EFFECTS OF SODIUM NITRITE-INDUCED HYPOXIA ON CEREBELLAR PURKINJE CELLS IN ADULT RATS

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ABSTRACT

Objectives: To explore the changes in Purkinje cells in adult rats after inducing chemical hypoxia by sodium nitrite

Methodology: Adult male albino rats, weighing 180-200 gm were used in this study. The animals were fasted for three hours prior to drugs administration. One hour after sodium nitrite (75 mg/kg) subcutaneous injection, rats were decapitated. The brains were removed and placed overnight in fixative containing 10% formalin. Cerebellum were paraffin-embedded for hematoxylin and eosin staining and cut at 5 µm in the coronal plane. Fifty Purkinje cells along the line of cells from two randomly chosen portions of each section were counted at a magnification of x20. Cells in a total of five sections were counted to compute the ratio of damaged cells to intact neurons.

Results: There was no marked difference in the general appearance and thickness of the cerebellar cortices of the control and the sodium nitrite treated rats. The principal findings in the treated group were that almost all the Purkinje cells showed autolytic changes. Their arrangement as a single layer was also deranged in certain areas. Some areas showed complete loss of Purkinje neurons.

Conclusion: Sodium nitrite-induced hypoxia results in severe damage to the Purkinje neurons.

KEY WORDS: Hypoxia, Cerebellum, Purkinje cells, Sodium nitrite, Autolytic cells.

INTRODUCTION

Oxidative stress is implicated as one of the primary factors that contribute to the development of neurodegenerative diseases like, Alzheimer’s, Parkinsonism and neurological conditions like epileptic seizures, stroke, brain damage, neurotrauma, hypoxia etc.1 The highest degree of oxidative damage usually occurs in organs like brain, heart and skeletal muscles, since these organs are composed primarily of post mitotic cells. The central nervous system shows increased susceptibility to oxidative stress because of its high oxygen consumption rate (20% of the total oxygen inhaled by the body) that accounts for the increase in generation of oxygen free radicals and reactive
oxygen substances like superoxide radical (O$_2^-$), single oxygen (O$_3$), H$_2$O$_2$ and hydroxyl radical (OH). Even brief lack of O$_2$ causes an almost instantaneous loss of consciousness. Some parts of the brain that are especially involved in higher cognitive functions (including consciousness) must be very dependent on a rich supply of energy.

It has been known that during hypoxia electrical activity disappears especially early in the hippocampus and the cerebellum. Purkinje cells play a vital role in the normal functioning of the cerebellum. They are highly sensitive to a variety of pathological conditions such as ethanol and ischemia. Cerebellar Purkinje cells represent a group of neurons highly vulnerable to ischemic and excitotoxic stress. Short-term IH exposure elicits dose-dependent cerebellar Purkinje and fastigial neuron damage. Cerebellar structures, and particularly Purkinje cells, are preferentially targeted during chronic hypoxic exposure with hypoxic damage possibly induced by excitotoxic mechanism. Purkinje cells, the pyramidal cells arranged in a single row between the molecular layer and the granular layers of the cerebellar cortex, integrate multimodal afferent inputs and, as the only projection neurons of the cerebellar cortex, are key to the coordination of a variety of motor- and learning-related behaviors. Purkinje cells receive two distinct excitatory inputs: climbing fibers (the axons of the inferior olivary neurons) and several parallel fibers (the axons of cerebellar granule cells), and represent the only output of the cerebellar cortex, and the only output of the entire vestibulocerebellum. Purkinje cells play essential roles in modulating activity of deep cerebellar nuclei.

Nitric oxide is a regulatory biological substance and an important intracellular messenger that acts as a specific mediator of various neuropathological disorders. In mammals and invertebrates, nitric oxide is synthesized from L-arginine in the central and peripheral neural structures by the endothelial, neuronal and inducible enzymatic isoforms of nitric oxide synthase. Nitric oxide may affect the function of various neurotransmitter-specific systems, and is involved in neuromodulation, reproductive function, immune response, and regulation of the cerebral blood circulation. However under certain conditions its excessive formation may be an important mediator of the nervous tissue damage. NO in high concentrations is indirectly neurotoxic through various mechanisms, including iron mediated lipid peroxidation. It also liberates iron from cell stores and depletes cell energy by disruption of mitochondrial enzymes and nucleic acids. Release of NO may also trigger neuronal apoptotic cells death. This makes nitric oxide the main candidate in brain responses to brain ischemia/hypoxia.

Several experimental models have been used to recapitulate the human cerebral hypoxia syndrome. Sodium nitrite (NaNO$_2$) is commonly used for induction of hypoxia in experimental animal models. The administration of sodium nitrite in high concentrations may cause brain inflammation, ischemia and impaired cerebral energy. These effects were attributed to excessive free radicals generation and impairment of oxidant / antioxidant balance that finally aggravate cellular brain damage. In the present study we explored the effects of sodium nitrite-induced hypoxia on the Purkinje cells in adult rats.

**METHODOLOGY**

*Chemicals:* All drugs and chemicals used in the present study were of high analytical grade and were obtained from Sigma-Aldrich Co. Sodium nitrite were dissolved in normal saline.

*Animals:* Adult male albino rats, weighing 180-200 gm obtained from the animal house of King Saud University, were used in this study. They were fed with a standard laboratory diet and tap water ad libitum and housed in cages (ten rats per cage). All animals were kept at standardized laboratory conditions (25±5°C, 55±5% humidity, and a 12 h light/dark cycle). One week after acclimatization the animals were fasted for three hours prior to drugs administration. All experiments were carried out according to recommendation of King Saud University of Experimental Animals Ethics
Hypoxia results in severe damage to cerebellar Purkinje cells

Committee which is matched with international ethics for handling of experimental animals. The dose of sodium nitrite used in the current study, was matched with those in the literature.14

Brain Tissue Preparation: Rats were divided into two groups and were treated as follows: Group I (n= 2): served as control and received normal saline, Group II (n= 6) served as hypoxic rats and received sodium nitrite (75 mg/kg). One hour after sodium nitrite subcutaneous injection, rats were decapitated. The brains were removed and placed overnight in fixative containing 10% formalin. Cerebellum were paraffin-embedded for hematoxylin and eosin (H and E) staining and cut at 5 µm in the coronal plane. Fifty Purkinje cells along the line of cells from two randomly chosen portions of each section were counted at a magnification of x20. Cells in a total of five sections were counted to compute the ratio of damaged cells in control and hypoxic rats.

Statistical analysis: Results are expressed as means ± S.E.M. Statistical analysis for the comparisons between control and treated groups was performed by Student t-test. A P < 0.05 was accepted as significant.

RESULTS

There was no marked difference in the general appearance and thickness of the cerebellar cortices of the control and the treated rats. The control rats showed Purkinje cells arranged as a single row between molecular and the granular layer (Fig.1a). The principal findings in the treated group were that sodium nitrite induced extensive cerebellar Purkinje cell damage. Almost all the neurons were affected. The damaged Purkinje cells were categorized according to simple standards including swollen, autolysed, dark and shrunken characteristics which have been employed by others.16 Cell swelling is described as a cell with visible intact nucleus, with an increased cytoplasm/nucleus ratio of 20% or more compared to adjacent cell (Fig.2a, b). Autolytic cell are cells with no nucleus and no distinct cellular morphology (Fig.2a). Dark shrunken cell are small rounded shrunken cell filled with dark stain and has a darkened outline compared to adjacent cell (Fig.2a, b). At places, there was complete loss of Purkinje cells (Fig.2a, b), and some regions showed many dark shrunken cells arranged in more than one rows (Fig. 1b, c). The counts of

Fig.1a: Purkinje cells from normal group (long black arrows) arranged in a single layer between molecular layer and granular layer. Fig.1b: Damaged Purkinje cells from treated group. Fig.1c: Darkly stained shrunken cells in more than one rows. WM=white matter. M=molecular layer, G= granular layer x20

Fig.2: Purkinje cells from treated group: swollen cells (black arrows), autolytic cells (Red arrow), and shrunken cells (black arrowheads). The region between the red arrowheads shows total loss of Purkinje cells. WM= white matter. M= molecular layer, G= granular layer x20
DISCUSSION

Cell death is a critical component of normal nervous system development; too little or too much results in abnormal development and function of the nervous system. The brain is the body’s single largest consumer of oxygen. The structural and functional integrity of brain depends on regular oxygen supply. Although the brain represents only about 2% of the body’s weight, it utilizes about 20% of the body’s oxygen. As a result, the brain is especially sensitive to hypoxia. Brain cells are extremely vulnerable to fluctuations in the extracellular environment, including ischemic stress, trauma and infectious challenges, and can begin to die within five minutes after oxygen supply has been cut off. Hypoxia involves the loss of specific neurological functions caused by reduced blood perfusion in corresponding brain areas, in which neuronal and synaptic electrical activities are silenced. Symptoms of mild cerebral hypoxia include inattentiveness, poor judgment, memory loss, and a decrease in motor coordination. When hypoxia lasts for longer periods of time, it can cause cognitive impairment coma, seizures, and even brain death. Brain tissue damage can also result from hypoxia associated with ischemia in conditions such as stroke. The brain tissue induces protective mechanisms within minutes to limit the damage. However, these protective mechanisms are lost within hours of the hypoxic-ischemic insult. Despite the many coping mechanisms that have evolved to combat the effects of hypoxia, once the tissue has been damaged it is at risk of triggering apoptosis, and while the brain is particularly susceptible, within minutes of a stroke or heart failure, delicate neural tissue is already beginning to shut down as the processes that initially protected the brain begin to fail and irreversible damage ensues.

Brain tissue exposed to the hypoxic insult responds by glycolysis, angiogenesis, vasodilatation and erythropoiesis. Hypoxia-ischemia induces the expression of hypoxia-inducible factor-1α (HIF-1) and its target genes like erythropoietin, glucose transporters, the glycolytic enzymes, vasoactive substances such as vascular endothelial growth factor (VEGF) and nitric acid synthase (NOS) in the brain. Enhanced NOS expression has been reported in cerebellum in response to hypoxia. Enhanced expression of NOS results in increased production of nitric oxide which may be toxic to the cells resulting in their death. Upgraded expression of HIF-1 in the brain following exposure of rats to hypoxia has been reported. One such mechanism often invoked to explain neuronal susceptibility to stress is their response to glutamate. Hypoxic–ischemic injuries cause a significant increase in NO production that contributes to cytotoxicity.

Acute hypoxia affects sensitive regions of the brain like the cerebellum. The cerebellum underlies the control of posture and balance, fine coordination of motor movement, adaptation of ocular responses, and learning of some conditioned behaviors. Acute exposure to severe or moderate hypoxia is known to impair muscular coordination and postural stability. A number of morphological alterations such as cytoplasmic darkening, nuclear condensation and autolytic necrosis with cytoplasmic vacuoles have been reported in the Purkinje cells exposed to hypoxic insult. Darkening of the Purkinje neuron cytoplasm with dilatation of cisternae of rough endoplasmic reticulum and Golgi apparatus has been observed following exposure of animals to hypobaric hypoxia. In the rat cerebellar slice preparation, exposure to hypoxia elicited by a 30 minutes exposure to artificial cerebrospinal fluid continuously gassed with 95% N₂: 5% CO₂ induced a characteristic type of toxicity of Purkinje cells resembling excitotoxic-mediated dark cell degenera-

<table>
<thead>
<tr>
<th>Damaged Neurons</th>
<th>Normal rats</th>
<th>Hypoxic rats</th>
</tr>
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<tbody>
<tr>
<td>Swollen cells</td>
<td>12.5±3.5</td>
<td>83±5.1*</td>
</tr>
<tr>
<td>Autolytic cells</td>
<td>9.0±3.0</td>
<td>143±12.4*</td>
</tr>
<tr>
<td>Dark shrunken cells</td>
<td>5.5±2.5</td>
<td>254±11.9*</td>
</tr>
<tr>
<td>Total number ofDamaged cells</td>
<td>27±3.2</td>
<td>484±2.3*</td>
</tr>
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Table-I: Damaged cerebellar Purkinje cell counts in normal and Hypoxic rats
tion. A decrease in Purkinje cell number was observed in the adult cat exposed to chronic hypoxia. A decrease in Purkinje cell number was observed in the adult cat exposed to chronic hypoxia.30 Excitatory mechanisms, high density of calcium channels, activation of AMPA receptors are thought to render Purkinje cells vulnerable to excitotoxic death. Purkinje cells are selectively vulnerable to \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-mediated delayed toxicity that is manifested as dark cell degeneration rather than necrosis.32

Purkinje cells exhibiting dark and vacuolated cytoplasm resemble the pathology seen following stroke, trauma, cardiac arrest, hypoglycemia and seizures.33 Morphologically, Purkinje cells exhibited marked rounded appearance with cytoplasmic darkening, nuclear condensation and cytoplasmic vacuoles. The cell loss in animal models of cardiac arrest was analogous to that seen after cardiac arrest in humans with loss of over 60% of Purkinje cells following a 10 minutes arrest.31 The functions of the Purkinje neurons may be altered in response to an acute exposure to hypobaric hypoxia resulting in impairment of motor coordination.23 Motor disturbances have been observed in Purkinje cell degeneration mutant mice.34 Loss of Purkinje neurons or an alteration in their morphology has been reported in various forms of ataxias.35

The vulnerability of Purkinje cells in hypoxic or ischemic insult may be related to their high metabolic demand. The Purkinje cell is an exceptionally large (50–80 µm) inhibitory neuron in the cerebellum that receives extensive excitatory input from both parallel fibers (from granule cells) and climbing fibers (from the inferior olivary nucleus). Parallel fibers make about 200,000 connections on each Purkinje cell and input from these neurons trigger calcium influx.36 Climbing fibers release glutamate or aspartate at all levels of the soma and dendrites of the Purkinje cell firing synchronously, forming one of the most powerful connections in the nervous system.3 As a result of the high level of excitatory amino acid synaptic connections and the response of the Purkinje cell that is mediated by voltage-gated and receptor-gated calcium channels, the Purkinje cell has an exceptionally high metabolic demand. A high metabolic demand combined with constant input from the inferior olive and large amounts of calcium stores and influx, makes the cell vulnerable. Excessive rises in intracellular calcium is associated with excitotoxicity, and can cause cell death.38

**CONCLUSION**

It is concluded that cerebellar Purkinje cells are very vulnerable to hypoxic insult and sodium nitrite induced hypoxia results in a significant excitotoxic degeneration of these cells.

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**REFERENCES**

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