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Does N-Acetyl Cysteine Protect Against Apoptosis in HL60 Cell Line?

[N-Asetil Sistein HL-60 Hücrelerini Apoptoza Karşı Korur mu?]

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ABSTRACT

Objectives: The primary objective of this study is to determine the role of glutathione depletion and N-acetylcysteine on apoptotic signal formation in HL60 cell line.

Methods: HL60 cells were exposed to t-BOOH which is a chemical used to induce oxidative stress. The effects of NAC on GSH in apoptotic cells were examined. Hence the GSH levels, caspase-3, 8, 9 activities and apoptosis percentages of the HL60 cells were determined in the presence or the absence of NAC.

Results and Discussion: Our results showed that pretreatment with NAC, eventhough it increases intracellular GSH content, does not protect against t-BOOH induced apoptosis. Caspase activities were decreased as compared with control group in the absence of NAC. In the presence of NAC, enzyme activities were similar to the control group. Therefore, cells may have gone apoptosis through caspase independent mechanism.

Key Words: HL 60 cell line, tertier-butylhydroperoxide, Glutathione, N-acetylcysteine, Apoptosis

ÖZET

Amaç: Bu çalışmada temel amaç HL-60 hücre dizisinde glutatyon tüketiminin ve N-asetilsisteinin apoptotik sinyal oluşumundaki rolünü belirlemektir.

Yöntem: HL-60 hücreleri oksidatif stres oluşturmak için tersiyer-butilhidroperoksite maruz bırakıldı. Apoptotik hücrelerde redükte glutatyon düzeyi, kaspaz-3, 8, 9 aktiviteleri ve apoptoz yüzdesi NAC yokluğunda ve varlığında belirlendi.

Sonuç ve Tartışma: Sonuçlarımız NAC ile önişleme maruz bırakılan hücrelerde, glutatyon içeriğindeki artışın t-BOOH ile indüklenen apoptoza karşı koruyucu olmadığını gösterdi. Kaspaz aktiviteleri NAC yokluğunda kontrol grubuna kıyasla düşük bulunurken, NAC varlığında, kontrol grubuna benzer aktivite sonuçları bulundu. Bu nedenle, hücrelerin kaspaz bağımsız bir mekanizma yoluyla apoptoza gitmiş olabileceği sonucuna varıldı.

Anahtar Kelimeler: HL 60 hücre dizisi, tersiyer-butilhidroperoksit, Glutatyon, N-asetilsistein, Apoptoz

Introduction

The homeostasis between life and death can be influenced by oxidative stress which is defined as disturbance of the oxidant/antioxidant equilibrium that results in cell damage. Cell damage is associated with depletion of cellular antioxidants leading to lipid peroxidation and alteration in protein and nuclei. Depletion of antioxidants renders the organism susceptible to ROS, leading to cell death. Cell death occurs through distinct pathways either by apoptosis, necrosis or autophagy. Apoptosis, the physiollogically programmed cell death mechanism, is characterized by chromatin condensation, membrane blebbing, cell shrinkage, lipopolisaccharide externalisation and DNA fragmentation [1].

Apoptosis is tightly regulated process, in which cells actively participate in their own destruction initiated either by activation through cell surface receptors or direct targetting of mitochondria. Depending on the initiation pathway, the process is accompanied by the activation of different caspases. Initiator caspase 8 activation is associated with the receptor dependent pathway whereas in the mitochondrial pathway the activated enzyme is caspase 9. Ultimately, both pathways activate the effector caspase 3 or 7 [2].

Prooxidants and redox cycling agents such as H_2O_2, diamide or t-BOOH can induce apoptosis. One of the defense mechanism developed by cell against apoptosis is reduced glutathione, the most abundant nonprotein sulfhydryl containing compound that constitutes the largest component of the endogenous thiol buffer [3]. The decrease in GSH levels might be attributable to several different mechanisms. One of them is the decreased activity of GSH related enzymes [4]. GSH is involved in many cellular functions which include the regulation of gene transcription as well as the modulation of apoptosis. The large GSH molecule is not transported into cells; several alternatives to increase intracellular levels of GSH have been developed. One of alternatives includes N-acetylcysteine (NAC). The thiol N-Acetyl-L-Cysteine (NAC) is readily deacetylated in cells to yield L-cysteine, promoting intracellular GSH synthesis and thus functioning as a GSH precursor. NAC is responsible for protective effects on both intra and extracellular environment, due to its nucleophilic and antioxidant properties. Usage of NAC use has been proposed for the treatment of a variety of disease which display alterations in the redox status and GSH depletion as common pathogenetic determinants [5].

Our objective was to investigate the connection between oxidative stress and apoptosis. To test our hypothesis, HL60 cells were exposed to t-BOOH which is a chemical used to induce oxidative stress. In one of our previous studies, we studied the effect of t-BOOH on GSH/GSSG levels [6]. In this study, the effects of NAC on GSH in apoptotic cells were examined. Hence the GSH levels and caspase-3, 8, 9 activities of the HL60 cells were determined in the presence or the absence of NAC.

Material and Methods

Materials

Reduced glutathione (GSH), Bovine serum albumin (BSA), N-(2-Hydroxyethyl)piperazine-N'-(4-butanesulfonic acid) (HEPES), tertiary butylhydroperoxide (t-BOOH), caspase substrates-(3, 8 and 9) were purchased from Sigma-Aldrich (St. Louis, USA), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), N-Acetyl-L-cysteine (NAC), metaphosphoric acid (MPA), glycerol, ethlyendiamino tetra acetic acid (EDTA), Triton X-100, dithiothreitol (DTT) were purchased from Sigma-Aldrich (St.Louis, USA); Annexin V (FITC kit) was obtained from Immunotech IM 3546 (Bekman Coulter, France); GSH, GSH buffer solution was supplied from Calbiochem (U.S and Canada) .

Methods

Cell and Culture Conditions

Human acute promyelocytic cell line HL60 was obtained from the American Tissue Culture Collection (USA). Cells were grown in RPMI 1640 media supplemented with 10% heat inactivated fetal bovine serum (Biochrom, Germany), penicillin (100 U/ml), and streptomycin (100 U/ml) (Bichrom, Germany) at 37°C in a humidified atmosphere with 5% CO_2, and were passaged twice weekly.

Analysis of Apoptosis by Flow Cytometry

HL60 cells cultured for 1, 2, 3, 4 and 4.5 hours in the absence (control; group 1) or presence of t-BOOH (group 2). In addition to these two groups, a third group was developed by addition of NAC (group 3). The cells were harvested in ice-cold PBS. The pellet was washed once with cold PBS and resuspended at $1x10^6$ cells/ml in 400µl lysing buffer (0.1 M HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After the addition of Annexin V fluorescein isothiocynate (FITC, Immunotech, Beckman Coulter) and propidium iodide (PI), the cells were incubated for 15 min in the dark at room temparature Two colour analysis was performed on a EPICS XL MCL (Beckman Coulter, USA) equipped with a single laser emmitting excitation light at 488 nm [7].

Fluorogenic assay of Caspase Activities

Control, t-BOOH and NAC + t-BOOH –treated cells ($1'10^6$/ml) were collected by centrifugation at 10,000 g for 1 min and washed with 1ml of ice-cold PBS. Cell pellets were resuspended in 50 mM Hepes buffer pH 7.4, containing 10 % sucrose, 0.1% Triton X-100 and 0. 5 mM EDTA and lysed for 20 min on ice. Cell lysates were cenrifuged at 10,000 g for 10 min at 4 °C to precipitate cellular debris and the supernatants were stored at 70°C, until used. The caspases activities were determined in reaction mixtures that contained 50 ml lysis buffer (blank) or supernatant (control or t-BOOH-treated samples) and 50 ml of lysis buffer and 10 mM DTT (fresh prepared) which were ini-

tiated by the addition of 5 mM Ac-DEVD-AFC, 1 mM Ac-LETD-AFC, 1 mM Ac-LEHD-AFC for caspase 3,8 and 9, respectively. After incubation for one hour at 37 °C, the fluorescence of the free AFC released upon proteolytic cleavage of the substrate by the appropriate caspase was detected at 400 nm excitation and 505 nm emission, using a Spectramax Plus 2 fluorimeter (Molecular Devices, UK). Arbitrary fluorescence units were quantified with reference to calibration curves ranging from 0.01 to 6 nmol AFC (from Sigma). The protein concentrations of supernatants were determined using the Bradford reagent and the DEVD, LETD, LEHD-spesific cleavage activities of the samples were expressed as nanomoles of AFC released/miligram of protein [8].

Measurement of GSH Levels

Cellular levels of GSH were measured using a nonenzymatic colorimetric assay kit spesific for GSH determination (Calbiochem, San Diego, CA). The cells were pelleted by low-speed centrifugation and resuspended in 5 % metaphosphoric acid. After a freze and thaw cycle to disrupt cells and subsequent centrifugation, aliquots of supernatant were assayed for cellular GSH at 405 nm using pure GSH as a standard. The remaining material in tubes was combined with ice-cold trichloroacetic acid. After centrifugation supernatant was decanted and, the pellet protein resuspended in 0.2 N NaOH. Cellular protein was determined using Bradford assay[9,10].

Statistical Analysis

Statistical difference between experimental groups was determined using Student's *t*-test where appropriate (GraphPad Instat Dr. Granger, LSU Medical Center, 1993). Differences were regarded as statistically significant when $p \leq 0.05$.

Results

Assessment of cell viability

The percentage of viable HL60 cells after 24 hours of pretreatment with GSH depleting agent t-BOOH was determined. The agent decreased the viability of cells in a time and concentration dependent manner. Cell viability was measured by trypan blue exclusion assay (Figure 1).

NAC and t-BOOH induces apoptosis in HL60 cells

The study consisted of three groups. The first is the control group formed of HL60 cells cultured in normal conditions without any incubation with additive chemicals. In the second group, the HL60 cells were incubated only with 0.1 mM t-BOOH. In the third group the HL60 cells were first incubated for 2 hours 20 mM NAC after which 0.1 mM t-BOOH was added to the cells. After these exposures, early apoptotic cells and necrotic cells were assessed by Annexin V FITC and Propidium Iodide (PI) double staining using flow cytometry (Figure 2). The

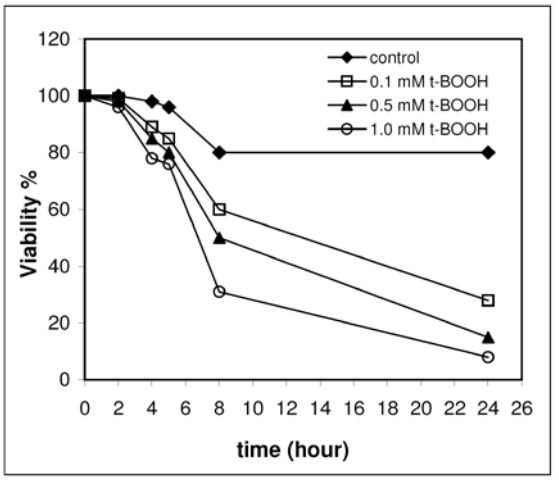

Figure 1. Percent of viable cells measured by Trypan blue exclusion after 0, 2, 4, 5, 8 and 24 h of t-BOOH (0.1 mM, 0.5 mM, 1.0 mM) treatment. Values are given as means ± SEM of three independent experiments.

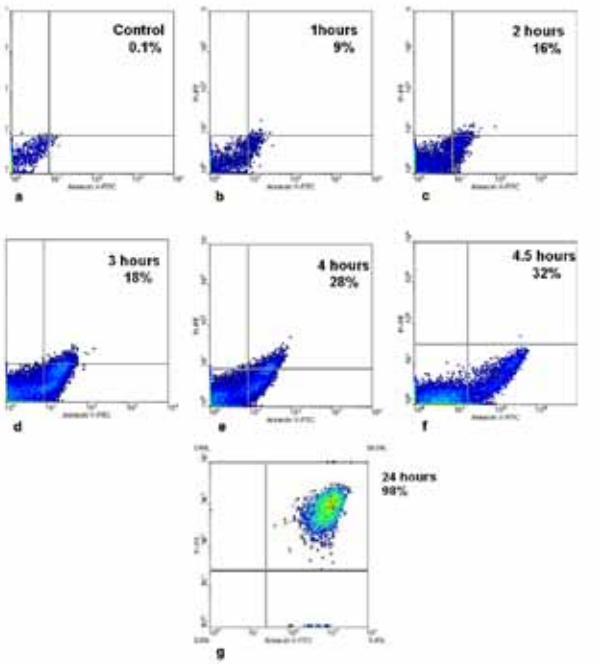

Figure 2. Representative flow cytometric analysis of NAC and t-BOOH induced cell death. Dot plot histograms represent apoptotic and necrotic cell population detected by Annexin V FITC and PI staining. Control HL60 cells (A). HL60 cells were treated with 20 mM NAC for 2 hours after which t-BOOH (0.1mM) was added to cells. Apoptosis was followed for 1, 2, 3 ,4, 4.5 h (b,c,d,e, and f). Apoptotic cells was significantly increased in a time dependent manner until 24 hrs of t-BOOH treatment ($p<0.05$). After this time point, late apoptotic and/or necrotic cells (g) were increased in the population of cells observed ($p<0.05$). The percentage of events for each experiment is indicated in the boxes which correspond to nonapoptotic (lower left), early apoptotic cells (lower right) and late apoptotic and necrotic cells (upper right) quadrants. Values are given as means ± SEM of three independent experiments.

percentage of apoptotic cells was increased when incubated with 0.1 mM t-BOOH, for 1-4.5 hours, respectively ($p<0.05$). Apoptosis was found to be increased in both t-BOOH and t-BOOH + NAC treated groups in a time

and dose dependent manner. At the same time, a parallel increase in necrotic cells following t-BOOH application after 4.5 hours was observed in both groups (Figure 2g). In the experiments, cells were first preincubated with 20 mM NAC for 2 hours after which 0.1 mM t-BOOH was added to cells. The time of t-BOOH addition was accepted as the start, or zero minutes. In cells treated this way apoptosis percentages were significantly higher in these cells than control group respectively ($p<0.05$) (Figure 3).

Caspase 3, 8 and 9 activities

Caspase activities were followed for 24 hours. Caspase 3 activity in control and t-BOOH + NAC treated cells stayed at the same level during the time period of observation. In only t-BOOH treated cells though, the caspase 3 activity declined rapidly in a time dependent manner (Figure 4a). On the other hand, both caspase 8 and 9 activities were found to be at the same level for all groups for a time period of 4.5 hours (Figure 4-b,c). After this time period, the activities decreased in a time dependent manner for the group of t-BOOH exposed cells alone. These results show that NAC treatment helps to protect the caspase 8 and 9 activities in t-BOOH + NAC treated cells and suggest that these cells undergo apoptosis in a caspase independent manner (Figure 3, Figure 4 a,b,c,).

GSH Levels

GSH concentration in HL60 cells was found to change between 0 and 4 minutes of the first hour of obsevation. The initial value of GSH in control group was 144 ± 8 nmol/mg protein. On addition of t-BOOH to cells, the observed GSH value decreased to 36 ± 4 nmol/mg protein at the 2nd minute. When NAC was added along with t-BOOH, GSH concentration was found as 105 ± 14 nmol/mg protein at the 2nd minute. At 60th minutes GSH was found as 125 ± 11 nmol/mg protein in the presence of NAC and t-BOOH. In the presence of NAC, the GSH values reached the control levels at 3 minutes but in its absence the GSH values stayed at 34 % of the control levels. On the other hand without NAC, GSH levels are decreased by 66 % of its initial value. Beginning at 4 minutes, the GSH starts to regenerate itself, and, its value is increased and conserved at this state during the observation period (Figure 5).

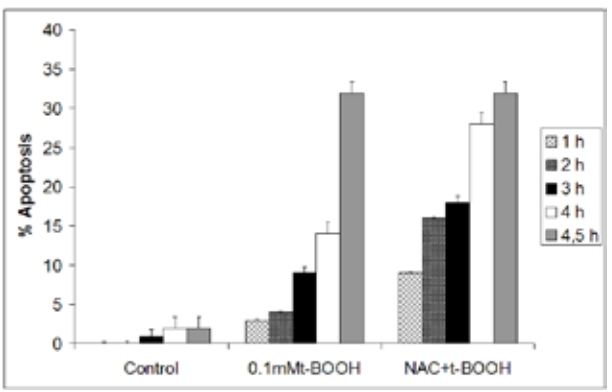

Figure 3. Evaluation of Apoptosis by Annexin V in HL60 cells pretreated with NAC and t-BOOH for 4.5 h Three groups are developed: First group is control. In the second group; t-BOOH alone was incubated with cells and in the third group; cells were incubated with 20 mM NAC for 2 hours, after which t-BOOH (0.1mM) was added to the cells. Apoptotic cells was significantly increased ($p<0.05$). Values are given as means ± SEM of three independent experiments.

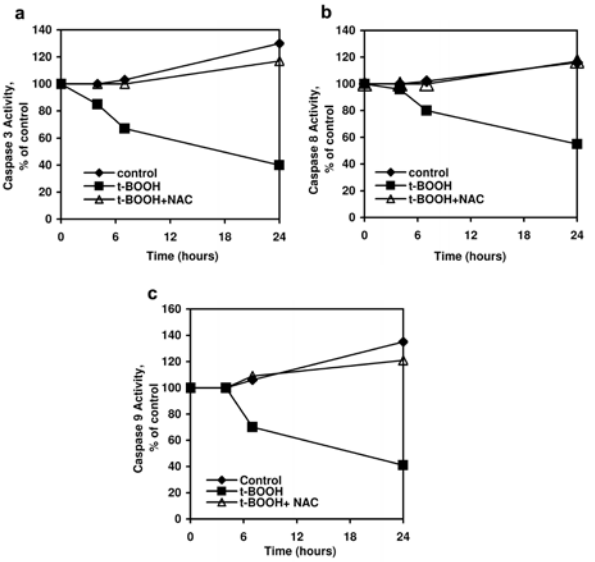

Figure 4. Caspase 3, 8 and 9 activities in HL60 cells. Caspase activities were followed in control, t-BOOH and NAC + t-BOOH treated HL60 cells. Control and t-BOOH treated HL60 cells ($1'10^6$/ml) were incubated for various time periods at 37 °C. First group is control. In the second group; t-BOOH alone was incubated with cells and in the third group; cells were incubated with 20 mM NAC for 2 hours, after which, t-BOOH (0.1mM) was added to the cells HL60 cells Caspase 3 activity in HL60 cells (a). Caspase 8 activity in HL60 cells (b). Caspase 9 activity in HL60 cells (c). In all the groups under study, the caspase 3, 8, 9 activities were found to be decreased as compared to the control group when treated with t-BOOH alone ($p<0.05$). In cells treated with t-BOOH + NAC the observed caspase 3, 8, 9 activities were found to be approximately same as the control group levels ($p>0.05$). All experiments were performed in triplicates.

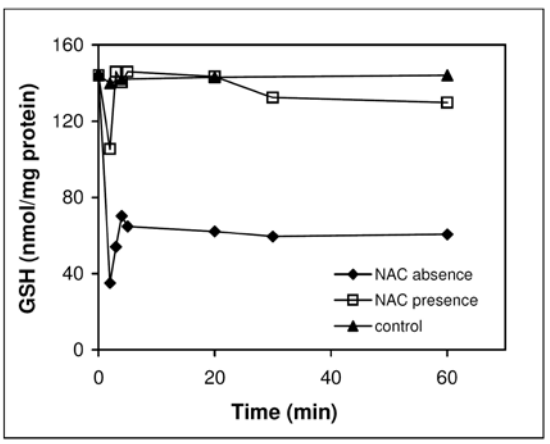

Figure 5. The effect of 0, 2, 3, 4, 5, 22, 32 and 62 min exposure of NAC and t-BOOH or t-BOOH alone on GSH levels in HL60 cells. The GSH values were found to be significantly decreased in t-BOOH treated cells in the absence of NAC as compared with the control group ($p < 0.05$). On the other hand, the GSH values were found to be equal to the control group levels in the NAC+ t-BOOH treated group ($p > 0.05$). Values are given as means ± SEM of three independent experiments.

Discussion

A wide range of anticancer drugs, including chemotherapeutic agents and hormones, induce apoptosis in malignant sensitive cells in vitro. Many chemotherapeutic agents have profound effects on the cellular redox status and, alteration of redox status may play an important role in the induction of apoptosis [11]. Cellular redox potential is largely determined by GSH, which accounts for more than 90 % of cellular nonprotein thiols [12]. The tripeptide glutathione (GSH) and its oxidized form glutathione disulfide (GSSG) comprise the major, low-molecular-weight thiol/disulfide redox buffer of most cells and their levels are strictly controlled. During oxidative stress GSH is rapidly oxidized to GSSG, which in turn may be reduced by GSSGGR in the presence ofNAD PH. In living cells to keep the ratio of GSH/GSSG within acceptable limits three different mechanisms (GSSG export, GSSG reduction by pentose phosphate pathway and by PSH) are used. the contribution of GSSG export to the maintenance oft he thiol redox status is negligible. In one of our previous studies, we studied the effect of t-BOOH on GSH/GSSG levels [6]. GSH exists in all cells in nature and is the only scavenger for hydrogen peroxide in mitochondria [13]. Depletion of cellular antioxidant defenses allows the generation of significant quantities of ROS, which may act as a signal for the induction of apoptosis [12]. At the same time ROS production is also accompanied by decreased activity of GSH relating enzymes [4]. Reduced glutathione, the most abundant intracellular thiol, acts as a major antioxidant by protecting against the damaging effects of free radicals and ROS, and may protect cells from apoptotic cell death [14]. Recent evidence suggests that the depletion of cellular glutathione, induced by several agents such as hydrogen peroxide, Buthionine Sulphoximine (BSO), tert-butylhydroperoxide is a stimulus leading to apoptosis in different cell types [15-17]. In the present study, we used t-BOOH to deplete the cells of GSH and demonstrate a time and dose dependent induction of early apoptosis with t-BOOH in HL60 cells. In our experiments initially, we used t-BOOH to deplete the cells of GSH and demonstrate a time (0-24 h) and dose dependent (0.1- 1.0 mM) induction of early apoptosis with t-BOOH in HL60 cells. However, with higher concentrations of t-BOOH, there was a significant increase in necrotic cell death (Figure 1). We also found that GSH showed a sharp decrease in first few minutes in the presence of high t-BOOH concentrations. We observed a similar result in our previous published data [6]. This mechanism of t-BOOH induced necrosis is still unknown. We speculate that the reaction of t-BOOH with GSH or specific thiols within cells can perturb the normal redox balance and shift HL60 cells into a state of oxidative stress, which induces apoptosis or necrosis. During the initial time period when cells encounter oxidative agents like t-BOOH, GSH begins to be oxidized. We observed a similar result in our previous published data [6]. In this study it was found that the assesment of the relative contribution of each one of the systems; intracellular glutathione, oxidized glutathione and their export in erythrocytes was very important. We also found that GSH showed a sharp decrease in first few minutes in the presence of t-BOOH. In parallel, oxidized glutathione concentration were increased by about 200-350 %.

This apoptotic process is then followed by the externalization of phosphatidylserine (PS) which is a signal of early apoptotic situation. After this time point even if GSH is regenerated, the PS re-location does not ocur. The apoptotic process from this point on is irreversible.

The caspase family genes encode proenzyme forms that require proteolytic cleavage for activation. Common to the caspase family is the presence of a reactive cysteine in the active site. This reactive cysteine is sensitive to the redox status of the cell. The redox status of the cell is regulated by the presence of glutathione [18]. Ghibelli showed that GSH depletion alone is not enough to lead cells to apoptosis [19].

Apoptosis and necrosis rates for t-BOOH were determined by flow cytometry. In the experiment, all of the cells underwent necrosis after “incubation with t-BOOH” after 4.5 hours. This observation caused the incubation interval with t-BOOH to be determined as 4.5 hours. Haidara K and Byrne AM reported that the effect of t-BOOH at different concentrations on apoptotic mechanism. These investigators have demonstrated that exposure of rat hepatocytes to t-BOOH cause disruption of mitochondria membrane potential (DYM). This situation is known to beget ROS production and consequently lipid peroxidation [20, 21]. The loss of mitochondrial membrane potential occurs earlier in the commitment phase of apoptosis. It results in the release of mitochondrial apoptogenic proteins like cytochrome c which binds to an adaptor molecule to form pro-caspase 9 activating complex [22-23]. Active caspase 9 subsequently processes the executioner caspase-3 and-7 into their catalytically active subunits [24].

In this study, we showed that caspase 3 activity decreased as a function of time of incubation with t-BOOH. These data are supported by recent study by Armstrong et al. who demonstrated that procaspase 3 cleavage could be induced with H_2O_2 [25]. Moreover, Yoshimura et al showed [26] hypoxia induced procaspase 3 activation is blocked by GSH. These results presented through t-BOOH treatment may play a role in these cleavage reactions and results in procaspase 3 activation. In addition, phosphatidylserine exposure was significantly but not completely activated by caspase 8 and 9 [26]. It is currently accepted that during Fas-induced apoptosis, caspase-8 becomes autoactivated in a death-inducing signaling complex (DISC) comprising of procaspase-8, Fas, and the Fas-associated death domain protein. This can lead directly to a sequential activation of effector caspases, such as caspase-3. Caspases may also

act downstream of $\Delta\psi_m$ changes. Active caspase-8 can cleave Bid, yielding a truncated species that inserts into the outer mitochondrial membrane and facilitates cytochrome *c* release and Apaf-1-mediated activation of caspase-9, which in turn activates caspase-3. To account for differences among individual cells in the apoptotic response to Fas ligation, it has been proposed that cells die through either extracelluler or mitochondrial pathways. The presence of active caspase-8 is sufficient to cleave Bid, which can then damage mitochondria, inducing cytochrome *c* release and caspase-9 activation via Apaf-1. It is suggested that both pathways contribute to different extents in various cell types [27-29].

We have observed that t-BOOH can inactivate caspases but when NAC is added this inhibition disappears. This result was similar to Borutaite's work which shows reversible inactivation of caspase-3, -8 and 9 with H_2O_2 [30].

Recent reports demonstrate that caspase dependent and independent pathways are involved in oxidative stres-induced apoptosis in cell [31]. Studies using the pharmacological caspase inhibitors have provided evidence for the coexistence of caspase dependent and independent pathways [32-34]. Inayat-Hussain and his group reported caspase independent processes in PS externalization that are induced by the polyphenolic metabolites of both remoxipride and benzene. At the same time they mentioned that these polyphenolic metabolites can undergo further oxidation either by myeloperoxidase (MPO) or autoxidation. These toxic semiquinones and/or quinones and ROS could cause direct or irreversible cellular damage [35]. Supporting evidence comes from a study where MPO-catalyzed redox cycling of phenol results in lipid peroxidation and thiol oxidation in HL60 cells [36]. Our results showed that caspase independent processes may orchestrate changes leading to PS exposure during apoptosis induced by NAC and t-BOOH.

The thiol NAC is a precursor of intracellular glutathione. Thus, it is used in the therapy of a variety of clinical conditions, mostly involving GSH depletion and alterations of the redox status. A number of studies report that NAC has the potential to prevent cancer [37-39]. The concentration with wide range of NAC (1-100 mM) have been used by different investigators [37]. Following on our experiments and our preliminary results for apoptosis were shown in figure 3, we decided to use 20 mM NAC. We found that pre-treatment with NAC, even when intracellular GSH content was increased could not protect HL60 cells against t-BOOH induced apoptosis. In contrast apoptosis was increased by NAC. We examined the effects of NAC on cellular GSH regeneration mechanism in the HL60 cell lines and saw that NAC have increased apoptosis induced by t-BOOH.

Kusano, Liu and their colleagues [40, 41] have shown that NAC can have different effects on apoptosis. 105 data were selected fom MEDLINE related with the effects of NAC on apoptosis. Of these 105 data, 91 (86.7 %) were coherent with the ability of NAC to inhibit apoptosis, 10 (9.5 %) showed no effect of NAC and 4 (3.8 %) showed an increase apoptosis by NAC. NAC was capabable of inducing apoptosis in several transformed cell lines and transformed primary cultures but not in normal cells [37, 41]. We can also also state that in our experimental setup NAC induces apoptosis in HL60 cells.

The active site cysteine residue for caspases is susceptible to oxidation, resulting in caspase inhibition [30, 42]. For this reason, it can be said that the intracellular GSH content is not important for the mechanism through which NAC increases apoptotic death. This effect may depend on inhibition of cell cycle progression or the induction of p53 expression.

In conclusion we present that oxidative stress induced by t-BOOH cause apoptosis due to GSH depletion. But even if GSH is regenerated by NAC, apoptosis could not be reversed. These findings suggest that in conditions like neurodegenerative diseases in which apoptosis is desired, usage of NAC is not applicable.

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