Supraphysioxic Room Air Adaptation of Human Lung A549 Cells Affects Silver Nanoparticle Toxicity Assays

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Abstract

Discrepancies between *in vitro* and *in vivo* toxicity assays confound prediction of human toxicities. Others have shown that CuO nanoparticle toxicity assays using A549 cells adapted to room air are plagued by oxygen artifact that helps account for the differences.This oxygen artifact was present even though lung cells naturally experience oxygen levels closer to room air than most tissues *in vivo*. Silver nanoparticles are found in as many as 400 consumer and medical products, yet show toxicity in murine pulmonary models. For this project, we hypothesized that adapting A549 cells to unbroken physioxic conditions (cell handling as well as incubation) would change silver nanoparticle toxicity profiles. For at least 14 days before assay, A549 cultures were maintained in two different oxygen conditions: conventional room air conditions (21% O2 for handling, 18% O2 for incubation) or full-time physiologic O2 for cell incubation and handling in the Xvivo System (10% or 3% at all times). Cell handling for all groups was performed in a controlled atmosphere at 37 degrees C and 5% CO2 with pre-equilibrated fluids. Critical cell parameters were monitored during cell handling and incubation including: O2, CO2, air temperature, chamber floor temperature and relative humidity. A549 cells, cultured in triplicate flasks, had longer cell doubling times at 10% O2 and had altered morphology. Cells were plated overnight before 10nm diameter silver nanoparticles (0-10 micrograms/ml) were introduced. Cell counts and viability assays at 24, 48, and 72 hours showed that cells adapted to constant physiologically relevant oxygen conditions showed significantly different toxicity profiles then those adapted to uncontrolled room air conditions. We concluded that full-time maintenance of in vitro cells in physioxic conditions is necessary to prevent adaptation of cells to supraphysiologic oxygen that can skew toxicology assay results, even for lung cells.

Background

- **Lung cells are normally exposed to much lower oxygen than room air (5-13% O2), and even lower in pathological states (3% or less).**
- **Silver nanomaterials are being incorporated into consumer products for anti-microbial, electrical, optical, and thermal properties**
- **Even after years in room air culture, oxygen levels affect lung cells used for tox tests[1]**
- **Inhaled nanoparticles can be toxic and induce generation of ROS and DNA damage[2,3]**

Objectives

Test silver nanoparticle toxicity on cells adapted to different oxygen levels:

- **Physioxic Lung (10% O_2)**
- **Standard Room Air Culture and Handling Conditions (18-21% O_2)**
- **Hypoxic Lung (3% O_2)**

References

1.Kumar A, Dailey LA, Swedrowska M, Siow R, Mann GE, Vizcay-Barrena G, Arno M, Mudway IS, Forbes B: **Quantifying the magnitude of the oxygen artefact inherent in culturing airway cells under atmospheric oxygen versus physiological levels.** FEBS Lett 2016, 590(2):258-269.
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3.Mittal S, Pandey AK: **Cerium oxide NP induced toxicity in human lung cells: role of ROS mediated DNA damage and apoptosis.** Biomed Res Int 2014, 2014:891934.

Conflict of Interest Disclosure

The authors all are employees of BioSpherix, Ltd.

Experimental Design

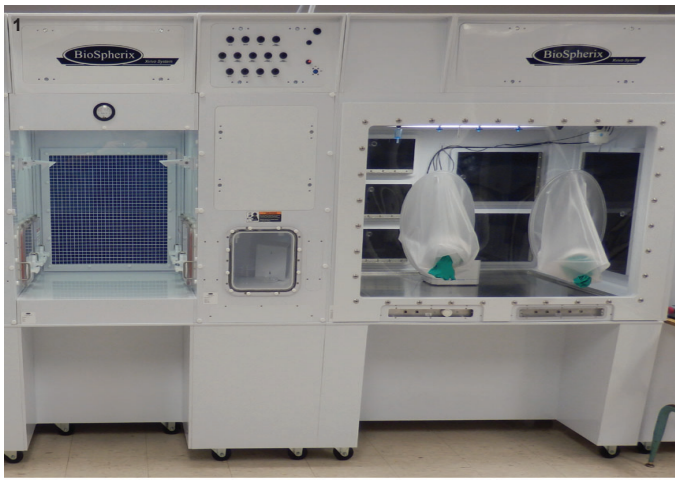


Figure 1. Experimental Design. All experiments were performed in the Xvivo System (BioSpherix) equipped with an entrance laminar flow module (module on left), airlock-like dual buffer chambers (center), and a cell processing chamber with multiple independent incubators (black doors on right). All chamber atmosphere was provided by tanked gases. Three incubators were used for this study, controlled to 37 degrees C, 5%CO_2, and 3%, 10%, or 18% O_2. The processing chamber conditions were set before an incubator door was opened so that the cells were exposed to continuously controlled conditions for cell handling as well as incubation. To better mimic poorly controlled standard room BSCs, the processing chamber was set to 21% O_2 for handling of the cells at 18% O_2 (room air incubator conditions). Human A549 lung cells were grown under these conditions for an initial set of data at 2 weeks. Studies were continued later at 4 months of adaptation.

	Incubation	Handling
Hypoxia	3%	3%
Physioxia	10%	10%
Hyperoxia (Room Air)	18%	21%

Table 1. Cell Conditions. Human lung A549 cells were maintained in unbroken oxygen-controlled conditions for four months.

Silver nanoparticles (10nm, Sigma-Aldrich cat #730785) (0-10micrograms/ml) were added to A549 that had been allowed to adhere in 96-well plates overnight (2x10e4 cells/well). Edge wells were excluded from assays. The cells were incubated for an additiional 24, 48, or 72 hrs, washed 3x with PBS (Gibco), fixed with 100% methanol for 15 minutes, and stained with 0.5% crystal violet in 20% methanol. After a tapwater wash, the residual dye was dissolved in 20%MeOH/20% acetic acid and absorbance read at 450nm in a BMG platereader.

This assay was chosen specifically as a measure of cell density rather than an MTT-based assay which measures mitotic activity, which varies under different oxygen levels and metabolic functions.

Results

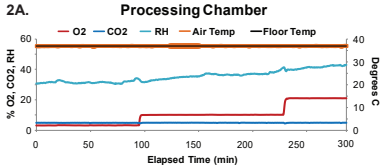
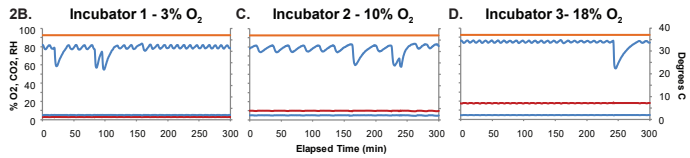


Figure 2. Cell Conditions Were Well-Controlled for Cell Handling as Well as Incubation. Chamber O_2, CO_2, Relative Humidity (RH), and temperature were controlled during cell handling in the processing chamber (A) and incubation (B-D). While O_2, CO_2, and temperatures were constant, RH can be seen to change when the incubator doors were opened and as open operations were conducted in the processing chamber with fluids. Humidity limit controls infused tanked N_2 to keep humidity levels below set levels (85% in the incubators, 50% in the processing chamber).



Doubling Time by Week

O_2	1	2	5	6
10%	37.13	28.24	24.06	23.78
18%	26.39	25.85	24.01	23.46

Table 2. Doubling Times (hrs). During these first two weeks, A549 at 10% O_2 grew more slowly than those at 18% O_2. After 5 weeks, the doubling times were the same.

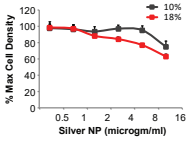
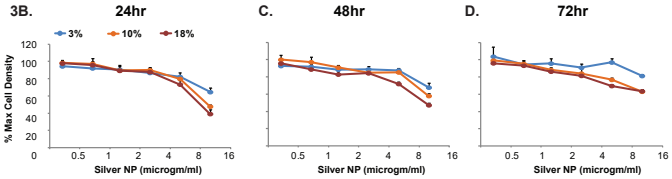


Figure 3. Adaptation to Different O_2 Levels Changed Cellular Response to Silver NP. There was a dose-dependent response of A549 to silver NP. Assays after 2 weeks of adaptation showed that A549 adapted to physioxic conditions were less sensitive to silver NP than those adapted to hyperoxic room air conditions (A). We added a third group, adapting A549 to 3% O_2 as well. After 4 months of adaptation, we found an oxygen-dependent difference between the groups' responses to Ag NP with bigger differences between the cells adapted hypoxia and those adapted to physioxia or standard hyperoxia.



Conclusions

Adaptation to different oxygen conditions takes more than 2 weeks
Adaptation of A549 cells to physiologic oxygen changes NP tox effects
Physiologically relevant cellular conditions should be maintained for physiologically relevant toxicological data