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Antidotal Efficacy of Antioxidants against Cyanide Poisoning *in vitro*

R. Bhattacharya , P. V. Lakshmana Rao, M. M. Parida and A. M. Jana
Defence Research & Development Establishment, Gwalior - 474 002.

ABSTRACT

Cyanide is a potent homicidal, genocidal and chemical warfare agent. Besides, its known inhibitory effects on various enzyme systems, its other pronounced toxic effects include lipid peroxidation (LPx), particularly in the central nervous system or neuronal cells *in vitro*. The present study assessed the cytotoxicity of potassium cyanide (*KCN*) in two non-neuronal mammalian cell cultures, viz., human embryonic lung epithelium (L-132) and baby hamster kidney (BHK-21) cells. In addition, the cytoprotective potential of two antioxidant agents, namely, curcumin (CMN) and *N*-acetylcysteine (NAC) against *KCN* (2 and 4 mM) *in vitro* was evaluated. In both the cell lines, *KCN* reduced cell viability as indicated by trypan blue dye exclusion, leakage of cytosolic lactate dehydrogenase and neutral red uptake. Protein content was unaffected in L-132 cells while cellular respiration (determined by MTT assay) was impaired in both the cells. A dose-dependent glutathione mediated LPx was observed in BHK-21 cells alone. The above cytotoxic changes produced by *KCN* were more effectively minimised by NAC as compared to CMN. Efficacy of CMN and NAC have therapeutic implications as adjuncts to existing cyanide antidotes.

1. INTRODUCTION

Cyanide is ubiquitously present in the environment and is regarded as a potent homicidal, genocidal and chemical warfare (CW) agent. Cyanide toxicity is due to complexity of effects on various enzyme systems as it is a potent inhibitor of numerous enzymes, predominantly the cytochrome -c-oxidase of mitochondrial respiratory chain[1]. The centrally-mediated toxic manifestations of cyanide have also been associated with induction of lipid peroxidation (LPx)[2,3]. Occurrence of cyanide-induced LPx has been demonstrated in mouse brain and kidney but not in heart and liver[4]. Membrane LPx and subsequent membrane dysfunction observed in cyanide poisoning has been related, in part, to a compromised antioxidant defence[3]. Critical role of calcium in cyanide-induced altered neuronal calcium homeostasis and subsequent LPx is well established. Therefore, pretreatment of calcium channel blockers like diltiazem is found to attenuate cyanide toxicity[4]. However, not much attention has been paid to explore antioxidant agents as antidotes to cyanide poisoning.

Most of the *in vivo* studies on cyanide toxicity have been limited to central nervous system (CNS) or neuronal cells *in vitro*. In the present study, two non-neuronal cell lines like human embryonic lung epithelium (L-132) and baby hamster kidney (BHK-21) cells have been used. Selection of the cell lines was dictated by the fact that cyanide-induced LPx has been demonstrated in the kidney and information on pulmonary cell type is insufficient. By employing these two cell lines, the antidotal efficacy of two antioxidant agents,

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namely, curcumin (CMN) and *N*-acetylcysteine (NAC) against cyanide poisoning *in vitro* has been evaluated. CMN (1,6-hepta-diene-3, 5-dione-1, 7-bis (4-hydroxy-3-methoxyphenyl) is an anti-inflammatory and antioxidant principle of *Curcuma longa* and has been shown to inhibit LPx *in vitro*[5-7]. NAC is a powerful antioxidant known to enhance glutathione levels both *in vitro* and *in vivo*[8,9]. The antidotal efficacy of NAC against cyanogenic compounds like acrylonitrile and methyl acrylonitrile has been reported earlier[9]. To assess the cytotoxicity of cyanide and the cytoprotective ability of the antidotes, a number of end points like trypan blue dye exclusion (TBDE), neutral red uptake (NRU). MTT assay, cellular protein content, and leakage of cellular lactate dehydrogenase (LDH) were determined. Besides, parameters for oxidative stress, viz., reduced glutathione (GSH) and malondialdehyde (MDA) levels in the cells were also measured.

2. MATERIALS & METHODS

2.1 Chemicals

Culture media and chemicals of highest purity were purchased from the following sources: Eagle's Minimum Essential Medium (EMEM), glutamine and gentamycin from Hi Media India Ltd., Bombay; foetal calf serum (FCS), dimethylsulfoxide (DMSO), CMN, NAC, Coomassie brilliant blue G-250 (CBB), MTT (3-[4,5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide), neutral red (NR), trypan blue (TB) and other chemicals for cell culture from Sigma Chemical Co., USA, and *KCN* from Ferrack, Germany. Ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), Triton -X-100 etc. were from E. Merck or Qualigen India Ltd.

2.2 Cell Culture

Continuous cell lines of human foetal lung epithelium (L-132) and baby hamster kidney (BHK-21) cells were obtained from the National Facility for Animal Tissue and Cell Culture, Pune (India). The cells were grown in EMEM supplemented with nonessential amino acids, 2 mM glutamine, 10 per cent FCS and gentamycin (80 µg/ml). Cells were maintained at 37 °C in a carbon dioxide incubator (Jouan Inc., France) set at 95 % air + 5 % CO_2 level. Cell monolayers were trypsinised to obtain single cell suspension and seeded in 24 well plates (Nunc Inc., France) at a density of 4×10^5 cells/ml in each well plate. After 48 hr of incubation, culture medium was decanted and fresh medium (without FCS) was added to each well plate.

2.3 Treatment

KCN and NAC were dissolved in phosphate buffer saline (PBS). CMN was dissolved in DMSO to a final solvent concentration of 0.033 per cent, which was well-tolerated by the cells. The stock solutions were diluted with EMEM. The 4 hr EC_{50} (effective concentration) of *KCN* was calculated for TBDE, NRU, reduction of MTT, cellular protein content and leakage of LDH. Considering the effect of *KCN* on all the parameters, 2 and 4 mM dose of *KCN* were selected for further studies. Preliminary range finding tests were conducted to determine the cytoprotective doses of CMN (2.5 mM) and NAC (1.0 mM). CMN and NAC were effective at 2 hr and 1 hr pretreatment, respectively. Various biochemical and morphological indices were assessed after 4 hr *KCN* exposure. Various treatment regimens were as follows:

Group 1 - PBS-treated (control)
Group 2 - CMN (2.5 mM)
Group 3 - NAC (1.0 mM)
Group 4 - *KCN* (2.0 mM)
Group 5 - *KCN* (4.0 mM)
Group 6 - CMN (2.5 mM) + *KCN* (2.0 mM)
Group 7 - CMN (2.5 mM) + *KCN* (4.0 mM)
Group 8 - NAC (1.0 mM) + *KCN* (2.0 mM)
Group 9 - NAC (1.0 mM) + *KCN* (4.0 mM)

2.4 Biochemical Indices

With the exception of extracellular LDH assay, the medium with test chemical was discarded after the exposure period. The cells were washed twice with medium (without FCS) and metabolic status of

the cells was assessed on the basis of the following parameters:

2.4.1 Trypan Blue Dye Exclusion

Each well plate was treated with 2.5 ml of medium containing 0.4 per cent TB dye. After 5 min, viable (excluding dye) and dead (stained) cells were counted under inverted microscope and values expressed as per cent viable cells.

2.4.2 Neural Red Uptake

The procedure of Borenfreund[10], *et al.* was adapted for NRU assay. To each well plate, 200 µl of EMEM (without phenol red) containing 50 µg of neutral red/ml was added. The cells were incubated at 37 °C for 3 hr. The media was removed and the cells were rapidly washed with 200 µl 1 per cent formaldehyde-1 per cent $CaCl_2$ to remove unincorporated dye. The formaldehyde solution was replaced by 1.25 ml 1 per cent acetic acid-50 per cent ethanol solution to extract the dye. Culture plates were kept in dark for 20 min and absorbance read at 540 nm on a multiwell plate reader (Dynatech, USA). The values were expressed as $Abs.^{570}/4 \times 10^5$ cells.

2.4.3 MTT Assay

To each well plate, 200 µl of EMEM (without phenol red) containing 1 mg MTT/ml was added. The cells were incubated at 37 °C for 4 hr. The medium with MTT was removed and 100 µl 0.4 *N HCl*-isopropanol (1:24, v/v) was added to extract and solubilise the formazon. After 30 min, absorbance was read at 570 nm[11]. The values were expressed as $Abs.^{570}/4 \times 10^5$ cells.

2.4.4 Protein Assay

The Bradford[12] method was followed to determine the cellular protein content. The CBB G-250 solution (100 µl) was diluted 1:1 (v/v) with distilled water and added to each well plate and incubated for 20 min in dark. Absorbance was measured at 570 nm and values were expressed as $Abs.^{570}/4 \times 10^5$ cells. For GSH and MDA assay, protein was measured in disrupted cell pellets and calculated from a standard curve.

2.4.5 Lactate Dehydrogenase Leakage

Extracellular LDH activity in 100 µl media aliquot was assayed using a diagnostic kit of Ranbaxy India Ltd. Cellular LDH was determined after lysing the cells with 50 µl of 20 per cent Triton-X-100. Leakage of LDH was expressed as per cent of total.

2.4.6 Glutathione Content

The cellular GSH content was determined by Ellman's reagent, following extraction with TCA[13]. The GSH content was expressed as n mol/mg protein.

2.4.7 Malondialdehyde Levels

MDA was measured in cellular homogenate after extraction with 2 ml of 11.5 per cent acetic acid containing 1mM EDTA (p*H* 3.5) and 1 ml of 1 per cent TBA. MDA levels were expressed as n mol/ mg protein[14].

2.5 Morphological Studies

The cells fixed in methanol were stained with Giemsa and photographed under inverted microscope.

2.6 Statistical Analysis

The tabulated values represent means ± SE of 2-3 experiments (each experiment replicated thrice). Analysis of data was performed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls multiple comparison test (Sigmastat version 1.0, Jandel Corp., USA). Probability of < 0.05 was considered as statistically significant.

3. RESULTS

The EC_{50} doses of *KCN* for different biochemical end points were: 2.8 ± 0.38 (TBDE), 2.1 ± 0.16 (NRU), 3.5 ± 0.14 (MTT), 5.0 ± 1.0 (protein) and 1.9 ± 0.13 mM (LDH leakage); and 2.3 ± 0.18 (TBDE), 1.1 ± 0.18 (NRU), 2.1 ± 0.32 (MTT), 4.2 ± 0.90 (protein) and 0.90 ± 0.14 mM (LDH leakage) for L-132 and BHK-21 cells, respectively. The data indicate that BHK-21 cells were more sensitive to *KCN* as compared to L-132 cells. In both the cell lines, the loss of cellular

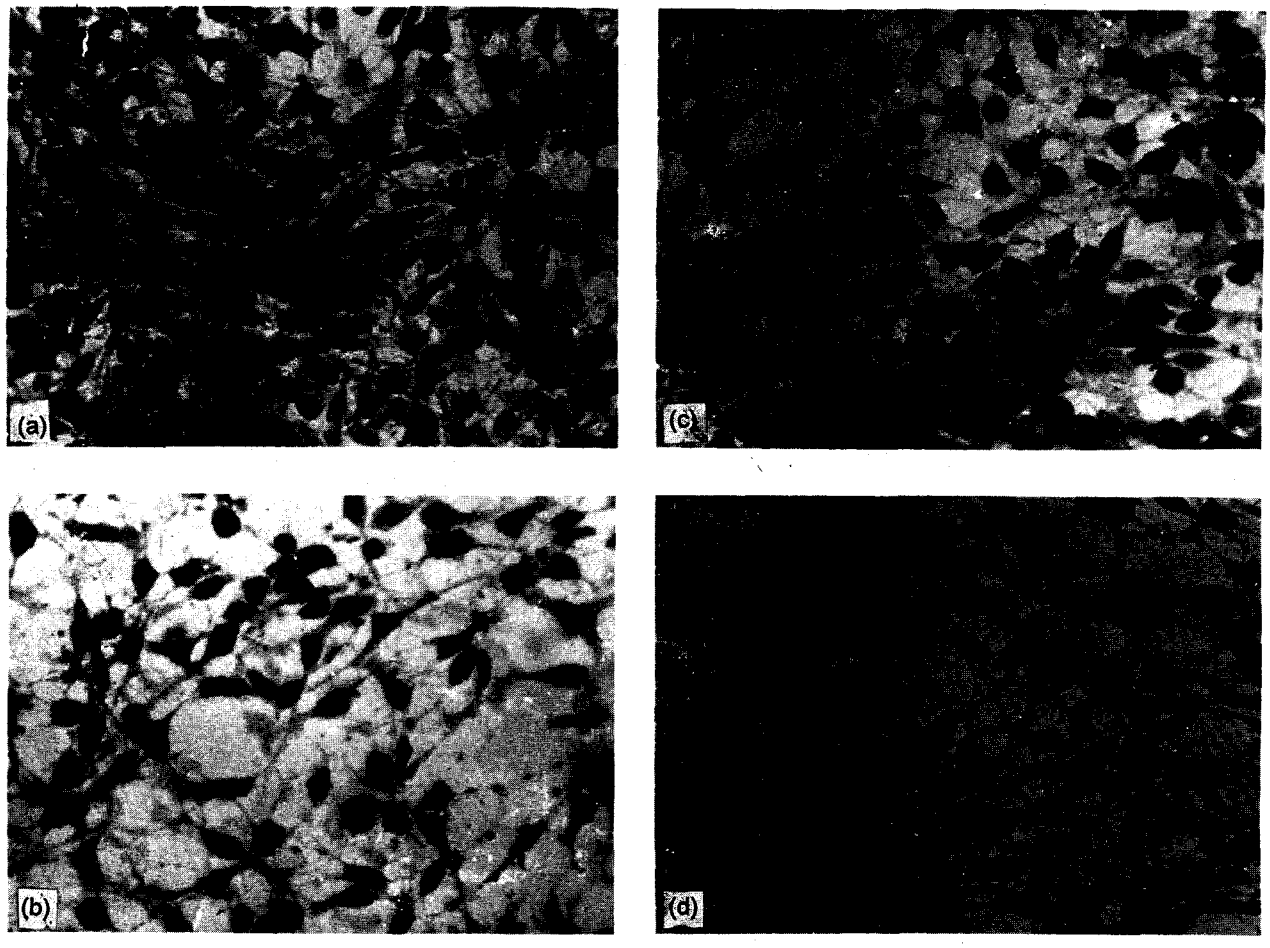

Figure 1. Cytotoxicity of *KCN* in human embryonic lung epithelium (L-132 cells) (a) PBS-treated control cells after 6 hr, (b) *KCN* (4 mM)-exposed cells after 4 hr, (c) *KCN* (4 mM)-exposed cells after 4 hr protected with CMN (2.5 mM, 2 hr pretreatment), (d) *KCN* (4 mM)-treated cells after 4 hr protected with NAC (1 mM, 1 hr pretreatment). Cells were stained with Giemsa (10X).

proteins occurred at a higher dose of *KCN* while the leakage of intracellular LDH was initiated at a lower dose. From the above EC_{50} values, two doses of *KCN* (2 and 4 mM) were selected for subsequent studies. The preliminary experiments showed that NAC up to a dose of 2.5 mM had no adverse effects on the cells while CMN at a dose of 10 mM produced cytotoxicity (data not shown). Based on these studies, the treatment protocol of both NAC and CMN was selected.

Table 1 refers to the effect of *KCN* on various end points in L-132 cells and the antidotal efficacy of both CMN and NAC. *KCN* at both the doses (2 and 4 mM) significantly altered all the parameters except cellular protein content. Protective efficacy of CMN was observed only in the higher dose of *KCN* while NAC proved beneficial against both the doses. Pretreatment of NAC was better than CMN for both the doses, particularly in TBDE and LDH leakages. Reduction of MTT was significantly inhibited by 4 mM *KCN* dose. This inhibition was significantly prevented by NAC but not by CMN. In comparison to control cells, *KCN*-exposed L-132 cells clearly demonstrate the cytotoxic effect as evident from disruption of confluent monolayer and decrease in cell density as shown in Figs 1 (a) and (b). CMN and NAC minimised these effects; cytoprotective effect of NAC was marginally better than CMN as shown in Figs 1 (c) and (d).

Table 2 indicates cyanide antagonism in BHK-21 cells. Here, unlike L-132 cells all the end

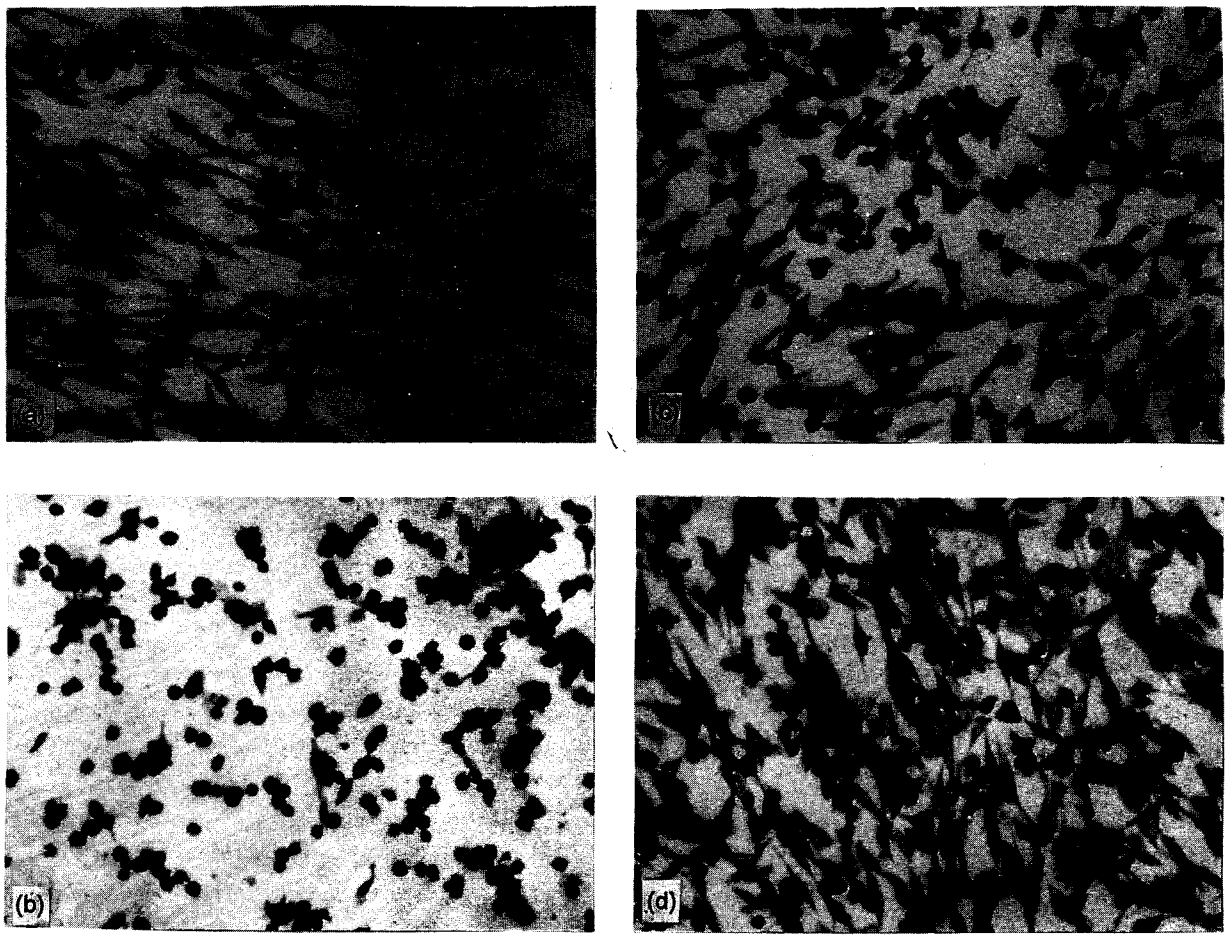

Figure 2. Cytotoxicity of *KCN* in baby hamster kidney (BHK-21) cells, (a) PBS-treated control cells after 6 hr, (b) *KCN* (4 mM)-exposed cells after 4 hr, (c) *KCN* (4 mM)-exposed cells after 4 hr protected with CMN (2.5 mM, 2 hr pretreatment), (d) *KCN* (4 mM)-treated cells after 4 hr protected with NAC (1 mM, 1 hr pretreatment). Cells were stained with Giemsa (10X).

points were significantly altered by both the doses of *KCN*. Both CMN and NAC were equally effective in ameliorating the toxicity of *KCN* at both 2 and 4 mM doses. In NRU and MTT assay, NAC was more effective than CMN against *KCN* (4 mM). Beneficial effect of CMN and NAC against *KCN*-induced depletion in cellular protein was significant only when treated by 4 mM *KCN*. As compared to control cells, *KCN*-treated BHK-21 cells are characterised by loss of cell-to-cell connection and rounding of cells, indicating severe cytotoxicity as shown in Figs 2 (a) and (b). These effects were significantly reduced by both CMN and NAC as shown in Figs 2 (c) and (d).

Both GSH and MDA levels were marginally altered in *KCN* (4 mM)-exposed L-132 cells. However, these changes were not statistically significant (Table 3). In contrast, alterations in BHK-21 cells were significant in a dose-dependent manner. Both CMN and NAC prevented these changes, although CMN was inferior to NAC treatment.

4. DISCUSSION

Besides many toxic manifestations, the propensity of cyanide to induce LPx has drawn attention of many workers lately[2-4]. Cyanide is reported to activate generation of reactive oxygen species (ROS), which impairs the cell's intrinsic antioxidant defences to produce cytotoxicity[15]. Since neural tissues are more vulnerable to oxidative stress, studies on such effects of cyanide are mainly limited to CNS[4]. In the present study, the cytotoxicity of *KCN* in two different cell lines

Table 1. Effect of *KCN* and its antagonism in human embryonic lung epithelium (L-132) cells

Groups	Parameters					
	TBDE (per cent viable cells)	NRU (Abs. ^{540}/ 4 × 10^5 cells)	MTT (Abs. ^{570}/4 × 10^5 cells)	Protein (Abs. ^{570}/4 × 10^5 cells)	LDH leakage (per cent of total)	
Control	84.8 ± 4.6	0.457 ± 0.009	0.341 ± 0.019	0.208 ± 0.012	19.8	± 2.15
CMN	82.1 ± 2.5	0.418 ± 0.018	0.304 ± 0.005	0.205 ± 0.004	19.1	± 0.67
NAC	77.9 ± 1.55	0.435 ± 0.013	0.335 ± 0.033	0.198 ± 0.002	19.6	± 2.30
KCN 2	46.4[a] ± 3.80	0.254[a] ± 0.015	0.259 ± 0.010	0.219 ± 0.012	39.7[a]	± 0.50
KCN 4	27.1[a] ± 0.70	0.163[a] ± 0.004	0.153[a] ± 0.005	0.167 ± 0.019	71.0[a]	± 1.60
CMN+*KCN* 2	61.0[a] ± 4.15	0.319 ± 0.020	0.336 ± 0.010	0.198 ± 0.012	29.4[a,b]	± 2.20
CMN+*KCN* 4	54.2[a,c] ± 4.0	0.257[a,c] ± 0.013	0.200[a] ± 0.004	0.184 ± 0.014	48.8[a,c]	± 4.15
NAC+*KCN* 2	72.8[b,d] ± 5.85	0.345[a,b] ± 0.012	0.316 ± 0.047	0.195 ± 0.013	37.5[a,d]	± 0.71
NAC+*KCN* 4	65.15[a,c] ± 1.75	0.315[a,c,e] ± 0.014	0.250[c] ± 0.002	0.176 ± 0.004	40.3[a,c]	± 0.45

Values are ± SE of three different experiments [a]group 1 *vs* all the groups, [b]group 4 *vs* 6 and 8, [c]group 5 *vs* 7 and 9, [d]group 6 vs 8 and [e]group 7 *vs* 9.

unrelated to CNS has been assessed, and also the cytoprotective efficacy of two antioxidants, namely, CMN and NAC has been evaluated. In *in vitro* studies, a single biochemical marker may not suffice to predict cytotoxic nature of the test compound precisely. Hence, in the present study, multiple end points were incorporated to assess the cytotoxicity of *KCN* and also to evaluate the cytoprotective potentials of the two antioxidants. Unrelated parameters can provide information on different mechanisms of action of the poison[16]. The uptake of 'vital dye' like TB and leakage of cytosolic LDH are associated with the interruptions in the continuity of the cell membrane[17]. Measurement of mitochondrial succinate dehydrogenase activity by the formation of dark blue formazon from reduction of tetrazolium salt (MTT) indicates the respiratory activity of the cell[10,11]. The NR assay measures incorporation of dye into lysosomes[10]. Decrease in total protein content of the cells has also been used to measure the toxicity of chemicals *in vitro*[16,18]. In addition, a

Table 2. Effect of *KCN* and its antagonism in baby hamster kidney (BHK-21) cells

Groups	Parameters					
	TBDE (per cent viable cells)	NRU (Abs.^{540} × 4 × 10^5 cells)	MTT (Abs. ^{570} × 4 × 10^5 cells)	Protein (Abs. ^{570} × 4 × 10^5 cells)	LDH (per cent of total)	
Control	82.1 ± 2.50	0.483 ± 0.005	0.515 ± 0.015	0.231 ± 0.001	21.8	± 4.20
CMN	74.5 ± 1.30	0.450 ± 0.006	0.489 ± 0.011	0.222 ± 0.002	19.9	± 0.37
NAC	78.1 ± 1.70	0.466 ± 0.007	0.467 ± 0.001	0.234 ± 0.008	20.4	± 2.20
KCN 2	37.3[a] ± 6.90	0.181[a] ± 0.005	0.215[a] ± 0.015	0.194[a] ± 0.006	68.1[a]	± 1.30
KCN 4	16.05[a] ± 0.85	0.107[a] ± 0.007	0.182[a] ± 0.014	0.152 ± 0.010	80.9[a]	± 1.50
CMN+*KCN* 2	66.1[b] ± 3.7	0.317[a,b] ± 0.017	0.366[a] ± 0.040	0.203[a] ± 0.007	44.7[a,b]	± 0.100
CMN+*KCN* 4	30.5[a] ± 1.90	0.198[a,c] ± 0.002	0.349[a,c] ± 0.051	0.194[a,c] ± 0.008	47.9[a,c]	± 3.05
NAC+*KCN* 2	69.8[b] ± 1.65	0.347[a,b] ± 0.001	0.426[b] ± 0.064	0.216[a,c] ± 0.015	44.0[a,b]	± 8.60
NAC+*KCN* 4	73.8[c,e] ± 8.60	0.291[a,c,e] ± 0.029	0.363[a,c] ± 0.064	0.194[a,c] ± 0.016	39.9[a,c]	± 4.30

Values are ± SE of three different experiments [a]group 1 vs all the groups, [b]groups 4 *vs* 6 and 8, [c]group 5 *vs* 7 and 9, [d]group 6 *vs* 8 and [e]group 7 *vs* 9.

Table 3. Effect of *KCN* and its antagonism based on GSH and MDA levels in human embryonic lung epithelium (L-132) and baby hamster kidney (BHK-21) cells

Groups	L-132		BHK-21	
	GSH (n mol/mg protein)	MDA (n mol/mg protein)	GSH (n mol/mg protein)	MDA (n mol/mg protein)
Control	21.92 ± 1.47	0.33 ± 0.01	30.39 ± 1.72	0.44 ± 0.03
CMN	21.02 ± 1.45	0.33 ± 0.45	28.54 ± 2.87	0.49 ± 0.56
NAC	24.63 ± 1.96	0.32 ± 0.04	32.87 ± 1.94	0.49 ± 0.08
KCN 2	20.00 ± 1.39	0.37 ± 0.04	20.88[a] ± 0.86	0.62[a] ± 0.04
KCN 4	17.42 ± 1.66	0.39 ± 0.06	16.93[a] ± 1.53	0.89[a] ± 0.06
CMN+*KCN* 2	21.06 ± 1.46	0.29 ± 0.05	18.96[a] ± 1.42	0.42 ± 0.06
CMN+*KCN* 4	22.19 ± 1.52	0.31 ± 0.02	18.06[a] ± 1.39	0.62[a] ± 0.08
NAC+*KCN* 2	22.46 ± 1.67	0.35 ± 0.01	29.13 ± 1.36	0.39 ± 0.06
NAC+*KCN* 4	22.35 ± 0.49	0.36 ± 0.06	23.75[a,c,e] ± 1.86	0.48[a,c,e] ± 0.06

Values are ± SE of two different experiments [a]group 1 *vs* all the groups, [b]group 4 *vs* 6 and 8, [c]group 5 *vs* p7 and 9 dgroup 6 *vs* 8 and [e]group 7 *vs* 9.

large number of chemicals were reported to react with the -SH groups of the cell, causing membrane damage. GSH content of the cells are also associated with the essential cytoskeletal proteins required for cell attachment. Both GSH and MDA levels in the cell are indicators of oxidative stress[19]. In view of the nature of cyanide toxicity, the selection of above parameters for the present study is appropriate. The choice of cell type also seems to be critical to the results from the point of view of *in vitro* toxicity assays[16]. This can be attributed to the fact that L-132 cells were more resistant to *KCN* as compared to BHK-21 cells. In L-132 cells, GSH and MDA levels were not altered to significant levels, whereas BHK-21 cells exhibited significant depletion in GSH with concomitant elevation in MDA levels. This indicates that GSH-mediated LPx did not occur in both the cells uniformly. The *KCN*-induced LPx in mouse kidney can be correlated to this observation on renal cells[4].

Antioxidant activities of CMN[5-7,18] and NAC[8,9,19] are well-documented. CMN treatment is reported to modulate GSH-mediated antioxidant defence mechanism in the cells[20]. This perhaps explains the lower efficacy of CMN in L-132 cells because both GSH and MDA levels were minimally altered here following *KCN* insult. However, besides antioxidant property, the ability of CMN to prevent leakage of enzyme can be attributed to its cytoprotective effects in L-132 cells[21]. The additional beneficial effect of NAC in both the cell lines can be ascribed to the fact that besides antioxidant potential, a sulfhydryl group is present in NAC. The effectiveness of NAC by direct reaction between *-SH* group and a cyanogenic compound (methyl acrylonitrile) has been reported earlier[9].

5. CONCLUSION

The present study shows that both CMN and NAC can antagonise *KCN*-induced cytotoxicity *in vitro*. As compared to CMN, the NAC treatment maintains the oxidative integrity of *KCN*-exposed mammalian cells more effectively. These findings have therapeutic implications, as these agents are likely to augment the efficacy of conventional antidotes to cyanide poisoning.

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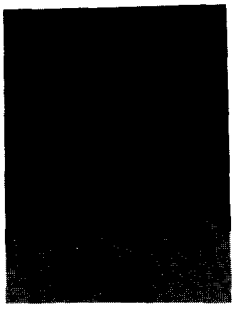
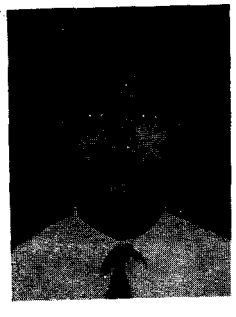
Contributors

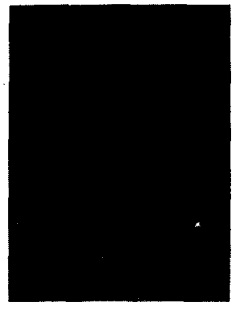


Dr R Bhattacharya obtained his PhD in Biosciences from Bhopal University in 1988. He joined DRDO at the Defence Research & Development Establishment (DRDE), Gwalior, as Scientist in 1988. His areas of research include toxicology and antagonism of cyanide and cyanobacterial toxins employing *in vitro* techniques. He has published 36 research papers in national/international journals.

Dr PV Lakshmana Rao obtained his PhD in Biotechnology from Indian Institute of Technology, Kharagpur, in 1986. He joined DRDO at the Defence Research Laboratory, Tezpur, where he worked on application of cell culture techniques for vegetable. crop improvement and high altitude agriculture. Presently, he is working at DRDE, Gwalior, in the areas of cyanobacterial toxins and genetic toxicology.



Dr MM Parida obtained his MVSc in Veterinary Virology from Indian Veterinary Research Institute, Izatnagar, in 1993. He joined DRDO at the DRDE, Gwalior, in 1994 and is presently working as Senior Scientist. His areas of research include immunodiagnosis of viral infections and studies on antiviral activity of certain indigenous medicinal plants.



Dr AM Jana obtained his PhD from Indian Veterinary Research Institute, Izatnagar, in 1977. He joined DRDO at the DRDE, Gwalior, in 1977 and is presently working as Dy. Director. His areas of research include virology with particular emphasis on isolation, identification and characterisation of various human and animal viral pathogens employing animal tissue culture and serology. He worked at the National Institute for Medical Research, London as Visiting Scientist under DBT Associateship from 1986-88, in the field of molecular virology with influenza virus. He has published 22 research papers in national/international journals.