, 6 and 12 h after plasma exposure times of 5, 10, and 15 s [71].

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As mentioned earlier, the second stage of wound repair is related to cell proliferation and migration as well as angiogenesis. Cell types involved in wound healing are mainly fibroblasts and keratinocytes, among which keratinocytes contribute to the major healing processes and fibroblast cells play a guiding role [69, 70]. It has already been reported that CAPs could increases fibroblast cell proliferation and migration by using N_2/Ar microplasma through simulated release of fibroblast growth factor-7 [71], as seen in **Figure 5**. Researchers from INP

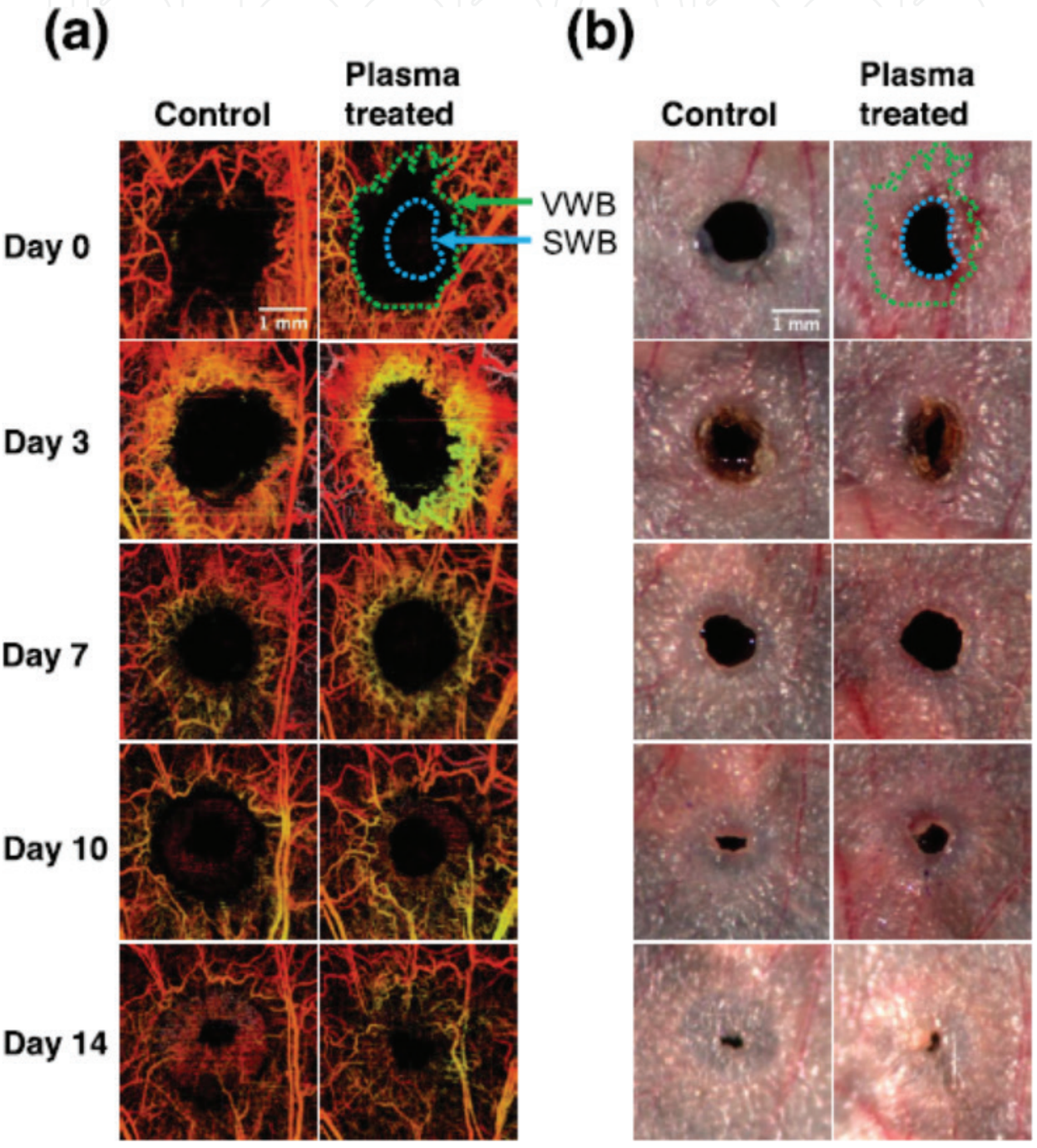

Figure 6. (a) Non-invasive angiographic OCT images and (b) stereoscopic images longitudinally acquired over 14 days after wound generation; relative depth in angiographic OCT images is color-coded as from yellow (superficial) to red (deep) [82].

have done series of investigations of plasma interaction of keratinocyte. They used different plasma sources including plasma jet (kINPen), SMD and DBD, and different keratinocyte cell models/tissues to study the response after treatment. They found increased b1-integrin expression and reduced E-cadherin and EGFR expression of HaCaT-keratinocytes after 30 s treatment [72]. Intracellular level of ROS increased after SMD treatment without dependence on the treatment time or different treatment regimens [73] and DBD and kINPen 09 plasma treatments could also induce oxidative stress in human keratinocytes [71, 72]. Plasma treatment could not only induce cell reactions of stress-sensing but also of proliferative nature, and they propose that stimulating doses of plasma treatment may protect epithelial skin cells in wound healing by promoting proliferation and differentiation through triggering hormesis-like processes [74–78]. Other groups also found the evidence that short-term plasma exposure could enhance keratinocyte proliferation [79].

Angiogenesis is a very important process in the second stage of wound healing involving with growth factors, cytokines, ROS, and NO which could be provided by CAPs. Studies of plasma-inducing angiogenesis have been reported by many groups. Arjunan et al. found that FE-DBD treatment could induce angiogenesis by FGF-2 release regulated by plasma-produced ROS [80]. Hirata et al. used a mouse burn model to investigate the healing process by plasma irradiation, and they found that healing process was improved and the quantity of neovascular vessels was increased after plasma treatment [81]. Kim et al. used angiographic optical coherence tomography (OCT), which successfully captured the plasma-induced angiogenesis process. **Figure 6** shows the en face vascular projections acquired from the angiographic OCT and matched stereoscopic images of the plasma and control wounds over 14 days. They found that the vascular wound area decease of plasma treated wound was more significant [82]. Up to date, very little is known about plasma-induced angiogenesis formation and a lot of work needs to be done in the future to understand the mechanism of plasma effect on angiogenesis.

5. CAPs treatment of in vivo animal m`odels

Based on previous fundamental research of plasma treatment on would healing, treatments on animal models with various wounds and clinical trials are also conducted. Ermolaeva et al. used an argon plasma, which tested the antibacterial effect on both vitro and on the animal model of infected wounds. They found that the 10-min treatment significantly reduced bacterial loads on wound surface and 5-day daily plasma treatment could eliminate bacteria from the infected surface 2 days earlier than the control. Wound closure was accelerated in the plasma-treated animals [41]. Nastuta et al. established a burned wounds model on Wistar rat's skin and used a helium plasma jet to stimulate the wound healing process. They found that both polyurethane wound dressing and plasma-assisted epithelization are positive for the recovery process of burned wounds [83]. Alcantara et al. and Hung et al. also found accelerated wound healing after argon and helium plasma needle and plasma jet treatment [84, 85]. Anke Schmidt used kINPen argon plasma jet device to investigate the wound healing activity on a murine model of full-thickness ear wound; a significant acceleration of

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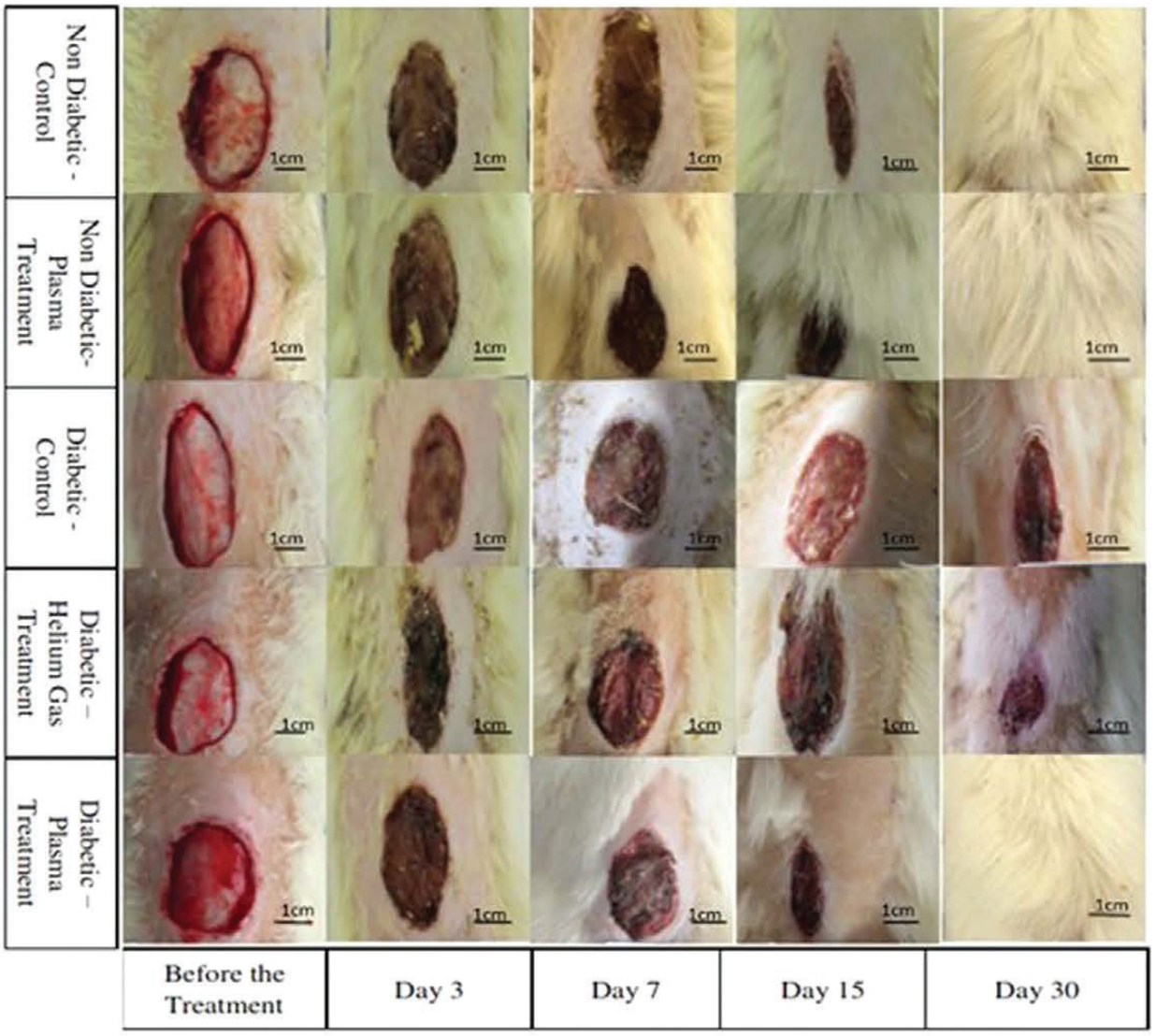

Figure 7. Wound observation for days 3, 7, 15 and 30 after the treatment [89].

wound re-epithelization was observed in days 3–9 [86]. Same results were found by Kubinova et al. without noticeable effects and concomitant activation of pro-inflammatory signaling [87]. Shao et al. investigated the efficacy of a nonthermal N_2/Ar treatment of a laser-induced partial thickness skin wound on a mouse model. Wound-closure kinetics, optical coherence tomography (OCT) and laser Doppler scanning methods were used to measure the healing efficiency and results also show the promotion of wound healing by micro-plasma treatment [88]. Wound healing process in diabetic patients is relatively slow and current therapeutic methods are not completely successful. Fathollah et al. studied the wound healing process by plasma in diabetic rats and found enhanced wound healing rate in the nondiabetic rats and significant wound contraction in diabetic rats after plasma treatment, as seen in **Figure 7**. And also histological analyses show the formation of epidermis layer, neovascularization, and cell proliferation [89].

6. Clinic trials and biosafety concern

Beside animal studies, clinical trials have also been done on patients, especially by using several commercialized products. The world's first plasma source used for clinical trials was the microwave plasma torch MicroPlaSter. Using the first-generation product named MicroPlaSter α, they treated 38 chronic infected wounds on 36 patients with 291 5-min daily treatments and standard wound care, and obtained a significant reduction (34%) of bacterial load without any side effects [90]. In the following study, they compared plasma treatment on various etiologies (Group A), all chronic ulcers (Group B) and Group C for 5-min plasma treatment of chronic venous ulcers. They found a greater reduction in width and length in Group A than control. In Groups B and C, significant reduction in width was found with plasma treatment but not in length [91]. **Figure 8** shows a modified version of MicroPlaSter β and results of treating inflamed ulcer [92]. Isbary et al. reported a successful treatment of

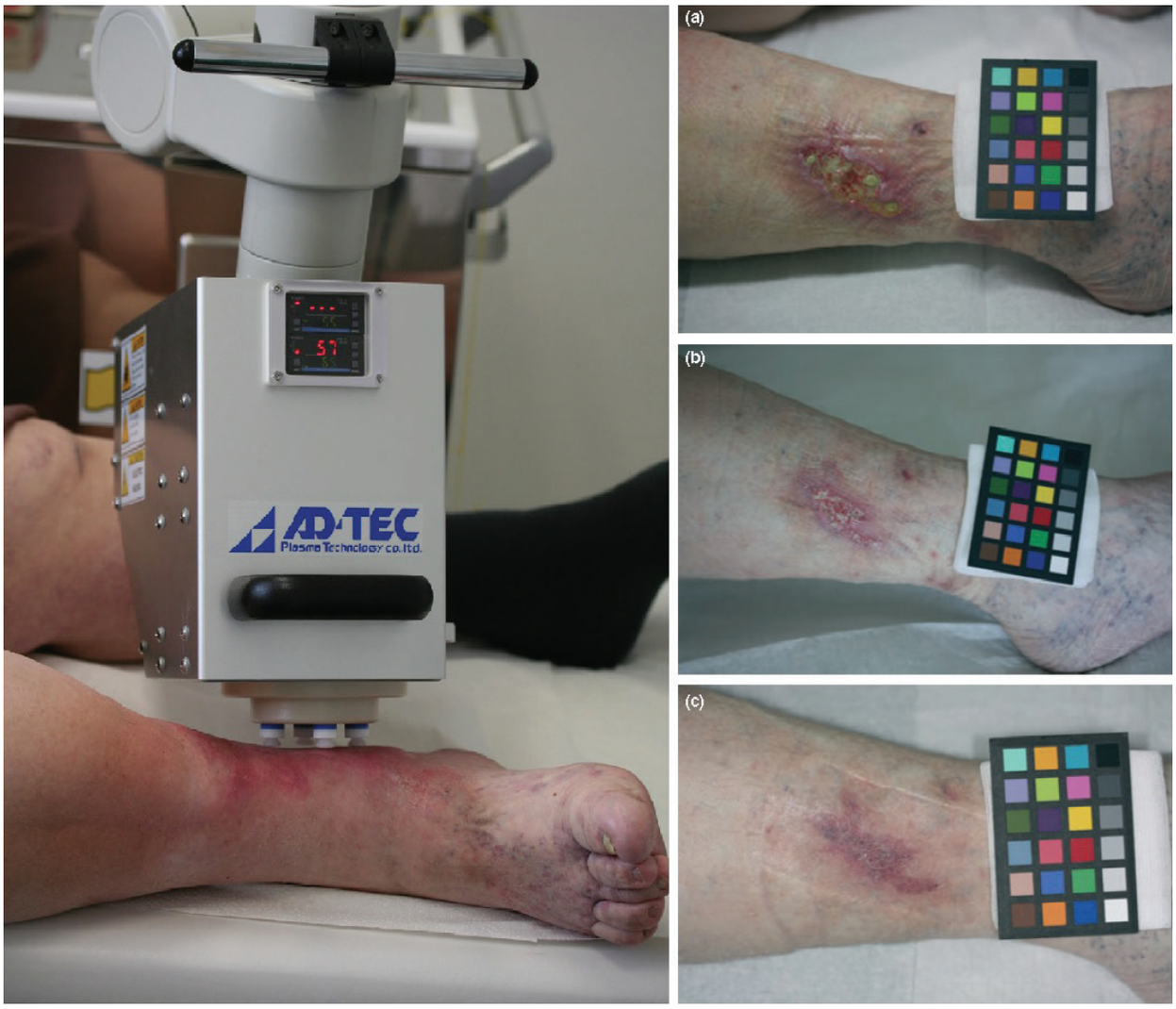

Figure 8. Inflamed ulcer of the right lower leg treatment with cold atmospheric argon plasma generated by MicroPlaSter β [92].

Hailey-Hailey disease by a daily 5-min cold plasma treatment and significant improvement was found after 11 treatments [93]. The argon plasma jet device kINPen MED and DBD plasma source PlasmaDerm successively got the CE marketing for medical devices in 2013. Based on the previous fundamental studies in vitro and on animals, they both reported series clinical trials on human beings focusing on wound healing treatment, especially for the treatment of chronic/infected wounds and microorganism-caused skin diseases. For example, PlasmaDerm was reported to reduced more than tenfold in bacterial colonization on an adult patient with atopic eczema by 30-day treatment of 1 min/day [94]. kINPen MED was reported to significantly reduce the wound volume compared to octenisept in 16 patients with ulcer [95].

Plasma biosafety is definitely a big issue in clinical application. Both of these commercialized products showed tolerable properties (temperature, UV radiation, reactive species, electrical currents, mutagenicity, penetration depth, subjective sensations, cytotoxicity, and histocompatibility) on human skin under controlled conditions and exhibited accelerated wound healing rate [95–98]. Systematic review work of these two devices could be found in [14, 92].

7. Conclusion

Atmospheric pressure cold plasmas could affect different stages of wound healing by helping to activate microorganisms in the first stage and stimulate skin-related cell proliferation and migration in the following period. CAPs have demonstrated high wound healing abilities and may become a promising therapy to replace or assist traditional methods in clinics for wound healing process, especially in chronic wounds. With the certification of several CAP products, more standards and procedures for clinical treatments should be cleared in the future to guide the plasma treatment under an effective and safe way.

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