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Apical Application of Nanosecond-Pulsed Dielectric Barrier Discharge Plasma Causes the Basolateral Release of Adenosine Triphosphate as a Damage-Associated Molecular Pattern from Polarized HaCaT Cells

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ABSTRACT: Promising biomedical uses for nonthermal plasma (NTP) in the fields of regenerative medicine, cancer therapy, and vaccine delivery involve the noninvasive application of uniform nonequilibrium plasma (including dielectric barrier discharge plasma) to living skin. Whereas most investigations have focused on achieving desired therapeutic outcomes, fewer studies have examined the mechanisms and pathways by which epithelial cells respond to NTP exposure. Using a transwell apical–basolateral-chambered system to culture the human keratinocyte HaCaT cell line, *in vitro* experiments were performed to demonstrate the effects of nanosecond-pulsed dielectric barrier discharge (nsDBD) plasma on polarized epithelial cell viability, monolayer permeability, intracellular oxidative stress, and the release of adenosine triphosphate (ATP). Application of nsDBD plasma at 60 Hz or below had minimal or no effect on HaCaT monolayer viability or permeability. nsDBD plasma exposure did, however, result in frequency-dependent reductions in intracellular glutathione (indicating direct induction of oxidative stress by nsDBD plasma) and increased extracellular ATP concentrations in the basolateral (subepithelial) media, which are indicators of cellular stress and an NTP-induced inflammatory response. These studies provide new insights into nsDBD plasma–induced inflammation and local innate immune responses initiated by polarized epithelial tissues.

KEY WORDS: dielectric barrier discharge plasma, polarized epithelium, inflammation, DAMP, oxidative stress, ATP, glutathione, keratinocyte

I. INTRODUCTION

Nonthermal plasma (NTP), applied directly to living tissue, has been used as an antimicrobial agent and, more recently, as a therapeutic alternative for regenerative medicine and cancer.[1–3] Many of these translational uses for NTP involve its application to epithelial tissues, such as skin and mucosal surfaces. Although the desired outcomes of NTP

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application, such as wound repair, skin sterilization, and tumor abatement, have been investigated and demonstrated, there is still much to be learned about the effects of NTP on epithelial tissues.

Human skin is an intricately organized arrangement of differentiated keratinocytes that respond to both mechanical and chemical stimuli.[4–6] The formation of stratified squamous epithelial structures is a crucial process of polarization that is necessary for protective barrier functions, nutrient absorption, and waste excretion.[4,7–9] Cell polarity directs keratinocyte morphology to an apical–basolateral arrangement that allows for the proper formation of cellular junctions necessary for efficient cell-signaling activity and epithelial structural integrity.[4,10] The spatial distribution of keratinocytes creates a barrier that defines internal and external environments and is influenced by either biotic (infectious agents) or abiotic (radiation or NTP) interventions. Because previous *in vitro* studies of the effects of NTP on epithelial cells have relied on the use of nonpolarized cells, there is a critical need for a greater understanding of the mechanisms of action that underlie the targeted effects of NTP application on polarized epithelial tissues.

During the application of NTP, the ionization of gas molecules in the environment by high-energy electrons generates a mixture of reactive species, including charged particles.[11] Through oxidative reactions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated (Fig. 1). Plasma-generated ROS can permeate the cellular membrane and induce cell death via DNA damage.[12] At lower subcytotoxic lev-

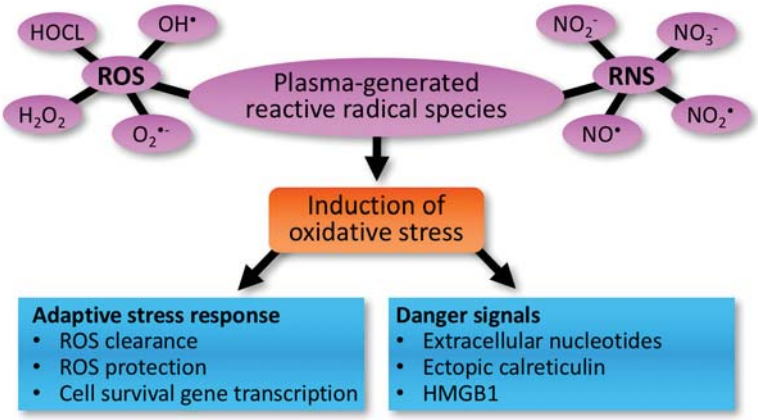

FIG. 1: Downstream effects of NTP-induced oxidative stress. Plasma-generated ROS and RNS are derived from the composition of air and amino acids that alters the redox potential of living cells. Mitochondria-associated stress involves the generation of intracellular ROS and dictates intrinsic death pathways to ensure optimal cell-survival conditions. Exposure to low levels of ROS induces an adaptive stress response that promotes ROS clearance, ROS protection, and cell-survival gene transcription. Another outcome of NTP-induced oxidative stress involves endoplasmic reticulum–associated pathways that result in local danger signaling. Danger molecules include extracellular nucleotides, ectopic calreticulin, and the HMGB1 protein

els of plasma-generated ROS, intracellular ROS (iROS) can augment cell proliferation, cell survival, and cell-mediated immune responses.[1,3,13–18] Cellular responses to iROS are usually signaled by oxidation of glutathione (GSH) to glutathione disulfide (GSSG). As GSH is depleted, excess iROS becomes a main cause of oxidative stress within a cell.[19] To counteract the effects of iROS, cells alter their gene expression profiles to facilitate survival and protection against oxidative stress.[20]

Activation of nuclear factor κ B (NF-κB), which is one of the transcription factors associated with these protective mechanisms, results in the up-regulation of cytokine and chemokine expression and release, causing local immune cell recruitment and activation.[21]

As a result of oxidative stress, cells also release a variety of stress factors, including adenosine triphosphate (ATP), S100, and high-mobility group box 1 (HMGB1), which are otherwise known as damage-associated molecular patterns (DAMPs).[22] These danger signals (Fig. 1) act as chemotactic mediators that also recruit and activate local innate immune cells (i.e., macrophages and neutrophils) to facilitate tissue repair. Extracellular ATP has been described not only as an immunostimulatory agent but also a potent autocrine and paracrine signaling molecule that binds to and acts through the P2 family of receptors.[23] These receptors are expressed in all eukaryotic cells and regulate a wide array of processes including junction formation and inflammation.[24,25] Activation of the P2Y receptor-mediated pathway also results in downstream activation of NF-κB.[25]

In a previous study, NTP induced intracellular danger signaling and stress-dependent release of ATP.[14] Current efforts investigated these findings in an *in vitro* model of the human epithelium. The model incorporates the human keratinocyte HaCaT cell line grown in a transwell culture system.[26] This arrangement mimics the topology of the skin, where keratinocytes form a barrier between the external (apical) environment and subepithelial (basolateral) tissues. As a HaCaT monolayer forms in the transwell, cells are able to form lateral connections (tight junctions) and polarize along the apical–basolateral axis. The polarity of the transwell culture provides the means to determine the basolateral effects of apical exposure to NTP, expanding our understanding of the effects of NTP on living skin.

Advances in plasma generation have resulted in the development of a floating electrode- dielectric barrier discharge (FE-DBD) system that is able to deliver NTP to living tissue without causing damage.[27,28] Nanosecond-pulsed dielectric barrier discharge (nsDBD) can generate uniform NTP while preventing the formation of potentially damaging streamers and filaments.[11,29] Direct plasma treatment generates the highest density of plasma at the tissue surface, encompassing both short- and long-lived radical species at exposure distances of ~1 mm from the tissue surface.[3,30] In experiments designed to examine the effects of nsDBD plasma on a polarized epithelium, cell viability and monolayer permeability assays established a sublethal exposure threshold, below which viability and integrity of the HaCaT monolayer were maintained. Plasma-induced oxidative stress in HaCaT monolayers was observed to cause transient depletion of GSH, but GSH levels recovered to baseline by 24 h postexposure. Inflammation was indicated

by increases in basolateral extracellular ATP to a degree dependent on plasma pulse frequency. These results reveal a mechanism of action by which NTP-induced oxidative stress can incite local inflammatory responses from a polarized epithelial monolayer.

II. MATERIALS AND METHODS

A. Cell Culture

Cells of the HaCaT human keratinocyte cell line were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.8% kanamycin, 0.8% penicillin streptomycin, and 0.74% sodium bicarbonate at 37ºC and 5% CO_2. Using a 24-well transwell system (polyester membranes with a 0.4-μm pore size; Sigma Aldrich), HaCaT cells were seeded at 1.5×10^5 cells/mL in 200 μL in the apical chamber with 600 μL of growth medium in the basolateral chamber. Cultures were maintained for 4 d before plasma treatment to allow the monolayers to reach confluence and low permeability.

B. nsDBD Plasma Exposure

nsDBD plasma was applied to HaCaT cells cultured in the upper chamber of the transwell culture system (Fig. 2A). Plasma energy delivered to the cells was altered by varying the frequency of nanosecond pulses controlled by an external function generator. The nanosecond pulser (FPG20-05NM, FID GmgH, Germany) generated a 31-kV pulse

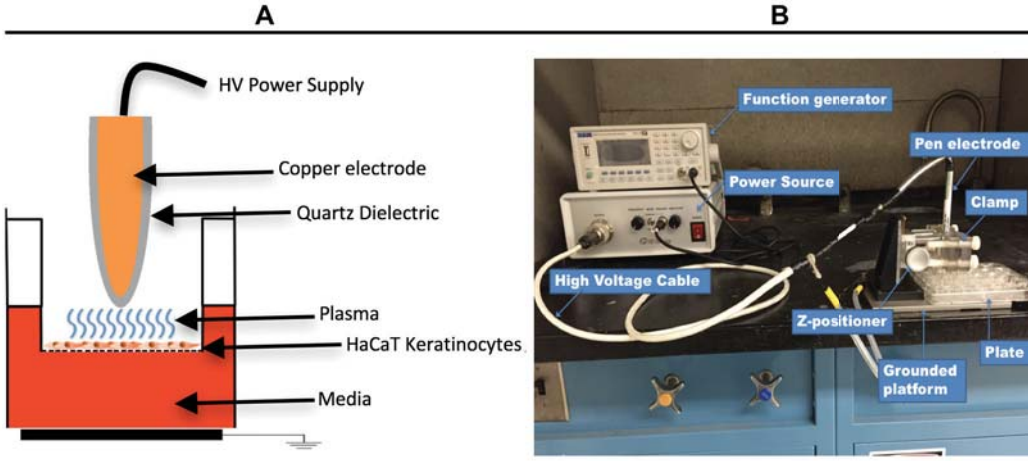

FIG. 2: Exposure of polarized human HaCaT keratinocytes to nsDBD plasma using a transwell-based epithelial model. (A) Schematic of the epithelial model and application of nsDBD. (B) Photograph showing the HV power source and function generator (left) as well as the electrode, z positioner, and transwell culture plate (right)

with 10-ns pulse width and 2–3-ns rise times. A z positioner was used to adjust the gap distance to 1–2 mm between the high-voltage (HV) electrode and the cells (Fig. 2B). After removing the growth media from the cells, nsDBD plasma was applied apically to cells for 10 s at frequencies ranging from 5 to 60 Hz. After plasma exposure, the cells were provided with new growth medium.

C. Monolayer Permeability and Cell Viability

To assess HaCaT cell monolayer integrity, phenol red-free medium (600 μL) was pipetted into each basolateral chamber followed by an introduction of 200 μL of media containing dextran (55 kDa) conjugated to fluorescein isothiocyanate (FITC) at a concentration of 4 mg/mL (Sigma-Aldrich) into each apical chamber. After 30-min incubation at 37ºC, the medium in the basolateral chamber was collected and analyzed with a fluorometer at 485 nm. The same cells were used to assess changes in viability subsequent to nsDBD plasma exposure.

Cell viability following 1-h post-nsDBD plasma exposure was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After aspirating the apical medium containing dextran-FITC and three washes using Hank's balanced salt solution (HBSS), phenol red-free medium (250 μL) was pipetted into each apical chamber followed by 150 μL of MTT solution (7.5 mg/mL in phenol-buffered saline), and the medium was then incubated for 45 min at 37ºC in 5% CO_2. After incubation, the MTT solution was aspirated. To solubilize the purple formazan crystals, acidic isopropanol (250 μL) was pipetted into each apical chamber and then constantly agitated for 20 min. The solubilized formazan solutions were subsequently collected, analyzed with a plate reader at 570 nm, and corrected for background at 690 nm.

D. GSH Detection

The GSH Glutathione-Glo assay (Promega) was used to detect intracellular GSH levels. The GSH Glo Reagent 2X was prepared by mixing a 1:1:50 solution containing luciferin-NT substrate, GHS *S*-transferase, and GSH-Glo Reaction buffer (respectively). The luciferin detection reagent was prepared by adding one bottle of reconstitution buffer with esterase to the amber bottle of lyophilized luciferin detection reagent. After plasma exposure, 50 μL of the GSH Glo Reagent 2X was added apically to the cells and then incubated for 30 min at room temperature. Cells were then scraped off the transwell inserts and collected in 1.5-mL Eppendorf tubes. The cells were lysed by two freeze–thaw cycles over dry ice and then centrifuged for 5 min at 14,000 rpm. After centrifugation, cell lysates were transferred into a 96-well black-walled plate and supplemented with 100 μL of the prepared luciferin detection reagent in each well. After a 15-min incubation, relative light units were measured with a GloMax Luminometer. GSH concentrations were determined using a standard curve generated with the provided GSH standard solution (5 nM) through 1:1 serial dilutions in pure water.

E. Extracellular ATP Detection

An ATP bioluminescent cell assay (Sigma-Aldrich) was used to detect extracellular ATP in basolateral conditioned medium from HaCaT epithelial cell transwell cultures. Basolateral conditioned medium was collected 1 h post-nsDBD plasma exposure. ATP reacts with luciferin, which is then catalytically oxidized by firefly luciferase to form oxyluciferin, adenosine monophosphate, carbon dioxide, inorganic phosphate, and light. The ATP assay mix was prepared by a 1:25 dilution of ATP assay mix stock solution to dilution buffer. A working solution (100 μL) was added in 12 × 55 test tubes (Hack) and incubated for 3 min at room temperature. Basolateral conditioned medium (50 μL) was then diluted in 100 μL of ultrapure water (Sigma-Aldrich), and 50 μL of the diluted sample was then added into the 12 × 55 test tube containing 100 μL of the ATP assay mix solution. Relative light units were measured immediately using a PhotonMaster luminometer (PM10146, LuminUltra). The luminometer was calibrated with UltraClear solution across a standard curve that was used to convert average relative light units to the ATP concentration (picograms/milliliter).

F. Statistical Analyses

All experimental data points were derived from duplicate experiments with duplicate samples and plotted as the mean ± standard error of the mean (SEM). Data were analyzed by a Student's *t*-test. A value of $p \leq 0.05$ was considered to be statistically significant.

III. RESULTS

A. Increases in nsDBD Frequency Associated with Modest Reductions in Cell Viability and Small Increases in HaCaT Monolayer Permeability

The use of NTP to effect changes in living tissue must strike a balance between the desired outcome and energy-dependent NTP toxicity. In addition, uniform nsDBD plasma delivered at higher energies (frequencies) can result in tissue damage and losses in cell viability that will hamper the efforts to identify any biochemical changes due to plasma exposure.[31] Before nsDBD plasma can be effectively used *in vitro* or *in vivo*, optimal dose ranges must be established that minimize the detrimental effects of plasma exposure. In applications involving nsDBD delivery to the skin, these types of studies are necessary to establish a therapeutic window.

To examine the effect of NTP frequency on polarized epithelial cells, HaCaT cells were exposed to nsDBD plasma generated at frequencies ranging from 10 to 60 Hz with a constant 1-mm gap distance (Fig. 3). In assays of cell viability, application of nsDBD plasma at 10 Hz reduced HaCaT cell viability to approximately 82% relative to mock-exposed cells (Fig. 3A). Although a slightly greater reduction in viability was observed at 20 Hz (~73%), no further reductions in viability were noted after application of nsDBD plasma generated at higher frequencies (up to 60 Hz).

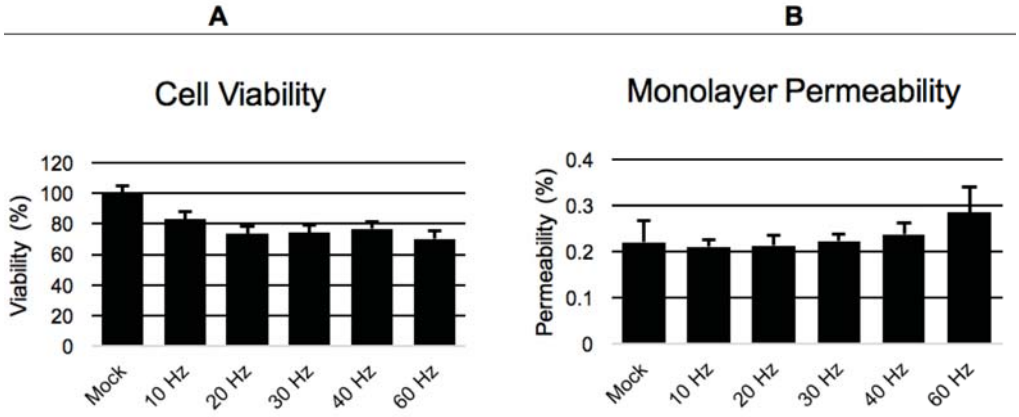

FIG. 3: Changes in polarized HaCaT cell (A) viability and (B) monolayer permeability following mock-plasma exposure or exposure to nsDBD plasma generated at 10–60 Hz and delivered at a 1-mm gap distance

The integrity of the cell monolayer, as measured by permeability to dextran FITC, was unchanged after exposure to nsDBD plasma generated at 10–30 Hz (Fig. 3B). Application of nsDBD plasma at 40 and 60 Hz resulted in modest frequency-dependent increases in permeability (~0.24% and 0.28%, respectively) relative to mock-exposed cells (0.22%).

B. Reduced nsDBD Uniformity Causes Frequency-Dependent Changes in Cell Viability and Increases in HaCaT Monolayer Permeability

The effect of nsDBD plasma is highly dependent on uniformity of plasma generation.[11] To study plasma-generated ROS as a source of metabolic and biochemical changes in living cells, uniform plasma is preferentially used. This ensures consistent exposure across a surface without any introduction of filaments that might damage living tissues and cells. Plasma exposure at a gap distance > 1 mm is characterized by a loss in uniformity and the formation of streamers with locally high electrical fields, resulting in losses in cell viability.[28] The detrimental effects of nonuniform plasma can complicate measurements of metabolic and biochemical changes that are attributable to nsDBD plasma exposure.

Experiments were performed to compare the changes in viability and monolayer permeability associated with the application of uniform (1 mm) and nonuniform (2 mm) nsDBD plasma (Fig. 4). HaCaT cells were exposed to nsDBD plasma generated at 10 and 30 Hz and delivered at gap distances of 1 and 2 mm. nsDBD plasma delivered at 1 mm and generated at either 10 or 30 Hz reduced cell viability to ~80% relative to mock-exposed cells (Fig. 4A). At a gap distance of 2 mm, cell viability was unaffected by nsDBD plasma at 10 Hz but was reduced to ~80% when frequency was increased

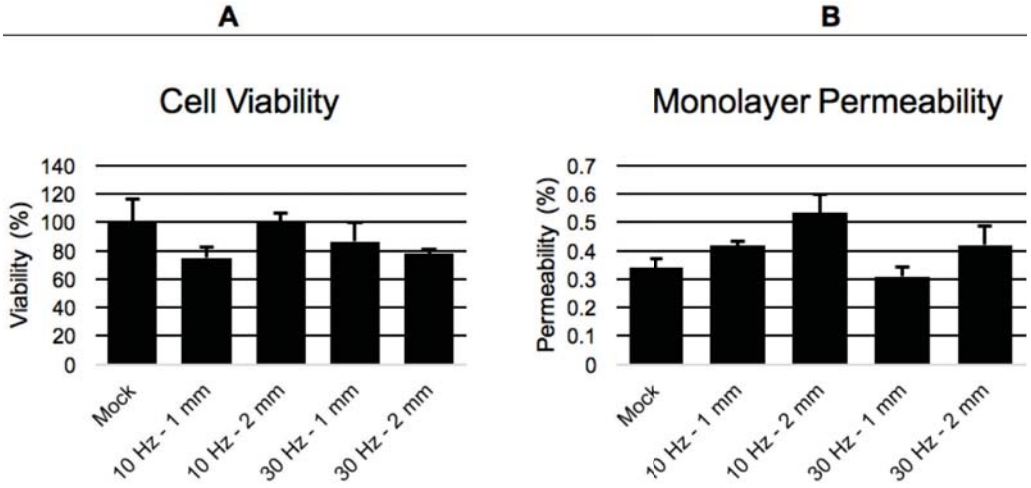

FIG. 4: Changes in polarized HaCaT cell (A) viability and (B) monolayer permeability following mock-plasma exposure or exposure to nsDBD plasma generated at 10 or 30 Hz and delivered at a gap distance of 1 or 2 mm

to 30 Hz. These results suggest that the effect of plasma uniformity on cell viability is dependent on pulse frequency.

Parallel assays of monolayer permeability were performed to determine the effect of uniform and nonuniform nsDBD plasma on intercellular connections between HaCaT cells and the integrity of the HaCaT cell layer. Minimal changes in monolayer permeability (relative to unexposed cells) were observed after exposure to plasma generated at 10 and 30 Hz and delivered at a gap distance of 1 mm (Fig. 4B). At a gap distance of 2 mm, however, nsDBD plasma exposure increased monolayer permeability from 0.3% (mock-exposed cells) to ~0.6% and 0.5% at 10 and 30 Hz, respectively. These results demonstrate a direct relationship between nsDBD plasma nonuniformity (gap distance) and cell permeability.

The results of these experiments demonstrate that nonuniform plasma altered the dependence of cell viability and permeability on plasma frequency. Furthermore, the changes in biological outcomes associated with variations in gap distance underscore the importance of controlling gap distance during *in vitro* experiments and suggest that the skin-to-electrode distance is a critical variable to consider in any *in vivo* uses of NTP. The modest frequency-dependent changes in cell viability and permeability after nsDBD plasma exposure illustrate the latitude available in establishing an optimal plasma dose during *in vitro* experiments. Additional experiments performed using an expanded range of frequencies demonstrated that polarized HaCaT cells tolerated nsDBD plasma generated at frequencies as high as 100 Hz (data not shown). During *in vivo* applications involving nsDBD plasma, effective dosages of NTP must be high enough to result in the desired outcome (e.g., wound repair, sterilization, or

immune cell recruitment) but low enough to mitigate undesirable consequences (e.g., cell necrosis or loss of epithelial integrity). Consideration of NTP frequency must be a critical component for establishing the parameters associated with therapeutic applications of NTP.

C. Increased nsDBD Plasma Frequency Results in a Greater Reduction in Intracellular GSH

ROS are believed to be the primary biochemical agents that are generated in living tissues subsequent to plasma exposure.[32] As suggested by numerous studies, ROS has roles in regulating a plethora of cellular responses including inflammation.[1,17,20] ROS generation within the cell is offset by a cycle of redox reactions designed to neutralize the cytotoxic effects of excessive intracellular ROS. One of the major reactions involves the oxidation of GSH by superoxide anion to GSSG. The depletion of GSH is indicative of cellular oxidative stress resulting from a variety of environmental factors.[19,33] GSH is also generated as a protective mechanism to combat persistent ROS presence within the cell. At a certain threshold of ROS imbalance, cells become tolerant to the generation of ROS and become relatively unaffected by ROS, compared to initial exposure.[34] In the context of application of nsDBD plasma to living tissues, the persistence of NTP-associated oxidative stress will be an important aspect of studies designed to establish the acute and chronic effects of nsDBD plasma exposure.

To determine the relationship between uniform nsDBD plasma and oxidative stress, HaCaT cells in the polarized epithelial model were exposed to nsDBD plasma at 10 or 30 Hz (1-mm gap distance) and assayed for changes in intracellular GSH levels (Fig. 5). Considerable frequency-dependent reductions in intracellular GSH

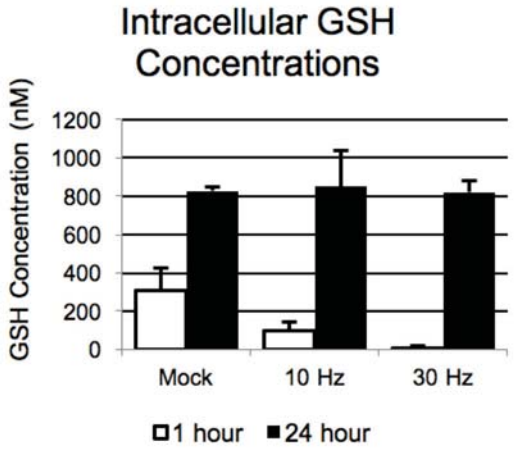

FIG. 5: Changes in intracellular GSH in polarized HaCaT cells at 1 and 24 hr following mock exposure or exposure to nsDBD plasma generated at 10 or 30 Hz and delivered at a 1-mm gap distance

were observed at 1 h postexposure. After exposure to nsDBD plasma generated at 10 Hz, GSH levels were reduced by more than 50% relative to unexposed cells. In cells exposed to 30-Hz nsDBD plasma, GSH levels were almost undetectable. By 24 h postexposure, however, unexposed and plasma-exposed cells had similar GSH levels, indicating a full recovery of the plasma-exposed cells during the 24-h postexposure maintenance period.

These experiments revealed an immediate increase in intracellular oxidative stress attributed to nsDBD plasma exposure, followed by a complete recovery within 24 h postexposure, presumably due to the replenishment of GSH pools that counteract the plasma-generated ROS. Protection from oxidative stress is driven by the up-regulation of cell survival and antiapoptotic pathways that allow cells to maintain their regulatory processes.[19] The transient nature of the plasma-associated changes in GSH and the capacity of the cells to mitigate the oxidative stress within 24 h suggest that plasma-associated ROS *in vivo* will cause short-term effects without invoking long-term damage. The cellular responses to nsDBD exposure in the polarized epithelial model also provide the foundation for more mechanistic studies of the mediators of plasma-associated oxidative stress, with an emphasis on the species involved in causing intracellular oxidative stress (ROS, RNS, neutral species) and the specific cellular pathways that are activated in response.

D. Extracellular ATP is Released into the Basolateral Chamber from Polarized HaCaT Cells Exposed to nsDBD Plasma

Release of extracellular ATP is an important mechanism of cell signaling used by neuronal, hepatic, and epithelial cells.[23,35] Large concentrations of ATP within the cell favor the movement of ATP out of the cell through ion channels.[36] Several studies have proposed mechanisms of ATP translocation and have identified a ubiquitous role of ATP in various cellular processes.[25,37–41] Cells are able to respond to extracellular ATP through ionotropic P2X receptors and G-protein–coupled P2Y receptors that bind ATP and are found on all cell types.[24] One downstream effect of P2Y receptor engagement is the activation of the transcription factor NF-κB, which is directly involved in the activation and recruitment of immune cells.[24] For this reason, ATP is considered to be an important DAMP involved in the process of inflammation and immune stimulation. Recent studies have demonstrated the release of ATP in response to environmental stress factors, such as imbalances associated with ROS.[22,35,36,42,43]

Because nsDBD plasma was shown to induce oxidative stress in polarized HaCaT cells, we hypothesized that ATP would be secreted in quantities proportional to the dose of plasma delivered. To investigate this hypothesis, polarized HaCaT cells were exposed to uniform nsDBD plasma generated at frequencies ranging from 5 to 30 Hz (1-mm gap distance) and assessed for the subsequent release of ATP into the basolateral medium (Fig. 6). Results of these experiments clearly demonstrated a direct relationship between nsDBD plasma frequency and basolateral ATP release. Exposure of cells to nsDBD plasma generated at 15 Hz caused a fourfold increase in extracel-

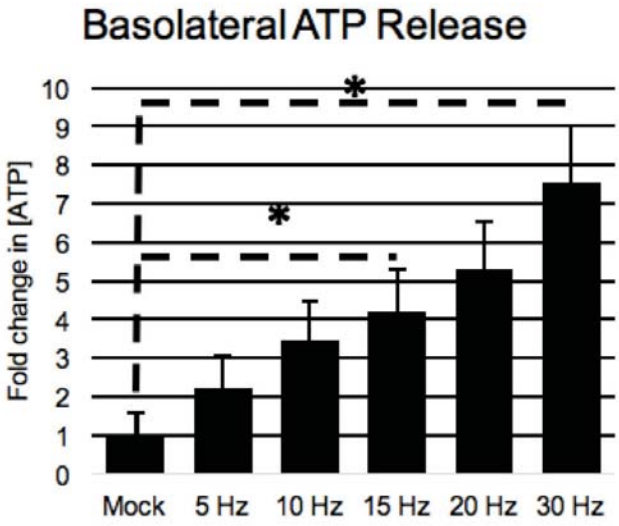

FIG. 6: Changes in extracellular ATP secretion from polarized HaCaT cells immediately after mock exposure or exposure to uniform nsDBD plasma generated at frequencies ranging from 5 to 30 Hz (1-mm gap distance). Mock-exposed cells released ATP at an average concentration of 45 nM. Asterisks indicate statistical differences ($p \leq 0.05$)

lular ATP release relative to mock-exposed cells (which also released ATP at lower, but detectable, concentrations). Furthermore, a doubling of plasma frequency (15 to 30 Hz) resulted in a further twofold increase in ATP release. These results demonstrate a clear correlation between nsDBD plasma–induced cellular stress and the release of ATP as a cellular DAMP.

The directional release of ATP into the basolateral chamber implies an effector mechanism in which polarized epithelial cells communicate with neighboring epithelial cells as well as immune cells residing in the subepithelial space. These results support the hypothesis that the effects of nsDBD exposure are a combination of the direct effects on keratinocytes within the epithelium and the indirect effects on nearby cells mediated by soluble signaling molecules such as ATP. To investigate this hypothesis, the transwell epithelial model is being expanded to include immune cells that would be subject to the indirect effects of nsDBD exposure transduced through the epithelial cell layer. In preliminary experiments, basolateral media conditioned by apical exposure of HaCaT cells to nsDBD plasma was shown to induce chemotaxis of primary human peripheral blood mononuclear cells (data not shown). Subsequent experiments are planned to (1) identify and characterize the specific immune cell types recruited by conditioned basolateral media and (2) identify the chemotactic factors that mediate the cell recruitment. Because ATP is itself a potent chemotactic factor,[44,45] it is likely that ATP released from HaCaT cells is at least partly responsible for the chemotaxis observed in these experiments.

IV. CONCLUSIONS

The development of nsDBD plasma for clinical use will require a deeper understanding of its effects on cells, tissues, and living organisms as well as the parameters of NTP delivery that modulate those effects. For example, the application of NTP to human skin will require a greater appreciation of the negative effects potentially associated with NTP, particularly at higher doses. The application of nsDBD plasma to living cells has been shown to induce significant cytotoxicity.[11,28] In experiments performed *in vitro*, nsDBD plasma produced levels of reactive radical species that induced apoptosis and necrosis in the cell.[28] Because of the cytotoxicity that can arise as a result of nsDBD exposure, *in vitro* experiments designed to explore the mechanisms that underlie the effects of plasma exposure can be difficult to interpret.[14,15] In addition, efforts directed toward the identification of optimal NTP application parameters (including plasma frequency and voltage, electrode distance, exposure time, and the selective delivery of specific NTP components such as ROS, RNS, and an electromagnetic field) for *in vitro* experiments and *in vivo* uses will need to consider plasma-associated toxicity.

Previous investigations into the biological effects of NTP typically involved exposure of submerged nonpolarized cells to NTP. Although these types of experiments produced reproducible results from functional assays,[14,15] they were not designed to emulate the application of NTP to the polarized, three-dimensional architecture of human skin. In contrast, the current investigations were conducted using polarized human HaCaT keratinocytes as a surrogate for human skin. Our experiments demonstrated that nsDBD exposure induced a significant but transient increase in oxidative stress and the release of ATP with minimal reductions in HaCaT keratinocyte viability or barrier function of the epithelial cell layer.

Future studies using this model system will examine other effects of NTP exposure on polarized cells, including inflammasome activation, changes in epithelial-cell gene transcription, and the effect of ATP as a DAMP on subepithelial immune cells (Fig. 7). With respect to nsDBD-induced changes in gene expression, we have already used this transwell model system to preliminarily demonstrate NF-κB–induced reporter gene expression subsequent to nsDBD plasma exposure (data not shown).

The polarized transwell model system mimics a typical human epithelium with HaCaT keratinocytes in the apical chamber and a basolateral chamber that can be sampled to study the subepithelial effects of nsDBD plasma. The results of these studies establish the polarized epithelial model as a more physiologically relevant system for studies of nsDBD plasma application to human skin and as an effective tool to study the role of NTP in inducing inflammation and immunomodulation. Importantly, they also support the conclusion that therapeutic, noninvasive applications of nsDBD plasma can be used to induce subepithelial inflammatory responses as effectors for wound repair, cancer therapy, and vaccine delivery (Fig. 7). Investigations in this area will provide new insights into the immunological consequences of nsDBD plasma on human skin and will also point to uses for nsDBD plasma that take advantage of the ability to induce innate immune responses in the skin through plasma exposure.

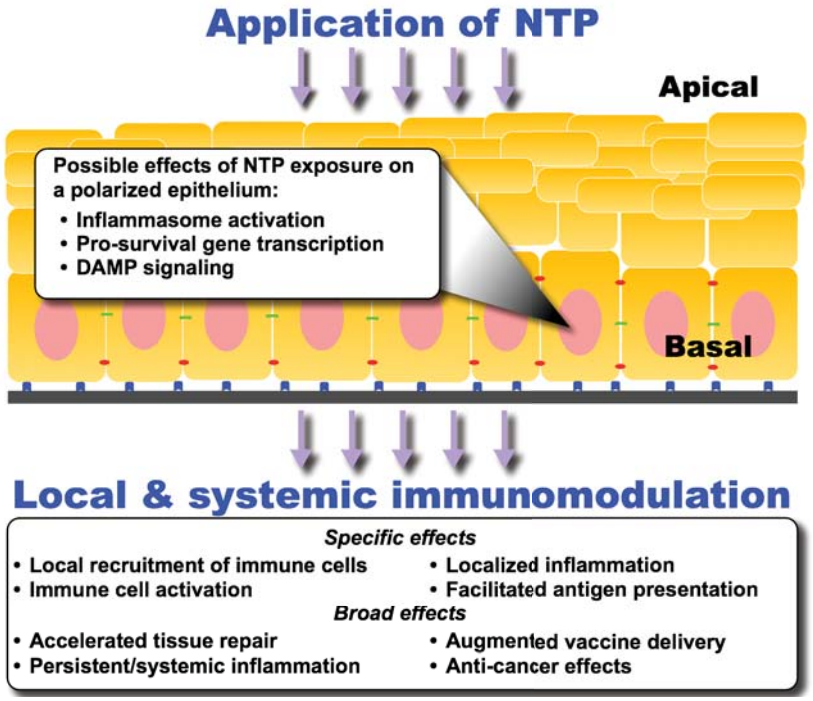

FIG. 7: Proposed model of the transduction of NTP effects by the polarized epithelium. Noninvasive apical exposure of the skin to NTP is hypothesized to stimulate polarized epithelial cells, resulting in inflammasome activation, proinflammatory changes in gene expression, and the release of DAMPs such as ATP. The basolateral release of DAMPs and other proinflammatory factors subsequently induces local innate immune responses, including immune cell recruitment and activation

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REFERENCES

1. Arjunan KP, Friedman G, Fridman A, Clyne AM. Non-thermal dielectric barrier discharge plasma induces angiogenesis through reactive oxygen species. J R Soc Interface. 2012;9(66):147–57.
2. Brulle L, Vandamme M, Ries D, Martel E, Robert E, Lerondel S, Trichet V, Richard S, Pouvesle JM, Le Pape A. Effects of a non thermal plasma treatment alone or in combination with gemcitabine in a MIA PaCa2-luc orthotopic pancreatic carcinoma model. PLoS One. 2012;7(12):e52653.

3. Haertel B, von Woedtke T, Weltmann KD, Lindequist U. Non-thermal atmospheric-pressure plasma possible application in wound healing. Biomol Ther (Seoul). 2014;22(6):477–90.
4. Basler K, Bergmann S, Heisig M, Naegel A, Zorn-Kruppa M, Brandner JM. The role of tight junctions in skin barrier function and dermal absorption. J Control Release. 2016;242:105–18.
5. Eberle T, Doganci B, Kramer H, Fechir M, Wagner I, Sommer C, Birklein F. Mechanical but not painful electrical stimuli trigger TNF alpha release in human skin. Exp Neurol. 2010;221(1):246–50.
6. Lourenco G, Caronni A, Cavallari P. Mechanical and focal electrical stimuli applied to the skin of the index fingertip induce both inhibition and excitation in low-threshold flexor carpi radialis motor units. Eur J Neurosci. 2007;26(8):2204–10.
7. Boer M, Duchnik E, Maleszka R, Marchlewicz M. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. Postepy Dermatol Alergol. 2016;33(1):1–5.
8. Engebretsen KA, Thyssen JP. Skin barrier function and allergens. Curr Probl Dermatol. 2016;49:90–102.
9. Lee T, Friedman A. Skin barrier health: regulation and repair of the stratum corneum and the role of over-the-counter skin care. J Drugs Dermatol. 2016;15(9):1047–51.
10. Norlen L. Current understanding of skin barrier morphology. Skin Pharmacol Physiol. 2013;26(4-6):213–6.
11. Fridman G, Friedman G, Gutsol AF, Shekhter AB, Vasilets VN, Fridman A. Applied plasma medicine. Plasma Proc Polymers. 2008;5(6):503–33.
12. Joshi SG, Cooper M, Yost A, Paff M, Ercan UK, Fridman G, Friedman G, Fridman A, Brooks AD. Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in Escherichia coli. Antimicrob Agents Chemother. 2011;55(3):1053–62.
13. Kalghatgi SU, Fridman A, Friedman G, Clyne AM. Cell proliferation following non-thermal plasma is related to reactive oxygen species induced fibroblast growth factor-2 release. Conf Proc IEEE Eng Med Biol Soc. 2009;2009:6030–3.
14. Lin A, Chernets N, Han J, Alicea Y, Dobrynin D, Fridman G, Freeman TA, Fridman A, Miller V. Nonequilibrium dielectric barrier discharge treatment of mesenchymal stem cells: charges and reactive oxygen species play the major role in cell death. Plasma Proc Polymers. 2015;12(10):1117–27.
15. Lin A, Truong B, Pappas A, Kirifides L, Oubarri A, Chen S, Lin S, Dobrynin D, Fridman G, Fridman A, Sang N, Miller V. Uniform nanosecond pulsed dielectric barrier discharge plasma enhances anti-tumor effects by induction of immunogenic cell death in tumors and stimulation of macrophages. Plasma Proc Polymers. 2015;12(12):1392–9.
16. Schmidt A, Dietrich S, Steuer A, Weltmann KD, von Woedtke T, Masur K, Wende K. Non-thermal plasma activates human keratinocytes by stimulation of antioxidant and phase II pathways. J Biol Chem. 2015;290(11):6731–50.
17. Vandamme M, Robert E, Lerondel S, Sarron V, Ries D, Dozias S, Sobilo J, Gosset D, Kieda C, Legrain B, Pouvesle JM, Pape AL. ROS implication in a new antitumor strategy based on non-thermal plasma. Int J Cancer. 2012;130(9):2185–94.
18. Wu AS, Kalghatgi S, Dobrynin D, Sensenig R, Cerchar E, Podolsky E, Dulaimi E, Paff M, Wasko K, Arjunan KP, Garcia K, Fridman G, Balasubramanian M, Ownbey R, Barbee KA, Fridman A, Friedman G, Joshi SG, Brooks AD. Porcine intact and wounded skin responses to atmospheric nonthermal plasma. J Surg Res. 2013;179(1):e1–e12.
19. Huet O, Cherreau C, Nicco C, Dupic L, Conti M, Borderie D, Pene F, Vicaut E, Benhamou D, Mira JP, Duranteau J, Batteux F. Pivotal role of glutathione depletion in plasma-induced endothelial oxidative stress during sepsis. Crit Care Med. 2008;36(8):2328–34.
20. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. 2014;24(10):R453–62.
21. Del Prete A, Allavena P, Santoro G, Fumarulo R, Corsi MM, Mantovani A. Molecular pathways in cancer-related inflammation. Biochem Med (Zagreb). 2011;21(3):264–75.
22. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer. 2012;12(12):860–75.

23. Dubyak GR. Signal transduction by P2-purinergic receptors for extracellular ATP. Am J Respir Cell Mol Biol. 1991;4(4):295–300.
24. Bours MJ, Dagnelie PC, Giuliani AL, Wesselius A, Di Virgilio F. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. Front Biosci (Schol Ed). 2011;3:1443–56.
25. Kobayashi T, Soma T, Noguchi T, Nakagome K, Nakamoto H, Kita H, Nagata M. ATP drives eosinophil effector responses through P2 purinergic receptors. Allergol Int. 2015;64 Suppl:S30–6.
26. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol. 1988;106(3):761–71.
27. Ayan H, Fridman G, Staack D, Gutsol AF, Vasilets VN, Fridman AA, Friedman G. Heating effect of dielectric barrier discharges for direct medical treatment. IEEE Trans Plasma Sci. 2009;37(1):113–20.
28. Fridman G, Shereshevsky A, Jost MM, Brooks AD, Fridman A, Gutsol AF, Vasilets VN, Friedman G. Floating electrode dielectric barrier discharge plasma in air promoting apoptotic behavior in melanoma skin cancer cell lines. Plasma Chem Plasma Proc. 2007;27(2):163–76.
29. Ayan H, Fridman G, Gutsol AF, Vasilets VN, Fridman A, Friedman G. Nanosecond-pulsed uniform dielectric-barrier discharge. IEEE Trans Plasma Sci. 2008;36(2):504–8.
30. Lloyd G, Friedman G, Jafri S, Schultz G, Fridman A, Harding K. Gas plasma: medical uses and developments in wound care. Plasma Proc Polymers. 2010;7(3-4):194–211.
31. Kalghatgi S, Kelly CM, Cerchar E, Torabi B, Alekseev O, Fridman A, Friedman G, Azizkhan-Clifford J. Effects of non-thermal plasma on mammalian cells. PLoS One. 2011;6(1):e16270.
32. Haertel B, Wende K, von Woedtke T, Weltmann KD, Lindequist U. Non-thermal atmospheric-pressure plasma can influence cell adhesion molecules on HaCaT-keratinocytes. Exp Dermatol. 2011;20(3):282–4.
33. Haton C, Francois A, Vandamme M, Wysocki J, Griffiths NM, Benderitter M. Imbalance of the antioxidant network of mouse small intestinal mucosa after radiation exposure. Radiat Res. 2007;167(4):445–53.
34. Graves DB, editor. Mechanisms of plasma biomedicine: what do we know? Proceedings of the 5th International Conference on Plasma Medicine; 2014 May 18–23; Nara, Japan.
35. Leong WS, Russell RG, Caswell AM. Stimulation of cartilage resorption by extracellular ATP acting at P2-purinoceptors. Biochim Biophys Acta. 1994;1201(2):298–304.
36. Schwiebert EM, Zsembery A. Extracellular ATP as a signaling molecule for epithelial cells. Biochim Biophys Acta. 2003;1615(1-2):7–32.
37. Aho J, Helenius M, Vattulainen-Collanus S, Alastalo TP, Koskenvuo J. Extracellular ATP protects endothelial cells against DNA damage. Purinergic Signal. 2016;12(3):575–81.
38. Feng H, Guan D, Bai J, Sun K, Jia L. Extracellular ATP: a potential regulator of plant cell death. Mol Plant Pathol. 2015;16(6):633–9.
39. Fuentes E, Palomo I. Extracellular ATP metabolism on vascular endothelial cells: a pathway with prothrombotic and anti-thrombotic molecules. Vascul Pharmacol. 2015;75:1–6.
40. Gan M, Moussaud S, Jiang P, McLean PJ. Extracellular ATP induces intracellular alpha-synuclein accumulation via P2X1 receptor-mediated lysosomal dysfunction. Neurobiol Aging. 2015;36(2):1209–20.
41. Zimmermann H. Extracellular ATP and other nucleotides—ubiquitous triggers of intercellular messenger release. Purinergic Signal. 2016;12(1):25–57.
42. Dubyak GR, el-Moatassim C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol. 1993;265(3 Pt 1):C577–606.
43. Sivaramakrishnan V, Fountain SJ. Evidence for extracellular ATP as a stress signal in a single-celled organism. Eukaryot Cell. 2015;14(8):775–82.
44. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science. 2006;314(5806):1792–5.
45. Honda S, Sasaki Y, Ohsawa K, Imai Y, Nakamura Y, Inoue K, Kohsaka S. Extracellular ATP or ADP induce chemotaxis of cultured microglia through $G_{i/o}$-coupled P2Y receptors. J Neurosci. 2001;21(6):1975–82.