

DISPOSITION INSTRUCTIONS:

Destroy this report when no longer needed. Do not return to the originator.

DISCLAIMERS:

The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).

The use of trade names does not constitute an official endorsement or approval of the use of such commercial hardware or software. This document may not be cited for purposes of advertisement.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) March 2005		2. REPORT TYPE Technical report		3. DATES COVERED (From - To) May 2002 to November 2002	
4. TITLE AND SUBTITLE Effects of rhCC10 as a Post-treatment in Mice Challenged with Phosgene				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Hess, ZA, Moran, TS, Lee, RB, Sciuto, AM				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-PN 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400				8. PERFORMING ORGANIZATION REPORT NUMBER USAMRICD-TR-05-02	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-RC 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In this study we evaluated the therapeutic efficacy of a post-exposure administration of rhCC10 (Claragen, Inc., Rockville, MD) against a toxic concentration of phosgene. rhCC10 is described as a 10kDa Clara Cell protein, an anti-inflammatory lung protein that is present on all mammalian mucosal surfaces. It protects against infection and environmental pollutants but apparently not against phosgene, which has a substantial effect in bronchioles and permeability in the deep alveolar lung. Supplementation with rhCC10 did not increase survival of animals exposed to phosgene compared with those treated with saline alone. In addition, the results of the bioassays show no significant differences between saline and the rhCC10 doses; therefore, there has not been any determination of rhCC10 as a beneficial post-exposure therapy for phosgene.					
15. SUBJECT TERMS rhCC10, lung protein, mucosal host defense proteins, phosgene, pulmonary edema, pulmonary inflammation, anti-inflammatory					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UNLIMITED	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON Theodore S. Moran
a. REPORT UNCLASSIFIED	b. ABSTRACT UNCLASSIFIED	c. THIS PAGE UNCLASSIFIED			19b. TELEPHONE NUMBER (include area code) 410-436-3080

Acknowledgements

Jeffry S. Forster, Diana L. Clapp, Laura B. Carpin, Erin A. Kane, Nichole D. Riggs,
David G. J. Mann.

Introduction

Recombinant human Clara Cell 10kDa protein (rhCC10) is an important anti-inflammatory lung protein that may be damaged and inactivated, or consumed, during phosgene exposure. rhCC10 is known to be protective against many types of pulmonary insults including infection and exposure to environmental pollutants such as PCB and ozone.¹ It is believed that supplementation with rhCC10 may reduce mortality as well as the severity of pulmonary inflammation that accompanies phosgene exposure.

rhCC10 is the most abundant member of a family of small anti-inflammatory proteins present on all mammalian mucosal surfaces. These proteins play a central role in regulating and modulating inflammatory and immune responses to chemicals, particles and antigens that routinely contact mucosal surfaces, and they are referred to as mucosal host defense (MHD) proteins. These proteins are part of an extracellular system that serves to clear a variety of environmental irritants at the mucosal surfaces of the respiratory, digestive and urogenital tracts, as well as from the eyes.¹

CC10 is chemically altered ("damaged") by contact with superoxides and nitric oxide. Damaged CC10 thus represents a quantifiable biomarker that can be measured, in sputum, after a suspected exposure to determine whether tissue damage or an inflammatory response is in progress.¹

Phosgene (abbreviated CG) is a toxic gas that was used extensively in chemical offense during World War 1, resulting in vast numbers of injuries and deaths. Today it is used commercially in the synthesis of insecticides, plastics, pharmaceutical drugs and dyes.² The National Institute for Occupational Safety and Health (NIOSH) reports that over one million tons of phosgene are produced annually in the United States and that thousands of workers may be involved with its production and usage.³ Phosgene is usually completely consumed in most of these processes; however, an accident or spill could lead to serious medical and occupational problems for industrial workers or firefighters. Inhalation of phosgene can cause non-cardiogenic pulmonary edema and possibly death, within 6-24 hours after exposure.⁴

In this study we evaluated the therapeutic efficacy of a post-exposure administration of rhCC10 (Claragen, Inc., Rockville, MD) against a toxic concentration of phosgene.

Methods

Male CD-1 (Charles River, Wilmington, MA) mice weighing 25-30g were subjected to whole-body exposure of 32 mg/m³ phosgene for 20 min (640 mg min/m³) in an approved laboratory fume hood. Ten percent phosgene and a balance of N₂ (Matheson Tri-Gas, Montgomeryville, PA) was metered through a Brooks mass flow controller (Brooks Instrument, Hatfield, PA) at a rate of 20 L/min. This was mixed with room air and passed through an infrared spectrometer (Miran 1A, Foxboro Co., Sharon, MA) to the animal exposure chamber. The Miran 1A was equipped with a real-time analog output. Concentration versus time graphs were developed, and the input concentration was calculated. The exposure occurred in a Plexiglas cylinder (25 cm in

height x 28 cm in diameter) with a total volume of 15.8 L. The chamber was divided into 4 quadrants with 10 mice per quadrant. Mice were exposed to phosgene for 20 minutes followed by a 5-minute chamber air washout. Outflowing gas from the chamber was passed through a second Miran 1A unit to determine the concentration of phosgene exiting the chamber. Following the exposure, the mice were injected (IP) with appropriate concentrations of rhCC10 (0.5, 1.5, or 5.0 mg/kg) or saline treatments at 20 minutes, 3, 6, and 12 hours after being removed from the chamber, to maintain a constant level of rhCC10. In all, each mouse received 4 injections of the appropriate concentration of rhCC10. Fresh rhCC10 was prepared in saline the day before the experiment and each injection consisted of 0.25 ml of solution. Mice were exposed in two groups of 40 for 20 minutes: one group receiving phosgene and the other group receiving air. This regimen was performed on six separate occasions and on one other occasion when both groups of 40 mice received phosgene (for survivability calculations). The number of mice for lavage and how they were grouped are displayed in Table 1 below.

Table 1

rhCC10 (mg/kg)	PHOSGENE	Air
0	39	32
0.5	38	33
1.5	36	34
5.0	44	33

Lavage samples of left lung for the wet/dry ratio was collected at 1, 4, 8, 12, 24, or 48 hours after the last injection. The left lung was weighed and placed on a tared planchete. These lungs, for the wet/dry ratio (LW/LD), were then placed in an oven at 100°C and reweighed eight days later for dry weight. The entire right lung was quickly frozen in liquid nitrogen and stored at -80°C for future analysis (data not shown here). Lavage samples consisted of 800 uL of saline washed into and out of the lung five times. The final volume of lavage collected was centrifuged at 14 K rpm for 20 seconds. The supernatant was removed and stored at -80°C. Portions were used for reduced glutathione (GSH), catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPX) determinations using OXIS International, Inc. (Portland, OR) assay kits.

Survival analysis data (Figure 1) were entered into SPSS, and a Life Table Analysis was first performed for each exposure week to visually assess the data and treatment group differences. In this assessment, mice exposed to phosgene and treatment of varying doses of rhCC10 were compared with mice exposed to phosgene and treatment with saline. It was noted that during the 5th exposure week all animals from each treatment group survived. This phenomenon was believed to be caused by lack of adequate levels of phosgene in the exposure tank. Data from these groups are considered outliers and excluded from further analyses. Data from all exposure weeks, except for week 5, were combined for further analyses, and a Cox regression was used to compare the three treatment groups with the saline group. Statistical significance was defined as p<0.05.

Also, a two-factor analysis of variance (ANOVA) was used to determine time and dose differences within each exposure group. Statistical significance was defined as $p < 0.05$.

Results and Discussion

The survival analysis (Figure 1) data employed the use of a Cox regression and showed no significant differences in the survival rates among the phosgene groups with three different levels of rhCC10 treatment, and also no significant difference in the phosgene with saline only group. The data were also entered into the Prizm (GraphPad Software, Inc. Version 3.02) survival analysis program. The results of the comparison of Survival Curves also showed no significant differences among the treatment groups and no significant differences from saline.

For GSH, SOD, and GPX assays (Figures 2, 3, and 4) there were significant differences ($p < 0.05$) observed within the phosgene exposure group across observation times, but not among rhCC10 doses. Therefore, the significant differences were due to the effect of phosgene exposure being expressed over a period of time rather than to treatment with varying doses of rhCC10.

For LW/LD (Figure 5) there were significant differences ($p < 0.05$) observed within the phosgene exposure group across observation times, but not among rhCC10 doses. Therefore again, the significant differences were due to the effect of phosgene exposure being expressed over a period of time rather than to treatment with varying doses of rhCC10.

For catalase (Figure 6) significant differences ($p < 0.01$) were again observed within the phosgene exposure group across observation times, but not among rhCC10 doses. At the 1.5 mg/kg dose, the catalase response increased slightly until the 24-hour time point, where a dramatic drop occurred; however, the response increased at the 48-hour time point. For the 5.0 mg/kg dose, differences among observation times were observed, with 1 and 24 hours having substantially lower catalase values.

Each type of assay completed aided in the determination of the effectiveness of rhCC10 as a (post-exposure) therapy against toxic concentrations of phosgene. GSH assay results are in the graph displayed as Figure 2. With the exception of the 0.5 and 1.5 dosages, there was not any consistent relationship between time and GSH levels. At these two dosages, the levels of GSH increased until the 24-hour time period, at which time they began to decrease. Overall, the shape of the graph is that of a normal (bell-curve) distribution, with the GSH levels rising to a certain point and then descending thereafter; the time points at which this occurred varied among the dosages. Since the saline and phosgene and all rhCC10 doses appear to have approximately the same levels of GSH, there was no indication that there were any differences in GSH levels due to rhCC10 treatment.

As Figure 3 illustrates, the SOD showed an early decrease at 4 hours and recovery by 48 hours. However, levels did not consistently increase or decrease as the dosage of rhCC10 was increased, nor did they stand out from the levels produced with saline. The pattern of the saline and phosgene and 5.0 mg/kg dosage is the same, with SOD levels decreasing until the 4- or 8-hour time period, respectively, and then increasing. The general pattern overall was inconsistent, once each dosage of the drug (and saline) caused SOD levels to decrease and increase at various time intervals. The variance among the change in SOD levels also fluctuated depending on the drug dosage; there appears to be a smaller fluctuation in the SOD levels for the 1.5 mg/kg and 5.0 mg/kg dosages.

Figure 4 represents the graph produced from the results of the phosgene groups. The GPX levels increased in each dosage group (including saline) until the 24-hour time period and then decreased drastically. While the GPX levels for each dosage remained similar to one another at each time period, the level for 1.5 mg/kg dosage at 24 hours was substantially lower. Nonetheless, the dosage of rhCC10 did not appear to influence the GPX levels.

The results of the Lung Wet Weight to Dry Weight Ratio show a significant difference between the phosgene and air groups. The phosgene groups displayed more than twice the ratio (approximately) of the air groups. The ratio of the phosgene group was consistently higher than the air group, but neither group demonstrated any effects of the absence of rhCC10 (saline) or varying the dosage. The results from the phosgene group are depicted in Figure 5, from which one can see the peak of the ratio at 8 hours for every dosage (including saline).

In Figure 6, the dosages of rhCC10 have peak catalase levels at varying times. The peak times appear to be inverse with dose. For the 0.5 mg/kg dosage, the peak is at 12 hours, at 8 hours for the 1.5 mg/kg dosage, and at 4 hours for the 5.0 mg/kg dosage, showing that as the dosage increased, the peak catalase levels shifted. Although this shift is evident in Figure 6, there is not a considerable difference in the actual concentration of catalase as the drug dosage increased.

Finally, after taking all of the data into consideration, it is concluded that post-exposure therapy with rhCC10 does not provide any benefit, regardless of the drug dosage. In the second exposure of week 6, there appears to be a slight benefit in the 0.5 mg/kg and 5.0 mg/kg dosages, but it was not determined to be statistically significant. More statistically significant results would be required to ascertain a benefit of rhCC10 at any dosage.

Conclusions

rhCC10 is described as a 10kDa Clara Cell protein, an anti-inflammatory lung protein that is present on all mammalian mucosal surfaces. It protects against infection and environmental pollutants but apparently not against phosgene, which has a substantial effect in bronchioles and permeability in the deep alveolar lung. Supplementation with rhCC10 did not increase survival of animals exposed to phosgene compared with those treated with saline alone. In addition, the results of the bioassays show no significant differences between saline and the rhCC10 doses; therefore, there has not been any determination of rhCC10 as a beneficial post-exposure therapy for phosgene. A previous experiment done with doses of 5 mg/kg, 10 mg/kg, and 20 mg/kg

of rhCC10 was also not able to show an improvement from saline in pulmonary edema formation.

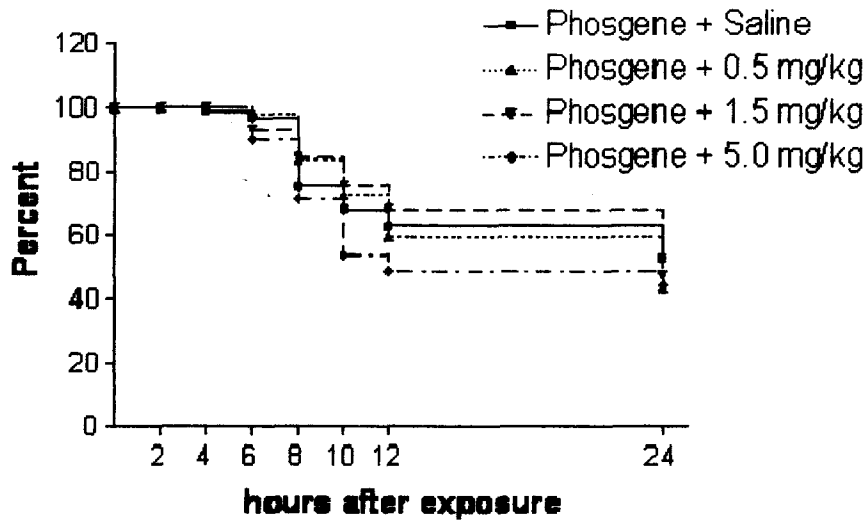


Figure 1. Total survival rates of mice treated with saline or varying doses of rhCC10 on 7 phosgene exposures (except week 5).

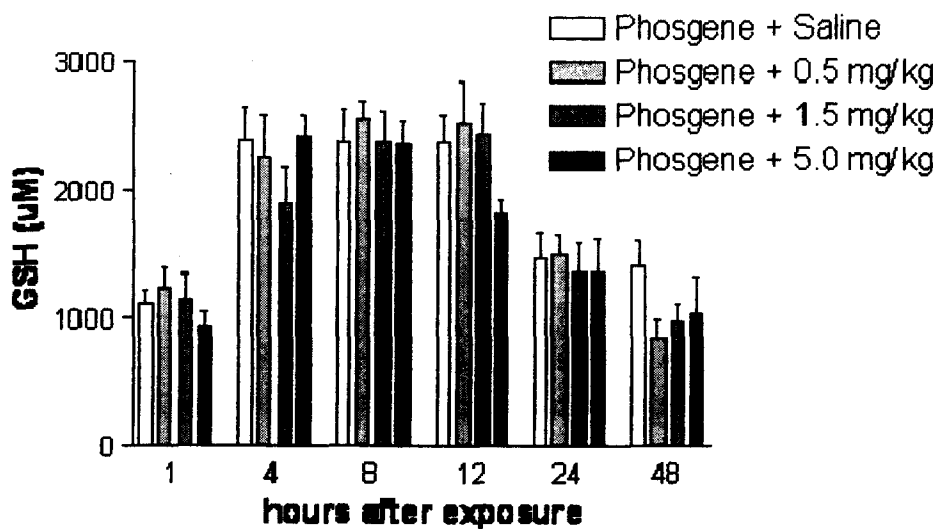


Figure 2. Reduced Glutathione in lavage fluid of phosgene-exposed mice treated with saline or rhCC10.

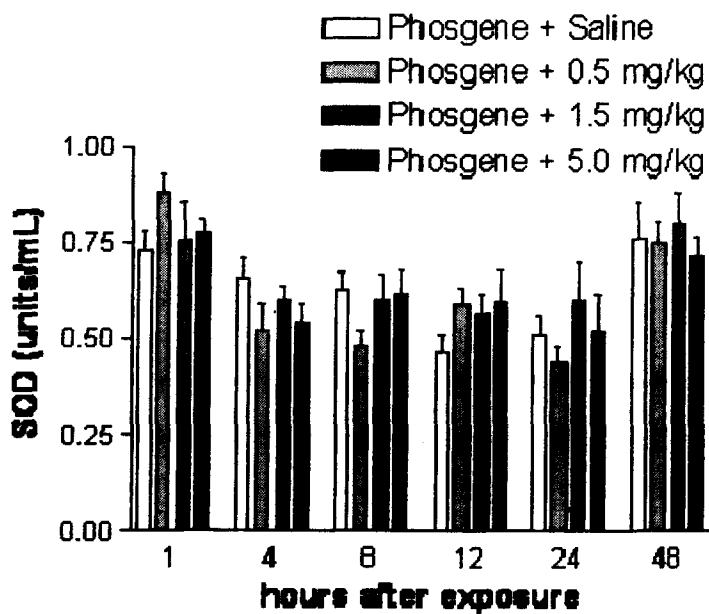


Figure 3. Superoxide Dismutase in lavage fluid of phosgene-exposed mice treated with saline or rhCC10.

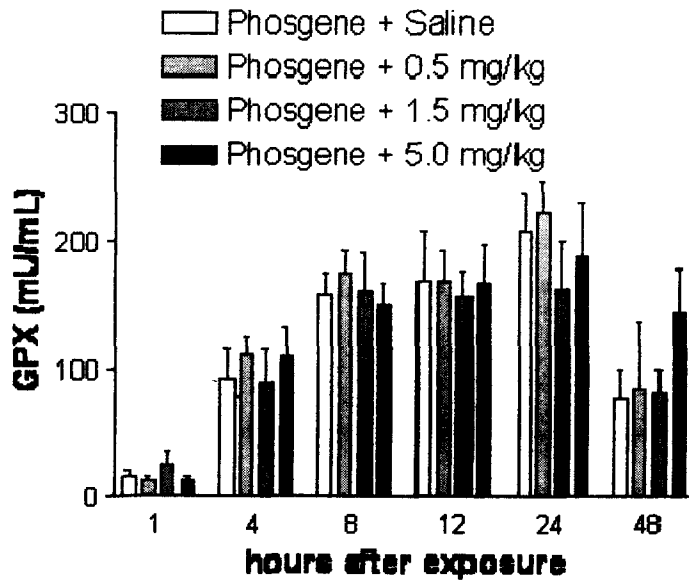


Figure 4. Glutathione Peroxidase in lavage fluid of phosgene-exposed mice treated with saline or rhCC10.

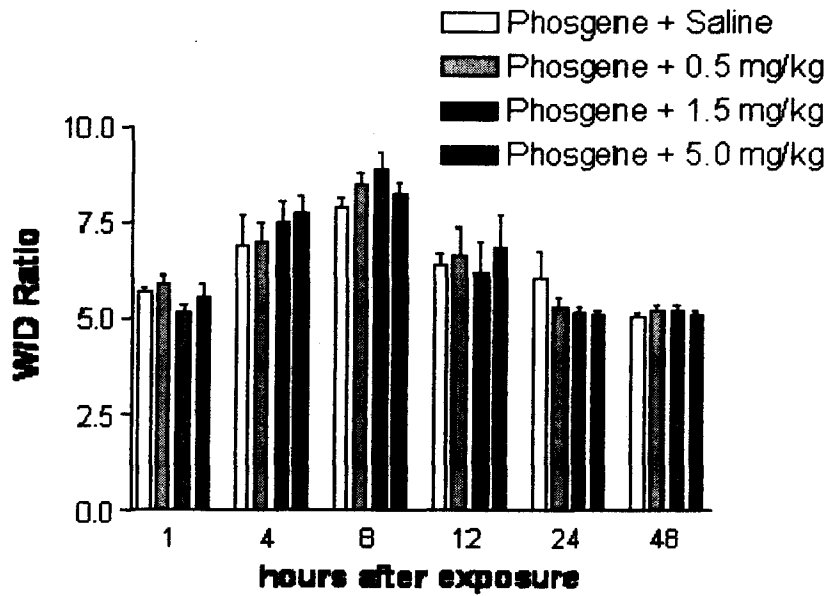


Figure 5. Temporal change in Lung Wet Weight to Lung Dry Weight Ratio in phosgene-exposed mice treated with saline or rhCC10.

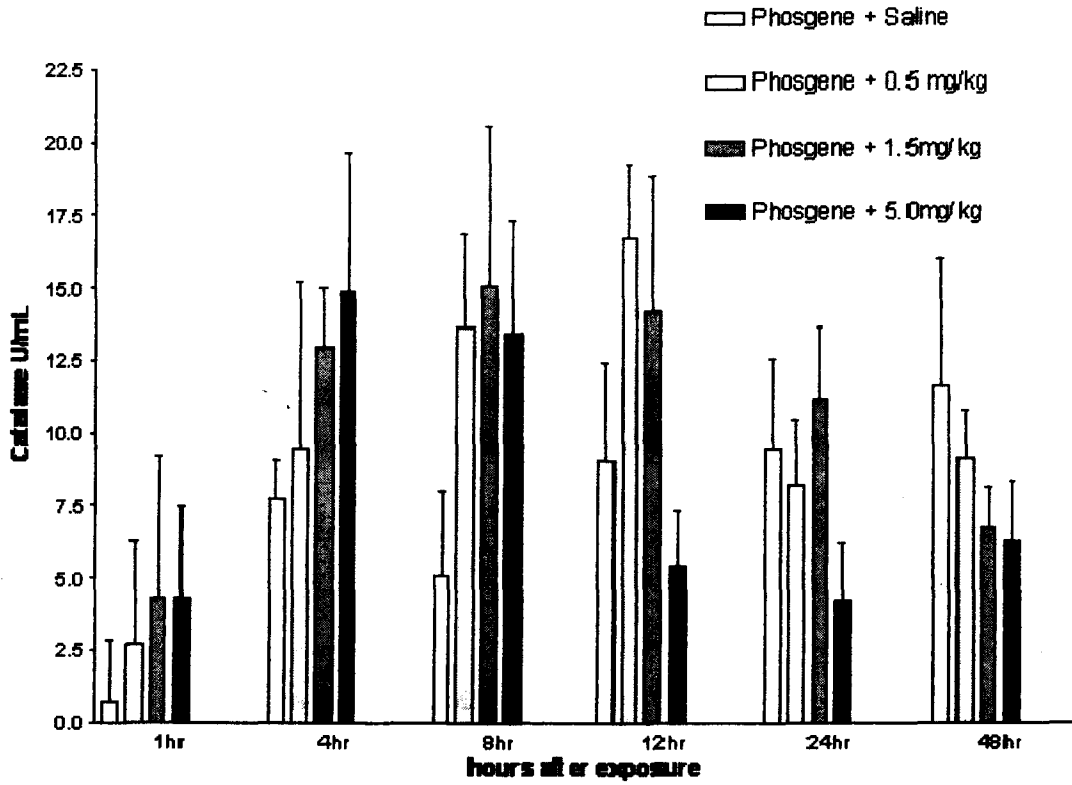


Figure 6. Catalase in lavage fluid of phosgene-exposed mice treated with saline or rhCC10.

References

1. Pilon AL: Detection of chemically altered forms of CC10 as indicators of exposure to chemical and biological warfare agents. Defense Threat Reduction Agency Small Business Innovation Research 2-005.
2. Babad H, Aeiler AG: The chemistry of phosgene. *Chem. Rev.* 1973;1:75-91
3. National Institute for Occupational Health and Safety. 1976 Criteria for a recommended standard: occupational exposure to phosgene. Department of Health, Education, and Welfare, Public Health Service, CDC publication 76137. Washington, DC.
4. Diller WF: Medical problems and their solutions. *J. Occup. Med.* 1978;20(3):189-193.