Chapter 5
Cold Plasma Based Wound Healing Application

Kai Masur

Abstract The healing of wounds displays a complex process of consecutive steps: homeostasis (stop the bleeding by forming a clot), followed by an inflammation phase (to kill micro-organisms), subsequently followed by a phase of cell proliferation and finally a phase of tissue remodeling. Usually human body is capable to heal acute wounds within a few days. However, these well orchestrated phases can be interrupted at any or multiple stages—causing a chronification of wounds. In most cases an underlying disease such as diabetes or the metabolic syndrome leading to such delays in wound healing—often accompanied by infections. Those wounds display a decreased support of nutrition and oxygen—weakening the body´s defense and repair abilities. This chapter will highlight the effects of cold atmospheric pressure plasmas (CAP) on the modulation cell activities and the support of wound healing. The complex mixture of reactive species, electric fields in combination with mild heat and various kinds of radiation—ranging from UV, over visible to near infrared light interacts with micro-organisms and human cells. CAP displays antimicrobial efficacy, support cell proliferation and migration by modulation cellular redox balance. And finally, clinical trials could show that CAP leads to an increased micro-circulation and therefore an elevated tissue oxygenation.

5.1 Background/Introduction

The development of life on our planet was influenced by many factors—one of which is oxygen. During the first billions of years, only single-celled life existed in the primordial oceans—and all developments initially took place under anaerobic conditions … the exclusion of oxygen. Due to the facts, that the first cyanobacteria began to photosynthesize, oxygen was released for the first time. However, even this oxygen did not reach the earth's atmosphere, but first oxidized everything that was freely available in the water. This is how most of today's iron ore deposits were

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formed—by freely available iron being oxidized to rust by the oxygen generated by cyanobacteria and deposited on the sea floor.

Only after this and similar oxidation processes had saturated over millions of years, the concentration of free oxygen did increase, first in the ocean and later in the Earth's atmosphere. This laid the foundation of life as we know it today, but initially also led to one of the greatest mass extinctions in the history of the Earth [1]. The background is that free oxygen—and the reactive oxygen species (ROS) derived from it—are highly reactive, and oxidize everything … including organic matter. Therefore, life had to adapt to the new conditions, and some organisms also managed this step. Numerous adaptations enabled a manageable number of single-celled organisms to come to terms with the highly reactive oxygen. They developed defence mechanisms to capture the oxygen—or the oxygen radicals, to make them harmless, or to repair the damage caused. The advantage of breathing air—containing free oxygen—led to development of multi-cellular organisms enabled to consume organic materials, which are metabolised by applying oxidative processes. In turn, they had to build up mechanisms to control/convert and repair the damages caused by oxygen and its reactive species.

In this context, aerobic cellular respiration also developed, and with it life as we know it today, which incorporates oxygen as an energy-rich compound into the processes of life. Thus, oxygen not only plays an essential role in the mitochondria in the cellular respiration of all eukaryotic organisms, these organisms also developed strategies to use oxygen as a weapon[2]. Besides this, ROS play a central in many physiological processes, orchestrating several signalling pathways and even influence tissue regeneration. However, the major function of reactive species in the human body this so-called oxidative or respiratory burst. The respiratory burst of phagocytes is required for the optimal killing of a wide variety of bacteria and fungi. The burst of 0_2 consumption is utilized by an NADPH-oxidase to generate highly-reactive oxygen species (ROS) starting with one and two electron reductions to generate superoxide anion $\left(O_2^-\right)$ and finally hydrogen peroxide (H_2O_2), respectively [3].

5.2 Wound Healing

5.2.1 Acute Wounds

Physiological wound healing is divided into four phases: hemostasis, inflammatory reaction, proliferation and remodelling phase—whose transitions are fluid [4]. In the first hours after wound formation, hemostasis begins, with constriction of blood vessels, platelet aggregation and finally thrombus formation. So the initial wound will be sealed to ensure that no further microorganisms might intrude the body. In the subsequent inflammatory phase the immune systems becomes activated and especially leukocytes such as monocytes, neutrophil granulocytes and lymphocytes infiltrate the wound. Usually this phase lasts between a few hours up to two or three

days. As a result, the wound has been cleared of the microorganisms and inflammation processes are turned down.

After the infection is deminished, the proliferation phase follows. Usually this phase lasts days to weeks, characterized by the re-epithelialization processes. This involves keratinocytes and fibroblasts, which are predominantly responsible for fibronectin, keratin, and collagen synthesis to ensure production of extracellular matrix (ECM) as well matrix contraction—necessary for correct wound closure. The proliferation phase also includes the beginning of angiogenesis: the formation of new blood vessels. However, the major part is the formation of granulation tissue in order to reduce the wound size and finally to close the wound. The proliferation phase transitions seamlessly into the remodelling phase, where vascularization of blood vessels and collagen formation is completed and reduction of scarring occurs.

5.2.2 Chronic Wounds

Alterations in any of these above mentioned phases can promote chronic wound development and may impede wound healing [5]. The pathological wound healing for example of the diabetic foot has to be distinguished from the physiological one. The dysfunction of the granulocytes is of central importance, but also fibroblasts are impaired in their function. The cause of this is often a disease-related shortage of tissue oxygenation and a reduction of nutrients. This is often accompanied by a permanent colonisation with microorganisms—causing a chronic infection. As a consequence, there is a reduced level of growth factors, e.g. Platelet-Derived Growth-Factor (PDGF), an increased level of proteases, especially an increased protein expression of matrix-metallo proteases (MMPs) [6]. The elevated level of proteases prevents coordinated wound healing by constantly degrading extracellular matrix, wound healing-promoting growth factors, and their receptors, and thereby preventing cell migration processes of fibroblasts and keratinocytes. As a result, subsequent re-epithelialization processes are prevented and immune cell persistence is increased.

However, physiological wound healing and a rapidly subsiding inflammatory response require a balanced concentration of growth factors and cytokines, as well as of proteases and extracellular matrix turnover, which interact with each other during the complex repair mechanisms (Fig. 5.1).

A major difference of wound healing in healthy individuals, in addition to the altered expression pattern of various endogenous factors, is the prolonged inflammatory phase in chronic wounds. Especially in diabetic foot, endotoxins released by pathogenic agents prolong inflammatory responses and worsen wound healing. In those wounds, the neutrophil count is upregulated, which maintains inflammation via the secretion of growth factors, such as TNF-alpha and interleukins—such as IL-6, and causes an insufficient supply of oxygen (ischemia and hypoxia).

Therefore, the development of innovative therapy options which significantly contribute to the healing of chronic wounds, by reducing microbial load, improving

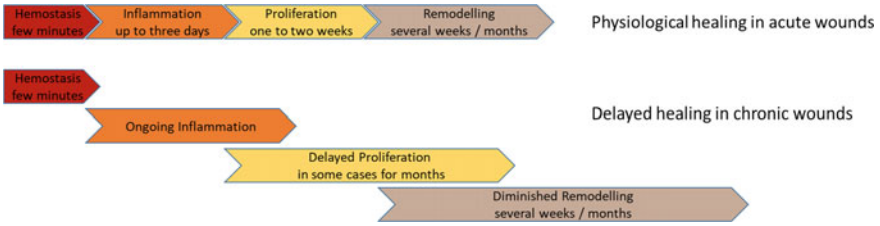

Fig. 5.1 Comparison of acute and chronic wound healing—delays in either inflammatory phase/proliferation phase and/or remodelling phase lead to chronification

tissue regeneration by enhanced cell proliferation and cell migration would be of tremendous importance.

5.3 Cold Atmospheric Pressure Plasma and Chronic Infected Wounds

In the recent years, a new field of research has been established: plasma medicine. Plasma medicine is an innovative research field combining plasma physics, life science, and clinical medicine. The main aspect in plasma medicine is the biological and clinical application of cold atmospheric pressure plasma (CAP). Applying energy to molecular or noble gases—can generate partially ionized gases that modulate biological response mediated by reactive oxygen and nitrogen species (ROS and RNS) in combination with electric fields and mild UV radiation [7]. Thereby these partially ionized gas plasmas contribute to wound healing by modulating several processes: reduction of microbial load, modulation of cell proliferation and cell migration and improving tissue oxygenation. In Europe, several plasma sources are already certified as class 2A medical devices. Based on their ability to generate cold atmospheric plasmas, which in turn are capable to inactivate microorganisms but also to stimulate tissue regeneration, current medical applications are focused on the treatment of wounds and skin diseases [7].

5.3.1 Anti-Microbial Effects

During the early years of plasma medicine the focus was led on the anti-microbial efficacy of CAP—which could be demonstrated for several plasma sources. Especially with focus on novel concepts to limit the spread of multidrug-resistant bacteria (MDR) the application of CAP was investigated intensively.

Usually, for each plasma source the appropriate treatment conditions have to be evaluated. Typically the distance and treatment time are crucial for a proper

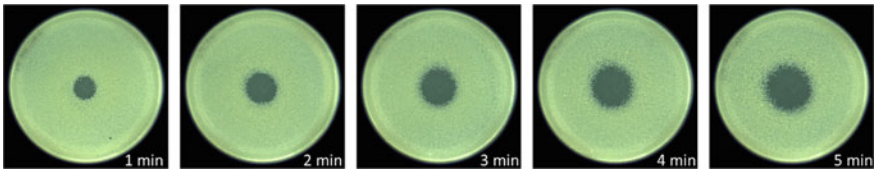

Fig. 5.2 Treatment time dependent increase of inhibition zones of Staphylococcus aureus seeded on agar plates and treated with argon plasma by kINPenMed ® for 1, 2, 3, 4 and 5 min per square centimetre

reduction of microorganisms. Most of the known plasma sources are capable to reduce bacteria within a few minutes or even seconds. A well established method to visualize the anti-microbial effects is to seed a distinct amount of microorganisms on an agar plate/perform the plasma treatment/incubate over-night and finally observe the inhibition zone—the area free of microorganisms (Fig. 5.2).

Daeschlein et al. evaluated the ability of CAP to eliminate MDR- compared to non-MDR-pathogens in chronic wounds. They could proof that a single CAP treatment reduced MDR in all wounds. In 14 treatments (63.6%) and for 16 pathogens (66.7%), a 100% reduction of the bacterial load was observed. For 11 of 17 (64.7%) MDR-pathogens a complete eradication was achieved [8].

However, there seems to be a discrepancy between the reduction rate of micro-organisms cultured and plasma treated on agar plates and micro-organisms treated on real wounds. While 5–6 log reduction (five to six orders of magnitude) are commonly found for plasma treated micro-organisms on agar plates—the same plasma sources used for wound treatment typically show reduction rates of one or two orders of magnitude (1–2 log reduction). This is mainly due to the fact, that on agar plates or generally in cell culture optimized conditions provide a better micro-environment compared to the situation found in real wounds. The wound fluid contains a huge amount of partially unknown proteins, buffering substances and contamination—ranging from bacteria and fungi to dead cells and debris of all kinds. This complex mixture of organic molecules will interact with plasma components and therefore diminish the effects on the micro-organisms themselves—finally leading to a smaller reduction rate (Fig. 5.3).

Besides the fact that reduction of bacterial load in patients wounds displays lower efficacy compared to clean room cultured organisms on agar plate—plasma treatment of wounds is most effective when combined with proper debridement. For well-orchestrated wound care, each wound needs to be assessed by a professional nurse or physician—including management of any complications and comorbidities. Therefore, plasma is a very helpful supporting technology and has the enormous potential to optimize a professional cleaning of wounds regarding the reduction of bacterial loads of chronic infected wounds.

However, this issue is of even greater importance, when patients are released from clinics and transferred to home care. In this case, a similar standard of wound care has to be ensured for a return to clinic for an ambulant plasma treatment. Figure 5.4 depicts the bacterial load of “patient 37” over several weeks of plasma treatment—with first nine treatments when “patient 37” was hospitalized followed by wound care

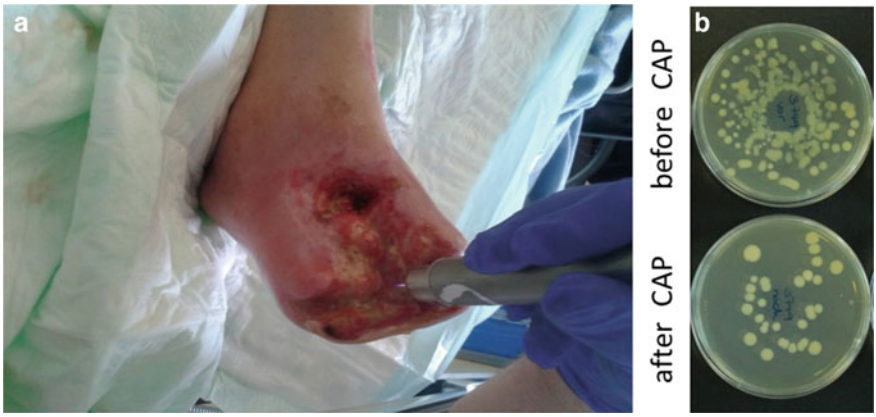

Fig. 5.3 Plasma treatment of a diabetic foot ulcer applying argon plasma of the kINPen Med ® for 30 s per square centimetre. **a** Over-night culture of micro-organisms **b** of a sample taken from the wound before (top picture) and after (bottom picture) argon plasma treatment

at home performed by day care and a accompanied ambulant plasma treatment at the clinic for several weeks. A less professional wound management led to an increase of bacterial load reaching levels higher that before the initial plasma treatment. Each follow-up plasma treatment in ambulant clinic procedure was able to reduce the bacterial load, but efforts were disrupted by bacterial re-growth in between the weekly plasma treatments.

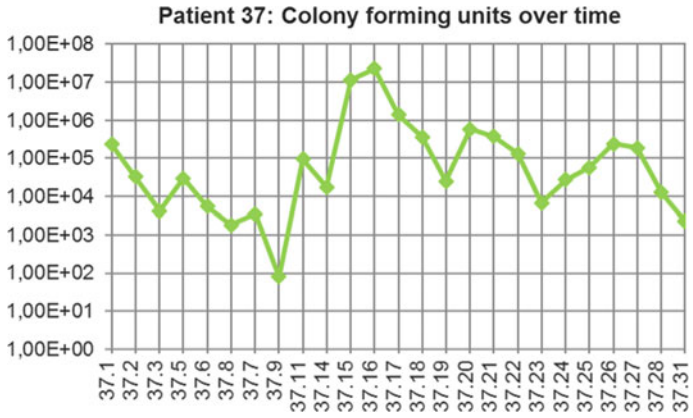

Fig. 5.4 Up and down of bacterial load (colony forming units) of "patient 37"—starting with stationary plasma treatments until treatment number nine—at the day of release from stationary wound care to day care. The following records of wound colonisation were dependent on the professionalism of wound care and the additional effects of cold plasma to diminish micro-organisms in wounds

In summary, plasma treatment is able to reduce bacterial load in chronic infected wounds—but success strongly depends on proper debridement and a state-of-the-art wound care management. There are differences between findings in the laboratory, with microorganisms cultured and treated under optimal conditions—and the real world treatment on the patient side. The ability of cold plasma to reduce bacterial load is affected by many factors—such as dead cells, components of extracellular matrix, body fluids (blood/lymph) containing large amounts of organic molecules, which scavenge reactive species generated in cold plasma. Therefore, cleaning wounds, removing necrotic tissue and visible bacterial films strongly supports an additive cold plasma treatment—and finally ensures a successful wound healing. The major advantage—from a antiseptic point of view—is the ability of CAP to kill antibiotic resistant bacteria in a same manner as non-resistant strains. So far, no report of any plasma-resistance has been reported.

5.3.2 Cold Plasma in Cell Culture

Besides its antimicrobial effects, CAP also modulates cell activities in dermis and epidermis. However, before starting applications on human beings, cell culture experiments have been performed in order to identify the mode of action. Applying CAP in a similar procedure compared to the tests for its capabilities on microorganisms, the focus now is on its stimulating effects. In most experiments keratinocytes and fibroblasts—in rare cases also immune cells are the focus of investigations. In first sight cell viability/cytotoxicity tests have been performed in order to find optimal treatment times and conditions for each plasma source. Usually a few seconds up to one or two minutes per square centimetre of CAP treatment are sufficient to modulate cellular activities. One major player identified is the NRF-2/Keap-1 system—which are sensors for oxidative stress in cells. Several authors showed that expression and cellular translocation of NRF-2 from cytosolic fraction into the nucleus could be detected after CAP treatment. Schmidt et al. could show that the cellular redox homeostasis was maintained and cells were defended from damage by a strong modulation of the nuclear E2-related factor (NRF-2) pathway [9]. As a transcription factor, NRF-2 binds to antioxidant response elements (AREs) in the nucleus leading to transcription of ARE genes [10]. In general, cells can overcome chronic oxidative stress by enhancing activities of anti-oxidant enzymes, thereby protecting cells from DNA damage [11]. While NRF-2/Keap-1 act as intra-cellular sensors for oxidative stress in order to prevent damage and to start repair mechanisms, when ROS and RNS are applied at higher concentrations—a short term treatment with cold plasma is capable to induce another transcription factor system: YAP/TAZ. This transcription factor is associated with the HIPPO pathway, known to activate genes for regeneration and proliferation [12, 13]. Shome et al. already demonstrated successfully that also short term plasma treatment of fibroblasts and keratinocytes led to an activation and translocation of YAP to the nucleus—leading to gene activation [14] (Fig. 5.5).

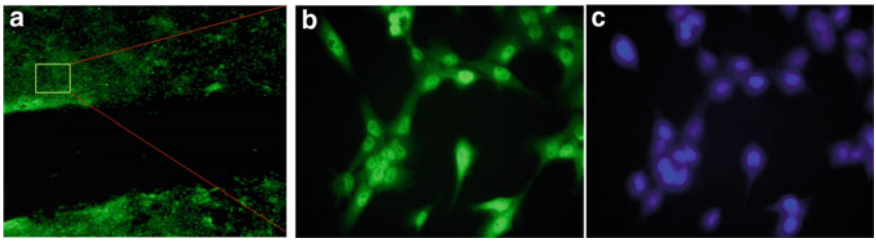

Fig. 5.5 Yap is translocated to nucleus of fibroblasts upon scratch wound and CAP treatment—indicated by green fluorescence in left figure (**a**) and in magnification in middle part (**b**). For comparison the DAPI counterstaining in the right figure (**c**) depicting nuclear staining in both cases

CAP treatment resulted in an upregulation of the HIPPO transcription factor YAP in both keratinocytes and fibroblasts. Downstream effectors of the HIPPO signalling pathway (CTGF and Cyr61) were upregulated mainly in fibroblast fraction. In addition, the administration of antioxidants such as N-acetyl-cysteine could inhibit CAP-mediated wound healing and abrogate the gene expression of the HIPPO downstream effectors. Furthermore, Shome et al. could also prove a paracrine signalling between fibroblasts and keratinocytes when co-cultured and plasma treated under this condition. In this case, a CAP treatment led to an activation of dermal fibroblasts—resulting in secretion of Cyr61 and CTGF—which in turn led to the paracrine stimulation of co-cultured keratinocytes (Fig. 5.6).

In addition, they could show that keratinocytes revealed an elevated cell migration when incubated with CAP-treated fibroblast-conditioned media compared to an incubation with untreated cell culture media—leading to a significantly improved cell migration of keratinocytes [14]. Taken these facts together, they could show an improved keratinocyte wound healing in co-culture—with CAP treated fibroblasts as central players. This also proves that experimental set-ups should be carefully

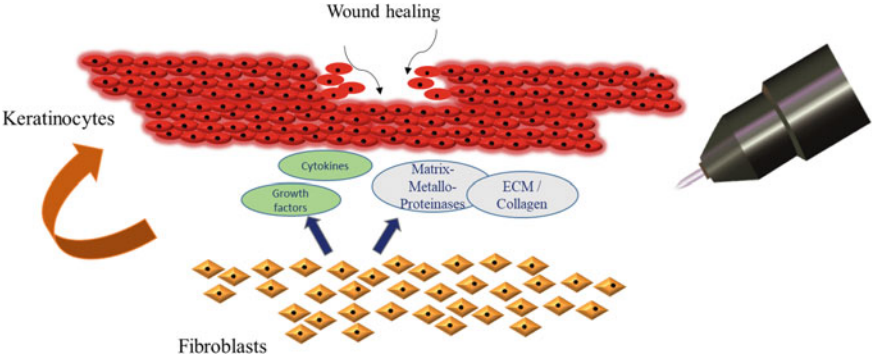

Fig. 5.6 Paracrine activation of keratinocytes by CAP modulated fibroblasts. Adapted from Shome et al. [14]. Oxidative Medicine and Cellular Longevity Plasma stimulated fibroblasts secret cytokines and growth factors, leading to increased keratinocyte migration and proliferation

planned and in best case should mimic real wound conditions. Co-cultured fibroblasts and keratinocytes were capable to tolerate a CAP treatment much better than the mono-cultured cells at same density. Cell communication after plasma treatment is more complex in co-cultured cells and therefore reflects real skin situation more close than mono-culture cell experiments.

5.4 Animal Studies Applying Cold Plasma

Another aspect in the CAP mediated effects on wound healing is reflected by animal experiments—mostly in mice. There are several groups showing an improved healing of acute wounds. In early studies Schmidt et al. examined the cold plasma's efficacy on dermal regeneration in a murine model of dermal full-thickness ear wound [15]. Within a period of two weeks, female mice received daily plasma treatment. Their results showed a significantly accelerated wound re-epithelialization at days 3–9 in comparison with untreated controls. But also cell communication, cell migration and cell attachment is influenced by cold plasma treatment. By combining in vitro analyses in primary dermal fibroblasts isolated from murine skin with in vivo studies in another murine wound model Schmidt et al. could demonstrate that plasma treatment changed phosphorylation of signalling molecules such as focal adhesion kinase and paxillin alpha in adhesion-associated complexes [16]. The same group also investigated the integrity of healthy skin of plasma treated mice by analysing tissue oxygenation, perfusion, hemoglobin, and water index by applying hyperspectral imaging. In this study Schmidt et al. could show a plasma based modification of the junctional network in skin, which promoted tissue oxygenation, and restricted penetration, implicating that plasma may provide a novel and sensitive tool of skin barrier regulation [17]. Furthermore, animal experiments also showed a direct activation of immune cells as another important fact in plasma mediated wound healing. Kupke et al. could prove a CAP-related induction of neutrophils in wound tissue from mice by investigating the functionality of human polymorphonuclear cells (PMN)/granulocytes through either a plasma-treated solution (PTS) or the direct CAP treatment [18]. They stated that the modification of PMN immunoreactivity by direct plasma treatment might be a main supporting mechanism for CAP-induced improvement in wound healing.

Besides the confirmation that CAP treatment led to significant improvements in wound healing—several studies also investigated the safety issues in long-term animal studies [19, 20]. Schmidt et al. studied in an one year follow-up risk assessment in SKH-1 mice the possible side effects of a CAP treatment applying an argon jet plasma. They applied quantitative PCR, to investigate expression levels of several cytokines and tumour markers in liver, lung, and skin. In addition, also histological and immune-histochemical analysis failed to detect abnormal morphological changes and the presence of tumour markers. Also magnetic resonance imaging and positron emission tomography confirmed the absence of neoplastic lesions in these mice [20]. Evert et al. investigated in their study the long-term risk assessment of

CAP treatment in the oral cavity. Histological analysis of 406 animals revealed that repeated CAP exposure did not foster non-invasive lesions or squamous cell carcinoma. In conclusion Evert et al. stated that a repeated CAP exposure of murine oral mucosa was well tolerated, and carcinogenic effects did not occur, motivating CAP applications in patients for dental and implant treatments in the future [19].

Similar results from cell culture experiments of various groups working with different plasma sources could confirm that none of the CE-certified plasma sources showed any mutagenic potential [20–23]. In summary, all certified plasma sources are safe in medical application, if the devices are handled according the manufactures advices. In the recent years, there are first efforts for a standardisation of plasma sources—with a German pre-standard DIN Spec91315. A first application of the DIN Spec91315 is published by Mann et al. and provides a basic approach how to test plasma sources for safety and efficacy [24]. Ongoing efforts recently started to transform this German pre-standard into a DIN norm, and parallel on an international mutual project the preparation of an IEC standard started in 2021.

Further information on safety aspects and standardisation can be found in chapter nine.

5.5 Clinical Application of Cold Plasma for Wound Healing

Based on very promising results from cell culture and microbiology first case reports were started in early 2012. Metelmann et al. investigated in a case report including five individuals with identical settings how a argon plasma can stimulate the healing of skin lesions of CO_2 laser and observed the recovery of these artificial acute wounds [25]. Those 20 laser lesions have been treated with argon plasma for 10, 30 s or three-times for 10 s, and compared to untreated control laser lesions. While in first approach, the scar formation was observed for 10 days, in a second follow-up study further evaluations of those lesions were evaluated after six and 12 months. As a result, Metelmann et al. stated, that plasma treatment shows superior aesthetics during scar formation. No precancerous skin features occurred up to 12 months [26].

Stratmann et al. started a clinical trial in order to determine whether the application of CAP accelerates wound healing in diabetic foot ulcers compared with standard care therapy [27]. This prospective, randomized, placebo-controlled, patient-blinded clinical trial was conducted at two clinics—and was the first study which could prove a positive influence of CAP on wound size reduction. Therefore, standard care treatment with eight applications of either CAP generated from argon gas of an atmospheric pressure plasma jet was compared to eight applications of placebo treatment in a patient-blinded manner. CAP therapy yielded a significant increase in wound healing, both in total mean (SD) area reduction and mean (SD) time to relevant wound area reduction [27]. Moreover, cutaneous blood flow and oxygen saturation can be improved in human skin—due to a clinical application of cold plasma [28]. As stated by Kisch et al., these effects are mostly explained by reactive oxygen species

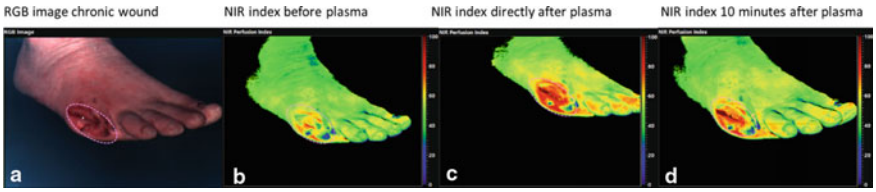

Fig. 5.7 Hyperspectral imaging of a plasma treated chronic wound **a** RGB coloured image—and **b** false colours indicated an increase in microcirculation indicated by shift to orange and red colour after plasma **c** which stayed elevated several minutes after CAP **d**

(ROS), but electric fields, currents and ultraviolet radiation may also have an impact on cells in the treated area [28].

Applying hyperspectral imaging we also could show that a treatment with the argon plasma jet improved tissue oxygenation and microcirculation in a chronic foot ulcer. Analysing the NIR perfusion index we could show a significant increased micro-circulation directly after plasma treatment (indicated by increase in orange and red colour—which stayed elevated after the procedure (Fig. 5.7).

In another clinical trial applying the argon jet plasma jet, wound exudate was investigated within a prospective, randomised, patient-blinded clinical trial. Hiller et al. recently published that those CAP-treated wounds showed increased levels of tumour necrosis factor-alpha, interleukins 1alpha and 8. They also found an induction of crucial growth factors, like FGF-2 and VEGF-A, and interleukins appears to be an important component of CAP-mediated promotion of granulation, vascularisation and reepithelialisation in the diabetic foot [29].

Taken exudate samples on a regular base allows a permanent control of wound progression. Besides the detection of inflammation markers such as cytokines, the analysis of matrix-metallo-proteinases (MMP) could be an hallmark for an evaluation of the stimulation by cold plasma. We could show, that CAP treatment led to a significant reduction of MMP-2, MMP-8 and MMP-9 levels over a duration of a 10 week plasma treatment (Fig. 5.8).

In summary, several case reports and clinical trails proved the positive effects of cold plasmas generated by different devises. Besides its antimicrobial efficacy cold plasma significantly induced wound healing—especially in diabetic patients often

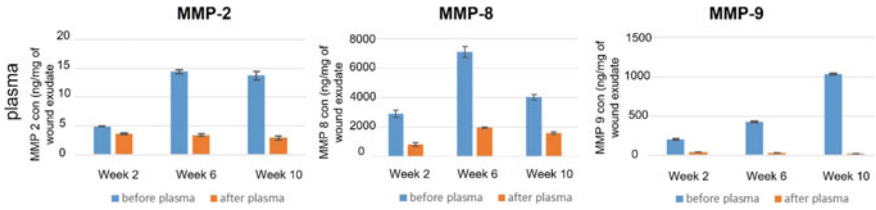

Fig. 5.8 Results from wound exudate analysis—CAP treatment reduces expression of matrix-metallo proteinases

suffering from chronic wounds for years. There is a clear tendency that CAP treatment is mediating growth factor induction and modulation of matrix-composition—resulting in a faster wound closure. A further surplus is the plasma modulation of tissue oxygenation and elevated levels of micro-circulation leading to an improved support with nutrients and oxygen. These data are in concurrence with results previously described in several in vitro and rodent experiments.

In chapter eight more detailed information are summarized about clinic trails and clinical applications.

5.6 Cellular Redox Balance Modulated by Cold Plasma

In order to heal, a wound is in need for energy—in form of nutrients (carbohydrates, amino acids etc.) and oxygen for an active metabolism to finally close the wound and regenerate the tissue. In chronic wounds, these processes are diminished—often caused by diseases like the metabolic syndrome—with all its side effects. Often reduced blood flow and dimished oxygenation are the reason for an insufficient supply with nourishment and oxygen, which have to be restored in first place. A lack of oxygen and nutrients not only hampers tissue regeneration, but also reduces the ability of immune cells to defend invading microorganisms finally leading to infected chronic wounds. Those missing natural ingredients—especially the reactive species (ROS/RNS) formed during immune defences but also tissue regeneration can be replaced by identical species generated in cold plasmas. Most reactive species generated in plasma are also known in biology—functioning in redox signalling and mammalian cells are equipped to interpret the plasma derived redox signal [30] (Fig. 5.9).

The hypothesis that a single plasma component could be traced to a specific cellular event or effect is somewhat difficult to disentangle in the case of cold plasma. Due to the fact, that a complex cocktail of reactive species and various kinds of radiation are formed during the plasma generation, and the fact that most of these species are very short lived—a lot of intermediates formed in the gas phase never will reach the cells or their liquid environment. To our knowledge, the long-lived redox-active species in combination with energy from various electromagnetic radiations dominate the biological effects, while moreover, plasma biological effects are significantly modulated by plasma modifications of the liquid environment. Furthermore, there is no such single receptor of plasma species inside (or outside) the cells—nothing like a drug that binds to a single receptor. As mentioned above, ROS and RNS are natural species employed in many cellular processes. There is a constant flux of reactive species formed by diverse occasions—such as respiratory chain or immune defence. Each cell type has—based on its function and metabolic state—its own redox balance. This means all cells produce their own reactive species—(roughly two percent of oxygen consumed for cellular respiration escape within mitochondria during the processes of respiratory chain). Hence, the need for counter reactions or repair mechanisms has to be active in all oxygen consuming cells in order to ensure a

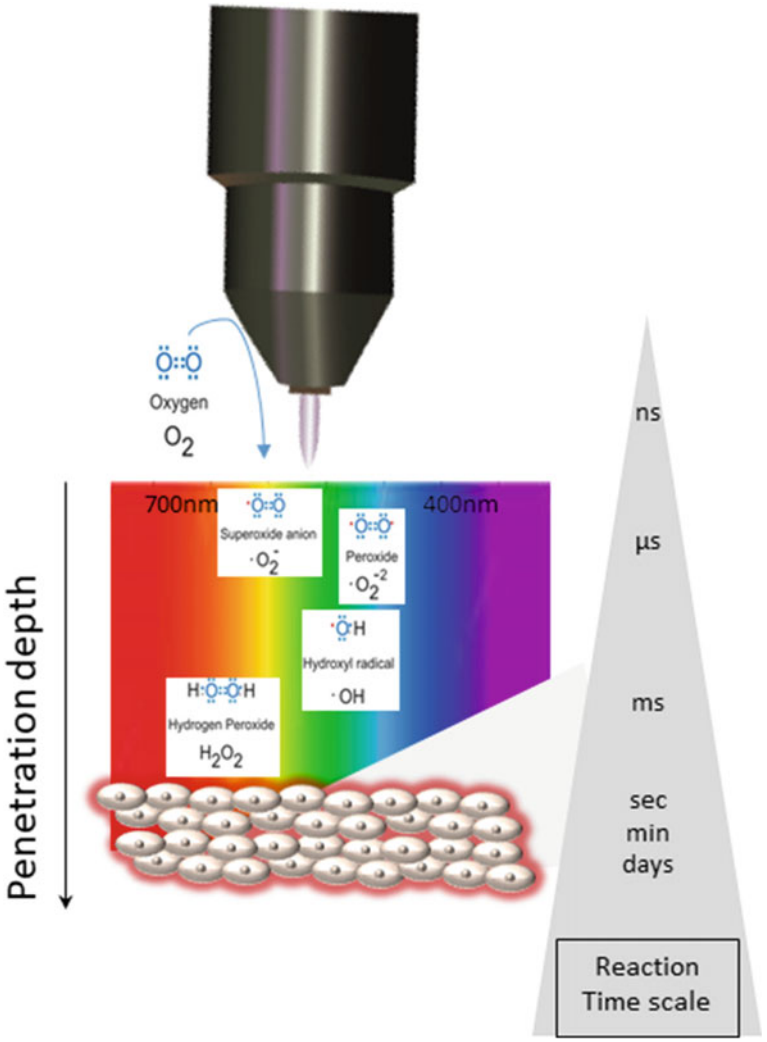

Fig. 5.9 The hub of plasma medicine. The addition of energy to gases leads to the formation of (partially) ionized gases—cold plasma—which are used to modulate the redox balance of living cells. Very short-lived radicals are formed in plasma—with half-lives ranging from nanoseconds to microseconds. Long-lived redox-active species (with half-lives up to several seconds) dominate the biological effects, while biological plasma effects are essentially modulated by plasma changes in the liquid environment, followed by secondary reactions within cells. The overall chain reaction—triggered by the plasma—can last for days and includes triggered effects such as cell proliferation and migration. Over time, the availability and penetration depth of the reactive species also change. The penetration depth of light—produced by the previously excited species—also depends on the wavelength, while UV light is absorbed by the air and fluids above the treated tissue—and only red and near IR light can penetrate multiple cell layers. Plasma reactions are very complex and cover a wide range in time. There are energy transfers occurring in the very first nanoseconds, triggering the generation of new reactive species. Some of them will react with an half-life of a few µs and milliseconds—finally leading to more stable species such as hydrogen peroxide, which is able to interact with living matter. The reaction triggered directly and indirectly at the cells are causing the next stage of plasma mediated reactions, such as the activation of transcription factors. Once transcription factors caused the activation of signalling cascades, and genes are transcribed, cell proliferation and cell migration are triggered within a time frame of hours. Finally the plasma directly and indirectly activates cellular activities which can last for up to a few days

proper cell cycle. However, there is a limit on the amount of reactive species, which can be handled by a single cell. For each cell type (based on metabolic state and function) there is a certain threshold—the **AOP anti-oxidative potential** of a cell, encircling all anti-oxidative possibilities and counter reactions (including repair).

For each oxidative influence (endogenous e.g. from respiratory chain/or exogenous like UV radiation, oxidants) a cellular counter reaction is induced by effectors such as NRF-2/TAZ—leading to a restoring of cellular redox balance. These modulations can be triggered by cold plasma—as an exogenous influence of the cellular redox balance like any other source from outside. Therefore, CAP can induce activation of the transcription factors NRF-2 and YAP and finally can lead to an activation of tissue repair and regeneration too. Those processes (as a sum of internal and external triggers) will lead to a cellular activation. By modulating the ROS/RNS based processes of the cells tissue regeneration but also tissue inflammation can be influenced by cold plasma. However, there is a certain limit for each cell type or tissue—which is defined by the anti-oxidative potential of the cells. Once this threshold is exceeded, the cellular redox balance is destroyed (Fig. 5.10d). This imbalance will lead to an accumulation of redox active species and compounds, which cannot be handled by the cellular anti-oxidants and repair mechanisms. A chronic elevation of ROS will lead to an excess of pro-oxidative effects, and finally will lead to an accumulation of cell damages, which in turn will start the cellular apoptosis program—the programmed suicide.

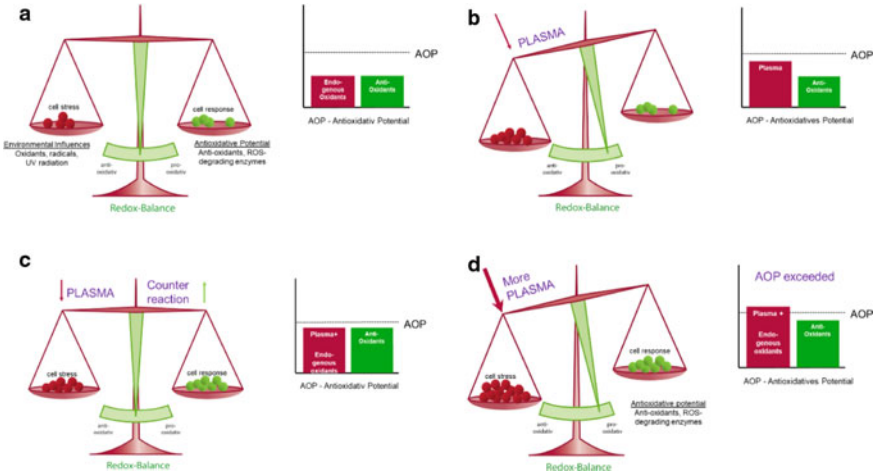

Fig. 5.10 The cellular redox balance: Each cell type has its own redox balance of exogenous and endogenous oxidative processes and its counter reactions of the cell. **a** Each increase on the oxidative side, **b** will lead to a redox balance restoration by cellular counter reaction. **c** This balance and counter-balance effects will ensure cell survival, as long the anti-oxidative potential is not exceeded. Once this threshold is exceeded, **d** cellular damage will occur—finally leading to the programmed cell death apoptosis

Therefore, each plasma source needs to be evaluated in detail, before an application on human beings is planned. Again, the German pre-standard DIN Spec 91,315 contains a selection of tests in physics, cell- and micro-biology in order to ensure a safe and efficient plasma treatment with a clinical focus. (more details in Chap. 9 on safety and standardisation.)

5.7 Summary

About two decades ago, new devices have been developed, capable to generate partially ionised gases—so-called cold plasmas. These energy-rich mixtures of reactive species (mainly ROS and RNS) in combination with mild heat and UV radiation are tissue-tolerable. These well prepared cocktails display several properties for a medical application with focus on wound healing: they show a high anti-microbial efficacy—with the surplus that they are effective on anti-biotic resistant strains as they are on normal skin microbes. Especially in chronic infected wounds with permanent bacterial load in combination with co-existing fungi an advantage to most antiseptics. However, besides anti-microbial effects, cold plasmas are capable to stimulate human tissue, by modulation the cellular redox balance. This well orchestrated signalling cascade of redox sensors (such as NRF-2/Keap-1) also other transcription factors will be activated upon CAP treatment. For example, the YAP/TAZ system which belongs to the HIPPO pathway is an important activator for tissue regeneration. Following an activation via NRF-2 or YAP cells start to synthesize cytokines and growth factors, enabling the plasma treated cells and surrounding tissues to elevate proliferation and cell migration activities. Both, animal and human trials proved that a third hallmark of cold plasma treatment is an increased tissue oxygenation and micro-circulation. This further supports a proper wound healing due to an improved support with nutrients and oxygen. All three facts: anti-microbial efficacy, modulation of cellular redox balance and thereby stimulation of cell migration and proliferation and third an elevated tissue oxygenation led to the superiority of cold plasma in healing infected chronic wounds. This could be proven in several case studies and clinical trials (Fig. 5.11).

CAP components are supporting cellular processes, which were reduced due to underlying diseases and limited supply of energy. Generating ROS/RNS as physiological components of immune defence and signalling cascades, plasma supports the diminished activities of a weakened immune system or tissue regeneration. There are hints that a plasma treatment of chronic wounds turns them back into acute wounds—where normal wound healing can start on its own. However, this plasma mediated support of wound healing can only be accomplished when a proper wound management is performed, and all other side effects of the underlying disease are corrected. Once the debridement is done, and blocked blood vessels are re-opened, an additional plasma treatment of such chronic wounds will be a great support for

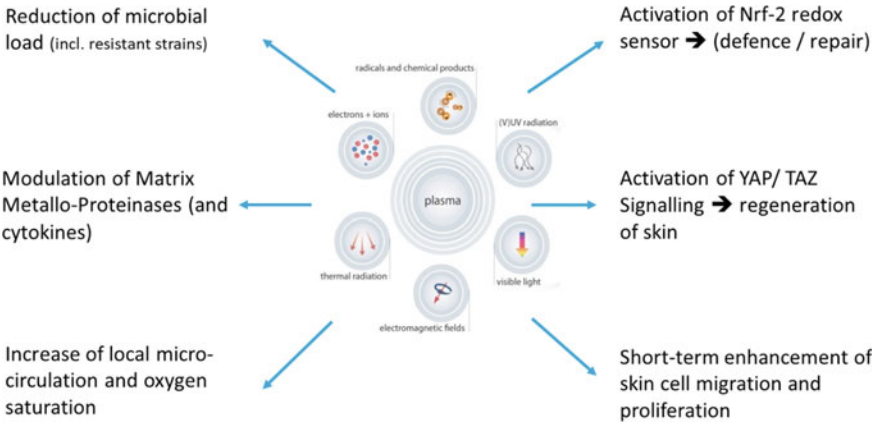

Fig. 5.11 Summary of the three hallmarks of cold plasma for wound healing: (1) anti-microbial efficacy; (2) modulation of cellular redox balance with subsequent stimulation of cell growth and migration and (3) increased tissue oxygenation and micro-circulation

most patients. Further studies and clinical trials will help to understand the underlying mechanisms in more detail, so that a more personalized CAP treatment will be even more effective.

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