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The Protective Effects of N-Acetl-Cysteine, Oxo-Thiazolidine-Carboxylate, Acetaminophen and Their Combinations against Sulfur Mustard Cytotoxicity on Human Skin Fibroblast Cell Line (HF2FF)

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ABSTRACT

Background: Using human skin-fibroblast cell line HF2FF, the efficacy of some drugs was evaluated against sulfur mustard (SM) cytotoxicity. The drugs were the sulfhydryl containing molecule including N-acetylcysteine (NAC), 2-oxo-thiazolidine-4-carboxylate (OTC) and acetaminophen as glutathione (GSH) stimulator pathway. **Methods:** The protective effects of NAC (0.1 mM), OTC (1.8 mM), and acetaminophen (25 mM) alone or in combination with each other were evaluated on SM (180 μM)-induced cytotoxicity. NAC and OTC were applied with SM simultaneously and acetaminophen 30 min before SM exposure, incubated for 1 h and then were rinsed and incubated with fresh medium. The efficacy was evaluated by determination of cells viability, intracellular GSH level and catalase activity 1 and 24 h post SM exposure or co-treatments. **Results:** The cells viability was decreased 21.8% and 55.2%, respectively for 1 and 24 h post SM (1 h exposure) incubation. So, the 1-h SM exposure and 24-h treatment incubation were selected for evaluation. While, NAC alone treatment increased the cells viability (25%), GSH level (320%) and catalase activity (18%), the most effective combination was NAC plus OTC and acetaminophen which increased more significantly the cells viability (about 40%), GSH level (470%) and catalase activity (100%). **Conclusion:** The most effective combination was NAC (0.1 mM) plus OTC (1.8 mM) and acetaminophen (25 mM) which should be used before or concomitant with SM exposure. These drugs may reduce SM toxicity possibly by increment of GSH level and catalase activity. This efficacy needs to be confirmed by *in vivo* study. *Iran. Biomed. J. 13 (4): 215-221, 2009*

Keywords: Sulfur mustard (SM), Skin fibroblast cells, N-acetyl-cysteine, 2-oxo-thiazolidine-4-carboxylate (OTC), Acetaminophen

INTRODUCTION

Sulfur mustard (SM, 2, 2-dichlorodiethyl-sulfide) is an alkylating agent that has been used as chemical weapon [1]. SM is a highly toxic chemical agent and still remains a threat to both civilians and military personnel. Although some beneficial effects have been observed with some drugs such as vitamins C, E, niacinamide [2], amifostine [3] and hexamethylene tetramine [4, 5] on tissue culture and animal models, the antidote activity of the tested compounds have been too weak to be used as protecting agents against SM [6]. Despite many years of research on this agent, the cytotoxicity mechanisms induced by mustard and the initial events leading to the cell death is not completely understood. The proposed biochemical mechanism for mustard-induced toxicity involves the process of alkylation of cellular targets. The DNA alkylation provokes the activation of poly (adenosine di-phosphate-ribose) polymerase (PARP) resulting in a rapid depletion of NAD^+/ATP metabolites leading to cell death [6, 7]. Moreover, the oxidative stress is likely involved in mustard-induced acute toxicity following glutathione (GSH) depletion [8-10]. Indeed, alkylating agents are known to induce GSH depletion [2, 11, 12] which strongly contributes to lipid peroxidation and cell

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death.

The human skin is one of the main targets of SM, and is the site of the most disabling lesions for exposed subjects [1, 6, 12]. Although few studies have been performed on the same biological model [13, 14], the main consequence of SM exposure of both human epidermis and tracheal epithelial cell cultures had been depletion of GSH [15, 16].

On the other hand, several drugs such as N-acetylcysteine (NAC) [1, 6] and 2-oxo-thiazolidine-4-carboxylate (OTC) [14], when administered individually, demonstrated moderate protective effect against SM via GSH increment. Based on the role of GSH in SM-induced toxicity, the aim of this study was to recruit molecules that capable to induce or stimulate GSH synthesis pathway. So, Sulfhydryl containing molecules such as NAC (precursor of GSH synthesis and GSSG reductase inducer) [17, 18], OTC (inducer of γ-glutamylcysteine synthetase, a rate limiting enzyme of GSH cycle) [18] and acetaminophen as GSH pathway stimulator [19, 20] were applied both individually and in combinations with each other (NAC plus either OTC, acetaminophen or all together) on HF2FF cell line against SM, to find the best drug or combination against SM intoxication. The efficacy against SM, of all the molecules investigated in this study was determined by measuring the viability of cells, GSH level and catalase activity in cell culture.

MATERIALS AND METHODS

Chemicals and reagents. NAC, OTC, 5,5'-Dithio-bis-2-nitrobenzoic acid), GSH, NADPH + H+, ATP, lactate dehydrogenase, pyruvate kinase, L-aminobutyrate and L-Glu were purchased from Sigma Chemical Co. (St Louis , Mo, USA). SM (purity>98%) was obtained from the Defense Medical Center (Ministry of Defense, Iran). Cell culture reagents were purchased from life Technologies (Gibco, Cergy-Pontoise, France), FCS, antibiotics (penicillin and streptomycin), RPMI medium, HBSS and Trypsin were purchased from Gibco, BRL, UK.

Cell cultures. HF2FF cell line was obtained from Pasture Institute of Iran (Tehran). The cell was routinely plated at a density of 100,000 cell/plate 60-mm coated, grown in RPMI (pH 7.4), supplemented with 10% FCS and antibiotics (7 U/ml penicillin and 100 μg/ml streptomycin) and cultured at 37°C in 95% air-5% CO_2.

Chemical treatment. All candidate molecules investigated in this study were chosen according to their reported efficacy against mustard. All these molecules were tested at non-cytotoxic concentrations and monolayer confluents were used in each assay. Before each treatment, the medium was removed and all treatments were carried out in HBSS medium. Acetaminophen was prepared in 25 mM concentration and stored at -20°C, NAC (0.1 mM) and OTC (1.8 mM) were prepared in sterile distilled water, and SM was stored in isopropanol (180 μM) at -20°C. The final isopropanol concentration was less than 0.1% (v/v) for SM exposure. Firstly, the final SM concentration in cell cultures was considered 180 μM as reported previously [17]. The cell cultures were exposed to SM (180 μM) for time ranging from 5 to 60 min to determine the best time exposure for maximum toxicity. Acetaminophen at the final concentration of 25 mM was added to HBSS medium 30 min before other exposures then, the medium was removed and cells were exposed to either SM, NAC (0.1 mM), OTC (1.8 mM) or their combination. After 60 min, the medium was removed and the cells were rinsed with HBSS and RPMI was added. The cytotoxicity parameters were determined after either 1 h or 24 h of incubation in RPMI.

Determination of SM cytotoxicity effects. In order to determine the appropriate incubation time, the cell cultures were exposed to SM (180 μM) for time ranging from 5 to 60 min. Then, they were rinsed and incubated with fresh medium for either 1 or 24 h to compare the toxicity of SM on the cells viability. The dose of 180 μM was chosen in order to obtain a significant cytotoxic effect (near 50% of control) as described previously [17]. The cells were exposed to 180 μM of SM for 60 min with or without adding candidate molecules. Two protection strategies were used to test the efficacy of prepared drugs against SM 1-NAC (0.1 mM) and OTC (1.8 mM) alone or in combination were added to the culture medium simultaneously with each other (control) or with SM (180 μM as test). 2-NAC, OTC and SM alone or in combination were added to the culture medium 30 min after the initial acetaminophen exposure (to stimulate GSH pathway). In both cases, the incubation time was 1 h. The cells were rinsed and incubated in fresh medium at 37°C for a further 24 h. Analyses were performed at 24 h from the initial exposure.

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Cytotoxicity studies. Cellular damage induced by SM was evaluated by Trypan blue assay method. The HF2FF cells were plated at 1×10^6 cells/dish in 60-mm culture dishes and cultured for 2 days. At the end of the incubation with SM, culture medium was aspirated and reserved. The cells were detached from culture dishes by treatment with 0.25% Trypsin/1 mM EDTA (Gibco, BRL). Then, the cells were suspended in RPMI 1640 and the culture medium was returned. The mixture was centrifuged at 800 × g for 3 min to concentrate the cells. Cellular suspension and 0.4% Trypan blue in Hanks' balanced salt solutions were mixed (final concentration of 0.07% Trypan blue), and the number of viable cells was calculated by optical density at 540 nm using an ELISA reader. The percentage of viable cells (cells viability) was calculated as 100 × (OD of treated cells)/(OD of control cells).

Biochemical assay:

Determination of intracellular GSH. The concentration of reduced GSH was determined according to the method of Tietze [21] with slight 5×10^5 cells/well in 6-well culture plates and cultured for 2 days. At the end of the incubation, cells were washed twice with Ca^{++} and Mg^{++} free PBS and lysed with 0.1 ml of 3% perchloric acid at 4°C for 15 min. After centrifugation at 800 × *g* for 5 min, supernatants were neutralized with 0.9 ml of 0.1 M sodium phosphate/5 mM EDTA buffer, pH 7.5 (phosphate/EDTA buffer). The reaction mixture contained 20 µl of the neutralized extract, 0.96 ml of phosphate/EDTA buffer and 20 µl of 60 mM 5, 5'-dithio-bis (2-nitrobenzoic acid). The increase of absorbance at 412 nm was monitored for 4 min. At each determination, a standard curve of GSH was prepared. The LD_{50} of SM in this study was chosen 180 µM as reported previously [17].

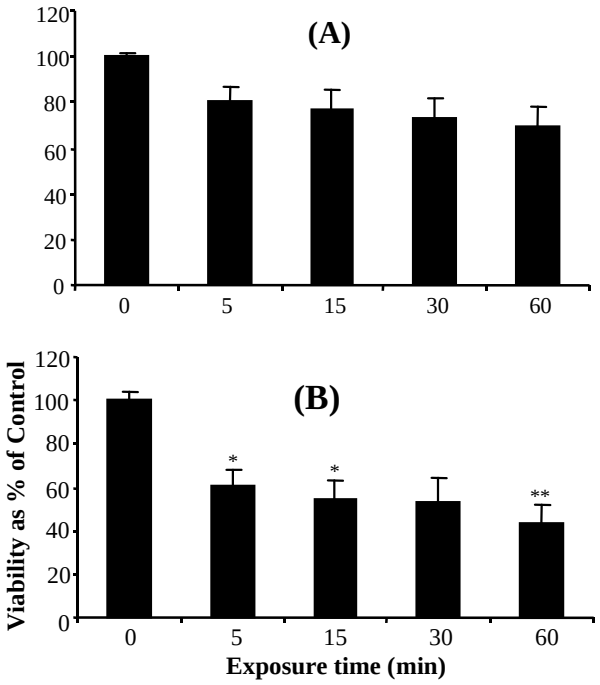

Fig. 1. Effect of time exposure to sulfur mustard on HF2FF cells. Cell cultures were exposed to SM (180 µM) for exposure time ranging from 5 to 60 min, and then rinsed and incubated in fresh culture medium prior to determining cytotoxicity for 1 h **(A)** or 24 h **(B)** after initial exposure. The cells viability was determined by Trypan blue method. *A significant difference (P<0.05) and **P<0.01 was observed between exposure times.

Catalase activity. Catalase activity was measured by monitoring enzyme-catalyzed decomposition of H_2O_2 [22]. Briefly, a solution of H_2O_2 (0.059 M) was added to the test tubes containing samples, a water blank, and H_2O_2 standard solutions (from 0-150 µl of 0.059 M as standards). After incubation for 3 min, the enzymatic reaction was terminated by the addition of $H_2SO_4.KMnO_4$ (0.05 M, 1.4 ml) was added to each tube (the final concentration of 0.054), vortexed and the absorbance recorded at 480 nm. One unit of catalase activity is defined as $k/(0.00693)$ [23], where $k = \log (S_o/S_2) \times (2.3/t)$, S_o is absorbance of standard minus absorbance of blank, S_2 is absorbance of standard minus absorbance of sample and t is time interval **.**

Statistical analysis. Data were analyzed using 2-Way Analysis of Variance. Then, the groups were compared with each other using Dunnett's test and significant differences among groups were assumed if the P value was <0.05.

RESULTS

SM toxicity on HF2FF cells. SM (180 µM) exposure induced a time-dependent cytotoxicity, and the cells viability was decreased with the time of exposure. The highest toxicity was obtained at 60 min post exposure. The typical time course effect of SM exposure on cells viability was obtained either after 1 h (Fig. 1A) or 24 h of incubation (Fig. 1B) with fresh medium after exposure. Based on these results, the exposure time of 1 h and incubation for 24 h were chosen for further experiments (Fig. 1B).

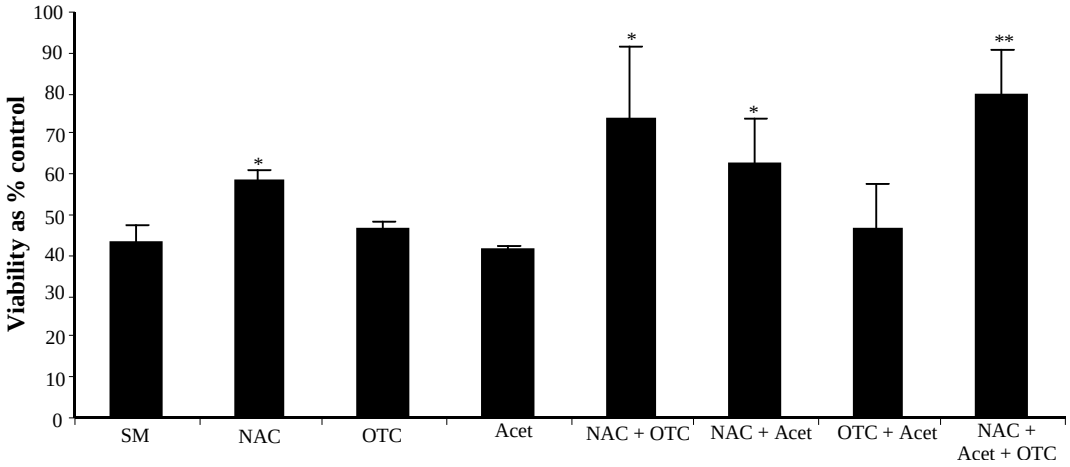

Fig. 2. The protective effects of N-acetyl-cysteine, 2-oxo-thiazolidine-4-carboxylate and acetaminophen alone or in combination against SM toxicity on viability of HF2FF cells. The cell cultures were exposed to 180 mM of SM for 1 h with or without treatments, and then were incubated for a further 24 h in fresh culture medium prior to determining the viability by Trypan blue method. * Indicates significant in comparison to the SM alone treatment group (P<0.05) and ** P<0.01.

Protection afforded by individual compounds. While the applied drugs alone had no toxic effect on HFf2FF cell line alone, among the candidate molecules, only NAC (0.1 mM) could increase significantly the cells viability (P<0.05, Fig. 2) and GSH level more than 5 folds when compared to the SM alone exposed group (P<0.01, Fig. 3A). However, the cells viability had no significant change under either OTC or acetaminophen treatment, but they could increase the GSH level more than 2 and 3 times respectively, when compared to the SM group. The catalase activity did not show any significant change with individual drugs **(**Fig. 3B). While, OTC (1.8mM) increased the GSH level significantly (P<0.05), acetaminophen (25 mM) provided relatively weak protection against SM in comparison to the SM treated group.

Protection afforded by combination of compounds against SM. No significant cytotoxic effects were observed with the drug combinations tested on HF2FF cells. Although, NAC plus OTC could significantly increase the cells viability (P<0.05, Fig. 2) and their GSH level (P<0.01, Fig. 3A) in comparison to SM group, but there was no significant difference between this combination and the NAC treatment alone group. The addition of acetaminophen to either NAC or OTC had no significant effect on their efficacy. Three drug combination (NAC + OTC + acetaminophen) presented higher degree of efficacy, with protective ratio of about 1.79 on cells viability (P<0.01, Fig. 2). The GSH level also increased by this combination to about 7 times higher than SM alone treated group (P<0.001, Fig. 3A). The catalase activity which was reduced by SM treatment to about 40 percent, increased significantly by the latter combination to about 80 percent (P<0.05, Fig. 3B).

DISCUSSION

In this study, we evaluated the protective effects of NAC, OTC, acetaminophen and their combination against SM-induced cytotoxicity on human skin fibroblast cell line. The cytotoxicity of SM on the HF2FF cell line was characterized by using the cells viability and biochemical factors assay at 1 and 24 h after initial exposure. SM exhibited greater toxicity after a longer time and its toxicity was time-of-exposure dependent. However, the toxicity for longer contact times could be explained by stability of the SM or its metabolites. The short contact time toxicity of SM could be explained by its high reactivity due to the presence and the availability of sulfhydryl groups, or fast penetration into the cells. SM is highly fat soluble which facilitates its passage across the cell membrane [1, 21].

The effects observed in our model of skin fibroblast confirm the fact that it is necessary to act rapidly to prevent SM-induced lesions. In this context, we performed immediate co-treatment with several candidate molecules reported to be effective via GSH cycle induction and their efficacy were

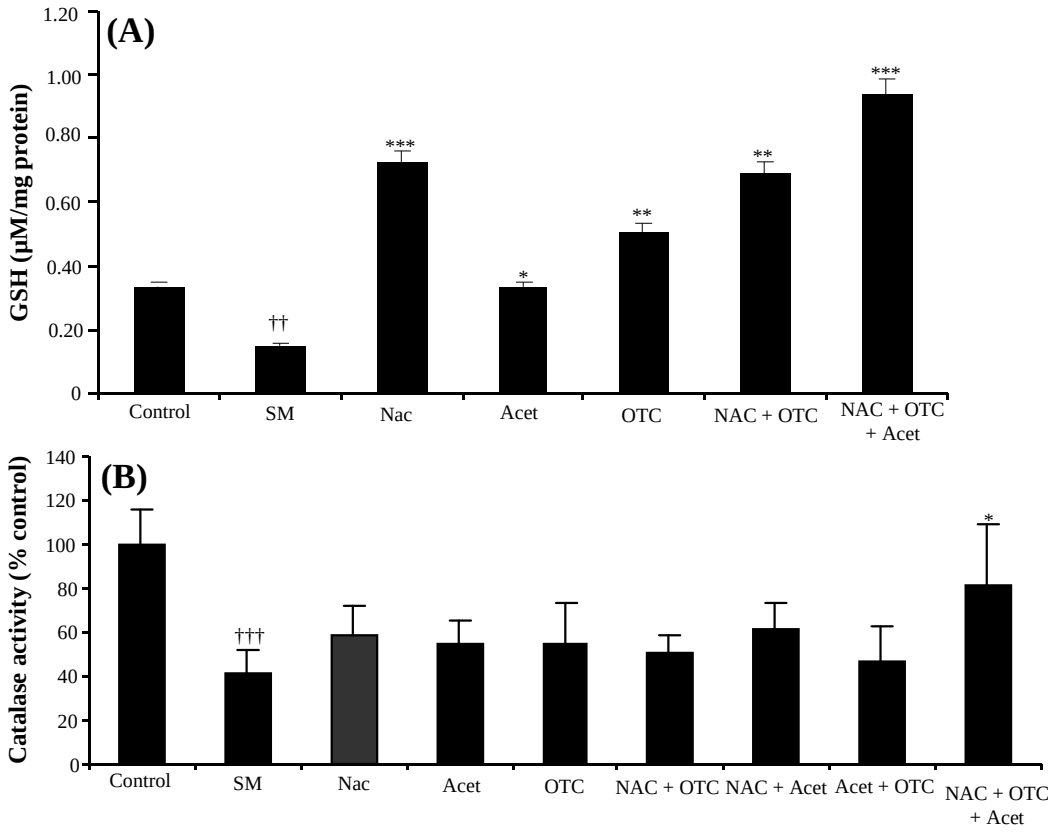

Fig. 3. Effects of N-acetyl-cysteine, 2-oxo-thiazolidine-4-carboxylate and acetaminophen alone or in combination against sulfur mustard (SM) toxicity on HF2FF cells. **(A)** Intracellular GSH concentration and **(B)** catalase activity. The cell cultures were exposed to 180 µM of SM for 1 h with or without a combination of molecules and were then incubated for a further 24 h in fresh culture medium. * indicates significant from SM treatment ($P<0.05$), ** $P<0.01$, *** $P<0.001$ and ††† indicates significant from control group, $P<0.001$.

tested 24 h after the initial mustard exposure or co-treatments. Our first strategy was the decrement of the bio-available amount of mustard in order to reduce the immediate lesions induction. For this purpose, NAC (sulfhydryl-containing molecule) as a scavenger was used to trap mustard. NAC was found to prevent SM cytotoxicity on HF2FF cells. NAC acts not only as a scavenger but also as a precursor of GSH synthesis and GSSG reductase inducer [17, 18]. Although OTC which can induce γ-glutamylcysteine synthetase (a rate limiting enzyme of GSH cycle) [18] could increase significantly the GSH level, but it was weakly effective against SM toxicity on cells viability. On the other hand, NAC and OTC were found to prevent SM toxicity on HF2FF cell line more effectively than NAC lonely. Our previous studies showed that pre-treatment of HF2EE fibroblastic cell line with hexamethylene tetramine or NAC before SM exposure is more effective than post treatment [4, 17]. In the present study, the protection provided by NAC or NAC plus OTC was remarkable at pre- or immediate exposure conditions, which is consistent with our previous findings [4, 17]. This effect may be due to a chemical interaction, in which NAC effectively binds SM and prevents its toxicity as previously described for hexamethylene tetramine [4, 5, 14, 17].

Our next strategy was to prevent the secondary biochemical consequences induced by mustard such as free oxygen radical scavenger to reduce oxidative stress. Although dimethylthiourea is classically described as a free radical scavenger [24], our study confirms that NAC and OTC in pre-exposure condition have significantly protective effect on HF2FF, as they could increase the cell's viability, GSH concentration and catalase activity probably via scavenger free radicals.

While, the level of protection of the cells from death afforded by NAC alone was about 25%, NAC, OTC or acetaminophen, could not induce any protection alone. Regarding to the GSH depletion from the cells as an important mechanism of SM toxicity, in order to improve the level of the protection by cell defense against oxidative stress cytotoxicity, combination of these drugs was recruited.

Based on the stimulation of GSH synthesis by oxidative stress induction in the cell [19] in the present study the acetaminophen as an oxidative agent was also applied as stimulator of GSH pathway [20]. However, acetaminophen alone could increase the GSH level about 2 fold in comparison to the SM exposed cells but it had no effect on cells viability. On the other hand, pre-treatment with acetaminophen before NAC and OTC administration had highly protective effect against SM, and could increase GSH concentration more than the conditions which used NAC alone or plus OTC. The probably protective mechanism of these agents is increasing the intracellular antioxidant systems such as GSH concentration and GSHpX activity [25, 18, 26].

Furthermore, the combination NAC, OTC and acetaminophen was found to be 20%, 150% and 80% more effective respectively on the cells viability, GSH level and catalase activity than NAC alone. In comparison to other drugs, these drugs are used clinically and, they have multiple beneficial properties that can be useful against the multiple effects of mustard without serious *in vivo* toxicity [14, 20, 26, 27], but dimethylthiourea as a free radical scavenger is potentially toxic *in vivo* [28-30] and is not suitable for treatment proposition.

Further investigations are warranted to determine the importance of the other pathways such as proteases, NOS inhibitors and so on. This efficacy, evidenced on *in vitro* model of human skin fibroblast cells, needs to be confirmed in other cell culture models, as well as *in vivo* condition.

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