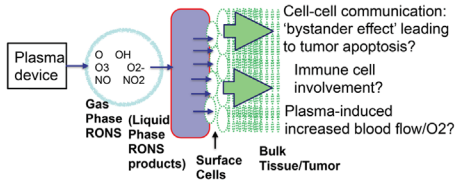
Reactive Species from Cold Atmospheric Plasma: Implications for Cancer Therapy

David B. Graves

Cold atmospheric plasmas (CAP) formed in air generate reactive oxygen and nitrogen species (RONS). RONS are biologically and therapeutically active agents and experimental evidence suggests that air plasmas shrink tumors by increasing oxidative and nitrosative stress on neoplastic tissue. Most mainline anti-cancer therapies – including ionizing radiation and chemotherapies – also operate primarily via this pro-oxidant, oxidative, and nitrosative stress mechanism. The main disadvantage of these conventional therapies is the development of treatment-resistant cells. A key question for plasma cancer therapies is therefore whether or not cold plasma will lead to similar oxidative stress resistance. However, there are hints that combining nitrosative stress with oxidative stress via air plasma might avoid this problem. Plasma-based cancer treatment may be a powerful and practical anti-cancer agent, acting either alone or in combination with other therapies.



1. Introduction

The major current therapies designed to eradicate or limit cancer are surgery, radiation (or radio-) therapy, and chemotherapy. More recently, gene-targeted therapies that attack specific oncogenes or tumor suppressor genes (and their associated biochemical pathways) have received much attention by pharmaceutical companies.[1,2] Cancer immunotherapy and stem cell transplants are also growing in importance. Local "focalized" therapies that have received considerable attention include laser ablation, thermal plasma coagulation, hyperthermia, focused ultrasound, and photodynamic therapy, among others.[3] Nevertheless, there remains an enormous need for better therapies to more effectively treat the many different forms of cancer with minimal side effects. The focus of the present article is to explore the possible mechanisms and opportunities of cold atmospheric plasma (CAP) as a novel anti-cancer therapy.

In a recent comprehensive review, Schlegel et al.[4] summarize progress in applying CAP to tumors, mostly for in vitro but also for increasing numbers of in vivo investigations. These authors point out that a variety of different CAP configurations have been explored, including rare gas jets and dielectric barrier discharges in air, both direct and indirect. They term the new field "plasma oncology," and point out that relatively low plasma "doses" appear to induce cell cycle arrest and higher doses lead to apoptosis and ultimately at the highest doses, to necrosis. Furthermore, a remarkable similarity of anti-tumor action for all types of cancers investigated has been observed: carcinomas, skin cancer, and brain tumors. In some cases, plasma treatment of cell culture medium has been shown to be an effective anti-tumor fluid, both in vitro and in vivo.[5]

The cellular effects of CAP appear to strongly involve, either directly or indirectly, the suite of reactive oxygen species and reactive nitrogen species (RONS) created by CAP in air environments.[6] The point of view of this article is that both these plasma-generated (gas phase) RONS and also their liquid phase/cellular reaction products, are probably the keys to understanding, controlling, and exploiting plasma oncology.

The important role played by oxidation–reduction ("redox") biochemistries in cancer generation, progression, and therapy is now a well established theme in the literature. No attempt is made in this article to provide a

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comprehensive survey of this extensive literature, especially the discussions of using increased oxidative stress for cancer therapy. This theme and its relation to plasma oncology are explored further in a subsequent section of this article. Renschler[7] provides an interesting historical perspective, pointing out that the idea to use RONS-generating drugs to treat cancer, primarily as radiation therapy sensitization, goes back to at least the early 1960s. Trachootham et al.[8] provide a particularly comprehensive (and highly cited) summary of the idea. Recent support for and further elaboration of this basic idea includes the papers by Mak et al. and Watson.[9,10]

The major focus of the current article is to attempt to establish a closer connection between plasma oncology and various current therapeutic modalities that also exploit oxidative and nitrosative stress. Of course, the way that RONS are generated or delivered to cells will be important in general. Ionizing radiation penetrates cells so it creates RONS directly in cells. Chemotherapeutic drugs (or their metabolite products) will generate RONS in cells through a series of biochemical reactions after entering cells and cell compartments like mitochondria. The manner in which RONS generated in CAP enter cells to affect cell biochemistry is still largely a mystery and it is obvious that progress in plasma oncology will require much more insight into this key step.

Conventional anti-cancer therapies often work well initially, leading to rapid shrinkage of the tumor or tumors. However, it is not uncommon that this initial effectiveness is relatively short-lived. The major problem with current therapies is the development of treatment-resistant cells, leading to regrowth of tumors, metastasis, and mortality in many cases. Tumor adaptation to oxidative stress-inducing therapies such as radiation and many chemotherapies is commonly invoked as a primary cause of resistance.[9,10] The fact that plasma oncology relies on oxidative stress suggests that plasma treatment could lead to similar problems with resistance. However, there are hints that RONS implicated in plasma oncology could eliminate this resistance.

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2. Basic Model of Plasma–Tissue Interaction

Before discussing the RONS aspects of current anti-tumor therapies, I address the question of what is happening when CAP is used to treat a tumor. The current model is still vague, but the basic ideas are illustrated in Figure 1.

It should be stressed that CAP is a surface or near-surface treatment modality, unlike for example penetrating ionizing radiation. One obvious question is how a surface or near-surface modality can affect deeper tissues, and some possibilities are listed in Figure 1. However it is done, the documented evidence of the presence of RONS and their products in both in vitro and in vivo experiments shows that CAP is somehow inducing oxidative and nitrosative stress in tissues, leading to apoptosis or necrosis depending on dose. The evidence for the role of RONS in plasma oncology, summarily documented by Schlegel et al.,[4] is related to fluorescent detection of RONS in treated cells as well as demonstrations of abrogation of the CAP effects with the addition of RONS/radical scavengers. Of course, strictly speaking, this evidence is circumstantial, and the observed intracellular RONS may have been generated at least partly within the cell. It is well documented that RONS can be generated

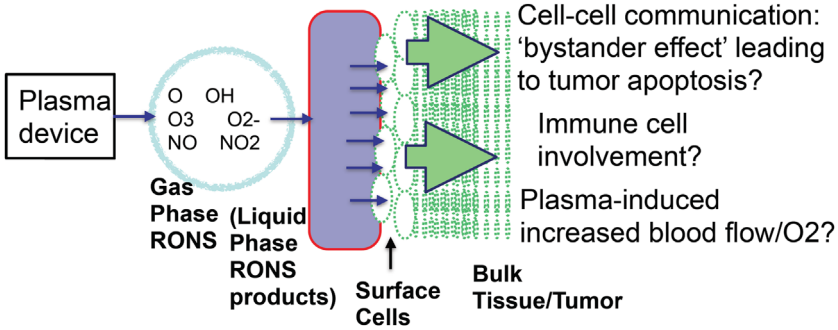

Figure 1. The plasma device operates to generate RONS that either enter a cell surface-covering liquid layer or enter the cells directly. Whatever the effects of the solvated RONS and their products are in the surface layer of cells that are exposed to them, the effects on deeper layers of tissue must involve some cell–cell communication. Some possibilities include mechanisms analogous to radiation-induced "bystander effects," the stimulation and involvement of the immune system, or possibly some effects associated with local blood flow and O_2 concentration.

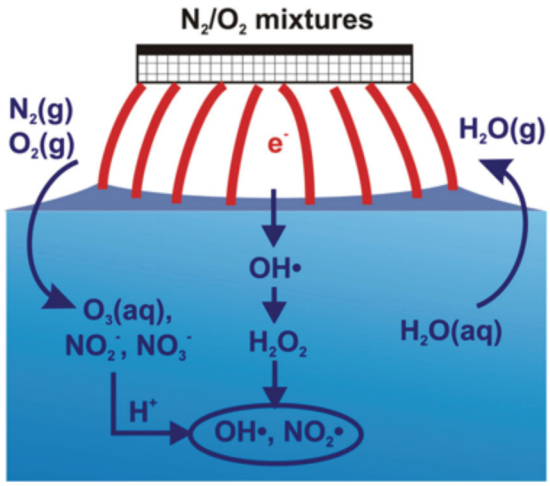

Figure 2. Following Lukes et al.[12] Air plasma creates various RONS that end up in the water in the form of dissolved O_3 (ozone); NO_2^- (nitrite); NO_3^- (nitrate), H^+ (acidity), OH (hydroxyl); H_2O_2 (hydrogen peroxide), and NO_2 (nitrogen dioxide). Reproduced with permission.[12] Copyright 2014, Institute of Physics.

intracellularly in response to many forms of stress as well as forming due to exogenous stimuli.[11]

Recent excellent work by Lukes et al.[12] identifies at least some of the chemistry occurring in water exposed to air plasma. Figure 2 is taken from this paper, summarizing some of these results.

The significance of this chemistry and these species is apparent in the context of the next several sections in which they are shown to be important in key biochemical processes related to known or postulated therapeutic effects. More details and discussion regarding air plasma--water interactions (sometimes referred to as "plasma-activated water" or PAW) is addressed in greater detail in the penultimate section of the paper.

3. Pro-Oxidant Anti-Cancer Therapies

As noted previously, the idea of increasing oxidative (and nitrosative) stress on cancer cells as a killing strategy has received wide support. It is generally acknowledged that many if not most existing anti-tumor therapies rely on this mechanism, including ionizing radiation, most chemotherapies and even some (gene-) targeted therapies.[8–10,13–17] The use of RONS-generating agents to attack cancer seems counter-intuitive at first because it has been strongly suspected for decades that RONS are implicated with inducing cancer. It is thought that both radiation and excessive inflammation are associated with cancer because they both involve the generation of RONS.[18,19] The dual nature of this redox biochemistry clearly complicates the story: therapy or damage can result, depending on the complex biological context as well as the duration and intensity of the exogenous application or endogenous generation of RONS.

Mak and co-workers[9] note that chemotherapeutic drugs that target the genes that turn cancer "on" (oncogenes) or "off" (tumor suppressor genes) have been relatively unsuccessful to date because of the genetic complexity of tumors and the ease with which they can mutate to become resistant to the drug. There are so many ways for the tumor to mutate to protect itself from specific genetic pathways that drugs that target a few genes are unable to remain effective for long enough to be truly therapeutic. These authors suggest that a more reliable strategy is to look for drugs that combine an increase in oxidative stress with attacks on anti-oxidants or anti-oxidant generating enzymes. Watson also supports this approach, emphasizing the role that anti-oxidants play in sustaining cancer cells and the need to address this weak point of tumors in order to attack late-stage cancer.[10]

The potentially important role of cancer stem cells (CSCs) in re-growing tumors after conventional RONS-based therapies have shrunk most of the tumor is related to this theme as well. Diehn et al.[20] showed that CSCs are more resistant to radiation than normal tumor cells at least in part due to their intrinsically low levels of RONS and correspondingly high concentration of antioxidants. The fact that plasma oncology relies on a similar oxidative stress mechanism raises the concern that anti-oxidant based resistance will also render plasma therapy ineffective as well. If this turns out to be true, CAP therapy may offer no real advantage over existing therapies. Some authors have pointed out that since cancer cells will tend to adapt to high RONS levels, thus leading to therapeutic resistance, the anti-resistance strategy should involve manipulating the tumor oxidative stress adaptation.[8,16,21]

One strategy that has received considerable attention with respect to chemo-sensitization and radio-sensitization is the use of nitric oxide-related compounds, either via NO-donor drugs or through gene therapy in which nitric oxide synthase (NOS) is enhanced for increased endogenous NO production. As I point out in the following section (and as seen in Figure 2 reproduced from Lukes et al.[12]), the fact that CAP methods can and usually do involve reactive nitrogen as well as reactive oxygen may offer hope that CAP will be able to escape this trap.

4. CAP-Generated NO_x Chemistry: A Way to Avoid or Reverse Resistance?

Although the present article is intended to be only the briefest introduction to NO_x biochemistry and its potential relation to plasma oncology, the scope of the field of nitric oxide-related compounds in biochemistry should not be underestimated. The comprehensive reviews of nitric oxide

(NO) and peroxynitrite ($ONOO^-$) by Pacher et al. and Szabó et al. will begin to give some idea of the size and scope of the field.[22,23] Pacher et al. reference nearly 1 500 papers. Nitric oxide has been the topic of on the order of 100 000 articles since its discovery as a biologically significant molecule in late 1980s. Understanding the role of CAP in generating NO_x-containing species that may be able to interact with complex biochemical processes that respond to these compounds will no doubt be an extended effort, to say the least. Nevertheless, there are some hints already that NO_x generated by CAP may be very important for a range of biomedical applications, and cancer therapy is one of them.

The special significance of NO_x chemistry for CAP is the fact that CAP operating in air can create biologically and therapeutically significant concentrations of NO_x locally. As noted above (cf. Figure 2) and as is described in further detail later in the article, this gas phase source of NO_x can be readily transferred to liquid media adjacent to the plasma. Hence we know for certain that CAP generates a chemistry (e.g., nitrite NO_2^- and peroxynitrite $ONOO^-$) that is closely related to the NO chemistry described by Hirst and Robson[24,25] and many other authors.

There is a substantial literature on the role of nitric oxide (NO) and related compounds as a direct cancer therapy or as an agent that re-sensitizes tumors to chemotherapy or radiation therapy (e.g., ref.[24–29] Figure 3; following[24]) illustrates the ways that nitric oxide can influence cancer therapy. These authors suggest that NO may be in some sense an "ideal" anti-cancer agent.

They note that the most potent and attractive aspects of NO therapy is in combination with other modalities, specifically taking advantage of chemo-sensitization and radio-sensitization.[25] The use of NO as a radio- (or radiation-) sensitizer was identified as early as the late 1950s. Later work showed that NO contributes to the cell–cell communication known as the "bystander effect."[30,31] This is potentially important in the context of plasma oncology as noted above since CAP is a tumor-surface application technique and apoptotic-inducing information must be transmitted from the site of application to interior tumor tissue.

Sonveaux et al.[26] list a series of reasons why NO can play an anti-tumor role: (i) vasodilation; (ii) inhibition of cell respiration; (iii) direct radio-sensitization; (iv) modulation of tumor immunity; and (v) modulation of angiogenesis. Recently, Oronsky et al. have proposed the concept of "hyponitroxia," or inadequately low levels of nitric oxide, in analogy with hypoxia (i.e., low levels of oxygen), as a promoter of tumor generation and growth.[32] These authors describe "...the role of persistently low NO concentrations or hyponitroxia as a key mediator in tumor progression." Bauer provides evidence of an important tumor apoptotic intercellular signaling mechanism involving NO and $ONOO^-$.[33]

NO has also been shown to be a potentially powerful agent to sensitize chemotherapeutic agents to remain active against a variety of tumors, although at least one exception was noted by Hirst and Robson.[24] The likely role of peroxynitrite was noted in the later review by Hirst and Robson.[25]

Sonveaux et al.[26] summarized the situation as follows:

> Exploitation of the biology of NO offers the opportunity to improve the efficacy of conventional anticancer treatments such as chemo- and radiotherapy. Different NO donors and NOS agonists (i.e., nitric oxide synthase enzyme promoters) have now been tested in

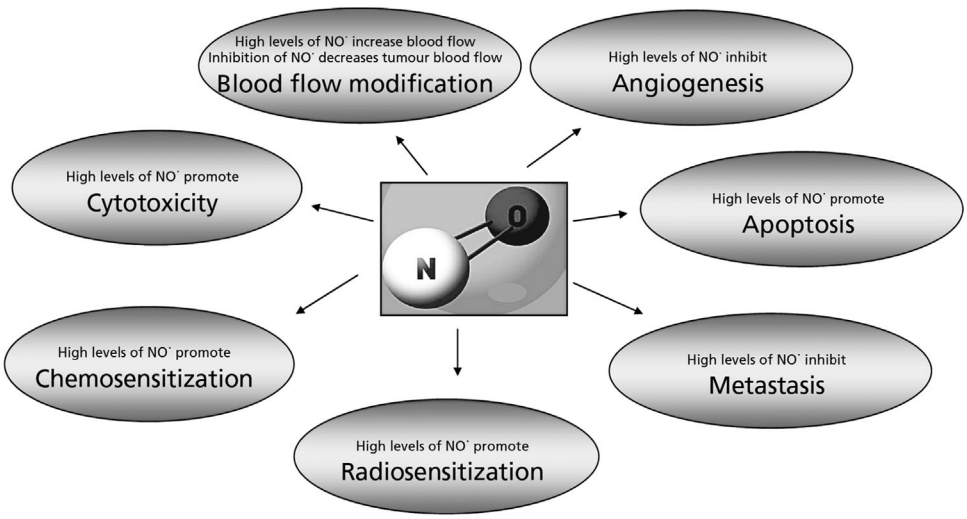

Figure 3. Following Hirst and Robson.[24] NO is at the center of a suite of positive therapeutic anti-cancer actions. Reproduced with permission.[24] Copyright 2014, Wiley-Blackwell.

preclinical settings, revealing a variety of mechanisms that contribute to transiently improve blood flow and pO_2 (i.e., tissue O_2 concentration), thereby increasing drug delivery and the stabilization of radiation-driven DNA damages.

The effects of NO donors or NOS-promoting agents on blood flow and tissue oxygen concentration are especially interesting in the light of very recent results from Collet et al.[34] These authors showed that applying a He plasma jet for 5 min to the skin of anesthetized mice induced significant increases in both local blood flow and tissue O_2 concentration. The increase in tissue O_2 concentration lasted on the order of several tens of minutes and was shown to be very localized to the point of application of the plasma jet. This is reproduced in Figure 4. Although the mechanism responsible for this effect is not certain, one possibility mentioned by the authors is the creation in the treated tissue of nitrite anion (NO_2^-), a well-known nitric oxide precursor in acidic environments. The possible direct role of NO generated in the plasma was eliminated when an experiment placing gauze impregnated by de-ionized water between the plasma jet and the skin showed similar effects. The fact that the same results as without the water-soaked gauze on the skin were observed strongly suggests that the effect is mediated through a compound like nitrite, known to be often formed in water adjacent to CAP. A key element of this scenario is that the plasma application also often creates nitrate (NO_3^-; the conjugate base of nitric acid, or HNO_3) with corresponding effects on lowering pH. Nitrite is known to form NO under acidic conditions (cf. the following section), and so the known effects of NO (listed above) on blood flow suggests this may be the cause of the observed effect on CAP-treated mouse tissue. The following section briefly summarizes the surprising number of therapeutic roles known to be played by nitrite.

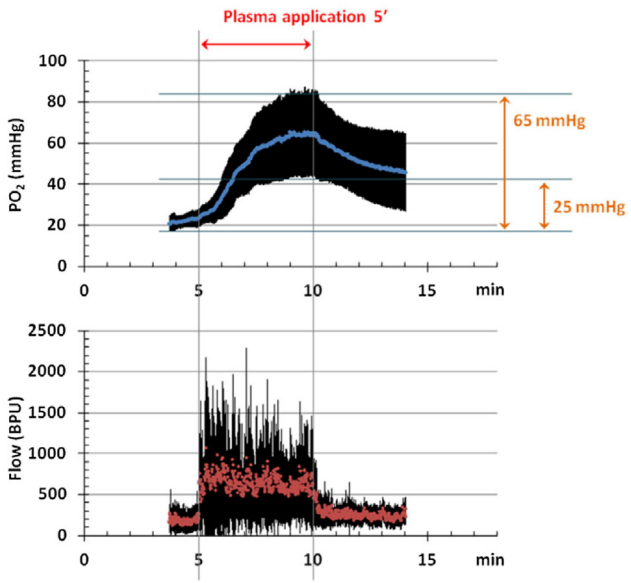

Figure 4. Following Collet et al.[34] A 5 min application of a He plasma jet to the skin of five mice (two locations each) resulted in a significant increase in sub-cutaneous tissue O_2 concentration (top) and blood flow (bottom). Averages are shown in the colored lines in the top and bottom panels. Reproduced with permission.[34] Copyright 2014, Institute of Physics.

5. Nitrite Therapies

It has been appreciated for at least a decade that nitrite anion (NO_2^-) can act therapeutically, most probably as a precursor source of nitric oxide. (e.g.,[35,36]) There are numerous ways that nitrite can be reduced to nitric oxide in the body, but they mostly involve low concentrations of oxygen and an acidic environment. The simplest example is the following reaction sequence, in which an acidic environment leads to the formation of NO and NO_2:

$$NO_2^- + H^+ \leftrightarrow HNO_2 \quad (1)$$

$$2HNO_2 \rightarrow NO^{\cdot} + NO_2^{\cdot} + H_2O \quad (2)$$

It should be noted that the reactions in liquid phase can be much more complex than this, especially in the presence of red blood cells and other metal-containing organic molecules. Nevertheless, the role of acidification in "activating" nitrite is well established.[37]

The most common therapeutic application of nitrite is in the form of aqueous solutions of sodium nitrite, delivered intravenously or even orally.[35] Figure 5, taken from Kevil et al., illustrates the point. The remarkable number of potential therapeutic uses of this simple molecule testifies to its power to modify blood flow and inflammation. Kevil et al. list seven different clinical trials under way (circa 2011) testing various aspects of nitrite therapy. The relation between these well documented and extensive applications of systemic nitrite and the effects of plasma biomedicine in general and plasma oncology specifically are still speculative, but the potential connection is compelling.

6. Air Plasma RONS and Plasma–Water Interaction

Generation of NO_x has been noted from the beginning of the use of CAP in biomedical applications. For example, Stoffels et al. showed that NO was one of the more important products for the helium "plasma needle" operating in air.[38] Bruggeman et al.[39,40] measured absolute NO densities in various atmospheric pressure rare gas plasma jets and found values on the order of a few to a few tens of ppm (about 10^{14}–10^{15} cm^{-3}). Higher values were reported by Vasilets and Shekter[41] in their air plasma "torch"

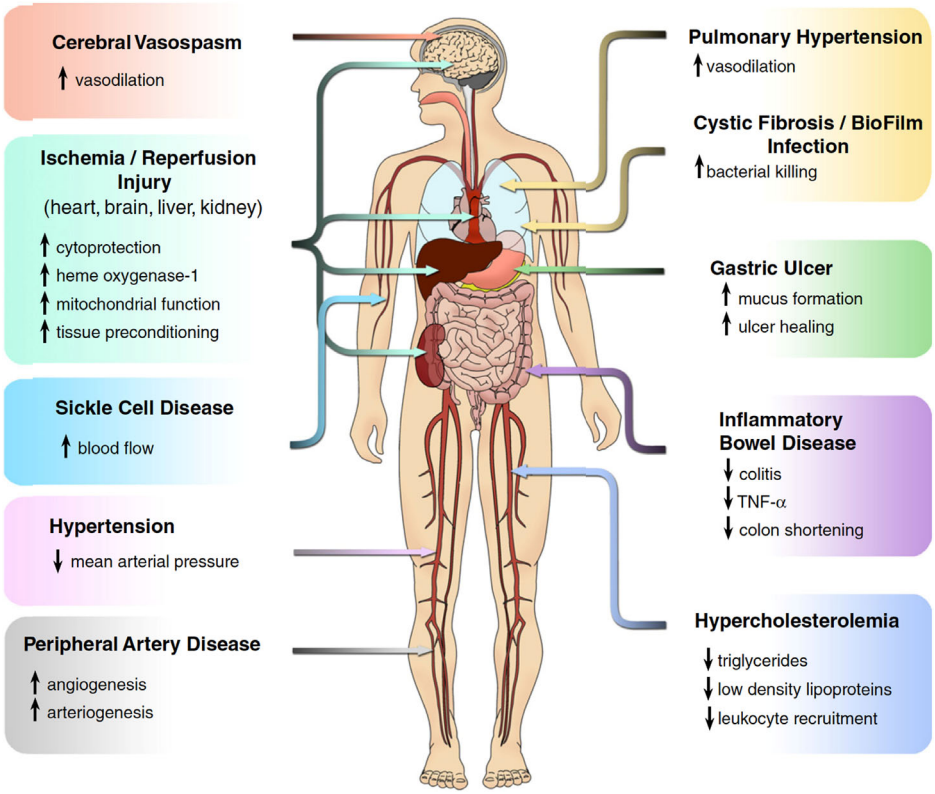

Figure 5. Following Kevil et al.[35] Illustration of the remarkable number of therapeutic applications of nitrite, delivered systemically. Reproduced with permission.[35] Copyright 2011, Elsevier.

configuration: depending on the distance from the hot plasma region and the air gas flow, they measured NO concentrations from several hundred to several thousand ppm ($\sim 10^{15}\,cm^{-3} - 10^{16}\,cm^{-3}$). This device has reportedly been used in Russia since about 2 000 for various wound healing and sterilization applications.[41] Liebman et al. report NO concentrations of up to 200 ppm for an air flow of 300 sccm through an atmospheric pressure plasma jet using a compressed air flow.[42] Surface dielectric barrier discharges operating in air were reported to generate on the order of hundreds to thousands of ppm of both NO and NO_2 in confined spaces next to the grounded electrode.[43] Spark and glow-type discharges in air can create even higher concentrations rapidly.[44]

CAP operated next to water create what is sometimes referred to as "plasma-activated water" or PAW. The RONS components and relative concentrations of the PAW of course depend on the type of plasma (e.g., rare gas jets, microwave torch, gliding arc, dielectric barrier discharge, etc.,) the nature of the mass transfer and mixing between the gas and liquid phase, the presence of other components in the water and so forth. In spite of the obvious differences in types and operating conditions, the presence of NO_x compounds, usually in the presence of hydrogen peroxide (H_2O_2) and under acidic conditions has been repeatedly identified in PAW.[12,45–50] The simultaneous presence of acidified conditions (i.e., H^+ or H_3O^+ concentration relatively high), hydrogen peroxide (H_2O_2) and nitrite (NO_2^-) results in the formation of peroxynitrite, and this compound can degrade rapidly to OH and NO_2:[12]

$$NO_2^- + H_2O_2 + H^+ \rightarrow O=NOOH + H_2O \quad (3)$$

$$O=NOOH \leftrightarrow OH^{\cdot} + NO_2 \quad (4)$$

Peroxynitrite is known to be a very important nitric oxide-related compound and it has received considerable attention in the literature, as noted above.[23] The mechanism noted above to form peroxynitrite from acidified nitrite and hydrogen peroxide has been known and utilized by biochemists interested in making and saving the compound. In this case, stop flow configurations are used with a strong base added immediately after formation of the unstable peroxynitrous acid.[51]

It is perhaps most appropriate to finish with a quote from the paper by Lukes et al. in reference to the summary diagram reproduced here in Figure 2:[12]

> Thus the overall post-discharge bactericidal effect of the air plasma in water involves the synergistic effect

of acidic conditions, nitrites, and peroxides through the cytotoxic activity of secondary reactive chemical species NO, NO_2, OH, and ONOOH, and possibly also contribution of ozone transferred from the plasma into the liquid.

Although Lukes et al. were discussing the effects of the bactericidal effects of PAW, the chemistry they describe is surely relevant to plasma oncology as well.

7. Concluding Remarks

The use of CAP for cancer therapy is in its earliest stages. Preliminary results seem promising, but progress will be most rapidly accelerated if we can identify at least some of the most important mechanisms through which plasma works to shrink tumors. CAP in, or mixed with, N_2 and O_2 creates copious quantities of various reactive oxygen and nitrogen species (RONS) in the gas phase. These species enter adjacent aqueous liquid phases to form solvated species that are known to engage in rapid reactions with important biomolecules such as proteins and lipids. The known importance of RONS (and their reaction products) in established anti-cancer therapies such as radiation therapy and many chemotherapies suggests that the observed anti-tumor effects of CAP are due to the same or a very similar biochemistry. It may prove fruitful, therefore, to focus on the creation and delivery of RONS via CAP to maximize anti-tumor effectiveness.

The fact that conventional pro-oxidant therapies such as radiation and chemotherapy are often limited by anti-oxidant based resistance suggests that CAP-based therapies might suffer the same fate. However, the apparent role of NO-related compounds in minimizing or reversing resistance suggests that CAP might exceed the capabilities of current cancer therapies since NO_x species are generally among the products of CAP. The recent results that show CAP increasing local blood flow and tissue O_2 concentration offers encouraging evidence that CAP could be a powerful adjuvant therapy for conventional cancer treatments.

Furthermore, the fact that nitrite is created in aqueous solution adjacent to CAP in air coupled with the multiple therapeutic applications of this compound suggest it might be significant in plasma oncology as well. Many of the uses for systemic nitrite therapy are related to the apparent role of this NO precursor in blood flow, vasodilation, ischemia, and/or inflammation. It is possible that this nitrite-blood flow connection is the cause of the observed increased local blood flow and O_2 concentration in the mouse model.

The character of CAP as a unique therapeutic modality seems most likely related to its ability to locally create, in situ, a host of important reactive species. It is, at least in principle, possible to deliver the same species that are created by CAP using other approaches such as NO donor drugs; NO and/or NO_2 gas; H_2O_2 in solution; and nitrite/nitrate in the form of aqueous nitrous and nitric acid solutions. However, the fact that CAP creates them all together in one place and under the control of the plasma generation device suggests that CAP may offer unique advantages in practice. In some CAP devices, the presence of physical effects such as electric fields, charges, and photons could synergize with reactive species, adding potentially important features to the technology.

It will be important in future studies to identify key plasma-generated RONS and track their effects on both normal and neoplastic cells. The issue of where these species originate, how they are transported to and within cells, what reactions they experience and finally how their effects are transmitted to other cells will all be important questions to explore in the future.

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