TECHNICAL REPORT

Toxicity study of Paerosol in the conditions simulating it application for volumetric disinfection of enclosed environment

1. INTRODUCTION

The results generated during Phase I demonstrated high decontamination efficacy of Microaerosol of Electrochemically Activated Solution (Paerosol) toward a variety of bioagents, including G-positive and G–negative microbial cells, bacterial spores, and viruses. It was shown that the contact of different bioagents with PAEROSOL resulted in serious morphology damage and irreversible death of microbial cells, spores and viruses.

First PAEROSOL toxicity study was conducted with outbreed white mice to observe toxicity of PAEROSOL. As the controls, microaerosols generated with dist water and with 1% NaCl were used. Minor and very similar variations were found in liver, spleen and lungs of the animals exposed to PAEROSOL and to the aerosol of 1% NaCl. That indicated that PAEROSOL was not more toxic than the aerosol of 1%NaCl.

Second study of PAEROSOL toxicity was conducted in the conditions simulating PAEROSOL application for volumetric disinfection of enclosed environment.

Both Toxicity Studies were performed at the Institute of Highly Pure Biopreparation and the Institute of Influenza (WHO Center for Influenza in Russian Federation).

2. MATERIALS AND METHODS

2.1. Animals

Swiss Webster specific pathogen-free (SPF) female mice were purchased from “Pushchino” Animal Breeding Facility, Moskovskaya obl., Russia.
Prior to the study, mice were kept in quarantine for 14 days with daily observation for behavior and general condition, and twice per a day - for sickness and death rates. After quarantine, only proper animals (healthy and with appropriate weight) were randomly split into the experimental and control groups.
Prior to and during the experiment, mice were kept in cages in a separate room. A 12-h light/dark cycle was employed and the animal room underwent 15 fresh air changes per an hour. The following conditions were controlled in the room with experimental and control groups: temperature 18-20^0 C, RH 50-70%; CO_2 concentration has not exceeded 0.15 % (v/v) and the ammonia concentration has not exceeded 0.001 mg/l.
The experiments with animals were conducted in compliance with the Protocols and Procedures approved by IHPBP Animal Care and Use Committee.

2.2. Electro-activated solution (EAS)

EAS was prepared from 1%NaCl solution with a STEL device (NPO “Ekran”, Moscow). The content of active chlorine in the EAS was (0.20 ± 0.02) % and pH =7.2 ± 0.1.

2.3. Protocol of PAEROSOL administration

Two hours prior to the experiment, mice were deprived of feed and water, weighed and randomly spread among the groups, each of 10 animals.

The mice were spread among the groups as follows:

1. Control group – mice not exposed to any aerosol
2. Placebo group – mice exposed to 2% NaCl/water aerosol during 20 hours
3. Mice exposed to PAEROSOL just after atomizing and remained inside the chamber in the PAEROSOL atmosphere for 1 hour
4. Mice exposed to PAEROSOL just after atomizing and remained inside the chamber in the PAEROSOL atmosphere for 4 hours
5. Mice exposed to PAEROSOL just after atomizing and remained inside the chamber in the PAEROSOL atmosphere for 20 hours
6. Mice positioned in the chamber 4 hours after PAEROSOL was atomized inside the chamber and remained in the PAEROSOL atmosphere for 20 hours
7. Mice positioned in the chamber 20 hours after EAS was atomized inside the chamber and remained in the PAEROSOL atmosphere for 20 hours

Mice were placed in a closed container, and positioned inside the aerosol chamber of 2.8 m^3 (~100ft^3). PAEROSOL was atomized with the VAG generator operating at 90 ml/min. In each experiment 650 ml of EAS was atomized in the chamber for 7.2 minutes to provide the initial PAEROSOL concentration 232 ml/m^3. According to the general protocol previously developed for decontamination of microbial spores, this concentration was shown to be effective for decontamination of ~ $1x10^6$ CFU/m^3 of the airborne or ~ $1x10^6$ CFU/cm^2 of the surface-absorbed *B.cereus* spores. The protocol allows for overall decontamination of airborne and surface-absorbed microbial spores, vegetative microbial cells and viruses.

Once PAEROSOL atomizing was terminated, the container with mice was opened with a tool located outside the chamber, and mice were exposed to PAEROSOL. The same protocol of atomizing and mice exposure was used when atomizing NaCl/water.

- Mice of Groups 3 to 5 remained inside a chamber in the PAEROSOL atmosphere during 1, 4, or 20 hours, respectively.
- Mice of Groups 6 and 7 were positioned in the chamber 4 or 20 hours after EAS was atomized inside the chamber and then remained inside the chamber during subsequent 20 hours.
- The placebo animals (Group 2) were positioned in the chamber, 2% NaCL/water was atomized inside the chamber, and the mice remained in the NaCL/water aerosol atmosphere during subsequent 20 hours.

All mice groups were observed during 14 consecutive days. Mice were monitored for general condition, behavior, motor activity, convulsions, irritant reactions, state of hair and skin, and changes in body weight daily.
On day 14 of the experiment, blood samples were obtained by retro-orbital puncture under anesthesia and animals were euthanized.

Pathology

Euthanized mice were inspected externally; subjected to necropsy, and abdominal and thoracic internal organs undergone extensive examination; the lungs were subjected to histological study. Simultaneously, bronchoalveolar lavage fluid (BALF) was taken in mice to study the BALF cellular composition and neutrophils activities in the test of zymosan-dependent chemiluminescence. The mice lungs were lavaged with 1 ml of PBS supplemented with 20 U/ml

of heparin solution. The lavage samples were centrifuged at 200*g* for 10 min. at 4^0C and the pellet re-suspended in PBS. The cell concentration was estimated in a Goryev's chamber. Simultaneously the smear preparations (thick smear) were prepared, dried, fixed in ethanol 96% and stained by Romanovsky using Azure-eosin. The BALF cell differentiation was examined under a light microscope, magnification 630 x. The number of monocytes/macrophages, neutrophils, and lymphocytes was counted per 100 cells. Data are presented in %.
The luminol-dependent chemiluminescence (CL) in BALF cells was measured using the Chemiluminometer Victor 2 (LKB Wallac). All measurements were made in 3 parallels in 96-well white non-transparent plates (Costar). Each well contained 20 µl of BALF sample, 40 µl of luminol at the concentration 10^{-4} M, and 20 µl of the opsonized zymosan used as a stimulant for CL reaction. CL was followed for 30 min. at 37^0C. Data were presented as cumulative counts.

Histological examination of the lung tissue

The lungs kept in 4% paraform were washed with a tap water for 24 hours and dehydrated in 70% ethanol. For histological study, lung pieces were cut from the diaphragmatic lobes and dehydrated in graded ethanol, 24-hour exposed in celloidin-castor mixture and embedded in Paraplast. Lung sections were cut at 6 µm thickness and stained with Hematoxylin and Eosin using a standard protocol, and analyzed using a visualizing instrument composed of a microscope Leica DMLB, a videocamera Leica DC 300 and a software Leica IM 50 (Germany), magnification was 20x for the objective and 16x for the camera.

Hematological and immunological studies

On day 14 of the experiment, blood samples were collected from the orbital sinus under chloroform anesthesia. Total leukocyte count was recorded. Blood smears were air-dried, and blood formula was analyzed (lymphocyte, neutrophil, monocyte, eosinophil and basophile cell counts were estimated; data are presented in %). Blood smears were fixed in 96% ethanol and stained by May-Grunwald and Romanovsky with Azure-eosin.
The cellular differentiation was examined under a light microscope, using oil immersion objective, magnification 630x. The number of lymphocytes, neutrophils, monocytes, eosinophils, and basophils was counted per 100 cells. Data are presented in %.

Spleens were removed under aseptic conditions from euthanized mice. The spontaneous and Con A-stimulated proliferation of spleen T-cells (the reaction of blast transformation) and of Concanavalin (Con) A-induced interleukin (IL)-2 synthesis were studied. For this purpose, spleens from mice throughout a group were pooled and cut with scissors. The cells were suspended in sterile PBS, filtered through double gauze and centrifuged. Further the cells were washed twice with PBS (erythrocytes were preliminarily lysed with 0.83% solution of NH_4Cl) and re-suspended in RPMI-1640 (Sigma) supplemented with 2.0 mM of L-glutamine, 50 µM of 2-mercaptoethanol and 20 µg/ml of gentamycine (complete medium). The cell counting was performed.
In 4 parallels, spleen T-cells (5 x 10^5cell/well) were incubated in 96-well flat-bottomed plates (Costar) with either 0 (spontaneous proliferation), 0.33 or 1.0 µg/ml of Con A (Sigma) in a complete medium, supplemented with 10% FCS (Sigma), in a CO_2 - incubator for 72 hours at 37^0C in absolute humidity. 24 hours prior to completion of incubation ^{3}H-labeled thymidine (the final concentration 5 µCi/ml) was added, the cells were harvested into fiberglass filters, type GF/C (Whatman) with a semi-automatic harvester (Flow Lab). The ^{3}H-labeled thymidine inclusion was measured with a scintillation liquid β-counter RackBeta 1217 (Wallac). The activity comes in the number of pulses/min (cpm).

To evaluate the Con A-stimulated IL-2 synthesis, spleen T-cells (1 x 10^6 cell/well) were incubated in 96-well flat-bottomed plates (Costar) with either 1 or 5.0 μg/ml of Con A (Sigma) in a complete medium, supplemented with 5% FCS (Sigma). The cell supernatants were collected 24 hours after incubation, and IL-2 activity was measured using the IL-2-dependent CTLL-2 cell line in accordance with the well-known protocol (Gillis S. et al, 1982).

Statistical analysis

Statistical analysis of the data was performed using the Microsoft Excel 2003 program. The meanings were presented as mean ± StD and the level of significance was set at $p < 0.05$. The parameters measured in mice exposed to PAEROSOL were compared with corresponding parameters in mice of both control groups. The differences were calculated using two-way unpaired Student's *t*-test with unequal deviations. The correlation was considered to be significant if $p<0.05$.

3. RESULTS

Body weights, general conditions, and internal organs

Throughout a whole period of observation (up to 14 days after a contact with MEAS), the mice general health, behavior and appetite were shown unaffected in each group.
Changes of body weight of the mice exposed to PAEROSOL and of control mice are presented in Table 1.

Table 1 Changes of body weight (g) of the PAEROSOL-exposed and control mice

Period of observation	Mice groups						
	Intact mice	Exposed to 2% NaCl aerosol	Exposed to PAEROSOL for:			Positioned in the chamber:	
			1 hour	4 hours	20 hours	4 hours after EAS atomizing	20 hours after EAS atomizing
	1	2	3	4	5	6	7
Prior to exposure	22,82±1,30	23,06±1,02	23,04±1,45	19,82±1,26	22,20±0,92	20,89±1,85	21,77±1,42
Day 3	23,04±1,01	23,12±1,27	23,10±1,76	20,14±1,32	22,81±1,57	21,38±1,46	22,58±1,37
Day 7	24,08±1,32	22,85±1,49	23,02±1,55	21,36±1,72	23,79±1,83	22,67±1,59	23,04±1,53
Day 14	24,70±1,13	23,22±1,04	23,79±1,83	23,18±0,94	24,21±1,41	23,22±1,74	23,53±1,26

The data indicated that the body weight gradually insignificantly increased, compared to initial weight, in all mice groups. No significant differences in the body weight were revealed among the groups and within one group at different time-points of observation.

After completion of the experiment, visual observation revealed that mice of all groups were of normal constitution, feed consumption, and with good hair and skin conditions. No excreta were observed. Mucous membranes were shine, smooth, pale, without pathology.

Mice necropsy revealed no exudates in thoracic and abdominal cavities, and the normal anatomic position of the internal organs.

In all mice groups: the submandibular lymph nodes and salivary glands were of spherical and oval forms, of white color and of moderate and dense consistency without changes. The thyroid

gland was of usual size and form, of reddish color, without pathology. The thymic gland was of triangular form, of whitish color and slightly dense consistency, without pathology.
The aorta intima was shine and smooth. The heart was without changes in size and shape; the heart muscle was of moderate thickness and of brownish color, without pathology. The trachea and large bronchi lumena were uniformly wide. Pulmonary collapse easily occurred on thoracotomy. The lungs were of uniform pale pink color. The lung tissue seemed gas-filled by touch, without pathology.
The esophageal mucosa was shine and smooth. The stomach was of normal size with the lumen filled with food debris. The gastric mucosa was folded, of uniform pinkish color, none irritation or hyperemia was observed. The large and small intestine mucosa were smooth and shine of grayish and pale pink color, respectively. The straight intestine mucosa was smooth, shine of grey-pink color without irritation.
Pancreas was of pale pink color, segmental. Liver and spleen were of normal color, size and fullness. None pathological changes in mice digestive system were revealed. A kidney capsule was easy to remove, the surface was smooth and of uniform brownish-grayish color. The cortical and medulla substances were well observed on incision. The adrenal glands were of spherical form, of whitish color and of moderate dense consistency, without changes.
The urinary bladder mucosa was shine, smooth and pale, without changes. The uterine body and horns were of normal appearance and size. The ovaries had uneven surface, were of reddish color and of moderate density. The urinary and genital systems organs were without pathological changes.
The brain tunics were shine, smooth and thin. On the front brain section, none brain ventricular ectasia was observed. Neither mouse revealed pathological changes.

Conclusively, macroscopic examination has not revealed pathological changes in the internal organs of mice exposed to PAEROSOL.

Changes of the inner organs (liver and spleen) weight in mice exposed to PAEROSOL and in control mice are presented in Table 2.

Table 2 Liver and spleen weight (g) in mice exposed to PAEROSOL and in control mice

Organs	Mice groups						
	Intact mice	Exposed to 2% NaCl aerosol	Exposed to PAEROSOL for:			Positioned in the chamber:	
			1 hour	4 hours	20 hours	4 hours after EAS atomizing	20 hours after EAS atomizing
	1	2	3	4	5	6	7
Liver	1.11±0.09	1.03±0.14	0.99±0.60	1.08±0.13	1.15±0.13	1.10±0.08	1.00±0.14
Spleen	0.13±0.02	0.11±0.02	0.11±0.01	0.11±0.01	0.12±0.01	0.13±0.01	0.10±0.02

As evident from Table 2, no significant changes of the liver and spleen weight were found in mice exposed to PAEROSOL, compared to control groups.

Lung tissue histology

Lung histology study revealed that the general lung tissue pattern was normal in all mice groups. The bronchi lumena were clear, and none peribronchiolar or perivascular cuffing was revealed. In most lung tissue specimens, the bronchial epithelium was of normal appearance. None pneumosclerosis foci or significant lung injuries were revealed. The airway tissue looked more

plethoric than normal one. The number of monocytes/macrophages was slightly increased however no typical signs of the acute inflammation were revealed.
No significant changes in the lung tissue pattern were found in mice exposed to PAEROSOL compared to control mice. Throughout one mice group, the lung tissue pattern slightly differed in the airway area, most probably due to different perfusion. One mouse from Group 2 demonstrated a sharp increase in the RBC counting and in monocyte/macrophage infiltrating that might be attributed to individuality of this mouse.
Fig.1 - Fig.7 present the micrographs of the lung tissue of mice exposed to PAEROSOL and control mice.
Conclusively, the lung tissue histology studies demonstrated no pathological changes in the lungs of mice exposed to PAEROSOL.

Hematologic and immunological studies

The peripheral blood differential cell counts and formula were analyzed in mice. The results are presented in Table 3.

Table 3 Changes of mean blood cell counts in mice of experimental and control groups

Animal Group	Total WBC count (10^6/мл)	Differential cell count, %				Absolute Lymph. Number x10^6/ml	Absolute Neutroph. number x10^6/ml
		Lympho-cytes	Neutrophils	Eosinophils	Monocytes		
1	5.90 ±1.70	86.20±3.44	12.11±3.52	1.31±1.02	0.43±0.56	5.07±1.41	0.74±0.32
2	6.10±1.24	86.60±2.01	12.33±2.31	0.32±0.42 *	0.82±0.64	5.29±1.10	0.75±0.20
3	7.40±1.20	86.60±2.76	11.04±2.61	2.03±1.18	1.04±0.35	6.42±1.06	0.79±0.17
4	4.24±1.40	86.81±2.42	11.24±2.28	1.71±0.70	0.32±0.48	3.71±1.27	0.45±0.17
5	6.25±1.70	88.82±2.24	9.62±2.52	1.12±0.74	0.51±0.60	5.53±1.48	0.62±0.27
6	6.60±1.60	87.41±4.41	10.30±3.31	1.61±1.08	0.72±0.84	5.78±1.45	0.67±0.25
7	4.90±1.40	83.03±5.43	15.22±4.84	1.23±1.08	0.64±0.60	4.06±1.13	0.75±0.33

* – $p < 0.05$

As can be seen from Table 3, no significant changes in the peripheral blood cell formula were found in experimental compared to control mice.

The eosinophile count in mice exposed to PAEROSOL looked like increased compared to that in mice of group 2 (exposure to NaCl aerosol) however did not significantly differ compared to that in intact mice. No other significant changes of peripheral blood parameters were revealed in mice of Group 2 compared to that in mice exposed to PAEROSOL and in intact mice. So, the difference in eosinophile count in mice of Group 2 may be ignored as insignificant for general results.

Based on our long-term experience, the morphological parameters of peripheral blood in healthy mice are the following: WBC - 7-15x10^6/ml, neutrophils – 10-40%, eosinophiles - 0-7%, basophils– 0-1%, lymphocytes – 35-90%, and monocytes – 0-3%.
As follows from the data presented in Table 3, the peripheral blood counts were within physiological norm in all mice groups, though slight differences have been revealed among the groups.

The influence of PAEROSOL on the BALF cell differentiation and on functional activities of leukocytes was studied by the luminol-dependent chemiluminescence method. The results are presented in Table 4 and Table 5.

Table 4 BALF cell differentiation in experimental and control mice

Animal Group	Total cell count (10^6/ml)	Differentiation cell count, %		
		Monocytic cells/macrophages	Lymphocytes	Neutrophils
1	0.21±0.11	95.52±1.12	4.02±1.01	0.50±0.50
2	0.26±0.22	94.01±2.23	3.62±1.60	2.40±1.91
3	0.17±0.10	96.82±0.81	3.02±0.81	0.20±0.31
4	0.47±0.31	94.83±2.91	3.51±1.90	1.67±1.62
5	0.27±0.11	96.11±1.72	3.62±1.43	0.22±0.41
6	0.15±0.10	96.51±1.63	3.03±1.31	0.44±0.60
7	0.26±0.11	96.93±0.71	3.02±0.61	0.26±0.10

It has been reported (Kaufmann,1999) that the BALF cellular composition in healthy mice for the most part consist of monocytes and macrophages with the small number of alveolar epithelial and blood cells: $1x10^5$ - $5x10^5$ of alveolar macrophages, about 5% of neutrophils, and about 6% of lymphocytes.

The results of this study demonstrated that the BALF cell differentiation in all mice was within the physiological range. No significant signs of inflammation were observed. No significant changes in BALF cell differentiation were found in mice exposed to PAEROSOL compared to that in control mice.

Table 5 Changes of the functional activities of leukocytes in mice exposed to PAEROSOL and control mice (10^{-4} pulses within 30 min)

Chemi-luminescence (CL)	Mice groups						
	Intact mice	Exposed to 2% NaCl aerosol	Exposed to PAEROSOL for:			Positioned in the chamber:	
			1 hour	4 hours	20 hours	4 hrs after EAS atomizing	20 hrs after EAS atomizing
	1	2	3	4	5	6	7
Spontaneous	13.80±0.32	13.72±0.33	11.52±0.40	14.05±0.44	14.26±0.49	14.38±0.54	11.28±0.35
Zymosan-stimulated	14.65±0.34	14.39±0.39	12.22±0.89	15.05±0.51	14.84±0.36	13.92±0.56	11.68±0.35

As can be seen from Table 5, no significant differences in spontaneous and zymosan-stimulated CL were revealed among the mice groups. Hence. no changes compared to the background indices were revealed in the functional activities of neutrophils.

The PAEROSOL effect on spontaneous and Con A-stimulated proliferation of spleen T-cells was studied. In addition, the PAEROSOL effect on the ability of these cells to produce IL-2 was studied. We used these tests as the routine immunotoxicity study and because of the high sensitivity of the immune cells to toxicants. Table 6 and Table 7 present the results.

Table 6 Spontaneous and Con A-stimulated proliferation of T-spleen cells in mice exposed to PAEROSOL and in control mice (pulses/min. x 10^{-3})

Dose of Con A (µg/ml)	Mice groups						
	Intact mice	Exposed to 2% NaCl aerosol	Exposed to PAEROSOL for:			Positioned in the chamber:	
			1 hour	4 hours	20 hours	4 hrs after EAS atomizing	20 hrs after EAS atomizing
	1	2	3	4	5	6	7
0	84.8 ± 3.3	98.6 ± 5.0*	73.4 ± 6.1	80.7 ± 8.3	110.7 ± 2.8*	76.7 ± 6.6	85.9 ± 4.2
0.33	122.5 ± 3.1	127.7 ± 8.3	129.5 ± 8.6	123.1 ± 6.6	161.9 ± 5.1*	117.8 ± 4.9	150.5 ±5.7*
1.0	135.4 ± 6.3	175.4 ± 4.5	148.9 ± 15.1	203.7±6.3	183.5 ± 1.2*	128.8 ± 10.5	150.8 ± 9.7

* - $p < 0.05$ compared to intact mice

Table 7 Spontaneous and Con A-stimulated IL-2 synthesis by T-spleen cells in mice exposed to PAEROSOL and in control mice (IU/ml)

Dose of Con A (μg/ml)	Mice groups						
	Intact mice	Exposed to 2% NaCl	Exposed to PAEROSOL for:			Positioned in the chamber:	
						4 hrs after EAS atomizing	20 hrs after EAS atomizing
			1 hour	4 hours	20 hours		
	1	2	3	4	5	6	7
0	1.4 ± 1.0	2.4 ± 0.3	2.5 ± 0.4	1.8 ± 1.1	3.1 ± 0.1	1.1 ± 1.1	3.8 ± 0.4*
1.0	16.4 ± 2.0	15.9 ± 0.5	16.1 ± 1.6	10.5 ± 3.2	13.9 ± 0.5	18.2 ± 2.3	30.8 ±0.6*
5.0	170.3 ± 9.5	190.2 ± 30.2	146.4 ± 18.5	165.4±18.5	201.2 ± 17.5	166.3 ± 11.5	188.7± 4.7

- $p < 0.05$ compared to mice exposed to NaCl aerosol

As can be seen from Table 6, a significant increase of the spontaneous proliferation was revealed in mice of Group 5 and Group 2, compared to that in intact mice. A significant increase of the Con A-stimulated proliferation was found in mice of Group 5 and Group 7, compared to intact mice. In mice exposed to PAEROSOL for different time intervals, the stimulated proliferation increased in accord with increased time. However, no significant differences were revealed between mice exposed to PAEROSOL and those exposed to NaCl aerosol (Group 2), suggesting that the above changes were nonspecific for PAEROSOL. We think this might be due to general stress effect of mice exposure that caused the proliferation increase.

A significant increase of the spontaneous and Con A-stimulated (1 μg/ml) IL-2 synthesis by T-spleen cells was found compared to that in mice exposed to NaCl aerosol. The mechanism of this effect requires further investigation; however, it cannot be attributed to PAEROSOL toxicity. As known, toxic effects usually cause a reduction of spontaneous and Con A-stimulated (1 μg/ml) IL-2 synthesis by T-spleen cells

According to the data presented in Table 6 and Table 7, the PAEROSOL had no toxic effect on the immune cells because the values of spontaneous and Con A (1 μg/ml)-stimulated cell proliferation and of IL-2 synthesis have not reduced in mice exposed to PAEROSOL, which is characteristic for toxic effect.

4. CONCLUSION

The results of the study of the PAEROSOL toxicity conducted in the conditions simulating the PAEROSOL application for volumetric disinfection of enclosed environment, revealed no toxic effects in mice exposed to the PAEROSOL during different time and those positioned in the aerosol chamber after visual aerosol disappearance.

The results of the study demonstrated that: the animal behavior and general conditions, the state of skin and hair, the body and the internal organs weight were not distorted after a contact with PAEROSOL. The macroscopic and microscopic examination of lung tissues revealed no pathology. All hematological parameters measured in mice remained within physiological range.

No toxic effect of the PAEROSOL toward the immune system of mice was revealed.

The mechanism, contributing to T-spleen cell proliferation and IL-2 production increase shown in mice exposed to PAEROSOL during 20 hours, is not clear and requires further investigations.

References

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