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In vitro cytocompatibility of antibacterial levels of polymer nitric oxide release

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ABSTRACT

Although the antibacterial property of nitric oxide (NO) has been well documented in gram positive and gram negative bacteria cultures, its cytotoxic effects are not completely clear. To limit potential *in vivo* cytotoxicity, our group recently investigated the effects of a range of NO fluxes on *S. epidermidis* and *S. aureus* to determine a minimum effective NO level. In this study, we report the antibacterial function of this minimum NO level also on gram negative *Pseudomonas aeruginosa* as well as its cytocompatibility effects on lung and kidney cells. Standardized bacterial cultures were treated with NO releasing PDMS substrates followed by plating, 24hr incubation, and colony analyses. Cytocompatibility or cell viability was conducted on WI-38 human lung fibroblasts and HEK-293 human embryonic kidney cells after their exposure to NO *in vitro*. NO flux of 21.18 ± 5.31 × 10−10 mol/ min/ cm2 significantly reduced *P. aeruginosa* growth compared to controls and PDMS-treated samples (p value < 0.0001). No significant differences was seen between control and cells treated at this flux (total moles delivered in 24hrs = 0.76 ± 0.18), but a significant reduction was observed at 45.1 ± 2.55 ×10−10 mol/ min/ cm2 (total moles delivered = 1.6 ± 0.09). The results suggest that at the lower NO flux level *pseudomonas aeruginosa* growth is significantly inhibited while maintaining cell viability.

Keywords: functional biomaterials, bactericidal surfaces, bacterial infection,communicable medical materials, nitric oxide

INTRODUCTION

Nitric oxide is a multifaceted biological molecule with many physiological functions including acting as an antibacterial agent[1-4] and a promoter of cell proliferation.[5, 6] However, it can be harmful to cells when delivered at levels incompatible with normal cellular function.[7] A crucial objective pertaining to functional materials formulated to deliver NO is to ensure inconsequential cell toxicity effects if any occurs at all. It is therefore critical that biomedical applications that incorporate these properties for developing functional and bioinspired biomaterials aim for the compatible concentrations.

At high and bactericidal concentrations, NO exerts bactericidal effects through many mechanisms via NO itself or its byproducts.[8, 9-11] The interaction of NO with superoxide (O2-) within or outside microbial cell yield reactive nitrogen species (RNOS), that are known to induce nitrosative and oxidative stresses to disrupt microbe's membranes through lipid peroxidation[9] as well as induce DNA damage. Moreover, NO can nitrosate cycteine and tyrosine leading to dysfunction of many key bacterial enzymes, proteins, and cell membrane adhesion proteins that mediate cell-substrate attachment.[5] Accordingly the exploitation of these properties have been pursued using several materials to deliver various levels of NO to bacteria cultures. While the research outcomes do show antibacterial activity, the exact therapeutic concentration of NO remain unclear. Nonetheless, such exact or range of bactericidal concentration(s) must also be cytocompatible to ensure selective elimination of bacteria after they are released by functional materials which may serve as surfaces of blood/tissue communicable devices.

Simultaneously, the interaction of high NO and its byproducts with cells, although bactericidal, can be detrimental to cells due to incompatibility and lead to many biological dysfunctions.[12-17]

Many cellular and tissue functions including DNA alterations and subsequent cell anti- proliferation activity[18, 19] can be impaired.

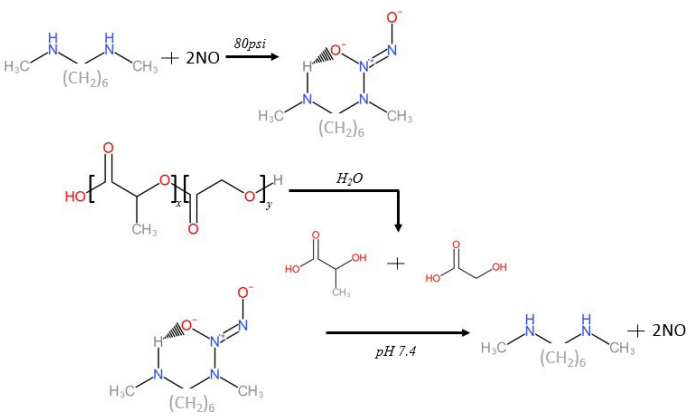
Therefore, studies that have investigated striking a balance between effective bactericidal activity and cytocompatibility using an optimal dose(s) of NO are being pursued for more conclusive data. Data from experiments conducted *in vitro* under conditions of NO gas flow[20-24] and *in vivo* using material-assisted NO delivery report the effects of different ranges of NO concentration on different compatibility outcomes.[25] In this study we investigate the effects of a wide range of NO fluxes (surface area and time normalized NO release), from low, protective, and proliferative fluxes to high levels, on cytocompatibility of lung and kidney cells *in vitro*. This evaluation method is important for the effective application of NO releasing polymers as coatings on implantable medical devices.

MATERIALS AND METHODS

Preparation of NO donor compounds for modification of PDMS substrates

Conjugation of NO to secondary amine linear polymers (dimethylhexanediamine or DMHD) to form diazeniumdiolated DMHD or DMHD/N2O2, which was then incorporated into polydimethylsiloxane (PDMS) along with poly (lactic-co-glycolic acid) or PGLA is shown in (Scheme 1).[26, 27] Hydrolysis of PLGA aids the catalysis of NO from preformed NO donor compounds embedded within the PDMS polymer.

As previously described,[28] synthesis of NO releasing PDMS substrates, as model materials for biological testing, was achieved via polymerization of a two-part silicone rubber (R21-2615, NuSil, CA). A 1:1 ratio of resin A (PDMS oligomer) and resin B (PDMS oligomer with platinum initiator) mixed in organic solvent (Mineral Spirit, Sigma-Aldrich, MO) was cured overnight at room temperature into 1mm thick base layers. 100 µl mixtures of THF solvent containing PDMS resin, 15 wt% of DMHD/N2O2, and 15 or 25 wt% of PLGA (Evonik, NJ) were



Scheme 1: Reaction of nitric oxide (NO) with linear dimethylhexanediamine to form diazeniumdiolate, and the subsequent release of the NO stimulated by hydrolysis of polylactide-glycolide acid and physiological conditions.

then casted atop a PDMS-only base layer, cured for 24hrs and followed by top casting 100 µl of PDMS solution and curing for another 24hrs.

Characterization of nitric oxide (NO) release

Real time NO release from modified PDMS and controls samples were quantified by chemiluminescence method using a GE 280i nitric oxide analyzer ((NOA), GE Instruments, CO). To measure NO release, samples were immersed in 10 ml PBS (pH 7.4) at 37°C inside the NOA reaction vessel. Nitrogen was used as a carrier gas to transport NO from the reaction vessel to the chemiluminescence detection chamber and NO flux from modified PDMS samples was calculated as the quotient of the products of NO concentration detected by NOA (ppb or ppm) and NOA calibration factor (mol/ppb*sec), and sample surface area (cm2) and duration NO detection (sec or min).

In vitro antibacterial test

Pseudomonas aeruginosa derived from ATCC 9027 was purchased from Microbiologics, and was cultured using Nutrient agar (NA) plates at 35°C. Overnight Bacterial cultures solutions were adjusted to make a final cell concentration at ~108 CFU/ml. Each NO releasing or control PDMS substrates was placed into a sterile 15-mL tube with 2 mL of the bacterial culture. The tubes were incubated at 37°C for 24 h while shaking (140 rpm). After incubation, the substrates were removed aseptically and bacterial culture was diluted in PBS buffer. 1 µL of each dilution was streaked onto NA plates for viable bacterial counting. The NCBI, Image J software was used for colony count. PDMS substrates and levofloxacin, 6mm paper disks saturated with 5 µg of levofloxacin (Hardy Diagnostics Inc), were used as additional controls.

In vitro cytocompatibility test

To determine the effect of the application of NO releasing PDMS on healthy cells, two human cell lines were exposed to NO release from PDMS and cell viabilities were quantified.

Cell culture

WI-38 human lung fibroblasts and HEK-293 human embryonic kidney cell lines were purchased from ATCC. WI-38 cells were maintained in MEM media (Sigma-Aldrich, Saint Louis MO) supplemented with 10% FBS (Gemini Biosciences, Denver CO), 1% Sodium Pyruvate, 1% l- glutamine, and 1% Penicillin/Streptomycin. HEK-293 cells were cultured in DMEM high glucose media supplemented with 10% FBS (Gemini Biosciences, Denver CO), 1% Sodium Pyruvate, and 1% Penicillin/Streptomycin. Both cell lines were maintained at 37°C, 5% CO_2.

Cell viability

MTT assay of WI-38 and HEK 293 treated with NO releasing PDMS-WI-38 and HEK239 cells were treated with a range of

PDMS disks that release different NO fluxes (Table 1). Cells were incubated in treatment for 24 hours. MTT dye (5 mg/mL) was added directly to media for an incubation period of two and half hours at 37oC. Immediately after incubation, media was aspirated and replaced with MTT solubilization buffer (0.04 M HCl in isopropanol). Cells were shaken at room temperature in solubilization buffer for at least ten minutes and then absorbance at a wavelength of 570 nm was detected using biotech microplate reader. Lower absorbance correlated to decreasing cell viability and viability was quantified as percent of control (absorbance from untreated cells).

Statistical Analysis

Data are expressed as mean ± SD (standard deviation of the mean). Comparison of results were analyzed by a comparison of means using Student's t-test or one way ANOVA. Values of $p < 0.05$ were considered statistically significant for all tests.

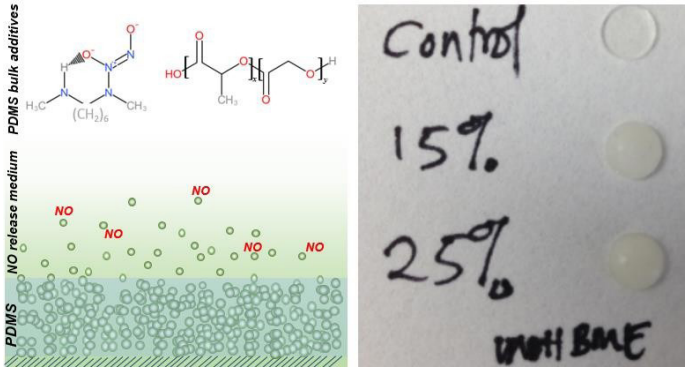
RESULTS AND DISCUSSION

Prepared samples and their nitric oxide release

Bulk composition of modified PDMS with stimulated NO release illustration (left) and representative samples for biological testing (right) are shown in Scheme 2. Control PDMS samples unmodified with any additives, top, were transparent while NO releasing samples, bottom two, appeared opaque due to the inclusion of DMHD/N_2O_2 and PLGA. The samples measured 6.35 mm ID and 1 mm in thickness.

Nitric oxide release from modified PDMS

To conveniently deliver different levels of NO to *P. aeruginosa*, WI-38 human lung fibroblasts, and HEK-293 human embryonic kidney cell, 15wt% DMHD/N2O2 +15wt% 5050DLG1A were stored at room temperature for various times before using them to treat bacteria and cells. The levels of NO released from PDMS as measured by chemiluminescence in terms of flux and total moles, and projected NO released into culture medium per minute is shown in (Table 1). All three levels of NO flux listed have been investigated on *S. aureus and S. epidermidis* strains in our previous study so in this work NO flux levels including the minimum effective flux were tested on *P. aeruginosa* and cells.



Scheme 2: Illustration of NO release from PDMS aided by its bulk composition of diazeniumdiolate (DMHD/N_2O_2) and polylactideglycolide, (left) and formulated samples showing PDMS (control) and PDMS-DMHD/N2O2 composites containing 15% and 25% of DMHD/N_2O_2.

Effect of nitric oxide on bacteria and cells

The effect of 21.18 ± 5.31 × 10−10 mol/ min/ cm2 flux of NO on *P. aeruginosa* is shown in (Figure 1). It can be seen in the left panel that qualitatively, there were no differences in colony growths between control (untreated bacterial culture streaks) and PDMS (PDMS-treated culture streaks) groups. Significantly less colonies were observed in the NO releasing PDMS group where the colonies were sparsely spotted on the plates. Colony counts in the NO releasing PDMS group (54 ± 7) × 2×104 CFU/mL was significantly different from count in no-treatment control (607 ± 52) × 2×104 CFU/mL and PDMS-treated control (602 ± 28) × 2×104 CFU/mL groups, $p < 0.001$. The response of bacteria growth to NO release at (45.1 ± 2.546 × 10−10 mol/ min/ cm2), Figure 2, shows a significantly ($p< 0.001$) lower colony counts of 80 ± 69 × 2×105 CFU/mL compared to 625 ± 75 × 2×105 CFU/mL and 700 ± 71 × 2×105 CFU/mL in the untreated control and PDMS-treated control groups respectively. Approximately 1 log/ 90% reduction in colony counts was observed at either level of NO flux while a higher reduction was seen in the Levofloxacin antibiotic positive control group, although at a level, (9 ± 2) × 2×105 CFU/mL, not significantly different compared to NO groups ($p > 0.05$).

Viabilities of WI-38 and HEK 293 cells responded differently to treatment with NO at various fluxes. At the low NO flux level of 0.48 ± 0.10 × 10−10 mol/ min/ cm2, the morphologies of WI- 38 and HEK-293 cells show no qualitative differences between their control and NO treatment groups. See (Figure 3). The adherent and spreading behavior of WI-38 controls can also be seen in the treatment group and the quantitative analysis of their viability showed no significant differences between controls [untreated cells and PDMS-treated cells (95 ± 10 %)] compared to the NO treatment group (117 ± 25%) ($p > 0.05$). The NO treatment group actually seem to improve in viability after the 24hr incubation period supporting the cell proliferative property of NO at low concentrations. In the HEK-293 cells, no observable difference in culture morphology was apparent, and no differences in viability was present between the controls and NO treatment groups ($p > 0.05$).

When the WI-38 cells were evaluated for their viability at 21.18 ± 5.31 × 10−10 mol/ min/ cm2, (Figure 4), again no statistically significant differences were observed among untreated control, PDMS-treated control (90 ± 12 %), and NO treatment groups (84 ± 15 %) ($p > 0.05$). Similarly, qualitative analysis of W1-38 cell culture, the only cell line evaluated here, showed no dissimilar adherent and spreading behavior between controls and the NO treatment groups.

Finally, treatment of cells with the highest NO flux tested

PDMS Active Layer Composition	Average NO flux (x 10-10 mol/min/cm2)	Average total NO (moles)	Average moles delivered to culture/ minute
15wt% DMHD/N2O2 +15wt% 5050DLG1A (24hr cured)	45.10±2.55	$1.60x10^{00} \pm 0.89x10^{-01}$	$1.16x10^{-03} \pm 6.69x10^{-05}$
15wt% DMHD/N2O2 +15 wt%5050DLG1A (24hr cured + 24hr storage)	21.18±5.31	$7.60\ x10^{-01} \pm 1.77\ x10^{-01}$	$5.47\ x10^{-04} \pm 1.28\ x10^{-04}$
5wt% DMHD/N2O2 +15wt% 5050DLG1A (24hr cured + 48hr storage)	0.48±0.10	$1.84\ x10^{-02} \pm 3.64\ x10^{-03}$	$1.29x10^{-05} \pm 2.58\ x10^{-06}$

Table 1: Averages of nitric oxide fluxes and averages of total nitric oxide moles released from NO releasing PDMS substrates used for in vitro testing on bacterial cells growth

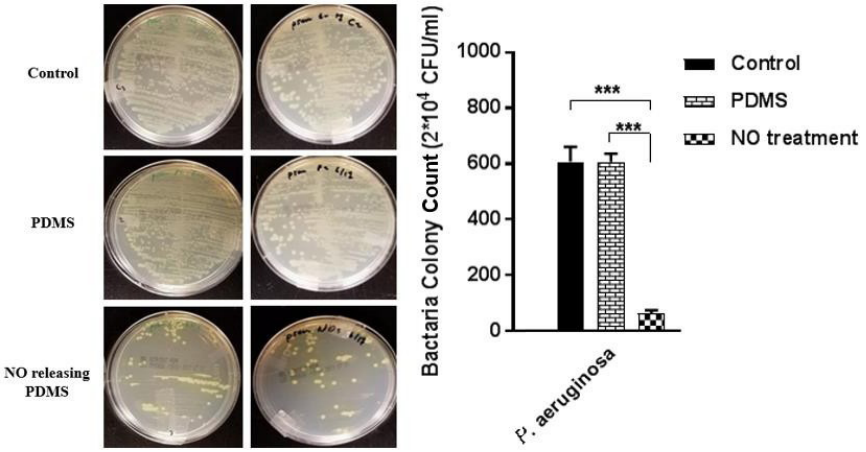


Figure 1: Antibacterial effect of 21.18 ± 5.31 × 10−10 mol/ min/ cm2 NO flux. Representative images of 1:10 dilution streaks of *P. aeruginosa* after 24 hours of NO treatment in comparison to control and PDMS alone (left). Quantitative analysis of streak plates, (n=12 per group), of *P. aeruginosa* shows significant reduction in colony count after NO treatment. Three asterisk denotes statistical significance (P value < 0.001)

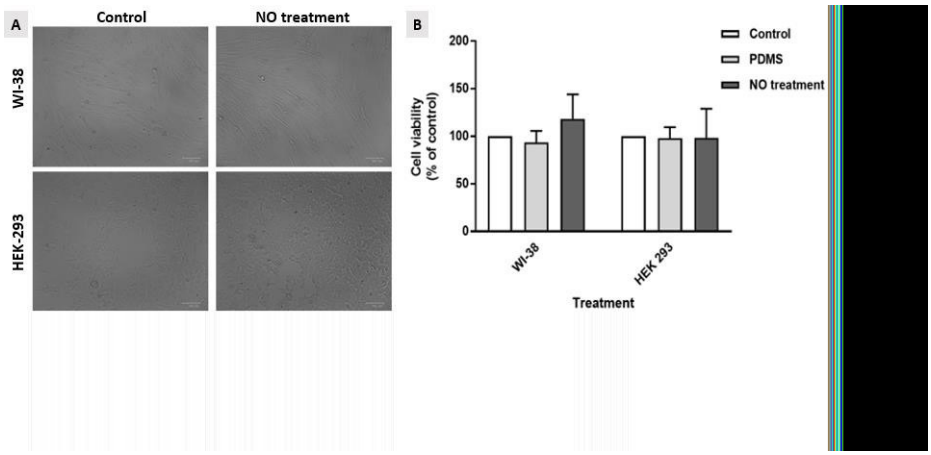


Figure 2: The effect of NO flux (45.1 ± 2.546 × 10−10 mol/ min/ cm2) on P. aeruginosa growth in nutrient broth. Representative images of 1:100 dilution streaks of P. aeruginosa after 24 hours of NO treatment (left) and colony counts (right) showing significant reduction in bacteria growth in the NO releasing group (***p < 0.001)

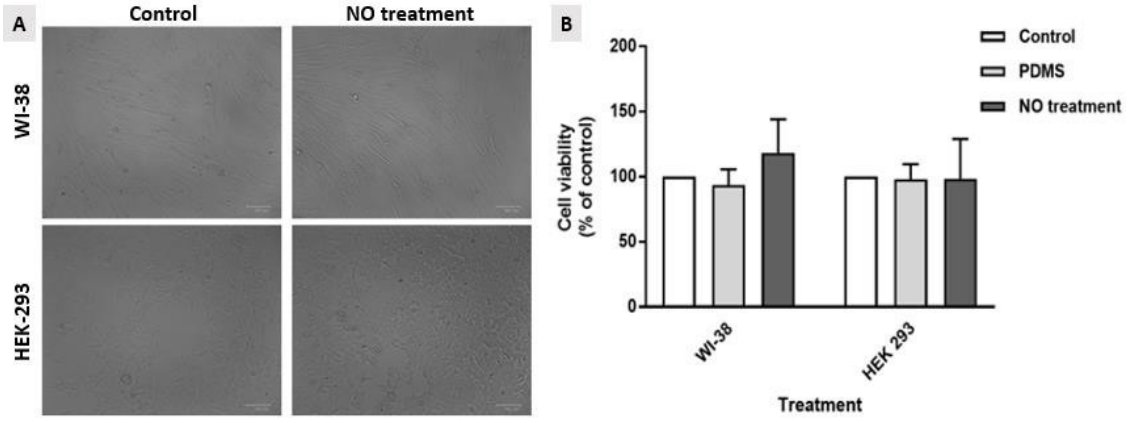


Figure 3: The effect of low NO flux on cell viability. (A) Representative images of both cell lines with NO treatment. (B) NO flux at 0.48 ± 0.10 × 10−10 mol/ min/ cm2 promotes the growth in WI-38 cells.

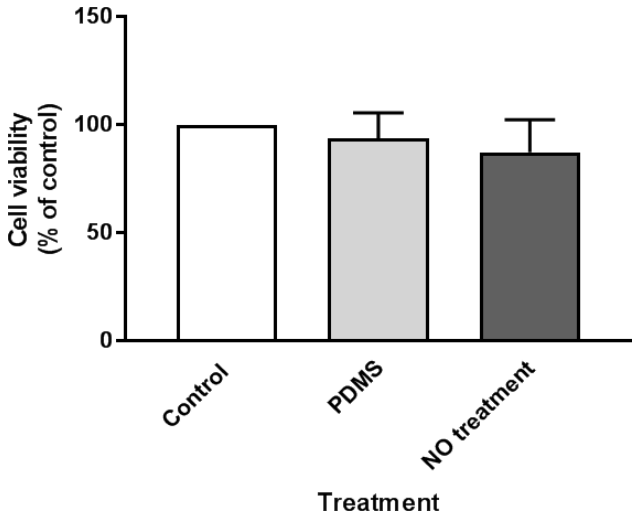


Figure 4: The effect of bactericidal NO flux on WI-38 human lung fibroblasts viability. No significant difference was found between groups. It shows no significant difference between control and cells treated with average NO flux of 21.18 ± 5.31 × 10−10 mol/ min/ cm2, average total NO moles delivered over 24 hours was 0.76 ± 0.177 mole.

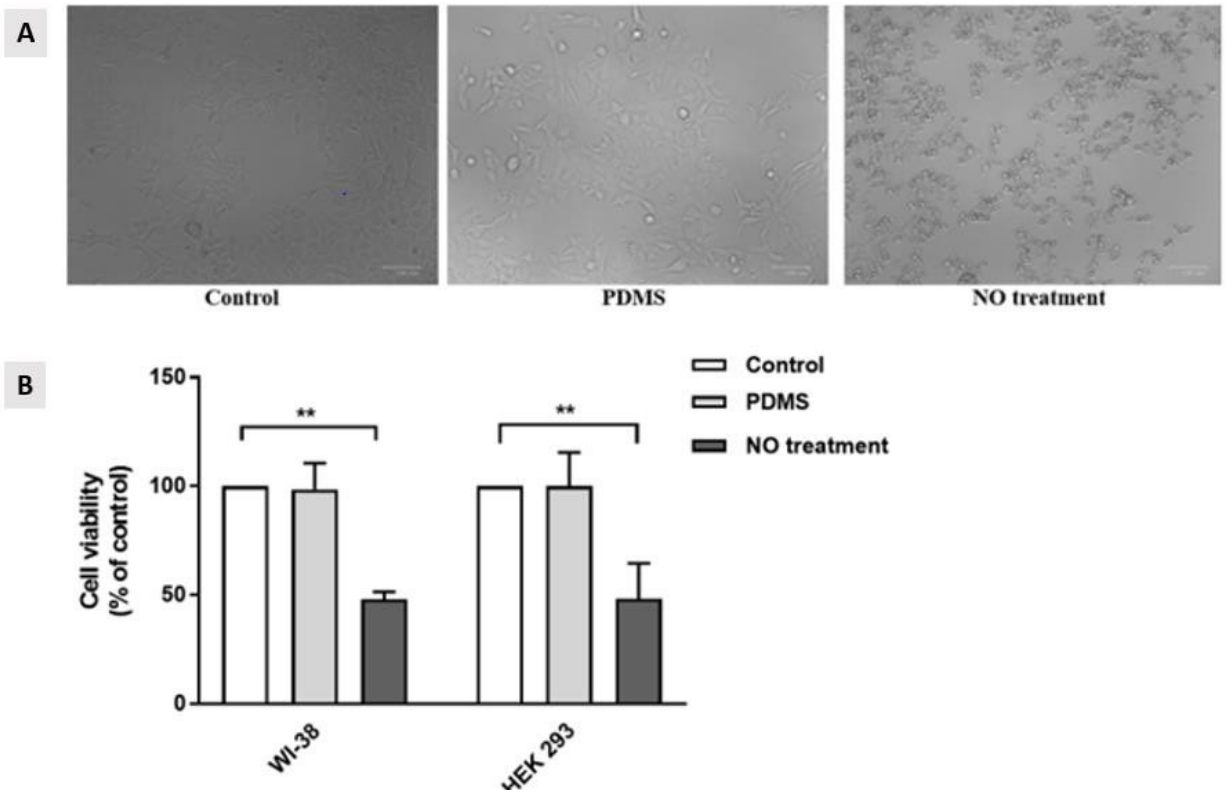


Figure 5A: The Effect of high NO dosage on cell viability. Representative images of HEK-293 cells with NO treatment in comparison to controls. **(5B)** Significant reduction in cell viability after treatment with higher dose of nitric oxide (45.1 ± 2.546 x × 10−10 mol/ min/ cm2). For WI-38 cells (p value =0.0023) and for HEK-239 (p value =0.0027).

(45.10±2.55 × 10−10 mol/ min/ cm2), a level which showed the highest reduction in *P. aeruginosa* growth although not significantly different from the 21.18 ± 5.31 × 10−10 mol/ min/ cm2 flux effect, affected viabilities as anticipated. It can be seen in Figure 5A that compared to untreated control WI-38 culture, cell viabilities after PDMS control and NO treatments were 94 ± 13% ($p > 0.05$) and 45 ± 07% ($p < 0.05$) respectively. With HEK-293 cells, viabilities were 100 ± 21% ($p > 0.05$) in PDMS control and 45 ± 18% ($p < 0.05$) in NO treatment compared to untreated controls. The morphology of HEK-293 cells elected for qualitative analysis of the effect of NO treatment revealed non- spreading and non-adherent properties. This was unlike the cell adhesion phenomenon seen in untreated and PDMS treated controls. The image in (Figure 5B) suggests the effect of such high level of NO flux can impair the spreading and adhesion of cells onto surfaces. Perhaps such levels significantly interact with cell adhesion molecules (CAMs) on cell membranes which are involved in cell adhesion, and may interfere with important CAMs properties such as maintaining tissue structure, function, and cell growth.

CONCLUSION

This study investigated the cytocompatibility of antibacterial property of nitric oxide at different concentrations to determine effective antibacterial level of NO that could also be safe to healthy cells. PDMS biomaterials formulated to release different levels of NO was characterized for their total levels of released NO, effect on gram positive (not included in this study) and gram negative bacteria growth as well as for an effective minimum flux with antibacterial yet cytocompatible properties. The effects of the NO fluxes, including the effective minimum, on lung and kidney cells viabilities were also determined. The results from this work suggest that marginal antibacterial benefits are gained at fluxes greater than 21.18 ± 5.31 × 10−10 mol/ min/ cm2 and most importantly, cell viabilities at this NO level are seemingly not affected. The largest NO flux tested, 45.10±2.55 × 10−10 mol/ min/ cm2, however had a negative effect on the viabilities of both cell lines.

Further studies including long term antibacterial NO effects on cytocompatibility using additional standard intracellular markers to evaluate cell function and the antibacterial functionalization of implantable devices associated with risk of infection are needed to fully understand NO effects and to ensure safe and effective applications.

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