Effects of N-acetyl-L-cysteine and Glutathione on Antioxidant Status of Human Serum and 3T3 Fibroblasts

The effectiveness of several sulphydryl compounds in the treatment of paraquat intoxication has been previously tested based on their antioxidant ability. However, practical guidelines for their clinical use remain to be determined. As a preliminary pharmacokinetic study on sulphydryl compounds, we attempted to establish the optimal concentration of N-acetyl-L-cysteine, glutathione, superoxide dismutase, and catalase. We measured the antioxidant effect of these antioxidants in normal pooled plasma and on intracellular reactive oxygen species (ROS) induced by paraquat. N-acetyl-L-cysteine begins to suppress the production of ROS in plasma at concentrations as low as 5 mM, with the suppression being maximal at 40 mM. In the same way, glutathione increased the total antioxidant status in plasma at concentrations of 5–40 mM in a dose-dependent manner. Complete suppression of ROS in plasma induced by exposure to 500 μM paraquat for 40 min was observed when using 40 mM N-acetyl-L-cysteine and 5 mM glutathione. These concentrations are comparable with 50 units of catalase, which reduced ROS at concentrations of 5–100 units. Further pharmacokinetic study into the systemic administration of these antioxidants is necessary, using effective concentrations of 5–40 mM for both N-acetyl-L-cysteine and glutathione, and 1–50 units of catalase.

Key Words: Antioxidants; Glutathione; Acetylcysteine; Paraquat; Poisoning

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridium dichloride) was introduced in 1962 as an effective herbicide that had low chronic toxicity because of its rapid deactivation upon soil contact (1). However, it has become notorious worldwide as a potent human poison. Despite the continuing decrease in the agricultural population, the incidence rate of paraquat poisoning is rapidly increasing in Korea with hundreds of deaths from this herbicide every year (2). In humans, intentional or accidental ingestion of paraquat is frequently fatal due to the failure of multiple organs (3). Early mortality of patients suffering from paraquat poisoning occurs as a result of vascular collapse, while delayed mortality is mainly due to lung damage.

Injury to pneumocytes is initiated by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of paraquat to monocation radicals (PQ+). Spontaneous reaction with molecular oxygen yields the superoxide radical (O2−) and reforms the paraquat dication, which can be reduced again. This process, known as a redox cycle, is sustained by the extensive supply of electrons and oxygen in the lungs (4). Dismutation of the superoxide species, which is catalyzed by superoxide dismutases (SOD), leads to the formation of hydrogen peroxide. This can in turn be metabolized to water by catalases or peroxidases. Superoxide and hydrogen peroxide also undergo a series of iron-catalyzed reactions to yield hydroxyl free radicals (OH•). These are highly toxic themselves and can also generate more free radicals by reaction with other biomolecules, such as proteins or membrane fatty acids (5).

In contrast to the rapid production of toxic oxygen species, the capacity of antioxidant systems, such as the enzyme SOD, catalase, glutathione (GSH) peroxidase, and vitamins C and E, is limited, and there is a lag time in their adaptation. This imbalance explains why the dose-response curve for paraquat toxicity is very steep (6).

Over the past 30 yr, several methods have been used in attempts to modify the toxicity of paraquat: (a) prevention of absorption from the gastrointestinal tract (7), (b) removal from the bloodstream (8, 9), (c) prevention of accumulation in the lung (10), (d) scavenging of oxygen free radicals (11), and (e) prevention of lung fibrosis (12). Unfortunately, all of these methods have not been proven effective, with the outcome being already determined by the degree of exposure at the time of the arrival of a patient at the clinic. The medical action taken immediately upon arrival is crucial, as is a multidisciplinary approach to assisting with impending death in the event that it is inevitable.

Recently, Suntres (13) presented an excellent review on the status of antioxidants in ameliorating or treating the toxic effects produced by paraquat. A number of sulphydryl compounds have been examined based on their antioxidant ability. An early observation by Bus et al. (14) was that depletion of...
GSH enhanced paraquat toxicity. Hagen et al. (15) and Brown et al. (16) showed that the addition of GSH to type-II alveolar cells protected against paraquat toxicity. Furthermore, the evidence that GSH peroxidase plays a key role in protecting animals against paraquat toxicity has been shown in recent studies on transgenic mice in which deletion of this enzyme enhances toxicity while addition of it affords some protection (17).

GSH provides the reducing equivalent for the reduction of hydrogen peroxide to H2O, and is oxidized to its disulfide (GSSG). Most of the GSSG is immediately reduced back to GSH through a GSH reductase with the cofactor NADPH. In the liver, GSSG is secreted mainly into bile, while in the heart and lungs it is released mainly into the perfusate of these organs (18).

N-acetyl-L-cysteine (NAC) is a precursor of GSH. Hoffer et al. (19) incubated NAC with type-II alveolar cells, and showed enhanced GSH content and prevention of paraquat-induced cytotoxicity. Moreover, it is used clinically for a broad spectrum of indications including mucolysis, detoxification after acetaminophen poisoning, adult respiratory distress syndrome, hyperoxia-induced pulmonary damage, HIV infection, cancer, and heart disease (20-22).

However, despite their frequent use and the considerable amount of clinical knowledge on these drugs, many questions still remain to be answered, including: What is the therapeutic window of the blood concentration of these drugs? What is the best antioxidant among the thiol compounds? Are they effective antioxidants for both intracellular reactive oxygen species (ROS) and/ or ROS formed in plasma?

In general, safe and effective drug therapy requires adequate delivery to their molecular targets in tissues at concentrations within the range that yields efficacy without toxicity. The purpose of this study was to establish the optimal concentrations of NAC, GSH, SOD, and catalase as a precursor to the pharmacokinetic study of sulfhydryl compounds for systemic administration in patients with acute paraquat intoxication.

**MATERIALS AND METHODS**

Study Design

This study consisted of two experimental designs. First, we measured the antioxidant effects of NAC, GSH, SOD, and catalase which were purchased from Sigma (St. Louis, Missouri, U.S.A.), in normal pooled plasma (NPP). Second, we measured the antioxidant effects of NAC, GSH, SOD, and catalase on the intracellular ROS induced by paraquat.

Normal Pooled Plasma and Basal Vitamin C Level

NPP was collected from 20 Koreans (10 males and 10 females) aged between 25 and 55 yr who had visited Soonchunhyang University Cheonan Hospital (Cheonan, Korea) for a medical examination. They had taken regular diet for 1 week prior to the examination. None of the subjects were alcoholics, and none had taken supplemental vitamins within 2 weeks of the study. Blood chemistry was measured by an automatic analyzer, and the results were as follows: total protein, 7.8 mg/dL; albumin, 4.6 mg/dL; fasting glucose, 93.9 mg/dL; total bilirubin, 1.0 mg/dL; blood urea nitrogen, 14.5 mg/dL; creatinine, 0.9 mg/dL; uric acid, 5.5 mg/dL; cholesterol, 171.1 mg/dL; and triglyceride, 97.8 mg/dL.

The concentration of vitamin C, as measured using a photometric method, was 0.48 mg/dL in NPP. The principle of this method is that the ascorbic acid in plasma is oxidized by Cu2+ to form dehydroascorbic acid, which reacts with acidic 2,4-dinitrophenylhydrazine to form a red bis-hydrazine, whose fluorescence is measured at 520 nm.

Total antioxidant status (TAS) in the NPP was measured using a commercial kit (BTS®, Randox Laboratories, U.K.) and a biochemical autoanalyzer (model 7150, Hitachi, Tokyo) according to the manufacturer’s instruction. The principle of TAS measurement is based on the quenching of the ABTS® [2,2’-azino-di-(3-ethylbenzthiazoline sulphonate)] radical cation, which is produced by the interaction of ABTS® with ferryl myoglobin radical species generated by the activation on metmyoglobin with H2O2. (23). The intra- and inter-assay coefficients of variation for this method were 7.5% and 8.7%, respectively. TAS was measured in NPP after addition of 0.1-100 mM NAC and GSH, and 0.1-100 unit/mL of both catalase and SOD. All samples were incubated for 30 min at 37°C before assay. The results were expressed as the percentage of TAS of NPP, where the basal TAS level of NPP was 1.2 mM/L. Tests for TAS were duplicated and the results are expressed as a mean value.

**Cell Culture**

Swiss 3T3 fibroblasts obtained from American Type Culture Collection (ATCC CCL 92) were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 25 mM HEPES, pH 7.4, 10% (v/v) fetal bovine serum, 100 unit/mL penicillin, and 100 mg/mL streptomycin (culture medium). For experiments, cells were cultured on round coverslips in 12-well plates and then stabilized for 30 min with Dulbecco’s modified Eagle’s medium supplemented with 5 mg/mL apotransferrin, 1 mg/mL bovine serum albumin, 25 mM HEPES (pH 7.4), 2 mM glutamine, 100 unit/mL penicillin, and 100 mg/mL streptomycin (serum-free medium).

**Measurement of Intracellular ROS**

Intracellular ROS was measured at a paraquat concentration of 500 μM after the incubation period of 40 min at various concentrations of catalase, SOD, NAC, or GSH. For the last 5 min of stimulation, 5 μM H2DCFDA was added to enable
measurement of intracellular ROS. The amount of intracellular ROS was measured according to the procedures of Koo et al. (24). Cultured cells on round coverslips were stabilized in serum-free medium without phenol red for at least 30 min and stimulated with paraquat for varying periods of time. Sometimes, cells were preincubated with NAC or catalase for 30 min. ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF; Molecular Probes, U.S.A.). For the last 5 min of stimulation, the membrane-permeable diacetate form of the dye (reduced DCF: DCFH-diacetate) was added to the perfusate at a final concentration of 5 μM. Within the cell, esterases cleave the acetate groups on DCFH-diacetate, thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. Intensity values are reported relative to the initial values, after subtracting the background. Then, the cells were immediately observed by a laser scanning confocal microscope (model LSM410, Carl Zeiss, Germany). The samples were excited by a 488 nm argon laser, and images were filtered by a long-pass filter with a cutoff wavelength of 515 nm.

In our preliminary study, paraquat produced ROS in Swiss 3T3 fibroblasts at concentrations of 50-500 μM in a dose-dependent manner at 30-60 min. The cells remained alive when the paraquat concentration was less than 500 μM. However, they detached within 30 min when the paraquat concentration was over 600 μM or the incubation time was over 60 min at lower concentrations. Therefore, we selected the optimal condition for our experiments to be 500 μM for 40 min.

Statistical Analysis

Results were expressed as mean ± SD unless stated otherwise. Intracellular ROS was measured in about 30 cells randomly selected from three separate experiments, and DCF fluorescence intensities of treated cells were compared with those of untreated control cells. Student’s t-test was used to detect differences in ROS between groups. Statistical significance was defined as probability values of \( p < 0.05 \).

RESULTS

Effects of NAC and GSH on the Production of ROS in NPP

NAC begin to suppress the production of ROS at a concentration of 5 mM, with the suppression being maximal at 40 mM. There was a dose-dependent relation between TAS and NAC as follows: 106.0% at 5 mM, 110.0% at 10 mM, 112.0% at 20 mM, and 118.0% at 40 mM. However, the level of TAS was flat when the concentration of NAC was over 40 mM (Fig. 1).

In the same way, GSH increased TAS for concentrations of 5–40 mM in a dose-dependent manner. But the slope of the dose dependency was greater for GSH than for NAC: 107.0% at 5 mM, 115.3% at 10 mM, 121.0% at 20 mM, and 141.6% at 40 mM. For NAC, the level of TAS was flat for GSH concentrations over 40 mM (Fig. 1). Catalase was observed to affect the production of ROS for concentrations from 0.5 unit/mL (106.5%) to 1 unit/mL (112.0%), with the effect being flat for catalase concentrations over 1 unit/mL. SOD at concentrations of 0.1–100 unit/mL did not affect the TAS of NPP (Fig. 1).

Effects of NAC and GSH on the Paraquat-induced ROS Production in Swiss 3T3 Fibroblasts

ROS was produced by paraquat at concentrations between 20-500 μM, for 40 min incubation (Fig. 2). The production of ROS is dependent on the paraquat concentration. The cells detached within 30 min when the paraquat concentration over 600 μM or an incubation time of over 60 min regardless of the concentration. NAC at 5–40 mM and GSH at 1-10 mM suppressed ROS in a dose-dependent manner (Fig. 3), with complete suppression of ROS being observed for 40 mM NAC and 5 mM GSH. These concentrations were comparable with 50 unit/mL of catalase, which reduced intracellular ROS at concentrations of 5-100 unit/mL in a dose-dependent manner (Fig. 4). SOD at concentrations of 0.1-100 unit/mL did not affect intracellular ROS.

DISCUSSION

The assessment of oxidant stress is complicated by the lack of universal oxidant stress products or markers. However, the
Fig. 2. Production of reactive oxygen species (ROS) by paraquat treatment in a dose-dependent manner in Swiss 3T3: After 40 min incubation of the cells with 20-500 μM paraquat, intracellular ROS generation was assessed with 2,7-dichlorofluorescein and a laser scanning confocal microscope (×400).

Fig. 3. Effect of N-acetyl-cysteine (NAC) and glutathione (GSH) on the production of reactive oxygen species (ROS) by paraquat (PQ) in Swiss 3T3: the signal intensities in Figure 2 were quantified by a quantification software. 5-40 mM of NAC or 1-10 mM of GSH suppress ROS in a dose dependant pattern. Complete suppression of ROS is observed at 40 mM of NAC or 5 mM of GSH. a or b denotes statistical differences (p<0.01) in comparison with control or PQ group, respectively.

Fig. 4. Effect of catalase on the production of ROS by PQ in Swiss 3T3: 5-100 unit/mL of catalase suppresses production of reactive oxygen species (ROS) in a dose dependant pattern. Complete suppression is observed at 50 unit/mL of catalase. The antioxidant effect of this concentration is compatible with 40 mM of N-acetyl-cysteine or 5 mM of glutathione. a or b denotes statistical differences (p<0.01) in comparison with control or PQ group, respectively.
most commonly used and specific indication is the presence of oxidizing molecules. Direct detection of excited species is possible by measurement of organ chemiluminescence. Also, evidence that antioxidant defenses are functioning can indicate the presence of oxidant stress. In our study, intracellular ROS was measured by DCF fluorescence intensity. By measuring the TAS, we could assess the change of the antioxidant status of the plasma. The principle of measurement in this assay system is quenching of a given radical cation. TAS in plasma is influenced by several substances, such as urea, thiol, vitamin C, and vitamin E, which is why we measured the effect of free-radical scavengers on TAS in NPP.

In our study, both NAC and GSH had dual antioxidant effects on intracellular and plasma ROS. NAC and GSH were effective on ROS in plasma at concentrations of 5–40 mM. When the target was intracellular ROS, the effective concentrations were 5–40 mM for NAC and 1–5 mM for GSH. Further pharmacokinetic study is necessary to identify a safe loading and maintenance dose of NAC and GSH for patients with paraquat poisoning. When administered at the same concentrations, GSH was a stronger antioxidant than NAC in NPP. In the same way, intracellular ROS was completely suppressed when using 40 mM NAC and 5 mM GSH, and these concentrations are comparable with 50 unit/mL of catalase. This finding suggests that GSH is a suitable treatment for patients with paraquat poisoning. However, other biologic roles of NAC have been reported, such as downregulation of the expression of transcription factor NF-κB, which is a major mediator of inflammatory responses and controls expression of a large variety of genes encoding cytokines, growth factors, acute phase proteins, and cell adhesion and immune-regulation molecules (25). It also has been reported to reduce the plasma concentration of homocysteine and improve endothelial function. Therefore, it seems reasonable to assume that both GSH and NAC can be prescribed for patients with acute paraquat poisoning.

Our results raise some questions about the transport action of NAC and GSH through the cell membrane. Several amino acid transporters have been identified during the past decade, such as heteromeric amino acid transporters (26). Griffith and Meister (27) and Meister and Larson (28) have reported that GSH is exported in the plasma to various cells and organs, and that it functions in the inter- and intra-organ transfer of cysteine as part of a pathway for membrane transportation of cysteine, cystine, and probably other amino acids (28).

Furthermore, there are evidences (15, 16) that endogenous GSH can be taken up by cells and protect against paraquat toxicity. Meanwhile, Smith et al. (29) have reported that in vivo administration of GSH is hindered by its inability to cross cell membranes and by rapid hydrolysis.

In our study, a slight increase in the concentrations of GSH and catalase, but not of SOD, in culture media suppressed intracellular ROS significantly. These findings demonstrate that GSH and catalase entered the cell with the same ease as does NAC.

In conclusion, both NAC and GSH were effective antioxidants for not only ROS in plasma but also intracellular ROS produced by paraquat. Intracellular ROS induced by exposure to 500 mM paraquat for 40 min were completely suppressed by 40 mM NAC and 5 mM GSH, and these concentrations are comparable with 50 unit/mL of catalase. In order to maintain these effective concentrations in plasma, further pharmacokinetic investigations are necessary to define the loading and maintenance dose of GSH and catalase.

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