Efficacy of glutathione in ameliorating sulfur mustard analog-induced toxicity in cultured skin epidermal cells and in SKH-1 mouse skin in vivo

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Running Title: Efficacy of GSH in ameliorating CEES-induced skin toxicity.

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Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia-Rad3-related; BrdU, 5-bromo-2'-deoxy-uridine; BSO, buthionine sulfoximine, CEES, 2-chloroethyl ethyl sulfide; DMSO, dimethylsulfoxide; GSH, Glutathione; HD, sulfur mustard; MAPKs, mitogen activated protein kinases; MMPs, matrix metalloproteases; MPO, myeloperoxidase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-cysteine; NF-κB, nuclear factor kappa B; 8-OH2dG, 8-oxo-2-deoxyguanosine; PARP, poly (ADP–ribose) polymerases; ROS, reactive oxygen species; SOD, superoxide dismutase.

Recommended Section: Toxicology.
Abstract

Exposure to chemical warfare agent sulfur mustard (HD) is reported to cause glutathione (GSH) depletion that plays an important role in HD-linked oxidative stress and skin injury. Employing HD analog, 2-chloroethyl ethyl sulfide (CEES), here we evaluated the role of GSH and its efficacy in ameliorating CEES-caused skin injury. Using mouse JB6 and human HaCaT epidermal keratinocytes, we observed both protective and therapeutic effects of exogenous GSH (1 or 10 mM) in attenuating CEES-caused decrease in cell viability and DNA synthesis, and S and G2-M phase arrest in cell cycle progression. However, the protective effect of GSH was stronger than its ability to reverse CEES-induced cytotoxic effect. The observed effect of GSH could be associated with an increase in intracellular GSH levels following its treatment before or after CEES exposure which strongly depleted cellular GSH levels. N-acetyl cysteine (NAC), a GSH precursor, also showed both protective and therapeutic effects against CEES-caused cytotoxicity. BSO, which reduces cellular GSH level, caused an increased CEES cytotoxicity in both JB6 and HaCaT cells. In further studies translating GSH effects in cell culture, pre-treatment of mice with 300 mg/kg GSH via oral gavage 1 h prior to topical application of CEES resulted in significant protection against CEES-caused increase in skin bi-fold and epidermal thickness, apoptotic cell death and myeloperoxidase (MPO) activity, which could be associated with increased skin GSH levels. Together, these results highlighting GSH efficacy in ameliorating CEES-caused skin injury, further support the notion for effective antioxidant countermeasures against skin injury by HD exposure.
Introduction

Sulfur mustard [HD, bis(2-chloroethyl) sulfide], a major chemical warfare agent, has been successfully deployed as a weapon in military conflicts (Graham et al., 2005; Paromov et al., 2007). Primary effect of HD exposure is cutaneous toxicity, in addition to ocular, respiratory and other tissue injuries (Dacre and Goldman, 1996; Kehe et al., 2008; Rowell et al., 2009). HD penetrates into skin tissue, attacks mainly dividing basal epidermal cells, and causes massive inflammatory response, apoptotic/necrotic cell death as well as delayed excruciating vesication (Ray et al., 2000; Henemyre-Harris et al., 2008; Hayden et al., 2009). HD is a powerful bi-functional alkylating agent, and its exposure causes depletion of cellular antioxidant thiols, mainly glutathione (GSH), which may lead to an increased production of ROS and membrane lipid peroxidation (Kehe and Szinicz, 2005; Mukhopadhyay et al., 2006; Paromov et al., 2007; Ruff and Dillman, 2007). These events lead to oxidative stress and macromolecular damage, including DNA, triggering various signaling pathways and modulating gene expression, causing a series of HD-associated toxic responses (Paromov et al., 2007; Ruff and Dillman, 2007; Kehe et al., 2009; Shakarjian et al., 2010). HD-induced increase in inflammatory cells including neutrophils in skin also generates ROS promoting oxidative stress (Shakarjian et al., 2010).

Apart from HD, its monofunctional analog 2-chloroethyl ethyl sulfide (CEES) also causes several skin toxic effects comparable to HD, and therefore CEES is extensively used to uncover the mechanism of action of HD and to screen effective therapeutic agents (Han et al., 2004; Paromov et al., 2007; Gould et al., 2009; Tewari-Singh et al., 2009; Tewari-Singh et al., 2010). HD/CEES-caused oxidative stress results in 8-oxo-2-deoxyguanosine (8-OH2dG) DNA adduct and lipid and protein oxidation which could cause inflammatory and other toxic responses in skin (Pal et al., 2009; Black et al., 2010). Exogenous addition of antioxidants such as GSH, N-
acetyl cysteine (NAC), vitamin E, superoxide dismutase (SOD), catalase, sulforaphane, quercetin, and catalytic antioxidants such as AEOL 10150 have been reported to attenuate lung and skin injury by HD/CEES (Gross et al., 1993; Smith et al., 1997; Amir et al., 1998; Han et al., 2004; Arfsten et al., 2007; Paromov et al., 2007; Paromov et al., 2008; Gould et al., 2009; Black et al., 2010; O’Neill et al., 2010). Whereas most of these efficacy studies with antioxidants indicate that they have beneficial effects in attenuating HD/CEES-caused lung injury, there are few, if any, reports of efficacy studies with GSH and associated mechanism of action in relevant skin epidermal cells and skin toxicity models of HD/CEES (Paromov et al., 2007).

Our recent study in mouse epidermal JB6 and human epidermal HaCaT cells suggests that the DNA damaging effect of CEES activates ataxia telangiectasia mutated (ATM)/ataxia telangiectasia-Rad3-related (ATR) cell cycle checkpoint signaling as well as caspase-poly(ADP–ribose) polymerases (PARP) pathways, which lead to apoptosis/necrosis (Tewari-Singh et al., 2010). In addition, our study in SKH-1 mouse model indicates that CEES induces oxidative stress and activation of interlinked complex mechanisms of CEES-mediated skin inflammation and injury (Pal et al., 2009; Tewari-Singh et al., 2009). To further analyze the role of GSH and oxidative stress in CEES-induced biological and molecular responses in these skin toxicity models, here we evaluated the efficacy of exogenous GSH in attenuating CEES-caused skin injury in both cell culture and mouse skin models. Our results further support the involvement of GSH and oxidative stress in molecular mechanisms that lead to CEES-caused skin injury, which form the basis for the development of effective antioxidant therapies alone or in combination with other identified countermeasures in attenuating HD-caused skin injury.
Methods

Chemicals, culture and assay materials. HD (CICH2CH2SCH2CH2Cl) analog, CEES (CH3CH2SCH2CH2Cl), buthionine sulfoximine (BSO; C8H18N2O3S), GSH (C10H17N3O6S), NAC (C5H9NO3S), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and saponin and propidium iodide (PI) were obtained from Sigma-Aldrich Chemical Co. (St. Louis., MO). Minimal essential medium (MEM) and Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin G, streptomycin sulfate and gentamycin for cell culture were obtained from Gibco BRL (Grand Island, NY). 5-bromo-2’-deoxy-uridine (BrdU) colorimetric kit was from Roche Applied Science (Indianapolis, IN). Fluorescent Myeloperoxidase (MPO) detection kit was purchased from Cell Technology (Mountain View, CA), eosinophil peroxidase and catalase (3-Amino-1, 2, 4-triazole) inhibitors were obtained from Sigma-Aldrich Chemical Co. (St. Louis., MO). GSH-Glo™, Glutathione Assay kit was from Promega (Madison, WI) and Bio-Rad DC protein assay kit was purchased from Bio-Rad laboratories, Hercules, CA.

Culture and Treatment of Cells. JB6 and HaCaT cell culture and treatments were carried out as described earlier (Tewari-Singh et al., 2010). Briefly, JB6 cells were cultured in MEM containing 5% heat inactivated FBS and 25 μg/ml gentamycin, and HaCaT cells were grown O/N in DMEM supplemented with 10% FBS and 100 U/ml penicillin G-100 μg/ml streptomycin sulfate. Cells grown O/N under standard culture conditions and treated with either DMSO, 1 or 10 mM GSH, 25 or 50 mM NAC as controls, with 1 or 10 mM GSH/25 or 50 mM NAC before or after exposure to 0.35 or 0.5 mM concentration of CEES in DMSO, or exposed to CEES in DMSO alone. These treatments and CEES exposures were carried out in the same growth medium without the removal of the GSH/NAC or CEES in the pre- or post-treatments. For BSO
studies, it was added in media at 0.2 mM concentration 6 h prior to CEES exposure. Required amount of CEES from the stock prepared in DMSO was mixed into the cell growth media and added to the 70-80% confluent cells immediately as reported earlier (Tewari-Singh et al., 2010). Desired GSH and NAC stocks were prepared in the cell growth media and pH adjusted to 7.0 before diluting to the concentration required for the treatment of cells. The final concentration of DMSO in the culture medium during treatments did not increase 0.1% (v/v).

**Measurement of cell viability and cell proliferation (DNA synthesis).** These measurements were carried out as reported earlier (Tewari-Singh et al., 2010). In short, cell viability (MTT) and cell proliferation (BrdU) assays were conducted at 24 or 48 h following the desired treatment of cells (2000 cells/well) cultured O/N in 96 well culture plates. For MTT assay, cells were incubated with 1 mg/ml of MTT in serum free medium for 4 h at 37°C, the MTT solution was removed thereafter and absorbance was read following addition of 100 µl DMSO at 540 nm. BrdU assay (based on measurement of thymidine analog, BrdU incorporation during DNA synthesis) was conducted following the manual instructions. Briefly, treated cells were incubated with BrdU, fixed and DNA denatured, and labeled with anti-BrdU mouse monoclonal Ab-Fab. The BrdU antibody bound to newly synthesized cellular DNA and complexes were detected via product quantification by measuring the absorbance at 370 nm (reference wavelength: 492 nm). For both MTT and BrdU assays, absorbance was read using Spectra max 190 micro plate reader (Molecular Devices, Sunnyvale, CA) and the blank control readings were subtracted from all the sample readings taken.
**Cell cycle analysis.** This was carried out as described previously (Tewari-Singh et al., 2010), employing 70-80% confluent cells cultured O/N in 60 mm petri dishes seeded at the density of 5000 cells/cm². Cells were treated with DMSO, GSH alone, GSH pre- or post-CEES exposures, or exposed to CEES alone for 24 h. Thereafter, cells were harvested, washed twice with 1X PBS and incubated in 0.5 ml of saponin/PI solution (0.3% saponin, 25 µg/ml PI, 0.1 mM EDTA, and 10 µg/ml RNase in PBS) at 4°C for 24 h in the dark. Cell cycle distribution was analyzed by flow cytometry using FACS analysis core services of the University of Colorado Denver Cancer Center (Denver, CO).

**Quantification of GSH in cells.** Equal number of cells was seeded in 96 well plates overnight and following desired treatments with DMSO, GSH alone, GSH before or after CEES exposure, or 0.5 mM CEES alone for 24 h, quantification of GSH was carried out using the GSH-Glo™ Glutathione assay kit from Promega, according to manufacturers’ protocol. Briefly, following desired treatments, medium was aspirated and well were washed with PBS, and then to the total number of cells/well, 100 µl of prepared GSH-Glo™ reagent (containing 1 µl Lucifern NT substrate and 1 µl Glutathione-S-Transferase) was added, mixed briefly and incubated at room temperature for 30 min. Thereafter, 100 µl of Lucifern Detection reagent was added to each well, mixed briefly and incubated for 15 min. Next, luminescence was read using luminescence plate reader (BioTek, Winooski, VT) and control readings were subtracted from all the sample readings. The GSH concentration was determined as pmol/1000 cells using the GSH standard curve.
Animal treatment. Female SKH-1 hairless mice (4 to 5 weeks of age from Charles River Laboratories, Wilmington, MA), were housed under standard conditions at the Center of Laboratory Animal Care, University of Colorado Denver, CO. The animals were acclimatized for one week before their use in experimental studies that were carried out according to the specified protocol approved by the IACUC of the University of Colorado Denver, CO. CEES was dissolved in acetone for dorsal skin topical application and GSH was administered in saline via oral gavage to mice. Mice were exposed to either 200 µl acetone alone, 2 mg CEES in 200 µl acetone, or treated with 300 mg/kg GSH in 200 µl saline, or with 300 mg/kg GSH 1 h before 2 mg CEES topical exposure. Untreated mice were also included as control group and five animals per group were taken for this study. CEES dose of 2 mg was chosen as this dose showed maximum changes in the studied biomarkers reported earlier by us in this mouse strain (Tewari-Singh et al., 2009). Also, CEES doses of 0.5-6 mg/kg/animal have been used in studies demonstrating its toxic responses in the skin tissue (Chatterjee et al., 2004), and skin injury could be observed following exposure to 10-40 µg/cm² of HD (Kehe et al., 2008). At the end of above defined treatments, following euthanizing mice, dorsal skin was collected and either snap frozen in liquid nitrogen or fixed in formalin as detailed earlier (Tewari-Singh et al., 2009).

Measurement of skin bi-fold thickness, epidermal thickness and quantification of apoptotic cells. The dorsal skin bi-fold thickness was measured (mm) at 3, 6, 9, 12 and 24 h following desired treatments using an electronic digital caliper (Marathon Inc. Belleville, ON, Canada), and Hematoxylin and eosin (H&E) staining of the mouse skin sections was carried as detailed earlier (Tewari-Singh et al., 2009). The epidermal thickness (µm) was measured in H&E stained skin tissue sections randomly in at least 5 fields per tissue sample under a microscope using...
Axiovision Rel 4.5 software (×400 magnification). Apoptotic cells were identified in H&E stained skin sections by virtue of their eosinophilic cytoplasm and dark purple pyknotic nuclei, and counted in randomly selected fields (×400 magnifications) of control and treated skin tissue sections. The apoptotic index was determined as number of apoptosis-positive cells ×100 / total number of cells.

**Measurement of myeloperoxidase (MPO) activity.** MPO activity was measured employing a kit from Cell Technology as published earlier (Tewari-Singh et al., 2009). In brief, ~100 mg of clean (with red blood cells removed) skin tissue samples from each group were utilized to prepare lysates and protein concentration was determined by the Lowry method using the Bio-Rad DC protein assay kit. For assay, a reaction mixture was prepared with detection reagent, 20 mM hydrogen peroxide, 1 µM eosinophil peroxidase inhibitor, 20 mM catalase inhibitor (3-Amino-1, 2, 4-triazole), and 1× assay buffer. Equal amounts of 50 µl reaction mixture and prepared sample (at conc. of 50 µg protein) or MPO standards were added in 96 well plates. After 1 h incubation at room temperature in the dark, the fluorescence was measured by a fluorescent plate reader using 530 nm excitation and 590 nm emission wavelengths. The blank control readings were subtracted from all the sample readings. The MPO activity was determined as mU/µg protein using the MPO standard curve.

**Determination of reduced GSH levels in skin samples.** Skin GSH levels were determined according to the protocol described earlier (Kariya et al., 2008). Briefly, skin tissues were weighed, sonicated on ice in KPBS buffer (50 mM potassium phosphate buffer, 17.5 mM EDTA, 50 mM serine, and 50 mM boric acid at pH 7.4) and incubated in the dark at room temperature.
for 30 min in the presence of monobromobimane (3 mM in acetonitrile). The reaction was stopped by the addition of 70% perchloric acid. The samples were then centrifuged at 16,000 x g for 10 min at 4 °C, the supernatant was removed, transferred to an HPLC vial and analyzed for GSH on a Hitachi high pressure liquid chromatograph (model L-2420; San Jose, CA) equipped with a fluorometric detector (model L-2480) as previously described with minor modifications using excitation and emission wavelengths at 390 and 480 nm, respectively (Kariya et al., 2008).

**Microscopic and statistical analyses.** The microscopic histopathologic analyses of skin samples were performed using a Zeiss Axioscop 2 microscope (Carl Zeiss Inc., Germany); photomicrographs were captured by a Carl Zeiss AxioCam MrC5 camera with the Axiovision Rel 4.5 software. The data were analyzed using SigmaStat software version 2.03 (Jandel Scientific) for statistical significance of difference between CEES treated group versus control and other treatment groups. Significance was determined by one-way analysis of variance (one-way ANOVA) with Bonferroni or Tukey t-test for multiple comparisons. \( P < 0.05 \) was considered statistically significant.
Results

**GSH protects and reverses CEES-caused decrease in JB6 and HaCaT cell viability.** In our recent studies using these skin epidermal cells, we have reported that decreased cell viability is as an important biomarker of CEES-induced skin toxicity (Tewari-Singh et al., 2010). To determine if exogenous GSH treatment was effective in reversing and/or preventing CEES-caused cell death, cells were treated with DMSO, 0.35 or 0.5 mM CEES, 1 or 10 mM GSH alone, or 1 or 10 mM GSH 1-120 min before or after 0.35 or 0.5 mM CEES; MTT assay was carried out 24 h after these treatments.

In JB6 cells, 0.35 mM CEES caused cell viability to decrease to 48% of control, which was significantly (p<0.05) attenuated when cells were treated with GSH 15, 30 or 120 min prior to or after 0.35 mM CEES exposure (Fig. 1A). Though 10 mM GSH was more effective causing almost complete protection/prevention in CEES-caused reduction in cell viability, pre- or post-treatment with 1 mM GSH also showed significant (p<0.001- p<0.01) protective and rescue effects (Fig. 1A). Increasing CEES concentration to 0.5 mM caused further decrease in viable JB6 cell number to 31% of control; however, GSH treatment 15, 30 and 120 min before CEES exposure increased the viable cells to 80, 90 and 91% of control, respectively (Fig.1B). GSH treatment (10 mM) 15-120 min after 0.5 mM CEES exposure also significant (p<0.001) reversed (90-67%) CEES-caused decreased cell viability t (Fig. 1B). Furthermore, 30 min post-treatment of 1 mM GSH also resulted in significant (p<0.001) reversal (51%) in CEES-caused cytotoxicity in JB6 cells (Fig. 1B). In HaCaT cells, pre-treatment of 10 mM GSH for 15 or 30 min resulted in a significant (p<0.001- p<0.05) protection against 0.5 mM CEES-induced reduction (37% of control) in cell viability, with a maximum protective at 30 min pre-treatment where 72% of control viable cells were evidenced (Fig. 1C). Treatment of HaCaT cells with 10 mM GSH, 1 or
30 min after 0.5 mM CEES exposure showed 68 and 55% of control viable cells, respectively (Fig. 1C). A 1 mM GSH treatment up to 30 min before 0.5 mM CEES exposure was effective in significantly (p<0.01- p<0.05) protecting the cells from reduction in cell viability (Fig. 1C); however, 1 mM GSH post-treatment was effective (p<0.05) only 1 min after CEES exposure in reversing CEES-caused decrease in HaCaT cell viability (Fig. 1C). A significant difference in the viability of cells between the DMSO control and GSH-treated groups was not observed in both JB6 and HaCaT cells (Fig. 1).

**GSH protects and reverses CEES-caused decrease in DNA synthesis in JB6 and HaCaT cells.** Our recent studies have shown that exposure of JB6 and HaCaT cells to CEES causes a decrease in DNA synthesis which is a measure of cell proliferation (Tewari-Singh et al., 2010). Accordingly, employing similar treatments as above for cell viability, next we assessed whether GSH is also effective against CEES-caused decrease in DNA synthesis in both cell lines. In JB6 cells, 10 mM GSH treatment 15-120 min before 0.5 mM CEES exposure caused a significant (p<0.001- p<0.01) increase in BrdU-incorporating cells (76-57% of control) as compared to CEES alone where BrdU-incorporating cells were reduced to 7% of control (Fig. 2A). Pre-treatment with lower concentration of 1 mM GSH for 15-120 min also resulted in significant (p<0.01- p<0.05) protection of JB6 cells from CEES-caused decrease in DNA synthesis (Fig. 2A). Treatment of JB6 cells with 1 or 10 mM GSH, 1 min after CEES exposure was also significantly (p<0.001) effective in reversing CEES-caused decrease in DNA synthesis (Fig. 2A); however, GSH treatment at later time points following CEES exposure was not effective. In HaCaT cells, 0.5 mM CEES alone reduced BrdU incorporating cells to 54% of control, but pre-treatment with 10 mM GSH for 15, 30 and 120 min significantly (p<0.01- p<0.05) increased
proliferating cells to 114, 104 and 90%, respectively (Fig. 2B). Fifteen min pre-treatment with 1 mM GSH resulted in maximum reversal with 91% (p<0.05) of control proliferating cells (Fig. 2B). Treatment of HaCaT cells with 10 mM GSH 1 min after CEES exposure was also effective in significantly (p<0.001- p<0.05) reversing CEES-caused decrease in DNA synthesis (Fig. 2B). A significant difference in the DNA synthesis of cells between the DMSO control and GSH treated groups was not observed in both cell lines. Together, above results indicated both protective and therapeutic effects of GSH in attenuating CEES-caused decrease in cell viability and DNA synthesis in both JB6 and HaCaT skin epidermal cells; however, the protective effect of GSH was more profound than its rescue ability.

**GSH protects and reverses CEES-caused S and G2-M phase arrest in cell cycle progression of JB6 and HaCaT cells.** CEES-induced decrease in DNA synthesis could be associated with the modulation in cell cycle progression which could either allow cells for DNA damage repair or, in case of extensive damage, cells consequently undergo apoptotic death. Our recent studies have demonstrated that DNA damaging effect of CEES in JB6 and HaCaT cells results in S and G2-M phase arrest (Tewari-Singh et al., 2010), prompting us to assess GSH effect on CEES-caused cell cycle arrest under the experimental conditions employed for cell viability and DNA synthesis studies. Exposure of JB6 cells to 0.5 mM CEES for 24 h caused 52% cells in S phase compared to 32% in control; however, treatment with GSH at 10 mM concentration 30 min before CEES exposure reduced S phase cell population to 19% (p<0.001, Fig. 3A). Similar GSH treatment of JB6 cells 30 min after CEES exposure resulted in only 17% (p<0.001) S phase cells (Fig. 3A). A significant (p<0.01- p<0.05) prevention in CEES-caused G2-M arrest in JB6 cells was also observed with 10 mM GSH pre-treatment for 30 min, but similar GSH post-treatment
did not show any significant reversal in CEES-caused increase in G2-M phase cell population (Fig. 3A). In other studies, only 1 mM GSH pre-treatment was significantly (p<0.001- p<0.05) effective in reversing CEES-caused S and G2-M phase cell cycle arrest in JB6 cells (Fig. 3A). In HaCaT cells, 0.5 mM CEES exposure for 24 h resulted in 31% G2-M phase cell population as compared to 16% in control (Fig. 3B), which was reduced to 18% (p<0.001) after 30 min pre-treatment with 10 mM GSH (Fig. 3B). Treatment with GSH 1 min after CEES exposure was also effective in significantly (p<0.01- p<0.05) reversing CEES-caused increase in G2-M phase HaCaT cell population (Fig. 3B). A significant difference in the percentage of cells in G1, S or G2-M phases between DMSO and GSH alone-treated JB6 and HaCaT cells was not observed. Together, these results also show that GSH pre- or post-treatment reverse CEES-caused cell cycle arrest in both JB6 and HaCaT cells; however, the protective effect of GSH was found to be more potent than its rescue ability.

**NAC protects and reverses CEES-caused decrease in JB6 and HaCaT cell viability.** Since GSH precursor NAC is more readily taken up by cells than GSH (Paromov et al., 2007), we next evaluated the protective and therapeutic effects of NAC in attenuating CEES-caused decrease in JB6 and HaCaT cell viability. Cells were subjected to MTT assay following exposure to either DMSO alone, 0.5 mM CEES, 25 or 50 mM NAC, or 25 or 50 mM NAC 5 or 1 h before, 30 min or 1 h after CEES exposure. As compared to 25 mM, treatment with 50 mM NAC before or after CEES exposure was more effective in ameliorating CEES-caused cytotoxicity in both JB6 and HaCaT cells (Fig. 4A and B). In JB6 cells, CEES exposure reduced cell viability to 22% of control, and 50 mM NAC treatment demonstrated complete protection and reversal (Fig. 4A). Treatment of HaCaT cells with 50 mM NAC for 1 h before CEES exposure showed 82% of
control viable cells compared to 28% following CEES alone exposure (Fig. 4B). Similar NAC treatment post CEES exposure resulted in complete reversal in CEES-caused cytotoxicity (Fig. 4B). Similar results were obtained with 50 mM NAC treatment given 5 h before or 30 min after CEES exposure (data not shown). A significant difference in the viability of cells between the DMSO control and NAC-treated groups was not observed in both JB6 and HaCaT cells (Fig. 4).

GSH prevents CEES-caused inflammatory responses in SKH-1 hairless mouse skin. Our recent findings have established dose- and time-dependent quantitative inflammatory biomarkers of CEES-caused skin injury in SKH-1 hairless mouse model (Tewari-Singh et al., 2009). Here, we utilized some of the prominent CEES-induced injury biomarkers, namely increased skin bi-fold thickness, skin epidermal thickness, apoptotic cell death, and tissue MPO activity to evaluate the protective effect of GSH. Though GSH exhibited both protective and therapeutic effects in reversing CEES-caused toxic consequences in cell culture studies, its protective effect was more prominent than rescue potential. Accordingly, we focused only on preventive effect of GSH in our SKH-1 mouse skin studies as summarized below.

Skin bi-fold thickness: Treatment with GSH 1 h before CEES exposure resulted in significant (p<0.001- p<0.01) protection against CEES-caused increase in skin bi-fold thickness (Fig. 5A). At 9 and 24 h, untreated controls showed 0.61 ± 0.04 and 0.598 ± 0.01 mm skin bi-fold thickness that increased to 2.11 ± 0.19 and 2.14 ± 0.07 mm (p<0.001) following CEES exposure, respectively (Fig. 5A). GSH pre-treatment reduced CEES-caused increase in skin bi-fold thickness to 1.29 ± 0.10 and 1.30 ± 0.27 mm (p<0.001- p<0.01) at these time points, respectively (Fig. 5A). No significant difference was observed in skin bi-fold thickness between untreated/vehicle control and GSH alone groups at any of the study time points (Fig. 5A).
Epidermal thickness and apoptotic cell death: Because we observed a significant effect of GSH in preventing the CEES-caused increase in skin bi-fold thickness, we next assessed its effect on other important histological changes related to CEES-caused inflammatory and toxicity responses (Tewari-Singh et al., 2009). Evaluation of H&E stained skin sections showed that GSH pre-treatment also resulted in a significant protection against CEES-caused increase in epidermal thickness (Fig. 5B, red arrowhead), upper epidermal cell necrosis and desquamation (Fig. 5B, black arrow), and cytoplasmic swelling with shrinkage or condensation of nuclei indicating apoptotic cell death (Fig. 5B, blue arrow). In terms of quantitative analysis, compared to untreated controls with epidermal thickness of 18.46 ± 0.28 µm, CEES treatment caused an increase to 33.52 ± 1.55 µm; however, GSH pre-treatment caused a significant (p<0.01) reduction in epidermal thickness to 23.16 ± 1.38 µm (Fig. 5C). We also quantified the apoptotic cells in epidermal layer where H&E stained skin sections from CEES-treated group showed 54% apoptotic cells in the epidermis compared to 14% in untreated controls; GSH pre-treatment decreased apoptotic cell number to 39% (p<0.01, Fig. 5D). No significant difference was observed in epidermal thickness or percent apoptotic cells between untreated/vehicle control and GSH alone groups at any of the study time points.

MPO activity: We also analyzed GSH efficacy on CEES-caused increase in skin tissue MPO activity, which is a measure of neutrophil infiltration at the sites of acute inflammation (Bradley et al., 1982). Following 24 h CEES exposure, 3.15 ± 0.42 mU/µg MPO activity was observed in skin tissue lysates compared to 1.22 ± 0.11 mU/µg protein in untreated controls (Fig. 5E). A significant decrease in MPO activity (1.80 ± 0.25 mU/µg protein activity, p<0.01) was observed in skin tissues from mice treated with GSH before CEES exposure (Fig. 5E). No
significant difference was observed in MPO activity between untreated/vehicle control and GSH alone groups at any of the study time points

**GSH treatment attenuates CEES-caused decrease in intracellular GSH levels in JB6 and HaCaT cells, and SKH-1 hairless mouse skin.** Since we observed that GSH treatment causes both protection and reversal of CEES-induced skin injury in cell culture and mouse skin (only protection in this case), we next assessed whether changes in intracellular GSH levels were associated with these effects. Following treatment of JB6 and HaCaT cells for above summarized cytotoxicity studies, intracellular GSH levels were measured. In JB6 cells, 0.5 mM CEES exposure decreased GSH levels to 3.2 pmol/1000 cells from 10.97 pmol/1000 cells in controls, and 10 mM exogenous GSH alone treatment increased the GSH level to 101.6 pmol/1000 cells (Fig. 6A). Importantly, GSH treatment 30 min pre- or post-CEES exposure resulted in significantly (p<0.001) elevated GSH levels of 110.1 and 149.4 pmol/1000 cells, respectively, compared to only 3.2 pmol GSH/1000 cells in CEES alone exposed JB6 cells (Fig. 6A). Similarly, exposure of HaCaT cells to 0.5 mM CEES resulted in GSH level of 7.0 pmol/1000 cells as compared to 26.7 pmol/1000 cells in respective controls. However, treatment with GSH alone, GSH 30 min pre- and 30 min post-CEES exposure resulted in elevated GSH levels of 53.3, 75.6 and 68.1 pmol/1000 cells, respectively (Fig. 6B). Together, these results clearly showed that exogenous GSH treatment before or after CEES exposure, increases intracellular GSH levels that were depleted by CEES alone exposure in both these cell types.

Based on our results showing protective and reversal effects of NAC against CEES-caused cytotoxicity, we next also measured GSH levels in NAC + CEES treated cells to decipher whether NAC treatment increases cellular GSH levels or it acts through antioxidant properties to
attenuate CEES-caused cytotoxicity. In JB6 cells, compared to DMSO control and CEES alone treated cells showing GSH levels of 13.5 and 6.3 pmol/1000 cells, 25 and 50 mM NAC treatments 60 min before CEES increased GSH levels to 16.93 and 16.91 pmol/1000 cells, respectively (Fig. 6C). In HaCaT cells, both 25 and 50 mM NAC treatments 60 min prior to CEES exposure showed an increase in cellular GSH level to 20.5 pmol/1000 cells, which was 18.1 pmol/1000 cells in vehicle controls and was reduced to 9.5 pmol/1000 cells following CEES alone exposure (Fig. 6D). In both the cell lines, treatment with NAC after CEES exposure did not show an increase in CEES-depleted cellular GSH levels.

Similar to cell culture findings, our animal study also showed GSH efficacy in protecting against CEES-caused skin injury, and therefore, we also asked the question whether topical application of CEES depleted GSH level in skin and that oral gavage feeding of GSH to mice indeed increases GSH level in skin which could be associated with the observed protective effect of GSH on CEES-caused inflammatory responses in SKH-1 hairless mouse skin. Our results show that 2 mg CEES exposure decreased GSH levels to 1.29 pmol/μg protein from 3.28 pmol/μg protein in vehicle control treated skin; however, GSH feeding 1 h before CEES exposure either retained or reversed CEES-caused depletion in GSH level in skin as we measured 3.79 pmol/μg protein GSH level which was comparable to that of vehicle alone-treated mouse skin samples (Fig. 6E). These results suggest that the changes in skin GSH levels could be related to the observed protective effect of GSH against CEES-caused skin injury in mice.

**BSO treatment further increases CEES-caused cytotoxicity in JB6 and HaCaT cells.** As eluted earlier, depletion of GSH and oxidative stress play an important role in CEES/HD-caused cytotoxicity (Kehe and Szinicz, 2005; Paromov et al., 2007; Black et al., 2010). In our present
study, treatment of skin epidermal cells with GSH or its precursor NAC attenuated CEES-caused cytotoxic responses in cell culture and skin injury in mouse skin together with the fact that CEES exposure decreased GSH levels in cell culture as well as in mouse skin systems. Accordingly, we also assessed the involvement of GSH in CEES-caused skin injury by asking the question whether a decrease in cellular GSH level would cause an increase in CEES cytotoxic effects in skin epidermal cells. In JB6 cells, 0.35 mM CEES exposure reduced viable cells to 57% of control; however, pre-treatment of cells with BSO followed by CEES resulted in viable cell number to be 3% of control (Fig. 7A). Similarly, following BSO treatment, 0.5 mM CEES exposure resulted in complete cell death as compared to 75% cell death by 0.5 mM CEES alone exposure (Fig. 7A). In HaCaT cells, 47 and 31% of control cell viability was observed following 0.35 and 0.5 mM CEES exposure, which was reduced to 18 and 3%, respectively, when the cells were exposed to BSO before CEES (Fig. 7B). A significant difference in cell viability between DMSO control and BSO alone-treated groups was not observed. Together, these results clearly show that pre-treatment with BSO, which is known to reduce GSH levels in cells, further increases CEES-caused cytotoxicity in both JB6 and HaCaT cells.
Discussion

The results of our present report demonstrate the protective and therapeutic efficacy of GSH in attenuating CEES-caused epidermal cell toxicity and skin injury, asserting at least protective role of GSH under *in vivo* condition against CEES/HD-caused skin injury and possibly vesication. This study also provides additional insights and further emphasizes the involvement of oxidative stress in biological and molecular mechanisms associated with CEES-caused skin injury reported by us recently (Pal et al., 2009; Tewari-Singh et al., 2010).

HD and CEES are alkylating agents that deplete GSH, which enhances lipid peroxidation and ROS generation causing damage to lipid, proteins and nucleic acids eventually leading to toxic responses (Kehe and Szinicz, 2005; Paromov et al., 2007; Ruff and Dillman, 2007; Black et al., 2010). CEES-caused mitochondrial oxidative damage and altered mitochondrial membrane potential are also reported in lung, liver and other tissues further indicating a role of oxidative stress in CEES-caused toxicity (Jafari, 2007; Gould et al., 2009; Black et al., 2010). Therefore, supplementing GSH or its precursors including NAC could help minimize this oxidative stress and reduce HD/CEES-caused toxicity though the associated mechanism and defined biological systems are not well-known (Amir et al., 1998; Atkins et al., 2000; Han et al., 2004; Arfsten et al., 2007; Paromov et al., 2007; Paromov et al., 2008). The protective role of extracellular GSH is highlighted in a report using the macrophage monocyte cell line J774 as well as in mitotically active SVK 14 keratinocyte cell line (Smith et al., 1997; Amir et al., 1998). Employing the biomarker established recently by us in JB6 and HaCaT cells (Tewari-Singh et al., 2010), present study demonstrates both protective and therapeutic efficacy of GSH in attenuating CEES-caused cytotoxic responses; however, the protective efficacy was greater than its rescue potential in terms of DNA synthesis and cell cycle progression parameters. Our results also showed that
exogenous GSH given before CEES exposure could avert CEES-induced GSH depletion and hence could diminish oxidative stress, which is, in part, responsible for CEES-caused DNA damage that leads to the observed decrease in DNA synthesis and cell cycle arrest. However, addition of GSH following CEES exposure, which possibly has already induced oxidative stress and DNA damage, could not effectively reverse these toxic effects of CEES.

The protection and reversal in CEES-caused cell viability by exogenous GSH could also be, in part, due to the involvement lipid peroxidation and/or protein damage reported previously in CEES-caused skin toxicity (Pal et al., 2009). Another mechanism by which extracellular GSH could protect the cells from CEES-induced damage is its possible extracellular conjugation with CEES, preventing CEES from entering the cell and causing the toxic effects (Salmen et al., 2005). Although protective effects of 10 and 50 mM NAC have been shown in LPS-stimulated macrophages and endothelial cells (Atkins et al., 2000; Paromov et al., 2008), respectively, both protective and therapeutic effects of NAC at 50 mM concentration were observed against CEES-caused decrease in cell viability by us. The efficacy of NAC in ameliorating CEES-caused toxicity was stronger than that of GSH in HaCaT cells, which could be due to better uptake of GSH in JB6 cells as compared to HaCaT cells as observed in this study even though the GSH levels in control JB6 cells were lower than that in HaCaT cells. Also important is our observation that NAC treatments before or after CEES exposure caused a strong reversal in CEES-caused cytotoxicity; however, the CEES-caused decrease in intracellular GSH levels was reversed only with pre-treatment of NAC and post-treatment was not effective. This finding suggests that the protective effect of NAC against CEES-caused toxicity is only in part through an increase in GSH levels, and the other mechanisms could be its activity as a strong ROS scavenger; NAC is also known to act via NF-κB pathway and inhibiting prostaglandin synthesis apart from being a
GSH precursor (Atkins et al., 2000; Arfsten et al., 2007). CEES-induced depletion of GSH and generation of ROS has been shown in human lymphocytes that induces cell death, and in human skin cells where treatment with GSH-depleting agent BSO showed an increased toxicity to HD (Han et al., 2004; Simpson and Lindsay, 2005). In the present study, we also observed that BSO pre-treatment to both the skin epidermal cells caused an increase in CEES-caused toxicity. We also observed that CEES exposure depletes cellular GSH levels and that GSH supplementation result in its elevated intracellular levels both in cell culture and mouse skin, further strengthening the role of GSH and in CEES-caused skin injury.

The role of oxidative and/or nitrative stress in HD/CEES-caused skin inflammation and injury is reported in various animal models (Paromov et al., 2007; Ruff and Dillman, 2007; Kehe et al., 2009; Pal et al., 2009; Black et al., 2010; Shakarjian et al., 2010). Our recent study in SKH-1 hairless mice demonstrated that induction of oxidative stress by CEES possibly leads to lipid, protein and DNA oxidation together with the activation of MAPKs and Akt pathways and AP-1 and NF-κB transcription factors (Pal et al., 2009) followed by inflammatory responses in mouse skin (Tewari-Singh et al., 2009). Utilizing the inflammatory biomarkers from this study, present report demonstrates the protective effect of GSH in attenuating CEES-caused increase in inflammation, which could be associated with the observed changes in the skin GSH levels. The observed reversal of CEES-induced increase in MPO activity by GSH feeding could also lead to a decrease in lipid peroxidation as the oxidative products of MPO could cause lipid peroxidation (Brennan et al., 2001). Treatment with NAC or lipoic acid was previously shown to significantly reduce skin, eye and lung toxicity associated with HD exposure in rats (Paromov et al., 2007), and SOD treatment to guinea pigs is shown to reduce skin lesion area caused by HD (Eldad et al., 1998; Arfsten et al., 2007; Paromov et al., 2007); however, most studies with antioxidants
have shown protection against CEES/HD-induced lung injury (Kumar et al., 2001; McClintock et al., 2006; Paromov et al., 2007; O'Neill et al., 2010). With inadequate reports on use of GSH and its precursors in attenuating HD/CEES-caused skin injury, the present study demonstrates protective role of GSH in decreasing CEES–caused inflammatory responses in mice, which further support our previous findings on the role of oxidative stress in CEES-induced signaling pathways that lead to inflammatory responses in the skin of SKH-1 mouse (Pal et al., 2009; Tewari-Singh et al., 2009).
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None.
Authorship Contributions

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Other: None.
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Footnotes

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.
Legends for Figures

Figure 1: Effect of GSH on CEES-caused decrease in JB6 and HaCaT cell viability. JB6 (A and B) and HaCaT (C) cells were seeded and grown O/N in 96 well plates as detailed under Methods; and thereafter treated with either DMSO alone (control), 0.35 (A) or 0.5 (B and C) mM CEES, 1 or 10 mM GSH, or 1 or 10 mM GSH before or after 0.35 (A) or 0.5 (B and C) mM CEES for the indicated time periods. Following the desired treatments, MTT assay was carried out 24 h after CEES exposure as described under ‘Materials and Methods’. MTT assay data shown are mean ± SEM of 4-6 independent samples for each treatment. *, p<0.05; **, p<0.01; and ***, p<0.001 as compared to CEES treated group. Similar results were obtained in two independent experiments.

Figure 2: Effect of GSH on CEES-caused decrease in DNA synthesis in JB6 and HaCaT cells. JB6 (A) and HaCaT (B) cells were seeded and grown O/N in 96 well plates as detailed under Methods; and thereafter treated with either DMSO alone (control), 0.5 mM CEES, 1 or 10 mM GSH, or 1 or 10 mM GSH before or after 0.5 mM CEES for the indicated time periods. Following the desired treatments, BrdU assay was carried out 24 h after CEES exposure as described under ‘Materials and Methods’. BrdU assay data shown are mean ± SEM of 5 independent samples for each treatment. *, p<0.05; **, p<0.01 and ***, p<0.001 as compared to CEES treated group. Similar results were obtained in two independent experiments.

Figure 3: Effect of GSH on CEES-caused S and G2-M phase arrest in cell cycle progression of JB6 and HaCaT cells. JB6 (A) and HaCaT (B) cells were seeded and grown O/N as detailed
under Methods; and thereafter treated with either DMSO alone (control), 0.5 mM CEES, 1 or 10 mM GSH, or 1 or 10 mM GSH before or after 0.5 mM CEES for the indicated time periods. Following the desired treatments, cells were collected and incubated with saponin/PI at 4°C for 24 h in the dark and subjected to FACS analysis as detailed under ‘Methods’. Data shown are mean ± SEM of 3 independent samples for each treatment. *, p<0.05; **, p<0.01 and ***, p<0.001 as compared to CEES treated group.

**Figure 4: Effect of NAC on CEES-caused decrease in JB6 and HaCaT cell viability.** JB6 (A) and HaCaT (B) cells were seeded and grown O/N in 96 well plates as detailed under Methods; and thereafter treated with either DMSO alone (control), 0.5 mM CEES, 25 or 50 mM NAC, or 25 or 50 mM NAC before or after 0.5 mM CEES for the indicated time periods. After the desired treatments, MTT assay was carried out 24 h after CEES exposure as described under ‘Methods’. Data shown are mean ± SEM of 5 independent samples for each treatment. *, p<0.05; **, p<0.01 and ***, p<0.001 as compared to CEES treated group. Similar results were obtained in two independent experiments.

**Figure 5: Effect of GSH on CEES-caused inflammatory responses in SKH-1 hairless mouse skin.** Mice were treated topically with either acetone or CEES (2 mg), given GSH (300 mg/kg) alone via oral gavage or given GSH (300 mg/kg) via oral gavage 1 h before CEES topical treatment. After 3, 6, 9, 12 and 24 h of the indicated treatments, skin bi-fold thickness was measured using a digital caliper (A) as detailed under ‘Methods’. Mice were sacrificed and dorsal skin tissue samples were collected at 24 h following the desired treatments, skin sections were processed for H&E staining and analyzed for histological changes, epidermal thickness (B;
400X magnification and C) and apoptotic cell death (D) as detailed under ‘Methods’. To determine the neutrophil infiltration, treated skin tissue samples were used for lysate preparation and MPO activity was determined by a fluorescence assay (E) as detailed under ‘Methods’. Data presented are mean ± SEM of 5 animals in each treatment group. Statistical significance of difference between the CEES exposed and control groups were determined by one way ANOVA with Bonferroni t-test for pair wise multiple comparisons. UC, untreated control; VC, vehicle control; e, epidermis; d, dermis; black arrows, epidermal necrosis; white arrows, dead cells; *, p<0.05; **, p<0.01 and ***, p<0.001 as compared to CEES treated group.

**Figure 6: Effect of CEES exposure and GSH treatment on intracellular GSH levels in JB6 and HaCaT cells, and SKH-1 hairless mouse skin.** JB6 (A and C) and HaCaT (B and D) cells were seeded, grown in 96 well plate overnight and treated with either DMSO alone (control), 0.5 mM CEES, 10 mM GSH (A and B)/25 or 50 mM NAC (C and D), or 10 mM GSH (A and B)/25 or 50 mM NAC (C and D) before or after 0.5 mM CEES for the indicated time periods. Following the desired treatments, intracellular GSH level was determined as detailed under ‘Methods’. Cellular GSH levels were calculated as pmol/1000 cells and estimated in three separate treatments per group. Mice (E) were treated topically with either acetone or CEES (2 mg), given GSH (300 mg/kg) alone via oral gavage or given GSH (300 mg/kg) via oral gavage 1 h before CEES topical application. Mice were sacrificed and dorsal skin tissue samples were collected at 24 h following the desired treatments and processed for the determination of total GSH levels in the skin (E) as described under ‘Methods’. The amount of GSH was calculated as pmol/μg protein. Data shown are mean ± SEM of 3-5 independent samples for each treatment. ***, p<0.001 as compared to CEES treated group. UC, untreated control; VC, vehicle control.
Figure 7: Effect of BSO on CEES-caused cytotoxicity in JB6 and HaCaT cells. JB6 (A) and HaCaT (B) cells were seeded and grown O/N as detailed under Methods; and thereafter treated with media without or with 0.2 mM BSO. After 6 h, media was removed, cells were exposed to either DMSO alone (control), or 0.35 or 0.5 mM CEES for 24 h in fresh media, and MTT assay was carried out as described under ‘Methods’. Data shown are mean ± SEM of 5 independent samples for each treatment. **, p<0.01; and ***, p<0.001 as compared to CEES-treated group. Similar results were obtained in two independent experiments.
Figure 1
Figure 2
Figure 3

A. JB6 cells

B. HaCaT cells

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

JB6 cells

Cell viability (% of control)

VC
BSO
CEES
BSO + CEES

0.35 mM CEES
0.5 mM CEES

B

HaCaT cells

Cell viability (% of control)

VC
BSO
CEES
BSO + CEES

0.35 mM CEES
0.5 mM CEES