Applied Plasma Medicine

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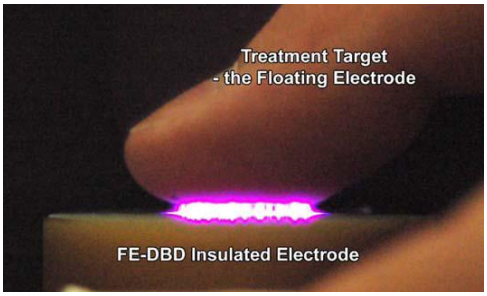
Table of contents (400 characters max):



Non-equilibrium atmospheric pressure discharges are becoming quite an intriguing new tool in a medical setting. Applications of non-thermal plasmas in medicine for sterilization of living tissue, in vivo and in vitro blood coagulation, wound healing and tissue regeneration, plasma-treatment of various skin diseases like Melanoma cancer, Leishmaniasis, various ulcers, and others are reported.

Summary (Abstract) (700 characters max):

In this review 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. The non-equilibrium plasma will be shown to be non-destructive to tissue, safe, and effective in inactivation of various parasites and foreign organisms.

Keywords: non-thermal plasma, non-equilibrium plasma, air plasma, dielectric barrier discharges (DBDs), atmospheric pressure glow discharges (APGDs), sterilization, disinfection, blood coagulation, skin diseases, wound healing, tissue regeneration, plasma medicine.

CONTENTS

1. 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. Lightning, 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 *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 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.

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 (also 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 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 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 vs. ozone produced in plasma can be tuned. It is also possible to tune micro-structure 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.

2. ANIMAL AND HUMAN LIVING TISSUE STERILIZATION

2.1. 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. Thus Figure 1 illustrates direct plasma treatment (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.

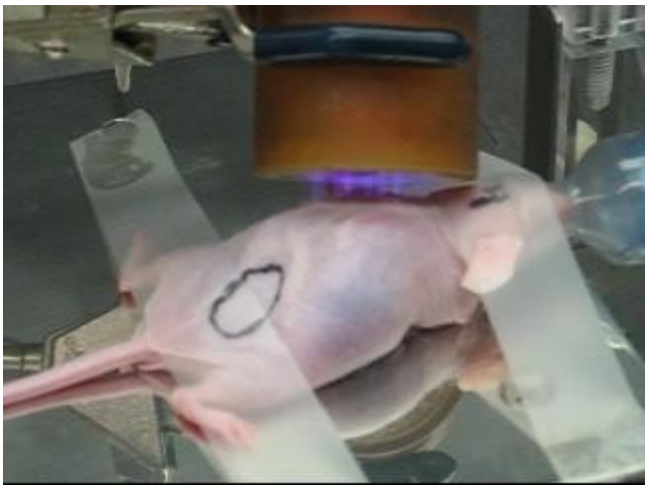


Figure 1. Non-damaging room temperature and atmospheric pressure FE-DBD plasma for treatment of living tissue: animal treated for up to 10 minutes remains healthy and no tissue damage is observed visually or microscopically [20].

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 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 dielectric barrier discharge (FE-DBD), which operates under the 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 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/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 thus far have been achieved by organization of the FE-DBD in the pulsed mode with pulse duration below 30-100 nsec [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.

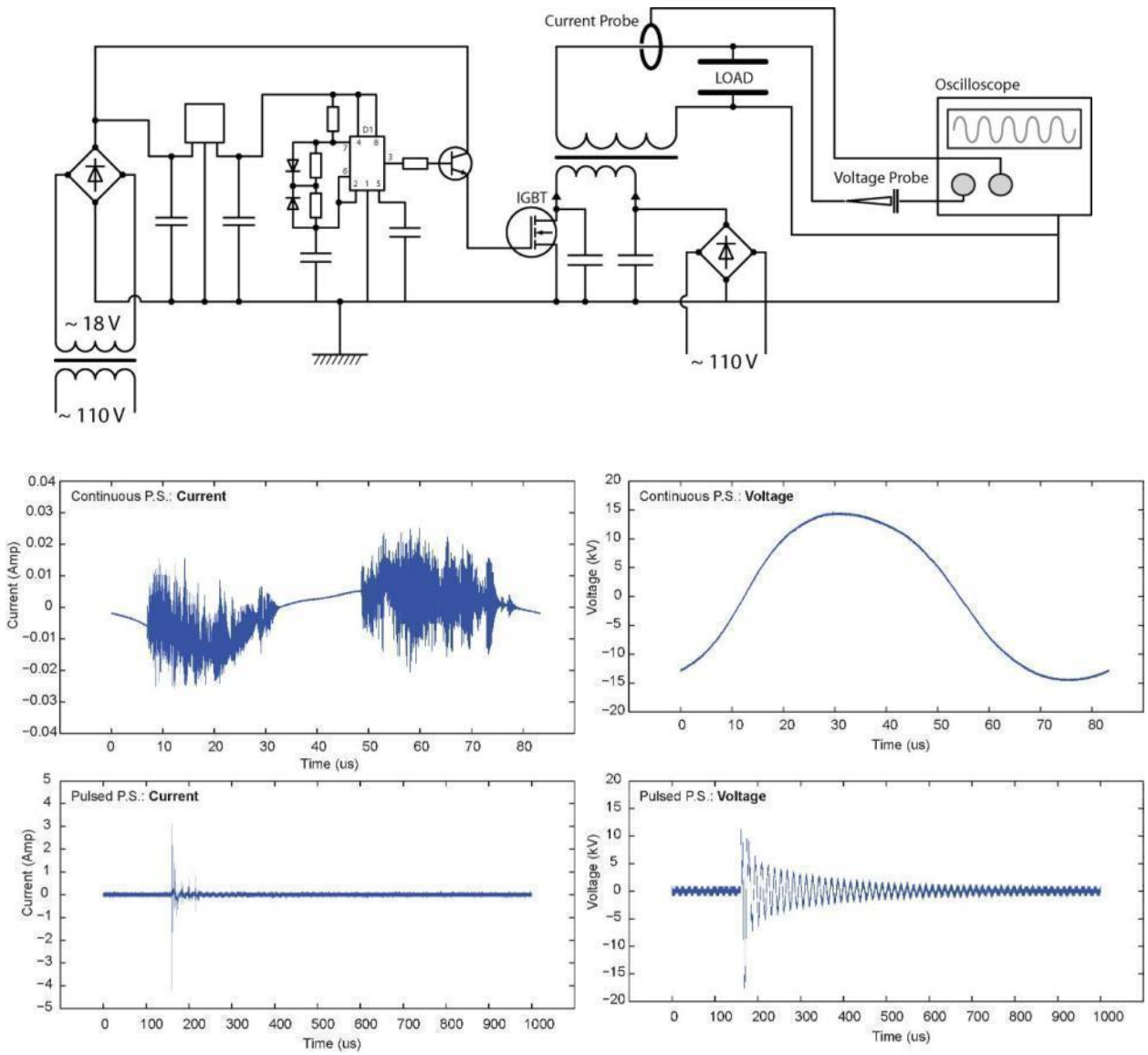


Figure 2. Schematic of FE DBD discharge plasma power supply (P.S.). Voltage and current oscillograms [19].

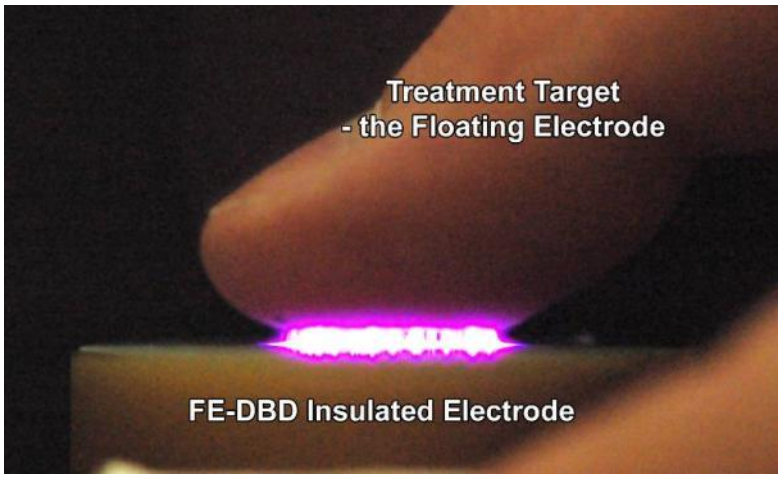


Figure 3. FE-DBD applied directly to the human body [20].

2.2. 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 seconds of treatment (Table 1). Similar level of the skin flora sterilization using *indirect* DBD approach requires 120 seconds and longer of plasma treatment at the same level of the discharge power [26].

Table 1. Bacteria sterilization results (in cfu/mL) [26].

Original Concentration	5 sec of FE-DBD	10 sec of FE-DBD	15 sec 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

Sterilization of the skin flora on cadaver skin samples occurred in the experiments generally after 4 seconds of treatment in most cases and 5-6 seconds 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, burns etc., as well as treatment of internal organs in gastroenterology.

2.3. 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 (in other words: problem of toxicity) is the key issue of all plasma medicine. Obviously, a topical treatment which damages 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 minutes without any visible or microscopic change in the tissue, as was verified with tissue sectioning and staining via Hematoxylin & Eosin (H&E) procedure, which is illustrated in Figure 4.

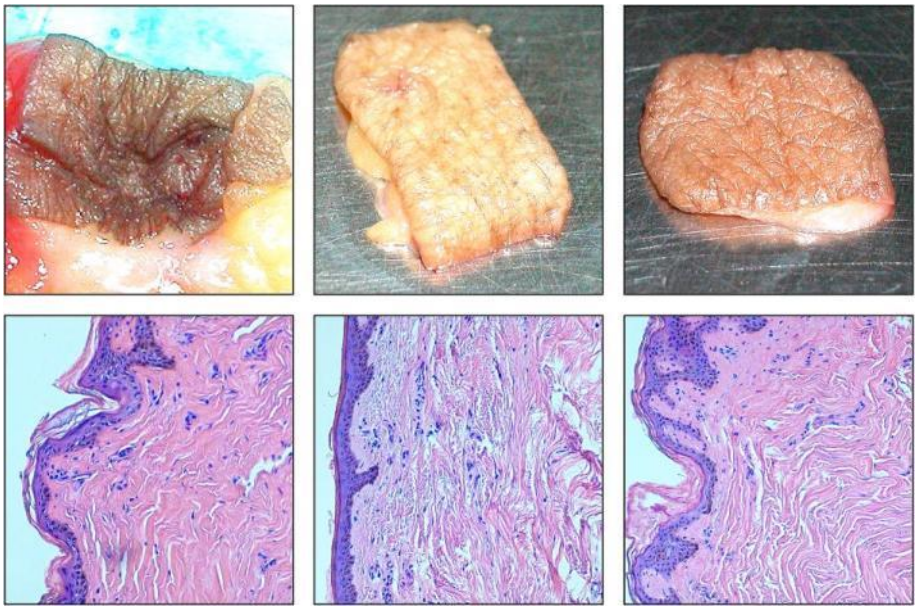


Figure 4. Photos (top) and tissue histology (bottom) of cadaver skin samples after FE-DBD treatment: control (left), 15 seconds of treatment (center), and 5 minutes of treatment (right) – no visible damage is detected [19].

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

Based on the experimental matrix, a dose of 10 minutes at 0.6 Watt/cm^2 was deemed maximum acceptable prolonged treatment and a dose of 40 seconds at 2.3 Watt/cm^2 was deemed maximum acceptable high-power treatment. Histological (microscopic) comparison of control SKH1 skin sample with toxic and non-toxic plasma doses shows 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 seconds at high-power treatment of 0.8 ±0.2 W/cm^2 and at 10 ±4 sec 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. Following the investigation on mice, an investigation on pigs was carried out achieving similar results.

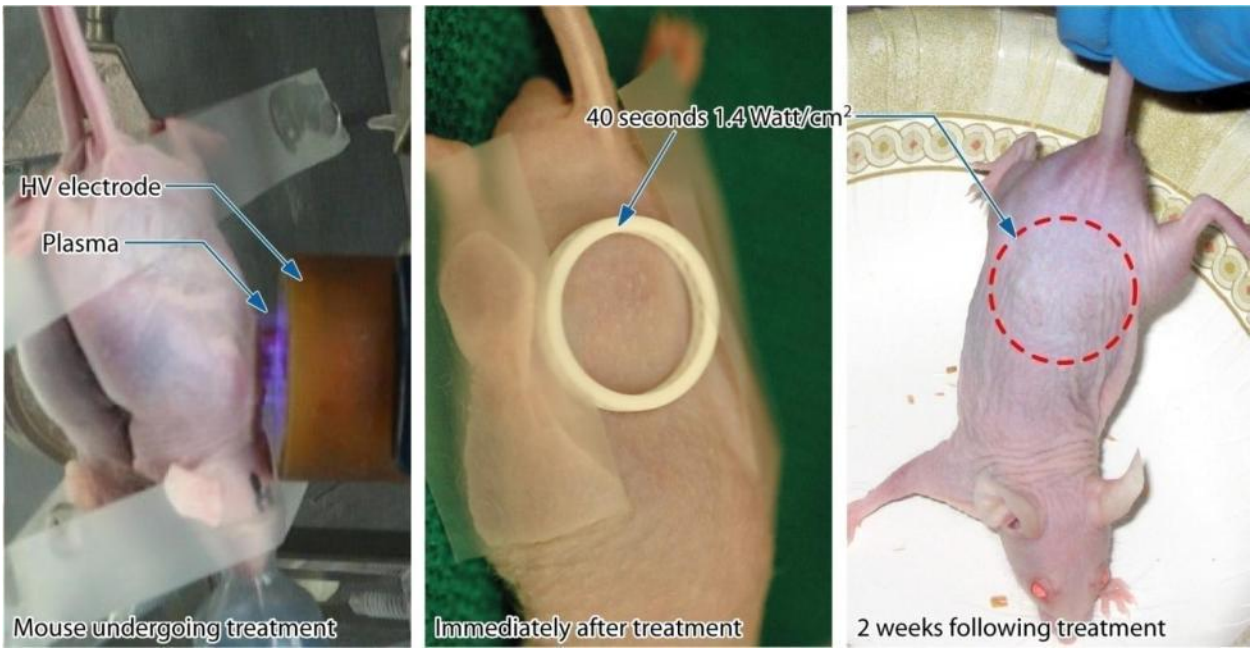


Figure 5. Animal remains fine after a reasonably high plasma dose (more than 10 times higher than needed for skin sterilization) [20].

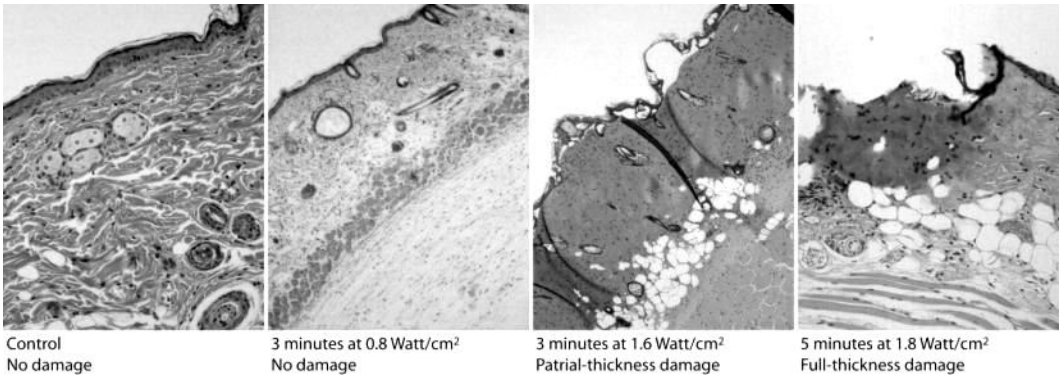


Figure 6. Histology of toxic and non-toxic to animal's skin plasma doses, compared to untreated skin [20].

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].

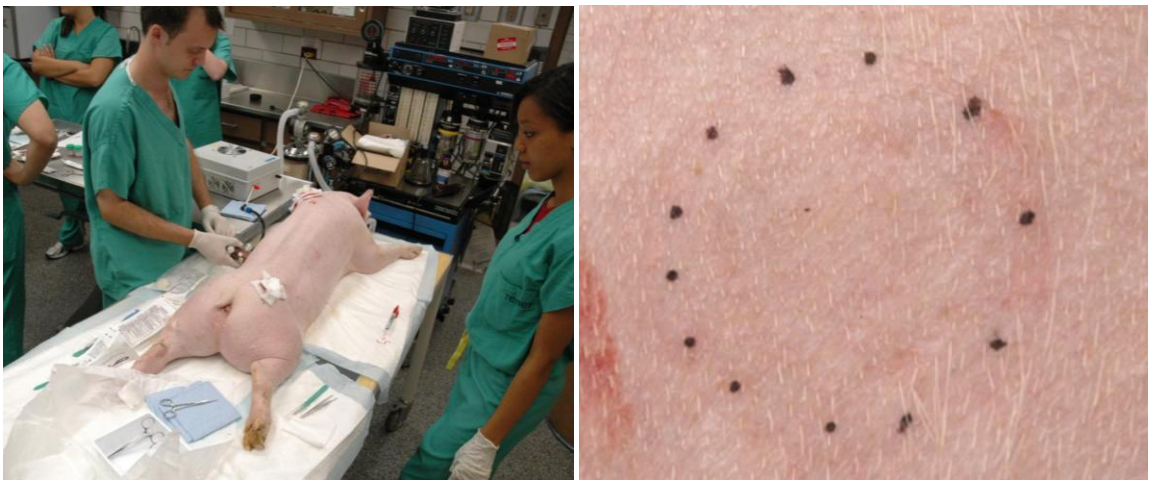


Figure 7. Live pig undergoing treatment (left) and appearance of pig skin immediately following 5 minutes of FE-DBD treatment (right). Animal survives the treatment and no visible or microscopic tissue damage is observed [32].

It should be especially noted that level of toxicity due to the FE-DBD plasma treatment of living tissue not only depends on the treatment dose (discharge power, and treatment duration), but also strongly depends on shape of voltage applied to the discharge. Pulsing of the DBD discharges can essentially decrease its damaging ability. Application of nanosecond pulses completely prevents formation of streamers and therefore the DBD micro-discharges, which helps significantly decrease toxicity of the direct plasma-medical treatment of living tissue [29, 30].

2.4. 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 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.

Drs. Alexeff and Laroussi and their colleagues reported a rather interesting modification of a conventional dielectric barrier discharge – a Resistive Barrier Discharge (RDB) [2, 45, 46]. Main feature of the RDB is that is 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 *E. Coli*, *B. 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 his colleagues [47-50] show that a barrier discharge in helium with

small additions of oxygen is not only able to sterilize bacteria but is able to influence metabolic changes in the organisms surviving the treatment [48]. This raises an intriguing question – can plasma-resistant bacteria emerge? Due to 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 Dr. Laroussi and colleagues: 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 micro-organisms.

Dr. Massines and her colleagues propose a DBD discharge in N_2/N_2O mixture for micro-organism 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 ultraviolet radiation on bacteria as apparently they cannot be neglected, even in plasmas where doses of UV are lower than in that proposed by Dr. Massines [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. Dr. Becker and colleagues [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 ultraviolet (UV) radiation, and sources suitable for micro-sized 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. Dr. Becker et al. show efficient inactivation of *B. subtilis* spores (1 log reduction in ~100 seconds) and *B. stearothermophilus* spores (1 log reduction in ~90 seconds) without damage to the substrate; more interestingly they are able to inactivate bio-films of *Chromobacterium violaceum* CV026 achieving 2 log reduction in ~ 5 minutes and 3 log reduction in ~60 minutes of plasma afterglow treatment [58]. In general, these microplasmas have not yet found a niche in medicine directly through 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].

Dr. Roth and his colleagues 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 increasing 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 volatile organic compounds 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 encourages to consult a recent publication by Dr. Roth et al. [64]. Of note, however, is a less recent publication from Dr. 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 micro-organism load, are ranging from 6 seconds for *E. coli* bacteria to 6.8 minutes for Bacteriophage Phi X 174 virus. Additionally survivability of these organisms on different substrates is addressed comparing glass, agar, and polypropylene 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].

Dr. Kong and his colleagues 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. Dr. 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 complete decontamination of them with removal not only of bacterial load but 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.

3. NON-THERMAL PLASMA-ASSISTED BLOOD COAGULATION

3.1. 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 form of the so-called cauterization devices: argon plasma coagulators (APC), 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].

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.

3.2. 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 minutes, while a similar drop treated for 15 seconds by FE-DBD plasma coagulates in under 1 minute (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 seconds – blood is coagulated and tissue surrounding the treatment area looks "cooked", however the temperature of the cut remains at room temperature (even after 5 minutes of FE-DBD treatment) and the wound remains wet, which could potentially decrease healing time as is the case with topical wound sealing agents [82, 83].

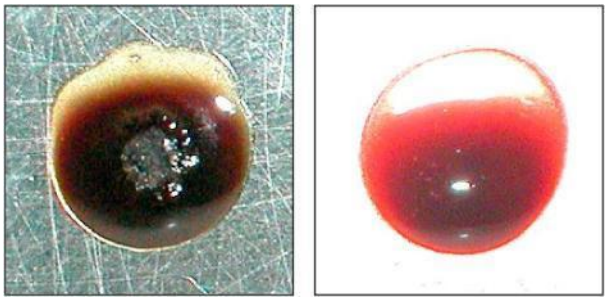


Figure 8. Blood drop treated by FE-DBD: 15 seconds of FE-DBD (left) and control (right); photo was taken 1 minute after the drops were placed on brushed stainless steel substrate; blood was treated immediately after it was placed on metal [19].

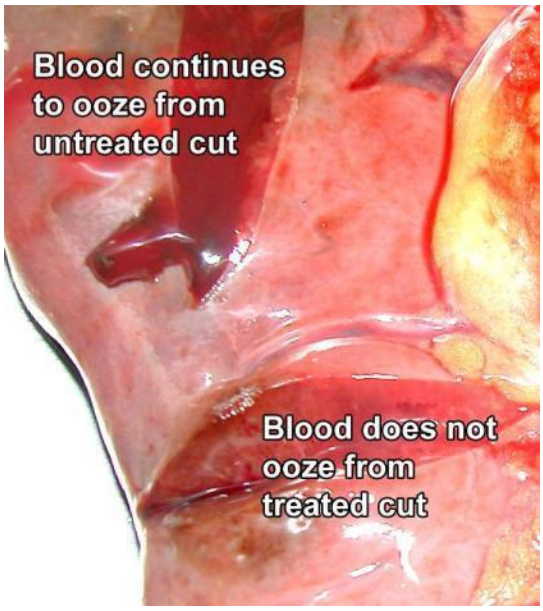


Figure 9. 30 seconds of FE-DBD treatment of human spleen: blood coagulates without tissue damage. Top cut: blood continues to ooze from an untreated area; bottom cut: blood coagulates while the wound remains wet [19].

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 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].

3.3. 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]. Fifteen seconds 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 in Fridman et al. and Balasubramanian et al. [20, 32, 86, 87].

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

3.4. 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: (1) plasma can coagulate both normal and anti-coagulated blood, but

the rate of coagulation depends on the anti-coagulant used; (2) 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; (3) plasma changes natural concentration of clotting factors significantly, thus promoting coagulation; (4) effects delivered by plasma are non-thermal and are not related to gas temperature or the temperature at the surface of blood; (5) 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.

(1) Anticoagulants like sodium heparin bind thrombin, in the coagulation cascade thus slowing coagulation; while sodium citrate or ethylene diamine tetraacetic acid (EDTA), are 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.

(2) 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 $[Ca^{2+}R^{2-}] + H^{+}_{(H2O)} \overset{k_{Ca}|k_{-Ca}}{\Longleftrightarrow} [H^{+}R^{2-}]_{(H2O)} + Ca^{2+}_{(H2O)}$ 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 micro-electrode. Calcium concentration was measured immediately after plasma treatment and remained almost constant for up to 30 s of treatment and then increased slightly for prolonged treatment times of 60 s and 120 s. Although, plasma is capable of coagulating anti-coagulated blood within 15 seconds, 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 sec) is less than the natural variation of pH, which indicates that the coagulation is probably not due to the pH change in blood.

(3) 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).

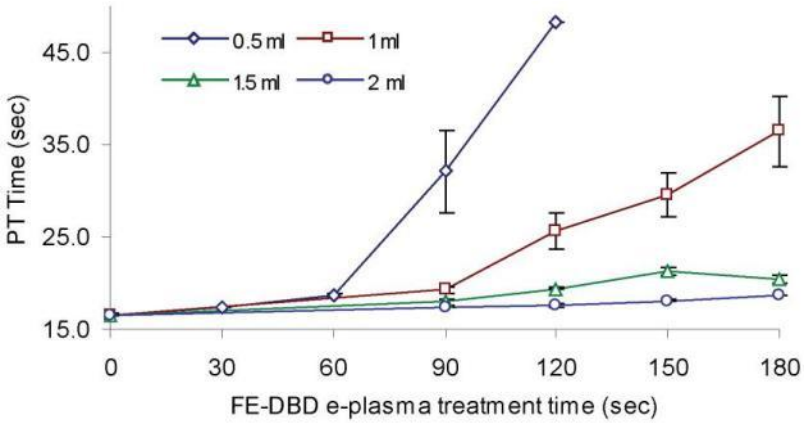


Figure 11. Prothrombin (PT) time for blood samples of different volumes with the same surface area of FE-DBD treatment [19].

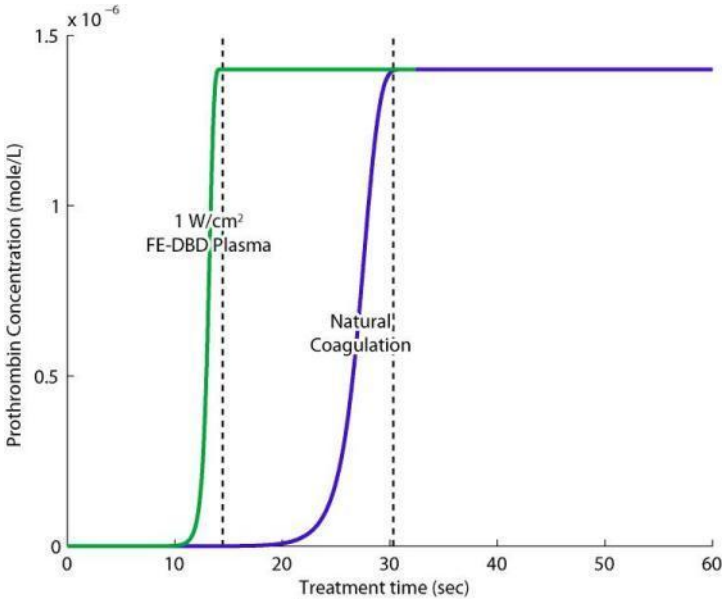


Figure 12. Prothrombin kinetics: two-fold decrease in clot formation time with plasma treatment [19].

(4) 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.

(5) 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. 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 dynamic light scattering (DLS). Thus non-thermal plasma selectively affects proteins (specifically, fibrinogen) participating in the natural coagulation mechanism.

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 minutes and its total protein weight and protein activity analyzed via fluorescence spectroscopy. Total protein weight, or the amount of protein in the treated solution, remains practically intact after up to 90 seconds of treatment (Figure 13); while the enzymatic (catalytic) activity of this protein drops to nearly zero after 10-15 seconds 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.

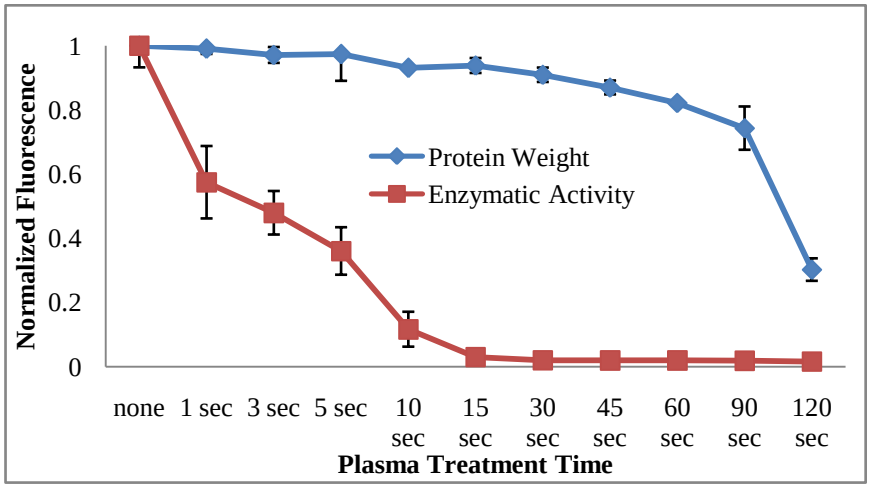


Figure 13. Total protein weight compared to enzymatic activity of Trypsin following plasma treatment [92].

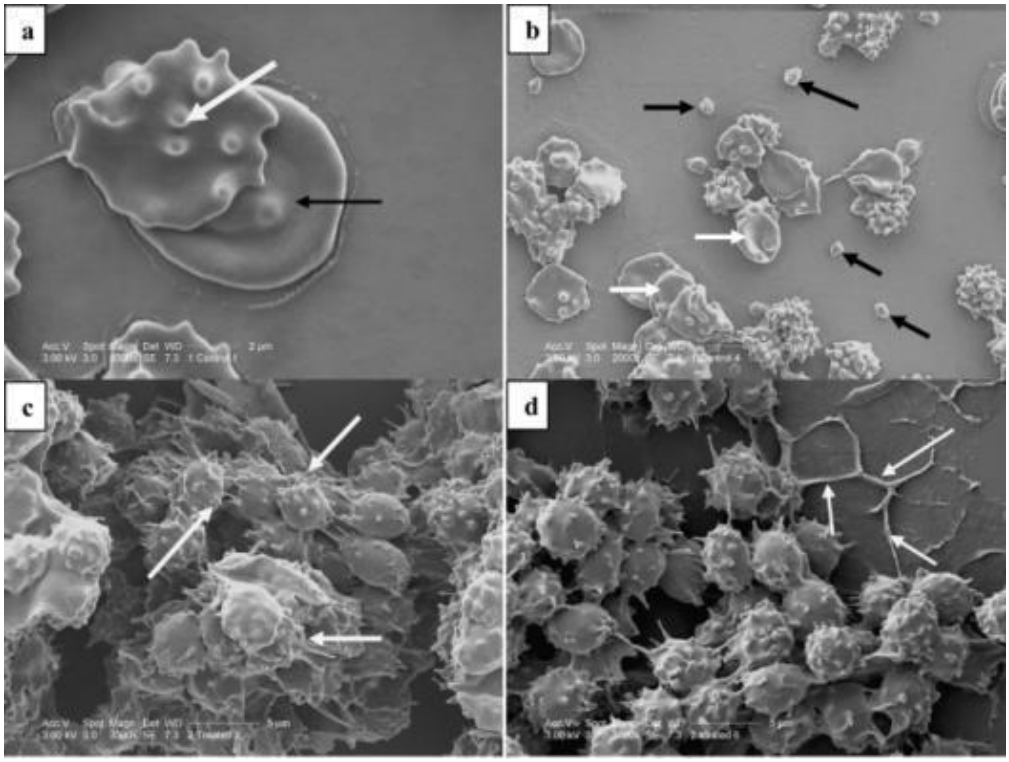


Figure 14. SEM images of untreated (a,b) and treated (c,d) anti-coagulated whole blood. (a) whole blood (control) showing single activated platelet (white arrow) on a red blood cell (black arrow); (b) whole blood (control) showing many non-activated platelets (black arrows) and intact red blood cells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

4. PLASMA-ASSISTED WOUND HEALING AND TISSUE REGENERATION

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

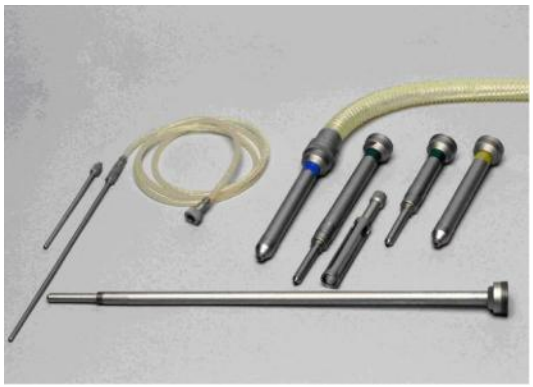
Effective use of plasma in surgery has been first demonstrated in 1960s: plasma afterglow jet of an inert gas has been applied for tissue sectioning with instant blood coagulation. Because of that plasma-surgical devices got a long-standing name of "plasma scalpel" in the hospitals (see, for example, 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 to 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 micro-compressor, 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 ~10^7-10^8 K/sec. Commonly, the cooling rate of plasma jets is on the order of ~10^6 K/sec. Thus, to achieve the cooling rate of ~10^7-10^8 K/sec, 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 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.

a b



c

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

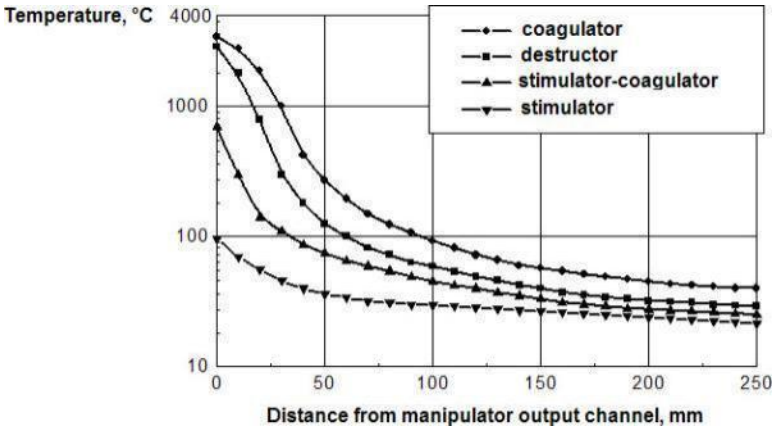


Figure 16. Temperature in the center of the gas flow jet for different manipulators.

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 the manipulator; second group of regimes – regimes of treatment of bio-tissues by completely cooled (20ºC) NO-containing gas flow, 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, broncoscope, cystoscope, rectascope, etc).

4.2. 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 in investigation of 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 antimicrobial and antitumor defense, etc. In pathology, NO plays a major role in adaptation, stress, tumor growth, immunodeficiency, cardiovascular, liver, and 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 antimicrobial 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 (2 orders of magnitude) than normal when NO is formed via constructional NOS: endothelial (eNOS) and neuronal (nNOS).

Exogenic NO is also crucial in trauma wound processes. Activity of inducible NO-synthase (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 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.

4.3. 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 five days 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-containing gas flow (500 ppm). Without the NO treatment, the results indicate high content of endogenic NO in wound tissues (10.3 ±2.3 μM). The liver of the animals with the wound contained 2.3 ±1.4 μM of DETC-ironmononitrosyl complex (IMNC); while the control group (without the wound) – only 0.06 ±0.002 μM.

Animals without the wound were used for investigation of penetration capability of gaseous exogenic NO through undamaged tissues of abdominal wall. Treatment by NO-containing gas flow was performed for 60 and 180 seconds. 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. Two minutes following the 180 second treatment a maximum signal was registered in the bowels 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 treatment by exogenic NO of the wound tissues. If the animal was euthanized 30-40 minutes 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-minute interval. This is probably due to 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 antioxidant defense mechanisms first via the increase in the levels of reducing agents (thioles, ascorbate, etc.), and then via activation of synthesis of antioxidant enzymes. Thirty to fourty minutes following the wound treatment by exogenic NO, activation of the first cascade of antioxidant 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, 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 micro-vessels under the influence of exogenic NO (500 ppm) lasts with varying intensity up to 10-12 hours, while the lifetime of NO molecules is no more than 10-15 seconds [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 of DNA synthesis (tested by inclusion of C^{14} thymidine) and to a lesser extent (1.5 times) increase of 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 hours following a 30-second treatment.

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

- direct bactericidal effect (through formation of peroxynitrite in the reaction: $NO+O_2^- \rightarrow ONOO^-$);
- induction of the phagocytosis of bacteria and necrotic detrite by neutrophils and by macrophages;

- inhibition of the free oxygen radicals, which exert pathogenic influence, and also possible activation of the antioxidant protection;
- normalization of microcirculation due to the vasodilatation, the anti-aggregation and anticoagulant properties of NO, that improves vascular trophicity and nutrient exchange;
- improvement of nerve conductance;
- regulation of immune-deficiencies, which are common in wound pathology;
- 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;
- direct induction of proliferation of fibroblasts and synthesis by them of proteins;
- increase in or regulation of collagen synthesis;
- regulation of apoptosis in remodeling of granular and fibrous tissues;
- influence on the proliferation of keratinocytes and thus on the epithelization of the wound.

4.4. 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 lower extremities with an area of 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 (pO_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 days in the NO-therapy regime. In the control group proteolytic and antimicrobial 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 of 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 to 4 times depending on the initial ulcer size (Figure 17). Larger ulcers tended to close faster than smaller ones. LDF investigation of microcirculation in the tissues of trophic ulcers showed that following the NO-therapy normalization of pathologic changes in the amplitude-frequency characteristics of the microvasculature and activation of regulatory mechanisms.

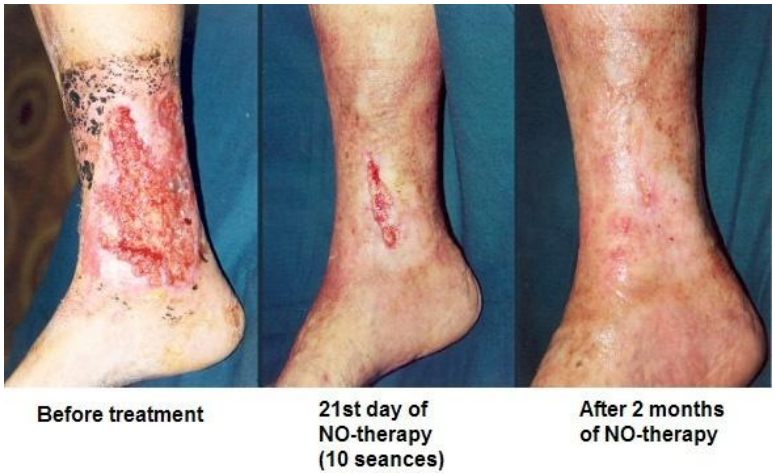


Figure 17. Dynamics of the healing of venous trophic ulcer during NO-therapy.

By 14-18 days 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).

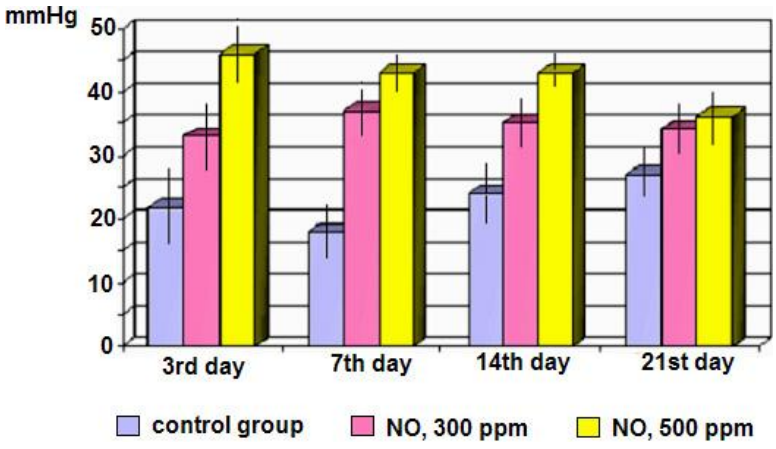


Figure 18. Dynamics of pO_2 level during NO-therapy of venous trophic ulcers.

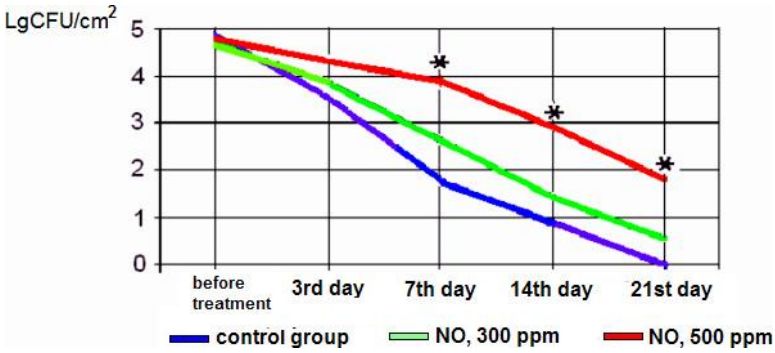
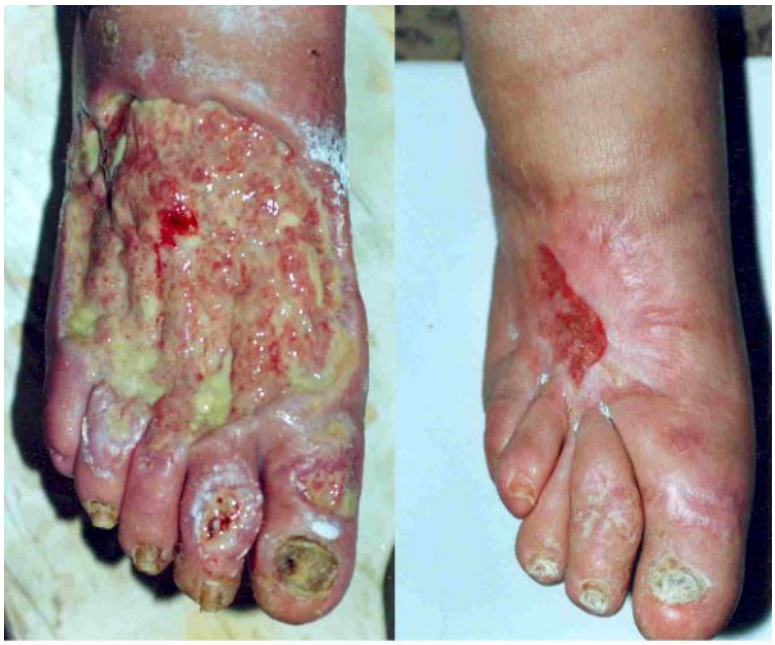


Figure 19. Dynamics of bacterial contamination of trophic ulcers during NO therapy (- statistical significance, $p<0.01$).*

Using the plasma-generated NO for local treatment of ulcerous and necrotic tissues in patients with diabetes (diabetic foot ulcer) has been demonstrated by Shulutko, Antropova, and Kryuger [101]. Patients were selected for this study following two 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 10 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 average by 2.3 times). In the cases of large ulcerating wounds, the necessity for amputation decreased 1.9 times (Figure 20).

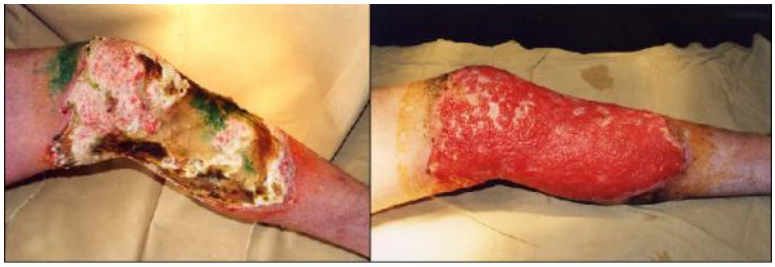


Before treatment

After 4,5 months of NO-therapy
(3 courses of NO-therapy;
12 seances per course)

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

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^{6-8} colony forming units (cfu) per gram of tissue to 10^{1-2}. 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 average 18.5 ±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 (Fig. 12.44), by day 7-10 of treatment were clear of the pyonecrotic exudate and were beginning to be covered by granular tissue, in other words these wounds we ready for dermautoplasty.



a b

Figure 21. Healing dynamics of the festered burn wound in process of NO-therapy: a) prior to the beginning of treatment; b) after 5 sessions of NO-therapy.

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 days of 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 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 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 postoperative hypertrophic and keloid scars, and for treatment of already formed ones: softening the scar tissue, decrease of fibrosis and preventive maintenance of their relapse with the surgical removal.

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

Possibility of directing of the plasma-generated NO-containing gas flows through puncture needles, vent lines, and endoscopic instruments, and also the inhalation method of action considerably enlarges prospects for the plasma NO-therapy in 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 (Shekhter et al., 1998, 2005):

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 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. The inhalation application in 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 of 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 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 treatment of open fractures.

Gynecology. Effectiveness of the plasma NO-therapy in combination with the coagulation by air plasma was shown with 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 days 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 postoperative 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 intraocular 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 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 ear, nose and throat (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 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 postoperative 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.

5. NON-THERMAL PLASMA TREATMENT OF VARIOUS DISEASES

5.1. 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 vs. 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 ~$1.5 \cdot 10^6$ per dish [27]. On days 3-5 of cell development, the cells were treated with the FE-DBD plasma for 5, 10, 15, 20, or 30 seconds. 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 following treatment, 1, 3, 24, 48, and 72 hours following 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 seconds. Following treatment, cells were harvested at 3, 24, 48 and 72 hours 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. Figure 22 demonstrates cell survival numbers after 5, 10, 20, and 30 seconds of 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 hours following treatment the total number of cells continues to decrease significantly (Figure 22).

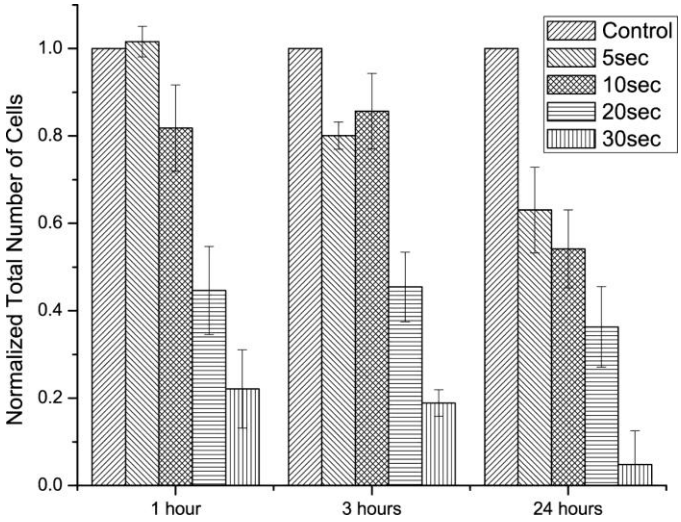


Figure 22. Results of FE-DBD treatment of Melanoma cancer cells: Control, 5, 10, 20, and 30 seconds, counted 1, 3, and 24 hours post-treatment [27].

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 seconds by plasma separately and then the cells were placed in this 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 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. Figure 23 presents an observation of groups of cells treated by plasma for 5 seconds and observed for a 3-day 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 seconds of plasma treatment does not inactivate cells immediately; however, cell growth slows down significantly, and the number of dead cells increases 24 hours after treatment, which is indicative of cell death occurring long after the treatment.

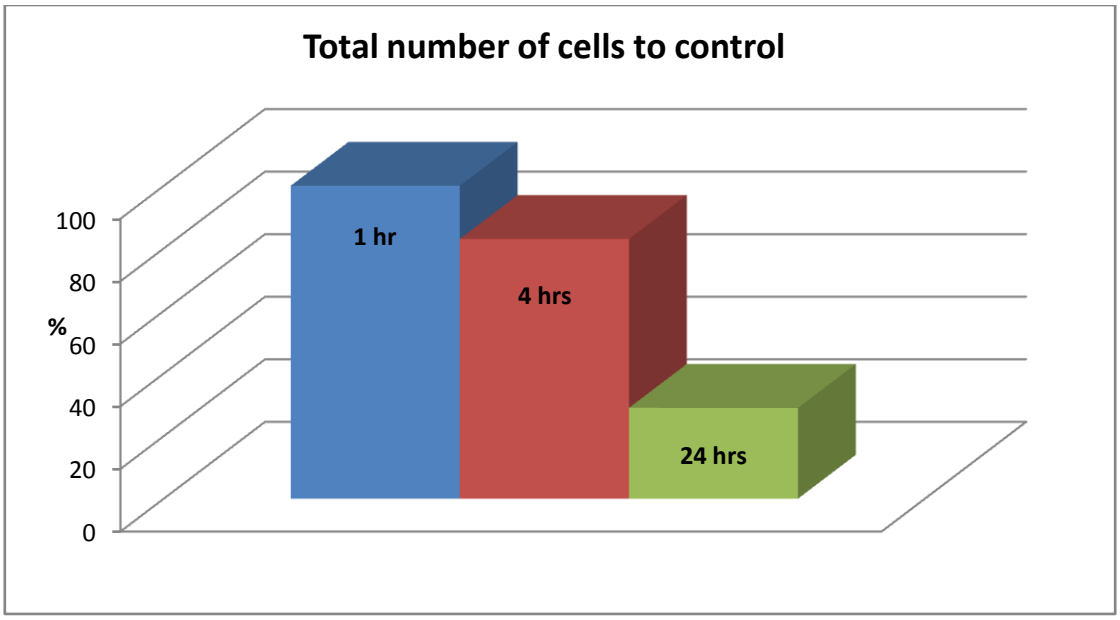


Figure 23. Results of observation of treated and untreated cells for a three-day period: total number of cells before and after FE-DBD treatment. Treatment time: 5 seconds [27].

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 seconds were then incubated and stained for DNA fractionation 24 hours 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 hours 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 vs. 3.2% in the control group 72 hours following treatment.

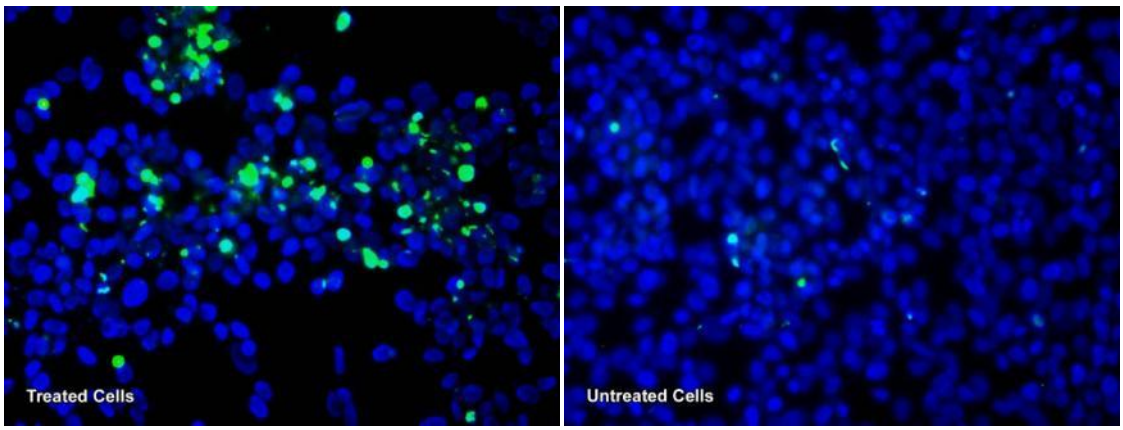


Figure 24. Images of treated (left) and untreated (right) Melanoma cancer cells stained following TUNEL assay protocol. All cells are stained blue (darker circles) and apoptotic cells are also stained green (bright spots) [27].

Thus, FE-DBD plasma can kill Melanoma skin cancer cells through necrosis at higher treatment doses (15 seconds and over at 1.4 W/cm^2) which are still significantly below the threshold of damaging healthy tissue [19, 20]. Very low doses of FE-DBD (5 seconds at 0.8 W/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 to 24 hours 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. Dr. Schoenbach and his colleagues [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 40 kV/cm pulses of 300 ns duration lead to 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 hours; 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 hours after 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 Dr. 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 hypothesis can potentially explain the complete tumor remission observed experimentally [116].

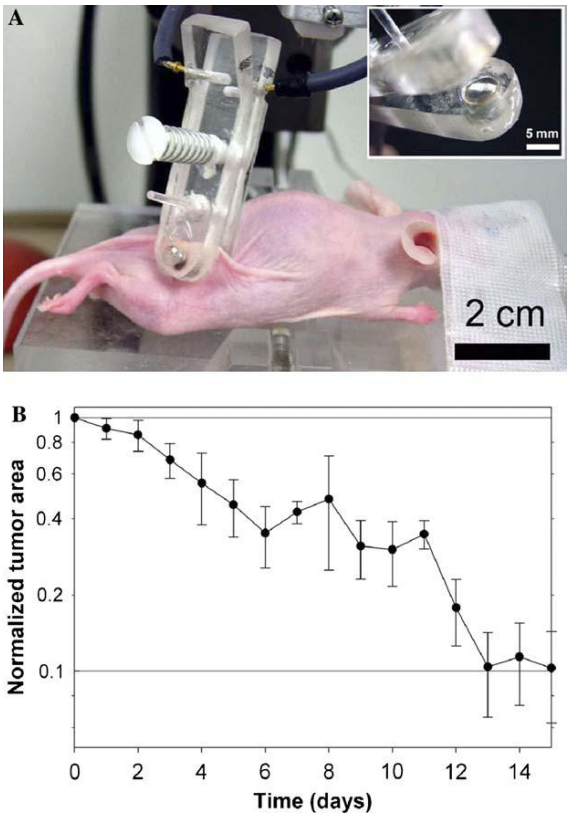


Figure 25. (A) Photograph of SKH-1 hairless mouse being treated with parallel plate electrode under isoflurane inhalation anesthesia. (Inset) Close-up of one of the plates of parallel plate electrode showing it recessed by 0.5 mm to allow a space for a conductive agar gel to be placed on it. (B) Mean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

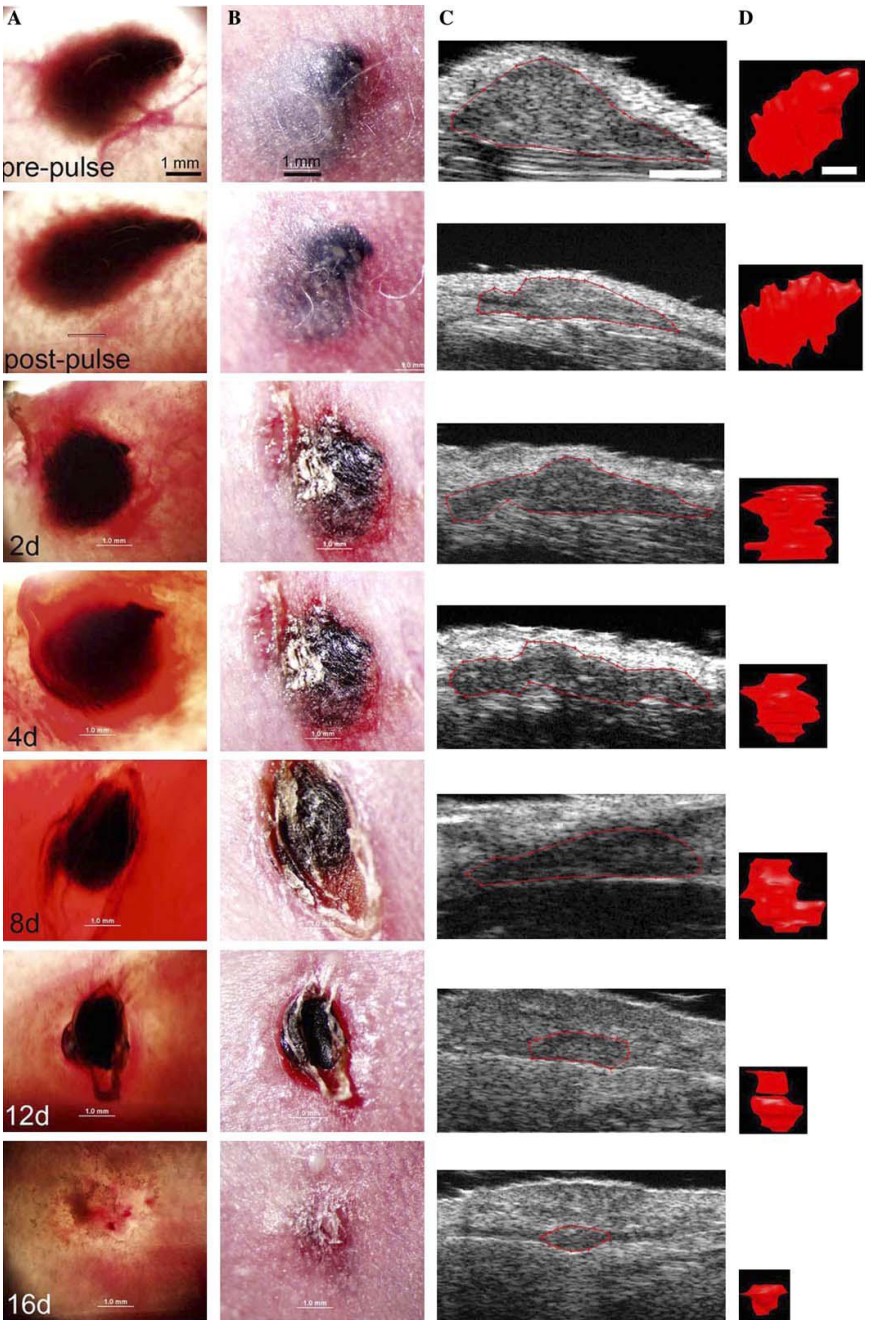


Figure 26. Typical response of a melanoma to three applications of 100 pulses (300 ns, 40 kV/cm, 0.5 Hz) 30 min apart on day 0 followed by a single application on day 4 using a 5 mm diameter parallel plate electrode on mouse #102. Collection of seven matched sets of images of the same tumor all taken 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].

5.2. 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 wounds and diseases. Cutaneous Leishmaniasis (C.L.) 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 Cutaneous Leishmaniasis 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 Cutaneous Leishmaniasis can transform to visceral Leishmaniasis – a systemic disease where parasites enter the blood stream and settle in vital organs. Ultra Violet (UV) radiation treatments of C.L. 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 minutes of plasma treatment, while 100% of promastigotes are inactivated in 20 seconds (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.

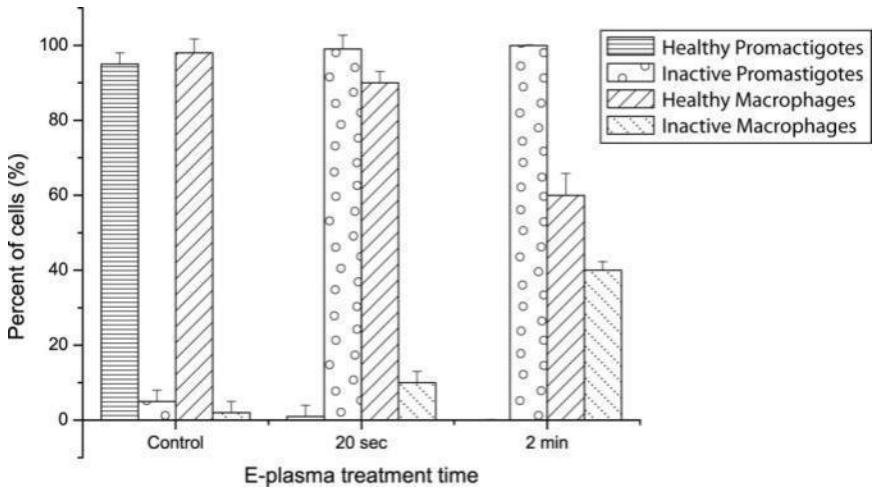


Figure 27. Inactivation of Cutaneous Leishmaniasis promastigotes [20].

Interestingly, C.L. 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 hours to grow and duplicate, so it is no surprise that number of alive parasites is not increasing 24 hours 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 hours post-treatment. This is indicative of the fact that the parasites that were inactivated continued to "kill" themselves and disintegrated in the 24 hour period following the treatment.

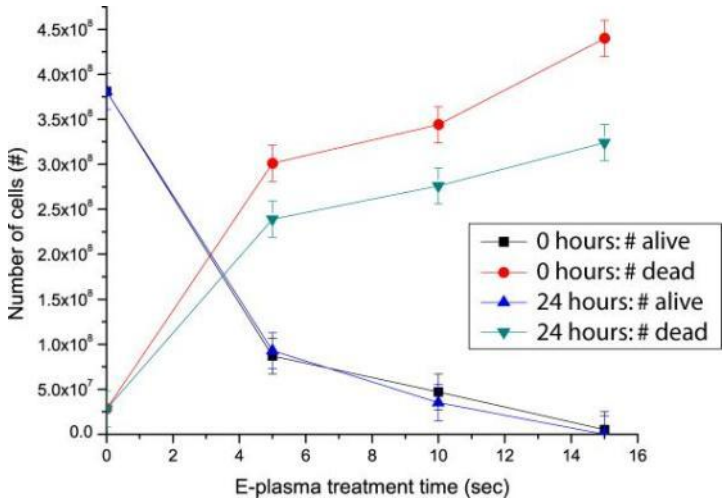


Figure 28. Apoptosis-like behavior of Cutaneous Leishmaniasis promastigotes following plasma treatment.

5.3. Non-Equilibrium Plasma Treatment of Corneal Infections

A special micro-plasma system has been developed for local medical treatment of skin diseases, and especially for 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 micro-plasma 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 able also 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 $\mu W/cm^2$, UVB (280-315 nm) 180 $\mu W/cm^2$, and UVC (200-280 nm) 330 $\mu W/cm^2$. UV-radiation of air plasma is: UVA (315-400 nm) 53 $\mu W/cm^2$, UVB (280-315 nm) 25 $\mu W/cm^2$, and UVC (200-280 nm) 90 $\mu W/cm^2$. Results of probe measurements of current density, velocity, and ion concentration at different distances from the exit nozzle are shown in Table 2.

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

Distance, mm	**1**	**1.3**	**2**	**3**
Current density, mA/cm^2	2040	2000	240	60
Velocity, cm/sec	$2\cdot10^4$	$1.8\cdot10^4$	$1.2\cdot10^4$	$7\cdot10^3$
Ion concentration, cm^{-3}	$6.4\cdot10^{14}$	$3.7\cdot10^{14}$	$1.5\cdot10^{14}$	$5.3\cdot10^{13}$

Ability of the medical micro-plasma system to sterilize surface has been demonstrated by Misyn et al. [125-128]. Staphylococcus cultures in liquid media (~$2\cdot10^6$ cfu/ml) have been treated by the air plasma plume of 3 mm diameter, incubated for 24 hours, and counted (Table 3).

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

Culture volume,	**Plasma exposure time, sec**

ml	0 (control)	25	50	100
1	$2 \cdot 10^6$ cfu	0 cfu	0 cfu	0 cfu
2	$4 \cdot 10^6$ cfu	25 cfu	0 cfu	0 cfu
3	$6 \cdot 10^6$ cfu	$1 \cdot 10^6$ cfu	680 cfu	460 cfu

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

A series of in vitro experiments on bacterial cultures and in vivo experiments on rabbit eyes [128] affirm the strong bactericidal effect of the micro-discharge 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: 1) plasma treatment has a pronounced and immediate bactericidal effect, and 2) 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 micro-plasma system for 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 seconds once every few days. By the 5th day of treatment (two 5-second 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 six days following the last treatment (Figure 29). The micro-plasma 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].

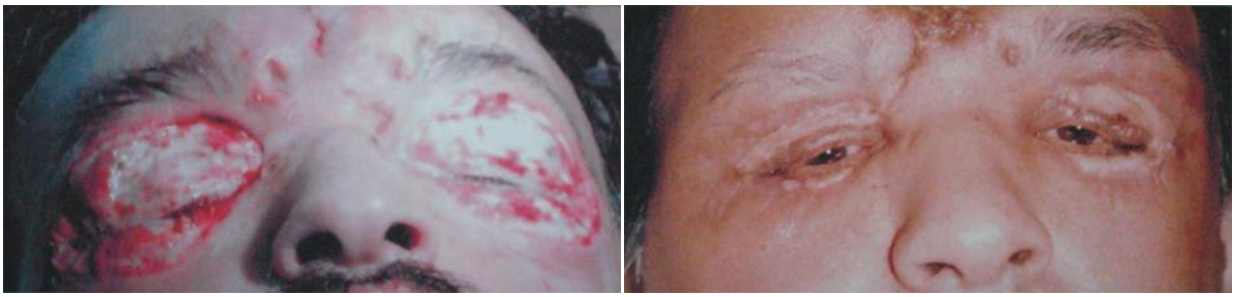


Figure 29. Result of six sessions of plasma treatment of the complicated ulcerous eyelid wound [23].

5.4. Non-Equilibrium Plasma Treatment of Dental Cavities

A radio frequency plasma source, a *plasma needle*, was recently developed by E. 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 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 treatment of various cell lines and inactivation of bacteria. Though the final goal of this plasma treatment is in 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]. Dr. Stoffels and her colleagues have thus-far worked with the following eukaryotic cells and bacteria [18]:

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

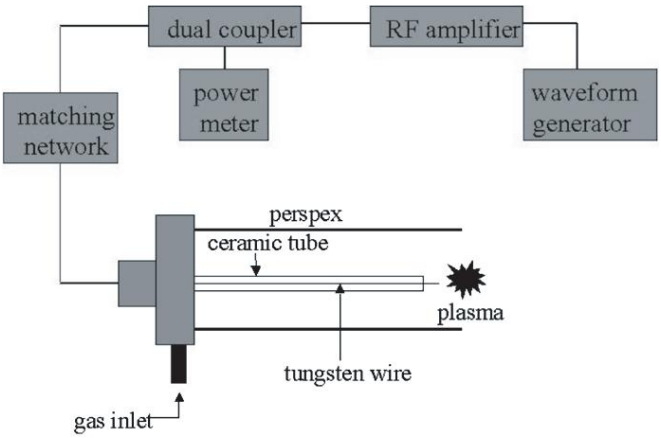


Figure 30. A principal schematic of the plasma needle setup [18].

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]. Dr. Stoffels hypothesizes that the dosage requirement induction of apoptosis are very narrow and further investigation onto the biochemical mechanisms of apoptosis induction are necessary. In treatment of *E. Coli* roughly a 2-log reduction in bacterial load was achieved in 60 seconds of plasma treatment at 100 mW, 1 cm away from the sample [18, 138].

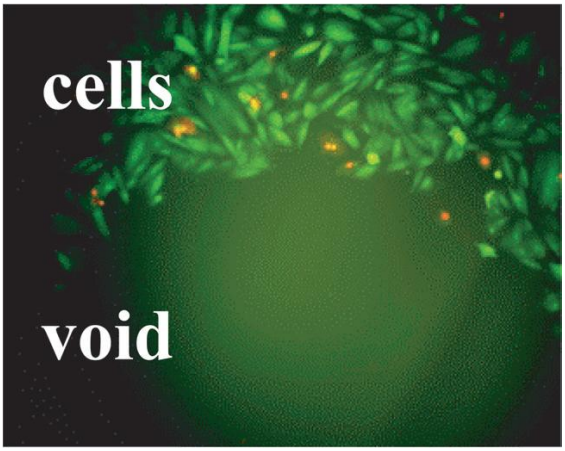


Figure 31. A void created in a cell culture, grown on a Petri dish. At the incidence of the plasma needle, the cells are removed (suspended in the medium and washed away) [18].

5.5. 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 U.S. markets in 2005 and European markets in 2006 [10, 11]. The Portrait® PSR^3 system is, basically, a radio frequency thermal plasma jet generated in nitrogen which impinges onto the tissue, damaging it slightly [10] (Figure 32). 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 treatment and a systemic anesthetic, administered orally, is recommended.

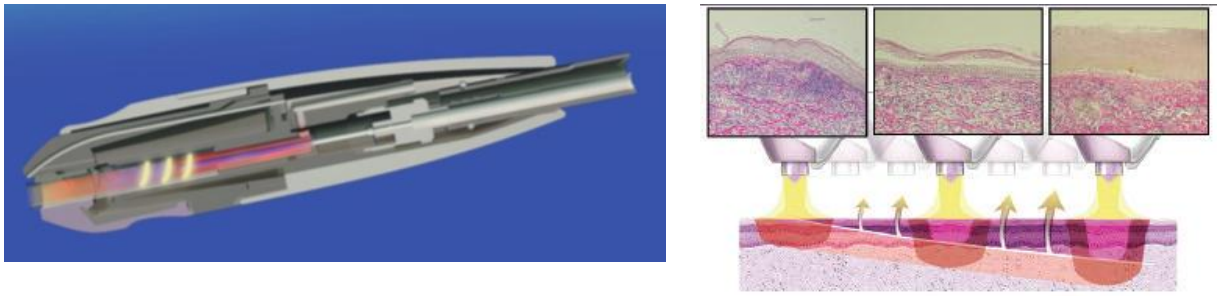


Figure 32. Portrait PSR^3 treatment head (left) and range of its treatment levels (right) which allow control of depth cleavage of skin and subsequent regeneration of skin architecture [11].

This technology was confirmed in clinical trials in both U.K. and U.S. 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 treatment [10, 139]. Histological analysis of full-thickness skin biopsies of 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 10 point scale) and reported over 60% improvement in their skin condition (Figure 33) [10].

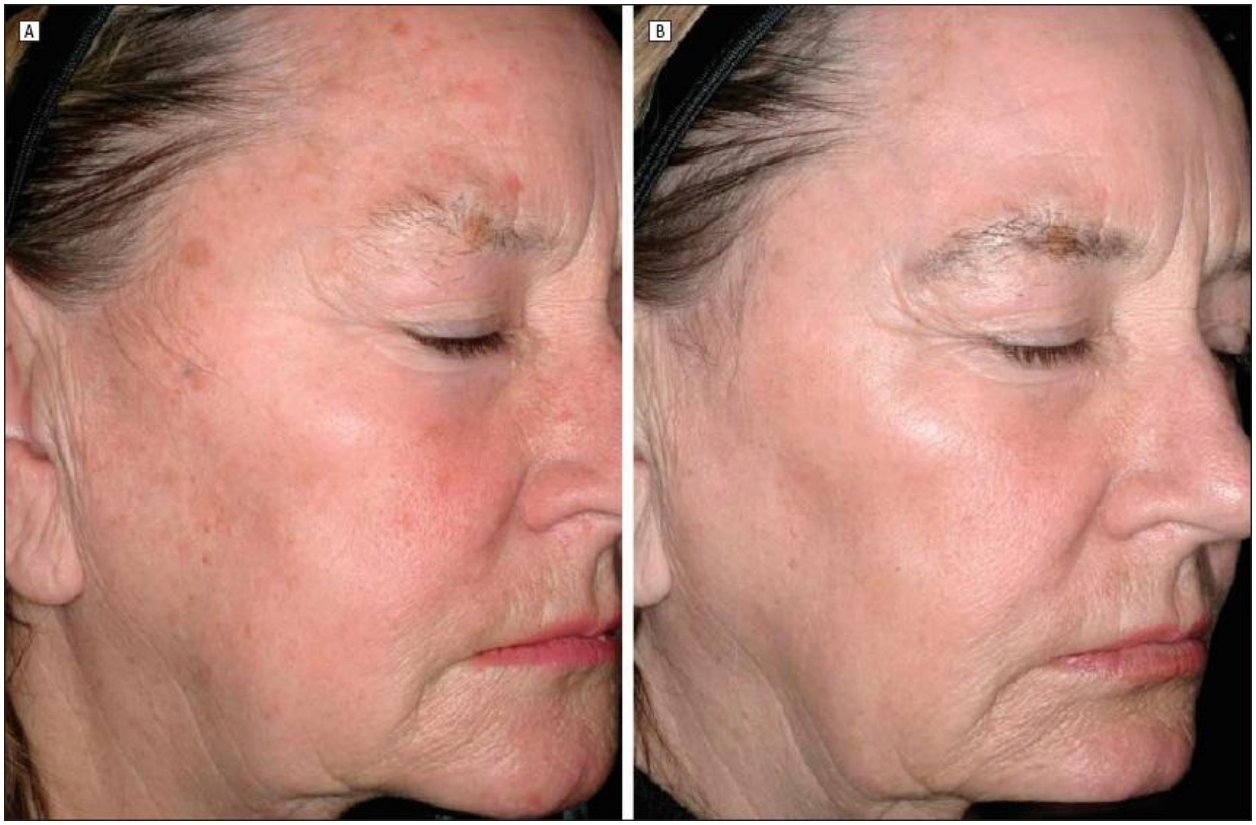


Figure 33. Facial appearance before (A) and 3 months after (B) plasma skin regeneration, with improvement in pigmentation and skin texture. Investigator-rated improvement on the 9-point facial rhytid scale changed from 7 (before regeneration) to 6 (after regeneration); patient-rated improvement in overall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

5.6. 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 (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 Watt [141]. Interestingly, the plasma afterglow generated in this way is able to sterilize many types of bacteria in minutes of treatment. Some of the bacteria tested were *Staphylococcus epidermidis*, *Escherichia coli*, *Streptococcus pyogenes*, *Bacillus cereus*, *Pseudomonas aeruginosa*, etc. Additionally *Candida 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].

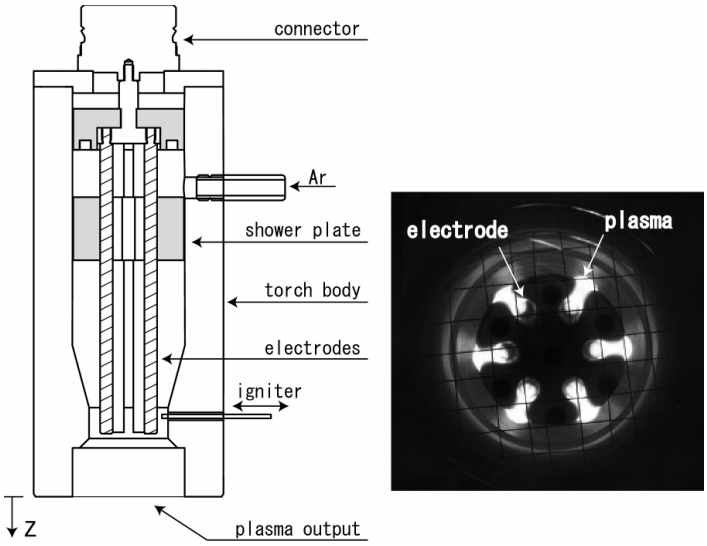


Figure 34. Microwave plasma torch schematic (left) and plasma output photo (right) [141].

Figure 35 shows an example of the results of a 2 minute 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 well [140, 141]. While the effect on bacteria is quite evident, Morfill et al. 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 minutes of 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 minutes of treatment which is quite sufficient to significantly reduce bacterial load.

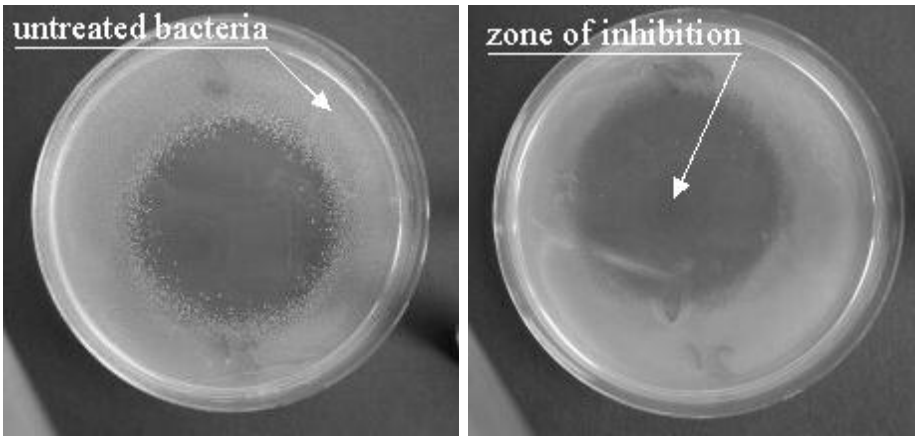


Figure 35. Bacterial cultures on agar plates after 2 minutes of plasma treatment. Left: methicillin-resistant Staphyolococcus aureus (gram positive) Right: Burkholderia cepacia (gram negative) [140].

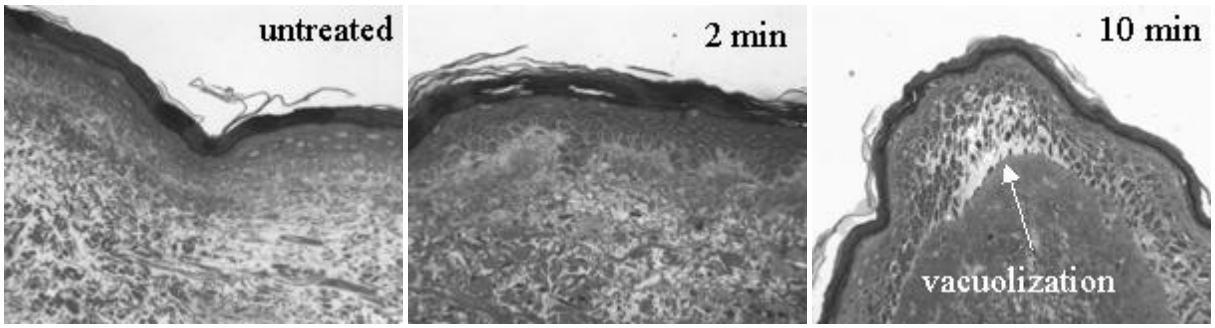


Figure 36. Histological images of skin samples, treated ex-vivo. After 2 minutes no changes could be observed with respect to the untreated control sample. Vacuolization of keratinocytes can be observed after 10 minutes [140].

6. CONCLUSIONS

Non-equilibrium plasmas were shown to be able to initiate, promote, control, and catalyze various complex behaviors and responses in biological systems. More importantly, it was 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 was achieved following various types of plasma treatment of a multitude of wound pathologies. The non-equilibrium plasma was shown to be non-destructive to tissue, safe, and effective in inactivation of various parasites and foreign organisms.

Treatment of various skin diseases by thermal plasmas and related afterglow plasma systems have been employed in a hospital setting for a long time; however, these treatments can be quite destructive. Depending on thermal capacity of the plasma flow there can be pyrolysis, coagulation, and destruction of biological tissue during the procedure. In treatment of especially sensitive areas, for example cornea, temperature should not exceed 43°C. Non-thermal plasma treatment opens in this perspective a whole new area of opportunities of plasma treatment without any tissue damage and undesirable medical consequences.

It is generally clear that Plasma Medicine is an emerging field with a great potential. Obviously, numerous open questions remain to be answered. Though plasma is shown to be selective and specific in medical treatment, mechanisms of this selectivity remain largely unknown. Mutagenic effects of repeated treatment also need to be analyzed in details. In dermatological applications for example, a patient can be subjected to a series of repeat treatments potentially spanning months before the desired effect is achieved (i.e. tattoo removal, or acne treatment).

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