Applied Plasma Medicine

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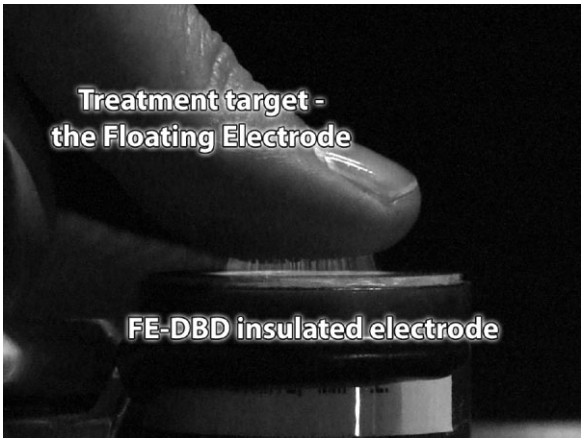
An emerging field of plasma medicine is discussed, where non-equilibrium plasmas are shown to be able to initiate, promote, control, and catalyze various complex behaviors and responses in biological systems. More importantly, it will be shown that plasma can be tuned to achieve the desired medical effect, especially in medical sterilization and treatment of different kind of skin diseases. Wound healing and tissue regeneration can be achieved following various types of plasma treatment in a multitude of wound pathologies. Non-equilibrium plasmas will be shown to be non-destructive to tissue, safe, and effective in inactivation of various parasites and foreign organisms.



Introduction

In physical sciences, ''plasma'' refers to the forth state of matter; while in medicine and biology plasma is known as the non-cellular fluid component of blood. Interestingly, the term plasma has been coined by Irving Langmuir to emphasize that the characteristics of ionic liquids ubiquitous in biology and medicine are analogous to plasma in the physical sciences.[1] Despite this historical connection, few applications of plasma in medicine have been explored until recently.[2] This situation is rapidly changing, and the main purpose of this review is to provide an update on the recent research related to applications of plasma in medicine and to possible mechanisms of interaction between plasma and living matter.

Plasma can exist in a variety of forms and can be created in different ways. In many technological applications, for example, plasma exists at low gas pressures. Lightening, on the other hand, is an example of atmospheric pressure thermal plasma. For the purpose of this article, it is important to distinguish between thermal and non-thermal plasma. In all plasmas supported by electric field, electrons receive the external energy much faster than the much heavier ions and have the opportunity to heat up to several thousands of degrees before their environment heats up. In *non-thermal* plasma, cooling of ions and uncharged molecules is more effective than energy transfer from electrons and the gas remains at low temperature. For this reason non-thermal plasma is also called non-equilibrium plasma. In a *thermal* plasma, on the other hand, energy flux from electrons to heavy particles equilibrates the energy flux from heavy particles to the environment only when temperature of heavy particles

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becomes almost equal to the electron temperature. Of course the terms thermal and non-thermal, equilibrium and non-equilibrium are not very precise. Sometimes even a few tens of degrees difference in the temperature of the heavier species can play a substantial role. This is particularly important when various plasma-chemical processes are considered. It is certainly important when plasma is used to treat heat-sensitive objects.

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Some of the earlier applications of plasma in medicine relied mainly on the thermal effects of plasma. Heat and high temperature have been exploited in medicine for a long time for the purpose of tissue removal, sterilization, and cauterization (cessation of bleeding).[3] Warriors have cauterized wounds by bringing them in contact with red hot metal objects since ancient times. Electrocautery is a more modern technique which applies controlled heat to surface layers of tissue by passing sufficiently high current through it.[4] However, contact of tissue with metal surface of a cautery device often results in adhesion of charred tissue to the metal. Subsequent removal of the metal can peel the charred tissue away re-starting bleeding. Some of the earlier applications of plasma in medicine provided an alternative to metal contact electrocautery. In argon plasma coagulation (APC, also sometimes called argon beam coagulation), highly conductive plasma replaced the metal contacts in order to pass current through tissue avoiding the difficulties with tissue adhesion. Hot plasma is also being employed to cut tissue,[3,5–8] although the exact mechanism by which this cutting occurs remains unclear. Heat delivered by plasma has also been employed recently for cosmetic re-structuring of tissue.[9–11]

What differentiates more recent research on applications of plasma in medicine is the exploitation of non-thermal effects. Why are non-thermal effects of plasma so interesting and promising? The main reason is that non-thermal plasma effects can be tuned for various sub-lethal purposes such as genetic transfection,[12–14] cell detachment,[15–18] wound healing,[19–23] and others (i.e.,[2,24,25]). Moreover, non-thermal effects can be selective in achieving a desired result for some living matter, while having little effect on the surrounding tissue. This is the case, for example, with recent plasma blood coagulation and bacteria deactivation which does not cause toxicity in the surrounding living tissue.[19,20] This review will concentrate mainly on these novel non-thermal effects and on possible non-thermal mechanisms of interaction between plasma and living organisms.

Most of the research focusing on the use of non-thermal plasma effects in medicine can be fit into two major categories: that are *direct* plasma treatment and *indirect* plasma treatment.[26] In direct plasma treatment, living tissue or organs play the role of one of the plasma electrodes. In many cases, voltage does not need to be directly connected to this living tissue electrode, but some current may flow through living tissue in the form of either a small conduction current, displacement current, or both. Conduction current should be limited in order to avoid any thermal effects or electrical stimulation of the muscles. Direct plasma treatment may permit a flux of various active uncharged species of atoms and molecules as well as ultraviolet (UV) radiation to the surface of the living tissue. These active uncharged species generated in plasma will typically include ozone (O_3), NO, OH radicals, etc. However, the most important distinguishing feature of the direct plasma treatment is that a significant flux of charges reaches the surface of the living tissue. These charges may consist of both electrons as well as positive and negative ions.

In contrast, indirect plasma treatment employs mostly uncharged atoms and molecules that are generated in plasma, but involves small, if any, flux of charges to the surface. In indirect treatment, the active uncharged species are typically delivered to the surface via flow of gas through a plasma region.

Both indirect and direct non-thermal plasma treatments permit some degree of tuning of the plasma properties.[26] For example, the amount of NO versus ozone produced in plasma can be tuned. It is also possible to tune microstructure of the plasma discharge which can be particularly relevant in direct treatment. The fact that direct plasma treatment involves substantial charge flux provides greater flexibility in tuning the non-thermal plasma effects. Indirect plasma treatment, on the other hand, may have an advantage when the plasma device needs to be at a substantial distance from the surface.

Animal and Human Living Tissue Sterilization

Direct Plasma Medicine, Floating-Electrode Dielectric Barrier Discharge (FE-DBD)

The *direct* plasma treatment implies that living tissue itself is used as one of the electrodes and directly participates in the active plasma discharge processes. For example, Figure 1 illustrates direct plasma treatment (for sterilization) of skin of a live mouse. Dielectric barrier discharge (DBD) plasma is generated in this case between the quartz-surface covered high-voltage electrode and the mouse which serves as a second electrode.

Direct application of the high-voltage (10–40 kV) non-thermal plasma discharges in atmospheric air to treat live animals and people requires a high level of safety precautions. Safety and guaranteed non-damaging regimes are the crucial issues in the plasma medicine. Discharge current should be obviously limited below the values permitted for the treatment of living tissue. Moreover, discharge itself should be homogeneous enough to avoid local damage and discomfort. Creation of special atmospheric discharges effectively solving these problems is an important challenge for plasma medicine.

Fridman et al. especially developed for this purpose the floating-electrode DBD (FE-DBD), which operates under the

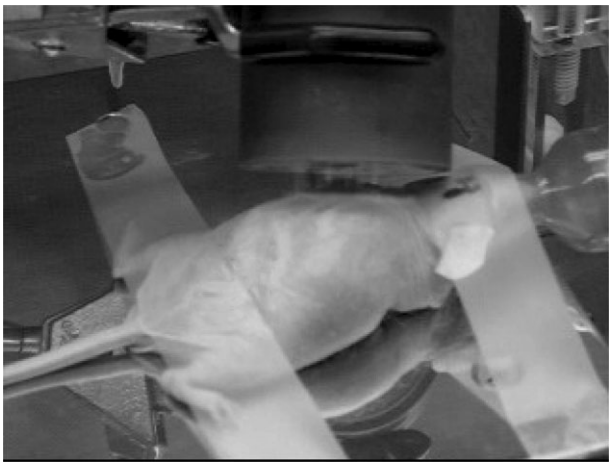

Figure 1. Non-damaging room temperature and atmospheric pressure FE-DBD plasma for the treatment of living tissue: animal treated for up to 10 min remains healthy and no tissue damage is observed visually or microscopically.[20]

conditions where one of the electrodes is a dielectric-protected powered electrode and the second active electrode is a human or animal skin or organ–without human or animal skin or tissue surface present discharge does not ignite.[19,20,26,27] In the FE-DBD setup, the second electrode (a human, for example) is not grounded and remains at a floating potential. Discharge ignites when the powered electrode approaches the surface to be treated at a distance (discharge gap) less than about 3 mm, depending on the form, duration, and polarity of the driving voltage.

Simple schematic of the FE-DBD power supply (PS) and voltage/current oscillograms are illustrated in Figure 2.[19] Typical value of plasma power in initial experiments was kept about 3–5 W, surface power density 0.5–1 $W \cdot cm^{-2}$. Further development of the FE-DBD discharge is related to optimization of shape of the applied voltage to minimize the DBD non-uniformities and related possible damaging effects. The best results so far have been achieved by organization of the FE-DBD in the pulsed mode with pulse duration below 30–100 ns,[28–30] which results in the no-streamer discharge regime, sufficient uniformity, and possibility of the non-damaging direct plasma treatment even when the second electrode is a living tissue and therefore wet, dirty, and essentially non-uniform.

As soon as the atmospheric discharge is safe, it can be effectively applied directly to human body as it is illustrated in Figure 3. Thus, the highly intensive and effective non-thermal plasma devices can be directly applied to living animal or human tissue for different types of medical and cosmetic treatment. As a first example, let us consider medical sterilization of living tissue.

Direct Plasma-Medical Sterilization of Living Tissue using FE-DBD Plasma

Sterilization of living animal or human tissue with minimal or no damage to this tissue is of importance in a hospital setting. Chemical sterilization does not always offer a solution. For example, transporting chemicals for sterilization becomes a major logistics problem in a military setting, while use of chemicals for sterilization of open wounds, ulcers, or burns is not possible due to the extent of damage they cause to punctured tissues and organs. Non-thermal atmospheric pressure plasma is non-damaging to the animal and human skin but quite a potent disinfecting and sterilizing agent,[20] which is to be discussed below.

Human tissue sterilization has been investigated by Fridman et al.[19,20] Bacteria in this case were a mix of "skin flora" – a mix of bacteria collected from cadaver skin containing Staphylococcus, Streptococcus, and Yeast. Direct FE-DBD plasma sterilization leads roughly to a 6-log reduction in bacterial load in 5 s of the treatment (Table 1). Similar level of the skin flora sterilization using *indirect* DBD approach requires 120 s and longer of plasma treatment at the same level of the discharge power.[26]

Sterilization of the skin flora on cadaver skin samples occurred in the experiments generally after 4 s of the treatment in most cases and 5–6 s in a few cases, depending on the initial bacterial contamination which varies greatly for different patients and different skin locations. Thus non-thermal atmospheric plasma, especially when it is applied directly, is an effective tool for sterilization of living tissue. It opens interesting possibilities for the non-thermal plasma applications in medicine including, in particular, pre-surgical patient treatment, sterilization of catheters (with points of contact with human body), sterilization of wounds and burns, as well as treatment of internal organs in gastroenterology.

Non-Damaging (Toxicity) Analysis of Direct Plasma Treatment of Living Tissue

Plasma has proven itself as an excellent sterilization tool for different surfaces.[2,20,24,31] One of the key questions in the direct plasma skin sterilization in medicine is if the skin remains intact after the sterilization. Moreover, the problem of non-damaging treatment (in other words: problem of toxicity[a]) is the key issue of all plasma medicine. Obviously, a topical treatment which damages

[a] In medicine, the term "toxic" in reference to a drug or a device usually implies some level of physical or chemical damage. We prefer to use the term "non-damaging treatment" to imply that the treatment and device used cause no immediate visible or microscopic physical damage to the tissue.

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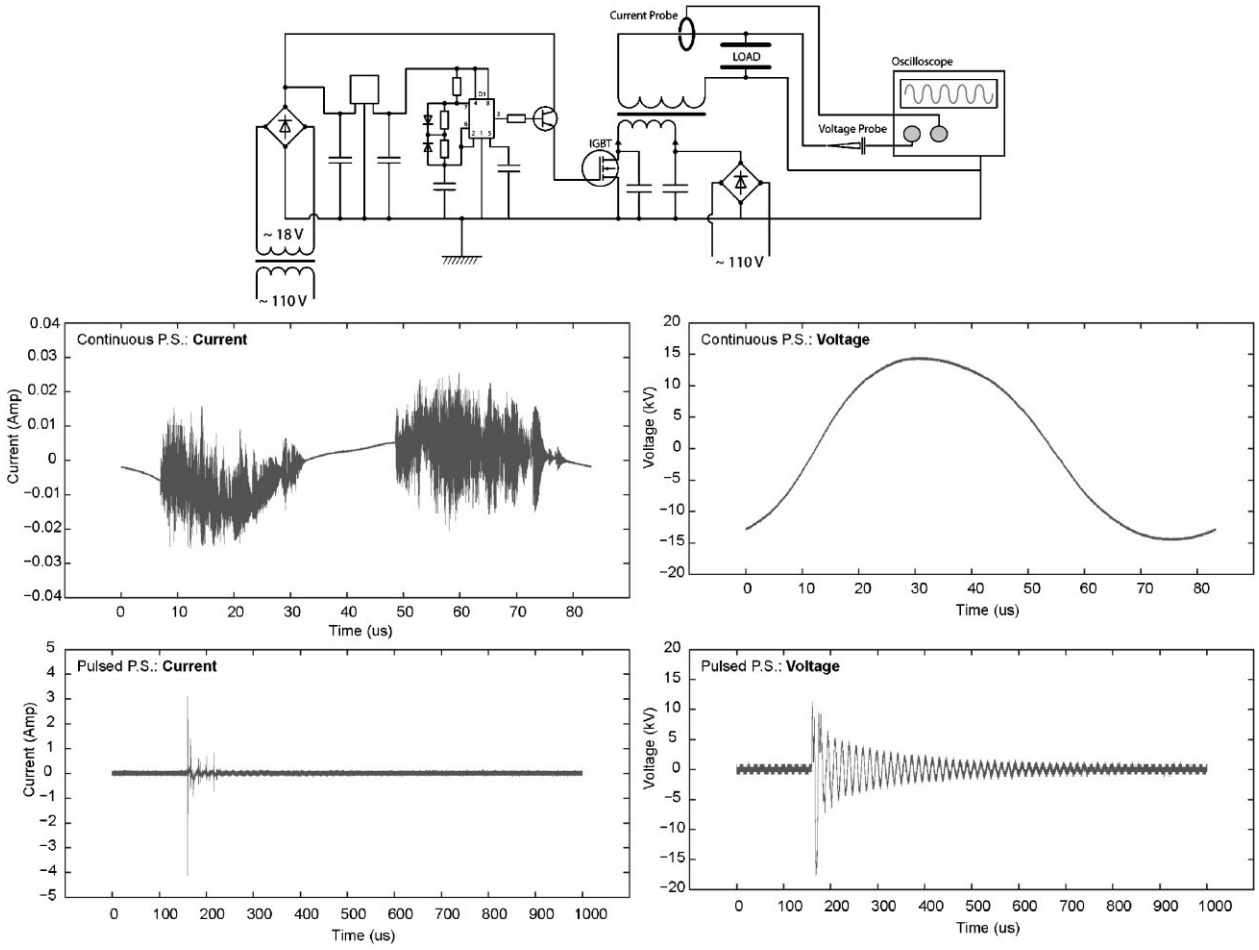

Figure 2. Schematic of FE DBD discharge plasma PS. Voltage and current oscillograms.[19]

the tissue surface would not be acceptable to medical community and thus first cadaver tissue was tested and then escalating skin toxicity trials were carried out on SKH1 hairless mice and pigs in the FE-DBD experiments of Fridman et al.[20] Cadaver tissue in these experiments was treated by FE-DBD plasma for up to 5 min without any visible or microscopic change in the tissue, as was verified with tissue sectioning and staining via Hematoxylin and Eosin (H&E) procedure, which is illustrated in Figure 4.

Based on the knowledge that FE-DBD plasma has non-damaging regimes, an animal model to assess this was constructed and accomplished in ref.[20] In an SKH1 mouse model, the skin treatment was carried out at varying

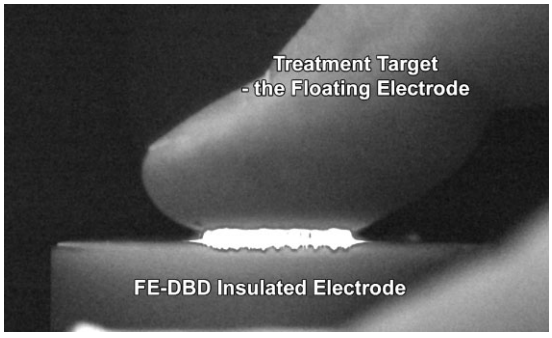

Figure 3. FE-DBD applied directly to the human body.[20]

Table 1. Bacteria sterilization results (in cfu · mL^{-1}).[26]

Original concentration	5 s of FE-DBD	10 s of FE-DBD	15 s of FE-DBD
10^9	850 ± 183	9 ± 3	4 ± 4
10^8	22 ± 5	5 ± 5	0 ± 0
10^7	6 ± 6	0 ± 0	0 ± 0

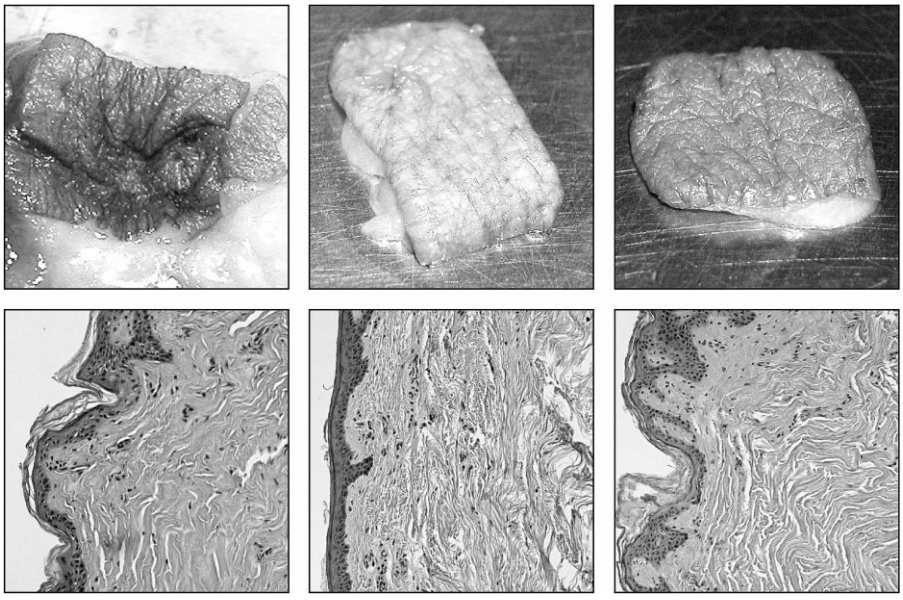

Figure 4. Photos (top) and tissue histology (bottom) of cadaver skin samples after FE-DBD treatment: control (left), 15 s of the treatment (center), and 5 min of the treatment (right) – no visible damage is detected.[19]

doses to locate damaging power/time (dose) combination and skin damage was analyzed in two stages. First, the animal was treated at what was deemed to be a toxic (damaging) dose based on trials with cadaver skin tissue (doses of >1 W $\cdot$ cm^{-2} and >10 min). Once the dose where the damage was visible was located, a new animal was treated at a lower dose. If no damage was observed at that dose, two more animals were treated and if no damage was observed in all the three the dose was deemed "maximum acceptable dose". Once the maximum dose was located, three animals were treated at that dose and left alive under close observation for 2 weeks.

Based on the experimental matrix, a dose of 10 min at 0.6 W $\cdot$ cm^{-2} was deemed maximum acceptable prolonged treatment and a dose of 40 s at 2.3 W $\cdot$ cm^{-2} was deemed maximum acceptable high-power treatment. Histological (microscopic) comparison of control SKH1 skin sample with toxic and non-toxic plasma doses show regions where plasma dose is fairly high while the animal remains unaffected (Figure 5, animal after the treatment, Figure 6, histological samples). Of note is that sterilization was achieved at 3 ± 1 s at high-power treatment of 0.8 ± 0.2 W $\cdot$ cm^{-2} and at 10 ± 4 s at half that power. Variation in time necessary for sterilization is attributed to the initial contamination level of the animal (same as for cadaver tissue); in other words, some skin samples are simply cleaner than others.

Ability of FE-DBD plasma to treat living animal skin without damage to this skin was also confirmed in a second differential skin toxicity trial following the same protocol used for SKH1 mice (see above) but this time with regular swine.[32] Experiments showed that non-damaging regimes exist and the animal skin exhibits no visible or microscopic damage (Figure 7). Detailed analysis of any biochemical changes and inflammatory response pathway alteration or initiation is currently underway.[32]

It should be especially noted that the level of toxicity due to the FE-DBD plasma treatment of living tissue not only depends on the treatment dose (discharge power, and

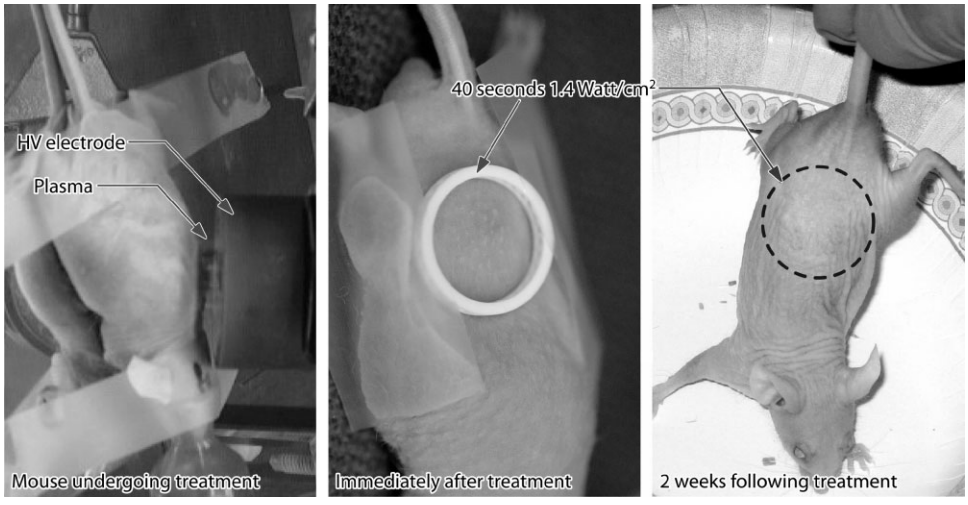

Figure 5. Animal remains fine after a reasonably high plasma dose (more than ten times higher than needed for skin sterilization).[20]

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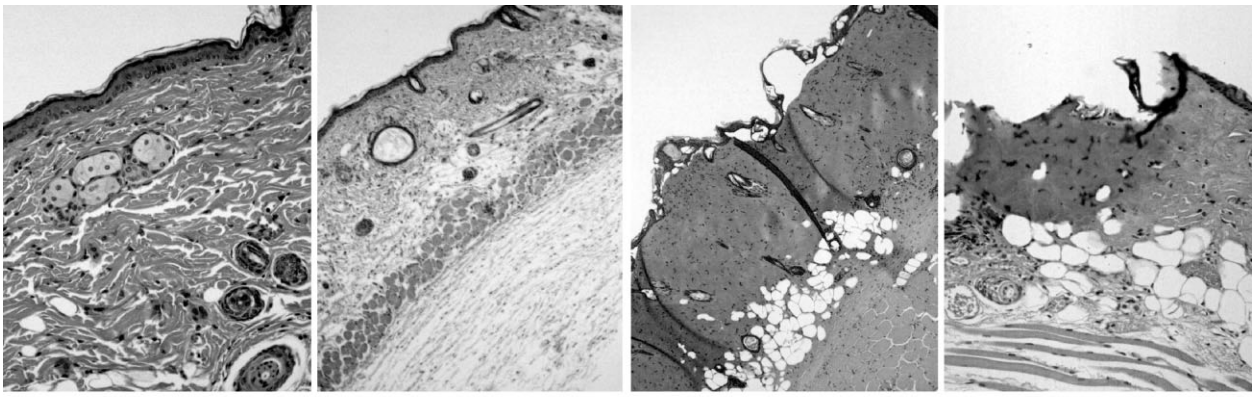

Figure 6. Histology of toxic and non-toxic to animal's skin plasma doses, compared to untreated skin.[20]

treatment duration), but also strongly depends on the shape of voltage applied to the discharge. Pulsing of the DBD discharges can essentially decrease its damaging ability. Application of nanosecond pulses completely prevents the formation of streamers and therefore the DBD microdischarges, which helps significantly, decrease toxicity of the direct plasma-medical treatment of living tissue.[29,30]

Sterilization of Non-Living Objects for Medical Applications

Traditionally, sterilization or treatment of non-living objects like metals, plastics, fabrics, and other surfaces have been carried out either by temperature (i.e., autoclaves[33–36]), liquid or gaseous chemistry (i.e., by ethylene oxide,[37,38] ozone,[39,40] chlorine,[41,42] etc.), or at reduced pressure by non-equilibrium plasmas.[43,44] Details of such approaches are widely available in the literature. In this paper, the focus is on medical application of non-equilibrium plasma at atmospheric pressure and surface sterilization of materials cannot be overlooked because, after all, these materials later come in contact with living tissue either as implants, dressings, tools, etc.

Alexeff and Laroussi and their coworkers reported a rather interesting modification of a conventional DBD – a resistive barrier discharge (RDB).[2,45,46] Main feature of the RDB is that it can function in both DC and AC modes, and rather than a dielectric a wetted high resistivity material is used. RDB has been shown to be effective in sterilization of *Escherichia coli*, *Bacillus subtilis*, and other organisms[2,31,46] without significant damage to the surface being processed. Going back to a more traditional DBD system, Laroussi and coworkers[47–50] show that a barrier discharge in helium with small additions of oxygen is not only able to sterilize bacteria but also able to influence metabolic changes in the organisms surviving the treatment.[48] This raises an intriguing question – can plasma-resistant bacteria emerge? Due to the synergetic effect of plasma constituents on bacteria, plasma-resistance might not be possible or statistically probable, however, the authors think that this issue might become rather important in the near future and should be addressed. Two more discharges are studied by Laroussi and coworkers: plasma plume (a helium jet),[51,52] and an arc-like discharge between metal and water in air.[53,54] Both discharges are also reported to efficiently inactivate various microorganisms.

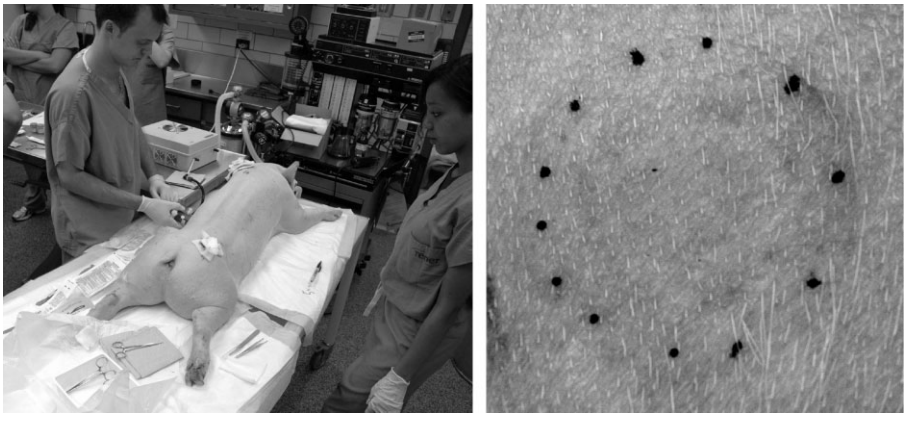

Figure 7. Live pig undergoing treatment (left) and appearance of pig skin immediately following 5 min of FE-DBD treatment (right). Animal survives the treatment and no visible or microscopic tissue damage is observed.[32]

Massines and coworkers propose a DBD discharge in N_2/N_2O mixture for microorganism

inactivation (i.e., *B. subtilis* spores).[55–57] Operated at atmospheric pressure, her results indicate a very high dependence of the inactivation efficiency on UV, which is somewhat contrary to results presented by other groups.[55] In fact, the difference is attributed to the fact that the gas composition necessary to achieve the best results is in a very narrow concentration range of the oxidant molecule, which might have simply been overlooked previously. Though this study offers good information on UV, a real-life environment might need a system that is slightly less picky as to the gas mixture concentration ranges. However, one needs to account for effects of UV radiation on bacteria as apparently they cannot be neglected, even in plasmas where doses of UV are lower than in that proposed by Massines and coworkers.[55]

Microplasmas have recently been gaining momentum in bio-medical applications. These systems of 10–500 μm characteristic dimensions capable of generating diffuse atmospheric pressure plasmas offer an interesting solution in, for example, medical diagnostics and environmental sensing. Becker et al.[58,59] offer a few different microplasma sources suitable for remediation of gaseous waste streams, removal of volatile organic compounds (VOCs), detection of trace contaminants in gas flow, generation of high intensity UV radiation, and sources suitable for microsized plasma-reactors. Though the temperature of these discharges can be at or near room temperature in noble gases, when a molecular gas (i.e., air) is used plasma temperatures can be high, on the order of 2 000 K. Becker et al. show efficient inactivation of *B. subtilis* spores (1-log reduction in ≈100 s) and *B. stearothermophilus* spores (1-log reduction in ≈90 s) without damage to the substrate; more interestingly they are able to inactivate biofilms of *Chromobacterium violaceum* CV026 achieving 2-log reduction in ≈5 min and 3-log reduction in ≈60 min of plasma afterglow treatment.[58] In general, these microplasmas have not yet found a niche in medicine directly though many potential applications are clearly possible and the reader is encouraged to take a look at a review of the recent developments in that field.[59]

Roth and coworkers have developed a one atmosphere uniform glow discharge plasma (OAUGDP) system capable of addressing a broad range of potential applications.[60–66] OAUGDP is a DBD-like bipolar RF plasma discharge operated in air or other gases. The list of potential applications where experimental evidence is very favorable includes increase in surface energy and wettability of fabrics, films, and solid surfaces; sterilization of various surfaces for healthcare and food processing; decontamination of surfaces compromised by chemical or biological warfare agents; a sterilizable air filter to deal with the sick building syndrome; removal of soot and VOCs from diesel engine exhaust; mercury-free atmospheric pressure fluorescent lamps; stripping of photoresist and directional etching in microelectronics; plasma-assisted chemical vapor deposition; and plasma aerodynamic flow control. For details on these applications reader is encouraged to consult a recent publication by Roth et al.[64] Of note, however, is a less recent publication from Roth's group comparing sterilization efficiency of their system against a multitude of bacteria, yeasts, and viruses.[61] *D*-values, or time to 90% reduction in microorganism load, are ranging from 6 s for *E. coli* bacteria to 6.8 min for bacteriophage Phi X 174 virus. Additionally survivability of these organisms on different substrates is addressed comparing glass, agar, and poly(propylene) with the later showing highest survival times. In general, OAUGDP was not reported to be used in medicine directly; however, sterilization of medical instruments and other surfaces found in the hospital as well as air sterilization in an operating room is on the list of potential medical applications.[64]

Kong and coworkers have investigated inactivation of various organisms by pulsed electric field,[67] and, primarily, by He/O_2 RF plasma afterglow (or jet).[68–77] Ability of their plasma setup to inactivate *B. subtilis* spores[68,72] and various *E. coli* mutants[73] does not come as a surprise, however the results on inactivation of biofilm-forming bacteria are quite intriguing. Vleugels et al.[75] have successfully achieved inactivation of biofilm-forming *Pantoea agglomerans* in sterilization of foods, specifically of bell peppers (*Capsicum annuum*). He/O_2 plasma afterglow was shown to effectively inactivate the biofilm without causing unacceptable levels of discoloration to the peppers.[75] Detailed analysis of this system reveals that the primary role in inactivation is played by reactive oxygen species (e.g., atomic oxygen and OH) with minor aid from UV photons, charged particles, heat, and electric fields.[68,71,73,74,76,77] Another interesting idea is not only sterilization of various surfaces but also complete decontamination of them with removal not only of bacterial load but also of the remaining protein debris. Deng et al. show that this RF plasma jet treatment can effectively remove proteins from surface of medical instruments, achieving up to 4.5-log reduction;[69,70] here, again, reactive oxygen species are deemed to be the major inactivation factors.

Non-Thermal Plasma-Assisted Blood Coagulation

General Features of the Plasma-Assisted Blood Coagulation

Blood coagulation is an important issue of medicine, in particular regarding wound treatment. Quasi-thermal plasma has been traditionally used for this application in the form of the so-called cauterization devices: APC,

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argon beam coagulators, etc.[3,5,8] In these devices, widely used in particular in surgery, plasma is just a source of local high temperature heating, which cauterizes and desiccates (actually cooks) the blood. Recent development of the effective non-thermal plasma-medical systems permits to achieve effective blood coagulation without any thermal effects. In such systems, which are to be discussed below, the cauterization effect is achieved through non-thermal plasma stimulation of specific natural mechanisms of blood coagulation without any "cooking" and damaging of the surrounding tissue.[19]

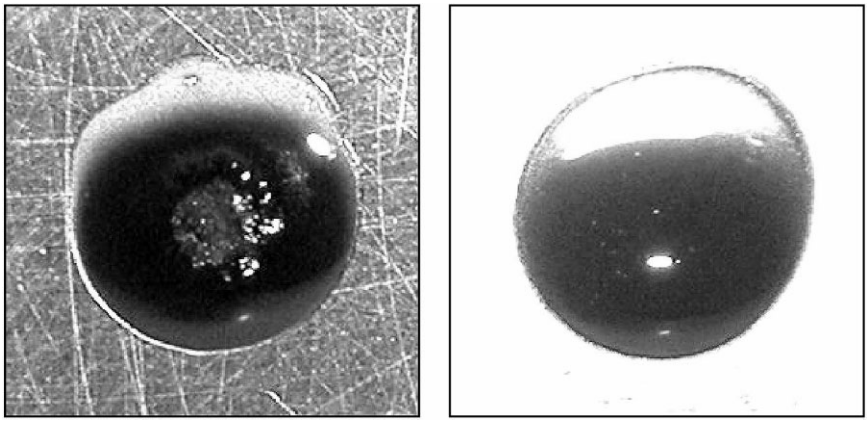

Figure 8. Blood drop treated by FE-DBD: 15 s of FE-DBD (left) and control (right); photo was taken 1 min after the drops were placed on brushed stainless steel substrate; blood was treated immediately after it was placed on metal.[19]

It should be mentioned that both coagulating the blood and preventing the coagulation could be needed, depending on the specific application. For example, in wound treatment one would want to close the wound and sterilize the surface around. Flowing blood, in that case, would prevent wound closure and create a possibility of re-introduction of bacteria into the wound. Where blood coagulation would be detrimental is, for example, in sterilization of stored blood in blood banks. There, a potential exists for blood to contain or to have somehow acquired bacterial, fungal, or viral infection which needs to be removed for this blood to be usable.[78,79] Here, of course, the treatment cannot coagulate the blood. Thus, clearly, an understanding of the mechanisms of blood coagulation by non-thermal plasma is needed. We are going to consider in this section the blood coagulation process stimulated by FE-DBD plasma.[19,20,80,81] Relevant *in vitro* and *in vivo* experiments will be followed up with discussion of the non-thermal plasma-stimulated blood coagulation mechanism.

Experiments with Non-Thermal Atmospheric Pressure Plasma-Assisted *in vitro* Blood Coagulation

FE-DBD plasma was experimentally confirmed to significantly hasten blood coagulation *in vitro*.[19,80,81] Visually, a drop of blood drawn from a healthy donor and left on a stainless steel surface coagulates on its own in about 15 min, while a similar drop treated for 15 s by FE-DBD plasma coagulates in under 1 min (Figure 8). FE-DBD treatment of cuts on organs leads to similar results where blood is coagulated without any visible or microscopic tissue damage. Figure 9 shows a human spleen treated by FE-DBD for 30 s – blood is coagulated and tissue surrounding the treatment area looks "cooked", however the temperature of the cut remains at room temperature (even after 5 min of FE-DBD treatment) and the wound remains wet, which could potentially decrease the healing time as is the case with topical wound sealing agents.[82,83]

Additionally, a significant change in blood plasma protein concentrations is observed after treatment by plasma of blood plasma samples from healthy patients, patients with Hemophilia, and blood samples with various anti-coagulants. Anti-coagulants, like sodium heparin or sodium citrate, are designed to bind various ions or molecules in the coagulation cascade thus controlling coagulation rate or preventing it all together. Analysis of changes in concentration of various blood proteins and clotting factors indicates that FE-DBD plasma aids in

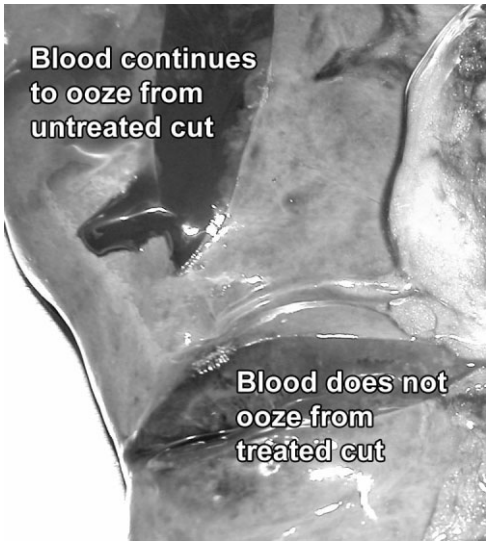

Figure 9. 30 s of FE-DBD treatment of human spleen: blood coagulates without tissue damage. Top cut: blood continues to ooze from an untreated area; bottom cut: blood coagulates while the wound remains wet.[19]

promoting the advancement of blood coagulation, or in other words, plasma is able to catalyze the complex biochemical processes taking place during blood coagulation.[19,20,80,81,84–91]

In vivo Blood Coagulation Using FE-DBD Plasma

Effective plasma stimulation of the *in vivo* blood coagulation has been demonstrated by Fridman et al. in experiments with live SKH1 mice.[20] 15 s of FE-DBD plasma treatment is able to coagulate blood at the surface of a cut Saphenous vein (Figure 10) as well as tail vein of a mouse. In these experiments only ability of direct non-thermal plasma treatment to coagulate blood was tested and the animal was not left alive to test improvement in healing times. Full investigation of ability of plasma to hasten wound healing through wound sterilization and blood coagulation is discussed by Fridman et al. and Balasubramanian et al.[20,32,86,87]

Mechanisms of Non-Thermal Plasma-Assisted Blood Coagulation

Detailed bio-chemical pathways of the non-thermal plasma-stimulated blood coagulation remain largely unclear. Several possible mechanisms, however, were investigated.[19,80,81] Firstly and most importantly, it was demonstrated that direct non-thermal plasma can trigger natural, rather than thermally induced, coagulation processes.[19] Secondly, it was observed that the release of calcium ions and change of blood pH level, which could be responsible for coagulation, is insignificant.[19,81] Instead, the evidence points to selective action of direct non-thermal plasma on blood proteins involved in natural coagulation processes.

Mechanisms of plasma interaction with blood can be deduced from the following facts observed in the experiments with FE-DBD plasma: (i) plasma can coagulate both normal and anti-coagulated blood, but the rate of coagulation depends on the anti-coagulant used; (ii) plasma is able to alter ionic strength of the solution and change its pH, but normal and anti-coagulated blood buffers these changes even after long treatment time; (iii) plasma changes natural concentration of clotting factors significantly, thus promoting coagulation; (iv) effects delivered by plasma are non-thermal and are not related to gas temperature or the temperature at the surface of blood; (v) plasma is able to promote platelet activation and formation of fibrin filaments, even in anti-coagulated blood. These experimental facts are discussed in further detail below.

(i) Anti-coagulants like sodium heparin bind thrombin, in the coagulation cascade thus slowing coagulation while sodium citrate or ethylene diamine tetraacetic acid (EDTA) is designed to bind calcium, an important factor in the cascade, thereby, preventing coagulation altogether.[88] Plasma treatment promotes visible coagulation for all of the above anti-coagulants.

(ii) Initial plasma coagulation hypothesis was focused on increase in concentration of Ca^{2+}, which is an important factor in the coagulation cascade. It was suggested that plasma stimulates generation of Ca^{2+} through the redox mechanism $[Ca^{2+}R^{2-}] + 2H^{+}_{(H_2O)} \underset{k_{-Ca}}{\overset{k_{Ca}}{\rightleftharpoons}} [H_2^{+}R^{2-}]_{(H_2O)} + Ca^{2+}_{(H_2O)}$, provided by hydrogen ions produced in blood in a sequence of ion/molecular processes induced by plasma ions.[19] Validity of the hypothesis was tested experimentally by measuring Ca^{2+} concentration in the plasma-treated anti-coagulated whole blood using a calcium selective microelectrode. Calcium concentration was

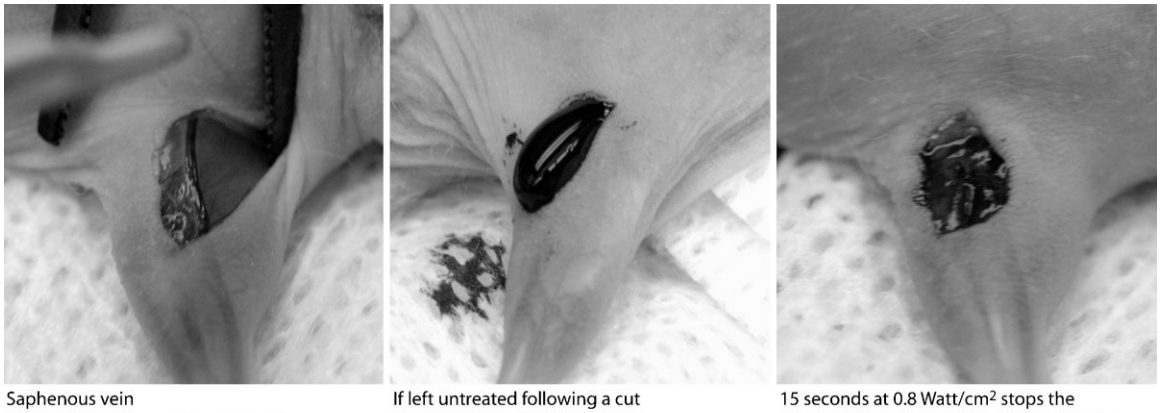

Figure 10. Blood coagulation on a live animal.[32]

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measured immediately after plasma treatment and remained almost constant for up to 30 s of the treatment and then increased slightly for prolonged treatment times of 60 and 120 s. Although, plasma is capable of coagulating anti-coagulated blood within 15 s, no significant change occurs in calcium ion concentration during the typical time of blood coagulation in discharge treated blood. *In vivo*, the pH of blood is maintained in a very narrow range of 7.35–7.45 by various physiological processes. The change in pH by plasma treatment (about 0.1 after 30 s) is less than the natural variation of pH, which indicates that the coagulation is probably not due to the pH change in blood.

(iii) FE-DBD treatment of whole blood samples was shown to change concentrations of various proteins participating in coagulation cascade. Plasma treatment is shown to "consume" coagulation factors (proteins and enzymes) and a visible film is formed on the surface of the treated samples. Increase in the sample volume and keeping the surface area fixed decrease the effect, indicating that plasma treatment initiates clot formation at the surface, not in the volume (Figure 11). Corresponding kinetic model of the plasma-assisted blood coagulation indicates a two-fold decrease in clot formation time with plasma treatment (Figure 12).

(iv) When the surface of blood is protected by small thin aluminum foil, which prevents contact between blood and FE-DBD plasma but transfers all the heat generated by plasma, no influence of blood is observed. This indicates a non-thermal mechanism of the plasma-stimulated blood coagulation.

(v) The final step in the natural biological process of blood coagulation is the production of thrombin which converts fibrinogen into fibrin monomers that polymerize to form fibrin microfilaments. FE-DBD plasma treatment of fibrinogen solution in physiological medium coagulates it, which is confirmed visually through a change in the color of the solution (from clear to milky-white) and through dynamic light scattering (DLS). Of note is that plasma does not influence fibrinogen through a pH or temperature change. FE-DBD treatment, however, is unable to polymerize albumin (directly not participating in coagulation cascade) as no change in its behavior is observed both visually and through DLS. Thus, non-thermal plasma selectively affects proteins (specifically, fibrinogen) participating in the natural coagulation mechanism.

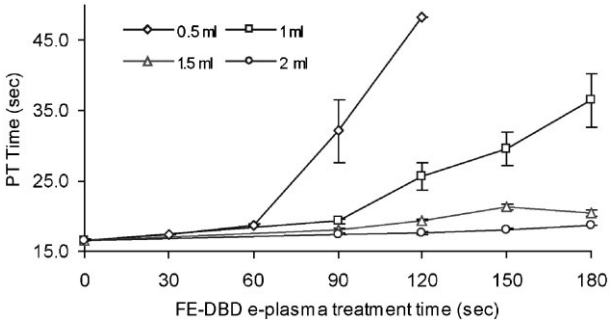

Figure 11. Prothrombin (PT) time for blood samples of different volumes with the same surface area of FE-DBD treatment.[19]

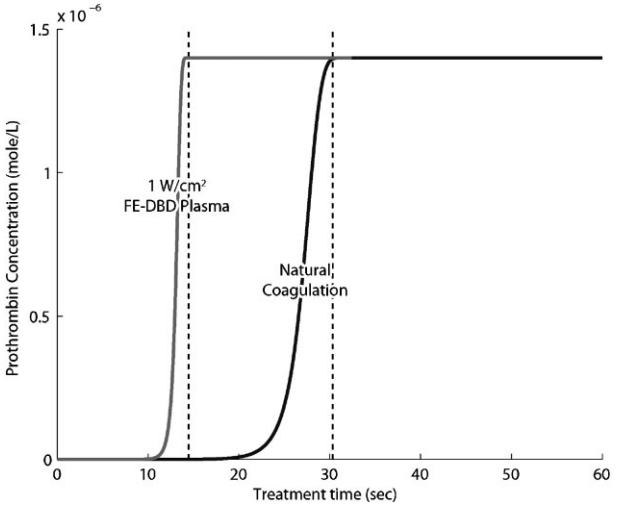

Figure 12. PT kinetics: two-fold decrease in clot formation time with plasma treatment.[19]

To assess plasma influence on protein activity, compared with plasma influence on the protein itself, trypsin [treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) to inhibit contaminating chymotrypsin activity without affecting trypsin activity] was treated by plasma for up to 2 min and its total protein weight and protein activity was analyzed via fluorescence spectroscopy. Total protein weight, or the amount of protein in the treated solution, remains practically intact after up to 90 s of the treatment (Figure 13); while the enzymatic (catalytic) activity of this protein drops to nearly zero after 10–15 s of treatment. Similar behavior is observed for albumin as well. This effect also proves that plasma effect on proteins is not just destructive but quite selective and natural.

Morphological examination of the clot layer by scanning electron microscopy (SEM) further proves that plasma does not "cook" blood, but initiates and enhances natural sequences of blood coagulation processes. Activation followed by aggregation of platelets is the initial step in the coagulation cascade and conversion of fibrinogen into fibrin is the final step in the coagulation cascade. Figure 14 shows extensive platelet activation, platelet aggregation, and fibrin formation following FE-DBD plasma treatment.

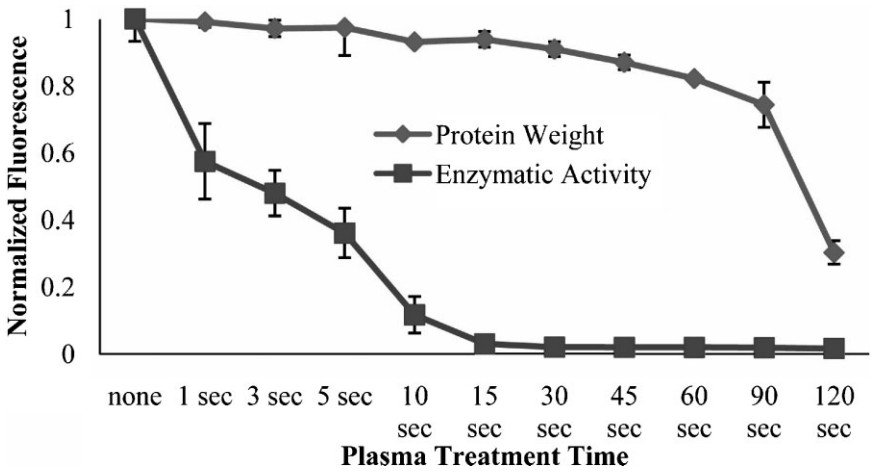

Figure 13. Total protein weight compared to enzymatic activity of trypsin following plasma treatment.[92]

Plasma-Assisted Wound Healing and Tissue Regeneration

Discharge Systems for Air-Plasma Surgery and Nitrogen Oxide (NO) Therapy

Effective use of plasma in surgery has been first demonstrated in 1960s; plasma afterglow jet of an inert gas has been applied for tissue sectioning with instant blood coagulation. Because of that plasma-surgical devices got a long-standing name of "plasma scalpel" in the hospitals (see, for example, Glover et al.[93]). Significant advancement in the plasma surgery, wound healing, and tissue regeneration is due to development of the "Plazon" system based on the jet of hot air plasma rapidly quenched and providing relatively high NO concentration with significant therapeutic effect.[94,95] This plasma device is used in two modes. In the first "hot mode" plasma jet is used for rapid coagulation and sterilization of wound surfaces, destruction and desiccation of dead tissue and pathologic growths, dissection of biological tissues. In the second "cold mode" NO-containing plasma gas flow with temperature of 20–40 °C is used for stimulation of regenerative processes and wound healing.

The Plazon generators[21,94,95] are the DC arcs with different configurations of the exit channels corresponding to the different applications (blood coagulation, tissue destruction, therapeutic manipulation/stimulation). Main and common elements of the system construction are the liquid-cooled cathode, intra-electrode insert, and anode. Atmospheric air enters the manipulator through the built-in microcompressor, passes through the plasma arc, heats up and thus accelerates, and exits through the hole in the anode of the plasma-generating module. Plasma temperature at the anode exit differs in different configurations of the device, corresponding to different medical applications (see Figure 15). Further away from the anode, temperature drops rapidly, and at 30–50 mm from the anode, the flow is composed simply of the warm gas and the plasma-generated NO. Nitrogen oxide content in the gas flow is mainly determined by the quenching rate. The necessary quenching rate for effective operation of the medical device is about $\approx 10^7$–10^8 K · s^{-1}. Commonly, the cooling rate of plasma jets is on the order of $\approx 10^6$ K · s^{-1}. Thus, to achieve the cooling rate of $\approx 10^7$–10^8 K · s^{-1}, it is necessary to utilize additional cooling of the plasma jet, which has been achieved by special construction of the plasma nozzles.

The therapeutic manipulator-stimulator configuration of the Plazon discharge system is used solely for therapeutic treatment by exogenic nitrogen oxide. The principle difference of this manipulator is that the air-plasma jet does not freely exit into the atmosphere, but rather it exits the anode into the two-step cooling system, gas channels of which are created in a maze scheme to force-cool the jet by the liquid circulating from the cooling system. This construction allows one to obtain NO-containing gas flow (NO-CGF) with sufficiently low temperature, and optimal concentration of nitrogen oxide molecules, which makes it possible to apply this manipulator for the treatment of external body surfaces by using the cooling hose of 150 mm length (temperature of NO-CGF at the exit ≈36 °C). Of course, NO content in the gas flow depends on the distance from the exit channel (Figure 16). Additionally, for laparoscopic operation, a special manipulator of 350 mm length and 10 mm diameter is utilized.

The possible operating regimes of the apparatus are defined by the characteristics of the gas flow exiting from the manipulator, main parameters of which are its temperature and the nitrogen oxide content. First group of regimes – regimes of free-flowing plasma off-gas exiting

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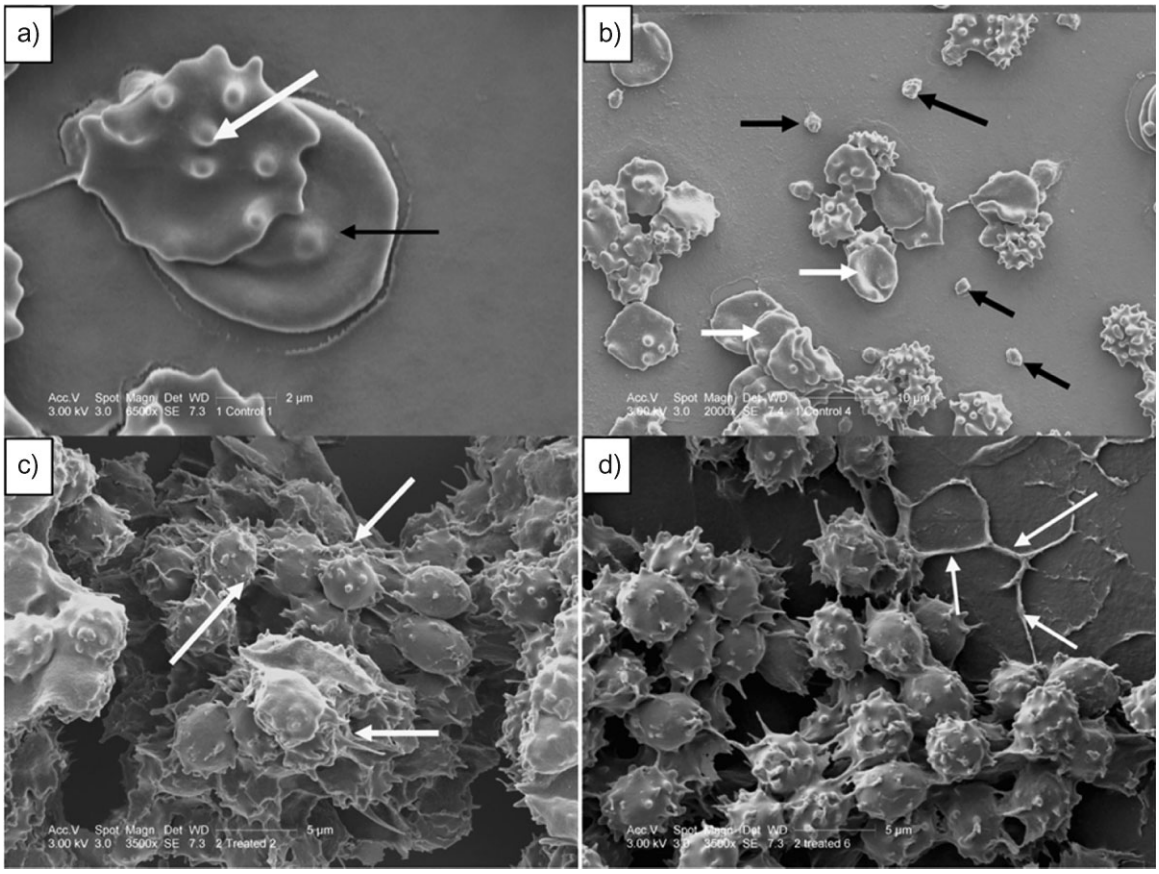

Figure 14. SEM images of untreated (a, b) and treated (c, d) anti-coagulated whole blood. (a) Whole blood (control) showing single activated platelet (white arrow) on a red blood cell (black arrow); (b) whole blood (control) showing many non-activated platelets (black arrows) and intact red blood cells (white arrows); (c) whole blood (treated) showing extensive platelet activation (pseudopodia formation) and platelet aggregation (white arrows); and (d) whole blood (treated) showing platelet aggregation and fibrin filament formation (white arrows).[81]

the manipulator; second group of regimes – regimes of the treatment of bio-tissues by completely cooled (20 °C) NO-CGF, to obtain which a manipulator is connected to the internal gas cooler, and delivery of NO-CGF to bio-tissues is achieved through a silicone tube with an attached tip of 130 or 390 mm length, and the exit channel diameter of 0.7 mm. This allows not only direct treatment of the bio-tissues by NO, but also its delivery to a pathologic center through drainage tubes, puncture needles, or any endoscopic devices (gastroscope, bronchoscope, cystoscope, rectascope, etc.).

Medical Use of Plasma-Generated Exogenic Nitrogen Oxide

The Nobel Prize in medicine and biology was awarded in 1998 to R. F. Furchgott, L. J. Ignarro, and F. Murad for their work on the function of nitrogen oxide as a signal molecule.[96] Today it is well known that in a human organism, NO serves a multitude of essential biological functions – it regulates blood vessel tone (via relaxation of flat epithelial cells) and blood coagulation, immune system and early apoptosis, neural communication and memory, relaxation of flat bronchial and gastrointestinal muscles, hormonal and sex functions, NO offers anti-microbial and anti-tumor defense, etc. In pathology, NO plays a major role in adaptation, stress, tumor growth, immunodeficiency, cardiovascular, liver, gastrointestinal tract disease, etc. This explains wide possibilities of the plasma-generated exogenic NO in multiple medical applications.

Importance of exogenic NO in infection and inflammation processes is also well studied and is linked with anti-microbial effects; stimulation of macrophages; induction of cytokines, T-lymphocytes, and many immunoglobulins; interaction with oxygen radicals; and influence on microcirculation, cytotoxic and cytoprotective role in different conditions. During inflammation, macrophages and some other cells (i.e., aibroblasts, epithelial cells, etc.) produce NO via inducible NO-synthase (iNOS) in quantities significantly greater (two orders of magnitude) than

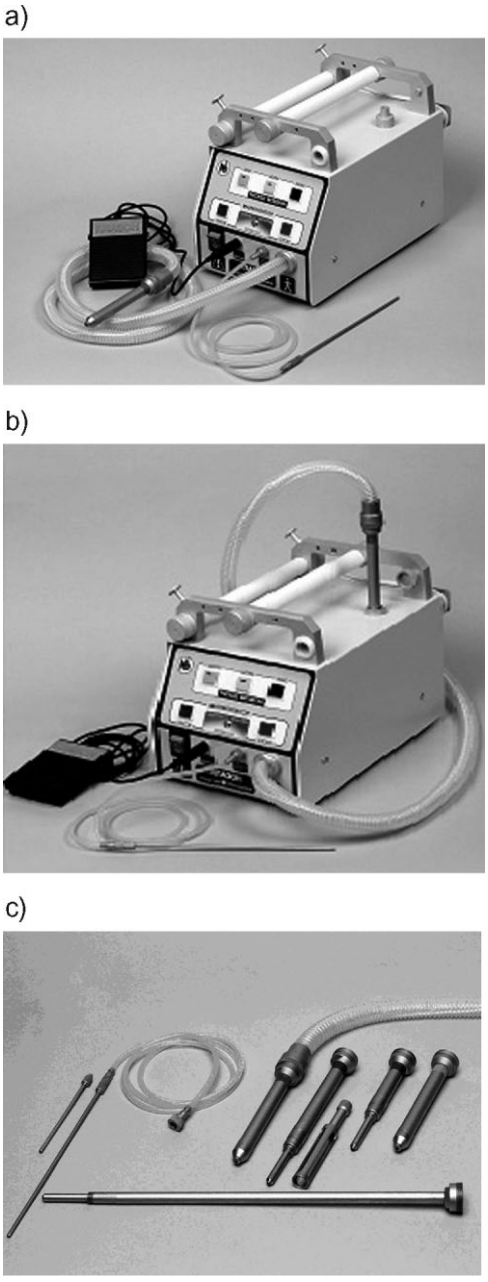

Figure 15. "Plazon" apparatus in two working modes: (a) hot mode (b) cold mode with manipulators and accessories (c).[94,95]

normal when NO is formed via constructional NOS: endothelial (eNOS) and neuronal (nNOS).

Exogenic NO is also crucial in trauma wound processes. Activity of iNOS grows substantially in trauma wounds, burn wound tissues, bone fracture site tissues, and others in the inflammatory and proliferation phases of the healing process. Activation of iNOS was also discovered in the cultivation of wound fibroblasts. Macrophage activation in a wound, cytokine synthesis and proliferation of fibroblasts, epithelization, and wound healing processes are all linked with the activity levels of iNOS. In animal models, injection of iNOS inhibitors disrupts all of these processes and especially the synthesis of collagen, while NO synthesis promoters increase the rate of these processes.

Animals with iNOS deficiency demonstrate significant decrease in wound healing rate, however this can be reversed by injection of iNOS gene. In complicated wound models, for example, in experimentally induced diabetes, protein deficiency, injection of corticosteroids, or immunosuppressants, and also in patients with tropic ulcers, lowered activity of iNOS is usually discovered which correlates to slowed healing processes. Exogenic delivery of NO-donors (nitrogen-containing compounds) to the wound promotes and speeds up healing processes in animals with complicated wounds and in animals with inhibited iNOS. This knowledge, coupled with theoretical and experimental data on NO generation in air plasmas, served as a basis for a series of bio-medical experiments focused on use of the plasma-generated exogenic NO, delivered directly to the pathologic site, for control of inflammatory processes and increase in the rate of wound healing.

Experimental Investigations of NO Effect on Wound Healing and Inflammatory Processes

EPR spectroscopy was utilized to investigate the dynamics of level of endogenic and exogenic NO in wound tissues and in organs in an animal model (70 rats).[21] NO "trap", diethylthiocarbamate (DETC), was injected into rats with a full thickness flat wound of 300 mm^2 area 5 d prior to EPR analysis. Following euthanasia, the samples were collected from the animals: blood, granular tissue from the bottom of the wound, and from internal organs (heart, liver, kidney, and the small intestine). For a portion of the animals, on the 5th day following the initial wound introduction, the wound surface was treated by the NO-CGF (500 ppm). Without the NO treatment, the results indicate high content of endogenic NO in wound tissues [$(10.3 \pm 2.3) \times 10^{-6}$ M). The liver of the animals with the wound contained $(2.3 \pm 1.4) \times 10^{-6}$ M of DETC-ironmononitrosyl complex (IMNC) while the control group (without the wound) – only $(0.06 \pm 0.002) \times 10^{-6}$ M.

Animals without the wound were used for investigation of penetration capability of gaseous exogenic NO through undamaged tissues of abdominal wall. Treatment by NO-CGF was performed for 60 and 180 s. A nearly linear dependence of the amount of DETC-IMNC produced in the liver and blood of the animal on the NO-containing gas treatment time was observed. 2 min following the 180 s treatment a maximum signal was registered in the bowels

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of the animal – 2.6 times higher than in the control group. In the heart, liver, and kidney the difference was 1.7 times. These results are indicative of the ability of the exogenic NO molecules to penetrate the undamaged tissues.

A more complex relationship was observed in the treatment by exogenic NO of the wound tissues. If the animal was euthanized 30–40 min following the treatment, then NO content in wound tissue and blood was observed to raise 9–11 times more than in the case of the 2-min interval. This is probably due to the formation of peroxinitrite, which can be formed through NO reacting with superoxide anions (O_2^-), as it is known that the superoxide levels are increased in the organism during the inflammatory processes. In response to the oxidative stress, the organism mobilizes the anti-oxidant defense mechanisms first via the increase in the levels of reducing agents (thioles, ascorbate, etc.), and then via activation of synthesis of anti-oxidant enzymes. 30–40 min following the wound treatment by exogenic NO, activation of the first cascade of anti-oxidant defense allowed for significant decrease in the level of superoxide anions. This considerably decreases its destructive influence on DETC-IMNC and the nitrosyl complexes of the hemoproteins, which leads to the increase in their concentration as is detected by the EPR spectroscopy. Additionally, the activation of NOS by the increase in endogenic NO cannot be neglected. It partially explains the discovered phenomena of stimulation of wound development processes via the influence of exogenic NO, when there is a deficiency of endogenic NO or excess of free radicals including superoxide.

In experiments on the cornea of rabbits, the mucous membrane of the cavity of the mouth of hamsters, and on the meninx membrane of rats, via lifetime biomicroscopy it was found that the effect of the expansion of the opening of the microvessels under the influence of exogenic NO (500 ppm) lasts with varying intensity up to 10–12 h, while the lifetime of NO molecules is no more than 10–15 s.[21,94,97] The experiments serve as additional evidence that single application of exogenic NO initiates a cycle of cascade reactions, including biosysnthesis endogenic NO, which leads to a long-lasting effect and explains the successes of the NO-therapy.

Action of the exogenic NO on the cellular cultures of the human fibroblasts and rat nervous cells was studied by Shekhter et al.,[21,94] Stadler et al.,[98] Ghaffari et al.,[99] and others. Single treatment by the plasma-generated NO of the cell cultures significantly increases (2.5 times) the cell proliferation rate via the increase in DNA synthesis (tested by inclusion of C^{14} thymidine) and to a lesser extent (1.5 times) increase in protein synthesis by the cells (tested by inclusion of C^{14} aminoacids). As expected, the stimulating effect is dose dependent. The action of exogenic NO on the phagocytic activity of the cultured wound macrophages from the washings of the trophic human ulcers, studied by the photochemiluminescence[100] revealed that a maximum increase in the luminous intensity (1.95 times in comparison with control) testifies about the activation of the proteolytic enzymes of macrophages under the effect of NO-CGF. Statistically significant increase in fluorescence of macrophages was observed in less than 24 h following a 30 s treatment.

In vitro investigation of the influence of NO-CGF on *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Candida albicans*, which are typically associated with many hospital infections, showed that 75 s of the treatment by NO-CGF significantly decreases viable colony forming units, 80 s practically removes them all, and no growth is detected at all following 90 s of the treatment.[101] Major mechanisms of the NO influence on various pathologic processes can be summarized as:[21,94]

(i) Direct bactericidal effect (through formation of peroxynitrite in the reaction: $NO + O_2^- \rightarrow ONOO^-$);
(ii) induction of the phagocytosis of bacteria and necrotic detrite by neutrophils and by macrophages;
(iii) inhibition of the free oxygen radicals, which exert pathogenic influence, and also possible activation of the anti-oxidant protection;
(iv) normalization of microcirculation due to the vasodilatation, the anti-aggregation, and anti-coagulant properties of NO, that improves vascular trophicity and nutrient exchange;
(v) improvement of nerve conductance;
(vi) regulation of immune-deficiencies which are common in wound pathology;
(vii) secretion of cytokines by the activated macrophages, which increase fibroblast proliferation, angiogenesis factors, chemokines, in particular, monocyte chemoattractant protein (MCP-1), G-protein, nuclear factor κB (NFkB), and other biologically active factors which regulate wound healing and inflammatory processes;
(viii) direct induction of proliferation of fibroblasts and synthesis of proteins by them;
(ix) increase in or regulation of collagen synthesis;
(x) regulation of apoptosis in remodeling of granular and fibrous tissues;
(xi) influence on the proliferation of keratinocytes and thus on the epithelization of the wound.

Clinical Aspects of Use of Air Plasma and Exogenic NO in Treatment of Wound Pathologies

Application of air plasma and exogenic NO in the treatment of the trophic ulcers of the vascular etiology in 318 patients showed high efficiency of NO-therapy in the treatment of the venous and arterial trophic ulcers of

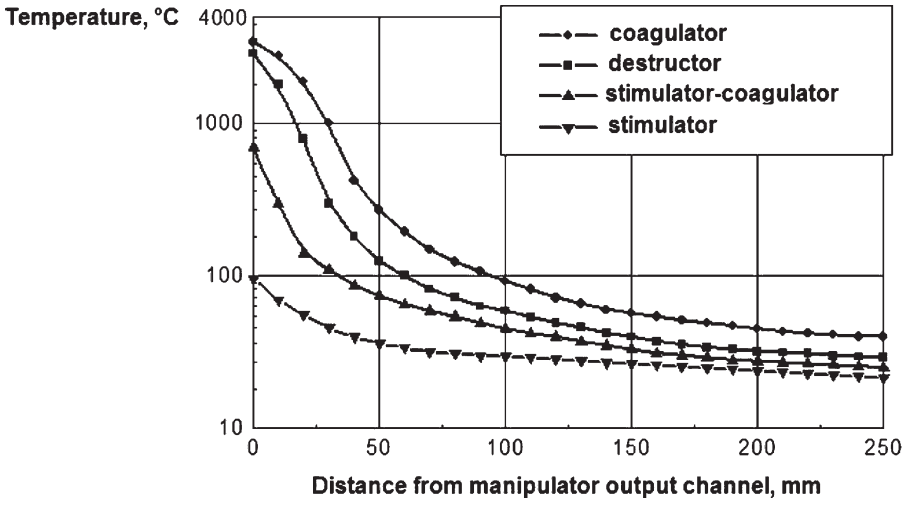

Figure 16. Temperature in the center of the gas flow jet for different manipulators.

lower extremities with an area from 6 to 200 cm^2.[21,94] For assessment of the effectiveness of the plasma NO-therapy, clinical and planimetric indices were analyzed in the course of the process of sanitation and epithelization of ulcers, a bacteriological study of discharge from the ulcer, cytological study of exudate, a histopathological study of biopsies from the boundary of a trophic ulcer, the indices of microcirculation [according to the data obtained by laser Doppler flowmetry (LDF)], and transcutaneous partial pressure of oxygen (TpO_2). In the main groups of observations, trophic ulcers were processed in the regime NO-therapy (500 and 300 ppm); or prior to beginning the therapy the ulcer surface was treated in the regime of coagulation until the evaporation of necrotic debris. Following initial treatment, the wounds were treated for 10–30 d in the NO-therapy regime. In the control group, proteolytic and anti-microbial drugs were used – in the phase of exudation and necrosis, and wound coatings – in the phase of tissue regeneration and epithelization.

Planimetric observation of the dynamics of decrease in the trophic ulcer area showed that, on average, traditional treatment methods applied in the control group lead to 0.7% per day decrease, while in the experimental group – 1.7% per day. Cleansing of ulcers from necrotic debris and exudate, and appearance of granulation and boundary epithelization were accelerated with NO-therapy on the average 2.5 times. The time to final healing was reduced 2.5–4 times depending on the initial ulcer size (Figure 17). Larger ulcers tended to close faster than smaller ones. Following the NO-therapy, LDF investigation of microcirculation in the tissue showed normalization of pathologic changes in the amplitude-frequency signal characteristics of the microvasculature and activation of regulatory mechanisms on those tissues.

By 14–18 d the average index of microcirculation, value of root-mean-square deviation, coefficient of variation, and index of fluctuation of microcirculation approached in its value those of the symmetrical sections of healthy skin. In the control group the disturbances of microcirculation remained. Against the background of treatment, normalization of the level of transcutaneous partial pressure of oxygen (TpO_2) happened at a higher rate in the experimental group than in the control group, especially at the NO concentration of 500 ppm (Figure 18). A bacteriological study of wound discharge from the trophic ulcers showed that in the experimental group, against the background, NO-therapy (especially in combination with the preliminary coagulation of ulcerous surface) reduced the degree of bacterial seeding (microbial associations) and already by days 7–14 it went below the critical level, necessary for maintaining the infectious process in the wound (Figure 19).

Using the plasma-generated NO for local treatment of ulcerous and necrotic tissues in patients with diabetes (diabetic foot ulcer) has been

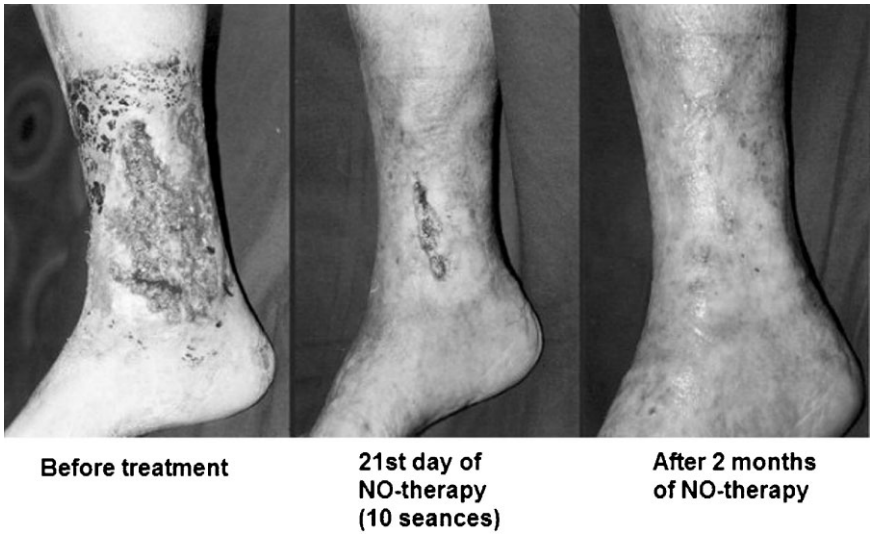

Figure 17. Dynamics of the healing of venous trophic ulcer during NO-therapy.

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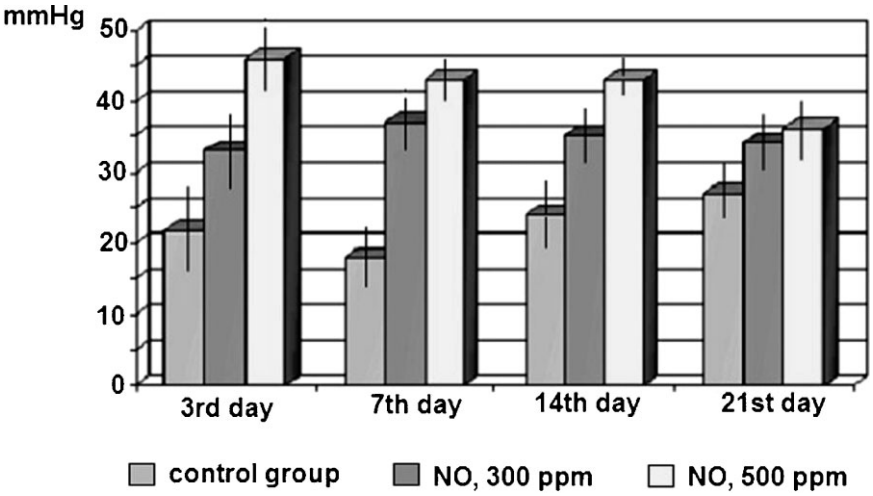

Figure 18. Dynamics of pO_2 level during NO-therapy of venous trophic ulcers.

demonstrated by Shulutko, Antropova, and Kryuger.[101] Patients were selected for this study following 2 months of unsuccessful treatments by the state-of-the-art techniques. Already from the first few sessions the difference was evident; inflammatory reaction was clearly reduced, patients reported decrease in pain, and cleansing of the ulcer surface was clearly visible. Following ten sessions, most patients expressed positive healing dynamics: ulcer size decreased to 1/3–1/4 of the original size. LDF markers, pO_2, and bacteriological investigation all showed a positive dynamic. In patients with relatively small-sized ulcers (initial diameter less than 1 cm), full epithelization occurred by 6–8 NO-treatment sessions. Period of stationary treatment and full clinical recovery of patients was noticeably shortened (on an average by 2.3 times). In the cases of large ulcerating wounds, the necessity for amputation decreased 1.9 times (Figure 20).

Effectiveness of the exogenic NO and air plasma on healing of the pyoinflammatory diseases of soft tissues has been demonstrated studying 520 patients with the purulent wounds of different etiology and 104 patients with the phlegmonous-necrotic form of the erysipelatous inflammation.[102,103] By the 5th day of therapy wounds on most of the patients in the experimental group (90%), contrary to the control group, were clear of necrotic tissue, and the wounds began to be covered by bright spots of granular tissue. Microbial infestation of the wound tissue had lowered from 10^6–10^8 colony forming units (cfu) per gram of tissue to 10^1–10^2. Data from complex analysis of microcirculation (LDF, pO_2) showed significant repair of the microvasculature and blood flow in the wound tissues in most of the patients in the experimental group. The predominant types of cytograms were regenerative and regenerative- inflammatory with a notable increase in fibroblast proliferation – on an average of $18.5 \pm 3.1\%$. Notable morphological changes in the biopsies were the significant development and maturing of the granular tissue and the regeneration of epithelial tissue at the edges. Large suppurated wounds, for example, suppurated burn wounds (Figure 21), by days 7–10 of the treatment were clear of the pyonecrotic exudate and were beginning to be covered by granular tissue, in other words these wounds were ready for dermautoplasty.

Effectiveness of the plasma NO-therapy is most apparent with the treatment of the pyonecrotic form of erysipelatous inflammation – patients who are considered the most severe cases of the purulent surgery departments.[102,103] The combination of surgical preparation of extensive pyonecrotic centers and local NO-therapy allowed in the majority of the patients with phlegmonous- necrotic erysipelas during 12–14 d of the treatment to liquidate heavy pyonecrotic process and to create conditions for completion of reparative procedures.

The plasma NO treatment has been also successfully applied to surgical oncology.[104,105] Inter-operative treatment in the coagulation regime ensures ablation, considerably decreases blood plasma and whole blood losses from extensive wound surfaces as a result of thin film formation over the wound surface, consisting of coagulative necrotic tissue. As a result of plasma NO-therapy of the post-operative wounds, a significant decrease in inflammation is observed

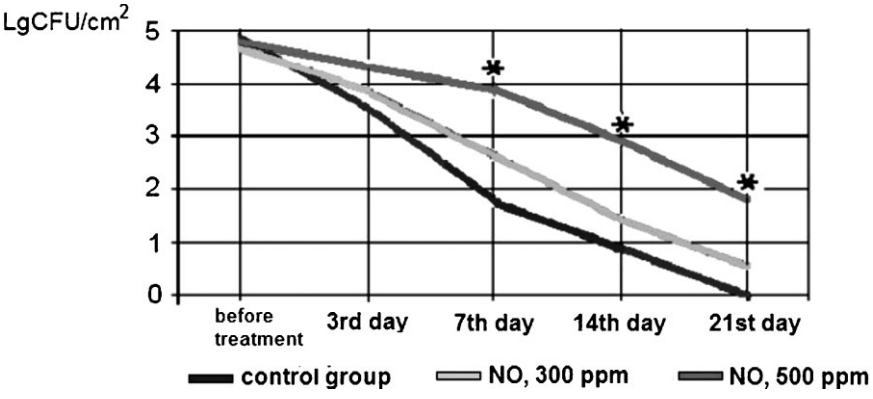

Figure 19. Dynamics of bacterial contamination of trophic ulcers during NO-therapy (* statistical significance, $p < 0.01$).

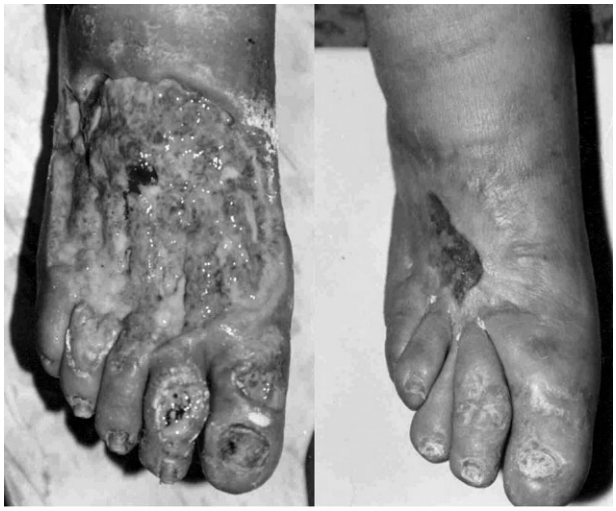

Figure 20. Extensive pyonecrotic ulcer of the foot (neuro-ischemic form of the syndrome of diabetic foot).

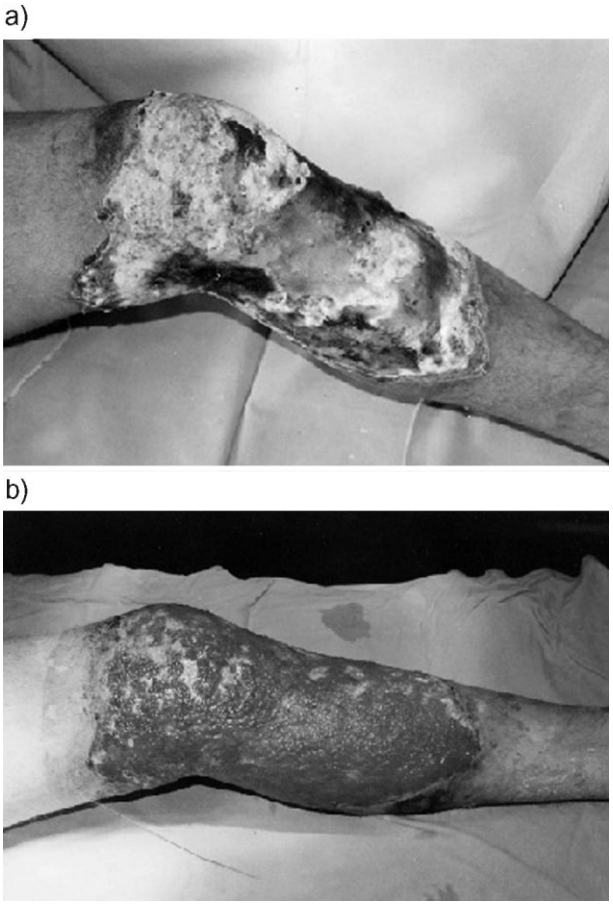

Figure 21. Healing dynamics of the festered burn wound in process of NO-therapy: (a) prior to the beginning of treatment and (b) after five sessions of NO-therapy.

along with stimulated proliferation of granular tissue and epithelization. Effect is observed independently of the location of the wound on the body and also of the plastic material used. Additional positive benefit of this treatment is the prophylactic treatment of the local relapses of the tumor, which allows for a wide application of this method in oncoplastic surgeries. Effectiveness of NO-therapy in the treatment of early and late radiation reactions allows for the surgeon to carry out a full course on radiation therapy in 88% of the patients. Treatment of radiation tissue fibrosis also yields a statistically significant improvement, confirmed in morphological investigation of these tissues. The plasma NO-therapy is successfully used both for the preventive maintenance of the formation of post-operative hypertrophic and keloid scars, and for the treatment of already formed ones: softening the scar tissue, decrease in fibrosis, and preventive maintenance of their relapse with the surgical removal.

Air Plasma and Exogenic NO in Treatment of Inflammatory and Destructive Illnesses

Possibility of directing of the plasma-generated NO-CGFs through puncture needles, vent lines, and endoscopic instruments, and also the inhalation method of action considerably enlarges prospects for the plasma NO-therapy in the treatment of the ulcero-necrotic, erosive, and inflammatory processes in the pleural and abdominal cavities, lungs, stomach, and bowels, ear, nose, and throat (ENT) organs (purulent sinusitis, purulent otitis media, paratonsillar abscesses), etc. Effectiveness of the plasma NO-therapy is already at present shown with a number of diseases in gynecology, traumatology, stomatology, ophthalomology, otorhinolaryngology, dermatology, gastroenterology, etc. Some specific relevant medical applications of the plasma system are summarized below.[21,94]

Pulmonology

Strong effect is demonstrated in the treatment of plural empyema via insufflation of the NO-CGF into the cavity of the pleura through the vent lines.[101] Therapy in the treatment of 60 patients with pleural empyema showed stimulative and regulative influence on the development of the wound tissues. Acceleration of the purification of pleural cavity from the microorganisms and the debris, stimulation of phagocytosis, and normalization of microcirculation accelerate the passage of the phase of inflammation during wound regeneration, which leads to a significant decrease in the drainage time for all patient categories in the experimental group, as compared to control, and to the reduction in the hospitalization time.

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The inhalation application in the treatment of patients with the complex chronic unspecific inflammatory lung diseases led to the clearly expressed positive dynamics of the endoscopic picture of the tracheobronchial tree: decrease in the degree of inflammatory changes in the mucous membrane of bronchi, reduction in the quantity, and the normalization of the nature of contents of the respiratory tract. During the study of biopsies of mucosa of bronchi it was verified for all cases that the liquidation or the considerable decrease in inflammatory changes occurred, in addition to a complete or partial restoration of the morphological structure of the bronchi.

Phthisiology

Plasma NO-therapy together with the specific treatment was used in patients with infiltrative and fibrous-cavernous pulmonary tuberculosis via NO insufflation through the bronchoscope or cavernostomy for cavernous tuberculosis, through the vent line with tubercular pleurisy or empyema. Through 8–10 therapy sessions, a significant acceleration of healing of the cavities, tubercular bronchitis, and pleurisy was achieved.[106]

Traumatology and Orthopedics

Positive effect has been demonstrated for the treatment of patients with the infected, prolongedly not healing wounds after sequestrectomy, osteomyelitic blowholes, etc.[21,107] It was shown that following the NO-therapy the bacterial load of wounds and blowholes was significantly reduced, inflammatory manifestations were reduced in the wound and the surrounding tissues, cleansing from the necrotic mass advanced, and active granulations appeared. All the participants of this study were previously treated by state-of-the-art methods for a long time without apparent success. Morphological investigations in these clinical observations showed that already by 3–4 plasma NO-therapy sessions a significant reduction in the infection was observed, weakening microcirculatory disorders and signs of inflammation were reduced, proliferation and differentiation of fibroblasts and angiogenesis were evident, and an increase in the granular tissue and the cicatrization of the wound were apparent. The therapy is being also used for the treatment of open fractures.

Gynecology

Effectiveness of the plasma NO-therapy in combination with the coagulation by air plasma was shown with the treatment of patients with purulent inflammation of appendages of the womb.[108–110] In surgery, where the abdominal cavity was opened, the purulent wound was processed by air plasma in the coagulation regime, and then at later time by the plasma NO-therapy they achieved by remote action through the front abdominal wall and vagina. With operational laparoscopy after dissection and sanitation of purulent center the region of surgical incision and the organs of small basin were treated by NO-CGF, which was delivered locally through the aspiration tube. The plasma NO-therapy was also continued in the post-operation period. The use of NO-CGF in surgical and therapeutic regimes aided the rapid decrease in the microbial load, decrease in swelling, lowered risk of post-operative bleeding, rapid development of reparative processes, and overall time the patients remained in the hospital was decreased by 6–8 d on average. The NO-CGF was also used in organ-saving surgical operations on the womb, the uterine pipes, and the ovary.

Dentistry

Effectiveness of the plasma NO-therapy has been demonstrated on the chronic gingivitis. After the first session of the therapy, gum bleeding ceased, after 1–2 weeks normalization of tissue and regional blood flow in the tissues of periodontium.[111] Normalization of cytological signs was observed in 2–3 months, however in the control group normalization was not observed at all. Utilization of NO-CGF in surgical intervention of generalized paradontitis (both in intra-operative and post-operative NO-therapy) showed that normalization of clinical and cytological signs occurs by 7th day in experimental group, while only on 14th day in the control group. Complications were not observed in the experimental group, unlike in the control group where they did occur.

Maxillofacial Surgery

The plasma NO-therapy was used to accelerate the healing of post-operative wounds and preventive maintenance of the formation of hypertrophic and keloid scars, treatment of the formed scars, treatment of pyonecrotic processes (abscesses, phlegmon, etc.). With the latter, preliminary coagulation of purulent centers was sometimes utilized.[21]

Ophthalmology

Treatment by NO-CGF (300 ppm) did not result in altering and/or toxic reaction, and does not cause changes in the intra-ocular pressure and morphological changes in the tissues of the eye, but considerably accelerated healing of the wounds and burns of cornea. The therapy was then used in the clinic for the effective treatment of burns, erosions and injuries of cornea, and burn ischemia of conjunctiva.[112]

Otorhinolaryngology

Effectiveness is demonstrated in the treatment of scar stenoses of larynx and trachea, chronic tonsillitis, relapsing nose hemorrhages, chronic and sharp rhinitis, pharyngitis, maxillary sinusitus, otitis, polypous purulent etmoidita, and other ENT pathologies.[113]

Dermatology

Therapy is effectively used with the treatment of psoriasis, eczemas, dermatitis, ulcerous injuries with local and systemic angiitises, scleroderma, red flat lishchaya, and a number of other skin illnesses.[114]

Gastroenterology

For the treatment of chronic ulcers and erosions of stomach and duodenum, and blowholes of small intestine, the therapy was delivered through endoscopic instruments.[115] Stomach ulcers healed twice as fast as in the control group. The proliferating activity of the epithelium according to the data of the immunomorphology of biopsies was strengthened 7.8 times.

Purulent Peritonitis

In the case of the purulent peritonitis caused by the diseases of the organs of abdominal cavity, the effect was achieved by the direct treatment of peritoneum, and in the post-operative period cooled NO-CGF was delivered through the vent lines.[107] NO carries bactericidal action, stimulates microcirculation, and lymphodrainage, normalizes the indices of cellular and humoral immunity, promotes inflammatory process, and serves as a factor in the preventive maintenance in the sealing of the abdominal cavity.

Non-Thermal Plasma Treatment of Various Diseases

Non-Thermal Plasma Treatment of Melanoma Skin Cancer

The FE-DBD plasma treatment was shown to initiate apoptosis in melanoma cancer cell lines – a threshold at which plasma treatment does not cause immediate necrosis but initiates complex cascade of biochemical processes leading to cell death many hours and even days following the treatment.[27] Melanoma cells, treated by plasma at doses significantly below those required for cell destruction, survive the plasma treatment but develop apoptosis many hours post-treatment and die (disintegrate) by themselves gracefully. This could potentially be an intriguing approach for cancer treatment, especially if by manipulation of plasma parameters the treatment could be made selective to cancerous cells over healthy cells, as was demonstrated before for bacteria versus healthy cells.[19,20]

Cellular macromolecules during apoptosis are digested into smaller fragments in a controlled fashion, and ultimately the cell collapses without damaging the surrounding cells or causing inflammation. With cancer cells, however, a problem arises with apoptosis as the tumor cells frequently "learn" how to turn off apoptosis as one of the processes they employ in evading the immune system and surviving under unfavorable conditions. A way to target apoptosis development only in specific areas of the body is needed, and can be achieved by the non-thermal plasma treatment.

Melanoma cancer cell line (ATCC A2058) was prepared in the Fridman et al. experiments to the total concentration of $\approx 1.5 \times 10^6$ per dish[b] (44 mm diameter, 12.5 mm height, fluted aluminum dish, Fisher Scientific).[27] On days 3–5 of cell development, the cells were treated with the FE-DBD plasma for 5, 10, 15, 20, or 30 s. The distance from the electrode surface to the fluid surface was 3 ± 0.5 mm. After the treatment media was removed from the dishes, the culture was allowed to propagate further by adding 2 mL of the fresh media or harvested by trypsinization for further testing. Trypan blue exclusion test was performed at different time periods after treatment: immediately 1, 3, 24, 48, and 72 h following the treatment.

Another group of experiments was performed, testing cells for the onset of apoptosis. For this set of experiments, cells were treated by plasma for 5 and 15 s. Following treatment, cells were harvested at 3, 24, 48, and 72 h after treatment. TUNEL apoptosis staining assay was performed which detects DNA breaks indicative of a late onset of apoptosis and cell's final preparations to disintegrate. This biochemical fluorescence-based staining technique, coupled with the careful analysis of the cell lifecycle is indicative of FE-DBD plasma's ability to initiate apoptosis development in these cells.

Melanoma cell growth patterns were noted to asses "background" cell death through lack of nutrition, cell age, or the influence of aluminum substrate on the cell's life cycle demonstrates cell survival numbers after 5, 10, 20, and 30 s of the treatment compared to control analyzed by Trypan blue exclusion test. Total cell numbers are normalized to 1 (100%) to account for cell growth between the counting sessions: controls are set to 100% and cell viability is expressed as percent to control to allow for comparison between experiments. It is of no great surprise that FE-DBD plasma is able to kill cells; what is unusual is that 24 h following treatment the total number of cells continues to decrease significantly (Figure 22).

It is important to distinguish between cell death by "poisoning" of the growth media the cells are in and the actual effect of direct plasma on these cells. To assess the difference, growth media was treated for up to 120 s by plasma separately, and then the cells were placed in this

[b] Aluminum dishes were selected as they are conductive and better mimic the situation in a live patient. Aluminum itself is mildly toxic so the dishes were specially pre-treated (details of this pre-treatment and further discussion can be found in ref.[27]).

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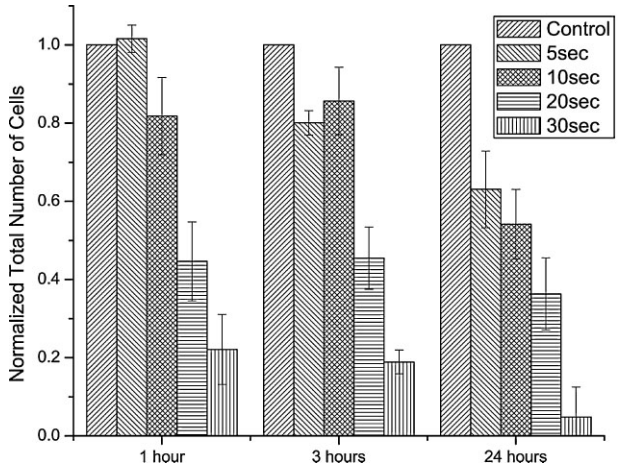

Figure 22. Results of FE-DBD treatment of melanoma cancer cells: Control, 5, 10, 20, and 30 s, counted 1, 3, and 24 h post-treatment.[27]

acidified media. Cells did not appear to react negatively to the acidified media. Additionally, cell inactivation under varying depths of growth media was investigated: FE-DBD plasma was able to inactivate cells under as much as 1 mm of cell growth media, though the time to achieve the same inactivation as without media increases.[27]

The general trend observed in treated cells is that they continue to die for days after the treatment presents an observation of groups of cells treated by plasma for 5 s and observed for a 24-h period following treatment. An emergent pattern appears where growth rate of treated cells is impaired as well as the number of inactivated cells grows substantially. Figure 23 shows the percentage of inactivated (dead) cells among treated and untreated populations. It was observed that 5 s of plasma treatment does not inactivate cells immediately; however, cell growth slows down significantly, and the number of dead cells increases 24 h after treatment, which is indicative of cell death occurring long after the treatment.

To analyze whether those plasma-treated cells that survive the initial insult die through an apoptosis-like process, TUNEL assays were performed. Cells treated for 5 s were then incubated and stained for DNA fractionation 24 h later. Following the TUNEL assay procedure it was observed that a significant percentage of these cells exhibit apoptotic behavior as is evident from Figure 24. Apoptosis develops 24 h following treatment, where 25.5% of cells are present in the treated group, compared with 2.2% in the control group. As time progresses, even more cells undergo apoptosis, further reaching 72.8% of apoptotic cells in the treatment group versus 3.2% in the control group 72 h following treatment.

Thus, FE-DBD plasma can kill melanoma skin cancer cells through necrosis at higher treatment doses (15 s and over at 1.4 $W \cdot cm^{-2}$) which are still significantly below the threshold of damaging healthy tissue.[19,20] Very low doses of FE-DBD (5 s at 0.8 $W \cdot cm^{-2}$ of plasma treatment) where no cell necrosis was observed were shown to initiate apoptotic behavior, or programmed cell death in melanoma cancer cells. Apoptotic behavior was deduced from the fact that treated cells do not initially die but stop growth and die en masse 12–24 h following treatment, while untreated cells continue to grow and proliferate. Apoptotic behavior was confirmed through DeadEnd™ Fluorometric TUNEL System apoptosis staining with subsequent flow-cytometry. It was shown that the plasma treatment initiates this behavior in cells not through poisoning of the growth media in which the cells reside or through interaction with the aluminum dishes the cells reside in, but through direct interaction with the cells.[27]

FE-DBD is not the only system shown to effectively inactivate melanomas. Schoenbach and coworkers[116] show an effective inactivation of this cancer *in vitro* and *in vivo* in an SKH1 hairless mouse animal model (Figure 25). Figure 26 shows a typical response of the tumor to the pulsed electric field treatment – tumor size decreases and melanoma simply vanishes. Many models have been proposed for such an efficient inactivation treatment; however, it seems that the fast rise time and short duration of these pulses are able to cause electro-deformation – open small pores and disrupt cellular membranes[116–123]: 100 pulses (40 $kV \cdot cm^{-1}$) of 300 ns duration lead to

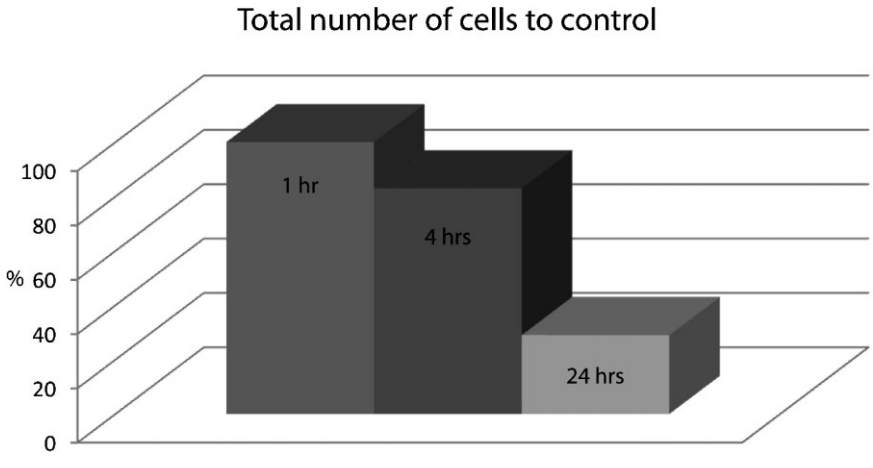

Figure 23. Results of observation of treated and untreated cells for a 24-h period: total number of cells before and after FE-DBD treatment. Treatment time: 5 s.[27]

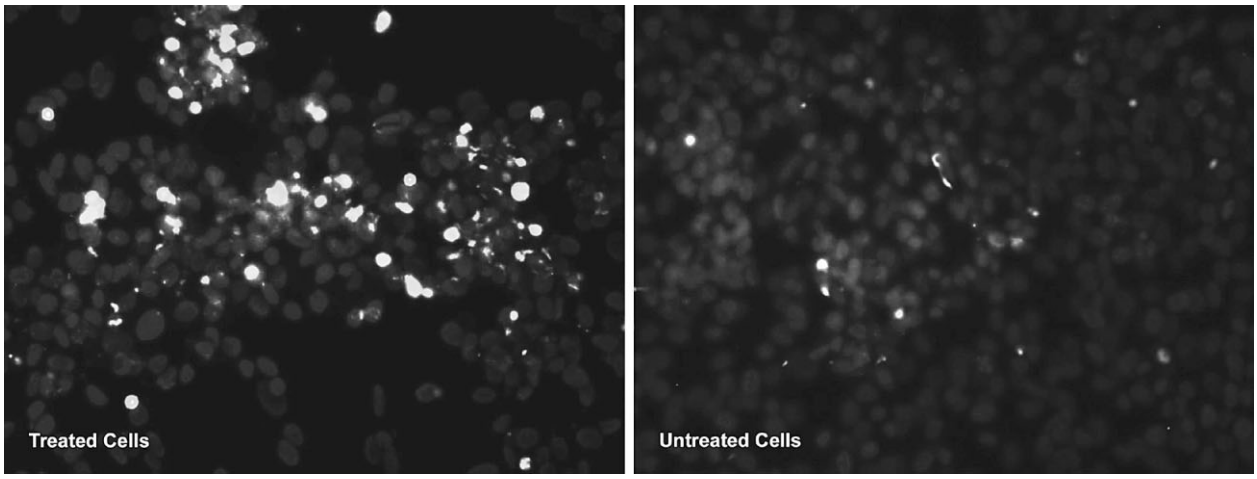

Figure 24. Images of treated (left) and untreated (right) melanoma cancer cells stained following TUNEL assay protocol. All cells are stained blue (darker circles) and apoptotic cells are also stained green (bright spots).[27]

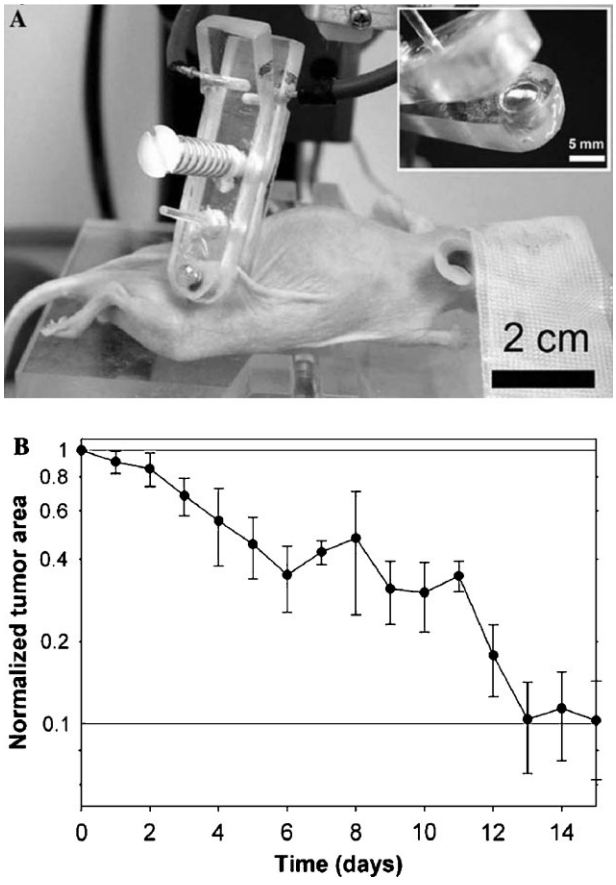

Figure 25. (A) Photograph of SKH-1 hairless mouse being treated with parallel plate electrode under isoflurane inhalation anesthesia. (Inset) Close-up of one of the plates of parallel plate electrode showing it recessed by 0.5 mm to allow a space for a conductive agar gel to be placed on it. (B) Mean change in normalized area of the transillumination image of six tumors from three mice treated with parallel plate electrodes using the same 4×100 pulse application (3×100 on day 0 and 1×100 on day 4). 40–80 kV · cm^{-1}, 300 ns pulses at 0.5 Hz.[116]

90% shrinkage of the tumor within 2 weeks.[116] Moreover, multiple treatments have resulted in complete tumor remission. Immediately after treatment, nuclei of the tumor cells shrink by 54% within minutes following the pulsing and by 68% within 3 h; while no further shrinkage was observed afterwards. Blood flow to the tumor was observed to cease as well.[116] Activity of caspases was measured using a flourogenic substrate Ac-DEVD-AFC at 0, 3, 6, and 9 h after the treatment with 100 pulses and was found to increase: 2.6-fold increase at 3 h after treatment. This can be linked to apoptosis development in the tumor cells, however this is not the only possible answer as apoptosis is an energy-dependent process and requires blood supply to the tumor. DNA damage in these cells was also observed following the treatment.

Nanoscale membrane fragmentation possibly with micelle formation through electrical charging of the lipids by these nanosecond high electric field pulses was predicted in an elegant model proposed by Pliquett et al.[117] Membrane vesicle formation (i.e., blebbing) was also observed.[124] Such occurrences can lead to temporary disruption of voltage-gated channels and ion-pump activity and make the cell temporarily permeable to molecules from the extracellular fluid – though the membrane might rapidly recover, cell might sustain too much external damage and turn on the self-suicide mechanisms, or apoptosis. These hypotheses can potentially explain the complete tumor remission observed experimentally.[116]

Non-Thermal Plasma Treatment of Cutaneous Leishmaniasis

Direct non-thermal FE-DBD plasma treatment is inherently a surface phenomenon and can be effectively applied to topical (surface) wounds and diseases. Cutaneous

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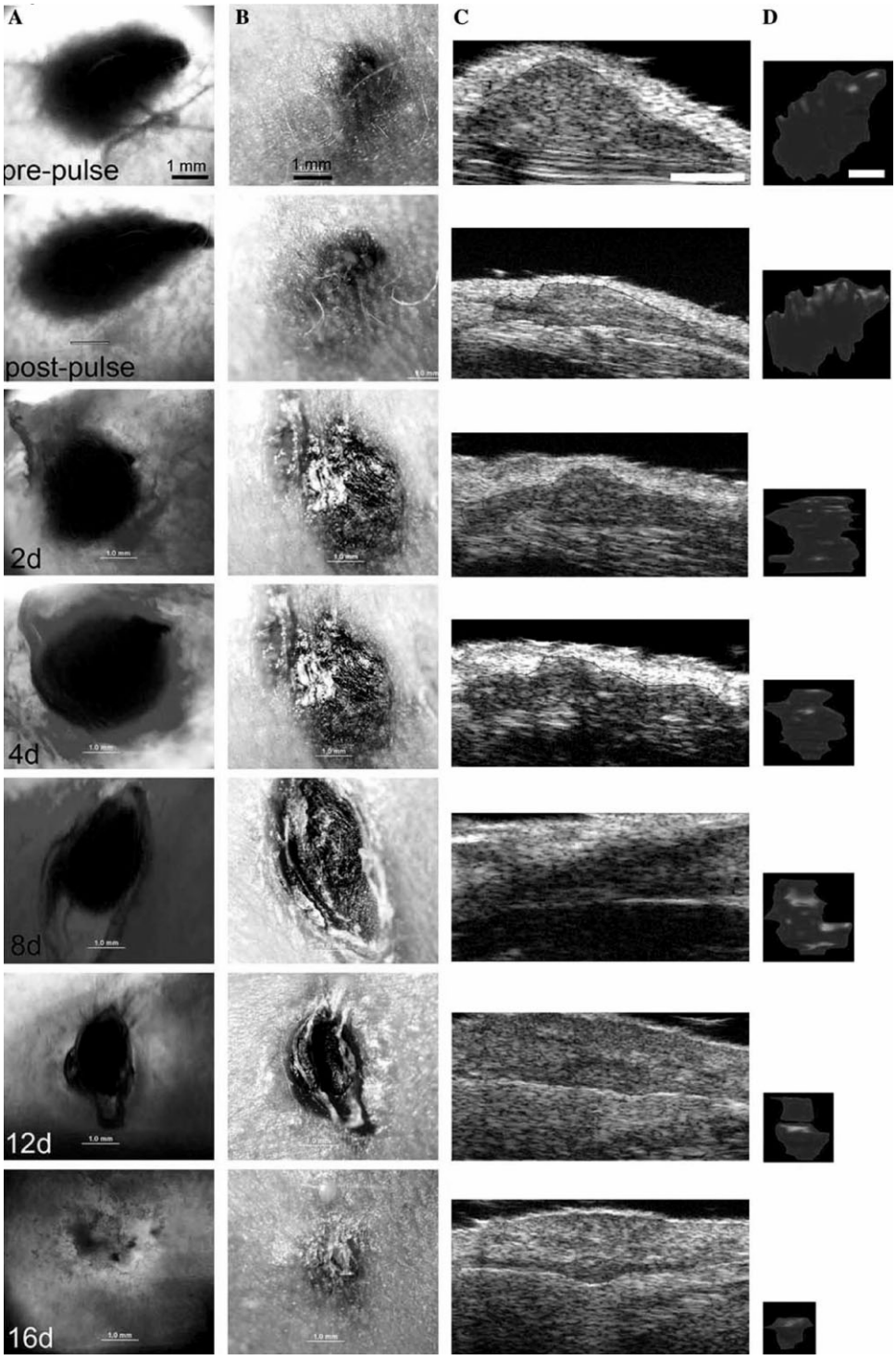

Figure 26. Typical response of a melanoma to three applications of 100 pulses (300 ns, 40 kV · cm^{-1}, 0.5 Hz) 30 min apart on day 0 followed by a single application on day 4 using a 5 mm diameter parallel plate electrode on mouse #102. Collection of seven matched sets of images of the same tumor all taken on the day indicated in the lower left corner of the transillumination image. (Column A) Transillumination image. (Column B) Surface view. (Column C) Ultrasound slice at center of tumor; (column D) 3-D reconstruction made from 100 serial ultrasound slices through tumor. Magnification is constant for each column and scale bar at top of each column represents 1 mm.[116]

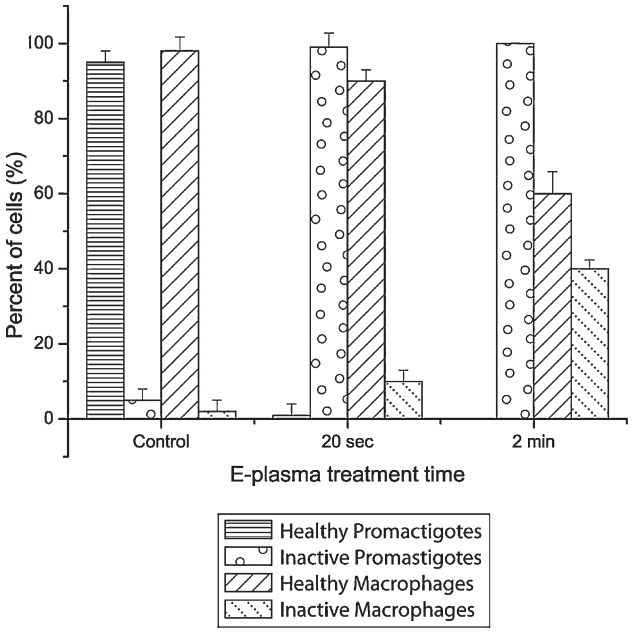

Figure 27. Inactivation of CL promastigotes.[20]

Leishmaniasis (CL) is a good example of such plasma treatment, specifically in the case of the post-Kala-azar dermal Leishmaniasis (PKDL). PKDL is a topical disease, and is a growing concern with 200 million people at risk and 500 000 cases of Leishmania per year. Few options are available today to treat the CL cases. Aside from surgical removal of the infected and surrounding tissue, there are two investigational drugs administered by the Center for Disease Control and Prevention (CDC): sodium antimony gluconate (Pentostam) and Amphotericin B (AmBisome). Both are very expensive, require frequent (in some cases daily for 2 months) visits to a trained physician with intravenous injections of the drug, and both are associated with reports of adverse side effects. Secondary infection of ulcers with skin flora is also common and must be treated. If prolonged, the CL can transform to visceral Leishmaniasis – a systemic disease where parasites enter the blood stream and settle in vital organs. UV radiation treatments of CL have been applied but the reported results indicate slow inactivation rates and high dose requirements which in turn may cause tissue damage. Non-thermal plasma is considered as an important possible solution to the medical problem.[20]

Leishmania promastigotes (parasites) have been treated at various FE-DBD plasma doses and separately human Macrophage cultures have been treated to assess both the difference in inactivation rates between two different cell lines and the dose required to inactivate the parasite.[20] About 20–30% of macrophages are inactivated in 2 min of plasma treatment, while 100% of promastigotes are inactivated in 20 s (Figure 27). Even though apoptosis is not possible in the promastigotes (they simply lack this mechanism), they did exhibit behavior indicative of something similar. For this study, promastigotes of Leishmania major were used.

Interestingly, CL promastigotes exhibit apoptosis-like behavior in a similar way that cancer cells do, though apoptosis is not possible in this type of cells. The promastigotes take longer than 24 h to grow and duplicate, so it is no surprise that the number of alive parasites is not increasing 24 h post-treatment as can be seen from Figure 28. [20] What is more interesting is that the number of metabolically inactive ("dead",' "non-viable", or simply "not moving") parasites decreases 24 h post-treatment. This is indicative of the fact that the parasites that were inactivated continued to "kill" themselves and disintegrated in the 24 h period following the treatment – a behavior similar to that of "apoptosis" in mammalian cells (although apoptosis is not possible in promastigotes similar mechanisms might exist that would drive the cell to self-disintegrate following plasma treatment).

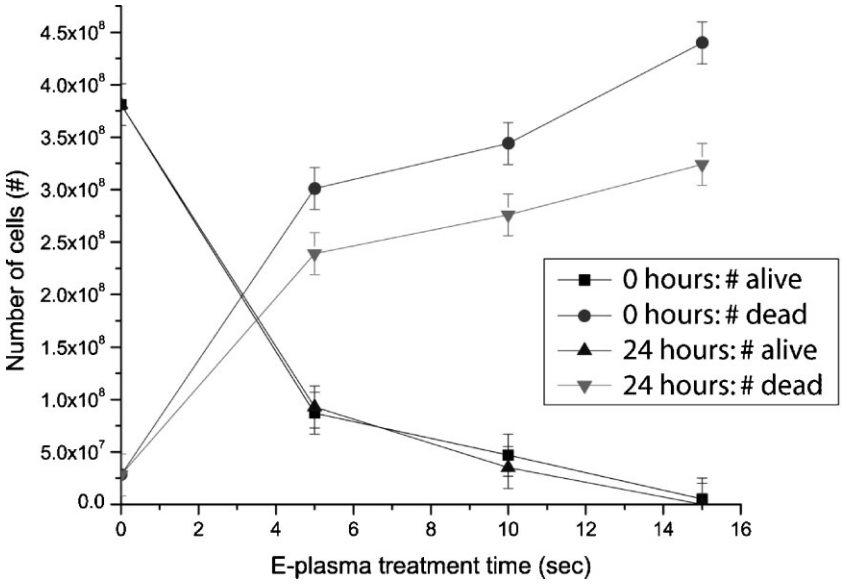

Figure 28. Apoptosis-like behavior of CL promastigotes following plasma treatment.

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Non-Equilibrium Plasma Treatment of Corneal Infections

A special microplasma system has been developed for local medical treatment of skin diseases, and especially for the treatment of corneal infections.[22] The device allows generation of plasma flows with average gas temperature not exceeding 30–40 °C. It consists of a coaxial cathode and needle-like anode, which is fixed in metal capillary. The gas is fed through the capillary to the discharge gap. The anode is connected with the positive lead of the power source and the cathode is grounded. The discharge appears on the nozzle output if the pressure on the nozzle input is higher than the atmospheric pressure. The discharge is a specific plasma sphere with the diameter ≈4 mm, atmospheric air or xenon are fed through the capillary at 0.2–0.5 atm, the discharge voltage is 1–3 kV, the pulse duration is about 50 μs, the total power was kept on the order of 1–2 W.

The medical microplasma operated in Xe radiates intensively in the UV range, and operated in air it generates excited oxygen species, ozone, oxides of nitrogen, and OH radicals.[22] Both regimes have bactericidal effects and air plasma is also able to aid in tissue regeneration via the NO-therapy mechanisms discussed above. UV radiation of Xe plasma in this case is: UVA (315–400 nm) 180, UVB (280–315 nm) 180, and UVC (200–280 nm) 330 $\mu W \cdot cm^{-2}$. UV radiation of air plasma is: UVA (315–400 nm) 53, UVB (280–315 nm) 25, and UVC (200–280 nm) 90 $\mu W \cdot cm^{-2}$. Results of probe measurements of current density, velocity, and ion concentration at different distances from the exit nozzle are shown in Table 2.

Ability of the medical microplasma system to sterilize surface has been demonstrated by Misyn et al.[125–128] Staphylococcus cultures in liquid media ($\approx 2 \times 10^6$ cfu $\cdot$ mL^{-1}) have been treated by the air plasma plume of 3 mm diameter, incubated for 24 h, and counted (Table 3).

A 6-log reduction in viable bacteria is achieved in 25 s of treatment; however the sterilization efficiency drops off with increase in volume of liquid which inhibits UV penetration and diffusion of active species generated in plasma. Nevertheless, the microplasma system should be a good solution for the treatment of living human and animal skin as the bacteria are normally at much lower concentrations on skin ($\ll 10^5$ cfu $\cdot$ cm^{-2} of skin surface[129]).

Table 2. Dependence of the current density and ion concentration of the Xenon plasma flow on the distance.[22]

Distance	Current density	Velocity	Ion concentration
mm	$mA \cdot cm^{-2}$	$cm \cdot s^{-1}$	cm^{-3}
1	2 040	2×10^4	6.4×10^{14}
1.3	2 000	1.8×10^4	3.7×10^{14}
2	240	1.2×10^4	1.5×10^{14}
3	60	7×10^3	5.3×10^{13}

Table 3. Results of Staphylococcus inactivation by air plasma.[22]

Culture volume	Plasma exposure time			
mL	s			
	0 (control)	25	50	100
1	2×10^6 cfu	0 cfu	0 cfu	0 cfu
2	4×10^6 cfu	25 cfu	0 cfu	0 cfu
3	6×10^6 cfu	1×10^6 cfu	680 cfu	460 cfu

A series of *in vitro* experiments on bacterial cultures and *in vivo* experiments on rabbit eyes[128] affirm the strong bactericidal effect of the microdischarge with minimal and reversible changes, if any, in biological tissues, even in such delicate tissues as cornea. During the investigation of plasma treatment of ulcerous dermatitis of rabbit cornea two important observations were made: (i) plasma treatment has a pronounced and immediate bactericidal effect and (ii) the treatment has an effect on wound pathology and the rate of tissue regeneration and wound healing process.

These results offered a strong ground for application of the medical microplasma system for the treatment of human patients with complicated ulcerous eyelid wounds, which is shown in Figure 29.[128] Necrotic phlegm on the surface of the upper eyelid was treated by air plasma plume of 3 mm diameter for 5 s once every few days. By the 5th day of the treatment (two 5-s plasma treatment sessions) the eyelid edema and inflammation were reduced; and by the 6th day (third session) the treated area was free of edema and inflammation and granular tissue appeared. Three more plasma treatments were administered (six total), and the patient was discharged from the hospital 6 d following the last treatment (Figure 29). The microplasma treatment is being further developed for stimulation of reparative processes in various topical wounds, tropic ulcers, chronic inflammatory complications, and other diseases of soft tissues and mucous membrane.[128]

Non-Equilibrium Plasma Treatment of Dental Cavities

A radio frequency plasma source, a *plasma needle*, was recently developed by Stoffels et al.[18,130–132] Plasma needle is a flexible hand-held device (Figure 30) consisting of a 0.3 mm diameter needle, 0.8 mm diameter Perspex

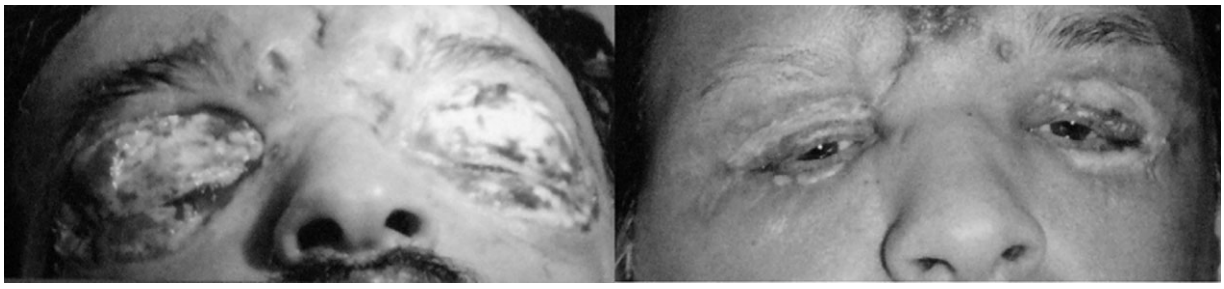

Figure 29. Result of six sessions of plasma treatment of the complicated ulcerous eyelid wound.[23]

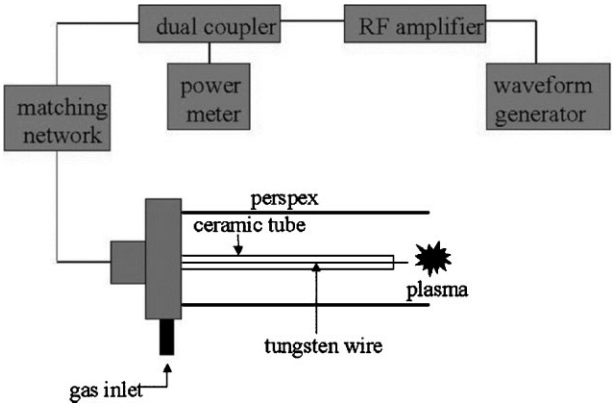

Figure 30. A principal schematic of the plasma needle setup.[18]

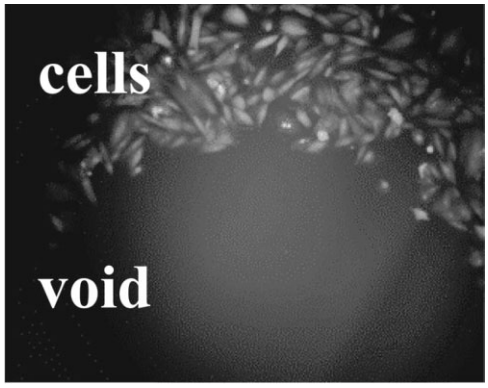

Figure 31. A void created in a cell culture, grown on a Petri dish. At the incidence of the plasma needle, the cells are removed (suspended in the medium and washed away).[18]

tube, 10 cm in length. The plasma is generated at the end of the needle at the applied frequency of 13.56 MHz. This device was successfully demonstrated in the treatment of various cell lines and inactivation of bacteria. Though the final goal of this plasma treatment is in the treatment of dental cavities, localized and precise inactivation of cancerous tissues, and in other medical applications, at the moment deeper understanding of biological mechanisms of plasma/cell interaction mechanisms is being pursued.[133] Stoffels and coworkers have thus-far worked with the following eukaryotic cells and bacteria:[18]

(i) Fibroblasts: Chinese hamster ovarian cells (CHO-K1),[134] 3T3 mouse fibroblasts,
(ii) muscle cells: rat aortic smooth muscle cells (SMC) (A7r5),[135]
(iii) endothelial cells: bovine aortic endothelial cells (BAEC),[135]
(iv) epithelial cells: human MR65 cells originating from non-small cell lung carcinoma (NSCLC),[136]
(v) Gram-positive bacteria: *Streptococcus mutans*,
(vi) Gram-negative bacteria: *E. coli*,[137,138] and
(vii) artery sections obtained from Swiss mouse (carotid and uterine arteries).[18]

Treatment by plasma needle of various cell lines causes these cells to lift off from the substrate and float away, without necrosis of these cells (Figure 31).[18] The penetration depth of this treatment is usually limited to a single cell layer when no necrosis is observed while deeper treatment is possible at higher doses where cell necrosis is also observed. In addition to a well-localized detachment, apoptosis-like behavior was observed in the detached cells; however, the level of apoptosis appears to be not too significant as about 3% of the human epithelial cells underwent apoptosis while 100% were detached.[18] Stoffels hypothesizes that the dosage requirement induction of apoptosis is very narrow and further investigation onto the biochemical mechanisms of apoptosis induction is necessary. In the treatment of *E. coli*, roughly a 2-log reduction in bacterial load was achieved in 60 s of plasma treatment at 100 mW, 1 cm away from the sample.[18,138]

Non-Equilibrium Plasma Use for Skin Regeneration

Plasma skin regeneration (PSR) is a novel skin treatment device already approved by the United States Food and Drug Administration and introduced to US markets in 2005 and European markets in 2006.[10,11] The Portrait® PSR^3 system is, basically, a radio frequency non-equilibrium plasma jet generated in nitrogen which impinges onto the tissue, damaging it slightly[10] (Figure 32). Thermal plasma here is rapidly cooled by

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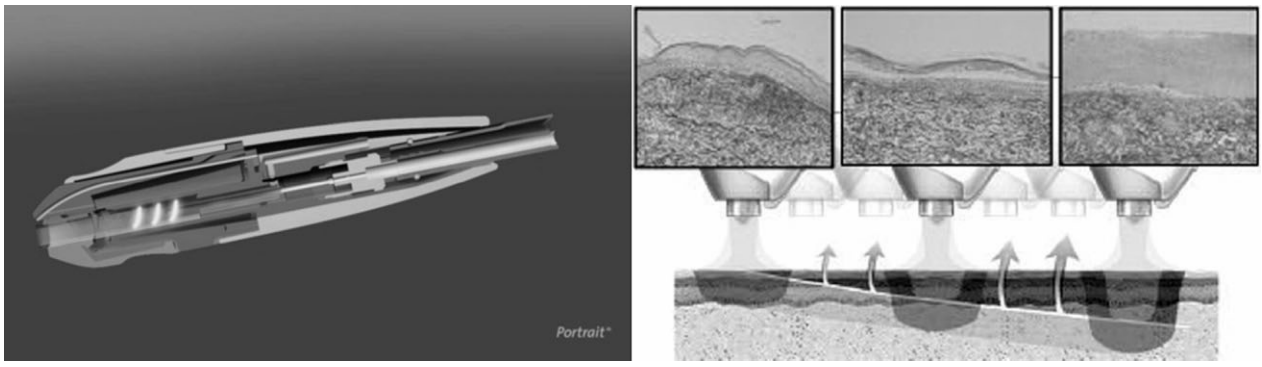

Figure 32. Portrait PSR^3 treatment head (left) and range of its treatment levels (right) which allow control of depth cleavage of skin and subsequent regeneration of skin architecture.[11]

the gas flow. This device does minimal damage to the tissue in a controlled way and was shown to be quite effective at stimulating skin regeneration, though a local anesthetic is required for the treatment and a systemic anesthetic, administered orally, is recommended.

This technology was confirmed in clinical trials in both UK and US to promote non-wounding skin rejuvenation in which superficial layers of skin are shed in the post-treatment phase.[11] Ablative-like effect, similar to that of laser skin resurfacing can also be achieved, but with higher doses (increase in either time or power).[10] The PSR^3 device was shown, at higher power (3–4 J per pulse), to induce significant skin tightening and textural improvement,[139] but with longer healing times that the treatment at a comparable dose (1–2 J per pulse, longer treatment time).[10] Overall facial rejuvenation of ≈50% and above was observed and silicone molds demonstrated ≈40% decrease in fine line depth 6 months following the treatment.[10,139] Histological analysis of full-thickness skin biopsies of the post-treatment skin confirmed the production of new collagen and remodeling of dermal architecture. Patients reported minimal discomfort following the procedure (2.3 on the ten-point scale) and reported over 60% improvement in their skin condition (Figure 33).[10]

Non-Equilibrium Plasma Treatment of Chronic Foot and Leg Ulcers

Treatment of chronic foot and leg ulcers was shown to be possible using a microwave argon plasma torch which is, as above, rapidly cooled by fast gas flow (Figure 34).[140,141] Argon is passed at 3 slpm through a 135 mm tube with six aluminum electrodes to which 2.45 GHz microwave power is allied. This torch operates at roughly 100 W.[141] Interestingly, the plasma afterglow generated in this way is able to sterilize many types of bacteria in minutes of the treatment. Some of the bacteria tested were *S. epidermidis*, *E. coli*, *S. pyogenes*, *B. cereus*, *P. aeruginosa*, etc. Additionally *C. albicans* yeast was tested. While the torch was effective in inactivation on all the organisms tested, the dose requirements and the size of the inactivation area varied by organism.[140]

Figure 35 shows an example of the results of a 2 min treatment of bacteria. An inactivation circle is clearly visible and is much larger than the diameter of the nozzle. Similar effects are observed on other types of bacteria as

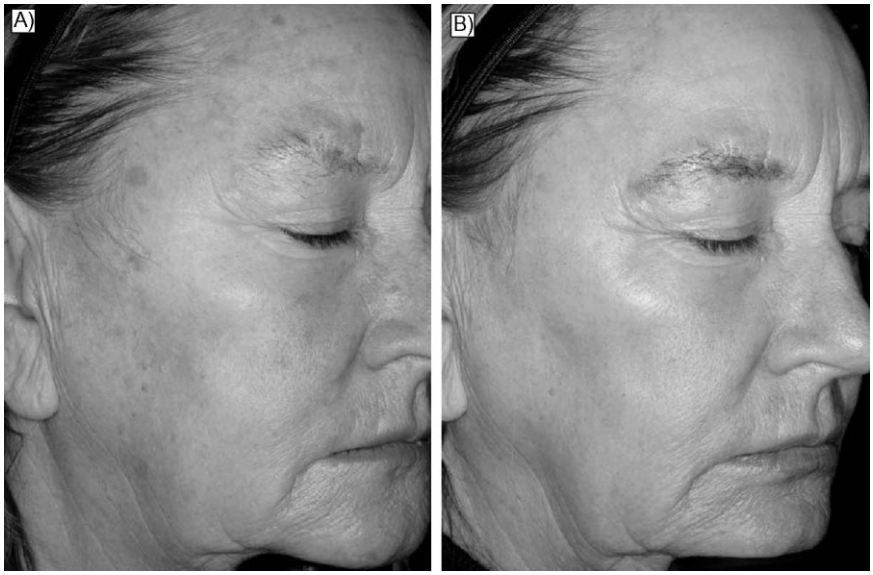

Figure 33. Facial appearance before (A) and 3 months after (B) plasma skin regeneration, with improvement in pigmentation and skin texture. Investigator-rated improvement on the 9-point facial rhytid scale changed from 7 (before regeneration) to 6 (after regeneration); patient-rated improvement in overall skin rejuvenation was 90%.[10]

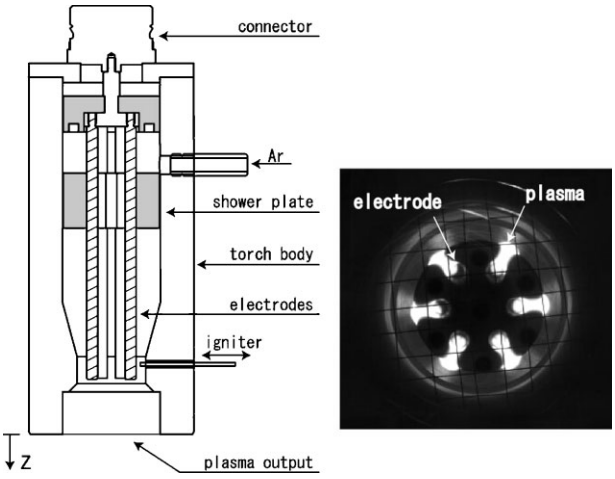

Figure 34. Microwave plasma torch schematic (left) and plasma output photo (right).[141]

well.[140,141] While the effect on bacteria is quite evident, Morfill and coworkers do not observe any effect on human blood or skin tissue (Figure 36).[140] Histological evaluation of the treated tissue revealed no or little difference as compared with untreated skin. Only after 10 min of the treatment vacuolization of keratinocytes of the basal epidermis becomes evident; curiously, these effects are observed *in vitro* on dead skin, only minutes following removal from live patient.[140] In any case, no effect is observed on blood or tissue in 2 min of the treatment which is quite sufficient to significantly reduce bacterial load.

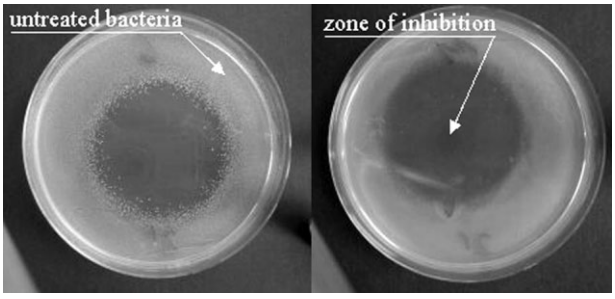

Figure 35. Bacterial cultures on agar plates after 2 min of plasma treatment. Left: methicillin-resistant *S. aureus* (Gram positive) right: *Burkholderia cepacia* (Gram negative).[140]

Conclusion

Although we attempted to provide a rather broad overview of the use of non-thermal atmospheric pressure plasma in medicine, we cannot be sure that all relevant work was included because of the rapid growth of publications in this important area. This review clearly demonstrates emergence of a new trend: the use of plasma in medicine not just for tissue ablation or cauterization, but also for much more subtle modalities of medical *therapy*. As shown above, there are clear examples of indirect "hot" plasma applications that are coming to therapeutic medical practice already: the use of NO generated in thermal plasma for skin regeneration using nitrogen and/or air plasma jets. Medical applications of "cold" plasmas such as tissue sterilization, treatment of corneal infections, blood coagulation, treatment of cancers, leishmaniasis, treatment of dental diseases, and others are in the beginning stages of their development. Though preliminary results are very promising, bringing cold plasmas into medical practice will not be very easy. Besides the usual safety concerns, the main obstacle remains the lack of fundamental understanding of physical, chemical, and biological mechanisms of interaction between non-thermal atmospheric pressure plasma and living cells, tissues, organs, and the whole organism. While it is clear that direct interaction with hot plasma occurs mainly through heat transfer, direct interaction with non-thermal plasma depends on a myriad of variables including various charged species, electric field, UV, radicals, electronically excited atoms and molecules. Complex synergies may be responsible for different effects. Not only dose, but also the effects of dose rate may be of

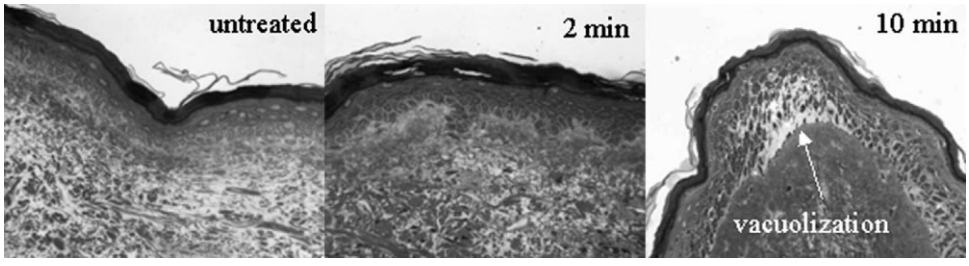

Figure 36. Histological images of skin samples, treated *ex-vivo*. After 2 min no changes could be observed with respect to the untreated control sample. Vacuolization of keratinocytes can be observed after 10 min.[140]

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critical importance for treatments. Fundamental studies in the area of plasma medicine are further complicated by the true interdisciplinary nature of investigations that are required. Despite all these challenges the number of researchers interested in plasma medicine is growing rapidly. This growth will probably continue in the near future not only because the payoffs are substantial, but also because plasma is one of the few remaining areas of modern technology whose application in medicine has been barely explored.

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