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Acrylonitrile Potentates Oxidative Stress in Rat Alveolar Macrophages

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Acrylonitrile (VCN), a widely used chemical in the production of plastics and synthetic fibers is known to be a potent acute toxin (Abreu and Ahmed 1980, 1982; Gut et al. 1981). Occupational exposure to VCN has been associated with an increased risk for developing lung cancer in humans (OBerg 1980; Strother et al. 1988). The potential carcinogenic effect of VCN has been strengthened by its ability to induce cytotoxicity, sister-chromatid exchange, and DNA-strand breaks in cultured human pulmonary endothelial cells (Chang et al. 1990; Ahmed et al. 1992). We have shown alterations in procoagulant activity of alveolar macrophages (AM) in rats following inhalation exposure to VCN (Bhooma et al. 1992).

VCN can be metabolically eliminated *in vivo* via two pathways (a) enzymatic and spontaneous conjugation with GSH and protein thiol groups and (b) cytochrome p 450 dependent oxidation of VCN to 2-cyanoethylene oxide (CEO), which is further metabolized to CN^- (Kopecky et al. 1980; Geiger et al. 1983); Acute toxicity of VCN has been associated with glutathione depletion with its consequences in the cellular redox equilibrium and with the inhibition of cytochrome oxidase by cyanide anion, the end metabolite of VCN (Szabo et al. 1977; Nerudova et al. 1981).

Although liver is the major site for VCN metabolic activation, rat and mouse lung epithelial cells and microsomes are capable of metabolizing VCN to CEO. Rat alveolar macrophages, however failed to convert VCN to CEO (Roberts et al. 1989). Therefore, the alternate route for the metabolic elimination of VCN in rat alveolar macrophages should be via its direct conjugation with glutathione. Following VCN administration, a significant depletion of glutathione in lungs of rats was observed (Pilon et al. 1988). Since macrophages are constantly exposed to an oxygen rich environment, the VCN induced thiol depletion is expected to potentiate the oxidative stress in macrophages. Effective detoxification of oxygen metabolites is essential for cell survival. Antioxidants serve primarily to protect the cells by scavenging and detoxifying oxygen metabolites. Involvement of reactive oxygen species in VCN-induced macrophage death has not been reported previously.

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The purpose of the present study is to investigate if VCN potentiates the formation of reactive oxygen species in rat alveolar macrophages under high oxygen tension. An attempt was made to reverse the VCN-induced cell death by exogenous addition of antioxidants.

MATERIALS AND METHODS

VCN was purchased from BDH chemicals, Bombay. Phorbol myristate acetate (PMA), superoxide dismutase (SOD), catalase, ethylene diamine tetra aceticacid (EDTA), d-mannitol, horse radish peroxidase, and hydrogen peroxide (H_2O_2 type III were obtained from Sigma Chemical Co., St. Louis, MO.

Adult male wistar rats were purchased from Forensic Sciences Department, Madras and kept in a room maintained at constant temperature. Alveolar macrophages (AM) from rats were obtained in a relatively high state of purity from broncho alveolar lavage (BAL), which samples the epithelial lung fluid of the lower respiratory tract. Animals were killed by cutting the jugular vein. After draining the blood, the thoracic cavity of rats was opened and lungs, heart, and trachea were removed en bloc. Trachea was cannulated and the lungs were lavaged 6 times with a total volume of 50 ml of cold sterile isotonic saline. The lavage effluent from 3 rats was pooled and centrifuged in the cold at 250 x g for 10 min, washed twice in phosphate buffered saline (PBS) and resuspended in RPMI- 1640 with penicillin, streptomycin and 10 % fetal calf serum (complete medium). Cells were counted and viability determined by trypan blue exclusion. This fraction had 74% macrophages, 23% lymphocytes and 3% polymorphonuclear lymphocytes. The lavage cells were added to glass coverslips in a 24 well plate. After 60 min incubation at 37°C, the nonadherent cells were removed and to the adherent AM, complete medium was added. The adherent cells (>95% of AM) were chosen for H_2O_2 assay and for the study of protective nature of antioxidant.

Each assay condition consisted of $0.5x10^6$ AM in a total volume of 1ml of complete medium in a 24 well plate. Controls received no further treatment. VCN at concentrations ranging from 200 nM to 20 µM was added to macrophages and allowed to incubate at 37°C for different time points in 95% O_2 and 5% CO_2 incubator. Duplicate wells were run for each condition. Trypan blue exclusion of macrophages was studied at one hour intervals up to 4 hr.

Method of Pick and Keisari (1980) was followed for the measurement of H_2O_2 in intact cells. The macrophages ($0.5x10^6$) in each well were washed with phenol red-free Hanks balanced salt solution (HBSS) followed by incubation with 500 µl of HBSS containing 19 units/ml horse radish peroxidase, 10 µM VCN (or 100nM PMA- positive control) and 0.02 % phenol red. Triplicate wells were run for each condition. After incubating the plate at 37°C for 1 hr, 100 µl supernatants were transferred to a 96-well plate. The enzyme reaction was arrested with 10 µl of lM

NaOH. Absorbance at 610 nM was determined using a Biotek microplate reader. For each experiment a standard was set up in the range of 0-10 nmol/ml of H_2O_2. After the preliminary experiments, the following levels of antioxidants were chosen and added to macrophages: SOD 100 units/ ml; Catalase 275 units /ml; EDTA 1 mM; d-Mannitol 100mM. Each condition was carried out in duplicate wells. The macrophages were incubated with antioxidants for 15 minutes followed by the addition of VCN. Viability was assessed at the end of 4 hr at 37°C under 95% O_2-5% CO_2 gas mixture. After the incubation period viability was assessed by exclusion of trypan blue dye.

Results are expressed as mean ± S.D (n=number of experiments). Student's t-test was used to determine the statistical significance of mean differences between control and VCN (or PMA) exposed cells (for dose kinetics and H_2O_2 assay). For the evaluation of protective nature of antioxidants, the mean difference between VCN without and with antioxidants was compared. Acceptable significance was attached to differences where $p < 0.05$.

RESULTS AND DISCUSSION

Alveolar macrophages, incubated with a level of VCN ranging from 200 nM to 20 µM showed a dose-dependent loss of viability (Fig 1). VCN at 200 nM did not affect the viability of macrophages whereas the viability was 65% ($p<0.02$), 46% ($p<0.01$), and 18% ($p<0.01$) at 2 µM, 10 µM and 20 µM, respectively at the end of 4 hr. Since the Ki 50 of VCN induced loss of viability at the end of 4 hrs was ~10 µM, this concentration was chosen to study the protective effect of antioxidants.

The control macrophages at the end of 1 hr released 2.5 ± 0.21 nmol $H_2O_2/0.5x10^6$ cells. PMA was used as a positive control. Stimulation of AM with 100 nM of PMA resulted in 28% increase in H_2O_2 ($p<0.05$), while VCN at 10 µM level resulted in 44% ($p<0.02$) increase in H_2O_2 level when compared to control (Table 1).

The antioxidants used in this study by themselves did not affect the percent viability of AM (90-94 ± 6.0); control AM showed 95% viability (Fig 2). While exposure of AM to VCN (10 µM) alone resulted in 42% viability, 100 units /ml SOD +VCN exerted a marked protective effect with a resultant viability of 79%0.

Demonstration of a toxic effect by VCN that is preventable by addition of SOD is consistent with cell injury mediated by a number of species, including superoxide radical (O_2-), hydrogen peroxide, hydroxyl radical (OH), and singlet oxygen although the possibility that a carbon-centered free radical might be involved. PMA, a superoxide generating system was taken as a positive control. PMA at 100 nM concentration caused 56.2 ±6.3 % viability at the end of 4 hr incubation period. SOD supplementation followed by PMA resulted in 90.1 ± 9.2 % ($p<0.01$, n=4) viability of macrophages.

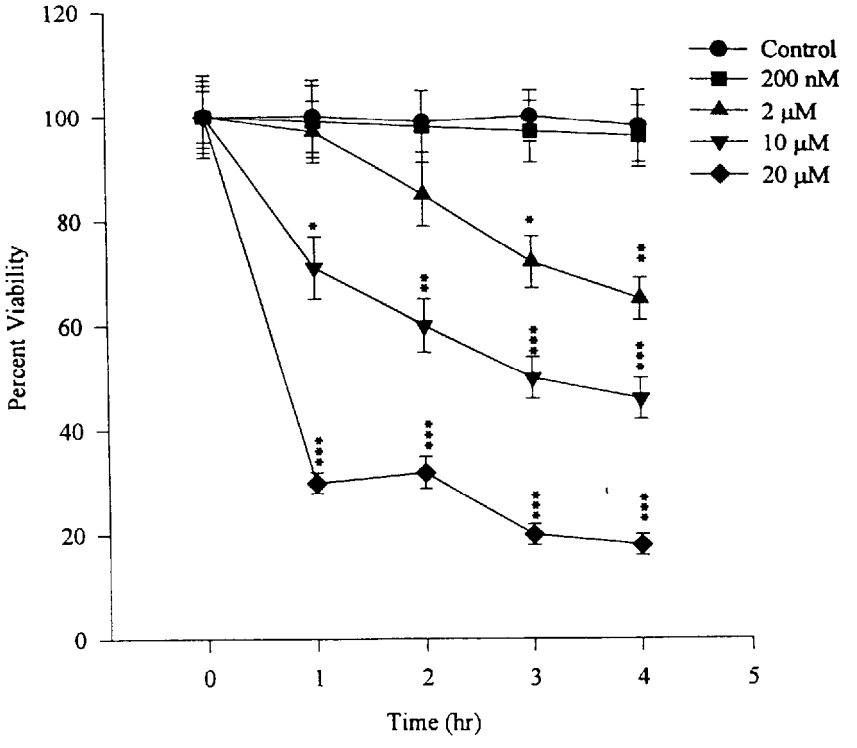

Figure 1. Percent viability of rat alveolar macrophages after incubation with varying concentrations of VCN at different time intervals. At times indicated, medium was removed, cells were washed and viability assessed. Each experiment consisted of exposing macrophages to VCN or complete medium alone (control). Significant variations when compared to VCN incubation and time- matched controls are expressed as ***p<0.01, **p<0.02 and *p<0.05 (n= 4 experiments).

Table 1. PMA and VCN-induced release of H_2O_2 by rat alveolar macrophages

Group	nmol H_2O_2 / $0.5x10^6$ AM	% increase from control
Control	2.5 ± 0.21	-
PMA (100 nM)	3.2 ± 0.25*	28.0
VCN (10 μM)	3.6 ± 0.30**	44.0

Values are mean ± S.D (n= 4 experiments). Each experiment consisted of exposing macrophages to VCN, PMA or complete medium alone (control). Statistically significant variations when compared to control are expressed as * p <0.05 **and * *** p <0.02.

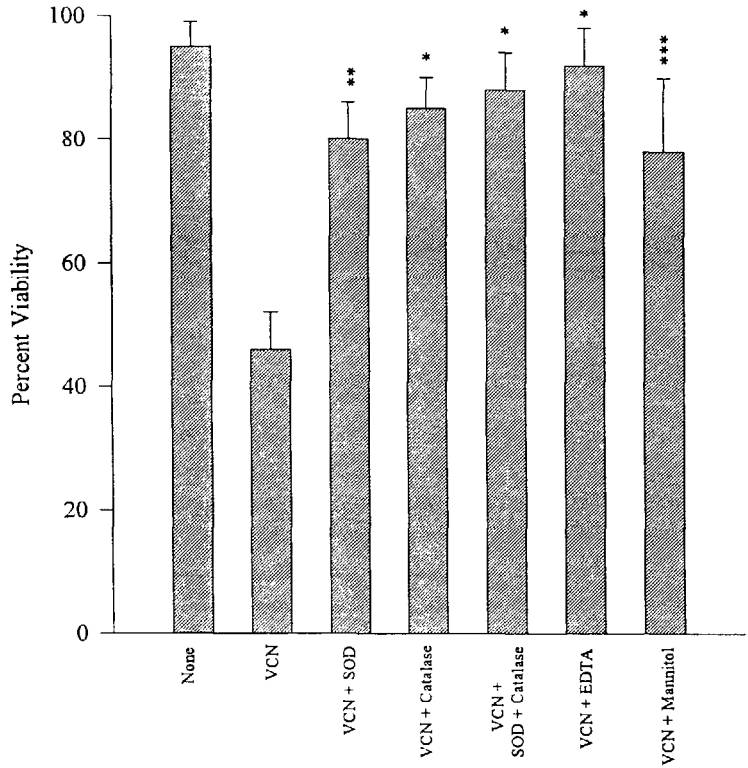

Fig 2: Effect of SOD-100 units/ml, Catalase- 275 units/ml, EDTA- 1 mM and d-Mannitol- 0.1 M on the viability of rat alveolar macrophages after 4 hours of incubation with VCN. The concentration of VCN in the medium was 10 µM. Values are Mean ± S.D (n= 3 experiments). Each experiment consisted of exposing macrophages to VCN without and with antioxidants. Significant variations when compared to VCN alone and VCN with antioxidants are expressed as ***$p<0.01$, **$p<0.02$, *$p<0.05$.

Catalase (275 units/ml) provided virtually complete protection against VCN-induced loss of viability. No difference in this result was seen when catalase and SOD were added together before incubation. Note that VCN at a concentration of 10 µM, when treated with alveolar macrophages resulted in a significant increase in H_2O_2 release when compared to control.

Because the nonenzymatic reaction which produces hydroxyl ion (OH) is of significance only in the presence of catalytic iron, the protective effect of an iron chelator (EDTA) was tested. Preincubation of macrophages with EDTA (0.1 M) provided marked protection (92%) against VCN-induced loss of viability (Fig 2).

The protective effect of SOD and catalase suggested an involvement of OH. Hence, d- mannitol a recognized scavenger of OH was added to the incubation medium before cells were incubated with VCN. Mannitol alone, at 0.1 M conc, caused little loss of viability, after 4 hr of incubation. It reduced the VCN toxicity by increasing the total viability to 78% (Fig 2).

The high concentration of molecular oxygen in the environment could lead to an incomplete reduction to superoxide anion radical instead of water in the cytochrome-mediated reaction (Heffener and Repine 1989). O_2^- is dismutated to H_2O_2 by SOD and H_2O_2 is inactivated to water primarily by glutathione peroxidase and catalase. OH^- is known to occur in *in vitro* by the nonenzymatic reaction of 0_2^- and H_2O_2. The combined action of SOD and catalase could be seen to eliminate the substrates which produce this highly reactive species (Freeman and Crapo 1982).

In this study, hyperoxic exposure for 4 hrs. did not affect the viability of rat alveolar macrophages. Toxic effect of VCN, preventable by SOD and catalase and EDTA affirms roles for O_2^-, H_2O_2 and OH" in *in vitro* VCN induced death of AM. A significant increase in H_2O_2 liberation from macrophages incubated with VCN further confirmed the involvement of reactive oxygen radicals. Catalase and Glutathione peroxidase are the two major enzymes which inactivate H_2O_2. Glutathione peroxidase needs GSH for the removal of H_2O_2. Since a major portion of VCN is eliminated by conjugation with glutathione (Kopecky et al. 1980), it is possible that GSH peroxidase cannot detoxifiy the excessively generated H_2O_2.

Intracellular catalase seems to play a lesser role in this condition because of its restricted cellular localization in the peroxisomes and its lower affinity for H_2O_2 (Heffner and Repine 1989). Thus, under conditions of high oxygen level, VCN may inhibit the degradation of reactive intermediates (via GSH depletion mediated inhibition of GSH peroxidase). It could be stated that H_2O_2 released into the extracellular space may be generated at or near the plasma membrane. This is applicable to O_2^- also since the enzymes catalase and SOD supposedly do not enter the cells.

In hepatocytes treated with VCN, high oxygen tension enhances rapid GSH depletion and lipid peroxidation (Nerudova et al. 1988). Further, short periods of lung glutathione depletion by VCN followed by exposure to 98% oxygen impaired the survival rate of rats (Vilim et al. 1988). During oxidant stress (evidenced by high oxygen tension, depletion of GSH) there is a rapid depletion of cellular energy state in macrophages which is later followed by evidence of membrane injury and loss of viability (Forman et al. 1986). Reversal of VCN induced cell death by SOD and catalase suggests the involvement of both O_2^- and H_2O_2 and therefore the production of highly toxic OH^- radicals. This could explain the induced loss of viability of macrophages after exposure to VCN.

Thus our experiments suggest that Alveolar macrophages which reside in oxygen rich environment could be an important target in acute toxicity of VCN. Human lung

tissue has a lower level of antioxidants when compared to rat lung (Slade et al. 1985). Thus this study confirms that oxygen ventilation and thiol depleting chemical in a work site could result in an additional risk.

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