Mechanisms of Interaction of Non-Thermal Plasma with Living Cells

A Thesis

Submitted to the Faculty

of

Drexel University

by

Sameer Ulhas Kalghatgi

in partial fulfillment of the

requirements for the degree

of

Doctor of Philosophy

March 2010

COPYRIGHT

DEDICATIONS

This thesis is dedicated to the following wonderful people in my life:

My wonderful parents, Ulhas and Suchita Kalghatgi who have raised me to the person I am today, who have been my role-model for hard work, persistence and personal sacrifices, and who instilled in me the inspiration to set high goals and the confidence to achieve them.

My dearest wife, Sheetal, who has been my pillar of strength and stood by me every step of the way through good times and bad, who constantly motivated me, had confidence in me when I doubted myself, boosted my morale whenever I felt low and who has shared the many uncertainties, challenges and sacrifices for completing my PhD.

Thank you Mom, Dad and Sheetal for your unconditional love, guidance, and support that you have always given me, helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to. Thank you for everything. I love you!

ACKNOWLEDGEMENTS

Though only my name appears on the cover of this dissertation, many people have contributed towards the creation and completion. I am grateful to all those people who have made this dissertation possible and because of whom my experience during the course of my PhD has been one that I will cherish forever.

First, I would like to express my deepest gratitude to my advisor Dr. Gary Friedman, for his constant encouragement, guidance and support throughout my PhD at Drexel University. He was highly instrumental in initiating me in to the exciting world of Plasma Medicine. He not only taught me how to express my ideas but also showed me different ways to approach a research problem along with the virtue of persistence to accomplish any goal. I have been fortunate to have an advisor who gave me the freedom to explore my own ideas and at the same time his patience and support helped me overcome many crisis situations and finish this dissertation. I really admire his tremendous knowledge and skills and I am grateful for his assistance in editing journal publications, peer reviewed conference publications and this thesis. He was always there to meet and talk about my ideas, to proofread and mark up my papers and chapters, and to help me think through my problems (whether philosophical, personal, analytical or experimental). Gary has truly been a friend, philosopher and guide.

I would like to specially acknowledge and appreciate Dr. Alexander Fridman, who played a highly important role in helping me complete my PhD and guiding me in the challenging research that lies behind it. Dr. Fridman has truly been a great mentor. He made me a better plasma physicist and brought out the good ideas in me. (More

importantly, he taught me how to work hard and play hard, and introduced me to his witty toasts which always ended with a shot of vodka and a slice of pickles).

I would like to acknowledge and appreciate Dr. Jane Azizkhan-Clifford who played a pivotal role in laying the foundations of my PhD by welcoming me, an electrical engineer, with open arms in her lab and in to the field of molecular and cell biology. Without her support and encouragement neither would have this research been possible nor could I have completed this dissertation. Her valuable insight, expertise and immense wisdom helped me tide over many a challenging situation and carry out scientific hypothesis driven research.

I would like to thank, Dr. Alisa Morss Clyne for the many fruitful discussions and her valuable assistance she provided at all levels of the research on understanding the effects of non-thermal plasma on proliferation of endothelial cells.

I would like to thank the members of my thesis committee, Dr. Timothy Kurzweg and Dr. Adam Fontecchio for agreeing to serve on my committee and for reviewing my dissertation and providing many valuable comments that improved the presentation and contents of this dissertation.

I am grateful to all my friends at Drexel University, in particular, Shubham, Suren, Hemang, and Sameet and fellow graduate student and medical residents, including Crystal, Ekaterina, Behzad, Krishna, Gregory Fridman, Moogega, Rachel, Danil, Jaqueline, Andrew Wu for many interesting and good-spirited discussions relating to my research and about life as a graduate student which really helped me to enjoy my time at Drexel University.

Most importantly, none of this would have been possible without the love and support of my parents and sister. My parents, to whom this dissertation is dedicated to, have been a constant source of love, concern & strength; and my sister Neha, for her love and affection and for believing in me the most. I would like to express my heart-felt gratitude to my family over all these years.

Last but not the least; I would like to thank my wonderful and loving wife Sheetal for her unwavering support and understanding during the course of my PhD. Her being there made all the difference and her support, encouragement and love were in the end what made the completion of my PhD and writing this dissertation possible.

Table of Contents

List of Tables

List of Figures

Abstract
Mechanisms of Interaction of Non-Thermal Plasma with Living Cells
Sameer Ulhas Kalghatgi
Gary Friedman, PhD

Thermal plasmas and lasers have been widely used in medicine to cut, ablate and cauterize tissues through heating; in contrast, non-thermal plasma produces various highly active molecules and atoms without heat. As a result, its effects on living cells and tissues could be selective and tunable. This makes non-thermal plasma very attractive for medical applications. However, despite several interesting demonstrations of non-thermal plasma in blood coagulation and tissue sterilization, the biological and physical mechanisms of its interaction with living cells are still poorly understood impeding further development of non-thermal plasma as a clinical tool. Although several possible mechanisms of interaction have been suggested, no systematic experimental work has been performed to verify these hypotheses.

Using cells in culture, it is shown in this work that non-thermal plasma created by dielectric barrier discharge (DBD) has dose-dependent effects ranging from increasing cell proliferation to inducing apoptosis which are consistent with the effects of oxidative stress. DNA damage is chosen as a marker to assess the effects of oxidative stress in a quantitative manner. It is demonstrated here that plasma induced DNA damage as well as other effects ranging from cell proliferation to apoptosis are indeed due to production of intracellular reactive oxygen species (ROS). We found that DNA damage is initiated primarily by plasma generated active neutral species which cannot be attributed to ozone alone. Moreover, it is found that extracellular media and its components play a critical

role in the transfer of the non-thermal plasma initiated oxidative stress into cells. Specifically, it is found that the peroxidation efficiency of amino acids is the sole predictor of the ability of the medium to transfer the oxidative stress induced by non-thermal plasma.

Phosphorylation of H2AX, a DNA damage marker, following plasma treatment is found to be ATR dependent and ATM independent, suggesting that non-thermal plasma may induce formation of bulky lesions unlike ionizing radiation (IR) or H_2O_2 which primarily produce DNA double strand breaks. Moreover, it is found that the pathway by which plasma generated oxidative stress is transferred across cellular membranes does not involve lipid peroxidation by-products, although lipid peroxidation does occur.

Chapter 1. Introduction

1.1. Plasma

Plasma is a partially ionized medium. Plasma will refer here to a partially ionized gas. It is characterized by a mixture of electrons, ions and neutral particles that, on average, is electrically neutral. Plasma can be created in several different ways. Heating is one of them. Electric field driven ionization is another. The term *plasma* was most likely coined by Irving Langmuir, because the multi-component, ionized gas reminded him of blood plasma in medicine [1]. This term is likely to create some confusion particularly in medical applications and especially during the process of plasma (the ionized gas) assisted blood coagulation. When necessary, the plasma in the form of ionized gas will be referred to as electrical plasma or non-thermal plasma to avoid the confusion.

Plasma occurs naturally and can also be man-made. Examples of natural plasma include solar corona, the Earth's ionosphere, lightning (Figure 1A) and Aurora Borealis (Figure 1B). Man-made plasma is employed in semiconductor electronics manufacturing, lighting, treatment of synthetic fabrics, plasma TVs, and many other areas including Plasma Medicine. Most man-made plasmas are created electrically. They are primarily used (1) to generate high temperature in gas phase; (2) to create electrically conducting gas medium; (3) to produce charged particles (ions or electrons) and, possibly, control their directed motion; (4) to create electronically excited molecules such as excited nitrogen or singlet oxygen $^1O_2$ for various purposes including generation of light and (5)

to produce radicals and other chemically active species such as ozone, molecular oxygen, $OH^{\bullet}$ radicals, nitric oxide (NO), and others.

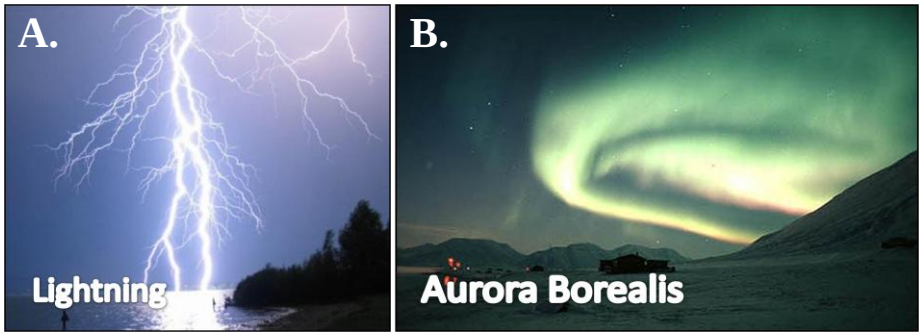

Figure 1. Plasmas in Nature. Examples of **(A)** Thermal and **(B)** Non-thermal plasmas.

For the purpose of this discussion it is convenient to classify all plasmas as low or atmospheric pressure. Low pressure plasmas are employed in electronics manufacture and lighting, for example. In this work, however, atmospheric pressure plasma will be the entire focus and we will not discuss anything related to low pressure plasmas. Another important characteristic of plasma in medical applications is its temperature. Most of the known uses of electrical plasma in medicine are based on the high temperatures generated on or within tissues by electrical discharges. Electrocautery [2, 3] and Argon Plasma Coagulator [4, 5], are widely used in medicine today to rapidly coagulate blood and ablate tissue [2, 3, 5-11] . However, they lead to significant thermal tissue damage. For these reasons, non-thermal room temperature electrical discharges, where thermal damage is eliminated and specific biochemical reactions can be initiated, are being sought [12-14]. It is such non-thermal discharges and their mechanisms of interaction with living tissues that are the sole focus of this work.

Different physical mechanisms of creating non-thermal plasma in electrical discharges are based on some common principles. In the very beginning of the electrical discharge most of the energy goes to electrons, rather than ions, because electrons are

much lighter and accept this energy faster. As a result, the temperature of electrons in plasma will rise quickly to around 10,000 K. Ions and neutral molecules, however, can remain at nearly room temperature for a short time. The resulting plasma is often called *non-equilibrium* because of the dramatically different temperatures of the electrons on the on hand and ions and neutral species on the other. Over time, however, heat is transferred from the electrons to ions and neutral species. Given the opportunity, temperature of heavier components of plasma can reach the same order of magnitude as the electron temperature. Such thermal plasmas are sometimes called *equilibrium* because of the temperature equilibration. Non-equilibrium or non-thermal plasma can, therefore, be maintained by not giving the opportunity for heat to transfer from electrons to ions and neutral species. This, in turn, can be accomplished in several ways. One is to limit the time average electrical current or equivalently the amount of time over which sufficient electric field is applied. Another is to transfer heat from the plasma region by limiting plasma volume and increasing heat transferring surface area around it.

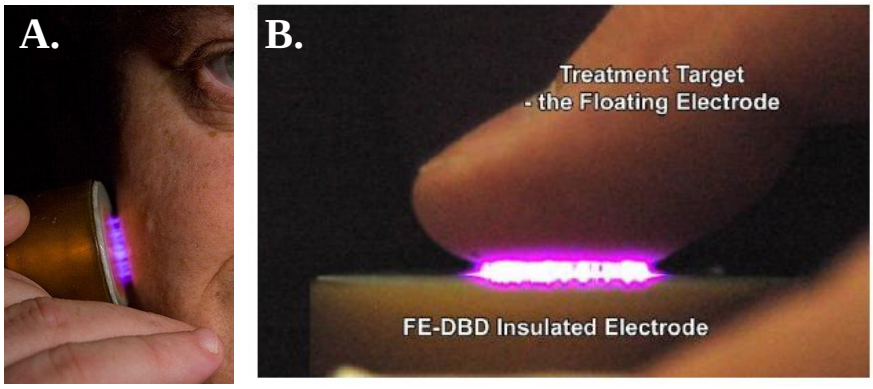

Figure 2. Floating electrode dielectric barrier discharge (FE-DBD) plasma. (a) Non-thermal plasma is safe to apply on living tissue. (b) The human body with its high dielectric capacity functions as the second electrode (floating electrode) [12].

1.2. Non-Thermal Dielectric Barrier Discharge Plasma

Non-thermal atmospheric pressure dielectric barrier discharge (DBD) plasma has recently emerged as a novel tool in medicine. The operating principle of this plasma discharge is similar to the Dielectric Barrier Discharge introduced by Siemens in 1862 [15]. Non-Thermal plasma occurs at atmospheric pressure in air or other gases when high voltage of sinusoidal waveform or short duration pulses is applied between two electrodes, with at least one electrode being insulated [16]. The insulator prevents build-up of current between the electrodes, creating electrically safe plasma without substantial gas heating. This approach allows direct treatment of living tissue (Figure 2) and biological systems without thermal damage observed in more conventional thermal plasma [5, 12].

In the case of direct application to a living tissue, the dielectric barrier discharge system (called Floating Electrode DBD or FE-DBD) is constructed similarly to conventional dielectric barrier discharge described above and is inherently non-thermal – it is able to operate at room temperature and atmospheric pressure [12, 15, 16]. Plasma operates under conditions where one of the electrodes is an insulated high voltage electrode and the second *active* electrode is human (Figure 2) or animal tissue or organ [12]. Plasma treatment exposes cells or the tissue surface to active short and long lived neutral atoms and molecules, including ozone (O_3), NO, OH radicals, and singlet oxygen ($O_2\ ^1\Delta_g$), and a significant flux of charged particles, including both electrons and positive and negative ions like super oxide radicals. Non-thermal plasma density, temperature, and composition can be changed to control plasma products to some extent [17].

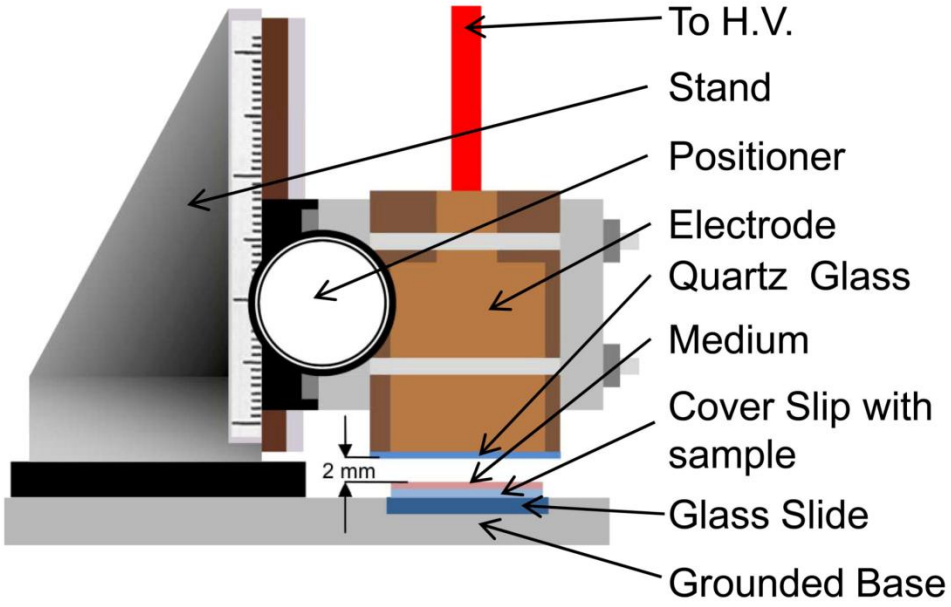

Figure 3. Non-thermal dielectric barrier discharge plasma treatment setup

For most of the work reported here, non-thermal atmospheric pressure dielectric barrier discharge plasma was produced using an experimental setup similar to one previously described [12] and schematically illustrated in Figure 3 and the actual setup itself is as shown in Figure 4.

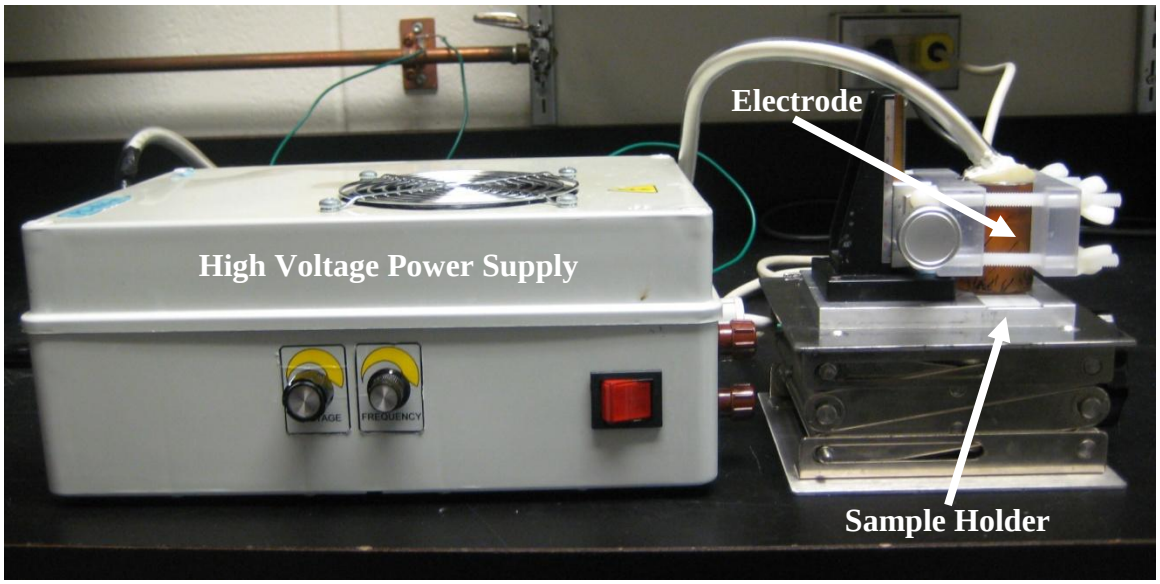

Figure 4. Non-thermal DBD plasma power supply, electrode and setup used for in vitro treatment of mammalian cells.

Non-thermal plasma was generated by applying alternating polarity microsecond pulsed voltage between the insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply (Quinta, Russia). 1 mm thick, polished clear fused quartz was used as an insulating dielectric barrier covering the 2.5 cm diameter copper electrode. The discharge gap between the bottom of the quartz and the treated sample surface was fixed at 2 mm. Discharge power density was measured to be roughly 0.13 Watts/cm^2 (at 500Hz) and 0.31 Watts/cm^2 (at 2 kHz) using both electrical characterization and a specially designed calorimetric system [18]. The values for typical discharge parameters are provided in Table 1.

Table 1. Operating parameters for the non-thermal atmospheric pressure dielectric barrier discharge (DBD) plasma used for all the studies in this dissertation

Parameter	Value
Excitation	Microsecond pulsed
Voltage	20 kV p-p
Rise Time	5V/ns
Pulsed Width (FWHM)	1.65 μsec
Frequency	0.5 – 1.5 kHz
Power Density	0.1 – 1 W/cm^2
Rotational Temperature	300 – 350 K
Vibrational Temperature	3000 – 4000 K

The plasma treatment dose in J/cm^2 is calculated by multiplying the plasma discharge power density by the plasma treatment duration. Non-thermal DBD plasma has a g-factor (number of ROS generated per electron volt or eV) between 0.3 and 0.5 [19]. For a plasma dose of 3.9 Joules cm^{-2}, 7.32 – 12.2 x 10^{16}/cm^3 ROS are generated in the gas phase. Table 2 shows typical densities of various active species generated by non-thermal dielectric barrier discharge plasma in gas phase [17, 20-22]. It is important to note that

DBD plasma at room temperature generates about 2 – 3 orders of magnitude more ozone than nitric oxide.

Table 2. Typical range of densities of various active species generated by non-thermal dielectric barrier discharge plasma in gas phase. (e^-: electrons; M^+: positive ions)

Plasma Generated Species	Density (cm^{-3})
Superoxide ($O_2^{\bullet-}$)	10^{10} - 10^{12}
Hydroxyl ($OH^{\bullet}$)	10^{15} - 10^{17}
Hydrogen Peroxide (H_2O_2)	10^{14} - 10^{16}
Singlet Oxygen ($^1O_2$)	10^{14} - 10^{16}
Ozone (O_3)	10^{15} - 10^{17}
Nitric Oxide (NO)	10^{13} - 10^{14}
Electrons (e^-)	10^{9} - 10^{11}
Positive Ions (M^+)	10^{10} - 10^{12}

1.3. Plasma Medicine

Plasma technology has been employed for some time in controlling microbial contamination. In fact, generation of UV light often employs lower pressure plasma discharges. Ozone which has been widely used as a disinfectant for water is typically generated by non-thermal plasma. However, broader spectrum of applications of non-thermal atmospheric pressure plasma for surface sterilization of inanimate substrates and modulation of inanimate surfaces for the purposes of cell attachment has been discovered relatively recently. In fact, the past ten years have resulted in significant developments of non-thermal plasma for various applications in medicine, where investigations are being carried out mostly at academic institutions and research centers on treatment of living tissues for the purpose of killing microbes on wounds, burns, within intestines and teeth, enhancing wound healing, coagulating blood without damage of surrounding tissue, and even treatment of malignancies. The most important finding so far is that non-thermal

plasma can be somewhat selective in killing microbes, while showing relatively little or no damage to surrounding living tissues. The reasons for this are not yet completely clear. What are the reasons for only recent emergence of applications of non-thermal plasma in life sciences? Some of the reasons can be related to only recent (past twenty years) emergence of power electronics technology which makes control of non-thermal plasma inexpensive. We can now make relatively small power supplies that control non-thermal air plasmas relatively cheaply. However, the main reason is probably socio-economic. Throughout 1970s, 80s and 90s plasma was being developed as one of the key technological tools for semiconductor industry. Many plasma scientists and engineers were focusing on electronics production at that time. These tools are now relatively well-developed. Moreover, there is a sense that the peak of electronics revolution has passed. For this reason many of the same scientists and engineers are seeking new applications for plasma. Applications of non-thermal plasma in medicine also pose a challenge because of their highly interdisciplinary nature.

1.3.1. Overview

Some of the earlier applications of plasma in medicine relied mainly on the thermal effects of plasma. Heat and high temperature have been exploited in medicine for a long time for the purpose of tissue removal, sterilization, and cauterization. Electrocautery is a more modern technique which applies controlled heat to surface layers of tissue by passing sufficiently high current through it [4]. However, contact of tissue with metal surface of a cautery device often results in adhesion of charred tissue to the metal. Some of the earlier applications of plasma in medicine provided an alternative to

metal contact electrocautery. In argon plasma coagulation (APC, also sometimes called argon beam coagulation), highly conductive plasma replaced the metal contacts in order to pass current through tissue avoiding the difficulties with tissue adhesion. Hot plasma is also being employed to cut tissue, [3, 5–8] although the exact mechanism by which this cutting occurs remains unclear. What differentiates more recent research on applications of plasma in medicine is the exploitation of non-thermal effects. Why are non-thermal effects of plasma so interesting and promising? The main reason is that non-thermal plasma can potentially be tuned to achieve various sub-lethal effects, not just indiscriminant tissue destruction.

1.3.2. Clinical Applications of Non-Thermal Plasma

Non-thermal plasma has been widely studied for sterilization of inert surfaces [12, 17, 20, 23-28] and treatment of inert substrates with the purpose of modulating cell attachment [14, 29]. Only recently it has been shown that non-thermal plasma can be applied to cells in sub-lethal doses to elicit specific biological effects, including gene transfection [30-32], cell detachment [14, 29, 33, 34], induction of apoptosis [35], cell proliferation [36] and wound healing [12, 13, 37-39]. Non-thermal plasma can even have selective effects. In recent studies on plasma initiated blood coagulation [12, 13], skin sterilization [12, 28] and tissue toxicity after plasma treatment [40, 41], plasma did not demonstrate effects visible to the eye.

1.3.2.1. Blood Coagulation

One of the areas where the use of plasma is being investigated is in blood coagulation to stop bleeding in critical life threatening situations. For example, coagulation is a vital issue in severely injured individuals in situations involving accident victims. Methods of inducing coagulation rapidly in such cases are critical for emergency medical responders to treat victims at the scene of an incident. Non-thermal plasma provides this opportunity to induce coagulation in a safe manner both at the accident site and in hospital emergency rooms [12, 13], particularly for slow bleeding. Similar applications might exist in brain surgeries, bleeding of vocal chords and other similar situations.

1.3.2.2. Sterilization

Another important area where the non-thermal plasma may have a significant impact on healthcare is related to the prevention of infection in skin wounds including burns and ulcerations that occur, for example, in patients with diabetes. The use of plasma has been demonstrated to have the capacity to kill bacteria that cause infections in such wounds without having an adverse affect on human tissues [12, 28, 38]. Such infections are often difficult to treat with conventional antibiotic treatment. Plasma treatment could potentially be used continuously in cases of severe burns as an adjunct to antibiotic therapy to attain sterilization of the affected areas thus preventing infections.

1.3.2.3. Cancer Therapy

Non atmospheric plasma is also being developed as a novel clinical tool for anticancer therapy. Non-thermal plasma has been tested for its ability to effectively treat melanoma, a cancer of the skin, by selectively killing cancer cells while leaving normal skin tissue unharmed. Experiments identified a dose of plasma that caused minimal immediate toxicity, but then went on to stimulate apoptosis in a melanoma cancer cell line [42]. In another study, Vandamme, et. al. at GREMI, University de' Orleans, France, demonstrated a marked antitumor effect of plasma treatment of human U87 malignant glioma xenografts in mice. They reported a tumor volume decrease of 56% for treated mice and also showed that the tumor volume reduction translated into an increase of mouse lifespan of 60% [43].

1.3.2.4. Wound Healing

Recently there is evidence that endothelial cells (cells which line all blood carrying vessels of our body) exposed to short duration (15 s – 30 s) of low power (~ 0.2 W/cm^2) non-thermal plasma treatment exhibit enhanced proliferation which may be due to observed release of Fibroblast Growth Factor (FGF) from plasma treated cells. Endothelial cells play a guiding role in angiogenesis, the growth of new blood vessels from existing vessels. In varied disease conditions, healing may result from promoting or inhibiting angiogenesis. This has further application to the effect of non-thermal plasma on the vasculature, which is exposed during plasma treatment of many tissues [44].

1.3.2.5. Modulation of Cell Transfection

Recent studies have investigated the role of non-thermal plasma treatment for gene therapy and researchers have come up with novel transfection methods for mammalian cells using gas plasmas. Introduction of foreign genes into target cells is a crucial step for achievement of gene therapy. Using an electric pulse-activated gas plasma generator one day after the 1–3 s plasma exposures with DNA concentration at 0.5 μg/μl, favorable transfection efficiencies (17.8–21.6%) and relatively low mortalities (0.65–2.86%) were obtained for HeLa-S3, HT-1080 and MCF-7 cells. The recipient cells became transiently permeable for plasmid DNA during the plasma exposure, suggesting that plasma-mediated transfection may involve similar mechanisms that accounts for electroporation [45]. In another study atmospheric pressure glow discharge torch (APGD-t), a miniature pulsed RF (13.56 MHz) plasma source was specifically designed to efficiently produce and transport reactive species to and interact with biological samples. Results showed that APGDt was capable of creating pores between 4.8 and 6.5 nm in radius in the cell membrane. Transfection of the plasmids in HeLa cells was possible with the APGDt and local efficiencies up to 35% were observed with low mortalities [46].

1.3.2.6. Skin Diseases

One example of plasma use in treatment of skin diseases is Cutaneous Leishmaniasis (CL, caused by the Leishmania parasite) which results from the bite of an infected sand fly when it injects the promastigote form of the disease into the host while feeding. There, the parasites are phagocytosed by the host macrophages, change into amastigote form, and break the host cell continuing the infection. A series of in-vitro

experiments comparing the effect of plasma on human macrophages and on the promastigote form of Leishmania parasite were conducted by Fridman et. al. [47]. Following plasma, 20% of macrophages are inactivated while 100% promastigotes appear inactive as observed through a phase contrast microscope with trypan blue exclusion test for macrophages and simple observation for the protozoa (they stop moving the flagellum and begin to disintegrate which takes about 48 h; the organisms do not appear to re-activate following treatment) [47].

1.4. Motivation For this Work

Although electrical discharges that generate non-thermal plasma (with gas near room temperature) have been known for a long time, their clinical potential has been largely ignored. Possible medical applications described above emerged only within the last 10-15 years. However, there is still a substantial lack of understanding of mechanisms by which non-thermal plasma could interact with living cells and tissues which limits further development of its clinical applications. The group of Eva Stoffels [14, 29, 33, 34, 48-52] did some pioneering work in trying to understand the effects of plasma on cells and they discussed the possible role of reactive oxygen species (ROS) and/or charged species in mediating the effects of plasma. However they have provided no direct evidence of ROS or the role of oxidative stress in any of their papers. Moreover, prior studies related to mechanisms of interactions focused mainly on bacteria. It is demonstrated here that, not only can non-thermal DBD plasma induce a variety of effects on mammalian cells ranging from increased cell proliferation to apoptosis, but also that oxidative stress is the primary mechanism of interaction between non-thermal

plasma and mammalian cells. This suggests that non-thermal plasma can be used to extend the list of medical tools, such as IR, photodynamic therapy, and various drugs, whose primary effects are mediated by Reactive Oxygen Species (ROS). It also raises questions about possible differences between oxidative effects created by plasma in comparison to IR, UV and hydrogen peroxide (H_2O_2).

1.5. Dissertation Overview and Organization

It is hypothesized that non-thermal plasma interacts with living cells via production of reactive oxygen species and resultant modulation of the oxidative state of the cells. The goal of this dissertation is to test this hypothesis and understand the mechanisms of interaction of non-thermal atmospheric pressure dielectric barrier discharge plasma with living cells. This involves investigating the effects of non-thermal plasma on living cells and cellular membranes when the cells are in a wet environment under various different mediums by carrying out a series of rigorous *in vitro* experiments. Non-thermal plasma treatment is essentially a surface phenomenon, unlike ionizing radiation, which penetrates through the volume of tissue or fluid being treated, but results have shown that non-thermal plasma treatment of mammalian cells induced DNA double strand breaks inside the cellular nucleus while the cell membrane appears to be intact. This leads to an interesting question, whether plasma has some subtle effects and somehow these effects are able to penetrate the cells? The objective of this dissertation is to investigate this very question and try to present a few potential mechanisms. The outline of the thesis is as follows:

Chapter 2 aims to provide a background on oxidative stress phenomena, effects of oxidative stress on various cellular components including DNA, lipids and proteins, antioxidant defense systems in the body, biological and clinical effects of oxidative stress, the role of oxidative stress in common diseases including diabetes, atherosclerosis, Alzheimers disease, etc. Commonalities between different clinical treatment modalities including, ionizing radiation, UV and hydrogen peroxide, which are known to interact with cells and tissue via the induction of and/or modulation of oxidative stress.

Since plasma is essentially a surface phenomenon, the effects of plasma are more likely to interact first with the cell membranes than DNA, we try to understand effects of plasma treatment on cell membranes of endothelial cells by analyzing the toxicity of non-thermal plasma and release of membrane bound growth factors. Further, low levels of oxidative stress are known to enhance cell proliferation. With the knowledge that non-thermal plasma produces reactive oxygen species in liquid medium, we tested the effects of low dose non-thermal plasma treatment on mammalian cells. The remarkable result that low dose non-thermal plasma enhances proliferation of endothelial cells *in vitro* forms the basis of **Chapter 3.** In this chapter we further try to understand the mechanism of low dose non-thermal plasma induced enhancement of proliferation of endothelial cells. Results indicate that low dose plasma induced oxidative stress may induce cell proliferation via release of FGF. In Chapter 3 we also present results on *in vivo* studies performed on animals to test the toxicity of low dose non-thermal plasma treatment on intact skin and wounded tissue. We were also interested in analyzing how deep do the effects of non-thermal plasma penetrate. This would be an important consideration when

non-thermal plasma would be developed as a clinical tool for skin sterilization and wound healing.

In **Chapter 4** we investigate the effects of high dose non-thermal plasma on mammalian cells. Initiation of apoptosis is an important issue in cancer treatment as cancer cells frequently have acquired the ability to block apoptosis and thus are more resistant to chemotherapeutic drugs. Targeted and selective destruction of cancer cells is desirable for many reasons, ranging from the enhancement of or aid to current cancer therapy modalities like IR and chemotherapy to problems currently lacking a solution, e.g., lung cancer. High level of oxidative stress is known to be detrimental to cell survival and in many cases leads to induction of apoptosis. We demonstrate the induction of apoptosis in a human melanoma cell line *in vitro* by exposure to high dose of non-thermal atmospheric pressure plasma. Our results show that exposure of tissue culture to atmospheric pressure non-thermal plasma induces apoptosis and this effect is likely related to production of ROS by non-thermal plasma.

Non-thermal atmospheric pressure dielectric barrier discharge plasma applied directly to living tissues is now being widely considered for various clinical applications. One of the key questions that arise in this type of topical treatment is if the skin or tissue remains undamaged after non-thermal plasma treatment. In **Chapter 5** we present results related to the in vivo studies aimed at investigating the toxicity of non-thermal plasma treatment of intact porcine skin and wounded porcine tissue.

In **Chapter 6**, we finally investigate and present results that elucidate some of the possible mechanisms of interaction of non-thermal plasma with living cells. To determine whether DBD plasma treatment of cells could induce DNA damage, we looked at

phosphorylation of H2AX, a histone variant that is phosphorylated in response to DNA damage [53]. Western blot with an antibody that detects H2AX phosphorylated at Ser139 (γ-H2AX) revealed that treatment of cells with DBD plasma induces a dose-dependent increase in γ-H2AX. Neutral active species play a major role in the interaction of non-thermal plasma with living cells and not charged particles. ROS produced by plasma in the medium mediate the interaction of plasma with living cells via production of organic hydroperoxides. These hydroperoxides may lead to lipid peroxidation and the byproducts of lipid peroxidation may bind to receptors on cell membranes and activate intracellular signaling pathways which may lead to subsequent DNA damage. We also show that phosphorylation of H2AX after non-thermal plasma treatment of MCF10A cells is primarily through ATR, in contrast to the ATM-dependent phosphorylation of H2AX after treatment of cells with IR or hydrogen peroxide. Our studies suggest that oxidative stress induced by non-thermal plasma treatment of cells leads to activation of ATR, suggesting that unlike IR and H_2O_2, non-thermal plasma primarily induces bulky lesions and stalled replication forks.

Chapter 7 concludes the thesis by summarizing the results elucidating some of the mechanisms of interaction of non-thermal plasma with mammalian cells and provides brief remarks on the future directions for this work.

1.6. Dissertation Contributions

Specific novel and useful contributions of the work described in this thesis are:

- Experimental confirmation that non-thermal DBD plasma affects genetic material within cells despite the fact that it is a non-penetrating form of treatment. This is important because most of cellular behavior as well as possible cellular transformation are controlled through DNA.
- Definitive test of the hypothesis that the primary agents of interaction between non-thermal DBD plasma and DNA within mammalian cells are reactive oxygen species.
- Experimental demonstration that non-thermal plasma created by DBD has dose-dependent effects ranging from increasing cell proliferation to inducing apoptosis which are consistent with the effects of oxidative stress.
- Discovery that neutral species produced in non-thermal DBD plasma are the primary plasma species which lead to oxidative stress and subsequent effects on DNA. Proof that these neutral species act very differently than ozone alone, although ozone is the major constituent of plasma produced neutral species in gas phase.
- Demonstration that specific composition of the extracellular medium plays the determining role in passing the oxidative stress between gas phase plasma species and cells.
- Proof that non-thermal DBD plasma may induce formation of bulky lesions unlike ionizing radiation (IR) or hydrogen peroxide (H_2O_2) which primarily induce DNA double strand breaks.
- Finally, initial results showing that mechanism by which oxidative stress passes through cell membrane does not require lipid peroxidation.

Chapter 2. Effects Of Oxidative Stress On Mammalian Cells

2.1. Overview

All organisms, except for some anaerobic and aerotolerant species, need O_2 for efficient production of energy by the use of electron transport chains that ultimately donate electrons to O_2. In the biosphere, molecular oxygen is the most important electron acceptor. By virtue of its biradical nature, molecular oxygen readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as reactive oxygen species (ROS) [54]. These include superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals which are involved in initiation and propagation of free radical chain reactions and are potentially highly damaging to cellular targets including DNA, lipids and proteins [54, 55]. In healthy aerobes, production of ROS is approximately balanced with antioxidant defense systems. This balance is not perfect, however, so some ROS-mediated damage occurs continuously. Antioxidant defenses control levels of ROS rather than eliminate them. Although some ROS perform useful functions, the production of ROS exceeding the ability of an organism to mount an antioxidant defense results in oxidative stress. Having too many ROS in relation to the available antioxidants is often said to be a state of *oxidative stress* [54]. Oxidative stress can be defined as the imbalance between cellular oxidant species production and antioxidant capability[56]. In a state of cellular imbalance, in which the levels of oxidants outweigh the levels of antioxidants, damage is caused to nuclear and mitochondrial DNA, proteins, and lipids. ROS are involved in a variety of different cellular processes ranging from proliferation, cellular

injury, apoptosis, necrosis, mutagenesis, and carcinogenesis, accelerated senescence and cell death. Oxidative stress is implicated in various diseases including some allergic and inflammatory skin diseases, Alzheimer's, atherosclerosis in diabetes patients, and Lou Gehrig disease. Cells are exposed to a large variety of ROS from endogenous sources like ROS enzymes, phagocytic cells, cellular metabolism and various diseases and from exogenous sources like radiation, UV radiation, electrophysical phenomena including non-thermal plasma and photodynamic therapy, food, drugs, pollutants and xenobiotics. This review aims to provide a background on oxidative stress phenomena, effects of ROS on various cellular components, biological and medical effects of ROS, differences and commonalities of different clinical treatments including, ionizing radiation, UV and hydrogen peroxide, which interact with cells and tissue via the induction of oxidative stress.

2.2. Reactive Oxygen Species (ROS)

Oxidative stress describes cell damage caused by an overabundance of oxidants, including ROS (ROS, e.g., oxygen ions, free radicals, and peroxide). ROS and other radicals are involved in a variety of biological phenomena, such as mutation, carcinogenesis, degenerative and other diseases, in inflammation, aging, and development. ROS are well recognized for playing a dual role as deleterious and beneficial species. ROS are harmful in excess, but some level of them is necessary for important cellular functions. Some cells produce ROS to kill invading microbes, and ROS are involved in cell signaling [55, 57]. Also, oxidative stress plays a role in cellular processes, such as aging and apoptosis. Oxidative stress can be defined as the imbalance

between cellular oxidant species production and antioxidant capability. ROS are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. In fact, molecular events, such as induction of cell proliferation, decreased apoptosis, and oxidative DNA damage have been proposed to be critically involved in carcinogenesis. Carcinogenicity and aging are characterized by a set of complex endpoints, which appear as a series of molecular reactions. ROS can modify many intracellular signaling pathways including protein phosphatases, protein kinases, and transcription factors, suggesting that the majority of the effects of ROS are through their actions on signaling pathways rather than via non-specific damage of macromolecules; however, exact mechanisms by which redox status induces cells to proliferate or to die, and how oxidative stress can lead to processes evoking tumor formation are still under investigation [54, 55].

In a balanced cell state, ROS are produced as a byproduct of metabolic processes and the level of ROS can be controlled with antioxidants, such as small molecular weight dietary supplements, including vitamin E and vitamin C; small molecular weight peptides and cofactors, including glutathione and pyruvate; and enzymes, including superoxide dismutase and catalase [58]. In a state of cellular imbalance, in which the levels of oxidants outweigh the levels of antioxidants, damage is caused to nuclear and mitochondrial DNA, proteins, and lipids. If this damage is irreparable, then injury, mutagenesis, carcinogenesis, accelerated senescence, and cell death can occur [58]. Oxidative stress has been linked to diseases, including some allergic and inflammatory skin diseases [59], Alzheimer’s [60] and atherosclerosis in diabetes patients [61].

2.2.1. Biologically Relevant Reactive Oxygen Species

Although molecular oxygen is a relatively stable radical, certain oxygen species are significantly more reactive. The different ROS present in the cell exhibit different chemical reactivities. Molecular oxygen exists in the triplet state; two of its valence electron unpaired in separate orbitals and having parallel spins. Ground state oxygen is not very reactive due to the inertness of its orbital structure: it would have to accept a pair of electrons with parallel spins to fill the vacancies of its orbitals. This is unlikely to occur since a pair of electrons in an atom or molecule usually have antiparallel spins [62]. In biological systems, this spin restriction can be accommodated by transition metal ions such as iron, manganese and copper. Transition metal ions undergo single-electron redox exchanges within their unoccupied 3d-orbitals [63]. Molecular oxygen can become more reactive when its outer valence electrons adopt antiparallel spins and occupy the same orbital. This state of molecular oxygen is known as the singlet state. Singlet oxygen ($^1O_2$) is the first excited state of molecular oxygen, and is commonly formed by absorption of light energy. Singlet oxygen is a powerful oxidant because the spin restriction is removed, which allows it to abstract electrons of either spin [62, 64]. The superoxide anion ($O_2^{\bullet -}$) is produced by a single electron reduction of molecular oxygen. Superoxide anion is both an oxidant and a reductant. It is more reactive than triplet state oxygen and tends to dismutate to hydrogen peroxide (H_2O_2) and molecular oxygen. This reaction can be catalyzed by superoxide dismutases such as Cu-Zn superoxide dismutases and Mn superoxide dismutase [62]. Hydrogen peroxide is not an oxygen radical because its valence orbitals become filled with a second electron. It is therefore more stable than any other reduction product of oxygen. Hydrogen peroxide can travel extensively into and

within the cell because of it inherent stability and because it may form hydrogen bound chelate structures with histidines and imidazole rings [65]. By itself hydrogen peroxide does not play a major role in cellular oxidative toxicity [66]. However in the presence of iron (Fe^{2+}) or superoxide anion, hydrogen peroxide can transform into the OH radical ($OH^{\bullet}$), the most reactive chemical species produced in living organisms. It is the third product of the stepwise reduction of oxygen in the electron transport chain and is also generated with superoxide anion and hydrogen peroxide react in the Haber – Weiss reaction. Hydroxyl radical can be produced by the radiolysis (x – rays, gamma – rays) or photolysis (~350 nm) of water, or the photolysis (UV light) of hydrogen peroxide or by laser irradiation of photosensitizers or by non-thermal plasma treatment of atmospheric pressure air. Xenobiotics such as bleomycin facilitate the formation of hydroxyl radicals on DNA by intercalating DNA and complexing with metal ions, forming a site for the Fenton reaction. Because it is so reactive, the hydroxyl radical does not travel for before it oxidizes a substrate.

Table 3. Radical and nonradical reactive oxygen species[55]

Reactive Oxygen Species	
Oxygen Radicals	**Nonradical Oxygen Species**
Hydroxyl ($OH^{\bullet}$)	Hydrogen Peroxide (H_2O_2)
Superoxide ($O_2^{\bullet-}$)	Ozone (O_3)
Peroxyl ($ROO^{\bullet}$)	Singlet Oxygen ($^1O_2$)
Alkoxyl ($RO^{\bullet}$)	Peroxynitrite (ONOOH)
Nitric Oxide ($NO^{\bullet}$)	Hypochlorous Acid (HOCl)

This review focuses on reactive oxygen species, which can cause damage to biological targets such as lipids, DNA, and proteins, and on the antioxidant defense systems of a cell. Reactive oxygen species (ROS) that can be classified into two groups

of compounds; radicals and nonradicals. The radical group, contains compounds such as nitric oxide radical ($NO^{\bullet}$), superoxide ion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$), and one form of singlet oxygen ($^1O_2$) as shown in Table 3. [67]. These species are radicals, because they contain at least 1 unpaired electron in the shells around the atomic nucleus and are capable of independent existence [54]. The occurrence of one unpaired electron results in high reactivity of these species by their affinity to donate or obtain another electron to attain stability The group of nonradical compounds contains a large variety of substances, some of which are extremely reactive although not radical by definition. Among these compounds produced in high concentrations in the living cell are hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic hydroperoxides, aldehydes, ozone (O_3), and singlet oxygen as shown in Table 3.

2.2.1.1. Hydroxyl Radical ($OH^{\bullet}$)

The reactivity of hydroxyl radicals is extremely high [54, 68, 69]. In contrast to superoxide radicals that are considered relatively stable and have constant, relatively low reaction rates with biological components, hydroxyl radicals are short-lived species possessing high affinity toward other molecules. Hydroxyl radical can be generated by reaction of meal ions with H_2O_2, or by UV induced hemolytic fission of the O – O bond in H_2O_2. Hydroxyl radicals can also be generated from ozone and peroxynitrite. Since the major constituent of cells is water, exposure to high energy radiation such as γ-rays will result in $OH^{\bullet}$ production by hemolytic fission of water. Sonication of aqueous solutions also produces $OH^{\bullet}$ radicals. $OH^{\bullet}$ is a powerful oxidizing agent that can react at a high rate with most organic and inorganic molecules in the cell, including DNA, proteins,

lipids, amino acids, sugars, and metals. $OH^{\bullet}$ radicals are responsible for much of the damage done to cellular DNA, proteins and lipids. If two $OH^{\bullet}$ radicals react, they can form H_2O_2. Although this reaction has a high rate contant and occurs in spurs during radiolysis, it is unlikely to occur *in vivo* because the steady state concentration of $OH^{\bullet}$ radicals is effectively zero. The three main chemical reactions of hydroxyl radicals include hydrogen abstraction, addition, and electron transfer [54]. $OH^{\bullet}$ is considered the most reactive radical in biological systems; due to its high reactivity, it interacts at the site of its production with the molecules closely surrounding it [68].

2.2.1.2. Superoxide Anion ($O_2^{\bullet -}$)

This species possesses different properties depending on the environment and pH. Superoxide is far less reactive than $OH^{\bullet}$ and does not react at all with most biological molecules in aqueous solutions. It does react quickly with other radicals such as $NO^{\bullet}$, iron sulfur clusters in certain enzymes and some phenoxyl radicals. Due to its pKa of 4.8, superoxide can exist in the form of either $O_2^{\bullet -}$ or, at low pH, hydroperoxyl ($HO_2^{\bullet}$) [54, 69, 70]. The latter can more easily penetrate biological membranes than the charged form. Hydroperoxyl can therefore be considered an important species, although under physiological pH most of the superoxide is in the charged form. In general, superoxide does not readily cross membranes, although it can pass through the anion exchange proteins present in some cells, for example erythrocytes and lung. The most important reaction of superoxide radicals is dismutation; in this reaction, superoxide radical reacts with another superoxide radical. One is oxidized to oxygen, and the other is reduced to hydrogen peroxide [71]. Although the rate constant at physiological conditions for this

spontaneous reaction is low, it can become much higher in acidic pH where the hydroperoxyl radical is formed [68, 69, 72].

In a hydrophilic environment both the $O_2^{\bullet-}$ and $HO_2^{\bullet}$ can act as reducing agents capable, for example, of reducing ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions; however, the reducing capacity of $HO_2^{\bullet}$ is higher. $O_2^{\bullet-}$ also acts as a powerful nucleophile, capable of attacking positively charged centers, and as an oxidizing agent that can react with compounds capable of donating H^+ ions (e.g., ascorbate and a-tocopherol). In organic solvents the solubility of $O_2^{\bullet-}$ is higher, and its ability to act as a reducing agent is increased. In general, however, in aqueous environments at physiological pH, superoxide is not highly reactive. Its rates of reaction with DNA, amino acids and most other biomolecules are low, or zero. Biological damage by $O_2^{\bullet-}$ is highly selective and usually involves its reactions with other radicals, for example $NO^{\bullet}$ [68, 69, 72, 73].

2.2.1.3. Hydrogen Peroxide (H_2O_2)

The result of the dismutation of superoxide radicals is the production of H_2O_2. There are some enzymes that can produce H_2O_2 directly or indirectly. Although H_2O_2 molecules are considered reactive oxygen metabolites, they are not radical by definition; they can, however, cause damage to the cell at a relatively low concentration (10 μM). H_2O_2 freely dissolved in aqueous solution and can easily penetrate biological membranes. Their deleterious chemical effects can be divided into the categories of direct activity, originating from their oxidizing properties, and indirect activity in which they serve as a source for more deleterious species, such as $OH^{\bullet}$ or HClO. Direct activities of H_2O_2 include degradation of haem proteins; release of iron; inactivation of enzymes; and oxidation of DNA, lipids, SH groups, and keto acids [54, 74, 75].

2.2.1.4. Singlet Oxygen ($^1O_2$)

Molecular oxygen can become more reactive when its outer valence electrons adopt antiparallel spins and occupy the same orbital. This state of molecular oxygen is known as the singlet state. Singlet oxygen ($^1O_2$) is the first excited state of molecular oxygen, and is commonly generated by photosensitization reactions. Singlet oxygen $^1O_2$ is not a radical; there are no unpaired electrons. Singlet oxygen is a powerful oxidant because the spin restriction is removed, which allows it to abstract electrons of either spin [54, 62, 64]. Singlet oxygen generated by photoexcitation and by chemiexcitation selectively reacts with the guanine moiety in nucleosides and in DNA. The oxidation products include 8-oxo-7-hydro-deoxyguanosine (8-oxodG) and 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). Singlet oxygen also causes alkali-labile sites and single-strand breaks in DNA. The biological consequences include a loss of transforming activity and mutagenicity and genotoxicity [76]. Singlet oxygen is also involved in inducing lipid peroxidation [77] and protein oxidation [78]

2.2.1.5. Peroxyl ($ROO^{\bullet}$) and Alkoxyl Radicals ($RO^{\bullet}$)

Peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals are usually very good oxidizing agents. $HO_2^{\bullet}$, protonated $O_2^{\bullet-}$, can be regarded as the simplest peroxyl radical. For example, peroxyl ($ROO^{\bullet}$) radicals oxidize ascorbate and NADH, the latter leading to $O_2^{\bullet-}$ formation in the presence of O_2. Peroxyl and alkoxyl radicals can abstract H• from other molecules, a reaction important in lipid peroxidation. Peroxyl radicals can react with each other, for example by the Russell mechanisms to generate $^1O_2$. Carbon centered radicals react with O2 to form $ROO^{\bullet}$ radicals. Decomposition of organic peroxides (ROOH)

generates both $ROO^{\bullet}$ and $RO^{\bullet}$ radicals. Most peroxides are stable at room temperature, but they can be decomposed by heating, exposure to UV light or by addition of transition metal ions [54, 68].

$$ROOH + Fe\ (III) \rightarrow ROO^{\bullet} + Fe^{2+} + H^{+} \quad (1)$$

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + OH^{-} + Fe\ (III) \quad (2)$$

Reactions shown in Equation (1) and Equation (2) account for much of the stimulation of lipid peroxidation by iron. Lipid peroxides also react with $HO_2^{\bullet}$ to form $ROO^{\bullet}$ as shown in Equation (3) [68]

$$HO_2^{\bullet} + ROOH \rightarrow ROO^{\bullet} + H_2O_2 \quad (3)$$

2.2.1.6. Nitric Oxide ($NO^{\bullet}$) and Peroxynitrite ($ONOO^{-}$)

The nitric oxide, or nitrogen monoxide, radical ($NO^{\bullet}$) is produced by the oxidation of one of the terminal guanido nitrogen atoms of L-arginine. In this reaction, catalyzed by the group of enzymes called nitric oxide synthase (NOS)s, L-arginine is converted to nitric oxide and L-citrulline. Three types of the enzyme exist: neuronal NOS, endothelial NOS (eNOS), and inducible NOS (iNOS). One-electron oxidation results in the production of nitrosonium cation (NO^{+}), while one-electron reduction leads to nitroxyl anion (NO^{-}), which can undergo further reactions, such as interacting with $NO^{\bullet}$ to yield N_2O and $OH^{\bullet}$. The half-life of the nitric oxide radical depends on the square of the radical concentration. $NO^{\bullet}$ can react with a variety of radicals and substances. One of the most important reactions under physiological conditions is that of superoxide and nitric oxide radicals resulting in peroxynitrite as shown in Equation (4). This reaction

helps to maintain the balance of superoxide radicals and other ROS and is also important in redox regulation [79, 80].

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^{-} \qquad k > 10^9\ M^{-1}s^{-1} \qquad (4)$$

The protonated form of peroxynitrite (ONOOH) is a powerful oxidizing agent that might cause depletion of sulfhydryl (−SH) groups and oxidation of many molecules causing damage similar to that observed when $OH^{\bullet}$ is involved. It can also cause DNA damage such as breaks, protein oxidation, and nitration of aromatic amino acid residues in proteins. Under physiological conditions, ONOOH can react with other components present in high concentrations, such as H_2O_2 or CO_2, to form an adduct that might be responsible for many of the deleterious effects seen in biological sites [54, 75, 79-81].

2.2.2. Sources of ROS

The cell is exposed to a large variety of reactive oxygen and nitrogen species from both exogenous and endogenous sources (Figure 5) [82]. The former include oxygen, gamma irradiation, UV irradiation, ultrasound, food, drugs and Xenobiotics, ozone etc while the later include phagocytic cells, enzymes, metabolism and diseases. Although the exposure of organism to ROS is extremely high from exogenous sources, the exposure to endogenous sources is much more important and extensive, because it is a continuous process during the life span of every cell in the organism.

2.2.2.1.Exogenous Sources

Oxygen and Ozone

The toxicity of the oxygen molecule itself is sometimes beneficial and used as a therapeutic aid as, for example, in hyperbaric oxygen therapy [83]. Ozone (O3) is essential in scavenging deleterious UV-C irradiation and extremely important with its presence in the upper atmosphere. On the other hand, it acts as a damaging species to biological tissues [84, 85]. Ozone is not a radical like oxygen, is characterized by its sharp odor, can damage lungs, and can serve as a powerful oxidizing agent that can oxidize biological components directly [86].

Radiation and Pollutants

Exposure of living organisms to ionizing and non-ionizing irradiation constitutes a major exogenous source of reactive species [87, 88]. Exposure of the cells to γ-radiation results in the production of a whole range of radical and non-radical species from the ionization of intracellular water (e.g., aqueous electron, $OH^{\bullet}$, H_2O_2). Even exposure to non-ionizing irradiation such as UV-C (< 290 nm), UV-B (290–320 nm), and UV-A (320–400 nm) can indirectly produce a variety of ROS including $^1O_2$, H_2O_2, and $O_2^{\bullet-}$ radicals; hemolytic cleavage of H_2O_2 by UV radiation yields $OH^{\bullet}$ radicals. Air pollutants such as car exhaust, cigarette smoke, and industrial contaminants encompassing many types of NO derivatives constitute major sources of ROS that attack and damage the organism either by direct interaction with skin or following inhalation into the lung [89, 90].

Drugs

Drugs are also a major source of ROS [91, 92]. There are drugs, such as bleomycin and adreamicine, whose mechanism of activity is mediated via production of ROS, those like nitroglycerine that are $NO^{\bullet}$ donors and those that produce ROS indirectly. Narcotic drugs and anesthetizing gases are considered major contributors to the production of ROS [93]. A large variety of Xenobiotics (e.g., toxins, pesticides, and herbicides such as paraquat) and chemicals (e.g., mustard gas, alcohol) [94-97] produce ROS as a by-product of their metabolism *in vivo*. The invasion of pathogens, bacteria, and viruses might result in the production of many ROS species by direct release from the invaders or an endogenous response induced by phagocytes and neutrophills.

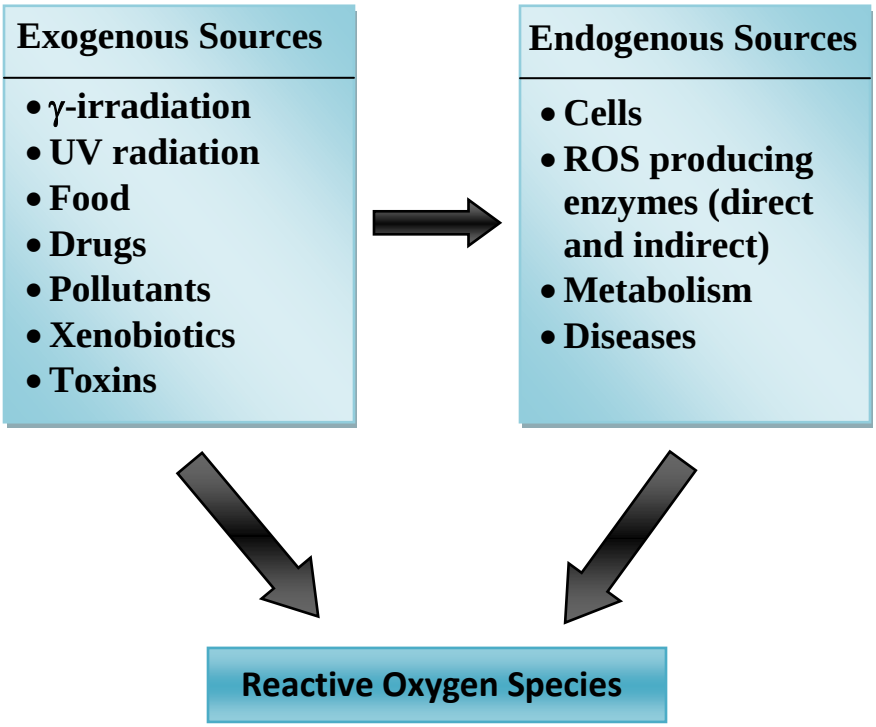

Figure 5. Sources of reactive oxygen species

Food

One of the major sources of oxidants is food [98-100], for a large portion of the food we consume is oxidized to a large degree and contains different kinds of oxidants such as peroxides, aldehydes, oxidized fatty acids, and transition metals [100]. Food debris that reaches the intestinal tract places an enormous oxidative pressure on the intestinal-tract mucosa [101].

2.2.2.2.Endogenous Sources

Enzymatic ROS Production

Enzymes comprise another endogenous source of ROS. While most enzymes produce ROS as a by-product of their activity, exemplified by the formation of superoxide radicals by xanthine oxidase, there are some enzymes designed to produce ROS, such as nitric oxide synthase that yields $NO^{\bullet}$ radicals, those that produce H_2O_2, and those responsible for hydroxylation [102-104]. White blood cells, including neutrophills, eosinophils, basophils, and mononuclear cells (monocytes), and lymphocytes, with their mechanisms to combat bacteria and other invaders [105, 106], are major producers of endogenous ROS and other factors that act synergistically with ROS [107, 108]. Following stimulation, these cells undergo a respiratory burst characterized by a 20-fold increase in oxygen consumption, which is accompanied by an increase in glucose utilization and production of reduced nicotinamide phosphate dinucleotide (NADPH) by the pentose phosphate pathway [109]. NADPH serves as a donor of electrons to an activated enzymatic complex in the plasma membrane. This NADPHoxidase complex utilizes electrons to produce superoxide radicals from the oxygen molecule. Following

dismutation, the production of H2O2 leads to the formation of OH. by the metal-mediated, Haber-Weiss reaction. The presence of the enzyme myaloperoxidase leads to the production of HClO by interaction between hydrogen peroxides and chlorides [110, 111]

ROS are also generated by cellular oxidase reactions catalyzed by enzymes such as NADPH oxidase, monoamine oxidase, and xanthine oxidase. NADPH oxidase functions in the cell membrane of neutrophills and other phagocytic cells to produce superoxide anion as part of a bactericidal mechanism known as the inflammatory burst [112]. Chronic granulomatous disease is an inherited disorder in which a component of the inflammatory burst is defective. Individuals with this disease suffer chronic infections due to the inability of neutrophills to kill microbes [113]. In addition to using ROS to protect the organism, ROS are generated as byproducts of other oxidase reactions. Monoamine oxidases catalyze deamination reactions, which are necessary to digest nitrogen containing compounds such as serotonin, tryptamine and 2-phenylethlyamine, as well as to inactive catecholamine-containing neurotransmitters such as dopamine, epinephrine, norepinephrine and histamine. In this process another ROS, hydrogen peroxide is generated [114]. Xanthine oxidoreductase catalyzes the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid. By-products of this reaction also include the ROS superoxide anion and hydrogen peroxide. Xanthine oxidase is implicated in the ischemia-reperfusion injury, which generated high levels of ROS. During ischemia, the cell loses its ability to maintaining ATP levels, resulting in the breakdown of transmembrane ion gradients and increased intracellular calcium levels. In addition, ATP catabolizes hypoxanthine, which accumulated along with xanthine

oxidase. Upon reperfusion, levels of oxygen increase, and the primed xanthine oxidase/hypoxanthine reaction reduces oxygen, generating high levels of superoxide and hydrogen peroxide. These ROS can form highly reactive hydroxyl radicals which cause injury to surrounding tissue.

Metabolism

Although the exposure of the organism to ROS is extremely high from exogenous sources, the exposure to endogenous sources is much more important and extensive, because it is a continuous process during the life span of every cell in the organism [84]. The reduction of oxygen to water in the mitochondria for ATP production occurs through the donation of 4 electrons to oxygen to produce water [115]. A major source of ROS (ROS) is generated during cellular respiration, when a molecule of oxygen accepts four electrons from reduced NADH and FADH2 generated by the glycolytic pathway and citric acid cycle, eventually forming H_2O. The free energy generated by this process drives the synthesis of adenosine triphosphate (ATP). The mitochondria regulate the stepwise transfer of electrons to oxygen by a series of electron carriers. The incomplete transfer of electrons to oxygen generates reactive intermediates that may oxidize lipid, proteins and nucleic acids. The reactive intermediates include superoxide anion (), hydrogen peroxide, or hydroxyl radical in order of the stepwise reduction of oxygen to water. During this process several major oxygen derivatives are formed [116]. In many cases there is a leakage of ROS from the mitochondria into the intracellular environment [116]. The mitochondrion serves as the major organelle responsible for ROS production and many events throughout the cell cycle [117]. The massive production of

mitochondrial ROS is increased further in the aging cell where the function of the mitochondrion is impaired and its membrane integrity damaged [118].

Diseases

Numerous pathologies and disease states serve as sources for the continuous production of ROS [119-128]. Many clinical disorders have been described in the literature in which ROS were important for the initiation stage of a disease or are produced during the course of a disease. ROS may be important initiators and mediators in many types of cancer [124, 129-132], heart diseases, endothelial dysfunction [133, 134], atherosclerosis and other cardiovascular disorders, inflammation and chronic inflammation [135], burns [136], brain degenerative impairments [137, 138], diabetes [131, 139, 140], eye diseases [141], and ischemic and post ischemic (e.g., damage to skin, heart, brain, kidney, liver, and intestinal tract) pathologies [104, 142]. In several normal conditions ROS are produced and play a role in the pathogenesis of the physiological condition.

2.2.3. ROS signaling

ROS are generated following ligand-receptor interactions and function as specific second messengers in signaling cascades involved in cell proliferation and differentiation. Although ROS are generated intracellularly by several sources, including mitochondria, the primary sources of ROS involved in receptor-mediated signaling cascades are plasma membrane oxidases, preferentially NADPH oxidases, with a rapid kinetics of activation and inactivation. This allows a tight up- and downregulation of intracellular ROS levels within the short time required for the transduction of signals from the plasma membrane

to the cell nucleus. The mode of action of ROS may involve direct interaction with specific receptors, and/or redox-activation of members of signaling pathways such as protein kinases, protein phosphatases, and transcription factors. Furthermore, ROS act in concert with intracellular Ca^{2+} in signaling pathways which regulate the balance of cell proliferation versus cell cycle arrest and cell death. The delicate intracellular interplay between oxidizing and reducing equivalents allows ROS to function as second messengers in the control of cell proliferation and differentiation [143].

Extracellular factors including ROS draw out a variety of responses, such as cell proliferation or cell death, through the cellular signaling system. Binding of growth factors to the receptors leads to the activation of receptor tyrosine kinases, which in turn stimulate downstream signaling systems such as mitogen-activated protein (MAP) kinases, phospholipase C gamma (PLCgamma) and phosphatidylinositol 3-kinase. These biochemical reactions finally reach the nucleus, resulting in gene expression mediated by the activation of several transcription factors. Various studies have revealed that cellular signaling pathways are regulated by the intracellular redox state. Generation of ROS, such as H_2O_2, leads to the activation of protein tyrosine kinases followed by the stimulation of downstream signaling systems. Cellular signaling pathways are generally subjected to dual redox regulation in which redox has opposite effects on upstream signaling systems and downstream transcription factors. Not only are the cellular signaling pathways subjected to redox regulation, but also the signaling systems regulate the cellular redox state. When cells are activated by extracellular stimuli, the cells produce ROS, which in turn stimulate other cellular signaling pathways, indicating that ROS act as second messengers. It is thus evident that there is cross talk between the

cellular signaling system and the cellular redox state. Cell death and life also are subjected to such dual redox regulation and cross talk. Death signals induce apoptosis through the activation of caspases in the cells. Oxidative radical stress induces the activation of caspases, whereas the oxidation of caspases results in their inactivation. Furthermore, some cell-death signals induce the production of ROS in the cells, and the ROS produced in turn stimulate the cell-death machinery. All this evidence shows that the cell's fate is determined by cross talk between the cellular signaling pathways and the cellular redox state through a complicated regulation mechanism [144].

2.2.4. Antioxidant Systems

Given that the cell is exposed to potentially dangerous ROS generated by aerobic respiration and by the enzymatic activity of oxidases, antioxidant systems are in place to neutralize the ROS. Redox homeostasis involves balancing cellular ROS with the antioxidants that neutralizes them. Antioxidants donate electrons to ROS safely, neutralizing them before they abstract electrons from sensitive targets such as lipids or DNA. Antioxidants include the enzymes superoxide dismutase, catalase, glutathione peroxidases, quinine reductase, heme oxygenase, thioredoxin and glutathione reductase, in addition to non-enzymatic compounds vitamin E (α-tocopherol), carotenoids, vitamin C (ascorbic acid) and glutathione. Glutathione is a tripeptide composed of glutamate, cysteine and glycine. It is present in high levels (5 – 10 mM) and serves as a cellular antioxidant buffer. The thiol groups in this cysteine containing peptide and other proteins such as thioredoxin act as basal reducing factors by undergoing reversible disulfide bond formation. The superoxide dismutase enzymes convert $O_2^{\bullet -}$ to the non-radical reactive

species hydrogen peroxide, and the catalase enzyme converts peroxide into water. In the presence of reduced iron however, hydrogen peroxide can be converted in highly reactive hydroxyl radical. Superoxide radicals can also react with nitric oxide (NO) to generate cytotoxic peroxynitirite anions ($ONOO^-$). When the cell cannot maintain a balance between the production of ROS and the neutralization of ROS by antioxidant mechanism, the cell is considered to be in a state of oxidative stress [54, 55, 131, 145-148].

2.3. Effects of Oxidative Stress

When the cell cannot maintain a balance between the production of ROS and the neutralization of ROS by antioxidant mechanisms, the cell is considered to be in a state of oxidative stress. Having too many ROS in relation to the available antioxidants is often said to be a state of oxidative stress [56, 74]. The term oxidative stress is widely used in free radical literature, but is not clearly defined. In 1991 Sies, H. defined oxidative stress as *a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage*. Such damage is often called **oxidative damage**. Halliwell and Gutteridge define oxidative damage as *the biomolecular damage caused by attack of ROS upon the constituents of living organisms* [54]. Increased oxidative damage can result not only from more oxidative stress, but also from more failure of repair or replacement systems. Consequences of oxidative stress can include any, or any combination of, the following to an extent that depends on the cell type and the severity of oxidative stress. Many cells respond to mild oxidative stress by proliferating. Cells may adapt to the oxidative stress by upregulation of defense systems, which may (a) completely protect against damage; (b) protect against damage to some extent or (c) overprotect the cells,

thereby making them resistant to oxidative stress imposed subsequently. Moderate levels of oxidative stress may lead to cellular injury. This involves damage to some or all molecular targets: lipids, DNA, proteins, carbohydrates etc. Such damage can sometimes be the trigger leading to cellular adaptations. At higher levels of oxidative stress, cells may enter a state of senescence, in which the cell survives but cannot divide. After cellular injury due to oxidative stress the cell may: (a) recover from the oxidative damage by repairing it or replacing the damaged molecules; or (b) it may survive with persistent oxidative damage; or (c) oxidative damage, especially to DNA, may trigger death by apoptosis, necrosis, or cell death [54]. Finally, chronic elevation in the ROS may play a role in the pathogenesis of cancer [124], diabetes [139], atherosclerosis [119, 126], neurodegeneration [120, 137, 138], ischemia/reperfusion injury [104, 134, 142] and other diseases. In the next section we elaborate the biological effects of oxidative stress.

2.3.1. Biological Effects of Oxidative Stress

The continuous efflux of ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components [149] and alters many cellular functions [150]. Among the biological targets most vulnerable to oxidative damage are enzymes [54, 151], lipid membranes [54, 152], and DNA [54, 153] (see Figure 6). Our understanding of the chemistry of radicals clarifies the interaction of these species in the locations at which they are being produced. For example, hydroxyl radicals, produced in mitochondrial compartments, are responsible for damage occurring in the mitochondrion but not the nucleus. Their high reactivity with biological molecules, which leads to their extremely short life span, does not permit their distribution within

the intracellular environment and limits their ability to cause damage a long distance from their site of formation. On the other hand, oxygen metabolites those are not extremely reactive, such as HO2 may exist in the intracellular environment for longer periods of time and reach locations far from their production site. For example, H_2O_2 produced in mitochondria may interact elsewhere in the cytoplasm or in the nucleolus.

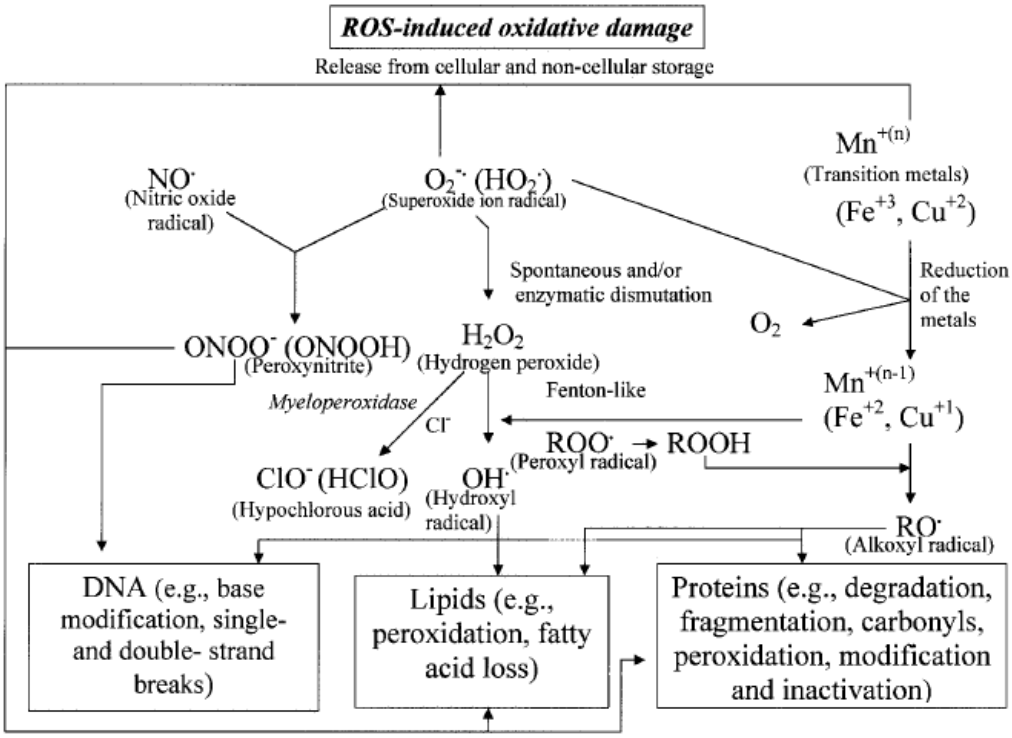

Figure 6. Oxidative damage induced by reactive oxygen species [55]

Although paradoxical, the oxidative stress phenomenon, while associated with deleterious processes and harmful events, is essential to the existence and development of the cell [131]. Oxidative conditions are the stimulating force for biochemical processes and events during the life span of the cell. While the resting-cell environment is highly reduced, it becomes more and more oxidized during proliferation and activation of cellular biochemical pathways until apoptosis and necrosis occur. In these latter stages the cellular environment becomes highly oxidized [154]. ROS and RNS play crucial roles

in gene activation [155, 156], cellular growth [157], and modulation of chemical reactions in the cell [158, 159] and function as major components of the defense against bacteria and viruses provided by neutrophills (phagocytes) and as agents responsible for dilation of blood vessels (e.g., NO) [160]. They also participate in blood pressure control [161]; are important mediators in the biosynthesis of other molecules, such as prostaglandins [162]; function in embryonic development [163]; and act as signaling molecules within the individual cell and among cells during their life spans [164].

2.3.2. Cell Proliferation

Low level oxidative stress (e. g. by adding small amounts of H_2O_2 [148], low dose non-thermal plasma [44], or exposure of cells to centrigray level doses of ionizing radiation [165-167]) stimulates the proliferation of several cell types in culture. The arrest of growth induced by cell confluence has been suggested by some to be related to a decrease in intracellular ROS and consequent loss of pro-proliferative stimuli[168]. Studies have further demonstrated that even exogenous ROS at sub-lethal levels can mimic certain growth factors to regulate, and activate the mitogenic-associated signal transduction pathways in the cells. It is now recognized that ROS at low concentrations are ubiquitous intracellular messengers. Recently a new physiological role of ROS was identified in several non-phagocytic cells, in which ROS generation was associated with the mitogenic stimulus action of growth factors [169]. A role for ROS production and oxidative stress has been proposed for the stimulation of cell proliferation. The effects of ROS and oxidative stress within cells appear to be cell specific and dependent upon the form as well as the intercellular concentration of ROS. ROS function to induce cell

proliferation during the tumor promotion stage of carcinogenesis (100). Both H_2O_2 and superoxide anion ($O_2^{\bullet-}$) induce mitogenesis and cell proliferation in several mammalian cell types (101). Furthermore, a reduction in cellular oxidants via supplementation with antioxidants such as superoxide dismutase, catalase, β-carotene, and flavenoids inhibits cell proliferation in vitro (102. Although no single mechanism explains the increased cell proliferation and/or inhibition of apoptosis observed following conditions that favor increased cellular oxidants, mounting evidence is emerging that links ROS with altered expression of growth regulatory genes [124]. Diminishing ROS levels can decrease cell proliferation; for example overexpression of catalase in rat aortic smooth muscle cells lowered H_2O_2 levels and blocked their proliferation. Pro-Proliferative effects of ROS may contribute to atherosclerosis, angiogenesis, rheumatoid arthritis and to fibrosis in several human diseases [168].

2.3.3. Cellular Adaptation

Oxidative stress involves responses to oxidants or agents that cause oxidation via intracellular redox reactions. As described earlier, a wide variety of agents and compounds, including oxygen itself, can cause oxidative stress if present in sufficient concentration. Severe oxidative stress can result in compromise of vital cell or organismal functions or even loss of viability. Cells usually tolerate mild to moderate oxidative stress, which often results in increased synthesis of antioxidant defences in an attempt to restore the oxidant/antioxidant balance. In many cases, mild oxidative stress can elevate defenses sufficiently to protect cells against more severe oxidative stress applied subsequently [168]. Studies have shown that cells can adapt to withstand extremely high levels of oxidative stress is first applied. Davies, K. J. A. et. al. have

reported a transient adaptation to the oxidative stress of hydrogen peroxide (H_2O_2) exposure in several mammalian cell lines including Chinese hamster ovary fibroblast cells, embryonic mouse fibroblasts, etc. They showed that very low concentrations of H_2O_2 from 3 – 15 μM result in 25 – 40% stimulation of cell growth while low H_2O_2 concentrations of 120 – 150 μM induced a temporary growth arrest and marked adaptive increase in H_2O_2 resistance, whereas higher H_2O_2 concentrations were strongly cytotoxic. They discovered that if the cells were pretreated with H_2O_2, however, the toxicity of subsequent H_2O_2 exposure was significantly reduced. The observed adaptation, though, comes at the cost of compromised replicative or divisional competence rather than gross destruction of cells. The adaptation may be explained by the fact that pretreatment with H_2O_2 induced the overexpression of antioxidants, such as catalases or glutathione peroxidases, which are capable of neutralizing the effects of H_2O_2. It is possible that mammalian cells normally adapt to fluctuating oxidative stress levels in vivo by transiently overexpressing a series of both antioxidant and non-antioxidant genes [148].

2.3.4. Cell Injury

All aerobic cells generate, enzymatically or non-enzymatically, a constitutive flux of $O_2^{\bullet-}$, H_2O_2, and possibly $OH^{\bullet}$. At the same time, the abundant antioxidant defenses of most cells, again both enzymatic and nonenzymatic, prevent these species from causing cell injury. Nevertheless, there are situations in which the rate of formation of partially reduced oxygen species is increased and/or the antioxidant defenses of the cells are weakened. In either case, oxidative cell injury may result [170].

2.3.4.1. Effects on Lipid Membranes

All cellular membranes are especially vulnerable to oxidation due to their high concentrations of unsaturated fatty acid. The damage to lipids, usually called lipid peroxidation, occurs in 3 stages. The first stage, initiation, involves the attack of a reactive oxygen metabolite capable of abstracting a hydrogen atom from a methylene group in the lipid. The presence of a double bond adjacent the methylene group weakens the bond between the hydrogen and carbon atoms so that it can easily be removed from the molecule. Following hydrogen abstraction, the remaining fatty acid radical retains 1 electron and is stabilized by rearrangement of the molecular structure to form a conjugated diene. When oxygen is in sufficient concentration in the surroundings, the fatty acid radical will react with it to form $ROO^{\bullet}$ during the propagation stage. These radicals themselves are capable of abstracting another hydrogen atom from a neighboring fatty acid molecule, which leads again to the production of fatty acid radicals that undergo the same reactions—rearrangement and interaction with oxygen. The $ROO^{\bullet}$ radical becomes a lipid hydroperoxide that can further decompose to an aldehyde or form cyclic endoperoxide, isoprotans, and hydrocarbons. The propagation stage allows the reaction to continue. A single initiation can lead to a chain reaction resulting in peroxidation of the entire unsaturated lipid in the membrane. An antioxidant that can stop this process is therefore defined as a chain-breaking antioxidant. Fatty acids with no double bonds or with 1 double bond can undergo oxidation but not a chain lipid peroxidation process; for example, oleic acid with 18 carbon atoms and 1 double bond (18:1) cannot undergo the lipid peroxidation process. The last stage, chain termination, occurs following interaction of one $ROO^{\bullet}$ with another radical or antioxidants [54, 171].

2.3.4.2. Effects on DNA

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage: modification of DNA bases, single- and double-DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage, and damage to the DNA repair system. Not all ROS can cause damage; most is attributable to hydroxyl radicals. For example, following exposure of DNA to hydroxyl radicals, like those induced by ionizing irradiation, a variety of adducts are formed. The $OH^{\bullet}$ radical can attack guanine at its C-8 position to yield an oxidation product, 8- hydroxydeoxyguanosine (8-OHdG) [172]. Other positions could be attacked, and other possible products could be formed. Hydroxyl radicals can also attack other bases like adenine to yield 8 (or 4-, 5-) - hydroxyadenine. Other products are the result of interactions between pyrimidines and hydroxyl radicals leading to the formation of thymine peroxide, thymine glycols, 5- (hydroxymethyl) uracyl, and other such products. The direct interaction of DNA with other less reactive ROS, such as $O_2^{\bullet-}$ and H_2O_2, does not lead to damage at their physiological concentrations; however, these species serve as sources for other reactive intermediates that can easily attack and cause damage. For example, H_2O_2 and superoxide might lead to the production of the OH. via the Haber-Weiss reaction, and NO and $O_2^{\bullet-}$ might lead to the formation of $ONOO^-$ that can easily cause DNA damage similar to that obtained when hydroxyl radicals are involved. Transition metals like iron that possess high-binding affinity to DNA sites can catalyze the production of $OH^{\bullet}$ in close proximity to the DNA molecule, thus ensuring repeated attack upon the DNA by an efflux of hydroxyl radicals [173-176].

The primary source of DNA oxidation is the hydroxyl radical. The hydroxyl radical abstract hydrogens from a base or sugar component of DNA, generating water and a carbon-centered radical. The carbon centered radical eventually becomes a hydroxylated carbon that may be further modified, generating a variety of products. Hydroxyl radical attack causes the degradation of bases, and breakage of the sugar-phosphate backbone either directly through oxidative degradation of the deoxyribosyl group or indirectly through the creation of an abasic site followed by a beta-elimination reaction [62]. If not repaired, DNA breaks can cause chromosomal aberrations or even cell death. DNA strand breaks may also indirectly cause cell death by depleting intracellular NAD+/NADH due to activation of the polyadenosine diphosphate (ADP) ribosyl transferase-dependent repair pathways [177]. The carbon-centered radicals may also react with amino acids residues in nuclear proteins bound to DNA, forming DNA protein crosslink's [62].

More than 50 different types of oxidatively modified purine and pyrimidine bases have been detected in nucleic acids exposed to ROS. Base modifications include addition of chemical groups such as hydroxyl groups and ring opening, which can lead to single base changes or base deletions [62]. Guanine is the most easily oxidized nucleotide, as it has the lowest redox potential among the DNA bases [178]. 8-oxo-7, dehydroguanine is the most abundant oxidative lesion of DNA [179]. 8-oxoguanin is formed when a hydroxyl radical abstracts an electron from the C8 position of guanine, generating a C8-OH adduct radical. Loss of an electron and proton generates 8-oxoguanine, while gain of an electron and proton generates 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy), the second major oxidation product of guanine [180]. Although 8-oxoguanine does not

block DNA synthesis, it can induce base mispairing during DNA synthesis because 8-oxoguanine can pair with both cytosine and adenine. This mispairing can lead to G: C → T: A transversions during DNA replication. In addition, oxidation of guanine can occur in the cellular pool of dGTP, forming 8-oxo-dGTO. This DNA substrate can be incorporated opposite adenine or cytosine residues of template DNA, resulting in A: T → C: G and G: C → T: A transversions [181]. The most prevalent modification to pyrimidines is the formation of thymine glycol which, unlike 8-oxoguanine, creates a bulky lesion on DNA that blocks replication and transcription. Hydroxyl radical abstraction at the C5=C6 double bond can result in the formation of C5-OH adduct radicals of thymine. Addition of OH^- or addition of water followed by deprotonation generates thymine glycol [179]. Also, oxidation of 5-methylcytosine (m^5C) results in the formation of thymine glycol [182].

2.3.4.3. Effects on Proteins

Proteins, also major constituents of membranes, can serve as possible targets for attack by ROS. Among the various ROS, the $OH^\bullet$ radical, $RO^\bullet$ radical, and nitrogen-reactive radicals predominantly cause protein damage. Hydrogen peroxide itself and superoxide radicals in physiological concentrations exert weak effects on proteins; those containing –SH groups, however, can undergo oxidation following interaction with H_2O_2. Proteins can undergo direct and indirect damage following interaction with ROS, including peroxidation, damage to specific amino-acid residues, changes in their tertiary structure, degradation, and fragmentation. The consequences of protein damage as a response mechanism to oxidative stress are loss of enzymatic activity, altered cellular

functions such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins. Protein oxidation products are usually aldehydes, ketocompounds, and carbonyls. One of the major adducts that can easily be detected and serve therefore as a marker for protein oxidative damage is 3-nitrotyrosine. This adduct is produced following the interaction between $ONOO^-$ and other nitrogen reactive radicals with the amino acid tyrosine. Following $OH^\bullet$ radical attack, a series of compounds can be formed, including hydroxyproline, glutamyl semialdehyde, and others. Following protein oxidation, modified proteins are susceptible to many changes in their function. These include chemical fragmentation, inactivation, and increased proteolytic degradation [152, 183-185].

2.3.5. Cellular Senescence

High levels of oxidative stress may induce a permanent state of non-division, known as senescence. For example human fibroblasts exposed to 100 – 300 μM H_2O_2 in culture developed a senescent phenotype, showing increased expression of proteins that inhibit the cell cycle. Senescence after prolonged cell culture is in part due to the oxidative stress of the culture process [54].

After a finite number of divisions, primary cell cultures enter a state of replicative senescence in which they are growth-arrested and refractory to further mitogenic stimulation. Although the relevance of *in vitro* senescence to organismal ageing remains controversial, several studies indicate that oxidants are important in the development of the senescent phenotype. Early studies with human diploid fibroblasts revealed that cells grown in low oxygen tension exhibit a prolonged life span. In contrast, cells grown in the

presence of high oxygen concentrations have a reduced life span and show an accelerated rate of telomere shortening per population doubling. Similarly, treatment of cultures of primary fibroblasts with moderate, non-lethal doses of exogenous hydrogen peroxide activates a rapid, senescence-like growth arrest [186].

The role of oxidants in cellular senescence was further underscored by recent observations that overexpression of an activated Ras gene can also induce a senescence-like state in human diploid fibroblasts. Subsequent analysis demonstrated that expression of activated Ras in diploid fibroblasts resulted in an increase in oxidant levels [187]. In addition, although expression of activated Ras in human diploid fibroblasts produced growth arrest, this arrest could be reversed either by reducing ambient oxygen or by treatment with a cell-permeable antioxidant. As such, these results raise the possibility that a moderate, sustained rise in oxidants may function as a common trigger for activation of the senescence program [188].

2.3.6. Cell Death

A cell exposed to severe oxidative stress may eventually die. Cell death can occur essentially by two mechanisms, necrosis and apoptosis, although death by mechanisms with features of both pathways is often seen (see Figure 7). Necrosis and apoptosis result from many causes, including oxidative stress. For example, in mammalian cells, adding mM levels of H_2O_2 can cause necrosis, whereas lower levels can trigger apoptosis [189]. H_2O_2 induces apoptosis via production of $OH^{\bullet}$ radicals [190]. Further there is evidence that intracellular H_2O_2 is an important mediator of the cytotoxic effects of several types of antineoplastic therapy including chemotherapy [191-193], ionizing radiation [194,

195], ultraviolet radiation [196, 197], and possibly photodynamic therapy [189, 198]. Lipid peroxides formed due to excessive oxidative stress are also known to trigger apoptosis [199]. Several observations suggest that ROS might mediate apoptosis. For example (a) the addition of ROS or the depletion of endogenous antioxidants can induce apoptosis. (b) apoptosis sometimes can be inhibited by endogenous or exogenous antioxidants; (c) apoptosis is sometimes associated with increases in intracellular ROS levels [200].

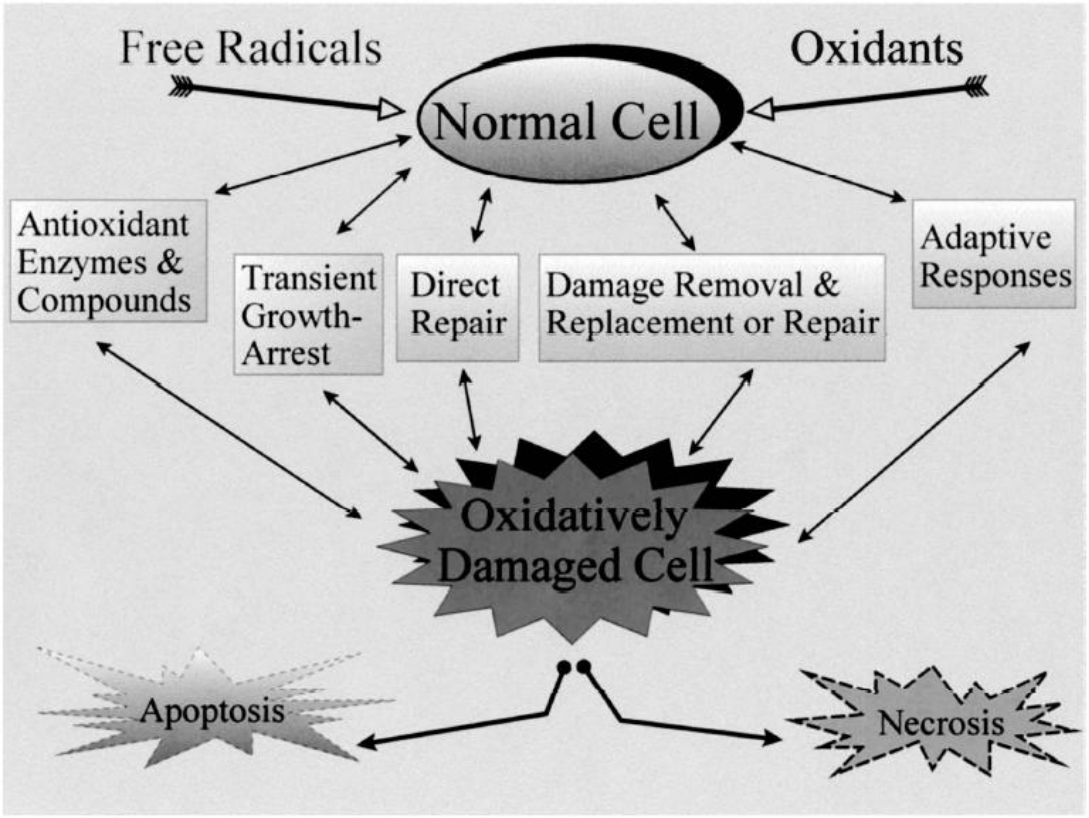

Figure 7. Range of cellular oxidative stress responses [148]

During necrotic cell death there is cell and organelle swelling, loss of integrity of mitochondrial, peroxisomal and lysosomal membranes and eventual rupture of the plasma membrane, releasing contents in to the surrounding area to affect adjacent cells [54]. In apoptosis, the cell's own intrinsic 'suicide mechanism' is activated. Apoptosis is

characterized by membrane blebbing, outer membrane leaflet inversion and exposure of phosphatidyl serine, cytosolic condensation, breakdown of nuclear DNA and finally, at least in vitro, the formation of well enclosed, apoptotic bodies [201]. Apoptosis is usually considered to involve two phases; (a) an activation phase, consisting of intracellular signaling pathways and (b) an execution phase, consisting of molecular machinery necessary for apoptosis to be completed. ROS have been shown to play an important role in the activation phase while not so much in the execution phase. In pathological circumstances ROS might trigger apoptosis by activating signaling pathways responsive to ROS themselves or damage caused by ROS [200].

One of the major insults that trigger apoptosis is oxidative stress, though higher levels of oxidative stress may also induce necrosis. Endogenously produced ROS play a key role in programmed cell death pathways of many cell types [201]. ROS and oxidative damage have been implicated in the induction of apoptosis. On the other hand, apoptosis triggered by mechanisms like ligand binding to death receptors is accompanied by oxidative stress. Releases of cytochrome c and caspase cleavage disrupt the mitochondrial electron transport chain, causing an excessive production of superoxide ($O_2^{\bullet -}$). Studies have shown that in many cases antioxidants slow down apoptosis induced via oxidative stress by a range of insults which include ionizing radiation, UV, hydrogen peroxide and more recently non-thermal atmospheric pressure plasma.

Generation of oxidative stress in response to various stimuli has also been implicated in the triggering of apoptosis. ROS and mitochondria play an important role in apoptosis induction under both physiologic and pathologic conditions. Cytochrome *c* release from mitochondria, that triggers caspase activation, appears to be largely

mediated by direct or indirect ROS action. Receptors of the tumor necrosis factor (TNF) family display various functions from cell growth to differentiation and cell death. A subset of this family, known as death receptors have been shown to induce apoptosis in some systems. Activation of these receptors by binding of TNF like ligands is sufficient and necessary to induce death via caspase dependent mechanisms. Interestingly studies have shown that N-acetylcysteine blocked the TNF-induced apoptosis suggesting a functional role of ROS in the process of apoptosis. Activation of the FAS receptor, which also belongs to the TNF receptor family, results in a signal transduction pathway that most likely uses ROS [202].

Necrotic cell death has long been considered an accidental and uncontrolled mode of cell death. But recently it has become clear that necrosis is a molecularly regulated event that is associated with numerous pathologies. Excessive oxidative stress can lead to necrosis via generation of intracellular ROS. ROS initiate damage to lipids, proteins and DNA and consequently results in mitochondrial dysfunction, ion balance deregulation and loss of membrane integrity which eventually leads to necrotic cell death [203]. In many cases caspases which have exposed –SH groups can be inactivated due to attack by high levels of ROS like H_2O_2, HOCl, $OH^{\bullet}$ and $ONOO^{-}$. These leads to a delay or halt in apoptosis and often cell death proceeds though the necrotic pathway [168]. ROS can initiate damage to proteins, nucleic acids and lipids [204, 205]. ROS can attack the disulfide bonds (-S-S-) and thiol groups (-SH) of proteins and thereby change their functions. Another effect of ROS during necrosis could be the modification of Ca'+ channels of the ER and plasma membrane [206]. Mitochondrial DNA is particularly susceptible to oxidative damage because of the absence of protective histones, limited

base excision repair mechanisms, and close proximity to the electron transport chain, all of which lead to mitochondrial genomic instability and consequent respiratory dysfunction followed by necrosis. Other important targets susceptible to ROS activity are the polyunsaturated fatty acids in the cellular membranes. Reactive aldehydes derived from lipid peroxidation can compromise the membrane function by interacting with both the protein and the lipid moieties in the membrane [207]. In mitochondria, the lipid peroxidation products negatively affect oxidative phosphorylation , inner membrane barrier properties, maintenance of mitochondrial membrane potential, and the mitochondrial Ca^{2+} buffering capacity, contributing in this way to necrosis [208]. Lipid peroxidation-mediated destabilization of both the plasma membrane and intracellular membranes, such as those of the lysosomes and the ER, results in leakage of proteases or efflux of Ca^{2+} into the cytosol, both of which participate in necrotic cell death processes [203].

2.3.7. Role of Oxidative Stress in Diseases

In the evolution of life on planet Earth was the acquisition of mitochondria, and thus of oxidative phosphorylation, by primordial cells. This achievement provided cells with larger amount of energy, which significantly contributed to the evolution of unicellular organisms into more complex and specialized living forms. However, this progress did not occur without any expenses. The use of oxygen in energy metabolism increased the production of energy as ATP, but toxic and potentially lethal substances were also produced, the ROS, which should be rapidly detoxified to prevent molecular and cellular damages. As a consequence, antioxidant systems developed to counteract the

negative effects of ROS, and living organisms adapted to strive by maintaining a specific redox state, i.e. a balance between ROS production and antioxidants' scavenger activity [123]

During the last two centuries, the life expectancy significantly increased, mostly because of improved nutrition, since medical advances contributed to this progress when mortality rates had already fallen substantially. However, longer life results in longer exposure to toxic agents, including ROS. Also, age- and/or disease-related inefficiency of antioxidant systems and reduced intake of antioxidants with the diet due to age- and/or disease-related anorexia increase oxidative stress, which in turn may promote molecular damages triggering the onset of degenerative diseases, worsening the clinical condition of patients and preventing healthy aging [123]. Chronic elevation in ROS may play a role in the pathogenesis of cancer, diabetes, atherosclerosis, neurodegenerative diseases including Alzheimers and Parkinson's, ischemia/reperfusion injury, and other diseases. Based on these premises, it is not surprising that considerable scientific efforts have been put into the investigation of the role of oxidative stress in various diseases, in aging process and in the pathogenesis of degenerative diseases. Leading researchers in their respective fields reviewed the available evidence to delineate the role of oxidative stress in cancer [124, 130], diabetes [209], atherosclerosis [119, 126], Alzheimer's [120] and Parkinson's diseases [125].

A linkage between an increase in cellular reactive oxygen radicals and the pathogenesis of several chronic diseases including cancer has been established. Cellular oxidants (reactive oxygen and nitrogen species) can be generated from endogenous (normal physiological processes) as well as exogenous sources (xenobiotic interaction).

When the antioxidant control mechanisms are exhausted or overrun, the cellular redox potential shifts toward an oxidative stress, in turn, increasing the potential for damage to cellular nucleic acids, lipid, or protein. Unrepaired damage to DNA may result in mutation, provided cell replication ensues prior to repair of modified bases. Although importance has been established for the role of oxidative nuclear DNA damage in neoplasia, formation of mitochondrial DNA damage, mutation, and alteration of the mitochondrial genomic function also appear to contribute to the process of carcinogenesis Besides direct modification of nuclear and/or mitochondrial DNA, interference with oxidative DNA repair mechanisms (in both nuclear and mitochondrial compartments) contributes to an increase in mutation frequency and persistent oxidative DNA damage. Aside from a role of oxidants in the induction of mutation, it is apparent that ROS and cellular redox status mediate cell signaling pathways that are involved in cell growth regulatory pathways and, thus, the carcinogenesis process [124, 130].

In diabetes oxidative stress plays a key role in the pathogenesis of vascular complications, and an early step of such damage is considered the development of an endothelial dysfunction. Hyperglycemia directly promotes an endothelial dysfunction inducing process of overproduction of superoxide and consequently peroxynitrite that damages DNA and activates the nuclear enzyme poly (ADP-ribose) polymerase. This process, depleting NAD+, slowing glycolysis, ATP formation and electron transport, results in acute endothelial dysfunction in diabetic blood vessels and contributes to the development of diabetic complications [209].

Oxidative stress seems to play a key-role in the pathogenesis of atherosclerosis. Intracellular ROS (ROS) have been increasingly appreciated to have a role in this

context. ROS seem to mediate various signaling pathways that underlie atherogenesis, from the initiation of fatty streak development through lesion progression to ultimate plaque rupture. Moreover enhanced oxidative stress has been found in many of the classical risk factors for cardiovascular disease [119, 126].

Research has shown that oxidative stress mediates an important role in the pathogenesis of various neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). In recent years, there has been an increased awareness of the seminal role that both oxidative stress and redox-active transition metals play in AD as well as other neurodegenerative diseases. Examination of Alzheimer's disease brain has demonstrated a great deal of oxidative damage, associated with both hallmark pathologies (senile plaques and neurofibrillary tangles) as well as in normal appearing pyramidal neurons. The oxidative damage found in AD includes advanced glycation end products, nitration, lipid peroxidation adduction products, carbonyl-modified neurofilament protein and free carbonyls. Current evidence points to the fact that changes in the balance of redox transition metals, especially iron and copper, are key in the process. Both Fe and Cu are present at significantly elevated levels in AD neutrophil. Iron catalyzes the formation of $OH^{\bullet}$ from H_2O_2 as well as the formation of advanced glycation end products. Furthermore, aluminum, which also accumulates in NFT-containing neurons, stimulates iron-induced lipid peroxidation. Advanced glycation end products in the presence of transition metals can undergo redox cycling with consequent production of ROS. Thus oxidative stress plays a major role in the pathogenesis of AD [120].

Oxidative stress is also implicated in the pathogenesis of Parkinson's disease. Oxidative stress contributes to the cascade leading to dopamine cell degeneration in Parkinson's disease (PD). Oxidative stress is intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, nitric oxide toxicity and inflammation Oxidative damage to lipids, proteins, and DNA occurs in PD, and toxic products of oxidative damage, such as 4-hydroxynonenal (HNE), can react with proteins to impair neuronal cell viability. The evidence for the occurrence of oxidative stress in PD is overwhelming, and there is little doubt that oxidative stress leads to an increase in oxidative damage in the brain cells [125].

2.4. Modalities Functioning via induction of Oxidative Stress

Currently various electrophysical effects like ionizing radiation, lasers, photodynamic therapy, pulsed electric fields and magnetic fields are being investigated or utilized in the treatment of various diseases and pathologies. Many of these devices are actually considered for similar types of medical applications. Non-thermal plasma and pulsed electric field, for example, are being viewed as potential treatments for cancer, an application where ionizing radiation has been employed for some time. In general, different electrophysical effects may have different advantages and disadvantages in a particular situation. However, exploiting advantages and circumventing disadvantages requires good understanding of biological mechanisms of interaction with cells and tissues. While such mechanisms are understood relatively well for modalities such as ionizing radiation, they remain poorly studied for non-thermal plasma, pulsed electric fields and to some extent for photodynamic therapy and laser treatment. In this section we consider the effects of and the mechanisms of interaction of three modalities of

treatment which are widely used in medicine; viz IR, H_2O_2 and UV, with cells and tissue, and all three of them are known to operate primarily via generation of intracellular oxidative stress. This section forms the basis of this thesis in trying to understand the mechanisms of interaction of non-thermal plasma with living cells. I specifically describe the commonalities and major differences between non-thermal plasma and IR/H_2O_2/UV later in this dissertation.

2.4.1. Ionizing Radiation

Ionizing radiation is a form of electromagnetic radiation that has the ability to create ions, directly or indirectly, as it passed through matter. Ionizing radiation is measured in units of Gray (Gy), a dosage value of 1 joule of energy absorbed by 1 kg of matter. Radiolysis, the breaking of one or more interatomic bond(s) due to exposure to high energy radiation, can occur in water to produce hydrogen peroxide, hydrogen atoms, hydrated electrons and hydroxyl radicals [210]. One of the important radiation-induced free-radical species is the hydroxyl radical which indiscriminately attacks neighboring molecules often at near diffusion-controlled rates. Hydroxyl radicals are generated by ionizing radiation either directly by oxidation of water, or indirectly by the formation of secondary partially ROS. These may be subsequently converted to hydroxyl radicals by further reduction (‘activation’) by metabolic processes in the cell. Secondary radiation injury is therefore influenced by the cellular antioxidant status and the amount and availability of activating mechanisms. The biological response to radiation may be modulated by alterations in factors affecting these secondary mechanisms of cellular injury.

Ionizing radiation has been shown to generate ROS in a variety of cells [211]. When water, the most abundant intracellular material, is exposed to ionizing radiation, decomposition reactions occur, and a variety of ROS, including superoxide, hydroxyl radicals, singlet oxygen, and hydrogen peroxide, are generated [212]. The secondary radicals formed by the interaction of hydroxyl radicals with organic molecules may also be of importance [211, 212]. These ROS have the potential to damage critical cellular components such as DNA, proteins, and lipids and eventually result in physical and chemical damage to tissues that may lead to cell death or neoplastic transformation [213]. In many cases, ionizing radiation-induced cell death has been identified as apoptosis [214].

2.4.1.1. Biological Effects of Ionizing Radiation

Ionizing radiation (IR) is an invaluable diagnostic and therapeutic tool that is widely used in medicine. Human beings are constantly exposed to low levels of natural background radiations. In addition, exposure to low dose ionizing radiation (LDIR) from medical and industrial sources is becoming a worldwide problem. The cellular response to LDR is quite complex; the balance between death, arrest, and survival is tipped by the presence or absence of signaling through specific pathways, the identities of which are not yet clear. The biological effects of LDIR have attracted a lot of attention, but the biological mechanisms of the effects of LDIR still remain unclear. In this section we provide an overview of the current understanding of the biological effects of ionizing radiation and focus on the IR induced oxidative stress in mammalian cells.

2.4.1.2. Cell Proliferation

While it has long been known that radiation leads to cell death, it has only recently become accepted that radiation has some potential to enhance proliferation in the surviving fraction of cells. Suzuki, et. al demonstrated that LDR stimulates the proliferation of normal human diploid cells. This effect was also observed in low-dose irradiated cells such as Raji lymphoma, Chinese hamster fibroblasts, and normal human diploid cells [165-167]. Exposure of cells to LDIR has been found to stimulate the activity of cell metabolism and enhancing cell growth. Irradiation at low doses of between 0.02 and 0.05 Gy caused stimulated proliferation of normal human diploid cells. Exposure to 0.5 Gy of IR stimulated induction of cell proliferation in mouse hematopoietic cells. 0.05 Gy of ionizing radiation enhances cell proliferation through the activation of ERK1/2 and p38 in normal human lung fibroblasts [165-167]. Suzuki, et. al. also found that 0.05 Gy of ionizing radiation enhanced cell proliferation and did not change the progression of the cell cycle. However, 2 Gy of ionizing radiation induced cell cycle arrest, phosphorylation of p53, and expression of p53 and p21 [167]. Thus low dose ionizing radiation enhances cell proliferation in various cell lines and it is possible that it does so by generating low concentration of ROS that may activate certain mitotic signal transduction pathways [165-167].

2.4.1.3. Cell Injury

2.4.1.3.1. Effects on Lipids

The interaction of ionizing radiation with biological matter induces complex and interconnected events. The excitation and ionization of atoms and molecules along

particle tracks result in physico-chemical reactions, initiating primarily hydroxyl and superoxide radicals [215]. For the past 30 years, the DNA molecule has been considered to be the essential target of ionizing radiation in the cell [216]. Less attention has been paid to biological membranes. The patho-physiological consequences of the exposure of biological tissue to ionizing radiation have usually been investigated in terms of cell death, with each cell considered as an independent entity. New concepts, however, increasingly take into account the importance of intercellular communication in determining the effects of radiation [217].

Ionizing radiation induces oxidative damage in phospholipids [218, 219], initiated mainly by $OH^{\bullet}$ and $O_2^{\bullet -}$ radical products and leading to the appearance of various degradation substances and modified molecules. When hydroperoxide residues are produced in the bilayer core, hydrophobic interactions between adjacent phospholipids become unstable, allowing easier penetration of water molecules [220, 221]. Effects of ionizing radiation at doses below 10 Gy, as these doses are more compatible with cell survival, on biological membranes include alterations in membrane proteins, peroxidation of unsaturated lipids accompanied by perturbations of the lipid bilayer polarity. Finally, significant degradation of membrane function and structure may favor apoptosis [222, 223]. Lipid peroxidation increased in the hours after radiation exposure, based on measurements of MDA and on the lipid peroxidation index after parinaric acid incorporation. (1) At 3 h after exposure to a dose of 2 or 4 Gy, the relative fluorescence of membrane-incorporated parinaric acid decreased significantly; (2) the MDA level also increased rapidly after exposure. Propagation of lipid peroxidation occurred in the hours after radiation exposure and returned to the basal level within 72 h. Radiation-induced

membrane damage may be a consequence not only of lipid peroxidation but also of protein oxidation [224]. Benderitter, et. al. showed that the carbonyl content of the membranes increased significantly in the hours after radiation exposure at doses as low as 2 Gy. This study indicates that some alterations of membrane fluidity are observed after low irradiation doses and for some time thereafter. One consequence of the development of this oxidative stress is the reorganization of the fatty acid pattern, particularly of the PE. Specifically, Benderitter, et. al. also observed that the lipid compartment of the membrane became more fluid while the lipid–protein membrane interface became more rigid. After γ-radiation exposure, alterations occur in the plasma membrane that affects the state of the lipid–protein phase, the secondary structure of proteins, and the environment of aromatic amino acids [224]. Protein–lipid interactions that are known to occur in specific domains within the membrane may play an important role in mediating such radiation effects. The changes in membrane fluidity might reflect oxidative damage, thus confirming a radiation-induced fluidization of biological membranes. An imbalance between the radiation-mediated oxidative damages and the antioxidant capacity of the cells was observed in the hours after radiation exposure. This suggests that radiation exposure increases the oxidative damage to the cell membrane. These findings reinforce our understanding that the cell membrane is a significant biological target of radiation [224, 225].

2.4.1.3.2. Effects on DNA

Damage to DNA occurs both directly, through the ionization of nucleotides, or indirectly, by the generation of ROS, or through the formation of DNA-protein

crosslinks. Radiolysis of water is thought to be the major potential cause of DNA damage in response to ionizing radiation. Ionizing radiation induces double strand breaks (DSB's), single strand breaks (SSB's), oxidized purines, oxidized pyrimidines, and abasic sites [226, 227]. In addition to inducing damage to isolated sites in the genome, ionizing radiation induces clustered lesions on DNA. Damage clusters have two or more lesions on opposing DNA strands within a few helical turns. These lesions are thought to be difficult to repair and may be a major source of IR induced cytotoxicity [228]. In human monocytes exposed to 1 Gy ionizing radiation, clustered DNA lesions represented 28% DSB's, 28% oxidized purines, 25% oxidized pyrimidines, and 20% abasic sites [229]. As cells attempt to repair DNA damage with glycosylases, double strand breaks can be induced [230]. Of the total damage to DNA caused by ionizing radiation, as much as 80% may result from radiation-induced water-derived free radicals and secondary carbon-based radicals [211]. In addition to the generation of hydroxyl radicals, the hydrated electrons formed by ionizing radiation can reduce it to $O_2^{\bullet-}$. $O_2^{\bullet-}$ can dismutate to H_2O_2 with the possibility of extra $OH^{\bullet}$ production by metal-catalyzed Fenton reaction [212].

2.4.1.3.3. Effects on Proteins

Ionizing radiation is a very widely used treatment modality for cancer treatment. It is important to know how free radicals generated by IR during treatment of tissue interact with biological macromolecules like proteins and amino acids. After water, which is unreactive, proteins are the most abundant constituent of cells and extracellular fluids by weight. The probability of interaction between free radicals generated in biological systems by ionizing radiation and proteins is high [231, 232]. Proteins are

readily attacked by free radicals generated in vivo and in the process trap the energy of radicals acing as antioxidants. Many studies have shown that the antioxidant action of proteins in many cases leads to protein damage. In the presence of oxygen, serum albumins exposed to ROS suffer polypeptide chain scission. With γ-irradiation, all constituent amino acids of BSA are affected, but some show especially high damage, as much as 50-80% modification of proline, lysine etc [233]. Exception of cysteine, all amino acids of the type NH2CH(R) COOH undergo oxidative deamination in aqueous solution to yield ammonia, a-keto acid, and hydrogen peroxides. Although the aromatic and heterocyclic constituents and, in particular, the sulfur moieties appear to be the most susceptible to indirect action, the evidence is that the over-all reaction of protein involves a diversity of amino acid residues even at the lowest irradiation levels. Increased susceptibility to proteolysis is also a common consequence of protein oxidation [234].

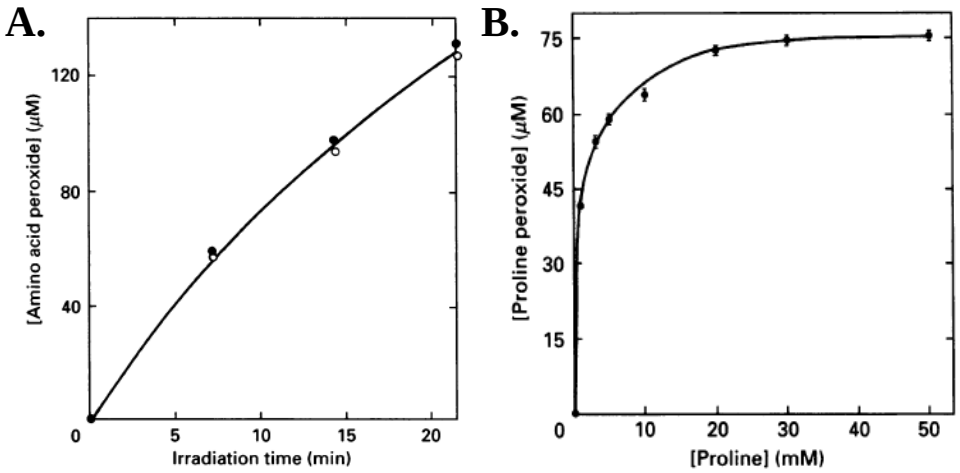

Figure 8. Peroxidation of amino acids by ionizing radiation. (A) Peroxidation of amino acids increase as the dose of ionizing radiation increases. (B) Peroxidation of proline increases as the concentration of proline increases [233].

Dilute aqueous solutions of BSA or lysozyme gave positive tests for hydroperoxides after exposure to ROS generated by γ-irradiation. Irradiation of dilute aqueous solutions containing oxygen leads to the production of both $OH^{\bullet}$ and $O_2^{\bullet-}$ and

free radicals in similar amounts. The oxygen free radicals effective in protein peroxidation were the hydroxyl and organic peroxyl, but not superoxide or its protonated form. Protein hydroperoxide yields corresponded to 1.2 –OOH groups per 100 eV of radiation absorbed for BSA and 0.8 for lysozyme. The peroxidation efficiency for BSA initiated by hydroxyl radicals was 40%. These protein hydroperoxides were relatively stable and decayed spontaneously with a half life of about 1.5 days. The rate of loss of hydroperoxides from the oxidized proteins shows that their lifetimes are long enough for reactions even at sites quiet remote from their point of formation [233].

Table 4. Peroxidation efficiency and peroxide yields of amino acids oxidized by free radicals produced by exposure of amino acid solutions to γ-irradiation [233]

Amino Acid	Peroxidation Efficiency	G-Value
Methionine	0	0
Serine	0	0
Threonine	0	0
Cysteine	0.4	0.01
Asparagine	2	0.06
Tyrosine	3	0.08
Phenylalanine	5	0.14
Aspartic Acid	6	0.17
Alanine	11	0.31
Arginine	13	0.37
Glutamine	16	0.44
Tryptophan	18	0.51
Glutamic Acid	28	0.77
Glycine	28	0.07
Histidine	34	0.12
Lysine	34	0.96
Isoleucine	43	1.21
Leucine	44	1.22
Proline	44	1.24
Valine	49	1.37

The amino acid composition of a protein is an important determinant of its susceptibility to peroxidation. Amino acids including Isoleucine, leucine, lysine, valine and proline were readily peroxidized by exposure to ionizing radiation and yields of

amino acid peroxides are dependent upon the concentration of amino acids and the dose of ionizing radiation (Figure 8). Exposure of common amino acids to hydroxyl radicals generated by ionizing radiation showed that six of them (glutamate, isoleucine, leucine, lysine, proline and valine) were peroxidized with similar efficiency to proteins, whereas the rest were inert or much less susceptible (Table 4). Further amino acid peroxidation was not linear with the duration of irradiation as seen in BSA and lysozyme and this might be attributed to progressive degradation of irradiated molecules [233].

Irradiation of organic biomolecules like protein and amino acids leads to free radical mediated peroxidation of these molecules [232]. The proteins and amino acids are not inert end-targets of free radical reactions and the formation of relatively stable hydroperoxides on molecules exposed to $OH^{\bullet}$ or $ROO^{\bullet}$ radicals in the presence of oxygen effectively traps some of their chemical energy, extending their lifetime and radius of operation. These amino acid and protein hydroperoxides may in turn act as second toxic messengers in cells and tissues leading to oxidation of GSH and ascorbate and depletion of primary antioxidants *in vivo,* which would eventually lead to oxidative cell and tissue damage.[231, 233]

2.4.1.3.4. Cell Death

Ionizing radiation (IR) at physiologically compatible doses of less than 10 Gy produces free radicals and ROS as the product of radiolysis of water. Radiation of tissue culture media generates electron paramagnetic resonance (EPR)-detectable hydroxyl and hydrogen radicals [235]. Recent evidence suggests a growing consensus that ROS such as hydroxyl radicals, superoxide anions, and organic hydroperoxides play an important role in cellular damage caused by ionizing radiation [212]. These ROS cause oxidative

damage to DNA, lipid peroxidation, and protein oxidation [213, 236, 237]. DNA is a particularly important target, suffering double and single strand breaks, deoxyribose damage, and base modification [74]. Cells may respond to such damage by growth arrest, cell senescence, and DNA repair or, if the damage is too severe to be repaired, undergo apoptosis [214, 238]. IR induced free radicals can also initiate a variety of cellular signal transduction pathways that may either aid the cell in coping with the excess oxidative stress resulting from radiation or set into motion pathways that lead to the destruction of cells damaged beyond the repair capabilities of the cell [239, 240]. Caspase-3 activation is generally associated with IR-induced apoptosis. Lipid metabolites have been implicated in IR-induced apoptosis. Deletion of the acid sphingomyelninase in transgenic mice rendered lymphocytes resistant to apoptosis induced by IR [241]. Ionizing radiation-generated ROS (ROS) resulting in oxidative damage to the cell membrane and its consequent role in the mechanism of apoptotic cell death have been receiving growing attention in cellular radiobiology. In recent years, evidence has accumulated to suggest that it is the damage to the cell membrane that contributes to the radiation cell killing. It has been demonstrated that degradation of membrane-bound sphingomyelinase (SMase) after irradiation of bovine endothelial cell produces ceramide, which initiates an apoptotic cascade, suggesting membrane-triggered events in the mechanism of cellular apoptosis [242]. Ionizing radiation induces the production of ROS, which play an important causative role in apoptotic cell death. Therefore, there are several reports to suggest that antioxidant enzymes play a key role in the cellular defense against IR-induced oxidative damage by supplying NADPH in the mitochondria and for the regeneration of mitochondrial GSH or thioredoxin [243, 244]. Mitochondrial GSH becomes critically

important against ROS-mediated damage because it not only functions as a potent antioxidant but is also required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidases. Mitochondria play central roles in cellular metabolism and apoptosis and are a major source of ROS. ROS play an important role in the radiation-induced apoptosis in multiple myeloma cells. It is well established that mitochondrial dysfunction is directly and indirectly involved in cell death including apoptosis and a variety of pathological states [245]. All the changes caused by ionizing radiation are compatible with mitochondrial failure, encompassing reduced production of ATP, generation of ROS, and accumulation of rhodamine 123 which reflect mitochondrial swelling or changes in the mitochondrial inner membrane [246].

Control of mitochondrial redox balance and the cellular defense against oxidative damage are some of the primary functions of mitochondrial $NADP^+$- dependent isocitrate dehydrogenase (IDPm). Two distinct levels of ROS were generated following irradiation: a small increase observed early, and a pronounced late increase, associated with depletion of reduced glutathione (GSH) and collapse of mitochondrial membrane potential. Late ROS production is associated with IR-induced apoptosis in malignant cells. Upon exposure to low dose-irradiation, there was a distinct increase in of morphological evidence of apoptosis, DNA fragmentation, cellular redox status, oxidative damage to cells, mitochondrial function, and the modulation of apoptotic marker proteins in IDPm (an important mitochondrial antioxidant enzyme) knockdown cells as compared to control cells. IDPm knockdown cells showed increased apoptosis after exposure to low dose ionizing radiation while cells overexpressing antioxidant enzymes were resistant to

IR induced apoptosis. Elevation of mitochondrial NADPH and GSH by antioxidant enzymes like IDPm in turn suppressed the oxidative stress and concomitant IR induced ROS mediated cell death. Therefore, antioxidant enzymes may play a role in preventing apoptosis caused by ionizing radiation in cells. Studies also demonstrate that antioxidant enzymes abrogate the ionizing radiation-induced early production of ROS, leading to protection against apoptotic cell death. In conclusion IR leads to apoptotic cell death due to production of ROS leading to an increase in oxidative damage which cannot be repaired by the cells [246].

In conclusion, since IR primarily causes DNA damage, the mechanisms for apoptosis likely are more closely related to those observed for other DNA damaging agents such as UVC radiation, for example, than to direct exposure to H_2O_2 [241].

2.4.2. Hydrogen Peroxide

Hydrogen peroxide is continually produced in many, if not all, tissues *in vivo*. Mitochondria may be significant contributors to cellular H_2O_2 generation, both by monoamine oxidases and by dismutation of $O_2^{\bullet-}$ from the electron transport chains Several enzymes generate H_2O_2, including xanthine, urate, glucose, monoamine and D-amine acid oxidases as well as superoxide dismutase. Hydrogen peroxide mixes readily with water and can diffuse within and between cells *in vivo*. Although water does appear to diffuse across the lipid bilayer, much appears to pass through membrane water channels, the aquaporins. Hydrogen peroxide can also traverse these channels, but this may not be the only mechanism by which it crosses membranes [54].

It is toxic to many cells at levels in the 10 to 100 uM, causing senescence or apoptosis, but at lower levels it can promote proliferation of certain cell types, and at higher levels it suppresses apoptosis and promotes necrotic cell death. Fortunately, given its widespread presence *in vivo*, H_2O_2 is only a weak oxidizing or reducing agent and is generally poorly reactive. This may allow it to play a role in signal transduction. No oxidation occurs when DNA, lipids or most proteins are incubated with H_2O_2, even at millimolar levels. Despite its poor reactivity, H_2O_2 can be cytotoxic and at high concentrations is often used as a disinfectant. Many animal cells in culture are injured or killed if H_2O_2 is added to the medium at concentrations at or above the 10 – 100 uM range [54].

2.4.2.1. Biological Effects of Hydrogen Peroxide

Hydrogen peroxide elicits a wide range of responses from proliferation to growth arrest, senescence and cell death (Figure 9). The proliferative response from low concentrations of peroxide (3 – 15 μM) probably acts as a signaling agent for mitosis. Peroxide treatment of fibroblasts at concentrations of 100 – 150 μM causes temporary growth arrest for 3 – 6 hours, during which time proliferating cells conserve energy through the reduced expression of non-essential genes, while the expression of genes involved in antioxidant defenses and damage removal or repair enzymes is increased. The transient growth arrest allows time for the cells to repair damaged DNA and build up an adaptive response to withstand further oxidant exposure. At concentrations of hydrogen peroxide between 200 - 400 μM, fibroblasts lose the ability to replicate and undergo a permanent growth arrest resembling cell senescence. At even higher concentrations of

peroxide, cells undergo apoptosis or necrosis depending on the dose. The mechanism of apoptosis appears to involve loss of mitochondrial membrane potential. Necrosis occurs by a breakdown in cell membrane integrity. In addition, peroxide exposure induces the activation of antioxidant genes such as catalase, glutathione peroxidases, hemeoxygenase, and mitochondrial Mn-superoxide dismutase in different cell types [148].

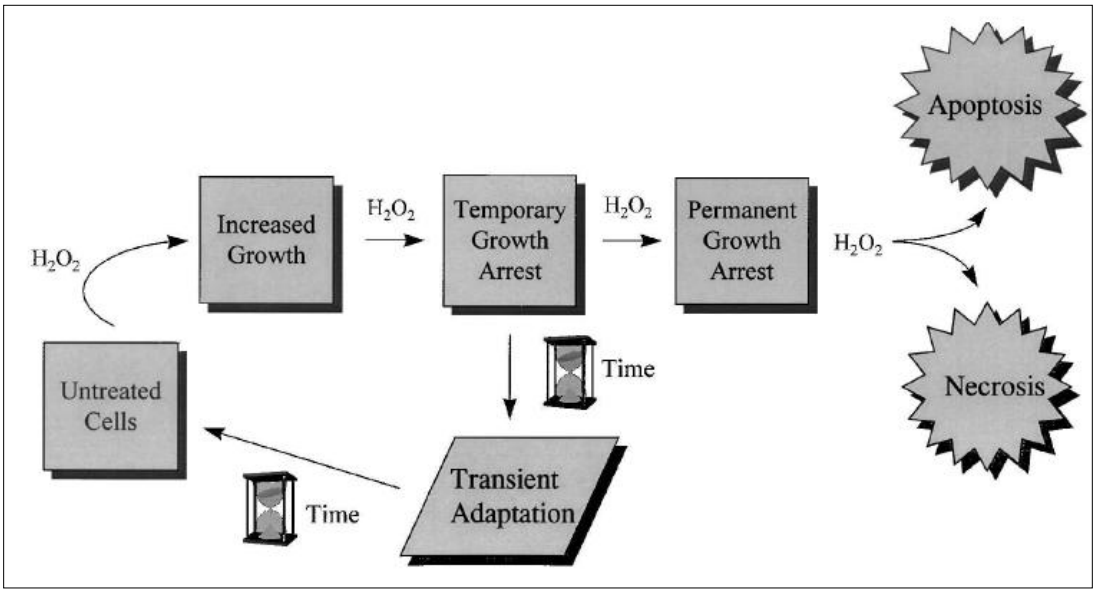

Figure 9. Range of cellular oxidative stress responses to increasing levels of H_2O_2 [241]

2.4.2.2. Cell Proliferation

Proliferating mammalian cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress level encountered. Exposure of dividing mammalian cells in culture to low concentrations of oxidants actually stimulates cell growth and division [247-249]. Very low levels of hydrogen peroxide, e.g., 3 to 15 μM, or 0.1 to 0.5 μmol/10^7 cells; cause a significant mitogenic response, 25% to 45% growth stimulation. Presumably at such low concentrations H2O2 does not cause a true oxidative stress. In all

probability, H_2O_2 in this concentration range actually acts as a signaling agent for mitosis [148].

2.4.2.3. Cell Injury

2.4.2.3.1. Effects on Lipids

Hydrogen peroxide (H_2O_2) is an important mediator of acute oxidative injury to vascular endothelium. Because the plasma membrane is the initial site of interaction between endothelial cells and extracellular H_2O_2 produced by stimulated neutrophills or macrophages, we evaluated the effect of H_2O_2 on the physical state, i.e., fluidity, and function of porcine pulmonary artery endothelial cell plasma membranes. The precise mechanism responsible for H_2O_2-induced cellular injury and the specific cellular sites of H_2O_2 attack are not completely defined. However, recent evidence suggests that membrane fatty acids and phospholipids may be early and critical targets of moderate dose H_2O_2-related cytotoxicity. For example, 50 uM H_2O_2 has been reported to cause peroxidative cleavage of polyunsaturated fatty acids, to induce phospholipid hydrolysis by activation of phospholipases, and to stimulate the synthesis and/or release of membrane-derived lipid mediators, such as platelet-activating factor and prostacyclin, in endothelial cells. The earliest abnormality observed in the present study was rigidification of the glycerol backbone region of the endothelial cell plasma membrane bilayer. Compared with control cells, H_2O_2 caused significant increases in LDH release and in 5-HT uptake 6 hr after exposure. These results indicate that H_2O_2 causes significant damage to the plasma membrane of pulmonary artery endothelial cells in vitro, leading to alterations in fluidity and leakiness of the membrane. First, H_2O_2 can react with iron via a

Fenton reaction to generate the highly reactive hydroxyl radicals ($OH^{\bullet}$). $OH^{\bullet}$ will react readily with the unsaturated bonds of plasma membrane fatty acids, resulting in the initiation of a lipid peroxidation cascade. A second possible mechanism responsible for H_2O_2 induced plasma membrane injury involves activation of pathways leading to hydrolysis of plasma membrane phospholipids. Thus H_2O_2 mediated injury is associated with reversible membrane lipid peroxidation, and can be prevented by pretreatment but not by post-treatment with lipophilic free radical scavengers like Vitamin E. A final possible mechanism to account for our observations in H_2O_2-treated endothelial cells is oxidation of plasma membrane protein sulfhydryl groups that leads, in turn, to altered plasma membrane protein lipid interaction, lipid packing, and membrane function [218, 250].

2.4.2.3.2. Effects on DNA

Although some cellular damage by H_2O_2 is direct, addition of H_2O_2 frequently leads to lipid, DNA and protein oxidation that cannot be mediated by H_2O_2. It may cross cell membranes and react with iron and probably copper ions to form more damaging species such as $OH^{\bullet}$. Indeed, $OH^{\bullet}$ accounts for most of all the damage done to DNA in H_2O_2 treated cells. H_2O_2 also interacts with haem proteins to cause oxidative damage. Sometimes, exposing cells to H_2O_2 can increase $O_2^{\bullet-}$ production, by activating NADPH oxidases [54].

2.4.2.3.3. Effects on Proteins

In addition, H_2O_2 can oxidize protein sulfhydryl groups, leading to protein dysfunction and in erythrocytes, H_2O_2-induced oxidation of heme proteins has been

shown to alter membrane protein-lipid interaction and lipid packing. Soluble proteins from the human lens nucleus were incubated for 12 weeks with 0, 10, 100 and 1000 μM H_2O_2. Progressive changes were observed in several of these parameters. Cysteine (up to 100 %) and methionine (up to 45 %) were rapidly oxidized but no significant alterations were found in any other amino acids. The gradually increasing solvent accessibility of tryptophan residues indicated that the proteins were undergoing conformational alterations. This was accompanied by the insolubilization of protein (up to 75 %). No methionine sulphoxide was detected in the control and very low levels in the 10 μM treatment. However, in the 1000 μM and 100 μM treatments, oxidation was rapid and appeared to reach a maximum at about 45 % of the total methionine content of the proteins. The control and 10 μM treated proteins did not show any significant accumulation of insoluble proteins. However, in the 100 and 1000 μM -treatments, the solubility of the proteins decreased until 65 and 75 % respectively was insoluble in water [251].

2.4.2.4. Cell Senescence

If dividing mammalian cells are exposed to greater concentrations of H_2O_2 than those that cause temporary growth arrest and transient adaptation, they can be forced into a permanently growth-arrested state. Thus, cells exposed to H_2O_2 concentrations of 250 to 400 μM, or 9 to 14 μmol/10^7 cells, will never divide again [249]. Studies of both cell populations and individual cells have revealed that cultured mammalian cells (with a normal doubling time of 24±26 h) can survive for many weeks (at least) after exposure to 100±200 μM H_2O_2 without dividing again [249].

2.4.2.5. Cell Death

Although H_2O_2 is a relatively weak oxidant compared to other ROS such as •OH, it has emerged as an important signaling molecule based on its unique biochemical properties: H_2O_2 is ubiquitously present in the biological system with a relatively long half-life; and more importantly H_2O_2 is soluble in both lipid and aqueous media [131]. Thus it easily diffuses to its cellular targets. Recent research has suggested that the apoptosis signal can be transduced extracellularly by soluble factors. A population of cells stimulated with cytokines transmitted the apoptosis signal to neighboring cells through a soluble catalase-inhibitable factor, suggesting that H_2O_2 may act as a paracrine mediator of apoptosis [148, 241, 252].

A fraction of cells exposed to higher concentrations of H_2O_2, 0.5 to 1.0 mM, or 15 to 30 μmol/107 cells, will enter the apoptotic pathway. The mechanism of oxidative stress- induced apoptosis appears to involve loss of mitochondrial transmembrane potential [253]; release of cytochrome *c* to the cytoplasm [254]; loss of bcl-2 [255]; down-regulation and degradation of mitochondrially encoded mRNA, rRNA, and DNA [256-258]; and diminished transcription of the mitochondrial genome [259]. Current thinking about toxicant-induced apoptosis suggests that in multicellular organisms, the repair of severely damaged cells represent a major drain on available resources [148].

At even higher concentrations of hydrogen peroxide, e.g., 5.0 to 10.0 mM, or 150 to 300 μmol/10^7 cells, cells simply disintegrate or become necrotic. Membrane integrity breaks down at such high oxidant stress levels, and all is then lost (*15, 59*). Studies that purport to examine cellular responses to 10.0 mM H2O2 in mammalian fibroblasts are not really looking at the responses of cells but rather at the release of components those

cells originally contained. At a high enough level of oxidative stress (e.g., >10 mM H_2O_2), all mammalian cell cultures will turn into a necrotic mess (*15*). Oxidation-induced necrosis may play a significant role in ischemia-reperfusion injuries such as heart attacks, strokes, ischemic bowel disease, and macular degeneration. Unfortunately, necrotic cells cause inflammatory responses in surrounding tissues. Such secondary inflammation (also an oxidant stress) may be particularly important in many autoimmune diseases such as rheumatoid arthritis and lupus [148].

2.4.2.6. Role of Hydrogen Peroxide in Diseases

Various studies have shown that the formation of extracellular or intracellular deposits of Amyloid beta (Aβ)-like protein fibrils is a prominent pathological feature of many different neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD). There is abundant evidence for the involvement of free radicals and oxidative stress in the pathogenesis of nigral damage in PD. One of the fundamental molecular mechanisms underlying the pathogenesis of cell death in AD and PD, and possibly other neurodegenerative or amyloid diseases, could be the direct production of hydrogen peroxide during formation of the abnormal protein aggregates. The possibility that hydrogen peroxide and, subsequently, hydroxyl radicals, might be formed in the immediate vicinity of Aβ could explain much of the oxidative damage observed when cells are exposed to this peptide. Studies have also shown that human Aβ directly produces H_2O_2 by a mechanism that involves the reduction of metal ions, Fe (III) or Cu (II), setting up conditions for Fenton-type chemistry. The degeneration and loss of nerve cells in the brain in both AD and PD could be due to the direct production of hydrogen

peroxide during extracellular or intracellular protein aggregation, which would induce oxidative damage, particularly in the presence of metals where the hydrogen peroxide would be converted, via Fenton's reaction, into the highly reactive hydroxyl radical. Thus H_2O_2 plays an important role on the pathogenesis of neural cell death in both AD and PD [260-262].

2.4.3. Ultraviolet Radiation

The sun emits ultraviolet (UV) radiation extending from the UVA band (315-400 nm) through the UVB (280-315 nm) down to the high energy UVC band (190-280) and vacuum UV (below 190 nm). Ultraviolet radiation (UVR) includes UVA, UVB and UVC radiation. The majority of solar radiation reaching the earth (90%) is UVA radiation, with a wavelength of 320 – 400 nm. UVB radiation makes up a smaller fraction of solar radiation, however it is much more mutagenic [263]. The UV radiation is very (photo-) chemically active. Conjugated bonds in organic molecules absorb radiation of wavelengths around and below 200 nm, and in linear repeats or in ring structures the absorption bands of these bonds shift to higher wavelengths (250-300 nm and higher, e.g., the broad absorption up to 550 nm by/3-carotene with its extended linear repeats and terminal rings). After absorbing UV radiation, a molecule may become altered (damaged) and/or affect (damage) other molecules, e.g., by producing ROS (ROS) [264]. Thus, UV radiation, especially in the short wavelength range, forms a direct threat to the stability of unprotected organic molecules, which are essential to life on earth: foremost, DNA with aromatic rings in all of its bases (absorption maximum around 260 nm). DNA is a chromophore of UVB, directly absorbing UVB's energy wavelength of 280 – 320 nm. As

a result of the energy absorption, chemical bonds may break in a process called photolysis. UVC radiation corresponds to a wavelength of 254 nm. This is close to the maximal absorption spectrum of DNA to initiate photochemical reactions and is used to study the effect of UV on DNA [265]. Both UVA and UVB generate ROS such as singlet oxygen, hydroxyl radicals and peroxynitrite radicals. The generation of singlet oxygen by the absorption of light energy is known as photosensitization. Further, UVR can cause photolysis of the chemical bonds of hydrogen peroxide forming hydroxyl radical [266].

2.4.3.1. Biological Effects of Ultraviolet Radiation

As a result of UV-induced damage, the cell undergoes growth arrest and can engage programmed cell death pathways in response to high levels of damage [267]. In the next few sections we describe in detail the range of biological effects of UV.

2.4.3.2. Cell Injury

The ultraviolet A (UVA) radiation component of sunlight (320-380 nm) has been shown to be a factor in many of the consequences of exposure of animals to sunlight including carcinogenesis and aging [268-270]. Although the mechanisms by which UVA radiation is involved in these processes are largely unknown, redox active species have been implicated [271, 272]. Solar irradiation depletes antioxidant compounds in skin cells in situ [273], adds to the evidence that solar radiation is a source of oxidant stress to skin[274]. Indeed, UVA irradiation of cellular constituents in vitro leads to the generation of redox active species. Hydrogen peroxide can be generated by the UVA irradiation of tryptophan [275], and both hydrogen peroxide and superoxide can be generated from the

UVA irradiation of NADH and NADPH [276, 277]. Singlet oxygen can be generated as a result of absorption of UVA radiation by endogenous photosensitizers such as porphyrins [278]. The involvement of ferrous iron (Fe^{2+}) in catalyzing redox reactions in vitro is well established. UVA radiation has been shown to reduce ferric iron (Fe^{3+}) associated with ferritin in vitro and in the presence of hydrogen peroxide the ferrous ions can catalyze the formation of hydroxyl radicals. The role of hydroxyl radicals in the biological effects of UVA radiation has not been thoroughly investigated. Ferrous ions are also good catalysts for the peroxidation of membrane lipids and give rise to membrane damage [279], but the role of iron in UVA radiation-dependent peroxidation of lipids is unknown. UVA radiation also enhances the ferrous ion catalyzed oxidation of protein sulphydryl groups in the presence of hydrogen peroxide [280-282].

Exposure of cultured human skin cells to UVA radiation immediately modifies cellular components and can eventually lead to cell death [283]. The peroxidation of lipids [284, 285] and the oxidation of glutathione [286] have been shown to be the immediate consequences of UVA irradiation of human skin cells and are typical of reactions of redox active species. Ferrous iron, singlet oxygen, and hydrogen peroxide have been identified as biologically important redox active species generated by UVA irradiation of human skin fibroblasts. These redox active species were shown to mediate a UVA radiation-dependent loss of protein sulphydryl groups in vitro and in human skin fibroblasts, and singlet oxygen and iron were shown to mediate UVA radiation-dependent peroxidation of phosphatidylcholine and fibroblast lipids. Because UVA radiation can reduce ferric iron associated with cellular molecules, it should be able to initiate biological redox reactions in which ferrous ions have been shown to have a catalytic role.

Such reactions include lipid peroxidation, sulphydryl oxidation, and the generation of subsequent redox active species such as the hydroxyl radical [287].

2.4.3.2.1. Effects on Lipids

Thiobarbituric acid-reactive species were formed from phosphatidylcholine after UVA radiation in vitro. By using iron chelators, this process was shown to involve iron. Ferric iron associated with potential physiological chelators was reduced by UVA radiation, but iron within ferritin was not. By enhancing the half life-time with deuterium oxide or by using scavengers, singlet oxygen was also shown to be involved in the UVA radiation dependent peroxidation of phosphatidylcholine. UVA radiation-generated singlet oxygen reacted with phosphatidylcholine to form lipid hydroperoxides, and the breakdown of these hydroperoxides to thiobarbituric acid-reactive species was dependent on iron. Vile et. al. have shown that iron and singlet oxygen are also involved in the UVA radiation-dependent formation of thiobarbituric acid-reactive species in human skin fibroblasts, and proposed that a similar concerted effect of iron and singlet oxygen was involved in UVA radiation-dependent damage to fibroblast lipids [287].

UVA radiation-dependent peroxidation of detergent dispersed phosphatidylcholine is dependent on iron in agreement with the general hypothesis that iron is an essential component in redox driven lipid peroxidation systems [279]. The role of iron in lipid peroxidation has been shown to be in the breakdown of lipid hydroperoxides (LOOH) according to reaction given in Equation (5).

$$LOOH + Fe^{2+} \leftrightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \quad (5)$$

An important feature of the biological effects of UVA radiation may be the ability of UVA radiation to interact with iron within lipid bilayers. Experiments performed by Vile et. al. were consistent with the involvement of singlet oxygen in the UVA radiation-dependent formation of thiobarbituric acid-reactive species from phosphatidylcholine. Further investigation also revealed that singlet oxygen ($^1O_2$) was involved at the level of the formation of phosphatidylcholine hydroperoxides [287]. This is probably via reaction described in Equation (6)

$$^1O_2 + LH \leftrightarrow LOOH \quad (6)$$

Vile et. al. proposed that UVA radiation can break down phosphatidylcholine to thiobarbituric acid-reactive species via the concerted action of singlet oxygen and ferrous iron. The involvement of iron and singlet oxygen in the UVA radiation-dependent peroxidation of fibroblast lipids is consistent with the possibility that a mechanism, similar to that seen with phosphatidylcholine, is involved in fibroblasts. They could find any evidence for the involvement of superoxide or the hydroxyl radical in the UVA radiation-dependent peroxidation of phosphatidylcholine or cell lipids [287]

2.4.3.2.2. Effects on DNA

The major effect of UV radiation on DNA is the formation of pyrimidines dimmers between adjacent pyrimidines on the same strand of DNA. Pyrimidine dimmers, also called cyclobutane-type dipyrimidines (CPD's) are formed when adjacent pyrimidines become covalently linked through the formation of a four-membered ring structure resulting from the saturation of their respective 5,6 double bonds. Of all possible bipyrimidines, TT bipyrimidines are the most photoreactive. However, TT

CPD's are quickly repaired and not mutagenic. Another common adduct to DNA is the pyrimidines-pyrimidone 6, 4 photoproduct. While TC and CC CPD's are not very photoreactive, there are mutagenic, forming TC → TT and CC → TT transitions. These mutations are found in the p53 gene of UV-induced cancer cells [266, 267, 288, 289].

In addition to forming CPD's, both UVA and UVB generate ROS such as singlet oxygen, hydroxyl radicals and peroxynitrite radicals. The generation of singlet oxygen by the absorption of light energy is known as photosensitization. Further, UVR can cause photolysis of the chemical bonds of hydrogen peroxide, forming hydroxyl radical. As a result of increased presence of ROS on DNA, oxidatively modified bases, especially 8-oxoguanine, can be detected following UV radiation [266].

2.4.3.2.3. Effects on Proteins

A concerted effect of iron and singlet oxygen was involved in UVA radiation-dependent damage to fibroblast lipids. Vile et. al. also showed that a concerted effect of iron and singlet oxygen was also involved in the oxidization of sulphydryl groups of bovine serum albumin and human γ-globulin upon UVA irradiation in vitro. The use of scavengers and deuterium oxide showed that UVA radiation dependent sulphydryl oxidation was dependent on singlet oxygen. By adding or chelating iron, UVA radiation-dependent oxidation of sulphydryl groups of bovine serum albumin and human γ-globulin was shown to be iron-dependent. The use of catalase and hydroxyl radical scavengers demonstrated that hydrogen peroxide, but not the hydroxyl radical, was involved. The oxidation of sulphydryl groups of proteins in human skin fibroblasts that occurs as a result of UVA irradiation was also shown to involve iron, singlet oxygen, and hydrogen

peroxide [287]. UVA irradiation of human skin fibroblasts at physiological doses has been shown to lead to a loss of intracellular glutathione, and Vile et. al. also show that similar doses of UVA radiation can lead to a loss of protein sulphydryl groups both in these cells and in vitro. The UVA radiation-induced protein sulphydryl loss was shown to involve iron and hydrogen peroxide, but not hydroxyl radicals. This is in agreement with other studies that have shown that the redox-dependent loss of sulphydryl groups requires iron and hydrogen peroxide, and that the hydroxyl radical is not involved [290, 291]. Hydrogen peroxide may mediate the oxidation of an iron-sulphydryl complex. Singlet oxygen readily reacts with protein sulphydryl groups, and we show that, in addition to the hydrogen peroxide/iron dependent mechanism, another pathway of UVA radiation-dependent loss of protein sulphydryl groups occurs via singlet oxygen both in vitro and in human fibroblasts. Loss of protein sulphydryl groups can lead to inactivation of protein function such as Ca^{2+} transport by Ca^{2+} - ATPase [281, 282]. Inactivation of sulphydryl containing proteins may be an important consequence of UVA irradiation. Indeed, UVA irradiation of skin cells in situ leads to inactivation of critical antioxidant enzymes.[287]

2.4.3.3. Cell Death

Ultraviolet radiation (UVR) has been divided into several functional wavelength ranges, the UVC (<280 nm) which is excluded by the earth's atmosphere; and the UVB (280–320 nm), the UVA (320–380 nm), and near visible (380–420 nm) components, which reach the earth's surface. UVA1 (340–400 nm) is a clinical definition of UVA for therapeutic applications [263]. The UVC and UVB regions overlap the DNA absorption spectra [292] and can cause direct DNA photodamage (*i.e.*, cyclobutane pyrimidine

dimers), associated with mutagenesis and tumorigenesis [293]. In contrast, the UVA region of the solar spectra is not absorbed directly by DNA. UVA exposure generates cellular oxidative stress, such that it stimulates the intracellular production of ROS by

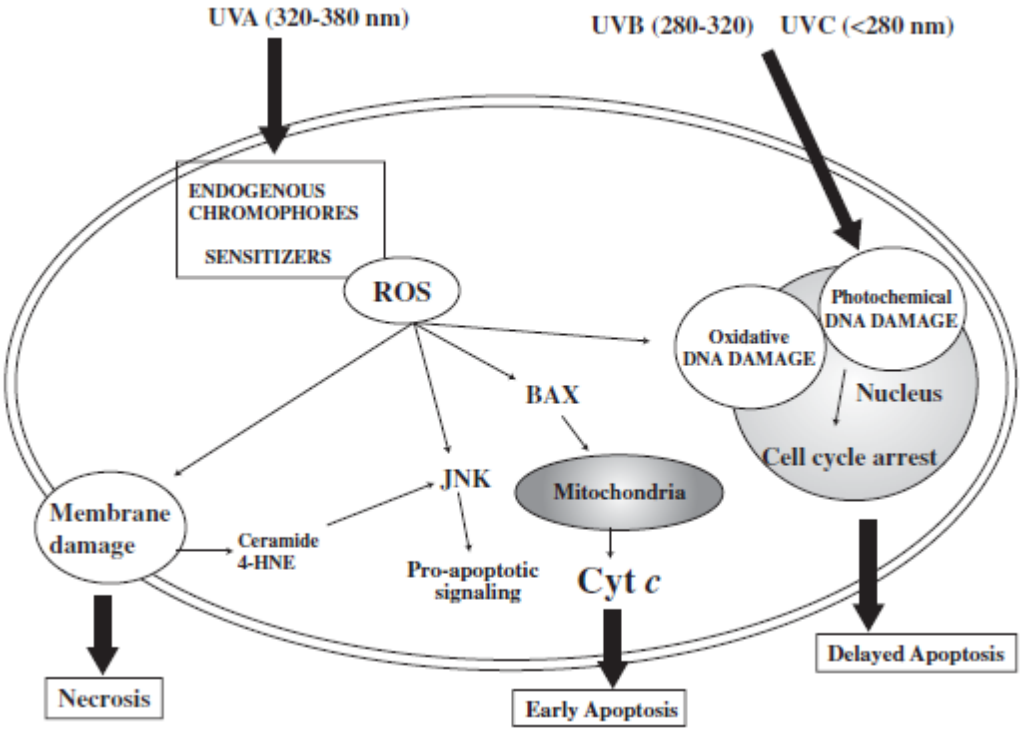

Figure 10. UV radiation induced cell death. UVA can cause lipid peroxidation and membrane damage, depletion of intracellular glutathione, and oxidative DNA damage. UVC and UVB cause direct photochemical damage to DNA. DNA damage and subsequent cell cycle arrest may lead to the delayed apoptosis observed after exposure to these agents.

photochemical reactions [263, 294]. The oxygen-dependence of UVA photokilling, and the specific involvement of $^1O_2$ in UVA mediated gene regulation have been demonstrated, though the precise endogenous chromophores which mediate UVA-induced ROS production remain poorly defined [263, 292, 294]. UVA cause cellular damage characteristic of oxidative stress, including membrane lipid peroxidation and oxidation of intracellular reduced glutathione (GSH). Furthermore, DNA damage can

occur after UVA exposure, including DNA strand breaks, and oxidative DNA damage such as 7, 8-dihydro-8- oxo-2-deoxyguanosine (8-OHdG) [285, 295-298].

The mechanisms of apoptosis following cellular exposure to UVR appear to be wavelength specific (Figure 10). To illustrate this, murine lymphoma cells were irradiated with UVA1, UVB, and UVC at isotoxic doses [299]. The UVA1 radiation (340–400 nm) induced both an immediate (<4 h) and a delayed (>20 h) apoptosis mechanism, while UVB or UVC radiation induced only the delayed apoptosis with no early component. The delayed apoptosis seen at all wavelengths was comparable to that observed with DNA damaging agents, whereas the early apoptosis associated with UVA1 was inhibited by membrane antioxidants and inhibitors of the mitochondria transition pore [299-301]. Apoptosis after UVA radiation has been observed in a number of physiologically-relevant skin cell models, including human dermal fibroblasts [302], epidermal keratinocytes [303],[304], melanocytes [305]; as well as transformed cell lines ([299, 306, 307]. Depending on the cell type, features of both extrinsic (Fas/caspase-8)-dependent and intrinsic (mitochondria-dependent) apoptotic pathways have been reported following cellular irradiation with UVA or UVB, involving modulation of Bcl-2 family proteins and typically culminating in caspase-3 activation [263, 298, 308]

2.4.3.4. Role of UV induced Oxidative Stress in Diseases

Ultraviolet A radiation of wavelength 320 – 400 nm penetrates the epidermis resulting in damage to the dermis. This causes skin to age prematurely, with effects that include roughening, blotchiness, sagging and wrinkles[309]. UVA also contributes to the development of skin cancer. Ultraviolet B is radiation between 290 – 320 nm and causes

sunburn and skin cancer [310]. UVB radiation inhibits DNA, RNA and protein synthesis and induces erythemal responses. UVC radiation from 100 – 290 nm is filtered out by the ozone layer and does not reach the earth's surface [311].

2.4.3.4.1. Photoaging

Premature aging of the skin is a well-documented consequence of exposure to Ultraviolet A (UVA) and Ultraviolet B (UVB) [309, 312]. Inflammation and the resulting accumulation of ROS play an important role in the photoaging of human skin in vivo. Environmental insults, primarily ultraviolet rays from the sun contribute to the generation of free radicals and ROS that stimulate the inflammatory process in the skin [313]. UV irradiation initiates and activates a complex cascade of biochemical reactions in the human skin. UV causes depletion of cellular antioxidants and antioxidant enzymes, initiates DNA damage leading to the formation of thymidine dimers, leads to immunosuppression and increased synthesis and release of pro-inflammatory mediators from a variety of skin cells. The net result of UV exposure of skin is inflammation and free radical generation. The inflammation and ROS lead to degradation of healthy skin and premature photoaging. In addition the inflammation and ROS cause oxidative damage to cellular proteins, lipids and carbohydrates which accumulate in the dermal and epidermal compartments, contributing to photoaging [311].

The acute effects of UV irradiation on human skin are well characterized. Immediately after UV exposure, keratinocytes release pro-inflammatory cytokines like TNF-α [314]. UV photons have a direct effect on DNA to cause DNA strand breakage [315]. UV also leads to depletion of cellular antioxidants and generation of ROS

including hydrogen peroxide, hydroxyl radical, singlet oxygen and peroxyl radicals [316]. These ROS play an important role in the UV induced photoaging of human skin *in vivo* [317]. UV radiation also triggers cytokine production and effects cellular mitosis, apoptosis and necrosis [318, 319]. Finally free radical induced peroxidation of membrane lipids contributes to further inflammation and oxidative damage to the skin [320]. Chronic UV exposure is also associated with protein oxidation in the human skin, both in the living layers and the stratum corneum [321, 322]. UVB also modulates several signal transduction pathway(s) leading to generation of ROS [317, 323]. Superoxide, hydrogen peroxide and hydroxyl radicals are primarily produced by phagocytic cells and lymphocytes in response to inflammation induced by UV exposure of skin [324]. Lymphocytes produce large quantities of RIS by expressing high amounts of NADPH oxidase. Skin is uniquely vulnerable to the damage caused by ROS, being rich in unsaturated fatty acids and exposed to high oxygen tension and UV, both of which promote ROS generation. ROS, especially $OH^{\bullet}$ radicals, are capable of damaging biological macromolecules such as DNA, carbohydrates, lipids and proteins. ROS initiates the process of lipid peroxidation in cellular membranes, causing cellular membrane damage, leakage of cellular components and ultimately cell death. ROS are also responsible for inflammation, tissue remodeling, oncogenesis and apoptosis, processes that orchestrate many of the degenerative processes associated with photoaging [324, 325].

Another effect of UV on skin is to induce pigmentation. In order to protect skin from further damage by UV, UV radiation also stimulates melanocytes directly to produce more melanin. Melanogenesis protects skin from further UV damage by

preventing apoptotic cell death following UV injury[326]. UV damage to cellular DNA induces skin melanogenesis by activating cellular-signaling pathways and increased DNA repair after irradiation enhances UV induced melanogenesis. Excessive melanogenesis by increased DNA repair has its downside too. Incorrect DNA repair and excessive melanogenesis often leads to melanoma, a form of skin cancer [309, 315, 326]

2.4.3.4.2. Skin Cancer

Aerobic life, UV solar radiation, genetic susceptibility, and immune status contribute collectively to the development of human skin cancers. In addition to direct DNA damage, UV radiation promotes the generation of reactive oxygen intermediates that can cause oxidative damage and inflammation [288, 327], and ultimately lead to tumor formation. Besides enabling us to breath, the production of oxygen is a very fortunate evolutionary event: by absorbing the most energetic UV radiation (wavelengths below 240 nm) in the outer reaches of our atmosphere and by forming ozone in the process, oxygen protects the earth's surface from the most damaging solar UV radiation. The rare stratospheric ozone absorbs most of the radiation with wavelengths below 310 nm. UV radiation below 290 nm is virtually undetectable at ground level. However, the fraction of UVB radiation that reaches us can still be absorbed by DNA and proteins and is enough to damage and kill unprotected cells (mainly through DNA damage). Hence, organisms on the earth's surface have to be well adapted, e.g., by forming UV-absorbing surface layers (skin and fur), by antioxidant defences to quench UV-generated ROS, by efficiently repairing damaged DNA, and by repairing or replacing damaged cells. Human skin--UV radiation does not penetrate any deeper--shows all of these adaptive features.

Nevertheless, the skin's defenses are not perfect and the continuous UV challenge leads to deterioration ("photoaging") in the long run and to an increasing likelihood of developing skin cancer [264, 310].

Ultraviolet B and A radiations (respective wavelength ranges 280-315 and 315-400 nm) are present in sunlight at ground level. The ultraviolet radiation does not penetrate any deeper than the skin and has been associated with various types of human skin cancers. The carcinogenicity of UVB radiation is well established experimentally and, to a large extent, understood as a process of direct photochemical damage to DNA from which gene mutations arise. Although UVA is generally far less carcinogenic than UVB radiation, it is present more abundantly in sunlight than UVB radiation (>20 times radiant energy) and can, therefore, contribute appreciably to the carcinogenicity of sunlight. In contrast to UVB, UVA radiation is hardly absorbed by DNA. Hence, the absorption by other molecules (endogenous photosensitizers) becomes more important, thus radicals and, more specifically, ROS can be generated that can damage DNA, membranes, and other cellular constituents [328].

Like UVB radiation, UVA radiation proved to be a complete carcinogen in experiments in which skin carcinomas developed in (hairless) mice under chronic exposure [269]. Unlike UVB radiation, UVA radiation initially induces mainly benign papillomas, which increase at a slow rate and are at a later stage outnumbered rapidly by carcinomas that increase at a much higher rate [329]. Chronic UVB exposure mainly induces a rapidly increasing number of squamous cell carcinomas (SCC) and actinic keratoses as precursors, with intermixed rare occurrences of benign papillomas, the chronic application of a (chemical) “initiator” of tumor development (generally a

mutagenic agent) will lead to carcinomas, whereas the repeated application of a "tumor promoter" (after a single application of an 'initiator") will generate benign papillomas; the promoting action appears to be related to the release of ROS. In this perspective, UVB radiation appears to exert predominantly a "tumor initiating" effect, whereas "tumor promotion" is relatively more pronounced with UVA radiation. Like UVB radiation, albeit differently and less efficiently, UVA radiation is clearly a mutagenic and carcinogenic agent. In contrast to UVB radiation, the fundamental photochemistry and pre-mutagenic DNA lesions are still not well determined for UVA radiation. Ten to 20% of sunburn and carcinogenic UV doses from sunlight stems from the UVA band[330]. UVA radiation is therefore an important toxic factor in our natural environment [264, 328].

2.5. Summary

Many modern treatment modalities including ionizing radiation, hydrogen peroxide, UV, photodynamic therapy, chemotherapy drugs, antibiotics, etc, operate via the induction of oxidative stress in cells and tissues by generating intracellular reactive oxygen species. The significance of the damage inflicted upon biological systems by ROS, produced exogenously or endogenously cannot be overestimated, as they have been implicated in numerous disease processes, including inflammation, degenerative diseases and tumor formation and involved in physiological phenomena, such as aging. It is imperative we harness the power of oxidative stress and we understand the mechanisms of interaction of oxidative stress with biological systems.

Chapter 3. Low Dose Non-Thermal Plasma Treatment

As described in Chapter 2 oxidative stress has a broad spectrum of effects on proliferating cells ranging from cell proliferation, cellular adaptation, and cell senescence to cell death via apoptosis and eventually necrosis. Studies have shown that low levels of reactive oxygen species enhance cell proliferation in mammalian cells. 3 – 15 μM of H_2O_2 induced a significant mitogenic response where 25 – 40% growth stimulation was observed [148]. Non-Thermal DBD plasma is known to produce a variety of ROS in liquid medium and the concentration of these ROS can be controlled by tuning the dose of plasma [17].

We divide the dose of non-thermal plasma treatment in to two major regimes; *low dose* (Treatment time ranging from 2 s to 30s or a dose of 0.2 – 5 J/cm^2) and *high dose* (treatment times greater than 30 s or a dose greater than 5 J/cm^2). In this chapter we investigate the effects of low dose plasma treatment on endothelial cells and present results related to enhanced cell proliferation. We show that the dose of non-thermal plasma treatment can be tuned to enhance proliferation in mammalian via release of growth factors like FGF-2.

3.1. Introduction

Non-thermal plasma interaction with the vasculature must be understood prior to treating vascularized tissue. We hypothesize that plasma can grow or regress blood vessels in a dose-dependent manner. Endothelial cells control many aspects of the vasculature from vascular tone to coagulation to inflammation. Endothelial cells also play

a guiding role in angiogenesis [331]. Endothelial cells produce and secrete angiogenic growth factors such as fibroblast growth factor-2 (FGF2), which in conjunction with many other signals induces cells to invade the surrounding tissue, proliferate, and develop into new blood vessels [332]. Angiogenesis can be both helpful and harmful. In wound healing, angiogenesis is required at the wound site for healing, whereas in cancer, angiogenesis blockade may prevent tumor growth[333].

Using an *in vitro* model, we investigated the effect of non-thermal DBD plasma on endothelial cells. Endothelial cell proliferation and death following plasma were measured. FGF2 release from endothelial cells and its effect on cell proliferation were quantified. Finally, mechanisms of non-thermal plasma effects were explored. We now show that while high dose non-thermal plasma induces endothelial cell death, lower doses induce endothelial cell proliferation. This proliferative effect is likely related to FGF2 release due to plasma-produced ROS.

3.2. Materials and Methods

Endothelial cell culture

Porcine aortic endothelial cells (PAEC) were isolated from porcine aortae by the collagenase dispersion method and used between passages 4 and 9 [334]. PAEC were maintained in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech) with 5% fetal bovine serum (Hyclone), 1% L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Medium was changed every two days. For plasma treatment, cells were washed with phosphate buffered saline, detached with 0.1% trypsin (Invitrogen), and seeded near confluence ($4x10^5$ cells per well) on 18 mm uncoated glass cover slips

(VWR) in 12-well plates (Corning). PAEC adhere well to bare glass, with close to 100% seeding efficiency. Cells were incubated for 24 hours prior to plasma treatment in 1.5 ml supplemented medium at 37°C and 5% CO_2.

Porcine tumor necrosis factor-α (TNF-α) was from R&D Systems. Recombinant human FGF2 was from Peprotech, and neutralizing FGF2 antibody was from Upstate Biotechnology. N-Acetyl-L-cysteine (NAC, Sigma), an intracellular ROS (ROS) scavenger and sodium pyruvate (Sigma), an extracellular ROS scavenger, were used to block plasma-produced ROS.

Endothelial Cell Plasma Treatment

Endothelial cells were treated with non-thermal plasma *in vitro* using the setup as illustrated schematically in Figure 3 and the operating parameters have been previously elaborated in section 1.2 on page 4. PAEC on glass cover slips were exposed to plasma for 5 to 120 seconds. Each cover slip was removed from the 12-well plate and placed on a microscope slide, which was positioned on the plasma device grounded base. 50 µl serum free medium was added to the sample to prevent drying. Following plasma, the cover slip was immediately placed in a new 12-well plate, 1.5 ml supplemented medium was added, and the samples were returned to the incubator.

Three approaches were used for plasma-treatment of cells: direct, indirect and separated. In direct treatment, the sample was one of the electrodes creating the plasma as shown in Figure 2 and Figure 3. Plasma discharge occurred between the quartz and the sample, which exposed the sample directly to neutral reactive species and charged particles. For indirect treatment, a grounded mesh was placed between the high voltage electrode and the sample to eliminate charged particles. In separated plasma treatment,

medium was plasma treated separately from cells and then immediately applied to cells. In this case, cells were not in direct contact with any plasma component.

Non-Thermal Plasma Induced Cell Death

Non-thermal plasma endothelial cell cytotoxicity was measured via cell counts and Live/Dead assay. For cell counts, PAEC were plasma treated as described. 3 and 24 hours following plasma treatment, attached (live) cells were trypsinized and counted using a Coulter counter (Beckman Coulter). These time points were selected to examine immediate and medium-term plasma toxicity effects. Since no change was detected between 3 and 24 hours, no longer time points were investigated. For the Live/Dead assay, 3 and 24 hours post treatment cells were labeled with 1 μM ethidium homodimer and 0.25 μM calcein (Invitrogen), incubated at room temperature for 45 minutes, and imaged by fluorescent microscopy (Olympus, USA) with a digital high performance CCD camera (Diagnostic Instruments). Live cells convert cell-permeant calcein to a FITC fluorescent form via intracellular esterases, whereas cell impermeant ethidium homodimer binds nucleic acids in membrane damaged dead cells to enhance TRITC fluorescence. Dead cells were manually counted in five distinct sample areas.

Endothelial cell apoptosis was measured via annexin V-propidium iodide labeling. Annexin V binds phosphatidylserine translocated from the inner to the outer cell membrane. Cells in early apoptosis are identified as annexin V-positive and propidium iodide-negative. PAEC were prepared by combining floating and trypsin-released attached cells. Samples were centrifuged to pellet cells, washed thoroughly, resuspended in annexin binding buffer, and labeled with annexin V-fluorescein and propidium iodide

as per manufacturer instructions (BD Pharmingen). Samples were analyzed immediately by flow cytometry (BD FACScanto).

Endothelial Cell Membrane Damage and FGF-2 Release

Endothelial cell membrane damage following non-thermal plasma was quantified through lactase dehydrogenase (LDH) release. PAEC were plasma treated as described, however DMEM without sodium pyruvate was used since sodium pyruvate interferes with the LDH assay. TNF-α (10 ng/ml) was the positive control. 0.5 ml conditioned medium was collected 2, 4, 6, 8, 12 and 24 hours after plasma, and LDH was quantified using the Cyototox-ONE Homogeneous Membrane Integrity Assay (Promega) as per manufacturer instructions. FGF2 release from plasma treated cells was measured in collected medium 0.5 to 24 hours after plasma treatment. FGF2 levels were quantified via FGF ELISA (R&D Systems).

Non-Thermal Plasma Induced Cell Proliferation

Endothelial cell proliferation was measured through cell counts and BrdU incorporation on treated cells or using conditioned medium. For cell counts, 10,000 PAEC were seeded on coverslips and plasma treated as described. Cell number was quantified on days 1, 3, 5 and 7 by counting trypsin-detached cells using a Coulter counter, with medium changes on days 2, 4 and 6. For directly treated cells, fold proliferation was determined by comparing cell number on day five to day one. For conditioned medium, confluent PAEC were plasma treated as described and incubated for 3 hours in 1 ml serum-free DMEM. Conditioned medium was collected and centrifuged to remove dead cells. 0.5 ml conditioned medium, along with 1 ml supplemented medium, was added to sub-confluent PAEC (10,000 cells per well) on days 2, 4, and 6

and cell proliferation was assessed. Conditioned medium from untreated cells and serum-free medium were controls. FGF2 effects were blocked by pre-incubating conditioned medium with FGF2 neutralizing antibody (1 µg/ml) for 30 min prior to adding it to cells.

DNA synthesis induced by plasma-treated cell conditioned medium was determined by BrdU incorporation. Thymidine analogue 5-bromo-2-deoxyuridine (BrdU) is incorporated instead of thymidine into newly synthesized DNA. 10,000 cells per well were seeded in a 96-well plate in supplemented medium. Conditioned medium was collected from plasma-treated cells as described and added to each well in a 1:2 ratio with supplemented medium. After 18 hours, 20 µl BrdU labeling solution (Chemicon) was added to each well for 3 hours. Cells were fixed and incubated with anti-BrdU conjugated with peroxidase. The optical density (450/570), which was directly proportional to DNA synthesis level, was determined using a microplate reader (TECAN).

Statistical Analysis

Statistical analyses were performed with Prism software (Graphpad). Data were normally distributed and expressed as the mean ± S.D. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than 2 groups were analyzed by ANOVA. A value of $p \leq 0.05$ was considered statistically significant and is indicated with a pound sign (#). $p \leq 0.01$ is indicated with an asterisk (*).

3.3. Results

3.3.1. Cell Proliferation in Response to Non-Thermal Plasma

Endothelial cell proliferation is enhanced by low dose non-thermal plasma treatment. Five days after treatment, cells treated with plasma showed greater viable cell

number than control up to 30 seconds of plasma. PAEC exposed to 30 seconds of plasma demonstrated twice as many viable cells as untreated controls (Figure 11A). However,

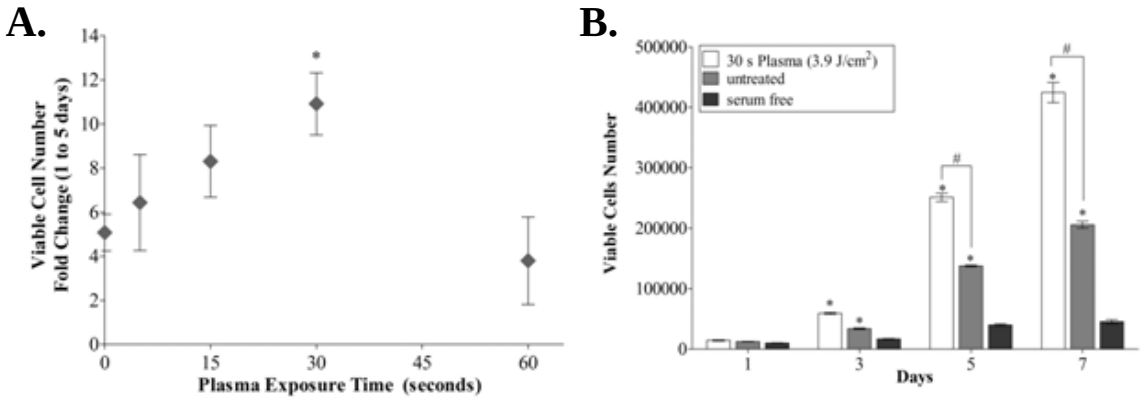

Figure 11. Plasma induces endothelial cell proliferation by direct treatment and through conditioned medium from treated cells. (A) PAEC were plasma treated on day 0, and counted on days 1 and 5, with medium changes on days 1 and 3. Data are presented as fold change, since plasma leads to some cell death on day 1. * $p < 0.01$ compared to untreated cells. (B) Conditioned medium was collected after three hours from untreated or plasma-treated cells and applied to untreated PAEC. Serum-free media, which does not contain soluble growth factors, was the negative control. Cell number was counted with a Coulter counter. * $p < 0.01$ compared to day 1; # $p<0.01$ comparing untreated cells with 30 seconds plasma.

plasma treatment beyond 30 seconds decreased cell number. A similar increase in cell number with 30 seconds of plasma treatment was observed for cells growth on collagen coated coverslips. To determine if increased cell number was related to a cell-secreted soluble factor, PAEC were incubated in conditioned medium from untreated or plasma treated cells (3.9 J/cm^2, 30 s) (Figure 11B). Serum-free media, which does not contain soluble growth factors, was the negative control. Plasma dose was selected based on maximal observed effect. Viable cell number was twice as high in cells incubated with plasma-treated cell conditioned medium on days 3, 5 and 7 compared to cells incubated with untreated cell conditioned medium.

3.3.2. Cell Death in Response to Non-Thermal Plasma

Decreased viable cell number was observed at high non-thermal plasma levels; therefore we investigated endothelial cell death in response to plasma. Plasma was relatively non-toxic to PAEC up to 60 seconds. While the number of live, attached cells decreased as plasma exposure increased, more than 75% of cells remained viable up to 60 seconds plasma (Figure 12A). There was no significant difference in cell viability 3 & 24 hours following plasma exposure, suggesting no long term plasma toxicity effects. A Live/Dead assay was used to confirm cell count results. Endothelial cells treated with plasma for short exposure times (up to 30 seconds) showed few dead cells (Figure 12B, quantified in Figure 12C), confirming that plasma is relatively non-toxic at short exposures. Dead cell number increased with increasing plasma exposure time ($p < 0.01$ by ANOVA). While dead cell number increased slightly with 60 seconds plasma, at 120 seconds a significant number of dead cells and few live cells were evident. This extensive cytotoxicity may be related to sample drying. Therefore, 120 seconds plasma was not used for subsequent assays.

To determine the endothelial cell death mechanism induced by plasma, PAEC were analyzed 24 hours post-plasma for apoptosis (Figure 12D). Apoptosis increased with plasma treatment ($p < 0.01$ by ANOVA). At 30 and 60 seconds plasma, 20% of cells were apoptotic compared to 10% of untreated cells. At 120 seconds, nearly 60% of cells were apoptotic. These data confirm that shorter plasma exposures are non-toxic, and apoptosis is one mechanism of plasma-induced cell death.

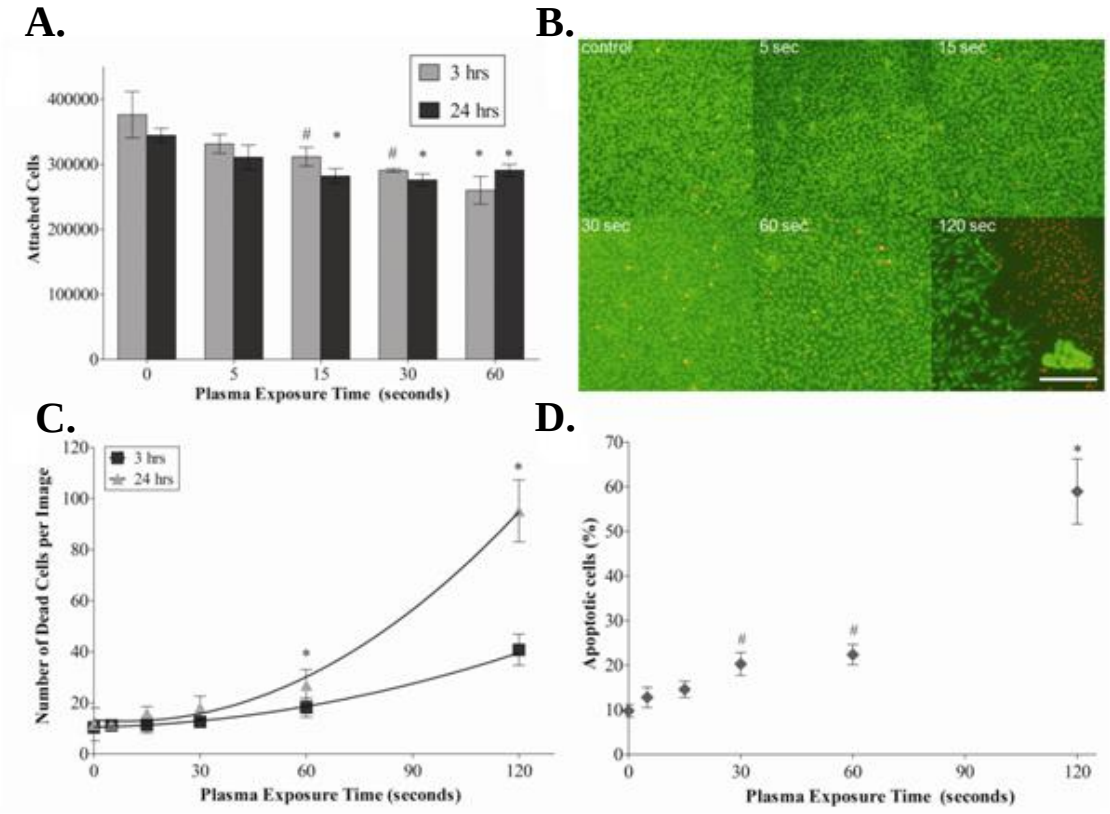

Figure 12. Low dose plasma is relatively non-toxic to cells, but high dose induces apoptotic cell death. (A) Attached cells, confirmed as alive by Trypan blue, were counted 3 and 24 hours after plasma ($p < 0.01$ by ANOVA). (B) Endothelial cell death was measured by Live/Dead assay. Live cells appear green, whereas dead cells appear red. Scale bar is 200 μm. (C) Quantified Live/Dead images (n=5). (D) Apoptosis was measured by Annexin V – propidium iodide 24 hours after plasma.

3.3.3. FGF-2 Release Post Non-Thermal Plasma Treatment

We next considered whether FGF2 was released from endothelial cells following plasma, and whether released FGF2 contributed to enhanced cell proliferation. FGF2 has no signal sequence for secretion, and therefore is primarily thought to be released during sub-lethal cell membrane damage. Cell-released FGF2 increased up to 3 hours after plasma treatment (3.9 J/cm^2, 30 seconds) and then rapidly decreased up to 24 hours after plasma (Figure 13A). In contrast, FGF2 levels for cells treated with 10 ng ml^{-1} TNF-α as a positive control rose more slowly but continued to rise up to 24 hours. Endothelial cell

membrane damage was assessed by LDH release following plasma. Medium LDH increased significantly by 4 hours after plasma and continued to rise throughout the first 24 hours (Figure 13B, $p < 0.01$ by ANOVA), comparable to TNF-α positive control.

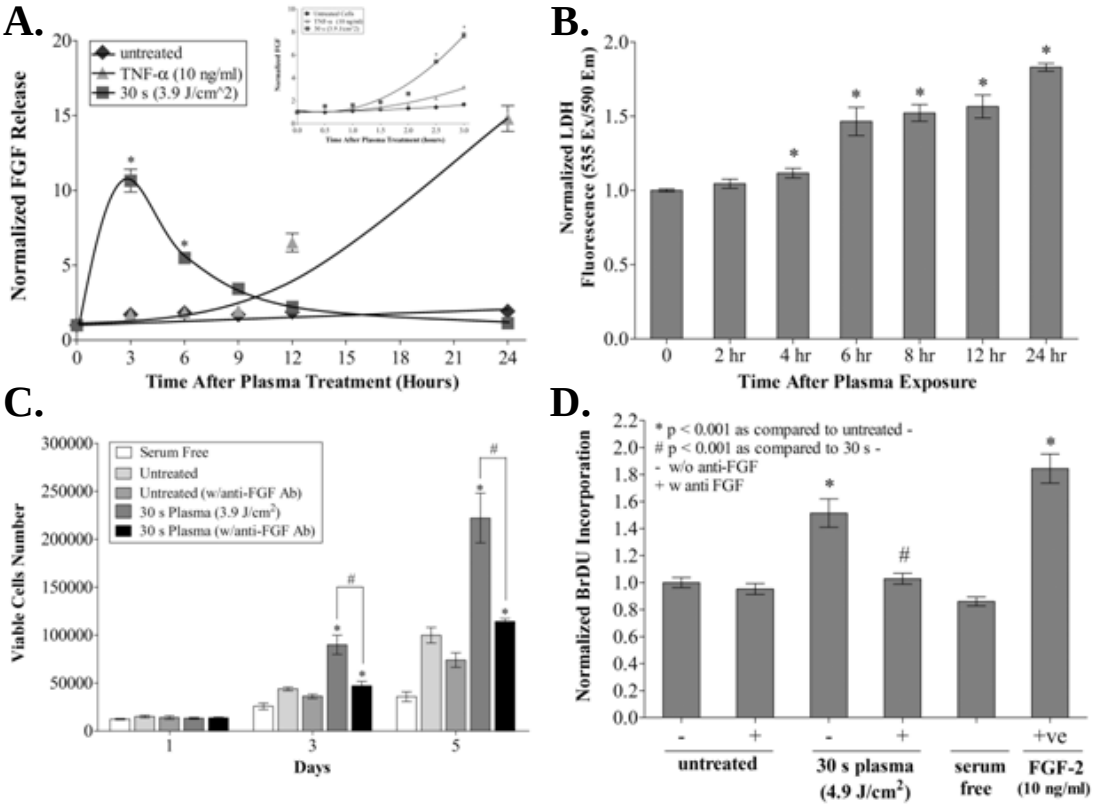

Figure 13. Endothelial cells release FGF2 after plasma, and cell-released FGF2 enhances proliferation. (A) FGF2 was quantified in cell medium after plasma by ELISA. Inset shows medium FGF2 up to 3 hours after treatment. (B) LDH release was measured in cell medium post-plasma. (C) FGF2 effects were blocked by incubating plasma-treated cell conditioned medium with FGF2 neutralizing antibody. (D) FGF-2 blockade reduced DNA synthesis in response to plasma-treated cell conditioned medium, measured by BrdU incorporation. Serum free media, which does not contain FGF2, was the negative control.

The role of released FGF2 in plasma-enhanced endothelial cell proliferation was investigated by treating conditioned medium from plasma-treated cells with an FGF2 neutralizing antibody to block FGF2 effects. Serum free media, which does not contain FGF2, was the negative control. The FGF2 neutralizing antibody significantly suppressed proliferation in PAEC exposed to plasma-treated cell conditioned medium (Figure 13C).

Viable cell number for samples with FGF2 blocked was similar to untreated cell conditioned medium. These data were confirmed with a BrdU assay (Figure 13D). BrdU incorporation was enhanced for cells incubated in plasma-treated cell conditioned medium, but the FGF2 neutralizing antibody abrogated the effect. These data suggest that plasma leads to FGF2 release, which contributes to enhanced endothelial cell proliferation following plasma.

3.3.4. Mechanisms of Release of FGF-2 Following Plasma Exposure

Plasma produces neutral short and long lived reactive species and charged particles like ions and electrons, yet which plasma component led to endothelial cell FGF2 release was unknown. To better understand FGF2 release mechanisms, PAEC were exposed to plasma directly, indirectly, or in a separated configuration. There was no statistically significant difference in FGF2 release between direct and indirect treatment (Figure 14A). Both direct and indirect cell plasma treatment induced significantly greater endothelial cell FGF2 release as compared to separated treatment, however separated treatment still produced significantly more FGF2 release than untreated control.

Non-thermal plasma produces large amounts of ROS. These ROS may interact with endothelial cells, leading to FGF2 release. To determine the role of ROS in plasma-induced cell FGF2 release, endothelial cells were pre-incubated in 4 mM NAC (intracellular ROS) and then plasma-treated in supplemented medium with or without 10 mM sodium pyruvate (extracellular ROS). Both NAC and sodium pyruvate significantly suppressed FGF2 release from plasma treated cells (Figure 14B), suggesting that intracellular and extracellular ROS may contribute to plasma effects.

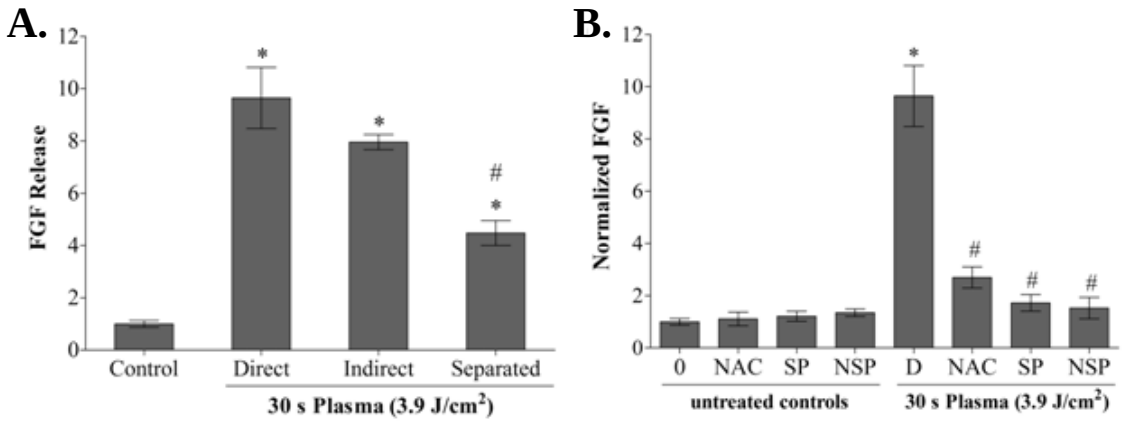

Figure 14. Endothelial cell FGF2 release is linked to neutral ROS. (A) PAEC were plasma treated directly, with a grounded mesh to remove charged particles (indirect), or medium was plasma treated and then applied to cells (separated). Medium FGF2 was measured by ELISA three hours after plasma. # $p<0.05$ compared to direct. (B) PAEC were pretreated with 4 mM (NAC, intracellular) or 10 mM sodium pyruvate (SP, extracellular), or both ROS scavengers (NSP). Samples were directly plasma treated, and cells pretreated with ROS scavengers were compared to cells directly treated with plasma alone (D). Medium FGF2 was quantified by FGF ELISA three hours after plasma.

3.4. Discussion

Non-thermal plasma interacts with the vasculature during tissue treatment, and plasma may be able to induce dose-dependent blood vessel growth and regression. We now show that high dose plasma induces endothelial cell death, whereas low dose enhances endothelial cell proliferation. The plasma proliferative effect is likely related to sub-lethal cell membrane damage by ROS, which leads to FGF2 release. FGF2, together with vascular endothelial growth factor and other critical signals, induces endothelial cell proliferation, migration, and tube formation [332]. Angiogenesis is a complicated process, and endothelial cell proliferation plays only a small initial role [331]. However, our data suggest that low-dose plasma could promote angiogenesis to accelerate wound healing, whereas high-dose plasma could inhibit angiogenesis to prevent cancer growth.

In cases where plasma cannot be used directly, fluid from plasma treated cells could also be used.

While FGF2 alone will likely not complete the angiogenic process, additional subsequent plasma treatments could be tuned to induce other angiogenic signals. For example, ROS play an important role in vascular endothelial growth factor signaling, and thermal plasmas that produce nitric oxide could also promote angiogenesis. Thus repeated plasma treatments of different doses or with different plasma types could be timed to maximize the angiogenic response. During these repeated treatments, plasma effects on the surrounding tissue must also be considered. In our previous work, high plasma doses did not induce gross or histological skin damage in an animal model, and malignant epithelial cells were more sensitive to plasma-induced apoptosis [335, 336]. These data suggest that plasma may induce angiogenesis without harming the surrounding tissue, whereas plasma inhibition of angiogenesis may synergistically kill malignant cells. However, since each of these studies were performed under slightly different plasma and cell or tissue conditions, additional studies should be performed to directly compare plasma sensitivity of various cell types.

Non-thermal plasma can induce endothelial cell death via the apoptotic pathway. In our previous work on ROS and endothelial cells, we showed that low ROS levels induce sub-lethal cell membrane damage, higher ROS levels induce apoptosis, and extremely high ROS levels induce non-specific cell death which is likely necrosis [337]. Our data suggest that plasma dose can similarly be used to modulate the cell death mechanism, which is an important consideration both *in vivo* and *in vitro*. Apoptosis is programmed cell death initiated by physiological or pathological signals. Apoptotic cells

are broken up into apoptotic bodies, which are engulfed by neighboring cells, leading to clean cell death without significant inflammatory response [338, 339]. On the contrary, necrosis is cell death accompanied by swelling, blebbing and increased membrane permeability leading to cytosolic content spillage. This typically leads to inflammation in surrounding tissue [338, 339]. By controlling plasma dose, we may be able to kill endothelial cells without significant necrosis and subsequent inflammation.

Plasma induces endothelial cell FGF2 release. FGF2 is thought to be released only at cell injury or death, since it has no signal sequence for secretion [332]. Since plasma effects occur shortly after treatment, plasma may induce sub-lethal endothelial cell membrane damage, rendering the cells leaky to intracellular contents like FGF2. Other stimuli which induce cell membrane damage lead to FGF2 release. Biochemical changes, such as high glucose, enhance FGF2 release also through ROS[337]. Transient plasma membrane disruption by mechanical forces leads to rapid cytosolic FGF2 release. This FGF2 release initiates growth required for tissue integrity maintenance and/or repair after injury [340]. Mechanical strain also stimulates a proliferative response in coronary vascular smooth muscle cells via FGF2 release, and strain can even enhance endothelial cell FGF2 mRNA expression [341, 342]. *In vivo,* FGF2 released into the coronary circulation after vascular injury promotes human vascular smooth muscle cell proliferation [343].

Similar to mechanical damage, cell membrane injury from ionizing radiation induces FGF2 expression and release in endothelial and epithelial cells *in vitro* [344-346] and *in vivo* [347]. FGF2 enhances endothelial, epithelial, and hematopoetic cell survival after ionizing radiation[345, 348-350], and FGF2 release is critical to radiation damage

repair [345]. Pulsed electromagnetic fields can stimulate endothelial cell growth, angiogenesis, and wound healing through endogenous FGF2 release [351-353]. Non-thermal plasma differs from irradiation and electromagnetic fields in that the latter are penetrating and injure surrounding tissue, or they need an expensive setup to be generated safely. Plasma provides a novel and safer means to induce FGF2 release and angiogenesis since it provides precise control of treatment area and depth. Non-thermal plasma devices are also small and relatively simple to produce.

When endothelial cells are exposed to plasma, the conditioned medium FGF2 level peaks three hours after treatment and then declines. In contrast, cells treated with TNF-α show a gradual increase in medium FGF2. Thus plasma FGF2 release kinetics is essentially different from TNF-α. One possible reason is that TNF-α remains in the medium continuously, whereas plasma treatment occurs over a short, finite time. An alternative is that while both plasma and TNF-α likely release FGF2 related to ROS, TNF-α takes longer to produce ROS. While TNF-α activates a variety of biochemical signaling pathways in endothelial cells, the most likely path for TNF-α FGF2 release is cell membrane damage, since FGF2 has no known signal sequence for secretion. TNF-α must bind the membrane-bound TNF receptor, which activates intracellular signaling cascades leading to ROS [354, 355]. Thus the indirect and extended FGF2 release from TNF-α induced cell damage differs greatly from the finite and direct nature of plasma-induced rapid FGF2 release. The released FGF2 may then be bound by remaining viable cells, which explains the drop in medium FGF2 level after 3 hours and enhanced endothelial cell proliferation. This brief and defined FGF2 release may be critical to

angiogenesis, since timing and local biochemical environment play important roles in FGF2 signaling [356].

Both FGF2 and LDH are released from cells due to cell membrane damage, yet in our experiments, FGF2 and LDH release from plasma treated cells followed different trends. Whereas FGF2 release peaked three hours post-plasma and then declined, LDH release became significant only four hours after plasma treatment but then increased up to 24 hours. This difference may be related to the relative LDH (134 kDa) and FGF2 (18 kDa) sizes. FGF2 may be released after early sub-lethal plasma membrane damage, whereas LDH release occurs gradually after more extensive cell membrane damage. Additionally the gradual post-plasma LDH release may indicate that non-thermal plasma does not lead to immediate irreversible membrane integrity loss normally associated with severe trauma or cell death. Viable cells release low LDH amounts without hampering cell function [357]. LDH also has no extracellular function, whereas FGF2 binds to cell membrane receptors, thus cell-released LDH is not metabolized by other cells whereas FGF2 is. This correlates with FGF2 decrease up to 24 hours after plasma. Non-thermal plasma may lead to sub-lethal membrane damage which is gradually repaired as living cells uptake released FGF2 and remain viable.

Plasma-induced FGF2 release is likely related to neutral ROS. Non-thermal plasmas produce long lived (O_3, NO, HO_2, H_2O_2) and short lived (OH, O, electronically excited O) neutral particles and charged particles (ions and electrons). Both charged and neutral particles can lead to ROS in treated fluid. When endothelial cells were treated directly or indirectly (excluding charged particles), endothelial cell FGF2 release was not significantly different. However, FGF2 release decreased in separated treatment, in which

medium was treated prior to applying it to cells. The time required to collect separately treated medium and apply it to untreated cells eliminated short lived neutral species and direct contact between plasma and cells. Direct plasma effects could include local heating by plasma streamers or UV radiation. Since separated treatment decreased FGF2 release by 50%, and FGF2 release remained significantly greater than in control cells, we believe that both short and long lived neutral species play a major role in plasma-induced FGF2 release. While a wide variety of plasma-produced ROS could affect cells, both atomic and singlet oxygen are short lived and therefore highly likely to recombine before reaching the sample surface during treatment. The plasma-produced ROS most likely to contribute to endothelial cell FGF2 release are OH radicals, hydrogen peroxide, and HO_2. Non-thermal plasma produces a large ROS concentration in extracellular medium during treatment. However, it is unclear if these ROS go inside cells. Both intracellular and extracellular ROS scavengers decreased FGF2 release following plasma. Combined scavengers reduced FGF2 release more than either scavenger alone. ROS produced by plasma extracellularly may move across the cell membrane through lipid peroxidation, opening transient cell membrane pores, or signaling pathways which modify ROS inside cells. Active species produced by plasma may also modify the cell medium, which in turn interacts with cells. Since many of active species have a short life span, they may immediately interact with medium components including amino acids and proteins, leading to production of long lived reactive organic hydroperoxides [358]. These hydroperoxides may then induce lipid peroxidation and cell membrane damage, or they may bind to cell membrane receptors and activate intracellular signaling pathways leading to FGF2 expression and release.

We believe that non-thermal plasma could be used *in vitro* and *in vivo* to stimulate angiogenesis. Potential plasma applications include vascularizing tissue engineering structures, enhancing transplanted tissue incorporation, and accelerating wound healing. Our two-dimensional treatment model – an endothelial cell monolayer on a glass substrate covered with a thin medium film (~100 μm) – is likely more severe than what would be experienced by cells either *in vivo* or in three-dimensional *in vitro* models. Both sample geometry and the amount of liquid covering the sample are crucial to plasma treatment efficacy. We previously showed that increasing media depth over malignant epithelial cells decreased plasma-induced cell death [336]. ROS are highly reactive and may be inactivated prior to reaching cells if the distance between the plasma and the cells is too great. In these situations, a higher plasma dose could be used to maintain plasma effects. In the future, we will examine plasma penetration depth variation with environmental conditions by treating endothelial cells within three-dimensional collagen gels. We observed similar plasma-induced proliferation results for cells seeded on uncoated and collagen-coated substrates in two dimensions, suggesting that plasma effects will be similar in a more tissue-like environment.

3.5. Summary

To summarize, mammalian cells were treated *in vitro* with a custom non-thermal plasma device. Low dose plasma treatment was relatively non-toxic to mammalian cells while treatment at high doses led to cell death. Endothelial cells treated with plasma at a dose of 4 J/cm2 demonstrated twice as much proliferation as untreated cells five days after plasma treatment. Release of fibroblast growth factor-2 (FGF2) peaked three hours

after plasma treatment. The plasma proliferative effect was abrogated by an FGF2 neutralizing antibody, and FGF2 release was blocked by ROS scavengers. These data suggest that low dose non-thermal plasma enhances cell proliferation due to ROS mediated FGF2 release. Plasma may be a novel therapy for dose-dependent promotion or inhibition of endothelial cell mediated angiogenesis.

Chapter 4. High Dose Non-Thermal Plasma Treatment

In Chapter 2, we saw that oxidative stress has a broad spectrum of effects on mammalian cells, ranging from cell proliferation to apoptosis. Studies have shown that high levels of oxidative stress leads to apoptosis in mammalian cells. 0.5 to 1 mM of H_2O_2 was shown to induce apoptosis in fibroblasts [148]. The dose of non-thermal plasma can be tuned to produce high concentrations of reactive oxygen species in medium [17]. As described in Chapter 3, plasma treatment exposures over 30 s of treatment or a dose of plasma greater than 5 J/cm^2 were classified as high dose plasma treatment. In this chapter our aim was to investigate the effects of high dose plasma treatment on mammalian cells. Malignant cells have frequently acquired the ability to block apoptosis and proliferate indiscriminately and it is important that treatment modalities address this concern by inhibiting proliferation and at the same time limit inflammation by primarily inducing apoptosis over necrosis in malignant cells. Earlier studies had shown that non-thermal DBD plasma treatment demonstrates apoptosis like behavior in melanoma cells [42]. In Chapter 4 we rigorously tested this claim and present results related to induction of apoptosis in malignant cells by high dose of non-thermal DBD plasma treatment.

4.1. Introduction

The ability of a cell to self-regulate is a vital function in multicellular organisms allowing for appropriate growth, development, and death at the necessary times. Apoptosis, or programmed cell death, is a critical element of this self-regulation [201,

359]. The non-functioning of a tumor-suppressor gene that facilitates apoptosis, or the over expression of an anti-apoptotic protein are both important pathways in cancer development. Many anti cancer therapies are aimed at modulating these factors with various bioactive agents in an attempt to target components of the apoptotic pathway [354, 360]. However, many of these approaches remain in preclinical development secondary to low efficacy and drug resistance [361, 362]. Our current research seeks to develop techniques to modulate apoptotic activity in cancer cells by evaluating an electro-physical approach to induce apoptosis.

In the present study, we sought to explore the effects of exposure of melanoma cells to non-thermal atmospheric pressure dielectric barrier discharge (DBD) plasma (non-thermal plasma) in cell culture. To date there has been no investigation of the interaction between non-thermal atmospheric plasma discharges and the induction of apoptosis in living cells. This information is critical if this is going to be a viable treatment strategy as a selective cytotoxic cancer therapy. We also present a few mechanisms underlying the induction of apoptosis by non-thermal plasma in melanoma cancer cells.

4.2. Materials and Methods

Cell Lines and Reagents

The Melanoma cell line (ATCC A2058) was cultured in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose (ATCC 30-2002), 10% fetal bovine serum (ATCC 30-2020) and 1% Penicillin/Streptomycin (Sigma-Aldrich, St Louis, MO). The cells were propagated in 100 mm plastic petri dishes (Greiner Bio One) in a 5% CO_2 incubator at 37 ºC. Media was changed every 2 – 3 days. After 4 – 5 days of incubation, the cells were trypsinized

(0.25% (w/v) Trypsin - 0.53 mM EDTA) harvested and transferred to 44 mm diameter aluminum Petri dishes (Fisher Scientific, Pittsburgh, PA). To improve the cell adhesion on aluminum petri dishes, the dishes were pre-treated with poly L-lysine for 1h. Experiments were begun 2 days after plating cells in aluminum dishes. The number of cells in each Petri dish at the beginning of each experiment was about 1×10^6 with 3-9% Trypan blue positive.

Actinomycin D (Biovision Inc., Mountain View, CA), a potent inducer of apoptosis, was used as a positive control. N-Acetyl-L-cysteine (2 mM, Sigma-Aldrich, St Louis, MO), an intracellular ROS (ROS) scavenger was used to block the ROS produced by non-thermal plasma treatment.

Non-Thermal Plasma Treatment of Melanoma Cells

Early experiments revealed that plastic Petri dishes prevented application of uniform plasma discharge to the cells. As an alternative, aluminum Petri dishes (Fisher Scientific) were used to plate and treat cells in-vitro. The only drawback was the inability to observe the cells directly on the plates. For this reason, additional controls were prepared in parallel with standard Petri dishes of the same size. At the start of each experiment all the media from each experimental or control Petri dish was aspirated and 200 µl of fresh media was added to keep the cells moist. The cells were exposed to non-thermal plasma and immediately after treatment; 2 ml of fresh media was added for further incubation until the time of analysis. Negative control dishes were treated as above without plasma exposure.

Trypan Blue Exclusion Test

Trypan blue exclusion was used in the initial dose response and time-course experiments using the following protocol. Cells were trypsinzed and scraped and collected in 15ml tubes, centrifuged at 1000 rpm for 10min in 4^0C. Fluid was aspirated and the cell pellet was re-suspended in 5 ml of 1x PBS. Then 10µl of suspension was mixed with 10µl of Trypan Blue dye (Cambrex) and transferred to a hemocytometer slide. The total number of cells and relative percentages of viable and dead cells were counted and calculated. The same procedure was performed for untreated (control) cells. Trypan Blue exclusion was used for cells treated with different doses of Plasma (5, 10, 15, 20, and 30 J/cm^2) and at different periods of time after treatment (immediately, 1 hr, 3 hr and 24 h later).

Colony Survival Assay

1 x 10^6 Melanoma cells were seeded in aluminum petri dishes two days before plasma treatment. Cells were plasma treated as described at various doses of plasma and then incubated for one day after plasma treatment. 300 cells were seeded onto 60-mm dishes after exposure to non-thermal plasma or H_2O_2 (positive control). Colonies, which formed 11 days after plating MCF10A cells, were fixed and stained with a crystal violet solution (0.5% in 20% ethanol) and then counted. Assays were done in triplicate.

Annexin-V/PI Assay

Cell propagation and Plasma treatment were done as above. In order to confirm apoptotic activity, cells were cultured as above and then exposed to non-thermal plasma at a dose of either 5 J/cm^2 or 15 J/cm^2. A negative control of untreated cells and a positive control of Actinomycin D treated cells were run in parallel. The cells were

harvested at 0, 24, 48, and 72 hours after treatment and stained using the Guava Nexin apoptosis kit (Guava Technologies, Hayward CA). Analysis was performed using the Guava® Personal Cell Analysis (PCA) 96 System. Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine (PS), a membrane component normally localized to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can readily bind them. This assay incorporates this PS-Annexin V binding as an indicator of early stage apoptotic cells. The Guava Nexin™ assay kit utilizes Annexin V-PE to detect PS on the external membrane of apoptotic cells. The cell impermeable dye, 7-AAD, was used as an indicator of membrane structural integrity. 7-AAD is excluded from live, healthy cells and early apoptotic cells, but permeates late stage apoptotic and dead cells. These populations of cells can then be distinguished in this assay. Numerous samples of each group were run consecutively at each time point to establish statistical validity.

Caspase-3 Cleavage

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by direct lysis of floating and attached cells in 2X SDS sample buffer containing β-mercaptoethanol. Samples were electrophoresed on a 1.5 mm 15% polyacrylamide gel at 150 V in Tris-glycine SDS running buffer [25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (pH 8.3)]. Following electrophoresis, proteins were transferred on to PVDF (Millipore, MA, USA) membrane for three hours at 65V in Tris-glycine transfer buffer [10% SDS, Deionized Water, Tris-Glycine and Methanol (VWR, PA USA)]. Immunoblotting was done by blocking membranes in 5% bovine serum

albumin (BSA, Fraction V, Fisher Scientific) in PBST for 30 min followed by incubation with primary antibody in 5% BSA in PBST for cleaved caspase-3 overnight for 10 to 12 h at 4ºC with rocking. Primary antibodies used for immunoblot included rabbit polyclonal antibodies specific for cleaved caspase-3 [Millipore, MA, USA]. The primary antibody was detected with fluorescently tagged goat anti-rabbit Alexa and Fluor 488 (Santa Cruz Biotechnology). Immunoblot was developed using Odyssey Infrared Gel Imaging system (LI-COR Biosciences, NE, USA).

TUNEL ® Assay

One of the hallmarks of late-stage apoptosis, or programmed cell death, is the fragmentation of nuclear chromatin. This generates DNA strands with exposed 3'-hydroxyl ends, which are enzymatically labeled in the TUNEL assay. Cells were harvested as above at 24, 48 and 72 hours after treatment in triplicate and were stained using the DeadEnd^{tm} Fluorometric TUNEL assay (Promega Co.). The DeadEnd™ Fluorometric TUNEL System is a classic TUNEL Assay designed for the specific detection and quantitation of apoptotic cells within a cell population. The DeadEnd™ Fluorometric TUNEL System measures nuclear DNA fragmentation, an important biochemical hallmark of apoptosis in many cell types. The DeadEnd™ Fluorometric TUNEL System measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP$^{(a)}$ at 3´-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. The fluorescein-12-dUTP-labeled DNA can then be visualized directly by fluorescence microscopy or quantitated by flow cytometry.

For fluorescence microscopy, cells were fixed on Lab-Tek slides for the TUNEL assay. Samples were analyzed in a fluorescence microscope using a standard fluorescence filter set to view the green fluorescence of Fluorescein at 520 ± 20 nm. Both control and treated cells were cultured in aluminum dishes to analyze the possibility of apoptosis developing from contact with aluminum.

For flow cytometry analysis, cell suspensions were treated with the DeadEndTM Fluorometric TUNEL System (Promega US Co), following the manufacturer's instructions. Counterstaining was done by incubating cells for 20 min at room temperature in the dark in phosphate-buffered saline containing 6 ng/mL RNAse (Roche Applies Sciences, Indianapolis, IN) and 5 ng/mL propidium iodide (Invitrogen, Carlsbad, CA) in PBS. Suspensions were washed once in PBS and resuspended in PBS for analysis. Flow cytometry was performed using a FACSORT flow cytometer (BD Biosciences, San Diego, CA). Data acquisition was done using Lysis II software (version 2.0, BD Biosciences). Fluorescence spill-over was removed by compensation. At least ten thousand events were acquired per sample. Data analysis was performed using WinMDI software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, available online from http://www.facs.scripps.edu). Apoptotic and necrotic cells were differentiated by plotting PI fluorescence over fluorescein in a bivariate plot. Quadrants were set to define TUNEL-negative cells with normal DNA content; TUNEL-positive cells were counted as apoptotic, TUNEL-negative cells with lower DNA content as necrotic.

4.3. Results and Discussion

4.3.1. Cell Viability

4.3.1.1. Trypan Blue

The first set of experiments sought to determine the short-term sub-lethal dose of non-thermal Plasma in our melanoma cell line. When control cells were compared to 5, 10, 20 and 30 J/cm^2 of Plasma exposure one hour after treatment as shown in Figure 15A, we found that doses greater than 10 J/cm^2 of exposure decreased the total number of cells in culture significantly (2.5 x10^6 vs. <1.1x x10^6; $p<0.05$). In addition, a dose 10 J/cm^2 reduced the percent of viable cells from 92% to 23% ($p<0.05$).

4.3.1.2. Colony Assay

Cell number and trypan blue exclusion gives an indication of cell growth; however, it does not indicate true long term survival which is better assessed by colony survival assays. In order to determine whether the decrease in cell number was due to induction of cell death and whether the remaining cells were viable, colony survival assay was performed at various time points after plasma treatment. Melanoma cell survival 11 days after plasma treatment was significantly decreased as the dose of plasma increased as shown in Figure 15B. Twenty eight percent of the cells treated at 5 J/cm^2 were viable 11 days after treatment ($p < 0.001$) and15% and 8% survival were observed at 10 J/cm^2 and 15 J/cm^2, respectively, ($p < 0.001$) as compared to untreated cells. Non-thermal plasma decreases long term viability and survival of melanoma cells. This indicates that non-thermal plasma is not only able to kill melanoma cells but many of the cells that appear to survive are not really viable, i.e. they cannot form a colony

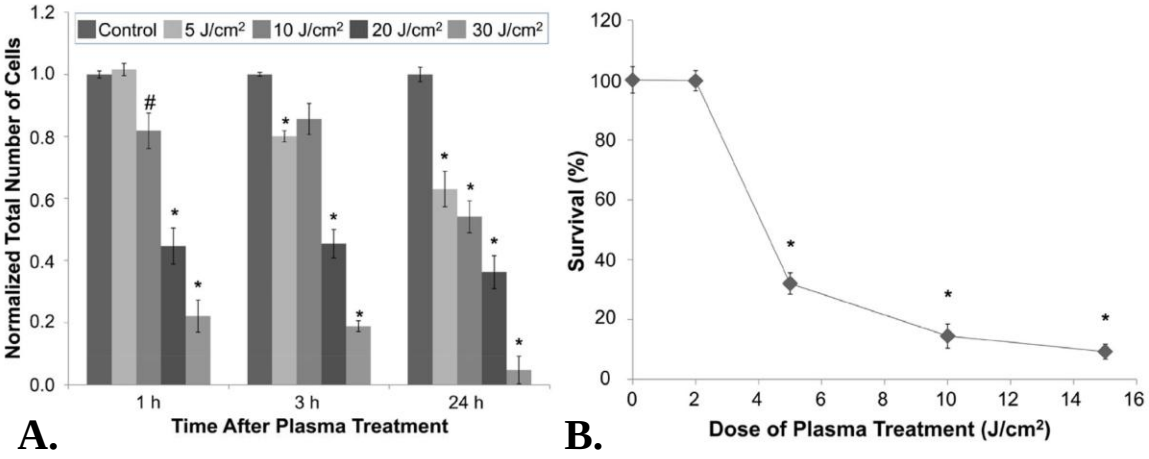

Figure 15. Dose dependent response of melanoma cells. (A) Data from triplicate samples (± S.E.M.) are expressed relative to total number of cells in untreated control. Trypan blue testing revealed that plasma treatment up to 10 seconds decreased the total number of cells 1 h, 3 h and 24 h after plasma treatment ($p < 0.001$). Also total number cells decreases linearly as the dose of plasma exposure increases from 5 J/cm^2 to 30 J/cm^2 ($p < 0.01$). # $p < 0.05$ as compared to untreated control * $p < 0.001$ as compared to untreated control (B) Cells were treated with the indicated dose of non-thermal plasma; one day after treatment, 300 cells were plated in a 6 cm dish and colonies were counted after 11 days. Data from triplicate samples (±S.E.M.) are expressed relative to the # of colonies in the untreated control. Survival of melanoma cells post plasma treatment decreases significantly as the dose of plasma exposure increases ($p < 0.001$). * $p < 0.001$ as compared to untreated control

4.3.2. Apoptosis

4.3.2.1. Annexin – V/PI

To confirm that non-thermal plasma was inducing apoptosis in melanoma cells, Annexin-V/PI staining was utilized. The cells were treated with non-thermal plasma at two different doses; viz, 5 J/cm^2 and 15 J/cm^2. Untreated cells were used as a negative control. Cells were treated with 4 mm Actinomycin D as a positive control. The cells were analyzed 3 h, 24 h, 48 h, and 72 h after treatment. As shown in Figure 16, Annexin-V/PI staining confirmed no significant increase in apoptosis early time points (3 h) after non-thermal plasma treatment, but a significant increase in apoptosis was observed in plasma-treated cells 24, 48, and 72 hours post-treatment ($p < 0.001$). As shown in Figure

16, 3 h after plasma treatment the number of total apoptotic cells in untreated cells was 8%, as compared with 12% and 10% after treatment with 5 J/cm^2 and 15 J/cm^2, respectively.

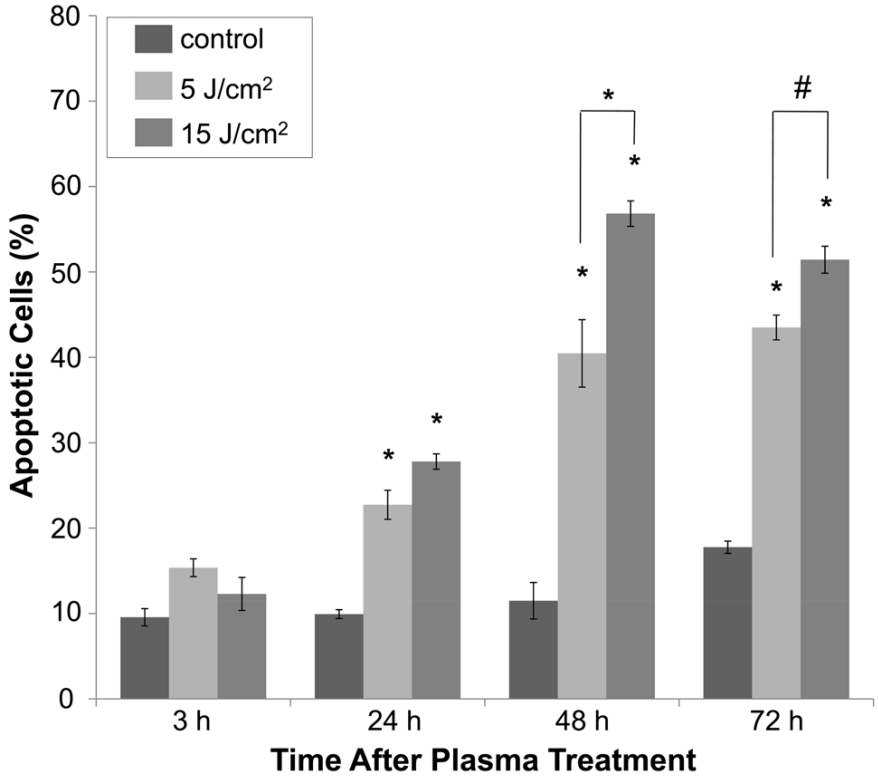

Figure 16. Annexin-V/Propidium Iodide Staining (A) Annexin staining confirmed no significant increase in apoptosis immediately after plasma treatment, but a significant increase in apoptosis in plasma-treated cells compared to untreated control cells 24, 48, and 72 hours post-treatment * $p < 0.001$. Cells treated at 15 J/cm^2 showed significantly more apoptosis as compared to cells treated at 5 J/cm^2 of plasma exposure at 48 and 72 h after plasma treatment # $p < 0.05$

Twenty four hours after treatment with 5 J/cm^2 and 15 J/cm^2, the amount of Annexin-V/PI positive cells increased to 22% and 27%, respectively, ($p < 0.001$) as compared to untreated cells, which had only 8% apoptotic cells. 48 h after plasma treatment total apoptotic cells in 5 J/cm^2 and 15 J/cm^2 increased to 40% and 57%, respectively ($p < 0.001$) as compared to untreated control which had only 10% apoptotic cells. Seventy two hours after plasma treatment, the total apoptotic cells were 42% and

50% respectively ($p < 0.001$) in cells treated with 5 J/cm^2 and 15 J/cm^2 as compared to 15% in untreated control. Thus, immediately following treatment with non-thermal plasma there was no significant increase in apoptotic cells. However, at later time points after treatment, non-thermal plasma induced significant apoptosis at both 5 J/cm^2 and 15 J/cm^2.

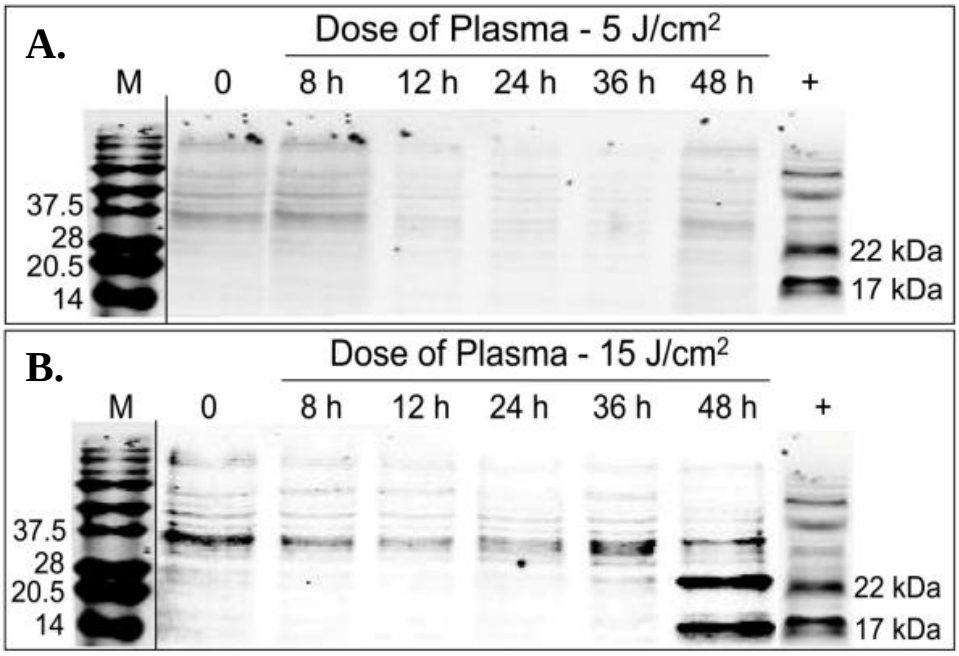

Figure 17. Caspase-3 cleavage assay. Cells were treated with non-thermal plasma at two different doses, 5 J/cm^2 and 15 J/cm^2. Caspase-3 cleavage was analyzed by immunoblot. Total cell lysates at 8 h, 12 h, 24 h, 36 h and 48 h after plasma treatment were prepared by direct lysis of floating and attached cells in 2X SDS sample buffer containing β-mercaptoethanol. Samples were electrophoresed at on 1.5 mm 15% polyacrylamide gels at 150 V in Tris-glycine SDS running buffer. Following electrophoresis, proteins were transferred to PVDF membrane for three hours in Tris-glycine transfer buffer. (A) No significant cleavage of caspase-3 was observed until 48 h after plasma treatment at a dose of 5 J/cm^2 while (B) we observed caspase-3 cleavage at 36 h after plasma treatment at a dose of 15 J/cm^2 and significant cleaved caspase at 48 hours.

4.3.2.2. Caspase – 3 Cleavage

To test whether non-thermal plasma treatment of mammalian cells indeed led to induction of apoptosis by initiating the caspase-3 mediated apoptosis, we determined

cleavage of caspase-3 post non-thermal plasma treatment by carrying out a western blot analysis of the cell lysates collected at various time points after plasma treatment. As shown in Figure 17A, no significant cleavage of caspase-3 is observed up to 48 h after plasma treatment at a dose of 5 J/cm^2 while as shown in Figure 17B, we see significant cleavage of caspase-3 48 h after plasma treatment at a dose of 15 J/cm^2. Thus non-thermal plasma induces cleavage of caspase-3 in melanoma cancer cells which may be related to the formation of intracellular ROS post non-thermal plasma treatment.

4.3.2.3. TUNEL® Assay

To determine whether the decreased cell viability at a dose of 5 J/cm^2 as assessed by trypan blue staining (Figure 15A) and colony survival (Figure 15B) was the result of apoptosis, terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assays were done in plasma treated melanoma cells. Cells treated for 5 s were then incubated and stained for DNA fractionation 24 h later. Following the TUNEL assay procedure it was observed that a significant percentage of these cells exhibit apoptotic behavior post non-thermal plasma treatment as is evident in Figure 18A while Figure 18B shows untreated cells. The number of TUNEL-positive cells (indicative of fragmented DNA) after treatment was significantly increased in cells treated with non-thermal plasma at a dose of 5 J/cm^2. The results of the flow cytometry tests performed 24, 48, and 72 h following treatment is presented as a representative in Figure 18C. As shown in Figure 18D, apoptosis develops 24 hours following treatment, where 25.5% of cells are present in the treated group, compared with 2.2% in the control group ($p < 0.001$). As time progresses, even more cells undergo apoptosis, further reaching 72.8% of apoptotic

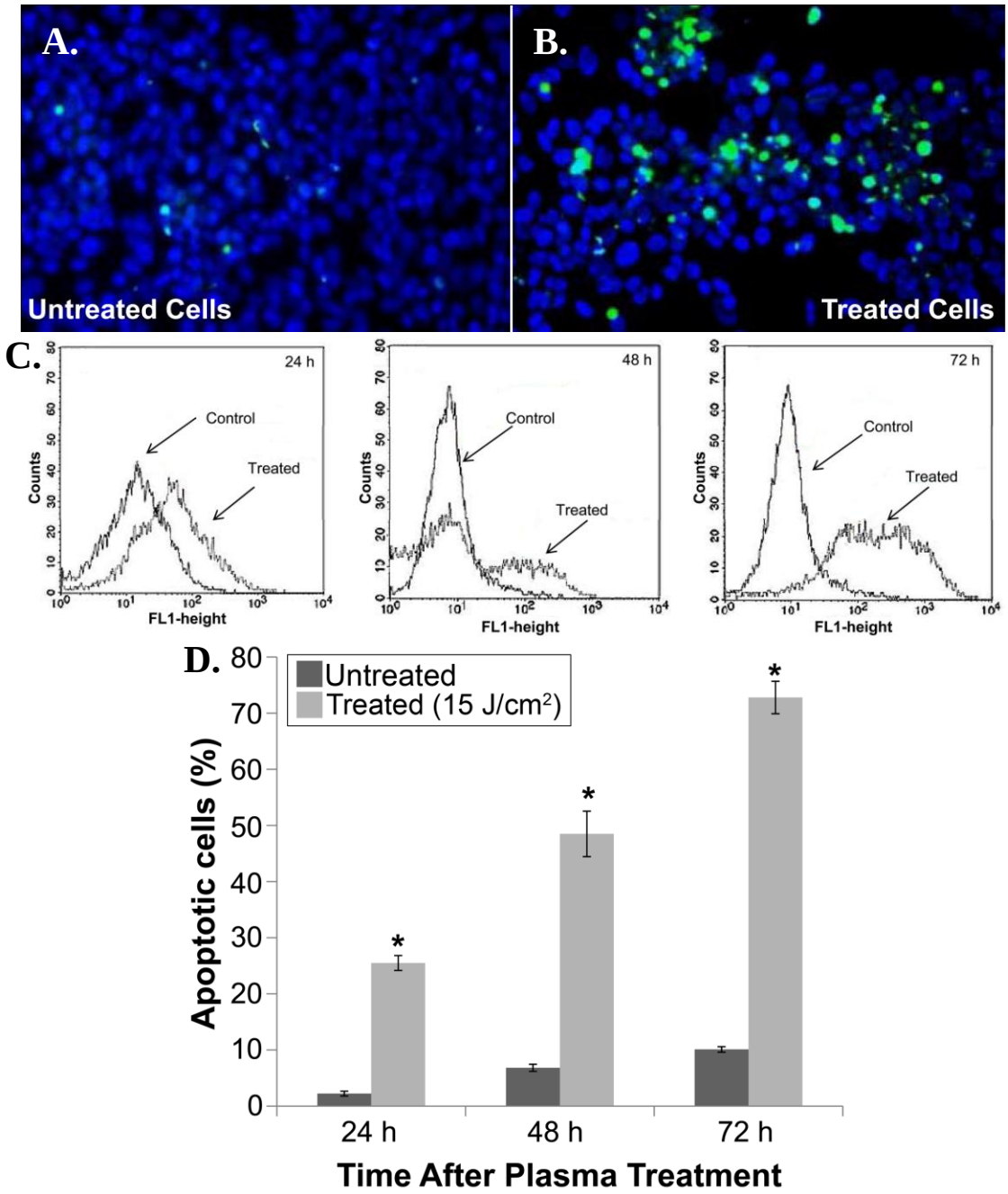

Figure 18. TUNEL® assay. Images of untreated (A) and treated (B) melanoma cells following TUNEL assay. All cells are stained blue (darker circles) and apoptotic cells are also stained green (bright spots). Assay performed 24 h following treatment with 5 J/cm^2. (C) TUNEL® analysis of cells treated at a dose of 5 J/cm^2 demonstrated a significant increase in apoptotic activity at 72 hours post plasma treatment. (D) Data from triplicate samples (± S.E.M.) analyzed via flow cytometry 24, 48 and 72 h after plasma treatment at a dose of 15 J/cm^2 show a significant increase in TUNEL positive cells as compared to untreated cells. (* $p < 0.001$) confirming late stage apoptotic behavior of melanoma cells.

cells in the treatment group vs. 3.2% in the control group 72 h following treatment ($p < 0.001$). This indicates that non-thermal plasma treatment induces apoptosis 24 h after plasma treatment and cells continue to die long after plasma treatment. TUNEL positive cells result from fragmentation of the nuclear chromatin, a hallmark of cells in the late stages of apoptosis.

4.3.3. Mechanisms of Induction of Apoptosis

4.3.3.1. Role of pH

It is known that non-thermal plasma treatment of cell culture medium leads to significant reduction of pH and this may be a cause for the observed cell death. As shown in Figure 19A, plasma treatment of cell culture medium at lead to drop in pH from 8.5 to 5.5 with increasing dose of plasma from 5 J/cm^2 to 30 J/cm^2. To test the role of change in pH post plasma treatment as a mediator of plasma induced cell death, plasma treatment of cells at a dose of 30 J/cm^2 was compared to incubation of melanoma cells in acidified medium (pH 5.4) for one minute. As shown in Figure 19B, the number of dead cells in plasma treated (30 J/cm^2) samples is significantly higher ($p < 0.001$) than those in untreated control or cells placed in acidified medium (pH 5.4) for 1 minute or untreated control. Change of pH of the medium after plasma treatment does not play a significant role in plasma induced apoptosis.

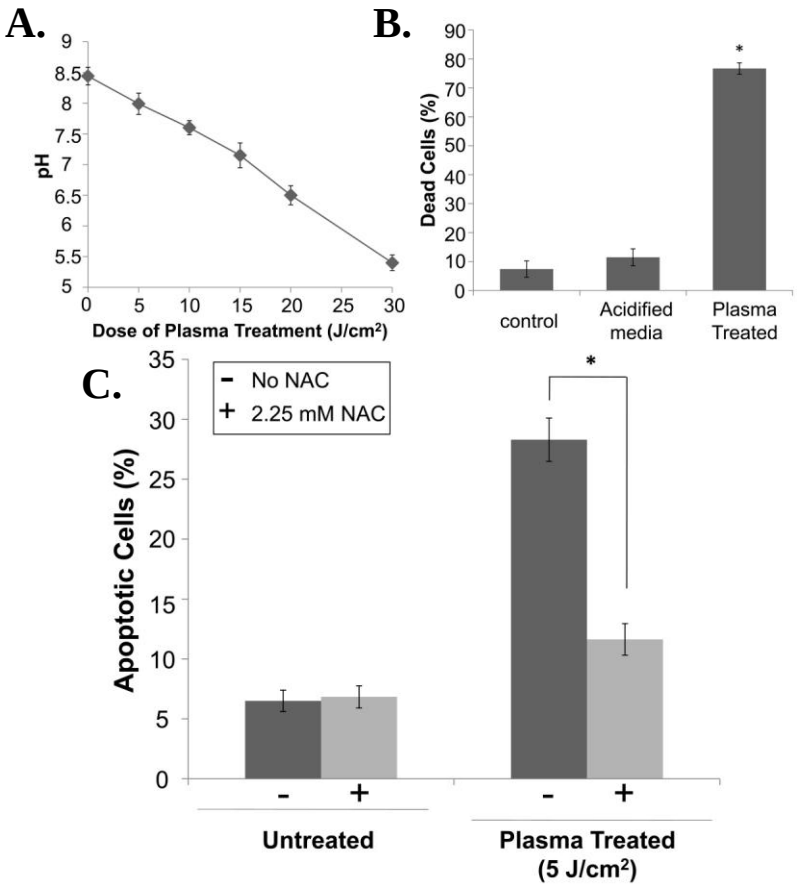

Figure 19. Mechanisms of non-thermal plasma induced apoptosis. (A) pH of the medium covering the cells during treatment drops from 8.5 to 5.5 as the dose of plasma exposure increases from 5 J/cm^2 to 30 J/cm^2 (B) Number of dead cells in plasma treated (30 J/cm^2) samples is significantly higher ($p < 0.001$) than those in untreated control or cells placed in acidified medium (pH 5.4) for 1 minute. Change of pH of the medium after plasma treatment does not play a significant role in plasma induced apoptosis. (C) Pretreatment with 2.25 mM N-acetylcysteine (NAC), a free radical scavenger, significantly decreased apoptosis in plasma-treated cells as analyzed by Annexin-V/PI staining 24 h after plasma treatment at 5 J/cm2 ($p < 0.001$) as compared to untreated or cells treated with N-acetyl cysteine alone.

4.3.3.2. Role of ROS

Non-thermal plasma is known to produce various active neutral short and long living ROS like (OH, O, electronically excited oxygen O (1D), O2 (1Δg)) and (O_3, HO_2, H_2O_2) in cell culture medium [363, 364]. To test the role of these ROS in non-thermal plasma induced apoptosis, we pre-incubated melanoma cells for 30 min in 2.25 mM N-

Acetyl cysteine (NAC), an intracellular scavenger of ROS, followed by treatment at a dose of 5 J/cm^2. Untreated cells and untreated cells with NAC alone were used as negative controls and cells treated at the same dose in the absence of NAC were used as positive control. As shown in Figure 19C, pre-treatment with 2.25 mM N-acetylcysteine (NAC), a free radical scavenger, significantly decreased the level of apoptosis induced in response to plasma-treatment by 50% as analyzed by Annexin-V/PI staining 24 h after plasma treatment ($p < 0.001$); plasma treatment incuced apoptosis in 28% of the cells, which was reduced to 12 % by pre-treatment with NAC, while NAC alone had no effect on the melanoma cells. These results indicate that ROS generated by non-thermal plasma are likely mediating non-thermal plasma induced apoptosis.

4.3.4. Discussion

The melanoma cell line (ATCC 2058) was chosen as our model because they are very rapidly proliferating and adherent. We did not aim to find a de novo treatment for skin melanoma, but rather investigate the influence of the non-thermal plasma on human cells. Initial experiments began by identifying the characteristics of a sub-lethal dose. This is the dose where we observed the ability of DBD plasma to induce apoptosis but not significant necrosis. We went on to follow the effects of this exposure on cell death over time. The cells were evaluated using standard approaches to measure apoptosis.

Non-thermal plasma was shown to induce apoptosis in melanoma cells at a dose of 5 and 15 J/cm^2 or higher. During apoptosis, cells undergo a series of complex biochemical changes leading to cell death without causing significant inflammation. That treated cells do not initially die but stop growth and die en masse 12–24 h following

treatment, while untreated cells continue to grow and proliferate, suggested that plasma treatment might induce apoptosis. Early apoptotic behavior was confirmed with Annexin-V/PI and Caspase-3 cleavage assays while late apoptotic behavior was confirmed through TUNEL staining with subsequent flow cytometry.

Previously we [12] and other groups [14, 23, 34, 50, 51, 365-369] have shown that plasma is able to destroy cells; however, it was also observed that plasma might be able to initiate or catalyze some biochemical processes in biological systems [12, 13, 38, 363, 370, 371]. Cell death after treatment of melanoma cells with non-thermal plasma occurs via the apoptotic pathway. The cell death mechanism post plasma treatment *in vivo* or *in vitro* is an important consideration. Apoptosis is a mode of programmed cell death initiated by physiological or pathological signals. Apoptotic cells are broken up into apoptotic bodies, which are engulfed by neighboring cells leading to a clean cell death without significant inflammatory response [201, 359, 360]. On the contrary, necrosis is a form of accidental cell death accompanied by cellular swelling, blebbing and increased membrane permeability leading to spilling of cytosolic content. This typically leads to inflammation in the surrounding tissue [201, 359, 360]. There is significant therapeutic advantage in cancer treatment to kill cells by apoptosis and avoid necrosis and the associated inflammation. By controlling plasma dose, it should be possible use plasma to induce apoptosis rather than necrosis. Although these results are promising, quite a few unanswered questions remain

By acidifying the media it was shown that the plasma treatment initiates this behavior in cells not through a pH change in the growth media or through interaction with the aluminum dishes, but through direct interaction with the cells. Pre-incubation of

melanoma cells with 4 mM N-acetyl cysteine significantly reduced the amount of apoptosis as compared to untreated control or cells without NAC. This indicates that intracellular ROS produced after plasma treatment are likely involved in plasma induced apoptosis. Plasma-induced apoptosis in melanoma cancer cells is likely related to neutral ROS. Here we have presented results indicating that adding N acetyl-cysteine reduces the apoptotic effect of plasma in our model. The source of the ROS is still unknown in this model, although interactions of the charged particles produced by plasma with atmospheric oxygen, water, lipid peroxides, carbohydrates and even amino acids are all possible sources in this system. Non-thermal plasmas produce long lived (O_3, NO, HO_2, H_2O_2) and short lived (OH, O, electronically excited O (1D), O2 (1Δg)) neutral particles and charged particles (ions and electrons). Both charged and neutral particles can lead to additional ROS production in the treated fluid [363, 364]. Direct effects of non-thermal plasma treatment could include local heating caused by plasma streamers or UV radiation. Non-thermal atmospheric pressure DBD plasma is able to produce a large concentration of ROS in the extracellular media during treatment [17]. However, it was unclear if these ROS go inside cells. Interestingly, the intracellular ROS scavenger NAC decreased induction of apoptosis in melanoma cells after plasma treatment. ROS produced by plasma extracellularly may move across the cell membrane through lipid peroxidation, active transport across the bilayer, transient opening of pores in the membrane, or activation of signaling pathways that modify ROS concentration inside cells.

Active species produced by non-thermal plasma may also modify the cell media, which in turn interacts with cells. Since many of these active species have a very short

life span, they may immediately interact with media components including amino acids and proteins, leading to production of long lived reactive organic hydroperoxides [233]. These long-lived hydroperoxides may induce lipid peroxidation and cell membrane damage, or they may bind to cell membrane receptors and activate intracellular signaling pathways leading to apoptosis. Various studies have shown that intracellular ROS can induce apoptosis in a caspase-3 dependent manner [372-375].

Ionizing radiation [376-378] and chemotherapy [379-381] are the most commonly used treatments for various types of malignancies, including melanoma, but both of the treatments have severe side effects since they not only kill the tumor but also damage healthy tissue surrounding the malignant tissue. Melanoma is the most aggressive form of skin cancer and is notoriously resistant to radiation and chemotherapy. Combination treatments utilizing immunotherapy and hyperthermia with radiation [382-386] or chemotherapy [387-391] have been somewhat more successful. Recently, pulsed electric fields have also been shown to be able to initiate apoptosis in melanoma cancer cell but pulsed electric fields need expensive equipment and skilled manpower [392]. Recently, photodynamic therapy (PDT), which combines a photosensitizing drug with a specific wavelength of light generated by lasers, is now being developed for treatment of melanoma and other skin cancers and has been shown to be somewhat successful [393, 394]. Non-thermal plasma differs from photodynamic therapy, ionizing radiation, chemotherapy and electromagnetic fields; chemotherapy has systemic side effects and electromagnetic fields are penetrating and therefore injure surrounding tissue. Ionizing radiation requires extensive and expensive equipment for administration. Various studies also suggest that melanoma cells frequently acquire radiation resistance [395] and

chemoresistance [396] by exploiting their intrinsic resistance to apoptosis and by reprogramming their proliferation and survival pathways during melanoma progression. Photodynamic therapy needs photosensitizers, which sensitize patients to light and the treatment itself can cause burns, swelling, pain, and scarring in nearby healthy tissue [394]. Non-thermal plasma on the other hand provides a novel and safer means to induce apoptosis since it is non-penetrating and therefore provides precise control of treatment area and depth and since it is cold, it causes no thermal damage to healthy tissue. Non-thermal plasma devices are also relatively inexpensive, small and simple to manufacture and operate. on-thermal plasma has the benefit that it can be applied directly to specific areas of interest potentially avoiding systemic side effects. Since this non-thermal plasma can be attached to the end of a probe it has the added benefit of potentially being able to target areas of the body that are technically difficult to reach such as the lungs.

Future work will endeavor to better delineate the pathways that are triggered by exposure to sub-lethal amounts of DBD plasma. Evaluation of key signaling components of the apoptotic pathway will be performed. The intrinsic and extrinsic pathway will be analyzed in order to determine how the non-thermal plasma induced effects lead to cell apoptosis. Molecular arrays and real time PCR can identify target pathways and proteins respectively that are intimately involved in the cellular response to Plasma exposure. A second avenue of research will focus on the actions of the various components of a Plasma discharge. Future investigations are warranted in order to better understand the interaction of biological tissues with non-thermal plasma exposure, with the ultimate goal of creating a non-thermal plasma discharge that would be selective for *in vivo* cancer cell killing while sparing the surrounding normal tissue.

From the results presented here, it is clear that plasma dose can be regulated and can be administered at doses that appear to induce apoptosis in cells to an equal or greater degree than a known apoptosis inducer.

4.4. Summary

To summarize, initiation of apoptosis is an important issue in cancer treatment as cancer cells frequently have acquired the ability to block apoptosis and thus are more resistant to chemotherapeutic drugs. Targeted and selective destruction of cancer cells is desirable for many reasons, ranging from the enhancement of or aid to current medical methods to problems currently lacking a solution, e.g., lung cancer. We demonstrated in this section the induction of apoptosis in a human melanoma cell line in vitro by exposure to non-thermal atmospheric pressure plasma. Non-thermal Plasma may provide a novel approach to treat malignancies via induction of apoptosis. The purpose of this study was to evaluate the apoptotic effects of non-thermal plasma on melanoma cells. Melanoma cells in-vitro were exposed to non-thermal plasma and evaluated by Trypan blue exclusion test, TUNEL® analysis, Annexin-PI staining and caspase-3 cleavage to determine viability and apoptotic activity. Trypan blue staining revealed that non-thermal plasma treatment significantly decreased the number of viable cells in a dose dependent manner. Annexin-V/PI staining revealed a significant increase in apoptosis at 24, 48, and 72 hours post-treatment. Caspase-3 cleavage was observed at 48 hours post plasma treatment at a dose of 15 J/cm^2. TUNEL® analysis of cells treated at 15 J/cm^2 demonstrated an increase in apoptosis at 48 and 72 hours post-treatment. Pre-treatment with N-acetyl cysteine (NAC), an intracellular free radical scavenger, significantly

decreased apoptosis in plasma-treated cells. Plasma treatment induces apoptosis in melanoma cells through a pathway that appears to be dependent on production of ROS (ROS) during treatment. ROS likely induce DNA damage resulting in the induction of apoptosis.

Chapter 5. Non-Thermal Plasma Treatment of Living Tissue – In Vivo Studies

In Chapter 3 we saw that low dose non-thermal plasma treatment of cells enhanced cell proliferation which would make plasma very attractive for applications related to wound healing and tissue regeneration. Further, in Chapter 4 we saw that higher doses of non-thermal plasma induce apoptosis in malignant melanoma cells *in vitro* without significant necrosis which would make plasma an interesting alternative to IR or it could be used in conjunction with other cancer treatment modalities. Before we start applying plasma directly on tissues and wounds we need to evaluate the toxicity of low dose non-thermal plasma treatment of intact skin and wounds *in vivo* and investigate the effects of plasma on and try to understand the mechanisms of interaction of plasma with cells in their native environment.

Non-thermal atmospheric pressure dielectric barrier discharge plasma applied directly to living tissues is now being widely considered for various clinical applications. One of the key questions that arise in this type of topical treatment is if the skin or tissue remains undamaged after non-thermal plasma treatment. The results from the previous rodent model provided strong evidence for the ability of non-thermal plasma to sterilize the surface of the tissue without any thermal damage to the tissue. It is well established that porcine (pig) skin closely resembles human skin; hence we evaluated the potential toxic effects of non-thermal plasma treatment on underlying skin cells and tissue on porcine skin. In a Yorkshire pig model, intact skin and wounded tissue treatment was carried out at varying doses of non-thermal plasma to determine a dosage regime where we observe damage to the skin or tissue and the resulting skin or tissue damage was

analyzed. In this dissertation we study the possible short term and long term toxic effects of the non-thermal plasma treatment on intact and wounded living tissue while identifying the boundaries of skin or wound toxicity after non-thermal electrical plasma treatment.

Moreover, in recent studies on plasma initiated blood coagulation [12, 13], skin sterilization [12, 28] and tissue toxicity after plasma treatment [40, 41], non-thermal plasma did not demonstrate gross toxicity in the surrounding living tissue. To test this claim vigorously we carried out non-survival and survival *in vivo* animal studies and in Chapter 5 we present results related to the *in vivo* studies aimed at investigating the toxicity of non-thermal plasma treatment of intact porcine skin and wounded porcine tissue.

5.1. Introduction

Over the past few years non-thermal atmospheric pressure plasma has emerged as a new promising tool in medicine. Non-thermal plasma has been widely used for sterilization of inert substrates [23-27] and is now being developed for treatment of inert substrates with the purpose of modulating cell attachment [397, 398]. Only recently has it been demonstrated that non-thermal atmospheric pressure plasma can be applied directly to living cells and tissues [29, 33, 34], killing bacteria without any gross histological changes in tissue [12] and inducing blood coagulation without any heating [12, 13]. It has also been shown that non-thermal plasma treatment can promote apoptosis in malignant cells [42] enhance cell transfection [30-32], enhance cell proliferation[36], sterilize root canals [399, 400], and possibly prove useful in wound healing [37-39]. Non-thermal

atmospheric pressure plasma is generated through direct contact with living tissue. Compared to the effects of the more conventional thermal plasma [5], non-thermal plasma can be selective in its treatment because of the ability to avoid burning healthy tissue [12]. In order for non-thermal plasma to be widely accepted by clinicians and doctors for various pathologies we have to prove that the dose of non-thermal plasma can be tuned to be able to operate it in a non-damaging regime while achieving beneficial effects. Hence, our goal in this section was to identify the boundaries of skin and wound toxicity after treatment of intact and wounded skin with non-thermal electrical plasma.

5.2. Materials and Methods

Non-Thermal Plasma Treatment

Non-thermal atmospheric pressure dielectric barrier discharge plasma was produced using an experimental setup similar to one previously described [12, 13] and the operating parameters have been previously elaborated in section 1.2 on page 4.

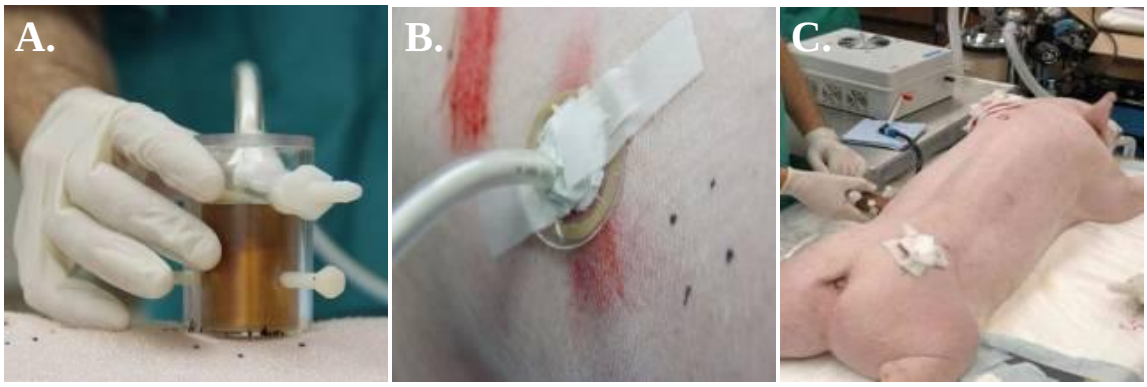

Figure 20. Non-Thermal Plasma Treatment of intact porcine skin and wounded porcine tissue. (A) Non-Thermal dielectric barrier discharge plasma treatment electrode held in a jacket which maintained the gap between the electrode and the surface of the intact skin. Setup shows the method used to treat intact skin. (B) Modified non-thermal dielectric barrier discharge plasma treatment planar electrode for treatment of wounded tissue. Setup shows the technique used to treat wounded skin. (C) The dorsum of the pig was separated into treatment areas for both intact and wounded skin.

The discharge gap between the bottom of the quartz and the treated skin or wound surface was fixed at 3 mm using a special electrode holder (Figure 20A) or a modified planar electrode (Figure 20B) respectively. Discharge power density was measured to be 0.13 W/cm^2 (at 500Hz) and 0.31 W/cm^2 (at 1.5 kHz) using both electrical characterization and a specially designed calorimetric system [18].

Study Design

We evaluated the potential toxic effects of the DBD plasma on both intact and wounded porcine skin in 7 Yorkshire pigs. Standard operative procedure included the following: The pig was anesthetized and the dorsum of the pig was marked and divided for treatment areas (Figure 20C). When intact skin was studied, the plasma electrode was placed 3 mm ± 1 mm above the skin by adjusting the holder, varying the power and time of treatment. When wounded skin was studied, a dermatome was used to create a skin abrasion, removing 1 square inch of the epidermis and dermis (approximately 1.5 mm ± 0.5 mm deep), followed by plasma treatment. In this case the planar plasma electrode was again placed 1.5 mm ± 1 mm above the skin by using spacers of the required height, varying the power and time of treatment. Pigs were then sacrificed immediately or 24 hours after surgical procedure. Tissue specimens from each treatment area were harvested and sent for histological analysis.

For the intact skin model (3 pigs), we had a total of 42 treatment areas on the three pigs that were harvested 24 hours after surgery. One area of intact skin (n=1) was treated with an electrocautery burn (positive control, Figure 21A, 17B and 17C), and one area of intact skin (n=1) was untreated (negative control). The remaining 40 areas were treated with 1 of 4 power settings: Highest power 0.31 Watt/cm^2 (12 specimens), 0.17

Watt/cm^2 (12 specimens), 0.15 Watt/cm^2 (12 specimens), and lowest power 0.13 Watt/cm^2 (4 specimens). For 0.31 Watt/cm^2 and 0.17 Watt/cm^2, there were 3 samples (n=3) treated with plasma for each of the following time points: 30 seconds, 1 minute, 2 minutes, and 3 minutes. For 0.15 Watt/cm^2, there were 3 samples (n=3) treated with plasma for each of the following time points: 1 minute, 2 minutes, 5 minutes, and 15 minutes. For 0.13 Watt/cm^2, there was 1 sample (n=1) treated with plasma for each of the following time points: 1 minute, 2 minutes, 5 minutes, and 15 minutes.

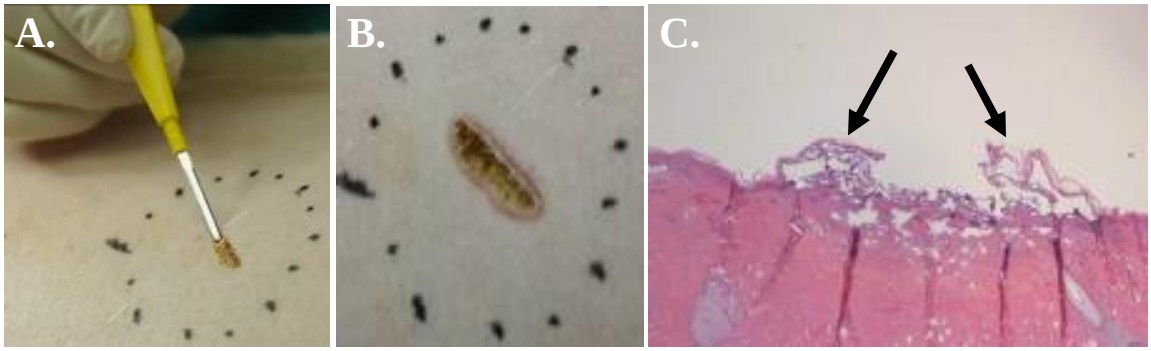

Figure 21. High frequency 'Bovie' electrocautery knife was used as a positive control. (A) doted circle indicates area of treatment. A half inch long burn was achieved with the 'Bovie' knife in the centre of the dotted circle. (B) Gross visual observation of the burn due to the 'Bovie' knife. (C) Microscopic histological observation of burnt tissue after treatment with the 'Bovie' knife. The arrow indicated disruption in the epidermis and we can observe collagen condensation indicative of severe burn.

For the wounded skin model (4 pigs), we had a total of 42 treatment areas on 2 pigs harvested immediately after surgery and 42 treatment areas on 2 pigs harvested 24 hours after surgery. In the non-survival pigs there were 21 specimens treated with low power (0.13 Watt/cm^2) and 21 specimens treated with high power (0.31 Watt/cm^2). In the 24 hour survival pigs, there were also 21 specimens treated with low power (0.13 Watt/cm^2) and 21 specimens treated with high power (0.31 Watt/cm^2). Each group of 21 specimens had a breakdown of 3 samples (n=3) treated for the following time points: no

treatment (negative control), electrocautery bovie (positive control), 30 seconds, 1 minute, 3 minutes, 5 minutes, and 15 minutes.

Histological Staining

In addition to recording all gross observations of the specimens, all specimens were analyzed with microscopic histological analysis. Specimens were longitudinally sectioned and fixed for 24 hours in formalin. Sections for histology were processed in a standard fashion and stained with hematoxylin-eosin. Our pathologists were blinded to all specimens and categorized each specimen into a burn grading system for the intact and wounded skin data analysis. For intact skin, as shown in Figure 22, the specimens were either classified as normal, minimal change, epidermal damage, or full burn through the dermis. For wounded skin, as shown in Figure 27, the specimens were either classified as normal, presence of a clot or scab, and full burn through the dermis.

Immunohistochemistry

Slides prepared in triplicates were used for immunohistochemical staining to analyze basement membrane integrity (laminin for immunostaining), and the demonstration of the DNA damage using γ-H2AX as a marker in our intact skin samples. Anti-laminin mouse monoclonal (Sigma Chemical Co, St. Louise, MI) or anti-γ-H2AX mouse monoclonal antibodies (Abcam, Cambridge, MA) were used. In brief, the slides were first warmed at 60^{o}C to melt the paraffin wax and then hydrated through a graded ethanol series. Heat-induced target retrieval was performed by heating the slides to 95^{o}C in a modified citrate buffer (Dako North America, Inc, Carpentaria, CA) to improve antibody binding. To block nonspecific binding sites, the tissue sections were incubated with heat-inactivated goat serum for 1 hour at room temperature. Slides were then

incubated with the primary antibody overnight at 4^{o}C. The next day, the slides were washed three times with PBS to remove excess primary antibodies and then incubated with a rhodamine-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for 2 hours at room temperature and protected from light. Three more washes with PBS were performed to remove excess secondary antibody, and then the slides were incubated for 3 minutes with a nuclear stain, Hoechst 33258 (0.05%) (Sigma). The slides were washed another 3 times with PBS and then mounted with Citifluor antifading mounting medium (Electron Microscopy Sciences, Fort Washington, PA) and examined for fluorescence microscopy using Leica DMRX fluorescence microscope with attached Leica DG300FX digital camera system, using appropriate band-pass filter (for corresponding blue and red dyes). The images were captured from 5 randomly selected areas for three different sets of experiments, saved as TIFF file and edited using Adobe Photoshop CS3.

5.3. Results

5.3.1. Intact Skin

The plasma device was tested on 3 pigs with intact skin at 4 different power settings, all harvested 24 hours after the procedure. Untreated skin appeared normal up on gross and histological observation. With minimal change, there was a small area of erythema on the skin. With epidermal damage, there was mild erythema that resolved itself usually within 20 minutes. With full burn through the dermis, there was diffuse erythema that remained until time of harvest (Figure 22 and Figure 23).

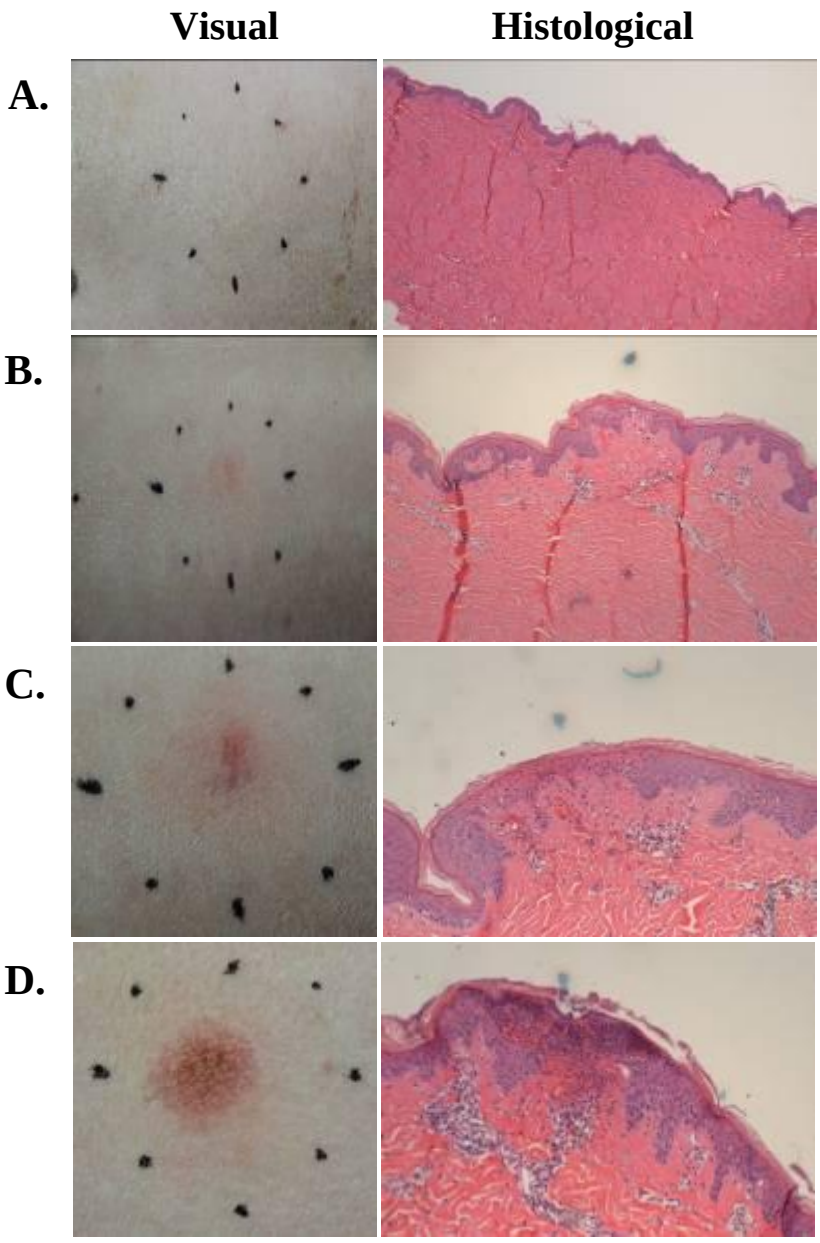

Figure 22. Gross and histological analysis of plasma treated intact skin. 3 and 24 h after plasma treatment, samples from treated and untreated skin were harvested and preserved in formalin until further analysis. H&E staining was used to evaluate the treated and untreated intact skin samples histologically. Representative histological classification of intact skin and associated gross (left column) and microscopic findings (right column). (A) Normal undamaged intact skin. (B) Minimal change and collagen condensation observed after low dose plasma treatment at 0.15 W/cm^2 up to 15 min. (C) Epidermal damage seen after moderate dose plasma treatment at 0.17 W/cm^2 up to 3 min and (D) Second degree burn observed after high dose plasma treatment at 0.31 W/cm^2 for 3 min.

At low power 0.13 Watt/cm^2 (4 specimens), 1 specimen treated with plasma for 1 minute was classified as normal skin. The remaining 3 specimens treated with plasma at 2, 5, and 15 minutes all showed epidermal damage on histology and mild erythema on gross examination (Table 5A).

At the next higher level of power 0.15 Watt/cm^2 (12 specimens), 3 specimens treated with plasma for 1 minute were classified as normal skin. In the samples treated for both 2 and 5 minutes, 2 samples showed epidermal damage and 1 with normal skin. In the 15 minute plasma treatment group, 1 sample showed epidermal damage and 2 with normal skin (Table 5B).

At power setting 0.17 Watt/cm^2 (12 specimens), 3 specimens treated with plasma for 30 seconds and 3 treated for 1 minute were classified as normal skin. In the samples treated for 2 minutes, 2 samples showed minimal change and 1 with normal skin. In the 3 minute plasma treatment group, 2 samples showed epidermal damage and 1 sample showed minimal change (Table 5C).

At high power 0.31 Watt/cm^2 (12 specimens), 3 specimens treated with plasma for 30 seconds were classified as normal skin. In the samples treated for 1 minute, 1 sample showed minimal change, 1 with epidermal damage and 1 with normal skin. In the 2 and 3 minute plasma treatment groups, 2 samples showed full thickness burn and 1 with epidermal damage each (see Table 5D).

For our controls, one specimen (n=1) served as our positive control, that of an electrocautery burn which yielded a full thickness burn. Another specimen (n=1) was intact skin without any plasma treatment, serving as our negative control.

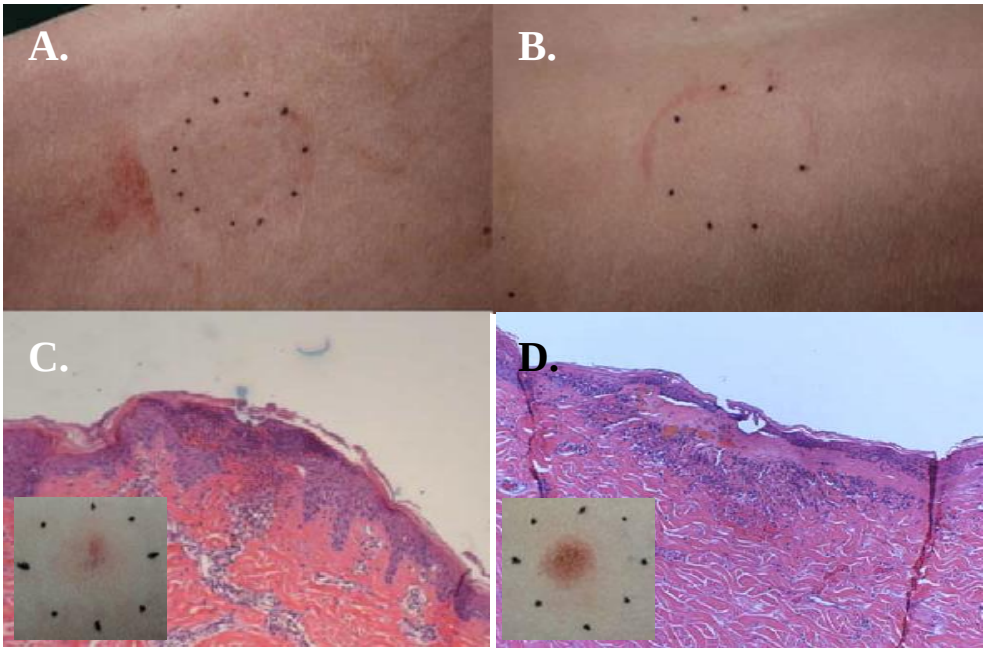

Figure 23. Summary of observations after plasma treatment of intact skin specimens (A) Intact skin before plasma treatment (B) Intact skin after plasma treatment at a dose of 0.13 W/cm^2 for 5 min (C) histological analysis of intact skin after plasma treatment at 0.15 W/cm^2 for 5 min (inset: gross observation of treated skin shows mild erythema which disappears after 20 min) and (D) histological analysis after plasma treatment at a dose of 0.31 W/cm^2 for 3 min (inset: gross observation of skin after plasma treatment shows a full thickness burn)

Table 5. Classification of results after non-thermal plasma treatment of intact skin at four different power settings.

(A) Low Power: 0.13 W/cm^2					
Time (min)	Normal	Minimal Change	Epidermal Damage	Full Burn	Total (n)
1	1				1
2			1		1
5			1		1
15			1		1
				4	
(B) 0.15 W/cm^2					
Time (min)	Normal	Minimal Change	Epidermal Damage	Full Burn	Total (n)
1	3				3
2	1		2		3
5	1		2		3
15	2		1		3
					12
(C) 0.17 W/cm^2					
Time (min)	Normal	Minimal Change	Epidermal Damage	Full Burn	Total (n)
0.5	3				3
1	3				3
2	1	2			3
3		1	2		3
					12
(D) High Power: 0.31 W/cm^2					
Time (min)	Normal	Minimal Change	Epidermal Damage	Full Burn	Total (n)
0.5	3				3
1	1	1	1		3
2			1	2	3
3			1	2	3
					12

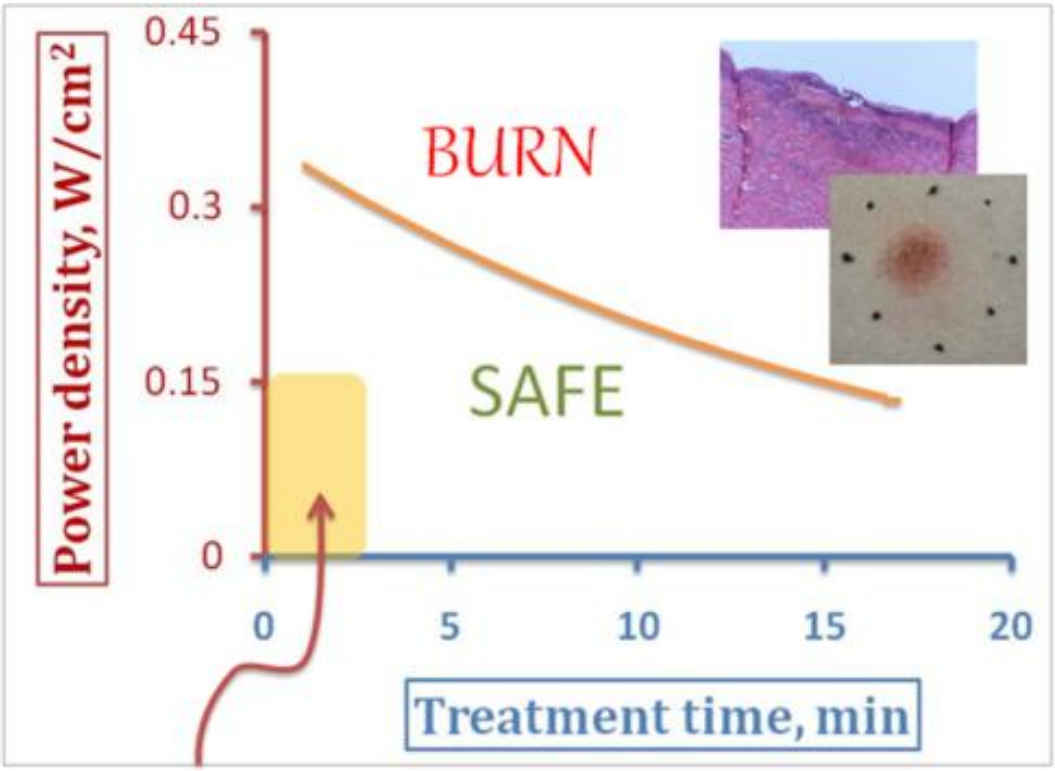

Figure 24. Schematic showing safe regimes of non-thermal plasma treatment of intact tissue. Plasma treatment at normal operating parameters does not cause tissue damage: Plasma doses up to 10 times longer than required for complete sterilization are safe. (Inset: We see a burn formation only after 10 min of continuous application of high power (> 0.31 W/cm^2) plasma dose).

In order to complement our gross and histological findings, we had performed immunohistochemical studies to looks at the architecture of laminin layers and nuclear damage in the form of DNA double strand breaks (DSB). This was performed for our intact skin samples. Integrity of laminin is essential for healthy state of skin tissues, and any breach or dissociation of the laminin layer indicates damage to skin epidermis and underlying structures. Figure 25 demonstrates laminin changes associated with non-thermal plasma treatment of intact skin. Normal skin showed integrity of the laminin layer (Figure 25A) whereas application of 'bovie' high frequency electrocautery caused burns, demonstrating classical disintegration of the laminin layer (Figure 25D), thus

serving as our controls. Upon comparison with controls, it is observed that non-thermal DBD plasma is safe to apply for duration of up to 5 minutes at low power (0.13 W/cm^2), after which slight laminin damage is observed (Figure 25B and 24C). These findings are consistent with H&E histological analysis done earlier.

To look at the change at the sub-cellular level, we carried out dual staining for nucleus using fluorescent dye Hoechst and antibody tagged with a fluorophore directed against γ-H2AX, a commonly used marker for determining DNA damage. During DNA damage, H2AX gets phosphorylated (at all 4 serine residues, and remarkably at serine 139) giving rise to γ-H2AX, which is considered as a marker for DNA damage, and is essential in DNA repair, acting as one of the signals for DNA repair initiation[401-403]. After plasma treatments, we analyzed the immunostained skin tissue sections for accumulation of γ-H2AX foci. Figure 26 shows the representative micrographs showing untreated control (Figure 26A) and positive control (Figure 26D) for DNA damage along with two plasma exposure time points. γ-H2AX foci formation was observed after 5 minutes of exposure to non-thermal plasma at low power (0.13 W/cm^2) (Figure 26C) and onwards. We did not see any DNA damage in lower plasma exposure-time points (Figure 26B)

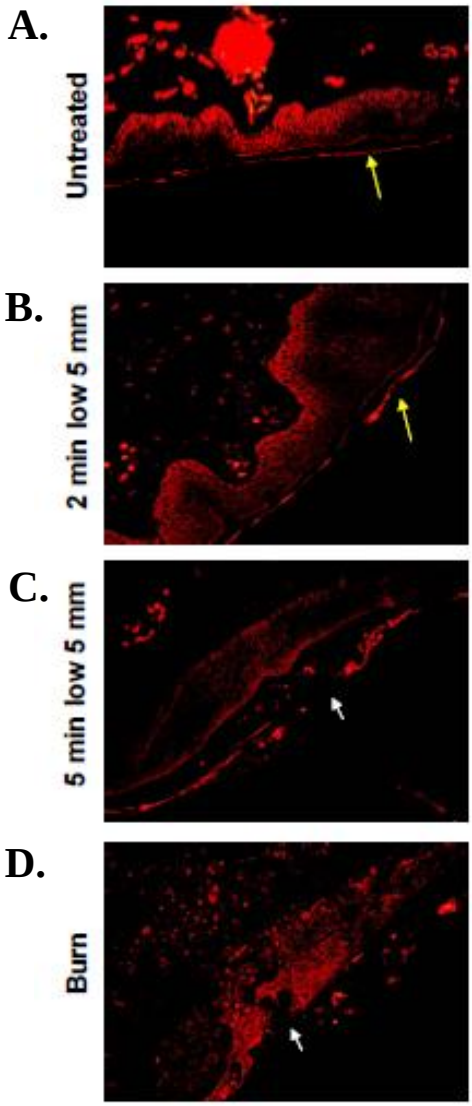

Figure 25. Laminin studies on intact skin samples. Untreated and plasma treated tissue samples were harvested 3 h and 24 h after surgery and snap frozen in liquid nitrogen and preserved until further analysis. Frozen samples were analyzed for presence of laminin using the procedure described in the methods section. (A) Normal skin showing integrity of laminin layer. (B) Laminin layer appears to be intact 2 min after exposure to non-thermal plasma at low dose (0.13 W/cm^2) (yellow arrows). (C) Damage to the laminin layer appears after 5 minutes of plasma treatment at low power (white arrows). (D) Classical disintegration of the laminin layer is observed due to burning of tissue after application of high frequency electrocautery device.

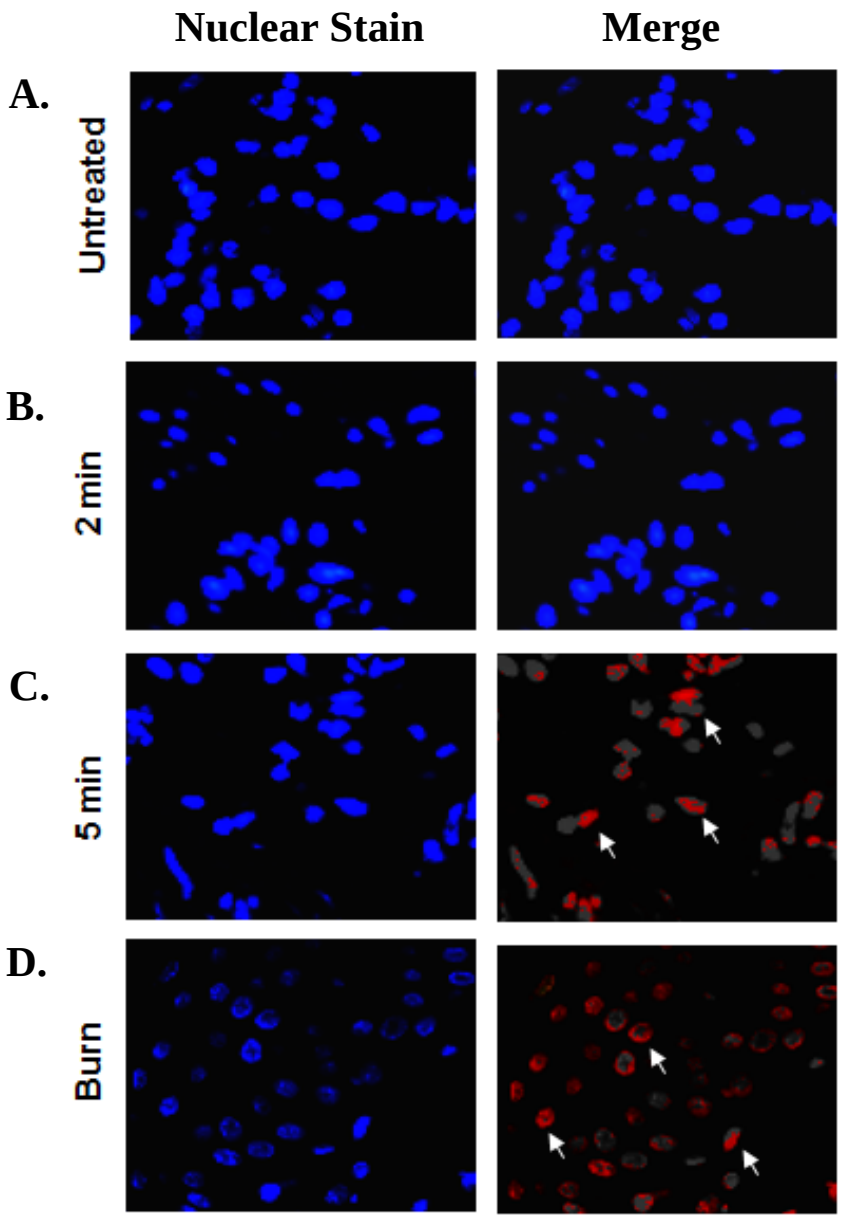

Figure 26. γ-H2AX studies on intact skin. Untreated and plasma treated intact skin samples were harvested 3 h and 24 h after surgery and snap frozen in liquid nitrogen and preserved until further analysis. Frozen samples were analyzed for γ-H2AX foci, which are commonly used markers for DNA damage, using the procedure described in the methods section. (A) Normal skin showing no DNA damage seen from the absence of red staining (γ-H2AX). (B) DNA appears to be intact 2 min after exposure to non-thermal plasma at low dose (0.13 W/cm^2) as seen by the absence of γ-H2AX staining. (C) γ-H2AX staining appears after 5 minutes of plasma treatment at low power indicating plasma induced DNA damage (white arrows). (D) uniform γ-H2AX staining indicating large scale DNA damage due to burning of tissue after application of high frequency electrocautery device.

5.3.2. Wounded Tissue

The plasma device was tested on 4 pigs with wounded skin with either low (0.15 Watt/cm^2) or high (0.31 Watt/cm^2) power settings. Two pigs were harvested immediately after the procedure, and two pigs were harvested 24 hours after the procedure. With normal histologic skin samples, the skin appeared normal grossly. With the presence of a

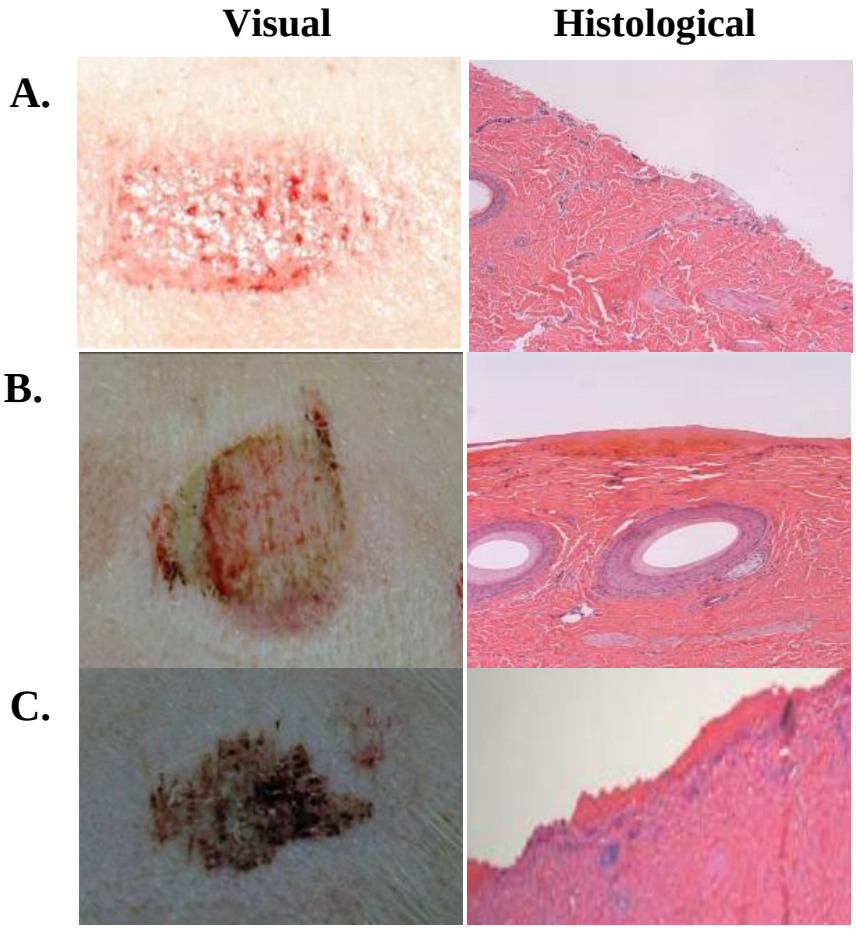

Figure 27. Gross and histological analysis of plasma treated wounds. 3 and 24 h after plasma treatment, samples from treated and untreated wound were taken and preserved in formalin until further analysis H&E staining was used to evaluate the treated and untreated wound samples histologically. Representative histological classification of wounded skin and associated gross (left column) and microscopic findings (right column). (A) Normal untreated wound. (B) Scab or Clot formation observed after low dose plasma treatment at 0.13 W/cm^2 up to 15 min and (C) Burns observed after high dose plasma treatment at 0.31 W/cm^2 up to 15 min.

clot seen on histology, the clot was visible on gross examination in pigs harvested after surgery. In pigs harvested at 24 hours, a scab was seen on gross examination instead of a

clot. With full burn through the dermis seen on histology, there was burned tissue seen grossly (see Figure 27 and Figure 28).

For the pig treated with low power 0.13 Watt/cm^2 (21 specimens) harvested immediately, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. 3 samples with 30 seconds treatment showed a clot on histology. With 1, 3, and 5 minute treatment groups, 2 showed a clot and 1 was normal in each group. With 15 minute treatment, all 3 samples showed a clot (see Table 6A).

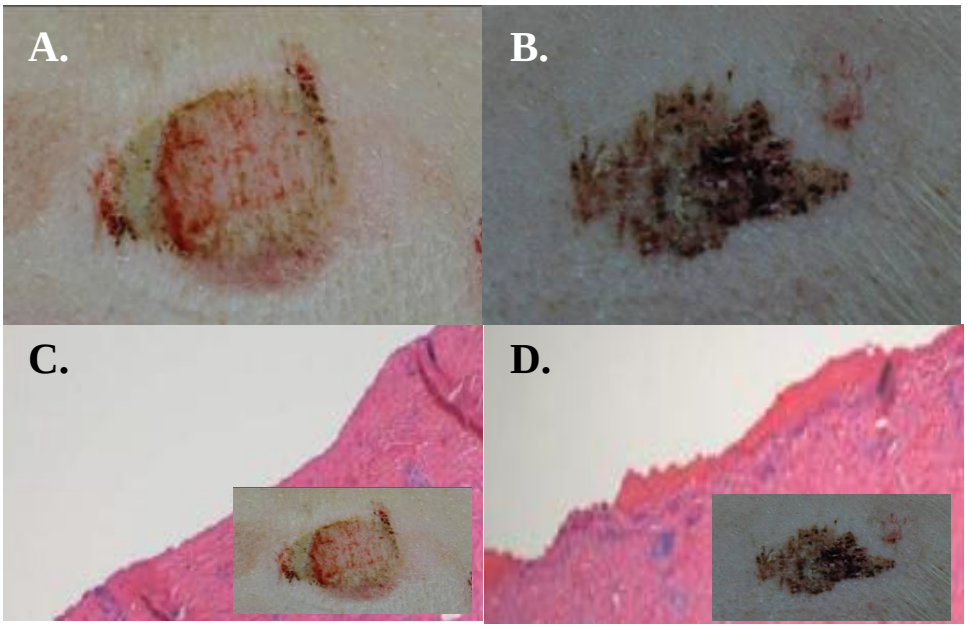

Figure 28. Summary of observations after plasma treatment of wounded tissue specimens (A) Wounded tissue after plasma treatment at a dose of 0.13 W/cm^2 for 1 min. (B) wounded tissue after plasma treatment at a dose of 0.31 W/cm^2 for 15 min. (C) histological analysis of wounded tissue after plasma treatment at 0.13 W/cm^2 for 1 min (inset: gross observation of wounded tissue shows normal wound without burn) and (D) histological analysis after plasma treatment at a dose of 0.31 W/cm^2 for 15 min (inset: gross observation of wounded tissue after plasma treatment shows scab formation due to blood coagulation).

For the pig treated with high power 0.31 Watt/cm^2 (21 specimens) harvested immediately, 3 samples of our untreated wounds (negative control) and 3 samples of

wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. With 30 seconds, 1 and 3 minute treatment groups, all three specimens showed a clot. With 5 and 15 minutes of plasma treatment, 2 showed a clot and 1 was normal in each group (see Table 6B).

Table 6. Classification of results of non-thermal plasma treatment of wounded skin at low and high power settings and harvested either immediately or 24 hours after plasma treatment.

(A) Low Power: 0.13 W/cm^2 (non-survival)				
Time (min)	Normal	Clot	Full Burn	Total
0.5		3		3
1	1	2		3
3	1	2		3
5	1	2		3
15		3		3
				15
(B) High Power: 0.31 W/cm^2 (non-survival)				
Time (min)	Normal	Clot	Full Burn	Total
0.5		3		3
1		3		3
3		3		3
5	1	2		3
15	1	2		3
				15
(C) Low Power: 0.13 W/cm^2 (24 h survival)				
Time (min)	Normal	Scab	Full Burn	Total
0.5	1	2		3
1		3		3
3		3		3
5		3		3
15	1	2		3
				15
(D) High Power: 0.31 W/cm^2 (24 h survival)				
Time (min)	Normal	Scab	Full Burn	Total
0.5	1	2		3
1	2		1	3
3	2		1	3
5			3	3
15		1	2	3
				15

For the pig treated with low power 0.13 Watt/cm^2 (21 specimens) harvested 24 hours after, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. With 30 seconds of treatment, 2 samples showed a burned scab and 1 with normal tissue. With 1, 3, and 5 minutes of treatment, all 3 samples in each group showed a burned scab. With 15 minutes of treatment, 2 showed a burned scab and 1 was normal (see Table 6C).

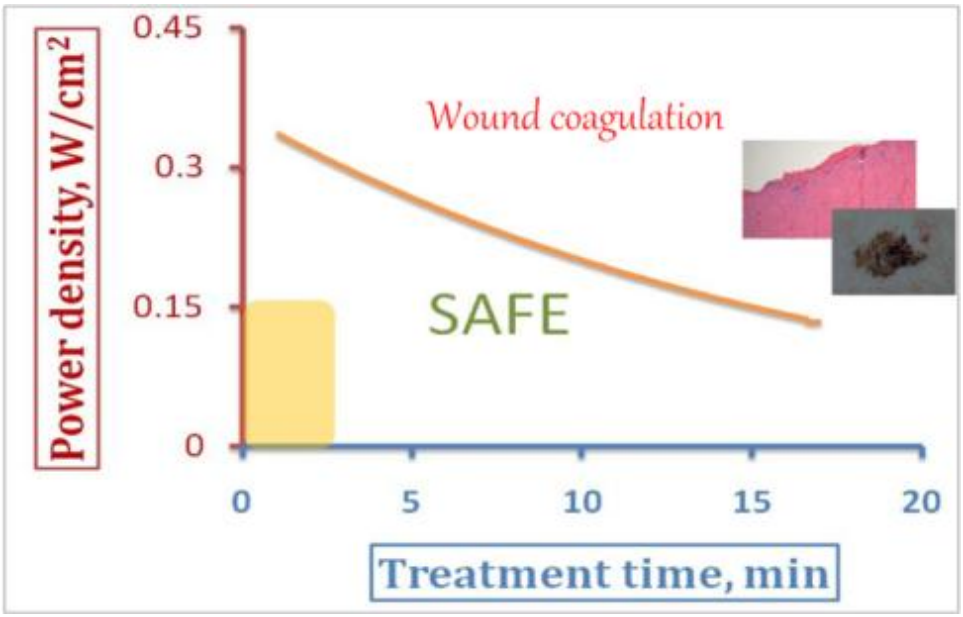

Figure 29. Schematic showing safe regimes of plasma treatment of wounded tissue. Plasma treatment at normal operating parameters does not cause tissue damage in wounds. Non-thermal Plasma starts coagulating an open wound after 3 min which protects the underlying wound from further damage by the plasma. (Inset: We see coagulated wounds at higher doses of 0.31 W/cm^2 or longer treatment times beyond 5 min).

For the pig treated with high power 0.31 Watt/cm^2 (21 specimens harvested 24 hours after, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. With 30 seconds of treatment, 2 samples showed a burned scab and 1 with normal tissue. With 1 and 3 minutes of treatment, 1 sample in each group

showed a burned scab and 2 with normal tissue. With 5 minutes of treatment all 3 samples showed a burned scab. With 15 minutes of treatment 2 samples showed a full thickness burn and 1 with a burned scab (see Table 6D).

5.4. Discussion

This animal study was the first *in vivo* study to assess the boundaries of non-thermal plasma toxicity on living tissue. From data of plasma treatment of intact skin, we can conclude that 2 minutes is the observed threshold for any signs of histological tissue damage created from non-thermal plasma treatment at all power settings except for high power (0.17 W/cm^2) treatment. When high power plasma was used, signs of minimal change, epidermal damage and full thickness burn were all seen beginning as early as 1 minute. The pathologists defined "minimal change" as the presence of epidermal and dermal cellular changes in both size of the cell and nucleus. Although almost appearing similar to normal skin, it was determined that due to the presence of some microscopic changes that an additional grading of "minimal change" be provided. With epidermal damage, there was notable increased vascular congestion and cellular disorganization. We determined a safe operating regimen for plasma treatment of intact skin. As shown in Figure 24, plasma treatment at normal operating parameters does not cause tissue damage. Plasma doses up to ten times longer than required for complete sterilization do not induce burn formation and is safe for application to intact skin.

From data of plasma treatment of wounded skin, we can conclude that the presence of a wound induced clot formation on all wounds which was protective to the underlying skin from plasma treatment damage. In non-survival pigs, a clot was seen on

the wounded skin, and in 24 hour survival pigs, a scab was seen on the wounded skin. All time points (30 seconds, 1, 3, 5, and 15 minutes) showed some type of clot or scab formation without disruption and/or damage to the underlying skin except for only one subgroup: high power treatment of wounded skin for 15 minutes (2 specimens showed full thickness burn). It has been reported in the literature that plasma treatment of tissue may sterilize tissue with as little as 30 seconds of treatment [12, 28]. Since tissue coagulation is a property of non-thermal plasma [12, 13], we can see that the ability to sterilize wounded tissue while stopping bleeding and inducing the formation of a protective clot may be of a clinical benefit in the field of medicine. We also determined a safe operating regime for plasma treatment of wounded tissue. As shown in Figure 29, plasma treatment at normal operating parameters does not cause tissue damage in wounds. Non-thermal Plasma starts coagulating an open wound after 3 min which protects the underlying wound from further damage by the plasma.

Continuous plasma application for five minutes at low power causes changes in skin tissue such as the dissociation of the laminin layer and changes in nuclear DNA (double stranded breaks) in our intact skin specimens. In harvesting skin tissue 24 hrs after plasma treatments, it is unlikely that tissues would have an adequate opportunity to undergo complete normal cellular repair. Under normal physiological conditions, injuries to cells and tissues occur every day in the body, but the body's repair and defense mechanisms takes time to heal completely. Our findings indicate that plasma exposure time of less than 5 minutes at low power in intact skin is safe, and does not cause any acute toxicity in skin tissues and no significant loss of architecture noted at lower time points. We have looked at both laminin and γ-H2AX in intact skin and studies looking at

the relationship between gamma-H2AX and laminin with wounded tissue are being currently being conducted.

5.5. Summary

Non-thermal plasma has only recently been applied within the field of medicine. It has been shown to induce apoptosis in malignant tissue and provide a means for tissue coagulation and sterilization while limiting damage to healthy tissue both grossly and microscopically. From our pig studies, we have shown that non-thermal plasma treatment of tissue can safely be applied to intact skin for up to two minutes at a dose of 0.31 W/cm^2 without inducing any gross or microscopic tissue damage. When applied to wounded skin, its coagulative properties helps induce a clot which acts as a protective layer preventing any underlying tissue damage for at least five minutes at a dose of 0.31 W/cm^2 and possibly as high as fifteen minutes at a dose of 0.13 W/cm^2. Once application of non-thermal plasma can be safely shown to prevent and/or limit toxicity in the animal model, its use may be studied and applied in humans for the sterilization of skin, treatment of various diseases including treatment of skin cancer and for wound healing.

Chapter 6. Mechanisms of Interaction of Non-Thermal Plasma with Living Cells

In this dissertation we have shown using mammalian cells in culture that non-thermal DBD plasma has dose-dependent effects that range from increasing cell proliferation at low doses as shown in Chapter 3 to inducing apoptosis at high doses as described in Chapter 4. In Chapter 5, we showed that non-thermal plasma is relatively non-toxic to intact skin at low doses and to wounded tissue at both low and high doses. In order to develop non-thermal plasma as a clinical tool it is important that we understand the mechanisms of interaction of non-thermal plasma with living cells and tissues. In Chapter 6 we try to investigate the mechanisms of interaction of non-thermal plasma with living cells.

6.1. Introduction

Devices, such as argon plasma coagulators, which are used clinically to cauterize living tissues, typically generate plasmas at temperature far exceeding room temperature. The effects of such hot plasmas on tissues are non-selective and difficult to control because they occur primarily through transfer of intense heat [5]. In contrast, in non-thermal plasmas gas can be maintained close to room temperature. Although electrical discharges that generate non-thermal plasma have been known for a long time, their clinical potential has been largely ignored, and applications have been confined to inert surfaces [12, 14, 20, 23-26, 50-52] for sterilization or to modulate cell attachment [29, 33]. Only recently has it been demonstrated that non-thermal atmospheric pressure plasma can be applied directly to living cells and tissues [29, 33], killing bacteria and

inducing blood coagulation without significant heating [12, 13]. It has also been shown that non-thermal plasma treatment can enhance cell transfection [404, 405], sterilize root canals [49, 399, 400] and possibly increase wound healing [406]. The simplicity and flexibility of devices required to generate non-thermal plasma and apply it to tissues is particularly appealing. However, there is still a substantial lack of understanding of mechanisms by which non-thermal plasma may interact with living cells and tissues, required to fully develop its clinical applications.

Prior studies related to mechanisms of interactions focused mainly on killing of bacteria [28]. It has also been shown that bactericidal effects require the presence of oxygen in plasma [26, 407], suggesting that oxidative stress may be mediating the interaction between non-thermal plasma and living organisms. Although it has been suggested that non-thermal plasma influences living systems through oxidative stress (among other factors) [14, 33, 52], to date there are no data to support this hypothesis. It was believed that, since non-thermal plasma does not produce sufficiently energetic particles or photons, cellular genetic material would not be affected.

Non-thermal DBD plasma produces various ROS in gas phase. A typical list of such ROS and their relative concentrations are provided in supplemental data (Table 2) [21, 22, 408]. The dependence of ROS concentration on plasma power density can be rather complex. Description of DBD plasma characterization employed in this work has been presented elsewhere [12, 18]. Measurement of ozone concentration at typical operating conditions and power level of 0.1 W/cm^2 yielded several hundred ppm, while nitric oxide (NO) concentration was measured to be below 1ppm (see methods section). Such measurements are entirely consistent with other published reports [14, 52, 409].

Several different methods of non-thermal plasma generation at atmospheric pressure are known [22]. The type of non-thermal plasma discharge employed in this study is called Dielectric Barrier Discharge (DBD), which was invented by Siemens in 1859 [15]. DBD occurs at atmospheric pressure in air or other gases when high voltage of sinusoidal waveform of microsecond or nanosecond duration pulses are applied between two electrodes, with at least one electrode being insulated [16]. The insulator prevents current build-up between the electrodes, creating electrically safe plasma without substantial gas heating. This approach allows direct treatment of biological specimens without the thermal damage observed in more conventional thermal plasma [5]. The human body, with its high capacity for charge storage as a result of its high water content and a relatively high dielectric constant can function as the second electrode [410, 411].

It is demonstrated here that non-thermal plasma induces a variety of effects on mammalian cells ranging from increased cell proliferation to apoptosis. Moreover, it can cause DNA damage. The DNA damage cascade activated by non-thermal plasma treatment is shown to result from oxidative stress and is different from that associated with IR or H_2O_2 [37].

6.2. Materials and Methods

Cell Culture

Mammalian Breast Epithelial Cells (MCF10A) were maintained in high glucose Dulbecco's Modified Eagle's Medium/Ham's F12 50:50 mixture (Cellgro, Mediatech, VA, USA) supplemented with 5% horse serum (Sigma Aldrich, St. Louis, MO, USA),

Epidermal Growth Factor (EGF, 100 µg/ml, Sigma Aldrich, St. Louis, MO, USA), Hydrocortisone (1 mg/ml, Sigma Aldrich, St. Louis, MO, USA), Cholera Toxin (1 mg/ml, Sigma Aldrich, St. Louis, MO, USA), Insulin (10 mg/ml, Sigma Aldrich, St. Louis, MO, USA) and Penicillin/Streptomycin (500 µl, 10000 U/ml penicillin and 10 mg/ml streptomycin, Sigma Aldrich, St. Louis, MO, USA). For plasma treatment, cells were washed with phosphate buffered saline (PBS), detached with 0.25% Trypsin (GIBCO, Invitrogen, CA, USA), and seeded near confluence (4×10^5 cells/well) on 22 x 22 mm square glass cover slips (VWR, PA, USA) in 6-well plates (Greiner Bio One, NC, USA). Cells were cultured for 24 hours prior to plasma treatment in 2.0 ml supplemented media in a 37°C, 5% CO_2 incubator to allow full attachment and spreading.

Amino acids; serine, methionine, cysteine, arginine, leucine, lysine, isoleucine, valine, proline, glutamic acid and glutamine, (100 µM, Sigma-Aldrich, St Louis, MO) were used to treat cells directly and separately to determine their role in inducing DNA damage after plasma treatment.

N-Acetyl-L-cysteine (NAC, 4 mM, Sigma-Aldrich, St Louis, MO), an intracellular ROS (ROS) scavenger was used as a scavenger for the ROS produced by non-thermal plasma treatment.

Non-Thermal Plasma Treatment

MCF10A cells on glass cover slips were exposed to non-thermal plasma at various doses (0.13 - 7.8 J/cm^2). Briefly, each cover slip was removed from the 6-well plate, drained, and placed on a microscope slide, which was then positioned on the grounded base of the plasma device. 100 µl of complete media was added to the glass cover slip before plasma treatment to prevent sample drying. Following plasma

treatment, the cells were held in the treated medium for one minute and then the cover slip was placed in a new 6-well plate containing 2 ml of supplemented media was added to the well, and the samples were returned to the incubator for one hour before analysis by immunofluorescence or western blot.

The plasma treatment dose in J/cm^2 was calculated by multiplying the plasma discharge power density by the plasma treatment duration. For example, plasma treatment of cells at a power density of 0.13 W/cm^2 for 15 s would correspond to a dose of 1.95 J/cm^2. The non-thermal DBD plasma has a g-factor (number of ROS generated per electron volt or eV) of between 0.3 and 0.5 [19]. For a plasma dose of 3.9 $Joules/cm^2$, $3.66x10^{16}$ – 6.1 $x10^{16}$ ROS are generated. Concentration of ozone produced by DBD plasma in the gas phase was measured using a calibrated optical ozone meter MedOzon-245/5 (MedOzone, Russia) and the concentration of NO was measured using an NO/NOx chemiluminescent analyzer Model 600 CLD (California Analytical Instruments, USA).

Immunofluorescence

MCF10A cells were plated onto glass cover slips 24 h before treatment. One hour after plasma treatment, cells were subjected to *in situ* cell fractionation as described [412], by incubation in pre-extraction buffer (1X PBS + 0.2% Triton-X + 1:50 PMSF) for 5 min at 4C, followed by one wash with PBS and incubation in fixation solution (3% paraformaldehyde + 2% Sucrose in PBS) for 10 min at room temperature. Cells were then washed in PBS, and incubated in permeabilization buffer (1X PBS + 0.5% Triton-X) for 5 min a 4C. Cells were washed twice with NaN_3 + PBST at room temperature and incubated overnight at 4C in primary antibody (mouse monoclonal γ-H2AX (serine 139, Upstate Biotechnology, 1:1000). After three washes in NaN3 + PBS, cells were incubated

for 1 h in the dark in secondary antibody (AlexaFlour594 donkey anti-mouse antibody, diluted 1:1,000). The secondary antibody solution was removed followed by incubation of slides in 1 μl DAPI + PBST + NaN_3 , three washes in NaN_3 + PBST and mounting using DAPI-free mounting media (Vector Labs) on glass microscope slides overnight. The slides were then frozen at -20 C for one day prior to imaging them on an upright fluorescence enabled microscope.

Western Blot

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by direct lysis of washed cells in 2X SDS sample buffer containing β-mercaptoethanol. Samples were electrophoresed at 150 V in Tris-glycine SDS running buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (pH 8.3)). Following electrophoresis, proteins were transferred to PVDF (Millipore, MA, USA) membrane for two hours in Tris-glycine transfer buffer (10% SDS, Deionized Water, Tris-Glycine and Methanol (VWR, PA USA)). Immunoblotting was done by blocking membranes in 1% nonfat dried milk (Carnation) in PBS with 0.1% Tween 20 (PBST) for α-tubulin or 5% bovine serum albumin (BSA, Fraction V, Fisher Scientific) in PBST for γ-H2AX followed by incubation with primary antibodies overnight for 10 to 12 h at 4ºC with rocking. Primary antibodies used for immunoblot included mouse monoclonal antibodies specific for γ-H2AX [phospho-histone H2AX (serine 139), clone JBW301; Upstate] and α-tubulin (Santa Cruz Biotechnology). The primary antibodies were detected with fluorescently tagged goat anti-mouse Alexa and Fluor 488 (Santa Cruz Biotechnology). Immunoblot was developed using Odyssey Infrared Gel Imaging system (LI-COR Biosciences, NE, USA).

Detection of Intracellular ROS

The intracellular generation of reactive oxygen species after plasma treatment was detected using the commonly used fluorescent probe 5-(and-6)-chlroromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes). Untreated cells, cells treated with 100 μM H_2O_2 and plasma treated cells were analyzed for changes in fluorescence. MCF10A cells (4×10^6) were plated on glass cover slips one day before plasma treatment. On the day of the experiment the cells were washed 2X with PBS, and then preincubated with 10 μM CM-H2DCFDA at 37°C for 30 min in the dark. After 30 min, the excess dye was washed off with PBS and cells were allowed recovery time of 30 min in full growth medium at 37°C in the dark. During the recovery time, the acetate groups on CM-H2DCFDA were removed by intracellular esterases, trapping the probe inside the cells. Production of ROS was measured by changes in the fluorescence of dichlorofluorescein (DCF) 5 min, 30 min and 1 h after plasma treatment using an upright fluorescence enabled microscope.

Cell Growth Assay

MCF10A cell proliferation was measured through cell counts on directly treated cells. 10,000 MCF10A cells were seeded on 22 x 22 mm square cover slips in 6-well plates one day before plasma treatment. Cells were plasma treated as described at various doses of plasma and incubated for an additional 3 days with a media change on day 2. Cell number was quantified on days 1 and 3 by counting Trypsin-detached cells using a Cell Viability Assay (Guava EasyCyte Plus, Millipore, MA, USA). Fold growth was determined by taking the ratio of the number of attached cells on day three to day one.

Colony Survival Assay

4 x 10^5 MCF10A cells were seeded on 22 x 22 mm square cover slips in 6-well plates one day before plasma treatment. One day after treatment with DBD plasma or H_2O_2 (positive control), 300 cells were seeded onto 60-mm dishes. Eleven days after plating, cells were fixed and stained with a crystal violet solution (0.5% in 20% ethanol) and colonies were counted. Assays were done in triplicate.

Apoptosis

Apoptosis was measured via Annexin V-propidium iodide labeling. Annexin V binds phosphatidylserine translocated from the inner to the outer cell membrane. Cells in early apoptosis are identified as Annexin V-positive and negative for the vital dye propidium iodide. Floating and trypsin-released cells were collected and centrifuged, washed thoroughly, resuspended in Annexin binding buffer, and labeled with Annexin V-fluorescein and propidium iodide as per manufacturer instructions (BD Pharmingen, San Jose, CA). Samples were analyzed immediately by flow cytometry (Guava EasyCyte Plus, Millipore, MA, USA).

Lentivirus Production and Cell Transduction

Lentivirus was prepared from MISSION shRNA (Sigma Aldrich, St Louis, MO, USA) following the manufacturer's instructions for targeting ATM (NM_000051.2-9380; NM_000051.2-2990) and ATR (NM_001184.2-231) and pLKO-Non-targeting (shC002), pLKO-GFP (shC104) were used as controls. The shRNA plasmids were then transfected into 293T cells with VSVG, RRE and RSV-Rev packaging vectors to generate corresponding pseudoviruses. Virus was collected 48 h post transfection. For stable

knockdown, 72 h after transduction, MCF10A cells were selected in 1 μg/ml puromycin for 48 h.

pH and Ca^{2+} Measurement

Changes in pH and Ca^{2+} concentration were determined immediately after plasma treatment. pH of the sample was measured using a pH meter (Lazar Research Labs 6230n pH/mV/Temp meter) and pH micro-electrode (Lazar Labs PHR146XS). Ca^{2+} concentration was measured after the treatment using the above meter with a micro-ion selective electrode (Lazar Research Labs LIS-146CACM) and micro reference electrode (Lazar Research Labs LIS 146DJM). The mV values obtained were converted to molar values using software (Arrow Lab Systems™).

SEM Analysis of Blood Samples

Morphological evaluation of the clot layer, formed on the surface of blood post e-plasma treatment was conducted at the Drexel Material Characterization Facility. The blood sample was treated for 30 s by e-plasma using the setup illustrated in Fig. 1. After the treatment, the clot layer was gently transferred onto a silicon wafer and immediately fixed overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 [413, 414]. The clot layer was then washed four times in distilled water and dehydrated in a graded series of increasing ethanol concentration (30-100%) over two hours. The specimens were mounted, sputter coated with platinum palladium in a SEM coating unit for 15 s, and examined in a Philips XL30 Field Emission Environmental Scanning Electron Microscope.

Dynamic Light Scattering

Dynamic Light Scattering (DLS) was used to detect aggregation of protein solutions (fibrinogen and albumin) due to non-thermal plasma treatment. DLS measured particle size distributions in fibrinogen and albumin solutions before and after e-plasma treatment were obtained using Malvern Instruments, Zetasizer NanoZS. The Zetasizer uses a 633 nm He-Ne laser to measure intensity of the light scattered due to the proteins and protein aggregates present in the solution.

Lipid Peroxidation

Bromotrichloromethane (BrCCl3, Sigma-Aldrich, St. Louis, MO, USA) was used as a known inducer of lipid peroxidation. N-acetyl-cysteine (Sigma-Aldrich, St. Louis, MO, USA), was used as an intracellular scavenger of reactive oxygen species and Diphenyl-phenyl-enediamine (DPPD, Sigma-Aldrich, St. Louis, MO, USA), a lipophilic synthetic alternative to Vitamin E was used as a lipid peroxidation inhibitor.

Malondialdehyde-thiobarbituric acid (MDA-TBA) levels were used as a measure of lipid peroxidation after non-thermal plasma treatment of mammalian cells. Cells were treated either directly or separately for 15 s at a dose of 1.95 J/cm2. Cells were held after plasma treatment for either 1 min or 10 min before adding butylated hydroxtoluene (BHT). The cells were scrapped with a rubber policeman and homogenized at 4°C. Whole lysates were used to measure the level of MDA following the manufacturer's protocol (OxiSelect^{TM} TBARS Assay kit, Cell BioLabs, San Diego, CA, USA). The TBA test was carried out under acidic (pH 3.5) conditions with a colorimetric 96-well microplate assay and the level of TBARS was expressed relative to the response of the assay to malondialdehyde (MDA) using a plate reader at 532nm.

Statistical Analysis

All experimental data points were from triplicate samples and are expressed and or plotted as the mean ± S.E.M. Data were analyzed by Student t test to establish significance between data points.

6.3. Results

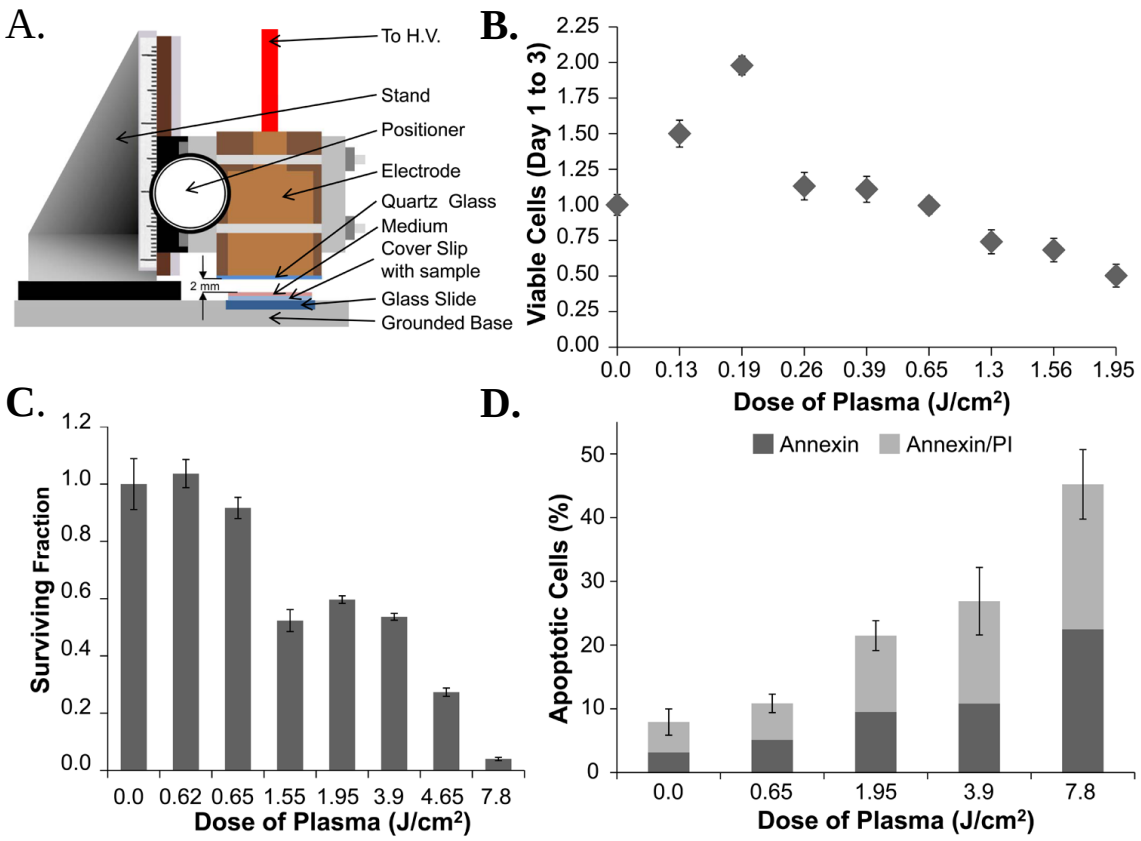

Figure 30. Dose-dependent effects of non-thermal atmospheric pressure dielectric barrier discharge (DBD) plasma on MCF10A cells. (A) Schematic of the apparatus for administration of DBD plasma. (B) 10^4 MCF10A cells plated on glass cover slips were treated with the indicated dose of DBD plasma as described. Cells were counted 24 and 72 hours after treatment. (C) Cells were treated with the indicated dose of DBD plasma; and colony survival assays were performed as described. Data are expressed relative to the # of colonies in the untreated control. (D) 3 days after treatment with the indicated dose of DBD plasma, cells were harvested and stained with Annexin V/ propidium iodide (PI) and analyzed by Guava.

In order to test the effects of plasma treatment on mammalian cells, DBD plasma was applied to human breast epithelial cells (MCF10A). Cells were grown on glass cover slips and exposed to non-thermal plasma as illustrated (Figure 30A). Cells were treated in 100 μl of media and incubated for 1 minute prior to placement in 2 ml of media. The initial experiment involved establishing the dose-dependent effects of plasma treatment on cell proliferation and survival by direct cell count and colony formation. At low doses, cell number increased, whereas at higher doses, cell number and survival decreased (Figure 30B, C). Annexin V/propidium iodide staining of cells treated with DBD plasma at doses ranging from 0.65 to 3.9 J/cm^2 revealed that apoptosis was induced at higher doses (Figure 30D).

6.3.1. Effects on DNA

One possible mechanism underlying these dose-dependent effects is generation of ROS, which at low levels is known to increase cell proliferation and at high levels induces cell death through DNA damage [413]. DNA damage induced by IR has been shown to result from formation of ROS [414, 415]. To determine whether DBD plasma treatment of cells could induce DNA damage, we looked at phosphorylation of H2AX, a histone variant that is phosphorylated in response to DNA damage [53]. Western blot with an antibody that detects H2AX phosphorylated at Ser139 (γ-H2AX) revealed that treatment of cells with DBD plasma induces a dose-dependent increase in γ-H2AX (Figure 31A). Indirect immunoflorescence also revealed foci of γ-H2AX (Figure 31B) which increased in number at higher doses. These data demonstrate that DBD plasma treatment of cells induces a dose-dependent increase in DNA damage.

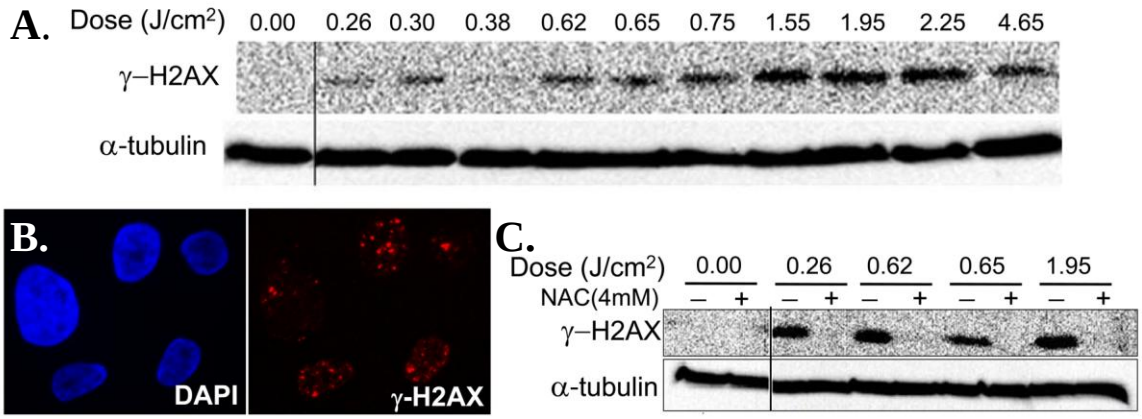

Figure 31. Reactive oxygen species mediate induction of DNA damage by DBD plasma. (A) MCF10A cells were treated with the indicated dose of DBD plasma as described. After one hour incubation, lysates were prepared and resolved by SDS-PAGE and representative immunoblots with antibody to γ-H2AX or α-tubulin are shown. (B) Indirect immunofluorescence of MCF10A cells was performed as described one hour after treatment with 1.55 J/cm^2 DBD plasma. (C) MCF10A cells were incubated for 2 hours with 4 mM N-acetyl cysteine (NAC) (+) or cell culture medium (-), followed by treatment with the indicated dose of DBD plasma. γ-H2AX (upper panel) or α-tubulin (lower panel) was detected by immunoblot of cell lysates prepared one hour after plasma treatment.

6.3.2. Role of ROS

We next sought to directly test whether the damage induced by DBD plasma is due to ROS (e.g. H_2O_2, $OH^{\bullet}$, $^1O_2$, atomic oxygen, $O_2^{\bullet-}$, etc.) generated in the media and/or cells by plasma treatment. Pre-treatment of cells with the ROS scavenger, N-acetyl cysteine (NAC), blocked induction of γ-H2AX even at high doses of DBD plasma treatment (Figure 31C), suggesting that the induction of DNA damage as measured by γ-H2AX is mediated by ROS.

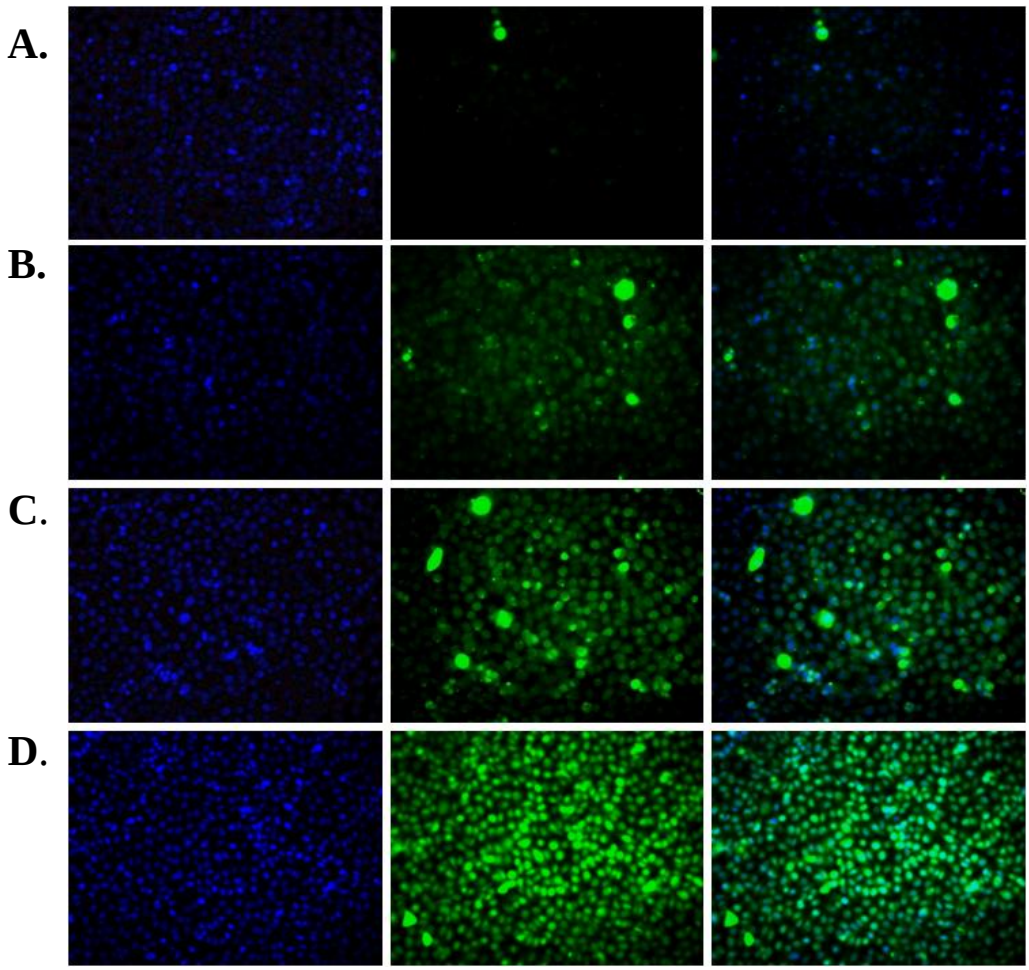

Figure 32. Non-Thermal plasma treatment of cells leads to intracellular generation of reactive oxygen species. Detection of intracellular ROS was performed as described 1 h after plasma treatment. Representative images are shown for (A) untreated control. (B) 200 μM H_2O_2. (C) Plasma treatment at a dose of 1.55 J/cm^2 and (D) Plasma treatment at a dose of 4.65 J/cm^2.

In order confirm the results of ROS scavenging and to evaluate whether plasma induces oxidative stress only in the extracellular environment or the effects of plasma are able to penetrate the cells and induce generation of intracellular ROS, we directly tested for intracellular ROS in MCF10A cells post non-thermal plasma treatment using a commonly used ROS detection dye CM-H_2DCFDA. Untreated cells were used as controls to evaluate baseline oxidative stress in cells (Figure 32A) while 200 μM $H_2O_2$was used as a positive control (Figure 32B). As shown in Figure 32C, 1 h after

treatment, non-thermal plasma induces generation of significant levels of intracellular ROS in MCF10A cells at a dose of 1.55 J/cm^2 as measured by fluorescence microscopy. The level of intracellular oxidative stress induced is higher as the dose of plasma is increased to 4.65 J/cm^2 as shown in Figure 32D. These results indicate that non-thermal plasma treatment of cells induces generation of significant levels of

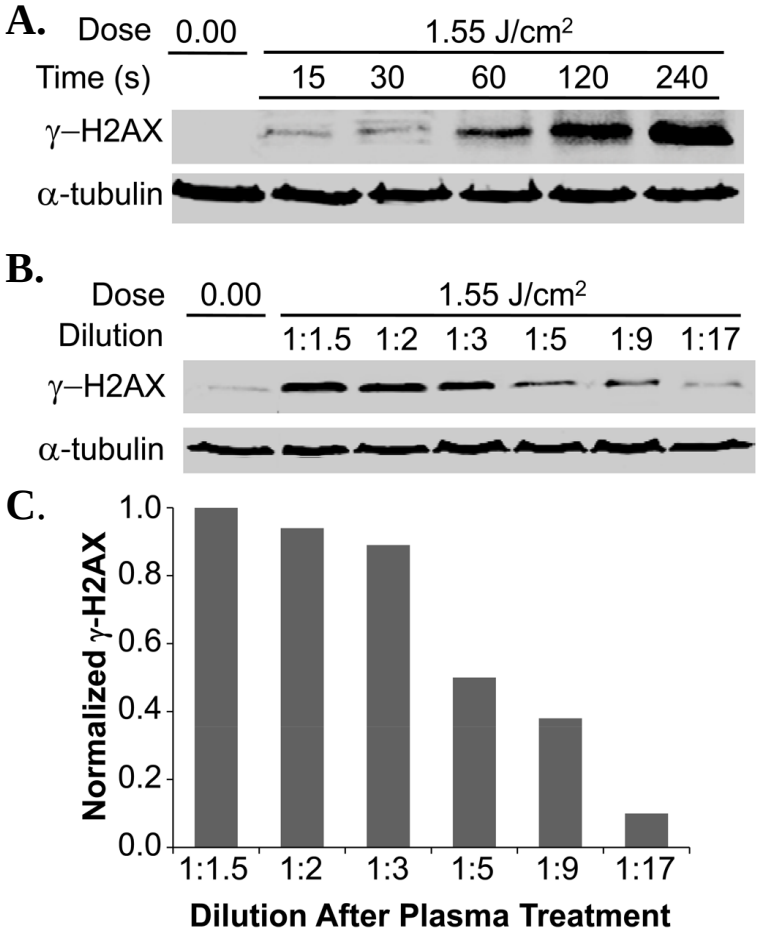

Figure 33. (A) ROS accumulate in media and effects on cells are dependent on their concentration. Cells on cover slips overlaid with 100 µl cell culture media were treated with 1.55 J/cm^2 DBD plasma, followed by dilution in 2 ml of media at the indicated holding time after treatment. Cell lysates were subjected to immunoblot after 1 hour of incubation. (B) Cells were treated in 100 µl cell culture media and immediately after treatment, it was diluted with media as indicated; all samples were incubated at the indicated dilution for one minute followed by incubation for 1 hour in 2 ml of medium. (C) The results of (B) are quantified.

intracellular ROS and the resultant oxidative stress may lead to observed DNA damage. Consistent with this finding, longer incubation in the 100 μl in which cells were treated before dilution into 2 ml of media resulted in higher levels of γ-H2AX (Figure 33A). Additionally, when the treated media was diluted immediately after treatment, the amount of damage was directly related to the dilution, i.e. damage was greater at the lowest dilution (Figure 33B, C) These data suggest that generation of intracellular ROS and induction of DNA damage is the result of interaction of plasma with extracellular media, the effect of which depends upon the concentration and the length of exposure. Moreover, these findings suggest that active species in the media are responsible for the effects of DBD plasma on cells.

6.3.3. Biochemical Interaction of Plasma

As seen in previous sections direct non-thermal plasma treatment has various dose dependent effects ranging from enhancing cell proliferation to inducing apoptosis. It was also apparent that reactive oxygen species produced by DBD plasma play a prominent role in mediating the biological effects of non-thermal plasma. Though, it was still unclear how the ROS produced by plasma would interact with cells. As plasma is primarily a surface phenomenon, ROS produced by plasma would very likely encounter the cell membrane before intracellular components of the cells. Further, as most of these ROS are short lived it seems unlikely that they could travel through the organic medium covering the cells during treatment to reach the surface of the cells and directly attack the cell membrane. We hypothesized that ROS produced by non-thermal plasma interact with organic content to produced long living organic species which eventually mediate the

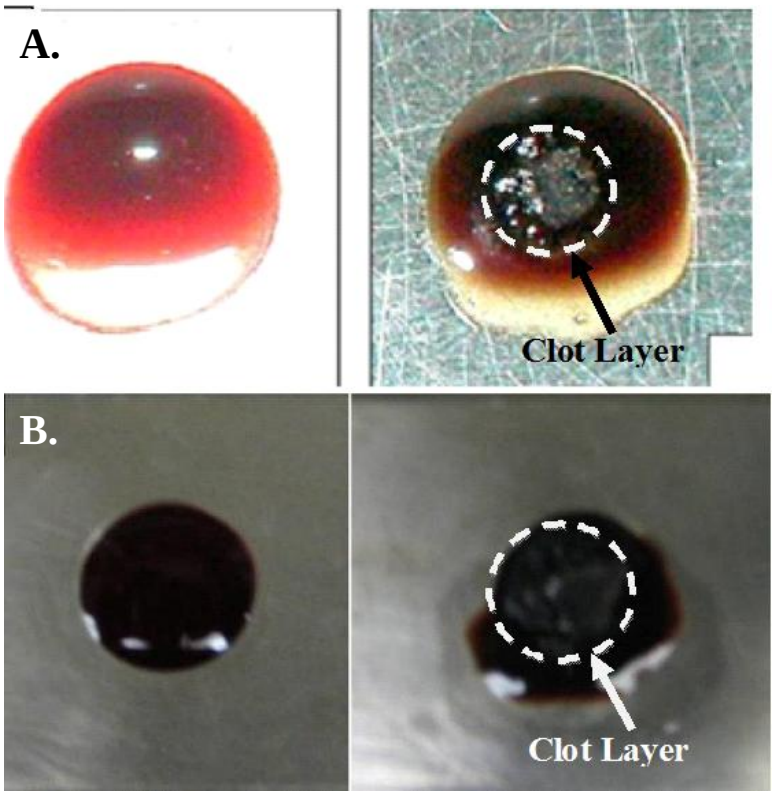

Figure 34. Coagulation of non-thermal plasma treated non anti-coagulated whole blood and citrated whole blood. (A) Non anti-coagulated donor blood treated with non-thermal plasma for 15 s exhibits immediate clot layer formation. (B) Citrated whole blood treated with non-thermal plasma for 15 s exhibits immediate clot layer formation.

interaction of plasma with cells. In order to test this hypothesis we decided to test the effects of non- thermal plasma on the most natural organic product of the human body, blood. We investigate mechanisms of blood coagulation by non-thermal atmospheric pressure plasma using an experimental setup similar to one previously described in [13] and schematically illustrated in Figure 3. For this study, de-identified whole blood samples with three different types of anticoagulants were obtained from Drexel University College of Medicine (DUCOM) Chemistry lab. 500 µl of the three different types of anti-coagulated whole blood, viz; heparinized whole blood; citrated whole blood and Ethylene Diamine Tetraacetic Acid (EDTA) whole blood were used. Each blood

sample was treated for 5 s, 15 s, 30 s and 60 s to study the effect of non-thermal plasma treatment on blood.

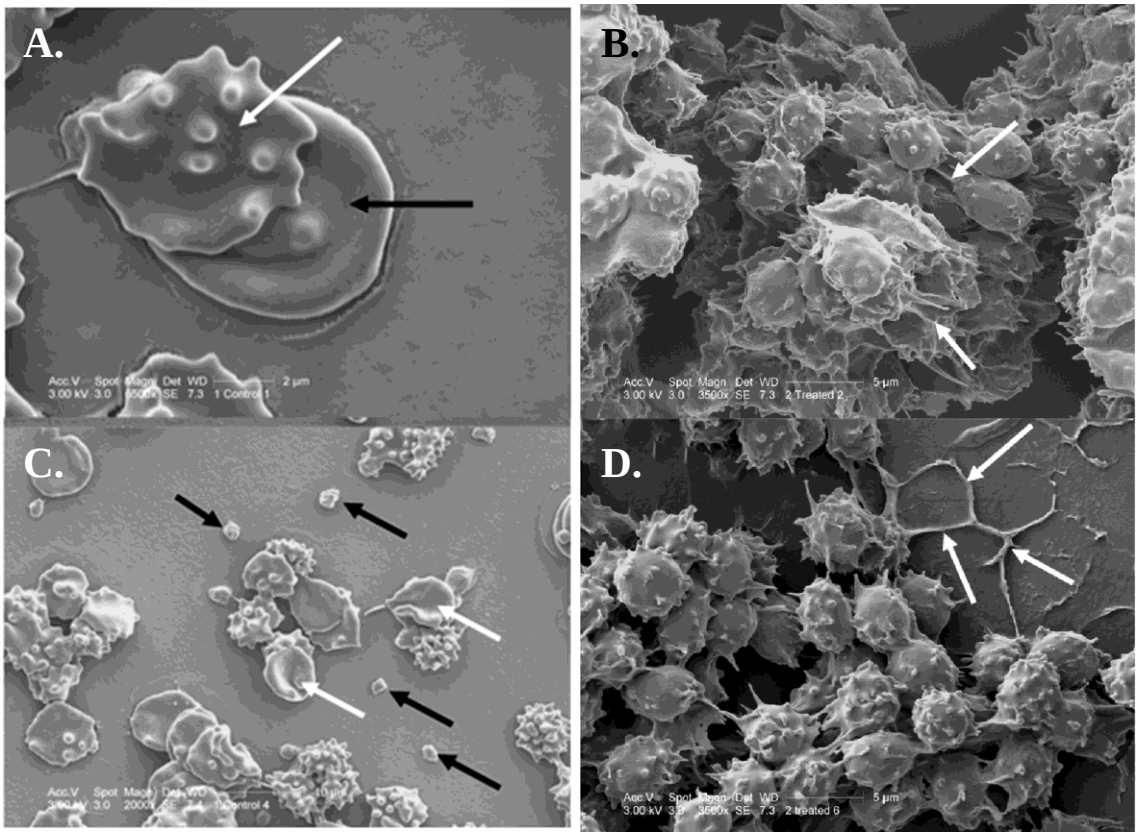

Figure 35. SEM observation of Non-Thermal plasma initiated blood coagulation. (A) Citrated whole blood (control) showing single activated platelet (white arrow) on a red blood cell (black arrow). (B) Citrated whole blood (control) showing many non-activated platelets (black arrows) and intact red blood cells (white arrows). (C) Citrated whole blood (treated) showing extensive platelet activation (pseudopodia formation) and platelet aggregation (white arrows). (D) Citrated whole blood (treated) showing platelet aggregation and fibrin formation (white arrows)

Evaluated visually, a drop of blood, with a volume of 500µl, drawn from a healthy donor and left on a stainless steel surface coagulates on its own in about 15 minutes, while a similar drop treated for 15 s by non-thermal plasma coagulates in under 1 minute as shown in Figure 34A. Similarly, 0.5 ml of citrated whole blood left in a well does not coagulate on its own even when left in the open air for well over 15 minutes,

while the same sample treated with DBD-plasma for 15 s exhibits immediate clot layer formation on the surface exposed to plasma discharge as shown in Figure 34B.

To gain further insight into the structure of the clot layer of the anti-coagulated blood samples, morphological examination of the clot layer was performed by Scanning Electron Microscopy. Figure 35A shows a single platelet [416] over a red blood cell in untreated (control) citrated whole blood. Figure 35B shows some activated, but many non-activated platelets and red blood cells in the untreated anti-coagulated whole blood. No platelet aggregation or fibrin strands are observed in these samples. On the other hand, extensive platelet activation (pseudopodia formation) and platelet aggregation was observed in the non-thermal plasma treated (30 s) whole blood as evident from Figure 35C. Figure 35D shows platelet activation, platelet aggregation and fibrin formation (white arrows) in the e-plasma treated anti-coagulated whole blood.

Natural blood coagulation is a complex process that has been studied extensively [417] and various non-thermal plasma products can affect this process. It was hypothesized previously that direct exposure to non-thermal plasma initiates coagulation of blood through increase in concentration of Ca^{2+}[12], an important factor in the coagulation cascade [418, 419]. Calcium circulates in blood in several forms. 45%-50% is free ionized, 40-45% is bound to proteins, mostly albumin, and rest is bound to anions such as bicarbonate, citrate, phosphate and lactate [420]. Bound calcium is in equilibrium with free calcium. pH has a significant effect on calcium ion binding to protein, with each unit decrease or increase in pH causing ionized calcium to change inversely by about 0.36 mmol/L [421]. It was proposed that non-thermal plasma is effective in increase of Ca^{2+} concentration through a redox (reduction/oxidation) mechanism. We tested the

validity of this hypothesis experimentally by measuring Ca^{2+} concentration in the non-thermal plasma treated anti-coagulated whole blood using a calcium selective micro-electrode. Calcium concentration was measured immediately after non-thermal plasma

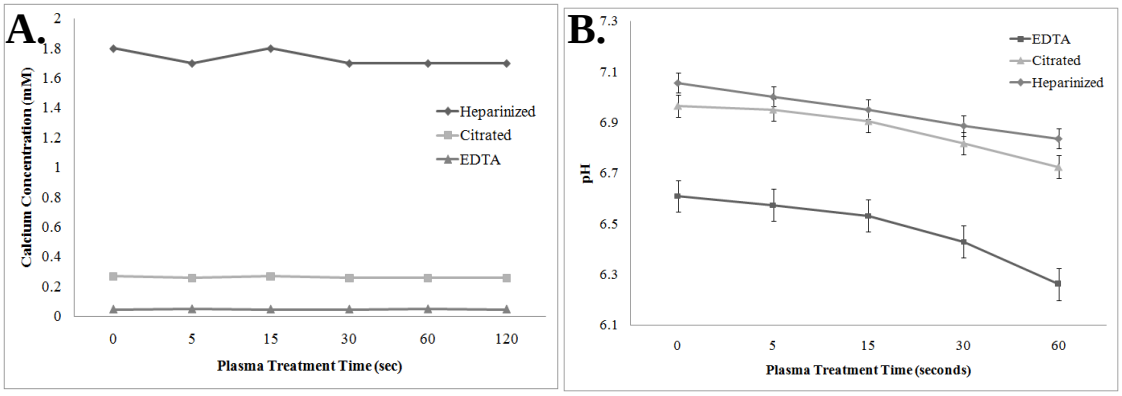

Figure 36. Coagulation of blood by non-thermal plasma treatment does not occur due to changes in pH or Ca^{2+} concentration. (A) Calcium concentration in different anti-coagulated whole blood treated with non-thermal plasma. There is no significant change in calcium ion concentration during the time non-thermal plasma treated blood coagulates. (Note: average error is less than 0.01 mM). (B) pH of whole blood after non-thermal plasma treatment for different durations. pH does not change significantly in the duration in which plasma treated blood coagulates.

treatment for 5 s, 15 s, 30 s, 60 s and 120 s. Figure 36A demonstrates that calcium concentration remains almost constant for up to 30 s of treatment and then increases very slightly for prolonged treatment times of 60 s and 120 s. Thus, although, non-thermal plasma is capable of coagulating anti-coagulated blood within 15 s, no significant change occurs in calcium ion concentration during this time in discharge treated blood. Therefore, we conclude that release of Ca^{2+} may not be the mechanism for non-thermal plasma triggered coagulation.

Changes in blood pH could also trigger blood coagulation through increase in calcium ion concentration due to redox mechanism [12]. In-vivo, the pH of blood is maintained in a very narrow range of 7.35-7.45 by various physiological processes [422]. Non-thermal plasma has been confirmed to generate a significant amount of hydrogen

ions which changes pH of water and phosphate buffered saline significantly within 30 s of treatment [12]. We tested the hypothesis that the non-thermal plasma treatment triggers coagulation through changes in the pH of blood. This was tested by measuring pH of each blood sample immediately after non-thermal plasma treatment as described in the methods section. Figure 36B shows that no significant change in pH occurs in the anti-coagulated blood samples during the time needed for non-thermal plasma treated blood to coagulate.

It was previously demonstrated that significant changes occur in blood plasma protein concentrations after treatment by non-thermal plasma of samples from non anti-coagulated and blood with various anti-coagulants [12]. Direct activation of intermediate protein factors by non-thermal plasma may be one of the mechanisms of coagulation of blood. Conversion of fibrinogen into cross-linked fibrin fibers is the final step in coagulation of blood [423]. Therefore, we postulated that one of the pathways by which non-thermal plasma treatment coagulates blood may be through direct effect on fibrinogen. To test this hypothesis we investigated the effect of the non-thermal plasma treatment (30 sec) on buffered fibrinogen solution (TRIS buffered saline solution, 20 mM) at physiological pH of 7.4 using the set up used for blood treatment. As compared to the untreated fibrinogen solution shown in Figure 37A, the opacity of the treated fibrinogen solution changes as shown in Figure 37B indicating that non-thermal plasma initiates changes in the fibrinogen solution. Dynamic Light Scattering (DLS) measurements on the treated and untreated fibrinogen solutions were carried out to test the possibility that the observed changes in opacity are due to conversion of fibrinogen into larger molecular structures.

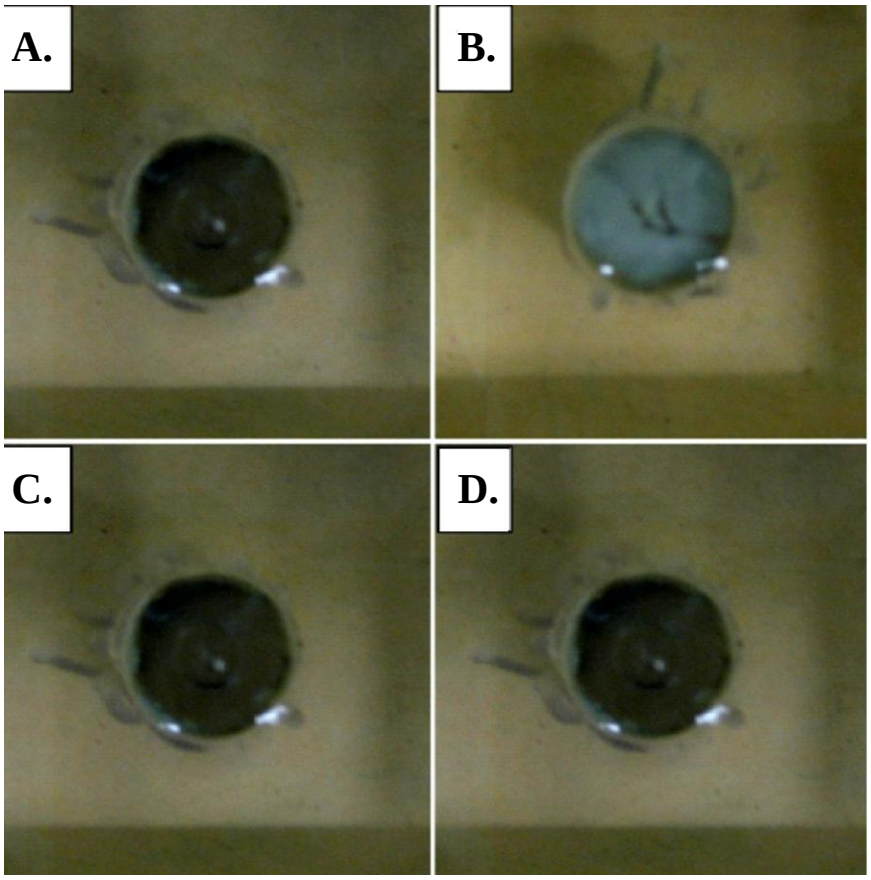

Figure 37. Treatment of buffered solution of fibrinogen (A) control, (B) 30 sec plasma treatment and buffered solution of albumin (C) control, (D) 30 sec plasma treatment. (B) shows change in opacity of fibrinogen solution after treatment whereas in (D) we see no change in opacity of albumin solution.

Figure 38A compares untreated (control) and treated fibrinogen solution. The diameter of a fibrinogen molecule is around 9 nm [423], which is demonstrated in Figure 38B. Treated fibrinogen exhibits a multimodal distribution of sizes with the largest increase in size by about two orders of magnitude as compared to the control. The largest fibrinogen aggregates have an average size of 2 μm. Thus coagulation of blood by non-thermal plasma treatment does not occur due to changes in pH or Ca^{2+} concentration. Non-thermal plasma appears to promote rapid blood coagulation by enhancing the natural coagulation processes. The results presented in this section indicate that direct conversion of fibrinogen into fibrin may be one of the mechanisms by which non-thermal plasma

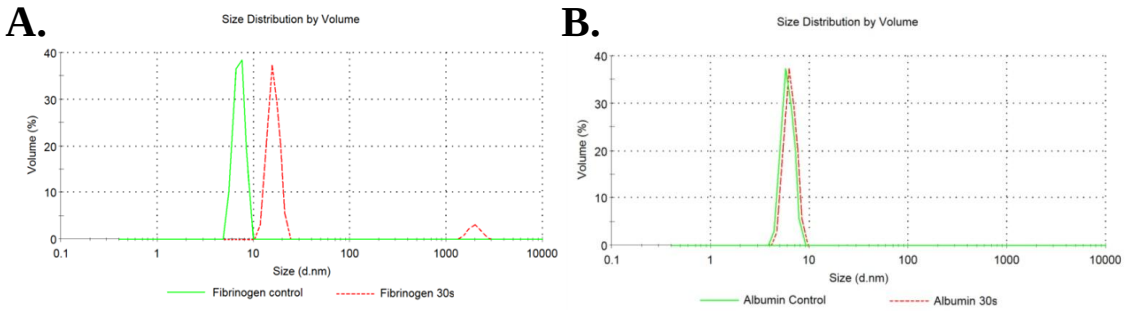

Figure 38. Dynamic light scattering measurements (A) Comparison of control and treated solution of fibrinogen. (B) Comparison of control and treated solution of albumin. Treated and untreated albumin show the same size distribution with average size of about 6nm, which corresponds well to the published albumin size of around 8 nm [424].

initiates coagulation. This indicates that non-thermal plasma not only has direct effects attributed to charges but also indirect biochemical effects which may be initiated by interactions of reactive oxygen species with organic content including proteins and amino acids.

6.3.4. Role of Plasma Generated Charged Species vs Neutral Active Species

Non-thermal plasma is known to produce many charged species (electrons and ions) and long living and short living neutral species in gas phase as well as in liquid phase [17]. To determine which of these species are responsible for inducing generation of intracellular ROS and the induction of DNA damage, DBD plasma was applied in different modes. Experiments shown in Figure 30, Figure 31 and Figure 32 utilized direct treatment, in which all the species produced by plasma come in contact with the surface of the medium covering the cells during treatment. Indirect treatment involves placement of a grounded mesh between the high voltage electrode and the medium, which blocks charged species and allows only neutral species to come in contact with the surface of the medium. Phosphorylation of H2AX was not significantly different in indirect treatment

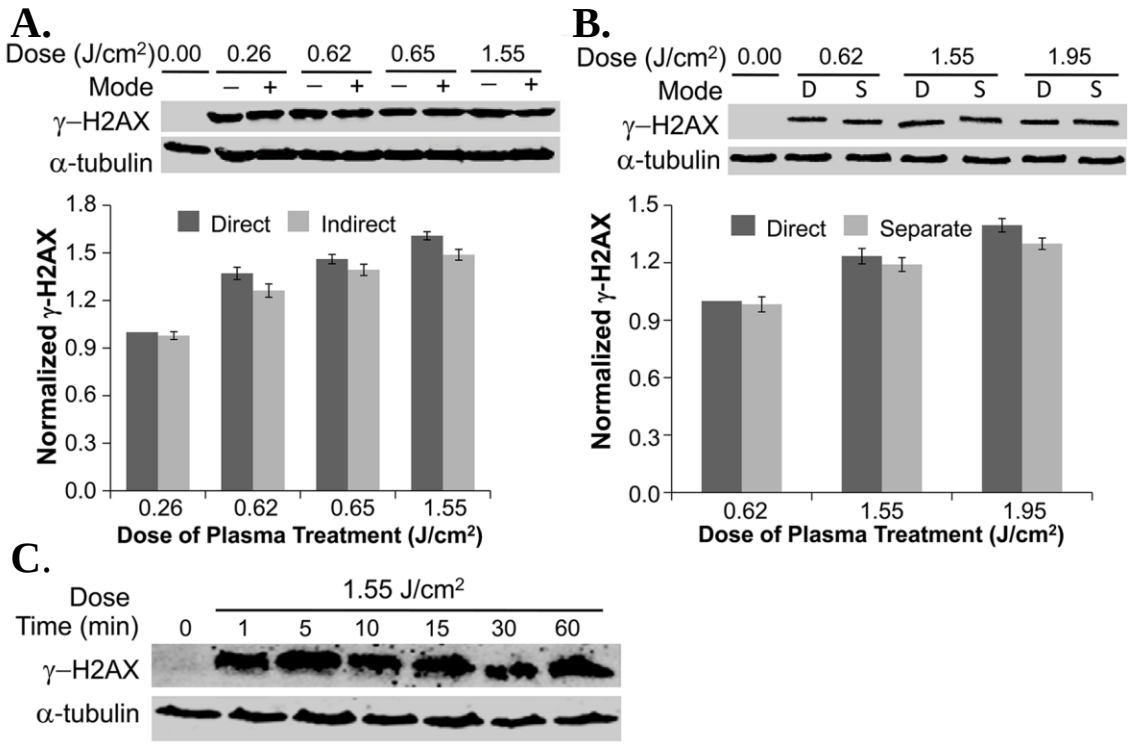

Figure 39. Effects of DBD Plasma are mediated by neutral species generated in the media. (A) Cells were subjected to DBD as described earlier (direct, D) or a grounded mesh was placed between the electrode and the medium (indirect, I). (B) Cells were subjected to direct treatment with DBD plasma (D) or media (100 μl) was exposed to DBD plasma and then transferred to the cells (separate, S). (A, B) Representative immunoblots with γ-H2AX or α-tubulin are shown. The graphs below the immunoblots show quantification using Odyssey. (C) Media (100 μl separated treatment) was subjected to DBD and was transferred to cells after holding for 1 to 60 min. After 1 minute incubation with cells, cover slips with treated media and cells were transferred to a dish with 2 ml of media.

as compared to direct treatment (Figure 39A), indicating that active neutral species produced by DBD plasma are responsible for the generation of ROS leading to induction of DNA damage.

6.3.5. Direct vs Separated Plasma Treatment

To determine whether the effects of DBD plasma are due to modification of the cell medium by the plasma treatment as opposed to a direct effect on the cells, the media

was treated separately and added to cells, 100 μl of medium on a coverslip (without cells) was treated with DBD plasma and then transferred to a coverslip with cells, which we have termed ‘separated treatment’. The effect of media separately treated with DBD plasma and added to cells was not significantly different from the effect of direct treatment of cells overlaid with media (Figure 39B). To begin to address what component of the medium is affected by the treatment, we initially addressed the stability of the separately treated medium. The medium was separately treated as described above and then held for increasing times before being added to cells. Induction of DNA damage by the treated medium was not reduced by holding the medium up to one hour prior to adding it to cells (Figure 39C), suggesting that stable component(s) such as organic peroxides [233] may be formed in the cell medium ,

6.3.6. Presence of Organic Content and Role of Amino Acid Peroxidation

To identify the active components, we compared the effect of separated treatment of complete medium vs. inorganic phosphate buffered saline (PBS). We observed no DNA damage in cells exposed to separately treated PBS (**Figure 40**A), whereas separately treated medium induced DNA damage as anticipated. This suggests that the presence of organic components in the medium is essential for the observed effects.

Cell culture medium used in the experiments described above is composed of amino acids, glucose, vitamins, growth factors and inorganic salts, as well as serum. Gebicki et al. have shown that γ-radiation (IR) induces formation of amino acid and protein hydroperoxides in aqueous solutions containing BSA or individual amino acids [233]. Equivalent levels of H2AX phosphorylation were induced in cells subjected to

C.

Amino Acid	Peroxidation Efficiency (%)
Methionine (M)	0
Serine (S)	0
Cysteine (C)	0.4
Arginine (R)	13
Glutamine (Q)	16
Glutamic Acid (E)	28
Lysine (K)	34
Isoleucine (I)	43
Leucine (L)	44
Proline (P)	44
Valine (V)	49

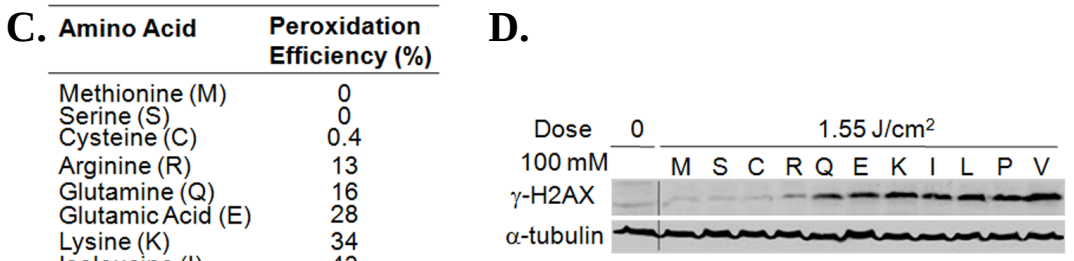

Figure 40. The amount of DNA damage induced is proportional to the peroxidation efficiency of the amino acid. (A) Cells overlaid with 100 µl of PBS were treated directly with DBD plasma (direct, D) or separately (S). (B) DNA damage is induced in cells subjected to DBD plasma treated serum-free media or PBS with BSA, but not PBS alone. 100 µl of PBS, media without serum, or PBS with 100 µg/ml BSA were treated with 1.55 J/cm^2 DBD plasma and immediately added to cells on a coverslip (S). Cells overlayed with 100 µl of the indicated solution were treated with 1.55 J/cm^2 DBD plasma (D). (C) Peroxidation efficiency of various amino acid components of cell culture medium when treated with IR [233]. (D) Solutions containing the indicated amino acid (100 µM) were separately treated with DBD plasma and then added to MCF10A cells. (A, B, D) After 1 minute incubation, cells on cover slips were diluted in 2 ml media, followed by lysis and Western blot for γ-H2AX or α-tubulin.

DBD plasma treated serum-containing media, serum-free media or PBS with BSA, but not PBS alone (Figure 40B), suggesting that amino acid peroxidation may be involved. Peroxidation efficiency is widely variable among different amino acids [233]. To determine whether the observed results were related to the peroxidation efficiency of organic components in cell culture medium, 11 different amino acids with a range of peroxidation efficiencies were dissolved individually in PBS and separately treated with DBD plasma and then added to cells. As shown in Figure 40, phosphorylation of H2AX (Figure 40D), there is a direct correlation between peroxidation efficiency and the amount of DNA damage, with valine producing the most significant level of damage and

serine and methionine producing no detectable DNA damage (Figure 40C),. These data provide strong support for the hypothesis that organic peroxides are produced in the medium by plasma treatment and are responsible for the observed effects on DNA, although further confirmation is required.

6.3.7. Role of Ozone

DNA damage is induced in cells treated directly in PBS (Figure 40 *a*), albeit less than that induced by direct treatment in media. Since separately treated PBS has no effect on cells and organic components are not present in PBS, it seems likely that the DNA damage induced by direct treatment of PBS must result from short-lived active species generated in PBS, like OH radicals or ozone. As shown in Figure 40, as interaction of plasma produced neutral active species with amino acids leads to DNA damage it is possible that ozone may play a role in mediating the interaction of non-thermal plasma with mammalian cells by modifying amino acids, producing long living organic hydroperoxides. [425] have shown that ozone is capable of reacting with various amino acids including methionine and cysteine with high rates of reaction at physiological values of pH. Although typically requiring much higher treatment time than the typical times reported here, ozonation is, in fact, widely employed to kill micro-organisms in water [426] and ozone therapy is now being widely studied as a novel treatment for various pathologies [427-430]. Since DBD plasma produces significant amounts of

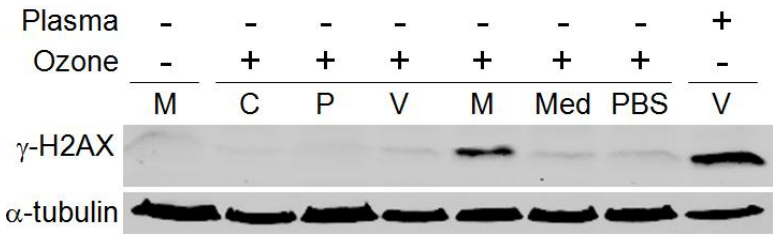

Figure 41. Ozone treatment is qualitatively different from Plasma treatment. Solutions containing the indicated amino acid (100 μM) or medium or PBS were ozonated for 15 s and then added to MCF10A cells. After 1 minute incubation, cells on cover slips were diluted in 2 ml media, followed by lysis and Western blot for γ-H2AX or α-tubulin. Cells exposed to separately plasma treated valine were used as control.

ozone [431, 432], it is possible that the DNA damaging effects of plasma may be mediated by ozone to some extent. To test the possibility, ozone plays a major role in mediating the DNA damage induced by plasma, we exposed cells to 100 μl of PBS containing cysteine, methionine, proline and valine dissolved in PBS, serum-containing medium or PBS alone that were separately treated with ozone. As shown in Figure 41, PBS containing amino acids cysteine, valine or proline, cell medium or PBS, separately treated with ozone resulted in no detectable DNA damage in MCF10A cells as measured by phosphorylation of H2AX, while we observed DNA damage in cells exposed to methionine separately treated by ozone. We, therefore, conclude that ozone treatment is qualitatively different from non-thermal DBD plasma and does not play a major role in these studies since it affects mostly methionine and cysteine, while not affecting valine and leucine [425] , which are most strongly affected in DBD treatment as shown in Figure 40B. Further investigation is necessary to determine which of the neutral species plays a major role in observed interactions of non-thermal plasma with mammalian cells

6.3.8. Lipid Peroxidation

Non-thermal plasma produces a large ROS concentration in the extracellular medium during treatment. However, it is unclear how these ROS go inside cells. N-acetylcysteine, an intracellular ROS scavenger completely blocked phosphorylation of H2AX after non-thermal plasma treatment of MCF10A cells, which indicates that, ROS produced by plasma extracellularly may move across the cell membrane through lipid peroxidation, opening transient cell membrane pores, or signaling pathways which modify the concentration of ROS intracellularly.. In this section we attempt to investigate whether non-thermal plasma induced lipid peroxidation is responsible for the observed DNA damage.

6.3.8.1. Mammalian Cells

Since many of active species produced by plasma including $OH^{\bullet}$, $O_2^{\bullet-}$, $RO^{\bullet}$, $ROO^{\bullet}$, $^1O_2$, have a short life span, they may immediately interact with medium components including amino acids and proteins, leading to production of long lived reactive organic hydroperoxides [233]. These hydroperoxides may then induce lipid peroxidation and the by-products of lipid peroxidation, like MDA [433] may lead to DNA damage [434], or they may bind to cell membrane receptors and activate intracellular signaling pathways leading to subsequent DNA damage.

It is well known that ROS like OH radicals, superoxide radicals, atomic oxygen, singlet oxygen, organic protein and amino hydroperoxides, etc. react with polyunsaturated fatty acid residues in membrane phospholipids to initiate lipid peroxidation which results in the production of a plethora of by-products, many of them

reactive toward protein and DNA[435]. One of the most abundant carbonyl products of lipid peroxidation induced by endogenous and exogenous ROS-inducing chemicals is malondialdehyde (MDA)[436]. It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine. The major adduct to DNA is a pyrimidopurinone called M_1G. Site-specific mutagenesis experiments indicate that M_1G is mutagenic in bacteria and is repaired by the nucleotide excision repair pathway [433, 435, 437, 438].

As described earlier, DNA damage induced by non-thermal plasma was mediated by ROS. ROS are known to induce lipid peroxidation in mammalian cells. Plasma produces ROS in the medium covering the cells during treatment. These ROS are likely to first encounter the cell membrane before DNA and it is possible that they either directly or by modifying organic content in the medium induce lipid peroxidation. To

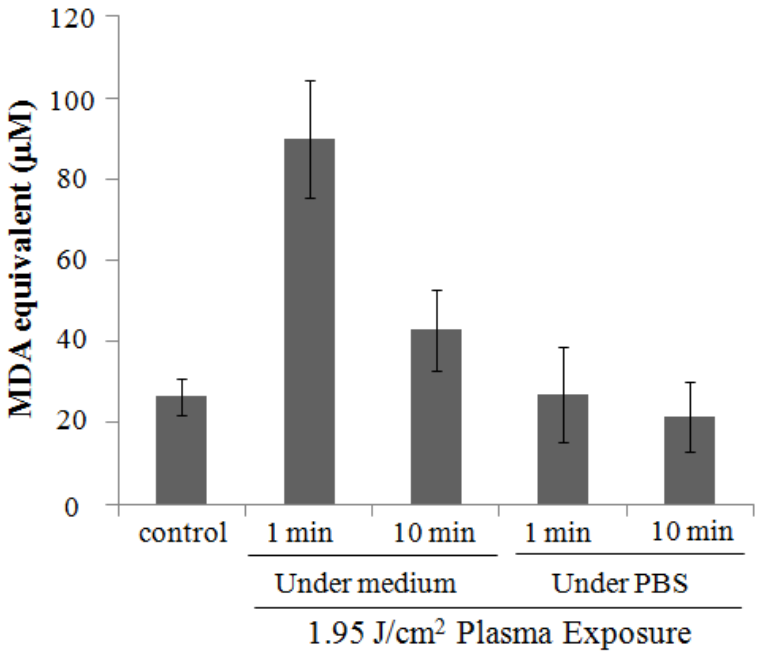

Figure 42. Non-Thermal plasma treatment leads to release of malondialdehyde (MDA), a commonly used marker for measuring lipid peroxidation in mammalian cells. Plot shows MDA equivalent for untreated cells and cells treated at indicated dose Data from triplicate samples (± S.D.) are plotted.

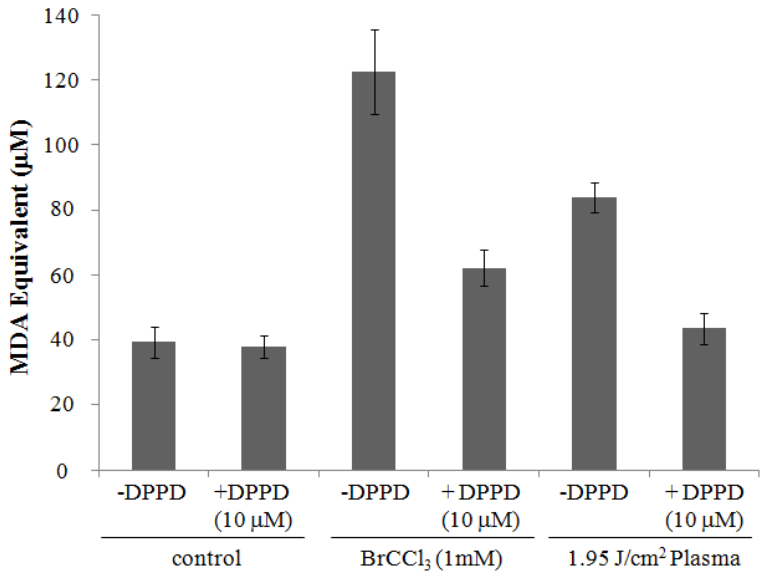

Figure 43. MCF10A cells were incubated for 1 hour with 10 μM N-diphenyl-phenyl-enediamine (DPPD) (+DPPD) or cell culture medium (-DPPD), followed by treatment at the indicated dose of DBD plasma or with 1 mM bromotrichloromethane ($BrCCl_3$). Lipid peroxidation was measured via release of malondialdehyde (MDA). Plot shows MDA equivalent for untreated cells (control) and cells treated at indicated plasma dose or with 1 mM $BrCCl_3$. Data from triplicate samples (± S.D.) are plotted.

determine whether DBD plasma treatment induced lipid peroxidation in cells, we looked at formation of malondialdehyde (MDA), a by-product of lipid peroxidation, which is commonly used as an index for measuring lipid peroxidation [439]. As shown in Figure 42, we see that plasma indeed induces lipid peroxidation in cells immediately after treatment, when treated under medium either directly or separately. In contrast, non-thermal plasma does not induce lipid peroxidation in mammalian cells treated under PBS, either directly or separately. This suggests that long living organic hydroperoxides, produced as a result of interaction of neutral active species produced by non-thermal plasma in medium with organic components of the medium like proteins and amino, may induce lipid peroxidation. These data are consistent with the fact that DNA damage is induced by organic peroxides formed as a result of ROS produced by neutral active species.

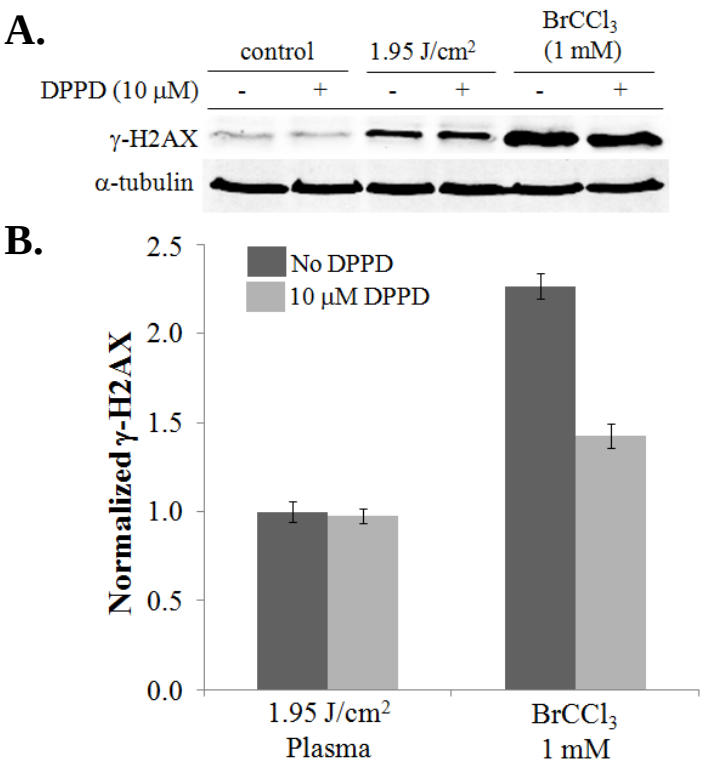

Figure 44. Non-Thermal plasma induced lipid peroxidation does not lead to the observed DNA damage. MCF10A cells were incubated for 1 hour with 10 μM N-diphenyl-phenyl-enediamine (DPPD) (+DPPD) or cell culture medium (-DPPD), followed by treatment at the indicated dose of DBD plasma or with 1 mM bromotrichloromethane ($BrCCl_3$). (A) Representative Immunoblot with γ-H2AX or α-tubulin are shown. (B) The graph below the immunoblot show quantification of triplicate data samples from three separate experiments.

We next sought to test whether plasma induced lipid peroxidation led to the observed DNA damage. In order to block lipid peroxidation in MCF10A cells after plasma treatment, we used N-Diphenyl-phenyl-enediamine (DPPD), a synthetic lipophilic antioxidant and bromotrichloromethane ($BrCCl_3$) a known inducer of lipid peroxidation as a pro- oxidant. DPPD is frequently used in cell culture and in *in-vivo* studies to inhibit lipid peroxidation by various chemical agents [440-443]. MCF10A cells were pre-incubated with DPPD for 15 min at 37°C before plasma treatment or addition of 1 mM $BrCCl_3$. As shown in Figure 43, DPPD significantly inhibits lipid peroxidation in MCF10A cells, both after plasma treatment and after incubation of cells with $BrCCl_3$ for

2 hours. Thus, DPPD is a potent inhibitor of non-thermal plasma induced lipid peroxidation. DPPD proving to be a potent blocker of lipid peroxidation, we next sought to investigate the role of non-thermal plasma induced lipid peroxidation in the observed DNA damage. As earlier, MCF10A cells were preincubated with 10 mM DPPD for 15 min before exposing the cells to non-thermal plasma at a dose of 1.95 J/cm^2 (15 s at low frequency) or to 1 mM $BrCCl_3$ for 2 h. After plasma treatment cells were incubated for 1h at 37°C prior to lysing for analyzing DNA damage. DNA damage was analyzed as earlier by western blot technique for measuring γ-H2AX. As shown in Figure 44, DNA damage after plasma treatment of MCF10A cells with or without pre-incubation of DPPD was same while DNA damage induced by $BrCCl_3$ was significantly reduced by DPPD. Non-thermal plasma induced lipid peroxidation does not lead to non-thermal plasma induced DNA damage. Non-thermal plasma produces a lot of ROS in the medium covering the cells during treatment which may lead to lipid peroxidation and DNA damage simultaneously, but DNA damage is not induced as a result of lipid peroxidation Further investigations are necessary to determine other pathways of non-thermal plasma induced DNA damage in mammalian cells, which may include ROS mediated cellular signaling or uptake of long lived ROS via active transport.

6.3.8.2. Fungi

As shown in the previous section, DNA damage in mammalian cells induced by non-thermal plasma is not mediated via non-thermal plasma induced lipid peroxidation. Lipid membranes in mammalian cells transiently allow transport of nutrients, water and other metabolites and small molecules across for proper functioning of the organism.

Reactive oxygen species produced by plasma may also be up taken by active transport mechanisms and thereby the effects of plasma could be transported across the cell membrane. To confirm that the effects of non-thermal plasma are not mediated via lipid peroxidation and physical membrane damage we chose to test the effects of non-thermal plasma on fungi which have multiple layers of membranes and cell walls as opposed to single bilayer in mammalian cells. We investigated the efficacy of plasma killing of fungi and visually studied morphological changes in the fungal membrane post non-thermal plasma treatment. 100 μl of fungal suspension of Alternaria Brassicicola is placed on a glass microscope slide and allowed to dry for about an hour. After drying the fungi are treated with non-thermal plasma and immediately after treatment the fungi are swabbed from the glass slide and spread on to an agar plate and incubated at room temperature for 3-5 days. Two approaches were used for plasma-treatment of fungi (Alternaria B.): direct and separated. In direct treatment, the sample was one of the electrodes creating the plasma. Plasma discharge occurred between the quartz glass dielectric and the sample, which exposed the sample directly to neutral reactive species and charged particles. In separated plasma treatment, DI water or other fluids like phosphate buffered saline (PBS) was plasma treated separately from the fungi and then immediately applied to fungi. In this case, the fungi were not in direct contact with any species produced by non-thermal plasma. 100 μl of fungal suspension is placed on a microscope slide and allowed to dry completely for about an hour. After drying the fungi are treated with plasma and immediately after treatment the fungi are swabbed from the glass slide and spread on to a sterile agar plate, the plate was sealed with parafilm and incubated at room temperature for five days. As shown in Figure 45A, Figure 45B, Figure 45C and Figure 45D we see

no colony formation after only 30 s of plasma treatment. Direct plasma treatment for 30 s completely inhibits growth of alternaria brassicicola. We see similar results after direct treatment for 60 s and 120 s. Figure 45E shows inactivation curves for directly treated fungi.

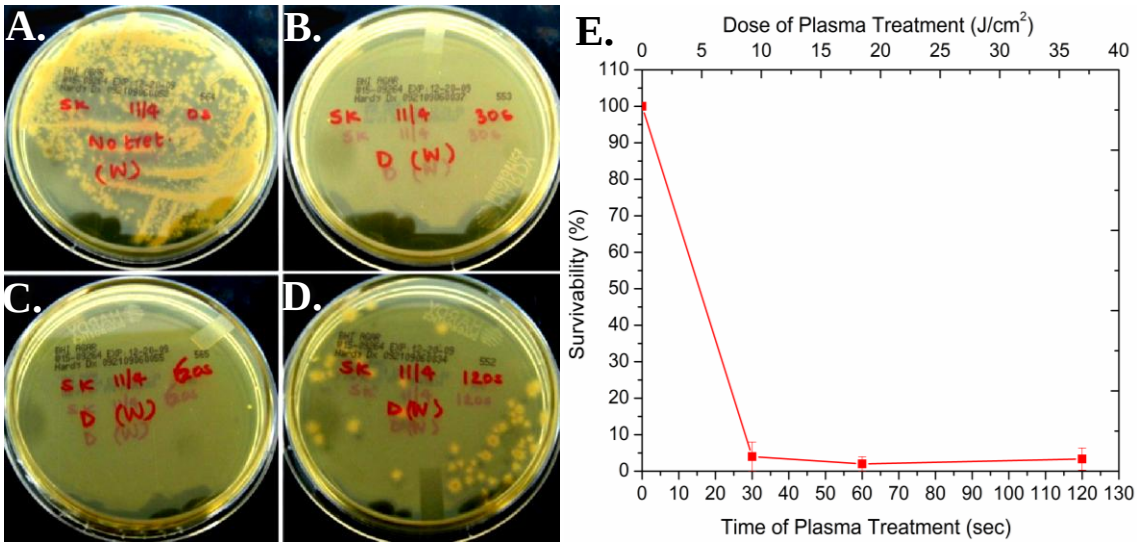

Figure 45. Direct plasma treatment of dried Alternaria Brassicicola. 100 µl of the fungal suspension was placed in a hanging drop microscope slide and allowed to sit for 1 h. After 1 h the dry fungi were directly treated with non-thermal plasma at increasing doses. (A) We see growth of untreated fungi 3 days after plating on an agar dish. (B), (C) and (D) Non-thermal plasma treatment for 30, 60 and 120 s significantly inhibits growth of fungus and we see no growth of fungi five days after plasma treatment. (E) Inactivation of growth of alternaria brassicicola after direct non-thermal plasma treatment

In the separated mode of plasma treatment, 100 µl of fungal suspension was placed on a microscope slide and allowed to dry for an hour. On a separate hanging drop slide 100 µl of DI water was treated at different doses of plasma and after treatment the separately treated water was poured on to the untreated dry fungi and allowed to sit for the same time as the duration of plasma treatment. The suspension was then pipetted on to a sterile agar plate. Any remaining fungi were swabbed with a sterile polystyrene applicator and transferred on to the same agar plate. The suspension was evenly spread

over the agar plate using a sterile spreader. The agar plate was sealed with parafilm and then incubated at room temperature for five days.

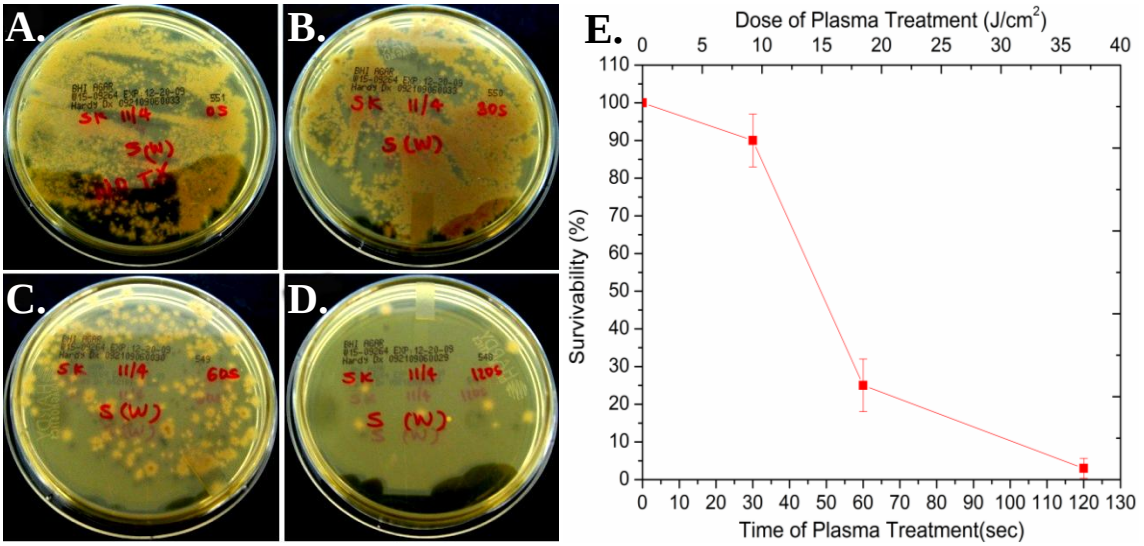

Figure 46. Separated plasma treatment of dried Alternaria Brassicicola on a hanging drop glass slide. 100 μl of the fungal suspension was placed on a microscope slide and allowed to sit for 1 h. After 1 h, DI water was separately treated for varying doses and immediately poured on the dried fungi. The fungi were then plated on agar dishes. (A) We see normal growth of untreated fungi 3 days after plating on an agar dish. (B) Fungi exposed to DI water separately treated with plasma for 30 s seem to grow normally, (C) As we increase the time of exposure to 60 s we start seeing significant inhibition in the growth of fungi after treatment and (D) after 120 s of separated treatment we see an almost complete inhibition of the growth of fungi. (E) Inactivation of growth of alternaria brassicicola after separated non-thermal plasma treatment.

As shown in Figure 46A, Figure 46B, Figure 46C and Figure 46D, the number of fungal colonies formed three days after plasma treatment decreases as the duration of plasma exposure increases from 30 s to 120 s. As shown in Figure 46E, where the number of colonies are quantified as a function of plasma exposure time, we see dose dependent inhibition of fungal growth after separated plasma treatment and we see complete inhibition of fungal growth after 120 s of separated treatment.

As shown in Figure 45 and Figure 46 above non-thermal plasma treatment can inactivate fungi after direct or separated treatment for 120 s. We wanted to test whether the fungal killing effects of plasma are mediated via membrane damage or not. Fungi

were treated as discussed in the above sections. Treated fungi were imaged immediately, 5 min, 30 min and 1 h after treatment with non-thermal plasma for 120 s and 300 s. As shown in Figure 47B and Figure 47C, 1 h after plasma treatment fungi look intact and the membranes do not seem to be ruptured even after treatment for as long as 300 s. 120 s of

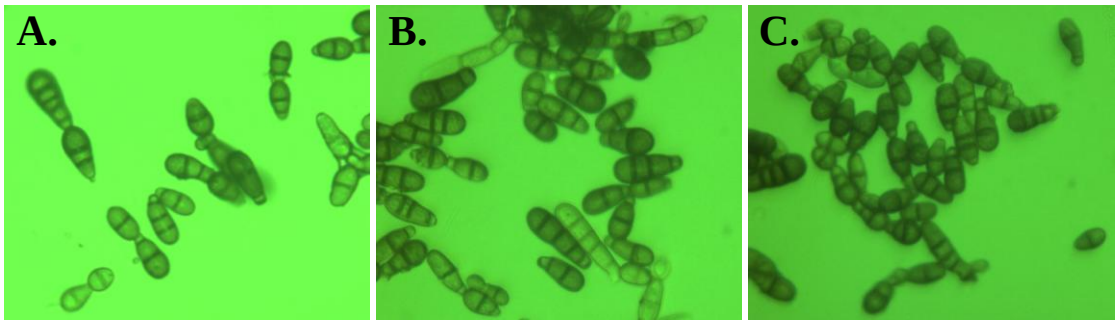

Figure 47. Plasma treatment of Alternaria Brassicicola. (A) Untreated fungi. Fungi were plasma treated on a glass cover slip for (B) 120 s and (C) 300 s, and imaged immediately, after 5 min, 30 min and 1 h. Representative images are shown for samples imaged 1 h after plasma treatment.

plasma treatment was sufficient to kill the fungi. This again confirms the fact that the effects of non-thermal plasma on living cells are not primarily due to physical membrane damage which may be induced by charged particles or lipid peroxidation known to be induced by reactive oxygen species produced by non-thermal plasma. We hypothesize that receptor mediated signaling (or Redox signaling) or active transport mechanisms may play a major role in the interaction of non-thermal plasma with living cells.

6.3.9. Type of DNA Damage

To more precisely identify DNA damage pathways, we next sought to identify the kinase that phosphorylates H2AX in response to non-thermal plasma. H2AX is phospho-

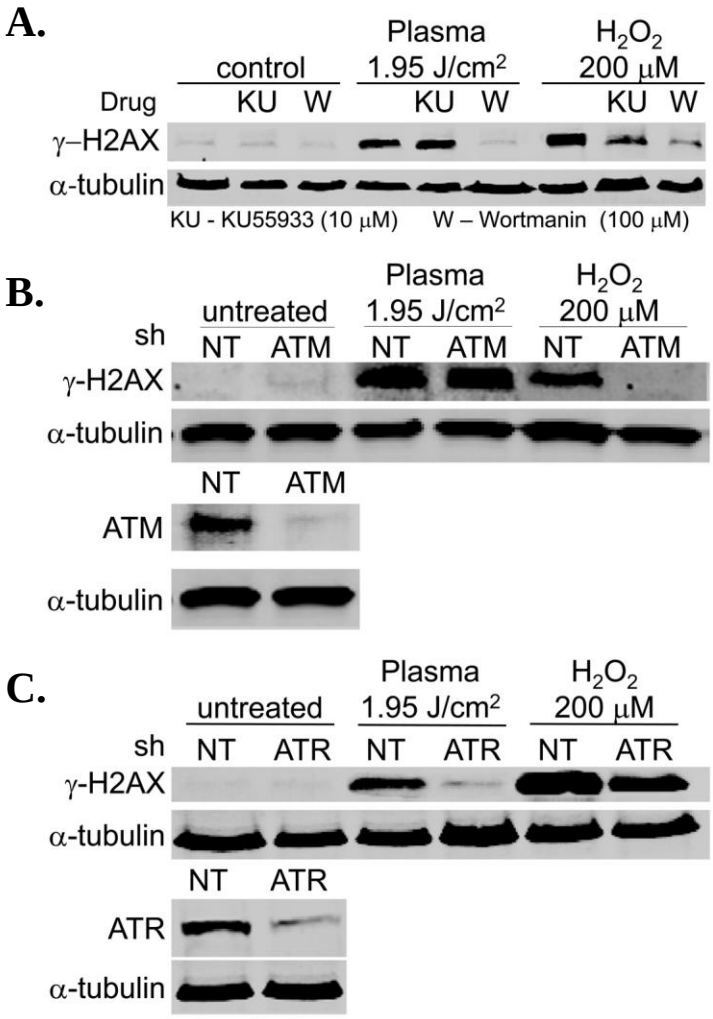

Figure 48. ATR dependence of Non-thermal plasma induced phosphorylation of H2AX. (A) Immunoblot of γ-H2AX (top), and α-tubulin (bottom) from MCF10As exposed to non-thermal plasma at a dose of 1.95 J/cm^2 or 200 μM H_2O_2 in the presence (+) or absence (-) of 100 μmol/L Wortmannin (Wort.) or 10 μmol/L KU55933 (KU). (B) MCF10As were depleted of endogenous ATM by shRNA (bottom, immunoblot of ATM from cells 72 h post transfection with ATM shRNA or nontargeting (NT) shRNA). Seventy-two hours after shRNA transfection, cells were trypsinized and plated on glass cover slips for 24 h. After 24 h, cells were exposed to DBD plasma at a dose of 1.95 J/cm^2 or 200 μM H_2O_2. After one hour incubation, lysates were prepared representative immunoblots with antibody to γ-H2AX or α-tubulin are shown. (C) MCF10As were depleted of endogenous ATR by shRNA (bottom, immunoblot of ATR from cells 72 h post transfection with ATR shRNA or nontargeting (NT) shRNA. Seventy-two hours after shRNA transfection, cells were trypsinized and plated on glass cover slips for 24 h followed by exposure to non-thermal plasma at a dose of 1.95 J/cm^2 or 200 μM H_2O_2. After one hour incubation, lysates were prepared and representative immunoblots with antibody to γ-H2AX or α-tubulin are shown.

rylated on Ser139 by the phosphatidyl inositol kinase-related kinases (PIKK), ataxia telangiectasia mutated (ATM), ATM and Rad - 3-related kinase (ATR) and -DNA protein kinase (DNA-PK). Initially, the effect of addition of KU55933, an ATM specific inhibitor [444] and Wortmannin, which inhibits ATM, ATR, and DNA-PK[445], was assessed. Pretreatment of MCF10A cells with 10 μM KU55933 for 1h did not significantly reduce the phosphorylation of H2AX in response to plasma treatment, whereas it significantly reduced it in response to H_2O_2 (Figure 48A). Phosphorylation of H2AX in response to non-thermal plasma (as well as H_2O_2) was markedly reduced in cells pretreated with 100 μM Wortmannin (Figure 48A). These findings indicate that ATR and/or DNA-PK is required for the phosphorylation of H2AX at Ser139 in response to non-thermal plasma, although they do not rule out that other kinases may involved. To more directly assess the role of ATM and/or ATR, shRNAs were utilized. ATM shRNA effectively reduced levels of ATM, but did not significantly affect the phosphorylation of H2AX induced by non-thermal plasma treatment (1.95 J/cm^2) as compared to non-targeting shRNA, whereas phosphorylation of H2AX was not detected in ATM knockdown cells treated with hydrogen peroxide (Figure 48B). These findings indicate that the ATM is not the primary mediator of H2AX phosphorylation in response to non-thermal plasma treatment. Depletion of ATR, [403], by shRNA reduced phosphorylation of H2AX by 92% relative to non-targeting shRNA; H2AX phosphorylation in response to hydrogen peroxide was reduced 40% by ATR shRNA (Figure 48C). Taken together, our findings demonstrate that non-thermal plasma treatment activates ATR.

6.3.10. Selectivity of Non-Thermal Plasma Treatment

Since effects of non-thermal plasma on mammalian cells seem to be mediated by ROS similar to ionizing radiation or photodynamic therapy, we wanted to compare the effects of non-thermal plasma on normal cells compared to malignant cells. Ionizing radiation and photodynamic therapy are effective anti-cancer treatment therapies because they exploit the susceptibility of malignant cells over healthy cells to oxidative stress by inducing formation of ROS primarily intracellularly which ultimately lead to significant DNA damage that cannot be repaired by malignant cells.

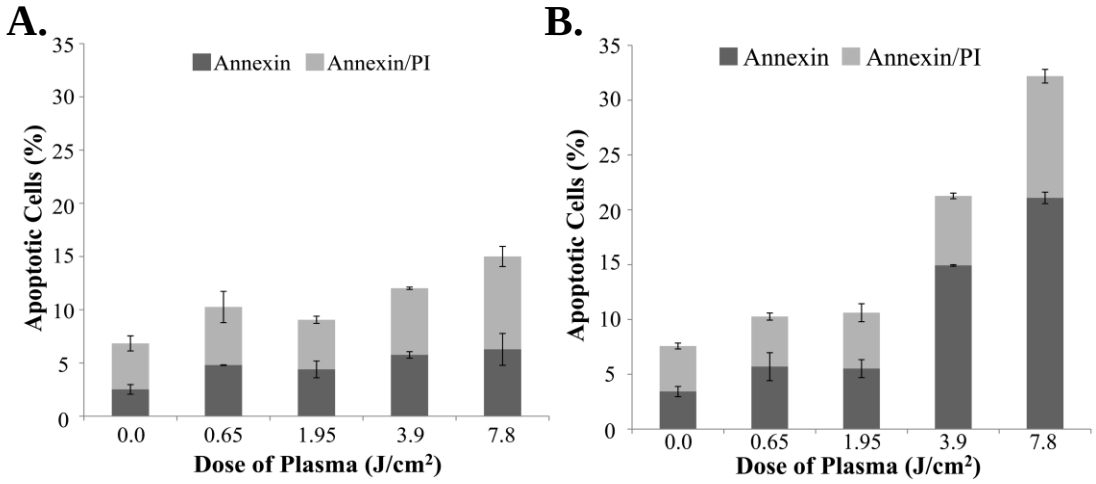

Figure 49. Selectivity of Non-Thermal plasma treatment in inducing apoptosis in malignant cells over healthy cells. (A) 48 h after treatment with the indicated dose of DBD plasma, MCF10A cells were harvested and stained with Annexin V/ propidium iodide (PI) and analyzed by Guava. (B) 48 h after treatment with the indicated dose of DBD plasma, MCF10A - NEUT cells were harvested and stained with Annexin V/ propidium iodide (PI) and analyzed by Guava. Data from triplicate samples (± S.D.) are plotted.

Non-thermal plasma on the other hand produces ROS primarily extracellularly. To see if ROS produced extracellularly by non-thermal plasma induces apoptosis in mammalian cells and to investigate whether malignant cells (MCF10A – NEUT) were more susceptible than healthy cells, normal mammary epithelial cells (MCF10A) and

transformed malignant cells (MCF10A-NUET) were exposed to increasing doses of DBD plasma and level of apoptosis was measured by Annexin-V/PI assay.

After DBD plasma treatment at doses ranging from 0.65 to 7.8 J/cm^2; a dose dependent increase in Annexin-V +ve, PI –ve cells was observed (Figure 49). Further, at low doses up to 1.95 J/cm^2 the level of apoptosis as seen by the Annexin-V +ve cells, in both cells lines (Figure 49) was similar, but at higher doses (> 1.95 J/cm^2) malignant MCF10A-NUET cells (Figure 49B) were more vulnerable to plasma treatment as seen by almost twice the number of apoptotic cells compared to that in normal cells (Figure 49A). These data suggest that malignant cells are more vulnerable to plasma than normal cells. By controlling plasma dose, we may be able to selectively kill malignant cells without inducing significant necrosis and subsequent inflammation in surrounding tissue.

6.4. Discussion

Based on its ability to produce dose-dependent effects from increased proliferation to apoptosis, non-thermal plasma offers the potential for therapeutic applications. In order to exploit the potential therapeutic applications, it is essential to understand the mechanism by which it can exert effects. The present study demonstrates that the dose- dependent effects of non-thermal plasma on mammalian cells are related to production of intracellular ROS, which induce a variety of effects, ranging from increased cell proliferation to apoptosis depending on concentration [413]. While the effects of IR and hydrogen peroxide are also primarily due to generation of ROS, these agents induce several different types of DNA damage directly, including DNA double strand breaks. A significant limitation of IR is the requirement for specialized equipment

and skilled personnel. Non-thermal plasma can be generated with a reasonably portable, compact and inexpensive device.

Cell death in response to non-thermal plasma treatment in the dose range examined is primarily though induction of apoptosis, which is an important therapeutic consideration. Apoptotic cells are broken up into apoptotic bodies, which are engulfed by neighboring cells, leading to cell death without significant inflammatory response [338, 339]. Controlled delivery of non-thermal plasma may provide a means to kill benign and malignant lesions in a defined area, without significant necrosis and subsequent inflammation. Delivery can be achieved by direct treatment of tissue surfaces or application of defined media treated with plasma.

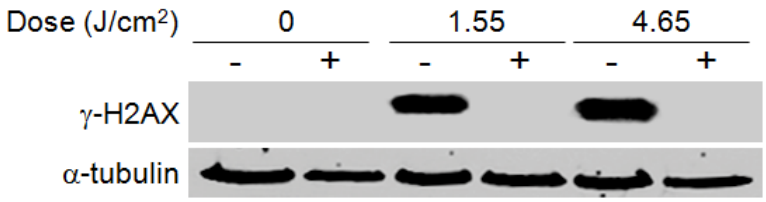

Figure 50. DBD plasma produced UV does not does not induce the observed DNA damage. Cells overlayed with 100 μl of medium were treated with DBD plasma at 1.55 J/cm^2 and 4.65 J/cm^2 with (+) and without (-) placing magnesium fluoride (MgF_2) glass on the cells during treatment. MgF_2 glass blocks all plasma species except UV from reaching the surface of the medium covering the cells during treatment. Representative immunoblot with γ-H2AX (upper panel) or α-tubulin (lower panel) is shown.

Plasma-induced DNA damage is likely initiated by neutral species which plasma produces in gas phase. As shown in Table 2, Non-thermal atmospheric pressure DBD plasmas produce long lived (O_3, NO, HO_2, H_2O_2) and short lived (OH, O, electronically excited oxygen O) neutral particles and charged particles which include ions and electrons [17]). Exclusion of charged particles (ions and electrons) by using a grounded mesh did not significantly affect H2AX phosphorylation, indicating that neutral species produced in the gas phase and not charged species are responsible for these effects.

Moreover, the results of the experiments comparing direct treatment with indirect and separated plasma treatment rule out any role of DBD plasma produced UV, temperature or electric field. DBD plasma is known to produce very weak UV and it has been shown to have no effect on living organisms [20, 28, 446]. Blocking of all plasma species, except UV by a magnesium fluoride glass placed on the cells during treatment completely blocked the phosphorylation of H2AX after DBD plasma treatment (Figure 50). This shows that UV does not play a role in DBD plasma induced DNA damage in mammalian cells. Further, the results of the experiments comparing direct treatment with indirect and separated plasma treatment rule out any role of heating as DBD plasma does not directly come in contact with medium and cells.

Mammalian cells undergo DNA damage as a result of ROS generated from endogenous and exogenous sources. The induction of DNA damage by non-thermal plasma is mediated through the formation of ROS. The intracellular ROS scavenger, NAC, completely blocked phosphorylation of H2AX after non-thermal plasma treatment of MCF10A cells. The role of ROS is further supported by direct measurement of intracellular ROS and by the formation of lipid peroxidation products, specifically MDA (Figure 42 and Figure 43).

We have provided several lines of evidence that the formation of intracellular ROS as a result of plasma treatment results from formation of organic peroxides in the media. Media separately treated with plasma remains active over extended periods of time, which would not be the case with inorganic peroxides (e.g. H_2O_2) and is consistent with published reports on organic peroxides [233]. Additionally, there is a direct correlation between the peroxidation efficiency of 11 different amino acids and the

amount of DNA damage induced in cells exposed to these amino acid solutions. Taken together, these data strongly support the conclusion that the effects of DBD plasma are mediated by the organic peroxides formed in the media, although formation of organic peroxides in plasma-treated media should be directly measured.

ROS–induced DNA damage includes small or bulky modifications to bases and sugars, interstrand and intrastrand cross-links, as well as single-strand breaks and double-strand breaks [62, 447]. Complex molecular networks that rapidly sense and repair DNA damage have evolved to maintain genomic stability and ensure cell survival [448]. Oxidative stress and IR activate the phosphatidylinositol 3-kinase–related kinases (PIKK), including ataxia-telangiectasia mutated (ATM), DNA-PK, and ATM and Rad3-related kinase (ATR) [445]. The histone variant H2AX is phosphorylated on Ser139 by ATM, ATR, and DNA-PK over a large region of chromatin surrounding a DSB [449, 450]. In this study, we have shown that phosphorylation of H2AX after non-thermal plasma treatment of MCF10A cells is primarily through ATR, in contrast to the ATM-dependent phosphorylation of H2AX after treatment of cells with IR or hydrogen peroxide. Our studies suggest that oxidative stress induced by non-thermal plasma treatment of cells leads to activation of ATR, suggesting that unlike IR and H_2O_2, non-thermal plasma primarily induces bulky lesions and stalled replication forks.

It has been shown earlier that reactive oxygen species mediate non-thermal plasma induced DNA damage in mammalian cells. Reactive oxygen species also interact with cellular membrane leading to lipid peroxidation. We investigated the role of ROS produced by non-thermal plasma in inducing lipid peroxidation in mammalian cells. It was hypothesized that non-thermal plasma induces DNA damage in mammalian cells via

lipid peroxidation. The results indicate that non-thermal plasma indeed leads to lipid peroxidation, but non-thermal plasma induced lipid peroxidation by-products is not what induces DNA damage. Further investigations are necessary to determine other pathways of non-thermal plasma induced DNA damage in mammalian cells, which may include reactive oxygen species mediated cellular signaling or uptake of long lived reactive oxygen species via active transport.

6.5. Summary

Clinical application of DBD plasma requires an understanding of its interaction with living tissues. We have shown here that DBD plasma generates ROS in gas phase, which can react with organic components such as serum or amino acids to produce long living reactive species, mostly likely amino acid and protein hydroperoxides. The amount of intracellular ROS produced by plasma can be controlled by varying the frequency and voltage waveform, allowing fine tuning of therapeutic effect, from stimulating cell proliferation to inducing apoptosis, essentially by modulating the level of ROS produced. Generation of organic peroxides in solution may provide an alternative means of administration. Understanding the mechanism underlying the effects of plasma is an essential first step in applying it to clinical use. Future work will involve investigating the mechanism by which peroxidized amino acids are processed by the cell. The only limitation to using DBD plasma for a wide range of clinical applications is designing devices for plasma delivery appropriate for the use. The potential clinical applications of DBD plasma include treatment of wounds to enhance healing and sterilize wound surfaces or controlled ablation of tissue, including benign lesions or cancers.

Chapter 7. Concluding Remarks

This thesis demonstrates that the dose-dependent effects of non-thermal plasma on mammalian cells are related to production of reactive oxygen species, which induce a variety of effects, ranging from increased cell proliferation to apoptosis depending on concentration. Non-thermal plasma offers the potential for therapeutic applications ranging from promoting wound healing to treating cancer. In order to utilize non-thermal plasma for therapeutic applications, it is essential to understand the mechanism by which it can exert these effects. While the effects of IR and hydrogen peroxide are also primarily due to generation or ROS, these agents induce several different types of DNA damage, including DNA double strand breaks. A significant limitation of IR or hydrogen peroxide is the lack of precise control over the area and depth of effects required to avoid injury to surrounding tissue. Exquisite control of treatment area and dose can be achieved with non-thermal plasma, which can be generated with a reasonably portable, compact and inexpensive device, in contrast to IR, which requires expensive facilities and highly skilled professionals.

Cell death in response to non-thermal plasma treatment is primarily though induction of apoptosis, which is an important therapeutic consideration. Plasma-induced DNA damage is likely related to neutral ROS which cannot be attributed to ozone alone. Moreover, we have shown that phosphorylation of H2AX after non-thermal plasma treatment of mammalian cells is primarily through ATR, in contrast to the ATM-dependent phosphorylation of H2AX after treatment of cells with IR or hydrogen peroxide. Our studies suggest that oxidative stress induced by non-thermal plasma treatment of cells leads to activation of ATR, suggesting that unlike IR and H_2O_2, non-

thermal plasma primarily induces bulky lesions. Further we also showed that although non-thermal plasma induces lipid peroxidation of the plasma membrane, the pathway by which plasma generated oxidative stress is transferred across cellular membranes does not involve lipid peroxidation by-products.

Clinical application of DBD plasma requires an understanding of its interaction with living tissues. We have shown here that DBD plasma generates ROS, which can react with organic components such as serum or amino acids to produce long living reactive amino acid and protein hydroperoxides. The amount of ROS produced by plasma can be tightly controlled by varying the frequency and voltage waveform, allowing fine tuning of therapeutic effect, from stimulating cell proliferation to inducing apoptosis, essentially by modulating the level of ROS produced. Generation of organic peroxides in solution may provide an alternative means of administration. Understanding the mechanism underlying the effects of plasma is an essential first step in applying it to clinical use.

Future work will involve investigating the mechanisms by which hydroperoxides are processed by the cell. It would be important to establish whether the effects of plasma are due to their uptake by active transport mechanisms or activation of intracellular signaling pathways due to binding of organic hydroperoxides to receptors on the cell membrane. The only limitation to using DBD plasma for a wide range of clinical applications is designing devices for plasma delivery appropriate for the use. The potential clinical applications of DBD plasma include treatment of wounds to enhance healing and sterilize wound surfaces or controlled ablation of tissue, including benign lesions or cancers.

Bibliography

1. Mott-Smith, H.M., *History of "Plasmas".* Nature, 1971. **233**(5316): p. 219.

2. Rappaport, W.D., et al., *Effect of electrocautery on wound healing in midline laparotomy incisions.* Am J Surg, 1990. **160**(6): p. 618-20.

3. Lord, M.J., J.A. Maltry, and L.M. Shall, *Thermal injury resulting from arthroscopic lateral retinacular release by electrocautery: report of three cases and a review of the literature.* Arthroscopy, 1991. **7**(1): p. 33-7.

4. Ginsberg, G.G., et al., *The argon plasma coagulator.* Gastrointestinal Endoscopy, 2002. **55**(7): p. 807-810.

5. Vargo, J.J., *Clinical applications of the argon plasma coagulator. Gastrointest Endosc*, 2004. **59**(1): p. 81-8.

6. Colt, H.G. and S.W. Crawford, *In vitro study of the safety limits of bronchoscopic argon plasma coagulation in the presence of airway stents.* Respirology, 2006. **11**(5): p. 643-7.

7. Ginsberg, G.G., et al., *The argon plasma coagulator: February 2002.* Gastrointest Endosc, 2002. **55**(7): p. 807-10.

8. Porter, K.A., et al., *Electrocautery as a factor in seroma formation following mastectomy.* Am J Surg, 1998. **176**(1): p. 8-11.

9. Puhlev, I., et al., *Desiccation tolerance in human cells.* Cryobiology, 2001. **42**(3): p. 207-17.

10. Raiser, J., Zenker, M., *Argon plasma coagulation for open surgical and endoscopic applications: state of the art.* Journal of Physics D: Applied Physics, 2006. **39**(16): p. 3520.

11. Simmons, P.D., F. Langlet, and R.N. Thin, *Cryotherapy versus electrocautery in the treatment of genital warts.* Br J Vener Dis, 1981. **57**(4): p. 273-4.

12. Fridman, G., et al., *Blood Coagulation and Living Tissue Sterilization by Floating-Electrode Dielectric Barrier Discharge in Air Plasma Chemistry and Plasma Processing*, 2006. **26**(4): p. 425-442.

13. Kalghatgi, S., et al., *Mechanism of Blood Coagulation by Nonthermal Atmospheric Pressure Dielectric Barrier Discharge Plasma. Plasma Science, IEEE Transactions on*, 2007. **35**(5): p. 1559-1566.

14. Stoffels, E., *Gas plasmas in biology and medicine. Journal of Physics D: Applied Physics*, 2006. **39**(16).

15. Siemens, C.W., *On the Electrical Tests Employed During the Construction of the Malta and Alexandria Telegraph, and on Insulating and Protecting Submarine Cables Journal of the Franklin Institute*, 1862. **74**(3): p. 166-170.

16. Eliasson, B., W. Egli, and U. Kogelschatz, *Modelling of dielectric barrier discharge chemistry. Pure and Applied Chemistry*, 1994. **66**(6): p. 1275-1286.

17. Fridman, A., *Plasma Biology and Plasma Medicine*, in *Plasma Chemistry*. 2008, Cambridge University Press: New York, NY. p. 848-857.

18. Ayan, H., et al., *Heating Effect of Dielectric Barrier Discharges for Direct Medical Treatment.* Plasma Science, IEEE Transactions on, 2009. **37**(1): p. 113-120.

19. Fridman, A., *Plasma Biology and Plasma Medicine*. 2008, New York: Cambridge University Press.

20. Laroussi, M. and F. Leipold, *Evaluation of the roles of reactive species, heat, and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure.* International Journal of Mass Spectrometry, 2004. **233**(1-3): p. 81-86.

21. Fridman, A., *Plasma Chemistry*. 2008, New York, NY: Cambridge University Press.

22. Fridman, A., Chirokov, A., Gutsol, A., *Non-thermal atmospheric pressure discharges.* Journal of Physics D: Applied Physics, 2005. **38**: p. R1-R24.

23. Laroussi, M., et al., *Inactivation of Bacteria by the Plasma Pencil.* Plasma Processes and Polymers, 2006. **3**(6-7): p. 470-473.

24. Laroussi, M., D.A. Mendis, and M. Rosenberg, *Plasma interaction with microbes. New Journal of Physics*, 2003. **5**: p. 41.1-41.10.

25. Laroussi, M., I. Alexeff, and W.L. Kang, *Biological decontamination by nonthermal plasmas. Plasma Science, IEEE Transactions on,* 2000. **28**(1): p. 184-188.

26. Laroussi, M., *Low Temperature Plasma-Based Sterilization: Overview and State-of-the-Art.* Plasma Processes and Polymers, 2005. **2**: p. 391-400.

27. Laroussi, M., Alexeff, I., Richardson, J.P., Dyer, F.F., *The resistive barrier discharge.* IEEE Transactions on Plasma Science, 2002. **30**(1): p. 158-159.

28. Fridman, G., et al., *Comparison of Direct and Indirect Effects of Non-Thermal Atmospheric-Pressure Plasma on Bacteria. Plasma Processes and Polymers*, 2007. **4**(4): p. 370-375.

29. Kieft, I.E., M. Kurdi, and E. Stoffels, *Reattachment and Apoptosis After Plasma-Needle Treatment of Cultured Cells. Plasma Science, IEEE Transactions on,* 2006. **34**(4): p. 1331-1336.

30. Coulombe, S. *Live cell permeabilization using the APGD-t*. in *1st International Conference on Plasma Medicine (ICPM)*. 2007. Corpus Christi, TX.

31. Coulombe, S., et al., *Miniature atmospheric pressure glow discharge torch (APGD-t) for local biomedical applications*. Pure and Applied Chemistry*, 2006. **78**(6): p. 1137-1146.

32. Léveillé, V. and S. Coulombe, *Design and preliminary characterization of a miniature pulsed RF APGD torch with downstream injection of the source of reactive species. Plasma Sources Science and Technology*, 2005. **14**: p. 467-476.

33. Kieft, I.E., et al., *Plasma treatment of mammalian vascular cells: a quantitative description. Plasma Science, IEEE Transactions on*, 2005. **33**(2): p. 771-775.

34. Stoffels, E., et al., *Plasma needle for in vivo medical treatment: recent developments and perspectives. Plasma Sources Science and Technology*, 2006. **15**(4): p. S169-S180.

35. Fridman, G., et al. *Nonequilibrium dielectric barrier discharge plasma promoting apoptotic behavior in melanoma skin cancer cells*. 2007. Piscataway, NJ, USA: IEEE.

36. Kalghatgi, S.U., et al., *Non-thermal dielectric barrier discharge plasma treatment of endothelial cells.* Conf Proc IEEE Eng Med Biol Soc, 2008. **2008**: p. 3578-81.

37. Gostev, V. and D. Dobrynin. *Medical microplasmatron*. in *3rd International Workshop on Microplasmas (IWM-2006)*. 2006. Greifswald, Germany. .

38. Kalghatgi, S., et al. *Applications of Non Thermal Atmospheric Pressure Plasma in Medicine*. in *NATO Advanced Study Institute on Plasma Assisted Decontamination of Biological and Chemical Agents*. 2007. Cesme-Izmir, Turkey: Springer.

39. Shekhter, A.B., et al., *Beneficial effect of gaseous nitric oxide on the healing of skin wounds Nitric Oxide-Biology and Chemistry*, 2005. **12**(4): p. 210-219.

40. Kalghatgi, S., et al. *Toxicity analysis of direct Non-thermal Plasma Treatment of Living Tissue*. in *IEEE 35th International Conference on Plasma Science*. 2008. Karlsruhe, Germany.

41. Kalghatgi, S., et al. *Toxicity of Direct Non-Thermal Atmospheric Pressure Plasma Treatment of Living Tissue*. in *Proceedings of the IEEE 17th International Pulsed Power Conference*. 2009. Washington DC, USA.

42. Fridman, G., et al., *Floating Electrode Dielectric Barrier Discharge Plasma in Air Promoting Apoptotic Behavior in Melanoma Skin Cancer Cell Lines* Plasma Chemistry and Plasma Processing, 2007. **27**(2): p. 163-176.

43. Vandamme, M., et al., *Response Of Human Glioma U87 Xenografted On Mice To Non Thermal Plasma Treatment.* Plasma Medicine,, 2010. **In Press**.

44. Kalghatgi, S., et al., *Endothelial Cell Proliferation is Enhanced by Low Dose Non-Thermal Plasma Through Fibroblast Growth Factor-2 Release.* Ann Biomed Eng, 2009.

45. Sakai, Y., et al., *A novel transfection method for mammalian cells using gas plasma.* J Biotechnol, 2006. **121**(3): p. 299-308.

46. Leduc, M., et al., *Cell permeabilization using a non-thermal plasma.* New Journal of Physics, 2009(11): p. 115021.

47. Fridman, G., et al. *Bio-Medical Applications of Non-Thermal Atmospheric Pressure Plasma*. in *37th AIAA Plasmadynamics and Lasers Conference*. 2006. San Francisco, CA.

48. Sladek, R.E.J.B., T.A.; Stoffels, E., *Plasma-Needle Treatment of Substrates With Respect to Wettability and Growth of Escherichia coli and Streptococcus mutans.* IEEE Transactions on Plasma Science, 2006. **34**(4): p. 1325-1330.

49. Sladek, R.E.J., et al., *Plasma treatment of dental cavities: a feasibility study.* Plasma Science, IEEE Transactions on, 2004. **32**(4): p. 1540-1543.

50. Sladek, R.E.J., Stoffels, E., *Deactivation of Escherichia coli by the plasma needle.* Journal of Physics D: Applied Physics, 2005. **38**: p. 1716-1721.

51. Goree, J., Bin l., Drake, D., Stoffels, E., *Killing of S. mutans Bacteria Using a Plasma Needle at Atmospheric Pressure.* IEEE Transactions on Plasma Science, 2006. **34**(4): p. 1317-1324.

52. Stoffels, E., et al., *Cold gas plasma in biology and medicine*, in *Advanced Plasma Technology*, R. d'Agostino, et al., Editors. 2008, Wiley-VCH: Weinheim. p. 301-318.

53. Modesti, M. and R. Kanaar, *DNA repair: spot(light)s on chromatin.* Curr Biol, 2001. **11**(6): p. R229-32.

54. Halliwell, B. and J.M.C. Gutteridge, *Free Radicals in Biology and Medicine*. 4th ed ed. 2007, New York: Oxford University Press.

55. Kohen, R. and A. Nyska, *Invited Review: Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification.* Toxicol Pathol, 2002. **30**(6): p. 620-650.

56. Sies, H., *Biochemistry of Oxidative Stress.* Angewandte Chemie International Edition in English, 1986. **25**(12): p. 1058-1071.

57. Decoursey, T.E. and E. Ligeti, *Regulation and termination of NADPH oxidase activity.* Cell Mol Life Sci, 2005. **62**(19-20): p. 2173-93.

58. Spitz, D.R., et al., *Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: a unifying concept in stress response biology.* Cancer Metastasis Rev, 2004. **23**(3-4): p. 311-22.

59. Okayama, Y., *Oxidative stress in allergic and inflammatory skin diseases.* Curr Drug Targets Inflamm Allergy, 2005. **4**(4): p. 517-9.

60. Moreira, P.I., et al., *Oxidative stress: the old enemy in Alzheimer's disease pathophysiology.* Curr Alzheimer Res, 2005. **2**(4): p. 403-8.

61. Lankin, V.Z., et al., *Oxidative stress in atherosclerosis and diabetes.* Bull Exp Biol Med, 2005. **140**(1): p. 41-3.

62. Roberfroid, M., Calderon, P.B., *Free radicals and oxidation phenomena in biological systems*. 1995, New York: Marcel Dekker, Inc.

63. Halliwell, B. and J.M. Gutteridge, *Oxygen toxicity, oxygen radicals, transition metals and disease.* Biochem J, 1984. **219**(1): p. 1-14.

64. Briviba, K., L.O. Klotz, and H. Sies, *Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems.* Biol Chem, 1997. **378**(11): p. 1259-65.

65. Oya, Y. and K. Yamamoto, *The biological activity of hydrogen peroxide. IV. Enhancement of its clastogenic actions by coadministration of L-histidine.* Mutat Res, 1988. **198**(1): p. 233-40.

66. Schubert, J. and J.W. Wilmer, *Does hydrogen peroxide exist "free" in biological systems?* Free Radic Biol Med, 1991. **11**(6): p. 545-55.

67. Kohen, R., E. Moor, and M. Oron, *Measurements of biological reducing power in health and diseases by voltammetric methods,* in *Redox Genome Interaction in Health and Disease*, J. Fuchs and L. Packer, Editors. 2004, Marcel Decker: New York. p. 13-42.

68. von Sonntag, C., *The Chemical Basis of Radiation Biology*. 1987, London: Taylor and Francis.

69. Bielski, B.H.J. and D.E. Cabelli, *Superoxide and Hydroxyl radical chemistry in aqueous solution*, in *Active Oxygen in Chemistry*, C.S. Foote, et al., Editors. 1995, Chapman and Hall: London. p. 66-104.

70. Schafer, F.Q. and G.R. Buettner, *Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple.* Free Radic Biol Med, 2001. **30**(11): p. 1191-212.

71. Bielski, B.H.J., et al., *Reactivity of RO_2/$O_2^{\cdot-}$ radicals in aqueous solution.* J Phys Chem Ref Data, 1985. **14**: p. 1041-1100.

72. Andrekopoulos, C., et al., *Bicarbonate enhances alpha-synuclein oligomerization and nitration: intermediacy of carbonate radical anion and nitrogen dioxide radical.* Biochem J, 2004. **378**(Pt 2): p. 435-47.

73. Sawyer, D.T. and J.S. Valentine, *How super is $O_2^{\cdot-}$?* Acc Chem Res, 1981. **14**: p. 393.

74. Halliwell, B.a. and J.M.C. Gutteridge, *Free Radicals in Biology and Medicine*. 3rd ed. 1999, Oxford: Oxford University Press.

75. Halliwell, B., M.V. Clement, and L.H. Long, *Hydrogen peroxide in the human body.* FEBS Lett, 2000. **486**(1): p. 10-3.

76. Sies, H. and C.F. Menck, *Singlet oxygen induced DNA damage.* Mutat Res, 1992. **275**(3-6): p. 367-75.

77. Kellogg, E.W. and I. Fridovich, *Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system.* Journal of biological chemistry, 1975. **250**(22): p. 8812-8817.

78. Grune, T., et al., *Protein oxidation and proteolysis by the nonradical oxidants singlet oxygen or peroxynitrite.* Free Radic Biol Med, 2001. **30**(11): p. 1243-53.

79. Goldstein, S. and G. Czapski, *The reaction of NO. with O2.- and HO2.: a pulse radiolysis study.* Free Radic Biol Med, 1995. **19**(4): p. 505-10.

80. Czapski, G. and S. Goldstein, *The role of the reactions of .NO with superoxide and oxygen in biological systems: a kinetic approach.* Free Radic Biol Med, 1995. **19**(6): p. 785-94.

81. Beckman, J.S. and W.H. Koppenol, *Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly.* Am J Physiol, 1996. **271**(5 Pt 1): p. C1424-37.

82. Kohen, R. and I. Gati, *Skin low molecular weight antioxidants and their role in aging and in oxidative stress.* Toxicology, 2000. **148**(2-3): p. 149-57.

83. Roth, R.N. and L.D. Weiss, *Hyperbaric oxygen and wound healing.* Clin Dermatol, 1994. **12**(1): p. 141-56.

84. Kohen, R., *Skin antioxidants: their role in aging and in oxidative stress--new approaches for their evaluation.* Biomed Pharmacother, 1999. **53**(4): p. 181-92.

85. Bhalla, D.K., *OZONE-INDUCED LUNG INFLAMMATION AND MUCOSAL BARRIER DISRUPTION: TOXICOLOGY, MECHANISMS, AND IMPLICATIONS.* Journal of Toxicology and Environmental Health Part B: Critical Reviews, 1999. **2**: p. 31-86.

86. Rao, M.V. and K.R. Davis, *The physiology of ozone induced cell death.* Planta, 2001. **213**(5): p. 682-90.

87. Shadyro, O.I., I.L. Yurkova, and M.A. Kisel, *Radiation-induced peroxidation and fragmentation of lipids in a model membrane.* International Journal of Radiation Biology, 2002. **78**(3): p. 211-217.

88. Pentland, A.P., *Active oxygen mechanisms of UV inflammation.* Adv Exp Med Biol, 1994. **366**: p. 87-97.

89. Victorin, K., *Review of the genotoxicity of nitrogen oxides.* Mutat Res, 1994. **317**(1): p. 43-55.

90. Koren, H.S., *Association between criteria air pollutants and asthma.* Environ Health Perspect, 1995. **103**: p. 235-242.

91. Ray, R.S., et al., *Evaluation of UV-induced superoxide radical generation potential of some common antibiotics.* Drug Chem Toxicol, 2001. **24**(2): p. 191-200.

92. Naito, Y., et al., *Role of oxygen radical and lipid peroxidation in indomethacin-induced gastric mucosal injury.* Dig Dis Sci, 1998. **43**(9 Suppl): p. 30S-34S.

93. Chinev, S., et al., *Lipid peroxidation in rat lung induced by neuroleptanalgesia and its components.* Eur J Anaesthesiol, 1998. **15**(6): p. 686-94.

94. Wormser, U., et al., *Topical iodine preparation as therapy against sulfur mustard-induced skin lesions.* Toxicol Appl Pharmacol, 2000. **169**(1): p. 33-9.

95. Obata, T., et al., *Release of dopamine by perfusion with 1-methyl-4-phenylpyridinium ion (MPP(+)) into the striatum is associated with hydroxyl free radical generation.* Brain Res, 2001. **906**(1-2): p. 170-5.

96. Jones, D.P., et al., *Redox state of glutathione in human plasma.* Free Radic Biol Med, 2000. **28**(4): p. 625-35.

97. Elsayed, N.M., et al., *Free radical-mediated lung response to the monofunctional sulfur mustard butyl 2-chloroethyl sulfide after subcutaneous injection.* Toxicology, 1992. **72**(2): p. 153-65.

98. Lijinsky, W., *N-Nitroso compounds in the diet.* Mutat Res, 1999. **443**(1-2): p. 129-38.

99. Kanner, J. and T. Lapidot, *The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants.* Free Radic Biol Med, 2001. **31**(11): p. 1388-95.

100. Ames, B.N., *Food constituents as a source of mutagens, carcinogens, and anticarcinogens.* Prog Clin Biol Res, 1986. **206**: p. 3-32.

101. Srigiridhar, K., et al., *Oral repletion of iron induces free radical mediated alterations in the gastrointestinal tract of rat.* Mol Cell Biochem, 2001. **219**(1-2): p. 91-8.

102. Shaul, P.W., *Regulation of endothelial nitric oxide synthase: location, location, location.* Annu Rev Physiol, 2002. **64**: p. 749-74.

103. Lewen, A., P. Matz, and P.H. Chan, *Free radical pathways in CNS injury.* J Neurotrauma, 2000. **17**(10): p. 871-90.

104. Canas, P.E., *The role of xanthine oxidase and the effects of antioxidants in ischemia reperfusion cell injury.* Acta Physiol Pharmacol Ther Latinoam, 1999. **49**(1): p. 13-20.

105. Forman, H.J. and M. Torres, *Redox signaling in macrophages.* Mol Aspects Med, 2001. **22**(4-5): p. 189-216.

106. Dhalla, N.S., R.M. Temsah, and T. Netticadan, *Role of oxidative stress in cardiovascular diseases.* J Hypertens, 2000. **18**(6): p. 655-73.

107. Ginsburg, I. and R. Kohen, *Synergistic effects among oxidants, membrane-damaging agents, fatty acids, proteinases, and xenobiotics: killing of epithelial cells and release of arachidonic acid.* Inflammation, 1995. **19**(1): p. 101-18.

108. Ginsburg, I., *Could synergistic interactions among reactive oxygen species, proteinases, membrane-perforating enzymes, hydrolases, microbial hemolysins and cytokines be the main cause of tissue damage in infectious and inflammatory conditions?* Med Hypotheses, 1998. **51**(4): p. 337-46.

109. Babior, B.M., J.D. Lambeth, and W. Nauseef, *The neutrophil NADPH oxidase.* Arch Biochem Biophys, 2002. **397**(2): p. 342-4.

110. Rodrigues, M.R., et al., *Macrophage activation includes high intracellular myeloperoxidase activity.* Biochem Biophys Res Commun, 2002. **292**(4): p. 869-73.

111. Hawkins, C.L., B.E. Brown, and M.J. Davies, *Hypochlorite- and hypobromite-mediated radical formation and its role in cell lysis.* Arch Biochem Biophys, 2001. **395**(2): p. 137-45.

112. Robinson, J.M., T. Ohira, and J.A. Badwey, *Regulation of the NADPH-oxidase complex of phagocytic leukocytes. Recent insights from structural biology, molecular genetics, and microscopy.* Histochem Cell Biol, 2004. **122**(4): p. 293-304.

113. Curnutte, J.T., D.M. Whitten, and B.M. Babior, *Defective superoxide production by granulocytes from patients with chronic granulomatous disease.* N Engl J Med, 1974. **290**(11): p. 593-7.

114. Jalkanen, S. and M. Salmi, *Cell surface monoamine oxidases: enzymes in search of a function.* EMBO J, 2001. **20**(15): p. 3893-901.

115. Fleury, C., B. Mignotte, and J.L. Vayssiere, *Mitochondrial reactive oxygen species in cell death signaling.* Biochimie, 2002. **84**(2-3): p. 131-41.

116. Ames, B.N., M.K. Shigenaga, and T.M. Hagen, *Mitochondrial decay in aging.* Biochim Biophys Acta, 1995. **1271**(1): p. 165-70.

117. Richter, C., et al., *Oxidants in mitochondria: from physiology to diseases.* Biochim Biophys Acta, 1995. **1271**(1): p. 67-74.

118. Brunk, U.T. and A. Terman, *The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis.* Eur J Biochem, 2002. **269**(8): p. 1996-2002.

119. Violi, F. and R. Cangemi, *Oxidative stress in the pathogenesis/treatment of atherosclerosis.* Curr Nutr Food Sci, 2007. **3**: p. 200-208.

120. Smith, M.A., et al., *Oxidative stress in Alzheimer's disease.* Biochim Biophys Acta, 2000. **1502**(1): p. 139-44.

121. McGeer, P.L. and E.G. McGeer, *Mechanisms of cell death in Alzheimer disease--immunopathology.* J Neural Transm Suppl, 1998. **54**: p. 159-66.

122. Levy, J.H., *The human inflammatory response.* J Cardiovasc Pharmacol, 1996. **27 Suppl 1**: p. S31-7.

123. Laviano, A., *Never Underestimate The Power of ROS - The Controversial Role of Oxidative Stress in Health and Disease.* Current Nutrition & Food Science, 2007. **3**: p. 183.

124. Klaunig, J.E. and L.M. Kamendulis, *The role of oxidative stress in carcinogenesis.* Annu Rev Pharmacol Toxicol, 2004. **44**: p. 239-67.

125. Jenner, P., *Oxidative stress in Parkinson's disease.* Ann Neurol, 2003. **53 Suppl 3**: p. S26-36; discussion S36-8.

126. Harrison, D., et al., *Role of oxidative stress in atherosclerosis.* Am J Cardiol, 2003. **91**(3A): p. 7A-11A.

127. Gutteridge, J.M., *Free radicals in disease processes: a compilation of cause and consequence.* Free Radic Res Commun, 1993. **19**(3): p. 141-58.

128. Baud, L. and R. Ardaillou, *Reactive oxygen species: production and role in the kidney.* Am J Physiol, 1986. **251**(5 Pt 2): p. F765-76.

129. Nyska, A., A. Dayan, and R.R. Maronpot, *New tools in therapeutic research--prostatic cancer and models.* Toxicol Pathol, 2002. **30**(2): p. 283-7.

130. Mantovani, G., et al., *Antioxidant therapy for the treatment of oxidative stress associated to cancer and cancer- related anorexia/cachexia.* Curr Nutr Food Sci 2007. **3**: p. 184-193.

131. Droge, W., *Free radicals in the physiological control of cell function.* Physiol Rev, 2002. **82**(1): p. 47-95.

132. Brown, N.S. and R. Bicknell, *Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer.* Breast Cancer Res, 2001. **3**(5): p. 323-7.

133. Najemnik, C., H. Sinzinger, and H. Kritz, *Endothelial dysfunction, atherosclerosis and diabetes.* Acta Med Austriaca, 1999. **26**(5): p. 148-53.

134. Aikawa, R., et al., *Reactive oxygen species in mechanical stress-induced cardiac hypertrophy.* Biochem Biophys Res Commun, 2001. **289**(4): p. 901-7.

135. Laroux, F.S., et al., *Role of nitric oxide in inflammation.* Acta Physiol Scand, 2001. **173**(1): p. 113-8.

136. Latha, B. and M. Babu, *The involvement of free radicals in burn injury: a review.* Burns, 2001. **27**(4): p. 309-17.

137. Giasson, B.I., et al., *The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases.* Free Radic Biol Med, 2002. **32**(12): p. 1264-75.

138. Floyd, R.A., *Antioxidants, oxidative stress, and degenerative neurological disorders.* Proc Soc Exp Biol Med, 1999. **222**(3): p. 236-45.

139. Mercuri, F., L. Quagliaro, and A. Ceriello, *Oxidative stress evaluation in diabetes.* Diabetes Technol Ther, 2000. **2**(4): p. 589-600.

140. Piconi, L., M.A. Ihnat, and A. Ceriello, *Oxidative stress in the pathogenesis/treatment of diabetes and its complications.* . Curr Nutr Food Sci 2007. **3**: p. 194-199.

141. Chiou, G.C., *Review: effects of nitric oxide on eye diseases and their treatment.* J Ocul Pharmacol Ther, 2001. **17**(2): p. 189-98.

142. Gross, G.J., J.R. Kersten, and D.C. Warltier, *Mechanisms of postischemic contractile dysfunction.* Ann Thorac Surg, 1999. **68**(5): p. 1898-904.

143. Sauer, H., M. Wartenberg, and J. Hescheler, *Reactive oxygen species as intracellular messengers during cell growth and differentiation.* Cell Physiol Biochem, 2001. **11**(4): p. 173-86.

144. Kamata, H. and H. Hirata, *Redox regulation of cellular signalling.* Cell Signal, 1999. **11**(1): p. 1-14.

145. Morel, Y. and R. Barouki, *Repression of gene expression by oxidative stress.* Biochem J, 1999. **342 Pt 3**: p. 481-96.

146. Davies, K.J., *An overview of oxidative stress.* IUBMB Life, 2000. **50**(4-5): p. 241-4.

147. Davies, K.J., *Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems.* IUBMB Life, 2000. **50**(4-5): p. 279-89.

148. Davies, K.J., *The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress.* IUBMB Life, 1999. **48**(1): p. 41-7.

149. Comporti, M., *Three models of free radical-induced cell injury.* Chem Biol Interact, 1989. **72**(1-2): p. 1-56.

150. Gracy, R.W., et al., *Reactive oxygen species: the unavoidable environmental insult?* Mutat Res, 1999. **428**(1-2): p. 17-22.

151. Levine, R.L. and E.R. Stadtman, *Oxidative modification of proteins during aging.* Exp Gerontol, 2001. **36**(9): p. 1495-502.

152. Davies, K.J., *Protein damage and degradation by oxygen radicals. I. general aspects.* J Biol Chem, 1987. **262**(20): p. 9895-901.

153. Beckman, K.B. and B.N. Ames, *Oxidative decay of DNA.* J Biol Chem, 1997. **272**(32): p. 19633-6.

154. Halliwell, B., *The antioxidant paradox.* Lancet, 2000. **355**(9210): p. 1179-80.

155. Kaltschmidt, B., T. Sparna, and C. Kaltschmidt, *Activation of NF-kappa B by reactive oxygen intermediates in the nervous system.* Antioxid Redox Signal, 1999. **1**(2): p. 129-44.

156. Engelhardt, J.F., *Redox-mediated gene therapies for environmental injury: approaches and concepts.* Antioxid Redox Signal, 1999. **1**(1): p. 5-27.

157. Shackelford, R.E., W.K. Kaufmann, and R.S. Paules, *Oxidative stress and cell cycle checkpoint function.* Free Radic Biol Med, 2000. **28**(9): p. 1387-404.

158. Sen, C.K., *Cellular thiols and redox-regulated signal transduction.* Curr Top Cell Regul, 2000. **36**: p. 1-30.

159. Sen, C.K., *Redox signaling and the emerging therapeutic potential of thiol antioxidants.* Biochem Pharmacol, 1998. **55**(11): p. 1747-58.

160. Gutteridge, J.M., G.J. Quinlan, and P. Kovacic, *Phagomimetic action of antimicrobial agents.* Free Radic Res, 1998. **28**(1): p. 1-14.

161. Wolin, M.S., *Interactions of oxidants with vascular signaling systems.* Arterioscler Thromb Vasc Biol, 2000. **20**(6): p. 1430-42.

162. Kuehl, F.A., Jr. and R.W. Egan, *Prostaglandins, arachidonic acid, and inflammation.* Science, 1980. **210**(4473): p. 978-84.

163. Saugstad, O.D., *Update on oxygen radical disease in neonatology.* Curr Opin Obstet Gynecol, 2001. **13**(2): p. 147-53.

164. Benhar, M., D. Engelberg, and A. Levitzki, *ROS, stress-activated kinases and stress signaling in cancer.* EMBO Rep, 2002. **3**(5): p. 420-5.

165. Kim, C.S., et al., *Low-dose of ionizing radiation enhances cell proliferation via transient ERK1/2 and p38 activation in normal human lung fibroblasts.* J Radiat Res (Tokyo), 2007. **48**(5): p. 407-15.

166. Kim, C.S., et al., *Low-dose radiation stimulates the proliferation of normal human lung fibroblasts via a transient activation of Raf and Akt.* Mol Cells, 2007. **24**(3): p. 424-30.

167. Suzuki, K., S. Kodama, and M. Watanabe, *Extremely low-dose ionizing radiation causes activation of mitogen-activated protein kinase pathway and enhances proliferation of normal human diploid cells.* Cancer Res, 2001. **61**(14): p. 5396-401.

168. Halliwell, B. and J.M.C. Gutteridge, *Cellular Responses to Oxidative Stress*, in *Free Radicals in Biology and Medicine*. 2007, Oxford University Press: New York. p. 189.

169. Huo, Y., et al., *Reactive oxygen species (ROS) are essential mediators in epidermal growth factor (EGF)-stimulated corneal epithelial cell proliferation, adhesion, migration, and wound healing.* Exp Eye Res, 2009. **89**(6): p. 876-86.

170. Farber, J.L., *Mechanisms of cell injury by activated oxygen species.* Environ Health Perspect, 1994. **102 Suppl 10**: p. 17-24.

171. Meral, A., et al., *Lipid peroxidation and antioxidant status in beta-thalassemia.* Pediatr Hematol Oncol, 2000. **17**(8): p. 687-93.

172. Jaffe, B., *Crucibles: The story of Chemistry. From Ancient Alchemy to Nuclear Fission*. 1976, Dover Publications: New York.

173. Poulsen, H.E., et al., *Antioxidants, DNA damage and gene expression.* Free Radic Res, 2000. **33 Suppl**: p. S33-9.

174. Kasprzak, K.S., *Oxidative DNA and protein damage in metal-induced toxicity and carcinogenesis.* Free Radic Biol Med, 2002. **32**(10): p. 958-67.

175. Halliwell, B., *Oxygen and nitrogen are pro-carcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen species: measurement, mechanism and the effects of nutrition.* Mutat Res, 1999. **443**(1-2): p. 37-52.

176. Ames, B.N., *DNA damage from micronutrient deficiencies is likely to be a major cause of cancer.* Mutat Res, 2001. **475**(1-2): p. 7-20.

177. Berger, N.A., et al., *NAD and poly(ADP-ribose) regulation of proteins involved in response to cellular stress and DNA damage.* Biochimie, 1995. **77**(5): p. 364-7.

178. Steenken, S. and S.V. Jovanovic, *How Easily Oxidizable Is DNA? One-Electron Reduction Potentials of Adenosine and Guanosine Radicals in Aqueous Solution.* Journal of the American Chemical Society, 1997. **119**(3): p. 617-618.

179. Dizdaroglu, M., *Chemical determination of oxidative DNA damage by gas chromatography-mass spectrometry.* Methods Enzymol, 1994. **234**: p. 3-16.

180. Slupphaug, G., B. Kavli, and H.E. Krokan, *The interacting pathways for prevention and repair of oxidative DNA damage.* Mutat Res, 2003. **531**(1-2): p. 231-51.

181. Sekiguchi, M. and T. Tsuzuki, *Oxidative nucleotide damage: consequences and prevention.* Oncogene, 2002. **21**(58): p. 8895-904.

182. Zuo, S., R.J. Boorstein, and G.W. Teebor, *Oxidative damage to 5-methylcytosine in DNA.* Nucleic Acids Res, 1995. **23**(16): p. 3239-43.

183. Grune, T., T. Reinheckel, and K.J. Davies, *Degradation of oxidized proteins in mammalian cells.* FASEB J, 1997. **11**(7): p. 526-34.

184. Davies, K.J., M.E. Delsignore, and S.W. Lin, *Protein damage and degradation by oxygen radicals. II. Modification of amino acids.* J Biol Chem, 1987. **262**(20): p. 9902-7.

185. Davies, K.J. and M.E. Delsignore, *Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure.* J Biol Chem, 1987. **262**(20): p. 9908-13.

186. Chen, Q. and B.N. Ames, *Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells.* Proc Natl Acad Sci U S A, 1994. **91**(10): p. 4130-4.

187. Lee, A.C., et al., *Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species.* J Biol Chem, 1999. **274**(12): p. 7936-40.

188. Finkel, T. and N.J. Holbrook, *Oxidants, oxidative stress and the biology of ageing.* Nature, 2000. **408**(6809): p. 239-47.

189. Wagner, B.A., et al., *Hydrogen peroxide-induced apoptosis of HL-60 human leukemia cells is mediated by the oxidants hypochlorous acid and chloramines.* Arch Biochem Biophys, 2002. **401**(2): p. 223-34.

190. Burdon, R.H., V. Gill, and D. Alliangana, *Hydrogen peroxide in relation to proliferation and apoptosis in BHK-21 hamster fibroblasts.* Free Radic Res, 1996. **24**(2): p. 81-93.

191. Arrick, B.A., et al., *Hydrogen peroxide from cellular metabolism of cystine. A requirement for lysis of murine tumor cells by vernolepin, a glutathione-depleting antineoplastic.* J Clin Invest, 1985. **76**(2): p. 567-74.

192. Komiyama, T., T. Kikuchi, and Y. Sugiura, *Interactions of anticancer quinone drugs, aclacinomycin A, adriamycin, carbazilquinone, and mitomycin C, with NADPH-cytochrome P-450 reductase, xanthine oxidase and oxygen.* J Pharmacobiodyn, 1986. **9**(8): p. 651-64.

193. Yang, M., et al., *Adriamycin stimulates proliferation of human lymphoblastic leukaemic cells via a mechanism of hydrogen peroxide (H2O2) production.* Br J Haematol, 1996. **95**(2): p. 339-44.

194. Otero, G., et al., *Altered processing of precursor transcripts and increased levels of the subunit I of mitochondrial cytochrome c oxidase in Syrian hamster fetal cells initiated with ionizing radiation.* Carcinogenesis, 1997. **18**(8): p. 1569-75.

195. Saran, M. and W. Bors, *Radiation chemistry of physiological saline reinvestigated: evidence that chloride-derived intermediates play a key role in cytotoxicity.* Radiat Res, 1997. **147**(1): p. 70-7.

196. Bertling, C.J., F. Lin, and A.W. Girotti, *Role of hydrogen peroxide in the cytotoxic effects of UVA/B radiation on mammalian cells.* Photochem Photobiol, 1996. **64**(1): p. 137-42.

197. Peus, D., et al., *H2O2 is an important mediator of UVB-induced EGF-receptor phosphorylation in cultured keratinocytes.* J Invest Dermatol, 1998. **110**(6): p. 966-71.

198. Buettner, G.R. and M.J. Need, *Hydrogen peroxide and hydroxyl free radical production by hematoporphyrin derivative, ascorbate and light.* Cancer Lett, 1985. **25**(3): p. 297-304.

199. Sandstrom, P.A., et al., *Lipid hydroperoxides induce apoptosis in T cells displaying a HIV-associated glutathione peroxidase deficiency.* J Biol Chem, 1994. **269**(2): p. 798-801.

200. Jacobson, M.D., *Reactive oxygen species and programmed cell death.* Trends Biochem Sci, 1996. **21**(3): p. 83-6.

201. Fiers, W., et al., *More than one way to die: apoptosis, necrosis and reactive oxygen damage.* Oncogene, 1999. **18**(54): p. 7719-30.

202. Simon, H.U., A. Haj-Yehia, and F. Levi-Schaffer, *Role of reactive oxygen species (ROS) in apoptosis induction.* Apoptosis, 2000. **5**(5): p. 415-8.

203. Vanlangenakker, N., et al., *Molecular mechanisms and pathophysiology of necrotic cell death.* Curr Mol Med, 2008. **8**(3): p. 207-20.

204. Estabrook, R.W., *Observations on the antimycin A inhibition of biological oxidations. I. Stoichiometry and pH effects.* Biochim Biophys Acta, 1962. **60**: p. 236-48.

205. Brand, M.D., et al., *Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins.* Free Radic Biol Med, 2004. **37**(6): p. 755-67.

206. Waring, P., *Redox active calcium ion channels and cell death.* Arch Biochem Biophys, 2005. **434**(1): p. 33-42.

207. Chen, J.J., H. Bertrand, and B.P. Yu, *Inhibition of adenine nucleotide translocator by lipid peroxidation products.* Free Radic Biol Med, 1995. **19**(5): p. 583-90.

208. Orrenius, S., V. Gogvadze, and B. Zhivotovsky, *Mitochondrial oxidative stress: implications for cell death.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 143-83.

209. Piconi, L., M.A. Ihnat, and A. Ceriello, *Oxidative stress in the pathogenesis/treatment of diabetes and its complications.* Curr Nutr Food Sci 2007. **3**: p. 194-199.

210. Friedberg, E.C., *DNA Repair*. 1985, San Francisco: W. H. Freeman.

211. Mikkelsen, R.B. and P. Wardman, *Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms.* Oncogene, 2003. **22**(37): p. 5734-54.

212. Ewing, D. and S.R. Jones, *Superoxide removal and radiation protection in bacteria.* Arch Biochem Biophys, 1987. **254**(1): p. 53-62.

213. Cerutti, P.A., *Prooxidant states and tumor promotion.* Science, 1985. **227**(4685): p. 375-81.

214. Chen, Q., et al., *The late increase in intracellular free radical oxygen species during apoptosis is associated with cytochrome c release, caspase activation, and mitochondrial dysfunction.* Cell Death Differ, 2003. **10**(3): p. 323-34.

215. Greenstock, C.L., *Redox processes in radiation biology and cancer.* Radiat Res, 1981. **86**(2): p. 196-211.

216. Iyer, R. and B.E. Lehnert, *Effects of ionizing radiation in targeted and nontargeted cells.* Arch Biochem Biophys, 2000. **376**(1): p. 14-25.

217. Cramp, W.A., M.B. Yatvin, and M. Harms-Ringdahl, *Recent developments in the radiobiology of cellular membranes.* Acta Oncol, 1994. **33**(8): p. 945-52.

218. Block, E.R., *Hydrogen peroxide alters the physical state and function of the plasma membrane of pulmonary artery endothelial cells.* J Cell Physiol, 1991. **146**(3): p. 362-9.

219. Wolters, H., C.A.M. van Tilburg, and A.W.T. Konings, *Radiation-induced Lipid Peroxidation: Influence of Oxygen Concentration and Membrane Lipid Composition.* International Journal of Radiation Biology, 1987. **51**(4): p. 619-628.

220. Parasassi, T., et al., *Membrane Oxidative Damage Induced by Ionizing Radiation Detected by Diphenylhexatriene Fluorescence Lifetime Distributions.* International Journal of Radiation Biology, 1992. **61**(6): p. 791-796.

221. Parasassi, T., et al., *Alterations in Erythrocyte Membrane Lipids Induced by Low Doses of Ionizing Radiation as Revealed by 1,6-diphenyl-1,3,5-hexatriene Fluorescence Lifetime.* International Journal of Radiation Biology, 1991. **59**(1): p. 59-69.

222. Lizard, G., et al., *Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis.* Cytometry, 1995. **21**(3): p. 275-283.

223. Vermes, I., et al., *A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V.* J Immunol Methods, 1995. **184**(1): p. 39-51.

224. Benderitter, M., et al., *The cell membrane as a biosensor of oxidative stress induced by radiation exposure: a multiparameter investigation.* Radiat Res, 2003. **159**(4): p. 471-83.

225. Berroud, A., A. Le Roy, and P. Voisin, *Membrane oxidative damage induced by ionizing radiation detected by fluorescence polarization.* Radiat Environ Biophys, 1996. **35**(4): p. 289-95.

226. Ward, J.F., *Radiation-induced strand breakage in DNA.* Basic Life Sci, 1975. **5B**: p. 471-2.

227. Ward, J.F., *Molecular Mechanisms of Radiation-Induced Damage to Nucleic Acids.* Adv Rad Biol, 1975. **5**: p. 181-239.

228. Gulston, M., et al., *Clustered DNA damage induced by gamma radiation in human fibroblasts (HF19), hamster (V79-4) cells and plasmid DNA is revealed as Fpg and Nth sensitive sites.* Nucleic Acids Res, 2002. **30**(15): p. 3464-72.

229. Sutherland, B.M., et al., *Low levels of endogenous oxidative damage cluster levels in unirradiated viral and human DNAs.* Free Radic Biol Med, 2003. **35**(5): p. 495-503.

230. Yang, N., H. Galick, and S.S. Wallace, *Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks.* DNA Repair (Amst), 2004. **3**(10): p. 1323-34.

231. Simpson, J.A., et al., *Long-lived reactive species on free-radical-damaged proteins.* Biochem J, 1992. **282 (Pt 3)**: p. 621-4.

232. Stadtman, E.R., *Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions.* Annu Rev Biochem, 1993. **62**: p. 797-821.

233. Gebicki, S. and J.M. Gebicki, *Formation of peroxides in amino acids and proteins exposed to oxygen free radicals.* Biochem J, 1993. **289 (Pt 3)**: p. 743-9.

234. Garrison, W.M., M.E. Jayko, and W. Bennett, *Radiation-induced oxidation of protein in aqueous solution.* Radiat Res, 1962. **16**: p. 483-502.

235. von Sonntag, C., *The chemistry of free-radical-mediated DNA damage.* Basic Life Sci, 1991. **58**: p. 287-317; discussion 317-21.

236. Repine, J.E., et al., *Dimethyl sulfoxide prevents DNA nicking mediated by ionizing radiation or iron/hydrogen peroxide-generated hydroxyl radical.* Proc Natl Acad Sci U S A, 1981. **78**(2): p. 1001-3.

237. Yamaguchi, S., S. Sakurada, and M. Nagumo, *Role of intracellular SOD in protecting human leukemic and cancer cells against superoxide and radiation.* Free Radic Biol Med, 1994. **17**(5): p. 389-95.

238. Gong, B., et al., *Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases.* Cell Growth Differ, 1999. **10**(7): p. 491-502.

239. Sen, C.K. and L. Packer, *Antioxidant and redox regulation of gene transcription.* FASEB J, 1996. **10**(7): p. 709-20.

240. Lander, H.M., *An essential role for free radicals and derived species in signal transduction.* FASEB J, 1997. **11**(2): p. 118-24.

241. Ryter, S.W., et al., *Mechanisms of cell death in oxidative stress.* Antioxid Redox Signal, 2007. **9**(1): p. 49-89.

242. Mishra, K.P., *Cell Membrane Oxidative Damage Induced by Gamma-Radiation and Apoptotic Sensitivity.* Journal of Environmental Pathology, Toxicology and Oncology, 2004. **23**(1): p. 60-65.

243. Chae, H.Z., S.J. Chung, and S.G. Rhee, *Thioredoxin-dependent peroxide reductase from yeast.* J Biol Chem, 1994. **269**(44): p. 27670-8.

244. Kwon, S.J., et al., *Inhibition of metal-catalyzed oxidation systems by a yeast protector protein in the presence of thioredoxin.* Biochem Biophys Res Commun, 1994. **201**(1): p. 8-15.

245. Green, D.R. and J.C. Reed, *Mitochondria and apoptosis.* Science, 1998. **281**(5381): p. 1309-12.

246. Lee, J.H., et al., *Regulation of ionizing radiation-induced apoptosis by mitochondrial NADP+-dependent isocitrate dehydrogenase.* J Biol Chem, 2007. **282**(18): p. 13385-94.

247. Burdon, R.H., *Superoxide and hydrogen peroxide in relation to mammalian cell proliferation.* Free Radic Biol Med, 1995. **18**(4): p. 775-94.

248. Burdon, R.H. and C. Rice-Evans, *Free radicals and the regulation of mammalian cell proliferation.* Free Radic Res Commun, 1989. **6**(6): p. 345-58.

249. Wiese, A.G., R.E. Pacifici, and K.J. Davies, *Transient adaptation of oxidative stress in mammalian cells.* Arch Biochem Biophys, 1995. **318**(1): p. 231-40.

250. Janero, D.R., D. Hreniuk, and H.M. Sharif, *Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): lethal peroxidative membrane injury.* J Cell Physiol, 1991. **149**(3): p. 347-64.

251. McNamara, M. and R.C. Augusteyn, *The effects of hydrogen peroxide on lens proteins: a possible model for nuclear cataract.* Exp Eye Res, 1984. **38**(1): p. 45-56.

252. Pletjushkina, O.Y., et al., *Hydrogen peroxide produced inside mitochondria takes part in cell-to-cell transmission of apoptotic signal.* Biochemistry (Mosc), 2006. **71**: p. 60-67.

253. Zamzami, N., et al., *Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis.* J Bioenerg Biomembr, 1997. **29**(2): p. 185-93.

254. Reed, J.C., *Cytochrome c: can't live with it--can't live without it.* Cell, 1997. **91**(5): p. 559-62.

255. Kane, D.J., et al., *Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species.* Science, 1993. **262**(5137): p. 1274-7.

256. Crawford, D.R., N.E. Abramova, and K.J. Davies, *Oxidative stress causes a general, calcium-dependent degradation of mitochondrial polynucleotides.* Free Radic Biol Med, 1998. **25**(9): p. 1106-11.

257. Crawford, D.R., et al., *16S mitochondrial ribosomal RNA degradation is associated with apoptosis.* Free Radic Biol Med, 1997. **22**(7): p. 1295-300.

258. Crawford, D.R., et al., *Down-regulation of mammalian mitochondrial RNAs during oxidative stress.* Free Radic Biol Med, 1997. **22**(3): p. 551-9.

259. Kristal, B.S., J. Chen, and B.P. Yu, *Sensitivity of mitochondrial transcription to different free radical species.* Free Radic Biol Med, 1994. **16**(3): p. 323-9.

260. Huang, X., et al., *The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction.* Biochemistry, 1999. **38**(24): p. 7609-16.

261. Tabner, B.J., et al., *Formation of hydrogen peroxide and hydroxyl radicals from A(beta) and alpha-synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease.* Free Radic Biol Med, 2002. **32**(11): p. 1076-83.

262. Turnbull, S., et al., *alpha-Synuclein implicated in Parkinson's disease catalyses the formation of hydrogen peroxide in vitro.* Free Radic Biol Med, 2001. **30**(10): p. 1163-70.

263. Ryter, S.W., et al., *Mechanisms of Cell Death in Oxidative Stress.* Antioxid Redox Signal, 2007. **9**(1): p. 49-89.

264. de Gruijl, F.R., *Photocarcinogenesis: UVA vs. UVB radiation.* Skin Pharmacol Appl Skin Physiol, 2002. **15**(5): p. 316-20.

265. Ichihashi, M., et al., *UV-induced skin damage*. Toxicology, 2003. **189**(1-2): p. 21-39.

266. Cadet, J., E. Sage, and T. Douki, *Ultraviolet radiation-mediated damage to cellular DNA.* Mutat Res, 2005. **571**(1-2): p. 3-17.

267. Hildesheim, J. and A.J. Fornace, Jr., *The dark side of light: the damaging effects of UV rays and the protective efforts of MAP kinase signaling in the epidermis.* DNA Repair (Amst), 2004. **3**(6): p. 567-80.

268. Setlow, R.B., et al., *Wavelengths effective in induction of malignant melanoma.* Proc Natl Acad Sci U S A, 1993. **90**(14): p. 6666-70.

269. van Weelden, H., F.R. de Gruijl, and J.C. Van der Leun, *The Biological Effects of UVA Radiation*, F. Urbach and R.W. Gange, Editors. 1986, Praeger: New York. p. 137.

270. Poulson, J.T., et al., *Dermal Elastosis in Hairless Mice after UVB and UVA applied simultaneuously, separately or sequentially.* Brit. J. Dermatol., 1984. **110**: p. 531-538.

271. Marks, F. and G. Furstenburger, *Tumor promotion in skin: Are active oxygen species involved?*, in *Oxidative Stress*, H. Sies, Editor. 1985, Academic Press: London. p. 437-469.

272. Pathak, M.A. and M.D. Carbonare, *Reactive oxygen species in photoaging and biochemical studies in the amelioration of photoaging changes*, in *Biological Responses to ultraviolet A irradiation*, F. Urbach, Editor. 1992, Valdenmar Publishing: Kansas. p. 189-207.

273. Shindo, Y., E. Witt, and L. Packer, *Antioxidant defense mechanisms in murine epidermis and dermis and their responses to ultraviolet light.* J Invest Dermatol, 1993. **100**(3): p. 260-5.

274. Tyrrell, R.M., *UVA(320-380 nm) radiation as an oxidative stress*, in *Oxidative Stress: Oxidants and Antioxidants*, H. Sies, Editor. 1991, Academic Press: San Diego. p. 57-83.

275. McCormick, J.P., et al., *Characterization of a cell-lethal product from the photooxidation of tryptophan: hydrogen peroxide.* Science, 1976. **191**(4226): p. 468-9.

276. Cunningham, M.L., et al., *Photosensitized production of superoxide anion by monochromatic (290-405 nm) ultraviolet irradiation of NADH and NADPH coenzymes.* Photochem Photobiol, 1985. **42**(2): p. 125-8.

277. Czochralska, B., et al., *Oxidation of excited-state NADH and NAD dimer in aqueous medium-involvement of O2- as a mediator in the presence of oxygen.* Biochim Biophys Acta, 1984. **801**: p. 403-409.

278. Thomas, J.P. and A.W. Girotti, *Photogeneration of singlet oxygen by membrane bound hematoporphyrin derivative.* Photochem Photobiol, 1988. **47**: p. 79s.

279. Aust, S.D., L.A. Morehouse, and C.E. Thomas, *Role of metals in oxygen radical reactions.* J Free Radic Biol Med, 1985. **1**(1): p. 3-25.

280. Hu, M.-L. and A.L. Tappel, *Potentiation of oxidative damage to proteins by ultraviolet-A and protection by antioxidants.* Photochem Photobiol, 1992. **56**: p. 357-363.

281. Kukreja, R.C., et al., *Oxygen radical-mediated lipid peroxidation and inhibition of Ca2+-ATPase activity of cardiac sarcoplasmic reticulum.* Arch Biochem Biophys, 1988. **261**(2): p. 447-57.

282. Scherer, N.M. and D.W. Deamer, *Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca2+-ATPase.* Arch Biochem Biophys, 1986. **246**(2): p. 589-601.

283. Danpure, H.J. and R.M. Tyrrell, *Oxygen-dependence of near UV (365 NM) lethality and the interaction of near UV and X-rays in two mammalian cell lines.* Photochem Photobiol, 1976. **23**(3): p. 171-7.

284. Punnonen, K., et al., *Effects of in vitro UVA irradiation and PUVA treatment on membrane fatty acids and activities of antioxidant enzymes in human keratinocytes.* J Invest Dermatol, 1991. **96**(2): p. 255-9.

285. Morliere, P., et al., *UVA-induced lipid peroxidation in cultured human fibroblasts.* Biochim Biophys Acta, 1991. **1084**(3): p. 261-8.

286. Lautier, D., P. Luscher, and R.M. Tyrrell, *Endogenous glutathione levels modulate both constitutive and UVA radiation/hydrogen peroxide inducible expression of the human heme oxygenase gene.* Carcinogenesis, 1992. **13**(2): p. 227-32.

287. Vile, G.F. and R.M. Tyrrell, *UVA radiation-induced oxidative damage to lipids and proteins in vitro and in human skin fibroblasts is dependent on iron and singlet oxygen.* Free Radic Biol Med, 1995. **18**(4): p. 721-30.

288. Cadet, J., et al., *Effects of UV and visible radiation on DNA-final base damage.* Biol Chem, 1997. **378**(11): p. 1275-86.

289. Daya-Grosjean, L., N. Dumaz, and A. Sarasin, *The specificity of p53 mutation spectra in sunlight induced human cancers.* J Photochem Photobiol B, 1995. **28**(2): p. 115-24.

290. Basu-Modak, S. and R.M. Tyrrell, *Singlet oxygen: a primary effector in the ultraviolet A/near-visible light induction of the human heme oxygenase gene.* Cancer Res, 1993. **53**(19): p. 4505-10.

291. Tyrrell, R.M. and M. Pidoux, *Singlet oxygen involvement in the inactivation of cultured human fibroblasts by UVA (334 nm, 365 nm) and near-visible (405 nm) radiations.* Photochem Photobiol, 1989. **49**(4): p. 407-12.

292. Tyrrell, R.M., *The Molecular and Cellular Pathology of Solar Ultraviolet Radiation*, in *Molecular Aspects of Medicine*, H. Baum, Editor. 1994, Oxford: Elsevier. p. 1-77.

293. Freeman, R.G., *Data on the action spectrum for ultraviolet carcinogenesis.* J Natl Cancer Inst, 1975. **55**(5): p. 1119-22.

294. Tyrrell, R.M., *Activation of mammalian gene expression by the UV component of sunlight--from models to reality.* Bioessays, 1996. **18**(2): p. 139-48.

295. Kvam, E. and R.M. Tyrrell, *Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation.* Carcinogenesis, 1997. **18**(12): p. 2379-84.

296. Kvam, E. and R.M. Tyrrell, *Artificial background and induced levels of oxidative base damage in DNA from human cells.* Carcinogenesis, 1997. **18**(11): p. 2281-3.

297. Salmon, S., et al., *A mechanistic study of the interaction of UVB radiations with human serum lipoproteins.* Biochim Biophys Acta, 1991. **1086**(1): p. 1-6.

298. Tada-Oikawa, S., S. Oikawa, and S. Kawanishi, *Role of ultraviolet A-induced oxidative DNA damage in apoptosis via loss of mitochondrial membrane potential and caspase-3 activation.* Biochem Biophys Res Commun, 1998. **247**(3): p. 693-6.

299. Godar, D.E. and A.D. Lucas, *Spectral dependence of UV-induced immediate and delayed apoptosis: the role of membrane and DNA damage.* Photochem Photobiol, 1995. **62**(1): p. 108-13.

300. Godar, D.E., *Light and death: photons and apoptosis.* J Investig Dermatol Symp Proc, 1999. **4**(1): p. 17-23.

301. Godar, D.E., *UVA1 radiation triggers two different final apoptotic pathways.* J Invest Dermatol, 1999. **112**(1): p. 3-12.

302. Didier, C., et al., *Induction of thioredoxin by ultraviolet-A radiation prevents oxidative-mediated cell death in human skin fibroblasts.* Free Radic Biol Med, 2001. **31**(5): p. 585-98.

303. Assefa, Z., et al., *Ultraviolet radiation-induced apoptosis in keratinocytes: on the role of cytosolic factors.* Biochim Biophys Acta, 2005. **1755**(2): p. 90-106.

304. Chouinard, N., et al., *UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53.* Biochem J, 2002. **365**(Pt 1): p. 133-45.

305. Bivik, C.A., et al., *UVA/B-induced apoptosis in human melanocytes involves translocation of cathepsins and Bcl-2 family members.* J Invest Dermatol, 2006. **126**(5): p. 1119-27.

306. Pourzand, C., et al., *Overxpression of Bcl-2 inhibits UVA-mediated immediate apoptosiinrat 6 fibroblasts: evidence for the involvement of Bcl-2 as an antioxidant.* Cancer Res, 1997. **57**(8): p. 1405-11.

307. Zhang, H., *p53 plays a central role in UVA and UVB induced cell damage and apoptosis in melanoma cells.* Cancer Lett, 2006. **244**(2): p. 229-38.

308. Zhuang, S. and I.E. Kochevar, *Ultraviolet A radiation induces rapid apoptosis of human leukemia cells by Fas ligand-independent activation of the Fas death pathways.* Photochem Photobiol, 2003. **78**(1): p. 61-7.

309. Gilchrest, B.A., *Skin aging and photoaging: an overview.* J Am Acad Dermatol, 1989. **21**(3 Pt 2): p. 610-3.

310. Armstrong, B.K. and A. Kricker, *The epidemiology of UV induced skin cancer.* J Photochem Photobiol B, 2001. **63**(1-3): p. 8-18.

311. Pillai, S., C. Oresajo, and J. Hayward, *Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation - a review.* Int J Cosmet Sci, 2005. **27**(1): p. 17-34.

312. Bernstein, E.F., et al., *Ultraviolet radiation activates the human elastin promoter in transgenic mice: a novel in vivo and in vitro model of cutaneous photoaging.* J Invest Dermatol, 1995. **105**(2): p. 269-73.

313. Katiyar, S.K. and H. Mukhtar, *Green tea polyphenol (-)-epigallocatechin-3-gallate treatment to mouse skin prevents UVB-induced infiltration of leukocytes, depletion of antigen-presenting cells, and oxidative stress.* J Leukoc Biol, 2001. **69**(5): p. 719-26.

314. Wood, L.C., et al., *Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis.* J Invest Dermatol, 1996. **106**(3): p. 397-403.

315. Gilchrest, B.A. and M.S. Eller, *DNA photodamage stimulates melanogenesis and other photoprotective responses.* J Investig Dermatol Symp Proc, 1999. **4**(1): p. 35-40.

316. Rhie, G., et al., *Aging- and photoaging-dependent changes of enzymic and nonenzymic antioxidants in the epidermis and dermis of human skin in vivo.* J Invest Dermatol, 2001. **117**(5): p. 1212-7.

317. Kawaguchi, Y., et al., *The effects of ultraviolet A and reactive oxygen species on the mRNA expression of 72-kDa type IV collagenase and its tissue inhibitor in cultured human dermal fibroblasts.* Arch Dermatol Res, 1996. **288**(1): p. 39-44.

318. Takashima, A. and P.R. Bergstresser, *Impact of UVB radiation on the epidermal cytokine network.* Photochem Photobiol, 1996. **63**(4): p. 397-400.

319. Bielenberg, D.R., et al., *Molecular regulation of UVB-induced cutaneous angiogenesis.* J Invest Dermatol, 1998. **111**(5): p. 864-72.

320. Hruza, L.L. and A.P. Pentland, *Mechanisms of UV-induced inflammation.* J Invest Dermatol, 1993. **100**(1): p. 35S-41S.

321. Thiele, J.J., *Oxidative targets in the stratum corneum. A new basis for antioxidative strategies.* Skin Pharmacol Appl Skin Physiol, 2001. **14 Suppl 1**: p. 87-91.

322. Sander, C.S., et al., *Photoaging is associated with protein oxidation in human skin in vivo.* J Invest Dermatol, 2002. **118**(4): p. 618-25.

323. Brenneisen, P., H. Sies, and K. Scharffetter-Kochanek, *Ultraviolet-B irradiation and matrix metalloproteinases: from induction via signaling to initial events.* Ann N Y Acad Sci, 2002. **973**: p. 31-43.

324. Trenam, C.W., D.R. Blake, and C.J. Morris, *Skin inflammation: reactive oxygen species and the role of iron.* J Invest Dermatol, 1992. **99**(6): p. 675-82.

325. Chlopicki, S., et al., *Functional role of NADPH oxidase in activation of platelets.* Antioxid Redox Signal, 2004. **6**(4): p. 691-8.

326. Gilchrest, B.A., et al., *Mechanisms of ultraviolet light-induced pigmentation.* Photochem Photobiol, 1996. **63**(1): p. 1-10.

327. Applegate, L.A., et al., *Endogenous glutathione levels modulate the frequency of both spontaneous and long wavelength ultraviolet induced mutations in human cells.* Carcinogenesis, 1992. **13**(9): p. 1557-60.

328. de Gruijl, F.R., *Photocarcinogenesis: UVA vs UVB.* Methods Enzymol, 2000. **319**: p. 359-66.

329. Kelfkens, G., F.R. de Gruijl, and J.C. van der Leun, *Tumorigenesis by short-wave ultraviolet A: papillomas versus squamous cell carcinomas.* Carcinogenesis, 1991. **12**(8): p. 1377-82.

330. Kelfkens, G., F.R. de Gruijl, and J.C. van der Leun, *Ozone depletion and increase in annual carcinogenic ultraviolet dose.* Photochem Photobiol, 1990. **52**(4): p. 819-23.

331. Folkman, J., *Angiogenesis.* Annual Review of Medicine, 2006. **57**: p. 1-18.

332. Nugent, M.A. and R.V. Iozzo, *Fibroblast growth factor-2.* International Journal of Biochemistry & Cell Biology, 2000. **32**(2): p. 115-120.

333. Folkman, J., *Angiogenesis in Cancer, Vascular, Rheumatoid and Other Disease.* Nature Medicine, 1995. **1**(1): p. 27-31.

334. Wong, M.K.K. and A.I. Gotlieb, *Invitro Reendothelialization Of A Single-Cell Wound - Role Of Microfilament Bundles In Rapid Lamellipodia-Mediated Wound Closure.* Laboratory Investigation, 1984. **51**(1): p. 75-81.

335. Fridman, G., et al., *Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air.* Plasma Chemistry and Plasma Processing, 2006. **26**(4): p. 425-442.

336. Fridman, G., et al., *Floating electrode dielectric barrier discharge plasma in air promoting apoptotic behavior in melanoma skin cancer cell lines.* Plasma Chemistry and Plasma Processing, 2007. **27**(2): p. 163-176.

337. Morss, A.S. and E.R. Edelman, *Glucose modulates basement membrane fibroblast growth factor-2 via alterations in endothelial cell permeability.* Journal of Biological Chemistry, 2007. **282**(19): p. 14635-14644.

338. Fiers, W., et al., *More than one way to die: apoptosis, necrosis and reactive oxygen damage.* Oncogene, 1999. **18**(54): p. 7719-7730.

339. Majno, G. and I. Joris, *Apoptosis, Oncosis, and Necrosis - an Overview of Cell-Death.* American Journal of Pathology, 1995. **146**(1): p. 3-15.

340. Muthukrishnan, L., E. Warder, and P.L. Mcneil, *Basic Fibroblast Growth-Factor Is Efficiently Released from a Cytolsolic Storage Site through Plasma-Membrane Disruptions of Endothelial-Cells.* Journal of Cellular Physiology, 1991. **148**(1): p. 1-16.

341. Ku, P.T. and P.A. Damore, *Regulation of Basic Fibroblast Growth-Factor (Bfgf) Gene and Protein Expression Following Its Release from Sublethally Injured Endothelial-Cells.* Journal of Cellular Biochemistry, 1995. **58**(3): p. 328-343.

342. Sudhir, K., et al., *Mechanical strain stimulates a mitogenic response in coronary vascular smooth muscle cells via release of basic fibroblast growth factor.* American Journal of Hypertension, 2001. **14**(11): p. 1128-1134.

343. Caplice, N.M., et al., *Growth factors released into the coronary circulation after vascular injury promote proliferation of human vascular smooth muscle cells in culture.* Journal of the American College of Cardiology, 1997. **29**(7): p. 1536-1541.

344. Chang, P.Y., et al., *Particle irradiation induces FGF2 expression in normal human lens cells.* Radiation Research, 2000. **154**(5): p. 477-484.

345. Haimovitzfriedman, A., et al., *Autocrine Effects of Fibroblast Growth-Factor in Repair of Radiation-Damage in Endothelial-Cells.* Cancer Research, 1991. **51**(10): p. 2552-2558.

346. Houchen, C.W., et al., *FGF-2 enhances intestinal stem cell survival and its expression is induced after radiation injury.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 1999. **276**(1): p. G249-G258.

347. Yamada, H., et al., *Cell injury unmasks a latent proangiogenic phenotype in mice with increased expression of FGF2 in the retina.* Journal of Cellular Physiology, 2000. **185**(1): p. 135-142.

348. Fuks, Z., et al., *Basic Fibroblast Growth-Factor Protects Endothelial-Cells against Radiation-Induced Programmed Cell-Death in-Vitro, and in-Vivo.* Cancer Research, 1994. **54**(10): p. 2582-2590.

349. Gallicchio, V.S., et al., *Basic Fibroblast Growth-Factor (B-Fgf) Induces Early-Stage (Cfu-S) and Late-Stage Hematopoietic Progenitor-Cell Colony Formation (Cfu-Gm, Cfu-Meg, and Bfu-E) by Synergizing with Gm-Csf, Meg-Csf, and Erythropoietin, and Is a Radioprotective Agent Invitro.* International Journal of Cell Cloning, 1991. **9**(3): p. 220-232.

350. Haimovitzfriedman, A., et al., *Protein-Kinase-C Mediates Basic Fibroblast Growth-Factor Protection of Endothelial-Cells against Radiation-Induced Apoptosis.* Cancer Research, 1994. **54**(10): p. 2591-2597.

351. Callaghan, M.J., et al., *Pulsed electromagnetic fields accelerate normal and diabetic wound healing by increasing endogenous FGF-2 release.* Plastic and Reconstructive Surgery, 2008. **121**(1): p. 130-141.

352. Tepper, O.M., et al., *Electromagnetic fields increase in vitro and in vivo angiogenesis through endothelial release of FGF-2.* Faseb Journal, 2004. **18**(9): p. 1231-+.

353. Yenpatton, G.P.A., et al., *Endothelial-Cell Response to Pulsed Electromagnetic-Fields - Stimulation of Growth-Rate and Angiogenesis Invitro.* Journal of Cellular Physiology, 1988. **134**(1): p. 37-46.

354. Rath, P.C. and B.B. Aggarwal, *TNF-induced signaling in apoptosis.* J Clin Immunol, 1999. **19**(6): p. 350-64.

355. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Tumor necrosis factor signaling.* Cell Death Differ, 2003. **10**(1): p. 45-65.

356. Clyne, A.M., H. Zhu, and E.R. Edelman, *Elevated fibroblast growth factor-2 increases tumor necrosis factor-alpha, induced endothelial cell death in high glucose.* Journal of Cellular Physiology, 2008. **217**(1): p. 86-92.

357. Danpure, C.J., *Lactate-Dehydrogenase and Cell Injury*. Cell Biochemistry and Function, 1984. **2**(3): p. 144-148.

358. Gebicki, S. and J.M. Gebicki, *Formation of Peroxides in Amino-Acids and Proteins Exposed to Oxygen Free-Radicals.* Biochemical Journal, 1993. **289**: p. 743-749.

359. Majno, G. and I. Joris, *Apoptosis, oncosis, and necrosis. An overview of cell death.* Am J Pathol, 1995. **146**(1): p. 3-15.

360. Jaattela, M., *Programmed cell death: many ways for cells to die decently.* Ann Med, 2002. **34**(6): p. 480-8.

361. Reed, J.C., *Apoptosis-based therapies.* Nat Rev Drug Discov, 2002. **1**(2): p. 111-21.

362. Hu, W. and J.J. Kavanagh, *Anticancer therapy targeting the apoptotic pathway.* Lancet Oncol, 2003. **4**(12): p. 721-9.

363. Kalghatgi, S., et al. *Penetration of direct non-thermal plasma treatment into living cells*. in *IEEE 35th International Conference on Plasma Science*. 2008. Karlsruhe, Germany.

364. Kalghatgi, S., et al. *On the Interaction of Non-Thermal Atmospheric Pressure Plasma with Tissues*. in *Proceedings of the IEEE 17th International Pulsed Power Conference*. 2009. Washington DC, USA.

365. Kuo, S.P., Tarasenko, O., Popovic, S., Levon, K., *Killing of Bacterial Spores Contained in a Paper Envelope by a Microwave Plasma Torch.* IEEE Transactions on Plasma Science, 2006. **34**(4): p. 1275-1280.

366. Sharma, A., Pruden, A., Stan, O., Collins, G.J., *Bacterial Inactivation Using an RF-Powered Atmospheric Pressure Plasma*. IEEE Transactions on Plasma Science, 2006. **34**(4).

367. Tarasenko, O., Nourbakhsh, S., Kuo, S.P., Bakhtina, A., Alusta, P., Kudasheva, D., Cowman, M., Levon, K., *Scanning Electron and Atomic Force Microscopy to Study Plasma Torch Effects on B. cereus Spores.* IEEE Transactions on Plasma Science, 2006. **34**(4): p. 1281-1289.

368. Williamson, J.M., Trump, D. D., Bletzinger, P., Ganguly, B. N., *Comparison of high-voltage ac and pulsed operation of a surface dielectric barrier discharge.* Journal of Physics D: Applied Physics, 2006. **39**: p. 4400-4406.

369. Zhang, R., Wang, L., Wu, Y., Guan, Z., Jia, Z., *Bacterial Decontamination of Water by Bipolar Pulsed Discharge in a Gas; Liquid; Solid Three-Phase Discharge Reactor*. IEEE Transactions on Plasma Science, 2006. **34**(4): p. 1370-1374.

370. Kalghatgi, S., et al. *Toxicity of non-thermal plasma treatment of endothelial cells*. in *IEEE 35th International Conference on Plasma Science*. 2008. Karlsruhe, Germany.

371. Kalghatgi, S.U., et al. *Non-thermal dielectric barrier discharge plasma treatment of endothelial cells*. in *30th Annual International Conference of the IEEE Engineering in Medicine and Biology Society*. 2008. Vancouver, BC, Canada: Inst. of Elec. and Elec. Eng. Computer Society.

372. Kawiak, A., et al., *Induction of apoptosis by plumbagin through reactive oxygen species-mediated inhibition of topoisomerase II.* Toxicol Appl Pharmacol, 2007. **223**(3): p. 267-76.

373. Ratha, J., et al., *Attenuated Leishmanial sphingolipid induces apoptosis in A375 human melanoma cell via both caspase-dependent and -independent pathways.* Mol Cell Biochem, 2007. **304**(1-2): p. 143-54.

374. Yang, J., Y. Su, and A. Richmond, *Antioxidants tiron and N-acetyl-L-cysteine differentially mediate apoptosis in melanoma cells via a reactive oxygen species-independent NF-kappaB pathway.* Free Radic Biol Med, 2007. **42**(9): p. 1369-80.

375. Zafarullah, M., et al., *Molecular mechanisms of N-acetylcysteine actions.* Cell Mol Life Sci, 2003. **60**(1): p. 6-20.

376. Berk, L.B., *Radiation therapy as primary and adjuvant treatment for local and regional melanoma*. Cancer Control, 2008. **15**(3): p. 233-8.

377. Schild, S.E., *Role of radiation therapy in the treatment of melanoma.* Expert Rev Anticancer Ther, 2009. **9**(5): p. 583-6.

378. Stevens, G. and A. Hong, *Radiation therapy in the management of cutaneous melanoma.* Surg Oncol Clin N Am, 2006. **15**(2): p. 353-71.

379. Eton, O., *Chemotherapy, cytokines, and biochemotherapy for melanoma.* Cancer Chemother Biol Response Modif, 2005. **22**: p. 739-48.

380. Flaherty, K.T., *Chemotherapy and targeted therapy combinations in advanced melanoma.* Clin Cancer Res, 2006. **12**(7 Pt 2): p. 2366s-2370s.

381. Mandara, M., et al., *Chemotherapy for metastatic melanoma.* Expert Rev Anticancer Ther, 2006. **6**(1): p. 121-30.

382. Trefzer, U. and W. Sterry, *Topical immunotherapy with diphenylcyclopropenone in combination with DTIC and radiation for cutaneous metastases of melanoma.* Dermatology, 2005. **211**(4): p. 370-1.

383. Miyato, Y. and K. Ando, *Apoptosis of human melanoma cells by a combination of lonidamine and radiation.* J Radiat Res (Tokyo), 2004. **45**(2): p. 189-94.

384. Strohmenger, U., et al., *Effects of hyperthermia, radiation and their combination on xenografts of human melanoma tumours in nude mice.* Strahlenther Onkol, 1989. **165**(7): p. 521.

385. Kim, J.H., E.W. Hahn, and S.A. Ahmed, *Combination hyperthermia and radiation therapy for malignant melanoma.* Cancer, 1982. **50**(3): p. 478-82.

386. Kim, J.H., E.W. Hahn, and N. Tokita, *Combination hyperthermia and radiation therapy for cutaneous malignant melanoma.* Cancer, 1978. **41**(6): p. 2143-8.

387. Currie, G.A. and T.J. McElwain, *Active immunotherapy as an adjunct to chemotherapy in the treatment of disseminated malignant melanoma: a pilot study.* Br J Cancer, 1975. **31**(2): p. 143-56.

388. Jacquillat, C., et al., *Adjuvant chemotherapy or chemoimmunotherapy in the management of primary malignant melanoma of level III, IV, or V.* Recent Results Cancer Res, 1978. **68**: p. 346-58.

389. Kaufman, S.D., et al., *Adjuvant therapy in malignant melanoma: a trial of immunotherapy. chemotherapy, and combined treatment.* Recent Results Cancer Res, 1978. **68**: p. 380-6.

390. Mitchell, M.S., M.B. Mokyr, and J.M. Davis, *Effect of chemotherapy and immunotherapy on tumor-specific immunity in melanoma.* J Clin Invest, 1977. **59**(6): p. 1017-26.

391. Newlands, E.S., et al., *Clinical trial of combination chemotherapy and specific active immunotherapy in disseminated melanoma.* Br J Cancer, 1976. **34**(2): p. 174-9.

392. Nuccitelli, R., et al., *A new pulsed electric field therapy for melanoma disrupts the tumor's blood supply and causes complete remission without recurrence.* Int J Cancer, 2009. **125**(2): p. 438-45.

393. Dolmans, D.E.J.G.J., D. Fukumura, and R.K. Jain, *Photodynamic therapy for cancer.* Nature Reviews Cancer, 2003. **3**(5): p. 380-387.

394. Vrouenraets, M.B., et al., *Basic principles, applications in oncology and improved selectivity of photodynamic therapy.* Anticancer Research, 2003. **23**: p. 505-522.

395. Pak, B.J., et al., *Radiation resistance of human melanoma analysed by retroviral insertional mutagenesis reveals a possible role for dopachrome tautomerase.* Oncogene, 2004. **23**(1): p. 30-8.

396. Soengas, M.S. and S.W. Lowe, *Apoptosis and melanoma chemoresistance.* Oncogene, 2003. **22**(20): p. 3138-51.

397. L. Detomasoa, R.G., R. d'Agostinoa, G.S. Senesib and P. Favia, *Plasma deposited acrylic acid coatings: Surface characterization and attachment of 3T3 murine fibroblast cell lines.* Surface and Coatings Technology, 2005. **2000**(1-4): p. 1022-1025.

398. Roberto Gristina, E.D.A., Giorgio S. Senesi, Antonella Milella, Marina Nardulli, Eloisa Sardella, Pietro Favia, Riccardo d'Agostino, *Increasing cell adhesion on plasma deposited fluorocarbon coatings by changing the surface topography*. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2008. **88B**(1): p. 139-149.

399. Jiang, C., et al. *Low Energy Nanosecond Pulsed Plasma Sterilization for Endodontic Applications*. in *IEEE International Power Modulators and High Voltage Conference, Proceedings of the 2008*. 2008.

400. Xinpei, L., et al., *An RC Plasma Device for Sterilization of Root Canal of Teeth.* Plasma Science, IEEE Transactions on, 2009. **37**(5): p. 668-673.

401. Burma, S., et al., *ATM phosphorylates histone H2AX in response to DNA double-strand breaks.* J Biol Chem, 2001. **276**: p. 42462 - 42467.

402. Rogakou, E., et al., *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139.* J Biol Chem, 1998. **273**: p. 5858 - 5868.

403. Ward, I.M., Chen, J., *Histone H2AX Is Phosphorylated in an ATR-dependent Manner in Response to Replicational Stress.* The Journal of Biological Chemistry, 2001. **276**(51): p. 47759-47762.

404. Coulombe, S., et al., *Miniature atmospheric pressure glow discharge torch (APGD-t) for local biomedical applications.* Pure and Applied Chemistry, 2006. **78**(6): p. 1147-1156.

405. Leduc, M., et al., *Cell permeabilization using a non-thermal plasma.* New Journal of Physics, 2009. **11**: p. 115021.

406. Shekhter, A.B., Serezhenkov, V. A., Rudenko, T. G., Pekshev, A. V., *Beneficial effect of gaseous nitric oxide on the healing of skin wounds.* Nitric Oxide: Biology and Chemistry, 2005. **12**(4): p. 210-219.

407. Weng, C.-C., et al., *Inactivation of bacteria by a mixed argon and oxygen micro-plasma as a function of exposure time.* International Journal of Radiation Biology, 2009. **85**(4): p. 362-368.

408. Fridman, A., Kennedy, L. A., *Plasma Physics and Engineering*. 2004: Routledge, USA 853.

409. Kogelschatz, U., et al., *Non-Equilibrium Air Plasmas at Atmospheric Pressure*. 1st ed. Plasma Physics. 2004: Taylor & Francis.

410. Benjamin, H., et al., *A planar micro-sensor for bio-impedance measurements* Sensors and Actuators B: Chemical, 2005. **111-112**: p. 430-435.

411. Greason, W.D., *Electrostatic discharge characteristics for the human body and circuit packs.* Journal of Electrostatics, 2003. **59**(3-4): p. 285-300.

412. Mirzoeva, O.K. and J.H. Petrini, *DNA damage-dependent nuclear dynamics of the Mre11 complex.* Mol Cell Biol, 2001. **21**(1): p. 281-8.

413. Lehnert, B.E. and R. Iyer, *Exposure to low-level chemicals and ionizing radiation: reactive oxygen species and cellular pathways.* Hum Exp Toxicol, 2002. **21**(2): p. 65-9.

414. Wiseman, H. and B. Halliwell, *Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer.* Biochem J, 1996. **313 (Pt 1)**: p. 17-29.

415. Leach, J.K., et al., *Ionizing Radiation-induced, Mitochondria-dependent Generation of Reactive Oxygen/Nitrogen.* Cancer Res, 2001. **61**(10): p. 3894-3901.

416. Kawasaki, J., et al., *Electron Microscopic Evaluations of Clot Morphology During Thrombelastography®.* Anesthesia & Analgesia, 2004. **99**(5): p. 1440-1444.

417. Giangrande, P.L., *Six characters in search of an author: the history of the nomenclature of coagulation factors.* Br J Haematol, 2003. **121**(5): p. 703-12.

418. Ataullakhanov, F.I., et al., *Calcium threshold in human plasma clotting kinetics.* Thromb Res, 1994. **75**(4): p. 383-94.

419. Butenas, S., C. van't Veer, and K.G. Mann, *"Normal" thrombin generation.* Blood, 1999. **94**(7): p. 2169-78.

420. McClatchey, K., *Clinical Laboratory Medicine, 2nd Ed*. 2nd Ed ed. 2002, New York: Lippincott Williams & Wilkins.

421. Wang, S., et al., *pH effects on measurements of ionized calcium and ionized magnesium in blood.* Arch Pathol Lab Med, 2002. **126**(8): p. 947-50.

422. Silverthorn, D.U., *Human Physiology, 4th Ed*. 2006, New York: Pearsons Education Inc.

423. Fuss, C., J.C. Palmaz, and E.A. Sprague, *Fibrinogen: structure, function, and surface interactions.* J Vasc Interv Radiol, 2001. **12**(6): p. 677-82.

424. He, X.M. and D.C. Carter, *Atomic structure and chemistry of human serum albumin.* Nature, 1992. **358**(6383): p. 209-15.

425. Pryor, W.A., D.H. Giamalva, and D.F. Church, *Kinetics of ozonation. 2. Amino acids and model compounds in water and comparisons to rates in nonpolar solvents.* Journal of the American Chemical Society, 2002. **106**(23): p. 7094-7100.

426. Lezcano, I., et al., *Ozone Inactivation of Pseudomonas aeruginosa, Escherichia coli, Shigella sonnei and Salmonella typhimurium in Water.* Ozone: Science & Engineering: The Journal of the International Ozone Association, 1999. **21**(3): p. 293 - 300.

427. Bialoszewski, D. and M. Kowalewski, *Superficially, longer, intermittent ozone theraphy in the treatment of the chronic, infected wounds.* Ortop Traumatol Rehabil, 2003. **5**(5): p. 652-8.

428. Martinez-Sanchez, G., et al., *Therapeutic efficacy of ozone in patients with diabetic foot.* Eur J Pharmacol, 2005. **523**(1-3): p. 151-61.

429. Nogales, C.G., et al., *Ozone therapy in medicine and dentistry.* J Contemp Dent Pract, 2008. **9**(4): p. 75-84.

430. Uysal, B., et al., *Efficacy of Hyperbaric Oxygen Therapy and Medical Ozone Therapy in Experimental Acute Necrotizing Pancreatitis.* Pancreas, 2009.

431. Ono, R. and T. Oda, *Ozone production process in pulsed positive dielectric barrier discharge.* Journal of Physics D: Applied Physics, 2007(1): p. 176.

432. Fridman, A., *Gas-Phase Inorganic Synthesis in Plasma*, in *Plasma Chemistry*. 2008, Cambridge University Press: New York, NY. p. 382-397.

433. Niedernhofer, L.J., et al., *Malondialdehyde, a product of lipid peroxidation, is mutagenic in human cells.* J Biol Chem, 2003. **278**(33): p. 31426-33.

434. Marnett, L.J., *Lipid peroxidation-DNA damage by malondialdehyde.* Mutat Res, 1999. **424**(1-2): p. 83-95.

435. Marnett, L., *Oxy radicals, lipid peroxidation and DNA damage.* Toxicology, 2002. **181-182**: p. 219-222.

436. Pryor, W.A. and J.P. Stanley, *Letter: A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation.* J Org Chem, 1975. **40**(24): p. 3615-7.

437. Marnett, L., *Lipid peroxidation—DNA damage by malondialdehyde.* Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 1999. **424**: p. 83-95.

438. Jeong, Y.C. and J.A. Swenberg, *Formation of M1G-dR from endogenous and exogenous ROS-inducing chemicals.* Free Radic Biol Med, 2005. **39**(8): p. 1021-9.

439. Draper, H.H. and M. Hadley, *Malondialdehyde determination as index of lipid peroxidation.* Methods Enzymol, 1990. **186**: p. 421-31.

440. Rubin, R. and J.L. Farber, *Mechanisms of the killing of cultured hepatocytes by hydrogen peroxide.* Arch Biochem Biophys, 1984. **228**(2): p. 450-9.

441. Ramsammy, L.S., et al., *Effects of diphenyl-phenylenediamine on gentamicin-induced lipid peroxidation and toxicity in rat renal cortex.* J Pharmacol Exp Ther, 1986. **238**(1): p. 83-8.

442. Farber, J.L., et al., *Peroxidation-dependent and peroxidation-independent mechanisms by which acetaminophen kills cultured rat hepatocytes.* Arch Biochem Biophys, 1988. **267**(2): p. 640-50.

443. Kirkland, J.B., *Lipid peroxidation, protein thiol oxidation and DNA damage in hydrogen peroxide-induced injury to endothelial cells: role of activation of poly(ADP-ribose)polymerase.* Biochim Biophys Acta, 1991. **1092**(3): p. 319-25.

444. Hickson, I., et al., *Identification and Characterization of a Novel and Specific Inhibitor of the Ataxia-Telangiectasia Mutated Kinase ATM.* Cancer Res, 2004. **64**(24): p. 9152-9159.

445. Abraham, R.T., *PI 3-kinase related kinases: 'Big' players in stress-induced signaling pathways.* DNA Repair, 2004. **3**(8-9): p. 883-887.

446. Dobrynin, D., et al., *Physical and biological mechanisms of direct plasma interaction with living tissue.* New Journal of Physics, 2009(11): p. 115020.

447. Evans, M.D., Dizdaroglu, M., Cooke, M. S., *Oxidative DNA damage and disease: induction, repair and significance.* Mutation Research, 2004. **567**(1): p. 1-61.

448. Shiloh, Y., *The ATM-mediated DNA-damage response: taking shape*. Trends Biochem Sci, 2006. **31**: p. 402-410.

449. Rogakou, E.P., Boon, C., Redon, C., Bonner, W. M., *Megabase chromatin domains involved in DNA double-strand breaks in vivo*. J. Cell Biol, 1999. **146**: p. 905-916.

450. Rogakou, E.P., Pilch, D. R., Orr, A. H., Ivanova, V. S., Bonner, W. M., *DNA doublestranded breaks induce histone H2AX phosphorylation on serine 139.* Journal of biological chemistry, 1998. **273**: p. 5858-5868.

Vita

Full Name: Sameer Kalghatgi
Place and Date of Birth: Mumbai, Maharashtra, India on 31st January 1982

Education and Experience

- Doctor of Philosophy, Electrical Engineering, Drexel University, Philadelphia. March 2010.
  - Graduate Research Fellow, 10/2005 – Present, A. J. Drexel Plasma Institute, Drexel University
  - Graduate Research Assistant, 9/2004 – 9/2005, ECE Department, Drexel University, Philadelphia
  - Teaching Assistant, 1/2006 – 12/2009, ECE Department, Drexel University, Philadelphia
- Bachelor of Engineering, Electrical Engineering, VJTI, Mumbai University, June 2003.

Awards and Honors

Highly Commended Citation for the Drexel University Excellence in Research Award (2009); Invited Talk, 36th IEEE ICOPS (2009); IEEE Student Travel Award, 17th IEEE PPC (2009); Honorable Mention, PhD Level Student Paper Competition, ASME Summer Bioengineering Conference (2009); IEEE Student Travel Award, 36th IEEE ICOPS (2009); Drexel University George Hill Jr. Endowed Fellowship (2009); Best Student Paper Award at the 36th IEEE ICOPS (2008); Highly Commended Citation for the Drexel University Excellence in Research Award (2008); NSF Student Travel Award, Gordon Research Conference on Plasma Processing Science (2008); Finalist for the Student Paper Excellence Award, 61st GEC (2008); Lee Smith Travelling Fellowship; 18th ISPC (2007); IEEE Student Travel Award, 35th IEEE ICOPS (2007); Drexel University Dean's Fellowship (2004)

Selected Journal Publications, Conference Presentations and Proceedings

- S. Kalghatgi, et. al., Endothelial Cell Proliferation is enhanced by Low Dose Non-Thermal Plasma Treatment through Fibroblast Growth Factor-2 Release, *Annals of Biomedical Engineering*, 2010, 38(3): p 748-757.
- S. Kalghatgi, et. al., Mechanism of Blood Coagulation by Non-Thermal Atmospheric Pressure Dielectric Barrier Discharge Plasma, *IEEE Transactions on Plasma Science*, 2007, 35(5): p1559-1566.
- D. Halverson, S. Kalghatgi, et. al., Manipulation of Nonmagnetic Nanobeads in Dilute Ferrofluid, *Journal of Applied Physics*, 2006, 99 (8): P504
- Sameer is also associated with 8 Journal papers in preparation, 45 posters at 16 conferences and has 14 conference proceedings. He has also given 17 oral presentations two of which were invited.

Patents

Plasma treatment for growth factor release from cells and tissues. Sameer Kalghatgi, Alexander Fridman, Gary Friedman, Alisa Morss Clyne (U. S. Provisional Patent Application Serial No. 61/160,496)

Teaching Experience

ECE Dept., Drexel University, Philadelphia PA	Teaching Assistant	1/2006 – 12/2010

Fund. of Intelligent Systems (Winter 06, Spring 06); Transform Methods (Winter 07); Electronic Devices (Spring 07, Fall 07); Lab 4 (Summer 07, Winter 08, Summer 08); Lab 1 (Spring 08); Electromagnetic Fields and Waves (Fall 08); Laboratory 2 (Winter 09); Solid State Devices (Fall 09).

Leadership Activities and Service

Treasurer	Graduate Student Association (GSA)	6/2008 – 6/2009
Vice President	Engineering Graduate Association (EGA)	6/2006 – 6/2008
Secretary	Engineering Graduate Association (EGA)	6/2005 – 6/2006
Reviewer	IEEE Transactions on Plasma Science	1/2007 – Present
Reviewer	Plasma Medicine Journal	3/2009 – Present

Professional Memberships

Member	American Association for Advancement of Science (AAAS)	1/2008 – Present
Member	Institute of Electrical and Electronic Engineers (IEEE)	1/2004 – Present