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Effect of exogenous melatonin administration on transient global cerebral ischemia and adult neurogenesis

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Chapter One

1.0: General Introduction and Literature Review

1.1: Introduction

Stroke is defined as the clinical syndrome of rapid onset of cerebral deficit (usually focal) lasting more than 24 hours or leading to death, with no apparent cause other than cardiovascular (Kumar and Clark, 2007). However, stroke to the public means weakness and in some cases, complete paralysis of one half of the body (Hemiplegia) and in very rare cases, involving the entire upper and lower limbs (Tetraplegia), either permanent or transient, often with loss of speech. Stroke is a major and leading cause of disability with one third of the survivors in nursing homes and institutional centres (Dobkin, 2008). Certain individuals are at risk of neurological stroke and this group includes individuals with hypertension, atherosclerosis, heart diseases, and other cardiovascular disorders and those who smoke cigarettes (Kawachi et al, 1993; Wolf, 1997). There are reported increase incidences of ischemic strokes in individuals on hormonal therapy especially when they are 35 years and above (MacMahon and Rodgers, 1994). Clinical ischemic stroke is increasingly recognized as a sexually dimorphic disease (McCullough et al, 2005).

The brain requires a constant supply of glucose and oxygen which is delivered by circulation and accounts for 15% of the resting cardiac output and 20% of the total body oxygen consumption (Kumar and Clark, 2007). Cerebral blood flow, normally about 50 ml per minute for each 100 gm of tissue remains constant over a wide range of blood pressure and intracranial pressure because of autoregulation

of vascular resistance (Cotran et al., 1999). There are regional considerations and variations between the white and gray matters and among different portions of the gray matter with respect to the central nervous system blood supply. Cessation of blood flow can result from a reduction in perfusion pressure, as seen in hypotension, or it may be secondary to small or large vessel obstruction. When blood flow to a portion of the brain is reduced, the survival of the tissue at risk depends on a number of modifying factors: such as the availability of collateral circulation, the duration of ischemia and the magnitude and rapidity of the reduction of flow. The peri-infarct zone (adjacent areas to stroke damage) is critical especially during rehabitation, as it shows heightened neuroplasticity allowing for sensorimotor functions to re-map from damaged areas (Cramer, 2008; Brown et al, 2009; Dijkuizen, 2003).

Cerebrovascular accident often has devastating neuropathological, neurophysiological, and behavioural consequences that commonly results in permanent disability or death. Diseases of the brain have singularly adverse effects on the quality and duration of life and hemiplegia following middle cerebral arterial thromboembolism is a typical example. The pathologic consequences of neurological damage that follow a transient ischemic episode to the brain are often devastating, and in most situations, extremely difficult to correct because damage to brain tissue is more or less permanent (Bernatt, 1997). Unlike many other tissues, the mature brain has limited regenerative capacity, and its unusual degree of cellular specialization restricts the extent to which residual healthily tissue can assume the function of the damaged brain (Jin et al, 2001). To date, there are no pharmacological therapies available, especially in the promotion

of recovery. Studies have shown that the brain has a limited capacity for repair after stroke, and neural repair after stroke involves re-mapping of cognitive functions in the tissue that surrounds or connects with the stroke (Carmichael, 2006; Nudo, 2006).

1.2: Epidemiology: Types and Pathophysiology of stroke

Stroke is the second common cause of non communicable diseases death worldwide (Murray and Lopez, 1997) but ranked the third commonest cause of death in developed countries with ischemic stroke being the most common type (Pei, 2002). The age-adjusted annual death rate from stroke is 116 per 100,000 populations in the USA and some 200 per 100,000 in the UK. It is higher in black Africa populations than in the Caucasian as two-third of these deaths occurs in developing regions of the world, such as sub-Saharan Africa. Stroke is the most important cause of death and disability (more than any other chronic condition) for people over 50 years of age in South Africa (Fritz, 2006), and ischemic stroke is associated with high morbidity and mortality accounting for about 88% of all strokes globally (Davalos et al, 2010). Studies have consistently shown that women have a lower stroke incidence relative to men until an advanced age; however, the early outcome from injury resulting from stroke may be more favourable in males (Sudlow and Warlow 1997; Wyller 1999). Stroke is relatively uncommon below the age of 40 years but relatively more common in males aged 40 and below, but the reasons for this are not clearly understood (Kumar and Clark, 2007).

Hypertension is the most treatable identifiable risk factor in any form of stroke and the incidence of stroke decreases in the 40-60 years age range. In the elderly, it remains a major cause of morbidity and mortality with thromboembolic infarction accounting for 80% of the cases, cerebral and cerebellar haemorrhage for 10% and sub-arachnoid haemorrhages are responsible for about 5% of the major cerebrovascular problems leading to stroke (Kumar and Clark, 2007). Cerebral ischemia is often caused by cardiac arrest, severe haemorrhage, and cerebral blood flow less than 25 % of the normal required values (Carvantes et al., 2008) with intracranial haemorrhage causes around 10% of strokes. The incidence of stroke in developing countries especially the sub Saharan Africa is also expected to rise in the future as the populations undergo what is widely refereed to as health transition (Reed, 1990).

1.2.1: Types of Stroke

Following the pathophysiology and pathologic anatomy, it is convenient to consider stroke as two processes:

A) Hypoxia/ischemia resulting from impairment of blood supply and oxygenation of central nervous system tissue. This occurs when there is a generalised reduction of cerebral perfusion resulting in global cerebral ischemia as seen in cardiac arrest, shock and severe hypotension. A reduction in blood flow or cessation of blood flow to a localised area of the brain due to large-vessel disease such as embolic or thrombolic arterial occlusion will result in focal cerebral ischemia.

B) Haemorrhagic stroke: Haemorrhages may occur at any site within the central nervous system. In some instances, they may be a secondary phenomenon. Primary haemorrhages within the epidural and subdural spaces are typically related to trauma. Haemorrhages within the brain parenchyma and subarachnoid spaces in contrast are often a manifestation of underlying cerebrovascular disease, although trauma may also cause haemorrhage in these sites (Rudd et al., 2000).

1.2.2: Pathophysiology of Ischemia/Reperfusion Injury

Global cerebral ischemia is induced when the common carotid arteries are bilaterally occluded following severance or when the occlusion of the common carotid arteries are intermittently interrupted with each episode lasting for not less than five minutes (Laas et al, 1983, Kameyama et al, 1985). This processes decreased the amount of oxygen available for metabolic activities in the body and in the brain generally. Focal cerebral ischemia occurs following the occlusion of a regional blood vessel such as the middle cerebral artery (Pulsinelli and Brierley, 1979). However, brain cell death is not instantaneous, and its pathophysiology is often complicated and bilateral transient common carotid artery occlusion could result in different ischemic lesions in rat brains (Iwasaki et al, 1989, Nakano et al, 1989). Several experimental methods have been advanced in the induction of ischemia in rats because of their rich collateral circulations in the brain (Payan et al. 1965, Pulsinelli and Brierley, 1979, Mendelow et al. 1984).

Morphological disruptions in cerebral cortex do occur in acute brain ischemia, resulting from the cell death, and this as a result of cell membrane swelling and

those of the other internal organelles of the cell (Petty and Wettstein, 2001). The immediate mechanisms responsible for the disruption and ischemic reactions was thought to involve excitotoxicity and free radical generations, leading to inflammatory reactions and apoptosis, which may be responsible for the late ischemic brain changes seen in the ischemic brains (Paschen, 2000; Iadecola and Alexander, 200). Because ischemic stroke is caused by abrupt and or near-total interruption of cerebral blood flow to the brain resulting in ischemic changes with long term sensorimotor deficits, the severity and consequences of the neuronal damage is directly related to the duration and severity of the cause of interruption of the blood supply to either the entire brain or the region(s) of the affected area(s) of the brain. Neuronal cell deaths following ischemic brain injuries are not instantaneous, and its pathophysiology is complicated. However, the immediate sequelae of events that occur from severe brain ischemia leads to rapid brain necrosis, characterized by failure of the cell membrane with swelling of the cell and internal organelles (Cham, 2001; Leker and Shohami, 2002). One of the important mechanisms of ischemic brain damage is excitotoxicity and disturbed calcium ion homeostasis with generations of free radicals mediated ischemic damage during the intermediate stage. The subsequent inflammation and apoptosis cause delayed ischemic damage in the brain (Paschew 2002, Iadecola and Alexander 2001, Petty and Wettstein 2001). Ischemia generally causes uncontrolled release of glutamate and aspartate, and this often leads to excessive stimulation of different types of glutamate receptors, leading to influx of calcium ion, sodium ion, and water into the cells resulting in cell swelling and bursting (Lee et al 1999, Leker and Shohami 2002). The elevated intracellular calcium ion

concentration causes cellular oxidative stress and activation of lipases, proteases, and endonucleases, leading to damaged DNA, cell proteins and lipids that cause the cell membranes to swell and subsequently burst with liberations of the internal organelles. The brain is more susceptible to this conditioning because of its vulnerability to oxidative damage, since, it has high rate of oxidative metabolic activity, intense mitochondrial production of reactive oxygen metabolites, relatively low antioxidant capacity, low activity of repair mechanisms, high membrane surface to cytoplasm ratio and non replicating nature of neurons (Reiter, 1995). Reactive oxygen species and nitric oxide also participate in the pathogenesis of cerebral injury induced by ischemia and reperfusion. The oxygen free radicals generated are elevated not only during the cerebral ischemia phases alone, but also during the reperfusion phases because of the failure of the metabolic reactions required to remove the reactive oxygen radicals (Fujimura et al., 2005; Moro et al., 2005). The contributory role of apoptosis to the brain cell death is very important since study had showed that it may be responsible for up to 50% of cellular death in ischemia (Choi, 1996) and mitochondrial dysfunctions provide the intercellular signals for apoptosis and activation of the tumour necrosis factor superfamily receptors which constitute the extra cellular signals for apoptosis to take place could be detected in ischemic injuries.

Neuronal injury after an ischemic attack is an evolving process that continues for days after injury might have occurred (Clark et al. 1993, and Clark et al. 1998). Strong evidences now abound indicating that active, delayed injury processes are involved in, and ultimately determine, the degree of brain cell survival after ischemic injury (Barinaga, 1998), and the inflammatory response is a complex

and multi-step delayed injury process that includes gene upregulation and release of chemotaxic agents into the blood stream with activation of the peripheral leukocytes. Inflammatory cells not only remove cellular debris but also cause major post-injury toxins such as reactive free oxygen radicals and pro-inflammatory cytokines that are injurious to the brain cells (Stanimirovic and Satoh, 2000). Hence, inflammation plays a significant role in brain injury and should be a major focus in neuroprotection and regeneration therapy.

Ischemic stroke causes local (focal) ischemia when the blood supply to a portion of the brain is interrupted, such as when the blood vessels rupture or by a thrombus (clot) as often seen in an atherosclerotic patient. This may be further complicated by bleeding that percolates into the brain substances since haemoglobin is neurotoxic (Pulsinelli, 1985, Ginsburg, 1997). Ischemic brain injury is the most common clinical expression of cell injury by oxygen deprivation. Complete occlusion of one of the end arteries to an organ and examination of the tissue supplied by the artery is the most significant way of studying the effect of ischemic injury. Complex pathologic changes occur in diverse cellular system during ischemia, and with time, these alterations progress in severely, ultimately compromising vital structural and biochemical components resulting in cell death (Kumar and Clark, 2007).

1. 3: Current Advances in Neuronal Repair

Generally, the central nervous system (CNS) has a relatively inefficient enzymatic oxidative defense system; hence, during a life-time, the central nervous system suffers more than its fair share of oxidative stress, due to stroke or other age

related diseases. Treatment or action that reduces the incidence of stroke includes the use of drugs and consuming diets rich in fruits and vegetables that are high in antioxidants. Because ischemic stroke often leads to the loss of neural function due to neural cell death, neurogenesis after ischemia should be important for compensation for, and recovery of, these functions (Abe, 2000). Hence, any therapeutic measures that will enhance the stimulations and production of neurogenic cells for replacement of the loss neural cells will be ideal in the treatment module of ischemic stroke and any other brain diseases where neural cells are lost. This is more so, since there is evidence to demonstrate that neuronal death within the hippocampus provides the stimulus for increased neurogenesis following ischemia, as seen in limbic seizures that cause apoptosis of granules cell and increase dentate neurogenesis (Parent et al, 1997). It has been shown that mice subjected to middle cerebral artery occlusion were able to maintain neurogenesis probably to replace the loss neural cells; however, there are genetic variations in response to this from stimulation among different species of mouse strain (Yandava et al, 1999).

Brain injuries are amenable to repair depending on the duration of ischemia and the type of neural cell that are lost in the process. Restoration of blood flow to the ischemic tissue can result in recovery of cells if the cells are reversibly injured, and in some conditions, it is not possible; especially when the affected neural cells are irreversibly damaged. However, depending on the intensity and duration of the ischemic insult, variable number of cells may proceed to die after blood resumes, by necrosis as well as by apoptosis (Choi, 1996; Lieberthal and Levine 1996; MacLallan and Schneider., 1997). The pathologic changes that characterised the

recovery of ischemic cells following restoration of blood supply are described in reversible ischemic injury. However, with further prolongation of the ischemic duration, the cell structures continue to deteriorate, the energetic machinery of the cell in the mitochondrial and the glycolytic pathway becomes irreparably damaged and this result in irreversible ischemic brain injury (Cotran et al., 1999). Since the neural damage that results from the two phases of ischemia (Ischemic and reperfusion phases) is a consequence of a variety of negative factors (free radicals), in order to prevent further extensive damages, it is important to restore the blood supply to the ischemic tissue as quickly as possible; but in doing so, adequate and necessary precautions must be taken to avoid further aggregations of the neural loss because reperfusion with oxygenated blood could causes further molecular destruction and neuronal/glial loss (Zhong et al, 2003). This reperfusion process could lead to generations of more free radicals ($O3^{-)}$ that are neurotoxic, thereby worsening the situations and leading to delayed recovery of the neural cells.

In response to the oxidative damage of the brain following ischemic injuries from overproduction of reactive free radicals, the brain triggers molecular and cellular repair mechanisms that contribute to recovery and this may include ischemic activation of neurogenesis in the adult brain (Zhong et al, 2003: Pulsinelli, 1997).The challenges of the modern management of ischemic stroke thus, lies in finding a suitable agent(s) that will help in the curtailment and removal of the free radicals generated that are responsible for the destruction and causation of the neural cells death. Melatonin provides this protection through its scavenging ability and its capability in the removal of the generated free radical (Reiter et al,

2005). In addition to the removal of the free radicals, there is urgent need to establish early return of blood supply to the regions of the brain affected. In conclusion, it should be appreciated that ischemic/reperfusion injury is a clinical important process seen in conditions such as myocardiac infarction and stroke and they may be amenable to therapeutic interventions.

1.4: Predisposing/aetiological factors to ischemic stroke

There are numerous factors that predisposed individuals and communities to the development of stroke and in particular: ischemic stroke. These factors are sometimes dependant on age, sex, occupation and standard of living of individuals and communities. The major aetiological factors in the causation of ischemic stroke ranges from thrombo-embolic phenomenal in the brain arterial blood, atherosclerosis of the blood vessels and arteromatous plug (Cotran et al, 1999). There are high incidences of ischemic stroke among smokers, obesity, chronic untreated hypertensives and diabetes mellitus sufferers. Other predisposing factors are thrombocythaemia and thrombophillia (Protein C deficiency, factor V Leiden) which are weakly associated with arterial stroke but predispose to cerebral venous thrombosis. Polycythaemia predisposes to stroke while anticardiolipin and lupus anticoagulant antibodies causes arterial thrombotic stroke especially in young patients. Endocarditis, vasculitis, and migraine are rare causes of cerebral infarction (Warlow, 2003). Drugs such as cocaine, anti-hypertensive agents and overdose of counter cold remedies containing vasoconstrictions in chronic use inhibit the COX II and may result in increased incidences of stroke (Dobkin, 2005).

Bleeding into the substances of the brain through rupture of microaneurysms (Chartcot-Bouchard aneurysm) and degeneration of small deep penetrating arteries is the principal pathology in some of the aetio-pathogenesis of ischemic stroke. Such haemorrhage is usually massive, often fatal and occurs in chronic hypertension and act at well defined sites; basal ganglia, pons, cerebellum and subcortical white matter. In normotensive patients; particularly over 60 years, lobar intracerebral haemorrhage occurs in the frontal, temporal, parietal or occipital cortex. Cerebral amyloid haemorrhages, and the tendency to rebel is associated with particular apolipoprotein E genotypes (Ellegala and Day, 2005).

1. 5: Melatonin

Melatonin was discovered in 1958 and named after it activities in skin bleaching effect upon melanin. Its isolation and identification of N-acetyl-5-methoxytryptamine (melatonin) from bovine pineal tissue set the foundation for modern pineal biochemistry (Lerner et al, 1958). Melatonin is not only a natural mammalian hormones found in all species of animals, it is also widely found in plants, foods and microbes (Caniato et al, 2003; Paredes et al, 2009). It may also be produced in some peripheral cells such as bone marrow, lymphocytes and epithelial cells (Conti et al, 2000; Maestroni, 2001). Usually, its concentrations in these cells are much higher compared to that found in the blood; however, no food has been found to elevate plasma melatonin concentrations in humans (Coates and Paul, 2005). The half life of melatonin was estimated to be 19.8 minutes in rats (Yeleswaram et al, 1997) and with advance in age; it could increase to 35-50 minutes with its oral pharmacokinetics influenced by marked first hepatic metabolism (Lane and Moss, 1985). The plasma melatonin concentration

following infusion reaches a steady state after 60-120 minutes and may be equal to the nocturnal melatonin concentration (Mallo et al, 1990). The secretion of melatonin in the pineal gland and other organs are associated with darkness, thus exhibiting large rises in pineal melatonin, and this is independent of whether the animals are normally nocturnally or diurnally active (Goldman et al, 1981; Reiter et al, 1981; Wilkinson et al, 1987). Nocturnal melatonin production in the pineal gland varies among species and the significance of the different patterns of production is thought to be correlated to the influence the pineal gland has on reproduction (Rollang et al, 1977; Reppert et al, 1978; Petterburg et al, 1981). Melatonin is released primarily into the blood vascular system but is not stored in appreciable quantities in the pineal gland, and in some cases it is released into the cerebrospinal fluid (Hedlund et al, 1977). Melatonin plasma concentrations in mammals declined with age after early childhood, which might be a factor in the vulnerability of old people to infections and some neurodegenerative diseases (Pierpaoli et al, 1999). Many biological effects of melatonin are produced through its interaction with melatonin receptors and in some cases, it has some perversive roles especially as a powerful antioxidant with particular reference to nuclear and mitochondrial protection.

1.6: Neuroprotective role of melatonin in ischemia: hypothetical and postulated mechanisms

Melatonin has pleiotrophic neurobiological actions mediated through cell membrane receptors. It also has the ability to modulate proliferating activity of neurogenic cells in the dentate gyrus in early postnatal rats (Dubocovich, 2007 and Kim et al, 2004). In addition to its antioxidant neuroprotective activity,

melatonin enhanced cell proliferation in ischemic brain, an indication that it could also stimulate endogenous neurogenesis in the brain after ischemic stroke (Kilic et al., 2008). Transient focal or global cerebral ischemia causes extensive destruction of neural tissue because of the high vulnerability of central nervous system to the transitory interruption of its blood supply (Samantaray et al., 2009). However, endogenously produced and exogenously administered melatonin have the ability to reduce the degree of tissue damage and limit the behaviour deficits associated with the conditions seen in experimental stroke models (Reiter et al., 2005). The ability of melatonin to reduce inflammation, couple with its capability to protect cytoskeleton organization and its anti-apoptotic activities without interfering with the thrombolytic pathway cascade in the brain makes it safe to use in therapeutic interventions designed for managements of experimental stroke models in animals (Carvantes et al., 2008). Its ubiquity and multimodal protective mechanisms is an important factor for neuroprotective activities which makes for it exploration in the neuronal injuries (Reiter et al., 2001). Melatonin has been found to have some protective effects against focal cerebral ischemia mainly via its potent direct and indirect antioxidant effects on the free radicals that are generated during pathogenesis of ischemic stroke. This protective activity of melatonin was linked to its ability to decrease the size of the infarct, reduce fragmentation of DNA, decrease mitochondrial cytochrome C release and inhibit the activity of the caspase-3 enzyme when administered during the acute phase of a stroke (Jou et al 2004). Interestingly, the ability of melatonin to scavenge free radicals is not in a ratio of mole to mole. Indeed one melatonin molecule scavenges two hydroxyl anions and its secondary and tertiary metabolites which are generated when

melatonin interact with these free radicals, are themselves referred to as effective free radical scavengers (Acuna-Casteroveijo et al, 2001).

Melatonin scavenges and neutralizes the most damaging free radicals, the hydroxyl radicals, five times more efficiently than glutathione and is twice more effective in deactivating the peroxyl radical than Vitamin E (Reiter, 2000). It also stimulates glutathione peroxidase activity and inhibits nitric oxide synthase, thereby reducing the production of the highly toxic hydroxyl and nitric oxide free radicals that are mostly responsible for the neuronal cell deaths in ischemic strokes (Tan et al, 2000). Melatonin also had been found to play an important role in many different processes such as sleep disorders, ageing and mood disorders. It is also involved in the regulation of the circadian rhythm, and reproduction (Reiter et al, 2005). Melatonin's powerful antioxidant properties and effects couple with it lack of serious toxicity has recently evoked hope that it might be a promising drug for the treatment of various diseases in which oxidative damage is involved as seen in ischemic stroke of the brain (Kilic et al, 2005), hence, the reason for its use in experimental stroke models (Cheung, 2003). The relative ease at which melatonin crossed the blood brain barriers (BBB) makes it an agent of choice and of particular interest for use in brain injuries especially in ischemic stroke (Menendez-Pelaez et al, 1993). It normally has high concentrations in the ventricular cerebrospinal fluid, (Tricoire et al, 2002; Reiter and Tan, 2002), ubiquity in its antioxidative actions in rats (Reiter, 2000; Allegra et al, 2003), high efficacy (Tan et al, 1998; Reiter, 2000; Poeggebr et al, 2002), general neuroprotective activity (Reiter 1995, 1998; Cuzzocrea and Reiter, 2001), and a virtual absence of toxicity in humans and other mammals (Jhanke et al, 1999; Jan

et al, 2000; Seabra et al, 2000). Furthermore, melatonin has been successfully used in humans, to reduce free radical (oxidative) damage, and found to be highly effective (Fulher et al, 2001; Gitto et al, 2001); however, the effect of melatonin on adult neurogenesis remains unclear (Moriya et al, 2007).

Melatonin increased the number of new neurons without altering the proliferation rate of precursor cells (Ramirez-Rodriguez et al, 2009). It increased the survival of newborn neurons in the dentate gyrus and might modulate reactive neurogenesis in the ischemic striatum of adult mice (Kilic et al, 2008). It may also have modulatory effects on the several types of cell genesis in the adult brain. The effects of exogenous melatonin in adult neurogenesis might partly result from its antioxidant and antiapoptotic capacities (Reiter, 1998). Therefore, preservation of cytoskeleton organization by melatonin might be important for migration, differentiation, survival, and maturation of newborn neurons in the adult brain (Benitez-King, 2006). Melatonin has been previously reported to enhanced neurogenesis in vitro but no data on adult neural precursor cells from the hippocampus were available (Kong et al, 2008, Moriya et al, 2007). Pre-treatment with single intraperitoneal injection of melatonin at doses between 5 and 50 mg/kg was found to lead to a drastic reduction in the relative infarct volume and confers excellent protection against focal cerebral ischemia without reperfusion (Carloni et al, 2008 and Pei et al, 2002). The available findings were difficult to extrapolate to adult neurogenesis because embryonic and adult neural stem cells have distinct properties, the details which are not clearly understood.

1.7: Adult Neurogenesis: Mechanisms and Potential Role in Stroke

Neural stem cells are self renewing and multipotent cells that generate the main phenotypes of the nervous system such as neurons, astrocytes and oligodendrocytes. Characteristics of these cells vary in different physiological systems and thus it is still debatable as to whether stem cells in the central nervous system adhere to all of these criteria (Taupin, 2005). The possible medical gains of embryonic stem cell research are apparently immense. However, adult stem cell research has proven to be just as vital if not better than embryonic stem cell research (Shah, 2004). The possibility, now confirmed, of using adult stem cells to attain the same goals as would be sought with embryonic stem cells indicates that adult stem cells represent a more reasonable and humane method for making correct and sound progress in this new field of research and in the therapeutic applications which it promises. These applications are undoubtedly a source of great hope for a significant number of suffering people.

Adult hippocampal neurogenesis occurs in various animals and humans (Taupin and Gage, 2002; Eriksson et al, 1998), and throughout life in the brain of adult mammals (Zhao et al, 2008), but 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). Neurogenesis in the adult hippocampus appears to contribute to hippocampal function by allowing lifelong adaptation processes in the network of the mossy fibre system (Kempermann, 2008). Taxonomic closely related species show similar forms of adult neurogenesis, though, levels of adult neurogenesis varies significantly among animals (Amrien et al, 2004). Neurogenesis occurs in discreet regions of the adult brain, including the rostral

subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the dentate gyrus (Altman 1962, Kaplan and Hinds 1977, Kaplan and Bell 1984). Isolated cells from the adult mammalian forebrain form colonies of undifferentiated cells in vitro that can be dissociated to form many more secondary colonies, demonstrating renewal. Moreover, these cells are multipotent since they can be induced to differentiate into neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss 1992, Weiss and van de Kooy 1998, Gage 2000, McKay 1999). Stem cells give rise to other stem cells as well as progenitor cells. Progenitor cells give rise to neurons, astrocytes, and oligodendrocytes. Microglia appears to be derived mainly from blood borne stem cells (Gage 2000, McKay 1999). Adult neurogenesis is regulated by a wide range of physiopathological and environmental factors. An enriched environment and activities of learning and memory have shown an increased neurogenesis (Gage, 2000), whereas alcohol consumption, social isolation, central nervous system disorders and stressful stimuli reduce neurogenesis (Taupin, 2006). Studies also show that adult neurogenesis plays a functional role in hippocampal maintenance (Bull and Bartlett 2005).

Quantitatively, it has been estimated that secondary neurogenesis produces several thousand granule neurons per day, or the equivalent of one new neuron for 2000 pre-existing granule cells per day (Zupanc 1999). It should be underlined that in all vertebrate models (fish, birds, and mammals), neurogenesis and apoptosis occur simultaneously and appear to be tightly linked together, cell death being a possible factor triggering neural precursor proliferation (Scharff et al 2000, Gould and Tanapat-1997). The occurrence of secondary neurogenesis implies the neural

stem cells are not only present in the developing nervous system but also in the adult nervous system. Genetic influence on the three stages of adult neurogenesis - proliferation, cell survival and cell differentiation into other neurons was observed to differ in the dentate gyrus of the hippocampus in the following strain of mice: C57SL/6, BAL B/C, CD1 and 129/SVJ. Neural stem cell proliferation was highest in C57BL/6 mice whilst a greater net neurogenesis (that is the number of cells that become neurons) was observed in the CDI mice (Gage and Kempermann 1999). The reasons why progenitor cells persist in the adult central nervous system and give rise to new neurons in some particular brain structure remain unclear, and this question is of great interest. Furthermore, the fact that secondary neurogenesis takes place in structures involved in learning and memory raises the possibility that newborn neurons could participate in mnemonic process and thus improve behaviour adaptation of the animal to its environment (Fuchs and Gould 2000).

It is worth noting that, in both vertebrates and invertebrates, secondary neurogenesis occur in important brain structures exhibiting a high degree of structural plasticity and displaying remarkable analogies, as they receive multiple sensory informations and play a central role in learning and memory processes (Laurent and Daridowitz 1994, Buzsaki 1997). Although the functional role of the newly formed neurons remains questionable, the recent discovery of neurogenesis in the adult human hippocampus has definitely ruled out the concept of immutability of the adult brain structures (Erikson et al 1998). This discovery of neurogenesis and neural replacement in adult brain is likely to affect the ways in which we think about neurological diseases and neuronal repair (Mendez-Otero et

al 2005) and strengthens the hope that there may be a therapeutic response (Ihunwo and Pillay 2007). To date there are few reports on adult neurogenesis and the effect of environmental and pathological factors on neurogenesis in the Sprague-Dawley rat animal model.

The occlusion of the bilateral common carotid arteries causes almost complete neuronal death in rodents within the hippocampal CA1 region by 3-7 days after the injury (Imai et al, 2007). The duration of ischemia affect the level of the progenitor proliferation in the SGZ but not by the severity of the CA1 death (Liu et al, 1998). Global ischemia enhanced neurogenesis in the SVZ area (Iwai et al, 2003, Pforte et al 2005) and newly generated progenitors in the SVZ express many immature neuronal markers (Tonchev et al, 2005). Stroke may also results in an increased cell proliferation which may lead to an increase in the subventricular zone (SVZ) and migration of the newborn cells into the striata projection neurons (Arvidsson et al, 2002, and Jin et al, 2001).

It is reported that in mouse with transient middle cerebral artery occlusion (tMCAO), there was induction of enhanced cell genesis in the SVZ of the lateral ventricle (Luo et al 2005). However, the ischemia induced neurogenesis produces cells that may have the capacity to replace the loss neurons since the new cells are found to be migrating to sites of brain ischemia (Jin et al 2003, and Nakatomi et al 2002). The mechanism by which ischemia induced neurogenesis is thought to be due to neuronal death within the hippocampus which then provide stimulus for increased neurogenesis following ischemia, while growth and mitogenic factors could also be said to play a role in the ischemia induced neurogenesis (Sharp et al 2002).

1.8: Main objective of this study

To investigate the effect of melatonin in transient global cerebral ischemia and adult neurogenesis in Sprague -Dawley rats

1. 8. 1: Specific objectives

1. To study histological changes associated with transient global cerebral ischemia in ischemic, pre-ischemic melatonin and post-ischemic melatonin groups.

2. Examine the ischemic-induced adult neurogenesis in the dentate gyrus of the hippocampus using Ki-67 as a marker for proliferating cells, and Giemsa staining for pyknotic cell and estimation of total granule cell number.

3. To study the rate of cell proliferation cell death and estimated total granule cell numbers following ischemia and exogenous melatonin administration.

1. 9: Rationale for the study

Stroke and other neurodegenerative diseases are seen more in our environment and they often leave the sufferer with chronic debilitating conditions. Treatment of these conditions had been mainly supportive in nature which in most cases are very expensive and time consuming.

There are reported modulatory activities of melatonin in the dentate gyrus in early post natal rats (Kim et al, 2004) and it attenuation of the proliferation and differentiation of embryonic neural stem cells (Moriya et al, 2007). Melatonin, a potent endogenous free radical scavenger and antioxidant play a significant role in reducing cerebral damage after ischemia/reperfusion injury (Tan et al 1998, and

Reiter, 1998). Melatonin influenced proliferation and differentiation of embryonic neural stem cells and increased differentiation of rat's midbrain stem cells (Kong et al, 2008).

The outcome of the work is to provide an insight into whether administration of melatonin prior to ischemia or post-ischemia can better reverse the effects of ischemic stroke. It will demonstrate the likely influence of melatonin on adult neurogenesis in the pre- and post-ischemic phases.

1.10: Limitation of the study

The study was limited to use of adult male Sprague-Dawley rats in creating an ischemic model of transient global cerebral ischemia, the use of Ki-67 as a marker of proliferating cells for adult neurogenesis; the use of optical fractionator techniques for estimation of total granule cells in the dentate gyrus and the effects of exogenous melatonin administration on all these parameters.

CHAPTER TWO

2.0: Materials and Methods

2.1: Pilot study to create an ischemic model

In order to establish and developed which time durations post induction of transient global cerebral ischemia will best create an ischemic model in adult male Sprague-Dawley rats, 10 adult male Sprague-Dawley rats was used for a pilot study. Haematoxylin-Eosin staining was used to examine ischemic area created in different brain regions (cerebrum, hippocampus and the cerebellum) with respect to the different time durations post induction of the ischemia. The rats were divided into four experimental groups with three rats allocated to each of the time durations of 24, 48 and 72 hours respectively, post induction of ischemia and the tenth animal used as the experimental sham control as recommended by the Animal Ethic and Screening Committee (AESC) of the University of the Witwatersrand. The animals were anaesthetized by intramuscular injection of 20 mg/kg of Ketamine. The neck was exposed through a midline ventral incision and both common carotid arteries (CCAs) identified. Both CCAs were occluded with aneurysm clips for 5 minutes (Tanaka et al, 2004), and the clips were then 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 during the operation. The surgical incision was closed and the animals were allowed free access to water and food at ambient temperature. The animals were observed for any symptom of neurological deficits. The animals were euthanized at the expiration of their time durations of 24, 48 and 72 hrs, but the sham control was

euthanized along with the 72 hours group. To perfuse and remove the brains, the rats were deeply anesthetized with intramuscular injection of 20 mg/kg of ketamine and transcardially perfuse with 0.9% normal saline. 4% paraformaldehyde in 0.1M PBS (pH 7.4) was then infused until generalised perfusion was achieved. The brains were carefully removed, weighed and post fixed overnight in 4% paraformaldehyde. The brains were then transferred to 30% sucrose for cryoprotection pending histological analysis of the sections.

2.2: Experimental animals use in second study

The study was carried out according to the guidelines of the University of the Witwatersrand Animal Ethics and Screening Committee (AESC), which parallel those set down by the National Institute of Health (NIH) for use of animals in scientific experiments (AESC No 2007/35/05). Fifty Sprague- Dawley rats were used for the study. Adult male rats with a weight in the range of 250-350 gm were obtained from the Wits Central Animal Service. The animals were allowed about 7-10 days in adjusting to the cages before being exposed to the experimental procedure. The rats were divided into 8 experimental groups each comprising of 6 rats (Table 2.2.1). The 2 point duration of 72 hours and 7 days post-ischemia was chosen because it represents the acute phase of ischemic stroke (Kumar and Clark, 2007).

Groups	Description	Reference term
Group A	Non Surgical Control (No incision, No occlusion)	Non surgical control
Group B	Skin incision only, No occlusion of common carotid arteries and no melatonin administered	No ischemia Sham control
Group C	Skin incision, 10 minutes bilateral occlusion of common carotid arteries without melatonin administration and the brain removed at 72 hours post surgery	Ischemic group 72 hours
Group D	Skin incision, 10 minutes bilateral occlusion of common carotid arteries without melatonin administration and the brain removed at 7 days post surgery	Ischemic group 7 days
Group E	Skin incision, 10 minutes bilateral occlusion of common carotid arteries with melatonin administration 30 minutes before surgery and the brain removed at 72 hours post surgery	Pre-ischemic melatonin 72 hours
Group F	Skin incision, 10 minutes bilateral occlusion of common carotid arteries with melatonin administration 30 minutes before surgery and the brain removed at 7 days post surgery	Pre-ischemic melatonin 7 days
Group G	Skin incision, 10 minutes bilateral occlusion of common carotid arteries with melatonin administration 30 minutes after the surgery and the brain removed at 72 hours post surgery	Post-ischemic melatonin 72 hours

Group H	Skin incision, 10 minutes bilateral occlusion of common carotid arteries with melatonin administration 30 minutes after the surgery and the brain removed at 7 days post surgery	Post-ischemic melatonin 7 days

Table: 2.2.1: Animal groups with the description of the experimental procedure for the study

2. 3: Pre and Post melatonin administration

The choice of a single dose 5 mg/kg of melatonin intraperitoneal administration in the study was based on reported observations that higher doses of melatonin demonstrate no significance difference in its protective ability especially in ischemic-reperfusion brain injuries (Pei et al., 2002 and Touzani et al., 2001). Melatonin (Sigma) was dissolved in 0.9 % normal saline and a single dose of melatonin at 5 mg/kg was given via an intraperitoneal (I.P) injection at 30 minutes before the onset of common carotid artery occlusion (CCAO) in the pre-ischemic melatonin groups (Carloni et al, 2008). For the post-ischemic melatonin groups, 5 mg/kg of melatonin was given intraperitoneally 30 minutes after ischemia had been induced. The animals were sacrificed either 72 hrs or 7 days post ischemia. The rats in the ischemic groups (72 hours and 7 days) had occlusion surgery but were not given any dose of melatonin.

2. 4: Surgical preparations and procedure

Based on the results of the pilot study where five minutes transient global cerebral ischemia was induced through the occlusion of the common carotid arteries and without any mortality, there was need to increase the possible infarct areas but

stay within the 30 minutes reported in literature (Rennie et al, 2008). The initial time duration of occlusion was therefore increased from five minutes to 10 minutes. The surgical procedure for the occlusion of the common carotid arteries is described below.

1. The animals were anaesthetized by intramuscular injection of Ketamine at 20mg/kg.

2. The neck was exposed through a midline ventral incision and both common carotid arteries (CCAs) were exposed and identified.

3. Both CCAs were occluded with aneurysm clips for 10 minutes, and the clips were then removed to restore cerebral blood flow (Iwai et al 2003).

4. The rectal temperature was monitored and maintained at 37.0 ± 0.5°C with a heating pad during the operation.

5. The surgical incision was closed and the animals were allowed free access to water and food at ambient temperature.

6. Sham-operated control animals were treated identically, except for the non occlusion of the CCAs.

Note: - All surgical procedures were done under aseptic conditions in the presence of a Veterinary Doctor and Nursing Assistant.

2.5: Collection of Blood samples

Blood samples were collected from the rats (both control and experimental groups) for the estimation of melatonin concentrations. To avert the diurnal changes associated with melatonin concentrations in the serum, all the blood samples were collected in the morning and this was kept constant throughout the duration of the study.

1. To minimize stress, singly housed males and pairs were transferred into their holding cages from the animal room to the blood collection room, where they were immediately anesthetized with 0.2 ml Xylazine and Ketamine (4:1 ratio).

2. Thereafter, 0.5– 1.0 ml blood was taken by cardiac puncture and the rats were then immediately euthanized.

3. Mean duration for blood collection (including transport between rooms) was about 4 minutes.

4. Blood samples were collected into non coagulant bottles and allowed to clots, centrifuged and the subsequent serum fraction was transferred to a polypropylene tube and frozen on dry ice. Adequate care was taken to prevent auto-haemolysis of the blood samples.

5. Samples were stored at − 80 °C prior to hormone quantification.

2.6: Perfusion Fixation and Removal of the Brains

1. The rats after blood collection were deeply anesthetized with intramuscular injection of 20 mg/kg of ketamine and transcardially perfuse with 0.9% normal saline.

2. 4% paraformaldehyde in 0.1M PBS (pH 7.4) was then infused until generalised perfusion was achieved.

3. The brains were carefully removed, weighed on sensitive chemical balance and post fixed overnight in 4% paraformaldehyde.

4. The brains were then transferred to 30% sucrose for cryoprotection.

2.7: Assay of melatonin concentration in the serum

The ELISA procedure for the quantitative measurement of melatonin in the serum samples after modification from commercial source (IBL, Hamburg, Germany) was used. Melatonin was measured in duplicate using a commercially prepared radioimmunoassay. Briefly, this assay is based on the competition principle and the microtiter plate separation.

1. First, each sample was passed through a C18 reversed phase column, extracted with methanol, evaporated to dryness, and reconstituted with distil water.

2. Then each sample was added to the corresponding well coated with the goat-anti-rabbit antibody of a microtiter plate.

3. An unknown amount of antigen present in the sample and a fixed amount of enzyme-labelled antigen competed for the binding sites of the antibodies coated on the wells.

4. After incubation for 1 hour at room temperature, the wells were washed to stop the competition reaction.

5. p-nitrophenyl phosphate (PNPP) substrate solution was added and the concentration of antigen which is inversely proportional to the optical density measured.

6. Melatonin standards were used to construct a calibration curve against which the unknown samples were calculated.

7. All samples were in a single assay run and the intra-assay coefficient of variation was less than 10%.

8. The sensitivity of melatonin assay was ~3.0 pg/mL.

2.8: Sectioning

Each brain was divided into a right and a left hemisphere with a sharp blade. The left hemisphere was used for histological staining and immunohistochemistry for each of the six rats used per experimental groups. The left hemisphere was frozen in dry ice and sectioned at 50 μm in sagittal plane using Leitz rotary microtome (Zeiss Microm HM 430, Germany). The sections were in series of six throughout the hemisphere used.

2.9: Haematoxylin and Eosin Staining

This staining was to demonstrate the cyto-architecture of the brain region of interest, namely the cerebrum and dentate gyrus of the hippocampus. The sections were immersed in Haematoxylin solution for about 5 minutes, washed in water and counter stained with Eosin solution for about 2 minutes. The sections were then decolourized in graded xylene and cover slipped using Depex.

2.10: Ki-67 Immunohistochemistry

To identify the proliferating cells in the dentate gyrus, every sixth section was selected for immunohistochemistry using Ki-67 using polyclonal rabbit NCL-Ki-67p (Novocastra). The procedure was as described below:

1. The Ki-67 immunohistochemistry was done using free floating sections of the brain incubated for epitope retrieval in citrate buffer, pH 6.0, at 90˚ C for 40 minutes in a water bath.

2. The sections were rinsed in TBS-Triton for three times of 10 minutes.

3. They are incubated in endogenous peroxidase blocking reagent, 0.6% H_2O_2 in TBS- Triton (0.05% Triton X-100 in TBS, pH 7.4) for 30 minutes at room temperature (RT).

4. The sections were rinsed in TBS-Triton for three times of 10 minutes.

5. Sections were incubated with primary antibody Ki-67 (polyclonal rabbit NCL-Ki-67p, Novocastra, 1:5000 in preincubation solution) overnight at 4˚ C.

6. The sections were then rinsed in TBS for three times of 10 minutes.

7. The sections were incubated with secondary antibodies using biotinylated goat anti-rabbit IgG 1:1000 + 2%NGS +0.1%BSA in TBS for 2 hours.

8. The sections were rinsed in TBS for three times of 10 minutes.

9. They are then incubated with streptavidin-biotin complex (Vectastain Elite ABC kit) and stained with DAB as chromogen (sections were treated in a solution containing diaminobenzidine (DAB) in 0.1 M PB for 3 minutes. Thereafter, 3 µl of 30% H_2O_2 per 50.5 ml of solution were added. Once an appropriate level of background staining observed, the reaction was stopped by placing sections in 0.1 M PB).

10. The sections were rinsed in TBS for three times of 10 minutes.

11. The sections were then mounted on glass slide and allowed to air dry over 2- 3 days.

2.11: Plastic Embedding Technique

The right hemisphere of the brains were embedded in glycolmethacrylate (Technovit 7100, Kulzer GmbH & Co, Wertheim, Germany) over a period of 9 days in accordance with the manufacturer instruction and described below:

1. Fixation of the brain in 4% paraformaldehyde

Day 1

2. The brain was washed in PBS for four times of 10 minutes each.

3. Serial dehydrations in graded alcohol

a. Ethanol 70% 4 hours

b. Ethanol 96% 4 hours

c. Ethanol 100% 26 hours

The long time duration of dehydration was employed because of the size of the brains.

Day 2

4. Pre-infiltration in Technovit base solution (100 ml Technovit 7100 + 1g hardener 1) in 100 ml of 100% ethanol for 2 hours (the mixture ratio 1:1)

5. Infiltration in Technovit base solution (100 ml Technovit 7100 + 1g hardener 1) for 48 hours

Day 5

6. Embedding in Technovit base solution + 1g hardener II (15 ml Technovit base solution + 1 ml hardener II). Only a small portion of the solution was made at a time as polymerization goes rather fast and shaking the solution gently for 1 minute, followed by these processes:

i. ¼ of the embedding solution was poured into the mould forms, with the bottom filled up 1 – 2 mm. The form was protected from light while waiting for the solution to get sticky.

ii. The tissue was put in the form in the desired orientation for cutting (medial surface to face the predetermined cutting surface) and was not allowed to sink to the bottom with enough space around the tissue.

iii. The form was filled up with the embedding solution, covered and incubated for 1 hour at 37^0C and allowed to harden at room temperature over night.

Day 6

iv. Labels were stuck to the plastic blocks for identification. The forms were subsequently removed and the blocks allowed to air dry at room temperature over a couple of days (minimum of three days).

2.12: Giemsa Staining

The processes for the Giemsa staining of the plastic embedded tissues are:

1. Glycolmethacrylate –embedded hemispheres were cut sagittally at 20 µm with a metal knife in a Leitz rotary microtome.
2. Every sixth section was stained according to Iniguez et al., 1985, summarized below
3. Incubation in Giemsa staining solution (Giemsa stock solution, Merck, Dermstadt, Germany) diluted 1:10 in buffer (67 mmol KH_2PO_4) at room temperature for 40 minutes.
4. Rinsed in 1 % acetic acid for 10 seconds and differentiated in 99 % alcohol for 3 times.

5. Cleared in xylanine and mounted with Eukitt.

2.13: Light Microscopy analysis

Light microscopy analysis of the general architecture of the cerebrum, cerebellum and dentate gyrus of the hippocampus was done using a Zeiss Axioskop 2 plus light microscope (Germany), fitted with a Zeiss Axiocam HRc camera (Germany).

2.14: Total Ki-67 Positive cell count

The proliferating cells were counted exhaustively in every sixth section on an axiovision light microscope using 100X oil-immersion lens and multiple 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 (SGL) and granule cell layer (GCL) of the selected hemisphere were counted (Hauser et al., 2009).

2.15: Total Pyknotic cell count

The pyknotic cells are located within the subgranular zone and granular layers of the dentate gyrus. They were identified by their strongly stained nuclei with condensed chromatin peripherally into a C or doughnut shape, solid and sometimes, with multiple cell bodies following Giemsa staining (Amrien et al., 2004). The pyknotic cells were counted exhaustively in every sixth section on an Axiovision light microscope using 63X eye lens and multiplied by the section sampling fraction to obtain the estimated total pyknotic cell number.

2.16: Total granule cell count

This procedure was conducted at the Institute of Anatomy, University of Zurich, Switzerland.

The Giemsa stained brain sections were analyzed using a Zeiss Axioplan 2 light microscope mounted with a sonny DXC-930P colour video camera.

1. The total granule cell number in the dentate gyrus was estimated using the optical fractionator's principle as described by West et al., 1991.

2. Stereo Investigator software 7.0 (MicroBrightField Inc, Williston, USA) couple to a Zeiss Axioplan 2 light microscope with a 63X oil-immersion lens was used.

3. The sum of the areas of the dentate gyrus was calculated for all sections, x-y steps of approximately 20 µm providing 265 sampling sites.

4. The optical dissector was set to a counting frame measuring 12 x 12 and the optical dissector height maintained at 10 µm.

5. Cell counts were performed using x63 oil-immersion lens and the section thickness was measured at every fifth sampling sites.

6. This sampling scheme was applied to all of the sagittal series.

2.17: Statistical Analysis

SPSS version 12 was used to analyse the result. All the tests were non-parametric, and the differences between groups were assessed by one-way analysis of variance and post hoc tests were carried out for ANOVA between the quantifiable

variables. $P \leq 0.5$ was considered to be significant using two-tail student's t-test distribution. Correlation was calculated between the variables (melatonin, granule cells, proliferating cells and pyknotic cells) to determine their relationship and strength of the correlation. Calculated ratios of log of proliferation and pyknotic cells number was demonstrated on a regression graph. Graphical representations were also used to present the data for ease of interpretation.

Chapter Three

3.0: Results

3.1: Pilot study report

All the rats used in the pilot study survived the surgery and effect of anaesthesia. There was no mortality recorded with the animals used in the pilot study after the bilateral occlusion of the common carotid arteries of 5 minutes. The mean average weight of the brains was 2.05 ± 0.15gm. Clinical manifestation of stroke such as weakness and paralysis of the limbs were not classically demonstrable in the 5 minutes of transient ischemic stroke in these animal models. The vulnerable rat brain regions investigated in the pilot study are those that have been reported to show some histological changes after reversible global ischemia, namely, the cerebral cortex, hippocampus and the cerebellum (Martinez et al, 2007). The layers of the cerebral cortex were not distorted and the neural cells were within normal shape and size. There were no marked histological, cyto-architectural and neuropil disruptions in the 24 and 48hours in all the slides. There were no significant differences in the 24 hours, 48 hours and normal control. Ischemic brain injuries in the brain begin to show from 72 hours post induction of ischemia. Histological findings demonstrated disruption of the cyto-architecture and neuropil in the cerebral cortex (figure 3.1A, B, C and D) and hippocampal regions of the brain (figure 3.1.2A, B and C). There are ischemic morphological changes and loss of the usual characteristics arrangement of the white and gray matter structures. There were predominant neurophic emigration and large vacuolated cells. Cytotoxic and vasogenic edema were evident (figure 3.1.3A, B, C and D).

Figure: 3.1.1: Representative photomicrograph of Haematoxylin and Eosin staining of the cerebral cortex from the pilot study following 5 minutes ischemia. (A) Normal cyto-architecture of the cerebral layers following ischemia, (B) Demonstrate no area of ischemic reaction after 24 hours post induction of transient brain ischemia (C) show no distortion in the neuropil of the cerebrum after 48 hours post ischemia, (D) Demonstrate areas of ischemic reaction after 72 hours post induction of 5 minutes transient global cerebral ischemia.
Magnification: A, B, C and D = X400

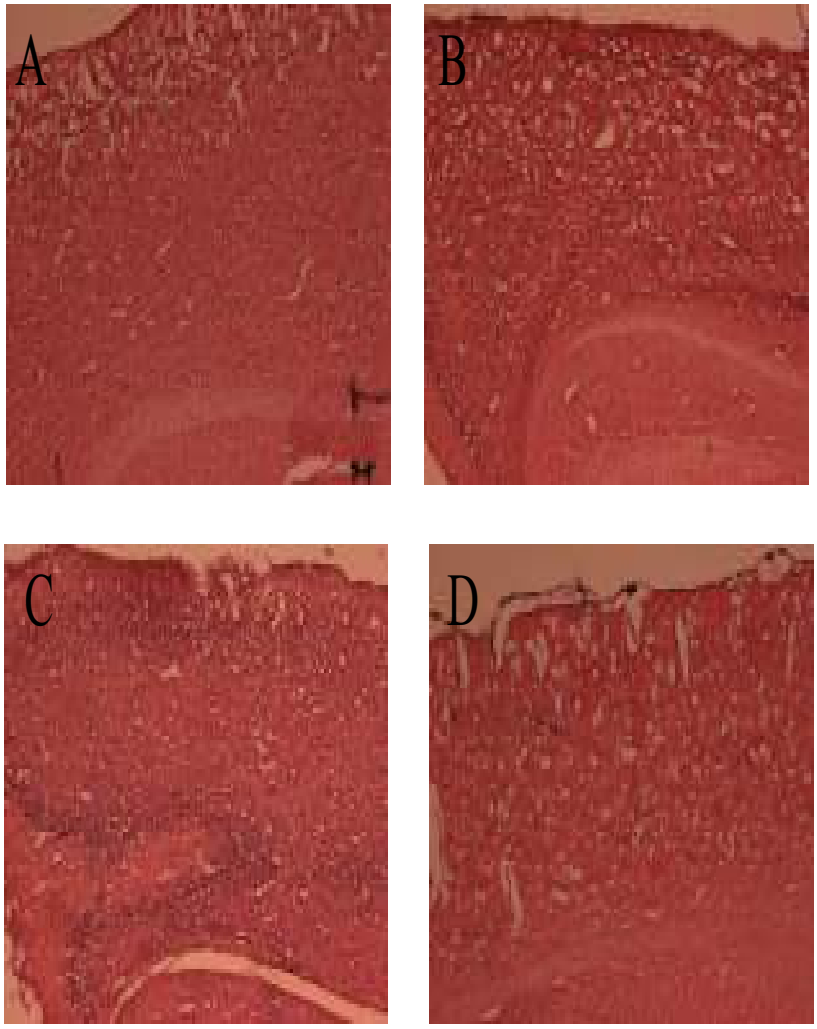


Figure 3.1.1(A, B, C and D)

Figure: 3.1.2: Representative photomicrograph of Haematoxylin and Eosin staining of the hippocampal region from the pilot study following 5 minutes ischemia (A) Demonstrate no area of ischemic reaction after 24 hours post induction of transient brain ischemia (B) show no distortion in the neuropil of the hippocampal region after 48 hours post ischemia, (C) Demonstrate areas of ischemic reaction after 72 hours post induction of transient global hippocampal region. Magnification: A, B and C = X400

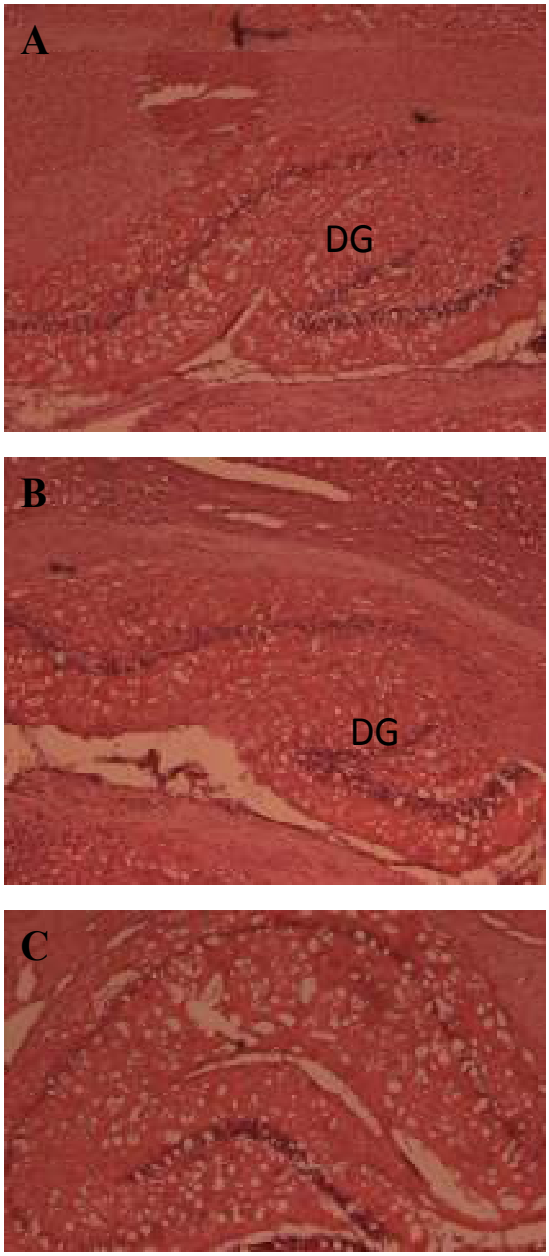


Figure 3.1.2(A, B and C)

Figure: 3.1.3: Representative photomicrograph of Haematoxylin and Eosin staining of the cerebellum from the pilot study following 5 minutes of ischemia (A) Normal cyto-architecture of the cerebellar layers following ischemia, (B) Demonstrate no area of ischemic reaction after 24 hours post induction of transient brain ischemia (C) show no distortion in the neuropil of the cerebellum after 48 hours post ischemia, (D) Demonstrate areas of ischemic reaction after 72 hours post induction of transient global cerebellum ischemia. Magnification: A, B, C and D = X400

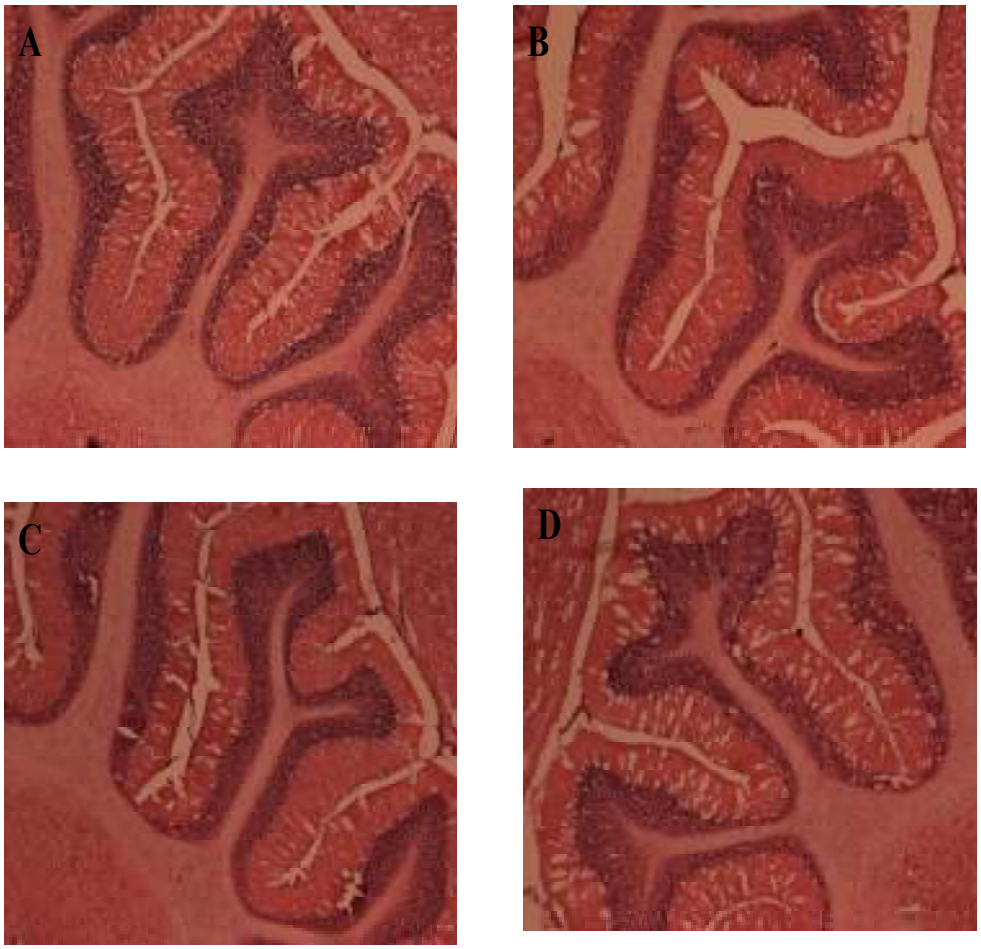


Figure 3.1.3(A, B, C and D)

3. 2: General Observations after surgery

All the rats recovered well after the effects of the anaesthesia weaned. The average time duration for each surgery was forty minutes and post operative care was excellently provided by the staff of the Centre for Animal Service (Medical school division). There were no neurological deficits noticed in the animals after their recovery from the surgery. However, following the 10 minutes transient occlusion of the bilateral carotid arteries, the mortality rate was 50 % in the ischemic group, 6.5 % in the pre-ischemic melatonin groups and 7.8 % in the post-ischemic melatonin groups. All the dead animals were replaced until the total numbers (6) of animals required for each experimental group were completed before the study was final terminated. The mean average weight of the brains was 2.1 ± 0.20 gm. There was no statistical significance difference between the various groups.

3. 3: Histology findings in the brains

In the cerebral cortex, the internal granular lamina (layer IV) showed widening of the cellular lamina and sparsely small, round cell bodies of non pyramidal cells, notably the spiny-stellate and the small pyramidal cells in the ischemic groups (figure 3.3.3B) but these are absent in both pre-ischemic melatonin and post-ischemic melatonin groups (figure 3.3.3A, B, and C). The internal pyramidal lamina (layer V) of the cerebral cortex demonstrate evidence suggestive of aggregation and neurophagia activities with neuronal cell death with scattered non pyramidal cell seen in the ischemic groups, 72 hours after pre-ischemic melatonin

and post-ischemic melatonin groups (figure 3.3.4C and D). All other cerebral layers showed normal histology.

However, normal structures of the hippocampus were seen in both the pre-ischemic melatonin and post-ischemic melatonin groups that were administered with intraperitoneal melatonin as evident by the presence of pyramidal neurons 72 hours after the pre-ischemic melatonin administration and the pyramidal neurons are more in the 7 days groups (figure 3.3.4C). There are marked ischemic changes associated with widening of Virchow-Robin (enlarged perivascular) spaces and densely stained chromatins (hyperchromasia) in neural cells in the ischemic groups (figure 3.3.4 D).

There were cellular and neuropil disruption in the dentate gyrus of the hippocampus in 72 hours post ischemia with increasingly disruptions in the cyto-architecture in the histology when the days progressed to 7 days post transient ischemia in the ischemic group. Microscopic examination of CA1 region of the hippocampus in the ischemic group showed increased neuropil disruption at 72 hours and this worsened as the days increased to 7 days period of the ischemia (figure 3.3.1A and B; figure 3.3.2A and B). All other CA regions presented little or histological changes. The arrows indicate the areas of disruptions and distortions following the ischemic injuries in the subgranular layer of the dentate gyrus of the hippocampus. The restoration of the normal cyto-architecture was evident in the 7 days post ischemia in both the pre-ischemic melatonin and post-ischemic melatonin administered groups. The differences between the pre- and post- administration of melatonin to the ischemic group are evident microscopically.

Figure: 3. 3. 1: Representative photomicrograph of Haematoxylin and Eosin staining of the dentate gyrus of Sprague Dawley rat brain. (A) 72 hours after ischemia with pre-ischemic melatonin administration and (B) 7 days post-ischemic melatonin administration (C) Restoration of cellularity and neuropil after 72 hours post ischemia with pre melatonin administration and (D) Cells recovery after 7 days post-ischemic melatonin administration. Arrows indicate reduced disruption caused by the ischemia. Scale bar A and B = 200 μm, C and D = 50 μm.

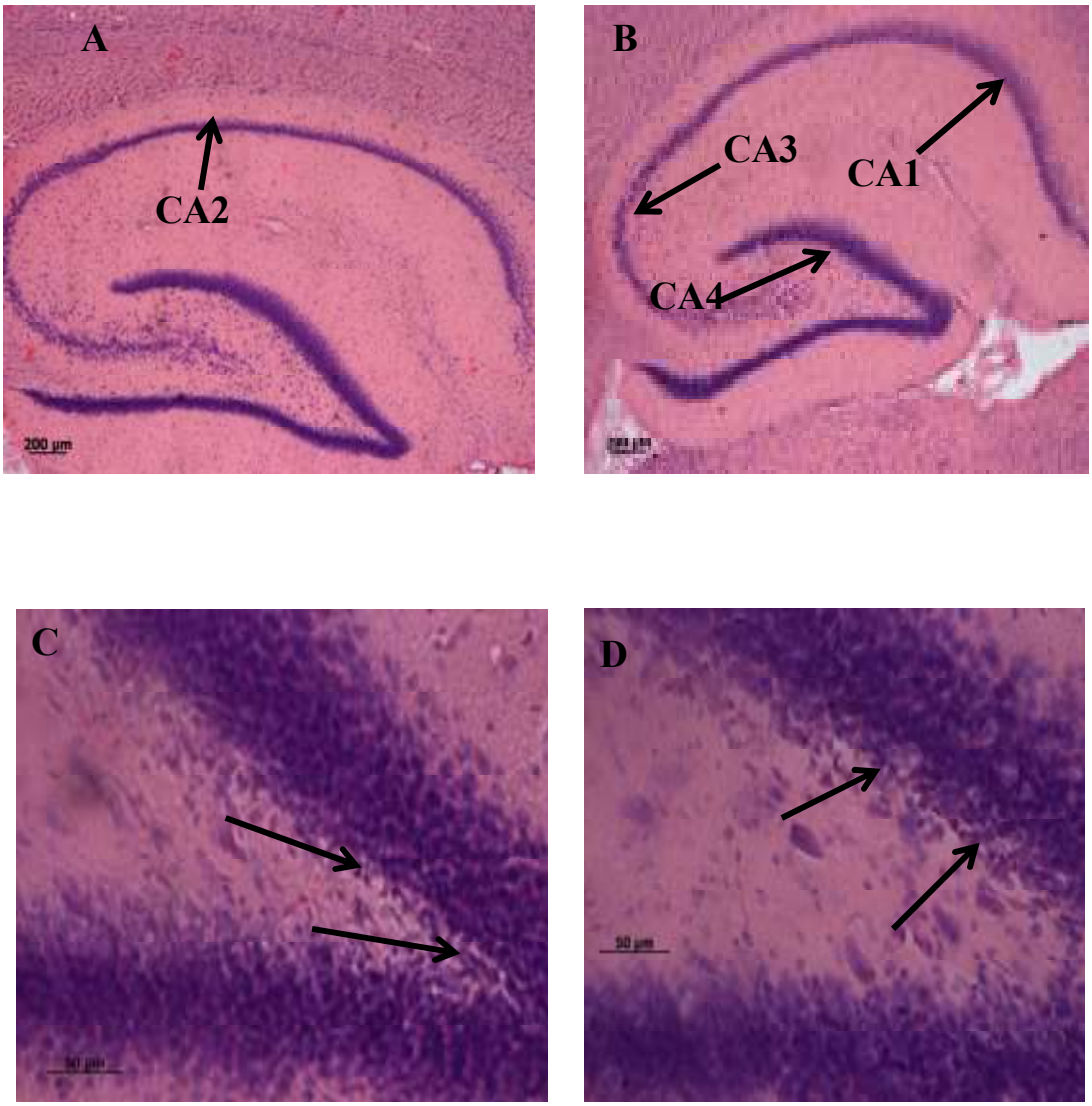


Figure: 3.3. 1 (A, B, C and D)

Figure: 3. 3. 2: Representative photomicrograph of Haematoxylin and Eosin staining of the hippocampus in the Sprague Dawley rat brain. (A) Distortion of the subgranular layer of the dentate gyrus after 72 hours post 10 minutes ischemia, (B) areas of late ischemic reaction after 7 days of ischemia (C) distortion in the subgranular layer after 72 hours post ischemia, (D) areas of late ischemic reaction after 7 days post, SGL: subgranular layer, GL: granule cell layer, and PL is the plexiform layer. Scale bar: A = 100 μm, B, C, and D = 50 μm.

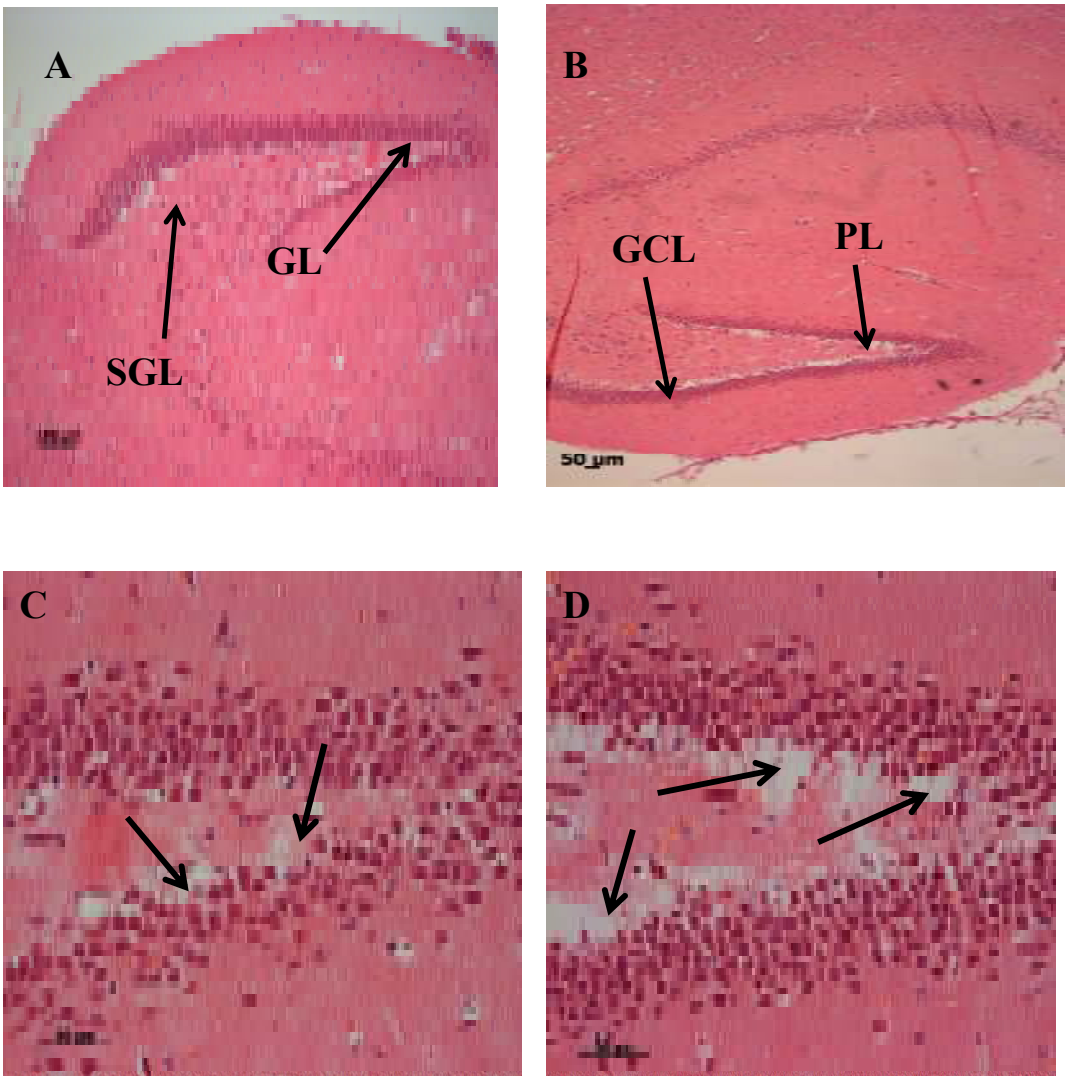


Figure: 3. 3. 2 (A, B, C and D)

Figure: 3. 3. 3: Representative photomicrograph of Haematoxylin and Eosin staining of the cerebral cortex of Sprague Dawley rat brain. (A) Sham control with normal brain histology, (B) patches areas of late ischemic reaction after 7 days of induction of 10 minutes ischemia, (C) 72 hours after ischemia with pre-ischemic melatonin administration and (D) 7 days post-ischemic melatonin administration. I-V indicate the layers of the cerebral cortex. Arrows in figure B indicate the ischemic sites. Scale bar A and B = 100 μm, C and D = 50 μm.

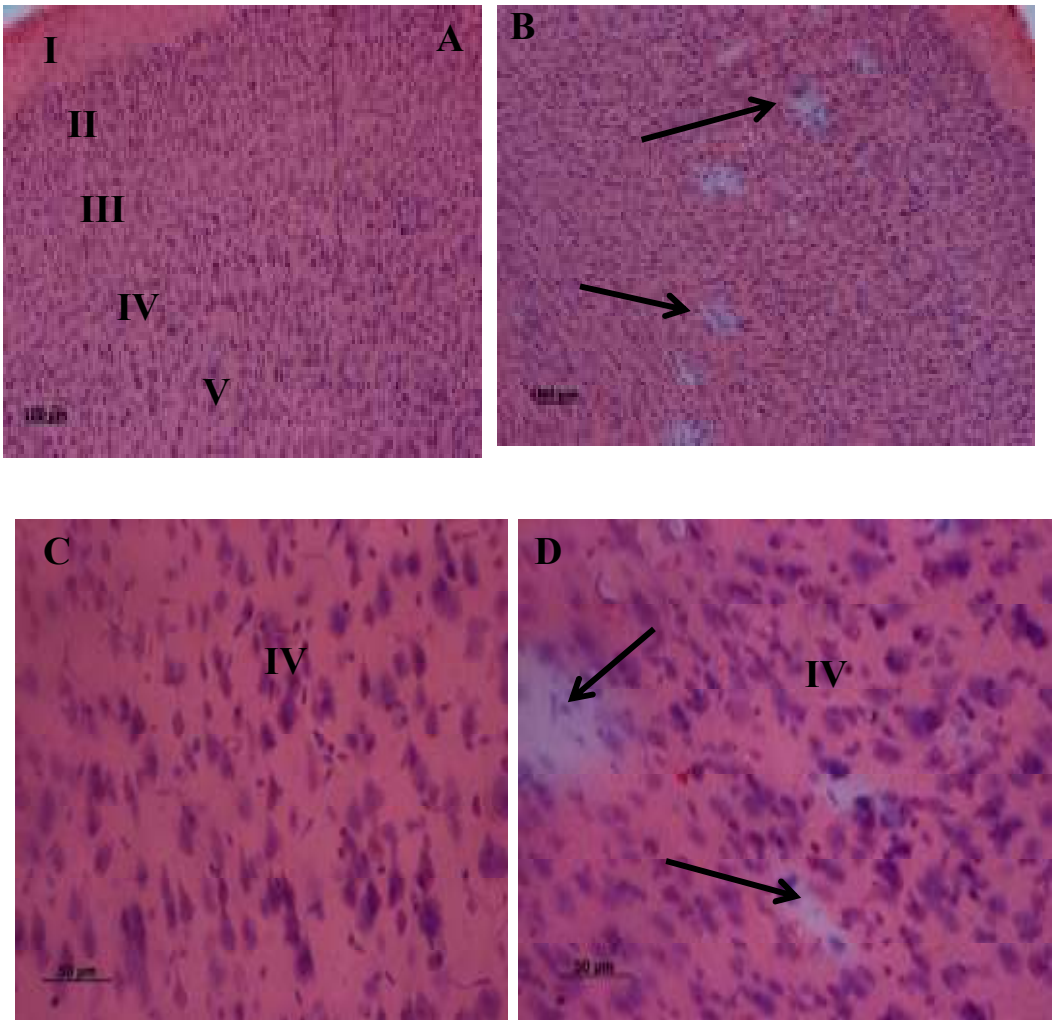


Figure: 3. 3. 3 (A, B, C and D)

Figure: 3. 3. 4: Representative photomicrograph of Haematoxylin and Eosin staining of the pyramidal layer of the cerebral cortex of Sprague Dawley rat brain. (A) Sham control with normal brain histology, (B) patchy areas of late ischemic reaction after 7 days of induction of 10 minutes ischemia in ischemic group, (C) 72 hours after ischemia with pre-ischemic melatonin administration showing marked reduced cellularity and (D) 7 days post-ischemic melatonin administration gradual restorations of normal brain tissues. IV and V indicate the cerebral layers of the cortex. Scale bar A and B = 100 µm, C and D = 50 µm.

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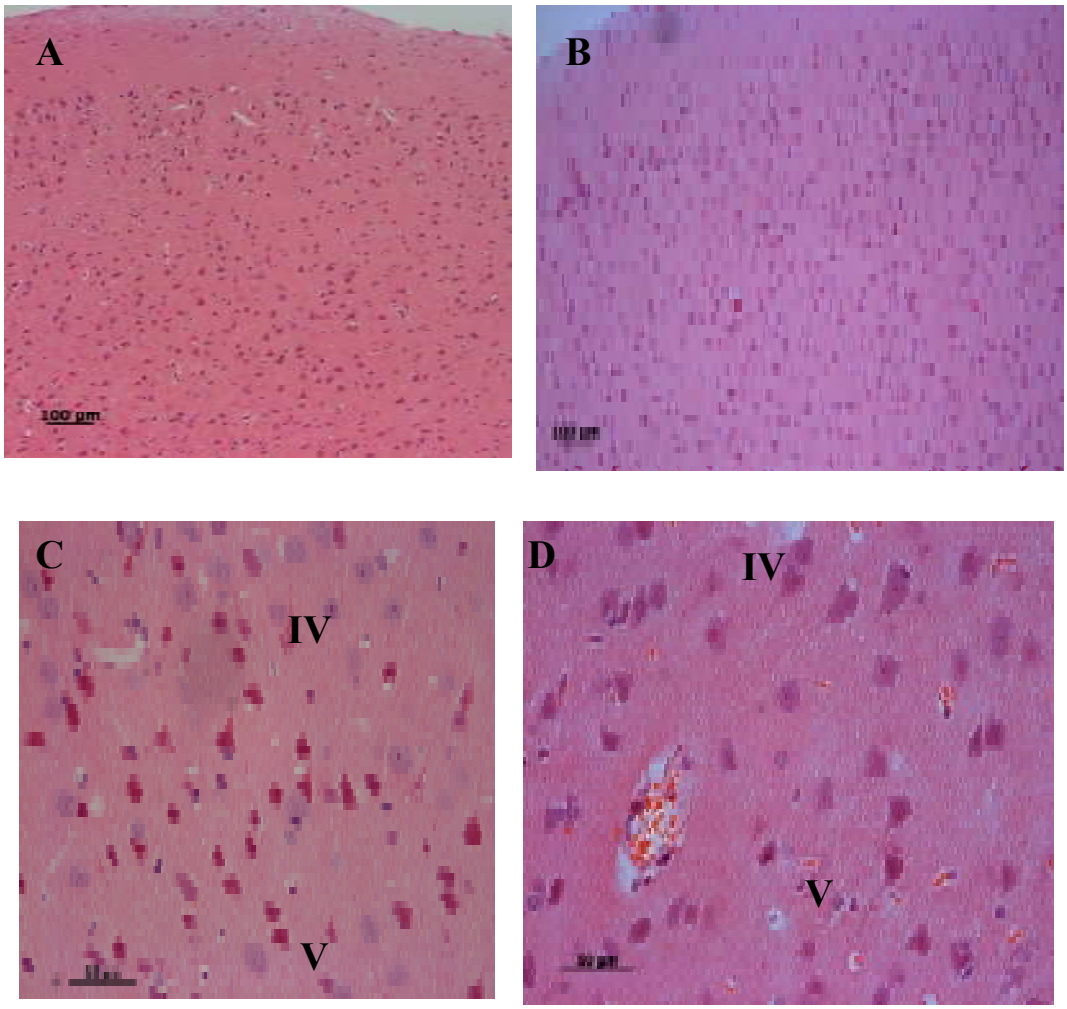


Figure: 3. 3. 4(A, B, C and D)

3. 4: Immunohistochemical staining of Ki-67

Immunochemical staining of Ki-67 positive cells was used as a marker for the proliferating cells in the subgranular and granule cell layers in dentate gyrus of the hippocampus. The Ki-67 positive cells were identified by their labelled nuclei and irregular shaped cluster appearing in both the no-ischemia sham control and ischemic brain. For each experimental group, no nuclear labelling was observed in the control sections in which the primary antibody had been omitted.

In the pre-ischemic melatonin groups, the proliferating cells are mostly discrete and spread widely within the granule cell and subgranular layers of the dentate gyrus (figure 3. 4. 1A, B and C). There are relatively few Ki-67 positive cells seen in the sham control group compared to the melatonin treated groups. The proliferating cells in the post-ischemic melatonin groups appear in clusters are more numerous mostly within the subgranular cell layer of the dentate gyrus (figure 3. 4. 2A, B and C).

Figure: 3. 4. 1: Representative photomicrographs of Ki-67 positive cells in the dentate gyrus of hippocampus: A) No-ischemic sham control; the line indicate the extent of the granular layer B) 72 hours pre-ischemic melatonin and C) 7 days pre-ischemic melatonin. Arrows indicate clusters of proliferating cells in the subgranular zone. Scale bar: A-C; 50 μm.

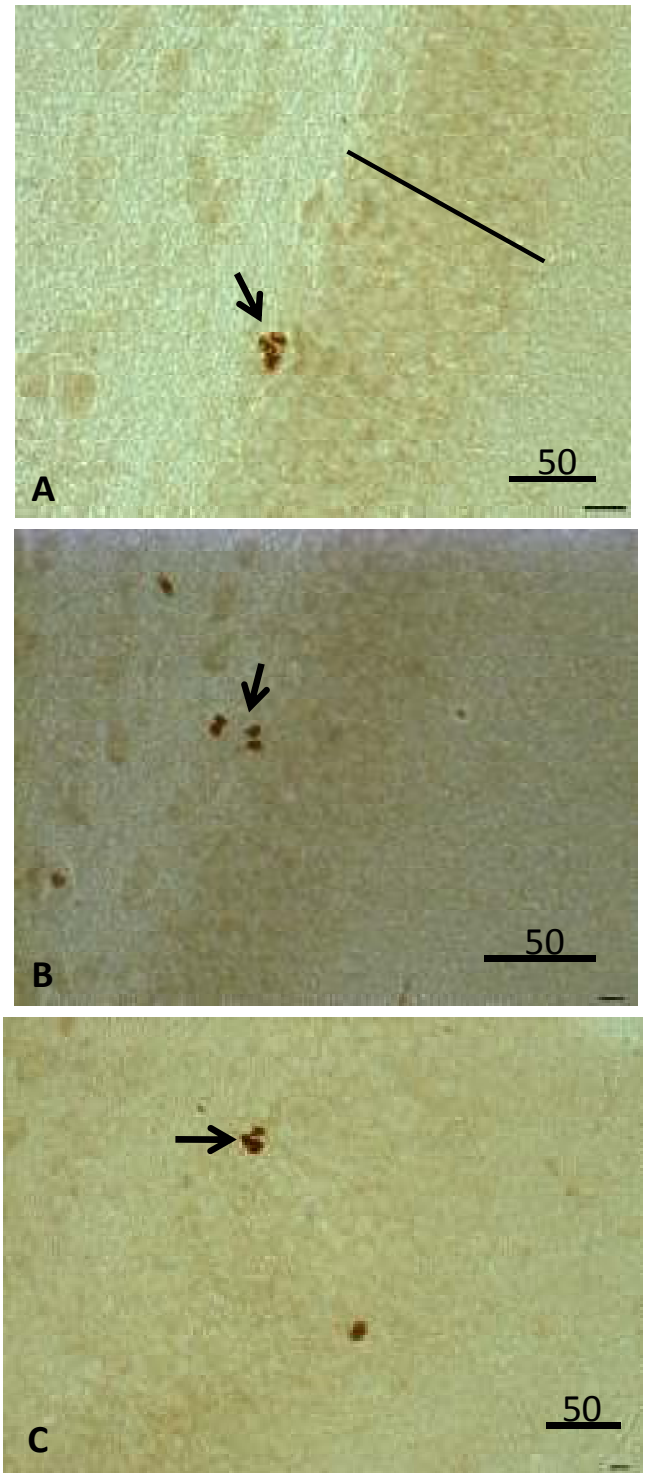


Figure: 3. 4. 1(A, B, and C)

Figure: 3. 4. 2: Representative photomicrographs of Ki-67 positive cells in the dentate gyrus of hippocampus: A) No-ischemic sham control; B) 72 hours post-ischemic melatonin and C) 7 days post-ischemic melatonin. Arrows indicate clusters of proliferating cells in the subgranular zone. Scale bar: A-C; 50 μm.

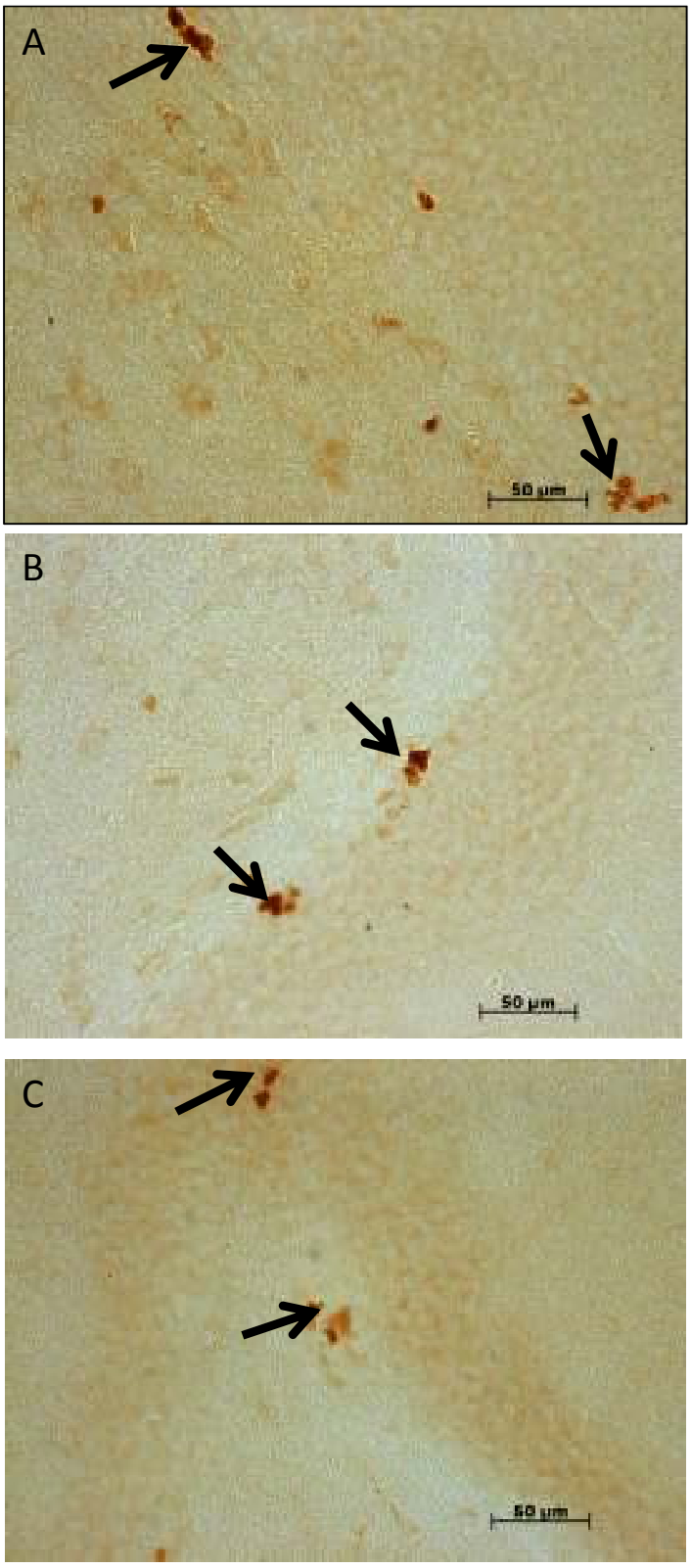


Figure: 3. 4. 2 (A, B, and C)

3. 5: Quantitative Results

3. 6: Total Proliferating Cell Number

Following 72 hours post-ischemic time duration, the mean total number of Ki-67 positive cell in the pre-ischemic melatonin group was 5964 ± 1894 more than twice those in both the ischemic and no-ischemia sham controls (Table 3.6.1). This showed a statistically significant difference ($p<0.05$) between the pre-ischemic melatonin and the no ischemic sham controls. The same observation was made within the 7 days post-ischemic melatonin groups. There was a three-fold statistically significant increase in the mean total proliferating cells (6900 ± 2011) in the pre-ischemia melatonin group compared to the ischemic and the no-ischemic-sham control (Table 3. 6. 1). The cell proliferation was sustained with time as the value after 7 days was higher than that of the 72 hours post ischemia (Figure 3.6.2).

Table: 3. 6. 1: Mean total proliferating cells number and mean total pyknotic cell number

Experimental Group	Proliferating Cells		Pyknotic Cells	
	No ± SD	P value	No ± SD	P value
72 hours ischemic time				
Sham control	2076 ± 583	ns	126 ± 62	ns
Ischemic	2016 ± 583	ns	462* ± 52	P<0.05
Pre-Ischemic melatonin	5964*±1894	P<0.05	288 ± 102	ns
Post-ischemic melatonin	2946 ± 698	P<0.05	276 ± 121	ns
7 days post ischemic time				
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
Post-ischemic melatonin	3108* ± 712	P<0.05	246 ± 119	ns

Mean total proliferating cell number and pyknotic cell number in the various experimental groups after administration of melatonin 30 min before (pre-ischemic melatonin groups) and 30 min after (post-ischemic melatonin groups). Asterisk (*) indicates significant values compared to other groups. SD = standard deviation and ns indicate not statistically significant.

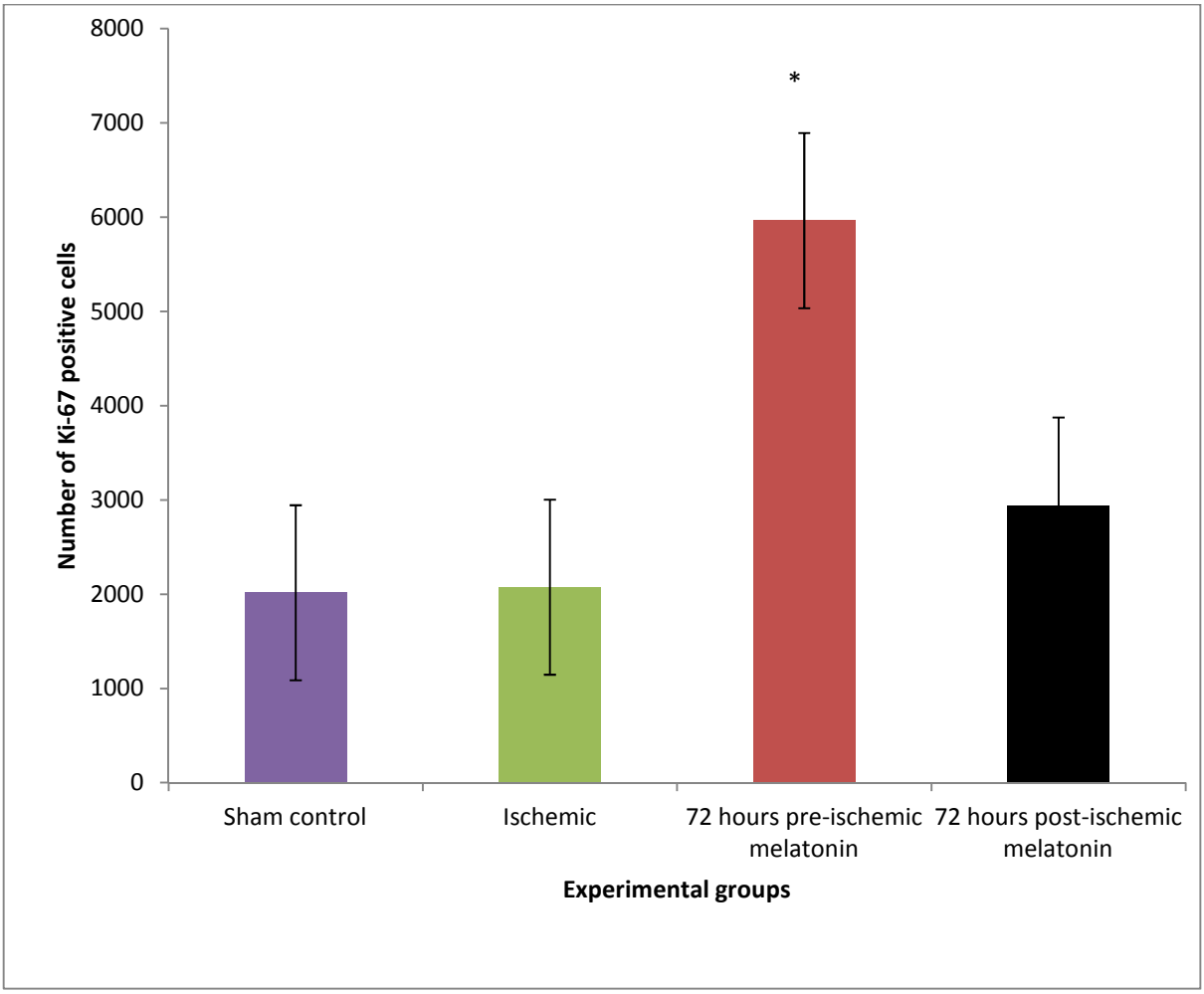


Figure: 3. 6. 1: Comparison of total proliferating cell number in pre-ischemic melatonin following transient ischemic brain injury. The asterisk indicates statistical significance at $P < 0.05$ in comparism to other experimental groups and the bars represent standard deviation (SD).

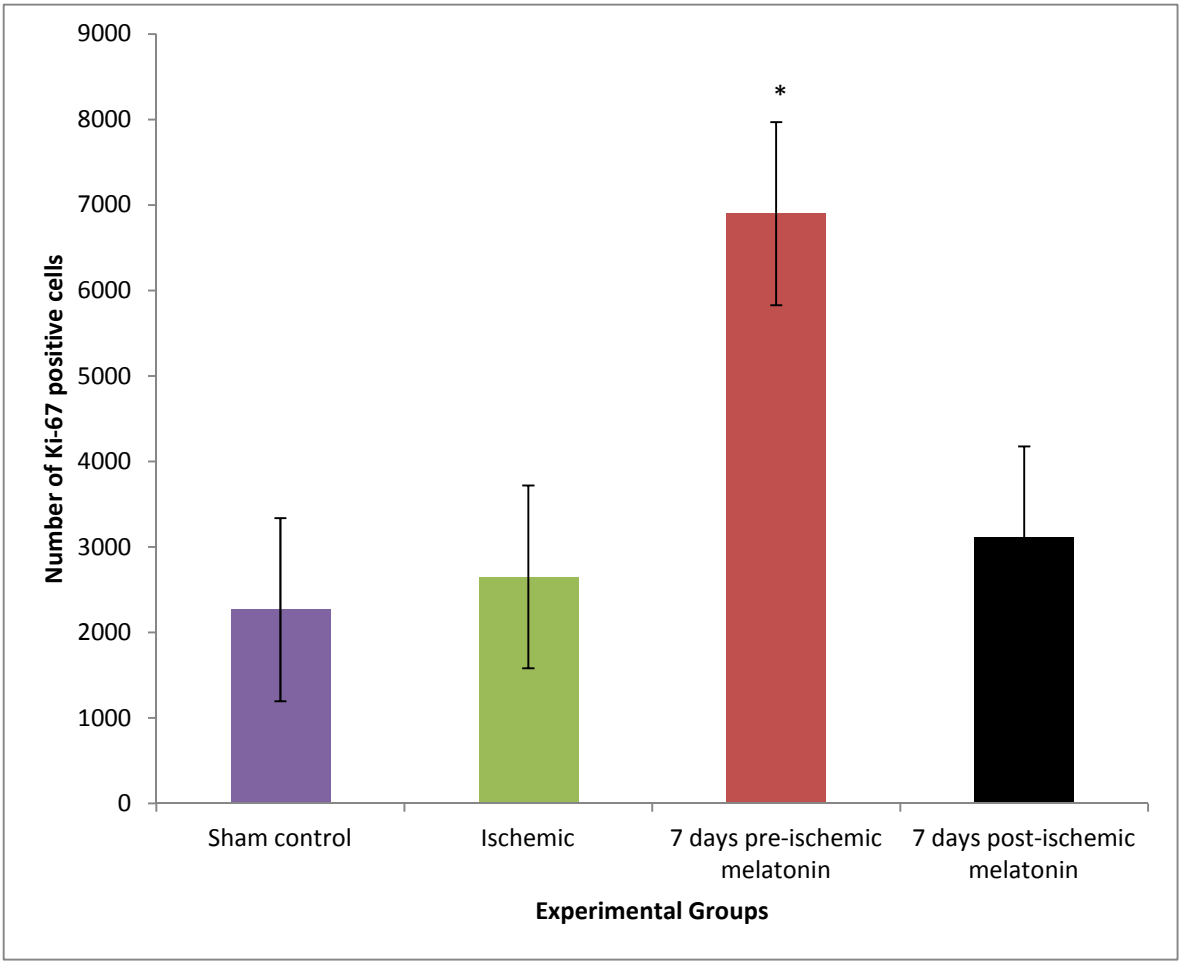


Figure: 3. 6. 2: Comparison of total proliferating cell number in post-ischemic melatonin following transient ischemic brain injury. The asterisk indicates statistical significance at $P < 0.05$ in comparism to other experimental groups and the bars represent standard deviation (SD).

However, in the post-ischemic melatonin groups, 72 hours post-ischemic time duration, the mean total number of Ki-67 positive cell was 2946 ± 276. This was higher than those in both the ischemic and no-ischemia sham controls (Figure 3.6.3). There was no statistically significant difference ($p<0.05$) between the post-ischemic melatonin and the no ischemic sham controls. The observation was different in the 7 days post-ischemic time duration. There was a one and half-fold increased in the mean total proliferating cells (3108 ± 246) in the post-ischemia melatonin group compared to the ischemic and the no-ischemic-sham control (Table 3. 6. 1). The difference was statistically significant. The cell proliferation seems to have been sustained with time as the value after 7 days was higher than that of the 72 hours post ischemia (Figure 3. 6. 4).

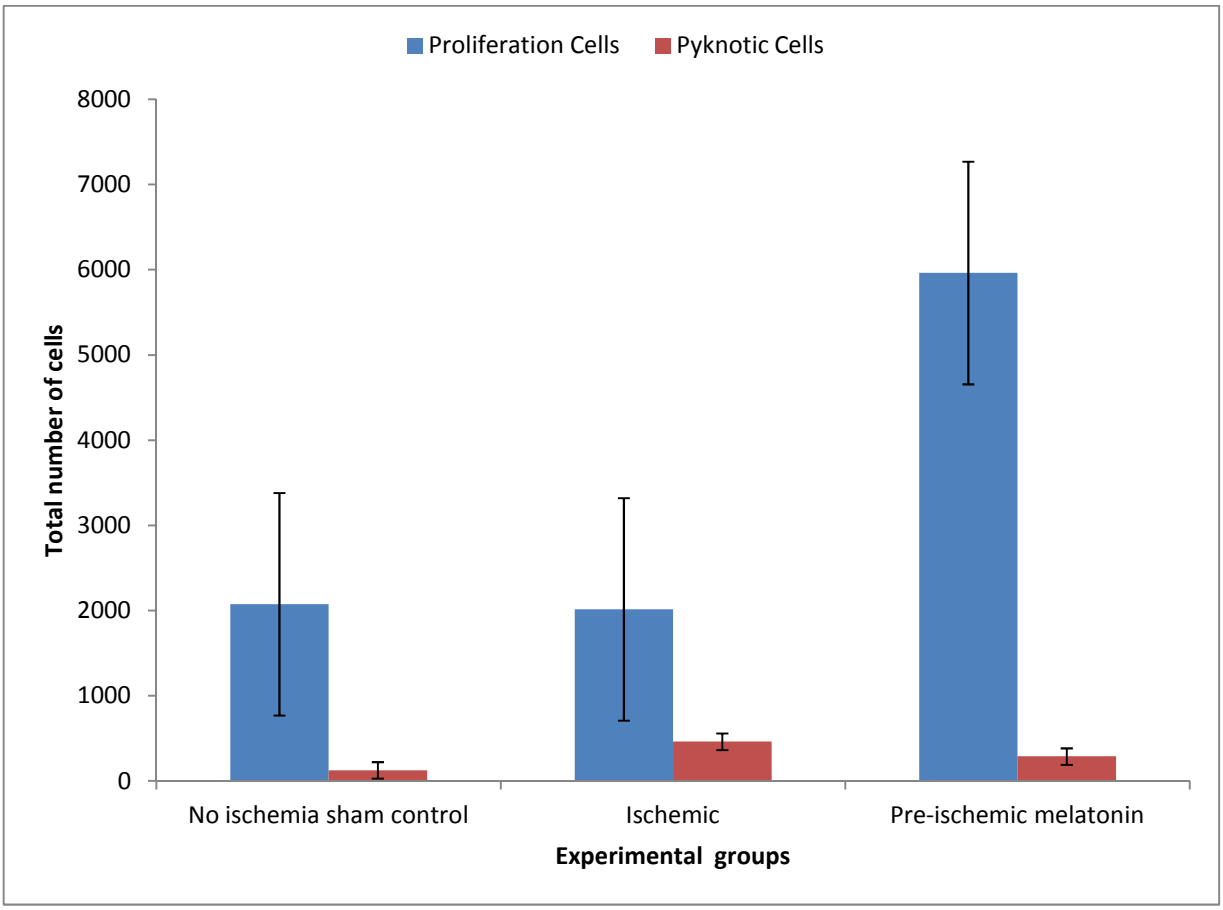


Figure: 3. 6. 3: Graph of the cell proliferation and pyknotic cells number in the pre-ischemic melatonin groups and different time duration representing the 72 hours post ischemic brain injury and the colour code indicates the type of cells counted.

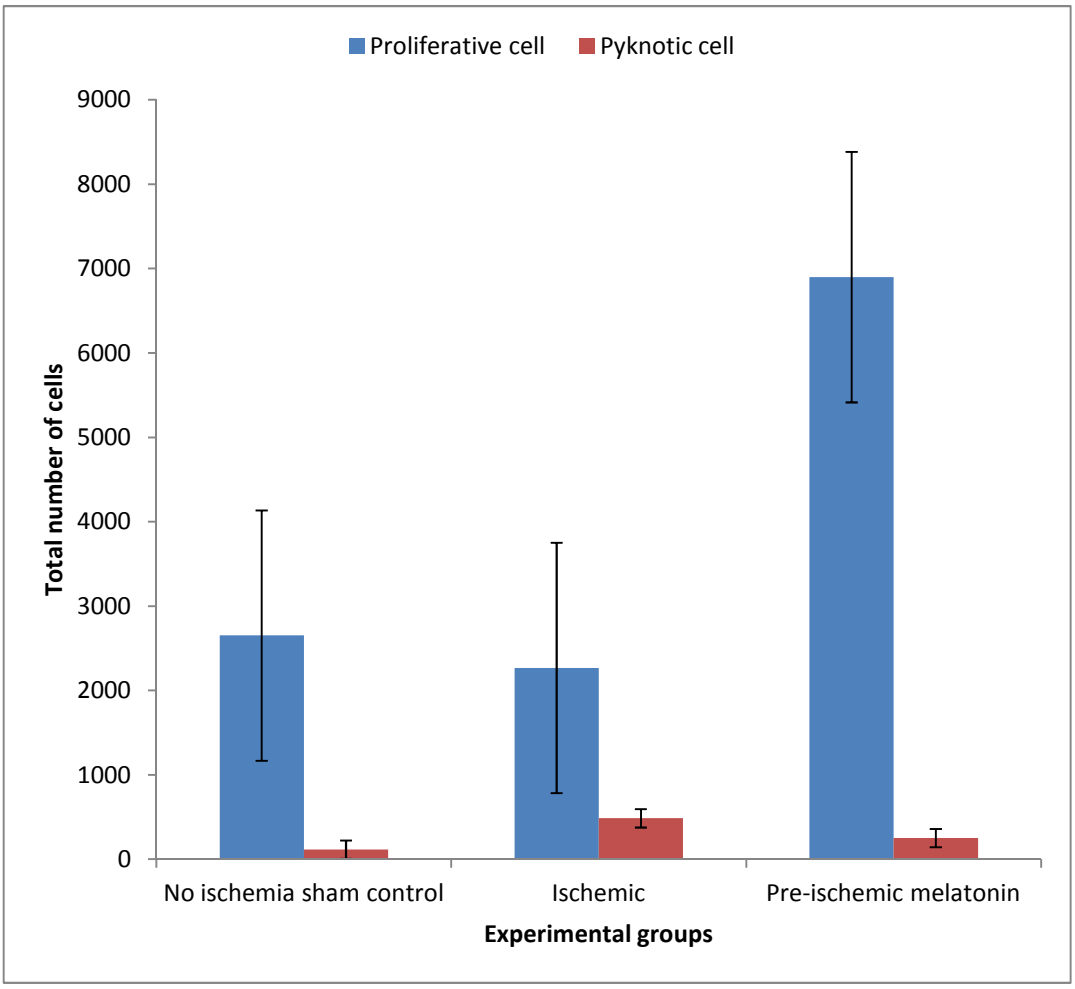


Figure: 3. 6. 4: Graph of the cell proliferation and pyknotic cells number in the pre-ischemic melatonin groups and different time duration representing the 7 days post-ischemia brain injury time duration while colour codes indicate the types of cell counted.

3. 7: Identification of Pyknotic cells

The pyknotic cells were identified in the subgranular and granular zones of the dentate gyrus of the hippocampus using Giemsa stained sections. The pyknotic cells presented strongly stained nuclei with condensed chromatin peripherally into a C or doughnut shape, solid and sometimes, with multiple cell bodies. The pyknotic cells in the pre-ischemic and post-ischemic melatonin groups were mainly found within the granule cell layers (figure 3. 7. 1A and B), while those of the ischemic group are scattered across the layers of the dentate gyrus (figure 3. 7. 1C).

Figure: 3. 7. 1: Representative photomicrographs of Giemsa staining of pyknotic cells. A; Granular zone in the pre-ischemic melatonin, B: Granular zone in the post-ischemic melatonin group and C: subgranular zone in the ischemic group. The cells are indicated by the arrows. Scale bar: A, B and C = 50 μm.

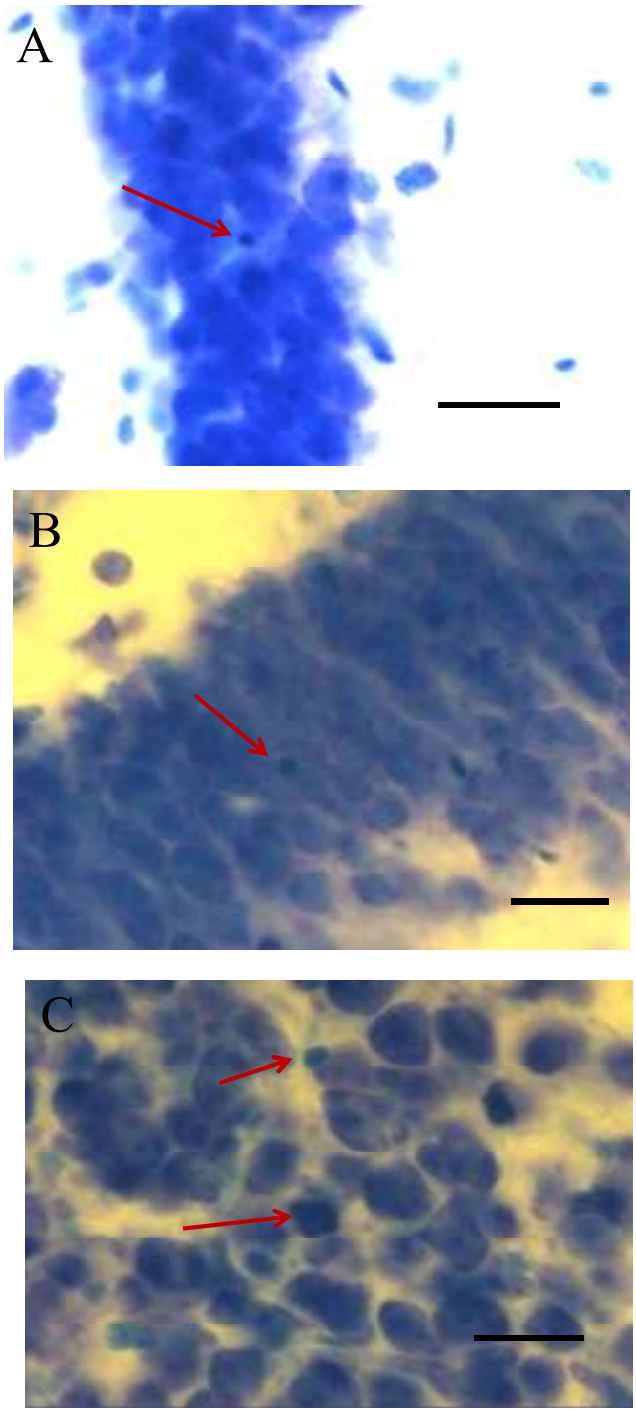


Figure: 3. 7. 1 (A, B, and C)

3. 8: Total pyknotic cell number

Under the 72 hours time duration of the pre-ischemic melatonin groups, the ischemic group had the highest number of pyknotic cells number (462 ± 52) more than two folds that of the no-ischemia-sham control and was statistically significant ($p<0.05$) compared to the no-ischemia-sham control. For the 7 days time duration, the pre-ischemic melatonin group estimated total pyknotic cell number was 252 ± 118 while the ischemic group mean total estimated value of 486 ± 46. This was statistically significant ($p<0.05$) compared to the no-ischemia-sham control (114 ± 58). The comparison of the estimated total pyknotic cells number for the pre-ischemic melatonin groups are demonstrated in figure 3. 8. 1.

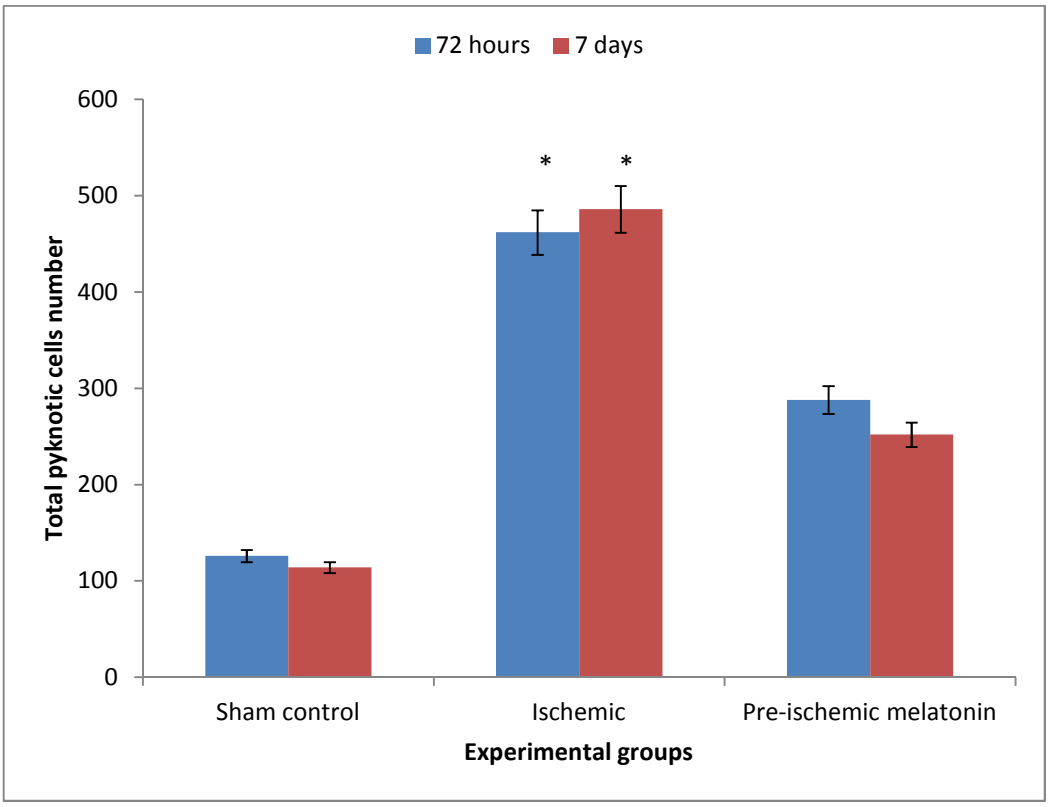


Figure: 3. 8. 1: Graph of the pyknotic cells number in the pre-ischemic melatonin groups at different time duration. The asterisk indicate statistical significance difference within the experimental groups and the bars represent the standard deviations and the asterisk indicate statistical significant.

The 72 hours time duration of the post-ischemic melatonin group had an estimated total pyknotic cells number of 276 ± 121 and it was statistically significant ($p<0.05$) compared to the ischemic group (462 ± 52). For the 7 days time duration, the post-ischemic melatonin group estimated total pyknotic cells number was 246 ± 119. This was also statistically significant ($p<0.05$) compared to the ischemic group (486 ± 46). The estimated total pyknotic cells number for 72 hours and 7 days time duration in the no-ischemic-sham control was low. The comparison of the total estimated pyknotic cells number for the post-ischemic melatonin groups are displayed in figure 3. 8. 2.

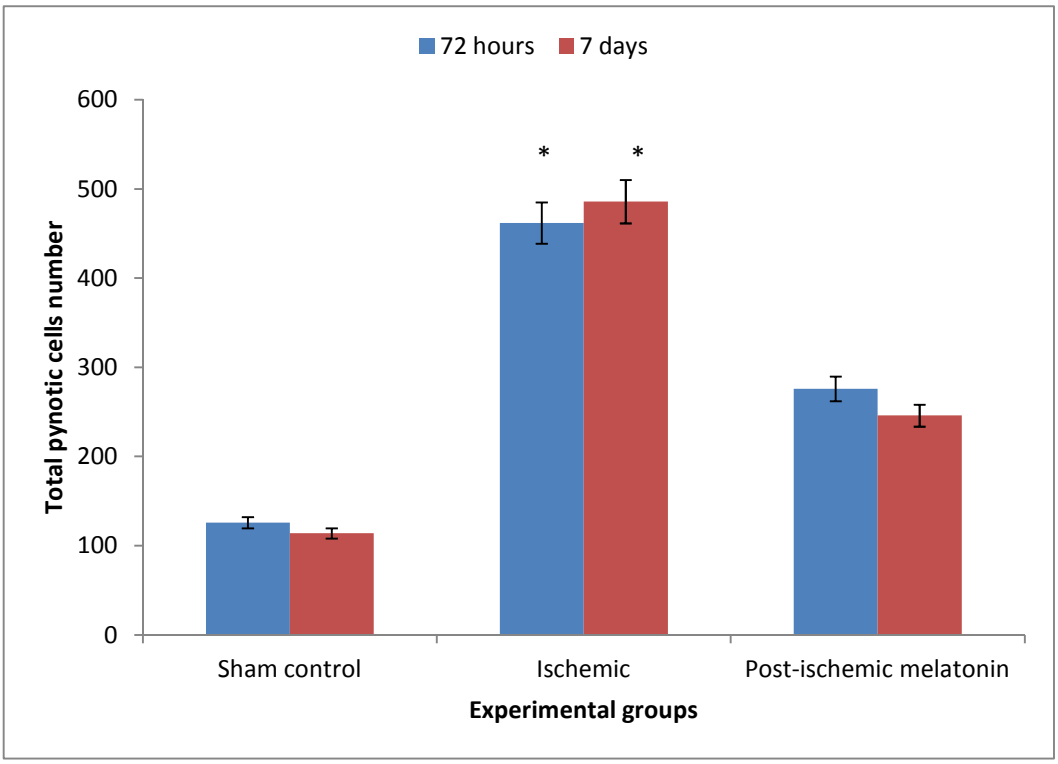


Figure: 3. 8. 2: Graph of the pyknotic cells number in the post-ischemic melatonin groups at different time duration. The asterisk indicate statistical significance difference within the experimental groups and the bars represent the standard deviations

3. 9: Total Granule Cell Number

The total granule cell number was estimated from the granule and subgranular zones of the dentate gyrus of the hippocampus using unbiased optical fractionator technique. The stereological parameters for optical fractionator analysis for all the groups are as shown in Table 3. 9. 1. The mean total granule cell number estimated in the dentate gyrus ranges from 986704 ± 197340.8 in the 72 hours sham control and 1110658 ± 222131 for the ischemic group. The Gunderson coefficient of error is 0.10 and 0.06 for the No-ischemic sham control and the ischemic group respectively.

The total granule cell number for the pre-ischemic melatonin was 1052639 ± 273686 following 72 hours post ischemia and for the 7 days after ischemic for the pre-melatonin group was 1061939 ± 286723 (Table 3. 9. 2). The coefficient of error during the stereological counting was 7.0 %. The estimated total granule cell number for the post-ischemic melatonin group was 1076619 ± 312219 for the 72 hours groups and 1053337 ± 273867 for the 7 days post ischemia. There was no statistical significant difference within the pre-ischemic melatonin and the no melatonin groups and between the two groups (Figure 3. 9. 2).

Experimental Groups	No of section analysed	Estimated total granule cells (10^6)	Gunderson Coefficient of Error
Sham control 72 hours	26 (26-36)	0.9867	0.10
Sham control 7 days	26 (26-33)	1.143	0.07
Ischemic 72 hours	26 (26-34)	1.110	0.06
Ischemic 7 days	26 (26-38)	1.141	0.10
Pre-ischemic melatonin 72 hours	26 (26-32)	1.052	0.07
Pre-ischemic melatonin 7 days	26 (26-34)	1.061	0.06
Post-ischemic melatonin 72 hours	26 (26-33)	1.076	0.10
Post-ischemic melatonin 7 days	26 (26-31)	1.053	0.07

Table: 3. 9. 1: Stereological parameters for optical fractionator analysis of the estimated total granule cell number (10^6), showing numbers of sections analysed, and Gunderson coefficient of error with experimental groups.

Experimental Group	Estimated total granule cells No ± SD	P-value
72 hours ischemic time		
Sham control	986704 ± 197340	ns
Ischemic	1110658 ± 222131	ns
Pre-ischemic melatonin	1052639 ± 273686	ns
Post-ischemic melatonin	1076619 ± 312219	ns
7 days post ischemic time		
Sham control	1143323 ± 320130	ns
Ischemic	1141938 ± 296903	ns
Pre-ischemic melatonin	1061939 ± 286723	ns
Post-ischemic melatonin	1053337 ± 273867	ns

Table: 3. 9. 2: Mean estimated total granule cell number in the dentate gyrus of the hippocampus showing estimated total granule cells number, p-values, ischemic time durations and experimental groups. SD = standard deviation and ns = not statistically significance.

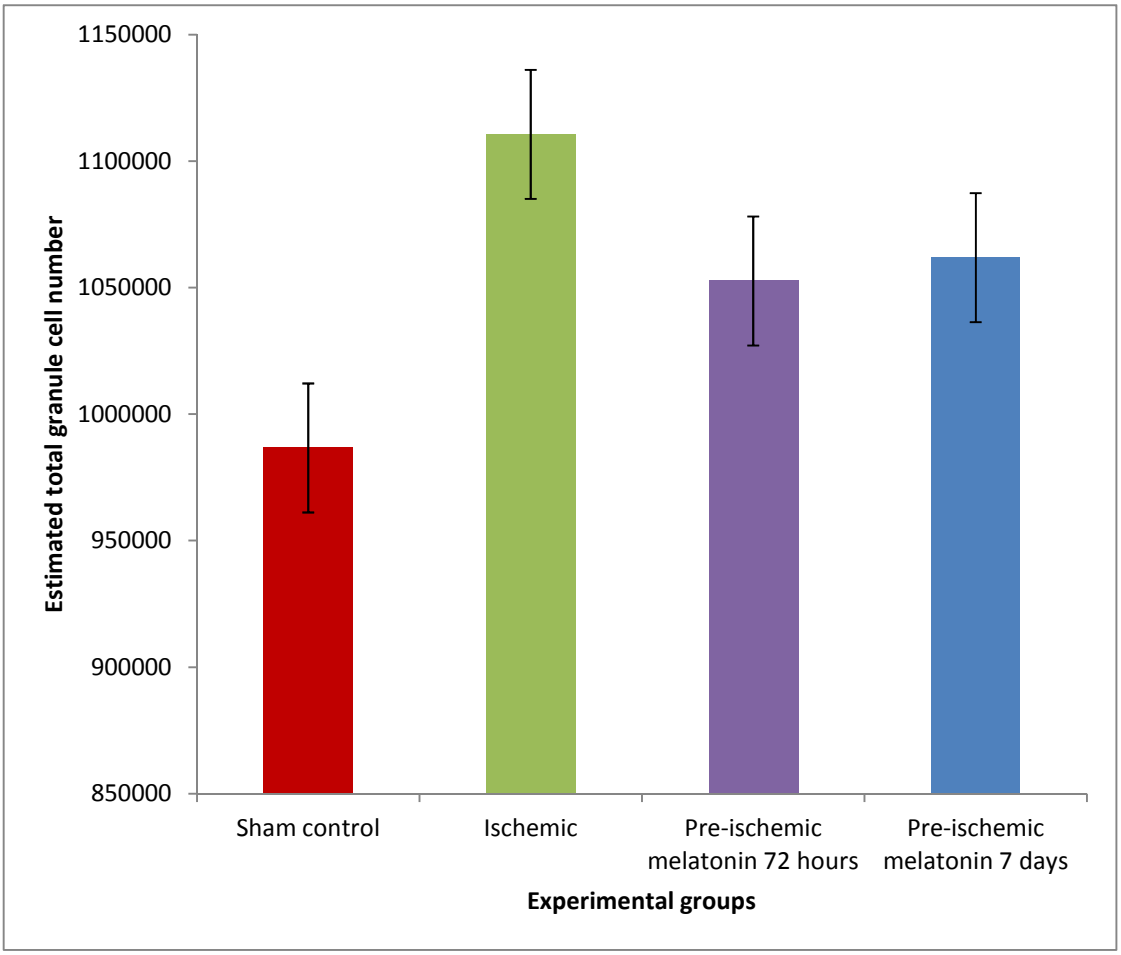


Figure: 3. 9. 1: Estimated mean total granule cells number of pre-ischemic melatonin in the dentate gyrus in various experimental groups. The bars represent the standard deviation

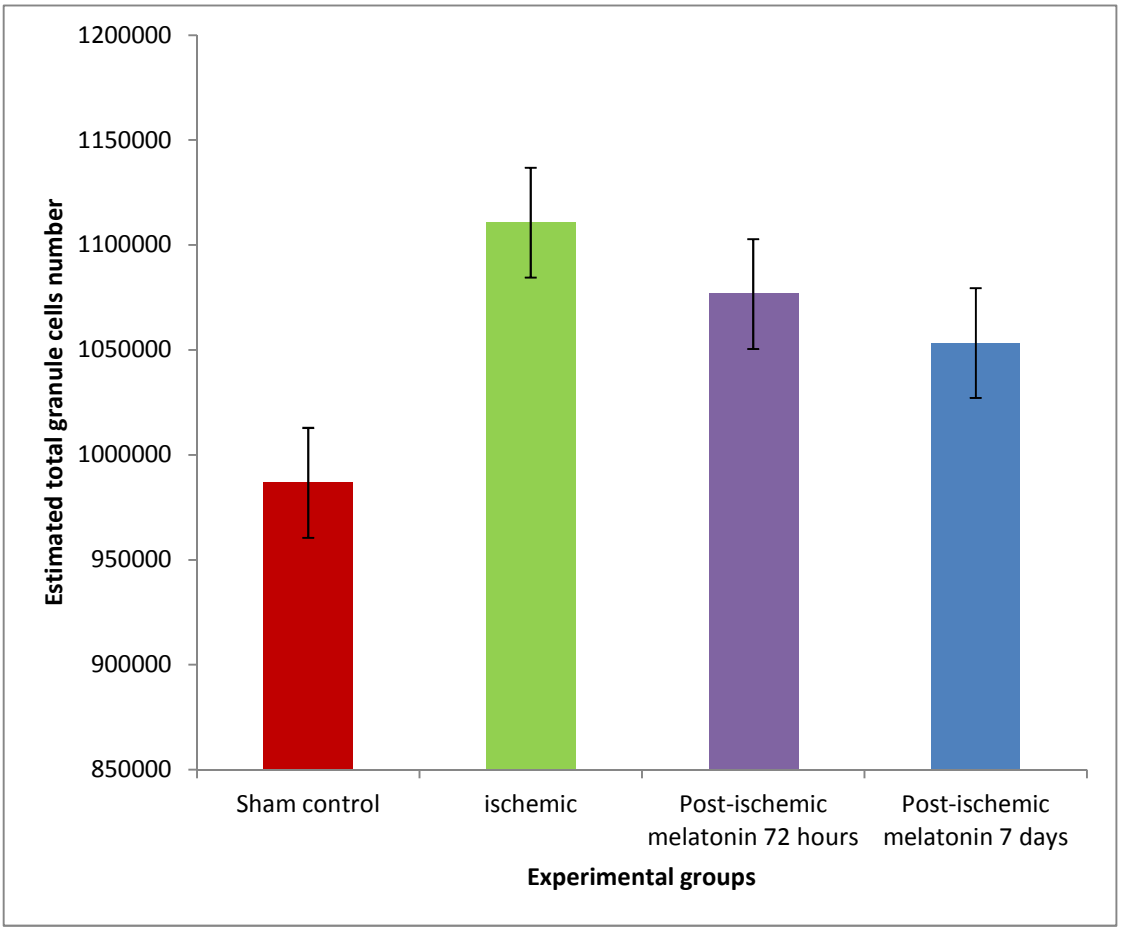


Figure: 3. 9. 2: Estimated mean total granule cells number of post-ischemic melatonin in the dentate gyrus in various experimental groups. The bars represent the standard deviation.

3. 10: Relationship between proliferating cells and pyknotic cells numbers

The relationship between the total pyknotic cells number and total proliferating cells number was linear in the no-ischemia sham control and the ischemic groups and very strong with positive correlation (R^2 = 0.6532 and 0.0943 respectively).

The presence of pyknotic cells augment the generations of the proliferating cells in the 72 hours after the induction of ischemia (figure 3. 10. 1). However, this was not observed in the pre-ischemic melatonin group with the coefficient of correlation at (R^2 = 0.004) indicating that the presence of proliferating cells in the pre-ischemic melatonin groups was independent of the melatonin availability at that phase (figure 3. 10. 1). In the 7 days post induction of ischemia, the proliferation of the neurogenic cells was dependent on the presence of melatonin (R^2 = 0.088), and independent of the total number of pyknotic cells present (figure 3. 10. 2).

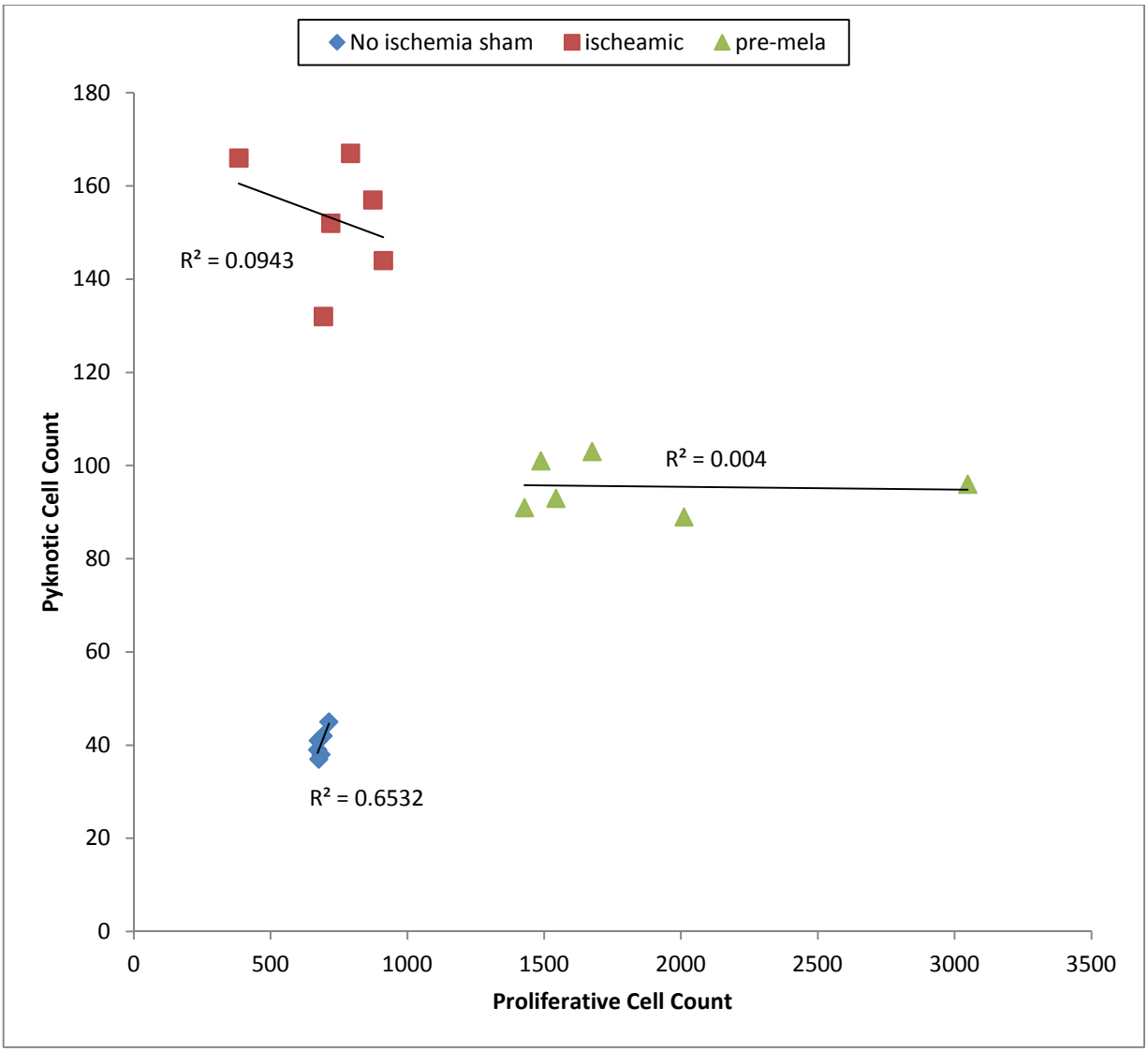


Figure: 3. 10. 1: Graph showing the correlation between the total pyknotic cell and total proliferating cells in the 72 hours experimental groups. R^2 indicates the strength of correlation between the pyknotic and proliferating cells in the pre-ischemic melatonin group.

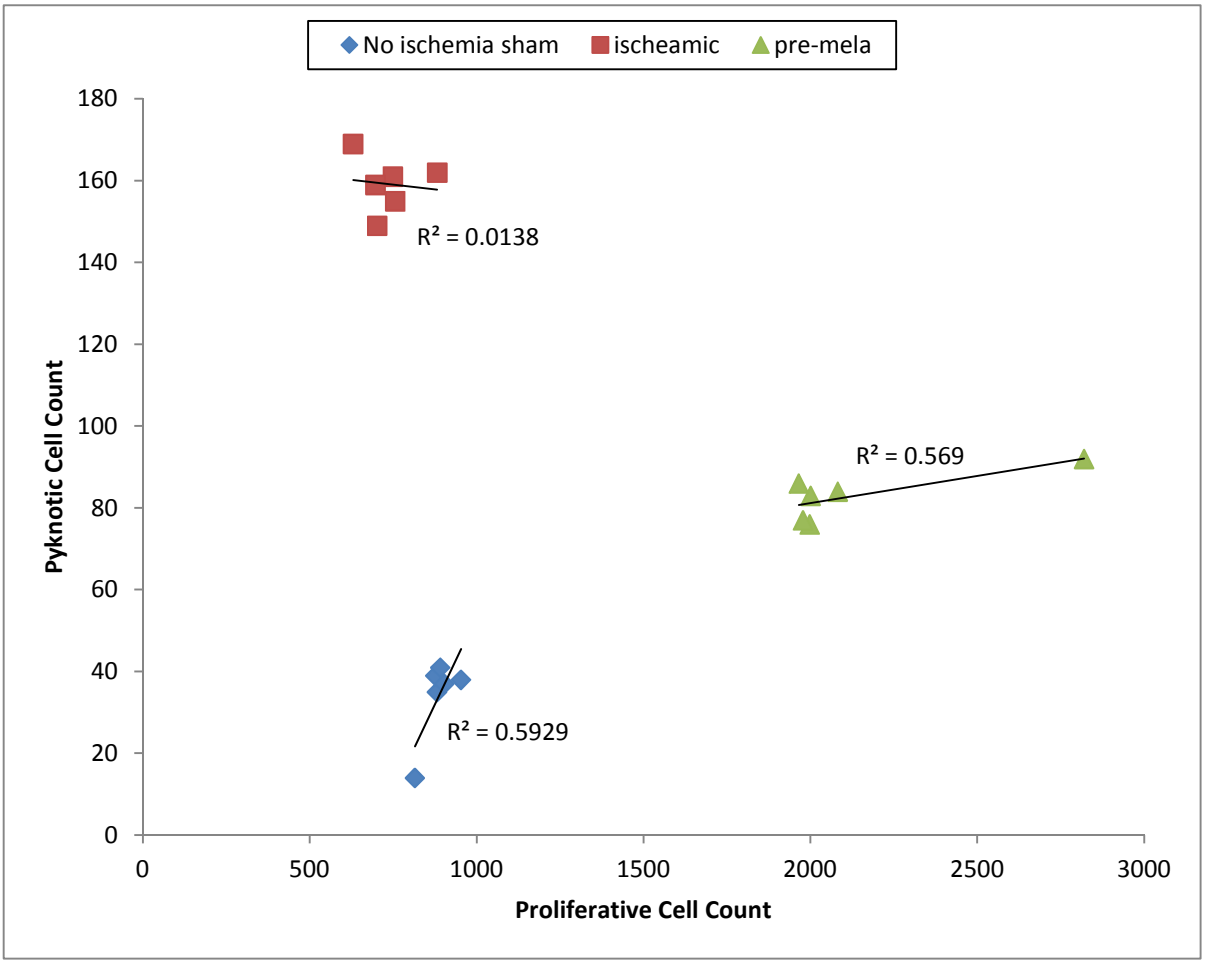


Figure: 3. 10. 2: Graph showing the correlation between the total pyknotic cell and total proliferating cells in 7 days post-ischemia. R^2 indicates the strength of the correlation between the pyknotic and proliferating cells in the pre-ischemic melatonin group.

Post ischemia administration of melatonin did not have any effect on the proliferating cells in both the 72 hours and 7 days after the induction of ischemic-reperfusion injury in the rats brains (figure 3. 10. 3-4). Similar trend was observed in the ischemic and no occlusion sham control groups in relation to the pre-ischemic melatonin groups (R^2 =0.5932 for sham control and 0.0032 for post-ischemic melatonin).

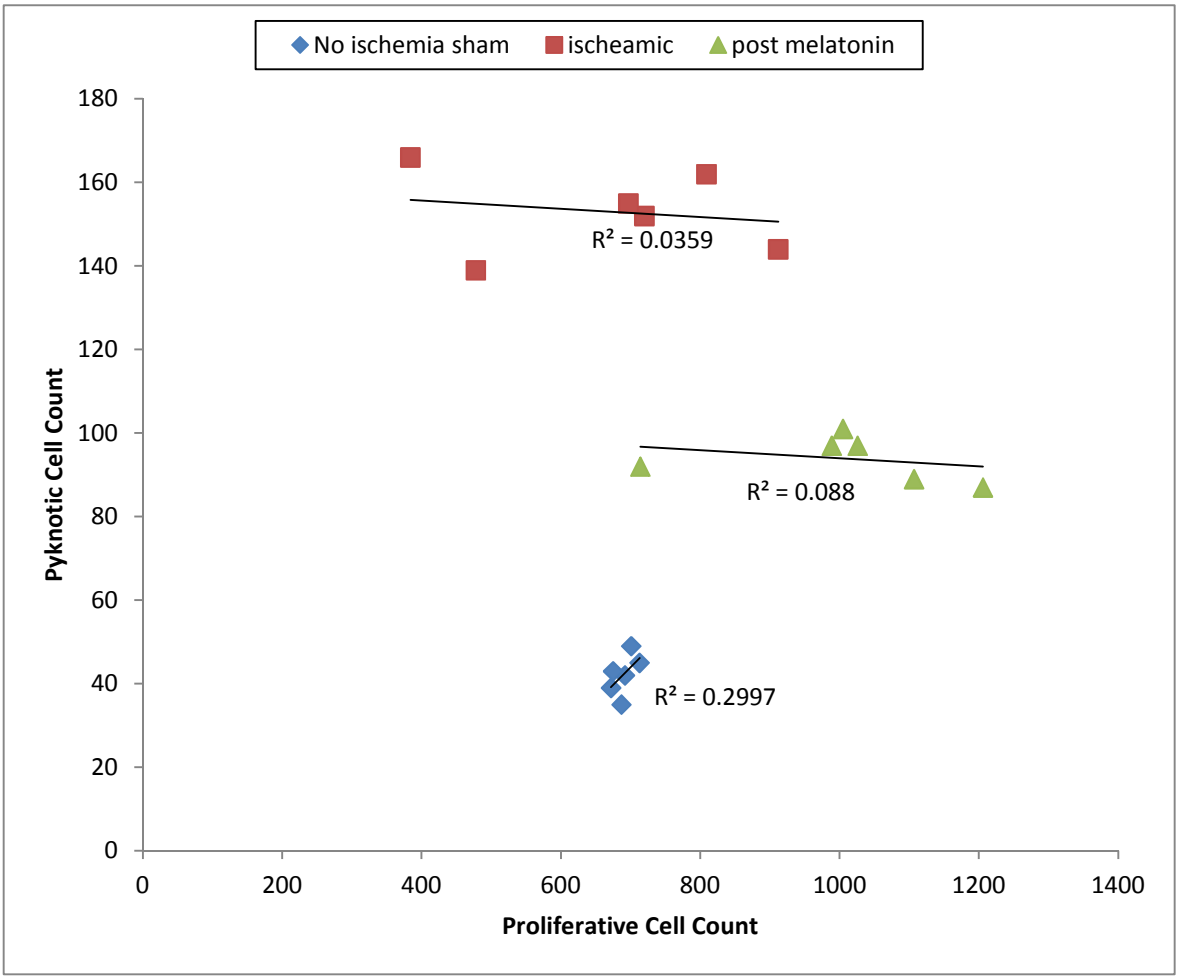


Figure: 3. 10. 3: Graph showing the correlation between the total pyknotic cell and total proliferating cells of the 72 hours experimental groups post-ischemia. R^2 indicates the strength of the correlation between the pyknotic and proliferating cells in the post-melatonin group.

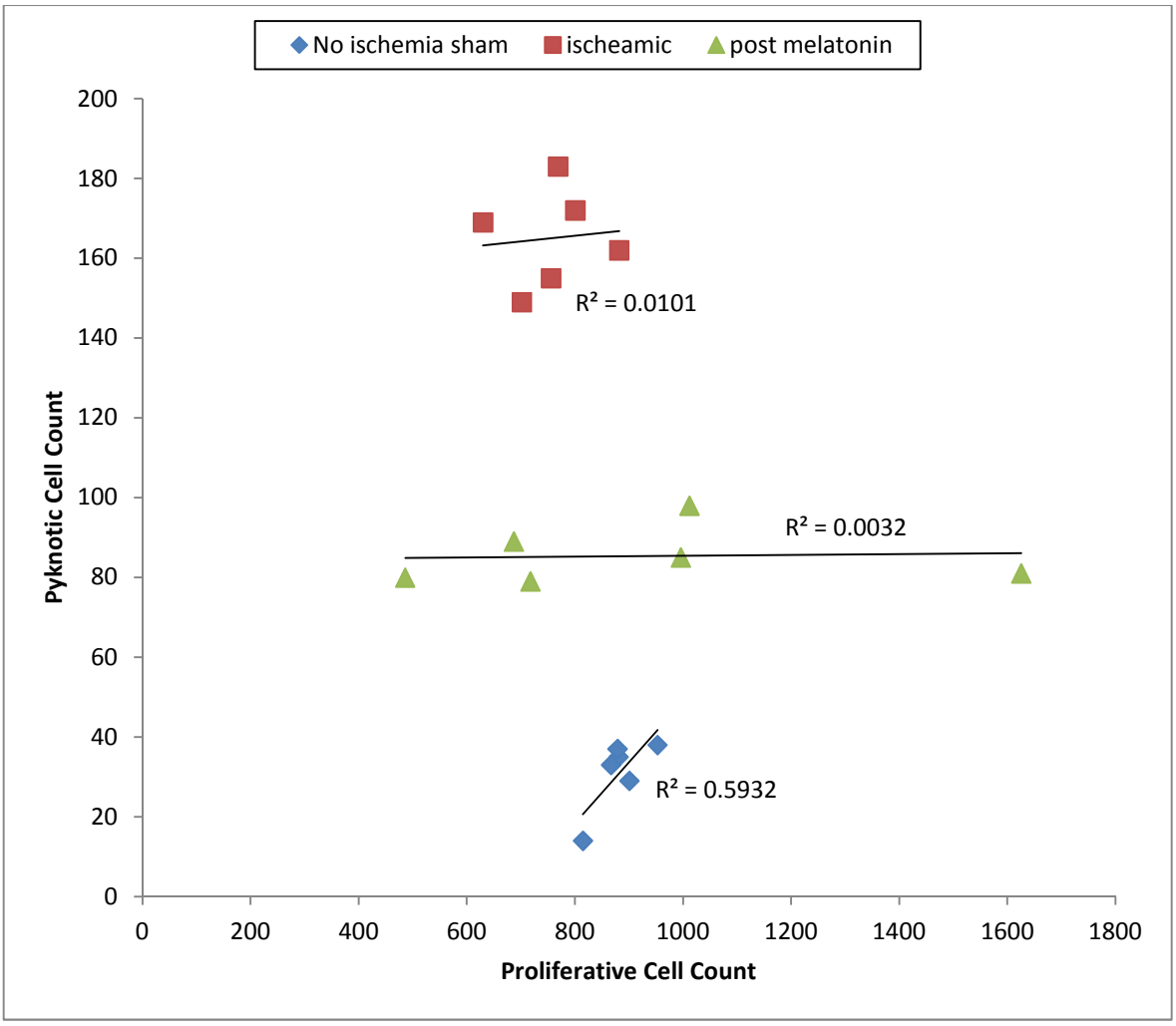


Figure: 3. 10. 4: Graph showing the correlation between the total pyknotic cell and total proliferating cells of the 7 days post-ischemia. R^2 indicates the strength of the correlation between the pyknotic and proliferating cells in the post-melatonin group.

3. 11: Ratio of proliferating per pyknotic cell numbers in dentate gyrus

The ratio for the 72 hours group was 16.47 for the No-ischemic sham control, 4.37 for the ischemic group, 20.71 for the pre-melatonin group and 10.67 for the post-ischemic group. The ratio for the 7 days post occlusion duration are 23.26 for the No-ischemic control, 4.67 for the ischemic group, 27.38 for the pre-melatonin group and 12.53 for the post-melatonin group (figure 3. 11. 1).

The results demostrate that there are consistent high ratios in the 7 days post ischemic groups over the 72 hours post ischemic melatonin groups in No-ischemic sham control, pre-ischemic melatonin and post ischemic melatonin except for the ischemic groups where the ratio was very close.

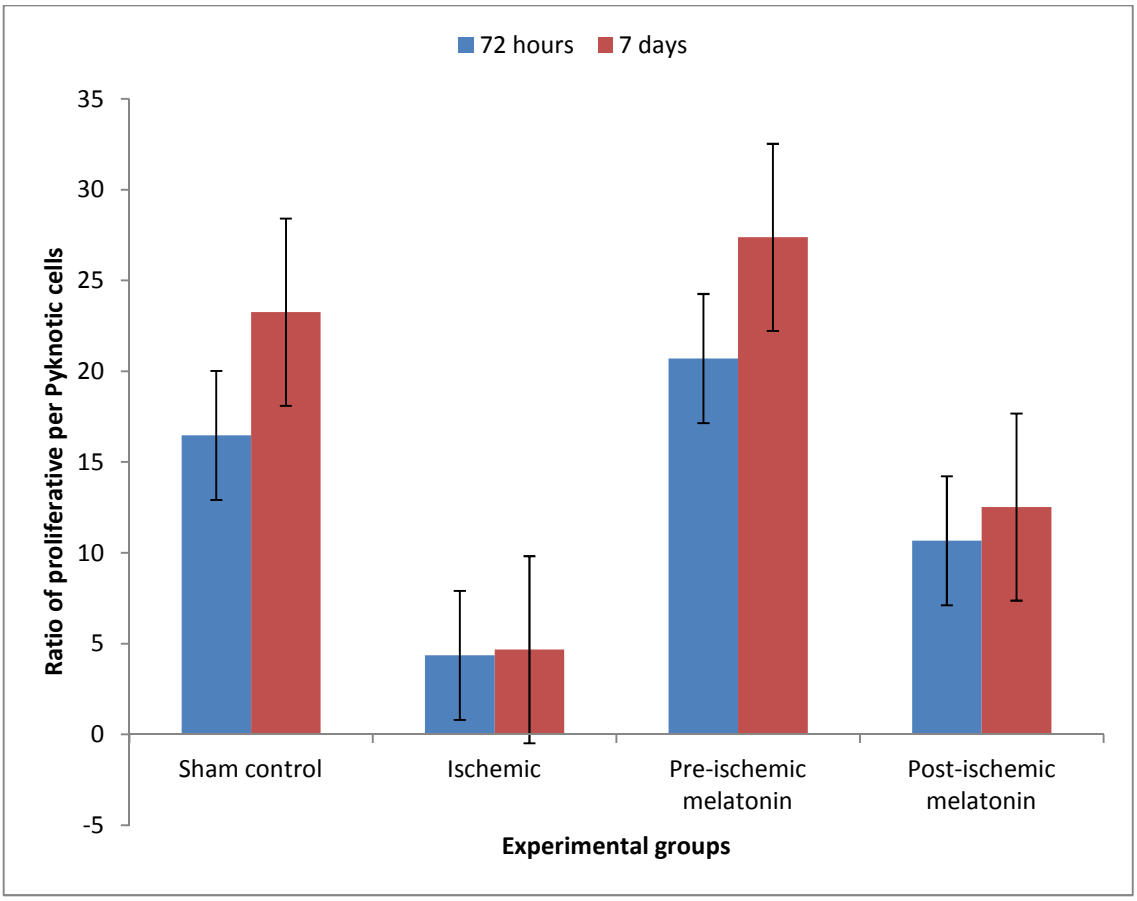


Figure: 3. 11. 1: Graph of ratio of proliferation per pyknotic cell number across the experimental groups in the dentate gyrus. The bar represents the standard deviation.

3. 12: Regression of coeffiicient of pyknotic and proliferating cell number

The regression coefficient (R^2) was 0.0639 for all the experiemntal groups. There are more proliferating cells in the pre-ischemic melatonin after 72 hours of induction of occlusion compared to other groups. The ischemic group has the least number of proliferating cells. The proliferating cells in the 7 days post induction of the ischemic are more in the pre-ischemic melatonin group and the least value was obtained in the No-ischemic sham control group. The estimated total pyknotic number are higher in the ischemic group compared to other groups in both the 72 hours and 7 days post exposure to the occlusion (figure 3. 12. 1). R^2 indicate positive regression coefficient correlation between total pyknotic cells count and total number of proliferating cells counted.

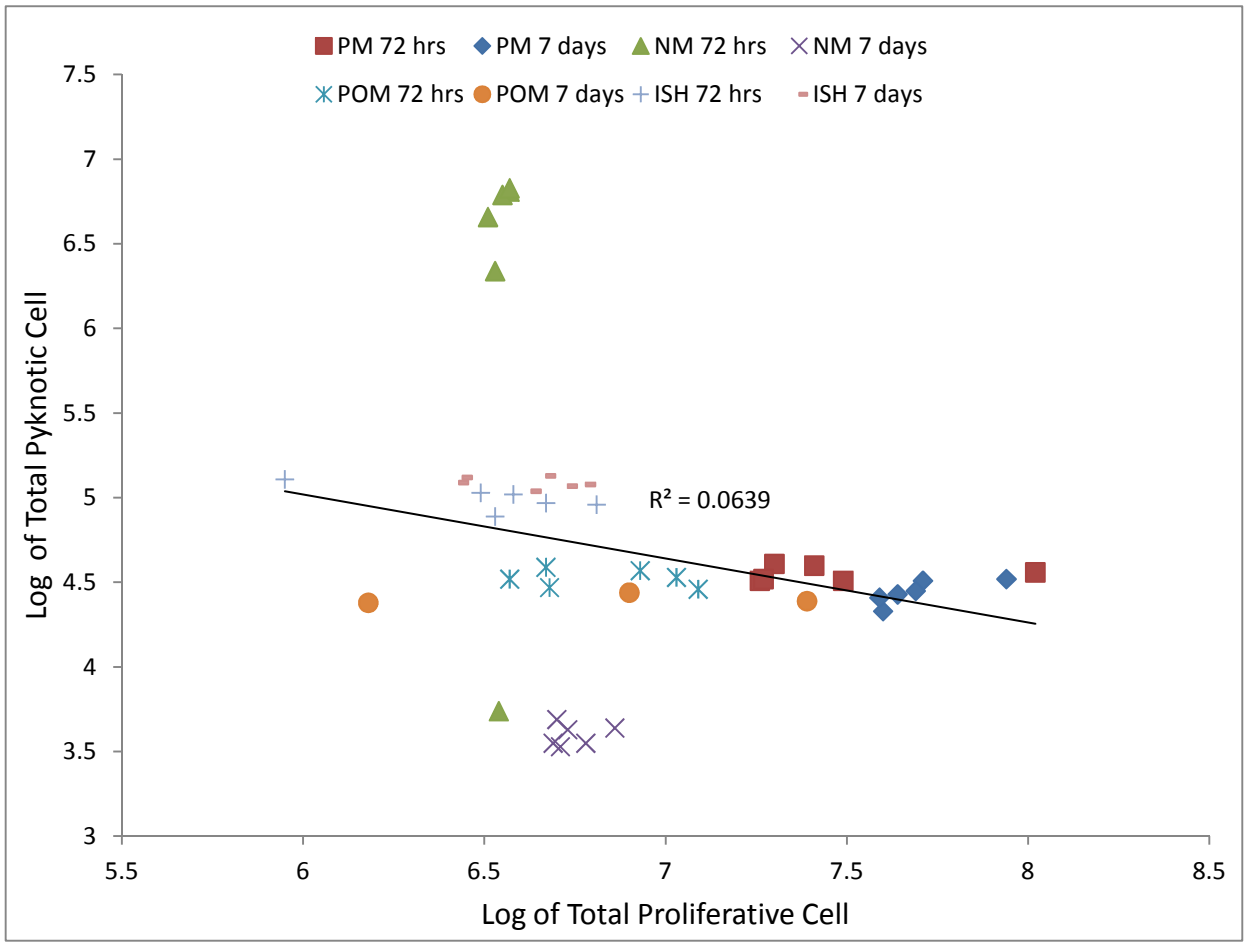


Figure: 3. 12. 1: Scattered diagram showing the regression line of log of total pyknotic cells against the log of total proliferating cells. PM: pre-ischemic melatonin, NM: No ischemia sham control, POM: post-ischemic melatonin and ISH: ischemic group.

3. 13: Estimated melatonin concentration

The serum melatonin concentration of the No ischemia sham control ranges from 216 – 311 pg/mol for the 72 hours duration, ischemic group range was 234 - 294 pg/mol. There was no statistical significant difference between them despite the elevated melatonin concentration in the sham control and pre-ischemic melatonin groups, 72 hours post ischemic time duration. The estimated serum melatonin concentration for the pre-ischemic melatonin groups 72 hours post ischemic was 223 – 308 pg/mol. This was statistically significant. However, the value for the sham control (251 – 311 pg/mol) was not statistically significant (Table 3.11.1).

The 7 days pre-melatonin estimated concentration ranges from 251 – 329 and the mean average melatonin concentration for the group was 277.83 ± 28.198 pg/mol (Table 3.13.2). The graphical representation of the values is shown in figure 3.13.1

Comparison between the post-ischemic time of 72 hours and 7 days across all groups did not present any statistically significant differences. However, comparison between the ischemic times with the pre-ischemic melatonin and post-ischemic melatonin groups against the controls did indicate statistical difference in the melatonin concentration (figure 3.13.1 and 3.13.2).

	Range	Mean pg/mol	SD	t-value	p-value
Sham control	216-314	266.94	12.02	1.472	ns
Ischemic	234-294	272.96	21.81	0.830	ns
Pre-ischemic	223-308	268.54	28.726	0.565	ns
Post-ischemic	251-365	291.58*	38.97	1.582	≤0.05

Table: 3. 13. 1: Serum melatonin concentration (pg/mol) for the groups at 72 hours post ischemia. The mean melatonin concentrations, the range of values measured standard deviation and calculated student t values. Asterisk indicates significant value at $p < 0.05$(s) and ns = statistical not significant.

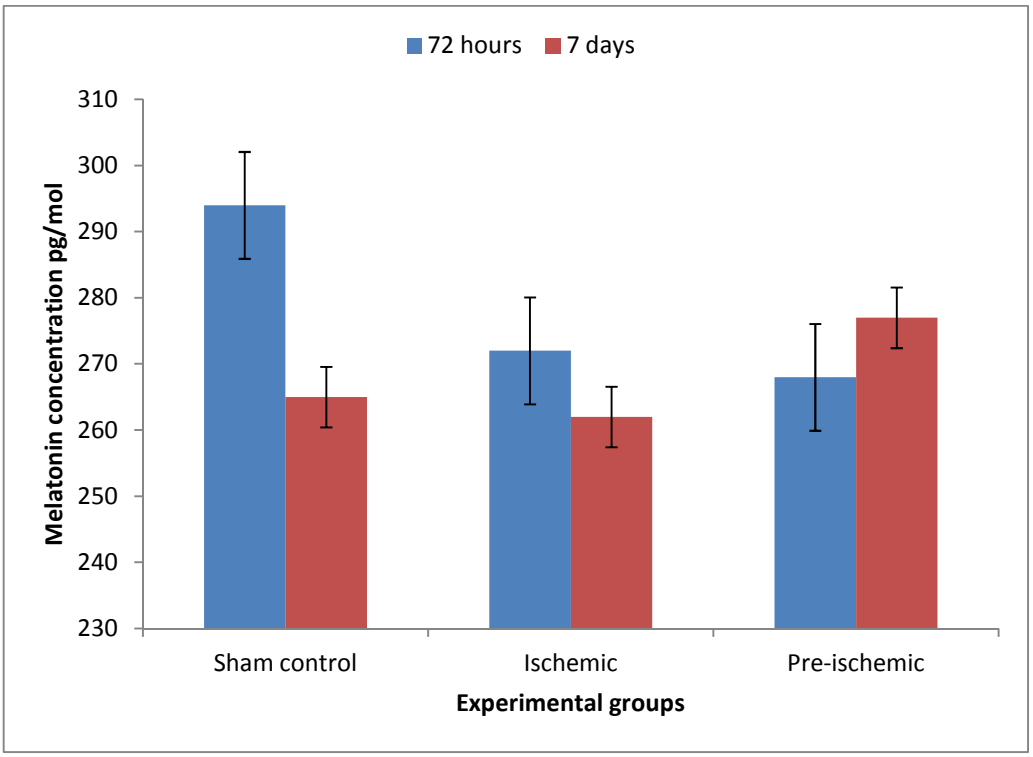


Figure: 3. 13. 1: Graph showing the estimated serum melatonin concentration (pg/mol) in the pre-ischemic melatonin groups. The bars represent the standard deviation.

Estimated melatonin concentration for the post-ischemic for the 72 hours ranged from 251 – 365 pg/mol and the values for the 7 days post-ischemic ranged from 214 - 286 pg/mol (Table 3. 13. 1). There are no statistical significant differences between the concentrations in the No-ischemic control, ischemic and melatonin administered (pre and post) groups. The mean melatonin concentration for the 7 days post melatonin group was 260.63 ± 27.90 pg/mol (Table 3.13.2). The graphical representation of the values is shown in figure 3.13. 2.

	Range	Mean pg/mol	SD	t-value	p-value
Sham control	209-281	251.50	12.02	1.472	ns
Ischemic	217-280	262.01	21.81	0.830	ns
Pre-ischemic	251-329	277.83	28.726	0.565	ns
Post-ischemic	214-286	260.63	38.97	1.582	ns

Table: 3. 13. 2: Serum melatonin concentration (pg/mol) for the groups at 7 days post ischemia. SD = standard deviation and ns = statistical not significant.

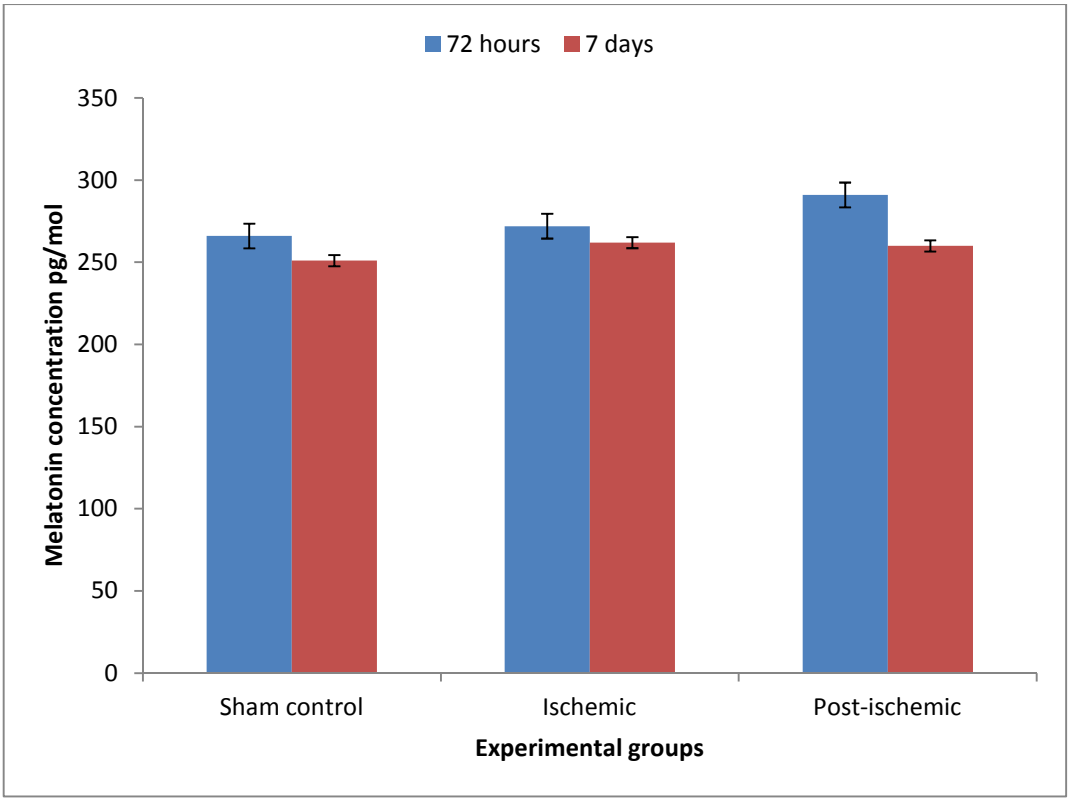


Figure: 3. 13. 2: Graph showing the estimated serum melatonin concentration (pg/mol) of the post-ischemic melatonin groups. The bars represent the standard deviations.

3. 14: Correlation between melatonin concentration and total proliferating cell number

Melatonin concentrations have a direct influence on the total number of proliferating cells generated in the dentate gyrus (positive correlation) where R^2 = 0.4485 in 72 hours post ischemia time within the pre-ischemic melatonin groups. Melatonin concentration in the post-ischemic melatonin groups had no influence with the proliferating cell counts in the 72 hours period after the induction of the ischemia but there was no significant influence within 72 hours post induction of the ischemic-reperfusion injury (figure 3.14.1).

There are indication of stimulations of proliferation as the days progressed to 7 days after the induction of ischemia in both the pre and post ischemic melatonin administered groups compared to the No-ischemic sham control and the Ischemic groups. The correlation indicates that melatonin concentration enhanced the proliferation of the neurogenic cells after 7 days post ischemia (figure 3. 14. 2).

The correlations in the ischemic and sham operated controls were not statistically significant. R^2 indicates significant correlate for the no-occlusion sham control and the ischemic groups but there is no correlation between the melatonin concentration and estimated total proliferating cells in the 72 hours post-melatonin group.

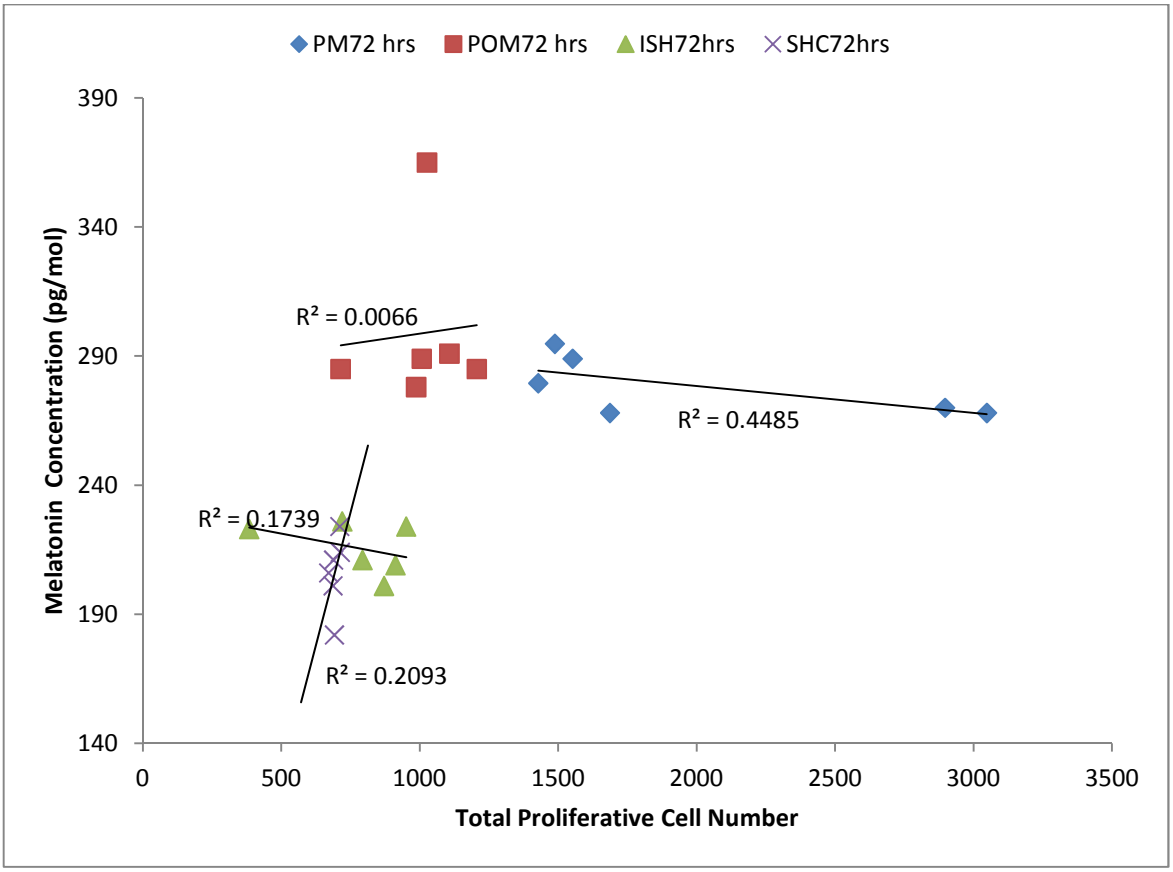


Figure: 3. 14. 1: Graph showing the correlation between the melatonin concentration and total proliferating cells after 72 hours post-ischemia. The R^2 indicates the strength of the correlation between the melatonin concentration and estimated total proliferative cell. PM: Pre-ischemic melatonin; POM: Post-ischemic melatonin; ISH: Ischemic group; SHC: No ischemic sham control

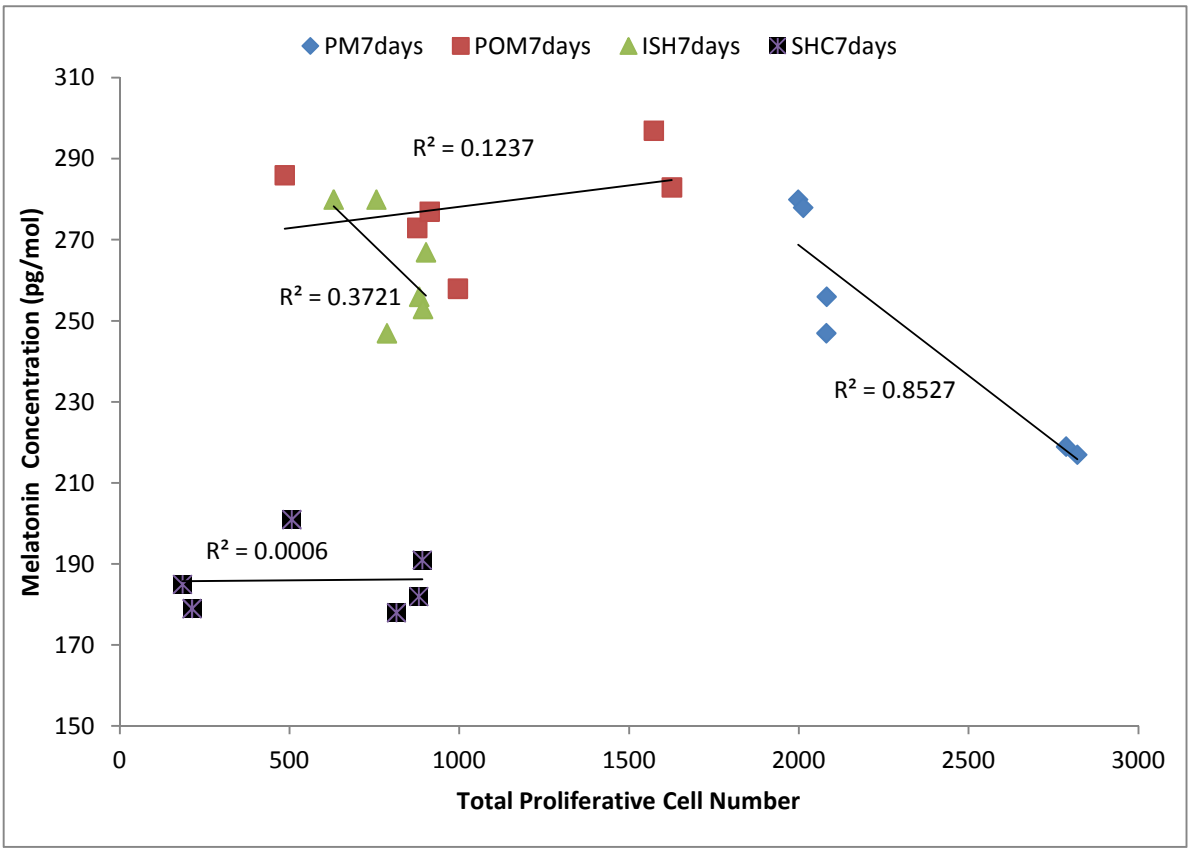


Figure: 3. 14. 2: Graph showing the correlation between the melatonin concentration and total proliferating cells after 7 days post-ischemia. The R^2 indicates the strength of the correlation between the melatonin concentration and estimated total proliferative cell number. PM: Pre-ischemic melatonin; POM: Post-ischemic melatonin; ISH: Ischemic group; SHC: No ischemic sham control

3. 15: Correlation between melatonin concentration and estimated total granule cell number

The estimated total estimated granule cell numbers were not influenced by the ischemia and same observation was recorded in the No-ischemic sham control group. Though, at 72 hours, post-ischemic melatonin increased total number of granule cell number (1076619) in dentate gyrus compared to the pre-ischemic melatonin groups (1052639) (figure 3. 15. 1). The difference was not statistically significant. However, there was correlation between melatonin concentration and estimated total granule cell number 72 hours after induction of ischemia for both pre-ischemic and post-ischemic melatonin, indicating that the presence of melatonin influence the production of granule cells.

At 7 days, post-ischemic melatonin, there was a slight reduction in the estimated total number of granule cell number (1053337) in dentate gyrus in the post ischemia compared to 72 hours pre-ischemic melatonin groups (1061939) (figure 3. 15. 2). R^2 indicates the strength of the correlation for the no-ischemic sham control and the ischemic groups and there is no correlation between the melatonin concentration and estimated total granule cell number in the post-ischemic melatonin group.

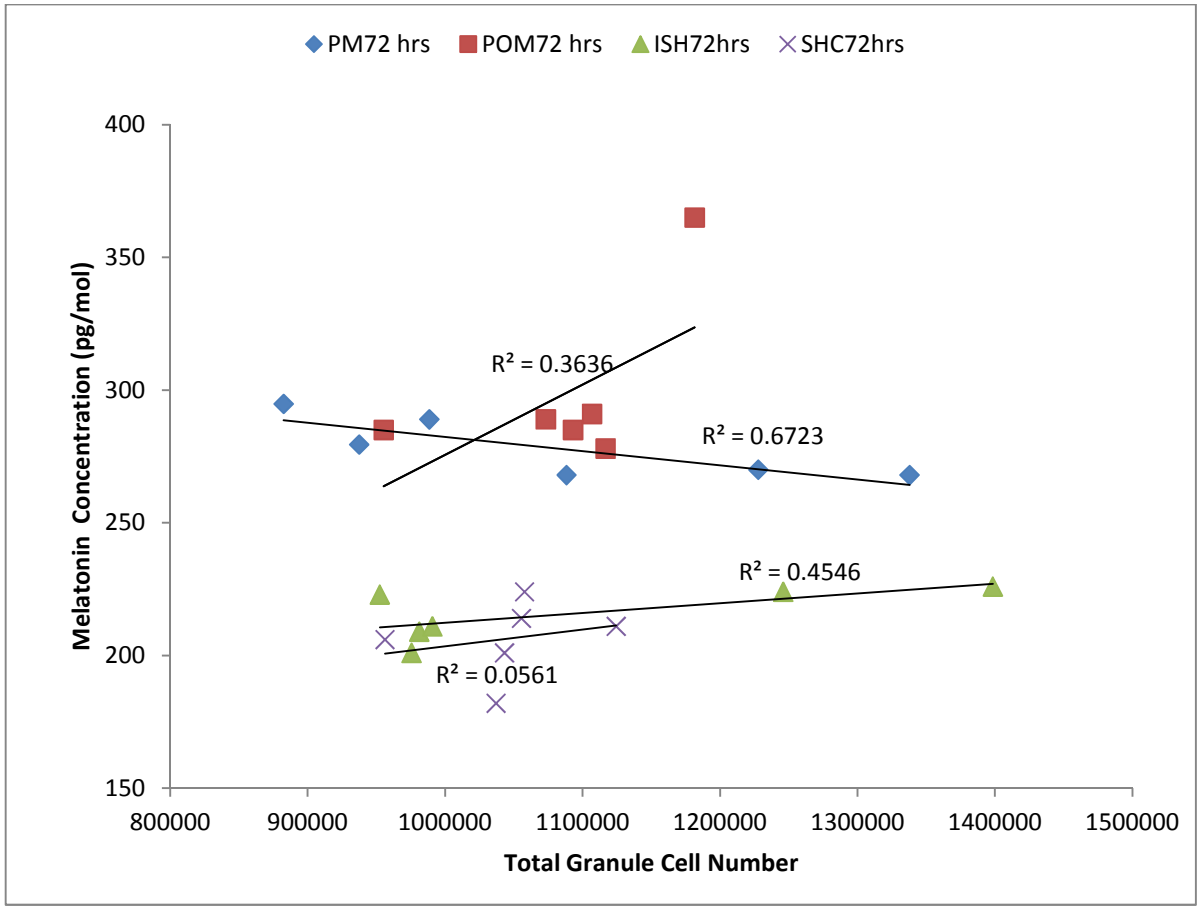


Figure: 3. 15. 1: Graph showing the correlation between melatonin concentration and estimated total granule cell number after 72 hours post-ischemia. The R^2 indicates the strength of the correlation between the melatonin concentration and estimated total granule cell number. PM: Pre-ischemic melatonin; POM: Post-ischemic melatonin; ISH: Ischemic group; SHC: No ischemic sham control

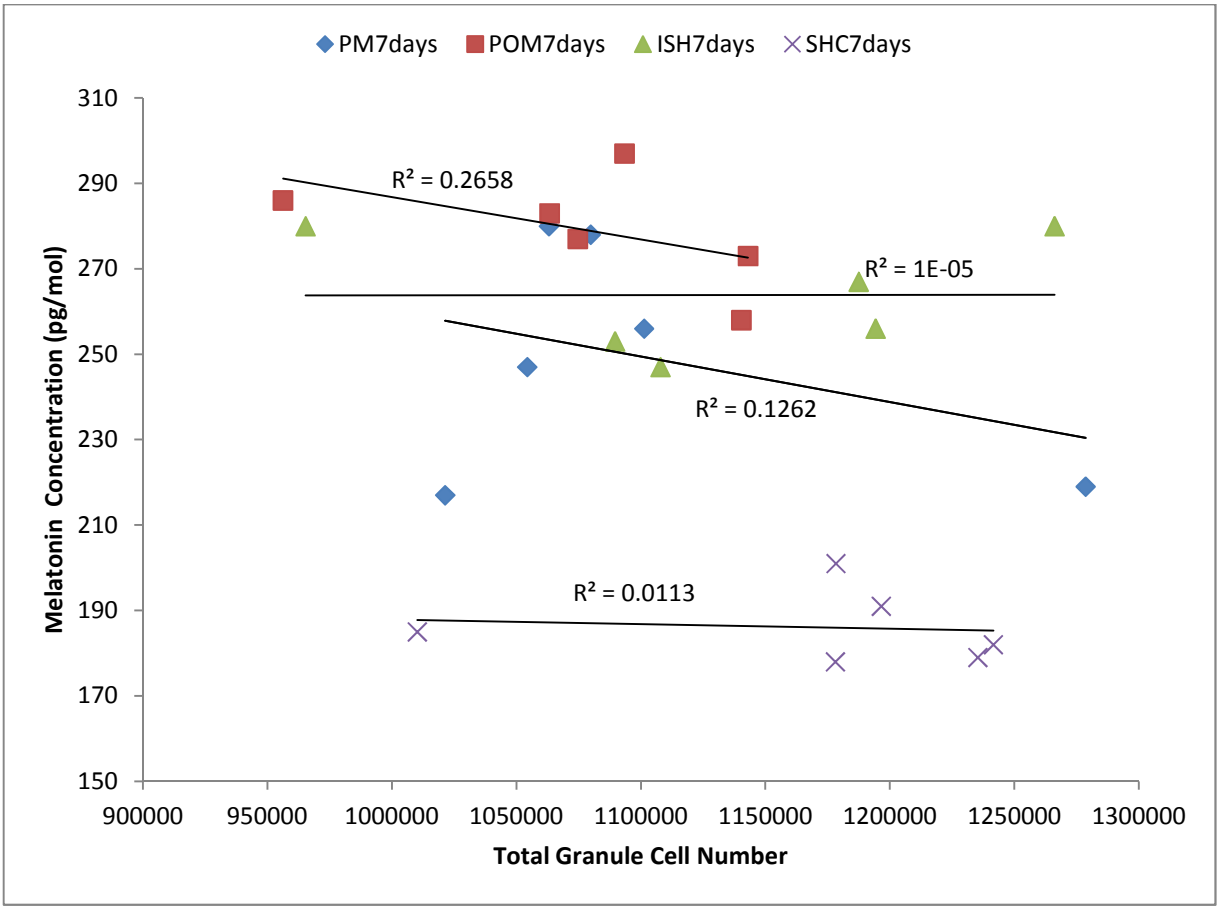


Figure: 3. 15. 2: Graph showing the correlation between melatonin concentration and estimated total granule cell number after 7 days post-ischemia. The R^2 indicates the strength of the correlation between the melatonin concentration and estimated total granule cell number. PM: Pre-ischemic melatonin; POM: Post-ischemic melatonin; ISH: Ischemic group; SHC: No ischemic sham control

3. 16: Correlation between melatonin concentration and pyknotic cell number

There was reduction in the estimated total pyknotic cell number in the pre-melatonin groups at 72 hours post ischemia and this reduction increased as the post ischemic-reperfusion injury days increased (figure 3. 16. 1) to 7 days. The correlation indicate that, exogenous administration of melatonin seem to be required for the reduction of pyknotic cells ($R^2 = 0.1627$).

In the post melatonin groups, there are sustained reduction in both the 72 hours and 7 days post ischemic-reperfusion injury of the total number of pyknotic cell counts ($R^2 = 0.5274 - 0.0325$). The reduction was days dependent since it increased as the days progressed after the induction of the ischemia.

Ischemia alone initially accounted for a slight increase in the total number of pyknotic cells in the 72 hours after the induction of the ischemia but this gradually dissipated as the post ischemia days increased to 7 days. The total number of pyknotic cells was independent on melatonin concentration in the sham controls (figure 3. 16. 1 and 2). R^2 indicates positive correlation for the no-occlusion sham control and the ischemic groups but there were correlation between melatonin concentration and estimated total pyknotic cell number in both the pre-ischemic and the post-ischemic melatonin groups.

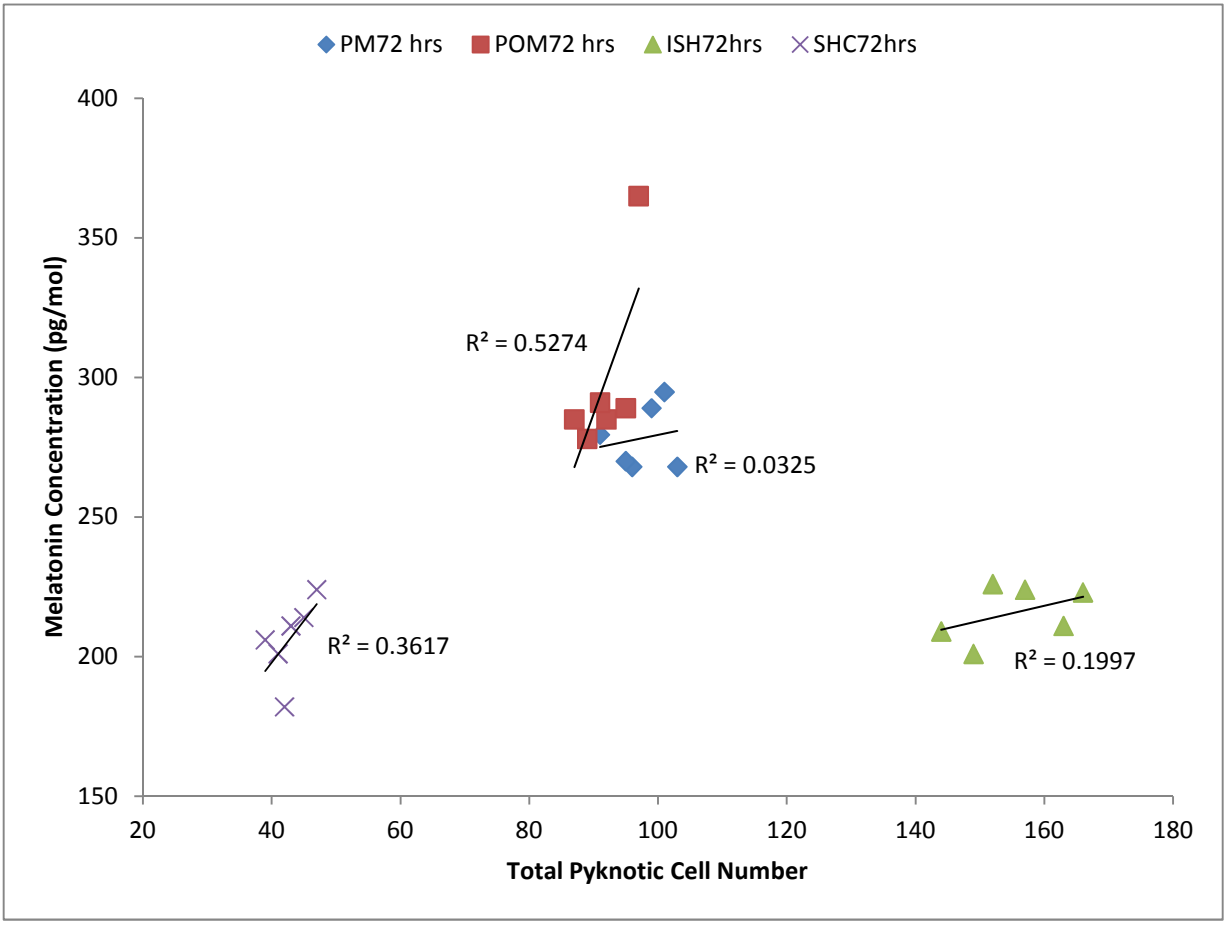


Figure: 3. 16. 1: Graph showing the correlation between melatonin concentration and total pyknotic cells number after 72 hours post-ischemia. The R^2 indicates the strength of the correlation between the melatonin concentration and estimated total pyknotic cell number. PM: Pre-ischemic melatonin; POM: Post-ischemic melatonin; ISH: Ischemic group; SHC: No ischemic sham control

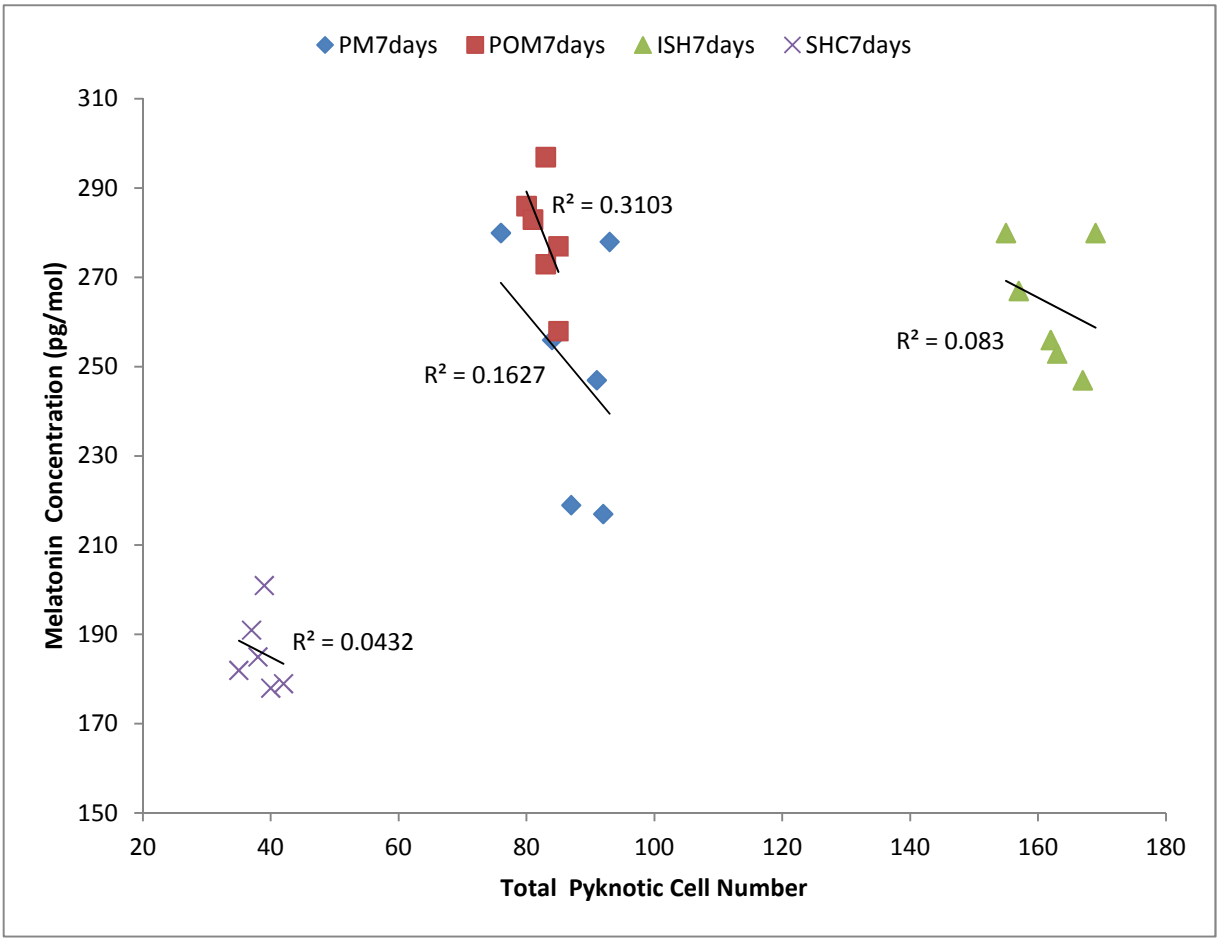


Figure: 3. 16. 2: Graph showing the correlation between melatonin concentration and total pyknotic cells number after 7 days post-ischemia. The R^2 indicates the strength of the correlation between the melatonin concentration and estimated total pyknotic cell number. PM: Pre-ischemic melatonin; POM: Post-ischemic melatonin; ISH: Ischemic group; SHC: No ischemic sham control

Chapter Four

4.0: Discussions

4. 1: Creating the ischemia animal model in Sprague-Dawley rats

The first experiment was to create an ischemic animal model using the Sprague-Dawley rats. This was achieved in the pilot study when 5 minutes ischemic time model was established. This preceded the full experimental induction of a 10 minutes transient global cerebral ischemia. The five minutes bilateral occlusion of the common carotid arteries model used produced no mortality and induced no visible motor deficits. This finding was important with respect to the coordination and locomotion, in which hemiparesis would invariably have resulted in pathologic findings that would have made interpretation much difficult. The finding of this study was at variance with the earlier report of Plum et al, (1963) where they reported 37% mortality in male Sprague-Dawley rats post unilateral occlusion of the common carotid arteries. The mortality recorded following the 10 minutes bilateral occlusion of the common carotid arteries in the present study was much lower compared to that reported by Fujishima et al, (1976) where they report 72% mortality within the first 24 hours after the bilateral occlusion of the common carotid arteries among spontaneous hypertensive rats and 25% among the normotensive.

The histological analyses of the brain showed disruption of the layering and neuropil in the hippocampal region and some regions of the cerebral cortex. The ischemic injuries were minimal in the hippocampal regions as evident by the mild distortions of the CA1, CA2 and CA3 areas of the hippocampus. Hence, there was

the need to increased the occlusion time from the initial period of five minutes to ten minutes to establish a sustained ischemic reaction, and at the same prevent mortality that are associated with induced global cerebral ischemia. The ten minute transient global ischemia was achieved in the subsequent procedures for creating the global cerebral ischemia. Unlike the five minutes ischemia, obvious brain infarcts were observed in the ten minutes ischemia. However, the increased transient occlusion time resulted in a considerable high mortality rate especially in the ischemic group that had no melatonin administration. The present study apparently demonstrated that bilateral carotid arteries occlusion caused severe and fatal damage to brain in ischemic groups resulting in the high mortality rate recorded. The reasons for these observations may be due to the marked brain oedema resulting from the ischemic injury which could have caused an additional reduction of cerebral re-perfusion, worsening of the injuries and increasing the infarct areas in the brain, thus, resulting in the deaths recorded. However, brain oedema was less marked in the pre-ischemic melatonin and post-ischemic melatonin groups allowing the animals to survive for a longer period of 7 days as designed in the subsequent experiment.

The rationale for this method of transient occlusion of the common carotid arteries is based on the incomplete arterial circle of Willis typical of the blood supply to the brain in rats that ensure continuous blood supply to the brain if proper occlusion is not carried out. The present method of occlusion used in the study ensured complete and total blockage of blood supply to the brain thereby preventing compensatory blood flow from the vertebral arteries, and creating global cerebral ischemia in the central nervous system of the rats. This method of

carotid arteries occlusion was employed by Levine and Sohn, 1969, Kahn, 1972 in their studies. Though, various methods have been used to induce cerebral ischemia in animal models with the aims of observing some physiological changes in behaviour of the rats and their response to some therapeutic exposures, little significant findings were reported in behavioural activities of the rats following ischemic brain injuries. The use of the ten minutes occlusion time was adequate for effecting transient global cerebral ischemia, resulting in morphological changes in the cortex and hippocampus of the Sprague-Dawley rat brains. Rennie et al, (2008) observed that transient (usually 5-15 minutes) bilateral carotid artery occlusion is a popular and simple method for induction of cerebral ischemia in animal's model. Cerebral ischemia is often caused by cerebral blood flow less than 25 % of the normal required values (Carvantes 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. Restoration of blood supply (reperfusion) after induction of ischemia must be carefully done because of its attendant risk of further worsening the injury since free radicals are also generated during the reperfusion phase.

The delayed in ischemic reaction infarct observed in this study may be due to the delay in inflammatory processes, couple with time-lag apoptosis dependent cell death which accompanied ischemic reperfusion injuries as corroborated by Paschew 2002 and Cham 2001. Other likely mechanisms of ischemic damage to the brain during these processes may result from the excitotoxicity of the organelles from the cell membranes and the release of free radicals generated during the ischemic process that are probably mediated during the intermediate

stage of ischemic-reperfusion injuries (Salford and Siesjo, 1974). Although leukocytes could accumulate in some ischemic-reperfusion brain injuries, their roles and mechanism in the whole processes is still unclear. The findings of oedema within the brains substances which further reduced blood flow to the various regions of the brain, will increased the brain infarct areas, thus exacerbating the ischemic injuries in the brain and thereby leading to neuronal cell death (Salford and Siesjo, 1974).

Though, apoptosis may be responsible for up to 50% of cellular death in ischemia (Choi, 1996), the presence of pyknotic cells findings in this study gives an indication of other mechanisms of cell death seen in ischemic brain injuries, more so, that few pyknotic cells were counted in the sham and normal controls compared to the 72 hours post induction of ischemia. Some of the ischemic lesions seen in the 72 hours and 7 days post occlusion may be associated with infarction which could be due to degeneration of cellular elements and accompanied by tissue permeation with intestinal fluids from the inflammatory cells often seen in ischemic brain injuries (Nagahori et al, 1994). Other contributory factor to this delayed neuronal cell deaths may be degeneration of some glial cells and impaired vascular elements. Severe brain ischemia rapidly causes necrosis, and this is characterized by lysis of the cell membrane following swelling of the cell and its internal organelles (Nowicki et al. 1991). The indication that ischemic brain damage continues to progress after the onset of ischemia was observed in the brain from 72 hours to 7 days post induction of ischemia correlated with the report by Heiss et al, (1992) and Chan, (1996). Interruption of cerebral blood flow transiently by vascular occlusion may provide

variety of features as seen in this study. Longer ischemic time injury causes severe and significant brain damage, while a brief ischemic insult that does not induce any neuronal death may render the brain to be more tolerant to subsequent and sustained ischemic attack. Accordingly, transient interruption of cerebral blood flow is sometimes necessary in preparing the brain to tolerance, especially during neurosurgery as reported by Yasargil and Fox 1985, Batjer et al. 1988, Batjer and Samson 1989 and generally, 5-15 minutes bilateral occlusion of the common carotid artery will not be hazardous, provided, the blood flow is maintain above 30 ml/100 g/min (Ohtomo et al. 1991). Takahashi et al, (1987) reported that adequate long interval is allowed in between interruptions of blood flow to the brain and if available, brain protective agents are used to enhance brain plasticity to recurrent ischemic insult. The present study indicates that a transient bilateral occlusion of the carotids could lead to severe brain injuries evident by severe histological disruptions of the neuropil. In conclusion, acute transient ischemia of 10 minutes duration of the bilateral common carotid arteries induced progressive neuronal changes in the cerebral cortex and the subgranular zone of the dentate gyrus of the hippocampus. The prolonged post-ischemic duration of 72 hours showed marked changes and the manifestation became progressively worse 7 days post the ischemic brain injury. This implies that the effects of acute or mini stroke may not manifest immediately but rather after a period of time.

4. 2: Dosage and estimation of melatonin concentration

Melatonin dosages used in the establishment of ischemic-reperfusion protective activities and abilities varies from 5-15 mg/kg. However, high dose of 50 mg/kg have also been used with no adverse side effects reported (Pei et al., 2002), but the result demonstrate no significant difference in the protective outcome of melatonin when compared to the smaller dosages used. Reiter et al, (2005) and Skinner and Malpaux, (1999) did report that melatonin exerts its effects across a wide range of concentrations depending on the targeted organ. Thus, pharmacological and physiological concentrations of melatonin are issues of concern, as serum melatonin levels do not correlate with levels in other fluids or cells. Though, melatonin concentration varies in different body fluids, cells types and subcellular compartment, its levels in the blood are normally low compared to other tissues or organs in the body that demonstrate significant high values of melatonin concentration. Nocturnal melatonin concentrations in plasma and cerebrospinal fluid are much higher (Longatti et al, 2007).

Since melatonin is not in equilibrium in the body fluids, studies have shown that endogenous melatonin levels are much higher in the cerebrospinal fluid compare to that of the blood in some animals and this is also true for humans (Skinner and Malpaux., 1999; Longatti et al., 2007). In a related study by Petri et al, (2010), this activity of melatonin may be mediated through the inhibition of the neuronal nitric oxide synthase (nNOS) isoform which was higher in the non surgical control, followed by the post-ischemic melatonin group. The increase in serum concentration levels in the 72 hours could also be associated with intra species variations in response to handling of the exogenous melatonin administration

coupled with the sporadic release of the endogenous melatonin in the animals. Since, the diurnal fluctuation is often reflected in its level in plasma, the effect of this on the serum concentrations of the measured melatonin are removed because all the blood samples were collected at a particular time of the day throughout the study. The significant role played by melatonin in the regulation of various neuroendocrine processes and in ischemic reperfusion injuries were as reported by Reiter (1998). The level of serum melatonin also oscillates in some species of mice (Kennaway et al, 2002 and Vivien-Roels et al, 1998). However, the concentration of melatonin diminished with reperfusion which could be associated with the increase in excretion through the kidneys.

4. 3: The role of melatonin in ischemia

The main stay in the treatment of cerebral ischemia lies in the restoration of blood flow to the ischemic tissue in the shortest possible time to prevent irreparable neuron damage and delayed recovery of the functions of the neurons. The alternative is to reduce the pathophysiological consequences of ischemic injury to the brain by preventing the damages caused by the free radicals especially during the reperfusion phase. Melatonin is noted for having the ability to remove these free radicals that are generated during the ischemic and reperfusion phases. One of the aims of this study was to investigate whether the possible protective effect of melatonin operates better in a pre-ischemic or post-ischemic scenario. The role of melatonin in ischemia in the study was analysed from the findings observed from the ischemic group, pre-ischemic melatonin and post-ischemic melatonin groups which received single dose of 5 mg/kg melatonin compared to the ischemic group that received no melatonin.The findings from this study indicate that melatonin

reduces cell death after stroke as evidence by the marked reduction in the estimated total pyknotic cells number observed. Furthermore, administering melatonin prior to transient ischemic stroke or at the onset of reperfusion seems to restore the cyto-architecture and physiological environment of the brain post injury as also reported by Lin and Lee (2008).

An important element in stroke treatment is the timing of drug delivery since most current therapies that promote functional recovery after stroke are limited to physical rehabilitation. Several neuroprotective activities of compound and metabolites have made limited progress in the prevention and treatment of ischemic stroke especially in humans over a very long period of time. Hence, early administration of any therapeutic agents directed at either prevention or cure will be important in the management of ischemic stroke. This partly explains why early administration of melatonin was crucial in this study. The mechanism by which melatonin exert it activity here may depend on the early events in ischemia-reperfusion injury by preventing the destructive actions of the free radicals, thereby preventing neuronal cell deaths either through necrosis or apoptosis. The anti-apoptotic activity of melatonin was thought to be due to its ability to inhibit mitochondrial permeability which is a necessary requirement for apoptotic cell death to take place (Lin and Lee 2009). The study confirmed that early pre-administration of exogenous melatonin provides protective effects on the acute phase of ischemic brain injury by demonstrating the ability of melatonin in preventing neuronal cell death from brain injury induced by occlusion of the blood vessels that supplied the brains and thus, significantly reduced the infarct size in the brain.

Melatonin represents an attractive option as a neuroprotective approach in ischemic stroke because of its potential efficacy and low toxicity in normal animals and experimental models. Administration of melatonin after the onset of ischemic stroke reduces the cerebral infarction as this study confirmed, thus indicating that melatonin may represent an important agent required in the long term reduction of the ischemic brain injury sequelae even after the injury must have occurred. The long lasting benefit of post ischemic administration of melatonin as observed in this study could give excellent outcomes in the reversal of the brain injury after the stroke, a postulation that corroborates with the observations of Killic et al. (2008). Through histological analysis of cerebral cortex and hippocampus and cell proliferations in the dentate gyrus, melatonin could be said to exert a neuroprotective activity after transient global cerebral ischemia when delivered within 30 minutes after the induction of ischemic stroke. Melatonin promotes the recovery of motor and coordination deficits and attenuates post ischemic hyperactivity and anxiety (Reiter et al, 2005). However, as a result of individual variability, the decreased brain damage with the single dose at 5 mg/kg did not show a statistical significant difference among the pre-ischemic melatonin and post-ischemic melatonin groups. This may be an indication that melatonin may be more effective and acts dose-dependently. This result is consistent with observations by Carloni et al, (2008) that melatonin administered after the onset of ischemic injury may require repeated doses or single bolus of a high dose. However, relative to several other antioxidants, much less melatonin is required to achieve significant molecular protection from

oxidative damage resulting from ischemic-reperfusion injuries (Yilmaz et al, 2002 and Montilla-Lopez et al, 2002).

The ability of melatonin in reducing cerebral inflammation after stroke (Carvantes et al, 2008), further strengthen the findings of the present study in which pre-ischemic and post-ischemic administration of melatonin decrease oedema and cellular infiltration in the ischemic regions of the brain. Lee et al, (2007) reported that post administration of melatonin following ischemic stroke does caused brain swelling and may not necessarily change the cellular immune response in the serum but play a significant role in the protective mechanisms of the brain post ischemia. The activity of post-ischemic administration of melatonin was evident in decreasing the infarct and thus reducing the penumbral areas in the brain and the cell infiltration in the ischemic regions of the brain. In conclusion, the results of this experiment indicate that pre-ischemic and post-ischemic melatonin administrations do offer protection against acute ischemic-reperfusion brain injury. However, the pre ischemic melatonin administration offered a better protective ability compared to the post ischemic group.

4. 4: The role of melatonin in adult neurogenesis

The total pyknotic cell numbers was used for the correlation involving melatonin and cell proliferation in the study. The correlation between the pyknotic total number and cell proliferation provides an indirect indication of the extent of the estimated total granule cell that becomes available in the hippocampus. This explanation has been included in the said section for clarity of the uniqueness of these findings in the ischemic brain developed as part of this study. The finding of

the present study demonstrated that the proliferation potentials of the dentate gyrus of the hippocampus in the ischemic brains can be enhanced by the presence of melatonin. This was correlated by the report of Ramirez-Rodriguez et al, (2009) that administration of exogenous melatonin in acute or chronic state can increase cell proliferation in the dentate gyrus of adult mice. However, these findings were at variance with a study by Kim et al, 2004 in which melatonin was reported to increase cell proliferation in the dentate gyrus in the brains of young rats only after seven days of treatment. Generally, it is believed that, the enhancement of post-ischemic neurogenesis and cell migration is expected to promote tissue repair and improve the functional outcomes of stroke (Zheng et al, 2007) and this could be augmented with administration of exogenous melatonin as demonstrated in the present study. The exact mechanisms by which post-ischemic neurogenesis occurred are not clearly understood. However, the effect of external and exogenous factors in the enhancement of stimulation of proliferating cells and their migrations to injured sites have been observed.

Ischemia alone does have some stimulatory effect on the proliferating cells of the hippocampus and this activity seems to be potentiated by the presence of melatonin (Ajao et al, 2010). This finding was consistence with Sharp et al, (2002) where they reported that neuronal death within the hippocampus could provide the stimulus for increased neurogenesis following ischemia. The mechanism by which melatonin does this may be related to its ability to reduce cell death after stroke (Lin and Lee, 2009). The above observations confirmed reduction in the estimated total number of pyknotic cells numbers in both the pre-ischemic and post-ischemic melatonin administered groups. This further correlate

with reports demonstrating melatonin ability to reduce apoptosis after stroke, hence, administering melatonin prior to transient global cerebral ischemia or at the onset of reperfusion could restore the cyto-architecture of the brain toward normalcy. Since, ischemia alone can stimulate proliferation and differentiation of neural/stems progenitor cells, the newly generated neurons will need to be integrated into the already existing neuronal networks, however, the mechanisms at which this is carried out is out of the scope of this study. The expansion of the pool of endogenous progenitors could augment regenerative capacity of the brain in response to the ischemic injury (Jin et al., 2001, and Arvidsson et al., 2002,). The cellular and molecular mechanisms that regulate post-ischemic appearance of newborn neurons in specific brain structures are not clearly understood, however, it is receiving attention (Arias-Carrion et al., 2007, Catts et al., 2008). The finding of the study shows that melatonin do not only exerts neuroprotective activity after transient global cerebral ischemia, but also stimulates endogenous neurogenesis and promote the recovery of neuronal functions; as observed by Rennie et al, (2008).

There were marked morphological changes in the pyramidal layer of CA1 sector during the initial 72 hours and 7 days after ischemia. Ki-67 positive cells were sparsely distributed within the granule and subgranular layers. However, the subsequent fates of these newly generated neurones were not part of this study. Wojcik et al, (2009) demonstrated that endogenous regenerative capacity appears to be very limited and not capable of restoring the lost neuronal circuit after brain injuries without the influence of exogenous factors, in this case, melatonin as in this study. Hippocampal neurogenesis was observed in the ischemic-stimulated

groups and this finding corroborates Ming and Song, (2005) finding of restriction of neurogenesis in the dentate gyrus subgranular zone of rodents. Melatonin enhanced cell proliferation in the ischemic brain in the present study and this may be an indication that the antioxidant also stimulates endogenous neurogenesis in the dentate gyrus of the hippocampus. However, the presences of abundant clusters of abundant neural progenitor cells in the areas following ischemic stroke and melatonin administration strongly suggest that, melatonin may be responsible for the stimulation of the proliferating cells. The presence of Ki-67 positive cells in the hippocampus may not fully be an explanation for the enhanced cell proliferation activities seen in these brains, but in rats, newly generated neurons may account for 6% of all granule cells in the cerebrum after global ischemia (Cameron and McKay, 2001). The estimated total granule cell numbers may not increase with age (Repp and Gallagher., 1996; Merrill et al., 2003), but, in rats, it seems the estimated total numbers of proliferating cells may have been balanced by cell death (pyknotic cells) as demonstrated in the present study. This is consistent with Amrein et al. (2004) findings that the number of proliferating cells correlates significantly with the number of pyknotic cells across several species. This, in general indicates cell generation in the dentate gyrus can be linked to cell death.

The estimated total granule cell numbers were not significantly increased by the exogenous administered melatonin except the post-ischemic melatonin group thus, suggesting that effects of melatonin are more pronounced in modulating the survivals of the new neurons from adult hippocampal precursor cells. This finding was at variance with Kempermann and Gage, (1999) report that granule cell

numbers can be increased in some experimental settings, and which was confirmed further by Ramirez-Rodriguez et al, (2009) in which they reported that melatonin in the hippocampus might affect the hippocampal microenvironment rather than influencing isolated precursor cells alone. In the present study, it was observed that the effects of melatonin in the protection of brain injury resulting from ischemic-reperfusion and survival of the neuronal cells occurred within the physiological parameters. As to why it does not modulate the production of granule cells was not clearly understood. Hence, the preservation of the microenvironment for the cell survivals and maturation of the new neurons in the adult brain may be an important potential medical intervention in the prevention and treatment of ischemic stroke. However, even though melatonin does not increase the total numbers of granule cells directly, it seems to modulate proliferation and reduce degeneration of granule cells in phases or immediately follow each other, possibly due to regulatory factors leading to proliferation in one cell population (neurogenic cells) and to the degeneration in another (Cameron and Gould, 1996). Despite marked differences in the total granule cell number, cell formation and cell death, it is interesting to find that no differences in the ratio of proliferating cells to pyknotic cells in all the experimental groups except in the ischemic group only. The finding of this study was consistence with Hsu and Buzaki, (1993) in which it was reported that granule cell loss is not a significant feature of moderate durations of global cerebral ischemia. This indicates that the mechanism regulating the formation and elimination of granule cells may be similar and closely related.

Ischemic brain injury frequently disrupts neural functions in the central nervous system (Yagita et al. 2001), it is therefore important to understand the response of the neural progenitor cells that are generated after ischemia. The findings from present study confirmed that ischemic brain injury lead to generations of more pyknotic cells in the ischemic groups compared to the non ischemic sham control, pre ischemic and post ischemic melatonin groups. The cellular and molecular mechanisms that regulate post-ischemic appearance of newborn neurons in specific brain structures are not clearly understood (Arias-Carrion et al., 2007, Catts et al., 2008). The finding of the present study indicated that administration of melatonin prior to induction of 10 minutes 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 hours post-ischemic duration. This increase was extended to a three-fold after 7 days post-ischemic duration in the pre ischemic melatonin group. 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. 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. Therefore the total numbers of pyknotic cells do have a direct correlation to the total numbers of proliferating cells in the presence of exogenous melatonin. The differences in the total pyknotic cells number 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. The view that expansion of the pool of endogenous progenitors

could augment regenerative capacity of the brain in response to the ischemic injury was reported by Jin et al., 2001, and Arvidsson et al., 2002 and this tend to correlate the findings of the present study. In conclusion, the findings of this study did demonstrate that melatonin does have some protective effects and modulatory role following ischemic-reperfusion brain injury and on proliferations of neurogenic cells respectively. Melatonin induces functionally relevant neurologic improvements in stroke and exerted an extended neuroprotective action on the brain after stroke and further stimulated endogenous neurogenesis.

Chapter Five

5. 0: Conclusions

Transient global cerebral ischemia was successfully created by a ten minute occlusion of the common carotid arteries in the adult male Sprague-Dawley rats. The ischemic animal model created was subsequently used to investigate the effect of pre- and post-ischemic administration of exogenous melatonin on transient global cerebral ischemia and adult neurogenesis. A 5 mg/kg exogenous administration of melatonin intraperitoneally, prior to ischemia, did not alter the serum melatonin concentration in the rats that had undergone 10 minutes transient global cerebral ischemia. There were alterations in the serum melatonin concentrations in early phase of post-ischemic that normalised as the post-ischemic days progressed.

Transient global cerebral ischemia of 10 minutes in rats caused ischemic changes in the brain evidenced by the disruptions of the neuropil and increased numbers of estimated total pyknotic cells starting from 72 hours after the induction of the ischemia and this became progressively worse with time. Most of the effects of the ischemic brain injury were found within the lamina IV and V of the cerebral cortex and in the hippocampus; the CA1 region and the subgranular zone of the dentate gyrus.

Global cerebral ischemia seems protected by exogenous administration of melatonin either pre or post induction of the global cerebral ischemia in rats and this protective ability of melatonin. The pre-ischemic melatonin administration

offered a better protective response compared to the post-ischemic melatonin administration.

Melatonin potentiated cell proliferations in the dentate gyrus of the hippocampus. There were strong indications that melatonin enhanced the generations of proliferating cells (positive correlation), and equally prevented cell death (reduced pyknotic cell number). Melatonin seems not to have significant influence on the estimated total granule cells number in the dentate gyrus of the hippocampus in the ischemic model using Sprague-Dawley rats.

5. 1: Further study

Electron microscopy studies of the extent of histopathological changes associated with transient global cerebral ischemic lesions is apt and the extent of the protective ability of melatonin is required especially on other cerebral layers, hippocampal region and other regions of the brain. This will help to determine the severity of the brain injury and if necessary, the extent of recovery following therapeutic interventions.

Determination of the fate of the newly generated proliferating cells and characterisation of their activities need to be documented. This is very important, because, the newly generated proliferating cells could serves as a veritable source of neurone cells for possible transplant in some neurodegenerative diseases of the brain in the near future.

The significance of the role of sexual dimorphism in transient global cerebral ischemia and melatonin activity is necessary to determine whether some of the female hormones will influence the outcome of the study, since, females are said to have better prognostic factors in ischemic stroke.

Conference attended and papers presented

The role of melatonin in transient cerebral ischemia in Sprague-Dawley rats: Preliminary report. International Conference on Immunopharmacology, Cuba, 2008.

Pre-administration of melatonin does not alter melatonin concentration in acute phase of ischemic rat brain. 22nd conference of the International Society of Neurochemistry and Asian Pacific Society for Neurochemistry: Busan, South Korea, 2009.

Delayed ischemic brain injury induced by transient occlusion of common carotid arteries in Sprague-Dawley rats. 9th conference of the Society of Neuroscientist of Africa, Egypt, 2009.

Transient ischemia and adult neurogenesis: Does melatonin play any role? 3rd International conference of INBR, Abuja, Nigeria, 2010

Melatonin potentiates cells proliferation in the dentate gyrus following ischemic brain injury in adult rats. 7th Annual conference of Anatomical Society of Nigeria, BUK, Kano, Nigeria, 2010.

Papers published and the journal

Ajao MS, Olaleye O. Ihunwo AO (2010): Melatonin potentiates cells proliferation in the dentate gyrus following ischemic brain injury in adult rats. J. Anim. Vet. Adv 9(11): 1633-1638

Petri ES, **Ajao MS,** Olaleye O, Ihunwo AO (2011): Effect of melatonin on neuronal nitric oxide synthase expressing cells in the brain following global cerebral ischemia. J. Anim. Vet. Adv.10 (4): 395 – 400.

The ischemic animal model created during the PhD research was used for this study and the author co-supervised the Honours project.

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