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Protective efficacy of various carbonyl compounds and their metabolites, and nutrients against acute toxicity of some cyanogens in rats: biochemical and physiological studies

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ABSTRACT

Cyanogens are widely used in industries and their toxicity is mainly due to cyanogenesis. The antidotes for cyanide are usually instituted for the management of cyanogen poisoning. The present study reports the protective efficacy of 14 carbonyl compounds and their metabolites, and nutrients (1.0 g/kg; oral; +5 min) against acute oral toxicity of acetonitrile (ATCN), acrylonitrile (ACN), malononitrile (MCN), propionitrile (PCN), sodium nitroprusside (SNP), succinonitrile (SCN), and potassium ferricyanide (PFCN) in rats. Maximum protection index was observed for alpha-ketoglutarate (A-KG) against MCN and PCN (5.60), followed by dihydroxyacetone (DHA) against MCN (2.79). Further, MCN (0.75 LD50) caused significant increase in cyanide concentration in brain, liver and kidney and inhibition of cytochrome c oxidase activity in brain and liver, which favorably responded to A-KG and DHA treatment. Up-regulation of inducible nitric oxide synthase by MCN, PCN and SNP, and uncoupling protein by PCN and SNP observed in the brain was abolished by A-KG administration. However, no DNA damage was detected in the brain. MCN and SNP significantly decreased the mean arterial pressure, heart rate, respiratory rate and neuromuscular transmission, which were resolved by A-KG. The study suggests a beneficial effect of A-KG in the treatment of acute cyanogen poisoning.

KEY WORDS: cyanogens; cyanide; acute toxicity; protection; biochemistry; physiology

Introduction

Cyanogens usually include aliphatic nitriles like acetonitrile (ATCN), acrylonitrile (ACN), malononitrile (MCN), succinonitrile (SCN), propionitrile (PCN), *etc.*, and inorganic compounds like sodium nitroprusside (SNP), potassium ferricyanide (PFCN), *etc.* (Borron, 2015). Cyanogens may exist naturally or produced synthetically and find wide industrial applications. Mostly after hepatic metabolism, these compounds are known to generate toxic levels of free cyanide that could be of serious health concern (Vesey *et al.*, 1985; Patnaik, 2007; Bhattacharya *et*

al., 2009). Cyanide is a rapid poison that impairs cellular respiration by inhibiting cytochrome *c* oxidase (CYTOX) activity. Inhibition of CYTOX (an end-chain enzyme of cellular respiration) leads to immediate morbidity or mortality (Bhattacharya & Flora, 2009). The non-nitrile portion of cyanogenic compounds may also produce notable toxicity, which could be independent of cyanide toxicity (Bhattacharya *et al.*, 2009). Aliphatic nitriles are implicated in various occupational accidents and have shown diverse toxicities due to resultant cyanide poisoning (Saillenfait *et al.*, 2000; Their *et al.*, 2000; Patnaik, 2007; Wu Jong-C *et al.*, 2009; Borron, 2015). We have earlier reported oral toxicity of six such compounds (ATCN, ACN, MCN, PCN, SNP, and SCN) (Bhattacharya *et al.*, 2009). Subsequently, we also reported the time-dependent cyanide generation and CYTOX inhibition in soft tissues after sub-acute oral intoxication with these cyanogens (Rao *et al.*, 2013).

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The toxicity of cyanogens is largely attributed to cyanide poisoning with disastrous consequences if not diagnosed and treated early and aggressively (Abraham *et al.*, 2015). Treatment of cyanide poisoning usually includes intravenous administration of sodium nitrite (SN) and sodium thiosulfate (STS) (Bhattacharya & Flora, 2009). The same regimen has been recommended also for the treatment of SNP poisoning (Hall & Guest, 1992; Abraham *et al.*, 2015). Hydroxocobalamin is also a safe and effective treatment for SNP poisoning (Hall *et al.*, 2007). In one report, successful treatment of life-threatening PCN exposure was achieved with SN/STS followed by hyperbaric oxygen (Scolnick *et al.*, 1993). Due to prolonged and sustained metabolism of nitriles to cyanide, multiple doses or continuous infusion of the antidotes are necessary (Borron, 2015; Mirkin, 2015). Antioxidants like *N*-acetyl-ʟ-cysteine (NAC) also confer protection against experimental or accidental ACN poisoning (Mirkin, 2015). In addition to SN, STS and hydroxocobalamine, protective efficacy of 4-dimethylaminophenol (4-DMAP), dicobalt-ethylenediaminetetraacetic acid (dicobalt-EDTA) and alpha-ketoglutarate (A-KG) has been reported against experimental nitrile poisoning (Bhattacharya *et al.*, 2009; Borron, 2015). The beneficial effect of A-KG against experimental cyanide poisoning is well recognized (Bhattacharya & Vijayaraghavan, 2002; Bhattacharya, 2004; Bhattacharya & Tulsawani, 2008; Bhattacharya & Flora, 2009). Thus A-KG has been shown to protect against several biochemical, oxidative and histological changes caused by acute and sub-acute MCN, PCN and SNP poisoning in rats (Bhattacharya *et al.*, 2009, 2014). Cyanide is known to react with carbonyl compounds to form cyanohydrin complex. For this reason various carbonyl compounds and their metabolites, as well as nutrients like A-KG, dihydroxyacetone (DHA), glyoxal, oxaloacetic acid (OAA), citric acid, succinic acid, fumaric acid, malic acid, maleic acid, glucose, sucrose, fructose, sorbitol, and mannitol were evaluated for their protective efficacy against experimental cyanide poisoning *in vitro* and *in vivo* (Bhattacharya & Tulsawani, 2008). The present study reports on the efficacy of these compounds against acute toxicity of seven cyanogens (ATCN, ACN, MCN, PCN, SNP, SCN, and PFCN) in Wistar rats. The study was based on (i) shift of median lethal dose (LD50) of cyanogens in the presence of antidotes, (ii) protection against cyanogen-induced elevated blood cyanide concentration, CYTOX inhibition, expression of inducible nitric oxide (iNOS) and uncoupling protein (UCP-2), and against DNA damage in brain tissue, and (iii) protection against cyanogen-induced physiological changes. The study highlights the therapeutic implications of various carbonyl compounds and their metabolites, and nutrients against acute cyanogen toxicity.

Materials and methods

Animals
Female Wistar rats (150–200 g) bred in the animal facility of the Defence Research and Development Establishment (DRDE), Gwalior, were used in this study. The care and maintenance of animals were as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India, New Delhi, India. The animals were kept in controlled environmental conditions of ambient temperature (22±2 °C) and relative humidity of 40–60%, in a 12:12 light/dark cycle. All the animals were fed standard pellet diet (Ashirwad Brand, Chandigarh, India) and water *ad libitum*. The animals were acclimatized for 7 d and fasted overnight prior to the experiment. The experimental protocol was approved by the Institutional Ethical Committee (TOX-25/50/RB dated 16 July 2012) approved by CPCSEA.

Chemicals
All the chemicals and antibodies, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-rabbit, anti-mouse and anti-goat horseradish peroxidase (HRP) conjugated antibodies were obtained from DAKO (Glostrup, Denmark). ATCN, ACN, MCN, PCN, and SCN were purchased from Acros Organics, New Jersey, USA. SNP, PFCN, and other chemicals of highest purity were from Merck, Darmstadt, Germany. 3-Methyl-1-phenyl-2-pyrazolin-5-on and bispyrazolone were procured from Fluka AG, Chemische Fabrik, Germany and BDH Laboratory, London, UK, respectively.

Treatments
All the solutions of cyanogens and antidotes were prepared fresh in triple distilled water, and administered orally in a volume <10 ml/kg body weight using a 16 gauge animal feeding needle (HSE-Harvard, March-Hugstetten, Germany).

LD50 determination
Twenty-four hour LD50 of various cyanogens was determined by Dixon's up and down method, using 4–6 rats for each LD50 value (Dixon, 1965). As in our previous report, the LD50 for ATCN, ACN, MCN, PCN, SNP, and SCN was found to be 5998.6 (5156.9–6977.7), 95.1 (81.7–110.6), 66.4 (57.1–77.2), 83.6 (71.8–97.2), 83.6 (71.8–97.2), and 378.5 (325.4–440.3) mg/kg, respectively (Rao *et al.*, 2013), whereas, the LD50 of PFCN was found to be 6636.7 mg/kg (5705.5–7719.9) (unpublished). The values in parentheses are fiducial limits at 95% confidence intervals. In the present study, LD50 of all the cyanogens was determined in the presence of various antidotes (1.0 g/kg; oral), given 5 min after the cyanogens. Animals were observed for 24 h for survival/mortality, and the Protection Index (PI) was calculated as the ratio of LD50 of cyanogens in the presence and absence of antidotes. The dose of antidotes was selected on the basis of our previous study, where 14 different compounds were tested for protective efficacy against cyanide *in vivo* and *in vitro*. Out of these compounds A-KG, followed by OAA and DHA were found to be most effective (Bhattacharya & Tulsawani, 2008). Therefore, in the present study, these three compounds were first tested against seven cyanogens. On the basis of maximum protection exhibited against MCN and SNP, the remaining 11 compounds were further tested against the two most toxic cyanogens.

Studies on biochemical changes

Forty-eight rats were divided into eight groups of six animals each and treated as follows: (i) Control (saline), (ii) MCN (0.75 LD50 ≈ 49.8 mg/kg), (iii) A-KG (1.0 g/kg), (iv) DHA (1.0 g/kg), (v) OAA (1.0 g/kg), (vi) MCN + A-KG (1.0 g/kg; +5 min), (vii) MCN + DHA (1.0 g/kg; +5 min), and (viii) MCN + OAA (1.0 g/kg; +5 min). Four hours after exposure, the animals were killed by cervical dislocation under ether anesthesia to measure cyanide concentration and CYTOX activity in excised brain, liver and kidney. Micro-diffusion of cyanide from 10% tissue homogenate was performed using Conway cells (Feldstein & Klendshoj, 1954). Tissue cyanide concentration was spectrophotometrically measured by the method of Epstein (1947) as elaborated elsewhere (Rao *et al.*, 2013). Cyanide values were expressed in nmol/g wet tissue. CYTOX activity in tissue homogenate was determined following the modified method of Cooperstein and Lazarow (1951). Briefly, a 10% mitochondrial preparation was used as the enzyme source for measuring CYTOX activity as discussed elsewhere (Rao *et al.*, 2013). The CYTOX activity was expressed in μmol of cytochrome *c* oxidized/min/g tissue.

Studies on iNOS and UCP-2 expression, and DNA fragmentation

Twenty seven rats were divided into nine groups of three animals each and treated as follows: (i) Control (saline), (ii) ACN (0.75 LD50 ≈ 71.33 mg/kg), (iii) MCN (0.75 LD50 ≈ 49.8 mg/kg), (iv) PCN (0.75 LD50 ≈ 62.7 mg/kg), (v) SNP (0.75 LD50 ≈ 62.7 mg/kg), (vi) ACN + A-KG (A-KG; 1.0 g/kg; +5 min), (vii) MCN + A-KG, (viii) PCN + A-KG and (ix) SNP + A-KG. Seven days after exposure, the animals were killed by cervical dislocation under ether anesthesia, and brain tissues were processed for Western blot (iNOS and UCP-2 expression) and DNA damage analysis. Tissues were processed for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot. The protein content in the extractions was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were subjected to electrophoresis in 12% SDS-polyacrylamide gel, followed by transfer to polyvinylidene difluoride (PVDF) transfer membrane (Pall Pharmalab (Mumbai, India). After blocking with phosphate buffered saline containing 5% nonfat dry milk, the PVDF membrane was exposed to primary anti UCP-2 antibody (Sigma-Aldrich, St. Louis, MO, USA) and anti iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for the whole night at 4°C on a shaker. The membranes were incubated for 2 h in HRP conjugated goat anti-goat secondary antibody (1:10,000) and HRP conjugated goat anti-rabbit secondary antibody (1:10,000), followed by signal amplification using chemiluminiscent peroxidase substrate (Sigma). The signals were detected using an enhanced chemiluminiscent detection system (Fusion SL Vilber Loumart, Cedex, France). Further, for densitometric analysis, data were normalized with internal control and expressed as relative density (% control) for each band using ImageJ 1.48V Wayne Rasband (NIH, MD, USA). The Western blot analysis was performed thrice and only representative blots with comparable results are shown

here. For the DNA fragmentation assay, DNA was isolated by phenol:chloroform method discussed elsewhere (Sambrook & Russell, 2000). DNA was electrophoresed on 1.2% agarose gel impregnated with ethidium bromide; 1 kb DNA served as standard.

Studies on physiological changes

Twenty rats were divided into five groups of four animals each and treated as follows: (i) Control (saline), (ii) MCN (0.75 LD50 ≈ 49.8 mg/kg), (iii) MCN + A-KG (A-KG; 1.0 g/kg; +5 min), (iv) SNP (0.75 LD50 ≈ 62.7 mg/kg), and (v) SNP + A-KG (A-KG; 1.0 g/kg; +5 min). In a separate study, twenty rats were divided into five groups of four animals each and treated as follows: (i) Control (saline), (ii) 0.75 LD50 PCN (≈62.7 mg/kg), (iii) 1.0 LD50 PCN (≈83.6 mg/kg), (iv) 2.0 LD50 PCN (≈167.2 mg/kg), and (v) 2.0 LD50 PCN (≈334.4 mg/kg). All the animals were anesthetized with urethane (1.6 g/kg; intraperitoneal) and various physiological parameters were recorded on Grass Polygraph (Model 7-16 P-35) at different time intervals (0–240 min), as discussed elsewhere (Tulsawani *et al.*, 2007). Briefly, the trachea was cannulated and connected to a pneumotachometer (Fleisch tube) to record the respiratory rate (RR) through a differential pressure transducer (Hugo Sachs Electronics, Germany). The carotid artery was cannulated to record blood pressure (B.P.) with a low level d.c. preamplifier (Model 7 P1) attached to a pressure transducer (P 23 1D, Gould, USA). Mean arterial pressure (MAP) was calculated from the recorded blood pressure. Pulse signals were also fed into a tachograph preamplifier (Type 7 P4) to record the heart rate (HR). The neuromuscular transmission (NMT) studies were carried out to record the twitch responses. The gastracnemius muscle was opened and the sciatic nerve was stimulated with a supramaximal voltage (1–10 V) of 0.2 msec duration at a frequency of 0.2 Hz using a Grass stimulator model S 88. The twitch response of muscle was recorded using a force transducer (Model FT0-3). Rectal temperature (RT) was measured using a rectal probe. Animals were allowed to stabilize prior to various treatments.

Data analysis

The results are expressed as mean±SE (n=4−6 animals). The data were analyzed by one way ANOVA followed by Student-Newman-Keul multiple comparison test. Statistical significance was set at $p < 0.05$ and $p < 0.01$ levels using SigmaStat software (Jandel Scientific Inc., CA, USA).

Results

Table 1 shows the protective efficacy of A-KG, DHA and OAA against acute toxicity of seven cyanogens (ATCN, ACN, MCN, PCN, SNP, SCN, and PFCN) in rats. On the basis of 24 h LD50, A-KG afforded maximum protection (PI 5.60) against both MCN and PCN, followed by marginal protection (PI 1.40) against SNP. DHA conferred the maximum protection (PI 2.79) against MCN alone,

Table 1. Protective efficacy of A-KG, DHA and OAA against acute toxicity of various cyanogens in rats.

		A-KG		DHA		OAA	
Sl. No.	Cyanogens	LD50 (mg/kg)	PI	LD50 (mg/kg)	PI	LD50 (mg/kg)	PI
1.	ATCN	3548.1 (1432.3–8789.4)	0.59 (0.23–1.46)	3700.8 (1493.9–9167.7)	0.61 (0.24–1.52)	1778.3 (717.9–4405.1)	0.29 (0.11–0.73)
2.	ACN	53.9 (21.8–133.6)	0.56 (0.22–1.40)	58.7 (23.7–145.3)	0.61 (0.24–1.52)	56.2 (22.7–139.3)	0.59 (0.23–1.46)
3.	MCN	371.9 (150.1–921.2)	5.60 (2.26–13.9)	185.5 (74.9–459.5)	2.79 (1.12–6.92)	82.5 (25.1–271.5)	1.24 (0.37–4.09)
4.	PCN	468.2 (188.9–1159.7)	5.60 (2.26–13.9)	107.6 (43.4–266.5)	1.28 (0.51–3.18)	56.2 (22.7–138.0)	0.67 (0.27–1.65)
5.	SNP	117.0 (47.2–289.9)	1.40 (0.56–3.46)	53.9 (21.8–133.6)	0.64 (0.26–1.59)	26.9 (10.9–66.6)	0.32 (0.12–0.79)
6.	SCN	67.5 (27.3–167.3)	0.17 (0.07–0.44)	33.9 (13.7–83.9)	0.08 (0.03–0.22)	70.8 (28.6–175.4)	0.18 (0.07–0.46)
7.	PFCN	4466.8 (1803.2–11065.2)	0.67 (0.27–1.66)	4659.1 (1880.8–11541.4)	0.70 (0.28–1.73)	4659.1 (1880.8–11541.4)	0.70 (0.28–1.73)

Rats received various doses of cyanogens (oral) in the presence of alpha-ketoglutarate (A-KG), dihydroxyacetone (DHA) or oxaloacetic acid (OAA) given at 1.0 g/kg (oral; + 5 min). Protection Index (PI) was determined as the ratio of LD50 of cyanogens in protected and unprotected animals. LD50 of various cyanogens in unprotected animals are 5998.6 (5156.9–6977.7), 95.1 (81.7–110.6), 66.4 (57.1–77.2), 83.6 (71.8–97.2), 83.6 (71.8–97.2), 378.5 (325.4–440.3), and 6636.7 (5705.5–7719.9) mg/kg for acetonitrile (ATCN), acrylonitrile (ACN), malononitrile (MCN), propionitrile (PCN), sodium nitroprusside (SNP), succinonitrile (SCN), and potassium ferricyanide (PFCN), respectively. The values in parentheses are fiducial limits at 95% confidence intervals.

Table 2. Protective efficacy of various carbonyl compounds, their metabolites and nutrients against acute toxicity of MCN in rats.

Sl. No.	Compounds	LD50 of MCN (mg/kg)	PI
1.	Glyoxal	85.4 (34.5–211.7)	1.28 (0.51–3.18)
2.	Citric acid	42.8 (17.3–106.1)	0.64 (0.26–1.59)
3.	Succinic acid	92.9 (37.5–230.3)	1.40 (0.56–3.46)
4.	Fumaric acid	42.6 (17.2–105.6)	0.64 (0.25–1.59)
5.	Malic acid	92.9 (37.5–230.3)	1.40 (0.56–3.46)
6.	Maleic acid	21.4 (8.6–52.9)	0.32 (0.12–0.79)
7.	Glucose	42.8 (17.3–106.1)	0.64 (0.26–1.59)
8.	Sucrose	42.8 (17.3–106.1)	0.64 (0.26–1.59)
9.	Fructose	92.9 (37.5–230.3)	1.40 (0.56–3.46)
10.	Sorbitol	21.4 (8.6–52.9)	0.32 (0.12–0.79)
11.	Mannitol	46.6 (18.8–115.4)	0.70 (0.28–1.73)

Rats received various doses of malononitrile (MCN; oral) in the presence of various carbonyl compounds, their metabolites and nutrients given at 1.0 g/kg (oral; + 5 min). Protection Index (PI) was determined as the ratio of LD50 of MCN in protected and unprotected animals. LD50 of MCN = 66.4 mg/kg (57.1–77.2). The values in parentheses are fiducial limits at 95% confidence intervals.

Table 3. Protective efficacy of various carbonyl compounds, their metabolites and nutrients against acute toxicity of SNP in rats.

Sl. No.	Compounds	LD50 of SNP (mg/kg)	PI
1.	Glyoxal	53.9 (21.8–133.6)	0.64 (0.26–1.59)
2.	Citric acid	28.2 (11.4–69.8)	0.33 (0.13–0.83)
3.	Succinic acid	112.2 (45.3–277.9)	1.34 (0.54–3.32)
4.	Fumaric acid	56.2 (22.7–139.3)	0.67 (0.27–1.66)
5.	Malic acid	53.9 (21.8–133.6)	0.64 (0.26–1.59)
6.	Maleic acid	56.2 (22.7–139.3)	0.67 (0.27–1.66)
7.	Glucose	56.2 (22.7–139.3)	0.67 (0.27–1.66)
8.	Sucrose	56.2 (22.7–139.3)	0.67 (0.27–1.66)
9.	Fructose	117.0 (47.2–289.9)	1.40 (0.56–3.46)
10.	Sorbitol	58.7 (23.7–145.3)	0.70 (0.28–1.73)
11.	Mannitol	117.0 (47.2–289.9)	1.40 (0.56–3.46)

Rats received various doses of sodium nitroprusside (SNP; oral) in the presence of various carbonyl compounds, their metabolites and nutrients given at 1.0 g/kg (oral; + 5 min). Protection Index (PI) was determined as the ratio of LD50 of SNP in protected and unprotected animals. LD50 of SNP = 83.6 mg/kg (71.8–97.2). The values in parentheses are fiducial limits at 95% confidence intervals.

followed by marginal protection (PI 1.28) against PCN. OAA afforded only a marginal protection (PI 1.24) against MCN alone. Table 2 shows the protective efficacy of the remaining eleven carbonyl compounds and their metabolites, and nutrients (glyoxal, citric acid, succinic acid, fumaric acid, malic acid, maleic acid, glucose, sucrose, fructose, sorbitol and mannitol) against acute toxicity of MCN in rats. Only minimal protection was observed in case of succinic acid, malic acid, fructose (PI 1.40) and glyoxal (PI 1.28). The remaining compounds did not offer any protection. Table 3 summarizes the protective efficacy of eleven compounds against acute toxicity of SNP in rats. Here again, only minimal protection was observed in case of fructose, mannitol (PI 1.40) and succinic acid (PI 1.34).

Figure 1 depicts the protective efficacy of A-KG, DHA and OAA against MCN-induced changes in brain cyanide concentration (A) and CYTOX activity (B) in rats. Four hours post exposure, the brain cyanide concentration was significantly high in MCN treated rats compared to control group. A-KG, DHA and OAA did not show any effect on cyanide concentration. Cyanide concentration in MCN treated rats was significantly reduced by A-KG. However, the levels were still statistically elevated compared to control. Although, treatments of DHA and OAA reduced the brain cyanide concentration in MCN treated rats, the levels continued to be significantly above control. MCN was found to significantly inhibit the brain CYTOX activity 4 h post exposure, which was significantly resolved by
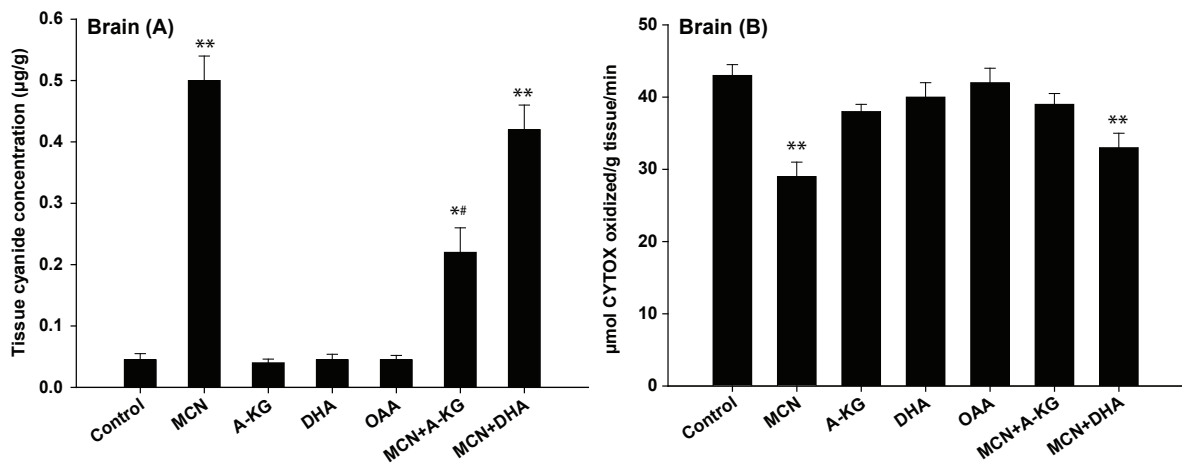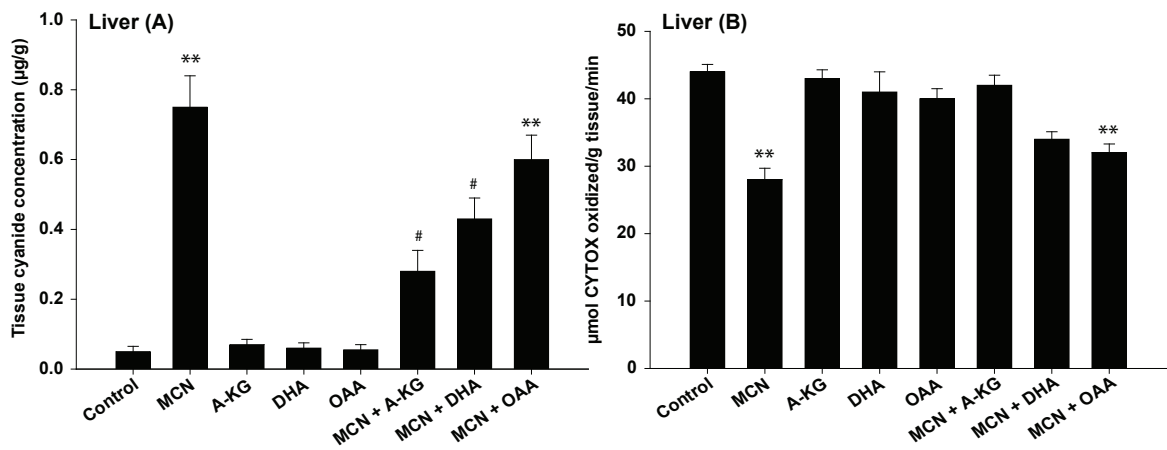
Figure 1. Protective efficacy of alpha-ketoglutarate (A-KG), dihydroxyacetone (DHA) and oxaloacetic acid (OAA; 1.0 g/kg; oral; +5 min) against malononitrile (MCN; 0.75 LD50; oral) induced changes in brain cyanide concentration **(A)** and cytochrome *c* oxidase (CYTOX) activity **(B)**, measured 4 h post exposure in rats. Values are mean±SE of 6 animals. Significance was set at *p<0.05 and **p<0.01 compared to corresponding control. # indicates that values are significantly different from MCN alone.
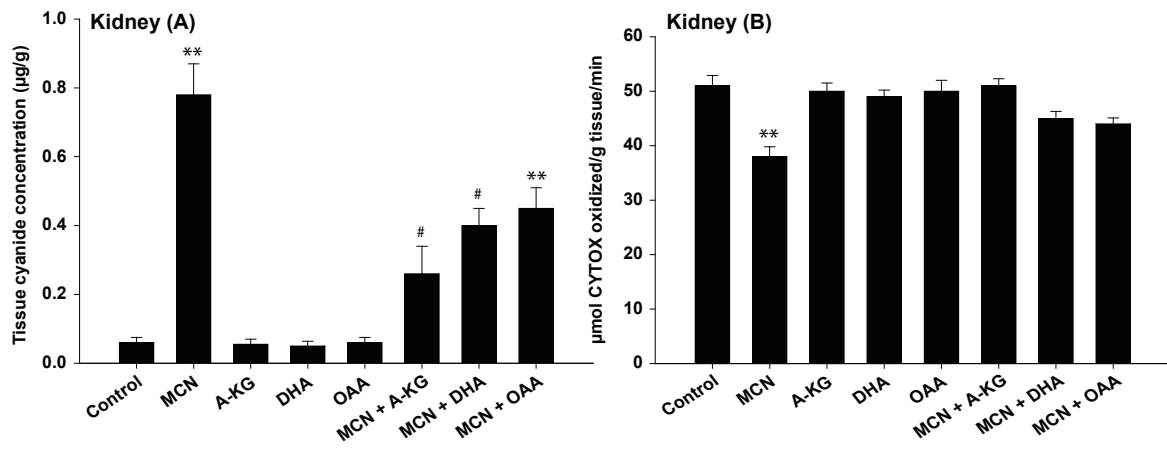

Figure 2. Protective efficacy of alpha-ketoglutarate (A-KG), dihydroxyacetone (DHA) and oxaloacetic acid (OAA; 1.0 g/kg; oral; +5 min) against malononitrile (MCN; 0.75 LD50; oral) induced changes in liver cyanide concentration **(A)** and cytochrome *c* oxidase (CYTOX) activity **(B)**, measured 4 h post exposure in rats. Values are mean±SE of 6 animals. Significance was set at *p<0.05 and **p<0.01 compared to corresponding control. # indicates that values are significantly different from MCN alone.


Figure 3. Protective efficacy of alpha-ketoglutarate (A-KG), dihydroxyacetone (DHA) and oxaloacetic acid (OAA; 1.0 g/kg; oral; +5 min) against malononitrile (MCN; 0.75 LD50; oral) induced changes in kidney cyanide concentration **(A)** and cytochrome *c* oxidase (CYTOX) activity **(B)**, measured 4 h post exposure in rats. Values are mean±SE of 6 animals. Significance was set at *p<0.05 and **p<0.01 compared to corresponding control. # indicates that values are significantly different from MCN alone.
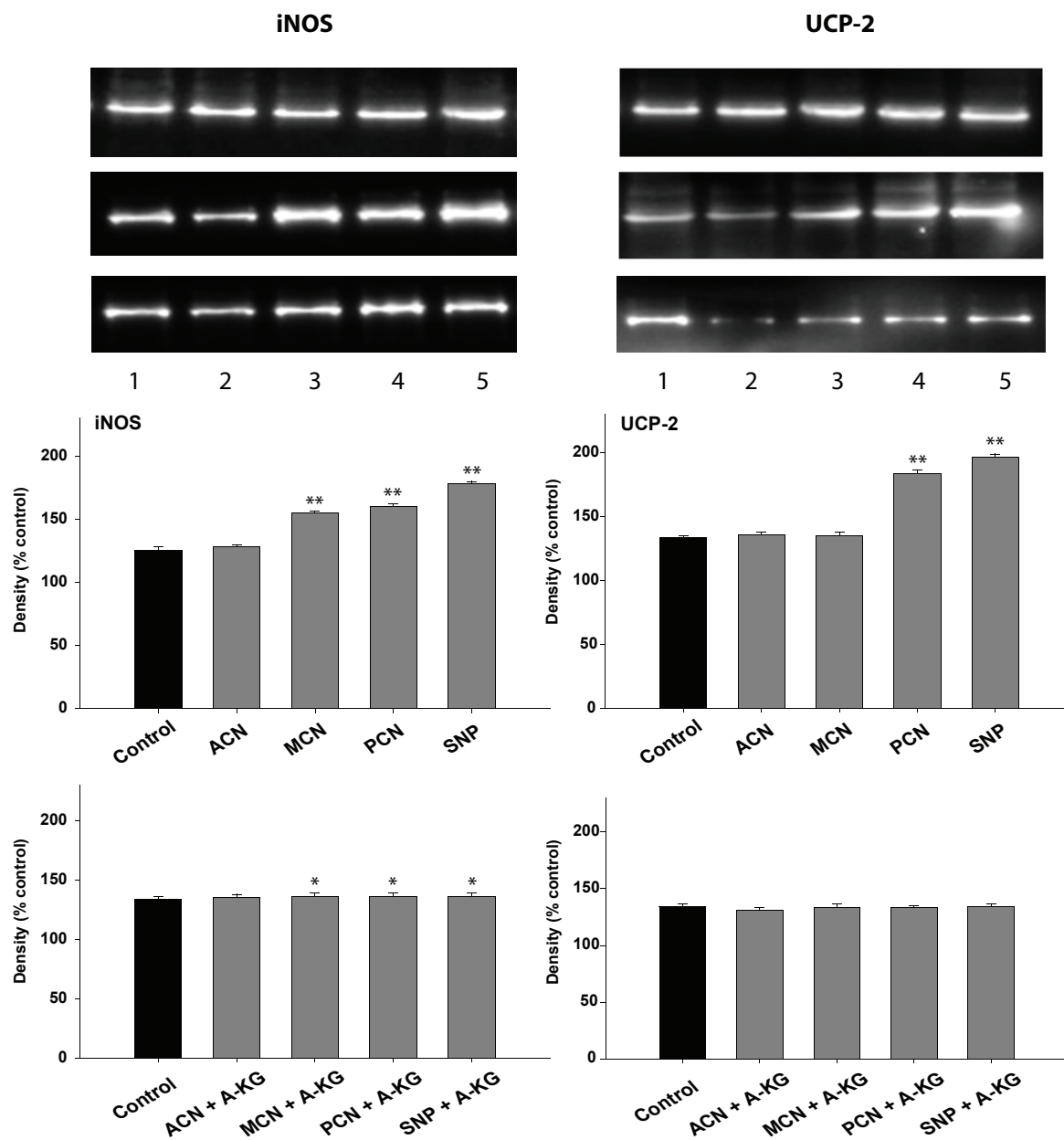
Figure 4. Western blot showing expression of brain iNOS and UCP2 in rats 7d after various treatments (oral). Vertical lanes represent: **(1)** Control, **(2)** acrylonitrile (ACN), **(3)** malononitrile (MCN), **(4)** propionitrile (PCN), and **(5)** sodium nitroprusside (SNP). All the cyanogens were given at respective 0.75 LD50. Horizontal lanes represent: **(A)** β-actin as an equal loading control, **(B)** various treatments alone, and **(C)** various treatments in the presence of alpha-ketoglutarate (A-KG; 1.0 g/kg; oral; +5 min). Figure represents blots of three experiments with similar results. The quantitative densitometric values are mean±SE of 3 experiments. Significance was set at *$p<0.05$ and **$p<0.01$ compared to corresponding control.

both A-KG and DHA treatment. However, treatment with OAA did not result in any significant protection. Figure 2 shows the protective efficacy of A-KG, DHA and OAA against MCN-induced changes in liver cyanide concentration (A) and CYTOX activity (B). The brain cyanide concentration was significantly elevated in unprotected animals compared to control group. Both A-KG and DHA significantly reduced the cyanide levels. Reduction in cyanide levels was also observed in OAA protected animals, however, it remained significantly different from control. MCN significantly inhibited liver CYTOX activity 4 h post exposure, which was significantly resolved by all

the antidotes except OAA. Protective efficacy of A-KG, DHA and OAA against MCN-induced changes in kidney cyanide concentration and CYTOX activity are shown in Figures 3A and 3B. The kidney cyanide concentration significantly increased in MCN treated animals was resolved by A-KG and DHA. However, MCN-induced CYTOX inhibition was resolved by all the antidotes.

Figure 4 shows the western blot of iNOS and UCP-2 in brain homogenate of rats, analyzed 7 d post exposure. Out of the four compounds tested (ACN, MCN, PCN and SNP), only MCN, PCN and SNP were found to up-regulate iNOS expression compared to control, which was partially

prevented by A-KG. However, up-regulation of UCP-2 by PCN and SNP treatments was completely normalized by A-KG. The Western blot analysis was also confirmed by densitometric analysis. Figure 5 represents the agarose gel electrophoresis for qualitative analysis of DNA extracted from whole brain of the rat 7 d after treatment with 0.75 LD50 of ACN, MCN, PCN and SNP. None of the treatments induced any DNA damage (necrotic or apoptotic).

Protective efficacy of A-KG against MCN-induced time-dependent changes in various physiological parameters in rats is depicted in Figure 6. Within 30 min of MCN treatment, a significant decrease in MAP, HR, RR and NMT was observed, which persisted till termination of the experiment (4 h). No significant change was observed in RT. Post treatment (+5 min) of A-KG significantly prevented all the changes caused by MCN, and the values were comparable to control. Physiological changes similar to MCN were also observed in animals treated with SNP (Figure 7). A transient decrease in RR was also observed 60 min post exposure, which resolved of its own. A-KG exhibited beneficial effects on all the physiological changes caused by SNP. Figure 8 illustrates the dose- and time-dependent effect of PCN on various physiological parameters in rats. PCN at 0.75 or 1.0 LD50 did not produce any change. However, 2.0 LD50 decreased both MAP and HR within 30 min post exposure and the decrease persisted up to 4 h. A transient decrease in RR was also observed between 3 and 4 h after the same dose of PCN. The 4.0 LD50 of PCN caused significant
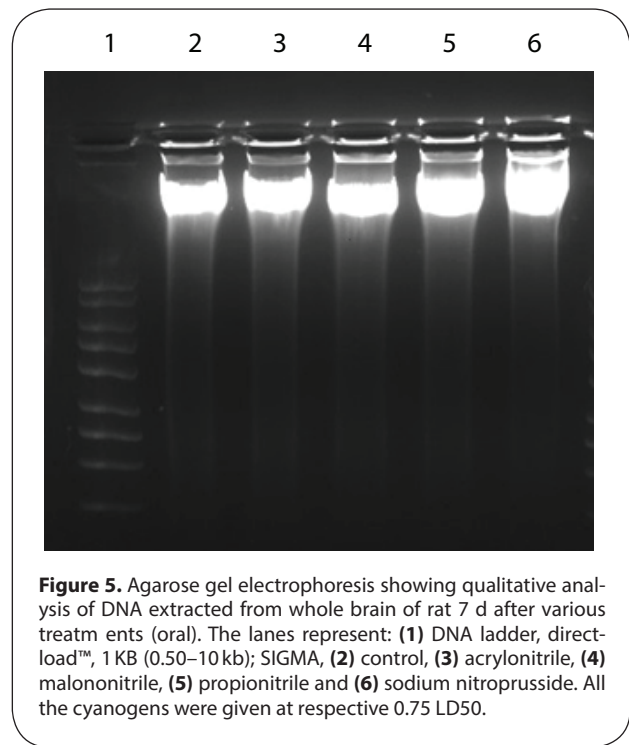

Figure 5. Agarose gel electrophoresis showing qualitative analysis of DNA extracted from whole brain of rat 7 d after various treatm ents (oral). The lanes represent: **(1)** DNA ladder, direct-load™, 1 KB (0.50–10 kb); SIGMA, **(2)** control, **(3)** acrylonitrile, **(4)** malononitrile, **(5)** propionitrile and **(6)** sodium nitroprusside. All the cyanogens were given at respective 0.75 LD50.

decrease in MAP, HR, RR and NMT between 30 min and 4 h post exposure. None of the doses of PCN produced any change in RT.
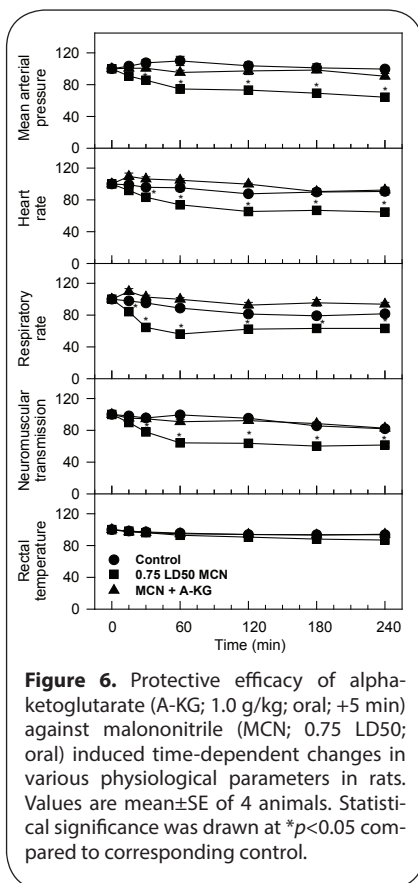

Figure 6. Protective efficacy of alpha-ketoglutarate (A-KG; 1.0 g/kg; oral; +5 min) against malononitrile (MCN; 0.75 LD50; oral) induced time-dependent changes in various physiological parameters in rats. Values are mean±SE of 4 animals. Statistical significance was drawn at *$p<0.05$ compared to corresponding control.
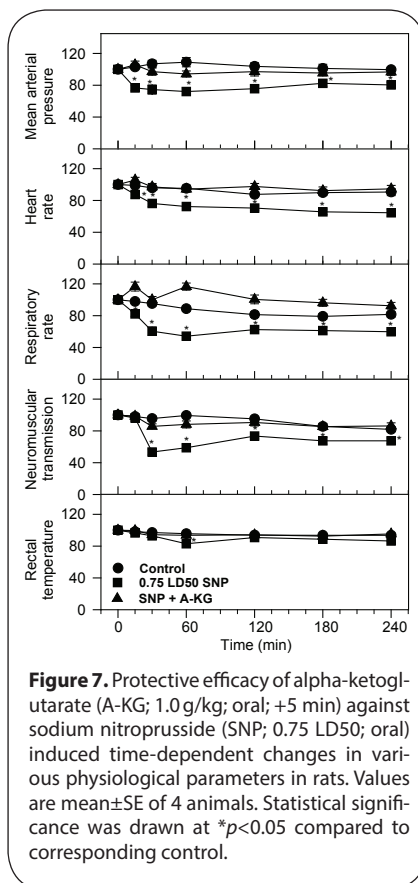

Figure 7. Protective efficacy of alpha-ketoglutarate (A-KG; 1.0 g/kg; oral; +5 min) against sodium nitroprusside (SNP; 0.75 LD50; oral) induced time-dependent changes in various physiological parameters in rats. Values are mean±SE of 4 animals. Statistical significance was drawn at *$p<0.05$ compared to corresponding control.
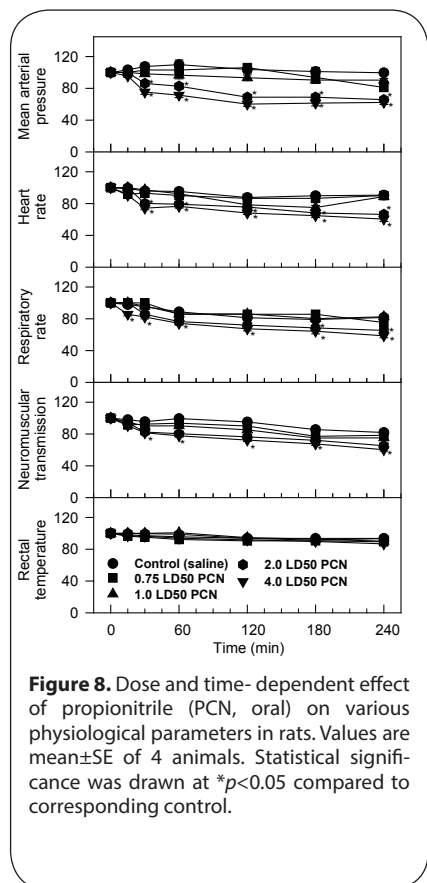

Figure 8. Dose and time- dependent effect of propionitrile (PCN, oral) on various physiological parameters in rats. Values are mean±SE of 4 animals. Statistical significance was drawn at *$p<0.05$ compared to corresponding control.

Discussion

Several cyanogenic compounds find wide industrial, domestic and therapeutic applications (Vesey & Cole, 1985; Patnaik, 2007; Borron, 2015). Many such chemicals have been implicated in cases of human poisoning, largely due to generation of toxic levels of cyanide ions (Vesey & Cole, 1985; Patnaik, 2007; Bhattacharya & Flora, 2009; Borron, 2015). Due to large variations in the time of cyanide generation and onset of toxic symptoms, suitable therapeutic measures have not evolved for the treatment of cyanogen poisoning. In most of the cases, only cyanide antidotes have been recommended (Hall *et al.*, 2007; Patnaik, 2007; Abraham *et al.*, 2015; Borron, 2015; Mirkin, 2015). One of our recent studies has demonstrated the acute oral toxicity of six different cyanogens like aliphatic nitriles (ATCN, ACN, PCN, SCN, and MCN) and inorganic compound (SNP), and their response to A-KG treatment (Bhattacharya *et al.*, 2009). The study revealed that A-KG reduced the levels of free circulating cyanide but did not show any appreciable protection against these compounds. This may have been due to variation in the therapeutic window of A-KG and the time of cyanogenesis by the compounds (Bhattacharya *et al.*, 2009). Our subsequent study revealed that the time of maximum blood cyanide concentration was 30 min, 1 h and 8 h for MCN, SNP and PCN, respectively, which almost coincided with their corresponding time for maximum CYTOX inhibition (Rao *et al.*, 2013). We determined the protective efficacy of various compounds considering the LD50 values reported earlier (Rao *et al.*, 2013). A variety of carbonyl compounds and their metabolites, as well as nutrients have been found to antagonize experimental cyanide poisoning *in vitro* and *in vivo*. Although, no unanimous conclusion could be drawn due to the large variations in the experimental protocols (Schwartz *et al.*, 1979; Delhumeau *et al.*, 1994; Niknahad *et al.*, 1994; Niknahad *et al.*, 1995; Niknahad & O'Brien, 1996), our subsequent study ascertained the protective efficacy of such compounds (particularly A-KG) in antagonizing cyanide poisoning (Bhattacharya & Tulsawani, 2008). All these compounds were now tested for their antidotal efficacy against different cyanogens. We established the effect of selected cyanogens on cyanide concentration, CYTOX activity (brain, liver and kidney), iNOS and UCP-2 expression, DNA fragmentation (brain), and various physiological indices. Cyanide-induced oxidative stress-mediated up-regulation of iNOS is known to occur due to depletion of mitochondrial glutathione (Prabhakaran, 2006). Implication of cyanide in up-regulation of UCP-2 (an inner mitochondrial membrane protein) is also known to mediate mitochondrial dysfunction (Zhang *et al.*, 2007). Previously, we have reported cyanide-induced DNA damage *in vitro* (Bhattacharya & Lakshmana Rao, 1997). We also reported on cyanide-induced various physiological changes and their attenuation by A-KG (Tulsawani *et al.*, 2007). In the present study all the above parameters were included in delineating the protective efficacy of various compounds against selected cyanogens.

The present study revealed that out of 14 compounds tested, A-KG afforded maximum protection, followed by marginal protection by DHA and OAA, particularly against MCN, PCN and SNP. These observations were comparable to previous studies with cyanide (Niknahad *et al.*, 1994; Niknahad *et al.*, 1995; Niknahad & O'Brien, 1996; Bhattacharya & Tulsawani, 2008). Many of these compounds participate in the citric acid cycle and as such are considered to be safe for human use (Bhattacharya & Tulsawani, 2008). The carbohydrate and amino acid nutrients were not found to be very effective in challenging the toxicity of cyanogens. However, alpha-keto acids like A-KG and glycolytic substrates like DHA exhibited moderate protection. The protection is usually facilitated by the carbonyl compound present in these molecules, which neutralizes the free cyanide ion by forming a cyanohydrins complex (Delhumeau *et al.*, 1994; Bhattacharya & Tulsawani, 2008).

Metabolism of cyanogenic compounds, particularly aliphatic nitriles, usually occurs via conjugation with reduced glutathione (GSH) or epoxidation by cytochrome *P*450 (CYPs), leading to the formation of epoxide intermediates. Subsequently, epoxide intermediates are metabolized by epoxide hydrolase to generate cyanide ions. Kinetics of this reaction would determine the appearance of cyanide ions in the blood and its toxicity (Chanas *et al.*, 2003). Kinetics of each antidote should coincide with the kinetics of blood cyanide, so that maximum protection could be achieved. This would help in designing the optimal prophylactic or therapeutic window for the antidote. The time of maximum cyanide generation by MCN and SNP falls within 1 h, and for the remaining compounds it is between 8 h and 72 h (Rao *et al.*, 2013). Although, there is a large variation among kinetics of cyanide generated by different cyanogens, we adhered to only +5 min as the treatment time for all the cyanogens tested. It is difficult to schedule the treatment time for different antidotes. The distribution half-life of A-KG is about 0.35 h (Bhattacharya, 2015), and possibly for this reason it has afforded maximum protection. The remaining compounds were notably ineffective. The choice of cyanide antidotes in the particular case of nitrile poisoning has not been clearly established. However, there is general consensus on the efficacy of STS (Borron, 2015). Arguably, addition of STS would further augment the efficacy of A-KG.

In the present study, out of 14 compounds, we first evaluated only those (A-KG, DHA and OAA) which were earlier found to be effective against cyanide (Bhattacharya & Tulsawani, 2008). Thereafter, we evaluated the remaining 11 compounds against only two cyanogens (MCN and SNP). Against these appreciable protection was afforded by A-KG DHA and OAA. However, MCN alone was included in the study on tissue cyanide concentration and CYTOX activity, where maximum protection was observed with A-KG. In the present study on iNOS and UCP-2 expression, ACN was also included in addition to MCN, PCN and SNP. These four compounds were found to be most toxic out of various cyanogens tested

(Bhattacharya *et al.*, 2009; Rao *et al.*, 2013). The study on physiological parameters revealed the protective efficacy of A-KG against MCN and SNP. However, PCN up to 1.0 LD50 did not show any toxicity within 4 h. This could be due to delayed toxicity of PCN (Rao *et al.*, 2013). As compared to MCN and SNP no toxicity was observed at 0.75 LD50 of PCN and therefore the protective efficacy of A-KG against it was omitted. Toxicity of PCN could be elicited within 4 h only when the dose was increased to 2.0 LD50 and above. In our previous study, A-KG was found to offer a PI of 7.6 against MCN compared to 2.7 and 3.6 against PCN and SNP, respectively (Rao *et al.*, 2013). However, in this study, A-KG was administered at 2.0 g/kg dose, 10 min after SNP and 2 h after MCN and PCN administration. In the present study, the time and dose of all the antidotes were fixed at +5 min and 1.0 g/kg, respectively.

Human interaction with various cyanogens cannot be overlooked due to their extensive industrial, medical and domestic use. However, we need to resolve their toxicity by adapting various treatment strategies. A-KG appears to be a promising antidote for the management of cyanogen poisoning, and incorporation of STS in the treatment regimen is likely to augment the protection.

Acknowledgement

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