

Melatonin Potentiates Cells Proliferation in the Dentate Gyrus Following Ischemic Brain Injury in Adult Rats

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Abstract: Melatonin, a potent free radical scavenger with neuroprotective property is thought to play some role in adult neurogenesis. The researchers investigated the influence of melatonin on cell proliferations in the adult dentate gyrus of the hippocampus in transient ischemia. About 36 adult male sprague-dawley rats were assigned into either the 72 h or the 7 days post ischemic duration groups. The pre-ischemic melatonin groups received 5 mg kg⁻¹ melatonin intraperitoneally prior to induction of ischemia. Rats were anaesthetised with ketamine and perfusion fixed with saline followed by 4% paraformaldehyde. Brains were removed with one hemisphere sectioned at 50 µm for Ki-67 immunohistochemical staining and the other hemisphere processed by plastic embedding, sectioned at 20 µm and stained with Giemsa stain. Ki-67 positive cells and pyknotic cells were counted in all stained sections with 100× oil-immersion objective to obtain the estimated total cell number. In the 72 h post-ischemic duration, the estimated total Ki-67 cells was 5964±1894 for the pre-ischemic melatonin group and lowest (2016±566) in the ischemic group. In the 7 day post-ischemic duration, the Ki-67 positive cells were 6900±2011 in the pre-ischemic melatonin and 2268±591 in the ischemic group. Pyknotic cell were highest in the ischemic group 462±52 and 486±46 in both the 72 h and 7 days durations, respectively. The results show that melatonin administration prior to ischemia enhances adult neurogenesis in the dentate gyrus of the hippocampus in adult sprague-dawley rat brain.

Key words: Ischemic brain, melatonin, cell proliferation, pyknotic cells, dentate gyrus, adult sprague dawley rats

INTRODUCTION

Adult hippocampal neurogenesis occurs in various animals and humans (Taupin and Gage, 2002; Eriksson *et al.*, 1998) and it occurs throughout life in the brain of adult mammals (Zhao *et al.*, 2008) but it is low or absent in bats (Amrien *et al.*, 2007). However, the functional roles of the newly generated neurons have remained controversial (Hauser *et al.*, 2009). Taxonomic closely related species show similar forms of adult neurogenesis though, levels of adult neurogenesis varies significantly among animals (Amrien *et al.*, 2004).

Focal and global ischemic stroke enhanced neurogenesis in the hippocampus and SubVentricular Zone (SVZ) and the newborn cells in the SVZ are able to migrate to the site of the injury (Lindvall and Kokaia, 2007). Cerebral ischemia alters normal pattern of adult neurogenesis in two ways: through its enhancement of cell proliferation within the SVZ and Sub Granular Zone (SGZ) and secondly by evoking the migration of neuroblasts into areas of damage (Thored *et al.*, 2006;

Zhang *et al.*, 2007). Melatonin, in addition to its neuroprotective activity, enhanced cell proliferation in ischemic brain indicating that the antioxidant activities also stimulate endogenous neurogenesis (Kilic *et al.*, 2008). There are reported modulatory activities of melatonin in the dentate gyrus in early post natal rats (Kim *et al.*, 2004) and its attenuation of the proliferation and differentiation of embryonic neural stem cells (Moriya *et al.*, 2007). Although, encouraging, the extent of cell replacement during ischemic brain injury is limited (Alagappan *et al.*, 2009). Hence, there is need to understand the adaptive mechanisms that occur in the brain as a result of the injury to enable therapeutic designs which will enable the brain to cope and achieve a more significant level of regeneration after the injury. Using a rat model of transient ischemic-reperfusion brain injury through bilateral occlusion of common carotid arteries. The researchers investigated at two time post ischemic durations, the extent of induced adult neurogenesis following administration of melatonin prior to ischemic brain injury in rats.

MATERIALS AND METHODS

Experimental animals and melatonin administration: The study was carried out according to the guidelines of the University of the Witwatersrand Animal Ethics and Screening Committee which parallel those set down by the National Institute of Health (NIH) for use of animals in scientific experiments. About 36 adult male sprague-dawley rats were assigned into either the 72 h or the 7 days post ischemic duration groups. Each of these time duration consisted of a no-ischemia-sham control group (n = 6) that was not subjected to Common Carotid Arteries Occlusion (CCAO) and no melatonin administered; an ischemic group (n = 6) that was subjected to CCAO but without melatonin administered and a pre-ischemic melatonin group (n = 6) that received 5 mg kg⁻¹ melatonin intraperitoneally 30 min prior to induction of global cerebral ischemia.

Surgical occlusion of common carotid arteries: The animals were anaesthetized by intramuscular injection of 20 mg kg⁻¹ of ketamine hydrochloride. The anterior neck region was exposed through a midline cervical incision and both common carotid arteries were identified and separated from the vagosympathetic trunk. The common carotid arteries were occluded with aneurysm clips for 10 min and the clips removed to restore cerebral blood flow (Iwai *et al.*, 2003). The rectal temperature was monitored and maintained at 37.0±0.5°C with a heating pad and the pulse monitored 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. The no-ischemia sham control animals were treated identically but without occlusion of the common carotid arteries. All surgical procedures were done under aseptic conditions.

Perfusion fixation: After surgery and subsequent recovery for 72 h or 7 days, the rats were euthanized with sodium pentobarbital, transcardially perfuse with 0.9% cold saline (4°C) and then fixed with 4% paraformaldehyde in 0.1 M Phosphate Buffered Saline (PBS) at pH 7.4. The brains were removed, weighed and post fixed overnight in same fixative and thereafter transferred to 30% sucrose in 0.1 M PB 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 brain for immunohistochemistry.

Ki-67 immunohistochemistry: The Ki-67 immunohistochemistry was done to estimate the rate of cell

proliferation. Free floating sections of the brain were incubated for epitope retrieval in citrate buffer pH 6.0 at 90°C for 40 min. This was followed by incubation in endogenous peroxidase blocking reagent, 0.6% hydrogen peroxide in TBS-Triton (0.05% Triton X-100 in TBS, pH 7.4) for 30 min at room temperature. Afterwards, sections were incubated with primary antibody Ki-67 (polyclonal rabbit NCL-Ki-67p, Novocastra, 1:5000 in TBS-Triton solution) overnight at 4°C. Incubation with secondary antibodies biotinylated goat anti-rabbit IgG (1:1000 + 2% NGS + 0.1%BSA in TBS) was performed for 2 h followed by incubation with streptavidin-biotin complex (Vectastain Elite ABC kit) and stained with DAB as chromogen. Until incubation with the primary antibody all rinses in between were made with TBS-Triton, afterwards with TBS alone. The primary antibody was omitted in the control tissue sections.

Plastic embedding: The hemisphere was dehydrated serially for a totally of 34 h in graded alcohol (4 h in 70%, 4 h in 96% and 26 h in 100% alcohol). The long dehydration time was employed because of the size of the brains. The brains were then embedded in glycolmethacrylate (Technovit 7100, Kulzer GmbH and Co, Wehrheim, Germany) in accordance with the manufacturer instruction and as described by Amrein *et al.* (2004). Sagittal sections of 20 µm thickness were cut using a metal knife on a Leitz rotary microtome.

Giemsa staining: Every 6th section was stained according to Iniguez *et al.* (1985). Incubation in Giemsa staining solution (Giemsa stock solution, Merck, Darmstadt, Germany) diluted 1:10 in buffer (67 mmol KH₂PO₄) at room temperature for 40 min, rinsed in 1% acetic acid for 10 sec and differentiated in 99% alcohol for 3 times, cleared in xylol and mounted with Eukitt.

Quantitative analysis

Proliferating cell count: Proliferative cells identified by Ki-67 immunohistochemistry were counted exhaustively in every sixth section on an Axiovision Light microscope using 100× oil-immersion lens and multiplied by the section sampling fraction to obtain the estimated total proliferating cell number in the study. Cells in the top focal plane of the section were not counted. All the Ki-67 positive cells in the subgranular layer and granule cell layer of the selected hemisphere were counted (Hauser *et al.*, 2009).

Pyknotic cell count: The pyknotic cells were identified in Giemsa stained sections by their strongly stained nuclei

with condensed chromatin peripherally into a C or doughnut shape, solid and sometimes with multiple cell bodies (Amrein *et al.*, 2004). The counting method used was same as for the proliferating cell count above.

Statistical analysis: Data were analyzed using one-way Analysis of Variance (ANOVA) followed by student t-test to detect significant differences between the means. All values are given as mean \pm Standard Deviation (SD) and $p < 0.05$ was considered statistically significant. Correlation analysis was performed between the number of proliferating cells against the no of pyknotic cells for the pre-ischemic melatonin and the ischemic group and also between the 72 h and 7 days time durations.

RESULTS AND DISCUSSION

Immunohistochemical staining of Ki-67:

Immunohistochemical staining of Ki-67 positive cells was examined in the subgranular and granular cell layers of the dentate gyrus of the hippocampus in the two each group. The proliferative cells were identified by their labelled nuclei and irregular shaped cluster appearing in both the no-ischemia sham control and ischemic brain which is characteristic of dentate gyrus progenitor cells (Fig. 1). For each experimental group, no nuclear labelling was observed in the control sections in which the primary antibody had been omitted.

Total proliferating cell number: In the 72 h post-ischemic time duration, the total number of Ki-67 positive cell in the pre-ischemic melatonin group (5964 ± 1894) was more than twice those in both the ischemic and no-ischemia sham controls (Table 1). This showed a statistically significant difference ($p < 0.05$) between the pre-ischemic melatonin and the no ischemia sham controls. The same observation was made within the 7 days post-ischemic time duration groups. There was a three-fold statistically significant increase in the proliferating cells (6900 ± 2011) in the pre-ischemic melatonin group compared to the ischemic sham control (Table 1). The cell proliferation seems to have been sustained with time as the figure after 7 days was higher than that of the 72 h post ischemia (Fig. 2).

Total pyknotic cell number: In the 72 h post-ischemic time duration the ischemic group had the highest number of pyknotic cells (462 ± 52) which was statistically significant ($p < 0.05$) compared to the no-ischemia-sham and the pre-ischemic melatonin groups (Table 1). In the 7 days post-ischemic time point, the ischemic group also had the highest number of pyknotic cells (486 ± 46). This was also statistically significant ($p < 0.05$) compared to theno-ischemia-sham and pre-ischemic melatonin group. In both the 72 h and 7 days post-ischemic between the rates of cell proliferation with that of cell

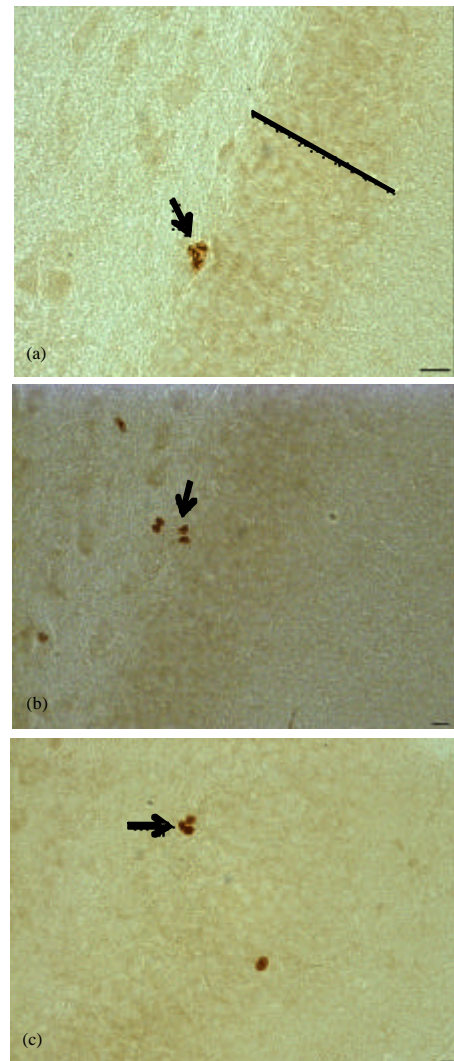


Fig. 1: Representative photomicrographs of Ki-67 positive cells in the dentate gyrus of the hippocampus. (a) is No-ischemia sham control; (b) 72 h pre-ischemic melatonin; (c) 7 days pre-ischemic melatonin. The clusters of proliferating cells in the subgranular zone are indicated by the arrows. The granular layer of the dentate gyrus is shown by the line in Fig. 1a. Scale bar: Fig. 1 a-c; 40 μ m

death as assessed by the pyknotic cell number. The relationship in the 72 h time duration appears linear in the no ischemia sham and the ischemic groups with positive correlation ($R^2 = 0.999$ and 1, respectively). However, there are no correlation between the total pyknotic cells count and the proliferative cells in the pre-ischemic melatonin groups ($R^2 = 0.001$), demonstrating that the stimulation

Table 1: Total proliferative cell count and pyknotic cell counts in the various experimental groups after administration of melatonin 30 min before the induction of 10 min ischemia

Experimental group	Proliferation cells		Pyknotic cells	
	No.±SD	p-value	No.±SD	p-value
72 h ischemic time				
No ischemia sham control	2076±583	NS	126±62	NS
Ischemic	2016±566	NS	462±52	p<0.05
Pre-ischemic melatonin	5964±1894	p<0.05	288±102	NS
7 days ischemic time				
No ischemia sham control	2652±673	NS	114±58	NS
Ischemic	2268±591	NS	486±46	p<0.05
Pre-ischemic melatonin	6900±2011	p<0.05	252±118	NS

No. = Mean total number of cells. SD = Standard Deviation. NS = Not Significant

required for the production of the proliferative cells is/are independent of the presence of pyknotic cells. This could be ascribed to the presence of the exogenous melatonin. The stimulation of cell proliferation 7 days following pre-ischemic melatonin administration enhanced the total number of pyknotic cells generated in the rat brains in the no ischemia sham and the pre-ischemic melatonin groups as indicated by the R^2 value ($R^2 = 0.823$ and 0.825 , respectively) but significantly weak in the ischemic group ($R^2 = 0.25$) showing a decline compared to 72 h group (Fig. 3a, b).

Cerebral ischemia is often caused by cardiac arrest, severe haemorrhage and cerebral blood flow <25 % of the normal required values (Carvanates *et al.*, 2008) and the longer the duration of occlusion, the greater the damage down to the brain and in most cases, it becomes irreversible. The enhancement of post-stroke neurogenesis and cell migration is expected to promote tissue repair and improve the functional outcomes of stroke (Zheng and Chen, 2007). Ischemia stimulates proliferation and differentiation of neural/stems progenitor cells and the newly generated neurons integrated into the already existing neuronal networks. Ischemia-stimulated neurogenesis in the dentate gyrus area is normally restricted to the dentate gyrus subgranular zone (Ming and Song, 2005).

Some Ki-67 positive cells are sparsely distributed within the distorted granule and subgranular layers and they are most likely to undergo apoptosis. Thus, the researchers agree with Wojcik *et al.* (2009) that endogenous regenerative capacity appears to be very limited and not capable of restoring the lost neuronal circuit. Melatonin enhanced cell proliferation in the ischemic brain, indicating that the antioxidant also stimulate endogenous neurogenesis. The presence of Ki-67 positive cells in the hippocampal region may not likely be an explanation for its enhanced cell proliferation activities but the clusters of abundant neural progenitor cells following ischemic stroke and melatonin administration strongly indicate that the melatonin may be

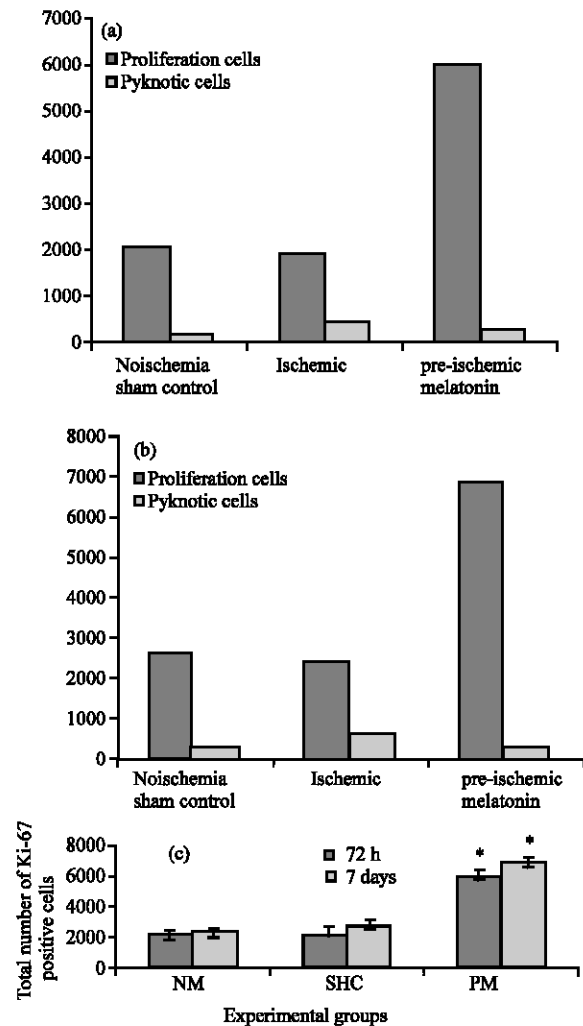


Fig. 2: Graph of the cell proliferation and pyknotic cells number in the different groups and time duration. Figure a and b represent the 72 post ischemic and 7 days post-ischemia time duration respectively. Figure c represents the number of cell proliferation in the different experimental groups. NM: No ischemia sham control group; SHC: Ischemic group; PM: Pre-ischemic melatonin group. The asterisk indicates statistical significance at $p<0.05$

responsible for the stimulation of the proliferative cells which are of neural origin. The findings indicate that melatonin do have some protective effects and modulatory role following ischemic-reperfusion brain injury and on proliferations of the neurogenic cells respectively. Though, ischemia alone does have some stimulatory effect on the proliferating cells of the hippocampus, this activity is potentiated by the presence of melatonin. The mechanisms by which melatonin does this may be related to its ability to reduce apoptosis after

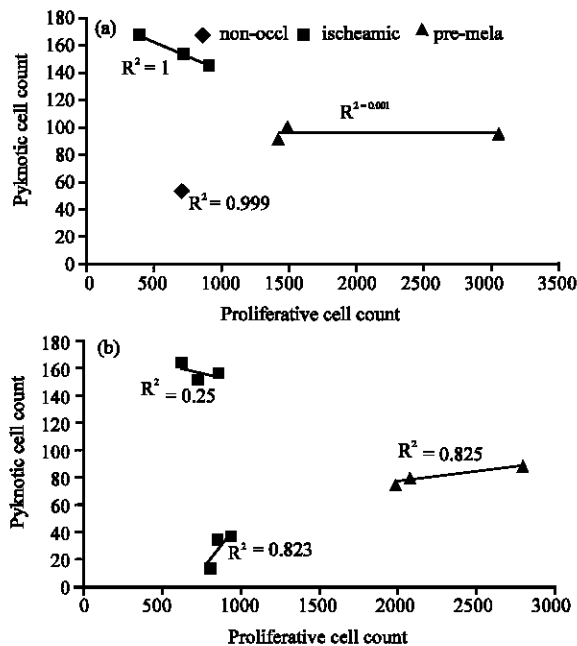


Fig. 3: Correlation between the rate of cell proliferation and rate of cell death. There was no correlation between the rate of cell proliferation cells and the rate of cell death. (a) is 72 h and (b) 7 days post ischemic time durations showing the correlation between total pyknotic cell count and the total proliferative cells count in the dentate gyrus. R^2 indicate the strength of the relationship between them

stroke as observed by Lin and Lee (2009) in which they found that melatonin reduces apoptosis after stroke and administering melatonin prior to transient ischemic stroke or at the onset of reperfusion restores the injury-induced by the ischemic-reperfusion injury in the brain. It is reported that across species the number of proliferating cells correlates significantly with the number of pyknotic cells indicating that in general, cell generation in the dentate gyrus is tightly linked to cell death (Amrein *et al.*, 2004). Since ischemic brain injury frequently disrupts neural functions in the central nervous system, it is important to understand the response of neural progenitor cells after ischemia (Yagita *et al.*, 2001).

The results do show that the ischemic injury resulted in more pyknotic cells compared to the no ischemia sham control. The presence of melatonin seems to have provided an improved environment such that cell death was reduced in both the 72 h and 7 days post ischemic groups. However, the severity of cell death was similar in the no ischemia sham control and pre-ischemia melatonin groups in both 72 h and 7 days post-ischemic time points. The cellular and molecular mechanisms that regulate post-ischemic appearance of newborn neurons in specific brain

structures are not clearly understood (Arias-Carrion and Ducker-Colin, 2007; Catts *et al.*, 2008). The researchers observed that the administration of melatonin prior to induction of 10 min transient global cerebral ischemia produced a more than 2-fold increase in cell proliferation compared to the ischemic and no-ischemic-sham control in the 72 h post-ischemic duration. This was extended to a three-fold increase in the pre-ischemic melatonin group after 7 days post-ischemic duration. The corresponding total number of pyknotic cells in the pre melatonin group was less compared to the sham control which did not receive any exogenous melatonin. This tends to indicate and also correlate with the neuroprotective activity of melatonin after focal cerebral ischemia in which there is endogenous neurogenesis which promotes recovery of neuronal functions as proposed by Rennie *et al.* (2008). The researchers therefore agree that the expansion of the pool of endogenous progenitors could augment regenerative capacity in response to the ischemic injury (Jin *et al.*, 2001; Arvidsson *et al.*, 2002). The low number of Pyknotic cells in the melatonin group is an indication of less cell death taking place and this may be related to the normal balancing out of proliferating and pyknotic cells during the normal process of adult neurogenesis. The total numbers of pyknotic cells do have a direct correlation to the total numbers of proliferative cells count in the presence of exogenous melatonin. Thus The belief was as a result of melatonin ability in potentiating the proliferations of the neurogenic cells in the hippocampal region of the ischemic brain. The differences in the total pyknotic cells count across the study groups was due to the activities of melatonin in reducing the cell death through inhibition of apoptosis and its protective activity following transient ischemia.

CONCLUSION

In conclusion, the results indicate that pre-ischemia melatonin administration do offer some protection against acute ischemic-reperfusion brain injury and seems to plays a modulatory role in stimulating the neuronal environment for proliferative cells to flourish.

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