Treatment of Influenza-Induced Lung Injury with Iron Oxide Nanoparticles using an Ischemic-Reperfusion Model

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Introduction

Influenza is a highly contagious viral infection[1]. One of the dangers of this illness is inflammation to the lungs, leading to a high risk of other complications such as pneumonia[2]. To prevent the development of such complications, a better treatment for lung inflammation must be found. One potentially effective solution may be to utilize iron oxide nanoparticles (IONs).

Nanoparticles have become increasingly used in the medical field. Because of their small size, nanoparticles can easily get into cells and deliver medication that is bound to the nanoparticles[3]. For example, IONs have been used in recent studies for targeted delivery of drugs. IONs also have the unique property of being magnetic[4]. Furthermore, IONs have previously been used in a study that investigates their potential in influenza diagnosis[5].

There have been mixed reports about the effect of IONs *in vitro*. Some studies have stated that IONs help to stabilize inflammation in certain cell lines[6], while other studies have used iron oxide to actually induce inflammation[7]. One study[8] in particular investigated the interaction between IONs and human monocyte-macrophages (HMMs). HMMs are involved with the signaling for inflammation within a cell. The study found that the IONs were retained within HMM lysosomes, which prevented them from inducing any inflammatory cytokines. Still other studies reported that all metal oxide nanoparticles are toxic to a variety of cell lines. That study[9] suggested that IONs may be even more toxic precisely because of their magnetic quality.

In this study, it was hypothesized that IONs stabilize inflammation. When the flu virus enters into a lung cell, an inflammatory process is initiated. One marker that is secreted is the protein tumor necrosis factor alpha (TNF-α) [10]. Nitric oxide acts as a mediator for the amount of inflammation; it gets used up as inflammation increases. Therefore cells studied *in vitro* stimulated with TNF-α would produce lower levels of nitric oxide when compared to stimulated cells saturated with iron oxide nanoparticles[11, 12]. It was also hypothesized that IONs would be non-toxic to the proliferation of cells. Cells with a higher concentration of IONs would have increased cell viability than cells with a lower concentration of IONs.

This hypothesis can also be studied *in vivo.* Students in a surgery training program were being taught a super mesenteric artery (SMA) ischemia reperfusion procedure[13, 14]. This procedure was part of an approved protocol and not associated with this study. Lipid peroxidation, overproduction of nitric oxide, and other complex mechanisms caused by SMA ischemia reperfusion results in lung injury and produce TNF-α in the lung[15, 16]. This mimics the mechanism that causes TNF-α to be secreted after the influenza virus enters a lung cell. Agents associated with influenza have been previously studied from models of ischemia reperfusion and lung injury in order to test vaccines and other potential treatments for influenza[17]. In lung samples, the anatomical distance between each alveoli sac can be measured[18]; a large distance between each alveoli sac indicates the collapse of the sacs, leading to capillary obstruction, and this is a mark of inflammation[19, 20]. Additionally, the number of edema-filled sacs within a lung is a further indicator of inflammation[14]. Levels of inflammatory cytokines can be measured from lung tissue for further analysis of inflammation. Specifically, malondialdehyde (MDA) is an indicator of inflammation in lung tissue. Therefore, *in vivo* samples obtained after ION inhalation should indicate a reduced amount of lung injury, which can be determined by measuring the distance between alveoli sacs, the number of edema filled sacs, and the amount of MDA.

Methods

***Cell Culture*:** Bronchial epithelial cells (SHP-77) (American Type Culture Collection, Manassas, USA) were cultured in Roswell Park Memorial Institute medium (RPMI) with 10% fetal bovine serum and 1% penicillin streptomycin. There were 18 cultures grown, 2 for each of the 9 concentrations of iron oxide nanoparticles.

***Tumor Necrosis Factor-Alpha*:** Recombinant TNF-α was obtained from Sigma Aldrich (St. Louis, USA). TNF-α was diluted to 10ng/ml in culture media and applied to cells in culture. Cells were kept at 10ng/ml TNF-α for 24 hours. All concentrations of iron oxide nanoparticles were applied to cells in 10ng/ml TNF-α.

***Iron Oxide Nanoparticles*:** 10 nanometer aqueous iron oxide nanoparticles were obtained from Sigma Aldrich (St. Louis, USA). The iron oxide nanoparticles were applied to cells grown in TNF-α and regular media. 9 concentrations were used: 0μg/ml, 5μg/ml, 10μg/ml, 20μg/ml, 50μg/ml, 100μg/ml, 200μg/ml, 300μg/ml, and 400μg/ml. After cells were grown and stimulated for the appropriate amount of time, each concentration was applied to the cells. The concentration remained in culture for 2 hours before nitric oxide levels were measured.

***Nitric Oxide Assay*:** Nitric oxide levels were measured using a colormetric nitric oxide enzyme-linked immunosorbent assay (ELISA) obtained from ABCam (Cambridge, USA).

***Vi-Cell Counter*:** The effect of iron oxide nanoparticles on cell viability and level of toxicity was measured using a Beckman Coulter Vi-Cell. The Vi-Cell utilizes trypan blue exclusion to approximate the number of viable cells and the number of total cells. Trypan blue is a stain that colors dead cells blue. Live cells are selective as to what is permitted to bypass the cell membrane and they do not absorb trypan blue.

***Animal Model*:** Subjects were twelve adult male albino Sprague-Dawley rats weighing between 350-500g that were used in a surgery training program and not associated with this study. The rats were randomized into four groups. The SHAM treatment indicates the group without ischemia reperfusion or iron oxide nanoparticle inhalation. The SHAM+ION treatment indicates the group with only iron oxide nanoparticle inhalation. The IR treatment indicates the group with only ischemia reperfusion. The IR+ION treatment indicates the group with ischemia reperfusion and the nanoparticle treatment. The rats were anesthetized via inhalation of 5% isoflurane for 4-7 minutes and were kept under anesthesia through the duration of the surgery via inhalation of 1.5-2% isoflurane. The students participating in the training program were instructed to perform an ischemia reperfusion procedure. The rat intestinal ischemia-reperfusion protocol used for training the students has been described previously[13]. An abdominal laparotomy was performed to expose the superior mesenteric artery (SMA). A microvascular clamp was placed on the SMA for two hours and then removed. The iron oxide nanoparticle treatment was started and continued for 15 minutes during the one-hour reperfusion. The animal was then euthanized by the instructor as part of a larger protocol approved by the animal committee and not associated with this study. Lung samples were taken and placed in 10% formalin or 4% glutaraldehyde/2%formaldehyde in 0.2M cacodylate buffer by the instructor for further histologic analysis.

***Aerosolized Iron Oxide Nanoparticles*:** The 10nm aqueous solution was diluted to a concentration of 100μg/ml using saline solution. The solution was placed in a compressor nebulizer chamber and connected to the isoflurane administrator. The treatment was given to the animal at the described time point.

***Histology*:** After fixation in 10% formalin, lung specimen were embedded in paraffin. Two microthin sections were taken and stained with hematoxylin and eosin and placed on slides for microscopy analysis. Ten randomly chosen high-powered fields (400X magnification) were examined and edema-filled alveoli in each field were quantified and recorded14. The distance between alveoli sacs was measured and determined for each specimen using a Nikon TS100 inverted microscope at 400X and NIS elements by Nikon.

***Transmission Electron Microscope (TEM)*:** A TEM was used to confirm that iron oxide nanoparticles were in the lung tissue. Lung tissue was taken from the lung and stored in a 4% glutaraldehyde / 2% formaldehyde in 0.2M sodium cacodylate buffer. Later, samples were diced, post-fixed with 2% osmium tetroxide, dehydrated in a graded series of acetone, embedded and cured in epoxy resin. 100nm sections were collected onto 200 mesh copper grids and post-stained with 2% uranyl acetate for 30 minutes and 0.5% lead citrate for 15 minutes. Samples were imaged with a JEOL JEM-2100.

***MDA ELISA*:** MDA levels in the lung were measured using an enzyme-linked immunosorbent assay (ELISA) obtained from MyBioSource (San Diego, USA).

In Vitro Results

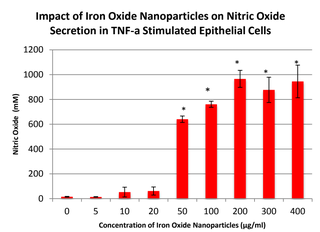

Figure 1: Nitric oxide levels in epithelial cell cultures with TNF-α. The data is represented by ± SD (n=3). Asterisk marks are statistical significance between the indicated concentration and the control p< 0.005*

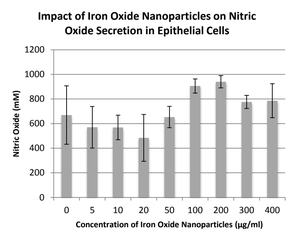

Figure 2: Nitric oxide levels in epithelial cell cultures. The data is represented by ⊥ SD (n=3). Asterisk marks are statistical significance between the indicated concentration and the control p< 0.05*

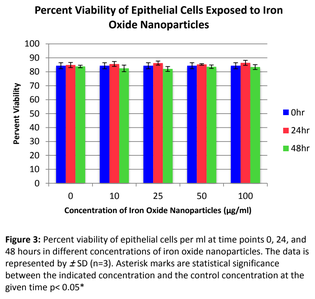

Figure 3: Percent viability of epithelial cells per ml at time points 0, 24, and 48 hours in different concentrations of iron oxide nanoparticles. The data is represented by ⊥SD (n=3). Asterisk marks are statistical significance between the indicated concentration and the control concentration at the given time p< 0.05*

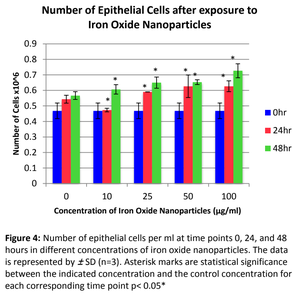

Figure 4: Number of epithelial cells per ml at time points 0, 24, and 48 hours in different concentrations of iron oxide nanoparticles. The data is represented by ⊥SD (n=3). Asterisk marks are statistical significance between the indicated concentration and the control concentration for each corresponding time point p< 0.05*

In Vivo Results

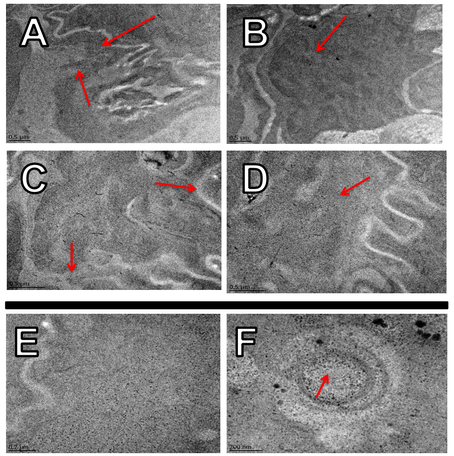

Figure 5: TEM Analysis of ION in Lung Tissue. Images A and B are from the SHAM lung. Images C, D, E, and F are from the SHAM+ION lung. Images A, B, C, and D are at the same magnification and shown for comparison. Images E and F are at a higher magnification to show the nanoparticles more closely. The red arrows point to areas with significant nanoparticle buildup.

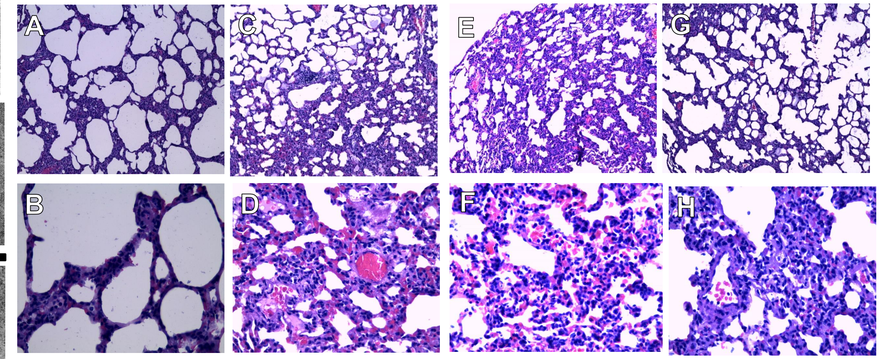

Figure 6: Light Microscopy Analysis of ION Treatment. Images A, C, E, and G are 100x magnification. Images B, D, F, and H are 400X magnification. Images A and B are from the SHAM group, demonstrating a normal lung. Images C and D are from the SHAM+ION group, demonstrating a lung after inhalation of iron oxide nanoparticles. Images E and F are from the IR group, demonstrating ischemia reperfusion induced lung injury. Images G and H are from the IR+ION group, demonstrating the effect of iron oxide nanoparticles on the ischemia reperfusion induced lung injury.

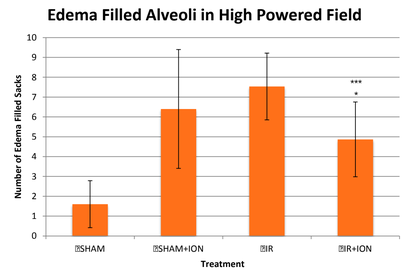

Figure 7: edema Filled Alveoli. The number of edema-filled sacs in each high-powered field was counted. The data is represented by ±SD (n=15). Asterisk marks are statistical significance between the IIR+ION treatment and all other treatments indicated above the bars); SHAM treatment indicated by p< $1x10^{-5}$*, SHAM+ION treatment indicated by p<0.05**, and IIR treatment indicated by p<0.0005***

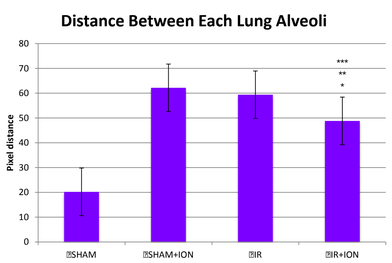

Figure 8: Lung Alveoli Measurements. NIS Elements by Nikon was used to measure the pixel distance between each alveoli sac. The data is represented by ±SE (n=80). Asterisk marks are statistical significance between the IIR+ION treatment and all other treatments indicated above the bars); SHAM treatment indicated by p< $3x10^{-13}$*, SHAM+ION treatment indicated by p<0.01**, and IIR treatment indicated by p<0.01***

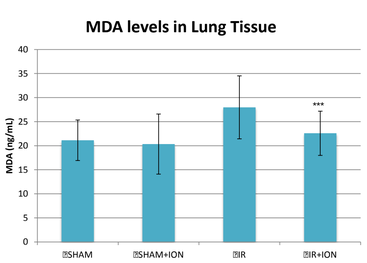

Figure 9: MDA ELISA in Lung. The data is represented by ±SD (n=9). Asterisk marks are statistical significance between the IR+ION treatment and all other treatments indicated above the bars); SHAM treatment indicated by p<.05*, SHAM+ION treatment indicated by p<0.05**, and IR treatment indicated by p<0.05***

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Results & Discussion

Nanoparticles had previously garnered significant publicity in the scientific literature for their potential toxicity. Cell viabilities of a lung cell culture over 24 hours and then 48 hours were used to test the hypothesis, and the results contradicted the reports of toxicity. The cell viability percentages, or percentage of living cells in a given volume, for the cell culture with no IONs were not statistically significant from any of the cells cultures with IONs introduced (Figure 3). This suggests that the IONs were not toxic to the cells and had no effect on how many survived or died in a given volume. In addition, approximate cell counts were analyzed. IONs increased the number of cells in a given volume, suggesting that the IONs actually promoted cell growth (Figure 4). This does not describe toxicity, but instead suggested a potentially useful application of the IONs: if cells are damaged from inflammation, nanoparticle-promoted cell growth could allow the body to repair the area of damage at a faster rate. This finding helped to increase the uncertainty about the effect of iron oxide nanoparticles on living cells. Previous studies reported that IONs were toxic to the cells and observed aspects of the cells to reach that conclusion[9]. However, the data from this experiment showed a promotion of cell growth, which is not likely if the nanoparticles were truly toxic to the cell.

In order to successfully confirm that the IONs were inside the lung *in vivo* and any measurable effect was caused from the IONs, a transmission electron microscope was used. The images taken confirmed that the inhalation method used in this study was successful in delivering the IONs into the lung tissue (Figure 5). The application of TNF-α onto lung cells simulated the inflammatory response to influenza in the lungs. Nitric oxide was used as a measure of inflammation and helped determine if the ION treatment was successful in reducing the inflammation. The results showed that the IONs were effective in reducing inflammation (Figure 1). There was a significant difference between the high and low concentrations of IONs with respect to their inducing different levels of secreted nitric oxide. The data suggested that at certain higher concentrations of IONs there would be no inflammation at all because the level of nitric oxide secretion in cells with the higher ION treatment matched the level of secretion in cells without any induced inflammation treatment. *In vivo* studies suggested a similar conclusion. In looking at average pixel distance between alveoli sacs and the average count of edema-filled sacs, the iron oxide nanoparticles were able to reduce the apparent inflammation and lung injury, but did not completely reverse the damage (Figure 7 & 8). Additionally, the pictures of the samples showed the IR+ION group resembling both the SHAM and the IR groups, supporting the hypothesis that the IONs helped reduce the lung injury but not consistently throughout the sample (Figure 6).

Overall, the data showed that IONs could be used a treatment for reducing the inflammation caused by influenza. The *in vivo* data suggests that the application of the IONs will not terminate the lung injury, as predicted from *in vitro* studies, but could reduce it. There were likely other mechanisms involved with inflammation and lung injury besides TNF-α and nitric oxide that IONs were not able to successfully suppress. Analysis of the MDA levels in the lung tissue suggests similar results (Figure 9). The IONs were able to reduce the levels of MDA (an indicator of inflammation) in the lung tissue. The IR group had a statistically significantly greater amount of MDA than the IR+ION group and the SHAM group. As with the other *in vivo* findings, the nanoparticles were able to reduce the levels of MDA. However, in contrast to the other *in vivo* data, there was no difference in the levels of MDA between the SHAM and the IR+ION, suggesting that IONs were able to reduce the levels of MDA to that of a normal lung.

The findings from this experiment demonstrate the potentially beneficial nature of the IONs and the plausible application of the IONs to help reduce lung inflammation. Previous studies[6] have shown that IONs that are coated with specific ligands to bind to mediators of inflammation did not initiate or compound apparent inflammation. However, the IONs used in this study were not coated with the ligands; some other mechanism within the lung caused the application of IONs to result in a reduction of inflammation. A different study[8] investigated the involvement of IONs on HMM. The IONs were retained by the HMM lysosomes and prevented them from inducing inflammatory cytokines. A similar mechanism was likely involved with the findings from this study. Even though there was inflammation already induced, the IONs may have also been retained by the lysosomes and prevented the monocyte-macrophages from continuing to induce inflammation.

In the SHAM+ION sample group, there was significant lung damage. The edema-filled sacs and the measurements of the average pixel distance confirmed that the nanoparticles actually caused lung injury in otherwise normal lung tissue (Figure 7). This damage was localized; some examined areas had extreme inflammation and lung injury, and other areas had barely any. The irregular filling of the lungs during inhalation is likely what caused the localization. Because there were still areas in the SHAM+ION sample that resembled the SHAM sample, the IONs are what likely caused the lung injury. This finding contradicts the *in vitro* studies (Figure 2). However, for levels of MDA, there was no statistically significant difference between the SHAM and the SHAM+ION (Figure 9). This suggests that the IONs had no effect on the MDA levels in the lungs. The images taken with the light microscope could have simply been localized injury that was not significantly harmful to the lung, or evidence of other lung injury pathways and mechanisms besides MDA. Overall, further investigation is needed to determine the true effect of IONs on the lung tissue.

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