

# Effect of Prophylactic Treatment with ASAP-AGX-32 and ASAP Solutions on an Avian Influenza A (H5N1) Virus Infection in Mice



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## Comment

### **Editorial Note by B.M.Hegde.**

A Preliminary Report on the effects of an ultra dilute silveraquisol on HIV positive patients

JSHO is a journal that, in its title reflects its focus on healing outcomes in human beings. The following study is published with a specific goal in mind, although it is a deviation from our "standard" practice. The new healing vector (silver aquasols) described in the following paper, which describes the nature of the material and in vitro data, also utilizes the same silver aquasol aka a nano-silver colloid, which is the pioneering representative of a new family of materials which may turn out to be a revolution in human therapeutics. Backed by centuries of Indian Ayurvedic experience, silver has had hoary past as a safe germ killer worldwide without producing drug resistance like antibiotics. There have been other studies in obscure venues, before the following study, as well as our own study in vitro, published elsewhere.

In 2007, we were confronted, in locality by a group of very, very poor HIV positive sufferers who could not afford even daily

food, leave alone retroviral drugs. They were taken up for a pilot study to see if this vector helps them. All the necessary formalities like informed consent were obtained. They were supplied with nutritious food, as soon as their appetite returned within a couple of weeks of silver treatment. The results below surprised us, and the demand for the drug has become almost impossible to contain what with many poor patients knocking at our doors since then. Now that we have the laboratory results of the drug's efficacy and safety\* we are planning a large scale controlled study of the application to HIV positive individuals in the near future. The following study\* provides all those laboratory findings in a different viral set up.

### **Treatment, Principal Findings and results**

7 HIV consecutive patients, confirmed by their Western Blot tests, were administered Nanosilver orally (15ml four times daily) for 4 months. The patients' pre-treatment and post -treatment CD4 Lymphocyte counts and weight are recorded in the table at the tail end of this note. All the patients showed a significant increase in their CD4 lymphocyte counts after the treatment. Clinically they were 100% fit and were feeling much better after the treatment period. We did not quantify viral loads though; this being a pilot study.

### **Discussion**

Interestingly, our recent laboratory work of the effect of nano silver in vitro on the HIV virus did show that the nanosilver particles could bind to HIV virus and could potentially block the viral entry into cells, eventually destroying the virus<sup>1</sup>. We are yet to publish this data.

S.no	Body weight ( Kg)		T-lymphocyte count ( Per micro liter)	
S.no	Body weight ( Kg)	Body weight ( Kg)	T-lymphocyte count ( Per micro liter)	T-lymphocyte count ( Per micro liter)
1	54	67	809	990
2	57	64	705	856
3	66	71	767	1050
4	48	55	540	670
5	66	70	870	1067
6	62	68	730	810
7	41	55	310	740

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## Introduction

Avian Influenza (H5N1, or Bird Flu) can be a fatal disease in humans and a serious threat to become a pandemic event. Since there is no pharmaceutical remedy 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), Antrax (5), Bubonic plague (6), Malaria (7), and numerous viruses such as Hepatitis (8).

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

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 gonorrhoeal ophthalmia (14).

The safe use of Silver 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). Dogs that inhaled Silver showed activity in the lung in one hour with 90% of the silver carried to the liver by the blood within 6 hours (15).

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. In this study, Silver Sol from American Biotech Labs demonstrates safe beneficial and preventive activity against H5N1 Bird Flu, when taken orally in mice.

The American Biotech Labs product, ASAP-AGX-32, as well as their product designated ASAP, 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<sub>10</sub> 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<sub>10</sub> 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 ASAP-AGX-32 and ASAP, to inhibit an avian influenza A (H5N1) virus infection of mice when administered orally to the animals beginning 1 week prior to virus exposure.

This report describes the results of this experiment.

## 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.

**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.

**Compounds:** ASAP and ASAP-AGX-32 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 ASAP solution

contained a colloidal silver at a concentration of 10 ppm, and the ASAP-AGX-32 contained the same colloidal silver at a concentration of 32 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°C until used.

**Arterial Oxygen Saturation (SaO<sub>2</sub>) Determinations:** SaO<sub>2</sub> was determined using the Ohmeda Biox 3800 pulse oximeter (Ohmeda, Louisville, OH). The ear probe attachment was used, the probe placed 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<sub>2</sub> 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 (16). Each lung homogenate was centrifuged at 2000 g for 5 min and the supernatants used in these assays.

**Experimental Design:** Groups of 19 mice were treated by oral gavage (p.o.) with either ASAP-AGX-32 or ASAP 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  $\mu$ l 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 ASAP 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, and SaO<sub>2</sub> 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.

**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<sub>2</sub> 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<sub>2</sub> declines (Figure 1), inhibiting lung score development (Figure 2), lung weight increase (Figure 3), and lung virus titer increases (Figure 4).

Treatment with ASAP-AGX-32 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<sub>2</sub> 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<sub>2</sub> 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 ASAP-AGX-32 were lower than the placebo controls on

days 3 and 6 of the infection (Figure 4).

Treatment with ASAP, which we understand is a less-concentrated version of ASAP-AGV-32, also provided some intriguing results. Especially of interest was the observation that 60% of the infected mice treated with this compound survived compared to the 30% in the placebo-treated controls. Although not statistically significant because of the number which survived in the latter controls, this effect is strongly suggestive a disease-inhibitory effect may have occurred. At two time points during the SaO<sub>2</sub> assays, days 3 and 6, the declines normally seen were significantly lessened ( $P < 0.01$ ), and there was a general lessening of decline throughout the times of assay (Figure 1). Modest inhibition in lung scores were seen in this treated group as well, especially on day 6 (Figure 2); the lung weight data did not correlate too well with the lung scores, however (Figure 3). Again, slight inhibition of lung virus titers were seen in the ASAP-treated, infected mice (Figure 4).

Both the ASAP formulations were well tolerated by the toxicity control mice as 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. It is also possible 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 ASAP material could be explained by immunomodulation, since the greatest immunologic effect is not necessarily at the highest dose used.

Another mechanism whereby the ASAP 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 ASAP 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-containing formulations ASAP-AGX-32 and ASAP 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<sub>2</sub> 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.

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**Table 1. Expt. ABLA-1. Effect of Oral Gavage Prophylactic Treatment with ASAP-AGX-32 and ASAP on an Influenza A (H5N1) Virus Infection in Mice.**

Animals: Female 18-21 g BALB/c mice

Virus: Influenza A/Duck/MN/1525/81 (H5N1)

Drug diluent: Company diluent

Treatment schedule: bid x 17 beg -7 days (Ribavirin: bid x 5 beg -4 h)

Treatment route: p.o.

Expt. duration: 28 days

Treatment	Dosage	Tox Controls		Infected, Treated Mice		
		Surv/Tot al	Mean Host Weight Change <sup>a</sup> (g)	Surv/Tot al	Mean Day to Death <sup>b</sup> ± SD	Mean Day 11 SaO2 (% ± SD)
ASAP-AGX-32	32 ppm	3/3	1.8	2/10	8.9 ± 1.4	75.4 ± 1.0
ASAP	10 ppm	3/3	1.4	6/10	7.3 ± 1.0	76.7 ± 2.1
Ribavirin	75 mg/kg/day	3/3	-0.4	10/10***	>21.0 ± 0.0** *	86.6 ± 2.5** *
H2O	—	—	—	6/20	8.4 ± 1.8	76.0 ± 1.9
Normal Controls	—	5/5	2.3	—	—	88.8 ± 3.0

<sup>a</sup> Difference between initial weight and weight 18 h after final treatment.

<sup>b</sup> Difference between initial weight and weight 18 h after final treatment.

\*P<0.05; \*\*P<0.01; \*\*\*P<0.001 compared to H2O -treated controls.

Figure 1. Expt. ABLA-1. Effect of oral gavage prophylactic treatment with ASAP-AGX-32 or ASAP on arterial oxygen saturation decline in avian influenza A (H5N1) virus-infected mice.

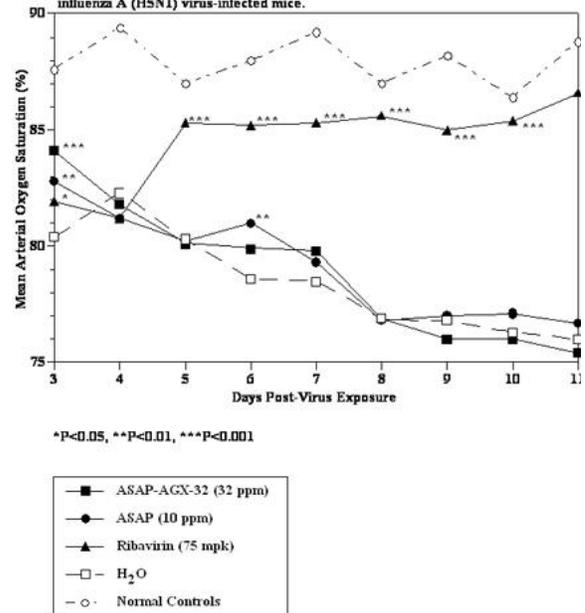


Figure 2. Expt. ABLA-1. Effect of oral gavage prophylactic treatment with ASAP-AGX-32 or ASAP on lung scores in avian influenza A (H5N1) virus-infected mice.

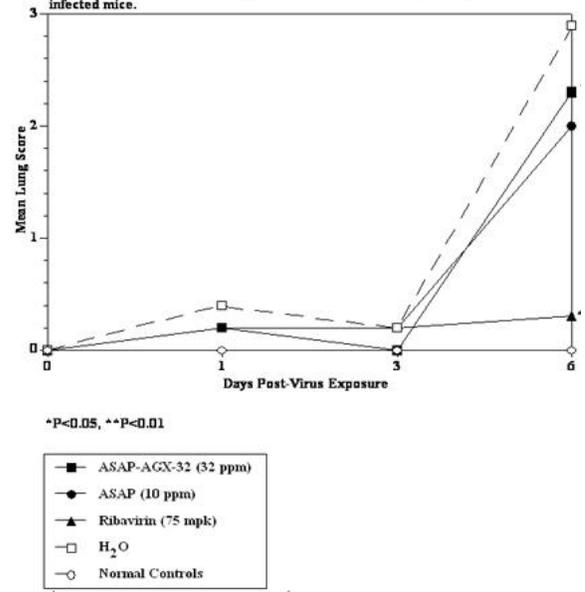


Figure 3. Expt. ABLA-1. Effect of oral gavage prophylactic treatment with ASAP-AGX-32 or ASAP on lung weight increase in avian influenza A (H5N1) virus-infected mice.

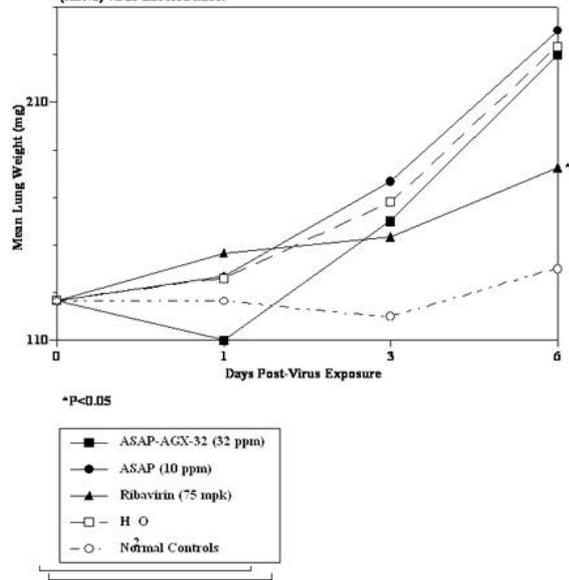


Figure 4. Expt. ABLA-1. Effect of oral gavage prophylactic treatment with ASAP-AGX-32 or ASAP on lung virus titers in avian influenza A (H5N1) virus-infected mice.

