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Cold Plasma in Medicine and Healthcare: The New Frontier in Low Temperature Plasma Applications

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Low temperature plasmas that can be generated at atmospheric pressure and at temperatures below 40°C have in the past couple of decades opened up a new frontier in plasma applications: biomedical applications. These plasma sources produce agents, such as reactive species (radicals and non-radicals), charged particles, photons, and electric fields, which have impactful biological effects. Investigators have been busy elucidating the physical and biochemical mechanisms whereby low temperature plasma affects biological cells on macroscopic and microscopic scales. A thorough understanding of these mechanisms is bound to lead to the development of novel plasma-based medical therapies. This mini review introduces the reader to this exciting multidisciplinary field of research.

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INTRODUCTION

Plasma medicine is about using low temperature atmospheric pressure plasmas to generate controllable amounts of specific chemically reactive species that are transported to react with biological targets including cells and tissues. The remarkable achievement of this plasma application is that it took only about 25 years to take it from initial discovery, to fundamental scientific investigation stage, and finally to applications on actual patients. How did this happen in a relatively short time? A brief answer to this question is that although the field started in a rather modest and unexpected way, it did not take long for the plasma physics community to realize its great potential and its revolutionary promise. This was accentuated by the recruitment of health science experts (biochemists, microbiologists, etc.) who joined the various research endeavors and greatly advanced the ongoing research. Up until the present the mechanisms of action of plasma on cells and tissues are still not fully understood but the body of knowledge has been steadily growing and our understanding has expanded significantly to include a relatively good grasp on the physical and biochemical pathways whereby plasma impacts biological matter.

The field started in the mid-1990s by few proof of principle experiments which showed that low temperature plasma (LTP) possesses efficient bactericidal property [1–5]. It was realized from the very beginning that the reactive species generated by LTP, which include reactive oxygen species (ROS) and reactive nitrogen species (RNS) played a pivotal role in the observed biological outcomes [1, 6]. It also became quickly apparent that LTP can not only be used to inactivate pathogens, such as bacteria, on abiotic surfaces but it can also be used to disinfect biological tissues and therefore can be employed for wound healing. In due time these early bold ideas, backed by some preliminary experimental data, resonated strongly within the LTP research community, which by then (around 2005) realized what these new, promising but not fully explored applications meant and joined

this emerging research field in droves. Consequently, advances and new milestones were reached at relatively “break neck” speed, and by the beginning of the second decade of the 2000s clinical trials on chronic wounds were conducted with some success [7]. In addition small doses of LTP were found to selectively kill cancer cells without harming healthy ones. This opened up another research avenue sometimes referred to as “plasma oncology.” Investigators from research labs around the world reported promising *in vitro* and *in vivo* results on the killing of various cancer cell lines (see review [8] and references therein). The cell lines included those associated with leukemia, carcinoma, breast cancer, brain cancer, prostate cancer, colorectal cancer, etc. [8]. In addition, more recently, cold plasma was used in Germany in limited preliminary trials as a palliative therapy for head and neck cancer patients [9]. The above described various efforts finally culminated in the US Food & Drug Administration (FDA) approval of the first clinical trials in the USA in 2019. This constitutes yet another major milestone for the efforts to develop novel LTP-based cancer therapies.

In this mini review, descriptions of LTP sources used in plasma medicine is first given, then some major medical applications are briefly described.

COLD ATMOSPHERIC PRESSURE PLASMA SOURCES

Two types of plasma discharges have been used extensively in biomedical applications: The dielectric barrier discharge (DBD) and the non-equilibrium atmospheric pressure plasma jet (N-APPJ). **Figure 1** illustrates two photographs showing a DBD ignited in argon gas (left photo) and the plasma plume emanating from a N-APPJ operated with helium (right photo).

Dielectric Barrier Discharge (DBD)

Dielectric Barrier Discharges are ideal for the generation of large volume non-equilibrium atmospheric pressure diffuse plasma. Extensive investigations allowed for a good understanding and improvement of their operation [10–23]. DBDs use a dielectric material, such as glass or alumina, to cover at least one of the electrodes. The electrodes are driven by high AC voltages in the kV range and at frequencies in the kHz. Plasmas generated by DBDs have been used for ozone generation, for material

surface modification, as flow control actuators, etc. DBDs most recent domain of application has been in biomedicine after their successful early use in the mid-1990s to inactivate bacteria [1]. Today they are used in various biomedical applications including wound healing and the destruction of cancer cells and tumors [24–27].

Sinusoidal voltages with amplitudes in the kV range and frequencies of few the kHz were originally used to power DBDs. However, since the early 2000s it was found that repetitive high voltage short pulses (ns - μ s) offered a more efficient way to enhance the chemistry of such discharges [28–30]. DBDs are able to maintain the non-equilibrium state of the plasma due surface charge accumulation on the dielectric surface as soon as a discharge is ignited. This creates an electrical potential that counteracts the externally applied voltage and results in a self-limited pulsed current waveform.

The electron energy distribution function (EEDF) defines/controls the chemistry in the plasma. Short repetitive high voltage pulses allow for preferential heating of the electrons population and therefore an increase of ionization and excitation [29]. Pulses with widths less than the characteristic time of the onset of the glow-to-arc transition maintain stable non-equilibrium low temperature plasma [29, 30].

To extend the operating frequency range below the kHz few methods were proposed. For example, Okazaki and co-workers used a dielectric wire mesh electrode to generate a discharge at a frequency of 50 Hz [16]. Laroussi and co-workers used a high resistivity layer/film to cover one of the electrodes in a device they referred to as the Resistive Barrier Discharge (RBD) [31]. The RBD can be operated with low frequencies extending all the way to DC. The film barrier usually has a resistivity of few M Ω .cm. The high resistivity film plays the role of a distributed resistive ballast which inhibits the discharge from localizing and the current from reaching high values.

Non-equilibrium Atmospheric Pressure Plasma Jets (N-APPJ)

Although plasma jets were previously employed for material processing applications [32, 33] biotolerant plasma jets developed specifically for plasma medicine have been in use only since the mid-2000s [34, 35]. These jets can emit low temperature plasma plumes in the surrounding air. Because they can maintain

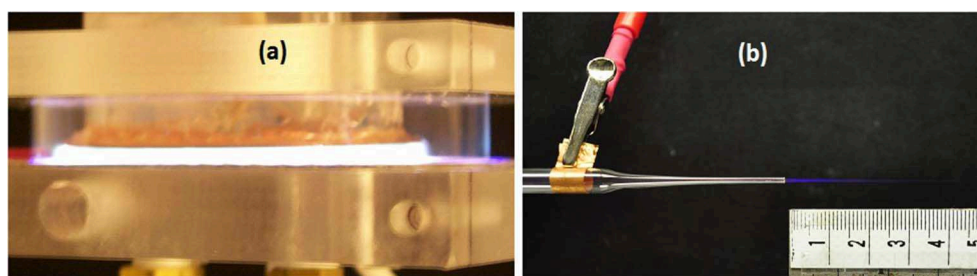


FIGURE 1 | Two sources of low temperature atmospheric pressure plasma: Dielectric Barrier Discharge in argon driven by repetitive short duration (ns - μ s) high voltage pulses **(a)**; A micro-jet using helium as operating gas, generating a cold plasma plume about 2.5 cm in length **(b)**.

temperatures below 40°C, they can come in touch with soft matter, including biological tissues, without causing thermal damage. These plasma sources proved to be very useful for various applications including biomedical applications [26, 34, 35]. Because the plasma propagates away from the high voltage electrodes and into a region free from high voltage the plasma does not cause electrical shock/damage to the target cells or tissues. However, the plasma plume does exhibit a very high instantaneous and local electric field at its tip. This field plays a role in the propagation of the plasma plume and can also affect the treated target.

Investigators discovered that the plasma plumes generated by N-APPJs are not continuous volumes of plasma but discrete plasma packets/bullets propagating at high velocities, up to 10⁵ m/s [36, 37]. The mechanisms governing the generation and propagation of these plasma bullets were reported by both experimental and modeling investigations [38–49]. A photoionization model was proposed by Lu and Laroussi who first investigated the dynamics of the plasma bullet [37]. Further investigations also showed that the high electrical field at the head of the plume plays a role in the propagation process. The average strength of this electric field was experimentally measured to be in the 10–30 kV/cm range [50–52].

The low temperature plasma sources described above produce chemically reactive species including reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are known from redox biology to play important biological roles [53]. Other agents generated by these plasma sources are also suspected to play active roles in biological applications. These include charges particles (electrons and ions), UV and VUV radiation, and electric fields. For example the electric field can cause electroporation of cell membranes, allowing molecules (including ROS and RNS) to enter the cells and cause damage to the cell's internal organelles (including mitochondria) and macromolecules such as lipids, proteins, and DNA. To learn more about the physics and design of LTP sources the reader is referred to the following references [23, 27, 54–57].

APPLICATIONS OF COLD PLASMA IN BIOLOGY AND MEDICINE

The early groundbreaking experiments using low temperature atmospheric pressure plasma for biomedical applications were conducted in a decade spanning from 1995 to 2004 [1–6, 58–60]. The earliest experiments involved the use of dielectric barrier discharge to inactivate bacteria on surfaces and in liquids [1, 58] and to generate pulsed plasma in saline solutions for surgical applications [61, 62]. Works on using cold plasma for the disinfection of wounds, enhancement of proliferation of fibroblasts, and cell detachment soon followed [25, 59, 60]. Eventually these seminal works attracted the interest of the low temperature plasma research community and the field witnessed a substantial growth in the years following 2005 and until the present. Applications in wound healing, dentistry, cancer treatment, etc. have since then been pursued in various laboratories and research centers around the world leading to a

remarkable increase in the number of journal manuscripts on the topic and to the publication of several books [63–66].

The ability of cold atmospheric plasma to inactivate bacteria recently gained more relevance because modern society has been facing several serious healthcare challenges. Amongst these are: (1) Antibiotic resistant strains of bacteria such as Methicillin Resistant Staphylococcus aureus (MRSA) and Clostridium difficile (C-diff) are sources of hospital acquired infection (HAI), which can be fatal to patients with a compromised immune system; (2) Chronic wounds, such as diabetic ulcers, do not heal easily or at all, and one of the problems is the high level of infection caused by a spectrum of bacteria. The inability of conventional methods to satisfactorily deal with these problems necessitated the need for novel approaches based on new technologies. Cold atmospheric plasma has been shown to effectively inactivate bacteria such as MRSA and to greatly reduce the bioburden in infected chronic wounds, making it a very attractive technology that can be used to help overcome the challenges listed above. In 2010, the first clinical trials on the treatment of chronic wounds with cold atmospheric plasma took place and yielded encouraging results [7]. Today there are several plasma devices on the market which have been licensed as medical instruments and which can be used in medicine, including the treatment of various dermatological diseases.

LTP can be applied in two different ways. The first is what is referred to as “direct” exposure. In this mode of application the plasma comes in direct contact with the biological target and therefore all plasma-produced agents act on the cells/tissues. The second mode is what is referred to as “indirect” exposure. In this case only the afterglow of the plasma is used or the plasma is first used to activate a liquid medium then the plasma-activated liquid is applied on top of cells/tissues. One of the advantages of the latter is that the plasma activated liquid (PAL) can be stored and used at a later time, giving a degree of flexibility that direct exposure does not offer.

Direct Exposure

As mentioned earlier under direct exposure the biological target is subjected to all plasma agents including charged particles, photons, electric field, and reactive species. These agents act alone and/or in synergy to produce certain biological outcomes. In the case of bacteria inactivation, all the above agents were reported to play a role. Lysing of vegetative cells as well spores were reported after direct exposure to LTP, but cell death without lysis was reported as well for gram-positive bacteria [67, 68].

The inactivation of bacteria by LTP has several applications ranging from sterilization of heat sensitive medical tools, to the destruction of biofilms, to disinfection of wounds, to decontamination of liquids, food, and agricultural products.

Direct exposure has also been used in a non-lethal way to affect eukaryotic cell functions, by modulating cell signaling pathways [69], and in a lethal way for the destruction of cancer cells and tumors [70–74]. Experiments using various cell lines have been reported which showed that under a certain exposure dose LTP can kill cancer cells in a selective manner [70–74]. Investigators reported that LTP exposure leads to an increase in intracellular ROS concentrations. Since cancer cells are under high oxidative

stress, the increase in ROS leads to severe redox imbalance, which can lead to one or more of the following: DNA damage, mitochondrial dysfunction, caspase activation, advanced state of oxidation of proteins, etc. Such acute stress ultimately leads to cancer cells death.

Indirect Exposure

In this section we limit the discussion to the case of plasma activated liquids (PAL). In this mode of exposure only long lived chemical species that diffuse and solvate into the aqueous state play a role. This eliminates the effects of photons, electric field, short lived species, and heat. Liquids that have been used include water to make plasma activated water (PAW) and biological culture media to make plasma activated media (PAM). The following discussion focuses on the use of PAM to destroy cancer cells. Over the past few years investigators have reported encouraging results on the use of PAM *in vitro* and *in vivo* to kill cancer cells and reduce tumors [75–81]. The anticancer characteristic of PAM has been attributed to the long lived species produced in the liquid phase after LTP exposure. These species include hydrogen peroxide, H_2O_2 , nitrite, NO_2^- , nitrate, NO_3^- , peroxyxynitrite, ONOO^- , and organic radicals.

The making of PAM involves the exposure of a liquid medium to an LTP source, most frequently the plasma plume of a plasma jet, for a certain length of time. Media used include Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM), Ringer's Lactate solution (RL), Roswell Park Memorial Institute medium (RPMI), with additives such as serum (e.g., bovine serum), glutamine, and antibiotics (e.g., mix of Penicillin/Streptomycin). As an example of producing PAM, a 24-well plate can be used where a few ml of fresh cell culture media is added to each well. Each well can be treated by LTP for a certain length of time, this way producing different PAMs with different "strengths." To illustrate the effects of PAM on cancer cells the following work done at the author's laboratory is summarized [81].

In this experiment, to make PAM, 1 ml of fresh cell culture media (MEM) was added to each well of a 24 well plate. Each well was exposed to the plume of the plasma pencil (a pulsed plasma jet) for a designated time. After exposure, the media on top of cells grown in a 96-well plate was replaced by 100 μl of PAM. After PAM application, cells were stored at 37°C in a humidified incubator with 5% CO_2 . Media not exposed by LTP was used for the control sample. The cancer cell line used was SCaBER (ATCC® HTB3™) cell line from a urinary bladder tissue with squamous cell carcinoma. Cell viability was quantified at different times of incubations using The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, MI, USA). To quantify the MTS assay results trypan blue exclusion assay was used [81]. **Figure 2** shows the results.

As can be seen in **Figure 2**, PAM that was created by longer LTP treatment times causes a greater cell kill. PAM created with an exposure time greater than 3 min induces more than 90% cell reduction. However, for the 2 min case, over time the proliferation of live cells overtakes the destruction of cells and therefore an increase in viability at 24 and 48 h was observed. To investigate the role of the reactive species in the killing of SCaBER

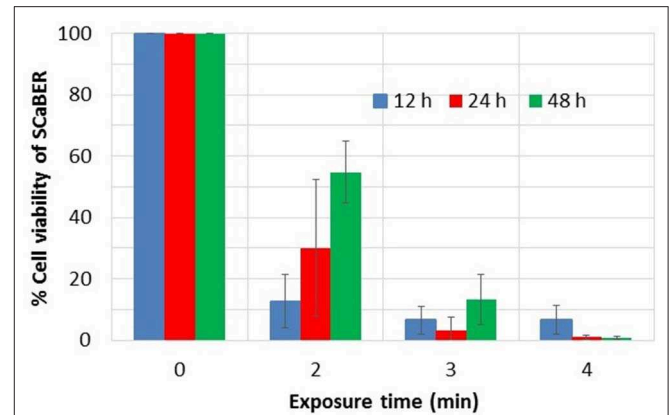


FIGURE 2 | Viability of SCaBER cells after PAM treatment, using MTS assay. Exposure time indicates the time the liquid media was exposed to plasma to make PAM. Measurements were made after 12, 24, and 48 h of PAM application. Data is based on three independent experiments using two replications each. This figure is plotted based on data previously published in Mohades et al. [81].

cells measurements of hydrogen peroxide, H_2O_2 , produced in PAM were made. It was found that the concentration of H_2O_2 in PAM increased with exposure time and correlated well with the reduction in cell viability of SCaBER [81]. This was in agreement with works of various investigators which have shown the key role H_2O_2 plays in the anticancer efficacy of PAM. Recently Bauer proposed the hypothesis that H_2O_2 and nitrite lead to the generation of singlet oxygen ($^1\text{O}_2$) which causes inactivation of catalase [82]. Catalase, which is normally expressed on the membrane of cancer cells, protects them from intercellular ROS/RNS signaling. With enough inactivation of catalase an influx of H_2O_2 via aquaporin occurs. Therefore the inactivation of the protective catalase causes ROS mediated signals that lead to apoptosis of malignant cells. Since healthy cells do not express catalase on their surface they are subject to an influx of ROS such as H_2O_2 or peroxyxynitrite. So if they are exposed to very high ROS concentrations they also can be damaged. Therefore, the applied dose of ROS/RNS has to be below a certain threshold to achieve selective killing of cancer cells.

CONCLUSION

The application of low temperature atmospheric pressure plasma in biomedicine opened up new frontiers in science and technology. On a scientific level, new fundamental knowledge (albeit still incomplete) regarding the interaction of plasma with soft matter has been created. Before the mid-1990s basic scientific understanding of the physical and biochemical effects of plasma on cells and tissues was simply missing. Today, 25 years later and after extensive and hectic scientific investigations, our knowledge has greatly grown and many of the mechanisms involved have been elucidated on the cellular and sub-cellular levels. This allowed remarkable advances in the quest of developing novel plasma-based therapies to overcome

various healthcare challenges. The recent approval of the US Food & Drug Administration of clinical trials using plasma for cancer treatment is a critical milestone and a sign that low temperature plasma may be on its way to be accepted as a promising and exciting healthcare technology. To learn more about the future directions of the field the reader is referred to references [83, 84]. In addition, low temperature plasma has obvious merits as a viable technology for space medicine. As deep-space long-duration space travel becomes a

reality it is crucial to have available adequate methods to meet medical emergencies in space. In this context plasma offers a practical “energy-based” and “dry” technology that can replace perishable drugs.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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