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14. ABSTRACT

The results of the first phase of HD study suggested that exposure to HD increased lung damage in rats by increasing the levels of oxidative damage and pro-inflammatory cytokines, whereas antioxidant treatment before HD exposure reduced lung damage by decreasing the levels of oxidative damage and inflammation. Among antioxidants, plasma levels of alpha-tocopherol were the most reliable indicator of antioxidant consumption. However, careful analysis of data revealed some problems that did not allow making any firm conclusion regarding the efficacy of antioxidants in reducing HD-induced lung damage. These problems included (a) high incidence of lung lesions in untreated animals, gavage route of administration aggravating lung lesions, and some doses of antioxidants aggravating HD-induced toxicity. These problems will be avoided in the second phase of the HD study.

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Introduction:

The threat of using chemical weapons in combat zones exists. Many chemical weapon agents, such as sulfur mustard (HD) and nerve agent (VX) mediate their primary actions by generating excessive amounts of free radicals and acute/chronic inflammation. One of the primary target organs after inhalation of sulfur mustard is the lung. The primary target of VX is the cholinergic neurons in the brain. The investigators at the USAMRICD have an extensive experience in the study of sulfur mustard inhalation and VX exposure in rats. All animal studies were performed by Dr. Jensen and his colleagues at the USAMRICD. They are responsible for collecting the samples, histological analysis, temporary storage and shipping the samples to PMC's laboratory (Antioxidant Research Institute, Novato, California) for biochemical analyses. These analyses included antioxidant levels in plasma and biomarkers of oxidative damage and inflammation in lung tissue samples (for HD study) and brain samples (for VX study). The data obtained from the present study may be useful for developing countermeasures against sulfur mustard agents as well as against nerve agents.

Body:

There were two studies; one involved evaluation of a preparation of multiple antioxidants in protecting mustard agent sulfur mustard (HD)-induced lung injury, and the other involved evaluation of the same antioxidant preparation in protecting against nerve agent O-ethyl-S-di-isopropylaminomethyl methylphosphonothiolate (VX)-induced neurological deficits. These studies were performed in collaboration with Drs. Neil Jensen and Todd Myers of the USAMRICD) at the Aberdeen Proving Ground, MD.

Hypothesis of HD study: For the first phase of the study, our hypothesis is that oral supplementation with a mixture of dietary and endogenous antioxidants before and/or after exposure to sulfur mustard may reduce damage to the lung in rats by decreasing oxidative damage and inflammation.

For the second phase of the study, our hypothesis is that oral supplementation with a mixture of dietary and endogenous antioxidants before and/or after exposure to sulfur mustard may improve the survival rate and survival time in rats by decreasing oxidative damage and inflammation.

Hypothesis of VX study: Our hypothesis is that oral supplementation with with a mixture of dietary and endogenous antioxidants before and/or after exposure to a nerve agent VX (O-ethyl-S-di-isopropylaminomethyl methylphosphonothiolate) when used in combination with standard therapy may reduce the incidence and severity of VX-induced seizures and improve the associated neurobehavioral dysfunction more than those produced by standard therapy alone by decreasing oxidative stress, inflammation, and release and toxicity of glutamate in rats.

Method

Species used: Rats

Protocol approval: The animals were individually or pair housed in the CRF Bldg., E-3156, and maintained according to the Animal Welfare Act and implementing Animal Welfare Regulations and the principles noted in The Guide for the Care and Use of Laboratory Animals as applied at USAMRICD. All animal experiments were initiated only after obtaining approval from USAMRICD Institute Animal Care and Use Committee (IACUC) and ACURO.

Antioxidants preparation: The one gram preparation of antioxidants contained vitamin A (retinyl palmitate, 45.429 mg); natural beta-carotene, 0.454 mg; d-alpha tocopheryl acetate, 136.289 mg; d-alpha-tocopheryl succinate ,136.289 mg; vitamin C (as calcium ascorbate), 454.297 mg; N-acetylcysteine (NAC), 68.144 mg; R-alpha lipoic acid, 68.144 mg; coenzyme Q10, 30.859 mg; and selenomethionine, 0.0908 mg

Doses of antioxidants and HD: For the first phase of HD study, doses of antioxidants 200 mg, 100 mg and 50 mg/kg of body weight were used, whereas a dose of HD (2.7 mg) in 100 µl ethanol was used.

Route of administration: For the first phase of HD study, HD was administered through inhalation, whereas antioxidants were given through gavage before and after treatment with HD.

Exposure to HD and antioxidant treatment: Rats (200-250 g) were anesthetized by injecting intramuscularly a combination of ketamine (80 mg/kg) and rompun (10 mg/kg). An electronic identification chip that codes an

animal ID number was implanted in the hind flank of each animal. Rats were intubated with a modified glass Pasteur pipette and connected to a custom embulizer device. HD (2.7 mg) in 100 µl ethanol or ethanol alone (control) was placed in a glass vapor generator (fabricated by Atmar Glass, Kennett Square, PA), and the rats were exposed for 10 min. This dose of HD consistently produces [8]LC_{50} (50% mortality within 8 days), After exposure the rats were provided supplemental heat for up to 4 hr while recovering from the anesthesia to protect them from hypothermia.

Animals were administered antioxidants daily for three consecutive days. One hour after the third antioxidant mixture dosing, rats were exposed to HD vapor using the inhalation system. After the third dosing with antioxidants, rats were anesthetized, and a tracheal intubation was performed. At 4 hours and 24 hours after HD exposure, blood samples were collected, added to 0.1 mM BHA and frozen. Lungs were perfused with heparinized PBS. The left lung was snap frozen, and the right lung was fixed with 4CF-1G. Frozen blood and lung samples were stored at -80^{o}C until shipment to PMC for analysis. Fixed lung samples were sectioned and H&E stained.

Two sets of seven dosing groups were used, one collected at 4 hrs post-exposure and the second at 24 hrs post exposure. Ten rats will be in each dose-time group for a total of 140 rats. Seven dosing groups include, (1) normal untreated – unexposed, (2) vehicle dosed – sham EtOH exposed, (3) 200 mg antioxidant mixture dosed - sham EtOH exposed, (4) vehicle dosed – HD exposed, (5) 50 mg antioxidant mixture dosed - HD exposed, (6) 100 mg antioxidant mixture dosed - HD exposed, and (7) 200 mg antioxidant mixture dosed - HD exposed

Assay of markers of oxidative damage and inflammation in the lung: The markers of oxidative damage include malondialdehyde, 8-hydroxy-deoxyguanosine, and oxidized and reduced glutathione, whereas the markers of pro-inflammatory cytokines included TNF-alpha and IL-6. In addition, the levels of alpha tocopherol, retinol, and beta-carotene were determined from plasma by high performance liquid chromatography (HPLC) using coulometric detection (C-18 column with gradient elution). 8-hydroxy-deoxyguanosine (8-OHdG) was determined in triplicate from plasma using an enzyme-linked immunometric assay (Oxis International). All other analytes were determined from lung tissue after homogenization and centrifugation to obtain a protein supernatant. In these cases, enzyme-linked immunometric assays were the method of quantitation (Oxis International and R&D Systems).

Histology analysis: The parameters of lung damage included perivascular edema (PE), alveolar exudates (AE); alveolar inflammatory cell infiltrates (AICI), alveolar hemorrhage (AH), alveolar epithelial necrosis (AEN), bronchiolar exudates (BE), bronchiolar inflammatory cell infiltrates (BICI), bronchiolar epithelial necrosis (BEN), BALT necrosis (BN), tracheal exudates (TE), tracheal inflammatory cell infiltrates (TICI), tracheal epithelial necrosis (TEN). The extent of damage was scores as follows 0= none; tr= trace; 1= mild (1-10%), 2= moderate (10-50%); 3= severe (≥50%)

Results of HD Study: malondialdehyde (MDA) levels in the lung tissue were undetectable. Irrespective of treatments, 40-70% of samples had undetectable levels of 8-OHdG. Table 1 showed that HD exposure increased the level of 8-OHdG) at 4 h after treatments compared to controls. Treatment of animals with antioxidants before HD exposure reduced the levels of 8-OHdG at doses 50 and 100 mg/kg of bod weight in comparison to those exposed to HD alone. After 24 HD exposure, the level of 8-OHdG decreased in all groups; however, except at a antioxidant dose of 50 mg/kg, it did not show any significant change, but at a dose of 100 mg/kg , it decreased the level of 8-OHdG in the lung. The levels of 8-OHdG in animals exposed to HD plus 200 mg/kg of body weight of antioxidants were slightly elevated in comparison to those exposed to HD alone 4 and 24 hours after exposure to HD. This suggested that a dose of 200 mg/kg of body weight may be toxic.

Tables 2 showed that the levels of reduced glutathione did not significantly change in experimental groups compared with control groups after 4 h of HD treatment; however, after 24 h of HD treatment, antioxidant treatment at doses of 100 mg and 200 mg/ kg of body weight increased the glutathione levels in the lung compared to HD treatment alone.

Table 3 showed that antioxidant treatment before HD exposure increased the levels of the ratio of reduced/oxidized glutathione in the lung tissue of rats at doses of 100 and 200 mg/kg of body weight at both 4 and 24 h after treatment with HD compared to those treated with HD alone.

Table 4 showed that exposure to HD enhanced the levels of a pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha) in the lung compared with vehicle control. Antioxidant treatment at all antioxidant doses reduced the levels of TNF-alpha at 4 h after exposure to HD. After 24 h of HD treatment, antioxidant treatment decreased the level of TNF-alpha only at a dose of 200 mg/kg of body weight.

Table 5 showed that exposure to HD increased the level of interleukin-6 (IL-6) at 4 h after treatment compared with vehicle control. Antioxidant treatment at doses 50 and 100 mg/kg of body weight reduced it, but it increased the levels of IL-6 at a dose of 200 mg/kg/body weight at both 4 and 24 h after HD exposure.

Table 6 shows that HD exposure alone did not change plasma level of alpha-tocopherol at 4 or 24 h after HD exposure compared to vehicle control; however, antioxidant treatment before exposure to HD showed dose-dependent increase in plasma vitamin E levels at all antioxidant doses and at both 4 and 24 after HD exposure.

Beta-carotene was not detectable in plasma. Retinol levels of highly variable; therefore no conclusion can be drawn (Table 7).

Histology of the lung: Untreated rats showed at least one lesion in 26 to 39 percent of the samples at 4 and 24 h, respectively (Table 8); therefore, the data must be interpreted with caution. This high incidence of lesion in control animals cannot be explained except possibly by the fact that this group of animals already had respiratory illness. It should be pointed out that respiratory distress is very common in rats.

Vehicle-treated animals showed at least one lesion in 15% of the animals at 4 after treatment and only 2 % after 24 h treatment. This in part could be related to administration of vehicle by gavage. Administration of antioxidants or vehicle by gavage may induce some trauma to the lung. Exposure to HD caused at least one lung lesion in 64 to 70% of the treated animals after 4 and 24 hours (Table 8).
Exposure to HD plus antioxidants at 50 and 100 mg/ kg of body weight reduced the number of animals with at least one lesion by 14-17%, whereas antioxidant treatment at a dose of 200 mg/kg of body weight increased it by 11% after 4 h of HD. Antioxidant treatment decreased the number of animals with at least one lung lesion by 19-27% 24 hours after HD exposure, the most effective dose being 100 mg/kg. The antioxidant dose of 200 mg appears to be toxic.

Table 9 described the number of animals with a specific lesion and the level of damage.

Table 10 describes a comparative analysis of major types of lung lesions among various groups revealed that antioxidant treatment was effective in reducing the number of animals with one or more major lung lesions, depending upon the dose and the time of assay after HD exposure. A dose of 200 mg/kg of body weight of

antioxidants reduced the number of animals with lung lesions by 27% at 4 h after HD exposure, whereas a dose of 50 mg/kg of body weight of antioxidants reduced it by 23% at 24 h after HD exposure.
Since tracheal epithelial necrosis (TEN) represented the common lung lesion in most animals, a comparative analysis of TEN among various groups revealed that antioxidant treatment at a dose of 50 mg/kg of body weight reduced the number of animals with this lesion by 49 %at 4 h after HD exposure, whereas at a dose of 100 mg/kg of body weight reduced it by 20% at 24 h after HD exposure. Antioxidants at a dose of 200 mg/kg of body weight increased the number of animals with TEN by 20% at 24 h after HD exposure (Table 11).

Key Research Accomplishments

Exposure of animals to HD increased the levels of 8-hydroxydeoxyguanosine (8-OHdG), one of the markers of oxidative damage in the lung. Antioxidant treatment at doses of 50 and 100 mg/kg of body weight reduced HD-induced elevated oxidative damage in the lung tissue at 4 h after HD exposure, but it slightly increased this damage after treatment with 200 mg/kg of body weight. These results suggest that HD exposure increases oxidative damage that is reduced by pre-treatment with antioxidants at 50 and 100 mg/kg of body weight in the lung. On the other hand, treatment with a dose of 200 mg/kg of body weight increased the oxidative damage in the lung. This dose of antioxidant was considered toxic, and will be avoided in future experiments.

Exposure to HD enhanced the levels of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) in the lung compared with vehicle control. Antioxidant treatment at all doses reduced the levels of TNF-alpha at 4 and 24 h after exposure to HD. Antioxidant treatment at doses 50 and 100 mg/kg of body weight reduced HD-induced elevated IL-6, but it at a dose of 200 mg/kg of body weight increased it. Again, the antioxidant dose of 200 mg/kg of body weight was found to be toxic.
Among antioxidants measured, plasma levels of alpha-tocopherol appear to be the most reliable indicator of consumption of antioxidants.
A comparative analysis of major types of lung lesions among various groups revealed that antioxidant treatment was effective in reducing the number of animals with one or more major lung lesions, depending upon the dose of antioxidants and the time of assay after HD exposure.

A comparative analysis of TEN, a common lung lesion in most animals, among various groups revealed that antioxidant treatment at a dose of 50 mg/kg of body weight reduced the number of animals with this lesion, depending upon the dose of antioxidants and the time of assay after HD exposure.

Reportable Outcomes

The first phase of HD study has been completed. Careful analysis of data suggests that exposure to HD increases lung damage by increasing the levels of oxidative damage and pro-inflammatory cytokines, whereas antioxidant treatment reduces it by decreasing the levels of oxidative damage and inflammation. However, analysis of data revealed some problems that do not allow making any firm conclusion regarding the efficacy of antioxidants in reducing HD-induced lung damage. For example, untreated rats showed at least one lung lesion in 26 to 39 percent of the samples. Thus, one cannot assess the effect of vehicle treatment on the lung lesion.
Multiple administration of antioxidants or vehicle though gavage appears to increase the risk of lung lesions; therefore they would be avoided in any future experiments. This mode of administration may also interfere with the absorption of antioxidants, because of repeated trauma to the upper gastrointestinal tract.
The antioxidant dose of 200 mg/kg of body weight appears to be toxic on several criteria.
Plasma levels of alpha-tocopherol appear to be the most reliable indicator of antioxidant consumption.

Conclusions: The results of the first phase of HD study suggested that exposure to HD increased lung damage in rats by increasing the levels of oxidative damage and pro-inflammatory cytokines, whereas antioxidant treatment before HD exposure reduced lung damage by decreasing the levels of oxidative damage and inflammation. Among antioxidants, plasma level of alpha-tocopherol was the most reliable indicator of antioxidant consumption. However, careful analysis of data revealed some problems that did not allow making any firm conclusion regarding the efficacy of antioxidants in reducing HD-induced lung damage. These problems included (a) high incidence of lung lesions in untreated animals, gavage route of administration aggravating lung lesions, and some doses of antioxidants aggravating HD-induced toxicity. These problems will be avoided in the second phase of the HD study.

Table 1 Plasma levels of 8-hydroxyguanosine (8-OHdG) after exposure to HD alone or HD plus antioxidants

Treatments	4 hours (ng/ml)	24 hours (ng/ml)
No treatment	1.40 (3)	0.78 (4)
Vehicle	0.94 (6)	0.95 (4)
HD	1.62 (5)	1.15 (5)
HD + 50 mg	1.44(3)	1.30 (4)
HD + 100 mg	1.20 (4)	0.90 (4)
HD + 200 mg	1.71 (4)	1.42 (4)
Vehicle + 200 mg	1.31 (10)	1.17 (9)

The number in parenthesis indicates number of samples showing detectable levels of 8-OHdG in the plasma samples. Each value is the average of number of samples indicated in the parenthesis.

Table 2 Levels of glutathione in the lung tissue of rat after exposure to HD alone or HD plus antioxidants

Treatments	4 hours (ng/mg of tissue)	24 hours (ng/mg of tissue)
No treatment	6.0 (5)	5.7 (3)
Vehicle	4.78 (9)	6.28 (10)
HD	6.00 (9)	4.30 (9)
HD + 50 mg	6.20 (12)	4.00 (14)
HD + 100 mg	6.00 (6)	5.50 (11)
HD + 200 mg	7.00 (6)	5.40 (7)
Vehicle + 200 mg	4.90 (11)	5.56 (12)

The number in parenthesis indicates number of samples showing levels of glutathione in the lung tissue in the plasma samples. The levels of glutathione in the lung tissues of rats were highly variable. Irrespective of treatments, 0-30% of samples had undetectable levels of glutathione.

Table 3 Ratio of Reduced/oxidized glutathione (GSH/GSSG) in the lung tissue of rat after exposure to HD alone or HD plus antioxidants

Treatments	4 hours	24 hours (ng/ml)
No treatment	17.0 (4)	4.5 (2)
Vehicle	3.4 (9)	3.6 (8)
HD	6.5 (8)	4.1 (6)
HD + 50 mg	6.4 (12)	4.5 (11)
HD + 100 mg	8.7 (5)	4.9 (11)
HD + 200 mg	30.2 (5)	16.6 (8)
Vehicle + 200 mg	5.6 (8)	7.7 (12)

The number in parenthesis indicates number of samples. Ratio of Reduced/oxidized glutathione (GSH/GSSG) in the lung tissue of rats was variable. Irrespective of treatments, 10-40% of samples had undetectable values.

Table 4 Levels of TNF-alpha in the plasma of rat after exposure to HD alone or HD plus antioxidants

Treatments	4 hours (pg/ml x 10^2)	24 hours (pg/ml x 10^2)
No treatment	67 (2)	5.7, 209 (2)
Vehicle	52 (4)	17 (5)
HD	141 (4)	42 ± (5)
HD + 50 mg	44 (4)	39 (6)
HD + 100 mg	43 (3)	45 (8)
HD + 200 mg	69 (5)	24 (5)
Vehicle + 200 mg	54 (4)	69 (1)

The number in parenthesis indicates number of samples. One sample with extremely high or low value in comparison with others was not included in determining the average value. Irrespective of treatments, high percentage of samples had undetectable levels of this biomarker of inflammation. Percentage of samples showing undetectable levels of TNF-alpha was similar in all groups except in group treated with 200 mg of antioxidants alone and assayed at 24 h later (90 % of samples had undetectable levels of TNF-alpha).

Table 5 Levels of IL-6 in the plasma of rat after exposure to HD alone or HD plus antioxidants

Treatments	4 hours (pg/ml x 10)	24 hours (pg/ml x 10)
No treatment	Undetectable	Undetectable
Vehicle	52 (2)	Undetectable
HD	67 (8)	64 (3)
HD + 50 mg	55 (5)	22 (4)
HD + 100 mg	46 (5)	38 (7)
HD + 200 mg	103 (6)	92 (4)
Vehicle + 200 mg	30 (2)	Undetectable

The number in parenthesis indicates number of samples. One sample with extremely high or low value in comparison with others was not included in determining the average value. Irrespective of treatments, 40-100% of samples had undetectable levels of this biomarker of inflammation.

Table 6 Plasma levels of d-alpha-tocopherol in the plasma of rat after exposure to HD alone or HD plus antioxidants

Treatments	4 hours (ng/ml x 10^3)	24 hours (ng/ml x 10^3)
No treatment	6.0 ± 0.8 (6)	6.8 ± 0.8 (4)
Vehicle	5.1 ± 1.9 (9)	5.9 ± 1.6 (10)
HD	4.7 ± (10)	5.9 ± 1.5 (10)
HD + 50 mg	7.5 ± 2.3 (12)	6.8 ± 1.9 (16)
HD + 100 mg	8.4 ± 2.6 (8)	8.6 ± 3.0 (13)
HD + 200 mg	10 ± 1.8 (10)	10.8 ± 2.4 (11)
Vehicle + 200 mg	9.8 ± 3.0 (11)	10.4 ± 3.0 (10)

The number in parenthesis indicates number of samples.

Table 7 Levels of retinol in the plasma of rat after exposure to HD alone or HD plus antioxidants

Treatments	4 hours (ng/ml x 10^2)	24 hours (ng/ml x 10^2)
No treatment	3.1 ± 0.8 (6)	3.5 ± 0.7 (4)
Vehicle	2.8 ± 0.7 (9)	2.9 ± 1.1 (10)
HD	2.6 ± 0.9 (10)	2.5 ± 1.1 (10)
HD + 50 mg	3.6 ± 0.9 (12)	2.9 ± 1.1 (16)
HD + 100 mg	2.1 ± 0.9 (8)	3.2 ± 1.1 (13)
HD + 200 mg	2.8 ± 1.0 (10)	4.3 ± 1.1 (11)
Vehicle + 200 mg	2.4 ± 0.8 (11)	2.3 ± 1.1 (10)

The number in parenthesis indicates number of samples.

Table 8 Effect of multiple antioxidants on HD-induced at least one lung lesion in rats

Treatments	treatment time (h)	Total number of animals	% of animals with at least one lesion	% change from HD exposure
None + None	4	27	26	
EtOH +Vehicle	4	45	15	
EtOH + 200 mg	4	44	18	
HD + vehicle	4	51	64	
HD + 200 mg	4	49	71	111
HD + 100 mg	4	32	53	83
HD + 50 mg	4	60	55	86
None + None	24	23	39	
EtOH + Vehicle	24	48	2	
EtOH + 200 mg	24	39	21	
HD + Vehicle	24	46	70	
HD + 200 mg	24	56	57	81
HD + 100 mg	24	55	51	73
HD + 50 mg	24	75	57	81

None- No treatment; EtOH – Ethanol; HD (mustard gas); mg (amount of antioxidant mixture per kg of body weight) Animal showing trace amounts of damage were not included.

Lung lesions: Perivascular edema (PE), Alveolar exudates (AE); Alveolar inflammatory cell infiltrates (AICI), Alveolar hemorrhage (AH), Alveolar epithelial necrosis (AEN), Bronchiolar exudates (BE), Bronchiolar inflammatory cell infiltrates (BICI), Bronchiolar epithelial necrosis (BEN), BALT necrosis (BN), Tracheal exudates (TE), Tracheal inflammatory cell infiltrates (TICI), Tracheal epithelial necrosis (TEN).

Table 9 Effect of multiple antioxidants on HD-induced major types of lung lesions

Treatments	Treatment time (h)	Total number of animals	Number of animals with a lesion (level of damage) TEN	BN	PE	BICI	BEN	BE
None + None	4	27	1(3)	0	0	0	0	0
None + None	24	23	3(1-3)	3(1)	2(1-3)	0	0	0
EtOH +Vehicle	4	45	3(2-3)	4(1)	1(1)	0	0	0
EtOH + Vehicle	24	48	1(2)	0	0	0	0	0
EtOH + 200 mg	4	44	4(1-3)	0	2(2-3)	0	1(2)	0
EtOH + 200 mg	24	39	1(1)	1(2)	4(2-3)	0	1(1)	0
HD + vehicle	4	51	6(1-3)	16(1-3)	6(1-2)	1(1)	0	0
HD + Vehicle	24	46	7(1-3)	0	19(1-3)	9(1)	4(1)	7(1-3)
HD + 200 mg	4	49	7(1-3)	0	7(1-3)	0	0	0
HD + 200 mg	24	59	10(1-3)	7(1-2)	11(1-3)	10 (1-2)	11(1-2)	10 (1-2)
HD + 100 mg	4	32	4(1-3)	11(1-2)	3(1-2)	0	0	0
HD + 100 mg	24	55	7(1-3)	7(1-2)	8(1-2)	10(1-2)	9(1)	9(1-2)
HD + 50 mg	4	60	4(1-2)	23(1-3)	9(1-2)	0	0	0
HD + 50 mg	24	75	10(1-3)	8(1-2)	19(1-3)	10(1)	0	11(1-3)

None- No treatment; EtOH – Ethanol; HD (mustard gas); mg (amount of antioxidant mixture per kg of body weight) Animal showing trace amounts of damage were not included. The number refers to the number of animal with a lesion and the number in parenthesis refers to the level of each lesion. The levels of AICI, TE, TICI, AH, AE were not included because they were absent in most groups, and if present, primarily in small number of animals at minimal level of 1.

Lung lesions: Perivascular edema (PE), Alveolar exudates (AE); Alveolar inflammatory cell infiltrates (AICI), Alveolar hemorrhage (AH), Alveolar epithelial necrosis (AEN), Bronchiolar exudates (BE), Bronchiolar inflammatory cell infiltrates (BICI), Bronchiolar epithelial necrosis (BEN), BALT necrosis (BN), Tracheal exudates (TE), Tracheal inflammatory cell infiltrates (TICI), Tracheal epithelial necrosis (TEN). The extent of damage was scores as follows 0= none; tr= trace; 1= mild (1-10%), 2= moderate (10-50%); 3= severe (≥50%)

Table 10 Effect of multiple antioxidants on the percentage of animals which developed one or more HD-induced major types of lung lesions

Treatments	Treatment time (h)	% of animals with lung lesions
None + None	4	3.7
EtOH +Vehicle	4	17
EtOH + 200 mg	4	16
HD + vehicle	4	56
HD + 50 mg	4	60
HD + 100 mg	4	56
HD + 200 mg	4	29
None + None	24	34
EtOH + Vehicle	24	2
EtOH + 200 mg	24	18
HD + Vehicle	24	100
HD + 50 mg	24	77
HD + 100 mg	24	91
HD + 200 mg	24	100

None- No treatment; EtOH – Ethanol; HD (mustard gas); mg (amount of antioxidant mixture per kg of body weight)

Table 11 Effect of multiple antioxidants on HD-induced tracheal epithelial necrosis (TEN) lung lesion

Treatments	treatment time (h)	Total number of animals	% of animals with a TEN lesion	% change from HD exposure
None + None	4	27	7	
EtOH +Vehicle	4	45	4	
EtOH + 200 mg	4	44	9	
HD + vehicle	4	51	14	
HD + 50 mg	4	60	8	51
HD + 100 mg	4	32	13	93
HD + 200 mg	4	49	14	100
None + None	24	23	17	
EtOH + Vehicle	24	48	2	
EtOH + 200 mg	24	39	3	
HD + Vehicle	24	46	15	
HD + 50 mg	24	75	16	106
HD + 100 mg	24	55	12	80
HD + 200 mg	24	56	18	120

None- No treatment; EtOH – Ethanol; HD (mustard gas); mg (amount of antioxidant mixture per kg of body weight)

Lung lesion: Tracheal epithelial necrosis (TEN)

Second phase of HD study

Because of problems, such as the high incidence of lung lesions in non-treated animals, administration route through gavage, and toxicity of some doses of antioxidants, associated with the first phase of the study, we have delayed the start of this experiment. In order to avoid the above problems, we will carefully monitor the health of the animals in consultation with our veterinarian, and if felt essential, we may decide to test the animals for any infection before using them in the second phase of the HD experiments. In our past experience, any sign of infection can increase the radiosensitivity of the animals. This could happen if infected animals are exposed to HD.

While working in collaboration with Dr. Ron Jackson of NHRC, we observed that rats consume a peanut butter pellet containing antioxidants within a few minutes efficiently without any significant loss of the materials. We will use this route of administration which is more pertinent to human usage as well as being less traumatic than the gavage that could cause some injuries after repeated doses.

In the second phase of the HD study, we plan to use 10, and 50 mg/kg of body weight of antioxidants.

Nerve agent (VX) Study

We have just started this study. The main reason for the delay in starting the experiment was the problems encountered during the first phase of the HD study. They included use of potentially infected animals, gavage route of administration and toxicity of antioxidants which would have created problems in data interpretation. We have now resolved these issues.