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Neuroprotective Properties of NMDA R1 Antagonist (*Ketamine*) in Cyanide Treated Neurons *in vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. OMO is the major author and contributor as he provided lab space equipments and initiated design. He also drafted the manuscript. All other authors took part in conducting the experiment and analyzing the results.

Research Article

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ABSTRACT

Aims: This study aims at investigating possible means of reducing cyanide toxicity by blocking NMDA R1 via ketamine (an NMDA R1 antagonist). This is to provide a template for quick arrest of cyanide toxicity in neurons under oxygen deprived condition.
Place and Duration of Study: Bingham University, Department of Anatomy, Karu, Nigeria. The duration of the study was100 minutes.
Methodology: Freshly harvested cortical tissue blocks were perfused in accessory cerebrospinal fluid (ACSF) containing all the necessary salts and glucose. The cultures were treated with ACSF (Control), ACSF+KCN (potassium cyanide), ACSF+KCN+Ketamine and ACSF+Ketamine for a total duration of 100 minutes at 37ºC.
Results: The Ketamine had a protective and reversal effects on the tissues both for oxygen deprivation and cyanide toxicity, The cells in tissues treated with

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ACSF+KCN+Ketamine showed normal appearance of cell body and axonal projections, the cells treated with ACSF+Ketamine showed fewer degenerating cells compared to those treated with cyanide.
Conclusion: Ketamine, an NMDA R1 antagonist is neuroprotective against the toxicity of cyanide.

Keywords: Cyanide; ketamine; NMDA R1; neurodegeneration; excitotoxicity.

1. INTRODUCTION

Genetic deletion of NMDA glutamate receptors disrupts development of whisker-related neuronal patterns in the somatosensory system. Independent studies have shown that NMDA receptor antagonists increase cell death among developing neurons (de Rivero et al., 2006). It has been reported that a dramatic feature of the developing somatosensory system **in** newborn NMDA receptor 1 (NMDA R1) knock-out mice is increased cell death in the ventrobasal nucleus (VB) of the thalamus (Isom and Way, 1984) as such neurons will fail to recognize the desired locale and will also not make connections with other neurons. In the adult neuronal system, however, NMDA R1 is one of the three glutamate receptors. The function of this receptor is major in memory, learning and vision (Pannese et al., 2012). Glutamate receptors are also important in the metabolism of the cell as the cell exchanges glutamate for glucose, while the exported glutamate is cycles into glutamine in the astrocytes. The role of cyanide as a potentiator of the glutamate system accounts for several resultant effects in the metabolism of the astrocytic-neuronal system (Pannese et al., 2012; Barneda-Zahonero et al., 2012).

Cyanide in this context refers to free cyanide generated from KCN dissolved in distilled water, as salts of cyanide like (Na, K and Ca) are highly reactive and dissociates readily in water to release free CN- (Isom et al., 1999). Cyanide, a known neurotoxin induces neuronal cell death via potentiation of glutamate receptor; specifically NMDA *R1*. In several knock out studies, NMDAR1 antagonists have been shown to reduce the severity of neuronal damage and the overall number of dead neuronal cells (Beutler et al., 2011). Toxicity of cyanide can be divided into two main parts, the first one entails blockade of cytochrome C oxidase (Heme a3-Cuβ) binuclear centre that will create a system where oxygen and electrons are in excess, thus leading to the formation of reactive oxygen species (ROS) that will in turn cause lipid peroxidation (Isom et al., 1999; Dong et al., 2011). In a second system, cyanide has been described to be a potent excitotoxin. This is so because cyanide can potentiate NMDA R1 (Pannese et al., 2012; Dong et al., 2011; Wu et al., 2011), a glutamate receptor. Glutamate is an amino acid neurotransmitter in the brain; it is widely distributed and has been described as the most abundant neurotransmitter system in the brain. Since cyanide can bind to the NMDA R1, it thus can prolong excitation of the neuron as there is no metabolic machinery to convert cyanide into non-harmful metabolites in the neuronal endings; leading to an excitotoxic effect (Isom and Way, 1984). Excitation of neuronal cells generally involves depolarization and repolarisation of the neuronal membrane by Ca^{2+} ions, thus toxicity of cyanide has been confirmed to increase in situations where cerebral Ca^{2+} ions level is elevated (Wu et al., 2011).

The question is, will inhibition of the NMDA R1 reduce the excitotoxicity of cyanide both in the normal state and in oxygen deprived conditions. If the NMDA R1 is inhibited, it can reduce cyanide toxicity in the following ways; first by preventing glutamate toxicity that will

normally result from cyanide potentiation of NMDA receptors, secondly since the NMDA R 1 is calcium gated, ketamine will prevent the calcium influx associated with NMDA R1 hyper activity due to cyanide toxicity, thus preventing prolonged excitation by cyanide. We suggest a structural similarity between cyanide, ketamine and glutamate as the three can bind to NMDA R1, but the difference is that ketamine is competitive with glutamate only to block the receptor while cyanide recognizes the receptor to over excite the receptor. In a third instance inhibition of calcium influx will help prevent the osmotic imbalance and cell swelling associated with cyanide excitotoxicity, thus we propose that NMDA R1 inactivation can be a temporary suppressor of cyanide toxicity in the neurons.

2. MATERIALS AND METHODS

2.1 NMDA R1 Antagonist

Ketamine ampoule containing 50mg of ketamine hydrochlorate in 2ml dextrose saline was procured from Standard Pharma. The solution was up to 50ml in dextrose solution such that the final concentration is at 1µg/µl. The reagent was freshly prepared and stored at 18ºC.

2.1.1 Induction of oxygen deprivation

All tissue blocks used in one experiment were prepared from adult male Wister rats (F1 generation) born on the same day from the same mother and exposed to similar environmental and diet for 50 days. The animals were sacrificed and the brain tissue exposed; the brain was gently removed and placed in cold ACSF (4ºC) bubbled with oxygen to contain 95% Oxygen and 5% CO_2. The dissecting blade was used to remove cortical tissue from the parietal lobe. Each cortical tissues removed were cut to uniform size. Before oxygen deprivation, tissues were washed in cold glucose-free medium and transferred to the anaerobic incubator. The ACSF was prepared so that it contains 18mM glucose; 119mM NaCl, 2.5mM KCl, 1.3mM $MgSO_4$, 2.5mM $CaCl_2$, 26.2mM $NaHCO_3$, 1mM NaH_2PO_4. The solution was prepared in distilled water to make up to 200ml. Oxygen was not included in the perfusion set up as we seek to study the activity of the pump blockers in cyanogenic-oxygen deprived environment (partial pressure for atmospheric dissolved oxygen in the solution was 5% O_2). For studies of glutamate receptor (NMDA R1), 50µmol/L non-competitive NMDA-receptor antagonist Ketamine was added to the ACSF before OGD (Dhanushkodi and McDonald, 2012). The cyanide concentration was 200µl of 1mg/ml solution of KCN dissolved in dextrose saline.

2.2 Treatment and Perfusion

Four separate perfusion set ups were treated as thus;

A: ACSF
B: ACSF+KCN
C: ACSF+KCN+*NMDA R1* Antagonist (Ketamine)
D: ACSF+KCN+*NMDA R1* Antagonist (Ketamine)

The tissue blocks were placed in 25ml perti dishes containing the appropriate treatment solution with 50 ml of the same solution contained in a perfusion chamber such that the culture is a continuous flow around the tissue block. The approximate dimension of the

tissue blocks are 60mm^3±10. The fluid flow was controlled using a syringe valve and the duration of the treatment is 100 minutes.

3. RESULTS AND DISCUSSION

3.1 Results

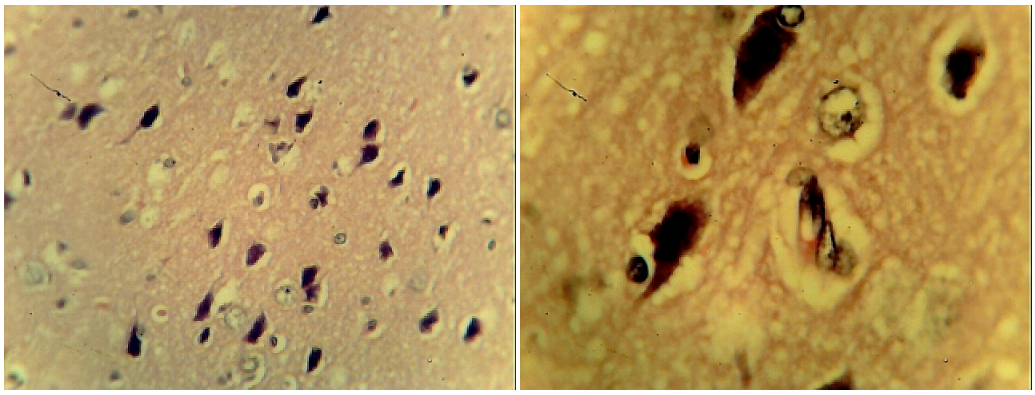

Fig. 1. General morphology of neurons sub-cultures in ACSF (Control) for 100 minutes. Fig 1A shows the appearance of the cell at X400 while 1B shows the neurons at X1, 000. The cells shows normal appearance of the neurons (n) at higher magnification the cytoplasm is densely stained. Spaces around the cells (x) are restricted to the immediate environment of the cell and are suspected to be the response to stress due to oxygen deprivation. Arrow heads shows the immediate connections of the cells are intact. (Staining method is H&E at X400 and X1,00)

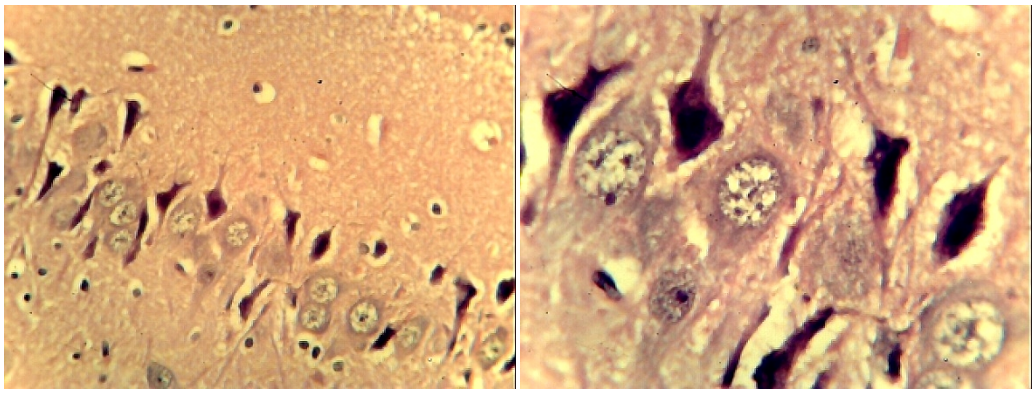

Fig. 2. The general morphology of cells in ACSF+KCN, the toxicity of cyanide was prominent in this group, large cells(a) with prominent axonal projections shows signs of excitotoxicity, while cell types (b) shows advanced state of neuronal degeneration possibly due to ROS formation coupled with excitotoxicity. (*Projections from cell body of neurons), Arrow heads shows the prominent axonal projections of the pyramidal cells compared to Fig. 1. Dashed line shows enlarged region. (Magnifications 2A: X400 and 2B: X1, 000; Staining method is H & E)

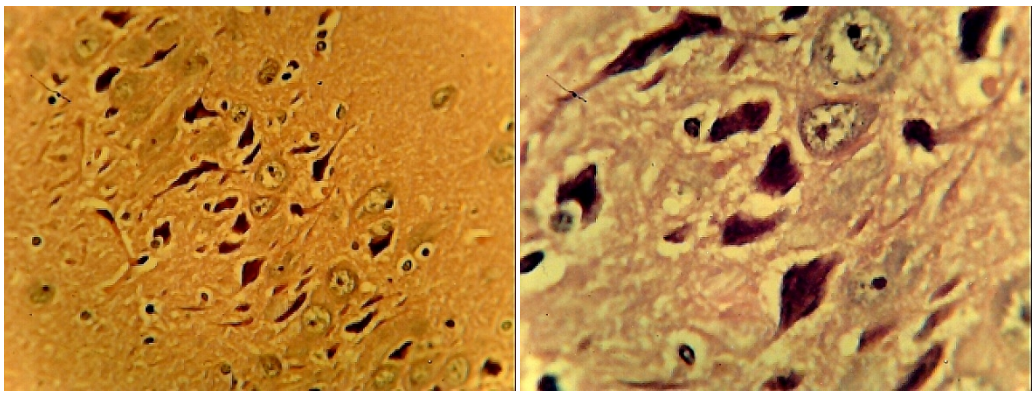

Fig. 3. Shows the general morphology of cells treated with ACSF+Ketamine under oxygen deprived condition. The appearance of the cells shows reactions similar to the control. This indicates that under oxygen deprivation, ketamine has some protective effect to reduce the extent of neuronal damage. (n) represents normal cells while (x) represents damaged cells, which are fewer in number compared to Fig. 2. ACSF+KCN. (Magnification 3A: X400, 3B: X1,000)

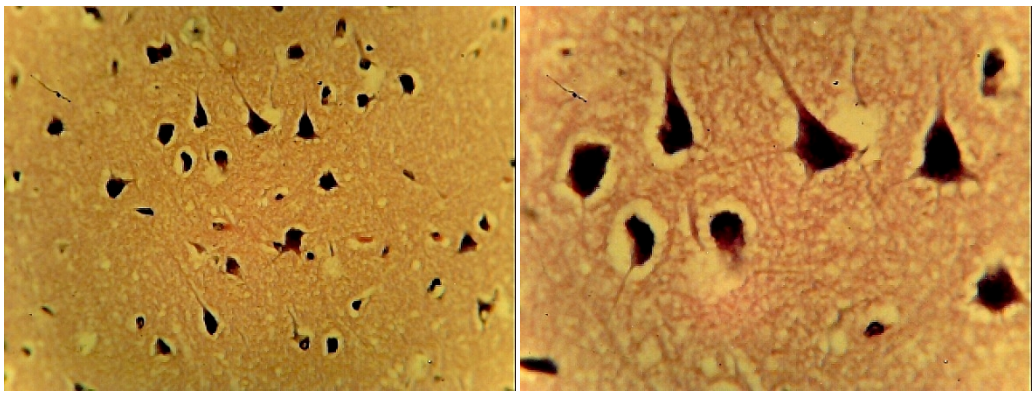

Fig. 4. General morphology of cells treated with ACSF+KCN+Ketamine (projections from cell body). The slides shows the protective effect of ketamine as the cell sizes were retained and the cells shows normal appearance. Although the axons were very prominent showing increased activity but it is not as prominent as those observed in Fig. 2. ACSF+KCN. Magnifications 4A: X400 and 4B: X1, 000). The appearances of the cells are closer to normal than those treated with ACSF only.**

3.2 Discussion

It is known that blocking of the NMDA R1 in neonates causes the neurons not to recognize their site or it could make them non-responsive to stimulus required for neuronal development post-natally (Pannese et al., 2012). We cannot over elucidate the role of calcium gated glutamate receptors (NMDA R1) in the toxicity of cyanide, since cyanide can potentiate this receptor by mimicking glutamate to induce excitotoxicity in the neurons (Wu et al., 2011). This study was conducted in oxygen deprived medium since cyanide is capable of

blocking Cytochrome *C* Oxidase to generate reactive oxygen species (ROS). However, for oxidative stress to occur in a system there are two possibilities;

Type I: A system where oxygen is present such that when cyanide inhibits cytochrome C oxidase, ROS is generated and causes lipid peroxidation in addition to excitotoxicity.

Type II: A system deprived of oxygen where minimal ROS is generated, thus the major toxicity effect of cyanide will be excitotoxicity via potentiation of NMDA R1.

Thus, for us to almost precisely focus on the excitotoxicity of cyanide independent from the toxicity induced by ROS, we have adopted the system Type II. This so because it is an utmost phenomenon in cyanide toxicity that oxygen utilization is suppressed via blockade of cytochrome *C* oxidase. This is so as cyanide can inhibit the three redox state of the enzyme Heme *a3-Cuβ* binuclear centre competing with oxygen for the binding site, thus inhibiting the conversion of molecular oxygen to water (Isom et al., 1999).

In this study it was observed that ketamine had a protective effect on the neurons under combined cyanide toxicity and oxygen deprivation (Fig. 4) and under oxygen deprivation only (Fig. 3). The protective effect of ketamine can be due to the fact potentiation of NMDAR1 is the major form of toxicity as against ROS formation or rather, the first effect is potentiation of the receptor while ROS formation, if it occurs will be a later event. Recapping on the possible modes of cyanide toxicity, Fig. 2 shows that a possible mechanism is increased size due to transient firing and osmotic pressure induced by calcium influx associated with NMDA potentiation, this will also represent the ability of cyanide to contest NMDA with glutamate and the fact that cyanide cannot be metabolized in the neurons.

Comparing the findings of this study with the work of Yamamoto and Tang, 1998, the neurons were treated with melantonin, NMDA or and Cyanide. It was observed that melantonin scavenged for free radicals thus reducing the toxicity of cyanide. In this investigation, no anti-oxidant was used rather NMDA R1 Blocker ketamine was used as a competitor against cyanide for the binding site on the NMDA R1. In both studies, the neurons were protected from the excitotoxicity of cyanide. This also confirms that cyanogenic toxicity will likely follow two major paths to include excitoxicity by potentiating NMDA R1 (as described in this study) and production of reactive oxygen species as described by Yamamoto and Tang, 1998).

Glutamate and glucose are exchange in a 1:1 stoichiometry following the principles and Magistretti and Pellirin, 1996, thus blockade of NMDA R1 or inhibition glutamate activity is an indirect method for inhibition of glucose (a sedative property of ketamine) (Moirera et al., 2012). In conditions of ischemia perfusion injury, glutamate is released in large quantities further causing excessive activation of NMDA R1 (a glutamate receptor). In the study of Ahlgren et al., 2011, NMDA R1 blocker MK-108 reduced the toxicity of glutamate in organotypic hippocampial cells under oxygen deprived conditions by competing with glutamate for the NMDA R1 binding site; thus further confirming that NMDA R1 blockade is effective both for cyanide toxicity, glutamate toxicity or a glutamate toxicity that occurs secondary to cyanide toxicity. When KCN was added to the medium alongside ketamine, the NMDAR1 antagonist reduced the activity of cyanide on the already inactivated receptor, thus cyanide is present in the system but the active sites of its activity has been covered thus preventing the toxicity in 3 main ways; firstly, inhibition of the glutamate receptor prevents cyanide potentiation and influx of calcium ions that can lead to transient firing and prolonged

excitation (Wang et al., 2012; Hall and Mudroch, 1990). Secondly, if prolonged excitation had occurred, osmotic imbalance would have occurred thus altering the electrochemical gradient and causing accumulation of fluid in the neurons (Rodriguez-Rodriguez et al., 2012). Thirdly inhibition of cyanide helps prevent glutamate toxicity; if the glucose transport is impaired due to osmotic imbalance, more glutamate is likely to be produced to request for more glucose, although glutamate level will rise, inhibition of the NMDA R1 will also helps protect the neuron not only against excitotoxicity of cyanide in the first instance but also the resultant glutamate toxicity in the second instance (Pannese et al., 2012).

4. CONCLUSION

Ketamine is seen to be neuroprotective under cyanide toxicity; by preventing excitotoxicity of cyanide via inactivation of NMDAR1, preventing calcium influx and osmotic imbalance and reducing the episodes of resultant glutamate toxicity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

Ahlgren, H., Henjums, K., Ottersen, O.P., Runden-Pran, E. (2011). Validation of organotypical hippocampial slice cultures as an ex vivo model of brain ischemia: Different roles of NMDA receptors in Cell death signalling after cyanide, NMDA and or Oxygen glucose deprivation. Cell Tissue Res., 345(3), 329-41.

Barneda-Zahonero, B., Servitja, J.M., Badiola, N., Minano-Molina, A.J., Fado, R., Saura, C.A., Rodriguez-Alvarez J. (2012). urr1 is required for NMDA receptor-mediated neuronal survival. J Biol Chem. 2012 Jan 31. [Epub ahead of print].

Beutler, L.R., Eldred, K.C., Quintana, A., Keene, C.D., Rose, S.E., Postupna, N., Montine, T.J., Palmiter, R.D. (2011). Severely impaired learning and altered neuronal morphology in mice lacking NMDA receptors in medium spiny neurons. PLoS One, 6(11), e28168.

de Rivero Vaccari, J.C., Casey, G.P., Aleem, S., Park, W.M. (2006). Corriveau RA. NMDA receptors promote survival in somatosensory relay nuclei by inhibiting Bax-dependent developmental cell death. Proc Natl Acad Sci USA, 103, 16971–16976.

Dhanushkodi, A., McDonald, M.P. (2011). Intracranial V. cholerae sialidase protects against excitotoxic neurodegeneration. PLoS One, 6(12), e29285.

Dong, Y., Zhang, W., Lai, B., Luan, W.J., Zhu, Y.H., Zhao, B.Q., Zheng, P. (2012). Two free radical pathways mediate chemical hypoxia-induced glutamate release in synaptosomes from the prefrontal cortex. Biochim Biophys Acta, 1823(2), 493-504.

Hall, R., Murdoch, J. (1990). Brain protection: physiological and pharmacological considerations. Part II: The pharmacology of brain protection. Can J Anaesth., 37(7), 762-77. Review.

Isom, G.E., Way, J.L. (1984). Effects of oxygen on the antagonism of cyanide intoxication: Cytochrome oxidase *in vitro*. Toxicol. Appl. Pharmacol, 74, 57–62.

Isom, G.E., Gunasekar, P.G., Borowitz, J.L. (1999). Cyanide and neurodegenerative disease. In Chemicals and Neurodegenerative Disease (S. C. Bondy, Ed.), 101–129. Prominent Press, Scottsdale, AZ.

Magistretti, P.J., Pellerin, L. (1996). Cellular mechanisms of brain energy metabolism. relevance to functional brain imaging and to neurodegenerative disorders. Annals of the New York Academy of Sciences Volume 777 Issue Bio artificial Organs: Science, Medicine and Technology, 380–387.

Moreira, T.A., Costa, P.S., Costa, L.R., Jesus-França, C.M/, Antunes, D.E., Gomes, H.S., Neto, O.A. (2012). Combined oral midazolam-ketamine better than midazolam alone for sedation of young children: a randomized controlled trial. Int J Paediatr Dent., 28. doi: 10.1111/j.1365-263X.2012.01246.x. [Epub ahead of print].

Pannese, R., Minichino, A., Pignatelli, M., Delle Chiaie, R., Biondi, M., Nicoletti, F. (2012). Evidences on the key role of the metabotrobic glutamatergic receptors in the pathogenesis of schizophrenia: a "breakthrough" in pharmacological treatment]. Riv Psichiatr., 47(2), 149-69.

Rodriguez-Rodriguez, P, Fernandez, E., Almeida, A., Bolaños, J.P. (2012). Excitotoxic stimulus stabilizes PFKFB3 causing pentose-phosphate pathway to glycolysis switch and neurodegeneration. Cell Death Differ., 16. doi: 10.1038/cdd.2012.33. [Epub ahead of print]

Wang, X., Chen, Y., Zhou, X., Liu, F., Zhang, T., Zhang, C. (2012). Effects of propofol and ketamine as combined anesthesia for electroconvulsive therapy in patients with depressive disorder. JECT, 28(2), 128-32.

Wu, Y.N., Munhall, A.C., Johnson, S.W. (2011). Mitochondrial uncoupling agents antagonize rotenone actions in rat *Substantia nigra* dopamine neurons. Brain Res., 1395, 86-93.

Yamamoto, H., Tang, H. (1998). Effect of 2-amino-7-phosphanoepatonic acid, melantonin or NG-nitro-L-Argenine on Cyanide or NMDA induced neurotoxicity in rat cortical cells. Toxicol. Lett., 16, 94(1), 13-8.