TECHNICAL REPORT

Initiate a series of Paerosol toxicity studies at the Institute of Highly Pure Biopreparation, and the Institute of Influenza (WHO Center for Influenza in Russian Federation), Saint Petersburg, Russia.
Conduct relevant toxicity tests according to Russian toxicity evaluation requirements

There are number of publications indicating no toxicity of Electrochemically Activated Solutions (EAS). However, toxicity of Micro Aerosol of Electrochemically Activated Solutions (Paerosol) has been never studied.

MATERIALS AND METHODS

Animals

Outbreed white mice males were purchased from "Rappolovo" Animal Breeding Facility, Leningradskaya reg. Russia.

Prior to the study, all animals were kept in quarantine for 14 days with daily observation for behavior and general condition, and twice per day - for sickness and death rates. After quarantine, only proper animals (healthy and with appropriate weight) were randomly split into experimental and control groups.

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 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.

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.

Experimental set-up

Mice were deprived of feed and water for 2 hours prior to the beginning of the experiment. weighed and randomly spread among the groups:

1. The groups received Electro-Activated Solutions (EAS) per oz consisted of 3 sub-groups, each of 9-10 mice. EAS was administered through the medicinal probe daily during 7 consecutive days. Each sub-group received different concentration of EAS day

- 0.5 ml of liquid EAS.
- 0.5 ml of twice diluted with water EAS
- 0.5 ml of 10 times diluted with water EAS

Control groups (9-10 mice each) consisted of two sub-groups:

- "water" control – received 0.5 ml of distilled water daily during 7 days *per oz*

- "NaCl control" – received 0.5 ml of 1% NaCl in distilled water daily during 7 days *per oz*

2. ***The groups exposed to PAEROSOL*** consisted of 3 sub-groups. each of 5 mice.
   PAEROSOL was generated in the presence of mice on the first day of the experiment
   - Group Aer 1: 100 ml of PAEROSOL was generated during 1 min inside the chamber of 109ft^3 in the presence of mice.
   - Group Aer 2: 500 ml of PAEROSOL was generated during 5 min inside the chamber of 109ft^3 in the presence of mice.
   - Group Aer 3: 1500 ml of PAEROSOL was generated during 5 min inside the chamber of 109ft^3 in the presence of mice.

   Control groups (5 mice each) consisted of two sub-groups:
   Aerosols were administered during 15 min on the first day of the experiment
   - "Water control": 1500 ml of distill water was atomized during 5 min inside the chamber of 109ft^3 in the presence of mice.
   - "1% NaCl": 1500 ml of 1% NaCl solution (not electrolyzed) was atomized during 5 min inside the chamber of 109ft^3 in the presence of mice.

Experimental procedure: To administer PAEROSOL, mice were placed in a closed container. and positioned inside an aerosol chamber of 109ft^3. PAEROSOL for experimental groups, or other micro aerosols (see above), were atomized with Vortex atomizer VAG-2 at the rate 90-100 ml/min. Then, a container with mice was opened inside a chamber with the tool located outside the chamber. Each group of mice remained in a contact with PAEROSOL during 30 min. Calculated aspiration dose for group Aer 1 was 22.37 µl/mouse; Aer 2 – 102.26 µl/mouse; and Aer 3 – 308.97 µl/mouse.
All groups were observed during 14 consecutive days, beginning from day 1 of the experiment. Every mice were daily monitored for: general condition, behavior, motor activity, convulsions, irritant reactions, state of hair and skin, and changes in body weight.
On day 14 of the experiment, blood was collected by orbital puncture under anesthesia, and animals were euthanized.

Pathology

Euthanized mice were inspected externally; subjected to necropsy, and abdominal and thoracic internal organs undergo extensive examination. Some individual organs were weighed.

Hematology

- Hematological parameters included white blood cell count (WBC) and blood count based on visual evaluation of blood smears prepared and stained by Romanovsky.
- EAS and PAEROSOL (liquid and aerosol. correspondingly) effects on phagocytes activity in whole blood were studied by luminol-dependent chemiluminescence (CL). Whole blood samples were used to measure the levels of spontaneous and the opsonized zymosan-induced luminol-dependent CL.

Determination of immune parameters

EAS and PAEROSOL effects on spontaneous and Con A-induced proliferation of thymus and spleen cells was assayed. Spleen and thymus were taken under the aseptic conditions from euthanized mice. Organs from mice of the same group were pooled and cut with scissors. The cell suspension in sterile PBS was filtered through double gauze and centrifuged. Then, the cells were twice washed with PBS (in the spleen cell suspension erythrocytes were first lysed with 0.83% solution of NH_4Cl), re-suspended in RPMI-1640 medium (Sigma) with 2.0 mM of L-glutamine, 50 μM of 2-mercaptoethanol and 20 μg/ml of gentamycine (complete medium) to count cell number.

In 4 parallels, thymus (1 x 10^6 cell/well) and spleen cells (5 x 10^5cell/well) were incubated in 96-well flat-bottomed culture plates (Costar) with either 0 (spontaneous proliferation), 0.33, or 1.0 μg/ml of Con A (Sigma) in a complete medium containing either 2% or 10% of FCS (Sigma), respectively. Incubation was conducted in a CO_2 - incubator for 72 hours at 37^0 C, and at absolute humidity. 24 hours prior to completion of incubation, ^{3}H-labeled thymidine (5 μCi/ml) was added, the cells were transferred with a semi-automatic harvester (Flow Lab) on fiberglass filters of GF/C type (Whatman). The intensity of ^{3}H-labeled thymidine inclusion was measured with a scintillation liquid β-counter RackBeta 1217 (Wallac) and expressed as the number of pulses per a minute (cpm).

Statistical analysis

Statistical analysis of the data received with experimental and control mice was performed using Microsoft Excel 2003. Unless otherwise specified, the meanings were presented as mean ± StD and the level of significance was set at $p < .05$. The differences were calculated using two-way unpaired Student's *t*-test with unequal deviations.

RESULTS

General toxicity

The experimental and control groups of mice were examined during 14 days after administering EAS as a liquid (*per oz*) and as PAEROSOL (aerosol). No changes in mice behavior, including appetite, the conditions of skin and hair were observed.

The changes in mice body weights after exposure to liquid EAS and PAEROSOL are summarized in Tables 1 and 2.

Table 1. Dynamics of mice weight after liquid EAS administering *per os*

Groups	Control (water)	1% NaCl	EAS 1:10	EAS 1:2	EAS undiluted
Prior to experiment	16.8±1.3	16.7±0.7	17.3±1.8	16.1±1.3	18.4±1.1
Day 3 Average weight change (%) of initial weight	20.0±1.6 +18.9% **	21.8±1.0 +30.0% **	20.8±1.7 +20.2% **	20.2±2.0 +25.3% **	22.6±1.5 +22.7% **
Day of sacrifice Average weight change (%) of initial weight	18.8±2.2 +11.6% *	21.9±2.0 +30.7% **	20.6±2.1 +18.9% **	20.4±3.0 +26.7% **	22.5±1.2 +22.0% **

* p<0.05 and ** p<0.01 vs. initial weight in a corresponding group

Table 2. Dynamics of mice weight after PAEROSOL administering

Groups	Control (water). aerosol	Control. 1% NaCl aerosol	Aer 1	Aer 2	Aer 3
Prior to experiment	25.74±2.5	26.9±3.5	19.2±1.2	17.4±2.4	17.5±0.9
Day 3 Average weight change (%) of initial weight	25.72±2.2 +0%	27.58±3.4 +2.5% **	22.6±1.1 +17.5% **	20.6±2.9 +18.1% **	20.7±1.1 +17.9% **
Day of sacrifice Average weight change (%) of initial weight	25.78±1.7 +0.23% *	27.64±3.6 +0.22% *	20.6±2.1 +18.9% **	20.4±3.0 +26.7% **	22.5±1.2 +22.0% **

* p<0.05 and ** p<0.01 vs. initial weight in a corresponding group

Within the first 3 days after the beginning of the experiment, reliable increase in body weights was observed in all groups received EAS liquid and exposed to PAEROSOL. No

additional weight increasing was registered at the end of the experiment, after 14 days of observation. No difference in weight increase dynamics was observed among the groups received different concentration of EAS liquid, as well as exposed to different concentration of PAEROSOL. Control mice (Table 2) have not demonstrated increase in body weight and this could be attributed to significant initial weight of control mice in comparison with experimental mice. So, these results showed no weight variations. The difference in the initial weight between control and experimental mice was taken in account for accurate calculations; the organ weight was expressed the as a percentage of body weight**.**

Visual inspection of mice in all the groups after completion of the experiment. demonstrated no changes in: animal build, feed consumption, excreta, hair, and skin. Visually inspected mucous membranes were normal.

Necropsy results revealed no exudates in thoracic and abdominal cavities, and anatomic position of internal organs was normal. The following organs were inspected and found in normal conditions: submaxillary lymph nodes, salivary, thyroid and thymus glands; aorta intima and heart; trachea and large bronchi lumen, lungs; esophagus mucous membranes and stomach; large and small intestine, rectum; pancreas; kidneys and adrenal glands; urinary bladder and testicles. No pathological changes were observed in brain.

However, noticeable changes in liver and spleen were observed in all experimental groups exposed to EAS and PAEROSOL, and in control groups exposed to NaCl (non-electrolyzed). The liver was pale and increased in size. The spleen was of moderate filling and longer than normal. There were no changes observed in “water control”; livers and spleens of these mice were of normal size and color. Table 3 shows the weight of some internal organs expressed as a percentage of a body weight.

Table 3. The organ weights expressed as a percentage of body weight in control and experimental mice

	Percentage liver/body weight				
Mice groups	Lung	Heart	Liver	Thymus	Spleen
Control "water"	0.95	0.46	4.60	0.363	0.711
1%NaCl	0.92	0.53	6.15*	0.341	0.922*
EAS 1:10	0.93	0.50	6.15*	0.394	0.835
EAS 1:2	0.85	0.49	5.68*	0.355	0.840
EAS undiluted	0.93	0.50	5.65*	0.379	1.051*
Control "MA of water "	0.93	0.53	5.45	0.231	0.553
Control " MA of 1% NaCl"	0.89	0.52	5.61	0.237	0.644
Aer1	0.83*	0.54	5.38	0.365	0.325
Aer 2	0.83*	0.49	6.18	0.332	0.663
Aer 3	0.75*#	0.44	5.52	0.368	0.538

* - $p < 0.05$ compared to "water" control

- $p < 0.05$ compared to control receiving 1% solution of NaCl

All the groups received EAS orally, including the control group that received 1% NaCl, demonstrated significant increase in liver weight in comparison with a control group received water. Noticeable increase in spleen weight compared to that in "water" control group was observed only in the group received undiluted EAS orally (Table 3).

The difference in liver weight observed in the mice exposed to PAEROSOL was insignificant, and seemed to be data scattering rather than reliable difference. For instance, noticeable difference is seen between controls groups received "MA of water"/"MA of %NaCl", and Aer2 group, which received PAEROSOL in concentration 102.26 µl/mouse. In the same time no difference is seen between control groups and groups Aer1 and Aer3, which received PAEROSOL in concentration 22.37 µl/mouse and 308.97 µl/mouse, correspondingly (Table 3). No increase in spleen weight was revealed in all groups exposed to PAEROSOL.

In groups Aer 1, Aer 2, and Aer 3, noticeable decrease in lung mass was identified, compared to that in "water" control group. In group Aer 3, this decrease was also observed compared to that in NaCl aerosol control. Visual inspection of the lungs in this group revealed no distinctions. There were no significant variations in heart and thymus weights among the groups.

Hematology

Data of quantitative and qualitative analyses of peripheral blood are presented in Table 4.

Table 4. Peripheral blood parameters

Groups	Total number of WBC ($x10^6$/ml)	Blood parameters. %						Absolute lymph. ($x10^6$/мл)	Absolute neutrophils ($x10^6$/мл)
		Lymphocytes	Neutrophils	PMN	Segmented	Eosinophils	M_o		
Control water	5.2±1.5	72.6±8.1	25.6±3.5	2.4±2.1	25.6±7.0	0	1.8±1.3	3.7±1.1	1.3±0.7
1%NaCl	3.6±1.1	67.0±6.8	30.4±6.2	2.6±1.3	27.8±5.2	0.8±0.8	1.8±1.1	2.4±0.9	1.1±0.3
EAS 1:10	3.6±0.9	66.4±7.0	31.6±7.2	3.2±1.5	28.4±7.1	1.3±1.5	1.0±0.7	2.4±0.5*	1.2±0.5
EAS 1:2	3.3±0.8	59.0±9.7*	40.0±8.7*	4.4±2.5	35.6±7.4*	0.6±1.3	0.4±0.9	2.0±0.6*	1.3±0.4
EAS undiluted	2.8±0.4*	65.6±5.8	32.2±6.3	4.0±4.2	28.2±7.0	1.0±0.7	1.75±1.5	1.8±0.3*	0.9±0.2
Control. water aerosol	5.3±1.7	75.0±3.4	23.2±5.1	3.4±1.0	19.8±3.3	0.8±0.4	1.0±0.5	4.0±0.2	1.2±0.2
1% NaCl aerosol	3.2±1.0	71.2±3.3	26.2±3.5	2.4±0.7	23.8±2.7	0.6±0.5	2.0±0.9	2.2±0.1*	0.8±0.1
Aer 1	2.7±1.0*	65.6±5.8	32.2±6.3	4.0±4.2	28.2±7.0	2.0±1.9	0.2±0.4	1.7±0.6*	0.9±0.4
Aer 2	3.2±0.7	68.0±8.6	30.6±6.5	4.2±1.9	26.4±6.3	0.4±0.9	1.0±1.7	2.2±0.7*	1.0±0.2
Aer 3	2.4±0.5*	66.4±7.1	29.8±5.9	2.0±2.3	27.8±4.4	1.8±1.3	2.0±1.0	1.57±0.2*	0.7±0.3

*- $p < 0.05$ as compared to that in 'water" control. both oral and aerosolized

The experimental groups received liquid EAS and exposed to PAEROSOL, and control groups received NaCl orally, or as an aerosol, demonstrated noticeable decrease in the total number of peripheral WBC, compared to that in "water" control group. This decrease was statistically true in the group received undiluted EAS orally and in Aer1 and Aer3 groups exposed to PAEROSOL. Correspondingly, there was a significant decrease in the percentage of lymphocytes and an increase in the percentage of neutrophils without a "left shift" in the WBC count. The absolute number of neutrophilic granulocytes remained almost the same in all the groups, but the absolute number of lymphocytes significantly reduced in all the groups received EAS and NaCl *per oz*, and in all groups exposed to MA of NaCl and to PAEROSOL, Thus, NaCl received by mouth and administered as microaerosol caused the same effects as EAS administered by mouth and as PAEROSOL.

The levels of spontaneous and opsonized zymosan-induced luminol-dependent CL measured in whole blood cells are summarized in Tables 5 and 6.

Table 5. Luminol-dependent chemiluminescence in whole blood samples taken 7 days after last administering of EAS liquid

Induction	Control "water"	Control 1% NaCl	EAS 1:10	EAS 1:2	EAS undiluted
Spontaneous	6.4±0.4	5.7±0.2	5.7±0.5	5.8±0.3	6.0±0.2
Zymosan	9.9±1.0	9.2±0.5	9.9±1.1	10.2±0.8#	10.7±1.1*

* $p<0.05$

Table 6. Luminol-dependent chemiluminescence in whole blood samples taken 14 days after mice exposure to PAEROSOL***

Induction	Control. MA of water	Control. MA of 1% NaCl	Aer 1	Aer 2	Aer 3
Spontaneous	5.7±0.4	5.7±0.2	6.0±0.3	5.6±0.4	5.8±0.6
Zymosan	6.7±0.1	7.7±0.4	9.7±1.2	8.9±1.2*	10.1±0.8**

*- $p<0.05$ compared to that in water control
** - $p<0.05$ compared to that in group exposed to 1 % NaCl
*** EAS aerosol was administered once during 30 min on the first day of the experiment

Practically no difference in level of spontaneous CL was observed among different experimental and control groups. Contrary, the level of zymosan-induced CL was somewhat higher in the groups received undiluted or twice diluted EAS orally. The groups exposed to EAS aerosol also demonstrated increase in Zymosan induced CL. While the results of chemiluminescent analysis are interesting and important itself, its interpretation is ambiguous today due to significant deviation in blood counts in different experimental and control groups. So that the results on chemiluminescent analysis should be considered as the data accumulated for the further, more detail study on EAS/PAEROSOL toxicity.

Study of immune parameters

Liquid EAS and PAEROSOL effects on spontaneous and Con A-induced proliferation of thymic and spleen cells was studied, because the immune cells, especially thymic cells, are known to be highly sensitive to different toxins. The results of this extensive and very laborious study are presented in the Tables 7 through 10.

Table 7. Effect of orally administered EAS on spontaneous and Con A-induced proliferation of thymic cells

	Water control	1% NaCl	EAS 1:10	EAS 1:2	EAS undiluted
Spontaneous	2372±1128	797±352	823±465	1198±1214	950±358
Con A 0.33	3620±1361	2045±314	2263±158	2183±714	1505±319*
Con A 1.0	53160±8170	37705±11085	48860±14509	38632±7529*	38114±5308*

* - $p < 0.05$ compared to water control

Table 8. PAEROSOL effect on spontaneous and Con A-induced proliferation of thymic cells

	Control MA of Water	Control MA of 1% NaCl	Aer 1	Aer 2	Aer 3
Spontaneous	2022±572	1559±287	505±139*#	703±305*#	884±115*#
Con A 0.33	6271±1311	6850±1678	1846±489*#	2715±1105*#	5515±1455
Con A 1.0	73622±24285	74444±8084	65454±20605	81641±6119	100204±10697*.#

* - $p < 0.05$ - $p < 0.05$ compared to water control
# - $p < 0.05$ compared to control with 1% NaCl

Table 9. Effect of orally administered EAS on spontaneous and Con A-induced proliferation of spleen cells

	Control "Water"	Control 1% NaCl	EAS 1:10	EAS 1:2	EAS Undiluted
Spontaneous	35.947±8231	15.904±1332*	14.719±2420*	12.713±2005*.#	13.965±2292*
Con A 0.33	56.429±9011	36.990±4659*	44.013±6381	32.584±1314*	45.445±3206#
Con A 1.0	83583±6072	95783±12137	95725±11833	83233±6883	102638±11384*

*- $p < 0.05$ compared to that in water control;
*# - $p < 0.05$ compared to that in control exposed to 1% NaCl
< 0.05 compared to water control

Table 10. PAEROSOL effect on spontaneous and Con A-induced proliferation of spleen cells

	Control MA of Water	Control MA of 1% NaCl	Aer1	Aer2	Aer3
Spontaneous	17011±2451	8648±701*	19604±937#	12746±4074	11611±2568*
Con A 0.33	138100±14411	89442±5605*	60867±7012*#	38308±6064*#	47673±5071*#
Con A 1.0	125650±14117	124500±18571	102146±13653	109750±6781	95921±8961*

* - $p < 0.05$ compared to that in water control
# - $p < 0.05$ compared to that in control exposed to 1% NaCl

To summarize the data presented in Tables 7 through 10 the following could be said:

1. Spontaneous proliferation of thymic cells was within normal, though in "water" control the level was above expected.
2. Spontaneous proliferation of spleen cells was significantly lower in all groups received NaCl as liquid, as M, as EAS, and as PAEROSOL compared to that in "water" control. However, in "water' control this parameter was higher than that expected for intact animals.
3. Con A-induced proliferation of thymic cells was lower in the groups exposed to oral undiluted and twice-diluted EAS, compared to that in "water" control.
4. Con A-induced proliferation of thymic cells in the groups exposed to PAEROSOL was lower than in the control groups and seemed to positively correlate with the time of mice exposure to aerosol (volume per Capito).
5. Proliferation of spleen cells stimulated with the suboptimal dose of Con A was significantly lower in the group received twice-diluted EAS, in all groups exposed to PAEROSOL, and the group exposed to oral NaCl, compared to that in "water" control;
6. The proliferation of spleen cells stimulated with the optimal dose of Con A was significantly higher in the group received oral undiluted EAS, while was significantly lower in Aer 3 group, compared to that in "water' control.

The lack of consistency in the results presented in Tables 7 through 10 does not allow for a strong conclusion about EAS and PAEROSOL toxicity. In spite of reliable differences in levels of spontaneous and Con A-induced proliferation of thymic and spleen cells in the groups received liquid EAS orally and exposed to PAEROSOL, no distinct dose-dependent relationships were observed. At the same time, the fact that individual parameters in experimental and control groups were noticeably different suggested that there was a certain "toxic" effect posed by EAS/PAEROSOL. However, this effect was very similar to that observed on the mice received either NaCl *per oz*. or exposed to an MA of NaCl.

CONCLUSION

1. First study was conducted to reveal toxic effects of EAS administered as liquid and PAEROSOL. No severe, statistically proved health effects were shown on mice.
2. The results of the study demonstrated that liquid EAS (electrolyzed 1%NaCl) administered orally caused no negative effect on mice behavior, appetite, and general conditions, while possibly caused moderate lymphopenia and had some effect on liver,

spleen and lung. The effects of EAS administered *per oz* were similar to the effects caused by MA of 1% NaCl (not electrolyzed NaCl).

3. PAEROSOL directly administered to experimental mice caused no sever effect on mice health conditions. The results of the study demonstrated that PAEROSOL caused no negative effect on mice behavior, appetite and general conditions, while possibly caused moderate lymphopenia and had some effect on liver, spleen and lung. However, the effects caused by PAEROSOL were similar to the effects caused by administering of MA of 1% NaCl aerosol (microaerosol of not electrolyzed 1%NaCl).
4. The variety of dubious data was observed during biochemical studies. Some effects were shown but its interpretation was not possible, because of significant lack in results consistency. Some data were in contradiction- showed no dose dependence and so that couldn't be interpreted as well.