

CATARACTOGENIC POTENTIAL OF CYANIDE-INDUCED OXIDATIVE STRESS IN RABBITS.

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ABSTRACT.

The relationship between chronic cyanide toxicity, oxidative stress and cataractogenesis was investigated in two groups of 4-month-old New Zealand White rabbits fed for 7 weeks on growers mash with or without 400ppm inorganic cyanide, after prior acclimatisation to chicken mash and laboratory conditions. Ophthalmoscopic examination of the rabbit ocular tissues was conducted prior to sacrifice, while the lens and retina tissues were assessed for antioxidant status with respect to superoxide dismutase, catalase, β -carotene, ascorbic acid and α -tocopherol. Results obtained show significant decreases in superoxide dismutase and catalase activities, as well as the antioxidant vitamins, in the lens and retina of the cyanide-treated rabbits relative to controls [$p < 0.05$]. The cyanide treatment also led to degenerative morphological changes viz pale fundus, weak retinal reflex and mild-to-pronounced lenticular opacification. These results suggest that cyanide imposes oxidative stress on ocular tissues, and thus may be a risk factor for development of cataracts.

Key words: Cyanide toxicity; oxidative stress; cataracts.

Running title: Cataractogenesis due to cyanide toxicity.

INTRODUCTION

Cyanide is a well-known respiratory poison in all aerobic organisms. Its toxicity arises from inhibition of cytochrome oxidase, the enzyme that catalyses terminal electron transfer to molecular oxygen in the respiratory chain. Thus cyanide blocks electron transfer, mitochondrial oxygen utilisation and cellular respiration [Jones *et al*, 1984; Borron and Baud, 1996]. In acute doses, death invariably results as a consequence of respiratory failure due to the high susceptibility of the nerve cells of the respiratory centre to hypoxia [Greer and Jo, 1995]. Chronic exposure to cyanide has been associated with pathogenesis of goitre [Cliff *et al*, 1986], tropical ataxic neuropathy [Osuntokun, 1981] and spastic paraparesis [Howlett *et al*, 1990]. Sources of exposure to cyanide are diverse, but are generally classified as either dietary or environmental or both [Adewusi and Akindahunsi, 1994]. Toxic cyanogenic glycosides are present in cassava [Nartey, 1968], legumes and cereal grains [Montgomery, 1969; Okolie and Ugochukwu, 1989]. Cassava constitutes a source of dietary calories for over 500 million people in the tropics [Padmaja, 1998]. One major source of environmental exposure to cyanide is smoke inhalation. This may be in the form of tobacco smoke or smoke from incinerators [Borron

and Baud, 1996; ATSDR, 1989]. It has for long been suggested that the dimness of vision in tobacco smokers (tobacco amblyopia) may be due to the toxic effect of cyanide in tobacco smoke [Wilson *et al*, 1966]. However, to our knowledge,

no attempts have been made to unravel the biochemical mechanism(s) by which cyanide interferes with visual acuity. Studies on other tissues have demonstrated the inhibitory effect of cyanide on $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ [Okolie *et al*, 1994] as well as superoxide dismutase, SOD and catalase [Izokun-Etiobhio, 1989]. $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ is vital for maintenance of lens transparency, while SOD and catalase combat oxidative stress by protecting biological membranes from the damaging effects of the highly reactive superoxide anion O_2^- [Halliwell and Gutteridge, 1985]. Oxidative stress is a high risk factor in pathogenesis of cataracts [Tessier *et al*, 1988; Lyle *et al*, 1999; Palisso *et al*, 1998].

The present study was carried out to investigate the relationship between cyanide intoxication, oxidative stress and cataract formation in rabbits. This is with a view to elucidating the biochemical mechanism by which cyanide interferes with vision.

MATERIALS AND METHODS

Animals and feeding

12 New Zealand White rabbits of both

sexes, aged about 4 months were housed singly in clean metal hutches and acclimatised to growers mash (Bendel Feed & Flour Mills BFFM Ltd., Ewu, Nigeria) for 4 weeks prior to the commencement of the experiment. One group of 6 rabbits was fed growers mash containing 400ppm inorganic cyanide (sodium cyanide, NaCN) for 7 weeks, while a control group (6 rabbits) received growers mash only. Prior to feeding the feed was mixed with water (10:1 w/v) to achieve a texture acceptable to the experimental animals, and to facilitate even distribution of cyanide in the feed to which NaCN was added. Each rabbit was presented with 100g of feed daily, along with clean drinking water. Stale feed remnants and spills were pooled daily and weighed before discarding. The feeding experiment was carried out under standard laboratory conditions at room temperature (28°C) and 12-hour daylight cycle. At the end of 7 weeks, ophthalmoscopic examination of the lens and retinas of rabbits in both groups were carried out. Subsequently the rabbits were weighed, anaesthetised with chloroform and sacrificed by swift decapitation. The lens and retina tissues were carefully removed and immediately analysed for SOD, catalase, β -carotene, α -tocopherol and vitamin C.

Enzyme assays

Preparation of tissue homogenates

0.10g of lens or 0.20ml of retina scrapings was thoroughly crushed with acid-washed sand and 5ml of cold physiological saline in a pre-chilled hand mortar for 10 minutes. The homogenate was allowed to stand for 24 hours at 4°C, and the supernatant fraction was used for assay of SOD and catalase. SOD was assayed indirectly, based on the inhibitory effect of the enzyme on the autooxidation of epinephrine [Misra & Fridovich, 1989]. The assay was carried out in a 3ml – disposable cuvette to which 2.20ml of 0.15mM carbonate buffer pH 10 containing 0.10mM EDTA; and 0.20ml of tissue extract were pipetted. An epinephrine reference cuvette was simultaneously

set up, with 0.20ml of the buffer in place of tissue extract. Both cuvettes were allowed to air – equilibrate at 28°C in a pye-Unicam UV spectrophotometer for 2 minutes so as to establish a stable baseline. Reaction was started (with one cuvette at a time) by addition of 0.30ml of freshly – prepared 0.30mM epinephrine. After mixing, increase in absorbance at 490nm was monitored for 1 minute against a blank containing only 2.70ml of 0.15mM carbonate buffer, pH 10. One unit of SOD activity was calculated in terms of amount of enzyme that causes 50% inhibition of epinephrine autooxidation under the assay conditions.

Catalase was assayed according to the method of Cohen et al, [1970], by calculating the first order rate constant of the decomposition of hydrogen peroxide, H_2O_2 by tissue extract. The assay tube contained 2ml of 0.05M phosphate buffer, pH 7 and 0.50ml of tissue extract, while a reference tube had 2.5ml of buffer instead. Both tubes were maintained at 0°C in an ice-bath. 3ml of 30mM H_2O_2 was added sequentially, and at fixed time intervals, to both tubes, and their contents were quickly mixed by inversion. Reaction was allowed to proceed for 1 minute and stopped by sequential addition of 1ml of 6M H_2SO_4 . Taking one tube at a time, 7.0ml of 0.01M potassium permanganate was added to the test and reference assays, and after mixing by inversion, absorbance at 480nm was read against a blank of distilled water. Catalase activity was calculated in terms of the first order rate constant for decomposition of H_2O_2 .

Estimation of antioxidant vitamins

Ascorbic acid

Vitamin C was assayed by the method of Roe & Cuether [1943]. 12 ml of 6% trichloroacetic acid, TCA was mixed with 3ml of tissue extract for 1 minute to produce a fine white suspension. This was centrifuged and the supernatant fraction was decanted into a clean test tube. Activated charcoal and acid-washed sand were added, with thorough mixing. The mixture was then filtered. This

Table 1. Feed intake, weight gains and urinary thiocyanate levels for rabbits fed KCN – diets and controls*

Feed Consumption	Test	Control
(g/rabbit/day)	88.45 \pm 4.3 ^a	76.57 \pm 6.1 ^b
Weight gain (g/rabbit)	592 \pm 15 ^a	507 \pm 11 ^a
Urinary SCN (μ mol/rabbit/day)	37 \pm 1.7 ^a	6 \pm 0.8 ^b

*Values are Mean \pm S.E.M. (n=6). Those that do not share common superscripts across differ significantly ($p < 0.05$).

Table 2. Catalase and superoxide dismutase activities in lens and retinas of cyanide treated rabbits and controls*

	Catalase (k min^{-1})		Superoxide dismutase (U/g)	
	Test	Control	Test	Control
Lens	2.47 ± 0.14^a	3.27 ± 0.39^b	234 ± 9^a	178 ± 11^b
Retina	3.35 ± 0.62^a	5.03 ± 1.1^b	87 ± 7^a	112 ± 10^b

*Values are Mean \pm S.E.M. (n=6). Those bearing different superscripts across differ significantly ($p < 0.05$). Unit of SOD in retina is U/ml of tissue.

procedure was repeated using 3 ml of standard ascorbic acid (1mg/ml). 4ml of the filtrate from both standard and test treatments were dispensed into separate test tubes, while a blank test tube contained 4ml of distilled water. One drop of thiourea and 1ml of 2% dinitrophenylhydrazine in concentrated H_2SO_4 were added to each tube, and mixed. The tubes were incubated at 37°C for 3 hours and transferred to an ice-bath. 5ml of 83% H_2SO_4 was then added to each tube. After a further incubation period of 30 minutes absorbance at 540nm was read against the blank in a Coleman Junior Colorimeter. Ascorbic acid levels were calculated with reference to standard values.

β -Carotene

The procedure of Jakutowicz et al, [1977] was used. Proteins were first precipitated with absolute ethanol, followed by extraction of tocopherol and carotenes using heptane. 3ml of absolute ethanol and 5ml of heptane were added to 3ml of tissue extract in a test tube and the tube was shaken vigorously for 5 minutes. On standing, 3ml from the heptane layer was taken up in a cuvette and read at 450nm against a blank of heptane. β -carotene levels were extrapolated from a β -carotene standard curve.

α -Tocopherol

The procedure used was identical with that described for β -carotene, except that the α -tocopherol in the heptane layer was treated with 4, 7- diphenyl 1,10 - phenanthralin to give a coloured complex which was read at 534nm. α -Tocopherol levels were extrapolated from a tocopherol standard curve.

Thiocyanate

Thiocyanate was estimated colorimetrically with ferric chloride according to the method of Bowler [1944].

Statistics

Mean \pm SEM of values obtained for tests and controls were analysed for statistically significant differences using students' t-test.

RESULTS

Table 1 shows mean feed intake, weight gains and urinary excretion for rabbits in both groups. The cyanide-treated rabbits had significantly higher feed intake and urinary thiocyanate than controls ($p < 0.05$) although weight gains for both group were comparable.

Table 2 shows catalase and superoxide dismutase activities in the lens and retinas of rabbits from both groups. These enzymes were significantly reduced in both tissues of the cyanide - fed rabbits compared to controls ($p < 0.05$).

Table 3 depicts the levels of the antioxidant vitamins in the retina and lens of the cyanide-treated rabbits and controls. Significant decreases ($p < 0.05$) were obtained in the levels of β -carotene, α -tocopherol and vitamin C in the lens and retina of the cyanide-toxified rabbits compared to controls.

Table 4 depicts results obtained in ophthalmoscopic examination of ocular tissues from rabbits in both groups. The cyanide-treated group manifested morphological degenerative changes including pale fundus, weak retinal reflexes and mild-to-pronounced lenticular opacification.

DISCUSSION

Cataracts are the end product of a degenerative ocular disease resulting in opacification of the lens. Evidence abounds suggesting a direct causal relationship between oxidative stress and cataractogenesis [Taylor, 1993; Taylor & Norwell, 1997; Meister, 1992], but so far none has been linked to cyanide intoxication. Oxidative stress may be precipitated by oxidising

Table 3. Antioxidant vitamin status in retina and lens of the cyanide-fed rabbits and controls*

	β -carotene (mg/g)		α -tocopherol (mg/g)		Vitamin C(mg/g)	
	Test	Control	Test	Control	Test	Control
Lens	30.98 \pm 1.49 ^a	49.45 \pm 1.86 ^b	21.13 \pm 2.06 ^a	50.1 \pm 2.5 ^b	60.05 \pm 1.85 ^a	83.8 \pm 1.2 ^b
Retina	0.65 \pm 0.04 ^a	1.01 \pm 0.3 ^b	0.48 \pm 0.2 ^a	0.99 \pm 0.04 ^b	1.86 \pm 0.4 ^a	1.30 \pm 0.03 ^b

*Values are Mean \pm S.E.M. (n=6). For each parameter and tissue, values that do not share same superscripts across differ significantly (p<0.05). Vitamin levels in retina are expressed in mg/ml of tissue.

Table 4. Morphological changes in lens and retinas of the cyanide-fed group and controls

	Cyanide-fed group (test)	Controls
Lens	Lenticular opacification (mild-to-pronounced)	Normal morphology
Retina	Weak retina reflex	High (normal) retinal reflex

agents such as the oxygen species superoxide anion, hydrogen peroxide and hydroxyl radical [Bhuyan & Bhuyan, 1984]; all of which are produced within the eye as a result of photo-oxidation and ionising irradiation [Reddy, 1990]. The lens contains a variety of antioxidants and antioxidant enzymes that convert these harmful reactive oxygen species to less damaging forms, thus preventing oxidative damage to lens proteins. These include superoxide dismutase, catalase, glutathione, β -carotene, α -tocopherol and ascorbic acid [Tessier et al, 1988; Lyle et al, 1999; Bunce & Hess, 1988]. In the present study, the cyanide-induced significant decreases in SOD, catalase and the antioxidant vitamins in the lens and retina would obviously have compromised the ability of these tissues to combat oxidative stress, thereby leading to cataract formation. This is consistent with the observed morphological degenerative changes in the lens and retina of the cyanide-toxified rabbits, especially lenticular opacification. Decreases in SOD and catalase activities are well-known cataractogenic factors [Jacques et al, 1988]. The cyanide-induced decrease in the levels of antioxidant vitamins may be attributed to the damaging oxidative effect of free radicals. Based on the observed oxidative stress arising from cyanide exposure, a relationship between cyanide, free radicals and antioxidant vitamins seems very

plausible. Tobacco smoke contains free radicals [Wang et al, 1999]; and it has been shown that the effect of oxidative stress induced by smoking can be mitigated by antioxidant vitamin supplementation [Howard et al, 1998]. In addition Wang et al [1999] have shown that free radicals reduce β -carotene levels through enhancement of its oxidation. Thus it can be reasonably argued that cyanide, by inhibiting SOD and catalase, increases the levels of free radicals in the lens and retina, and hence the rate of inactivation of β -carotene. This may be responsible for the observed significant decrease in β -carotene levels of lens and retina of the cyanide-treated rabbits relative to controls.

Dehydroascorbic acid, the toxic oxidation product of ascorbic acid, has been implicated in the pathogenesis of senile cataract [Ortwerth et al, 1988]. Reduced glutathione; GSH plays a protective role in the reduction of dehydroascorbic acid to ascorbic acid. Although GSH levels were not measured in the present study, it has been demonstrated in a previous study that cyanide inhibits glucose-6-phosphate dehydrogenase [Okolie & Osagie, 1998], the key enzyme of the Hexose Monophosphate Shunt that generates NADPH needed to maintain glutathione in the reduced state. Thus GSH levels should be expected to decrease in cyanide intoxication,

leading to a block in the pathway for reduction of dehydroascorbate and shortfalls in ocular content of vitamin C. Moreover it is known that vitamin C can re-generate the active form of vitamin E after the latter has reacted with a free radical, and both vitamins appear to function synergistically [Jeng et al, 1996]. Thus a decrease in the level of active vitamin C by cyanide – induced oxidative stress would elicit concomitant decrease in the levels of vitamin E.

Elevation of serum and urinary thiocyanate is an accepted index for assessing cyanide exposure. The significant increase in urinary thiocyanate SCN of the cyanide-fed rabbits over controls is a clear manifestation that the animals were exposed to cyanide insult. This is further evident from the fact that the cyanide-fed rabbits had a significantly higher feed intake than controls. This elevated feed intake is an adaptation to shortfalls in ATP availability due to the inhibition of aerobic metabolism by cyanide. Similar results have been reported elsewhere [Okolie et al, 1994; Okolie & Osagie, 2000].

In conclusion, the results obtained in this study have shown clearly that cyanide intoxication and cataractogenesis are linked by oxidative stress arising from depression of ocular antioxidant system by cyanide. Obviously it would not be practicable to repeat this experiment with human subjects. However if animal extrapolation to man is allowed, we have demonstrated a biochemical basis for cyanide-induced ocular degeneration. This finding is considered vital, in view of the fact that millions of people in the tropics are constantly exposed both to dietary and environmental cyanide such as may arise from cassava processing, cassava diets, tobacco smoke, vehicle exhaust fumes, and gas flaring during crude oil explorations.

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