Nitric oxide releasing two-part creams containing *S*-nitrosoglutathione and zinc oxide for potential topical antimicrobial applications

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ABSTRACT

Currently, most antimicrobial topical treatments utilize antibiotics to prevent or treat infection at a wound site. However, with the ongoing evolution of multi-drug resistant bacterial strains, there is a high demand for alternative antimicrobial treatments. Nitric oxide (NO) is an endogenous gas molecule with potent antimicrobial activity, which is effective against a wide variety of bacterial strains. In this study, the potential for creating NO releasing creams containing the naturally occurring NO carrier, *S*-nitrosoglutathione (GSNO), are characterized and evaluated. GSNO is shown to have prolonged stability (> 300 days) when mixed and stored within Vaseline at 24 °C. Further, enhanced proliferation of NO from GSNO using zinc oxide nanoparticles (ZnO) is demonstrated. Triggering NO release from the GSNO/Vaseline mixture using a commercial zinc oxide-containing cream exhibits first-order NO release kinetics with the highest %NO release over the first 6 h. Significant killing effects against *S. aureus*, *S. epidermidis*, and *P. aeruginosa* are demonstrated for the GSNO/Vaseline/ZnO cream mixtures in a proportional manner dependent upon the concentration of GSNO in the final mixture.

1. Introduction

Infected wounds can lead to serious issues such as delayed wound healing, necrosis, limb or life-threatening situations, and spread of infection not only in the patient's body but to others in a hospital setting [1–3]. The most common topical treatments to prevent or treat infections often utilize antibiotics [4]. Consequently, the effects of these treatments over time diminish as multi-drug resistant bacterial strains continue to evolve [5–7]. Over the past decade, the rate of antibiotic resistance has increased rapidly, caused by the misuse of antibacterial agents [6,8,9]. Organizations such as the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) have declared antibiotic resistance as a global health concern [8,9].

When considering topical antimicrobial treatments, either prescription or non-prescription type, the treatment must display several characteristics to be an effective agent. First, active ingredients must be safe and stable. According to U.S. Food and Drug Administration (FDA) standards, new cosmetics with drugs must report an extensive amount of safety data that also demonstrate stability of the ingredients [10,11]. Topical antimicrobial treatments fall within this category. Second, the active components (e.g., antibacterial agent(s)) should exhibit activity

towards a wide range of microbes (non-selective) [6]. This will ensure broad application potential of the antimicrobial treatment. Third, the treatment must be practical and effective over a designated application period. For example, treatments that would require application every 30 min would be considered impractical; however topical application once or twice daily would be much more acceptable.

An example of an antimicrobial treatment that is capable of meeting all of the above criteria is one that incorporates nitric oxide (NO) as an active ingredient [3,12–18]. Nitric oxide (NO) is a simple diatomic free radical that has drawn much attention in the biomedical field due to its potential as a therapeutic agent [14,19,20]. NO is naturally synthesized *in vivo* by the enzyme nitric oxide synthase (NOS) and plays a key role in several biological functions such as neurotransmission, prevention of platelet activation and adhesion, and serving as a potent antimicrobial/antiviral agent produced by immune system macrophages to fight infection [21–24]. As a free radical, NO can facilitate a multitude of reactions leading to microbial cell death, making it ideal for therapeutic applications [14,19,25]. To date, NO has displayed broad activity, killing many different types of bacteria [14,25].

Free radical molecules like NO are highly reactive and therefore NO donor/carrier molecules are often utilized to stabilize NO, and then

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release NO locally upon demand. Some NO donors that have been used for antimicrobial/wound healing applications include nitrite (converted to NO by a reduction reaction), *N*-diazeniumdiolates (NONOate), and *S*-nitrosothiols (RSNO) [3,12–17]. A major concern for NO donors is the toxicity of byproducts that are generated upon NO proliferation. NONOates have been shown to form *N*-nitrosamines by back-reaction of some fraction of the NO released, and most nitrosamines are carcinogenic [26,27]. RSNOs, on the other hand, are NO releasing agents that have the least toxicity-related issues. Most concerns arise from the use of synthetically derived RSNOs and the concentration that humans can be exposed to, which is also an issue with other types of NO donors [21].

To circumvent any toxicity-related issues, this work utilizes an endogenous RSNO, *S*-nitrosoglutathione (GSNO), that is present in human blood at a concentration between 0.02 and 0.20 μM, serving as a carrier of NO [20,28,29]. Upon liberation of NO, the two most common products are glutathione (GSH) and glutathione disulfide (GSSG), which are also found in human blood at approximately 17 μM and 3 μM, respectively [29]. GSNO is a primary RSNO that can decompose and liberate NO in the presence of certain reducing agents, including trace metal ions (e.g., Cu(I)), as well as by thermal degradation and photolysis [28,30–39]. Hence, maintaining stability of GSNO as well as other RSNOs over long periods of time can be challenging.

Herein, the potential long-term stability issues of GSNO are solved by storage within a highly viscous and hydrophobic matrix, Vaseline. Further, a novel method for initiating and accelerating the release of NO from GSNO stored within the Vaseline matrix is achieved by mixing with a cream containing zinc oxide particles. Zinc oxide (ZnO) particles are widely utilized in pharmaceuticals, cosmetics, textiles, and electronics [40]. For example, ZnO is used in most topical sunscreens because it can absorb UVB (290–320 nm) and UVA (320–400 nm) sunlight radiation [41]. It is also employed within diaper rash creams as a stringent and antimicrobial agent [34,42]. The commercial ZnO cream matrix used in this work to initiate/accelerate NO release from the GSNO/Vaseline mixture is screened to confirm that the ZnO particles within are primarily responsible for the accelerated NO release from GSNO. In addition, antimicrobial studies with the GSNO/Vaseline/ZnO cream mixtures are shown to exhibit significant killing effect against *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, which are commonly associated with wound or burn-wound infections [43,44].

2. Materials and methods

2.1. Materials

ʟ-Glutathione reduced (GSH), hydrochloric acid (HCl), sodium nitrite, zinc chloride, copper chloride, glycerol, poly(ethylene glycol) (avg. MW = 300) (PEG-300), magnesium sulfate, alpha tocopheryl acetate, 1,2-octanediol, 1,2-hexanediol, tropolone, Trizma base, and potassium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). Acetone and xylenes was purchased from Fisher Scientific Inc. (Pittsburgh, PA). LB agar and 10 mM phosphate buffered saline (PBS) (pH 7.2) were purchased from ThermoFisher Scientific (Grand Island, NY). 30 nm zinc oxide nanoparticles were purchased from EPRUI Biotech Co. Ltd. (ShangHai, China). Medical grade silicone sheeting (non-reinforced, gloss, 0.0127 cm thick) was purchased from BioPlexus Corp. (Ventura, CA). Vaseline® Jelly, Unilever, Lot 08226JB00 (Vaseline); Neosporin® + Pain Relief Cream, Johnson & Johnson Consumer Inc, Lot 0058LZ (Neosporin cream); Avalon Organics® Intense Defense with Vitamin C Oil-Free Moisturizer, The Hain Celestial Group Inc, Lot 618747 (vitamin C cream); and Desitin® Rapid Relief Cream: Zinc Oxide Diaper Rash Cream, Johnson & Johnson Consumer Inc, Lot 1577LZ/2 (zinc oxide cream) were purchased from a local CVS Pharmacy. Osmotics Cosmeceuticals Blue Copper 5®, Osmotics LLC, Lot 4248D7 (copper cream) was purchased from Amazon.com. All aqueous solutions were prepared with 18.2 M Ω deionized water using a Milli-Q

filter (Milli-q purified water) from EMD Millipore (Billerica, MA). 10 mM Trizma hydrochloride (Tris-HCl) buffer, pH 7.4, was used for all aqueous experiments. *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the American Type Culture Collection (Manassas, VA).

2.2. S-Nitrosoglutathione (GSNO) synthesis

An adapted method of the procedure reported by Hart et al. was used to synthesize GSNO [45]. Synthesis was completed in the absence of light. Reduced glutathione (GSH) (4.59 g) was dissolved in aqueous HCl (0.5 M, 31.5 mL), cooled to 0 °C using an ice bath, and continuously purged under nitrogen. An equal molar amount of sodium nitrite (1.035 g) was added directly to the GSH solution. After stirring for 40 min at 0 °C, ice-cold acetone (10 mL) was added into the reaction mixture to precipitate the GSNO. After stirring for another 10 min, the pink precipitate was separated by vacuum filtration. The resulting pink powder was washed by ice-cold water (3 × 10 mL) and acetone (3 × 10 mL), respectively. Finally, the desired GSNO product (3.85 g, 77% yield) was obtained after drying under vacuum at room temperature (24 °C) for 24 h. GSNO was stored at freezer temperature (−20 °C) in the dark for any further use. The synthesized GSNO was characterized in D_2O at room temperature (24 °C) by ^1H NMR spectroscopy (Varian 400 MHz spectrometer) (see Fig. S1 for the ^1H NMR spectrum). ^1H NMR (400 MHz, D_2O): δ 4.63 (app. t, *J* = 6.2 Hz, 1 H), 4.02 (dd, *J* = 51.2, 12.8 Hz, 2 H), 3.91 (s, 2 H), 3.76 (t, *J* = 6.4 Hz, 1 H), 2.41 (t, *J* = 7.6 Hz, 2 H), 2.08 (app. nonet, *J* = 7.6 Hz, 2 H). The purity of prepared GSNO was > 98% based on the characterization of the ^1H NMR results.

2.3. Preparation of various wt% GSNO in Vaseline

The entire preparation of each formulation was completed in the absence of direct light. A mortar and pestle were used to grind GSNO crystals into a fine powder. The desired mass of fine GSNO powder was then weighed out into a mixing vessel. In the same vessel, the desired mass of Vaseline was added. The fine GSNO powder was then mixed thoroughly with the Vaseline using a wooden stirrer for 2.5 min. The resulting mixture was designated as a primary matrix.

2.4. Preparation of various wt% of GSNO in Vaseline for long-term stability studies

The desired wt% of primary matrix was prepared. The sample was placed into a 20 mL amber glass vial with septum cap and wrapped in aluminum foil. The vial was purged with nitrogen gas via the septum top for 5 h. Samples were then stored in the dark at 24 °C for extended time periods.

2.5. Evaluating stability of GSNO in Vaseline using UV-Vis spectroscopy

The entire procedure was completed in the absence of direct light. An aliquot of the GSNO/Vaseline primary matrix from the stability study samples was weighed out into a separate 20 mL amber glass vial. Five mL of xylenes was added to the vial. A mixture of xylenes was used to dissolve the Vaseline while not dissolving the GSNO. The vial was shaken on a horizontal shaker (Shaker 30, National Labnet Company, Woodbridge, NJ) at 400 RPM for 10 min. After that time, the contents were transferred into a 60 mL separation funnel. The original sample vial was further washed twice with 1.5–2.5 mL of Milli-q purified water and the contents were added to the separation funnel. The total volume of Milli-q purified water used was noted. The contents in the separation funnel were gently mixed for 1 min. Shaking or agitating the solution too much caused the Vaseline to crash out of the xylenes/organic layer. The bottom aqueous layer that contained dissolved GSNO was then

collected. The appropriate volume of aqueous sample was pipetted into a quartz cuvette such that the absorbance measured would be < 1 at 334 nm. Beer's Law (A = εbc) and an extinction coefficient of ε_{334} $_{nm}$ = 922 $M^{-1}cm^{-1}$ for the GSNO was used to calculate the theoretical absorbance for each sample [45]. The sample was diluted further if necessary to achieve an absorbance of < 1. UV-Vis spectra were collected using a UV-Vis Spectrophotometer (Lambda 35, PerkinElmer, MA). The spectra were obtained by scanning from 300 to 500 nm at a scanning speed of 240 nm/min. Milli-q purified water was used as the blank to standardize the baseline absorbance. The absorbance at 334 nm was used to calculate the %recovery of GSNO from the stored GSNO/Vaseline primary matrix [35,45].

2.6. Preparation of matrices for NO release measurements

The entire procedure was completed in the absence of direct light. The GSNO/Vaseline primary matrix was prepared as described above. In the same mixing vessel, the desired mass of a secondary matrix was weighed out such that the ratio of primary to secondary matrix (e.g., commercial ZnO cream, Neosporin cream, copper cream, or vitamin C cream) was 27/73. Both matrices were mixed together using a wooden stirrer for 2.5 min. A small aliquot of the resulting matrix was placed inside of a plastic (polystyrene), circular stencil (diameter 5.85 mm, height 0.33 mm), on top of a glass slide. The excess matrix was scraped away from the top and the stencil was removed, leaving the matrix at a defined sample size.

2.7. Measuring NO release from matrices

NO release from various mixed matrices was measured using a Sievers Chemiluminescence Nitric Oxide Analyzer (NOA) 280i (Boulder, CO). The NOA was calibrated before via a two-point calibration of N_2 gas passed through a NOA zero air filter and a standard of 44.3 ppm NO in N_2 gas. The test matrix mixture was prepared and put onto a small glass slide as described above. The glass slide was then place into an amber NOA sample cell. The bottom of the amber NOA sample cell was filled with Milli-q purified water and the glass slide was placed on top of a stage above the water line such that the matrix mixture did not come in contact with the water. The water reservoir was bubbled with N_2 gas at a rate of 50 mL/min to humidify the NOA sample cell to prevent the matrix from drying out. The NO generated from the GSNO within the Vaseline/cream mixture was swept into the NOA by N_2 sweep gas. For measuring NO release of samples at 34 °C, the amber NOA sample cell was placed in a 34 °C water bath. All amber NOA sample cells were wrapped in aluminum foil to shield the samples from light exposure.

2.8. Measuring NO release from GSNO in the presence of Zn^{2+} ions

All solutions were made with 10 mM Tris-HCl buffer, pH 7.4. NO release was measured using the chemiluminescence analyzer. The reaction mixtures were analyzed for NO release in the absence of light at 24 °C. Two mL of a 1 mM GSNO solution in buffer was added to an amber NOA sample cell (bulk). After a 1 min equilibration period, 100 μL of 1 mM $ZnCl_2$ was added to the bulk. For the control, 100 μL of the buffer (10 mM Tris-HCl, pH 7.4) was added into the test solution. After 9.5 min, 100 μL of 1 mM $CuCl_2$ was added to the bulk as a positive control. Each solution was bubbled with N_2 gas at a rate of 50 mL/min. NO generated was carried into the NOA by a N_2 sweep gas.

2.9. Measuring NO release from GSNO for component studies

All solutions were made with 10 mM Tris-HCl buffer, pH 7.4. NO release was quantitated using a NOA in the absence of light at 34 °C. The components studied were the same or similar to those known to be present in the commercial zinc oxide cream (Desitin® Rapid Relief

Cream: Zinc Oxide Diaper Rash Cream). These included 30 nm ZnO nanoparticles, glycerol, magnesium sulfate, tocopheryl acetate, PEG-300, 1,2-octanediol, 1,2-hexanediol, tropolone, and potassium hydroxide. The experiment scheme employed was as follows: 2 mL of a solution containing one component was first placed inside of an amber NOA sample cell (bulk solution) at 34 °C. Then, 100 μL of a GSNO solution was added to the same sample cell. The bulk solutions contain 10 μmol of the given test component and the GSNO solution contains 1 μmol of GSNO. The final mole ratio of test component to GSNO was 10:1 μmol. For each of the aqueous-soluble components, 5 mM bulk solutions were made in advance in buffer (e.g., glycerol, magnesium sulfate, PEG-300, 1,2-octanediol, 1,2-hexanediol, tropolone, and potassium hydroxide). The 30 nm ZnO nanoparticles and tocopheryl acetate are not soluble in aqueous buffer and therefore prepared in a different manner to make the bulk solutions. For the ZnO nanoparticles, 0.82 mg were added to 2 mL of the buffer (0.41 mg ZnO/mL buffer) inside the amber NOA sample cell and sonicated for 20 s. For testing tocopheryl acetate, 4.92 μL was added to 2 mL of the buffer (2.36 mg tocopheryl acetate/mL buffer) inside the NOA sample cell and sonicated for 20 s. The amber NOA sample cell containing the bulk solution (s) was submerged in a 34 °C water bath and allowed to equilibrate for 1 min. After 1 min, 100 μL of a 10 mM GSNO solution was added into the bulk solution. As previously described, the solutions inside the sample cells were bubbled with N_2 gas at a rate of 50 mL/min and the NO generated was carried into the NOA by a N_2 sweep gas.

2.10. Antimicrobial studies

Overnight grown bacteria cultures were diluted with 1 × PBS buffer (10 mM, pH 7.2) to 1 × 10^5 CFU/mL. Fifty μL of the diluted culture was spread on to a 6 cm diameter LB agar plate and allowed to air dry for 10 min. Squares, 1.5 cm × 1.5 cm, were cut from the inoculated LB agar and place in an empty Petri dish. Separately, medical grade silicone sheeting (0.0127 cm thick) was cut into 2.5 cm × 2.5 cm squares. Using a plastic (polystyrene) stencil to define the matrices area (2 cm × 2 cm × 0.033 cm), different mixtures (e.g., GSNO at different wt% in Vaseline mixed with commercial zinc oxide cream (with 13 wt% ZnO)) were spread onto the square silicone sheets and the excess was scraped away. The average mass of matrix on each silicone sheet was 84.9 ± 3.0 mg (n = 6). The square silicone sheets with the given matrix mixture were placed on top of the inoculated LB agar squares and the cover was lightly place on the Petri dish. Samples were incubated at 34 °C, absent from light, for 6 h. After incubation, the silicone sheets were removed from the top of the LB agar squares. A circle (diameter 0.8 cm) was punched out using a biopsy punch (Tru-punch, Fisher Scientific, Hampton, NH) from the center of the LB agar squares. This punched out circle was homogenized in 2 mL of 1 × PBS (10 mM, pH 7.2) in a 15-mL tube using a homogenizer (OMNI TH, OMNI International, Kennesaw, GA) at full speed, and 10-fold serially diluted. Five μL of the dilutions were spread on fresh LB agar plate for overnight culturing and single colonies were numerated. A schematic of the antimicrobial study set-up is shown in Fig. 5a (below).

3. Results and discussion

3.1. Preliminary stability study of 10 wt% and 33 wt% GSNO in Vaseline

Vaseline was chosen as the primary matrix to store and stabilize GSNO. Vaseline is a commercially available matrix consisting of 100% petroleum jelly/petrolatum (white). Petrolatum (white) is insoluble in water because of its extremely hydrophobic nature consisting of saturated hydrocarbons [46]. Having zero water content is a key factor when considering the storage and stability of GSNO. In the presence of water, GSNO can decompose in several manners. A transnitrosation reaction can occur between GSNO and other thiols to yield GSH and the corresponding nitrosated thiol [47,48]. Singh et al. reported that GSNO

will decompose in the presence of its parent thiol GSH (and there is always a tiny amount of this species present in the GSNO preparation) and that the decomposition rate is dependent on the concentration of GSH [35]. In aqueous solution, GSNO can also react with other reducing agents such as ascorbate (ascorbic acid) or catalysts such as iron and copper ions [30–32,36,38].

Two different concentrations of GSNO in Vaseline, 10 wt% and 33 wt% GSNO, were prepared to evaluate the long-term stability of GSNO in the Vaseline matrix at 24 °C, in the dark, and in dry storage. UV-Vis spectroscopy was used to determine the stability of GSNO in Vaseline because GSNO has a strong absorption peak in the UV region at 334 nm [35,45]. Absorbance (ABS) versus wavelength was measured for both 10 wt% and 33 wt% GSNO in Vaseline stability samples on day 583 and day 313, respectively (Figs. S2a–b). The % recovery for the 10 wt% GSNO in Vaseline on day 583 was 89.8 ± 3.4%. The %recovery for the 33 wt% GSNO in Vaseline on day 313 was 87.6 ± 3.5%. The samples were stored in the dark to avoid any photochemical decomposition of GSNO [37]. Traditionally, RSNOs are most stable at colder temperatures. Shishido et al. reported increased thermal stability of CysNO in a PEG 400 matrix because a higher viscosity imposes a cage effect on the thiyl and NO radical pair such that geminate recombination occurs more often [33,49]. Hence, the longevity of GSNO stability at 24 °C suggests that the dry GSNO being sequestered within the viscous Vaseline matrix may increase GSNO's thermal stability due to favorable geminate recombination of the thiyl and NO radical pair.

3.2. Initiating NO release using various secondary matrices

Four different commercially available secondary matrices were chosen for testing because they have ingredients that have the potential to assist with NO proliferation from GSNO when mixed with the GSNO/Vaseline primary matrix. The non-prescription matrices selected were Neosporin® + Pain Relief Cream (Neosporin cream), Avalon Organics® Intense Defense with Vitamin C Oil-Free Moisturizer (vitamin C cream), Osmotics Cosmeceuticals Blue Copper 5® (copper cream), and Desitin® Rapid Relief Cream: Zinc Oxide Diaper Rash Cream (zinc oxide cream). The primary matrix (33 wt% GSNO in Vaseline) was mixed with each secondary matrix at a 27/73 ratio. This ratio was chosen primarily based on consistency of the final mixture. NO release for these mixtures at two temperatures was investigated, room temperature (24 °C) and a temperature closer to the surface of human skin (34 °C) [50]. After mixing the primary and secondary matrices, NO release was monitored over a 6 h period using a NOA. Only the first 6 h of NO release were monitored to simulate a theoretical application period. Fig. 1 shows the total %NO released from the known amount of GSNO present during the 6 h period for each secondary matrix at 24 °C and 34 °C. It is clear from the data shown in Fig. 1 that the zinc oxide cream provides a significantly enhanced rate of NO release when mixed with the GSNO/Vaseline mixture compared to the other three commercial creams tested.

In general, for each final matrix mixture there is a minimum of two known mechanisms by which NO is proliferated from GSNO, thermal and via reaction with its parent thiol GSH (a trace amount of GSH is always present in the GSNO preparation) [35,51]. Only these two reactions take place to generate NO in the case where Neosporin is involved. Based on the ingredients present in the Neosporin cream, there are no additional reactions that could take place to proliferate NO from GSNO. When Neosporin was mixed with the GSNO/Vaseline mixture, only as small percentage of NO was released after 6 h at both temperatures (Fig. 1a). This data shows that Neosporin is not capable of releasing an appreciable amount of NO from GSNO because the NO generation pathways are limited to the two mentioned above.

In the case involving the vitamin C cream, there is an additional reaction that could take place to increase NO release. The active ingredient ascorbic acid/ascorbate (serving as a reducing agent) is known to enhance NO release from GSNO [30,32]. However, an extremely low

%NO release was observed (Fig. 1b). The concentration of ascorbic acid in this commercial cream is proprietary and unknown. Therefore, there may not be enough ascorbic acid in the vitamin C cream to release an appreciable amount of NO from GSNO because ascorbic acid is a reactant, not a catalyst for the NO release reaction [30,32,52]. Further, the reaction rate between GSNO and ascorbic acid may be hindered by other species present in the Vitamin C cream. It is known that ascorbic acid can be readily oxidized by moisture, light, heat, and metal ions [53,54]. For this reason, cosmetic formulations use different chemicals or additives to stabilize ascorbic acid such as tocopheryl acetate, citric acid, boric acid, tartic acid, and glycerine [54].

The %NO released by the copper cream was much lower than expected because copper ions are well-known to be a very good catalyst for NO proliferation from RSNOs (Fig. 1c) [31,36,55]. Similar to the vitamin C cream, there may be a component in the commercial copper cream that inhibits the reaction between the copper ions and GSNO. One ingredient present in the copper cream is protocatechuic acid (PCA). PCA is known to be a metal ion chelator as well as free radical scavenger [56,57]. Therefore, the NO produced may be scavenged or the copper ions are likely strongly bound to PCA, not enabling them to serve as a catalyst for NO generation from GSNO.

The zinc oxide cream released significantly more NO from the GSNO/Vaseline mixture than any other secondary matrix tested, at both 24 °C and 34 °C (Fig. 1d). The zinc oxide cream was chosen because McCarthy et al. showed that Zn^{2+} ions had the capability of releasing NO from another RSNO (*S*-nitroso-*N*-acetylpenicillamine (SNAP)) [58]. However, with no reports on Zn^{2+} ions significantly enhancing the proliferation of NO from GSNO, further experimentation was needed to determine the true source of NO proliferation from GSNO.

3.3. NO release characteristics and kinetics from GSNO using zinc oxide cream

The first step to better understand the exact agent in the ZnO cream that accelerates the NO release from GSNO was to investigate the NO release kinetics using three different starting concentrations of GSNO. An aliquot of the appropriate wt% of GSNO in Vaseline was mixed with the zinc oxide cream at a 27/73 ratio such that the final wt% of GSNO in the combined matrix was 3, 6, or 9 wt%. NO release was measured at 34 °C for 6 h revealing that the NO release profile for each concentration was fairly similar (Fig. 2a). Cumulative NO release plotted versus time reveals a similar NO release trend for all three concentrations of GSNO tested, exhibiting apparent first-order kinetics (Fig. 2b). Integration of Fig. 2a showed that 77.9 ± 3.8%, 75.4 ± 3.8%, and 74.6 ± 2.2% of the total theoretical NO available was released when employing the 3, 6, and 9 wt% GSNO final mixtures over a 6 h test period, respectively (Table 1). The observed first order rate constant for the overall NO release kinetics was determined by the cumulative moles of NO release for 3, 6, and 9 wt% GSNO in the final mixtures versus time, giving k_{obs} = 0.58 ± 0.01 h^{-1} ($t_{1/2}$–1.18 h), k_{obs} = 0.63 ± 0.01 h^{-1} ($t_{1/2}$–1.10 h), and k_{obs} = 0.64 ± 0.01 h^{-1} ($t_{1/2}$–1.08 h), respectively (see Figs. S3a–c, Table 1).

While it is possible that there could be several different reactions taking place to proliferate NO from GSNO, overall the total %NO release, half-life, and rate constant values are very similar for all three concentrations of GSNO in the final mixtures (Table 1). Indeed, the measured rate constants for each concentration of GSNO are very similar, leading to the conclusion that the rate constant is independent of GSNO concentration. However, the actual rate of reaction is time and concentration dependent according to the first-order rate law.

All of the studies described above were completed in the absence of direct light. However, for practical topical application, the creams have a high chance of being exposed to light. Therefore, the NO release kinetics of the highest GSNO concentration in Vaseline/ZnO cream (9 wt% GSNO final cream) were measured in the presence of ambient laboratory light. More specifically, the light source was from fluorescent
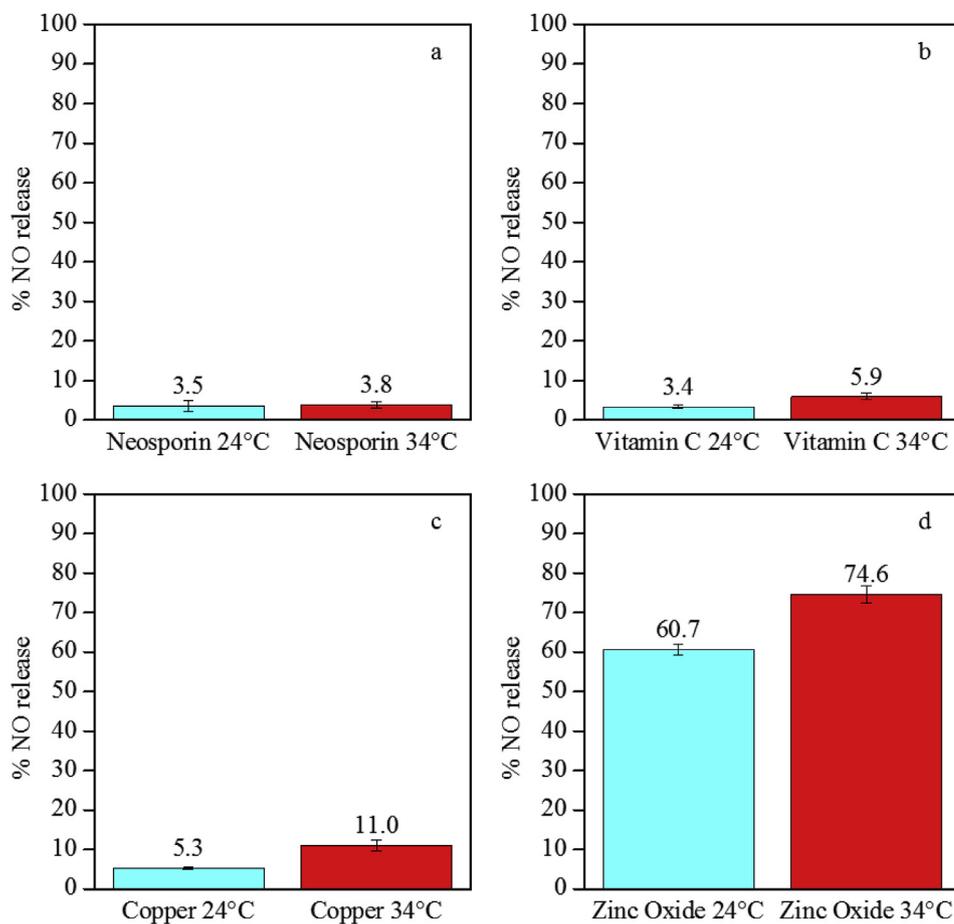
Fig. 1. %NO released from 33 wt% GSNO/Vaseline primary matrix mixed with several secondary matrices: (a) Neosporin cream; (b) vitamin C cream; (c) copper cream; and (d) zinc oxide cream at a 27/73 ratio to yield a final 9 wt% GSNO matrix. NO release was measured in the dark over a 6 h period at both 24 °C and 34 °C. Data represents the mean ± SEM (n = 3).

ceiling lights approximately 2 m away from the samples tested. In general, the same procedure outlined in Section 2.7 was followed. The only changes were that the laboratory light was on during the 6 h NO release measurements, the NOA sample cell was clear glass not amber, and no aluminum foil was used to cover the cell. A direct comparison of the real-time NO release in the dark versus in ambient room light shows a negligible difference in the rate of NO generation (see Figs. S4a–b). The %NO released over the first 6 h in the presence of light was 75.1 ± 3.2%, which is very similar to that observed under dark conditions (Table 1). The observed first order rate constant in the presence of light was determined in the same manner as described previously, giving k_{obs} = 0.63 ± 0.01 h^{-1} ($t_{1/2}$–1.10 h), which again is very similar to the in dark scenario (see Fig. S4c, Table 1). One plausible reason why these different scenarios yield similar results could be because only the surface of the opaque cream is exposed to light and not

Table 1
Summary of %NO release in 6 h, first order rate constant (k_{obs}), and half-life ($t_{1/2}$) for 3, 6, and 9 wt% GSNO final matrices measured in the dark at 34 °C. GSNO/Vaseline primary matrices were mixed with commercial Desitin zinc oxide cream at a 27/73 ratio. (n = 3 separate preparations).

Matrix	% NO release in 6 h	k_{obs} (h^{-1})	$t_{1/2}$ (h)
3 wt% GSNO	77.9 ± 3.8	0.58 ± 0.01	1.18
6 wt% GSNO	75.4 ± 3.8	0.63 ± 0.01	1.10
9 wt% GSNO	74.6 ± 2.2	0.64 ± 0.01	1.08

the bulk of the cream. This data concludes that the NO release kinetics are not significantly impacted by the presence of ambient laboratory lighting.
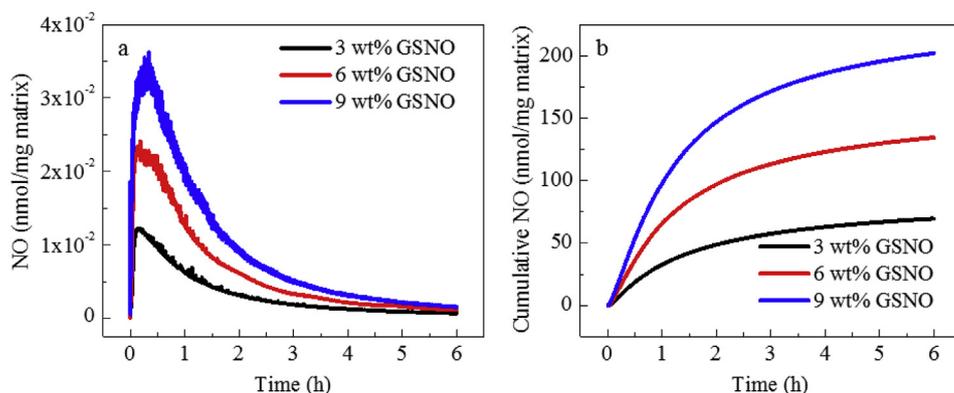

Fig. 2. (a) Real-time NO release of 3, 6, and 9 wt% GSNO matrices in the dark at 34 °C. (b) Cumulative NO release vs. time for 3, 6, and 9 wt% GSNO matrices in the dark at 34 °C. GSNO/Vaseline primary matrices were mixed with commercial Desitin zinc oxide cream at a 27/73 ratio to achieve 3, 6, and 9 wt% GSNO final matrices. Data represents mean (n = 3).
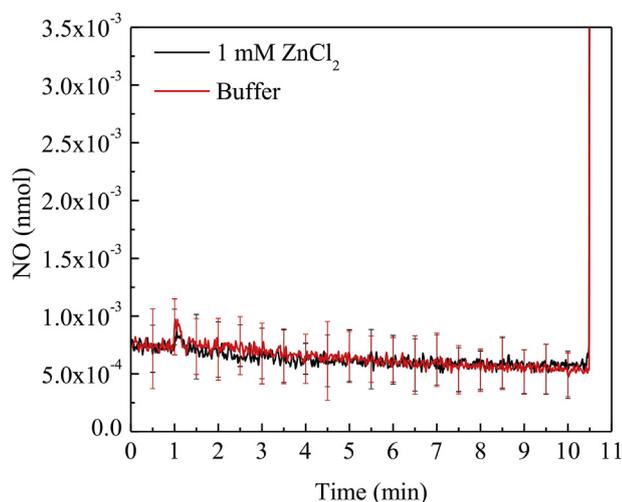
Fig. 3. Real-time NO release from 2 mL of 1 mM GSNO after addition of either 100 µL of 1 mM $ZnCl_2$ or 100 µL of the buffer. Completed in the dark at 24 °C. All solutions made with the buffer 10 mM Tris-HCl (pH 7.4). At t = 10.5 min, 100 µL of 1 mM $CuCl_2$ was added as a positive control. Data represents the mean ± SEM (n = 3).

Fig. 4. Real-time NO release after addition of 100 µL of 10 mM GSNO into 2 mL bulk solutions containing a given component. Completed in the dark at 34 °C. All solutions were made with a 10 mM Tris-HCl buffer (pH 7.4). The final ratio of component to GSNO was 10:1 µmol. Data represents the mean ± SEM (n = 3).

3.4. Investigation of NO release from GSNO in the presence of Zn^{2+} ions

An aqueous phase study was undertaken to determine if Zn^{2+} ions can increase NO proliferation from GSNO in the dark at 24 °C. As stated above, McCarthy et al. reported the NO generation potential of several different metal ions from SNAP under physiological pH conditions [58]. They discovered that three transition metal ions including Co^{2+}, Ni^{2+}, and Zn^{2+} increased NO release rates, while Fe^{3+}, Mg^{2+}, Mn^{2+}, and Pt^{2+} displayed significantly less NO release enhancement [58]. Krężel et al. reported that the stability of GSNO in HEPES buffer (pH 7.4) actually increases in the presence of Zn^{2+} due to the formation of simple coordination complexes [59]. Askew et al. observed no catalysis capability of Zn^{2+} on SNAP to proliferate NO, although no data was shown to assess whether a non-catalytic reaction can occur [60]. More recently, Lutzke et al. reported the NO releasing capabilities of over twenty different metal ion species when mixed with GSNO, and Zn^{2+} ions showed no activity [61]. In light of these conflicting reports, further investigation was needed to better understand the substantial increase in the rate of NO release when the zinc oxide cream is mixed with the GSNO/Vaseline mixture.

For the current study, $ZnCl_2$ was chosen as the source of Zn^{2+} ions. Fig. 3 shows NO release from GSNO in solution as a function of time with and without Zn^{2+} ions present in solution. At t = 1 min, 100 µL of 1 mM $ZnCl_2$ was added to the bulk solution containing 2 mL of 1 mM GSNO resulting in a very small, insignificant burst of NO. The same insignificant burst was observed when the buffer (10 mM Tris-HCl, pH 7.4) was added to the bulk solution. As a positive control, 100 µL of 1 mM $CuCl_2$ was added to each scenario at t = 10.5 min and consequently a very large burst in NO release was observed. A comparison of these two scenarios reveals that Zn^{2+} ions do not appreciably increase NO proliferation from GSNO. This data supports the findings of Krężel et al. and Lutzke et al. [59,61].

3.5. Component study of Desitin® Rapid Relief Cream: Zinc Oxide Diaper Rash Cream

An aqueous phase study was undertaken to determine which agent or agents within the Destin cream were responsible for NO release from GSNO. A simplified system was employed to monitor and measure NO release when GSNO is mixed with various components/ingredients present in the commercial zinc oxide cream. Some ingredients present
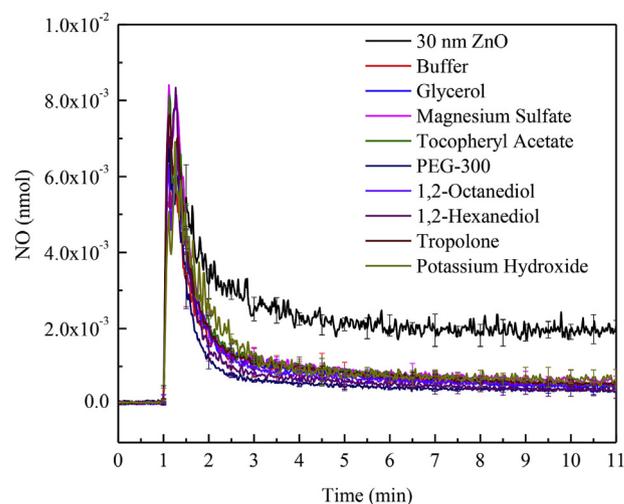
in the Desitin cream were omitted due to their low water solubility (mineral oil, petrolatum, beeswax, microcrystalline wax). The experiments were performed in the presence of 10 mM Tris-HCl buffer, pH 7.4, at 34 °C. Nine components were studied at a 10:1 µmol ratio with GSNO in aqueous solution. Fig. 4 shows NO release versus time when 100 µL of 10 mM GSNO was added into different bulk solutions containing a given component. Only the first 10 min of each reaction was monitored to determine which component is responsible for the enhanced NO release from GSNO. As shown clearly in Fig. 4, which compares the NO release profiles of each of the components, only the 30 nm ZnO nanoparticles yield a significantly enhanced and prolonged NO release from GSNO. The NO release profiles of the other 8 components demonstrate a similar profile to the buffer control, where no added components are present. Moreover, this study demonstrates that the other 8 components present in the Desitin cream are not significantly involved in the enhanced NO release from GSNO, as observed in Sections 3.2 and 3.3.

Promoting NO release from GSNO using ZnO nanoparticles has not been reported previously. However, very recently Singha et al. demonstrated enhanced and prolonged NO release from SNAP-doped CarboSil films with an additional top coating consisting of CarboSil and ZnO nanoparticles [62]. This demonstrates that ZnO nanoparticles could be capable of promoting NO release from other RSNOs. There also have been reports of gold and platinum nanoparticles catalyzing NO generation from RSNOs [63–65]. Jia et al. reported the enhanced rate of NO release from GSNO via reaction of gold nanoparticles in a dose-dependent manner [63]. Later, Taladriz-Blanco et al. showed that due to the affinity between gold and thiols, the RS-NO bond breaks when in the presence of gold nanoparticles, and subsequently the gold surface is functionalized with the corresponding thiol species (as a submonolayer) [64]. More recently, Cao et al. reported enhanced NO generation from SNAP and GSNO using 3 nm platinum nanoparticles, and suggested evidence of Pt–S bond formation during the reaction [65]. The data shown here (Fig. 4) clearly demonstrates that zinc oxide nanoparticles can enhance NO release from GSNO. Investigation into the exact mechanism is not the main focus of the current work, but is being studied (using various surface analysis and other methods) and will be reported in the near future.
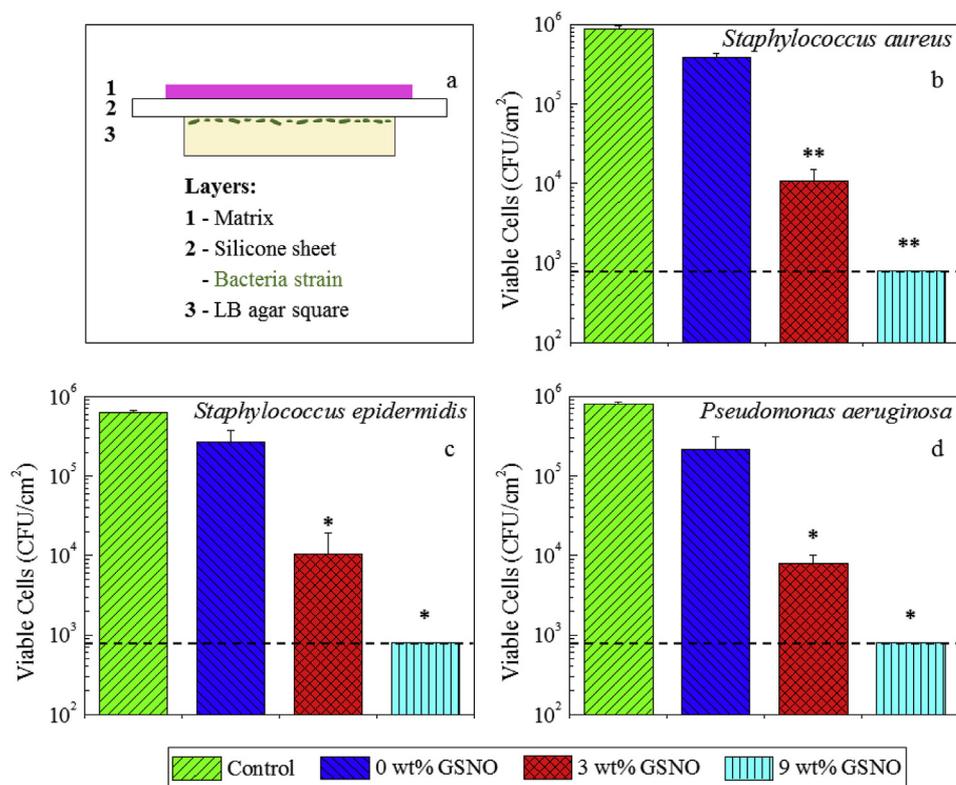
Fig. 5. (a) Side view schematic of antimicrobial study configuration using agar square with bacterial growth cut from Petri dish. (b–d) Antimicrobial study results for Control and 0, 3, and 9 wt% GSNO final matrices indirectly applied (with silicone sheet between) to (b) *S. aureus*, (c) *S. epidermidis*, and (d) *P. aeruginosa* inoculated LB agar plates in the dark at 34 °C for 6 h. Control means nothing was applied to the surface of the inoculated LB agar. GSNO/Vaseline primary matrices were mixed with Desitin zinc oxide cream at a 27/73 ratio to achieve 0, 3, and 9 wt% GSNO final matrices. Viable cells were determined via plate counting. Horizontal dashed line represents the LOD (7.96×10^2 CFU/cm^2). No error bar means the LOD was reached for each trial. Data represents the mean ± SEM ($n = 3$). *$p < 0.025$, **$p < 0.01$, 0 wt% GSNO vs. 3 and 9 wt% GSNO.

3.6. Antimicrobial studies

Antimicrobial studies were conducted to demonstrate the killing effect of NO released from the 3 and 9 wt% GSNO test mixtures with the Desitin cream containing 13% ZnO against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*. These three bacteria strains were chosen because they have been shown to be common bacteria associated with wound or burn-wound infections [43,44]. The initial concentration of bacteria (1×10^5 CFU/mL) was chosen because it is often associated with infection for open wounds and hindering wound closure [66]. Fig. 5a shows a schematic of how the antimicrobial studies were performed. A silicone sheet was employed to keep the matrices from coming into direct contact with the bacteria on agar plates such that no additional killing effects from the matrix itself (e.g., ZnO particles) would be observed [42]. Silicone films were chosen because Ren et al. reported that silicone rubber polymers have the highest rate of NO diffusion compared to several other biomedical grade polymers [67]. The antimicrobial studies were completed in the dark at 34 °C with an incubation/application period of 6 h.

The matrices studied in these antimicrobials experiments (Fig. 5) were the same as those employed in Section 3.3. "Control" in Fig. 5 means nothing was applied to the surface of the inoculated LB agar. The 0 wt% GSNO sample used in this study served as a second control to determine how much bacteria were lost when removing the silicone sheeting from the surface of the LB agar plate (Fig. 5b–d). All log reduction values of bacterial counts are reported in the order *S. aureus*, *S. epidermidis*, *P. aeruginosa*. Comparing the "Control" columns in Fig. 5 versus the "0 wt% GSNO" columns in Fig. 5b–d, the average log reduction after removing the silicone sheeting was calculated to be 0.36, 0.37, and 0.57, respectively. Therefore, when no NO is present and the bacteria are only in contact with the silicone sheet, very little killing effects are observed. In Fig. 5b–d, the columns that do not display an error bar means that all of the bacteria of that strain were killed for each trial (n = 3), therefore reaching the limit of detection for this study (7.96×10^2 CFU/cm^2). The average log reduction between the 0 wt% GSNO and 3 wt% GSNO matrices were 1.55, 1.41, and 1.44; and

between the 0 wt% GSNO and 9 wt% GSNO matrices were 2.68, 2.53, and 2.44, for each of the bacteria examined, respectively.

In general, the amount of NO released from the 3 and 9 wt% GSNO samples were able to kill each bacteria in a proportional manner (more NO equals greater log reduction). However, the amount of NO released from the 9 wt% GSNO matrix always killed all of the bacteria, therefore reaching the limit of detection for this antimicrobial assay. The true killing potential of the 9 wt% GSNO matrix is, therefore, likely greater than that illustrated in Fig. 5b–d. Nonetheless, the current study demonstrates that NO produced from the ZnO particle enhanced reaction with GSNO is capable of killing both gram-positive (*S. aureus* and *S. epidermidis*) and gram-negative (*P. aeruginosa*) bacteria to a significant degree. This phenomenon is not surprising and has been reported several times because there are multiple mechanisms by which NO can be toxic to bacteria [68–73]. Future directions will include direct application of these combination matrices on to bacteria grown on the surface of LB agar or pig skin (no silicone membrane), and then ultimately using an animal burn wound healing model and/or an animal wound closure model, to examined the antimicrobial effectiveness of the new GSNO/Vaseline/ZnO cream for potential medical applications.

4. Conclusion

The characteristics and capabilities of preparing different NO releasing topical matrices based on storing GSNO as the NO donor in Vaseline have been evaluated. Long-term storage stability was observed for 10 wt% and 33 wt% GSNO mixed in commercial Vaseline at 24 °C. The enhanced stability of GSNO in Vaseline was attributed to the extremely dry storage conditions and the high viscosity of the matrix leading to geminate recombination of the radical pair. Out of four secondary matrices tested to promote NO release when mixed with the GSNO/Vaseline primary matrix, the commercial Desitin zinc oxide-based cream released the highest percentage of NO at both 24 °C and 34 °C over a 6 h period. The unexpected low rates of NO release from GSNO in the presence of commercial ascorbic acid and copper-containing creams under the same experimental conditions were attributed

to interferences in the NO release reaction from other ingredients present in these matrices. First-order NO release kinetics were reported for 3, 6, and 9 wt% GSNO final matrices using the zinc oxide cream as the NO release promoter. It was also determined that Zn^{2+} ions have little or no effect on NO proliferation from GSNO in pH 7.4 buffer at 24 °C. Further, a thorough component study of the Desitin zinc oxide cream suggests that the ZnO nanoparticles present are primarily responsible for increased NO proliferation from GSNO. Antimicrobial studies demonstrated the killing effect of NO released from the 3 and 9 wt% GSNO final matrices prepared by mixing Desitin zinc oxide cream with the GSNO/Vaseline mixture in the dark at 34 °C with an application period of 6 h. *S. aureus*, *S. epidermidis*, and *P. aeruginosa* were all killed in proportion to the concentration of GSNO employed in the final mixture. Future studies will include using surface sensitive techniques (e.g., X-ray photoelectron spectroscopy, etc.) to investigate the mechanism of NO release from GSNO in the presence of ZnO nanoparticles, direct application of NO releasing creams to bacteria grown on the surface of LB agar or pig skin, and studies of the effectiveness of the GSNO/Vaseline/ZnO cream formulation in a suitable animal model for wound healing/closure.

Declaration of interest

The authors report no conflict of interest.

Acknowledgment

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.05.009.

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