A Supersaturated Oxygen Emulsion for the Topical Treatment of Ocular Trauma

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ABSTRACT Introduction: Roughly 13% of all battlefield injuries include some form of ocular trauma. Ocular tissue preservation is critical for wound healing for warfighters with ocular injuries. Our team hypothesized that oxygen plays a vital role in ocular tissue preservation and wound healing and has developed a supersaturated oxygen emulsion (SOE) for the topical treatment of ocular trauma. Materials and Methods: The partial pressure of oxygen (PO_2) was measured in the SOE. Safety and efficacy studies were carried out in primary human corneal epithelial (HCE) cells, as the outermost layer is the first barrier to chemical and mechanical injury. Western blot, scratch assay, and MTT assays were conducted to determine the effect of the SOE on various molecular markers, the rate of scratch closure, and cellular viability, respectively. Results: Data indicate that the SOE releases oxygen in a time-dependent manner, reaching a partial pressure within the emulsion over four times atmospheric levels. Studies in HCE cells indicate that application of the SOE does not lead to DNA damage, promote cell death, or hinder the rate of scratch closure and enhances cellular viability. Preliminary studies were carried out with chloropicrin (CP; developed as a chemical warfare agent and now a commonly used pesticide) as a chemical agent to induce ocular injury in HCE cells. CP exposures showed that SOE treatment reverses CP-induced DNA damage, apoptotic cell death, and oxidative stress markers. Conclusions: Maintaining adequate tissue oxygenation is critical for tissue preservation and wound repair, especially in avascular tissues like the cornea. Further studies examining the application of the SOE in corneal injury models are warranted.

INTRODUCTION

Ocular injuries have historically accounted for roughly 13% of battlefield trauma.[1,2,3] Injuries to the eyes most frequently occur as a result of blast exposure from improvised explosive devices and rocket-propelled grenades, leading to closed (blunt trauma) or open globe (rupture, penetrating or perforating injuries, and intraocular foreign bodies) injury patterns.[1,4,5] Thermal and chemical ocular injuries from various battlefield hazards or chemical weapons can be devastating and may accompany and compound mechanical trauma to the eyes. Because there are few ways to diagnose and treat ocular injuries in the field, medical protocol typically involves protecting the injured area and evacuating the casualty to a hospital capable of definitive ophthalmic care. This traditional "shield and ship" method has been an effective way to manage ocular trauma during the previous wars in Iraq and Afghanistan where our country's aeromedical support teams had extensive networks in country and were able to respond to a medical evacuation call during the critical "Golden Hour"[6]. However, the operational situation is changing and the battlefield is becoming more remote, especially considering our increased reliance upon the U.S. Military's Special Operations Forces who typically deploy in small teams to austere environments without access to first-world infrastructure.[7] Thus, casualties may be hours or days away from definitive care and managing both medical and trauma patients in these types of settings requires extensive medical training and knowledge in prolonged field care. [7,8,9] Ocular injuries, especially those resulting from chemical exposures, that are not quickly treated or surgically repaired can lead to complications that may ultimately result in irreversible vision impairment or total loss. Thus, there is a critical need to address ocular injuries in a prolonged field care setting through measures that promote tissue preservation and wound healing.

Oxygen plays a vital role in wound healing, and oxygen therapy has been shown to be beneficial in treating acute ocular chemical or thermal burns.[10] Specifically, oxygen therapy improved limbal ischemia, accelerated epithelialization, increased corneal transparency, and decreased corneal vascularization as compared to conventional medical treatment.[10] Increasing the PO_2 at the corneal surface may lead to the prevention of neovascularization and corneal clouding, improved tissue preservation, and enhanced wound healing following injury and is something that could be leveraged by medics to treat ocular trauma on the battlefield to improve visual outcomes. However, extra oxygen tanks and hyperbaric chambers are cumbersome and not likely to be used in austere environments.

To address this need, our team has developed a lightweight, ruggedized, and portable solution that could deliver oxygen to

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MHSRS, Kissimmee, FL, 2017, Abstract MHSRS-17-1793; MHSRS, Kissimmee, FL, 2018, Abstract MHSRS-18-1367

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doi:10.1093/milmed/usz337

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the corneal surface following injury in austere environments. This technology, a supersaturated oxygen emulsion (SOE), derives from work originally funded by the Defense Advanced Research Project Agency to develop a novel topical therapeutic for wound healing. Early formulations of the SOE showed great success in improving the rate of wound healing and quality of newly formed tissue in skin mechanical and thermal injury models.[11,12,13] Due to the properties of an ingredient called perfluorodecalin (PFD), the formulation is capable of carrying dissolved oxygen up to six times that of atmospheric levels and releasing this oxygen in a time-dependent manner to the surrounding environment. PFD is biocompatible, inert, and has been used safely as an intraocular retinal tamponade.[14,15] We hypothesized that the SOE would be safe to use on the ocular surface and would support corneal wound healing.

In this study, we used chloropicrin (CP, PS, CCl_3NO_2, nitrochloroform, trichloronitromethane), an aliphatic nitrate compound employed during World War I as a warfare agent,[16,17] as a model agent to induce chemical injury in human corneal epithelial (HCE) cells. CP is a colorless liquid with irritating, choking, and lacrimating properties and exposure primarily affects the eyes, skin, and respiratory system. CP is commonly used in agriculture as a broad-spectrum fumigant and pesticide, and there are clear risks associated with occupational exposure.[16,18] Its widespread availability and lack of approved countermeasures make CP a potential agent for chemical warfare and terrorism.[16,19] CP ocular exposure causes eye irritation, associated with lacrimation and inflammation, which involves corneal edema, ocular tissue damage, and visual damage.[16,20] The cornea is the outermost layer of the eye and is highly sensitive to chemical exposures and injuries; thus, studies were conducted using HCE cells. The effect of SOE treatment on CP-induced toxicity in HCE cells was investigated. The results of this study suggest that the SOE is safe to use on HCE cells and may improve wound healing, as assessed via in vitro assays.

METHODS

Chemicals and Reagents

Chloropicrin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anti-beta-actin antibody, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Primary HCE cells and culture media were obtained from ATCC (Manassas, VA), and TrypLE Express was from Thermo-Fisher Scientific (Waltham, MA). Primary antibodies for phosphorylated H2A.X (Ser139), phosphorylated p53 (Ser15), p53, cleaved-poly (ADP-ribose) polymerase (PARP), and anti-mouse and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). The detergent compatible protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence kit (ECL) was from GE healthcare Bio-Sciences (Pittsburgh, PA). For scratch wound assay, 35-mm culture-insert μ-dish was purchased from ibidi (ibidi USA, Fitchburg, WI). Cyclooxygenase-2 (COX-2) antibody was from Cayman chemicals (Ann Arbor, MI). Unoxygenated and oxygenated (30 and 55% w/v PFD) formulations of the SOE were manufactured by Suite-k (Edison, NJ) for Roccor.

Preparation and Evaluation of SOE

All formulations of the SOE were manufactured by Suite-k (Edison, NJ) and shipped to Roccor. The PO_2 in each sample of SOE (unoxygenated and oxygenated, 30 and 55% w/v PFD) was evaluated using the Microx 4 oxygen meter and oxygen dipping probe (PreSens Precision Sensing GmbH, Regensburg, Germany) according to manufacturer's instructions during batch production and upon arrival at Roccor. Samples were then transported to the University of Colorado for laboratory testing.

Cell Culture

Primary HCE cells (ATCC, Manassas, VA) were grown in corneal epithelial cells basal medium (ATCC) supplemented with corneal epithelial cell growth kit (ATCC) under standard cell culture conditions, as previously published.[21]

CP Exposure and SOE Treatment

HCE cells were cultured in 100-mm culture plates. At 60 to 70% confluency, media were removed and the cells were either exposed to CP (50 μM CP for 30 minutes) or left unexposed (Control). After the exposures, the cells were washed and either fresh media or media containing 0.25 g/mL of SOE (55% PFD) were added and cells were cultured for 24 hours. After 24 hours of exposure, cell lysates were prepared and analyzed by Western immunoblotting as described previously.[22] Briefly, after SDS-PAGE, samples were transferred to nitrocellulose membrane. Upon blocking with nonfat dry milk, membranes were probed with appropriate primary antibodies (overnight at 4°C) followed by incubation with peroxidase-conjugated secondary antibody. Protein loading was confirmed by stripping and reprobing the membranes with β-actin antibody.

Cell Viability (MTT) Assay

HCE cells (5×10^4 cells/well) were seeded in 24-well plates and grown overnight under standard culture conditions. The following day, culture media were changed, and the cells were cultured with either 1 mL of media only (Control) or media containing 0.0625 or 0.25 g/mL of the unoxygenated vehicle or media containing 0.0625 or 0.25 g/mL of the SOE with either 30 or 55% PFD (6 wells/each) for 24 hours. Culture media were then removed, and the cells were incubated with 0.5 mg/mL of MTT (Sigma-Aldrich St. Louis., MO) for

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4 hours at 37°C. After removing MTT solution, dimethyl sulfoxide was added to the wells and absorbance was read at 540 nm using Spectra max 190 micro plate reader (Molecular Devices, Sunnyvale, CA).

Scratch Wound Assay

HCE cells (70 µL of 5 × 10^6 cells/mL) were seeded in 35-mm culture-insert µ-dish (ibidi USA, Fitchburg, WI). After overnight incubation, the culture inserts were removed. Images were taken (0 hour) and the plates were then cultured with either 1 mL of media only (Control), media containing 0.0625 or 0.25 g/mL of the unoxygenated vehicle, or media containing 0.0625 or 0.25 g/mL of the SOE (55% PFD) in triplicate. At 2 and 4 hours after the addition of the emulsions, media from each well were aliquoted to a 24-well plate. After removing the media, the wells were washed twice, fresh media were added, and images were taken. After imaging, the aliquoted media were added back to the wells. The images were analyzed using ImageJ software and the percent increase in total cell number or area at 2 or 4 hours was calculated for each plate ($n = 3$).

Western Blot Analyses

Cell lysates were prepared after the CP exposure and treatments in HCE cells, and protein estimation was carried out. About 60 µg of the samples were subjected to SDS-PAGE as described earlier, and Western blot analyses were carried out as described previously.[21,22] Briefly, after SDS-PAGE, samples were transferred to nitrocellulose membrane. Upon blocking with nonfat dry milk, membranes were probed with appropriate primary antibodies (overnight at 4°C) followed by incubation with peroxidase-conjugated secondary antibody. Protein loading was confirmed by stripping and reprobing the membranes with β-actin antibody.

Statistical Analysis

Data were analyzed using a one-way analysis of variance with Tukey or Bonferroni t-test for multiple comparisons (Sigma Stat 2.03). Differences were considered significant for P values <0.05. Data are presented as the mean ± standard error of mean (SEM; $n = 3$).

RESULTS

SOE Oxygen Concentration

The PO_2 in each sample of SOE (unoxygenated and oxygenated, 30 and 55% w/v PFD) was evaluated using the Microx 4 oxygen meter and oxygen dipping probe (PreSens Precision Sensing GmbH, Regensburg, Germany) according to manufacturer's instructions during batch production and upon arrival at Roccor. A time-dependent test of oxygen release was conducted using a small (golf ball-sized) bolus of the 55% PFD formulation (Fig. 1). Immediately after being dispensed, the PO_2 in the SOE rose to over 550 mm Hg (Torr) and remained over 500 mm Hg for longer than 22 hours.

FIGURE 1. PO_2 in the SOE over time. The PO_2 in the oxygenated 55% PFD formulation was evaluated using the Microx 4 oxygen meter and oxygen dipping probe (PreSens Precision Sensing GmbH, Regensburg, Germany) according to manufacturer's instructions during batch production and upon arrival at Roccor. A time-dependent test of oxygen release was conducted using a small (golf ball-sized) bolus of the 55% PFD formulation. Immediately after being dispensed, the PO_2 in the SOE rose to over 550 Torr and remained over 500 Torr for longer than 22 hours.

SOE Increases HCE Cell Viability

The effect of SOE treatment on HCE cell viability was assessed via MTT assay. Following SOE exposure, there was an observed increase in HCE cell proliferation. Cell viability in the 0.0625 and 0.25 g/mL SOE-treated groups (55% PFD) increased significantly as compared to Control (Fig. 2A and B), but there was no change in cell viability in the 0.0625 and 0.25 g/mL SOE-treated groups (30% PFD). Cell viability increased by 1.5- and 1.4-fold in the 0.0625 and 0.25 g/mL SOE-treated group (55% PFD), respectively, in comparison with untreated cells (Control). Since an increase in cell viability was only associated with exposure to the SOE with 55% PFD, the SOE with 30% PFD was not tested further. All remaining experiments were conducted using the SOE with 55% PFD.

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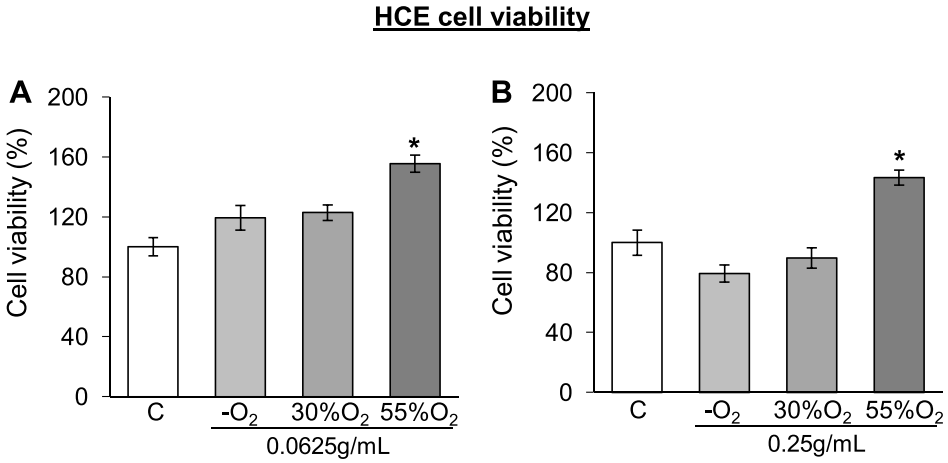

FIGURE 2. MTT assay demonstrates that the SOE promotes increased HCE cell viability even in the face of optimum growth conditions. HCE cells were cultured and colorimetric MTT assay was performed using primary HCE cells as described in Methods section. MTT assay with culture media alone (C), unoxygenated SOE ($-O_2$), and oxygenated SOE at 30 and 55% PFD. (A) 0.0625 g/mL and (B) 0.025 g/mL. Data expressed as percent cell viability normalized to control (mean ± SEM, $n = 6$); $^*P < 0.05$ compared to control.

SOE Promotes Wound Healing in Cultured HCE Cells

The SOE-induced increase in cell viability could also be accompanied with an increase in cell proliferation. SOE exposure promoted wound healing (closure of the scratch) in HCE cells, which was investigated via wound scratch assay (Fig. 3A–E). After 2-hour incubation with the SOE, a 1.4-fold increase in cell coverage (area) was observed for both concentrations of exposure (0.0625 and 0.25 g/mL) as compared to the Control (Fig. 3B and D). After 4 hours of SOE exposure, a 1.6- and 1.3-fold increase in cell coverage in the 0.0625 and 0.25 g/mL groups, respectively, was observed compared to Control (Fig. 3C and E).

SOE Treatment Reverses CP-Induced Markers of DNA Damage and Apoptosis in HCE cells

Treatment with SOE (55% PFD, 0.25 g/mL) did not lead to the expression of markers of DNA damage or apoptotic cell death in HCE cells (Fig. 4A). There was no induction of H2A.X (Ser139), a marker for double-stranded DNA breaks; p53 (Ser15), a key molecule involved in DNA repair; and PARP, the executor of apoptosis (Fig. 4A). However, an increase in COX-2 expression was observed (Fig. 4C). Following CP exposure, HCE cells showed enhanced expression of proteins related to DNA damage and cell death, as indicated by a strong increase in the phosphorylation of H2A.X (Ser139), p53 (Ser15), and accumulation of cleaved-PARP (Fig. 4B). Treatment with SOE (55% PFD, 0.25 g/mL) led to a complete reversal in H2A.X (Ser139) and p53 (Ser15) phosphorylation (Fig. 4B), as well as cleaved-PARP. However, as SOE exposure alone led to increased COX-2 expression, no change in COX-2 levels was observed when SOE was added after CP exposure. In fact, the increase in COX-2 expression following SOE alone was higher than that following CP alone (Fig. 4C).

DISCUSSION

Ocular trauma is common among combat injuries and in the absence of prompt treatment outcomes can be devastating. Close to one-third of service members who experience ocular trauma become legally blind.[1] As the cornea is devoid of any blood vessels, oxygen delivery from the atmosphere is critical for cornea health.[23] Atmospheric oxygen dissolves in tear film and diffuses throughout the cornea. In cases of corneal injury with epithelial damage, the exposed corneal surface must be covered by the epithelial cells to initiate healing, and the cells undergo a morphologic transformation to normal-appearing corneal epithelium under adequate oxygen concentrations.[24] Oxygen is crucial for general wound healing following injury, as tissues often become ischemic and subsequently hypoxic, leading to further tissue damage, delayed wound healing, and even necrosis. Oxygen therapy appears to blunt this pathophysiological cascade and improve healing outcomes in skin and ocular tissues.[10,25] Hyperoxia is shown to act as a potent stimulus for growth factor and cytokine production, which play a role in the tissue repair process. For example, oxygen therapy has been shown to stimulate vascular endothelial growth factor, a growth factor mediating cellular proliferation, migration, and differentiation—key processes in angiogenesis and wound repair. Additionally, oxygen is antimicrobial, and increased tissue oxygen tension may assist in preventing infection in damaged tissues.[25]

In order to increase tissue oxygen tension, hyperbaric oxygen therapy has traditionally been used. However, this therapy is expensive and cumbersome and is unsuitable for use in the field for treatment of combat trauma. Because of these limitations, we developed a new topical therapy for ocular trauma that involves application of a liquid SOE to the eye. We propose that an SOE being used as a topical therapeutic may therefore be a critical tool for military personnel to deliver

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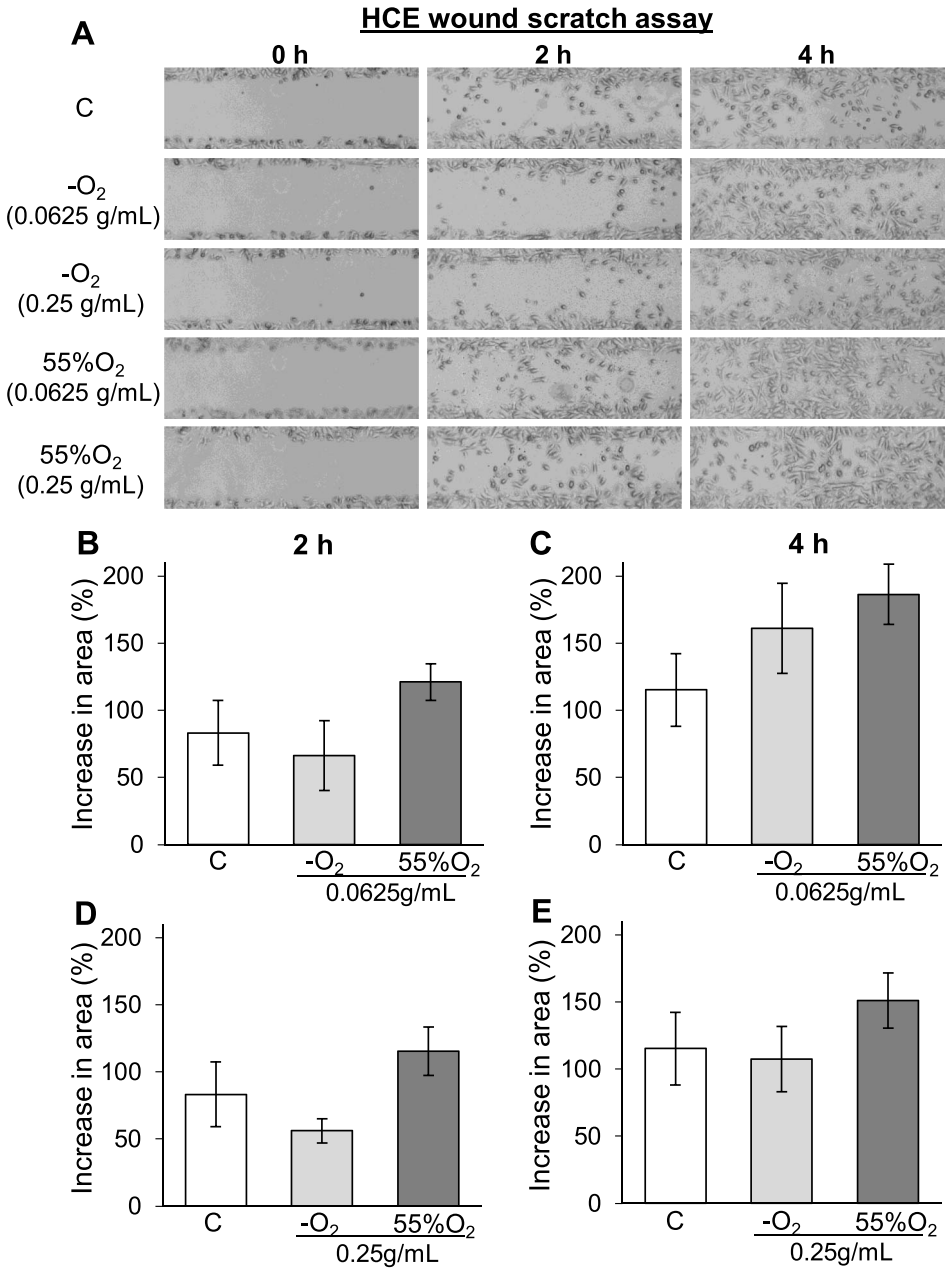

FIGURE 3. Wound closure assay demonstrates that the SOE promotes increased rate of wound closure in HCE cells even in the face of optimum growth conditions. HCE cells were cultured in wound scratch assay dishes as described in Methods section. Cells were grown with either media alone (C), 0.25 g/mL of unoxygenated SOE (-O_2), or oxygenated SOE (55%O_2). Representative images of the scratch wounds were taken at 0, 2, and 4 hours (A) and analyzed using image J to determine the percent increase in total cell area at the acellular zone. Quantification of the rate of wound closure normalized to 0-hour time point for 0.0625 g/mL concentrations (B and C). Quantification of the rate of wound closure normalized to the 0-hour time point for 0.25 g/mL concentrations (D and E). Data presented as the mean ± SEM, ($n = 3$).

oxygen to ocular wounds in the field, ultimately preserving tissue and promoting healing.

Oxygenated perfluorocarbon emulsions with high stability and reliable oxygen release have been used as artificial oxygen carriers in medical applications for decades.[14] Of all the perfluorocarbons, PFD has seen the most interest in medical applications because it is chemically and biologically inert and it can dissolve extremely large amounts of oxygen. For example, Hindryckx et al. showed that the PO_2 within PFD can be increased from about 170 mm Hg to approximately 700 mm Hg by bubbling with 100% O_2 at a rate of 3 L/min for 5 min.[26] When stored in ambient air (21% O_2), the PO_2 of the PFD gradually decreased and returned to normal after 6 hours.

Experiments herein were carried out to test the effects of SOE treatment on HCE cells, as corneal epithelial cells

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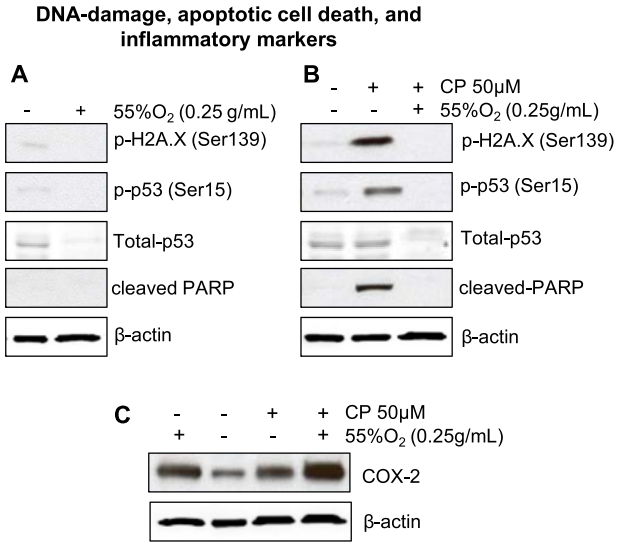

FIGURE 4. Western immunoblot assay provides evidence that the SOE does not alter the expression of proteins involved DNA damage and cell death pathways. HCE cells were either treated SOE (55%O_2, 0.25 g/mL) or left untreated, and Western blot analyses were carried out for phosphorylated H2A.X, p53, and accumulation of cleaved PARP (A), as described in the Methods section. HCE cells were exposed to CP (50 μm, 30 min) then cultured for 24 hours with either media alone (CP) or oxygenated SOE (55%O_2, 0.25 g/mL). Lysates were analyzed for phosphorylated H2A.X/p53, and accumulation of cleaved PARP, and COX-2 (B and C). Protein loading was verified by stripping and reprobing the membranes with β-actin antibody.

are most sensitive to chemical and mechanical injuries. The MTT assay is widely used to measure cell viability/proliferation, and MTT analyses of HCE cells following SOE treatment indicate that the SOE promotes cell viability/proliferation. The MTT assay is designed to evaluate the mitochondrial reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan product by viable cells and provides a quantitative and sensitive detection of cell proliferation.[27] Minute changes in the metabolic activity of the cells can be detected using MTT, thereby permitting one to analyze the effect of a toxic agent on the cells even in the absence of cell death. In the MTT analyses carried out with the SOE, an increase in cell viability suggests that SOE treatments do not diminish HCE cell viability but rather have proliferative effects on HCE cells.

An increase in wound closure was also observed upon SOE treatment in HCE cells, providing further evidence in support of the cellular growth promoting ability of the SOE. The wound scratch assay is a well-established, easy, low-cost in vitro assay used to measure cell migration that mimics the in vivo cell migration during wound healing.[28,29]

CP, a toxic chemical employed during World War I as a chemical warfare agent, has harmful effects on the respiratory system, eyes, and skin. Our results indicate that SOE treatment alone does not cause DNA damage and does not induce apoptotic cell death and oxidative stress markers. CP exposure caused an increase in DNA damage and apoptotic cell death marker in HCE cells. However, the CP-induced increase in markers of DNA damage and apoptotic cell death were abrogated upon SOE treatment. These results are of relevance, as DNA damage and cell death are the first steps in the injury process associated with exposure to several chemical agents. However, no effect on CP-induced increase in COX-2 levels was observed, indicating that inflammation is likely not abrogated by the SOE and that a different treatment regimen may be required to reduce the chemical-induced inflammation. The effects of SOE treatment need to be studied in ex vivo and in vivo corneas to further confirm these findings and to elucidate the molecular mechanism involved.

CONCLUSION

The primary finding of this study indicates that the SOE is safe when diluted in culture media and applied topically to HCE cells. Additionally, the SOE may promote wound closure and enhance cellular viability. SOE treatment was found to be effective in reversing the chemical agent-induced increases in markers of DNA damage and apoptotic cell death in HCE cells, suggesting its potential efficacy against DNA damage- and cell death-inducing agents. However, this needs to be further evaluated. There may be a slight inflammatory response in the HCE cells following SOE exposure, as indicated by an increase in COX-2 expression. Thus, exploring the therapeutic effects of the SOE alone and in combination with an anti-inflammatory agent in a more advanced ocular model with and without injury is warranted.

FUNDING

This work was supported by the U.S. Army Medical Research and Materiel Command under contracts W81XWH-15-C-0138 and W81XWH-17-C-0008.

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