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Effect of Melatonin on Neuronal Nitric Oxide Synthase Expressing Cells in the Brain Following Global Cerebral Ischemia

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Abstract: Nitric oxide can be either neuroprotective or neurotoxic depending on which isoform is expressed during global cerebral ischemia. The chronological and spatial distribution and expression of neuronal nitric oxide synthase cells in the brain following ischemia and melatonin administration was studied in Sprague-Dawley rats. Global cerebral ischemia was induced by common carotid artery occlusion for 10 min followed by reperfusion. The rats were divided into three experimental groups. One group received 5 mg kg^{-1} melatonin 30 min before ischemia, another group received the same dose of melatonin post-ischemia and a third NO ischemia NO melatonin control group. All animals were euthanized 72 h post ischemia, perfusion-fixed with 4% paraformaldehyde in phosphate buffer and the brains removed. Immunopositive neuronal Nitric Oxide Synthase (nNOS) expression was observed in the cerebral cortex, putamen, caudate nucleus, substantia reticularis, olfactory bulb, nucleus caudatus, hippocampus and subcallosal cortex. No nNOS positive cells were observed in the cerebellum in any group. The nNOS expression was higher in the noischemia NO melatonin group (220) followed by the post-ischemia melatonin group (179) and the lowest (148) in the pre-ischemia melatonin group. A neuroprotective role by melatonin in the post-ischemic phase seems to be the mechanism of action associated with NOS activity in ischemic brain injury.

Key words: Neuronal nitric oxide synthase, immunohistochemistry, ischemia, melatonin, brain, Sprague-Dawley rats

INTRODUCTION

Nitric Oxide (NO) is a critical player in pathological and physiological processes in the Central Nervous System (CNS) (Szabo, 1996). NO is produced by the activation of three distinct nitric oxide synthases namely: neuronal NOS (nNOS/Type I/NOS-1), inducible NOS (iNOS/Type II/NOS-2) and endothelial NOS (eNOS, Type III/NOS-3). NO exerts neuroprotective and neurotoxic effects after focal cerebral ischemia (Dalkara and Moskowitz, 1994; Samdani *et al*., 1997).

NO is a potent vasodilator and an inhibitor of platelet aggregation and leukocyte adhesion that might improve post-ischemic blood flow by enhancing collateral circulation and preventing microvascular plugging by platelets and leukocytes (Kubes, 1995). On the other hand as suggested by Beckman *et al.* (1990), NO is a well-known cytotoxin which might adversely affect the ischemic brain by several mechanisms. In neuronal cultures NO, one of the redox forms of NO is toxic by producing peroxynitrate while Nitrosonium (NO^+) is protective (Lipton, 1993). It has been demonstrated that the different isoforms also produce different neurological effects, for example iNOS produces NO with cytotoxic effects whereas eNOS plays a protective role (Leon *et al*., 2005). Therefore, the biological properties of NO suggest that this agent can either be protective or destructive in cerebral ischemia.

Furthermore, reports have documented the neuroprotective actions of melatonin in experimental models of ischemia/reperfusion injury (stroke). In these investigations which used three species (rat, gerbil and cat), melatonin was universally found to reduce brain damage that normally occurs as a consequence of the temporary interruption of blood flow followed by the reflow of oxygenated blood to the brain (Reiter *et al*., 2003). Guerrero *et al.* (1997) used the gerbil model to examine the potentially beneficial effects of melatonin against an ischemic insult. The pineal gland and its major hormone melatonin is capable of translating

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environmental lighting information into signals that modulate reproductive, adrenal and other neuroendocrine interactions as well as immune functions. A considerable body of evidence suggests that the mammalian pineal gland is capable of influencing the central nervous system function. Reiter *et al.* (1980) showed that pinealectomy induces convulsions that seem to parallel alterations in midbrain dopamine, serotonin and norepinephrine levels.

Melatonin plays a neuroprotective role against transient or permanent ischemic brain injury. Little data is available regarding the levels of the nNOS isoform in acute ischemia followed by melatonin administration in pre- and post-ischemia. There are two periods of time after injury when NO accumulates in the brain, immediately after injury and then again 7 h or days later. The initial immediate peak in NO after injury is probably due to the activity of endothelial NOS and neuronal NOS (Cherian *et al.*, 2001). The aim of this study was to investigate the expression pattern of the neuronal nitric oxide synthase following global cerebral ischemia and melatonin administration in experimental rats.

MATERIALS AND METHODS

All procedures used were approved by the University of the Witwatersrand Animal Ethics Screening Committee which parallels the National Institute of Health (NIH) guidelines. About 18 Sprague-Dawley rats weighing between 250-350 g were divided into three experimental groups (n = 6 each). A NO ischemia and melatonin sham control group had only skin incision but NO melatonin administered. A pre-ischemic melatonin group had melatonin administered prior to ischemia. A post-ischemic melatonin group had ischemia followed by melatonin administration.

Melatonin administration: Melatonin (Sigma-Aldrich) was dissolved in 0.9% normal saline and a single dose of 5 mg kg^{-1} was administered intraperitoneally by injection at least 30 min before the induction of ischemia via the Common Carotid Artery occlusion (CCAo) in the pre-ischemic melatonin group. The post-melatonin group received 5 mg kg^{-1} of melatonin 30 min after the ischemia.

Surgical occlusion of the bilateral common carotid artery: This was as previously described (Iwai *et al.*, 2003; Ajao *et al.* 2010). In summary, all animals were anaesthetized by an intramuscular injection of ketamine hydrochloride at 20 mg kg^{-1}. The neck was exposed through a midline ventral incision and both Common Carotid Arteries (CCAs) identified. Bilateral CCAs were occluded with aneurysm clips for 10 min and the clips removed to restore cerebral flow. The rectal temperature was monitored and maintained at 37.0±0.5°C with a heating pad and pulse monitor during the operation. The surgical incision was closed and animals allowed to recover and then given free access to water and food at ambient temperature. Sham-operated control animals were treated identically, except for NO occlusion of the CCAs. All surgical procedures were done under aseptic conditions.

Perfusion fixation: After CCA occlusion and subsequent recovery for 72 h, the rats were euthanized with sodium pentobarbital, transcardially perfused with 0.9% cold saline (4°C) and then fixed with 4% paraformaldehyde in 0.1M Phosphate Buffered Saline (PBS) at pH 7.4. The brains were removed, weighed and post-fixed overnight in same fixative and then transferred to 30% sucrose in 0.1M phosphate buffer until equilibrated for subsequent cryoprotection. The brains were frozen in dry ice, sectioned in the sagittal plane at 50 μm thickness using a sliding microtome and sections placed in vials containing PBS. A one in six series of sections were taken from each rat brain for immunohistochemistry.

Immunohistochemistry for neuronal Nitric Oxide Synthase (nNOS): Brain sections were rinsed 3 times for 10 min in 0.1 M Phosphate Buffer (PB) while under gentle shaking, at room temperature. Brain sections were placed in a solution containing 50% methanol, 50% PB as well as 1.66% H_2O_2. Tissues was then pre-incubated for 2 h at RT in a blocking solution containing 3% Normal Goat Serum (NGS), 2% Bovine Serum Albumin (BSA) and 0.25% Triton x 100 in 0.1 M PB.

The sections were placed in the blocking solution containing the primary rat anti-neuronal Nitric Oxide Synthase (nNOS) antibody (1:600; Millipore, USA, Cat #: AB 1529) for 24 h at 4°C. The sections were rinsed three times for 10 min in 0.1M PB under gentle shaking at RT and then incubated in the blocking solution that contained the IgG secondary antibody (1:500; Millipore, USA) for 2 h at room temperature. Sections were washed 3 times for 10 min in 0.1M PB under gentle shaking at room temperature and then incubated for 1 h in AB solution at a ratio of 1-40 μL for reactive A added to reactive B followed by another 3 times rinsing. Sections were then treated in a solution containing diaminobenzidine (0.05% DAB in 0.1M PB) for 5 min at room temperature. Thereafter, 3 μL of 30% H_2O_2 per 0.5 mL solution was added. The reaction was stopped by placing the section in 0.1M PB and sections mounted on 0.5% gel-coated glass slides and air-dried overnight.

Sections were dehydrated through a graded series of alcohols, cleared with xylene and then cover-slipped with an Entellan mounting.

Definition of positively stained NOS neurons: The criteria developed by Trump *et al.* (1981) outlining the morphological features (light and stereo microscopy) of positively stained nNOS using immunohistochemistry techniques was adopted. Using light microscopy, positively stained nNOS was identified as a darkly stained nucleus and well defined long, slender dendrites which synapse with others. According to Otto *et al.* (2006), these features can be encompassed in these designations: pyknosis/eosinophilia (red neurons) or complete loss of hematoxylinophilia (ghost neurons). Other cellular alterations such as nNOS only showing the nucleus and not including completely illustrated dendrites were included in the neuronal counts.

Cell count of nNOS positive cells: For each brain, 8 sections containing the brain regions which had been stained for neuronal NOS were analysed. To avoid bias the slides were numbered according to the experimental classification number unique to the treatment with melatonin as well as the brain section from where it was taken. Using this manner of classification it was unknown to the examiner whether this animal had received melatonin before or after or had not received melatonin at all. This information was only recalled after the quantitative examination and morphometric analysis were completed. After the areas of interest were located, a grid was applied on the image and neurons were individually marked to avoid duplication of counting.

Images of each region considered were recorded with a Zeiss Axiocam HCR digital camera fitted to a Zeiss Axioskop 2 plus microscope and reversed on an Acer computer by the Axiom software. The number of positively stained cells was evaluated by three independent investigators on the digital photographs using the NIGH image program (Scion image), set as follows: threshold limits in the LUT window between 200 and 245; minimal particle size 5 megapixels. These conditions allowed the counting of all positively stained cells in the selected areas excluding occasional cell or fiber debris.

RESULTS AND DISCUSSION

Expression of neuronal NOS: With immunohistochemi stry, there were nNOS positive stained cells in the cerebral cortex, subcallosal cortex, putamen, olfactory bulb, caudate nucleus and hippocampus (Fig. 1). Stained neurons were randomly distributed or grouped into small clusters of 5-6 cells. The cells were of medium size, fusiform and lacked spines but with long defined dendrites. In the cerebral cortex and hippocampus, neurons were either scattered or in clusters (Fig. 1). Darkly stained neurons arranged in clusters or grouped together were found in the hippocampus. Within the substantia reticularis, a few scattered cells of moderate staining were present. In all 3 groups, there were NO positively stained nNOS cells in the cerebellum (Fig. 1e).

The morphology of positive nNOS cells presented as unipolar, bipolar or multipolar neurons (Fig. 1b-d). When visible as isolated neurons they showed characteristics of multipolar neurons. Whereas when neurons were expressed as clusters of cells they showed features of being mainly bipolar. This trend was observed throughout all the brain locations.

Quantitative analysis of nNOS: Positively stained neurons were most numerous in the subcallosal cortex, followed by the olfactory bulb. The area with the least number of nNOS cell was the caudate nucleus (Fig. 2). Figure 3 shows that the number of positively stained nNOS neurons was highest in the NO ischemia and melatonin group (220) followed by post-ischemic melatonin group (179) and then lastly by the pre-ischemic melatonin group (148).

In this study, the expression of nNOS cells following cerebral hypoxia-ischemia was determined. Since awarding the Nobel prize to R. Fuchgott, L. Ignarro and F. Murad for their discovery of nitric oxide as a biological mediator, a rapidly expanding body of data have indicated the importance of nitric oxide in the physiology of the central nervous system (Zhou and Zhu, 2009). NOS isoforms vary considerably in their distribution, regulation and function. The regional distribution patterns of nNOS positive cells were similar in the different experimental groups. The area where there was positively stained neuron was the same for all three groups except the fact that if the number of positively stained neurons tend to decrease in specific brain region.

Neuronal NOS plays a prominent role in neurodegeneration. Levels of nNOS increase in CCAo-induced brain injury and melatonin prevents the increase of nNOS levels. This hypothesis of Dalkara and Moskowitz (1994) correlated with the findings as there was a decrease in numbers of nNOS expressed in animals that received pre-and post-ischemic melatonin. This phenomenon could be due to two contributing factors. Firstly, melatonin easily crosses the blood-brain barrier and can thus be of effect immediately to counteract the formation of NOS by binding to appropriate receptors.

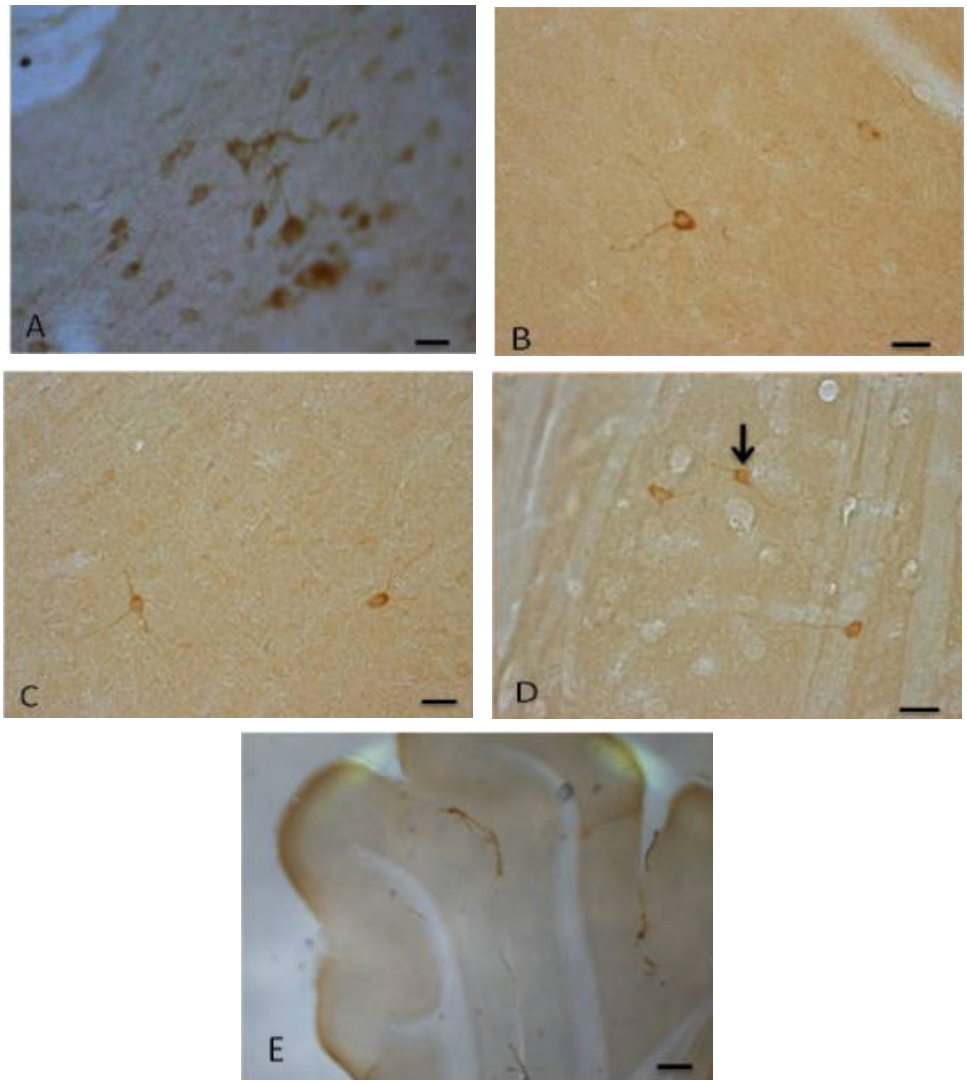

Fig. 1: Representative photomicrographs showing morphological characteristics of nNOS cells after global cerebral ischemia and melatonin administration in the Sprague Dawley rat. (a) Clusters of nNOS cells in the cerebral cortex. (b) Darkly stained cell body with well-defined bipolar dendrites visible. (c) Two isolated nNOS positively stained multipolar neurons in the olfactory bulb. (d) Bipolar nNOS neuron (arrow) in the hippocampus. (e) Cerebellum with NO staining for nNOS. Scale bar; a-d = 10 μm, e = 20 μm

Secondly, Van den Tweel *et al*. (2005) had determined that levels of nNOS change according to the time period after which ischemia has been induced. They reported a significant change in nNOS expression over time.

Even though, melatonin is administrated after ischemia induced brain injury, there would still be receptors to bind with thereby preventing the formation of NOS. This correlates with the findings which showed a decrease in nNOS expression in both groups receiving melatonin with very little variation between the groups. Moreover, nNOS knockout mice show decreased neuronal death after a cerebral ischemia (Eliasson *et al*., 1999).

A 10 min ischemic period due to bilateral occlusion of the carotid arteries followed by 5 min reperfusion was followed by a rise in cerebral nitrite/nitrate levels and cGMP concentrations. Melatonin given in pharmacological concentrations prior to interruption of the cerebral blood supply, prevented the changes reported above suggesting that melatonin inhibited NO production. This would be consistent with the observations of others (Bettahi *et al*., 1996; Pozo *et al*., 1997) who reported an inhibitory effect of melatonin on NOS. Cho reported that melatonin given 30 min prior to reperfusion onset was ineffective in reducing neuronal loss due to ischemia/reperfusion.

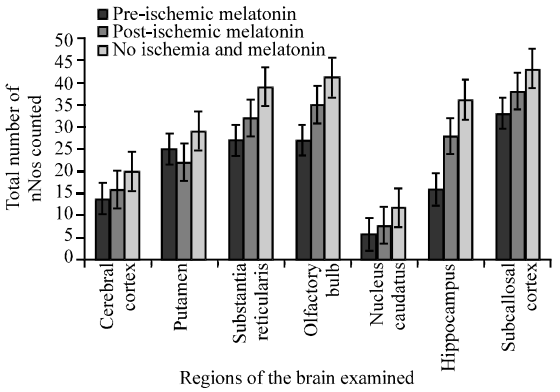

Fig. 2: Histogram showing the number of nNOS positive cells counted at different regions of the brain

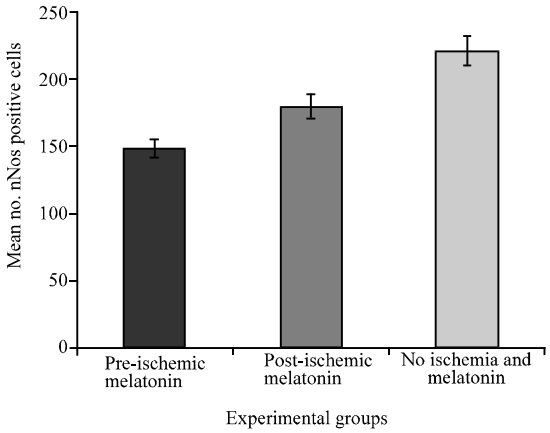

Fig. 3: Histogram showing the mean total nNOS cell count in each experimental group

When one takes into consideration the report that as early as 30 min after hypoxia-ischemia nNOS expression increased and this increase was maintained until 3 h after hypoxia-ischemia, the data tends to be in conflict as after 3 h the nNOS expression returned to baseline levels. The present study noted less nNOS in animals receiving melatonin before induction of ischemia than those receiving melatonin after ischemia. In addition to these findings, there is a clear difference between the two groups that did receive melatonin against that which did not receive any melatonin.

Another interesting observation was the absence of positively stained nNOS expression in the cerebellum of any of the experimental groups. This finding could be associated with the dose of melatonin given after CCAo-induced brain injury. The observation of lack of staining in the rat cerebellum correlates with research done by Pozo *et al*. (1994, 1997). These researchers were also the first to describe this phenomenon. Pozo reported a significant inhibition of enzyme activity (<22%) that was observed with 1nM melatonin which is in the range of the physiological serum concentrations of the hormone in the rodent and human sera at night (Reiter, 1991). The inhibitory effect of melatonin was observed exclusively in the presence of Ca^{+} whereas there was no effect in the presence of Ethylene Glycol-bis (Beta-Aminoethyl ether (EGTA). This corresponds with the production of Ca^{+} after CCAo-induced brain ischemia (which the experimental animals were exposed to). They then concluded that melatonin inhibits rat cerebellar NOS activity at physiological concentrations of the pineal hormone.

On the contrary, Barjavel and Bhargava (1995) using kinetic analysis of the rate of conversion of [3H]arginine to [3H]citruline in brain regions, observed highest activity of NOS in the cerebellum followed in decreasing order by midbrain, hypothalamus, cortex, striatum, pons-medulla, hippocampus and spinal cord in normal rat.

Although, the mechanism remains to be clarified, these results open a new area of investigation which could clarify further the physiological action of melatonin in the CNS.

Melatonin itself may be involved in the pineal effect on brain excitability as suggested by the ability of this hormone to suppress seizure activity in humans, a concept supported by the generation of epileptiform activity in rats that have received intraventricular injections of anti-melatonin sera (Fariello *et al*., 1977). Various aspects of human performance have also been found to be influenced by the administration of melatonin, i.e., reduced vigor and increased fatigue and confusion as measured by the Profile Of Mood States (POMS), Stanford Sleepiness Scale (SSS) and the Wilkinson auditory vigilance task (Lieberman and Lea, 1988). These and other functions of melatonin are consistent with the existence of the high affinity melatonin receptors in different areas of the CNS.

CONCLUSION

Overall, the results suggest that melatonin do reduce cell death caused by ischemic brain injury in the post-ischemic phase and the neuroprotective effects of melatonin are mediated through the inhibition of the nNOS isoform.

The hypothesis that melatonin protects neuronal cells in ischemic brain injury with the regulation of NOS isoforms by contributing to the protective effect of melatonin seems to be the mechanism which require further investigation.

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