

Intracellular Acidification: The Initiating Event in Sulphur Mustard Induced Cytotoxicity

Thomas W. Sawyer^{*}, Cory Vair, Peggy Nelson, Stephen Bjarnason,
Catherine Tenn, Michael McWilliams and Mercy Villanueva

Defence R&D Canada – Suffield
PO Box 4000 STN MAIN
Medicine Hat, Alberta T1A 8K6, Canada

Thomas.Sawyer@drdc-rddc.gc.ca

ABSTRACT

The dependence of sulphur mustard (HD) toxicity on intracellular (pH_i) and extracellular pH was examined in CHO-K1 cells. HD produced an immediate and significant concentration-dependent decline in cytosolic pH, and also inhibited the mechanisms responsible for restoring pH_i to physiological values. This concentration response closely paralleled the acidification of the extracellular buffer through HD hydrolysis. A viability study was carried out in order to assess the importance of HD-induced cytosolic acidification. Cultures were exposed to HD for 1 hr in media that was adjusted through a pH range (pH 5.0 – pH 10), and the 24 hr LC_{50} values were assessed using the viability indicator dye alamarBlue™. The toxicity of HD was found to be dependent on extracellular pH, with a greater than eight-fold increase in LD_{50} obtained in cultures treated with HD at pH 9.5, compared to those treated at pH 5.0. Assays of apoptotic cell death, including morphology, soluble DNA, caspase-3 activity and TUNEL also showed that as pH was increased, much greater HD concentrations were required to cause cell death. The modest decline in HD half-life measured in buffers of increasing pH, did not account for the protective effects of basic pH. We propose that HD causes an extracellular acidification through chemical hydrolysis and that this, in both a concentration and temporally related fashion, results in cytosolic acidification. Furthermore, HD also acts to poison the antiporter systems responsible for maintaining physiological pH_i , so that the cells are unable to recover from this insult. It is this irreversible decline in pH_i that initiates the cascade of events that results in HD induced cell death.

1.0 INTRODUCTION

The chemical warfare (CW) agent sulphur mustard (bis (2-chloroethyl) sulphide, NATO Standard Agreed designation; HD) was extensively used in the First World War and most recently during the 1984/1985 Iran/Iraq War. It exerts direct deleterious effects on a variety of tissues including skin, eyes and the respiratory tract and can also cause profound systemic intoxication (Dacre and Goldman, 1996; National Academy Press, 1993; Papirmeister *et al.*, 1991; Smith and Dunn, 1991). Although HD has been well documented as having potent cytotoxic, mutagenic, and vesicant properties, its mechanism of vesicant action is not well understood and treatment of the mustard casualty is entirely symptomatic (Dacre and Goldman, 1996; Kehe and Szinicz, 2005; National Academy Press, 1993; Papirmeister *et al.*, 1991; Smith and Dunn, 1991; Willems, 1989).

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The regulation of intracellular pH (pH_i) within a closely defined physiological range is vital for a wide spectrum of cellular processes, including cell metabolism, calcium homeostasis, gene expression, cell motility and contractility, cell adhesion and cell death (Lagadic-Gossmann *et al.*, 2004; Pucéat, 1999). In order to maintain constant cytosolic pH, eukaryotic cells employ a variety of mechanisms that include plasma membrane proton pumps and channels, as well as ion transporters (Pucéat, 1999). Perturbation of these processes has been implicated as one of the mechanisms in the cytotoxicity of a variety of toxicants (Huc *et al.*, 2004; Lagadic-Gossmann *et al.*, 1999; Mahnensmith and Aronson, 1985).

In this study, the effect of HD on the pH_i of CHO-K1 cells was investigated using the pH sensitive fluorophore SNARF-1. The results of this work also led us to examine the effects of extracellular pH on HD-induced cell death.

2.0 MATERIALS AND METHODS

2.1 CHO-K1 Cell Culture

Seed cultures were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in 10% FCS in F-12 culture medium supplemented with streptomycin (100 $\mu\text{g}/\text{ml}$) and penicillin (100 IU/ml), with the medium being changed as required. Stock cultures were closely monitored and not allowed to grow to confluency prior to subculture. Test cultures were seeded so that cells were used just prior to, or at confluency (2 – 3 days).

2.2 Chemical Treatment and Cytotoxicity Studies

On the day of experimental treatment cultures were aspirated and re-fed with test medium adjusted to the desired test pH. The cells were then immediately treated with freshly prepared HD treatment medium (at the same test pH) so that the final HD concentration was reached at 0.25% solvent vehicle (ethanol, v/v). The cultures were incubated for 1 hr at 37°C and then the treatment medium was aspirated and replaced with routine culture medium, pH 7.4. Twenty-three hr later, cell viability was assessed using the alamarBlue™ (AccuMed International Inc., Westlake, OH) cytotoxicity assay. The dye was added (10%, v/v) to the cultures and incubated for the last 2 – 3 hr of treatment. The absorbences (570 nm - 600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing 6 wells per data point. All experiments were carried out at least 3 times. Sulphur mustard was prepared at DRDC Suffield at greater than 99% purity.

2.3 Apoptosis Studies

In all studies that examined the effect of pH on HD-induced apoptotic endpoints, cells were treated with varying concentrations of the toxicant at the desired test pH for 1 hr. The cultures were then re-fed with fresh medium and apoptotic cell death was assessed 5 hr after the initiation of HD exposure. Assays of morphology, TUNEL, soluble DNA and caspase-3 were carried out as previously described (Sawyer *et al.*, 2007)

2.4 Intracellular pH

Suspended CHO-K1 cells (5×10^5) were incubated with 0.02% Pluronic-F127 and 5 μ M carboxysemnaphthorhodafluor-1 (SNARF-1 AM, Molecular Probes, Eugene, OR) in 1 ml assay buffer (Hanks Balanced Salt Solution, Invitrogen, Carlsbad, CA) with 10 mM HEPES, 1 mM CaCl_2 and 250 μ M sulfipyrazone at the pH being investigated for 30 min at 37°C. Cells were then rinsed with 1 ml fresh assay buffer twice and incubated for at least 20 min on ice prior to analysis. Intracellular pH was monitored at 37°C using 514 nm excitation of SNARF-1, and ratiometric analysis of 580 nm and 640 nm emission using a Deltascan fluorometer and Felix 32 software (Photon Technology International, Trenton, NJ). The fluorescence ratio of 580/640 was converted to pH units using a calibration curve. pH calibration was performed on SNARF-1 loaded cells in depolarizing buffer (135 mM KCl, 15 mM NaCl, 1 mM CaCl_2 , 1 mM KH_2PO_4 , 0.5 mM MgSO_4 , 10 mM HEPES) using 0.5 pH unit steps between pH 6.0 and pH 9.0. Cells were treated with 10 μ M nigericin (Molecular Probes) to equilibrate intracellular and extracellular H^+ .

2.5 Extracellular pH

For the determination of HD-induced changes in extracellular pH, suspensions of CHO-K1 cells were treated in an identical fashion to those being prepared for intracellular pH determination (above). At time zero, HD was added and extracellular pH was measured at 37°C using a VWR Symphony SB70P pH meter.

2.6 Ammonium Chloride Cytosolic Acidification

Cells (5×10^5) were incubated with 0.02% Pluronic-F127 and 5 μ M SNARF-1 AM in 1ml of assay buffer (Hanks Balanced Salt Solution (HBSS), 10mM HEPES, 1mM CaCl_2 and 250 μ M sulfipyrazone, pH 7.0) for 30 minutes at 37°C. The cells were then centrifuged and the cell pellet rinsed once with, and then resuspended in the ammonium chloride loading buffer (HBSS with 20 mM NaCl replaced by 20 mM NH_4Cl , 10mM HEPES, 1mM CaCl_2 and 250 μ M sulfipyrazone, pH 7.0). After a 30 min incubation, the cell suspension was centrifuged and the cell pellet was then resuspended in 2 ml of assay buffer and immediately monitored for changes in pH_i .

2.7 Rate of HD Hydrolysis

Test buffer solutions (130 mM NaCl, 40 mM sucrose, 10 mM HEPES, 10 mM glucose) at pH 5.0, 7.0 and 9.5 were incubated at 20°C in a Haake re-circulating water bath for at least 20 min prior to each sample being spiked with HD (200 μ M HD in 0.5% absolute ethanol final concentration). The solutions were vigorously hand shaken for 30 sec and incubated at 20°C in a circulating water bath. Two ml samples were taken at 3, 10, 15, 20, 30, 40, 50 and 60 min and transferred into one dram glass vials, containing 0.6 ml dichloromethane. The vials were vigorously hand shaken for 30 sec. The dichloromethane layer was allowed to settle and 150 μ l sample aliquots were transferred into sample vials for GC analysis. A Varian 3800 gas chromatograph equipped with an 8400 autosampler was used to determine the HD concentration in all dichloromethane extracts. The chromatograph was equipped with a flame ionization detector (FID) for quantitative HD analyses and a pulsed flame photometric detector (PFPD) for sample peak identification. A 15 metre DB-1701 column (JW Scientific, Folsom, CA) with an internal diameter of 0.32 mm and a stationary phase film thickness of 0.25 μ m was used.

3.0 RESULTS

Figure 1A shows the change in pH_i (ΔpH_i) of cells incubated in buffer at pH 7.0 and exposed to HD (200-800 μM). A concentration-response was observed, with a maximal decline in pH_i of $\sim 0.25 - 0.3$ pH units, when compared to control values. Figure 1B shows representative measurements of tracings of HD-induced cytosolic acidification. Maximal acidification occurred by ~ 15 min and then plateaued. The effects of ammonium chloride induced intracellular acidification is depicted in Figure 2. Acidification of pH_i to \sim pH 6.6 (from pH 7.0) was obtained within seconds, after which the cells rapidly recovered to a pH_i of ~ 6.9 . Incubation with HD not only prevented pH_i recovery, but also caused further acidification. No elevation of pH_i towards physiological levels was observed during the 30 min test period.

Incubation of HD in cell suspensions incubated at $37^\circ C$, caused a concentration-dependent decrease in extracellular pH (Fig. 3A), that very closely paralleled the concentration response of pH_i caused by this agent. The decline in extracellular pH caused by 800 μM HD was only slightly larger than the decline in pH_i (Fig. 3B).

Figure 4 shows the effect of pH on HD toxicity in CHO-K1 cells as assessed using the indicator dye alamarBlue™. At pH 7.5, cell viability rapidly declined at lower HD concentrations and then only gradually decreased at HD concentrations greater than 200 μM . The concentration-response curve shifted to the left when HD exposure was carried out at pH 5.5, with 300 μM HD producing almost 100% cell death. In contrast, when cultures were exposed to HD in a medium of pH 9.5, just over 50% cell death was observed at 800 μM HD (Fig. 4A). This trend is more fully illustrated in Figure 4B, where the LC_{50} of HD is clearly increased as the exposure is carried out in an increasingly basic environment. The viability of vehicle-treated control cultures incubated in medium at pH 5.0 to pH 10.0 was unaffected. However, incubation of cultures in medium outside of this pH range resulted in toxicity (data not shown).

The effect of HD on apoptotic DNA fragmentation was quantitated using two assays; soluble DNA and the TUNEL reaction. Figure 5A depicts the concentration-dependent HD-induced DNA fragmentation as measured by soluble DNA. At pH 7.5 fragmentation increased with concentration up to 600 μM , and then decreased. At pH 9.5, the percent fragmentation for control cultures was similar to those of cells incubated in pH 7.5 medium. In contrast to those cells incubated in pH 7.5 medium, HD did not induce any further DNA fragmentation over control values. Very similar trends were obtained when DNA fragmentation was measured using the TUNEL assay (Fig. 5B). The HD-induced effects on apoptotic and necrotic cell death was assessed using morphological criteria (Fig. 6A). While necrotic cells were detected, in no case were they a statistically significant HD-induced phenomenon, and therefore their quantitation was not included. At both pH 7.5 and 9.5, a concentration-dependent increase in apoptotic cells were induced by HD. This trend was much reduced at pH 9.5, with 800 μM HD inducing a little over 10% apoptosis, as compared to $\sim 70\%$ when cells were exposed to HD in pH 7.5 culture medium. The effect of HD on caspase-3 activity is depicted in Figure 6B. At both pH 7.5 and 9.5, HD induced a concentration-dependent increase in activity, although maximal levels were significantly reduced at pH 9.5, as compared to those obtained at pH 7.5. At pH 7.5, induced activity was maximal at 400 μM HD and then declined, compared to cells exposed to HD at pH 9.5, where the maximal induction occurred at 600 μM HD.

Table 1 shows the LC_{50} of HD on CHO-K1 cells exposed in media of various pH. In addition, it depicts the half-life of HD when incubated at $37^\circ C$ in physiological buffer at these pHs, and the calculated one hour concentration x time integral of these half-lives. Cells incubated in pH 9.5 medium were almost eight times more resistant to the toxicity of HD than when they were incubated at pH 5.0. The half-life of HD slightly,

but significantly decreased as the pH was increased. This was also reflected in the one hour concentration x time integral, which only decreased by a factor of 1.2 from pH 5.0 to pH 9.5.

4.0 DISCUSSION

An early theory of HD toxicity postulated that cell death was due to the direct toxic action of hydrochloric acid, produced through the hydrolysis of HD in an aqueous environment. Although this hypothesis was rapidly dismissed at the time (Papirmeister *et al.*, 1991), it is now known that xenobiotic-induced alterations in pH_i , particularly acidification, can have profound implications with respect to the regulation of diverse biochemical pathways including calcium metabolism, apoptosis, cell growth, lymphocyte activation and neoplastic transformation (Benedetti *et al.*, 2001; Grinstein *et al.*, 1988; Lagadic-Gossmann *et al.*, 2004; Puc at, 1999; Putney *et al.*, 2002; Reshkin *et al.*, 2000; Vaughan-Jones *et al.*, 1983). In order to examine the possibility that HD exerts its toxicity by interfering with intracellular pH regulation, we investigated the effect of HD on the pH_i of CHO-K1 cells using the pH sensitive dye, SNARF-1.

In cells incubated in buffer at physiological pH, HD induced a significant and rapid concentration-dependent decline in pH_i . The acidification induced by 800 μM HD was ~ 0.25 pH units compared to controls, was complete in ~ 15 min and then plateaued within the 30 min test period. Cytosolic pH is maintained by intracellular buffers such as organic acids, bases and proteins, as well as by several plasma membrane-bound antiporters. It is possible that at higher concentrations of HD, these intracellular buffering systems were overcome, eventually resulting in toxicity due to intracellular acidification. We further examined the effect of HD on pH_i using the ammonium chloride prepulse technique. Pulsing the cells with ammonium chloride rapidly produced a decline in pH_i of ~ 0.4 units. In control cells, the antiporter systems responsible for maintaining physiological pH_i , returned the cells back to physiological levels. However, in addition to causing a further and very significant decline in cytosolic pH, HD also prevented any recovery from the combined ammonium chloride/HD acidification. Thus, although this cell culture system has robust mechanisms with which to protect itself against acidic pH_i , HD not only causes cytosolic acidification, but also poisons the very mechanisms that are in place to counteract this kind of insult.

The hydrolysis of HD has been shown to result in acidic degradation products (Brimfield *et al.*, 2006) and we therefore examined the effect of varying HD concentrations in cell suspensions incubated at 37°C . Sulphur mustard induced a concentration-dependent decline in pH that very closely paralleled the acidification in pH_i that it causes in this same cell type. It must therefore be concluded that HD induced- pH_i acidification is a direct result of the extracellular acidification caused by HD hydrolysis.

In order to gain additional understanding of the importance of HD-induced cytosolic acidification, we examined the effect of extracellular pH on HD toxicity, since it has been shown to modulate pH_i (Prigent *et al.*, 1997; Vaughan-Jones and Wu, 1990). The range tested was pH 5.0 – pH 10. In this range, control cultures showed no measurable loss in viability. We observed a very gradual decrease in HD toxicity from pH 5.0 to pH 8.5, a slightly steeper decrease at pH 9.0 and then a dramatic increase in LC_{50} at pH 9.5 and 10.0. Similar protection was also obtained when we examined the effects of basic pH on assays of apoptosis. It is likely that basic pH buffers HD-induced acidification. In those test regimens where pH_i did not enter into apoptosis/cell death-permissive conditions, cell death was prevented.

Although HD hydrolysis has been reported not to be pH dependent (Papirmeister *et al.*, 1991), we nevertheless determined the half-lives of HD in buffer at pH 5.0, 7.0 and 9.5 to rule out the possibility that our cytotoxicity findings were simply the result of pH-induced alterations in HD exposure. We found that HD half-life decreased slightly as the buffer became increasingly basic. Integration of the decay curves over the

one hour time period that we treated our cultures, did not explain the almost eight-fold decrease in HD toxicity from pH 5.0 to pH 9.5. It appears that the effect of pH on HD-induced toxicity must be biochemical/pharmacological in nature and not an effect on the rate of HD hydrolysis.

The data obtained in this study supports the hypothesis that HD causes an extracellular acidification through chemical hydrolysis and that this, in both a concentration and temporally related fashion, results in cytosolic acidification. Sulphur mustard also acts to poison the antiporter systems responsible for maintaining physiological pH_i , so that the cells are unable to recover from this insult. It is this irreversible decline in pH_i that initiates the cascade of events which results in HD induced cell death.

Table 1: Effect of pH on HD LC_{50} values, half-life, and CT exposure*

pH	LC_{50} (μM) (x +/- SD)	Half-life (x +/- SD, min)	CT (1 hr) (AUC, ng-min.ml) (x +/- SD)
5.0	76.9 +/- 2.6*	25.3 +/- 0.7	29.5 +/- 0.5
7.0	126.1 +/- 5.0	22.4 +/- 0.4	27.3 +/- 0.3
9.5	593.7 +/- 53.7	19.1 +/- 0.5	24.4 +/- 0.5

* Half-life values were determined using 200 μM HD in buffer at 20°C. Concentration x time (CT) values were calculated based on a 60 min exposure. Results represent the mean +/- SD of three experiments and were analyzed by ANOVA and *post-hoc* Tukey HSD Multiple Comparison testing.

* Not significantly different from value obtained at pH 7.0 ($p > 0.05$). All other values within columns were significantly different from each other ($p > 0.05$).

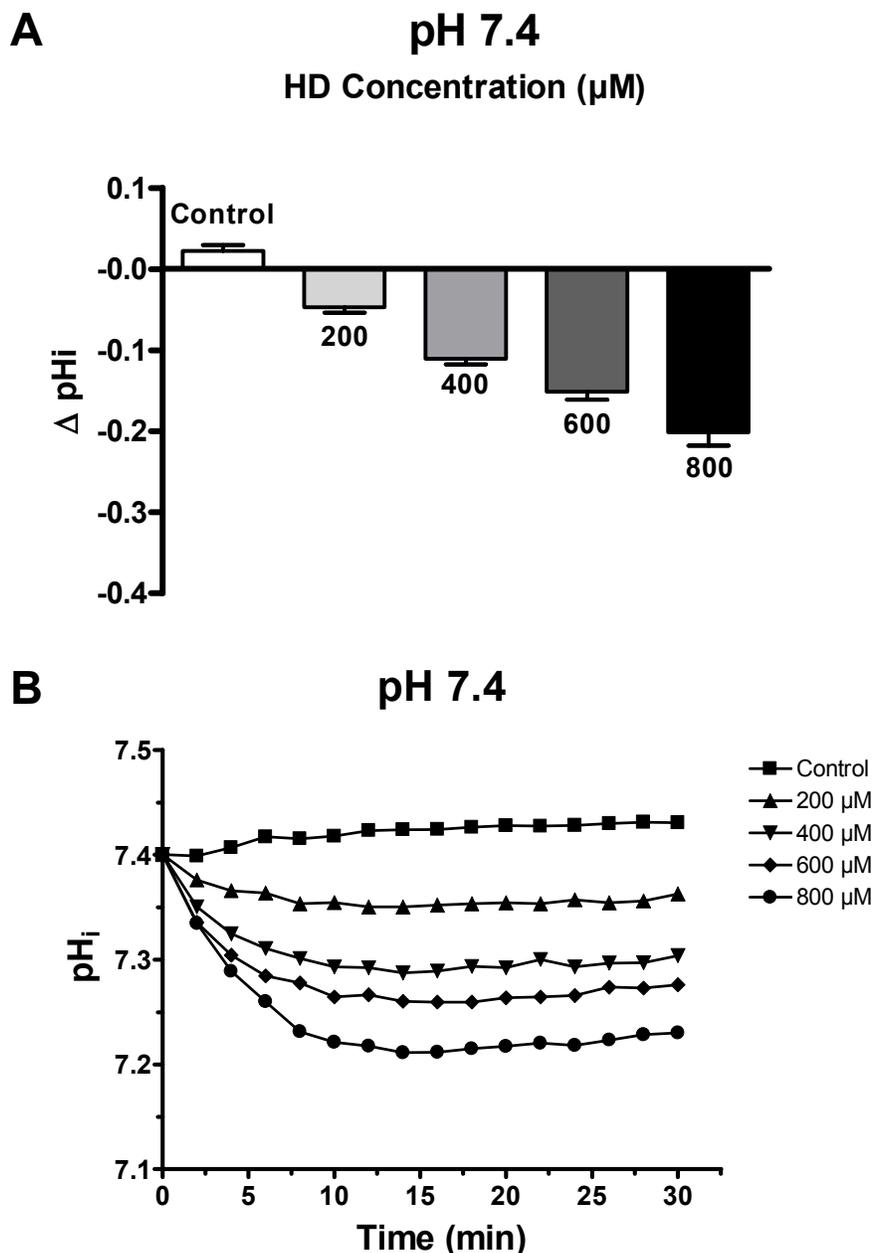


Figure 1 Sulphur mustard induced changes in intracellular pH in CHO-K1 cells. Cells were loaded with the intracellular pH dye indicator SNARF-1 prior to HD exposure. The top panel shows results representing the mean \pm standard deviation of three experiments (A), while the bottom panel shows representative tracings of HD-induced cytosolic acidification (B).

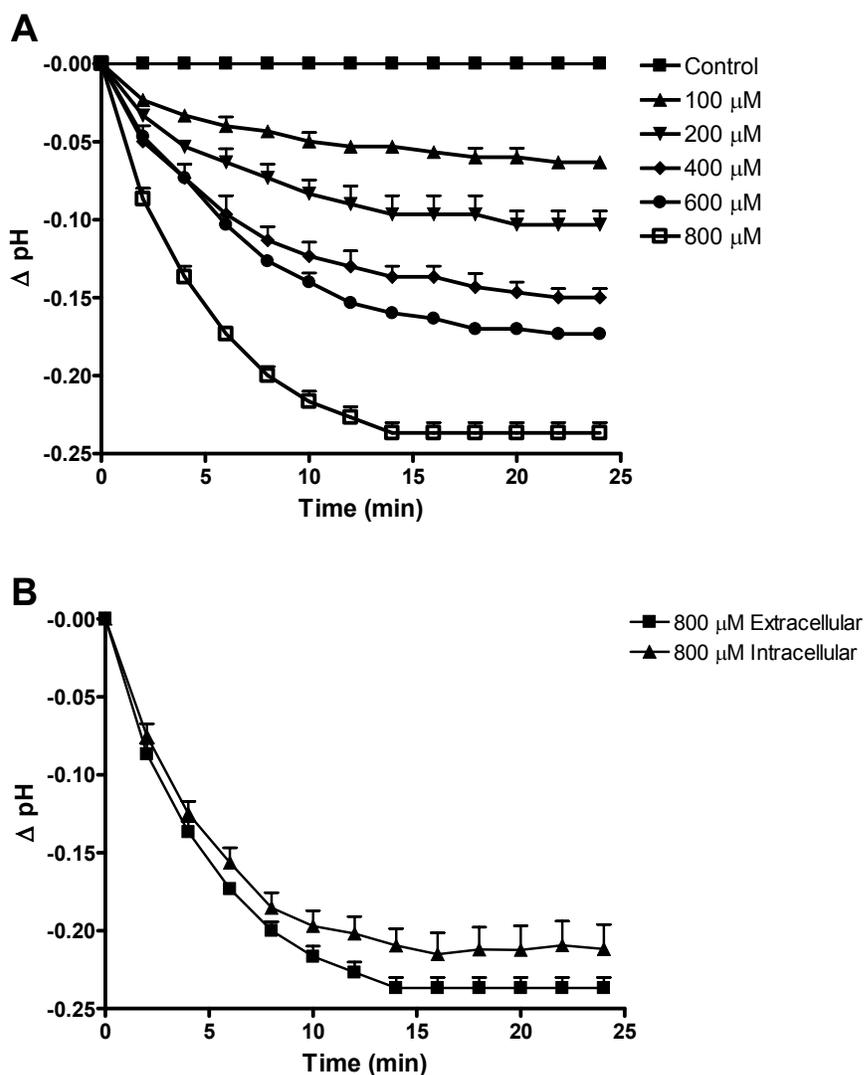


Figure 2 Ammonium chloride cytosolic acidification of CHO-K1 cells at pH 7.0. The cytosolic pH of CHO-K1 cells was acidified using the ammonium chloride prepulse technique, and the effect of 800 μM HD on the recovery of pH_i was monitored. Results represent the mean +/- standard deviation of 3 separate experiments.

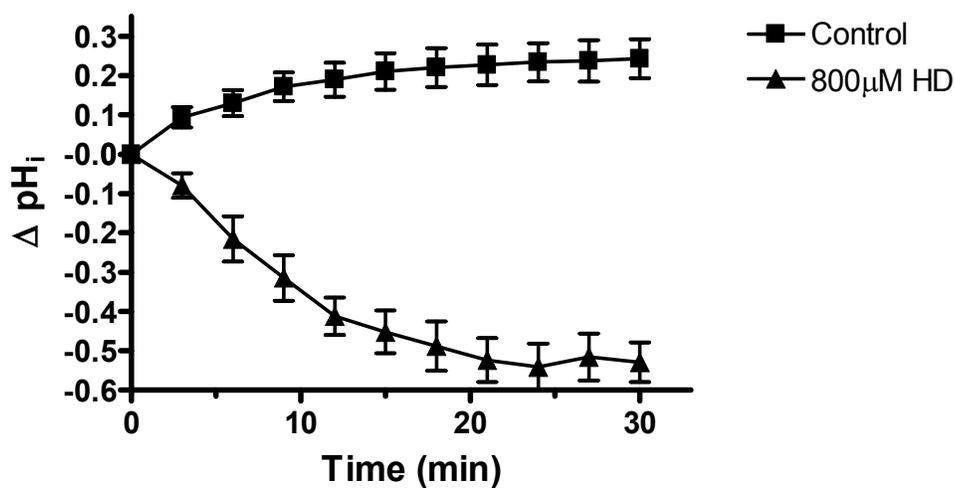


Figure 3 Effect of HD hydrolysis on extracellular acidification.

Cell suspensions (pH 7.4) were treated identically to those that were prepared for pH_i determinations. The suspensions were then monitored immediately after HD treatment for changes in extracellular pH (A). Sulphur mustard (800 μM) induced an extracellular acidification that very closely paralleled its effects on pH_i (B). Results represent the mean \pm standard deviation of 3 separate experiments.

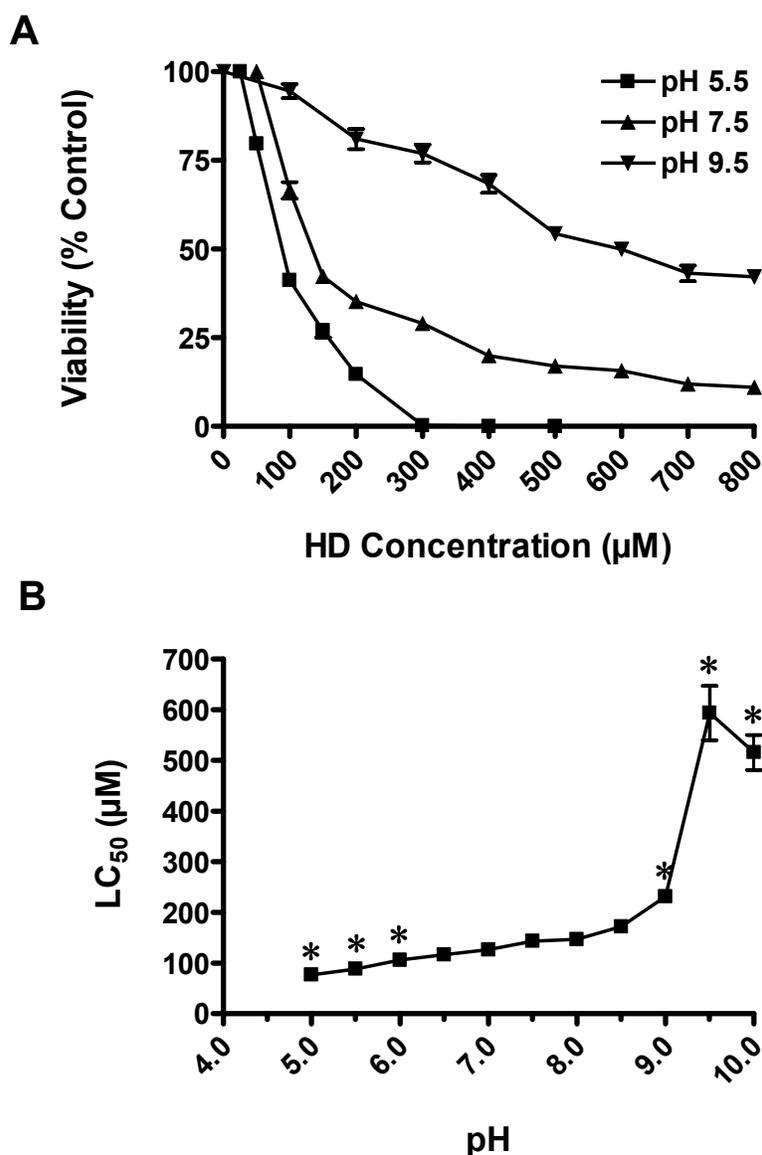


Figure 4 Effect of pH on the cytotoxicity of HD in CHO-K1 cultures. Just-confluent CHO-K1 cultures were treated with varying HD concentrations in culture medium of different pH. After 1 hr, the test medium was removed and replaced with routine culture medium (pH 7.4). The top panel shows representative concentration-responses of cells treated with HD at three different pHs (A). The bottom panel shows the change in HD LC₅₀ values as a function of treatment medium pH (B). Cytotoxicity was determined at 24 hr using the alamarBlue™ cytotoxicity assay. LC₅₀ values were obtained graphically. Results represent the mean +/- standard deviation of 3 separate experiments. Data was analyzed by ANOVA and *post-hoc* Dunnett's Method Comparison test using pH 7.5 as the control group (* $p < 0.05$ vs. pH 7.5).

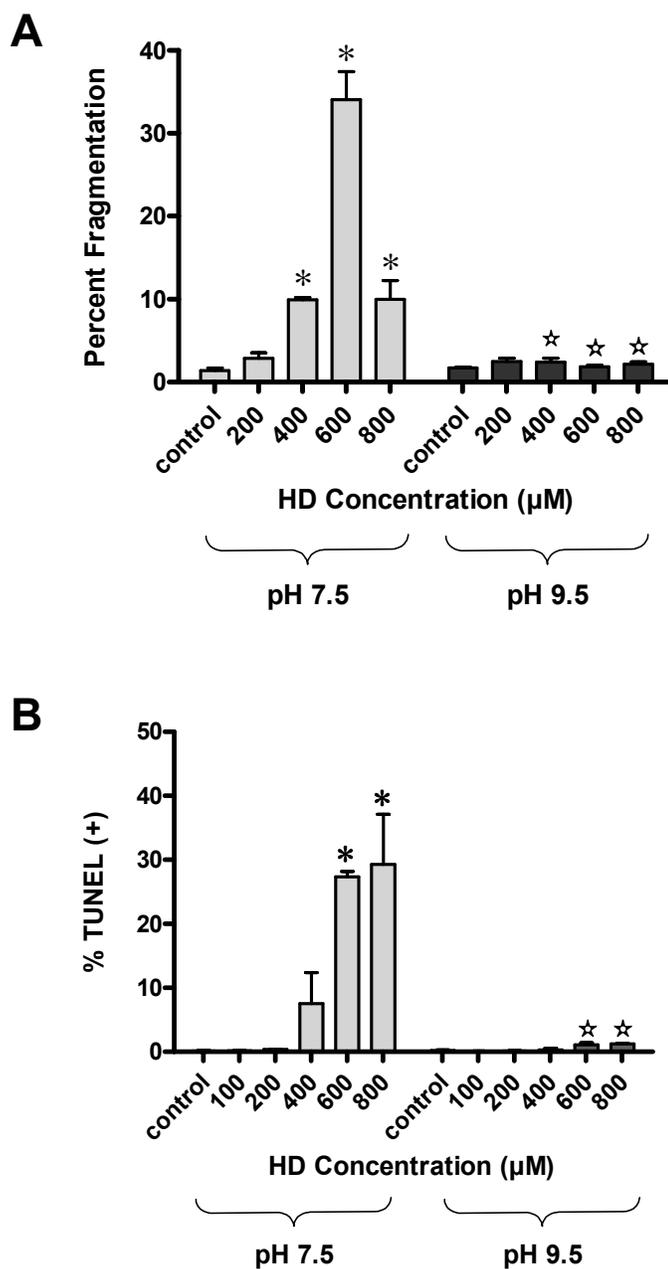


Figure 5 Effect of pH on HD-induced DNA fragmentation in CHO-K1 cultures. Cultures were treated with varying HD concentrations in culture medium of different pH for 1 hr. Following the 1 hr incubation, the test medium was removed and replaced with routine culture medium (pH 7.4). DNA fragmentation was assessed as soluble DNA (A), or determined using the TUNEL assay (B), 5 hr post-treatment. Results represent the mean \pm standard deviation of 3 separate experiments. Data was analyzed by ANOVA and *post-hoc* Tukey HSD Multiple Comparison testing (* $p < 0.05$ vs. respective controls; * $p < 0.05$ vs. corresponding HD concentration at pH 7.5).

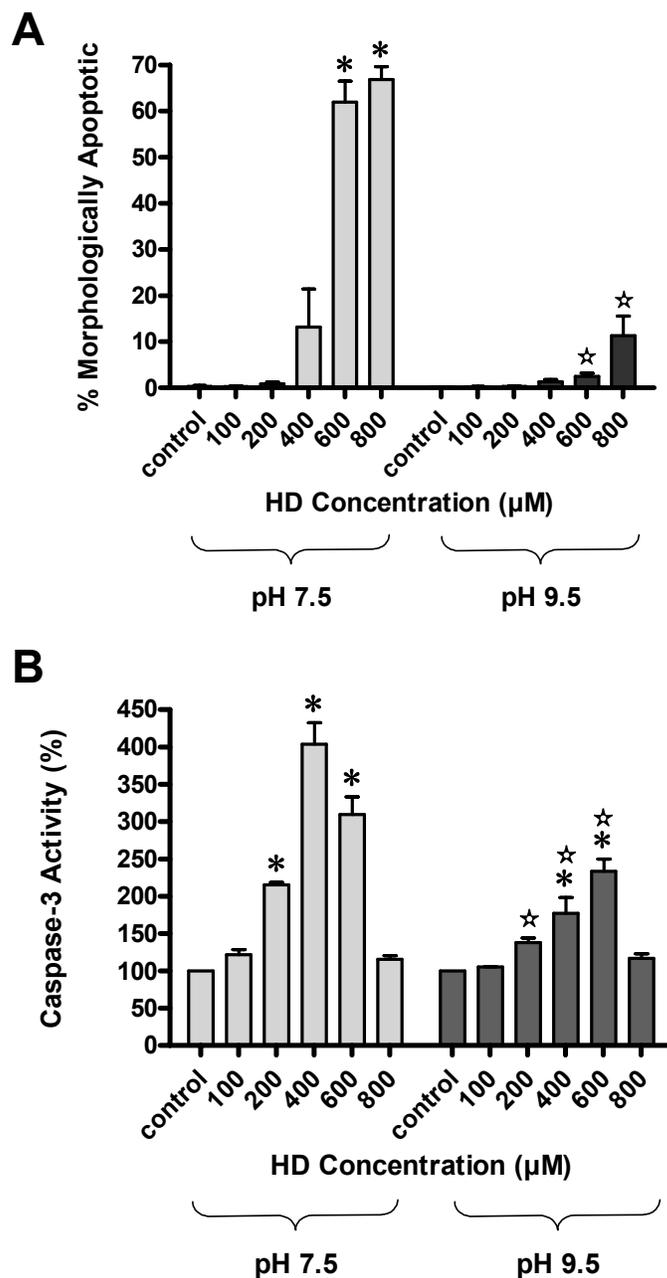


Figure 6 Effect of pH on HD induced morphology and caspase-3 activity in CHO-K1 cultures. Cultures were treated with varying HD concentrations in culture medium of different pH for 1 hr. Following the 1 hr incubation, the test medium was removed and replaced with routine culture medium (pH 7.4). Sulphur mustard induced changes in morphology (A) and caspase-3 activity was assessed 5 hr post-treatment. Results represent the mean \pm standard deviation of 3 separate experiments. Data was analyzed by ANOVA and *post-hoc* Tukey HSD Multiple Comparison testing (* $p < 0.05$ vs. respective controls; ** $p < 0.05$ vs. corresponding HD concentration at pH 7.5).

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