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Low temperature plasma biomedicine: A tutorial reviewa)

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Low temperature plasma biomedicine: A tutorial review[a)]

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Gas discharge plasmas formed at atmospheric pressure and near room temperature have recently been shown to be potentially useful for surface and wound sterilization, antisepsis, bleeding cessation, wound healing, and cancer treatment, among other biomedical applications. This tutorial review summarizes the field, stressing the likely role of reactive oxygen and nitrogen species created in these plasmas as the biologically and therapeutically active agents. Reactive species, including radicals and non-radical compounds, are generated naturally within the body and are now understood to be essential for normal biological functions. These species are known to be active agents in existing therapies for wound healing, infection control, and cancer treatment. But they are also observed at elevated levels in persons with many diseases and are associated with aging. The physical and chemical complexity of plasma medical devices and their associated biochemical effects makes the development of safe, effective plasma medical devices and procedures a challenge, but encouragingly rapid progress has been reported around the world in the last several years. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4892534]

I. INTRODUCTION

The use of near-room temperature ("cold") gas discharges at atmospheric pressure for various biomedical applications is now well established, although not well understood. For the purposes of this review, I will refer to these discharges as "cold atmospheric plasmas," or CAP. Most devices use either rare gas (He, Ne, or Ar) jets in air or some type of dielectric barrier discharge in air. Other variations exist. These simple CAP-generating devices have been shown to readily kill various microbes (bacteria, viruses, and fungi) on surfaces and in solution. Antimicrobial effects are usually at least somewhat attenuated on skin or in the presence of biological fluids like cell culture medium. This is presumably due to competing reactions that consume the active species generated by the plasma, thus reducing their impact on adjacent microbes. However, mechanisms of killing are not fully understood. Obvious possibilities include: reactive chemical species, photons, electric fields, and electrical charge (ions and electrons).

Other recent medical/therapeutic investigations have explored applications ranging from treating dental infections, various dermatological applications, promoting wound sterilization, healing and bleeding cessation, and cancer treatment, among others. A recent review by Von Woedtke *et al.*[1] points out that three clinical trials of CAP devices have been conducted in Germany and two CAP devices received CE marking in Europe ("CE" signifies the device conforms to European Union regulations) in 2013, both for treating skin wounds and ulcers. This is an important milestone in the field, and it is anticipated that similar approvals will be granted soon in other countries.

It is important to distinguish CAP, a set of new and rapidly developing biomedical technologies, from existing plasma-based electrosurgical devices and technologies. Surgeons have used plasma devices for decades for applications including tissue cutting, coagulation (stopping bleeding), dessication (water removal) and cauterizing or fulgurization. The Argon Plasma Coagulator (APC) is an example of this kind of device.[2] These technologies operate via heating the tissue with electrical current and so the effects are primarily thermal. Low temperature plasma, by contrast, transfers little heat and the effects are primarily non-thermal.

The effects of CAP exposure on mammalian cells and tissue is generally dose-dependent, although "dose" is not yet rigorously defined since we do not yet know for certain which effects or chemical species are most biologically important. What has been observed is that in general, if the plasma is operated at higher delivered power, for longer times and at positions nearer the treated tissue, the effects are more pronounced and can change character. For example, at "low" CAP dose, mammalian cells growing in cell culture will tend to proliferate; but at higher doses, they will undergo apoptosis (controlled cell death); and at even higher doses, necrosis (uncontrolled cell death) is observed. Similar comments apply to the effects of plasma exposure on cellular DNA and other measures of toxicity.

The point of view taken in this tutorial review is that the most likely "active agent" underlying CAP biomedicine are the reactive chemical species, especially reactive oxygen and nitrogen species (RONS) created in the plasma. Over the course of about the last 20–30 years, the field of aerobic biology has seen enormous growth of interest in and study of the biological effects of reactive chemical species in general and RONS in particular.[3] The topic is sometimes called redox (for "oxidation-reduction") biology or biochemistry.[4] These species were originally suspected to be *only* deleterious and compounds known as "antioxidants" were thought to be therapeutic in countering excess levels of "oxidative stress" from reactive species such as oxygen and other kinds of radicals.

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The well known free radical theory of aging postulated that the aging process is primarily due to the accumulated damage caused by radicals that are the inescapable by-product of metabolism.[5,6] It is now understood that this is a too simplistic point of view, and that RONS are key players in normal physiology and even in therapies such as anti-microbial and anti-cancer therapies. The title of a recent review outlining the arguments critical of this theory is titled: "When a theory of aging ages badly."[7] These authors conclude that decades of study have proven that strictly speaking, radicals, and other reactive species are *not the cause of aging*, but it is true that elevated concentrations are *associated with aging and other disease*. The implications of this important distinction are not fully understood yet, but nutritional antioxidant supplements are now thought to be mostly ineffective in preventing disease. Fruit and vegetable consumption is generally associated with somewhat lower rates of overall mortality, but the degree to which this is specifically related to antioxidant activity is unknown.[8]

CAP operated in air is certainly creating copious quantities (as defined by their biological activity) of RONS and these species can strongly influence cellular biochemistry. This means that plasma biomedicine is therefore unavoidably connected closely to biochemistry, and even a brief introduction to this field requires some understanding of biochemistry. This field is far from simple, especially for scientists trained exclusively in physics. Furthermore, the field is advancing rapidly, increasing the challenge to newcomers to the field.

The fact that RONS are known to be centrally important in animal and plant immune systems is one reason why it is suspected that they are key players in CAP therapeutics. Plant and animal immune systems use RONS in their fight against invading microbes and parasites; against tumors; and in response to wounds. It seems likely that the demonstrated success of CAP in each of these areas is due in at least some measure to similar reactive species biochemistry. In addition, existing drug therapies, including antibiotic, antifungal, and anticancer chemotherapies are known to directly or indirectly involve RONS. Cancer radiation therapy and photodynamic therapy (PDT) utilize RONS. At the very least, the putative connection between RONS biochemistry and CAP biomedical efficacy offers a promising set of research hypotheses.

The main goals of this paper are to briefly summarize a small subset of the recent biomedical results from CAP use, to identify and briefly characterize some of the plasma-generating devices used, and to provide some evidence, mostly circumstantial, that CAP might be working primarily via the actions of RONS. Since RONS typically react via oxidation-reduction ("redox") chemistry, I will highlight some of what is known about redox biochemistry, mention briefly its role in the immune system and describe known or suspected RONS actions in existing therapies. It should be stressed, however, that other mechanisms (i.e., electric fields, charges, and photons) may well be important in at least some cases. It should also be noted that synergies involving coupled effects of different aspects of the plasma (e.g., chemistry plus photons) can be important in some cases.

There have been numerous recent reviews on various aspects of plasma biomedicine, e.g., Refs. 1 and 9–11. A more complete review on the role of RONS in plasma biomedicine was recently published.[12] The present article aims to lay out in a more tutorial fashion some of the basic ideas for newcomers, and especially plasma physicists, to this field. The paper will address a few important points but it is not intended to be comprehensive and many important aspects, details and exceptions are not included. Any serious student of the field should plan to read the many other reviews and monographs, as well as the original research literature, in order to begin to gain a more complete picture.

II. STRUCTURE AND COMPOSITION OF PLASMA JETS

One of the most common configurations used to create CAP for biomedical applications is the rare gas plasma jet. There are usually composed of jets of He (and sometimes Ar) either pure or with small amounts ($\sim$1%) molecular gas such as O_2 and/or N_2. Typical configurations are shown in Fig. 1: plasma jets are shown in Figs. 1(a) and 1(b); Fig. 1(c) (following Emmert *et al.*[13]) is an image of a floating electrode dielectric barrier discharge (FE-DBD). The dielectric tube used for the plasma jet discharge is usually on the order of 1 cm in diameter; one or two ring electrodes are placed around the tube periphery; the flow of He is $\sim$5–10 lpm; the excitation voltage is usually $\sim$5–20 kV; and frequency $\sim$1–30 kHz. Applied powers are typically on the order of $\sim$1 W. Gas temperature is usually on the order of 300–350 K. The plasma in the FE-DBD configuration is similar. These conditions can of course vary. For example, pulsed dc discharges are also used; higher powers generally lead to higher gas temperature and so forth. Von Woedtke *et al.*[1,10] include a much more complete set of discharge configurations used in CAP plasma biomedicine. The visible plasma plume extends on the order of several cm from the tip of the tube. It may or may not touch the surface to be treated.

Even though the plasma is sustained primarily in He (or Ar), the fact that the plasma extends into and is mixed with the air environment means that O_2, N_2, and H_2O are dissociated and ionized to some extent, leading to many different reactive charged and neutral species. Charged species generally recombine close to the visible plasma plume but reactive neutrals can travel significant distances—cms to perhaps 10 s of cm or even farther. Of course, highly reactive radicals such as O, N, and OH tend to react with other species relatively rapidly. For example, O atoms will react rapidly with O_2 and N_2 to form O_3 and NO, respectively. Figure 2 (Ref. 14) is a plot of measured O and O_3 profiles as a function of distance from the tip of a He/O_2 jet. Typical air plasma chemical reaction mechanisms are reported by Sakiyama *et al.*[15] and Zhang *et al.*,[16] for example. Figure 3 plots the predicted neutral species as a function of distance from the tip of an Ar/O_2 plasma jet. Note that the O and O_3 profiles measured in Fig. 2 and predicted in Fig. 3 are approximately the same, even though the plasmas are different.

Details of jet plasma chemistry are far from fully understood, but commonly observed chemical species at

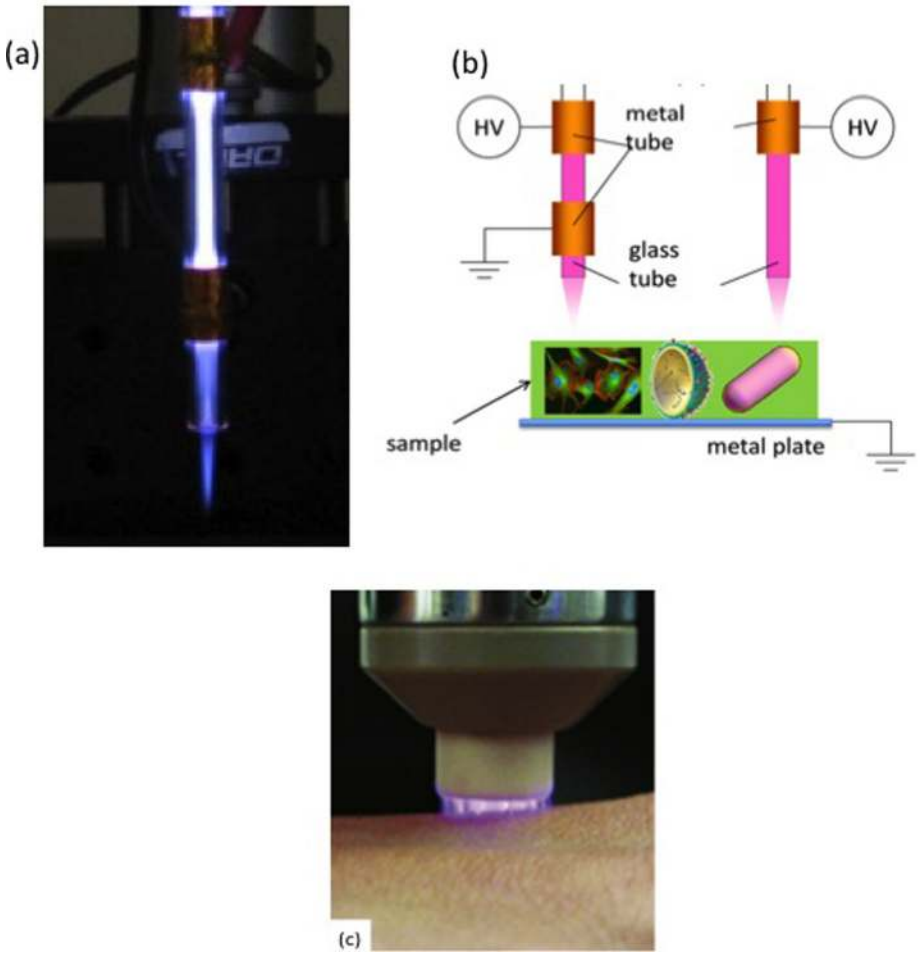

FIG. 1. (a) Image of laboratory He plasma jet with ~1 cm diameter quartz tube, ~1–4 slm He flow, and ~1 W dissipated power; (b) sketch of configuration; (c) Image of direct FE-DBD in air, treating human skin. Reprinted with permission from S. Emmert, F. Brehmer, H. Hanble, A. Helmke, N. Mertens, R. Ahmed, D. Simon, D. Wandke, W. Maus-Freidrichs, G. Daschlein, M. P. Schon, and W. Viol, Clin. Plasma Med. **1**(1), 24–29 (2013). Copyright 2013 Elsevier.

significant concentrations include ozone (O_3), singlet delta oxygen (O_2 $a^1\Delta_g$), atomic nitrogen (N), hydroxyl radical (OH), various nitrogen oxides (e.g., NO, NO_2, and N_2O), hydrogen peroxide (H_2O_2) and nitric and nitrous acid (HNO_3, HNO_2), among others. In some cases, these species are similar or identical to known RONS of importance in biology, as discussed below.

An important feature of rare gas jets is that they are often observed to operate by launching a series of rapidly propagating ionization waves that appear as moving luminous structures sometimes referred to as "plasma bullets." Recent model results have explained much of the physics of these discharges.[17–19] A typical set of images is illustrated in Fig. 4. Note that the apparent "bullet velocity" reaches $\sim 10^7$ cm/s in this image.

One implication of the propagating ionization wave is that surfaces in contact with the end of the plasma plume are subject to a charge flux. This plasma-deposited charge has been directly measured.[20] Typical surface charge densities observed were on the order of several nC/cm^2. It is possible that the charges have effects when the plasma plume interacts with tissue or other biological surfaces.[21]

As noted above, there are several other types of CAP devices that have been tested over the last several years, including dielectric barrier discharges in air and microwave plasmas.[22,23] Regardless of the type of plasma source, however, they all produce significant quantities of reactive species, and especially reactive oxygen and nitrogen species. These species are important players in normal biochemistry, as summarized in Sec. III.

III. REDOX BIOCHEMISTRY

One class of important biochemical reactions involves electron transfer between reactants. Included in this class are reactions between oxygen and other species ("oxidation" reactions), acid-base reactions, ionic reactions, and free radical reactions, among others. Chemists often use the concept of "oxidation state" to characterize the valence electrons in species that are involved in such reactions. If a species has been *oxidized*, its oxidation state (an integer index) is increased (e.g., iron in its 2^+ oxidation state, or Fe^{2+}, if oxidized, loses an electron, thereby entering the oxidation state 3^+, written Fe^{3+}). Since O_2 tends to be more electronegative than other species, it tends to *gain* an electron from its reaction partner; oxidation for its reaction partner involves *loss* of an electron. Oxygen is then *reduced* by its reaction partner, since every oxidation must involve a corresponding reduction, in order to conserve electrons in the electron transfer reaction.

In aerobic biology, species engage in *respiration,* the process by which glucose is oxidized to water and carbon dioxide (H_2O and CO_2, respectively). The reactions involved in glucose oxidation are generally redox reactions. Aerobic

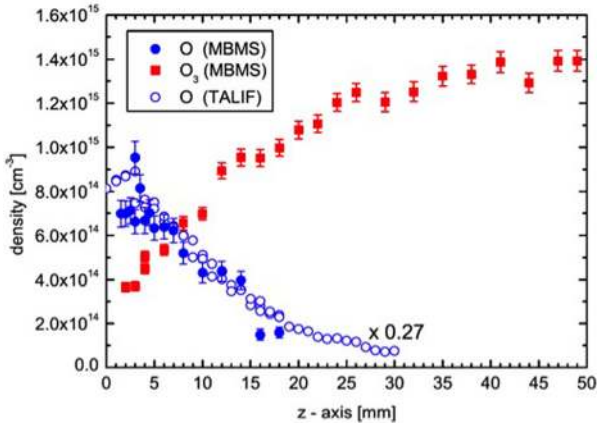

FIG. 2. Measured density of O and O_3 as a function of distance from the tip of a He/O_2 plasma jet, using two different methods: molecular beam mass spectrometry (MBMS) and two-photon absorption laser-induced fluorescence (TALIF). Reprinted with permission from D. Ellerwig, J. Benedikt, A. von Keudel, N. Knake, and V. Schulz-von der Gathen, New J. Phys. **12**, 013021 (2010). Copyright 2013 IOP.

respiration generates energy for the organism, generally in the form of molecules of adenosine triphosphate (ATP). The majority of ATP molecules are generated in a process known as *oxidative phosphorylation*. This process occurs in the part of the cell known as the *mitochondrion*, and it is driven by the thermodynamically favored transfer of electrons to oxygen, leading ultimately to water. Sometimes, this is referred to as the *electron transfer chain* (ETC). It is known that the ETC can "leak" electrons that react with dissolved O_2 to form an important source of a special kind of reactive oxygen species (ROS), the superoxide anion (O_2^-). More on this key reactive oxygen species is discussed below.

Superoxide anion is one of an important class of chemical species, termed 'radicals' or 'free radicals,' that have one or more unpaired valence electrons. Reactions that involve radicals are usually electron transfer or redox reactions.

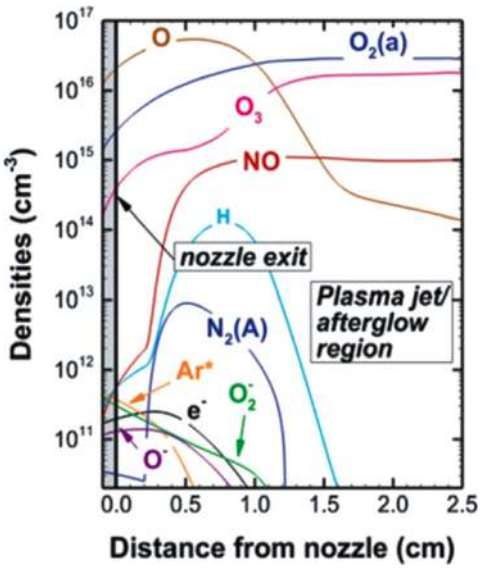

FIG. 3. Model predictions of chemical species as a function of distance from an Ar/O_2 plasma jet tip. Reprinted with permission from S. Zhang, W. van Gaens, B. van Gessel, S. Hofman, E. van Veldhuizen, A. Bogaerts, and P. Bruggeman, J. Phys. D **46**, 205202 (2013). Copyright 2013 IOP.

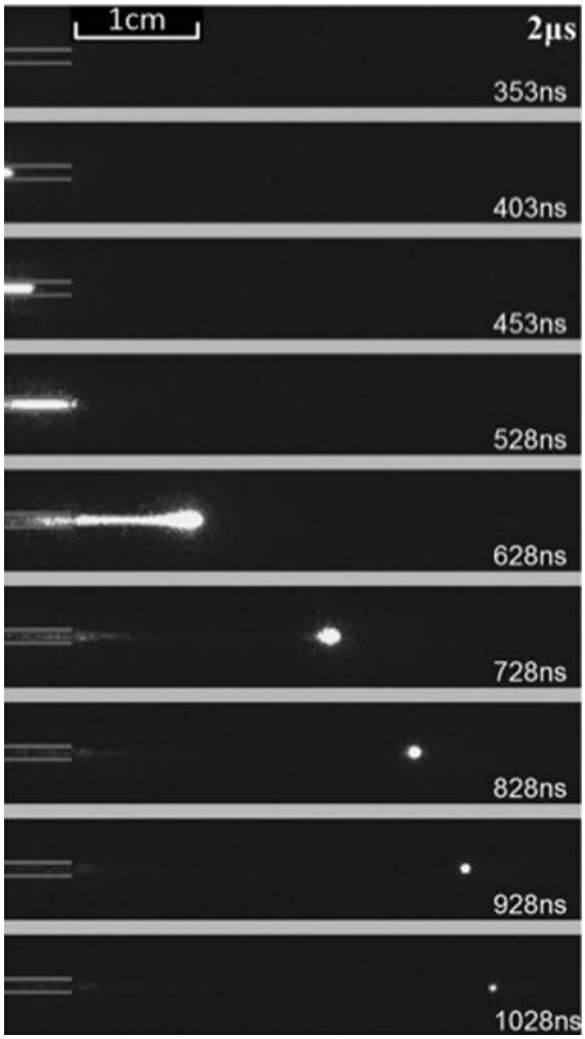

FIG. 4. Sequential plasma emission images showing the existence of a visible, propagating ionization wave in the plasma jet (jet flow left to right and horizontal orientation here); sometimes referred to as the "plasma bullet." Reprinted with permission from S. Zhang, W. van Gaens, B. van Gessel, S. Hofman, E. van Veldhuizen, A. Bogaerts, and P. Y. Xian, P. Zhang, X. P. Lu, X. K. Pei, S. Q. Wu, Q. Xiong, and K. Ostrikov, Sci. Rep. **3**, 1599 (2013). Copyright 2013 Macmillan Publishers Limited.

Radicals are usually relatively unstable and react readily with other species. Oxygen is a di-radical with two unpaired electrons, although it is not particularly reactive at room temperature, except with other radicals. Radicals can be formed when any covalent (electron-sharing) bond is broken. This can happen at higher temperatures (e.g., in combustion); by absorbing electromagnetic radiation (e.g., photolysis or photo-ionization); and in various chemical reactions, including certain enzymatic reactions, among other possible routes. Of course, in the context of this article, energetic gas phase electrons in CAP readily create radicals via electron-impact molecular dissociation; or ionization to form an electron-positive ion pair; or attachment of an electron to form a negative ion. CAP-generated photons (especially in the vacuum ultraviolet regime) can also create radicals, but this is usually thought to be less likely than electron-impact processes. Radicals in biochemistry are generally in the liquid phase, but gas phase radicals created in CAP can dissolve into an adjacent liquid.

A. Reactive oxygen species

ROS are created from reactions involving O_2, and a key set of biochemical reactions in aerobic biology involve oxidative respiration, as noted above. Biochemists point out that one way to look at the conversion of O_2 ultimately to H_2O in respiration is to think of the process as a series of electron transfer reactions. This is illustrated in Fig. 5.[4] In this figure, molecular oxygen with the addition of one electron becomes superoxide anion, O_2^-. There are also several enzymatic paths to form O_2^- in solution. Two of the more important ones are via enzymes *xanthine oxidase* and *NADPH oxidase* (nicotinamide adenine dinucleotide phosphate-oxidase). Superoxide anion will convert to hydroperoxy radical (O_2H) at low pH (acidic conditions), since the extra H^+ present in solution under acidic conditions will combine with the anion to form it.

O_2 can also be converted to ozone (O_3) if O atoms can be formed in the presence of O_2, a process that requires enough energy to dissociate O_2 into O atoms. O_3 can be formed in various natural processes, including photolysis in the atmosphere or in lightning discharges. In plasma, it can form via electron impact dissociation of O_2 to form O atoms, followed by a three-body recombination of O and O_2 to make O_3. An electronically excited state of O_2 (singlet delta) can also be formed with energy supplied by electron impact or a photon of the proper wavelength. As noted below, there is another way to make singlet O_2 that involves molecules that absorb a photon ("photosensitizers"), creating an electronically excited state that can transfer this energy to an adjacent O_2 molecule. This reaction is the basis of PDT, and this is an important example of how creation of ROS is known to be potentially therapeutic.[24]

Reaction of O_2 with a second electron plus two protons (H^+ ions) will make hydrogen peroxide (H_2O_2). H_2O_2 with a proton and electron can, under the right conditions, split off a water molecule and form hydroxyl (OH) radical, one of the most reactive chemical species known. This reaction can be catalyzed in the presence of an iron atom in its Fe^{2+} oxidation state in a reaction termed a "Fenton" reaction. OH will form water with the fourth electron and another H^+ ion. It should be noted that the extreme reactivity of OH radical makes it an obvious suspect in the well-known correlation between ROS and various diseases and aging. Kell postulates that many diseases are related to this reaction.[25]

There are two important catalytic reactions that convert reactive oxygen species to less reactive forms. *Superoxide dismutase* (SOD) converts superoxide to O_2 and H_2O_2; and *catalase* converts H_2O_2 to water and O_2. Other enzymatic reactions act (via species sometimes referred to as "antioxidants") to eliminate other reactive radicals, and some of these are discussed below.

B. Reactive nitrogen species (RNS)

The second major class of reactive species is termed reactive nitrogen species (sometimes also referred to as RNI or "reactive nitrogen intermediates"). A few of the key RNS reactions and species are shown in Fig. 6. Figure 6(a) shows how the key species nitric oxide (NO) is made enzymatically: the amino acid L-arginine reacts with one of the versions of the nitric oxide synthase (NOS) enzyme to form NO. Subsequent reaction with O_2 yields NO_2; NO_2 in turn will react with NO to form N_2O_3. If NO reacts with O_2^-, another key reactive species, peroxynitrite ($ONOO^-$) forms. This will form peroxynitrous acid (ONOOH) at low pH (by reacting with the extra H^+ present at low pH), and this in turn decomposes to NO_2 and OH.

Another key set of reactions involving NO_x species is shown in Fig. 6(b); sometimes this is referred to as the "nitrate-nitrite-nitric oxide" pathway.[26] Nitric oxide can be formed starting with nitrate (NO_3^-), then reducing it to form nitrite (NO_2^-). Under low pH conditions, nitrous acid (HNO_2) forms from nitrite and this in turn decomposes to form NO and NO_2. This sequence can be important in the body since, for example, nitrate enters the bloodstream via various foods. It can be reduced to nitrite by commensal bacteria (that is, bacteria that live in and on the body symbiotically) on the tongue. Swallowing the nitrite allows antimicrobial nitric oxide to form from the gastric acid in the stomach.[26] Nitrite is also known to be present in human perspiration and the slightly acidic skin environment will release a certain amount of NO, thus protecting against microbial infection. Nitrite in skin is activated by photolysis from near-UV photons from sunlight to form NO and this in

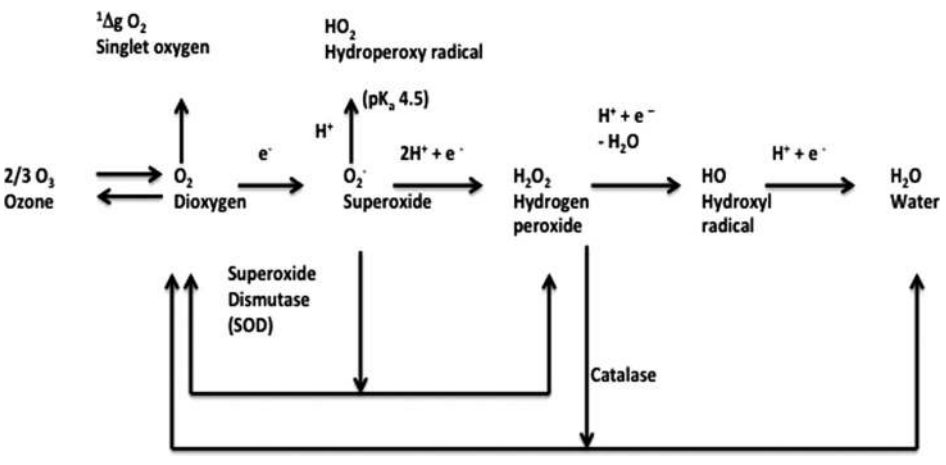

FIG. 5. Four electron, four proton transfer reactions transform O_2 into H_2O. O_2 can also form ozone (O_3) and the singlet delta excited state of O_2. Superoxide becomes hydroperoxy radical (HO_2) if the pH is less than about 4.5. Enzymatic reactions between superoxide and the enzyme SOD create O_2 and H_2O_2; H_2O_2 in turn is catalytically degraded to O_2 and water via enzymatic reaction with catalase.

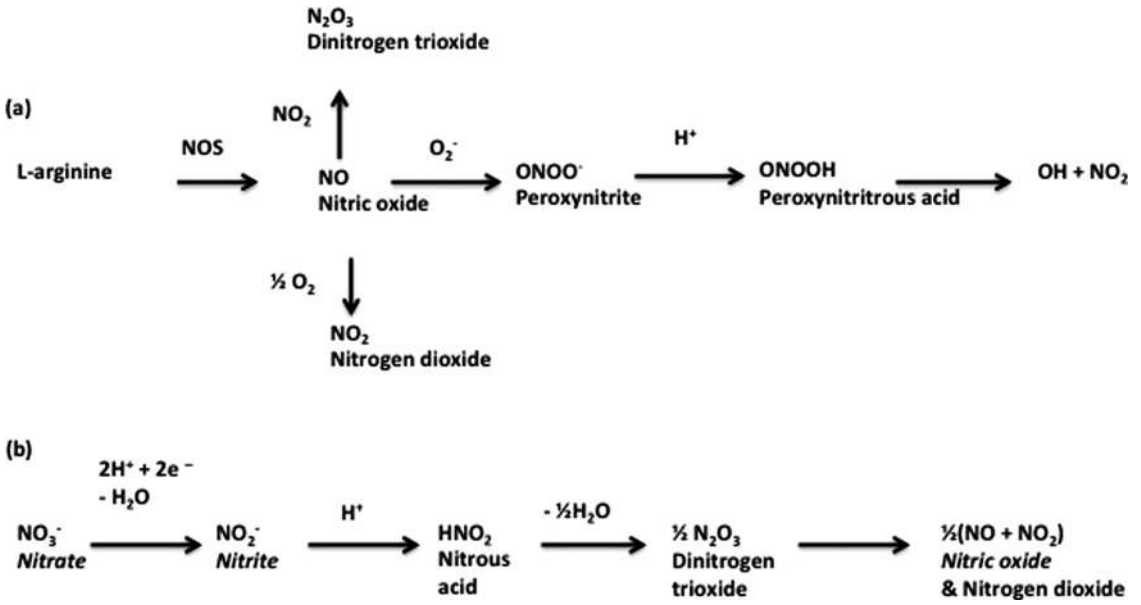

FIG. 6. Typical reactive nitrogen species reactions. (a) NO is formed enzymatically via L-arginine (acting on NH_2-containing species, not shown) and nitric oxide synthase (NOS). NO reacts with O_2^- to form peroxynitrite, which in turn forms peroxynitrous acid under acidic conditions. This compound can decompose to form OH and NO_2 (nitrogen dioxide); (b) the nitrate-nitrite-nitric oxide (NO_3^- - NO_2^- - NO) pathway. The reduction of nitrate to nitrite may be enzymatic or non-enzymatic. Note the conversion of nitrite (NO_2^-) to nitric oxide (NO) requires acidic conditions.

turn has recently been shown to lead to a significant lowering of blood pressure.[27,28]

Nitric oxide (NO) is an extremely important molecule in biochemistry; it is estimated that there are now over 100 000 articles on this compound in the literature. And the 1999 Nobel Prize in Physiology or Medicine was granted for research showing that NO is a key species in cell signaling, the immune system and vasodilation, among many other roles.

The relevance of RNS in plasma biomedicine comes from the fact that NO and NO_2 (among other nitrogen oxides) are often formed in air-containing CAP. Once again, important chemical species from the biochemical point of view are known to be created in atmospheric pressure air plasmas.

C. RONS in biochemistry

Entire monographs are devoted to this topic (e.g., Ref. 3), so only a few reactions will be highlighted here to serve as representative examples. Figure 7 illustrates reactions between RONS and polyunsaturated fatty acids (PUFAs). These species, sometimes referred to as "lipids," have a hydrophilic ("water-loving") end: the –COOH carboxylic acid group; the other part is a hydrocarbon and is therefore hydrophobic. "Unsaturation" refers to the fact that there are carbon-carbon double bonds present, and therefore the molecule has not been "saturated" with hydrogen. Both reactive oxygen and reactive nitrogen will react with PUFAs to form various products, as illustrated in the figure.

The biochemical significance of oxidized and nitrated lipids can be enormous, as for example when they react with proteins, thereby altering their subsequent reactions.[29,30] Reactions that alter proteins are referred to as "post-translational modifications" and these can include change in the protein structure as well as addition of a variety of chemical groups onto the protein, typically requiring enzymatic processes. Proteins are generally created in cells by first copying ("transcribing") the segment of DNA (sequence of nucleic acids) corresponding to the protein into an

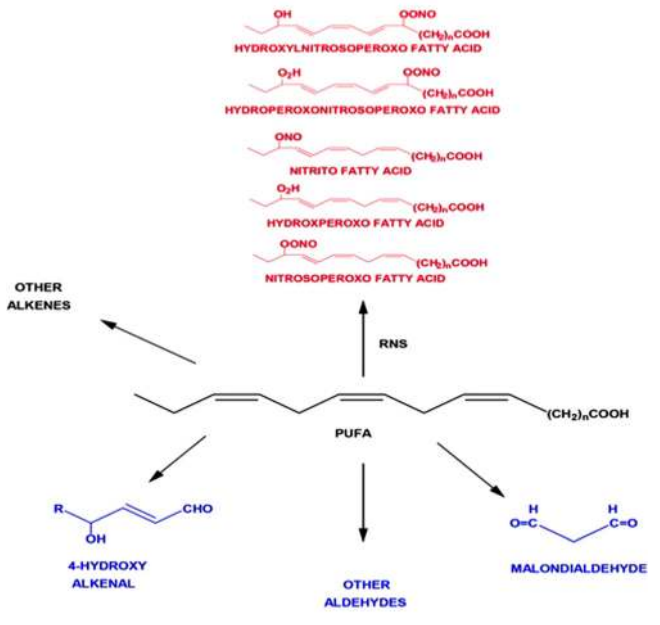

FIG. 7. Some important reactions between RONS and PUFAs. PUFAs are acids because there is a -COOH (carboxylic acid) group at one end. The other end (the "fatty" part) consists of a hydrocarbon chain of -CH_2- or -CH-, depending on whether the C-C bond is single or double, respectively. The term "unsaturated" refers to the fact that there are several (three in the image above) carbon-carbon double bonds and these bonds are not "saturated" with H at these locations. ROS reactions with these species generate, e.g., aldehydes and hydroxylated species; RNS react to form various nitrogen oxide-containing products such as nitrated fatty acids. These products react further, creating a cascade effect in biochemical cycles. Reprinted with permission from I. Acworth, *The Handbook of Redox Biochemistry* (ESA Biosciences, Chelmsford, MA, USA, 2003). Copyright 2003 ESA, Inc.

intermediate RNA molecule, which is then used as a template allowing protein (sequence of amino acids) production via "translation." Post-translational modification of proteins allows variation of function and can result in accumulation of protein due to increased stability or reduction of amount of protein due to degradation.

The chemical species created by plasma are usually so reactive that they will not live long in solutions containing macromolecules and other biological components. However, lipids and proteins modified by plasma-created species are likely to have much longer lifetimes and may participate in important biochemical cycles. It seems likely that some of the biochemically relevant species created in this way will be similar to species that arise naturally when the immune system creates RONS in its inflammatory response to infection, tumors, or wounds.

Reactions that form radicals in lipids are also known to cause problems. Figure 8 illustrates a lipid peroxidation chain reaction that is initiated by a Fenton reaction between Fe^{2+} and H_2O_2, liberating the extremely reactive OH radical. This OH radical can abstract a hydrogen atom from a carbon, creating the lipid radical, denoted $L^{\bullet}$ Reaction with O_2 in solution creates the lipid peroxidation product $LOO^{\bullet}$, and this can be propagated in various ways, creating new radicals. The radical chain reaction will terminate in several ways, as illustrated in the figure.

Figure 9 illustrates several ways that special molecules, sometimes referred to as "*anti-oxidants*," act to eliminate radicals, thus "protecting" the organism. In Fig. 9, two antioxidants, namely α-tocopherol ("vitamin E") and ascorbate ("vitamin C"), are shown acting in concert. Tocopherol is fat- (or lipid-) soluble and resides in the lipid bilayer membrane of the cell whereas ascorbate operates in the aqueous

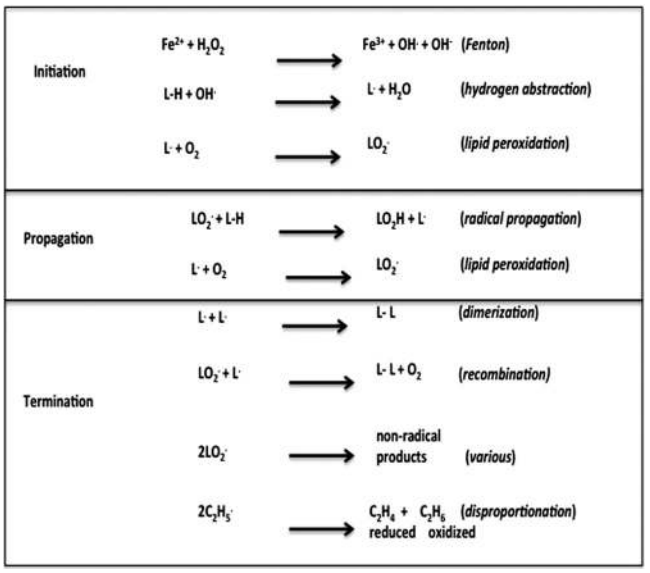

FIG. 8. Lipid peroxidation chain reaction. Initiation occurs when a $OH^{\bullet}$ radical, created for example by the Fenton reaction, abstracts a H atom from a lipid molecule ('L') to form a lipid radical (denoted ('$L^{\bullet}$'). This radical reacts quickly with dissolved O_2 to form the lipid peroxyl radical $LO_2^{\bullet}$, which in turn will create another radical. The radical chain can be terminated through one of a series of reactions that result in non-radical products, or possibly a less reactive radical (cf. Fig. 9).

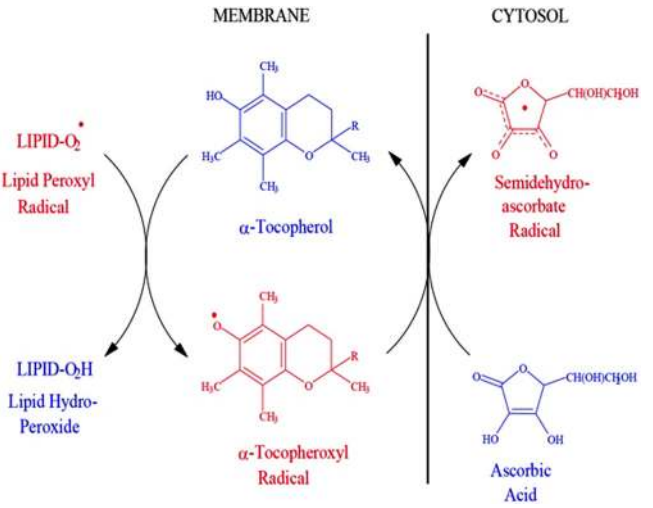

FIG. 9. Schematic diagram of coupled antioxidants protecting against the potentially damaging effects of a lipid peroxyl radical. In this scenario, a radical lipid peroxyl is converted by cell membrane-based antioxidant α-tocopherol to a more stable product (lipid hydroxy-peroxide), but creating an α-tocopherol radical in the process. This radical is in turn converted back into the non-radical parent molecule by reacting with cytosol-based anti-oxidant ascorbic acid, creating a more stable radical structure that is much less reactive and therefore less damaging. The cytosol is the liquid inside cells; it is separated into compartments by membranes. Membrane lipids are compounds which form bilayers defining the cell boundaries. Reprinted with permission from I. Acworth, *The Handbook of Redox Biochemistry* (ESA Biosciences, Chelmsford, MA, USA, 2003). Copyright 2003 ESA, Inc.

solution of the cytosol. The radical that originated as a lipid peroxyl radical is transferred to ascorbate via these reactions, ultimately creating a product that is stable enough to not do further damage in the cell.

The older literature emphasized strongly the idea that RONS act mostly as a kind of "molecular hoodlum," responsible only for damaging reactions such as lipid peroxidation. There is an enormous literature with considerable evidence that associates elevated levels of RONS in the body with a vast array of diseases and aging (e.g., Refs. 3, 12, and 31). However, the more recent view is that this purely negative perspective is at least incomplete: RONS play many key positive roles as well, some of which are described later.[32]

IV. RONS IN THE IMMUNE SYSTEM

The immune system is designed to identify and eliminate invading microorganisms, parasites, and even cancer cells. Mammalian immune systems generally involve two separate but interacting components: the innate immune system and the adaptive system. The *innate* immune system reacts immediately following a detected wound formation or some tissue damage and involves attracting phagocytic cells (e.g., white blood cells) to the cite of tissue damage and likely microbial attack. The innate immune system results in local inflammation, including release of RONS, and this must resolve fairly quickly to avoid excessive damage to host tissue. Inflammation that never turns off—that is, *chronic inflammation*—can lead to auto-immune disorders, cancer, and many other maladies.[33]

The *adaptive* immune system is based on the exquisitely sensitive and selective character of antibodies responding to

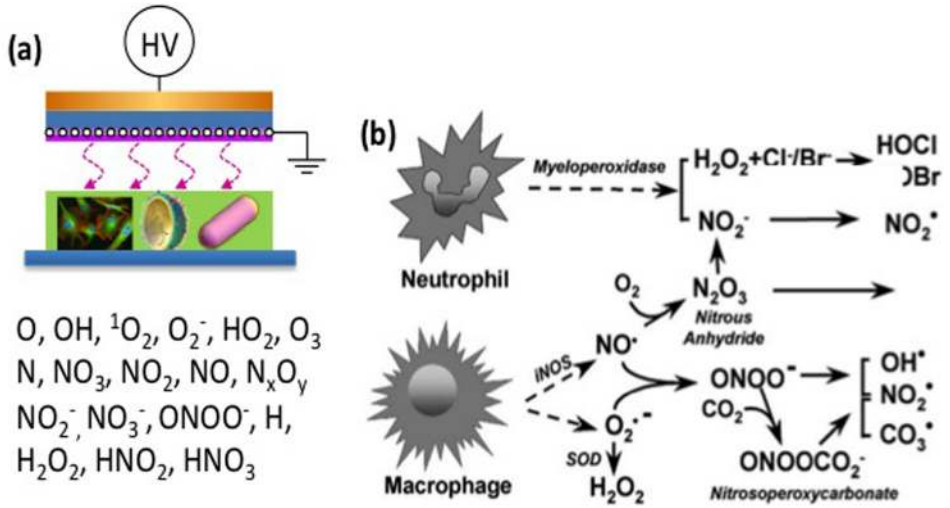

FIG. 10. (a) Illustration of typical chemical species created in CAP in air surface microdischarge (SMD); (b) corresponding set of chemical species known to be created in mammalian innate immune systems. Reprinted with permission from P. C. Dedon and S. R. Tannenbaum, Arch. Biochem. Biophys. **423**, 12–22 (2004). Copyright 2004 Elsevier.

perceived antigens, then activating T cells and B cells to attack and eliminate these threats. It is activated later than the innate system and can act much longer. Vaccines, for example, are intended to activate the adaptive immune system to provide long-term protection against infective targets. RONS are known to be involved in both parts of the immune system, but they have been most thoroughly studied in the context of the innate immune system.

White blood cells such as macrophages and neutrophils seek to identify and eliminate invading microorganisms via phagocytosis. An important part of the action of these cells is the creation and use of various RONS. An illustration of this is shown in Fig. 10.[34]

The fact that air plasma creates a set of chemical species that are identical or closely related to species created by white blood cells suggests that the observed therapeutic successes of CAP may be due, at least in part, to these species. Furthermore, the use of therapies that rely explicitly on RONS to treat infectious agents has become more widely accepted in the last several years. A recent review highlighting this principle lists the following methods that are known to rely on this mechanism in addition to CAP: standard antibiotics and disinfectants; photodynamic therapy; photocatalysis (e.g., using TiO_2); medicinal honey; and NO-releasing molecules and nanoparticles, among others.[35] Another recent review summarizes the use of redox-active drugs to fight parasite infections such as Leshmaniasis, Chagas disease, and malaria, among many others.[36] The postulated mechanisms of action of CAP for cancer therapy is addressed below.

V. CAP AND CANCER THERAPY

One of the most exciting and promising area of plasma medicine is the treatment of cancer. Schlegel *et al.*[37] recently summarized progress in applying CAP for cancer therapy. Most of the 41 studies listed in this review are *in vitro* (studying the response of various cancer cell lines growing in dishes), but about half a dozen are *in vivo* studies, mostly in mice. Several other studies have been published since this paper was published in mid-2013, giving some idea of the rapid pace in the field. I will focus below mainly on recent *in vivo* results here since they are key to demonstrating that plasma techniques have genuine potential for practical cancer therapy. Schlegel *et al.*[37] observe that many different types of cancer cell lines have been tested and they all show sensitivity to plasma treatment. Many authors observed cells undergoing programmed cell death ("apoptosis") and that the plasma treatment appeared to stop the normal cell cycle, leading to enhanced cellular vulnerability. Several researchers noted that radiation-resistant and chemo-resistant cell lines seem especially sensitive to plasma treatment.[38,39]

One recent dramatic example, in this case in the form of a He plasma jet created in a thin capillary tube in an open air environment, was reported by Brulle *et al.* (2012).[40] These authors implanted mice with human pancreatic cancer cells onto the mouse pancreas and tested the plasma device acting alone or in combination with the drug gemcitabine, a common pancreatic cancer chemotherapeutic agent. The effects of the plasma, drug, and combination treatment as a function of days post-tumor implantation are shown in Fig. 11.

The fact that the plasma seems to combine effectively with chemotherapy suggests that plasma treatment could be used clinically as an "adjuvant," or combination therapy with other therapies.

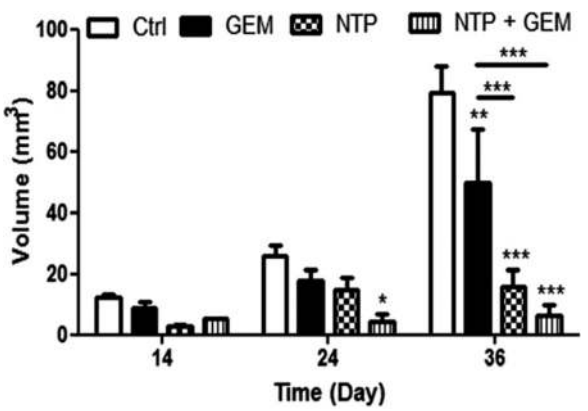

FIG. 11. Table indicating the effects of the gemcitabine drug treatment ("GEM"), application of the plasma ("NTP" for non-thermal plasma), and the combination of the two treatments on tumor volume in a set of mice. The mice were followed for a maximum of 36 days. The results show promising evidence of a positive additive effect of plasma and drug treatment (Ref. 40).

Similarly positive results have been reported for other types of cancer cells injected under the skin of mice by Vandamme *et al.*,[41,42] Keidar *et al.*,[43] Walk *et al.*,[44] Partecke *et al.*,[45] and Utsumi *et al.*,[39] among others.

An obvious question for potential plasma cancer therapies is how to apply the plasma if the tumor is internal to the body? Access for external organs like the skin (e.g., melanoma) or treatment within body cavities such as colorectal or bronchopulmonary cancer are fairly obvious, but what about other possible tumor locations? Walk *et al.*[44] note that if a surgeon has sufficient physical access to a tumor to remove it, then plasma treatment could follow surgical resection. In this case, the surgeon could use the plasma to "paint" the resected tumor bed, potentially limiting cancer recurrence. These authors also suggest that plasma-generating devices could be miniaturized sufficiently to fit on the tip of a catheter that could be applied using small holes to access various internal cavities or even be able fit inside veins.

The recent results of Utsumi *et al.*[39] show that plasma treatment of cell culture medium can yield a fluid that is itself tumoricidal. Utsumi *et al.* focused on ovarian cancer, and this type of cancer can manifest as tumors that coat the inside of the peritoneal cavity. It is generally difficult for surgeons to treat this "bed" of tumors as they try to "de-bulk" all of the tumors to a diameter of less than 1 cm. Utsumi *et al.* suggest that this anti-tumoral plasma-treated liquid medium could be conveniently injected into the peritoneal cavity.

As a final prospective example, consider prostate cancer treatment. Current prostate cancer treatment often involves non-surgical "focal" treatment if the tumor remains localized, but existing methods are not fully satisfactory. These methods include focused ultrasound therapy, photodynamic therapy, cryotherapy, and radiation therapy. CAP therapy is being envisioned as a possible replacement or adjuvant for these conventional non-surgical therapies. Fig. 12 shows an image of a plasma device that might be applied to treat a localized prostate tumor, following Hirst *et al.*[46] The TRUS (trans-rectal ultrasound) device is used to identify the tumor location and the plasma delivery device is inserted through a small hole in the perineum to contact the tumor.

VI. CAP THERAPEUTIC MECHANISMS

It is natural to ask what biochemical mechanism or mechanisms might be responsible for the *selective* effects of plasma against tumors (or against infecting microrganisms or parasites for that matter) as compared to their effect in damaging non-cancerous on uninfected tissue. It is trivially easy to kill cells of any kind so the question is always about selectivity: how does the putative therapy minimize damage to normal tissue while killing tumors or infectious pathogens? As noted above, the chemical similarity between species created in air plasma and those known to be involved with the actions of the innate immune system suggest that CAP-generated RONS are somehow causing the observed successes. Nathan and Shiloh[47] addressed this important question of specificity related to the action of RONS in the context of the immune system. They wrote:

Perhaps the most striking example of non-specificity in the immune system is its reliance on the production of chemically reactive micromolecules (i.e., *RONS*) that do not discriminate the genomic source of their chemical targets.

One possible answer to this puzzle, Nathan and Shiloh assert, is that the multicellular organism (animal or plant) will locally sacrifice some of its own cells at the site of an apparent infection that if uncontained could spread and kill the entire organism.[47] But this action must be turned off fairly soon, or unacceptable damage to the host will ensue, as noted above. We might postulate that some degree of selectivity for CAP therapies should be analogous to the way the innate immune system operates: they should be *spatially localized* and *relatively short* treatments at known sites of infections or tumors.

There is another postulated mechanism that has been proposed to provide some degree of selectivity with RONS used for cancer therapy. This is sometimes referred to as "prooxidant therapy," and, quite apart from CAP, has received

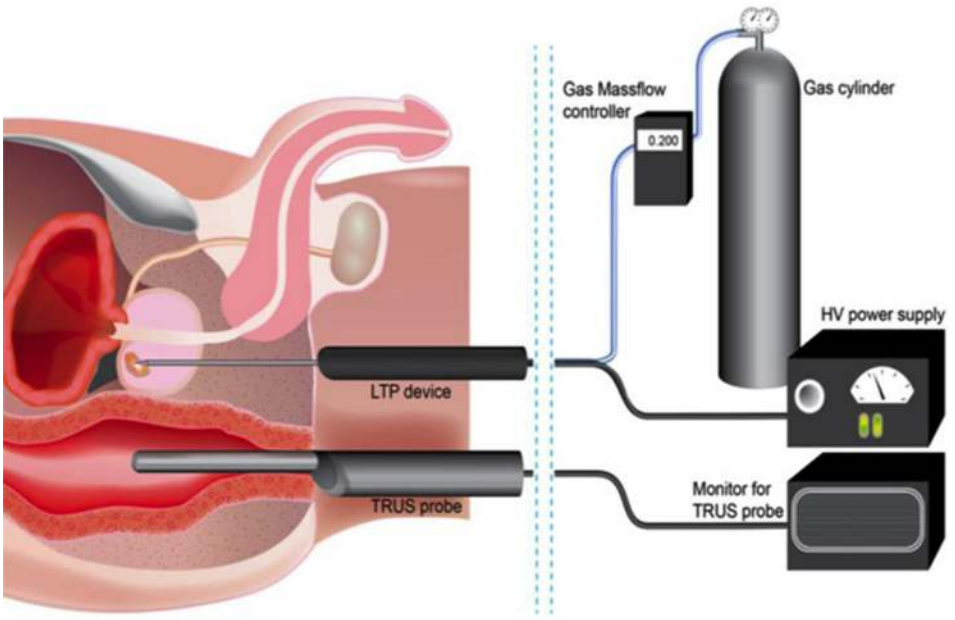

FIG. 12. Proposed configuration for treating localized prostate tumor using a low temperature plasma (LTP) device, with positional guidance provided by ultrasound (TRUS probe) (Ref. 46).

considerable attention in the literature over the last 5–10 years. (e.g., Refs. 48–52) The basic idea is to selectively attack the tumor cell by raising oxidative stress on all cells and rely on the differences in metabolism and signaling between cancerous and normal cells to selectively kill the cancer cells. This strategy is based on the fact that most tumors have a higher base level of RONS and oxidative stress than non-cancerous tissue, and they are already expressing all of the antioxidants they can in order to avoid death.

The fact that ionizing radiation kills cancer cells mostly via the generation of oxidative stress in cancer cells is well established.[53] PDT, as noted above, involves the use of photosensitizer drugs that ideally segregate to tumors or infection sites. These compounds are activated by externally applied light to excite oxygen in solution to a singlet delta state. This reactive oxygen species then reacts with adjacent biomolecules, causes various biological effects such as programmed cell death ("apoptosis"), and leads to tumor shrinkage. PDT is also a USA Food and Drug Amdinistration- (FDA) approved therapy for some forms of macular degeneration, a serious eye disease that often afflicts the elderly.[54]

Many chemotherapies also operate by raising oxidative stress in cells or by somehow restricting antioxidants or the enzymes that make the antioxidants. Gorrini *et al*.[52] describe in detail why and how cancer cells generally have much higher levels of reactive oxygen species than normal cells due to their metabolic and signaling aberrations. This makes them vulnerable to further increases in oxidative stress since at some point, this excessive oxidative stress will result in cell death. This concept is illustrated in Fig. 13: normal cells have low base levels of ROS and can survive if oxidative stress is increased via chemotherapy, but cancer cells start at higher levels of ROS and they cannot protect themselves as well, resulting in cell death if drugs or some other source increases cellular oxidative stress.[49]

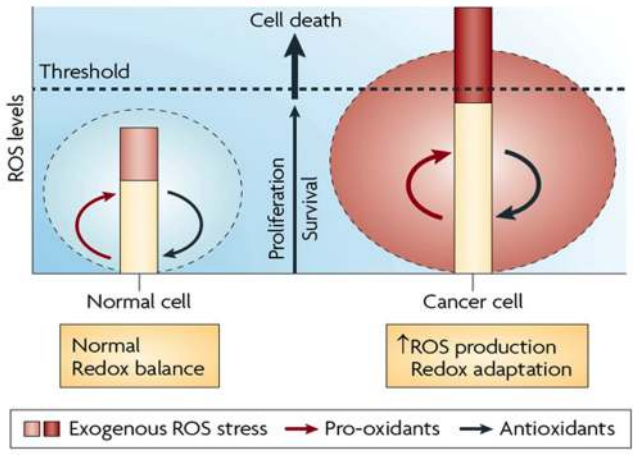

FIG. 13. Proposed model for selective, therapeutic effects of increasing reactive oxygen stress on normal cells and cancer cells. The normal cell (left) can sustain an increase in oxidative stress induced by "exogenous" (external) sources such as chemotherapy and remain below the threshold for cell death (dashed line). The cancer cell (right) starts at a higher base level of ROS and increases of oxidative stress from drugs or other external agents will force it above the threshold, resulting in cell death. Reprinted with permission from D. Trachootham, J. Alexandre, and P. Huang, Nat. Rev. Drug Discovery **8**, 59–591 (2009). Copyright 2009 Macmillan Publishers Limited.

Most reports of CAP on cancer cells have reported observing increases in ROS concentrations in cells exposed to plasma and the cell death mode is generally consistent with a ROS-based mechanism (e.g., Refs. 41, 42, 55, and 56). Addition of ROS-scavenging agents generally eliminates the anti-cancer cell actions of the plasma. The available data therefore seem to support the hypothesis that CAP cancer treatment can be thought of as a kind of oxidative stress modulating therapy.

VII. CONCLUDING REMARKS

CAP biomedical applications have advanced rapidly in the last several years and have been successfully applied in a variety of applications, ranging from treating infected tissue to promoting wound healing to shrinking tumors. Two CAP devices have received CE marking in Germany in 2013 for wound healing applications. There are many different types of CAP devices and the ways they are employed can be varied; but in general, the progress in biomedical applications of CAP seems quite promising.

A better understanding of the mechanisms of biomedical therapeutic action will allow further refinement and extensions of the technology. The point of view taken here is that the reactive oxygen and nitrogen species created by CAP are probably central to their effectiveness. Analogies with the mammalian and plant immune systems and similarities with existing therapies support this hypothesis, but more work must be done to base our understanding on solid science. The effects of RONS are probably virtually always indirect: the reactive species created in the gas phase enter the liquid phase and then react with bio-macromolecules (proteins, lipids, carbohydrates, amino acids, etc.) and these relatively stable species are the ones that alter cell signaling processes, modify gene expression, influence immune system response, disrupt cell cycle, induce apoptosis (programmed cell death), and no doubt get involved in other cellular processes.

And although the emphasis in this article has been on the likely role played by RONS in plasma therapeutics, it must be kept in mind that other mechanisms may, at least in some cases, be important. These include things like the effects of electric fields, photons, and charge accumulation.

Assuming the plasma acts primarily by creating and delivering biochemically active chemical species, it may be that plasma is not unique. The plasma is creating at least some chemical species that may be created and delivered therapeutically in other ways. A key question is: why use plasma? The flexibility and power of plasma technology suggest that there will be many reasons why plasma creation and delivery is preferred, and working out these details will require close cooperation between plasma specialists, biochemists, microbiologists, molecular and cell biologists, and medical researchers. Plasma medicine promises to be one of the most important applications of CAP technology ever developed and it is expected to mature significantly over the next decade or so.

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