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Evaluating a novel oxygenating therapeutic for its potential use in the advancement of wound healing

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ABSTRACT

Non-gaseous oxygen therapeutics are emerging technologies in regenerative medicine that aim to sidestep the undesirable effects seen in traditional oxygen therapies, while enhancing tissue and wound regeneration. Using a novel oxygenating therapeutic (Ox66™) several *in vitro* models including fibroblast and keratinocyte monocultures were evaluated for potential drug toxicity, the ability of cells to recover after chemical injury, and cell migration after scratch assay. It was determined that in both cell lines, there was no significant cytotoxicity found after independent treatment with Ox66™. Similarly, after DMSO-induced chemical injury, the health parameters of cells treated with Ox66™ were improved when compared to their untreated counterparts. Particles were also characterized using scanning electron microscopy and electron dispersive spectroscopy both individually and in conjunction with fibroblast growth. The data in this study showed that the novel wound healing therapeutic has potential in advancing the treatment of various types of acute and chronic wounds.

1. Introduction

Dermal wounds are a seemingly inevitable element of today's world. Injury to skin occurs regularly in everyday life and can otherwise be inflicted by a number of medical procedures. The vast majority of these wounds are classified as acute and will heal within several weeks of injury, however chronic wounds can take years to heal and are associated with a number of complications (Gottrup et al., 2000; Tandara and Mustoe, 2004; Schreml et al., 2010). Typically wound healing is characterized by three overlapping, continuous stages: inflammation, proliferation, and wound remodeling (Reinke and Sorg, 2012). Within each of these stages, there is complex system of coordinating mechanisms that ultimately leads to the closure of the site of injury; each of these phases have been determined to be heavily dependent on the presence or absence of oxygen (Tandara and Mustoe, 2004; Gorlach et al., 2000; Sen et al., 2002).

Oxygen is a fundamental building block in tissue repair. It functions as a nutrient, antibiotic, supports angiogenesis, cell motility, and extracellular matrix formation (Schreml et al., 2010; Bishop, 2008; Chambers and Leaper, 2011). Conversely, hypoxic conditions generally impair wound healing. However, the relationship between wound healing and oxygen is not a simple one and has been discussed and debated in numerous studies (Sen, 2009; Rodriguez et al., 2008; Gordillo and Sen, 2003; Hunt et al., 2004). For example, the initiation of wound healing is said to be stimulated by hypoxia. The inflammatory phase is dependent upon reactive oxygen species (ROS), whose activity are initiated by an absence of oxygen (Gorlach et al., 2000; Sen et al., 2002). ROS are considered critical to wounds at low concentrations as they are capable of stimulating growth factors and angiogenesis, acting as scavengers to destroy bacteria, and debriding damaged tissue (Bishop, 2008). However, as hypoxia onsets, the production of ROS becomes increasingly improbable due to a lack of available oxygen available for creating the compounds. In combination with increasing hypoxia, a lack of ROS prevents wounds from advancing through subsequent stages of wound healing causing them to become infected or chronic. In general, as tissue repair progresses, the demand for oxygen increases and the supply decreases. This crisis in the availability of oxygen is due to metabolic processes consuming large amounts of the resources as they attempt to repair the wound site. This explains why supplemental oxygen delivery to the wound site is vital and why many studies have attempted to fill this therapeutic gap in wound healing technologies (Bishop, 2008).

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Chronic wounds are a major target for medical technological development. In the United States, there are 6.5 million patients affected by chronic wounds each year with an estimated $25 billion spent annually on their treatment (Sen et al., 2009). Chronic wounds are defined as being arrested in one of the stages of wound healing, usually the inflammatory or proliferative phase. Typically, a wound becomes chronic in the presence of foreign material, bacteria, or pathogens which invoke the production of cellular constituents and impede wound healing by using or destroying building blocks such as oxygen, causing the wound to remain hypoxic (Attinger and Bulan, 2001). A supply of oxygen to wounded tissue *via* microcirculation is critical for both wound healing and resistance to infection. Chronic wounds are particularly compromised in this regard and therefore require supplemental oxygen administration in order to heal. As such, the administration of supplemental oxygen has shown significant beneficial impact on the treatment of chronic wounds by providing cells with sufficient oxygenation for progression through subsequent wound healing phases (Niinikoski, 1969; Sander et al., 2009; Sano et al., 2012).

Using oxygen as a wound healing therapeutic is not a novel scientific breakthrough. In fact, its advent occurred accidentally in 1965 with the use of hyperbaric oxygen treatment (HBOT), a method to deliver oxygen systemically at increased atmospheric pressure, to treat victims of carbon monoxide poisoning (Cianci et al., 2013). At the time of the discovery, Wada observed the unintended improved healing of burns in patients treated with supplemental oxygen (Cianci et al., 2013). This led to the advent of the use of oxygen as a wound healing therapeutic, with HBOT spearheading the way. However, the use of HBOT as a therapeutic is limited due to the tissue and/or systemic toxicity that can be caused by HBOT-induced hyperoxia, the physical inconvenience of a hyperbaric chamber, mental stress on patient, and cost of the treatment (Sen et al., 2002; Gordillo and Sen, 2003; Kalliainen et al., 2003). An alternative method of supplemental oxygen treatment emerged in the form of topical oxygen therapy (TOT), where instead of the patient inhaling oxygen (as in HBOT), it is locally administered to the site of injury at atmospheric pressure using a balloon-like device. This type of treatment has several advantages in comparison to HBOT, including a more simple administration with no systemic toxicity, however, it too has its drawbacks (Kalliainen et al., 2003). TOT relies on a continuous supply of gaseous oxygen and requires the patient to be physically attached to the administration device, therefore inhibiting their mobility during treatment. These shortcomings emphasize a need for continued research in alterative oxygen administration strategies that are both convenient and cost effective.

The goal of the present study is to examine the novel oxygenating therapeutic Ox66™ as a potential alternative strategy to treat wounds using oxygen. Ox66™ is a polyoxygenated aluminum hydroxide composed of approximately 66.2% oxygen and organized as a true clathrate, allowing for the capture of molecules within its lattice structure. The idea behind the design of this potential therapeutic was to avoid the applicational complications associated with conventional oxygen therapeutics, such as reliance on gaseous oxygen, systemic toxicity, and patient immobility. Theoretically, this material could be used in combination with existing wound care technologies such as antimicrobials, foam or bandage-type dressings, or vacuum-assisted closure (VAC). In the current study, we began investigating the drug as a potential therapeutic by using *in vitro* toxicity studies to answer the question of whether or not the Ox66™ is toxic to certain skin cells. In a step further, we simulated a chemical injury with the intent of recovering the viability and proliferation of human skin cells using varying concentrations of Ox66™ as a therapeutic for potential recovery. The obtained data shows that Ox66™ was able to facilitate recovery of cells from injury while showing little to no significant toxicity. If Ox66™ proves to be a successful therapeutic, the potential significance exists in the idea that it could advance modern wound healing techniques by filling a niche left empty by HBOT and current TOT therapeutics.

2. Materials and methods

2.1. Cell culture

2.1.1. Keratinocytes

Normal human primary epidermal keratinocytes from neonatal foreskin were obtained from American Type Culture Collection, Manassas, VA, USA (ATCC PCS-200-010) and cultured in Dermal Cell Basal Media supplemented with Keratinocyte Growth Kit components (ATCC PCS-200-030, ATCC PCS-200-040).

2.1.2. Fibroblasts

Normal human foreskin fibroblasts (ATCC CRL-2429) were cultured in Iscove's modified Dulbecco's medium (IMDM) obtained from ThermoFisher Scientific (REF 12200036) supplemented with 1% penicillin-streptomycin and 7.5% fetal bovine serum (FBS).

2.1.3. Cell culture conditions

Both cell lines were grown in a humid atmosphere with 5% CO_2 at 37 °C until they reached the appropriate confluency for the specific experiment (~90% for Toxicity tests, ~60% for Injury/Recovery tests). The health and morphology of cells was monitored daily, with medium changes every other day.

2.2. Ox66™ particles

Ox66™ (Hemotek, LLC, Plano, TX, USA) is a polyoxygenated aluminum hydroxide that is composed of approximately 66.2% oxygen, aluminum, and chlorine. It is organized as a true clathrate that allows for the capture of elements (such as oxygen) within its lattice structure. The compound was used as test concentrations in both toxicity and injury-recovery tests (see below) where the original, powdered form was dissolved in either fibroblast or keratinocyte cellular media to a specified concentration, as dictated by experimental design.

2.3. Characterization of particles and cells using SEM

2.3.1. Particle characterization

The surface topography, shape, and size of the Ox66™ particles were analyzed using scanning electron microscopy (SEM). The particles were attached to carbon tape and mounted on stubs, then sputter-coated with 20 nm of gold (EM ACE 600, Leica Microsystems), followed by SEM imaging using a Versa 3D SEM (FEI, company, Hillsboro, OR, USA).

2.3.2. Treated cell characterization

Electron microscopy and electron dispersive spectroscopy (EDS) was used to visualize fibroblast growth when cells were treated with Ox66™ and to confirm particle composition on cells. Briefly, cover glass (18 × 18 mm) with attached fibroblasts (grown to ~100% confluency using cell culture conditions previously described in the above section) was fixed with 2.5% glutaraldehyde in 0.06 M PBS (pH 7.2) at room temperature for 90 min, then washed four times for 10 min each with PBS at room temperature. The samples were then dehydrated by being passed through ascending ethanol concentrations (50%, 70%, 90%, 100%) for two times 10 min each at room temperature. After critical point drying (EM CPD300, Leica Microsystems, Wetzlar, Germany), samples were mounted onto stubs using carbon tape and sputter-coated with 20 nm of gold (EM ACE 600, Leica Microsystems). Subsequently, specimens were observed using a Versa 3D SEM (FEI, company). EDS was performed with an Octane Pro Silicon Drift Detector (EDAX, Mahawah, NJ, USA) at 20 kV, spot size 7, and at a working distance of 10 mm.

2.4. Toxicity tests

Fibroblasts and keratinocytes were cultured separately in 96-well

plates until they reached approximately 95% confluency. Cell culture media was then removed and replaced with various concentrations of Ox66™. Due to the low solubility of Ox66™, 100 ppm was the highest consistent concentration achieved in lab, and thus 0.01, 0.1, 0.2, 0.5, 1, 10, 20, 50, 100 ppm Ox66™ was prepared in respective cell culture medium and used in the toxicity tests. Cells were allowed to incubate for 24 h, after which a proliferation or viability assay was performed (see below). The experiment was validated using cell culture media as a cellular control and a 1% Triton-X solution as a positive control.

2.5. Injury/recovery tests

Fibroblasts and keratinocytes were cultured separately in 96-well plates until they reached approximately 60% confluency. At this point, cell media was removed and replaced with a 7% dimethysulfoxide (DMSO) solution and allowed to incubate for 24 h. This simulated a chemical injury that decreased cellular viability by 60–80% as determined by 5-CFDA, AM (data not shown). After 24 h in DMSO, the solution was removed and replaced by various concentrations of Ox66™ (1, 10, 20 ppm) and allowed to incubate for another 24 h. This was followed by the performance of a viability or proliferation assay. The experiment was validated using several controls including: a cellular control; a positive control where cells were grown in a 1% Triton-X solution for 24 h (in lieu of DMSO) then replaced with fresh media for 24 h; an injured, untreated cell set where cells were incubated with 7% DMSO for 24 h then replaced with fresh media for 24 h (as an untreated control).

2.6. Methods of analysis

2.6.1. 5-Carboxyflourescin diacetate, Acetoxymethyl Ester (5-CFDA, AM)

Dye was purchased from Molecular Probes, Eugene, OR, USA and used to determine cellular viability after injury/recovery tests on both keratinocytes and fibroblasts. The method used to conduct this assay was adopted from F. Zhang et al. (2015). (Zhang et al., 2015) Briefly, the cells were washed with phosphate buffered saline (PBS) before the addition of 4 μM 5-CFDA, AM dye dissolved in ACAS media (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, FBS free, phenol red free) and incubated for 30 min at 37 °C. After incubation, dye was removed and cells were washed twice with ACAS to remove excess dye. Finally, fluorescence was measured using a Fluoroskan Ascent™ FL Microplate Flourometer and Luminometer (Thermo Scientific, Waltham, MA, USA) at 485 nm excitation/535 nm emission. The assay was verified using the controls previously described.

2.6.2. Lactate dehydrogenase (LDH)

The integrity of cell membranes from toxicity tests on both fibroblasts and keratinocytes was assessed by measuring extracellular LDH activity (OPS Diagnostics, Lebanon, NJ, USA). The method used was adopted from F. Zhang et al. (2015). Briefly, 50 μL of cell culture medium was collected from culture wells and added to 150 μL of LDH reagents, whereby the solutions were mixed and incubated for 5 min at room temperature. The optical intensity was then read at 490 nm on a microplate reader ELx800 (Biotek Instruments, Inc., Winooski, VT, USA). The assay was validated using controls previously mentioned.

2.6.3. Janus Green B

Dye was purchased from Sigma Aldrich, St. Louis, MO, USA and used to measure the proliferative activity of the fibroblast and keratinocyte cell lines in both the toxicity and injury/recovery experiments. This protocol was adopted from F. Zhang et al. (2015). Briefly, cell culture media was removed and cells were washed twice with PBS, followed by a 90 s incubation period in absolute ethanol. Cells were then incubated in Janus Green B dye for 60 s and were again twice washed with PBS to remove excess dye. The dye was then extracted using absolute ethanol and an additional equal part of water was added to prevent evaporation. Optical intensity was read by a microplate reader ELx800 (Biotek Instruments, Inc., Winooski, VT, USA) at 630 nm.

2.7. Scratch assay

Fibroblasts were grown on Lab-Tek chambered coverglass (Thermo Fisher Scientific, Rochester, NY, USA) according to cell culture conditions previously described. Although the scratch assay is a well-developed method, our protocol was adapted from Liang et al. (2007). Cells were grown to ~100% confluency, then scratched in a straight line using a p200 pipet tip to create a scratch of approximately 0.5–0.8 mm in width. After scratching, cellular debris was removed by gently washing the cells with PBS, then media was replaced with either fresh media (control) or a solution of 10 ppm Ox66™ (treated). All scratch assays were executed in paired quadruples. Imaging was performed in order to evaluate cell migration into the “wound site” by using an inverted light microscope, Axio Observer A1 (Carl Zeiss Microscopy, LLC., Thornwood, NY, USA), at 0, 4, 8, 16 and 24 h. The width of each wound site was measured at 5 locations along the length of the scratch, to best describe the wound size.

2.8. Statistical analysis

2.8.1. Toxicity and injury recovery results

All results (Figs. 1–4) are presented as the mean values ± SEM of untransformed data of three independent biological replicates where within each biological replicate there were sixteen independent technical replicates. Statistical comparisons between groups were performed using one-way ANOVA and post-hoc multiple comparisons were made using a Bonferonni correction test. P values indicate statistical significance where $^{*}P < 0.05$.

2.8.2. Scratch assay results

Results (Fig. 7) are presented as the mean values ± SEM of untransformed data of 4 independent biological replicates where within each biological replicate there were 5 technical replicates (measures of the distance across the scratch site). Statistical comparisons between

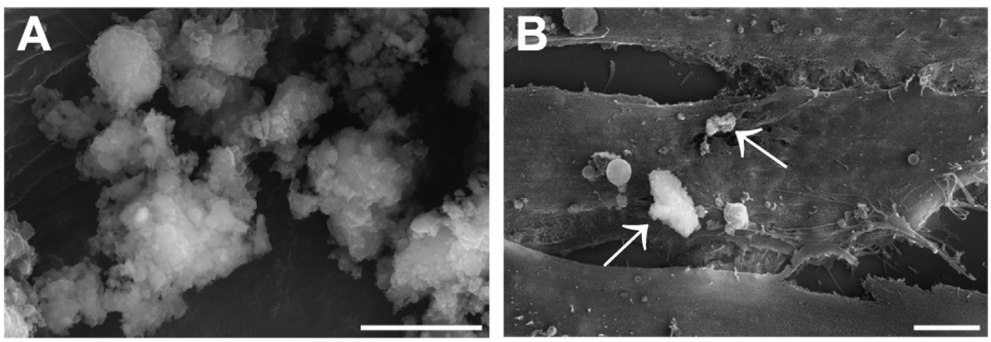

Fig. 1. Ox66™ particle topography visualization using SEM. Image A shows an overview of dry particles mounted onto carbon tape, emphasizing their inconsistency in size and shape. Image B shows the particles (indicated by white arrows) attached to the exterior of fibroblasts. Particles were confirmed as Ox66™ using EDS. Scale bars = 5 μm.

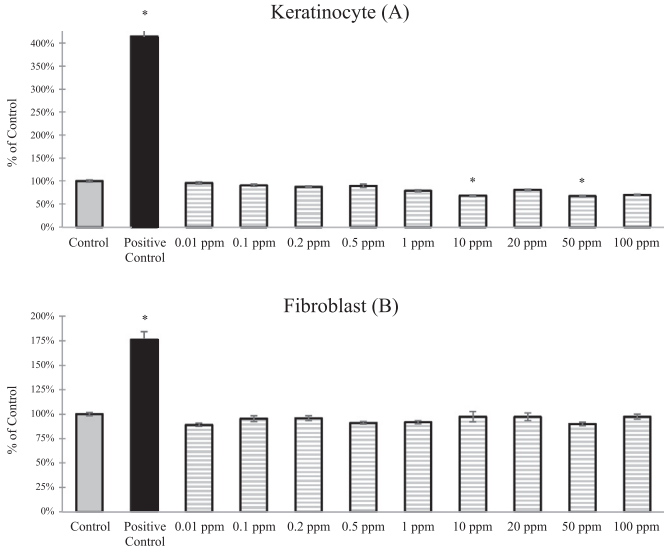

Fig. 2. Extracellular lactate dehydrogenase (LDH) activity for viability using fibroblast and keratinocyte cell lines. Keratinocytes (A) and fibroblasts (B) were treated with varying concentrations of Ox66™, then extracellular LDH release was measured to determine cellular disruption. In keratinocytes, cells dosed with 10 ppm and 20 ppm of Ox66™ showed significantly less ($*p < 0.05$) LDH in their cellular media as compared to the control group, indicating that the viability of these cells improved when exposed to Ox66™. In fibroblasts, although none of the dosed concentrations a showed significant decrease in LDH, all LDH measurements were below that of the control group. In both cell lines, there were no LDH measurements above those of the controls, indicating that Ox66™ is non-toxic to these cell lines.

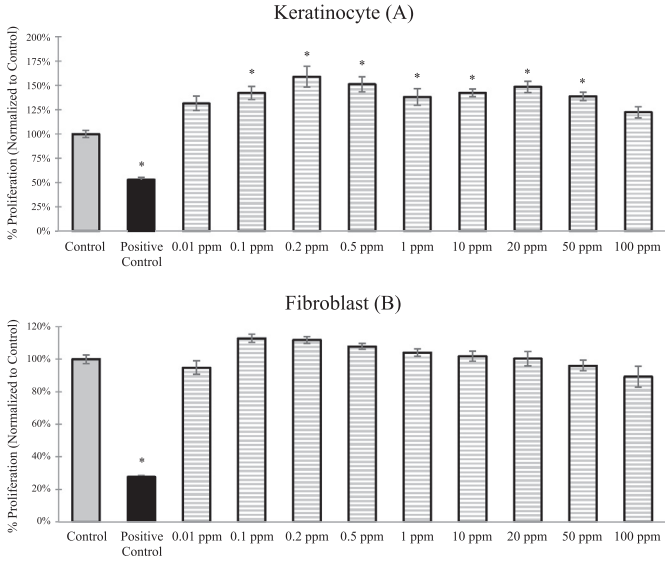

Fig. 3. Proliferation for fibroblasts and keratinocytes using Janus Green B dye. Keratinocytes (A) and fibroblasts (B) were treated with varying concentrations of Ox66™, then Janus Green B dye was used to measure the proliferative capability of the cells after exposure. In keratinocytes, cells dosed with Ox66™ concentrations between 0.1 ppm and 50 ppm showed a significantly higher proliferative ability than the control ($*p < 0.05$). In fibroblasts, there was no significant decrease ($*p < 0.05$) in proliferative ability as compared to the control. However, fibroblasts dosed with Ox66™ between the range of 0.1 ppm and 10 ppm showed higher proliferative ability than the control, although not significant. These data indicate that when cells are exposed to certain concentrations of Ox66™ they have the potential to increase in proliferation, which may lead to a more effective wound closure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

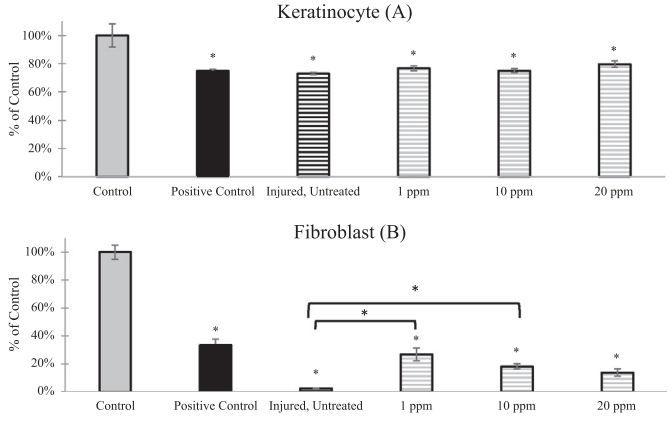

Fig. 4. Injury/recovery viability for fibroblasts and keratinocytes using 5-CFDA, AM fluorescence dye. Keratinocytes (A) and fibroblasts (B) were dosed with DMSO to induce a chemical burn, then treated with varying concentrations of Ox66™ to recover the cells. Keratinocytes showed no significant recovery ($*p < 0.05$) in comparison to injured, untreated cells. In fibroblasts, cells exposed to 1 and 10 ppm Ox66™ showed significant recovery ($*p < 0.05$) in comparison to injured, untreated cells.

groups were performed using a repeated measures ANOVA analysis. *P* values indicate statistical significance where $*P < 0.05$.

3. Results

3.1. Characterization of particles and cells using SEM

The topography of individual Ox66™ particles and dosed fibroblasts was examined using SEM (Fig. 1). The particles were polydispersed in size and shape and had an average size of 1.87 μm. They exhibited a rough exterior texture similar to the topography of the two-dimensional layer structure observed in the mineral mica (Fig. 1A). Particles attached to fibroblasts (white arrows in Fig. 1B) presented the same topography and remained roughly within the same size range as dry particles (Fig. 1A) before they were applied to the fibroblasts.

EDS confirmed the expected composition (data not shown) of the particles as defined by manufacturer information held by Hemotek, LLC (Plano, Texas). The Ox66™ production is a trade secret and its uses are patented and patent pending, therefore its full composition cannot be disclosed at this time.

3.2. Toxicity tests

Several assays were used to evaluate the effects of Ox66™ particles on the cellular health of both keratinocytes and fibroblasts, including Janus Green B for cellular proliferation and LDH for viability. LDH is a cellular enzyme found in the cytosol which is released to culture medium during cell death or membrane failure. Thus, measuring the amount of released LDH has become a commonly used viability technique for monitoring cell death or disruption (Bopp and Lettieri, 2008). When higher amounts of extracellular LDH are measured, cells are less viable, and *vice versa* for lower amounts. In this experiment, cellular viabilities after a 24 h exposure to Ox66™ are shown in Fig. 2. In both cell lines, a general trend of decreased LDH release was shown in comparison to controls when both cell lines were exposed to Ox66™. Hence, generally, an exposure to Ox66™ increased viability, however not all increases were statistically significant. In keratinocytes, cells exposed to concentrations of 10 and 50 ppm Ox66™ showed statistically significant decreases in LDH as compared to the control. Keratinocytes with the greatest significant decrease in LDH were exposed to 50 ppm and showed a 33% decrease as compared to the control. In fibroblasts, none of the tested concentrations of Ox66™ showed a statistically significant decrease in the amount of LDH released in comparison to control cells. However, although insignificant, all tested concentrations

of Ox66™ demonstrated lower extracellular measurements of LDH than the control. Specifically, the greatest LDH decrease in fibroblasts was seen with exposure to a concentration of 0.01 ppm, with an 11% overall decrease as compared to the control.

In a similar test of cellular health, after a 24 h exposure to Ox66™ particles, the cellular population was evaluated using the Janus Green proliferation assay (Fig. 3). In keratinocytes, none of the populations of cells treated with Ox66™ significantly declined as compared with the control. Interestingly, in comparison to the control, nearly all cellular populations of keratinocytes increased when exposed to test concentrations of Ox66™, the largest of which as much as 59% (0.2 ppm) and the smallest as little as 38% (1 ppm). Only keratinocytes exposed to concentrations of 0.01 and 100 ppm did not show a significant increase in proliferative ability. Fibroblasts exposed to the test material did not show any significant differences from the control. However, cells exposed to a certain range (between 0.1 and 10 ppm) of concentrations did cause cells to proliferate at a greater rate (up to 13% as in 0.1 ppm) than the control group. Only concentrations at the tail ends of our testing concentrations (0.01, 50, and 100 ppm) showed lower proliferative abilities than the control group.

3.3. Injury/recovery tests

This experiment was designed to simulate a chemical burn for which Ox66™ was then used as a therapeutic to stimulate cellular recovery. We evaluated recovery in the form of cellular viability and proliferation using the 5-CFDA, AM and Janus Green assays.

5-CFDA, AM is a quantitative fluorogenic dye which is commonly used to measure cellular viability in cytotoxicity studies. When living cells are exposed to membrane-permeable, nonpolar, non-fluorescent 5-CFDA, AM dye they convert it into polar, fluorescent carboxyfluorescin (*CF*) thereby allowing a fluorescence measurement, where the fluorescence is an indicator of both the active enzymes and the plasma membrane integrity (Bopp and Lettieri, 2008). Cellular viability after injury using DMSO and recovery using Ox66™ is shown in Fig. 4. In both cell lines, the viability of all the test conditions were significantly decreased in comparison to the control, which indicated that the injuries were significant. However, in fibroblasts treated with 1 and 10 ppm of Ox66™, the viability increased in comparison to the injured, untreated set. Of these, the most significant improvement in fibroblasts was seen in cells treated with 1 ppm, where a 25% increase in viability was seen in comparison to the injured, untreated set. In keratinocytes, we saw no significant recovery after injury in any of the concentrations tested. However, all concentrations tested on keratinocytes showed improvement in viability (ranging from 2 to 8%) in comparison to the injured, untreated set.

The Janus Green proliferation assay was used to assess the proliferative capability of cells after a 24 h injury with DMSO and a 24 h recovery period using Ox66™ (Fig. 5). In both cell types, there were significant decreases in all of the injured cell populations (both Triton-X and DMSO) in comparison to the control, indicating that the injury mechanisms were successful. When Ox66™-dosed keratinocytes were compared to the injured, untreated set, there were slight increases (2–3%) in each cell population, however the increases were not statistically significant. In fibroblasts, all cells exposed to Ox66™ after injury showed a population increase in comparison to injured, untreated cells, although this increase was not significant. The greatest increase was seen in cells exposed to 1 ppm Ox66™, which showed a 12% proliferative recovery in comparison to the untreated cells. Other concentrations of Ox66™ on fibroblasts showed increases of 11% (10 ppm) and 8% (20 ppm).

3.4. Scratch assay

The scratch assay is a convenient and commonly used method for the analysis of cell migration *in vitro* (Liang et al., 2007). Hence, a physical injury was simulated in a monolayer of fibroblasts and cell migration was visually monitored (selected images shown in Fig. 6) until 24 h after initial injury. At 0 h, in both the control and dosed cell set, scratches were visible with clear boundaries and with little to no cellular debris remaining in the scratch site after PBS rinse. Although the average width of the initial scratches in each set varied (between 0.5 and 0.8 mm), they were normalized to the mean of the initial width for each time point measured. As time progressed, cell migration was evident in both sets as the width of the scratches became smaller and the boundary of the sites became blurred (Figs. 6 and 7). After 4 h, a mean closure of 26% was observed in the control set while the dosed cells recorded a mean closure of 28%. After 8 h, a statistically significant difference ($*p < 0.05$) in closure rates was observed between the control cells and the dosed cells with a 38% mean closure in the control set *versus* a 52% mean closure in the dosed cells. Similarly, a statistically significant difference in closure between the two data sets after 16 h, where a 52% mean closure was observed in the control set while a 75% mean closure was observed in the dosed cells. Treated cells completely closed the wound 24 h after the initial injury, in comparison to 73% mean closure observed in untreated cells. From 8 hours post-injury on, at each monitored time point, Ox66™ treated fibroblast cells exceeded the control cells by approximately 24% in migrational closure rate.

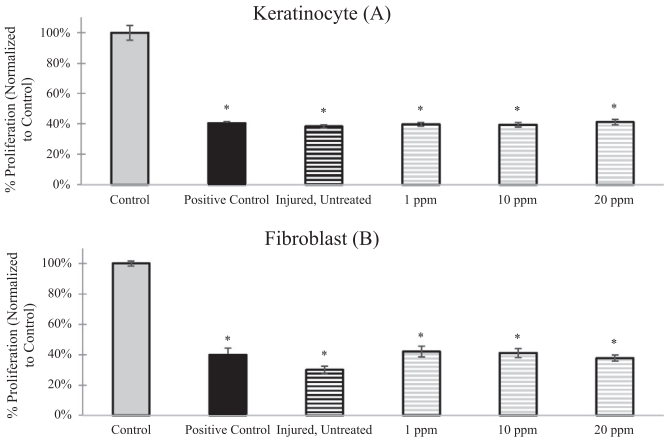

Fig. 5. Injury/recovery proliferation for fibroblasts and keratinocytes using Janus Green B dye. Keratinocytes (A) and fibroblasts (B) were dosed with DMSO to induce a chemical burn, then treated with varying concentrations of Ox66™ to recover the cells. Keratinocytes showed no significant recovery in their proliferative capabilities in comparison to injured, untreated cells when dosed with any of the test concentrations of Ox66™. Similarly, we saw no significant ($*p < 0.05$) recovery after injury and treatment with Ox66™ in fibroblasts. However, although not significant, both cell lines showed higher proliferative abilities in treated cells than in untreated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

A novel topical oxygenating therapeutic as an effective treatment alternative for multiple wound types would ideally satisfy the characteristics of Gottrup et al. (2000): simple application (Tandara and Mustoe, 2004); no systemic or localized toxicity; (Schreml et al., 2010) provide a prolonged supply of oxygen without a reliance on gaseous oxygen; and (Reinke and Sorg, 2012) exist as a cost-effective option available to the general public. The current study details the *in vitro* cytotoxicity testing of the novel wound healing therapeutic Ox66™. The potential wound healing drug utilizes a chemical-based source of oxygen, is minimally toxic or nontoxic to relevant human skin cells, can conceivably facilitate recovery in the viability of cells after injury, and has the potential to be used in conjunction with existing treatment options in a cost-effective manner.

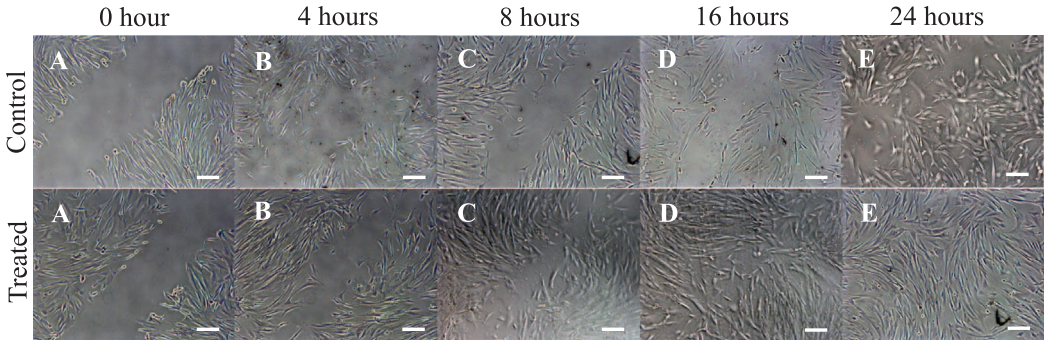

Fig. 6. Selected images of fibroblasts after scratch assay (control – top, dosed - bottom). Image A shows the cells immediately after scratch injury, showing clear boundaries between the cells and the scratch site. Images B – E show the scratch 4 h (B), 8 h (C), 16 h (D) and 24 h (E) after injury. As time advances, the gap created by the scratch begins to narrow. Images D and E show the most advanced stage of treated cell migration in comparison to the control set. Scale bars = 200 μm.

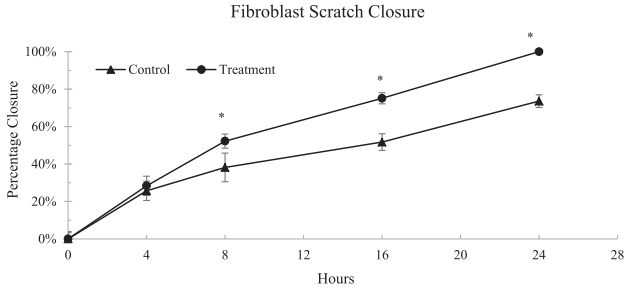

Fig. 7. Comparison of scratch closure rates between Ox66™ treated and untreated (control) fibroblasts. The chart compares the rate of closure at hours 0, 4, 8, 16 and 24 between treated and untreated fibroblasts in the scratch assay (selected images shown in Fig. 6). Between treated and untreated cells, there is no statistical difference between the rates of closure at 4 h (*$p < 0.05$). However, hours 8, 16 and 24 show statistically significant differences between treated and untreated cells. Treated cells seem to follow a more linear closure rate, while the untreated cells migrational closure rate slowed down 4 h after the injury.

4.1. Toxicity tests

To date, many studies have evaluated the acute and chronic toxicity effects of oxygen as a wound healing therapeutic on various organs in the human body (most notably including the skin). However, there have been few studies that have focused on the effects of non-gaseous oxygen. In a noteworthy study by Chandra et al. (2015), an oxygen-generating wound dressing was developed that improved wound closure and re-epithelialization on average 10–12% better than the control in a full thickness porcine model (Chandra et al., 2015). The release of oxygen in their dressings was based on a chemical reaction involving hydrogen peroxide decomposition and was one of the first evaluations of a non-gaseous oxygen therapeutic. The success achieved in the research by Chandra et al. (2015) is echoed here with the use of a novel clathrate, oxygen-capture method for topical application, where no oxygen generation is necessary.

In this study, various Ox66™ concentrations were tested for toxicity on two human skin cell lines, demonstrating that even in high-dose exposure scenarios Ox66™ did not have a significant impact on cellular viability, extracellular LDH activity, or proliferation compared to control groups. Interestingly, in some cases, a 24 h exposure to Ox66™ significantly improved the viability and/or proliferative ability of keratinocytes in comparison to their controls. Keratinocytes were more responsive to Ox66™ exposure, in that nearly all concentrations (except 0.01 and 100 ppm in the proliferation study) showed increased proliferation. We also observed an improved response in keratinocytes in the viability study, where concentrations of 10 and 50 ppm demonstrated increased viability. Although fibroblasts did not show significant increases in either viability or proliferation when exposed to Ox66™, we observed the best improvement in midrange-dose exposures with concentrations between 0.1 and 1 ppm where there were increases in both viability and proliferation. Wilson et al. (2005) demonstrated in a toxicity index study using 17 dermal cleansers that fibroblasts experienced more cytotoxic effects from test materials than keratinocytes *in vitro* (Wilson et al., 2005). They found this to be unsurprising due to the fact that fibroblasts in their natural role as connective tissue are not directly exposed to chemical insult, but are rather protected by a topical layer of different cell types. The conclusions found by Wilson et al. (2005) support those found herein, explaining why fibroblasts in our study showed more sensitivity than keratinocytes and reemphasize that *in vivo* fibroblasts would be more protected from direct exposure to Ox66™. However, the data presented here still shows promise for Ox66™ to function as a successful therapeutic. In these studies, we have shown that Ox66™ does not pose any significant threat to the health of fibroblasts or keratinocytes *in vitro* and have developed a potential therapeutic dose range for using the drug in further study. Additionally, these preliminary studies provide evidence for the perusal of future studies involving three-dimensional or *in vivo* tissue where the effects of Ox66™ can be more accurately elucidated in a more relevant model.

4.2. Injury/recovery tests

The procedure behind any potential wound healing study naturally must first involve some sort of wound. While there are many different types of dermal wounds commonly inflicted on human skin (incisional, non-penetrating, chemical, and thermal injuries, *etc.*), in this of study we chose to evaluate chemical wounds on monolayered cells due to the ease and feasibility of mimicking such an injury in a non-coculture model. The aim of this study was to determine if Ox66™ was capable of recovering various criteria of cellular health after injury. Future studies will survey different types of injuries using more advanced models.

Data from preliminary toxicity tests, as described in the previous section, revealed a potential therapeutic dose range for Ox66™. This range in combination with a chemically-induced injury demonstrated that the viability of fibroblasts exposed to concentrations of 1 and 10 ppm of Ox66™ showed both significantly increased viability in comparison to injured, untreated cells. Keratinocytes did not show significant recovery in viability or proliferation when exposed to the tested concentrations of Ox66™ when compared to an injured, untreated control. However, their recovery in both proliferation and viability proved to be greater than that of the injured, untreated control. These conclusions contrast with those found in our cytotoxicity studies, in that without injury, keratinocytes were most responsive to Ox66™ alone, while they were least responsive to Ox66™ after injury. This phenomenon is described Van de Sandt et al. (1999). They explain that keratinocyte monolayers are particularly sensitive to toxicity *in vitro* due to the lack of protective stratum corneum layer that is found *in vivo* (Wilson et al., 2005; van de Sandt et al., 1999). The lack of a protective layer may therefore explain why undifferentiated keratinocytes in our injury study were less capable of recovery than their fibroblast counterparts. Additionally, this data suggests that a potentially higher therapeutic dose range needs to be used for keratinocytes in order to elicit significant differences in the recovery in comparison to control groups. This gap in understanding potential therapeutics makes obvious the need for three-dimensional human skin models in future testing.

However, data presented here warrants a continuation of the study of Ox66™ as it shows potential healing capabilities in relevant cell lines *in vitro*.

4.3. Scratch assay

A scratch assay is a well-developed, *in vitro* alternative for studying cell migration. One of the foremost advantages of this method is that it is able to mimic the migration of cells *in vivo* where an incisional wound might be studied. The scratch assay functions as an *in vitro* alternative to a physical injury (Liang et al., 2007). In our treatment groups, the percentage closure of the scratch in comparison to its initial width roughly increased at similar rates after each time point with 28% after 4 h, 24% after 8 h, 17% after 16 h and 25% after 24 h. Based on the data observed, cells migrated at an approximately constant rate showing linear closure at each measured time point. Contrarily, cells in the control groups started at a higher rate of migration for the first 4 h with a 26% mean closure than the subsequent time points. Migration rate slowed down to between 12 and 14% of mean closure from 4 h post-injury to 16 h post-injury. During the final observation period, the mean closure rate resumed to 21%, and concluded in an overall 73% mean closure at the end of experiments. Tompach et al. (1997) partially explains this conundrum in their study to elucidate the effects of hyperbaric oxygen on cells involved in wound healing. They found that fibroblasts required a 120-min exposure to supplemental oxygen in order to produce a proliferative or migrational response (Tompach et al., 1997). Their data however, does not explain why cells exposed to Ox66™ required longer exposure period (> 4 h) to elicit significant improvement over the control in migrational rate. Nevertheless, we hypothesize that due to the nature and chemical composition of the Ox66™ compound, it may take longer for oxygen to absorb across the membrane of cells, where presumably in Tompach's study cells experienced rapid diffusion. Future studies with additional measurements of oxygen levels both in the extracellular matrix and on surface of the tissues will be employed to elucidate the mechanisms of action required in order to fully understand this singularity.

In both sets, as time progressed, the buildup of cellular debris became more evident. This is believed to be due to the sloughing of dead cells during migration and regeneration in the wound healing process. However, in some of the dosed cell set, an abundance of debris (small particulate matters) is present at 0 h and persists through 24 h. The majority of this type of debris is not attached to the cells and is caused by the visualization of Ox66™ particles in solution.

5. Conclusion

These studies provide evidence for the continued study of Ox66™ as a potential wound healing therapeutic. Data herein conclude that exposure to Ox66™ in varying concentrations showed no toxic effects on the cellular health of fibroblasts and keratinocytes *in vitro* and indicated that such exposure can potentially aid in the closure of incisional wounds and in the recovery of cellular health after a chemical wound. While some of the toxicity data indicated that cellular health improved with exposure to Ox66™, exposure to few concentrations showed a decline in cellular health, though none of these declines proved to be statistically significant. Additionally, injury/recovery data showed that while fibroblasts showed better recovery than keratinocytes, there was no statistically significant decline in cellular health seen in keratinocytes. Generally, the scope of the data supports a continued investigation into more relevant tissue models in the potential wound healing therapeutic Ox66™.

The next steps in the future of Ox66™ research dictate the use of a three-dimensional model to test the efficacy of the drug on wounds in a full thickness human skin model or in an animal model.

Conflicts of interest

Funding for this research was provided in part by Hemotek, LLC, a company with patents and patent pending interest in the Ox66™ compound.

Transparency document

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