

Full Length Research Paper

Blockade of Na⁺/K⁺ ATPase Pump Amplifies Toxicity of Cyanide in Oxygen Deprived Neurons

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Cyanide is a potent neurotoxin capable of potentiation NMDA R1 (N-methyl-D-aspartate Receptor 1) a form of glutamate receptor that is calcium gated, thus causing excitotoxicity. It is also well established that the glutamate-glucose exchange is dependent on the activity of the Na⁺/K⁺ ATPase pump, thus we examine the role of the Na⁺/K⁺ pump in the metabolism of the neuron during cyanide toxicity. Six separate perfusion set up of the rat brain cortical tissues were made with ACSF (Accessory cerebrospinal fluid) (ACSF, ACSF+KCN (potassium cyanide), ACSF+KCN + pump blocker, ACSF + pump blocker). The tissues were perfused for duration of 180 minutes. The tissues were processed histologically to study the membrane and axonal structure. The pump blockers (methyl digoxin and promethazine) induced excitotoxicity when used in culture, and amplified cyanide toxicity when combined with KCN. Cell enlargement was characteristic of both cyanide and pump blocker treated cultures, the pattern of cell enlargement observed in the methyl digoxin/promethazine treated tissues shows more of osmotic imbalance induced cell enlargement while the Cyanide treated sections showed moderate enlargement as osmotic imbalance is believed to be a latter event in this tissue.

Keywords: Cyanide, Neurotoxin, Excitotoxicity, Glutamate-glucose, ATPase pump, Tissue, Membrane, Axonal structure.

INTRODUCTION

For cyanide to exert its excitotoxic effects, potentiation of glutamate receptor called *N-methyl-D-Aspartate receptor* (NMDA R1) is an essential step in the toxicity process (Ahmed *et al.*, 2002). For this potentiation to occur, several movements of ions across the membrane are imminent. For us to accurately present the picture of the events we must first describe the significance of Na⁺/K⁺ exchange as a facilitator of glutamate-glutamine cycle across the astrocytic and neuronal membrane, also the rate of glucose consumption in relation to the glutamate-glutamine cycle.

Following the proposition of Pellerini and Magistretti, 2011, glutamate surge was suspected to be the primary stimulus for glucose synthesis in neurons, however, our recent findings on the tricarboxylic acid cycle (TCA) reveals otherwise but complimentary to the findings of Magistretti and co-workers. They also proposed that

glutamate is cycled into glutamine from the astrocyte to the neuron and that for this cycling to occur, Na⁺ and K⁺ ions are exchanged via the ATPase pump against glutamine going into the neuron to be converted into glutamate in the cytoplasm (Ogundele *et al.*, 2012). This process is however energy consuming and has been found to require 2 molecules of ATP to cycle glutamine into the neuron and glutamate out of the neuron and to exchange Na⁺ and K⁺ ions across the neuronal membrane. Considering the fact that 2 molecules of ATP are required Magistretti and Pellirini suggested it balances the energy production capacity of the glycolytic pathway which will yield 2 molecules of ATP. In our recent study of this phenomena in oxygen deprived neuronal cells of the primary visual cortex in an *in vivo* experiment where adult wistar rats (wild type: WT) were treated with 20 mg/KgBW of KCN (*potassium cyanide*) for a duration of 30 days (representing an acute and sub-lethal dose), we observed that the primary driver for ATP production in oxygen deprived condition is the step 6 of the TCA cycle rather than glucose consumption itself. For us to achieve the desired representation of the

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phenomena, certain rate limiting steps in the glucose metabolic pathway of the neurons were selected as indicators for the processes (glycolysis and TCA cycle). The obtained parameters in the glucose metabolic pathway were used in constructing a metabolic pathway against glutamate levels (the adopted unit was in $\mu\text{mol/g}$) in homogenized brain tissues at 4°C (Ogundele *et al.*, 2012). Glucose-6-phosphate dehydrogenase (G6PDH) was assayed as an indicator of the pentose phosphate pathway which determines whether glycolysis proceeds into pyruvate production for TCA cycle or ribose sugar for DNA repair. LDH (lactate dehydrogenase) was assayed as a measure of the shift from aerobic to an aerobic metabolism, thus, an indirect indicator of the ATP/ADP ratio. SDH (succinate dehydrogenase) was measured as an indicator of the vital step 6 of the TCA cycle. This step is catalyzed by succinate dehydrogenase and it involves the co-conversion of succinate to fumarate and ubiquinone to ubiquinol. This step uses FADH_2 and donates $2e^-$ to the electron transport chain (possibly Complex V). We also measure the level of SOD (superoxide dismutase) as an indicator for the level of superoxide anions/oxygen radicals ($\text{O}^{\cdot-}$), otherwise reactive oxygen species (ROS) using the methods of Isom and Way, 1984 to depict a switch in metabolism in the neurons under cyanide induced oxygen deprived condition.

We observed that in glutamate levels in cyanide toxicity/oxygen deprivation followed a proportionate 1:1 pattern with the level of SDH rather than the fall in G6PDH, rise in LDH and perhaps G6PDH/LDH ratio. For us to interpret these findings, we analyzed the pathway as thus;

We deduced three possibilities;

1. That glucose consumption and glutamate-glutamine cycling follows a 1:1 form in C6-glioma cells as it concerns the 2 ATP molecules from the glycolytic pathway is correct. But in oxygen deprived neurons toxicity response will require shunting glucose metabolism to the step 6 of the TCA cycle and follows 1:1 with this step as the energy yield of step 6 is approximately 2ATP. This can also be supported by the fact that the adult neurons only possess aerobic machinery for ATP assembly.
2. For the first assumption to occur, the step 8 of the TCA cycle is incorporated into a portion of the ornithine cycle; especially those involving the conversion of glutamate to α -ketoglutarate then fumarate; which is the energy product of step 6.
3. For hypothesis I&II to occur, the Na^+/K^+ ATPase pump must be kept active to constantly exchange Na and K ions across the neuronal membrane to generate the action potential in the neurons which seem to be a driving force in the cycling of glutamate to glutamine and glucose consumption.

In this study, we seek to investigate the role of Na^+/K^+ ATPase pump in governing the toxicity response of the

neurons to the assault (oxygen deprivation), and how the pump maintain the action potential of the neurons to drive energy production in response to the cells ATP requirement for the house keeping jobs. We seek to use a pump blocker to determine the hallmark of the Na^+/K^+ exchange in the excitotoxic process by measuring morphological parameters in treated perfused cortical tissue perfused in ACSF.

METHOD

All reagents were procured from Sigma Aldrich, Na^+/K^+ pump blockers were procured from Standard Pharma, Nigeria)

Preparation of ACSF

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 100ml (Khakhalin and Aizenman, 2012). 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.

Na^+/K^+ Pump Blockers

Digoxin and Promethazine were procured from Standard Biochem Co, Nigeria. 250 μg of powdered Digoxin was dissolved in 50ml dextrose saline solution (Kano *et al.*, 2011). 2ml ampoule of promethazine contains 50mg promethazine in dextrose saline; the solution was diluted by making up to 50ml in a clean glass measuring cylinder such that the new concentration is 1mg/ml (Adolph *et al.*, 2012).

Preparation of tissue for perfusion

Adult male wistar rat weighing 250gm was dissected to expose the brain using the method of Svendsen and Hau, 1994. Cortical tissue blocks of 0.5gms were obtained and washed in dextrose saline and was immediately transferred into a test tubes. Six tubes labeled A, B, C₁, C₂, D and E such that A contains ACSF only Control I), B (ACSF + KCN), C₁ (ACSF + KCN + Digoxin), C₂ (ACSF + KCN + Promethazine), D (ACSF + Promethazine), E (ACSF + Digoxin). KCN was added to the perfusion fluid at a concentration of 25mg/Kg of tissue, Digoxin concentration in ACSF was 0.3571 $\mu\text{g}/\text{Kg}$ while Promethazine concentration was 0.714mg/Kg. C₁ and C₂ were re-perfused with the blockers every 30 minutes while the overall process lasted for 180 minutes such that

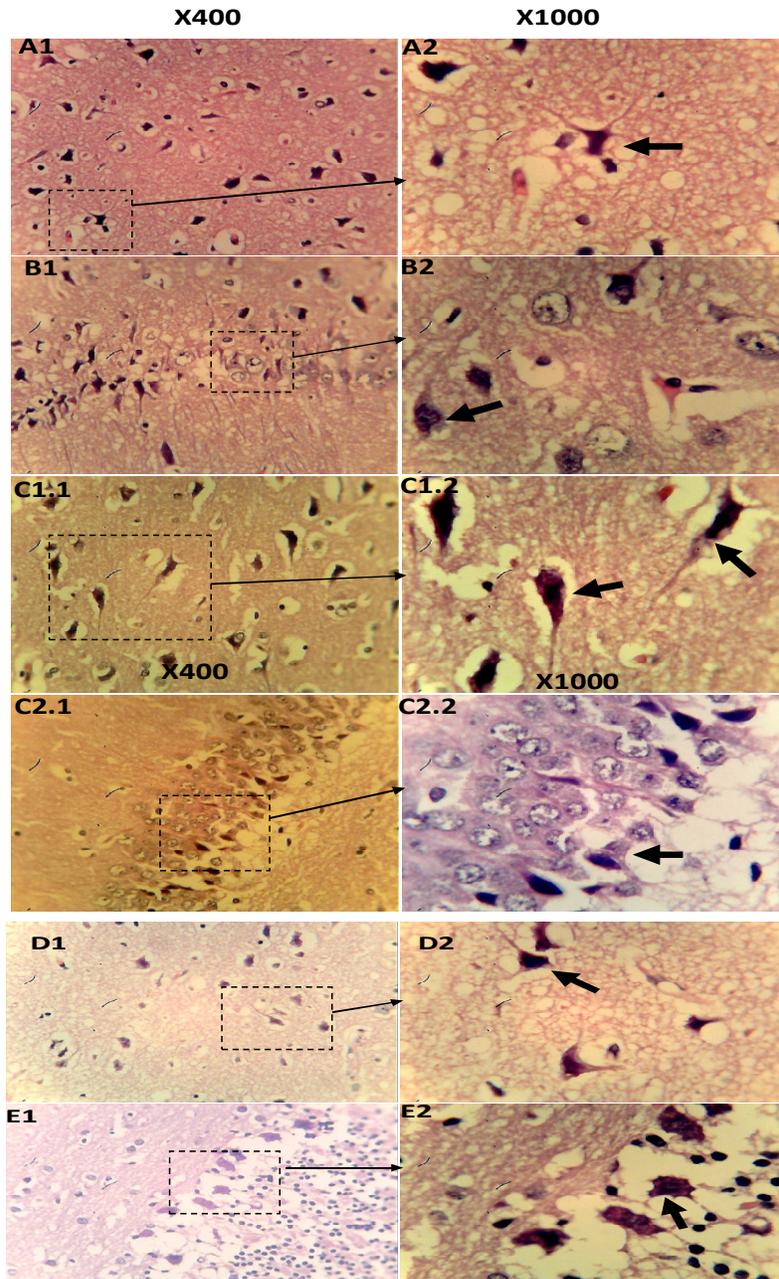


Figure 1. Demonstration of the general morphology of neurons of tissue treated *in vitro*; 1A (ACSF), 1B (ACSF+KCN), 1C1 (ACSF+KCN+Digoxin), 1C2 (ACSF+KCN+Promethazine), 1D (ACSF+Promethazine), 1E (ACSF+Digoxin) (Magnification X 400). Hematoxylin and Eosin staining using the method of Pearse, 1960.

the total volume of blocker perfused is between 150-250 μ m.

Perfusion

The test tubes A, B, C₁, C₂, D and E were connected to a

perfusion set up to run the respective ACSF (as described above for each tube) through the tube for 180 minutes, the set up was rocked gently at intervals to aid circulation and exchange in the tissue block. 10 μ l metronidazole was added to each tube as an antibiotic to prevent bacteria action. The set up was incubated at 37 $^{\circ}$ C.

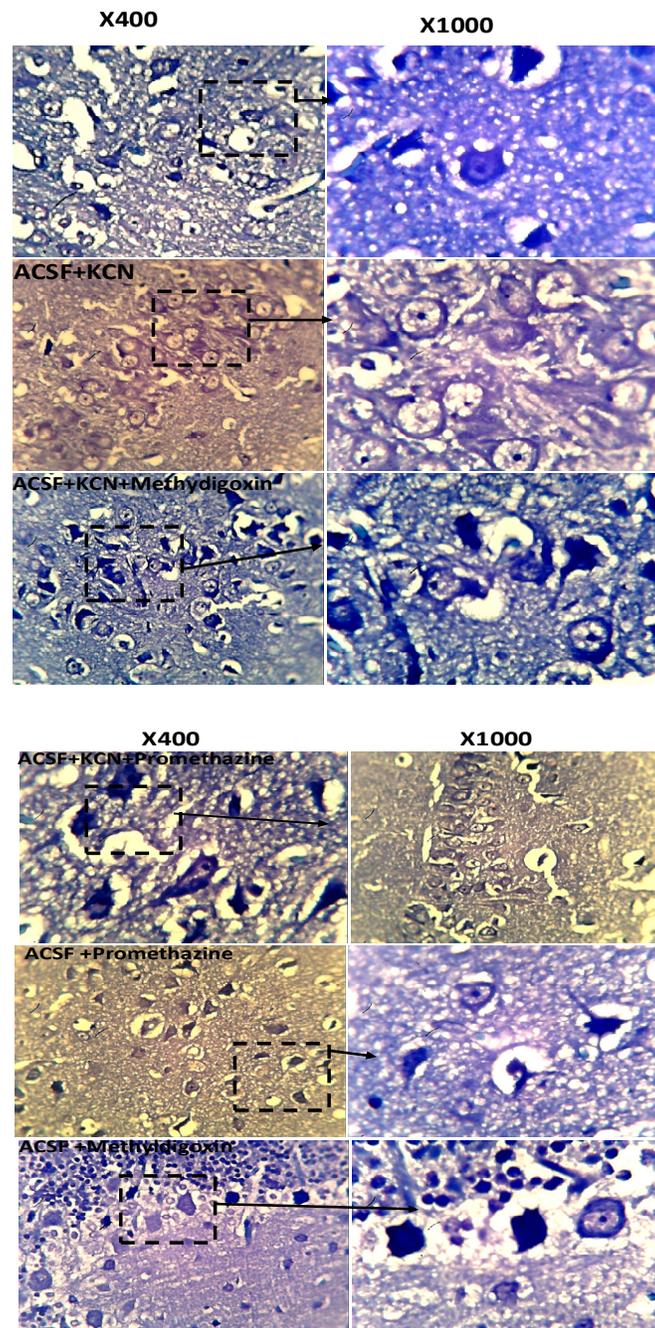


Figure 2. Loss of Nissl substances in the treatment groups as compared with ACSF only. The most drastic change was observed in ACSF+KCN+ Na/K Blocker (promethazine and Mathyldigoxin). Staining in Toluidene blue to demonstrate Nissl and cytoplasmic fragments in neurons.

Histology

At the end of the 180 minutes, the tissue blocks were obtained from the tubes and fixed in formalcalcium (Freshly

prepared) to be processed for histology and immunohistochemistry. Hematoxylin and Eosin staining was done using the methods of Pearse, 1960.

RESULT

ACSF Only

The tissue cultured in oxygen deficient ACSF was observed to have shown little structural changes. The most prominent change was presence of spaces around the neurons which are suspected to be stress or inflammatory response from the neurons and surrounding astrocytes. The cell diameter was about $3.5\mu\text{m}$ at a magnification of X100 (oil immersion). The axons were prominent and the cell body was well stained with hematoxylin showing the presence of cytoplasmic materials and a relatively normal appearing cell (Figure 1A).

ACSF+KCN

The excitotoxic effect of cyanide was prominent in this tissue; the cells are enlarged up to $5.5\mu\text{m}$ such that the width of the axon at the hillock was approximately $1.1\mu\text{m}$. Other cells were observed with ruptured membrane and distorted axons (labeled x and y in Figure 1B). This shows the excitotoxic effect of cyanide in oxygen deprived state; thus little ROS generation is possible in this state. This also justifies the major role of the excitotoxicity in cell changes induced by cyanide.

ACSF+KCN+Methyldigoxin

The toxicity response is seen to be amplified in this group, the cells are enlarged and highly distorted, cytoplasmic fragments were observed, axons are prominent, the rate of cellular degenerative changes proceeded at a much faster rate than ACSF+KCN (Figure 1C1).

ACSF+KCN+Promethazine

Excitotoxicity was observed as the neurons shows drastic changes (Figure 1C1) compared to ACSF+KCN (Figure 1B). The cytoplasm is fragmented and pale stained. The nucleus is eccentric and enlarged, at some point fragmented; this are possible signs of apoptotic cell death.

ACSF+Promethazine and ACSF+Methyl Digoxin

This is to investigate the effects of the Na^+ , K^+ pump blockers on the morphology of the neurons in oxygen deprived condition. The cellular changes includes cell enlargement ($6\mu\text{m}$) (Figure 1D and 1E), the nucleus was enlarged and eccentric, The cytoplasm was pale stained

but shows no sign of fragmentation as observed in Figure 1C1 and 1C2. thus, this shows that Na^+ pump blockers can mimic the effect of cyanide on neurons as cyanide is capable of excitotoxicity first before ionic imbalance, thus the major cell change is moderate cell enlargement believed to be induced by excitotoxicity. When over excitation occur, the membrane channels become blocked thus causing the imbalance at a much later stage of the excitotoxic process. In contrast both excitotoxicity and ionic imbalance can be achieved by the Na^+ pump blocker in a first instance, thus the cell enlargement is more prominent in Figure 1D and E (Na^+ , K^+ Pump blocked) than Figure 1B (KCN treated).

DISCUSSION

In this study, we inferred that for cyanide to induce excitotoxicity, it must potentiate a glutamate receptor (NMDA R1) mimicking glutamate to cause continuous calcium influx that drives action potential in the neuron (Rosenstock *et al.*, 2010). This is however possible since NMDA R1 is a calcium gated glutamate receptor (Ahmed *et al.*, 2002). The transient firing of the glutamate system is imminent since the neuron has no machinery to metabolize cyanide into an inactive form. Prolonged excitation of the neuron thus causes cell enlargement due to prolonged and continuous excitation (stress) (Tauskela *et al.*, 2003). In case of Na^+/K^+ pump blockade, K^+ is trapped in the ICF and Na^+ in the ECF thus causing ionic concentration imbalance such that the high concentration of salts in the ICF creates a system whereby water molecules diffuse into the cytoplasm thus causing the cell to enlarge (Kimmelberg, 2004). If this were so, the cell did not burst because the neuronal cell membrane like other membranes have been found to contain membrane in pleating that irons out under such conditions as observed in Figure 1D and 1E. In a second instance, blockade of the pump will impair glucose transport, since Na^+ is co-transported with glucose (Lee *et al.*, 2002). Following the propositions of Magistretti and Co-workers, 1996, a one ration one stoichiometry was observed for glutamate release and glucose consumption, thus they concluded that glutamate surge is the primary requirement for glucose consumption (Pellirini *et al.*, 1996). However in our own study in an oxygen deprived system, we deduced that the cell is dependent on glutamate cycling via the Krebs Tri-cycle to generate ATP at step 6 of the TCA cycle via the cycling of succinate-fumarate-oxaloacetate-glutamine-GABA-Succinate and back to fumarate (depicting the TCA, ornithine and GABA/glutamine/Succinate cycle; the latter is believed to have occurred in GABAergic nerve endings (Ehalge *et al.*, 2011). Thus we concluded that the primary requirement for glucose consumption is the energy need of the cell itself as against glutamate surge. In view of this, if the Na^+ pump is blocked, the co-transport of

glucose is also blocked such that the cell raises alarm for energy need. This will in turn facilitate the release of glutamate; since sodium cannot bring in glucose; transient release of glutamate is imminent, thus causing excitotoxicity as if cyanide were potentiating a glutamate receptor, thus excitotoxicity.

When a cell is under stress, the metabolic machinery of the neuron (glycolysis) will favor the conversion of glucose-6-phosphate to pyruvate/lactate as against proceeding into the pentose phosphate pathway (PPP) for formation of ribose sugar for DNA repair (Zhao *et al.*, 2008). Thus when the cell is deprived of glucose, transient glutamate release is imminent to request for glucose. Cyanide activates the NMDA R1 causing divalent calcium to flow into the cell, this also causes ionic imbalance when the sodium pump is blocked thus initiating accumulation of fluid. Since glucose cannot be transported into such a neuron, evidence of tissue respiration was not seen in the perfusion fluid (result not shown). On comparison with tissues where the sodium pump was blocked and no KCN was added under similar condition gave the same result as if cyanide were added.

At this point, we will like to critically evaluate the role of the Na^+/K^+ pump in excitotoxicity of cyanide. Cyanide will potentiate NMDA R1 as if it were glutamate to cause the influx of divalent calcium ions while, the Na^+/K^+ pump blockade will cause the cell to release glutamate in request for glucose following the co-blockade of glucose with Na^+ ions; this will create the same effects using two different mechanisms. In both cases the up regulation of Glutamate (NMDA R1) is a common factor either by introducing cyanide to bind to the NMDA R1 or depriving the neuron of glucose that causes glut release. The Na^+/K^+ pump blockade will inhibit the feedback mechanism necessary for respiration switch (to step 6 of TCA) in cyanide toxicity (Ogundele *et al.*, 2012). In another scenario of CN toxicity and no blockade of the sodium pump, the respiration can proceed up to step 6 of the TCA cycle by cycling succinate to glutamate back to succinate via the Krebs tricycle. Thus the excitotoxicity due to Na pump blockade is independent and can occur in the absence of cyanide toxicity. We therefore deduce that the blockade of the pump will compliment excitotoxicity of cyanide.

The cells generally will have two mechanisms of maintaining homeostasis in response to salt imbalance such as which can be caused by blockade of the Na^+/K^+ pump. Without these mechanisms in place (the pump being 1), osmotic forces take over. With more water and less salt outside the cell makes water flood the inside of the cell (Kimmelberg, 2004). Cell swelling will normally not occur except the pumps are blocked. In a second mechanism, overstimulation of receptors causes feedback blockade of membrane receptors. In this case, neuronal membrane reserves begins to flatten out, thus the channels in the membranes are affected by rapid exocytosis caused by the prolonged calcium influx.

CONCLUSION

Cyanide and pump blockers can induce excitotoxicity;

1. Cyanide potentiation of the NMDAR1 causes releases of calcium and prolonged AP
2. Blockade of the Na pump, impairs glucose transport thus causing, glutamate releases in another mechanism inducing excitotoxicity.
3. In both scenarios, ionic imbalance is affected causing swelling due to osmotic pressure.

We conclude that, excitotoxicity of CN was potentiated by the potentiated by the Na pump blockade, although they can occur as independent excitotoxic processes by upregulation of glutamate and disruption of ionic imbalance.

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