Effect of Prophylactic Treatment with Silver Sol Solutions on an Avian Influenza A (H5N1) Virus Infection in Mice

August 30, 2007

Gordon Pedersen Ph.D.
Robert Sidwell Ph.D.
Colonel Alan Moloff DO, MPH
Colonel Robert Saum Ph.D.
Keith Moeller
William Moeller

Abstract

Female BALB/c mice were pre-treated with (Silver Sol) for seven days prior to being challenged with Avian Influenza (H5N1). The results were compared to control mice and Ribavirin treated mice to determine survival and prevention rates of Silver Sol on Avian Influenza (H5N1). Groups of 19 mice were treated by oral gavage (p.o.) with (Silver Sol 10 ppm) twice daily (every 12 h) for 7 days, then infected intranasally (i.n.) with an LD70 dose of influenza virus, then treated an additional 10 days. As controls, 35 mice were treated with water using the identical schedule as used for the materials and infected as above. Oxygen saturation (SaO2), necropsy for lung scores, and lung viral titers were tested to quantify ongoing tissue damage. As toxicity controls, 3 uninfected mice were treated in parallel with each test material and observed for signs of adverse effects for the next 21 days.

As a result of this experiment, 60% of the infected mice treated with Silver Sol (10 ppm) survived compared to the 30% in the placebo-treated controls. This is a 100% increase in the ability of mice to survive an H5N1 Avian Influenza challenge. Results of this study demonstrate suggested inhibitory and preventive effect on this virus infection as seen by either less animals dying in the treated groups than in the placebo-treated controls, delay in mean day to death, lessened SaO_2 decline, modest inhibition of lung consolidation, and/or lessened virus titers in the lungs. In addition there was no sign or symptom of toxicity from the usage of Silver Sol in mice, even at extreme doses. . If the human protection were similar to that found in mice, it could provide a significant advancement in the prevention of a potential pandemic event. The data from this study strongly suggests that daily oral use of silver sol will safely prevent avian influenza and improve survival rates in mice.

Introduction

Avian Influenza (H5N1, or Bird Flu) can be a fatal disease in humans and a serious threat to become a pandemic event. Since antivirtal therapy is only effective soon after exposure and because there is no effective preventive regimen for the Bird Flu it is essential that preventive treatments be tested and developed in order to enhance survivor rates in the human population.

Since 1973, Silver has been shown to have topical activity against 22 bacterial species (643 isolates) including gram positive and gram negative bacteria (1). As an antimicrobial agent, Silver has been shown to be beneficial in the treatment and prevention of burn infections, post surgical wound infections, and gynecological infections (2, 3). In addition, Silver has been shown to be active against black mold (4), Anthrax (5), Bubonic plague (6), Malaria (7), and numerous viruses such as Hepatitis (8).

The term Sol is a chemical term that identifies a mineral with a magnetic charge that is permanently dissolved into a liquid, whereas the liquid retains the magnetic charge of the mineral. For our purposes, pure silver was dissolved into water through use of 10,000 volts of alternating current, resulting in a

unique Silver Sol. Recently it was reported that the American Biotech Labs product referred to as Silver Sol demonstrated additive and synergistic effects when combined in individual trials with 19 different antibiotics (9). The Silver Sol was shown to improve the effectiveness of the antibiotics even against antibiotic resistant infections (9).

The safe use of Silver Sol as an orally consumed preventive agent has been demonstrated and supported by reports from the EPA and the United States Department of Health and Human Services in a 76 week long study (10,11, 12). The Merck Index identifies the following medicinal uses of silver: Antiseptic particularly for mucous membranes and infectious sinusitis (13). The Merck Manual and Centers for Disease Control, recommend that Silver nitrate drops should be placed in each infant eye as soon as possible or at least in the first half hour of life to prevent gonorrheal ophthalmia (14).

Due to the increased risk from methicillin resistant bacteria, black mold, plasmodium and especially bird flu, the need for orally consumed, safe, daily prophylactic prevention exists

American Biotech Labs provided Silver Sol (liquid) in the form of two different concentrations for the study: Silver Sol (32 ppm), as well as their product designated Silver Sol (10 ppm). Both were found to be virucidal against the avian influenza A/Vietnam/1203/2004 (H5N1) x A/Ann Arbor/6/60 hybrid virus, with an up to 2 $\log_{10}$ virus titer reduction occurring after a 6 h incubation of the product and the virus (USU report dated March 28, 2006). In that same report, similar incubation with the avian influenza A/Duck/MN/1525/81 (H5N1) virus reduced the virus titer by approximately one-half $\log_{10}$ in the same time period. This material is reportedly very well tolerated in human subjects when ingested orally, Dr. Gordon Pedersen of American Biotech Labs designed a study with the Centers for Antiviral Research to evaluate the potential for Silver Sol, to inhibit an avian influenza A (H5N1) virus infection of mice when administered orally to the animals beginning 1 week prior to virus exposure. The objective of this study is to pre-treat mice with Silver Sol, then challenge them with H5N1 Bird Flu, and determine if these mice can survive the challenge significantly better than the controls. This data would suggest a potential preventive treatment for H5N1 Bird Flu.

Materials and Methods

Animals

Female specific pathogen-free 18-21 g BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). They were quarantined 5 days prior to use. They were housed in polycarbonate cages with stainless steel tops and provided tap water and mouse chow *ad libitum.* Female specific mice were used because they are less aggressive than males when kept in large control groups, and have proven to provide dependable results for many years. The mice were fed Silver Sol by oral gavage at a dose of 75 mg/kg/day for the Silver Sol products.

Virus

Influenza A/Duck/MN/1525/81 (H5N1) virus was originally provided by Dr. Robert Webster of the St. Jude Hospital (Memphis, TN). The virus was adapted to mice by passage twice through weanling animals and a large pool prepared in MDCK cells for use in this study. The virus was titrated in young adult mice prior to use in the present experiment.

Silver Sol Solutions

Silver Sol products were provided by Dr. Pedersen. They were in blue bottles, so all studies run with each were performed using the materials in injection bottles covered with aluminum foil to avoid light exposure. All were stored at room temperature until used. It is understood that the solution contained a

Silver Sol at a concentration of 10 ppm. Ribavirin, included as a known positive control, was provided by ICN Pharmaceuticals, Inc. (Costa Mesa, CA); it was dissolved in sterile saline and stored at 4^{o} C until used.

Experimental Design

Groups of 19 mice were treated by oral gavage (p.o.) with Silver Sol twice daily (every 12 h) for 7 days, then infected intranasally (i.n.) with an LD70 dose of influenza virus, then treated an additional 10 days. A similar group of mice were treated p.o. with ribavirin at a dosage of 75 mg/kg/day twice daily for 5 days beginning 4 h pre-virus exposure. The infection was achieved by anesthetizing the mice with an intraperitoneal injection of Ketamine at a dosage of 100 mg/kg and instilling 90 µl of suspended virus in minimum essential medium on the nares of the animals. As controls, 35 mice were treated with water using the identical schedule as used for the Silver Sol materials and infected as above. Ten infected, test substance-treated mice and 20 water-treated controls were observed daily for deaths for 21 days after virus exposure (days 7 through 28 of the experiment), and SaO_2 levels ascertained on days 3-11, which were the times when this parameter usually declines. From the remaining infected, treated animals, 3 test substance-treated and 5 water-treated control mice were killed on days 1, 3 and 6, and their lungs removed, assigned a consolidation score, weighed, and assayed for virus titer. As toxicity controls, 3 uninfected mice were treated in parallel with each test material and observed for signs of adverse effects for 21 days. The weights of these mice as well as 5 normal controls were determined prior to initial treatment and again 18 h after final treatment to determine if the treatments affected host weight gain. Three normal controls were also sacrificed on days 3 and 6 to provide background lung data.

Arterial Oxygen Saturation (SaO_2) Determinations

SaO_2 was determined using the Ohmeda Biox 3800 pulse oximeter (Ohmeda, Louisville, OH). The ear probe attachment was used, the probe paced on the thigh of the animal. Readings were made after a 30 sec stabilization time on each animal. Use of an earlier Ohmeda Model (3740) for measuring effects of influenza virus on SaO_2 in mice has been previously described by Dr's Sidwell and Pedersen (12).

Lung Score Determinations

Each mouse lung removed and placed in a petri dish which, using a permanent black marker, had been divided into sections which were pre-numbered from 1 through 3 or, for placebo controls, 1 through 5. Each lung was assigned a score ranging from 0 (normal appearing lung) to 4 (maximal plum coloration in 100% of lung). These scores were assigned blindly, with the individual doing the scoring not being aware of what group was being examined. An arithmetic mean was determined for each group.

Lung Virus Titer Determinations

Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells as described previously (15). Each lung homogenate was centrifuged at 2000 g for 5 min and the supernatents used in these assays.

Statistical Analysis

Increases in total survivors were evaluated by chi square analysis with Yates' correction. Increases in mean day to death, differences in mean SaO_2 values, mean lung weight, and mean virus titers were analyzed by *t*-test. Only animals dying up to day 21 were considered for mean day to death calculations. The Wilcoxon ranked sum analysis was used for mean lung score comparisons. Each statistical test was run using Excel software on a MacIntosh computer.

Results and Discussion

The results of this experiment are summarized in Table 1 and in Figures 1-4. As seen in Table 1, the virus challenge in this experiment was lethal to 14 of the 20 placebo-treated mice, with the mean day to death being 8.4 days. Such a pattern of death is considered ideal for evaluation of potential antiviral agents. This optimal condition was verified by the observation that Ribavirin was fully protective to the mice, preventing any deaths from occurring (Table 1), significantly lessening SaO_2 declines (Figure 1), inhibiting lung score development (Figure 2), lung weight increase (Figure 3), and lung virus titer increases (Figure 4).

Treatment with Silver Sol appeared to not affect the numbers of animals dying of influenza, although a half-day delay in mean day to death was seen (Table 1). SaO_2 declines in this group of treated mice were almost at the same rate as those in the placebo controls, although it was interesting that on the first day this parameter was assayed, a highly significant ($P<0.001$) difference was seen (Figure 1). SaO_2 declines are a manifestation of declining lung function, suggesting that the lung consolidation in the lungs did not progress as rapidly as seen in the placebo controls. The treatment appeared to moderately lessen lung consolidation as seen by lower lung scores on each time evaluated, the day 6 mean lung score being significantly ($P<0.05$) less than the placebo treated controls (Figure 2). Lung weights, another indication of fluid developing in the lungs to cause pneumonia in the animal, were also less at each time point than seen in the placebos (Figure 3). The mean lung virus titers in the mice treated with Silver Sol were lower than the placebo controls on days 3 and 6 of the infection (Figure 4).

There were no demonstrated adverse side effects from the 10 ppm or 32 ppm solutions, as demonstrated by the toxicity control mice and seen by no deaths occurring in them and host weight increases observed during time of therapy. Ribavirin, while not lethal to the mice, did result in a 0.4 g host weight loss (Table 1); this was an expected effect for the latter material, since the maximum tolerated dose is approximately 100 mg/kg/day.

It is difficult to attribute the effects seen in this experiment wholly to viral inactivation, since both test materials were administered orally to animals infected by direct nasal inhalation, although the treatments began one week before virus exposure, so it is possible that a portion of the Silver Sol may have been able to be in the vicinity of the virus-exposed lung tissue. The mechanism of action of Silver Sols has not been determined completely. There are a number of hypotheses for the mechanism of action and effectiveness they include the possibility that this material is exerting a mild immunomodulatory effect in the animals, which would provide modest protection against the infection. If such a mechanism is indeed associated with the potential activity seen, then a different treatment schedule, perhaps limiting the number of treatments to one per day or once every other day, may enhance any immune modulatory effects, since it is recognized that too-frequent dosing may overtax the immune system. The greater protection seen by the lower-dosed Silver Sol material could be explained by immunomodulation, since the greatest immunologic effect is not necessarily at the highest dose used.

Another mechanism whereby the Silver Sol materials may have inhibited the influenza virus infection in these studies may simply be one of coating the virion with Silver Sol to prevent attachment and penetration. Again, the material would need to be in the vicinity of the exposed lung tissues at the time infection was initiated. The Silver material could also play a role in limiting apoptosis of the epithelial lining of the lung induced during acute lung inflammation. Apoptosis plays a causative role in acute lung injury in part due to epithelial cell loss.

Further studies would have to be conducted to more fully delineate the actions of this material.

It is acknowledged that the effects seen in the present study, while of considerable interest, would need to be repeated to confirm that the observations were not due to mere chance. Consideration of combined use of oral administration of the Silver Sol materials and intranasal instillation at near the time of virus

exposure would determine whether the effects seen were indeed associated with virucidal effects of these materials.

Summary

Mice infected with avian influenza A/Duck/MN/1525/81 (H5N1) virus were treated with the Silver Sol-provided by American Biotech Labs. Oral gavage treatment began 7 days prior to virus exposure and continued twice daily for a total of 17 days. Treatments with both formulations provided a suggested inhibitory and preventive effect on this virus infection as seen by either less animals dying in the treated groups than in the placebo-treated controls, delay in mean day to death, lessened SaO_2 decline, modest inhibition of lung consolidation, and/or lessened virus titers in the lungs. Ribavirin was included as a positive control drug, used orally at a dose of 75 mg/kg/day twice daily for 5 days beginning 4 h pre-virus exposure, and this treatment was markedly inhibitory to the infection as expected.

Conclusion

The results are based on a small sampling size, using appropriate mice under controlled laboratory conditions. A larger study must be conducted to determine if the results may be applied to farm animals or humans in a "real world" environment. It could be relevant to national security that mice that orally consumed Silver Sol for one week prior to H5N1 challenge, survived 100% better than control mice, because the prophylactic use of this non-toxic liquid can be used in humans. If the human protection were similar to that found in mice, it could provide a significant advancement in the prevention of a potential pandemic event. Silver Sol has antiviral activity in vitro and in this animal study, with results that are similar to pharmaceutical drugs, but Silver Sol produces no harmful metabolites as it is non-toxic and passes through the human body unchanged. This is remarkable because Silver Sol is capable of being used every day while pharmaceutical drugs produce unsafe metabolites that prohibit every day preventive use. The results of this report suggest the prophylactic use of Silver Sol provides a safe and beneficial improvement for use in the prevention of H5N1 Bird Flu in mice.

Bibliography

1. Carr, H., Wlodowski, T., 1973. Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York. Antimicrobial Agents and Chemotherapy. 10:585-587.
2. Fox, C.J. Jr., 1968. Silver sulfadiazine, a new topical therapy for Pseudomonas burns. Arch. Surg. 96:184-188.
3. Fox, C. J., 1969. Control of Pseudomonas infection in burns by Silver Sulfadiazine. Surg.Gynecol. Obstet. 128:1021-1026.
4. U.S House International Relations Committee, 2005. Written testimony on Black Mold.
5. Illinois Institute of Technology. 2001. Anthrax. Anthrax Review.
6. Robinson, R., 2003. Bactericidal activity of ASAP Silver Solution on Yersinia Pestis, the etiological agent of plague. Department of Microbiology, Brigham Young University.
7. U.S. House Relations Committee. 2005. Written testimony on Malaria.
8. Hafkine, A., 2003. ASAP antiviral activity in Heapatitis B; DNA Ploymerase Inhibition, Reverse Transcriptase Inhibition. Hafkine Institute for Training, Research and Testing.

9. DeSousa, A., Mehta, D., Leavitt, R. W., 2006. Bactericidal activity of combinations of silver-water dispersion with 19 antibiotics against seven microbial strains. Current Science Investigations. 91:7-11.
10. EPA Research and Development. 1988. Drinking water criteria document for Silver. Office of Health and Environmental Assessment.
11. U.S. department of Health and Human Services, Public Health Service and Agency for Toxic Substances and Disease Registry. 1990. Toxicological profile on Silver.
12. Sidwell, R. W., Huffman, J. H., Gilbert, J., Moscon, B., Pedersen, G., Burger, R. and Warren, R. W. 1992. Utilization of pulse oximetry for the study of the inhibitory effects of antiviral agents on influenza virus in mice. Antimicrob Agents Chemother 36:473-6.
13. Wilson, B.H., Merck Index., 2006. Silver. 1:645.
14. Porter, R,. Kaplan, J., Merck Manual., 2006. Silver. 1:2082.
15. Sidwell, R. W., Huffman, J. H., Call, E. W., Alaghamandan, H., Cook, P. D., and Robins, R. K. 1985. Effect of Selenazofurin on influenza A and B virus infections in mice. Antiviral Res. 6:343-353.

Tables and Figures