FINAL REPORT

BIOLOGICAL EFFECTS OF LOW INTENSITY ELECTROMAGNETIC FIELDS AND ELECTROSTATIC MASSAGE IN THE MODEL OF QUARTZ-INDUCED ACUTE LUNG INFLAMMATION

Institutions-participants – Dept. Molecular Biology, Russian State Medical University, Moscow, Russia

Scientific supervisor –Professor Ludmila Korkina, MD, PhD, Dr. Sci., Dept. Molecular Biology, Russian State Medical University, e-mail: korkina_l@mail.ru el/Fax: +7095 434 8740 (office) and Tel/Fax: +7095 438 5237 (home)

BACKGROUND AND OBJECTIVES

The physiotherapeutical device HIVAMAT 200 manufactured by PHYSIOMED targets to treat a number of human disorders basing on the biological effects of low intensity (non-thermal) electro-magnetic radiation (LIEMR) and electro-mechanical action of static electricity as a tissue massage. The numerous positive clinical effects have been shown such as improved lymphatic drainage and venous insufficiency, improved functions of pulmonary, gastrointestinal and neuro-muscular systems. HIVAMAT has beenwidely used as a pain killer, anti-edematous and anti-inflammatory medical device [1, 2, 3].

For the time being, the common scientifically proven opinion considers that the biological LIEMR effects occur through several pathways including nervous, endocrine, reproductive, and immune systems [4,5]. It has been shown previously that LIEMR led to modulation of both humoral and cell-mediated immune reactions in both human and animals. At the cellular level, the major targets for LIEMR are granulocytes, macrophages, and then, T- and B-lymphocytes [6]. Some data have demonstrated that LIEMR affected the conformation of

proteins on the cellular surface. Due to it, the interaction between receptors and agonists was modulated. The changes in the receptor-agonist interaction inevitably lead to the modulation of signal transferring and responsive induction of nuclear factors and enzymes [7]. Calcium transporting system was recognized as a sensor of the interaction of electro-magnetic field with living matter [8]. As a result, the main cellular reactions such as oxygen and nitric free radical, inflammatory mediators production, ATP synthesis and protein phosphorylation are effected by LIEMR [9].

On the other hand, imbalance of all above mentioned cellular processes seems to play a key role in the pathogenesis of small airway and pulmonary diseases such as asthma, chronic obstructive pulmonary disease, acute and chronic lung inflammation, interstitial fibrosis, induced, for example, by inhaled dust particles. One of the most spectacular examples of such agents are crystalline silica (quartz) leading to acute lung inflammation, lung fibrosis (silicosis), and lung cancer.

Inflammatory cells like alveolar macrophages, granulocytes, and lymphocytes are thought to be crucial in the pathogenesis of quartz-induced and some other inflammatory lung diseases. In asthma, for example, the increase in the number of eosinophils correlates with the development of bronchial hyper-responsiveness and following constriction [10]. In the lung inflammation and obstructive bronchitis, neutrophils appear to play a leading role since their number is highly increased, and the release of a variety of inflammatory mediators including the most deleterious hydroxyl radicals and peroxynitrite has been reported in numerous publications [11].

Recent findings have indicated that these inhaled quartz particles themselves can cause the production of reactive oxygen species (ROS) that leads to the depletion of antioxidants causing oxidative stress in the epithelial cells in the lung and that this is an important mechanism for the initiation of inflammation [12]. Following this initial oxidative stress, the epithelial cells produce inflammatory mediators that cause infiltration of inflammatory cells, such as

macrophages and neutrophils in the lung tissue. The inflammatory cells that migrate to the lung may eventually contribute further to the oxidative stress at the site of particle deposition and, in the case of generalization of the process on systemic level – in the blood. The major functions of macrophages are to engulf and digest pathogens (phagocytosis). During the complex process of phagocytosis, macrophages generate ROS and proteases and control the extracellular environment through the release of cytokines. The oxidative stress that arises in epithelial cells from overproduction of ROS may initiate damage to cell membranes in the form of lipid peroxidation. As a result, a depletion of the major antioxidant in the lung, reduced glutathione (GSH) occurs. Oxidative stress leads to activation of redox sensitive transcription factors such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) that control the transcription of pro-inflammatory and proliferative genes [13]. Thus, inflammation started from quartz-induced oxidative stress contributes to fibrosis and cancer.

In the present work, some cellular and molecular effects of low intensity electromagnetic fields and electrostatic massage provided by HIVAMAT 200 were studied in the well-established experimental model of quartz-induced acute lung inflammation

MATERIALS AND METHODS

Equipment under investigation: HIVAMAT 200 Physiomed Elektromedizin, Germany.

Reagents of analytical grade ***and quartz particles*** were purchased from Sigma Co. (Italy).

Animals. 28 male Wistar rats were maintained under controlled environmental conditions with a 12 h dark/light cycle. Food and water were available ad libitum.

Study design. Animals were divided into three groups: normal (6 animals), control (12 animals) – with quartz inflammation, experimental (12 animals) – quartz + HIVAMAT treated. When 7 week old (weighing 180-220g), animals were lightly anaesthetized with ethyl ether and intratracheal instillation of quartz (50 mg/ml; 1 ml per rat) was performed. Normal rats were

instilled with only PBS. Animal of experimental group were treated with HIVAMAT (3 min: 60 sec rise from 20 to 80 Hz, 60 sec – 80 Hz, 20 sec return to 20 Hz). The procedure was repeated daily from the second till last day of experiment without any anaesthesia.

At day 4 were analyzed six animals from control and six animals from experimental groups. At day 9 – six animals from each group including normal. At day of analysis animals were given ether anaestesia. Then, the lungs were cannulated via the trachea and perfused for 10 times with 5 ml aliquots of PBS, and bronchoalveolar lavage (BAL) was obtained. The BAL fluid was drained passively by gravity and the procedure was repeated two times, giving a total BAL volume of 10 ml. Then animals were sacrificed, and blood and lung tissue were collected. BAL fluid was centrifuged twice: 400 g, 15 min to collect cells, followed by 1000 g to obtain supernatant which was analyzed for lung injury parameter (total protein), MDA content and GPx activity. Lung tissue was homogenized, and the MDA and GSH content were analyzed. Part of the blood was used for CL analysis, plasma was separated by centrifugation (400 g, 15 min) and the MDA content was determined.

Methods of BAL analysis

a) Number of neutrophils in BAL cells

Total cell number per ml of the BAL fluid was calculated using hemocytometer chamber and viability was assessed by trypan blue dye exclusion.

b) Level of protein in BAL

Total protein was analysed according to the method described by Lowry [14] using Sigma Kit no.690-A.

c) Determination of MDA in BAL

The estimation of MDA was carried out by the TBARS method [15]. TBA reacts with MDA, one of the aldehyde products of lipid peroxidation in BAL, to give a colored product which was extracted in butanol and absorbance measured spectrophotometrically at 530 nm. Briefly, to 0,1 ml of BAL, 1,5 ml of orto-phosphoric acid and 0,5 ml of 0,2% TBA were added. The mixture

was heated in a boiling water bath for 30 minutes. After cooling, the resultant chromogen was extracted with 2 ml of n-butyl alcohol and the separation of the organic phase was done by centrifugation at 800 g for 10 minutes. Absorbance of the butyl alcohol extract of samples was measured at 532 nm against distilled water as blank. The concentration MDA in the sample was calculated taking into account the value of molar extinction coefficient ($1{,}56 \times 10^5$).

d) Activity of glutathione peroxidase (GPx) in BAL

Lavage GPx activity was determined using method [16]. This method is based on that of GPx catalyses the oxidation of gletathione by tert-butyl-hydroperoxide in the presence of glutathione reductase and NADPH. The oxidised gletathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured. GPx activity was expressed in as units/mg protein.

Method of lung tissue analysis

a) Determination of GSH level in lung tissue

GSH concentration in lung tissue was analyzed using method [17]. According to this method GSH reacts with Ellman's reagent (5,5'-dithiobis(nitrobenzoic acid) or DTNB) to produce a chromophore TNB with maximal absorbance at 412 nm. The concentration of GSH was determined by calculating from the linear equation generated from several standards of glutathione and expressed as umol GSH/mg protein.

Methods of blood analysis

a) Evaluation of chemiluminescence (CL) of whole blood

Whole blood generated oxygen species were studied by luminol-dependent chemiluminescnce [18]. 10 µl of freshly drawn human blood were mixed with 0.980 ml of Hanks' balanced salt solution containing 5×10^{-5} M luminol and spontaneous CL was determined. The free radical generation was induced by adding 10µl of PMA (final concentration, 10 nM). The chemiluminescence response to the phorbol ester was registered continuously for 30 min at $t^o=37^oC$ and stirring. The results were expressed as relative units.

b) Determination of MDA in plasma was carried out as for BAL, but 100 ul of plasma instead of 100 ul of BAL was taken to analyze.

RESULTS AND DISCUSSION

Inflammatory Activity of Quartz and Effect of HIVAMAT 200

Neutrophils migrate in the lung tissue when inflammation is started. As shown in Table 1, quartz induced more than 8-fold increase of neutrophils content (expressed as percent of PMN in BAL total cell number) of the control animals as compared to normal value. The number of neutrophils in BAL demonstrated the significant inflammatory potential of the quartz particles ($p<0.05$), which had maximal value at 4^{th} day of the experiment and remained at an almost constant level until the end of observation. HIVAMAT 200 treatment resulted in remarkable decreasing of lung inflammation: neutrophils percentage dropped in 1,6 times at 4^{th} day and in 3,36 times at 9^{th} day of the experiment.

Table 1. Number of neutrophils in BAL cells of normal, quartz and quartz+HIVAMAT-treated animals.

Group	Neutrophils contents, %	
	4 day	9 day
Control	39.1±11.0*	36.8±6.3*
Experimental	24.5±6.5*,**	11.5±3.1*,**
Normal	4.5±2.1	

*p<0.05 vs. norma

**p<0.05 vs. control group

Protein Level in BAL

Another sign of inflammation is a protein level in BAL. The results of its analyses are collected in Table 2. We registered that quartz inhalation leads to accumulation of the protein in lavage

fluid which depend on increased permeability of blood vessels induced by inflammatory mediators. Level of protein in BAL increased during the experiment. It is necessary to stress that there was significant difference between the animals treated with quartz alone and quartz+HIVAMAT. Investigated equipment had ability to reduce pathological effect of quartz but not to normal value.

Table 2. Protein in BAL

Group	Protein in BAL, mg/ml	
	4 day	9 day
Control	0.42±0.27*	0.66±0.20*
Experimental	0.31±0.19*,**	0.49±0.21*,***
Normal	0.29±0.14	

*p<0.05 vs. norma

**p<0.05 vs. control group

*** p=0.08 vs. control group

Since our study of the neutrophils number and protein level in BAL of control animals indicated that quartz induced strong inflammatory reaction in lung tissue and HIVAMAT 200 significantly reduced this process we analyzed redox status of organism of the experimental animals because well known that it must reacts to ROS-production during the inflammation. Under normal circumstances, there is a delicate balance between ROS formation and antioxidant defenses in lung. When the generation of ROS is overwhelming, as in the case of quartz exposure, or the antioxidant defense mechanism is impaired, an oxidative stress is induced, resulting in cell injury.

GSH content in lung tissue.

GSH is among the most important antioxidants in organism due to its potent antioxidant capacity, close involvment in many cellular functions, and abundance in tissues and cells [19]. It

is well known that GSH plays an important role in the antioxidant mechanism in lung. For instance, it has been noted that the epithelial lining fluid of normal lungs contains very high concentrations of this tripeptide, - 100 times higher than that found in the extracellular fluid of many other tissues [20]. Some recent studies showed the involvement of GSH in silica-induced lung injury [21]. Our data indicated that lung tissue had depleted GSH content after quartz inhalation (Table 3). HIVAMAT 200 leaded to the rise of GSH level and therefore strengthened the antioxidant defense on the organ level.

Table 3. Concentration of GSH in lung tissue

Group	GSH in lung tissue, umol/mg protein	
	4 day	9 day
Control	5.11±0.64*	5.05±0.21*
Experimental	7.55±1.16*,**	7.94±0.88*,***
Norma	9.72±1.10	

*p<0.05 vs. norma

**p=0.02 vs. control group

***p<0.05 vs. control group

Activity of Glutathione Peroxidase in BAL

Mechanism of defense action of GSH tightly connects to activity of GPx. This enzyme together with GSH participates in the lipid peroxides utilization. Table 4 illustrates a statistically significant decrease of the GPx activity in the control group at 4^{th} day after quartz exposure. HIVAMAT 200 improved the situation and increased GPx activity but not to normal value.

Table 4. Glutathione peroxidase (GPx) in BAL

Group	GPx, U/mg protein	
	4 day	9 day

Control	0.10±0.06*	0.23±0.11
Experimental	0.15±0.14	0.18±0.07
Norma	0.27±0.12	

*p<0.05 vs. norma

Lipid Peroxidation in BAL and plasma

The result of disbalance between ROS production and processes of ROS neutralization in lipid phase of lung tissue is a peroxidation of its lipids. In our study we found that quartz activated peroxidation and induced accumulation of the peroxidation products in BAL fluid and, in the case of deep disturbance, in the blood. All animals from the control and experimental groups had very high content of MDA (more then 5 times as compared to) both in BAL and in blood at 4^{th} day of the observation. By the end of the experiment level of lipid peroxidation did not differ significantly of normal value (Tables 5 and 6).

Table 5. MDA in BAL

Group	MDA, umol/g protein	
	4 day	9 day
Control	2.15±1.14*	0.49±0.06
Experimental	2.42±0.45*	0.68±0.29
Normal	0.48±0.14	

*p<0.05 vs. norma

Table 6. MDA in plasma

Group	MDA, umol/ml	
	4 day	9 day
Control	2.01±0.60*	1.34±0.16*

Experimental	1.95±1.02	1.55±0.26*
Normal	1.07±0.26	

*p<0.05 vs. norma

ROS-generating activity of whole blood

In this study, luminol-dependent CL test was used for the determination of free radical formation in blood. The results are summarized in Fig.7. As shown, quartz induced more then 4-fold increase of CL value that means significant rise of ROS-production by blood cells. Treatment of experimental quartz-inhaled animal with HIVAMAT 200 did not influence on such high level of free radical formation.

Table 7. PMA-activated luminol-dependent CL of whole blood

Group	CL of whole blood, units	
	4 day	9 day
Control	162±58*	77±25
Experimental	206±104*	183±88
Normal	55±7	

*p<0.05 vs. norma

CONCLUSIONS

The present study showed that the physiotherapeutical device HIVAMAT 200 substantially suppressed both local inflammation in the lungs and redox imbalance induced by quartz in the lungs of experimental rats evidenced by the decrease of neutrophil content, protein level, and GPx activity in the BAL fluid as well as by the increase of GSH concentration in the lung tissue. At the same time, HIVAMAT 200 did not affect generalized oxidative stress in the blood.

HIVAMAT could be recommended for the treatment of acute lung inflammations of different origin in combination with conventional therapies.

ABBREVIATIONS

BAL - bronchoalveolar lavage, CL – chemiluminescence, DTNB - 5,5'-dithiobis(nitrobenzoic acid), GPx – glutathione peroxidase, GSH – glutathione, reduced, MDA – malonyl dialdehyde, PBS – phosphate buffer saline, PMN – polymorphonuclear cell, ROS – reactive oxygen species, TBA – thiobarbituric acid, TBARS – TBA reactive substances, TCA – trichliroacetic acid

REFERENCES

1. Aldrich T.E., Easterly C.E. Electromagnetic fields and public health. Environ.Health Perspect. 1987;75:159.

2. Lushnikov K.V., Shumilina Y.V., Yakushina V.S. et al. Effects of low-intensity ultrahigh frequency electromagnetic radiation on inflammatory processes. Bull Exp Biol Med. 2004; 137(4):364-6.

3. Johnson M.T., Waite L.R., Nindl G. Noninvasive treatment of inflammation using electromagnetic fields. Biomed Sci Instrum. 2004;40:469-74.

4. Walleczek J. Electromagnetic effects on cells of the immune system. FASEB J. 1992;6:3177.

5. Scardino M.S., Swaim S.F., Sartin E.A. Evaluation of treatment with a pulsed electromagnetic fields on wound healing. Am.J.Vet Res. 1998;59:1177-81.

6. Cadossi R., Bersani F., Cossarizza A. et al. Lymphocytes and low-frequency electromagnetic fields. FASEB J. 1992;6:2667.

7. Goodman R., Bassett C.A., Henderson A.S. Pulsing electromagnetic fields induce cellular transcription. Science, 1983;17;220(1603):1283-5.

8. Baureus Koch C.L., Sommarin M., Persson B.R. et al. Interaction between weak low frequency electromagnetic fields and cell membranes. Bioelectromagnetics. 2003;24(6):395-402/

9. Diniz P., Soejima K., Ito G. Nitric oxide mediates the effects of pulsed electromagnetic field stimulation on the osteoblast proliferation and differentiation. Nitric Oxide.2002;7(1):18-23.

10. C. Henderson, E. P. Ingenito, H. Atileh et al. Selected Contribution: How does airway inflammation modulate asthmatic airway constriction? An antigen challenge study. J Appl Physiol 2003;95: 873-882.

11. Atsushi Y., Mami Ohba, Xia Wu. Accumulation of platelets in the lung and liver and their degranulation following antigen-challenge in sensitized mice. British Journal of Pharmacology 2002;137: 146−152.

12. Anna Clouter[*,1], David Brown[*], Doris Höhr Inflammatory Effects of Respirable Quartz Collected in Workplaces versus Standard DQ12 Quartz: Particle Surface Correlates Toxicological Sciences 2001;63:90-98.

13. Chen F., Lu Y., Demers L.M. at al. Role of hydroxyl radical in silica-induced inflammatory mediator production in macrophages. Biochem.Biophys.Res.Commun. 1995;214:985-992.

14. Lowry at al. J.Biol.Chem. 1951;193:265-275.

15. Uchiyama M., Mihara M. Analyt.Biochem. 1978, v.86, 271

16. Greenwald R.A. Handbook of methods for oxygen radical research – CRC Press, Florida, 1986, 291-302.

17. Beutler E., Duron O., Kelly B.M. Improved method for the determination of blood glutathione. J Lab Clin Med, 1963; 61(5):882-8.

18. Korkina L.G., Samochatova E.V., Maschan A.A. et al. J Leuk Biol. 1992;52(3):357-362.

19. Meister A., Anderson M.E. Glutathione. Annu.Rev.Biocjem. 1983;52:711-760.

20. Van Klaveren R.J., Demedts M., Nemery B. Cellular glutathione turnover in vitro, with emphasis on type II pneumocytes. Eur.Respir.J. 1997;10:1392-1400.

21. Boehme D.S., Maples K.R., Henderson R.F. Glutathione released by pulmonary alveolar macrophages in response to particles in vitro. Toxicol.Lett. 1992;60:53-60.

Analysis of BAL

Table 1. Number of neutrophils in BAL cells (%).

Group	**Neutrophils contents, %**	
	4 day	9 day
Quartz	39.1±11.0*	36.8±6.3*
Quartz + HIVAMAT	24.5±6.5*,**	11.5±3.1*,**
Control	4.5±2.1	

*p<0.05 vs. control group

**p<0.05 vs. Quartz group

Table 2. Protein in BAL, mg/ml

Group	**Protein in BAL cells, mg/ml**	
	4 day	9 day
Quartz	0.42±0.27*	0.66±0.20*
Quartz + HIVAMAT	0.31±0.19*,**	0.49±0.21*,***
Control	0.29±0.14	

*p<0.05 vs. Control group

**p<0.05 vs. Quartz group

*** p=0.08 vs. Quartz group

Table 3. MDA in BAL, μm/g protein

Group	**MDA, μm/g protein**	
	4 day	9 day
Quartz	2.15±1.14*	0.49±0.06
Quartz + HIVAMAT	2.42±0.45*	0.68±0.29
Control	0.48±0.14	

*p<0.05 vs. Control group

Table 4. Glutathione peroxidase (GPx) in BAL, U/mg protein

Group	**GPx, U/mg protein**	
	4 day	9 day
Quartz	0.10±0.06*	0.23±0.11
Quartz + HIVAMAT	0.15±0.14	0.18±0.07
Control	0.27±0.12	

*p<0.05 vs. Control group

Analysis of lung tissue

Table 5. Glutathion (GSH) in lung tissue, μm/mg protein

Group	**GSH in lung tissue, μm/mg protein**	
	4 day	9 day
Quartz	5.11±0.64*	5.05±0.21*
Quartz + HIVAMAT	7.55±1.16*,**	7.94±0.88*,***
Control	9.72±1.10	

*p<0.05 vs. Control group

**p=0.02 vs. Quartz group

***p<0.05 vs. Quartz group

Analysis of blood

Table 7. Chemiluminescence of whole blood (CL) (luminol-PMA), arb. units

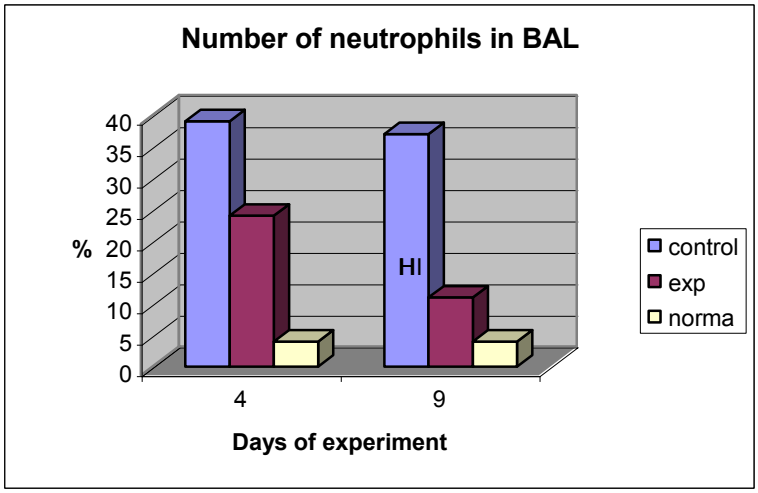
Group	**CL of whole blood, arb. units**	
	4 day	9 day
Quartz	162±58*	77±25
Quartz + HIVAMAT	206±104*	183±88
Control	55±7	

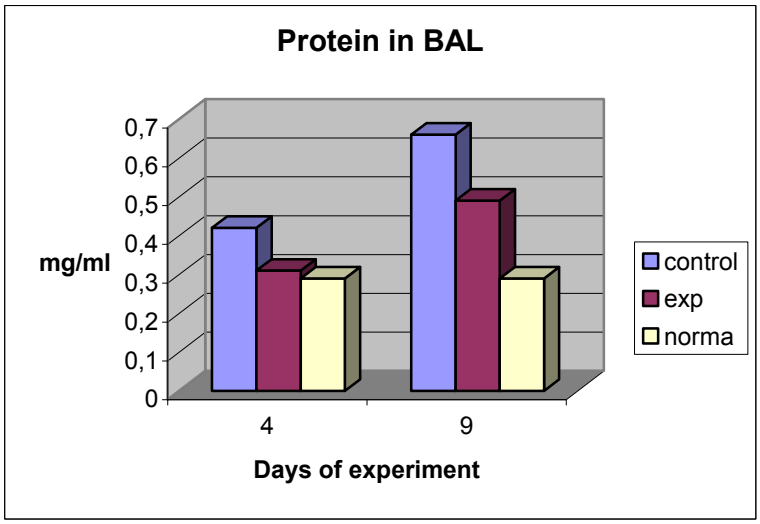
*p<0.05 vs. Control group

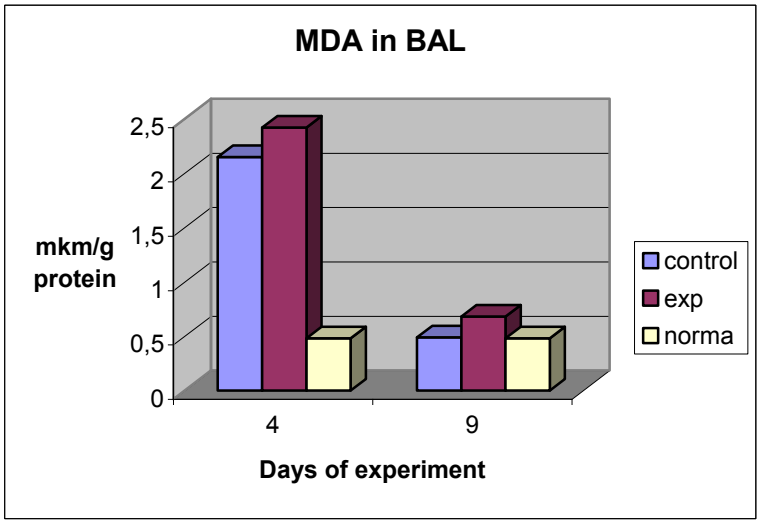
Table 8. MDA in plasma, μM

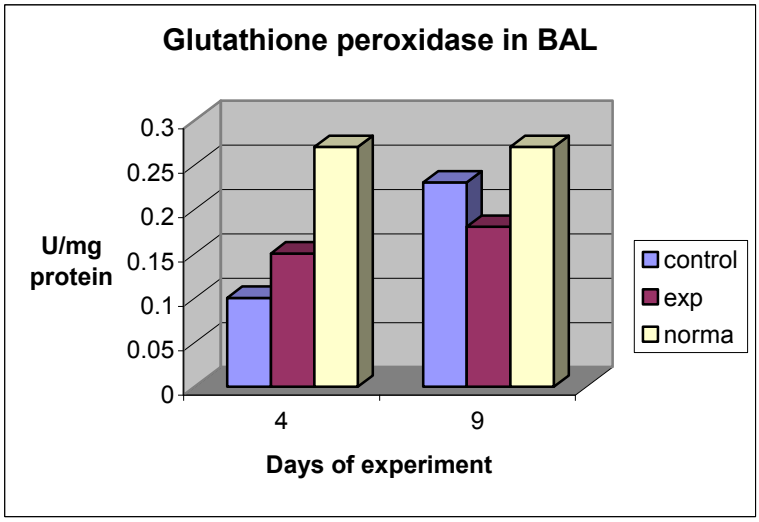
Group	**MDA, μM**	
	4 day	9 day
Quartz	2.01±0.60*	1.34±0.16*
Quartz + HIVAMAT	1.95±1.02	1.55±0.26*
Control	1.07±0.26	

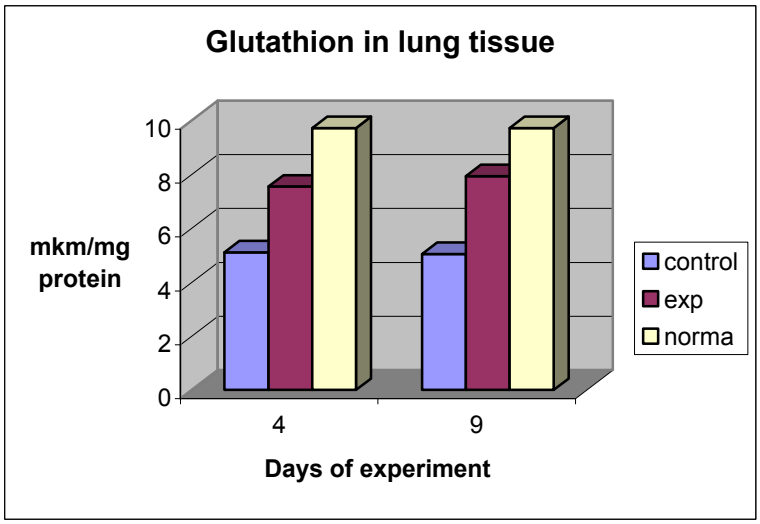
*p<0.05 vs. Control group

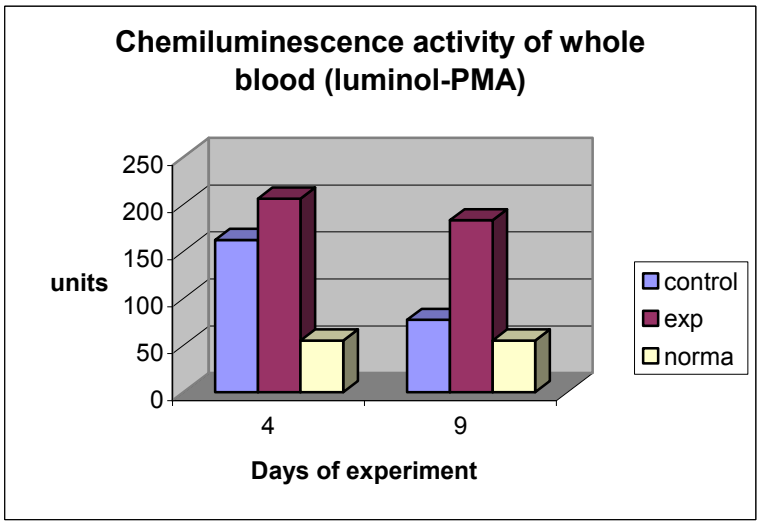

Number of neutrophils in BAL
40
35
30
25
% 20
15
10
5
0
HI
4
9
Days of experiment
control
exp
norma


Protein in BAL
0,7
0,6
0,5
0,4
mg/ml
0,3
0,2
0,1
0
4
9
Days of experiment
control
exp
norma


MDA in BAL
2,5
2
1,5
mkm/g
protein
1
0,5
0
4
9
Days of experiment
control
exp
norma


Glutathione peroxidase in BAL
U/mg protein
0.3
0.25
0.2
0.15
0.1
0.05
0
4
9
Days of experiment
control
exp
norma


Glutathion in lung tissue
mkm/mg protein
10
8
6
4
2
0
4
9
Days of experiment
control
exp
norma


Chemiluminescence activity of whole blood (luminol-PMA)
units
250
200
150
100
50
0
4
9
Days of experiment
control
exp
norma