EFFECT OF MELATONIN ON HIPPOCAMPAL NEUROGENESIS IN THE SPINAL CORD INJURY MODEL

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IN
ANTI-AGING AND REGENERATIVE SCIENCE

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Independent Study Title	Effect of Melatonin on Hippocampal Neurogenesis in the Spinal Cord Injury Model
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ABSTRACT

Stress defined as a condition that seriously perturbs the physiological and psychological balance of an individual. Stress-related psychopathologies such as major depressive disorder (MDD), anxiety, conduct disorders, and posttraumatic stress disorder (PTSD). In humans, dysfunctions of glutamatergic neurotransmission, maladaptive structural and functional changes in hippocampal circuitry, and decreased hippocampal volume have been associated with stress-related conditions such as PTSD. In several studies, the animal models of PTSD resemble the animal models of neurodegenerative disease. To date, one of all neurodegenerative diseases causes permanent paralysis in patients was traumatic spinal cord injury (SCI). One of the neurotrophic factors in our body is melatonin. It has been reported that melatonin has also been shown to ameriorate in adult hippocampal neurogenesis, suggested to occur through its ability to scavenge free radicals.

Currently, we try to investigated the interventions to reduce hippocampal cell loss (dentate and cornu ammonis) after SCI by melatonin treatments immediately following the trauma and continuously administered for 14 days. Female mice were used in this study and were induced SCI via performing laminectomy at T12 vertebrae. The spinal cord was then compressed by using Dumont forceps to made

severe crush injury models. Melatonin was deliver by intra-peritoneal injection after SCI induction. After 14 days mice were sacrificed and brain tissue was removed for the immunohistochemical procedures study for neuronal density in hippocampus.

The previous study was consistent with this finding that melatonin can protecting the brain from prolong oxidative stress and loss of cells resulting from the inflammatory after traumatic of spinal cord injury as seen in neuronal density of hippocampus (dentate gyrus with cornu ammonis) differed significantly between melatonin treatment group and SCI group (16.00 ± 3.16 versus 1.25 ± 0.96) $p < 0.05$. These result can evaluated the neuroprotective effect of melatonin to elevated levels of inflammation on hippocampal in mice with spinal cord injury.

For further study, concerning with the chronically elevated concentration of melatonin within the large experimental group in prolong duration of melatonin uptake are necessary.

Keywords: Neurogenesis/Hippocampal Cell Density/Spinal Cord Injury/Posttraumatic Stress Disorder (PTSD)/Melatonin

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION AND SYMBOLS

SCI	Spinal cord injury
PTSD	Post traumatic stress disorder
MDD	Major Depressive Disorder
HPA	Hypothalamic-Pituitary-Adrenal Axis
DG	Dentate Gyrus
CA	Cornu ammonis
CNS	Central Nervous System
PNS	Peripheral Nervous System
IL	InterLeukin
TNF	Tumor Necrosis Factor
EC	Entorhinal Cortex
PFC	PreFrontal Cortex
NMDA	*N*-methyl-D-aspartate
SVZ	subventricular zone
SGZ	subgranular zone
PBS	Phosphate buffered saline
BPD	bipolar disease
N	Number of sample population
SD	Standard deviation
*	$p< 0.05$
**	$p< 0.01$
***	$p< 0.001$

CHAPTER 1

INTRODUCTION

1.1 Research Background

Central nervous system insults, such as spinal cord injury (SCI) or traumatic brain injury (TBI), are among the leading causes of mortality and morbidity worldwide. They produce profound neurological dysfunction, due to the progressive destruction of local and distal neuronal networks, resulting from injury-induced tissue damage and subsequent local, cellular, and biochemical reactions. Interestingly, significant spontaneous neuroplasticity occurs over the weeks and months following SCI or TBI, leading to some functional recovery (Raineteau, 2008; Darian-Smith, 2009; Kernie & Parent, 2010; Richardson et al., 2010). Post traumatic stress disorder (PTSD) is a consequence of the traumatic event resulting in SCI. The PTSD is unique among psychiatric diagnoses because it emphasizes a causative event, with actual or threatened death or serious injury or other threat to a person's physical integrity (Criterion A). The characteristic symptoms of PTSD include persistent re-experiencing of the event (Criterion B), avoidance of stimuli associated with the trauma and numbing of general responsiveness (Criterion C), and increased arousal (Criterion D). The full symptom picture must be present for more than 1 month (Criterion E), and the disturbance must cause clinically significant distress or impairment in social, occupational, or other important areas of functioning (Criterion F) (American Psychiatric Association [APA], 1994). A hallmark feature of PTSD is reduced hippocampal volume. The hippocampus is implicated in the control of stress responses, declarative memory, and contextual aspects of fear conditioning. Not surprisingly, the hippocampus is one of the most plastic regions in the brain. Prolonged exposure to stress and high levels of glucocorticoids in laboratory animals damages the hippocampus, leading to reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis (Fuchs & Gould, 2000). Bremner et al. (1995) show that the small hippocampal volumes were associated with the severity of trauma and memory

impairments in these studies. Injury to the spinal cord is well-known to induce the formation of reactive astrocytes and the infiltration of immune cells in the vicinity of the lesion site (Horky, Galimi, Gage & Horner, 2006; Yang et al., 2006; Rolls, Shechter & Schwartz, 2009; Beck et al., 2010), but whether SCI also induces the production of new neurons in vivo remains controversial. Studies have shown that spinal neurogenesis occurs to a limited extent after SCI (Chi et al., 2006; Ke et al., 2006; Shechter, Ziv & Schwartz, 2007), but that it could be stimulated by experimental intervention (Ohori et al., 2006). Overall, available data suggest that the extent of neurogenesis at the spinal level after SCI depends on the localization and severity of the injury (Ohori et al., 2006; Vessal, Aycock, Garton, Ciferri & Darian-Smith, 2007). However, previous research showed the reduced number of newly generated neurons in the forebrain accounts for reduction in cell proliferation rather than an increase of cell death after SCI.

Melatonin is a neuroprotective compound and its metabolites are potent radical scavengers protecting cells from damage induced by a variety of oxidants, including hydroxyl radicals and lipid peroxidation products (Hardeland, 2009; Akbulut, Gonül & Akbulut, 2008). Melatonin is not only inhibiting inflammation by reducing the activation of microglia and macrophages, but also reducing tissue alterations. (Samantaray et al., 2008; Esposito et al., 2009). Moreover, it also significantly improved the recovery of limb function (evaluated by motor recovery score) and anti-inflammatory benefit in an animal model of SCI (Genovese et al., 2005). Spinal cord injury (SCI) leads to irreversible neuronal loss and glial scar formation, which ultimately result in persistent neurological dysfunction. Neuroprotective drug could be an ideal approach to replenish the lost cells and repair the damage. Therefore, this study was undertaken to examine the effect of the melatonin on neurogenesis in the adult rat spinal cord injury by immunohistochemical methods.

1.2 Research Objectives

Study the effects of melatonin treatment on neurogenesis in the hippocampus after post-traumatic stress disorder (PTSD) caused by SCI in animal model.

1.3 Hypothesis

The Melatonin may be responsible for neuroprotective effects on neurogenesis in PTSD after SCI study in adult mice.

1.4 Scope of Research

This independent study emphasize on the neuroprotective effects of melatonin to post PTSD after spinal cord injury by induced crush SCI in mices. To determine the newly born cells after SCI, double-fluorescent immunolabeling was performed, newborn neurons were identified by Ki67 and colocalization with neucleus stain with DAPI. Analysis was done by fluorescent microscopy. Net neurogenesis was calculated multiplying the total number of Ki67-labeled cells per the percentage of in the hippocampus area (dentate gyrus and cornu ammonis areas)

1.5 Operational Definitions:

This study divided into 3 groups;

1.5.1 Control group (n=1, 4 slides sample), Female mice (3 months old) will purchase from National Laboratory Animal Center (Mahidol University, Thailand) and without any treatment.

1.5.2 SCI Group (n=1, 4 slides sample), To study of PTSD, induction of SCI performed following the standardized severe crush method Briefly, mice will be anesthetized and a laminectomy performed at the T12. Using Dumont forceps to compress the cord laterally from the sides for 5 sec made crush injury model as previously described by Krityakiarana et al. in 2010.

1.5.3 SCI with Melatonin (n=1, 4 slides sample), mice were induced with SCI and 10 mg/kg doses of melatonin will deliver by intra-peritoneal injection after SCI induction in several timing.

1.6 Conceptual Framework

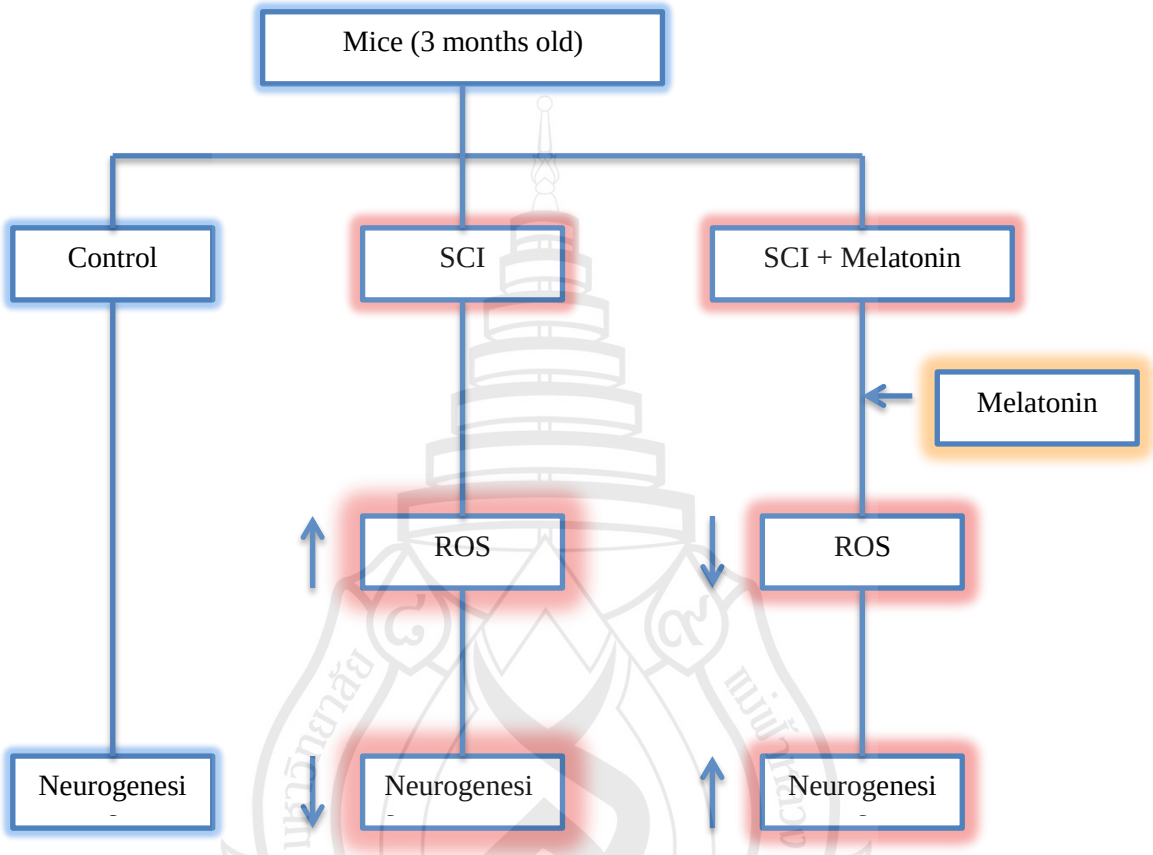


Figure 1.1 Conceptual Framework

1.7 Term of Definitions

1.7.1 Immunohistochemical methods: Phenotypic analysis of newly formed cells in the hippocampus of mice by immunofluorescent staining.

1.7.2 PTSD: The Post traumatic stress disorder after induced with spinal cord injury

1.7.3 SCI: Spinal cord injury disrupts ascending and descending connections below the lesion. Depending on the nature and level of the injury, patients can suffer severe disability and a high economic burden with rehabilitation being the only current treatment option

1.7.4 Melatonin: The main product of the pineal gland is cyclically synthesized in synchrony with the dark period of the circadian cycle (Reiter, 1991).

1.7.5 Neurogenesis: is the process by which neurons are generated from neural stem cells and progenitor cells. It plays a central role in neural development. Neurogenesis is most active during pre-natal development and is responsible for populating the growing brain with neurons. In mammals, adult neurogenesis has been shown to occur in multiple brain structures, including the dentate gyrus of the hippocampus

1.7.6 Ki67: The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation. It is strictly associated with cell proliferation. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes.

1.7.7 DAPI: (4', 6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy.

CHAPTER 2

LITERATURE REVIEW

2.1 Spinal Cord Injury (SCI)

Spinal cord injury (SCI) results in the loss of function below the lesion. usually induced by direct bone fracture, dislocation of spinal disc and ligament in forms of compression or contusion. The compression of the spinal cord that follows vertebral displacement and edema is considered to be a very frequent cause of traumatic spinal cord lesion. (Mautes et al., 2000). Primary traumatic mechanical injury to the spinal cord causes the death of a number of neurons that cannot be recovered or regenerated. Some neurons continue to die for hours after SCI (Christie et al., 2008; Kaptanoglu et al., 2000) following the primary impact includes a number of biochemical and cellular alterations, localized edema, hemorrhage, thrombosis, vasospasm, and loss of vasculature autoregulation. Blood vessels are damaged, which leads to hemorrhage and ischemia at the lesion site and including to epicenter within minutes after trauma. Disruption of spinal tissue causes membrane depolarization and results in glutamate release to activate NMDA and AMPA receptors. This process can increase the toxic levels, which effected to the tissue. The Secondary injury neuronal death may be caused by substances released from cells in response to the primary injury. After SCI has damage on tissue by tissue inflammation, myelin degeneration, and glial cell death at the injury epicenter and adjacent areas. And induce the formation of reactive astrocytes and the infiltration of immune cells in the vicinity of the lesion site (Horky et al., 2006; Yang et al., 2006; Rolls et al., 2009; Beck et al., 2010), but whether SCI also induces the production of new neurons in vivo remains controversial. Studies have shown that spinal neurogenesis occurs to a limited extent after SCI (Chi et al., 2006; Ke et al., 2006; Shechter et al.,2007), but that it could be stimulated by experimental intervention (Ohori et al., 2006). Several studies data suggest that the extent of neurogenesis at the spinal level after SCI depends

on the localization and severity of the injury (Ohori et al., 2006; Vessal et al.,2007). Central nervous insults, such as spinal cord injury (SCI) or traumatic brain injury (TBI), are among the leading causes of mortality and morbidity worldwide. They produce profound neurological dysfunction, due to the progressive destruction of local and distal neuronal networks, resulting from injury-induced tissue damage and subsequent local, cellular, and biochemical reactions. Interestingly, significant spontaneous neuroplasticity occurs over the weeks and months following SCI or TBI, leading to some functional recovery (Raineteau, 2008; Darian-Smith, 2009; Kernie & Parent, 2010; Richardson et al., 2010).

The underlying cellular mechanisms remain unclear, but may comprise dendritic remodeling, axonal sprouting, as well as local neuronal circuit reorganization (Darian-Smith, 2009). Another feature that could contribute to this post injury neuroplasticity is injury-induced neurogenesis (Kazanis, 2009). In adult neurogenesis in physiological conditions is sustained by neural stem and progenitor cells that persist in two main regions of the mammalian brain, the subventricular zone (SVZ) bordering the lateral ventricle (Alvarez-Buylla & Garcia-Verdugo, 2002), and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Seaberg & vander Kooy, 2002). Several studies have shown that the proliferation of neural progenitors in the adult forebrain is stimulated after various types of brain injury, including stroke, seizure, or TBI. Following TBI, cell proliferation and neurogenesis increase in the hippocampal dentate gyrus (Kernie, Erwin & Parada, 2001; Rola et al., 2006; Urrea et al., 2007; Yu, Dandekar, Monteggia, Parada & Kernie, 2005), and a recent study using a mouse model of TBI showed that newly generated neurons in this region persist over time and might participate in the cognitive recovery (Blaiss et al., 2011). In addition to these two principal brain niches, other areas enriched in markers of morpho-functional neuroplasticity contain adult neural stemcells, albeit to considerably lower levels compared to SVZ and SGZ regions. One such area is the dorsal vagal complex (DVC) of the hindbrain (Bauer, Hay, Amilhon, Jean & Moyse, 2005; Charrier et al., 2006). Like for the SVZ and SGZ, neuronal markers of plasticity such as PSA-NCAM and BDNF are continuously expressed in the adult DVC (Conner, Lauterborn, Yan, Gall & Varon, 1997; Bouzioukh, Tell, Jean & Rougon, 2001). Interestingly, the DVC is responsive to injury since vagotomy produces an increase of microgliosis and astrogliosis in this hindbrain area (Bauer et al., 2005).

The situation becomes more complex for the adult mammalian spinal cord, which is non-neurogenic in physiological conditions. Although it retains multipotent neural stem cells that could generate functional neurons in vitro (Dromard et al., 2008; Meletis et al.,2008), their potential is mainly restricted to the glial lineage in vivo (Horner et al., 2000; Wrathall & Lytle, 2008). Injury to the spinal cord is well-known to induce the formation of reactive astrocytes and the infiltration of immune cells in the vicinity of the lesion site (Horky et al.,2006; Yang et al.,2006; Rolls et al., 2009; Beck et al., 2010), but whether SCI also induces the production of new neurons in vivo remains controversial. Studies have shown that spinal neurogenesis occurs to a limited extent after SCI (Chi et al., 2006; Ke et al., 2006; Shechter e tal., 2007), but that it could be stimulated by experimental intervention (Ohori et al., 2006). Overall, available data suggest that the extent of neurogenesis at the spinal level after SCI depends on the localization and severity of the injury (Ohori et al., 2006; Vessal et al.,2007). However, previous research showed the reduced number of newly generated neurons in the forebrain accounts for reduction in cell proliferation rather than an increase of cell death after SCI. (Felix et al., 2012)

2.2 Spinal Cord Injury in Animal Model

Spinal cord injury (SCI) models have proved indispensible not only for investigating the efficacy of therapeutic interventions but also for better understanding the molecular pathways involved. These models result in distinct neural deficits than thoracic injuries and should be considered in terms of therapeutic approaches. Based on the mechanism of injury, SCI models can be classified as contusion, compression, distraction, dislocation, transection or chemical. Compression models are characterized by compression of the spinal cord over an extended period of time (Dunham, Siriphorn, Chompoopong, & Floyd, 2010)

The majority of behavioral outcome studies in mice with SCI used only a single strain and focused on locomotor recovery using a 5- or 6-point rating scale. The features of locomotor evaluation include weight supported plantar stepping, coordination of the

forelimbs (FL) and hindlimbs, consistent position of the paw during stepping, adequate toe clearance, and maintenance of a stable trunk.

Locomotion in normal mice, regardless of strain, is typified by consistent weight supported stepping, a steady trunk and hindlimbs (HL) movement. Normal mice typically display a reproducible stepping pattern in which a step of the FL is coincident with a step of the contralateral HL and tail, and an easily recognizable pattern of FL (Basso et al., 2006)

The three phases of locomotor recovery depending on the severity of the injury. The first phase of locomotion was characterized by a period of either paralysis characterized by no hindlimb (HL) movement or paresis wherein only isolated joint movements below the level of the injury occurred. In the second phase, plantar placing of the paw and weight support in stance was followed by the onset of stepping and increasing frequency of stepping. In the third phase, improvements in the fine details of locomotion such as coordination and paw position were evident. Based on frequency analysis of the number of mice exhibiting these type of behaviors collapsed across the 42-day recovery period (Basso et al., 2006).

2.3 The Post Traumatic Stress Disorder (PTSD)

The hypothalamic-pituitary-adrenal (HPA) axis is the central coordinator of the mammalian neuroendocrine stress response systems, and as such, it has been a major focus of scrutiny in patients with PTSD (Figure 2.1) In short, the HPA axis is made up of endocrine hypothalamic components, including the anterior pituitary, as well as an effector organ, the adrenal glands. Upon exposure to stress, neurons in the hypothalamic paraventricular nucleus (PVN) secrete corticotropin-releasing hormone (CRH) from nerve terminals in the median eminence into the hypothalamo-hypophyscal portal circulation, which stimulates the production and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH in turn stimulates the release of glucocorticoids from the adrenal cortex. Glucocorticoids modulate metabolism as well as immune and brain function, thereby orchestrating physiological and organismal behavior to manage stressors. At the same time, several brain pathways modulate HPA axis activity. In

particular, the hippocampus and prefrontal cortex (PFC) inhibit, whereas the amygdala and aminergic brain stem neurons stimulate, CRH neurons in the PVN. In addition, glucocorticoids exert negative feedback control of the HPA axis by regulating hippocampal and PVN neurons. Sustained glucocorticoid exposure has adverse effects on hippocampal neurons, including reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis.

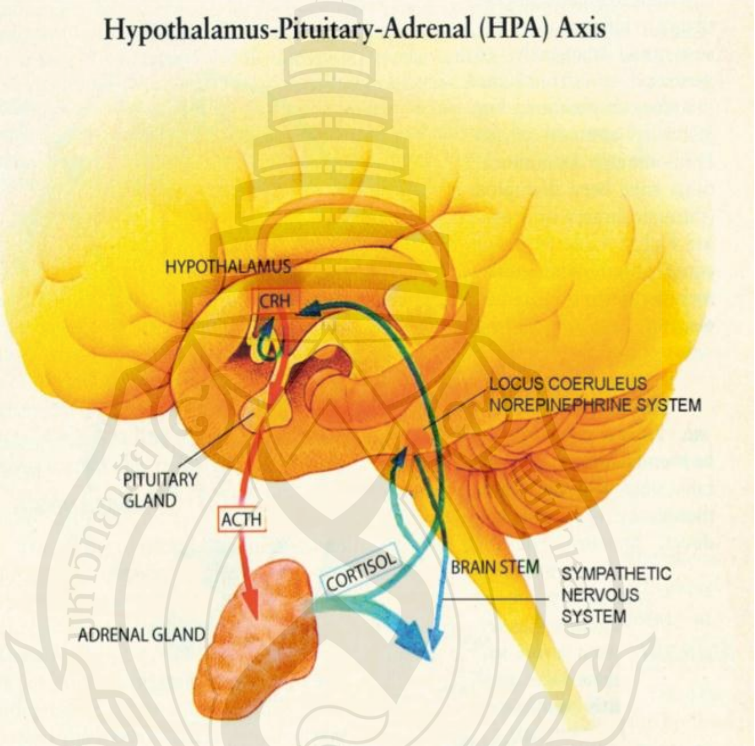


Figure 2.1 The Hypothalamic-Pituitary-Adrenal Axis is the Body's Major Response System for Stress

The hypothalamus secretes CRH, which binds to receptors on pituitary cells, which produce/release ACTH, which is transported to the adrenal gland where adrenal hormones such as Cortisol are produced/released. The release of Cortisol activates sympathetic nervous pathways and generates negative feedback to both the hypothalamus and the anterior pituitary. This negative feedback system appears to be compromised in

patients with post-traumatic stress disorder. CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin

2.4 Melatonin

Melatonin (5-methoxy-N-acetyltryptamine) is a neuroprotective compound. synthesized from L-tryptophan in the pineal gland, retina and gastrointestinal tract, released into the blood with a circadian rhythm that peaks during the night in mammals (Reiter, 2008). Melatonin is rapidly absorbed because of its lipophilic nature, and readily crosses the blood-brain barrier (Reiter, 1991). Reduction of endogenous melatonin after pinealectomy makes rats more vulnerable to trauma and exogenous melatonin administration has an important neuroprotective effect. The spinal cord is particularly vulnerable to free radical oxidation because of its high lipid content. Pharmacological doses of melatonin do not produce in vivo toxic effects and seem to support some features of the antioxidant defense systems (Reiter, 1991).

Melatonin and its metabolites are potent radical scavengers protecting cells from damage induced by a variety of oxidants, including hydroxyl radicals and lipid peroxidation products (Hardel, 2009; Akbulut et al., 2008). melatonin is not only inhibiting inflammation by reducing the activation of microglia and macrophages, but also reducing tissue alterations. (Samantaray et al., 2008; Esposito et al., 2009). Moreover, it also significantly improved the recovery of limb function (evaluated by motor recovery score) and anti-inflammatory benefit in an animal model of SCI (Genovese et al., 2005) Likewise, melatonin effects in reactive astrocytes and scar formation are never been elucidate. Since the increased expression of growth factors, axonal guidance factors, extracellular matrix molecules, such as changes of chondroitin sulfate proteoglycans (CSPG), and angiogenic factors can be observed in the chronic phase (days to years) following SCI, oxidative stress-related protein and gene are still increased (Bareyre & Schwab, 2003; Di Giovanni et al., 2003).

Melatonin is able to prevent oxidative stress both through its free radical scavenging effect and by directly increasing antioxidant activity (Reiter & Tan, 2003), It

is known that endogenous melatonin production diminishes in elderly persons (Reiter, 1992) and that the total antioxidative capacity of serum correlates well with its melatonin levels in humans (Reiter, Garcia. & Pie, 1998). Moreover, melatonin shows beneficial anti-aging effects in rats, preventing lipid peroxidation and other mechanisms related to oxidative stress (Poeggeler, 2005; Paredes et al., 2009).

Central nervous insults, such as spinal cord injury (SCI) or traumatic brain injury (TBI), are among the leading causes of mortality and morbidity worldwide. They produce profound neurological dysfunction, due to the progressive destruction of local and distal neuronal networks, resulting from injury-induced tissue damage and subsequent local, cellular, and biochemical reactions. Interestingly, significant spontaneous neuroplasticity occurs over the weeks and months following SCI or TBI, leading to some functional recovery (Raineteau et al., 2008; Darian-Smith et al., 2009; Kernie & Parent, 2010; Richardson et al., 2010).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Research Design

Experimental design

3.2 Study Design

Mice were divided into 3 groups as Control (n=1, 4 slide sample), SCI (n=1, 4 slide sample), and SCI+Melatonin (n=1, 4 slide sample). The SCI groups all mice were induced by spinal cord injury (SCI) and/or treated with melatonin. This study was undertaken to examine the neuroprotective effect of melatonin to post traumatic stress after induced SCI and the number of newly dividing cells was quantified in hippocampus area. To visualize the nucleus, cells were counterstained with DAPI and Co-label with Ki-67, neural progenitor cells.

3.3 Sample Size

In this study, the total sample size is 12 calculated by power analysis program, different mean and SD refer from the research "Alteration of forebrain neurogenesis after cervical spinal cord injury in the adult rat" (Felix M-S, Popa N, Djelloul M, et al., 2012).

1. Kruskal-Wallis Test - One-Way Design Power Analysis

The formula for the Kruskal-Wallis Test is

$$T = \frac{\frac{12}{N^2+N}\sum_{k=1}^{g}\frac{R_k^2}{n_k} - 3(N+1)}{\left[1 - \frac{\sum_{i=1}^{G}(t_i^3 - t_i)}{N^3 - N}\right]}$$

R_k is the sum of the ranks of the data k^{th} group ($Rk = \Sigma^{nk}_{j=1} r_{kj}$)

G is the number sets of tied ranks

T_i is the number of tied values within set i

$n_1, n_2, \ldots, n_g$ denote the number of subjects in each group

N denote the total sample size of all groups

2. Simulation Summary

Number of Groups 3

Random Number Pool Size 10000

Number of Simulations 500

3. Numeric Results

Table 2.1 Numeric Results

Row	Power	Mean		Target Alpha	Actual Alpha	Std Dev of H1 μ's	Mean of H1 σ's	SD
		Group Sample Size n	Total Sample Size N					
1	0.000	2.0	6	0.050	0.000	0.0	7.9	7.8
2	0.040	4.0	12	0.050	0.060	0.0	7.7	7.8
3	0.044	6.0	18	0.050	0.038	0.0	7.9	7.8
4	0.052	8.0	24	0.050	0.038	0.0	7.8	7.8
5	0.038	10.0	30	0.050	0.058	0.0	7.9	7.8

4. Power and Alpha Confidence Intervals from Simulations

Table 2.2 Power and Alpha Confidence Intervals from Simulations

Total Sample Size			Lower Limit of 95% C.I. of Power	Upper Limit of 95% C.I. of Power	Target Alpha	Actual Alpha	Lower Limit of 95% C.I. of Alpha	Upper Limit of 95% C.I. of Alpha	SD
Row	N	Power							
1	6	0.000			0.050	0.000			7.8
2	12	0.040	0.023	0.057	0.050	0.060	0.039	0.081	7.8
3	18	0.044	0.026	0.062	0.050	0.038	0.021	0.055	7.8
4	24	0.052	0.033	0.071	0.050	0.038	0.021	0.055	7.8
5	30	0.038	0.021	0.055	0.050	0.058	0.038	0.078	7.8

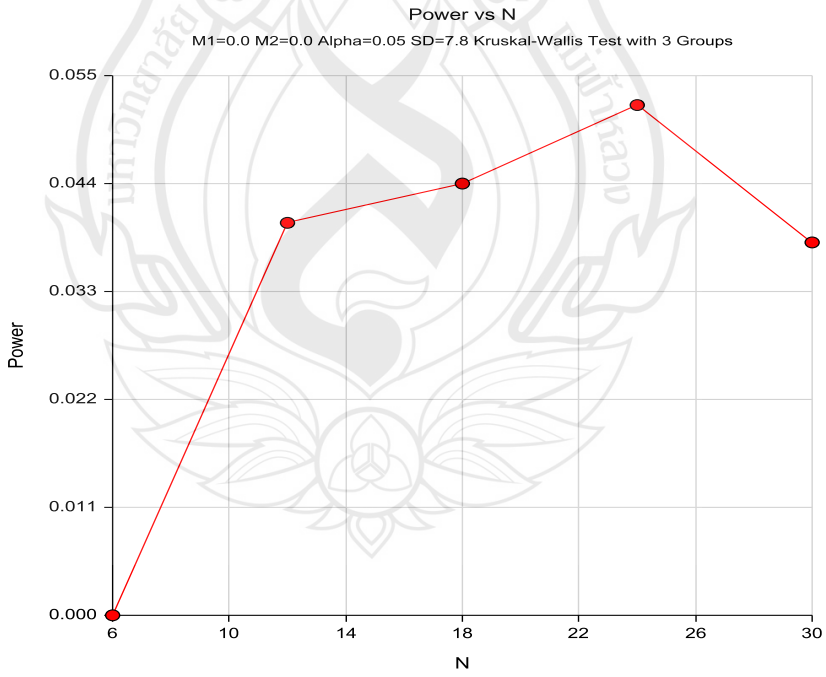


Figure 2.2 Chart of Power and Alpha Confidence Intervals from Simulations

3.4 Material and Method

3.4.1 Animal care

Female mice (3 months old) were purchased from National Laboratory Animal Center (Mahidol University, Thailand) and will provide with water and food pellets ad libitum. All care, surgery, and injury induction will perform in accordance with the guide for the National Institute of Health (NIH) and Faculty of Medicine, Srinakharinwirot University, Thailand, under supervision of Asst.Prof.Dr.Nopporn Jongkamonwiwat and were approved by the Animal Care and Use Committee at the Faculty of Medicine, Srinakharinwirot University, Thailand. (Ethical approval number 11/2555)

Mice were randomly and divided into 3 groups: Control (n=1, 4 slide sample), SCI (n=1, 4 slide sample), and SCI+Melatonin (n=1, 4 slide sample). Each Control, SCI and SCI+Melatonin mices is kept individual cage by group. The sedentary mice are kept in standard cage.

3.4.2 Inclusion and Exclusion Criteria

3.4.2.1 Inclusion criteria

1. Female mice 3 months old
2. Mice with complete spinal cord injury, evaluated by the movement of mice without left and right hind limbs movement
3. Uncontrolling bladder injured mice

3.4.2.2 Exclusion criteria

Mice with incomplete spinal cord injury, evaluated by the voluntary movement of mice with left or right hind limbs

3.4.3 Spinal Cord Injury

Induction of SCI wills performed following the standardized severe crush method as previously described (Krityakiarana, Maggio et al., 2010; Krityakiarana, Espinosa-Jeffrey et al., 2010). Briefly, rats were anesthetized and a laminectomy will perform at the T12. Using Dumont forceps number 5 made crush injury model by ground down to the tips contact. The forceps are used to compress the cord laterally from the sides for 5 sec. Mice were then housed individually by group in standard cages and the bladder voided manually three times a day. Following surgery, all mice were

randomized to 2 experimental SCI groups: SCI (no Melatonin-therapy), SCI+Melatonin (Melatonin-therapy started on day 1 after SCI, For melatonin treated mice, the melatonin (10 mg/kg) will deliver by intra-peritoneal injection 10 min after lesion (Erten et al., 2003; Beni, Kohen, Reiter, Tan & Shohami, 2004; Korkmaz, Horwitz, Jenne & Gauthier, 2010). Mice are maintained in a temperature-controlled environment during recovery and testing. Manual bladder expression will perform 3 times a day. Antibiotics and pain-killer are administered after operation for 7 days and 3 days, respectively. After 14 days mice were sacrificed and brain tissue was removed for the histochemical procedures study for numbers of newly generated cells.

3.4.4 Immunohistochemical Methods

3.4.4.1 Tissue preparation and sectioning

Isofulrane is used for anesthetized the mice. Then, perfusion transcardially with phosphate buffer saline (PBS, pH 7.4) and 4% paraformaldehyde in phosphate buffer is performed. After perfusion, The whole brain is post-fixed in the later solution overnight at 4^{o}C. The brain is transferred to 30% sucrose in 0.1 M phosphate buffer overnight at 4^{o}C. The brain tissue will dissect out of the brain and keep in the 30% sucrose in 0.1 M phosphate buffer overnight at 4^{o}C. The brain tissue is then frozen in freezing media and cut by cryostat at 20 μm for thickness (Horizontal section and Cross-section).

3.4.4.2 Immunohistochemical staining

Tissues are transferred to the room temperature and keep at room temperature for 1 hour. Tissues are incubated in 0.1% triton-X 100 for 30 min at 37^{o}C and blocked for 1 hour at room temperature with 10% NGS and 0.1% triton-X in PBS. Primary antibodies (Ki67) are mixed in the proper dilution in 5% NGS and 0.1% triton-X 100 in PBS and incubate overnight on 4^{o}C. After rinsing, appropriated secondary antibodies (5% NGS in PBS) to visualize the primary antibodies above are incubated for 1 hour at room temperature. After incubation with primary antibody, the sections were washed and incubated for 1 hour with Ki67 Mouse anti-Rat diluted 1:100 in PBS at room temperature. Blocking: 2-5% normal serum to reduce unspecific background staining; 0.5-3% H_2O_2 to block endogenous peroxidase activity; avidin/biotin to block endogenous biotin activity if necessary. The sections were stained (after all other

staining) with DAPI for nuclear staining. The sections were then rinsed in PBS three times and covered with a cover glass. Serial images stained sections are examined and photographed using fluorescent microscope for visualize fluorescent signals from the labeled cells with appropriate filters or a Olympus BX51 fluorescence microscope.

3.4.4.3 Fluorescence microscope analysis

Fluorescence images were taken on an Olympus Microscope BX51 by Serial image of each channel using appropriate filters and later manipulated using CellSens Software. Antibody penetration was assessed in whole image by imaging areas of hippocampus through the entire the dentate gyrus layer and CA1 and CA4 layer with magnification at 10X. Optical slice thickness was 20 micron for phenotypic antibody studies for neurogenesis study

3.5 Data Collection

Cell Counting

Quantitation of unbiased stereological cell counting neurogenesis was made by computerized image analysis. Images at 10x magnification were acquired as digitized tagged-image format files to retain maximum resolution using an Olympus BX51 microscope with an attached digital camera system (DP-70, Olympus). Digital images were transferred into PC for quantitative analyses using CellSens software (Olympus). Images of four sections (180 μm apart) were captured from serially sectioned hippocampus. Fluorescence color images were separated into green, red, and blue channels. The monochrome channel for new cell proliferation (Ki67) or nucleus staining (DAPI) was taken by setting the threshold to discriminate staining from background. Each field of interest area in each image was manually counted to eliminate artifacts.

For the Ki67 analyses, data are reported as the number of labeled area captured the positive pixels that co-localized with the area of DAPI. The mask of the neurogenesis cell produced by the same area counted and superimposed on adjacent area stained with DAPI which activated as cell counting stain. Unbiased estimates of the number of neurogenesis neurons in Dentate Gyrus (DG) and Cornu Ammonis (CA)

areas were made by counting fluorescent staining-cells in serially sectioned of hippocampus according to the previously described by Shors, Wood and Bevlin (2001) and Shors, Townsend, Zhao, Kozorovitskiy and Gould (2002) Briefly, positively labeled cells were counted in every 10th section (each section separated by 180 μm) using a modification to the optical dissector method; cells on the upper and lower planes were not counted to avoid counting partial cells. The number of neurogenesis (Ki67 cells) was counted in every 10^{th} section and multiplied by 10 to get the total number of neurogenesis cells in the DG, CA1 and CA4 cells in hippocampus.

For the Dentate Gyrus (DG) and Cornu Ammonis (CA) volume of both sides were calculated by multiplying the outlined area in every 10^{th} section and multiplied by 10 to get the total volume. All volumetric data represent average ratings from both sides of hippocampus in each animal. In experiment, analyzing all images represented by the sampling of group eliminated bias and the total of mices were analyzed for each group.

3.6 Statistical Analysis

The Kruskal-Wallis Test will be used for determining the significance of the results with *P-values* less than 0.05 will consider as significant and *P-values* less than 0.01 are considered very significant difference. The analysis of variance test (ANOVA) will be applied to compare inter- and intra-group among the studies. The values are given as the mean of measurements as well as the standard deviation of the mean.

3.7 Study Location

Srinakharinwirot University, Bangkok, Thailand.

CHAPTER 4

RESULTS

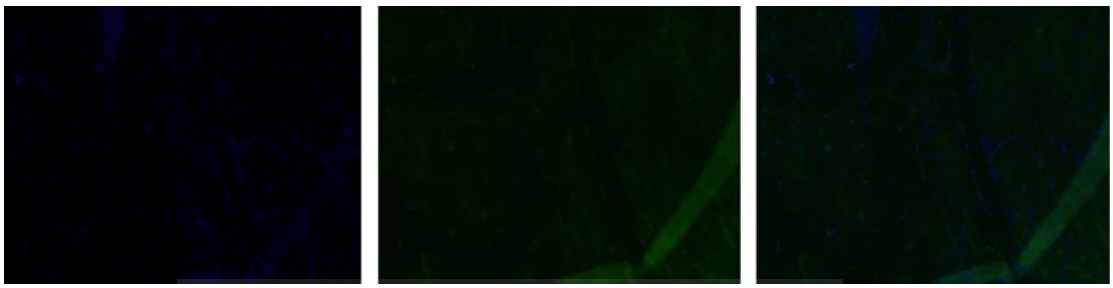
In this study, we investigated the effects of Melatonin on Spinal cord injury in mice. Underwent three groups, in the experimental group mice has spinal cord injuries one group has melatonin after spinal cord injuries.

Cell counting, Quantitation of unbiased stereological cell counting neurogenesis was made by computerized image analysis are presented in Figure 4.1 and Table 4.1. The comparison between Control group (n=1, 4 slide sample) and Spinal cord injury (n=1, 4 slide sample) Control group (n=1, 4 slide sample) and Spinal cord injury with Melatonin (n=1, 4 slide sample), Spinal cord injury (n=1, 4 slide sample) and Spinal cord injury with Melatonin (n=1, 4 slide sample), the mean value of cell counting analysis were computed and used in statistical analyses. The analyses of student t test were performed using statistic analysis software. Data are expressed as means ± standard division (SD), *P-value* < 0.05 was considered significant.

To assess the cell counting number of hippocampus by measurement the cellSens Software of The Olympus Microscope, shown in Table 4.1

a b c

Figure 4.1 a: DAPI Staining of Brain Section from Control Group, b: Ki67 Staining of Brain Section from Control Group, c: DAPI + Ki67 Staining of Brain Section from Control Group



a b c

Figure 4.2 a: DAPI Staining of Brain Section from SCI Group, b: Ki67 Staining of Brain section from SCI Group, c: DAPI + Ki67 Staining of Brain Section from SCI Group

a b c

Figure 4.3 a: DAPI Staining of Brain Section from SCI with Melatonin group, b: Ki67 Staining of Brain Section from SCI with Melatonin Group, c: DAPI + Ki67 Staining of Brain Section from SCI with Melatonin Group

Table 4.1 The Table of Cell counting Number. (means ± standard division (SD), *P-value* < 0.05 was considered significant.)

Group	**Dentate Gyrus (DG)**	**Cornu Ammonis (CA)**	**Hippocampus area (DG + CA)**
Control Group	5.75 ± 3.77	14.75 ± 3.30	20.50 ± 2.08
SCI Group	0.25 ± 0.50	1.00 ± 0.82	1.25 ± 0.96
SCI with Melatonin Group	6.25 ± 2.87	9.75 ± 3.50	16.00 ± 3.16

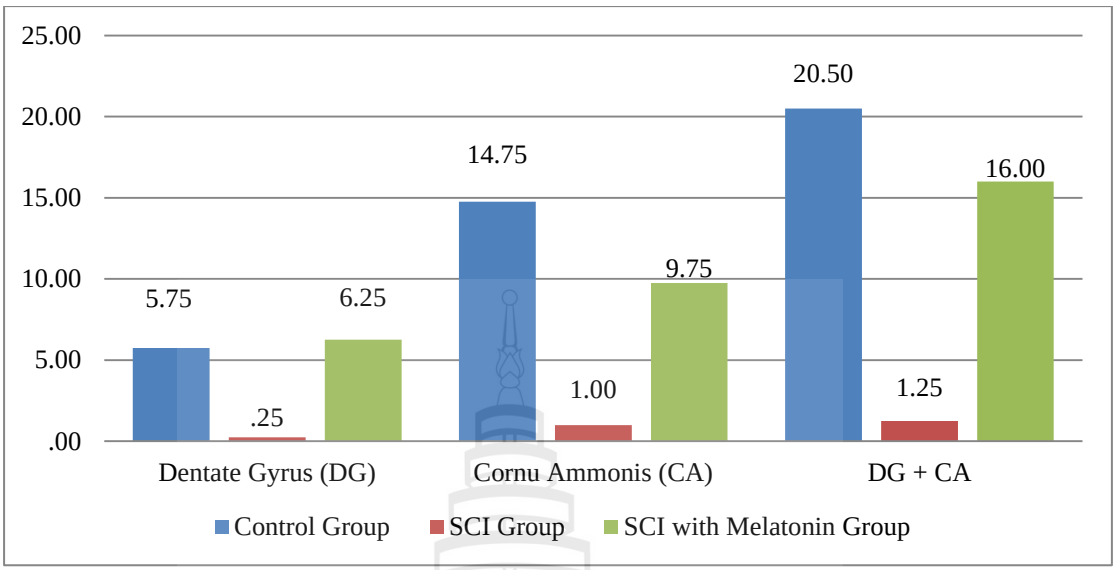


Figure 4.4 Cell Counting Number in Hippocampus Across Groups

According to table 4.1, the result shows the cell counting number of Dentate Gyrus (DG) and Cornu Ammonis (CA) from three groups of mice, consist of control group (n=1, 4 slide sample), spinal cord injury (n=1, 4 slide sample), and spinal cord injury with melatonin treatment (n=1, 4 slide sample). It shows cell counting number of Dentate Gyrus (DG) from control group is equal to 5.75 ± 3.77, SCI group of 0.25 ± 0.50, and SCI with melatonin treatment of 6.25 ± 2.87. For Cornu Ammonis (CA), the cell counting number of control group is equal to 14.75 ± 3.30, SCI group 1.00 ± 0.82, and SCI with melatonin treatment is 9.75 ± 3.50. For Hippocampus (DG + CA) the cell counting number of control group is equal to 20.50 ± 2.08, SCI group 1.25 ± 0.96, and SCI with melatonin treatment is 16.00 ± 3.16

Table 4.2 The Comparative Analysis of Cell Counting in Hippocampus Across Group of Mice with Kruskal-Wallis Test

Group	Control group	SCI group	SCI with Melatonin group	P-value
Dentate Gyrus (DG)	5.75 ± 3.77	0.25 ± 0.50	6.25 ± 2.87	0.021
Cornu Ammonis (CA)	14.75 ± 3.30	1.00 ± 0.82	9.75 ± 3.50	0.013
Hippocampus (DG + CA)	20.50 ± 2.08	1.25 ± 0.96	16.00 ± 3.16	0.011

As seen in the table 4.2, the Kruskal-Wallis Test was applied for determining the difference in cell counting number of hippocampus from two areas across three groups of mice, which is of control group (n=1, 4 slide sample), spinal cord injury (n=1, 4 slide sample), and spinal cord injury with melatonin treatment (n=1, 4 slide sample). There are differences in the number of cell counting in Hippocampus area (DG + CA) across group of mice with Kruskal-Wallis Test ($p = 0.011$, Chi-square = 9.073).

According to the Dentate Gyrus (DG), the result shows there are a difference in cell counting number of Dentate Gyrus among control group, SCI group, and SCI with melatonin treatment ($p = 0.021$, Chi-square = 7.700). Furthermore, there are difference in cell counting number of Cornu Ammonis (CA) across experimental groups at 0.05 significant level ($p = 0.013$, Chi-square = 8.731).

Table 4.3 The Comparative Analysis of Cell Counting Number in Dentate Gyrus (DG) Across Difference Group of Mice with Kruskal-Wallis Test

Groups	Control	SCI	SCI with Melatonin	P-value
Mean ± SD	14.75 ± 3.30	1.00 ± 0.82	9.75 ± 3.50	P < 0.05
	a*		b*	
	c			

From table 4.3, the Kruskal-Wallis Test was selected for comparing cell counting number of Dentate Gyrus (DG) between groups. The result found a difference between control group and SCI group at 0.05 significant level (p = 0.017) (a*). Furthermore, SCI group and SCI group with melatonin treatment has a difference in cell counting number (p = 0.017) (b*). Lastly, the researcher found no significant difference when compare the cell counting number of Dentate Gyrus (DG) between the control group and SCI group with melatonin treatment at 0.05 significant level (p = 0.559) (c).

Table 4.4 The Comparative Analysis of Cell Counting Number in Cornu Ammonis (CA) Across Difference Group of Mice with Kruskal-Wallis Test

Groups	**Control**	**SCI**	**SCI with Melatonin**	**P-value**
Mean ± SD	14.75 ± 3.30	1.00 ± 0.82	9.75 ± 3.50	P < 0.05
		a*	b*	
		c		

According to table 4.4, the Kruskal-Wallis Test was selected for comparing the cell counting number of Cornu Ammonis (CA) between groups. The result found a difference between control group and SCI group at 0.05 significant level (p = 0.020) (a*). Furthermore, SCI group and SCI group with melatonin treatment has a difference in cell counting number (p = 0.017) (b*). Lastly, the researcher found no significant difference when compare the cell counting number of Cornu Ammonis (CA) between the control group and SCI group with melatonin treatment at 0.05 significant level (p = 0.102) (c).

Table 4.5 The Comparative Analysis of Cell Counting Number of Hippocampus Area Across Difference Group of Mice with Kruskal-Wallis Test

Groups	**Control**	**SCI**	**SCI with Melatonin**	**P-value**
Mean ± SD	20.50 ± 2.08	1.25 ± 0.96	16.00 ± 3.16	P < 0.05
	a*	b*		
	c			

Regarding to table 4.5, the Kruskal-Wallis Test was selected for comparing the cell counting number of hippocampus between groups. The result found a difference between control group and SCI group at 0.05 significant level (p = 0.020) (a*). Furthermore, the SCI group and SCI group with melatonin treatment has a difference in cell counting number (p = 0.020) (b*). Lastly, the researcher found no significant difference when compare the cell counting number of hippocampus between control group and SCI group with melatonin treatment at 0.05 significant level (p = 0.059) (c).

CHAPTER 5

DISCUSSION

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenously generated molecule whose level in the blood normally exhibits a circadian rhythm due to its exclusive nighttime production in and release from the pineal gland (Reiter, 1991). In addition melatonin is also a powerful free radical scavenger and antioxidant that can crosses the blood-brain barrier so, it can protects both neurons from oxidative injury and death (Reiter et al., 1998). Melatonin also aids in the transport of electrons through the mitochondrial electron transport chain thereby reducing free radical generation and the loss of brain cells via apoptosis (Jou et al., 2007). *Reduced cell density in hippocampus of SCI model*

SCI caused primary and secondary injuries which will cause further damage in many systems. The consequences of secondary injury include mitochondrial dysfunction, neurotransmitter accumulation, blood brain barrier and blood spinal cord barrier disruption, apoptosis, excitotoxic damage, initiation of inflammatory and immune processes (Bains & Hall, 2012; Khalatbary et al., 2010).

Secondary injury involves the production of highly reactive species, reactive oxygen species (ROS) reactive nitrogen species (RNS) or free radicals will cause damage to protein structure, DNA and cell membrane leads to oxidative stress which result in tissue damage (Naseem, Srivastava & Dandekar, 2006).

Moreover, recent evidences indicate that ROS may involve in inducing apoptosis. Various studies on antiapoptotic activity of melatonin are reported in which the administration of exogenous melatonin has been shown to be effective in preventing normal cells from apoptosis in vivo (Muñoz-Casares et al., 2006). From the above reasons was in line with the reduction of cell density in hippocampus of SCI group (1.25 ± 0.96) comparing with control (20.50 ± 2.08) in the current study.

Increased cell density in hippocampus on SCI-M group

Oxidative stress by free radical ROS and RNS are secondary injury caused by SCI. Recently melatonin have been documented to be an effective direct free radical scavenger and also stimulate the activities of several endogenous antioxidative enzymes (Reiter, Manchester & Tan, 2010).

In this study a significant increase in cell density in hippocampus was observed in SCI-M group (16.00 ± 3.16) comparing with SCI group (1.25 ± 0.96). It can conclude that melatonin treatment was effective in reducing tissue injury in SCI model.

The previous study was consistent with this finding that melatonin can protecting the brain from prolong oxidative stress and loss of cells resulting from the inflammatory after traumatic of spinal cord injury as seen in neuronal density of hippocampus (dentate gyrus with cornu ammonis) differed significantly between melatonin treatment group and SCI group (16.00 $\pm$ 3.16 versus 1.25 $\pm$ 0.96) $p < 0.05$. These result can evaluated the neuroprotective effect of melatonin to elevated levels of inflammation on hippocampal in mice with spinal cord injury.

For further study, concerning with the chronically elevated concentration of melatonin within the large experimental group of prolong duration of melatonin uptake are necessary.

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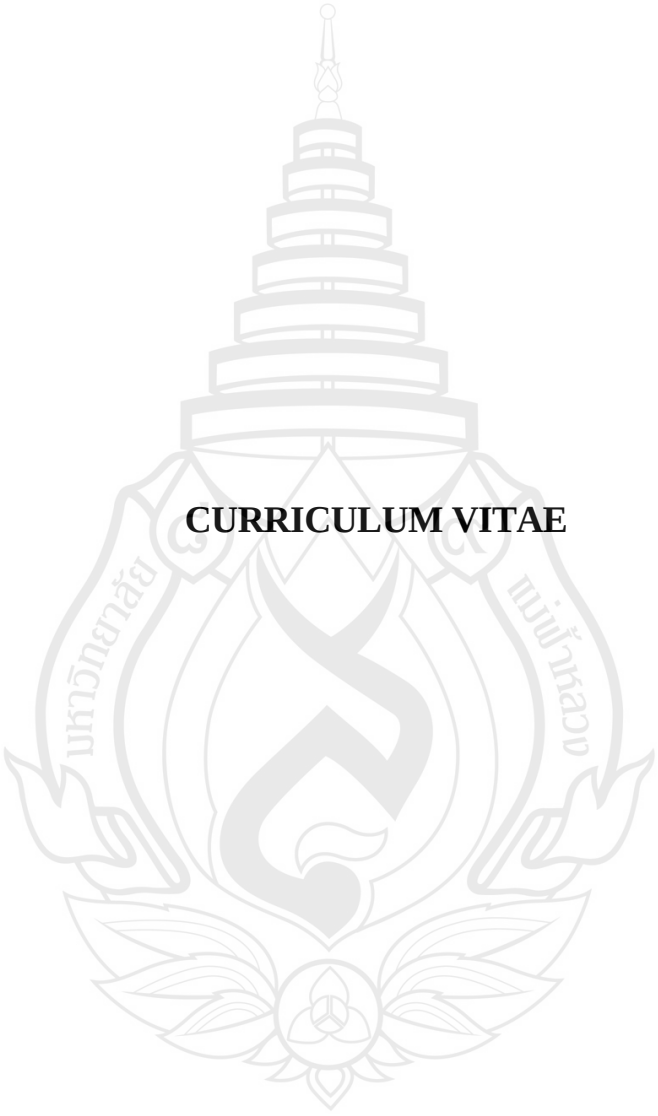

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