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MODULATORY ROLE OF ANTIOXIDANT VITAMINS (A AND E) IN CYANIDE INDUCED LIVER AND BRAIN DAMAGE IN ADULT MALE ALBINO MICE

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ABSTRACT

Cyanide is a potent neurotoxic substance that can initiate series of intracellular reactions leading to oxidative stress. To evaluate the effect of sub lethal administration of potassium cyanide (KCN)on lipid peroxidation and some antioxidant enzymes in adult malealbino mice and possible ameliorative role of vitamins A and E. Thirty five adult male mice weighing between 18-22 g were used. An acute toxicity study was carried out to determine LD_{50} using Locke method. The animals were randomly divided into five groups (n = 7) as follows; group I (control, received deionized water), group II (1.5 mg/kg KCN), group III (1.5mg/kg KCN + 25 mg/kg vitamin A), group IV (1.5 mg/kg KCN + 50 mg/kg vitamin E) and group V (1.5 mg/kg KCN + 25 mg/kg vitamin A + 50 mg/kg vitamin E). Treatment groups was carried out daily through oral gavage for administration of potassium cyanide while vitamins A and E were administered intraperitoneal (IP) for 28 days and on the last day, the animals were sacrificed and isolation of tissues for biochemical assays of malondialdehyde, superoxide dismutase, catalase, acetyl cholinesterase and serum liver enzymes. From acute toxicity studies, LD_{50} was calculated to be 15 mg/kg. Number of deaths was observed along with asphyxiation, diarrhoea and convulsion. Vitaminspre-treatedgroups ameliorated some of the toxic signs observed.The results obtained indicated significant ($p < 0.05$) increase in MDA levels, indicating lipid peroxidation in the cyanide group (3.30±0.19 nMol/mg) compared to the vitamin treated group V (1.82±0.21 nMol/mg). There was a significant ($p < 0.05$) decrease in inhibition of superoxide dismutase in group II (1.30±0.07 µ/mg) as compared to control (2.43±0.32 µ/mg). Furthermore, a decrease in catalaseinhibition was recorded in group II (30.81±1.43 mmol/min/ µ/mg) in comparison to group III (41.60±1.96 mmol/min/ µ/mg).Acetylcholinesterase enzyme activity was significantly increased in group II (32.10±0.90 nmol/min/mg) as compared to the vitamin treated group V (16.20±0.90 nmol/min/mg). Furthermore, liver enzymes AST recorded high levels in the potassium cyanide treated group (155.20±6.44 U/L) when compared to control (45.80±1.77U/L) a decreased was observed in ALT for group V (20.20±1.77 U/L)in contrast to group II (58.80±3.57 U/L). There was an increase in ALP level in vitamin treated group V (42.00±3.74 U/L) as compared to saline group (27.00±2.55 U/L) that was not exposed to potassium cyanide. Antioxidant vitamins (A and E) played an important role in ameliorating the oxidative stress poised by cyanide through stimulating the antioxidant defence system.

INTRODUCTION

Cyanide is a rapidly acting, potentially deadly chemical that can exist in various forms (Gracie and Shepherd, 2004). It is a chemical substance that exists freely in nature either as free cyanide (CN-), hydrocyanic acid (HCN) or cyanogen chloride (CNCl), which can be found in gaseous or aqueous state (Abdel *et al.,* 2014), or a crystal form such as sodium cyanide (NaCN) or potassium cyanide (KCN). It is sometimes described as having a "bitter almond" smell, but it does not always give off an odor (Ayuba, 2014).Cyanide is toxic to humans and animals and exposure can occur in various ways. Many substances are potential sources of cyanide exposure, including edible and non-edible plants (e.g., cassava), industrial operations (e.g. plastics

processing), fires, and cigarette smoke (Ogundele and Olubolaji, 2012). Although the primary natural source of cyanide poisoning is from plants, other natural sources include volcanoes, bacteria, and fungi (Barone *et al*., 2003).Its toxicity is well known but it is still used in surgical dressing, metal-plating, mining, chemical and agricultural industries (Al-Ghanim and Mahboob, 2012).

The toxic effects of potassium cyanide have traditionally been attributed to inhibition of cytochrome C oxidase, the terminal enzyme of respiratory chain, which compromises oxidative phosphorylation leading to cytotoxic hypoxia (Baskin *et al*., 2010). However, numerous other biochemical effects of cyanide not directly related to the inhibition of the respiratory chain have been reported in the recent past (Alvandi and Hosetti, 2014).Neurological disturbances have been reported from parts of Africa in protein-deficient populations attributed to cyanide (CN^-) exposure from prolonged dietary use of cassava (Osuntokun, 1981; Mathangi and Namasivayam, 2000). Furthermore, the involvement of apoptosis in cyanide induced neural death has been identified (Caxton *et al*., 2012) while elevation in blood amino-transferases and alkaline phosphatases has been reported (Okafor *et al*., 2002).

Antioxidant is a molecule that inhibits the oxidation of other molecules (Mates *et al*., 1999). They are man-made or natural supplements that prevent or delay cell damage in the body (Behr *et al*., 2011). Antioxidants terminate these chain reactions by removing radical intermediates and inhibiting other oxidation reactions by being oxidized themselves (Moreira *et al*., 2008). The antioxidant defence systems function through,

- Blocking initial production and scavenging the oxidants,
- Converting the oxidants to less toxic compounds,
- Blocking the secondary production of toxic metabolites or inflammatory mediators, and
- Finally, blocks the chain propagation of the secondary antioxidant defence system of the target (Gonzalez *et al*., 2001).

Vitamin A (retinol) and its congeners (the retinoids) participates in panoply of biological events, such as cell differentiation, proliferation, survival, and death, necessary to maintain tissue homeostasis (Behr *et al*., 2011). Furthermore, such molecules may be applied as therapeutic agents in the case of some diseases, including dermatological disturbances, immunodeficiency, and cancer (mainly leukemia).Vitamin E compounds (tocopherols and tocotrienols) are well recognized for their effective inhibition of lipid peroxidation in foods and living cells (Allard *et al.,* 2009). Antioxidative activity of the tocopherols is related to scavenging the free radicals of unsaturated lipids.

Cyanide-induced cellular oxidative stress, i.e. increase of superoxide anions, lipid peroxide, hydroxide radicals, hydrogen peroxide and others, appears to arise through multiple pathways (Alvandi and Hosetti, 2014). As oxidative damage is mediated by free radicals, it was necessary to investigate the status of endogenous antioxidant enzymes. Due to high reactivity of reactive oxygen species (ROS), most components of the cellular structure and function may become potential targets of oxidative damage (Aigbiremolen *et al.,* 2011). The present study was aimed at evaluating the sub lethal effect of cyanide and role of vitamins A and E on the activity of some biochemical parameters, antioxidant enzymes, acetylcholinesterase and serum liver enzymes in adult male albino mice.

Materials and Methods

This present research was carried out in the Department of Human Physiology Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University Zaria, Nigeria.

Animals handling

Thirty five male albino mice (weighing 18-22 g on the average) bred in animal house of Department of Human Physiology, Ahmadu Bello University Zaria, Nigeria was used. All animals were kept at room temperature (27-

30°C) and had free access to drinking water and their diets ad libitum.

Treatment of Animals

The animals were randomly grouped into five groups (n = 7). The animals were exposed to sub lethal concentration of KCN 1.5 mg/kg (10% of LD_{50}; which was calculated to be 15 mg/kg), vitamin A 25 mg/kg (Behr *et al.,* 2004) and vitamin E 50 mg/kg (Aliyu *et al.,* 2012). The groupings were as follows; Group I control (received deionized water only), group II (1.5 mg/kg KCN only), group III (1.5 mg/kg KCN + 25 mg/kg vitamin A), group IV (1.5 mg/kg KCN + 50 mg/kg vitamin E), and group V (1.5 mg/kg KCN + 25 mg/kg vitamin A + 50 mg/kg vitamin E). Potassium cyanide was administered orally while vitamins were administered via intra-peritoneal (IP) once daily for 28 days. The animals were observed for clinical signs and possible deaths throughout the study period. Ethical clearance was sought from animal Research Ethic Committee of the Ahmadu Bello University, Zaria for conducting the experiment.

Drugs and Chemicals

Ellman's Reagent [5^1, 5^1 -Dithios-nitrobenzoic acid (DTNB)], acetylthiocholine, thiobarbituric acid (TBA), Potassium cyanide were obtained from Sigma Chemicals Co., (USA). Vitamin A (1000IU) and E (100 mg DL alpha tocopherol) were obtained from Patterson Zoochonis Ltd, Nigeria, and were reconstituted in soy oil to 1% solution and 20 mg/ml respectively.

Acute toxicity test

Median lethal dose (Ld_{50}) of potassium cyanide (KCN)

The LD_{50} of potassium cyanide was determining using the method as described by Lorke (1983) in mice. This test was carried out in two phases; in the first phase, three are three groups of three animals, the animals were treated with potassium cyanide at the doses of 10, 100 and 1000 mg/kg body weight and observed for signs of toxicity (e.g. salivation, convulsion, asphyxia, piloerection, soft faecal bolus etc.) and death within 48 hours. In the second phase; three (3) groups with each containing one mouse were administered with three more specific doses of KCN based on the results of the first phase (2, 4 and 9 mg/kg respectively). The LD_{50} value was determined by calculating the geometric mean of the lowest dose that cause death and the highest dose at which all the animals survived.

Preparation of blood serum and brain tissues for biochemical studies

Following the completion of treatment, animals were deeply anaesthetized by ether and blood was collected through the retro orbital vein into plain tubes. The blood was centrifuged in a bench top centrifuge at 3000 rpm for 15 min to obtain the serum. The brain was removed, wiped clean and kept in cold container. The whole brain was homogenized in sodium phosphate buffer (0.1 M, pH 7.4), centrifuged for 10 min at 10,000 rpm at 4 °C, and the supernatants were separated into aliquots. The brain supernatants was used for determination of brain acetylcholinesterase (AChE), and oxidative stress biomarkers namely, superoxide dismutase, catalase, acetylcholinesterase and malondialdehyde (MDA).

Oxidative stress and antioxidant enzymes

Biochemical Estimations

Lipid peroxidation: Malondialdehyde (MDA)

The level of thiobarbituric acid reactive substance, malondialdehyde (MDA), as an index of lipid peroxidation was evaluated in brain samples .The principle of the method was based on spectrophotometric measurement of the colour developed during the reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA). The MDA concentration in each sample was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5/cm/M and expressed as nMol/mg of tissue protein. The concentration of protein in the brain homogenates was evaluated using the Lowry method (Lowry *et al.,* 1951).

Determination of brain superoxide dismutase and catalase enzyme activity

The assessment of superoxide dismutase and catalase activities was used to determine antioxidant enzymes activity using the method described by Freitas *et al*., (2005). The 10% (w/v) homogenate obtained was centrifuged at 800g for 20 min and the supernatant was used to

assay for superoxide dismutase and catalase activities.

Determination of brain acetylcholinesterase enzyme activity

Acetylcholinesterase enzyme as a brain biomarker of cholinergic function and memory was assessed by the Ellman's method. Briefly, 100 µLs of brain supernatant was diluted 10 times in phosphate buffer saline (0.1 M, pH 7.4) followed by addition 50 microliters of DTNB (0.01 M). The reaction mixture was incubated for 5 min and baseline absorbance measured at 412 nm in a cuvette. Then, 25 µL of acetylcholine iodide (0.028 M) was added for the reaction to proceed for 3 min, and final absorbance was measured again. The change in absorbance per minute was determined and using the molar extinction coefficient (1.36×10^4), the rate of AChE activity was calculated and expressed as nmol/min/ mg tissue protein.

Determination of blood serum liver enzyme activity

The assay liverenzyme as indicator of damages to some organs such as the liver was carried out using bloodserum of the animals. The plasma liver enzymes assayed includes aspartate amino transferase (AST) and alanine amino transferase (ALT) as recommended by Reitman and Frankel (1957) and alkaline phosphatse (ALP) as described by Klein*et al*. (1960).The enzyme activity, which was expressed in /L, was obtained from a calibration curve using the values supplied along with the kits.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a *post hoc* test according to the statistical package program (SPSS version 17.0). All values $p < 0.05$ was considered as significant for all statistical analysis in this study.

Results:

Acute Toxicity Test

Signs and symptoms of toxicity such as urination, tremor, depressed locomotion and deaths were observed in acute toxicity test. There were behavioural alterations and a significant reduction in spontaneous locomotor activity in the male mice. Potassium cyanide at a single oral dose of 10-1000 mg/kg showed a percent mortality of 100% in adult male mice but no mortality was recorded in a single dose of 2 mg/kg.

Table 1.1: Results of acute toxicity study showing the different doses of potassium cyanide administered orally to mice, number of deaths and the percentage mortality.

Phase I

Group (n=3)	Dose (mg/kg)	Number of Death	% Mortality
1	10	1/1	100
2	100	1/1	100
3	1000	1/1	100

Table 1.2: Results of acute toxicity study showing the different doses of potassium cyanide administered orally to mice, number of deaths and the percentage mortality.

Phase II

Doses (mg/kg)	No of animals in each group				
	1	2	3	4	Total
2	0/1	0/2	0/3	0/5	0/11
4	0/1	0/2	1/3	1/5	2/11
9	0/1	½	3/3	5/5	10/11

The LD_{50} was estimated in the following manner: The Geometric mean of the doses for which 0/1 and 1/1 were found.

LD_{50} of KCN = $\sqrt{16 \times 14}$

$= \sqrt{224}$

$= 15$ mg/kg

Therefore LD_{50} of KCN = 15 mg/kg.

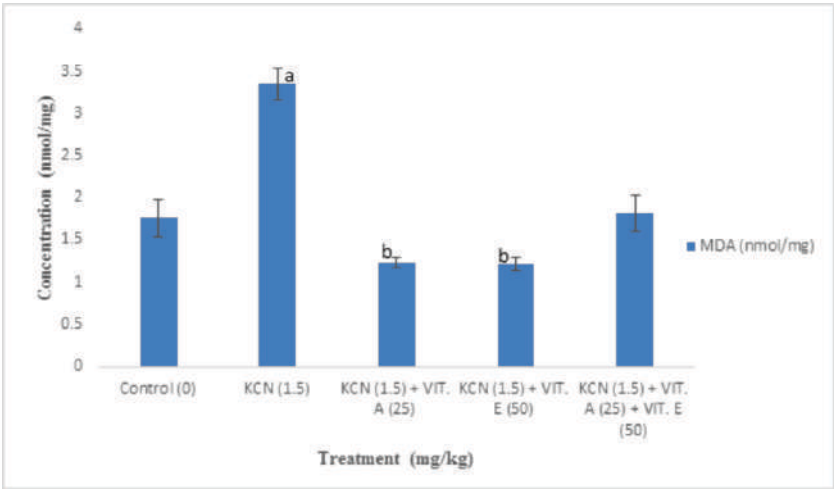

Fig. 1: Effect of sub lethal Administration of Potassium Cyanide (KCN) and Administration of Vitamins A and E on brain MDA concentration in albino mice. Superscript[a] shows statistical significance ($p < 0.05$) when control is compared to vitamin pre-treated groups while superscript[b] shows statistical significance ($p < 0.05$) when KCNtreated group is compared to vitamin pre-treated groups.

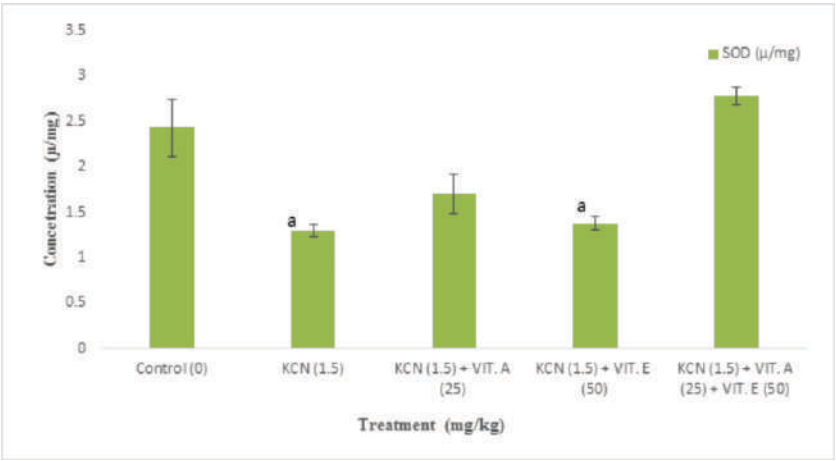

Fig. 2: Effect of sub lethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain SOD activity in albino mice. Superscript[a] shows statistical significance ($p < 0.05$) when control is compared to vitamin pre-treated groups while superscript [b] shows statistical significance ($p < 0.05$) when KCN treated group is compared to vitamin pre-treated groups.

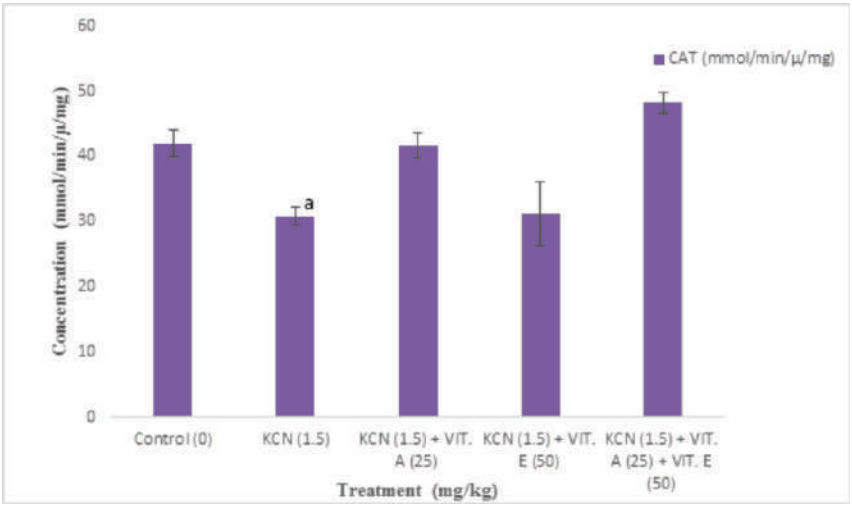

Fig. 3: Effect of Sub lethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain CAT activity in albino mice. Superscript[a] shows statistical significance ($p < 0.05$) when control is compared to vitamin pre-treated groups while superscript [b] shows statistical significance ($p < 0.05$) when KCN treated group is compared to vitamin pre-treated groups.

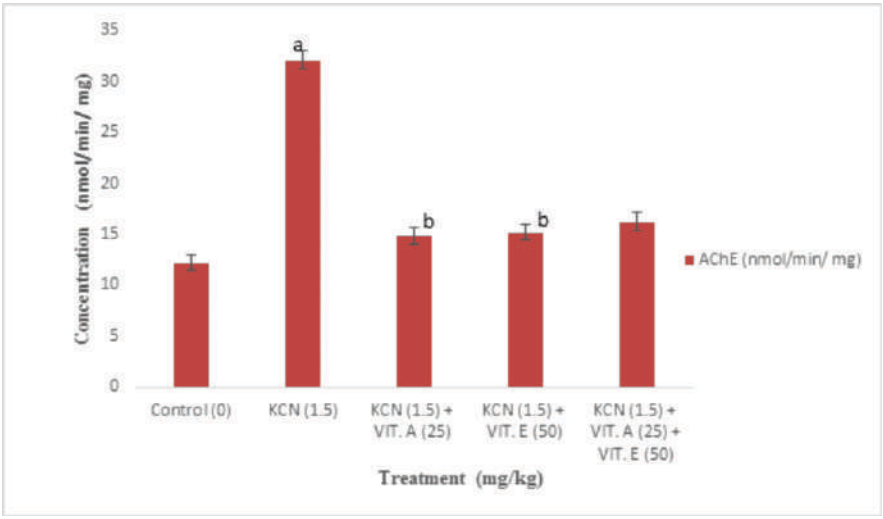

Fig. 4: Effect of Sub lethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain acetylcholinesterase activity in albino mice. Superscript[a] shows statistical significance ($p < 0.05$) when control is compared to vitamin pre-treated groups while superscript [b] shows statistical significance ($p < 0.05$) when KCN treated group is compared to vitamin pre-treated groups.

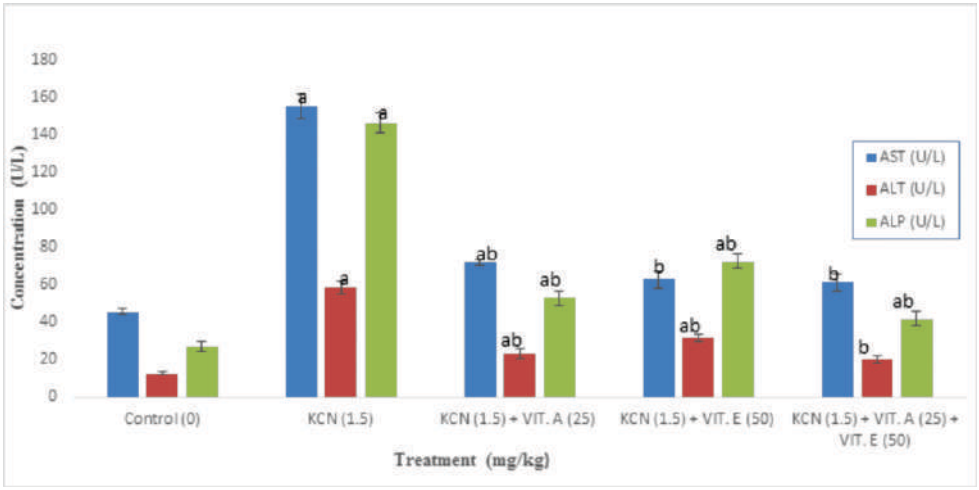

Fig. 5: Effect of Sub-lethal Administration of Potassium Cyanide and Co-administration of Vitamin A and E on Liver enzymes in Albino Mice. Superscript[a] shows statistical significance ($p < 0.05$) when control is compared to vitamin pre-treated groups while superscript[b] shows statistical significance ($p < 0.05$) when KCN treated group is compared to vitamin pre-treated groups.

Discussion

The increased level of malondialdehyde (MDA) concentration in the potassium cyanide treated group is an indicator that cyanide is a neurotoxin that evoked lipoperoxidative changes in brain tissues through free radical induction. These findings agree with results obtained in the works of Baskin *et al*., 2010. After 28 days of administration, the MDA levels significantly ($p<0.05$) increased in potassium cyanide (KCN)treatment as compared to the control. This increased demonstrates that oxidative damage occurred in brain tissue as a result of lipid peroxidation attributed to cyanide induced toxicity. Membrane lipids are rich in polyunsaturated fatty acids are vulnerable to peroxidation by oxidants (Hyldegaard *et al.,* 2011). The activities of different membrane-bound enzymes were alteredresulting in the degeneration of cell membrane (Ardelt *et al.,* 1994).In agreement with our findings, potassium cyanide led to higher MDA content with an increase in lipid peroxidation activity (Kadiri and Samuel, 2019).

The low MDA levels in vitamins pre-treated groups (III, IV and V) showed their ability to significantly ($p < 0.05$) reduce cyanide induced tissue lipoperoxidative damage which is in agreement with the work of Anorou *et al.,* 2008. It has also been reported that antioxidant vitamins administration leads to a decrease in MDA levels (Asonye and Okolie, 2004) and brain tissue (Behr *et al.,* 2011). The present findings showed that vitamin E treated group caused further decrease in the MDA levels in the brain as compared to co-administration of vitamins A and E, suggesting single administration is more potent in overcoming lipid peroxidation in the brain tissue. This may have been responsible for the mitigative role of cyanide-induced clinical and biochemical deficits (Al-ghanim *et al.,* 2012). Moreover, studies have shown that free radical mediated lipid peroxidation in cigarette smokers can be mitigated by antioxidant vitamins supplementation (Asonye and Okolie, 2004). These vitamins act synergistically as antioxidants by neutralizing oxidizing free radicals, including the superoxide and hydroxyl radicals. Their redox properties also allow them to act as reducing agents and are excellent scavengers of superoxide, hydroxyl ion and peroxyl radicals there by inhibiting lipid peroxidation (Kapur *et al.,* 2004).

Cyanide is a known potent inhibitor of superoxide dismutase (SOD) activity as it was seen in previous studies (Allard *et al.,* 2009).

Thus some of the numerous biochemical activities of cyanide either from dietary source or otherwise could be mediated through depletion in the antioxidant status of the body (Hussain *et al.,* 1999). From the results obtained in this study, there was a significant ($p < 0.05$) decreased level of brain SOD activity by cyanide treated group. This strongly suggests that cyanide imposes oxidative stress in the brain tissue. This finding corroborates with works of Bhatcharya *et al.,* 2010; which showedthat cyanide toxicity caused increased generation of superoxide anion and lipid peroxidation with inhibition of antioxidants enzymes. Similarly, in the present study, superoxide dismutase as intracellular antioxidant was depleted after exposure to 1.5 mg/kg of KCN, making the cell environment less reducing and vulnerable to oxidative stress.Decreased in the activity of SOD also portends reduction in the capacity of the mice to handle reactive oxygen species. The changes in brain tissues may be induced by cyanide due to the imbalance in the antioxidant enzymes and oxidative stress (Aparicio and Sotoblanco 2002; Abe *et al.,* 2005). Reactive oxygen species generated in the brain tissues induced by cyanide were significantly scavenged by enzymatic antioxidant system.From the results obtained, brain superoxide dismutaseactivity was increased through administration of non-enzymatic antioxidants such as vitamins A and E which may be responsible for the restoration of SOD activities. These findings are considered vital for the importance of antioxidant vitamins supplements especially amongst populations routinely exposed to cyanide through habits, diets and occupations

Catalase is a prospective biomarker of cyanide toxicity; this indicates that sub lethal concentration of cyanide can cause changes in activity of key enzymes (Alvandi and Hosetti, 2014). From the results, exposures to sub lethal levels of cyanide significantly ($p<0.05$) decreased catalase brain activities, indicating a typical response to stress in group pre-treated with KCN as compared to control mice. This suggested that cyanide is a potential toxicant and induces oxidative stress and may bring serious potential health risks (Okolie and Uaseonge, 2013). The reduction in the activity of this enzyme in the organs may be due to the inhibitory effect of cyanide on the enzyme. Works of Bhattacharya *et al.*, (2009) also reported that brain is a highly sensitive organ and is one of the most important targets under cyanide toxicity.

The antioxidative enzymes are important in controlling the oxidative stress in animals. The activity of one or more of these enzymes is generally increased or decreased in animals exposed to stressful conditions (Al-ghanim and Mahboob, 2011). In the present study, catalase activity in the brain tissues of mice was increased by vitamins pre-treated groups (III, IV and V). The results suggests that antioxidant defence system biologically scavenged these toxins by converting them into metabolically non-destructive cellular molecules when co-administered (vitamins A and E)than single administration. This further showed protective roles of antioxidant vitamins as was seen in the study which corroborates with the works of Asonye and Okolie, 2004.

Acetylcholinesterase is an enzyme that regulates neurotransmitter acetylcholine at neurone junctions (Ahmed *et al.,* 2015). Cyanide treated mice showed significant ($p < 0.05$) increase in brain acetylcholinesterase activity as compared to control. This finding suggests an involvement of brain AChE in chronic cyanide toxicity. The increase AChE activity can be attributed to the stress induced by cyanide. Furthermore, it has been reported that AChE activity was regarded as a significant parameter in assessing complex toxicogenic effects of cyanide (Anyawu *et al*., 2010). The ability of cyanide to phosphorylate the enzyme results in impairment of its activity (Caxton *et al*., 2012). Various studies in mice have successfully reported that increase acetylcholinesterase enzymes activity leads to excessive decrease of ACh in the brain tissue which is associated with cyanide induced neurotoxicity (Lambark and Cabeza, 2006).Groups pre-treated with vitamins (A and E) showed significantly decrease activity of the enzyme. This treatment prevented the cyanide-induced rise in AChE activity. This finding suggests the neuroprotective potential of

antioxidant vitaminswhich is involved in cholinergic system modulation (Aliyu *et al.,* 2012). The sub lethal administrations of potassium cyanide for the period of 28 days adversely affected AChE activity in the brain tissue which can be attributed to high lipid solubility and persistence of cyanide in the brain.

Liver enzymes (AST and ALT) are considered an important biomarker for the detection of cyanide hepatotoxicity (Abdel *et al*., 2014). From the results, cyanide caused a significant increase ($p < 0.05$) in AST and ALT levels when compared to the control group. Increasing levels of AST and ALT in the plasma of treated mice is due mainly to the leakage of these enzymes from the livercytosol into the blood stream (Concepcion *et al*., 1993). The AST level rises significantly in the plasma as a consequence of enzyme leakage from the injured hepatic cells into the circulation. ALP also increases in plasma when cellular degeneration or destruction occurs in the organ (Hassoun *et al.,* 1995). Increase in serum liver enzymes is another implication of cyanide exposure due to detoxification and depletion of antioxidant enzymes (Haousas *et al*., 2014) as was seen from the present study. We can deduce that high levels of transaminases, normally located in the hepatocyte cytosol, are signs of cells damage leading to liver dysfunction in treated mice.Thus, some of the numerous biochemical activities of cyanide either from dietary source or otherwise could be mediated through increase in the serum enzymes (Anyawu *et al.,* 2006).The antioxidant properties can directly or indirectly reduce oxidative damage by preventing the excessive generation of free radicals (Aigbiremolen *et al*., 2011).The toxic effects of cyanide induced neurotoxicity was seen in the increasedlevels of the serum enzymes. Pre-treatment with vitamins resulted in significant improvement in the liver enzymes level (AST, ALT and ALP) as compared to potassium cyanide treated group. This decrease could be attributed to the antioxidant vitamins (A and E)which have excellent scavenging property. More so, they acted as reducing agents to free radicals and in turn able to ameliorate the oxidative damage caused by cyanide induced toxicity. The significant reduction in serum liver enzymes pre-treated with vitamins A and E clearly demonstrated their protective role over cyanide-induced tissue damage, probably as a result of their antioxidant properties.

Conclusion

The biochemical findings revealed potential neurotoxicity mechanisms via induction of lipoperoxidation, acetylcholinesterase and oxidative stress mediators, which showed that potassium cyanide possesses neurotoxic effects on brain and liver tissues. While antioxidant vitamins (A and E) supplementation can mitigate these anomalies under potassium cyanide induced oxidative stress.

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