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An *In Vivo* Human Time-Exposure Investigation of a Commercial Nano-Particle Solution

Mark A. Munger, Pharm.D.^{*†}, Przemyslaw Radwanski, Pharm.D., Ph.D.[‡], Greg Hadlock, Ph.D.[‡], Greg Stoddard, M.S.[†], Akram Shaaban, M.D.[¶], David Grainger, Ph.D.[§], and Garold Yost, Ph.D.[‡]

Departments of Pharmacotherapy^{*}, Pharmacology and Toxicology[‡], Pharmaceutics and Pharmaceutical Chemistry[§], Internal Medicine[†], and Radiology[¶], University of Utah, Salt Lake City, Utah.

Corresponding Author

Mark A. Munger, Pharm.D.
University of Utah
30 South, 2000 East, Rm #201
Salt Lake City, Utah 84112-5820
Phone: (801) 581-6165
Fax: (801) 585-7316
mmunger@hsc.utah.edu

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nanotechnology – nanotoxicology
safety research

35 **Abstract**

36 Background: Biodistribution, bioprocessing with possible toxicity of nanoscale silver is receiving
37 increasing attention to human health.

38 Methods: We prospectively studied several time exposures of a commercial 10- and 32-ppm nanoscale
39 silver solution in a double-blind, controlled, cross-over phase design. Healthy subjects (60) underwent
40 complete metabolic, blood and platelet count, urinalysis, sputum induction, and chest and abdomen
41 magnetic resonance imaging. Silver serum and urine content was determined.

42 Results: No clinically important changes in any metabolic, hematologic, or urinalysis measure identified
43 were determined. No morphological (or structural) changes were detected in the lungs, heart (cardiac
44 function) or abdominal organs. No significant changes were noted in pulmonary reactive oxygen species
45 or pro-inflammatory cytokine generation.

46 Conclusion: *In vivo* oral exposure of a commercial nanoscale silver solution does not exhibit clinically
47 important changes in metabolic, hematologic, urine, vital sign changes, physical findings or imaging
48 changes visualized by MRI. Further study of increasing time-exposure, dose, and additional organ
49 systems is warranted.

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No Negative
effect in any
System

66 **Introduction**

67 The age of nanotechnology is driving potentially the most important engineering revolution since
68 the industrial age. There are over 1,300 manufactured nanotechnology enabled consumer products in the
69 marketplace today.¹ This revolution has afforded silver a reemergence as a medical modality.²⁻⁴
70 Nanoscale silver makes up approximately a quarter of the inventory of commercially available
71 nanoproducs. Silver exhibits physiochemical attributes and biological activity broadening its application
72 as an antibacterial, anti-viral, and anti-inflammatory therapy.⁴⁻⁹ With greater systemic applications to the
73 human condition, penetration of silver nanoparticles across cell membranes could result in vascular
74 redistribution presenting the potential for evasion of immune cellular clearance, leading to systemic acute
75 or chronic cytotoxicity or illness.⁴

Assets
What
Silver
does

76 A growing body of in vitro evidence supports that silver nanoparticles, in concentrations
77 primarily between 5-50 µg/ml, may be toxic to mammalian cells. A variety of tissues including the
78 lung,¹⁰⁻¹³ liver,^{11,14-17} brain,¹⁸ vascular system¹⁹ and reproductive systems²⁰⁻²¹ may be negatively
79 influenced. The lung and liver may be the most targeted with prolonged exposure.^{10, 13} Given the
80 trajectory of new nanoscale products, especially silver-based, being introduced into the consumer
81 marketplace it is important to understand whether these in vitro findings translate to human toxicity.²²

82 To this end, we studied 60 healthy volunteers through several time-length exposures in a
83 prospective, randomized, placebo-controlled, single-blind, dose-monitored, cross-over design to
84 quantitate changes in metabolic, hemotologic, and suptom morphology and to qualitate changes in
85 physical findings and organ imaging from a commercially available silver nanoparticle 10 and 32 ppm
86 solution.

87 **Methods**

88 *Study Population*

89 The two studies were conducted at the University of Utah Lung Health Study Clinic and Center
90 for Clinical and Translational Sciences at University Hospital. Each prospective subject underwent a
91 screening evaluation to assess eligibility for enrollment. Sixty healthy volunteers were subsequently
92 enrolled in two separate studies (i.e., 10 ppm [36 subjects] and 32 ppm [24 subjects], respectively)
93 between 18-80 years of age. Subjects were excluded with a history of any heavy metal allergy; asthma,
94 chronic bronchitis or emphysema; or renal impairment defined by a creatinine clearance ≤ 30 ml/minute;
95 or significant acute or chronic disease as determined by the investigators. Females of child-bearing
96 potential, defined as women physically capable of becoming pregnant, including women whose career,
97 lifestyle, or sexual orientation precludes intercourse with a male partner and women whose partners are
98 using 2 barrier birth control methods or hormonal contraceptive method. Any female subject who was
99 nursing was excluded. Subjects who were unable to complete the study were excluded from analysis.

60
People
18-80

100 All patients were provided written informed consent before participating in any study procedures.
101 The study was conducted in accordance with the International Conference on Harmonisation of Technical
102 Requirements for Registration of Pharmaceuticals for Human Use Guidelines for Good Clinical Practice

103 and the Declaration of Helsinki, and received approval from the University of Utah Institutional Review
104 Board. The trials are registered with Clinical-Trials.gov (Identifier: NCT01243320 and NCT01405794).

105 Both studies were overseen by an independent Data Safety and Monitoring Board which
106 reviewed each individual subject's and the study population data in aggregate, granting approval of safety
107 from the lesser time-period before the study was allowed to proceed to the next period.

108 *Study Product*

109 The study product was manufactured by American Biotech Laboratory (Alpine, Utah) as a
110 reduced elemental silver colloidal dispersion in water (USP 7,135,1095). The silver is in the form of
111 zero-valent elemental silver coated with silver oxide (Ag_4O_4). The reduced silver ions have a particle size
112 average between 5-10 nm (10 ppm) or 25-40 nm (32 ppm), respectively. The average daily ingestion of
113 elemental silver used in this study is estimated to be 100 mcg/day for 10 ppm and 480 mcg/day for 32
114 ppm.

Silver
Metallic
 Ag_4O_4

daily dose daily Dose

115 *Study Design for Metabolic, Silver Concentrations, Induced Sputum, and MRI*

116 Each subject initially received the silver nanoparticle solution diluent (e.g. sterile water [no silver
117 nanoparticles]) followed by the active silver nanoparticle solution for a period of 3-, 7- and 14-day
118 periods, respectively. The 10 ppm solution was provided in 3-, 7-, and 14-day time periods, the 32 ppm
119 for a 14-day period only. There was a 72-hour washout period prior to study drug cross-over. Each
120 subject received 15 milliliters of study medication daily from a pre-mixed oral syringe. Each dose was
121 observed daily by study personnel to ensure compliance. Subjects were blinded to the study product
122 received.

123 At baseline and the end of each time-period, subjects underwent a medical and drug history, a
124 complete physical examination, comprehensive metabolic panel, blood count with differential and
125 complete urinalysis. Blood and urine were collected for serum and urine silver concentrations at trough
126 concentrations (≥ 24 hours post-dose) for the 3- and 7-day time periods at 10 ppm and at peak
127 concentration (≤ 2 hours post-dose) for the 14-day 10 ppm dose and for the 32 ppm study population.
128 Silver serum or urine concentrations were determined by Inductively Coupled Mass Spectrometry (NMS
129 Laboratories, Willow Grove, Pennsylvania). Calibration samples are in dilute nitric acid and controls are
130 in human serum. The quantization limit of the samples for this study were 0-40 mcg/L. Silver
131 concentrations were determined at either trough (24 hours post-dose) or at peak (≤ 2 hours post-dose).

Peak @
2 hrs
out in
24 hrs

132 Sputum was collected by induction protocol within 24 hours of the last dose of each time-period,
133 as previously described.²¹ Briefly, subjects inhaled increasing concentrations of nebulized hypertonic
134 saline of 3-10% solution from Devilbiss Pulmo Aide Compressor (Specialty Medical Supply, Milford,
135 Ohio) fitted with Sidstream High Efficiency Nebulizer (Cardinal Health, Dublin, Ohio) without valve or
136 nose clips. Sputum induction time between 15-30 minutes was used for all subjects. The protocol was
137 stopped at 30 minutes, if the FEV₁ dropped by $\geq 20\%$, or whenever a quality sample was obtained.

138 *Sputum Analysis:*

139 *Determination of hydrogen peroxide production.*

140 Hydrogen peroxide concentrations were determined using a modification of the method described
141 by Cameron et al. (1999). Briefly, after cells were removed from the sputum samples (described
142 previously), 5 units of horseradish peroxidase (HRP) were added to the samples, and then the total sample
143 volumes were brought to a final volume of 1.0 mL with 1mM 2,2-azino-bis(3-ethylbenzthiazoline-6-
144 sulfonic acid) (ABTS). After incubating for 20 min at room temperature, samples were filtered using a
145 Kimwipe to remove mucus and other debris and ABTS radical formation was quantified
146 spectrophotometrically at 650 nm.

147 *Measurement of peroxiredoxin (Prx) protein expression.*

148 Cells collected from the induced sputum samples were pelleted, resuspended in TRIzol
149 (Invitrogen, Carlsbad, CA) and GlycoBlue (Invitrogen), and frozen at -80 °C until processing. After all
150 patient samples were collected, RNA and protein was isolated using TRIzol according to the
151 manufacturer's instructions. The isolated protein was resuspended in 1% sodium dodecyl sulfate and 4 M
152 urea and heated for 1 h at 50 °C. The isolated RNA was retained for quantitative PCR (described below).
153 Protein concentrations were determined using the Bicinchoninic Acid assay kit (Pierce, Rockford, IL).
154 Equal quantities of total protein (~50µg) were electrophoresed through a 12% NuPAGE gel (Invitrogen)
155 and subsequently transferred to a polyvinylidene difluoride membrane. The membranes were blocked
156 using 5% (w/v) dry milk for 24 h at 4°C. The membrane was then incubated with a polyclonal rabbit anti-
157 PrxSO₃ antibody (ab16830; Abcam, Cambridge, MA), which recognizes both the sulfinic and sulfonic
158 forms of Prx, diluted in 2.5% (w/v) dry milk (1: 2000) overnight at 4°C. The membranes were then
159 washed 2 times in phosphate buffered saline with Tween (PBST) and incubated with a goat anti-rabbit
160 HRP conjugated secondary antibody (ab6721; Abcam) diluted in 2.5% (w/v) dry milk (1: 2000) for 1 h at
161 room temperature. After washing 3 times in PBST, the bands were visualized using the Pierce ECL plus
162 chemiluminescent Western blotting substrate (ThermoScientific, Waltham, MA) on a Kodak image
163 station 440 (Kodak, Rochester, NY). Relative band intensities were quantified using the Kodak 1D image
164 analysis software (Kodak).

165 *Determination of RNA expression using quantitative real-time PCR (qPCR).*

166 Total RNA was extracted as described above. Total RNA (0.5 µg) was converted to cDNA using Superscript
167 III (Invitrogen). The resultant cDNA was diluted 1: 50 in RNase-free ddH₂O for analysis by qPCR using the
168 LightCycler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN) on a LightCycler 480 system (Roche)
169 using the LightCycler 480 software (Roche). The PCR program consisted of a 10 min incubation at 95 °C,
170 followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Experiments were performed in
171 triplicate and standardized to the β2 macroglobulin (β2M) gene. Primer sequences were (5' → 3'): h β2M
172 sense-GATGAGTATGCCTGCCGTGTG and antisense-CAATCCAAATGCGGCATCT; hIL-1α sense-
173 CGCCAATGACTCAGAGGAAGA and antisense-AGGGCGTCATTCAGGATGAA; IL-1β sense-
174 CTGTCCTGCGTGTTGAAAGA and antisense-TTGGGTAATTTTTGGATCTACA; hIL-8 sense-
175 ACTGAGAGTGATTGAGAGTGGAC and antisense-AACCCTCTGCACCCACTTTTC; hMCP1 sense-
176 TTCTGTGCCTGCTGCTCAT and antisense-GGGGCATTGATTGCATCT; hNQ01 sense primer:
177 ATGTATGACAAAGGACCCTTCC hNQ01 anti-sense primer: TCCCTTGAGAGAGTACATGG
178 hCRP sense-CTTGACCAGCCTCTCTCATGC and antisense-CGTGTAGAAGTGGAGGCACA; hiNOS
179 sense-GTCACCTATCGCACCCGAGATG and antisense-GCCACTGACACTCCGCACAAAG; and
180 hIL-6 sense-AACCTGAACCTTCCAAAGATGG and antisense-TCTGGCTTGTTCCTCACTACT.

181 *MRI Protocols*

182 A cardiac and abdominal MRI were obtained at the end of each phase of each time-period.
183 Patients were examined on a 1.5-T, 32-channel superconducting MR system (Magnetom Avanto, Siemens
184 Medical Solutions) with high-performance gradients (maximum gradient strength, 45 mTm⁻¹; maximum
185 slew rate, 200 mTm⁻¹s⁻¹).

186 Cardiac MRI studies were carried out using breath-hold acquisitions prospectively triggered by
187 the ECG. The imaging protocol included multiplanar fast imaging with steady-state precession (SSFP)
188 and delayed enhancement imaging.

189 Cine SSFP (TrueFISP) images were acquired in multiple standard short-axis and long-axis views,
190 including specific RVOT and LVOT orientations, using; slice thickness 8 mm, echo time 1.2 ms, pixel
191 bandwidth 1.150 Hz, repetition time 3.0 ms, temporal resolution about 43 ms, matrix 256 × 202). The
192 field of view was 340 mm on average and adapted to the size of the patient, leading to a spatial resolution
193 of about 2.4 × 1.4 × 8 mm. Abdominal MRI protocol included a transverse T1-weighted fast gradient-
194 recalled dual-echo sequence (TR/in-phase TE/out-of-phase TE, 129/4.36/2.0; flip angle, 70°; matrix, 134
195 × 256; section thickness and intersection gap, 6 and 0.6 mm; signal average, 1; field of view, field of view,
196 220–340 mm [depending on body habitus])) and a transverse T2-weighted HASTE (TR/TE, 1,000/89;
197 refocusing angle, 180°; slices, 20; slice thickness, 6 mm with a 10% gap; matrix, 168–192 × 256; field of
198 view, 220–340 mm [depending on body habitus]).

199 A dynamic contrast-enhanced 3D gradient-echo volumetric interpolated breath-hold examination
200 (VIBE) sequence was performed in the arterial, venous, and delayed, after the injection of 0.1 mmol/kg of
201 body weight of Gadopentetate dimeglumine (Magnevist; Bayer HealthCare Pharmaceuticals, Berlin,
202 Germany) at rate of 2 mL/s using a pressure injector. This was followed by delayed contrast-enhanced
203 cardiac study using a segmented inversion–recovery sequence in the same views used for cine cardiac
204 MRI 10–20 min after contrast administration.

205 All images were deidentified and transferred to a 3D postprocessing workstation (Leonardo,
206 Siemens Healthcare). LV function and volumes were calculated by planimetry of the endocardial and
207 epicardial borders from the serial short-axis views (usually 8–14) with no gap between the slices. Ejection
208 fraction, end diastolic volume and end systolic volume were analyzed.

209 *Statistical Analysis*

210 The power of the study to detect toxicity was determined by the hypothesis that there would be no
211 toxicity observed at any time-period. There were a total of 36 subjects in the 3-, 7-, and 14-day periods at
212 10 ppm and 24 subjects at the 14-day period for 32 ppm in a crossover design. This enrollment provides
213 an 80% probability, of indentifying a toxicity problem, if toxicity is as common as 6.5%, based on a
214 binomial probability and using 1-Prob (observed incidence=0|true incidence=0.065). Combining the 10
215 ppm and 32 ppm solutions, for a total sample size of n=60, based on a binomial probability, there was an
216 80% probability of observing toxicity in at least one study subject if the true toxicity incidence is 2.7%.
217 For the analysis examining paired sample mean differences between placebo and active solution, the n=24
218 subjects with maximum exposure, 32 ppm for 14 days, provided 80% power to detect a mean difference
219 of 0.48 standard deviations (SD), using a two-sided alpha 0.05 comparison and assuming a correlation of
220 r=0.70 between the placebo and active phases. For the total sample of n=60, there was 80% power to

221 detect a 0.30 SD difference. Assuming the placebo phase is centered in the normal laboratory reference
222 range, with 2 standard deviations in either direction for the mean of the active solution phase to go outside
223 of the normal reference range, or a 2 SD difference, the effect sizes of 0.48SD and 0.30SD represented
224 detectable effects well within any given normal laboratory reference range.

225 The analysis used two approaches for the data analysis: 1). A mixed effects linear regression with
226 measurements nested within each subject used to examine an average effect, and 2). Individual
227 observations examined descriptively with standard reference ranges (± 2 standard deviations[SD]) and
228 extended reference ranges (± 4 SD) to assess toxicity in individual subjects. To account for skewness and
229 outliers, analogous intervals with equivalent inclusion probabilities were formed from the median and
230 appropriate multiples of the interquartile range. The primary analysis of clinical toxicity was determined
231 if in any subject, from any of the three exposure time-periods, and for each of the outcomes measured, by
232 any value that changes by 2 times the upper limit of normal (ULN), as defined by the normal range from
233 Associated Regional University Pathologists Laboratory, (ARUP) Salt Lake City, Utah of the mean
234 baseline value of the subjects. The clinically significant toxicity bound was set at twice the ARUP ULN
235 or mean+4SDs. If a subject crossed this bound during either phase of the time-period, it was concluded
236 that the subject experienced a toxicity event. Any MRI event will be qualitatively described.

237 Results

238 A total of 62 subjects were enrolled in the study. Sixty subjects completed the study. Two were
239 discontinued due to inability to draw blood (subject never received study product) and hospitalization for
240 pulmonary embolism (subject only received 12 days of placebo-diluent), respectively. The population is
241 described in Table 1.

242 *Changes in Body Mass Index (BMI), Blood Pressure and Heart Rate*

243 Changes in subject hemodynamics are listed in Table 2. No clinically important changes in
244 weight, BMI, systolic or diastolic blood pressure or heart rate were noted. However, heart rate
245 significantly declined by 2.3 beats per minute for the total group, but not for the individual dosing groups.

246 *Comprehensive Metabolic Panel, Complete Blood Count with Differential and Urinalysis Findings*

247 Results of the complete metabolic panel are listed in Table 3. There were no statistically
248 significant or clinically important changes in any laboratory finding through in the total population
249 studies, independent of time-period exposure. Blood urea nitrogen and alanine aminotransferase tests
250 from the 10 ppm dose were statistically significant, but nothing in the 32 ppm dose were noted. However,
251 when the 95% CI limits of these significant tests are added to the mean value of the active period,
252 representing a statistical comparison to the normal reference range limits, all values remain within these
253 limits, without statistical important inference. To further understand whether toxicity may be related to
254 exposure time, we conducted a mixed effects linear regression analysis while controlling for the baseline
255 value for all 36 subjects in the 10 ppm. There was no statistical significance in any comprehensive
256 metabolic panel test, suggesting increasing exposure time does not increase toxicity response at the 10 or
257 32 ppm dose. Chronological age or gender was not associated with any change in any clinical metabolic
258 test.

No changes
over
time

Age as gender did
NOT matter

259 The results of the complete blood count with differential are displayed in Figure #1.
260 Comparison of the red blood cell count (RBC) between active vs. placebo solutions was statistically
261 significant in the 10 ppm dose, without significance in the 32 ppm or the total sample analysis. When the
262 linear regression model was applied to the comparison, there was no significance. There were no
263 clinically important changes in any blood count value including erythrocytes, granulocytes, or
264 agranulocyte counts. Exposure time did not show significance with any blood count value, nor was age
265 or gender a determinate of change.

266 There were no statistically significant or clinically important changes in the complete urinalysis,
267 including continuous variables of urine specific gravity, pH, or urine urobilogen. Although there were
268 individual subject positive tests for urine ions, proteins, blood cells, and some other molecules, these
269 changes remained unchanged in comparison between the active and placebo solutions at 10 ppm, 32 ppm
270 or in the total sample.

271 *Serum Silver and Urine Concentration Findings*

272 Serum silver and urine silver concentrations were determined at different time variables related to
273 the time of dose. There was no detection of serum silver from any subject at trough concentrations
274 throughout the 3- and 7-day time periods of 10 ppm. Peak serum silver concentration was detected in
275 42% of subjects in the 14-day 10 ppm showing a mean of 1.6 ± 0.4 mcg/L. The 32 ppm dose mean
276 concentration was detected in 92% of subjects at 6.8 ± 4.5 mcg/L. Four 32 ppm subjects showed
277 concentrations \geq than the lower limit of quantitation for toxic concentrations as determined by NMR
278 Laboratory (range: 11-40 mcg/L). No detection of silver was determined in the urine, independent of
279 dose or time period.

280 *Sputum Reactive Oxygen Species and Pro-Inflammatory Cytokine RNA Findings*

281 Quality paired samples allowing determination of reactive oxygen species concentrations and
282 pro-inflammatory cytokine RNA expression from induced sputum samples were analyzed in 72% and
283 83% of 10 ppm and 32 ppm study population, respectively (Table 4). There was no statistically
284 significant change in hydrogen peroxide production or Prx expression based on dose, study population or
285 time period. Analysis of IL-8, IL-1 α , IL-1 β , MCP1 and NQO1 also showed no statistical difference
286 between the active silver and placebo solutions at either dose, total study population, or based on time
287 frame.

288 *MRI Findings*

289 Eighteen 10 ppm and eleven 32 ppm subjects underwent a post 3-14 day, respective active and
290 placebo solution cardiac and abdominal MRIs. No morphological or structural changes were noted with
291 active compared to placebo in any subject. There was no detection of silver coalescence or aggregation
292 on any MRI image.

No build up in any systems

293 **Discussion**

294 Nanoscale silver has the widest degree in integration of commercialization including consumer
295 products, medical devices, and pharmaceuticals.¹ Therefore, the study of this nanoscale source is critically

296 important to our understanding of potential human toxicity of this field. Sources of nanoscale silver
297 include, but are not limited to, the textile, food, cosmetic, biosensor and electronic industries.
298 Furthermore, the potential for use of silver nanoparticles in the disease detection and treatment may lead
299 to increased exposure to the human state. To determine the human risk of a commercial nanoscale silver
300 product we conducted a prospective, controlled, parallel design systematic *in vivo* study of two doses of a
301 commercial silver nano-particle solution over a 3-14 day monitored exposure. Our findings show that this
302 product is distributed into human serum but does not demonstrate clinically significant changes in
303 metabolic, hematologic, urine, vital sign changes, physical findings, sputum morphology or imaging
304 changes as visualized by MRI. To our knowledge, this is the first systematic *in vivo* study of any
305 systemic nanoscale product.

Silver
used
in

No negative effect in any system

↑ First *in vivo* human study

306 Many authors have detailed the potential for human toxicity from nano-particles. Suggested and
307 defined target organ systems which may result from nano-particle adverse health effects include the
308 pulmonary, cardiovascular, neurologic, reticuloendothelial, renal, or reproductive systems.²³⁻²⁶ To this
309 end, the literature has called for a new toxicological science to establish procedures to test the use of
310 nanoparticles in the marketplace, as well as the critical need for *in vivo* studies.²⁷⁻²⁸ Our study starts to
311 answer existent human toxicity in a systematic way.

312 Abdominal organ system toxicity has been shown to occur from exposure to silver nanoparticles.
313 The liver, in particular, has been noted as a toxicity target, possibly due to oxidative stress.¹³ Non-
314 cytotoxic doses of silver nanoparticles reduced mitochondrial function, cell proliferation, and induced
315 apoptosis in rat and human liver, and human mesenchymal cell lines, respectively.^{13-14, 29} We have shown
316 in MDR1.C and Hep G2 cell lines that after 24 hour exposure of a commercial 32 ppm silver nanoparticle
317 solution, where cell viability was maintained, that nanoscale silver maybe a potential source of drug-drug
318 interactions.³⁰ Potential interactions may occur through reductions in NADPH cytochrome c reductase
319 activity (citation?). In contrast, a prospective, controlled 90 day exposure of 56 nm silver nanoparticles at
320 30 mg/kg *in vivo* rat study did not show any clinical chemistry, hematological, body weight, food
321 consumption, or water intake changes.³¹ Our human clinical findings correlate closely with these results.
322 Hence, potential *in vitro* hepatocellular toxicity could occur from non-cytotoxic doses of nanoscale silver
323 but these results do not correlate with *in vivo* rat or our human findings. Reasons for this disconnect may
324 be *in vitro* direct cell exposure time or incomplete understanding of *in vivo* bioavailability, distribution, or
325 liver blood flow dynamics of the nanoscale silver. MRI results from this study were not able to
326 differential any abdominal changes from exposure to nanoscale silver.

327 The lung is another major target of silver nanoparticle exposure, particularly through inhalation.
328 Silver nanoparticles (15-18 nm) may bind to lung epithelial cells and alveolar macrophages, producing
329 reactive oxygen species, potentially limiting function of cells.^{9-10, 12, 32} Histopathological examination from
330 inhaled 18 nm silver nanoparticles for 90 days in Sprague-Dawley rats show dose-dependent alveolar
331 infiltration, thickened alveolar walls and small granulomatous lesions.¹² These histology changes were
332 associated with reductions in tidal and minute volumes. Congruence of results from multiple *in vitro* and
333 a single *in vivo* animal model support cellular and functional toxicity from inhaled nanoscale silver.
334 However, oral dosed nanoscale silver from our study failed to induce changes in reactive oxygen species
335 or pro-inflammatory cytokine RNA from induced sputum samples. This probably is due to a lack of
336 translocation of these particles to the respiratory system from the gastrointestinal route.

337 Further studies are necessary to understand the potential of nanoscale silver toxicity on the
338 human reproductive system, systemic toxicity from subcutaneous delivery systems or from leaching of
339 imbedded silver in catheter-based medical equipment, and to the central nervous system from multiple
340 delivery systems. We did not specifically study these other physiologic systems or other delivery
341 systems. Our study time frame of 14 days, although a moderate time, needs to be extended in order to
342 determine whether longer exposure leads to accumulation of nanoscale silver in human lipid
343 compartments, which may result in chronic toxicity.

344 In summary, nanoscale silver is an important biomaterial in the current engineering nano-
345 revolution. We have demonstrated that monitored dosing over a 14-day period of a commercially
346 available oral nano-silver product does not realize in clinically important toxicity across a wide variety of
347 physiological parameters. Further study of longer durations is clearly warranted.

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435 **Tables**

436 Table 1: Study Population Demographics

Demographic/Clinical Variable	10 ppm (n=36)	32 ppm (n=24)	Total Sample (n=60)
Age, years	52±11	41±15	47±14
Range	26-76	20-67	20-76
Gender, n M/F (%)	17/19 (47/53)	18/6 (75/25)	35/25 (58/42)
BMI (kg/m ²)	29±6	29±6	29±6
mean±SD (max-min)	20-45	21-43	20-45
SBP (mmHg)	127±19	127±13	127±17
mean±SD (max-min)	176-84	150-102	176-84
DBP (mmHg)	83±11	81±11	82±11
mean±SD (max-min)	106-55	107-62	107-55
Heart Rate (bpm)	69±9	65±7	68±8
mean±SD (max-min)	84-42	79-52	84-42

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438 Table 2: Study Population Changes in Hemodynamics

Hemodynamic Variable	10 ppm Mean Change [95% CI: min, max] (<i>p</i> value)	32 ppm Mean Change [95% CI: min, max] (<i>p</i> value)	Total Sample Mean Change [95% CI: min, max] (<i>p</i> value)
Weight (kg)	-1.1[-2.6, 0.4] (0.17)	-0.2[-0.1, 0.8] (0.14)	-0.5[-1.4, 0.4] (0.30)
BMI (kg/m ²)	-0.4[-0.9, 0.1] (0.15)	-0.1[-0.04, 0.3] (0.14)	-0.2 [-0.5, 0.1] (0.26)
SBP (mmHg)	1.3[-1.7, 4.3] (0.40)	-1.3[-3.0, 5.6] (0.54)	1.3[-1.2, 3.8] (0.30)
DBP (mmHg)	-2.4[-5.5, 0.7] (0.13)	-0.7[-2.5, 3.8] (0.67)	-1.2 [-3.4, 1.1] (0.31)
HR (bpm)	-1.9[-5.0, 1.3] (0.25)	-1.2[-6.0, 3.5] (0.61)	-2.3 [-4.6, -0.93] (0.05)

439 BMI: Body Mass Index; SBP: Systolic blood pressure; DBP: Diastolic Blood Pressure; HR: Heart Rate

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447 Table 3: Study Population Comprehensive Metabolic Panel (10 ppm [n=36], 32 ppm [n=24], Total
 448 Sample [n=60])

Comprehensive Metabolic Panel	10 ppm Mean Change [95% CI: min, max] (p value)	32 ppm Mean Change [95% CI: min, max] (p value)	Total Sample Mean Change [95% CI: min, max] (p value)
Sodium [mmol/L]	-0.1[-0.7, 0.6] (0.87)	0.2 [-0.7, 1.0] (0.71)	0.03 [-0.5, 0.6] (0.90)
Potassium [mmol/L]	-0.1[-0.3, 0.02] (0.10)	-0.03 [-0.2, 0.1] (0.74)	-0.08 [-0.2, 0.03] (0.13)
Chloride [mmol/L]	-0.4[-1.2, 0.3] (0.23)	0.04[-1.1, 1.2] (0.94)	-0.3[-0.9, 0.4] (0.44)
Carbon Dioxide [mmol/L]	0.5[-0.5, 1.6] (0.33)	-0.04[-1.1, 1.0] (0.94)	0.3[-0.5, 1.1] (0.44)
BUN* [mg/dL]	-0.9[-1.7, -0.1] (0.03)**	0.5 [-1.1, 1.8] (0.41)	-0.3 [-1.1, 0.4] (0.37)
Creatinine [mg/dL]	0.01 [-0.02, 0.03] (0.60)	-0.02 [-0.04, 0.01] (0.21)	-0.003 [-0.02, 0.01] (0.74)
Glucose [mg/dL]	3.6 [-1.6, 8.7] (0.17)	-0.7 [-4.2, 2.9] (0.71)	1.9 [-1.5, 5.3] (0.28)
ALP [U/L]	-1.4 [-3.9, 1.1] (0.28)	2.0 [-1.0, 5.0] (0.18)	-0.03 [-2.0, 1.9] (0.97)
AST [U/L]	-0.44 [-2.1, 1.2] (0.60)	2.0 [-2.6, 6.5] (0.40)	0.5 [-2.0, 2.5] (0.61)
ALT [U/L]	-2.6 [-4.8, -0.3] (0.03)**	2.3 (-1.6, 6.3) (0.25)	-0.6 (-2.7, 1.5) (0.58)
Total Protein [g/dL]	-0.02 [-0.3, 0.2] (0.87)	0.2 [-0.001, 0.4] (0.051)	0.1 [-0.1, 0.3] (0.45)
Total Bilirubin [mg/dL]	-0.02 [-0.08, 0.3] (0.43)	-0.03 [-0.11, 0.05] (0.49)	-0.02 [-0.07, 0.02] (0.29)
Albumin [g/dL]	-0.07 [-0.1, 0.0004] (0.06)	0.11 [-0.01, 0.23] (0.09)	0.002 [-0.07, 0.07] (0.96)
Calcium [mg/dL]	-0.1 [-0.2, 0.04] (0.20)	0.1 [-0.003, 0.2] (0.06)	0.0 [-0.1, 0.1] (0.99)

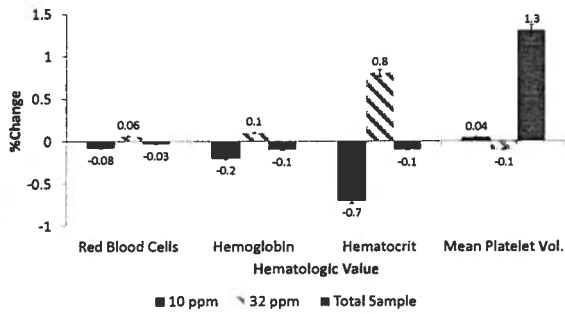
449 *BUN- Blood Urea Nitrogen; ALP: Alkaline Phosphatase; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase
 450 **p≤0.05 Comparison of 10 ppm or 32 ppm active solution vs. placebo solution, controlling for baseline value, in a mixed effects
 451 linear regression model.
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453 Table 4: Reactive Oxygen Species and Pro-inflammatory Cytokine Analyses

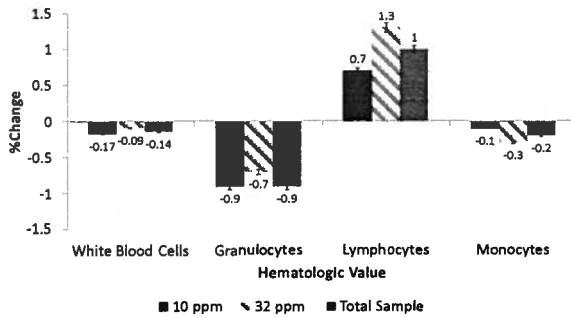
ROS or Cytokine Parameter	10 ppm Mean Change [95% CI: min, max] (p value)	32 ppm Mean Change [95% CI: min, max] (p value)	Total Sample Mean Change [95% CI: min, max] (p value)
ROS μM	0.89 [-0.6, 2.38] (0.24)	-0.44 [-1.23, 0.35] (0.28)	0.52 [-0.56, 1.60] (0.34)
IL-8 (copies/1000 B2M)*	2.19 [-1.53, 5.91] (0.25)	6.39 [-5.83, 18.60] (0.31)	4.52 [-2.47, 11.51] (0.21)
IL-1α (copies/1000 B2M)	-0.0005 [-0.0007, 0.0006] (0.88)	0.0197 [-0.0014, 0.0408] (0.07)	0.0128 [-0.0014, 0.0269] (0.08)
IL-1β (copies/1000 B2M)	0.017 [-0.011, 0.044] (0.24)	0.027 [-0.058, 0.112] (0.53)	0.022 [-0.027, 0.072] (0.38)
MCP1 (copies/1000 B2M)	-0.028 [-0.084, 0.028] (0.34)	-0.004 [-0.026, 0.017] (0.69)	-0.015 [-0.046, 0.015] (0.33)
NQO1 (copies/1000 B2M)	-0.0043 [-0.0115, 0.029] (0.24)	-0.0279 [-0.4671, 0.4114] (0.90)	-0.0182 [-0.2850, 0.2487] (0.89)

454 ROS: Reactive Oxygen Species; B2M: Beta-2 microglobulin; MCP1: Monocyte chemoattractant protein -1; NQO1:
 455 NADH quinine oxio-reductase-1
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459 **Figures**
 460 **Figure #1: Complete Blood Count with Differential Panel**
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 462 **Panel A: Change in Erythrocyte Counts**
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 466 **Panel B: Change in Granulocyte and Agranulocyte Counts**