HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY ON THE EFFECT OF MELATONIN IN RAT CEREBELLAR CORTEX INJURY AFTER TRANSIENT BILATERAL COMMON CAROTID ARTERY LIGATION

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ABSTRACT

Brain ischemia is a condition in which there is insufficient blood flow to meet its metabolic demands leading to many functional and structural changes. The cerebellum is considered one of the vulnerable regions for brain ischemia. Melatonin, produced by the pineal gland, has antioxidant, anti-inflammatory and anti-apoptotic effects. Therefore, the current study has been performed to evaluate the effect of melatonin administration on induced ischemic reperfusion injury (I/R) in rat cerebellar cortex. Thirty adult male albino rats were used and divided into three groups (10 rats / group). Group A contained the sham-control rats,

while group B included I/R rats which were subjected to transient ischemia by ligation of both common carotid arteries simultaneously for 10 minutes and subdivided into 2 subgroups; B1(3 days after I/R) and B2 (one month after I/R). Group C included I/R rats (as in group B) with intraperitoneal melatonin administration (in a dose of 5 mg/ kg) 30 minutes before performing ischemia and one & three hours after release of ligation respectively and also was subdivided into 2 subgroups; C1 (3 days after I/R with melatonin administration) and C2 (one month after I/R with melatonin administration). The cerebellum was dissected and prepared to be stained with haema-

toxylen and eosin (H&E). Also, immunohistochemical study was performed for detection of glial fibrillary acidic protein (GFAP) and p53 positive cells. Subgroup B1 sections showed shrunken Purkinje cells and apoptotic granular ones which were markedly increased in number in subgroup B2. These histological alterations were ameliorated in subgroup C1 sections with melatonin administration. The histological architecture of cerebellar cortex of subgroup C2 appeared more or less similar to the control group. Few p53 and GFAP positive cells in rat cerebellar cortex of subgroup B1 were observed with significant increase in subgroup B2. However, sections of subgroups C1 and C2 showed an apparent decrease in the number of positive cells of both immunohistochemical markers as compared to the corresponding B subgroups.

It could be concluded that injection of melatonin could ameliorate I/R associated changes in rat cerebellar cortex through a possible neuroprotective role.

Keywords: Melatonin, I/R, Cerebellar cortex, Apoptosis, Astrocytes

INTRODUCTION

Ischemia refers to a condition produced by decreased perfusion whereby tissue receives less than the necessary amount of blood, oxygen, glucose, and other essential nutrients (1). The brain, including cerebellum, is one of the vulnerable regions subjected to damage due to ischemia. The severity of brain damage during ischemia is dependent on the thoroughness and duration of the interruption of neural blood flow. Alterations in flow thresholds cause different degrees of physiological disruption and tissue destruction (2). Ischemia may be a result of arterial embolism, vascular disease or anomalies of the coagulation process (3,4). Furthermore, heart function and body temperature are critical determinants of the severity of neuronal damage and functional recovery following cerebral ischemia (5,6).

Two types of ischemia can be induced in animals; global and focal. Global ischemia affects widespread brain areas but typically gives rise to neuronal alterations within selectively vulnerable brain regions. The cerebellum is regularly affected in global cerebral ischemia, and can be

devastated (7). By contrast, models of focal ischemia, either with or without reperfusion, leads to histopathology characterized by focal necrosis or infarction to a specific area of the brain (1).

Cellular damage after reperfusion of previously viable ischemic tissues is defined as ischemia–reperfusion (I/R) injury (8). Brain I/R injury leads to a complex series of pathophysiological events leading to neuronal death and subsequent neurological dysfunction (9). It involves oxidative stress damage (10,11), inflammatory pathways (12), ionic imbalances, and apoptosis (13,14). Ischemia initiates a complex process in which inflammation contributes to brain injury. The inflammatory response to neural tissue injury, including neutrophilia, lymphocytopenia and monocytosis, has been shown to be initiated as early as one hour after onset of ischemia and persists up to three months (15,16,17).

Individual ischemic neurons may die from a mixed form of concurrent apoptosis and necrosis named hybrid death (18). Apoptosis and necrosis are probably triggered in parallel, and furthermore, the apoptotic path-

way has been suggested to lead to necrosis (19). The morphological criteria for necrosis and apoptosis are well defined. Apoptosis refers to a process that involves an inherent cellular program leading to cell death (20). Necrosis is accidental cell death that results entirely from circumstances outside the cell and is manifested by karyolysis and cell swelling. Ischemic cell death is also characterized by a long delay between the insult and manifestation of major cell damage. This delay varies greatly, depending on the nature of the insult and the brain region being affected. In some cases it is as long as several days or even weeks, whereas in others it is a few hours or less (19). Reperfusion is the restoration of blood flow to the ischemic tissue. Despite its benefits to an ischemic tissue, reperfusion can elicit a cascade of adverse reactions that paradoxically injure tissues (21).

p53 induces apoptosis by controlling the translation of proapoptotic genes such as Bax, Puma and Noxa which trigger mitochondrial pathways, including cytochrome-C release and Caspase -3 accumulation. In addition, it also controls the down-regulation of antiapoptotic gene such

as Bcl 2 (22,23). In the nucleus, p53 is a crucial transcriptional regulator and controls the program of transcription-dependent apoptosis in response to various signals, including DNA damage, oxidative stress, and ischemia (24).

Brain injury triggers an extensive glial cell response and activation (25). The glial response, collectively referred to as reactive gliosis, involves mainly activated astrocytes and microglia. Reactive gliosis is characterized by hypertrophic and proliferating astrocytes, and proliferating microglia (26). Reactive astrocytes are commonly observed in basically all pathologies in the central nervous system. They are characterized by cellular hypertrophy, an increase in number and upregulation of intermediate filament components, in particular GFAP and vimentin (27,28). GFAP is the major component of intermediate filaments of mature astrocytes. Because of its specificity and abundance, expression of GFAP has become the most common marker used for astrocytes to recognize the astrocyte population in response to brain injury (29).

Melatonin (N-acetyl- 5-methoxytr-

yptamine) is the chief indolamine produced by the pineal gland (30). It is a naturally occurring compound found in animals, plants and microbes (31). Melatonin has a molecular weight of 232 and is both lipid (32) and water soluble, although its solubility in lipid is clearly greater (33). It has two important functional groups which determine its specificity: the 5-methoxy group and the N-acetyl side chain. Once synthesized, the majority of melatonin diffuses directly towards the cerebrospinal fluid of the brain third ventricle, while the other fraction is released into the blood stream where it is distributed to all tissues within a very short period (34,35).

Melatonin has two major properties, antioxidant and anti-inflammatory, which make it a viable candidate for treating various pathologies, including neurodegeneration as Alzheimer's disease and Parkinson's disease (36). Melatonin is a potent free radical scavenger as well as an indirect antioxidant. It has been shown to be protective in gastrointestinal, myocardial and cerebral models of I/R injury when it is given before ischemia and/or repeated doses started before reperfusion

(37). Moreover, Melatonin has several mechanisms in conferring neuroprotection after I/R by activation of its receptors in the cerebral blood vessels leading to vasodilation and normalising the perfusion of the ischemic region, preventing neurons from excitotoxic damage (38,39) and prevention of subsequent apoptosis in neurons (40).

Therefore, the current study was designed to investigate the histological and immunohistochemical changes of I/R in rat cerebellar cortex and to evaluate the possible protective effect of melatonin.

MATERIALS AND METHODS

Animals and Experimental design:

30 adult male rats weighing about 150 - 200 gm were used in the present work. The animals were kept in adequate ventilation and temperature with regular 12 h light/dark cycle. All animals were given free access to standard laboratory food and water. The animals were equally divided into 3 groups (10 rats each). Group A was the sham-control rats that were subjected to a midline cervical incision with exposure of both

common carotid arteries followed by closure of the incision. Group B included rats subjected to brain (I/R) to induce transient global brain ischemia. Both common carotid arteries were clamped simultaneously for only 10 minutes using "Bull dog" clamps through a midline neck incision followed by release of ligation and closure of the incision. This procedure was previously described by some researchers (41,42,43,44). This group was further subdivided into subgroup B1 (3 days after I/R) and subgroup B2 (1 month after I/R). Group C included rats subjected to I/R injury (as group B) and intraperitoneal melatonin administration (Sigma- Aldrich Company) in a dose of 5 mg/kg, dissolved in absolute ethanol and diluted in isotonic saline to a final concentration of 1:10 then administered half an hour prior to ischemia, one and two hours after reperfusion (45). Group C was further subdivided into subgroup C1 (3 days after I/R) and subgroup C2 (one month after I/R).

Histological and Immunohistochemistry Study:

The cerebellum was dissected and fixed in 10% neutral buffered formalin and processed to obtain

paraffin sections of 5 μ thickness for light microscopic study. Sections were stained with H&E stain (46). In addition, Paraffin sections of 4-5 μ m thick were prepared for immunohistochemical staining for p53 and GFAP for detection of apoptotic cells and astrocytes respectively (47). In addition, a specimen of human ovary with high grade serous papillary adenocarcinoma was obtained from the pathology department of Mansoura Faculty of Medicine and was prepared for p53 immunostaining as a positive control section.

Morphometric and Statistical Analysis:

Slides were photographed using Olympus® digital camera installed on Olympus® microscope using 40 X objective. The result images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in automated object counting routine for both p53 and GFAP positive cells. Data were then tabulated, coded and analyzed using the computer program SPSS (Statistical package for social science) version 17.0 to obtain:

- Descriptive data:

Descriptive statistics were calculated in the form of mean and standard deviation ($\pm$ SD).

- Analytical statistics:

In the statistical comparison between the different groups, the significance of difference was tested using ANOVA (analysis of variance) to compare between more than two groups of numerical (parametric) data followed by post-hoc tukey test for multiple comparisons.

A P value <0.05 was considered statistically significant and P value <0.001 was considered highly significant in all analyses.

RESULTS

1) Haematoxylen and Eosin-stained paraffin sections (H&E):

Light microscopic examination of H&E stained sections of the rat cerebellum of the control group showed its characteristic folia. It was composed of an outer cortex and an inner white matter. The cerebellar cortex was formed of outer molecular, middle Purkinje and inner granular layers (Fig.1). The molecular layer showed a sparse population of neurons including; basket cells near the

Purkinje cell layer and stellate cells near the surface. Also, it contained dendritic arborizations, non-myelinated axons and radial fibers of neuroglial cells. Purkinje layer was formed of a single layer of large flask shaped cells with rounded euchromatic nuclei and a characteristic apical dendritic tree. They were surrounded by perineuronal neuroglia cells. The granular layer showed a large number of small cells with deeply stained nuclei of the granular cells, vesicular nuclei of Golgi cells and acidophilic non cellular areas in between the cells formed of synaptic complex of axons and dendrites, representing the cerebellar islands (Fig. 2).

In the cerebellar cortex of subgroup B1 (three days after I/R), empty spaces appeared around deformed and shrunken Purkinje cells which had condensed nuclei. Moreover, some apoptotic granular cells with small dark eccentric nuclei and an acidophilic cytoplasm were observed (Fig.3). Many distorted Purkinje cells surrounded by empty spaces as well as numerous apoptotic granular and neuroglial cells were encountered in subgroup B2 (one month after I/R) (Fig.4). However, in

subgroup C1 (three days after I/R and melatonin treatment), the majority of Purkinje and granular cells appeared normal, while few apoptotic ones were seen in the granular layer (Fig.5). The histological architecture of cerebellar cortex in subgroup C2 (one month after I/R and melatonin treatment), appeared more or less similar to the control group except for few apoptotic cells in the granular layer (Fig.6).

2) Immunohistochemical staining for p53:

Negative immunohistochemical staining for p53 was observed in sections of rat cerebellar cortex of control group A (Fig.7). In subgroup B1, there were few p53 positive cells in the granular layer of rat cerebellar cortex (Fig.8), while in subgroup B2; there was also an apparent marked increase in p53 positive cells (Fig.9). In Subgroup C1, there was an apparent decrease in the number of positive cells in the granular layer of rat cerebellar cortex (Fig.10). The number of positive cells declined markedly in cererbellar cortex in subgroup C2 as compared to subgroup B2 (Fig. 11).

3) *Immunohistochemical staining for Glial Fibrillary Acidic Protein (GFAP):*

Few positively stained cells were seen in the control cerebellar cortex after immunohistochemical staining for GFAP. This reaction was compared with control positive one in the white matter of cerebellum (Fig. 12). Subgroup B1, showed relative increase in GFAP- positive cells (Fig.13), whereas in subgroup B2, there was an apparent marked increase in positive cells in the granular layer of the cerebellar cortex (Fig.14). In the cerebellar cortex of subgroup C1, relatively few positive cells were observed (Fig. 15), while in subgroup C2, there was an apparent decrease in positive cells as compared to subgroup B2 (Fig.16).

4) *Morphometric and statistical results:*

The mean value, standard deviation and probability of both p53 and GFAP positive cells for all groups were shown in tables (1). ANOVA test revealed significant changes

among different groups of the experiment for both p53 and GFAP; among (control group A, subgroup B1 and subgroup C1) and also among (control group A, subgroup B2 and subgroup C2). Post-hoc tukey test showed the following:

The number of p53 and GFAP positive cells showed a statistically significant increase in B1 and C1 subgroups as compared to the control. In addition, the number of p53 positive cells in subgroup C1 was still significantly higher than the control group, while GFAP positive cells showed a non significant increase in C1. Similar results were obtained when comparing B2 with the control group and C2 subgroup in both p53 and GFAP. On the other hand, there was a significant increase in the number of p53 and GFAP positive cells in B2 as compared to B1 subgroups. The number of p53 positive cells was significantly higher in C2 than C1 subgroups, while GFAP positive cells showed a statistically non significant increase.

Table (1): p53 and GFAP positive cells in control and experimental groups:

		Control -ve	B		C	
			B1	B2	C1	C2
Mean±SD	P53	.00	15.67±2.73 [*]	42.77±4.41 ^{*◇}	6.53±2.00 ^{♦♦‡}	15.74±3.86 ^{♦♦◇†}
	GFAP	9.57±1.71	16.73±3.70 [*]	31.50±4.72 ^{*◇}	10.95±1.79 ^{♦‡}	12.2±2.59 ^{†◇}

SD: standard deviation

Test used: ANOVA followed by post-hoc tukey

* : significance between Control-ve & B groups

♦ : significance between B & C groups ?

● : significance between Control-ve & C groups

♂ : significance between B1 & B2 subgroups

○ : significance between C1 & C2 subgroups

‡ : significance between B1 & C1 subgroups

† : significance between B2 & C2 subgroups

Fig 1: A photomicrograph of a paraffin section in a control rat cerebellar cortex demonstrating a part of cerebellar folium formed of an inner white matter (WM) covered by an outer cortex formed of molecular (M), Purkinje cell (P) and inner granular layer (G). The insert is the lower magnification of the cerebellar folia. (H&E x 100, insert x 40) **Fig 2:** A photomicrograph of a paraffin section in the control rat cerebellar cortex showing molecular layer (M), Purkinje layer (P) and granular layer (G). The molecular layer shows nuclei of stellate cells (S) and of basket cells (B). The Purkinje layer is formed of large flask shaped cells (arrows) with rounded euchromatic nuclei (arrow heads). Perineuronal neuroglia cells (wavy arrows) are seen around Purkinje cells. The granular layer contains numerous small granular cells (tailed arrows) with darkly stained nuclei; Golgi cells (go) with vesicular nuclei and cerebellar islands (thick arrows) in between

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Fig 3: A photomicrograph of a paraffin section in rat cerebellar cortex three days after I/R. Shrunken and deformed Purkinje cells surrounded by empty spaces (asterisks) having condensed nuclei (arrows) are seen. The granular layer shows many apoptotic cells with small eccentric nuclei and an acidophilic cytoplasm (crossed arrows). (H&E x 1000)

Fig 4: A photomicrograph of a paraffin section in rat cerebellar cortex, one month after I/R, showing many distorted Purkinje cells with little acidophilic cytoplasm and dark nuclei (arrows). These cells are surrounded by empty spaces (asterisks) and numerous neuroglial cells (arrow heads). In the granular layer, many apoptotic cells (crossed arrows) are also seen. (H&E x 1000)

Fig 5: A photomicrograph of a paraffin section in cerebellar cortex of melatonin treated rat three days after I/R. Some Purkinje cells appear shrunken (arrows), while others are normal (wavy arrows). The majority of granular cells appear normal (tailed arrows), while a few ones are apoptotic (arrow heads). (H&E x1000)

Fig 6: A photomicrograph of a paraffin section in cerebellar cortex of melatonin treated rat one month after I/R. Normal Purkinje cells (p), granular cells (arrows) and cerebellar islands (tailed arrows) are seen, while few apoptotic cells (crossed arrows) are also demonstrated. (H&E x 1000)

Fig 7: Photomicrographs of p53 control sections. A positive control section in human ovary with high grade serous papillary adenocarcinoma (A) showing numerous p53 positive cells with brownish coloration of the nuclei (arrows). Control rat cerebellar cortex (B) shows negative p53 reaction. (Anti p53 immunostaining A x1000, B x 400)

Fig 8: A photomicrograph of a section in a rat cerebellar cortex, three days after I/R, demonstrating few p53 positive cells mostly of the granular layer (arrows). (Anti p53 immunostaining x 400)

Fig 9: A photomicrograph of a section in a rat cerebellar cortex, one month after I/R, demonstrating numerous p53 positive cells. (Anti p53 immunostaining x 400)

Fig 10: A photomicrograph of a section in cerebellar cortex of melatonin treated rat, three days after I/R, demonstrating few p53 -positive cells in the granular layer (arrows). (Anti p53 immunostaining x 400)

Fig 11: A photomicrograph of a section in cerebellar cortex of melatonin treated rat, one month after I/R, demonstrating apparent decrease in p53 -positive cells (arrows). (Anti p53 immunostaining x 400)

Fig 12: A Photomicrograph of positive control section in a rat cerebellar white matter (left side) showing cells (arrows) with strong positive reaction for GFAP and control rat cerebellar cortex (right side) with few GFAP positive ones (arrows). (Anti GFAP immunostaining x 400)

Fig 13: A photomicrograph of a section in a rat cerebellar cortex, three days after I/R, demonstrating an increase in GFAP -positive cells (arrows). (Anti GFAP immunostaining x 400)

Fig 14: A photomicrograph of a section in a rat cerebellar cortex, one month after I/R, demonstrating an apparent marked increase in the number of GFAP -positive cells (arrows). (Anti GFAP immunostaining x 400)

Fig 15: A photomicrograph of a section in cerebellar cortex of melatonin treated rat, three days after I/R, demonstrating few GFAP -positive astrocytes (arrows). (Anti GFAP immunostaining x400)

Fig 16: A photomicrograph of a section in cerebellar cortex of melatonin treated rat, one month after I/R, demonstrating an apparent decrease in GFAP -positive astrocytes (arrows). (Anti GFAP immunostaining x 400)

DISCUSSION

The adult brain is extremely vulnerable to various insults. Ischemia is considered the most serious one that may result in both short and long term neurodegenerative and functional disorders (48,49). The experimental studies concerning these issues are mostly confined to the first hours or days following the initiation of ischemia and observations on long-term or permanent ischemia-induced modifications are relatively rare (50).

Despite the dependence of cerebellar cortex in its blood supply mainly on vertebro-basilar system, deformed and necrotic Purkinje cells and many apoptotic granular cells with small eccentric nuclei were observed in the present study. These changes could be attributed to the connection networks between the damaged forebrain and cerebellum. In non-human primates and humans, the cortical areas of the forebrain make several axonal connections with the cerebellum via the pallidum, the thalamus and the pons (51). Moreover, it was mentioned that unilateral injury confined to the preterm cerebral hemisphere was associated with a significantly decreased vol-

ume of the contralateral cerebellar hemisphere (52). In addition, indirect cerebellar injury may result from traumatic brain injury resulting in selective cerebellar Purkinje neuron loss (53). The indirect damage can be transmitted by several methods, such as mechanical forces, metabolic changes and presynaptic hyperexcitability (54).

Possible mechanical or functional injury of corticopontocerebellar pathway could affect the activity in the ipsilateral pons, and also the contralateral cerebellar cortex, a phenomenon known as crossed cerebellar diaschisis (55). Diaschisis was commonly observed in acute brain disorders, such as ischemic stroke, causing clinical deficits that exceed the functional consequences expected from the focal lesion with regard to its size and location in the brain. The experimental and clinical studies revealed decreased blood flow and metabolism in anatomically intact brain regions distant from a cerebral lesion, e.g., the cerebellar hemisphere contralateral to a lesion of the cerebral cortex. Reduced neuronal activity in the cerebral cortex led to a decrease in Purkinje cell activity in the contralateral cerebellar

hemisphere, resulting from reduced efferent excitatory activity in crossed projections (56). In a model of I/R, crossed cerebellar diaschisis was shown to be a result of functional deactivation as well as transneuronal degeneration (57). Six hours after middle cerebral artery occlusion in a mouse model of I/R, increased immunoreactivity for biliverdin reductase, a sign of neuron damage, was found not only in the supratentorial ischemic core, but also in the Purkinje layer of the cerebellum (58).

Neuronal factors seem to be involved in impaired autoregulation not only in the cerebrum but also in the cerebellum in experimentally induced ischemia in the cerebrum. Sympathetic nerves are known to constrict cerebral blood vessels in response to changes in perfusion pressure. Cerebral ischemia activates the noradrenergic system of the brain, with large amounts of catecholamines being released in the ischemic site as well as from non ischemic nerve terminals, which in turn affect vasoreactivity to various stimuli such as autoregulation and CO₂ response (59).

In the current study, a rat model

of bilateral common carotid artery occlusion (BCCAO) for 10 minutes followed by reperfusion was used to examine the effect of the antioxidant melatonin on indirect cerebellar injury due to transient global cerebral ischemia. Some researchers suggested that this model required a more simple surgical preparation, that reperfusion could be readily accomplished, and that this model was easily suitable for chronic survival studies (60,61).

The results of this study revealed the presence of many apoptotic granular cells and shrunken necrotic Purkinje ones in the cerebellar cortex three days after I/R injury. Some authors reported that apoptotic cell death may occurred in addition to necrosis and apoptotic neurons could survive for some time but will continue to die for many hours or even days after ischemia (9,62). Some authors assumed that the strongest pathophysiological activity could be observed 28 days after middle cerebral artery occlusion in a model of focal cerebral ischemia. They added that, an imbalance in cortical function persisted in the borderzone of the infarct up to six months after injury. These persistent changes in cel-

lular function might be best explained by a permanent reorganization of the neocortical network, such as a loss of inhibitory control mechanisms and/or an augmentation of excitatory action (50).

Some authors described apoptosis as a program preferentially triggered by a microcirculatory disturbance (63,64). Accordingly, apoptosis would significantly contribute to the neuronal damage in transient ischemia (65). Apoptotic cell death revealed characteristic morphological changes in H&E staining, such as acidophilic change of cytoplasm and pyknosis of cell nuclei (66), whereas neuronal necrosis after global cerebral ischemia had been developed between the second and fourth days (67) and was manifested by karyolysis and cell organelles swelling (20).

The changes in the cerebellum after BCCAO are supported by some authors who demonstrated that chronic BCCAO in adult wistar rats leads to reduction of blood flow to the cerebellum and dilation of the basilar artery which ensures blood flow to the forebrain, changes in cortical astrocytes and decrease the number of Purkinje cells. This could

be attributed to the remodeling of the basilar artery resulting in secondary vascular perturbations and an enlargement of its lumen over the course of days to weeks in young rats to become partially responsible for providing blood flow to the forebrain as a compensation for BCCAO (68).

In humans, many factors might play a role in delayed cell death observed after ischemic stroke including the neuronal excitability (69), released inflammatory mediators from reactive astrocytes (70) and reactive oxygen species which might be involved in creating the detrimental environment (71). In addition, post ischemic modifications in neuronal gene expression were thought to play a crucial role with regard to cell recovery or cell death. Different induction patterns of members of c-jun and c-fos genes, after global ischemia, are involved in postischemic selective vulnerability and apoptosis especially in the neocortex, pyramidal neurons of hippocampus and Purkinje cells of the cerebellum (72,73).

Apoptosis was reported in many studies in ischemia (74,75). p53 pro-

tein plays a role in several modes of cell death depending in part upon its subcellular distributions (76,77). It was suggested that apoptotic cells increased as early as 0.5 hour, peaked at 24 to 48 hours and persisted for 4 weeks after the onset of reperfusion and the process of apoptosis could be triggered at any time either during or after the insult (78). Similar results were also obtained by who declared that positive apoptotic cells were observed at 2 weeks and 1 month after injury (79).

In this study, rat cerebellar cortex showed relative increase in GFAP-positive astrocytes which was observed after three days and increased up to one month. These results are in agreement with some investigators (80,81). Some of them reported that in experimental I/R, gliosis began at 72 hours post-stroke or later (80), while others mentioned that astroglial response in the core of the lesion started at 4 hours after the trauma and activation was observed up to 28 days after stroke onset (81).

Reactive changes in intermediate filaments in astrocytes, especially GFAP, had been shown to occur after various stimuli, including global

ischemia (82). An early increase in GFAP immunoreactivity corresponded to the development of neuronal tolerance to subsequent ischemia after sublethal brief global ischemia (83). GFAP-upregulated astrocytes are able to uptake harmful substances, such as extracellular ions or excitatory neurotransmitters and to produce neurotrophic factors under pathological conditions (84).

Ischemic brain neuroprotection might be defined as any strategy, or combination of strategies, that antagonizes, interrupts or slows down the sequence of injurious biochemical and molecular events that eventually resulted in irreversible ischemic injury (85). Acceptance of the ROS and inflammatory theory has contributed to an increasing interest in the use of free radical scavengers and anti-inflammatory agents as potential neuroprotective agents (9). In addition, long-term preservation of the neuronal population in cerebral vulnerable structures and their functions after acute global cerebral ischemia is a major end-point of neuroprotective strategies (86). Several studies have shown the neuroprotective effects of melatonin in adult models of I/R both when the

drug was administered before or after ischemia (87,88).

In the present study, the histological architecture of cerebellar cortex appeared more or less similar to control group with few sporadic shrunken cells after injection of melatonin intraperitoneally, before and after I/R. These effects were maintained from three days up to one month. Another important finding of this study was the long lasting neuroprotective effect of melatonin. In addition, the number of p53 and GFAP positive cells showed statistically significant decrease, especially for GFAP positive cells, in melatonin-treated subgroups as compared to the corresponding I/R subgroups in the cerebellar cortex.

It was found that pinealectomy led to greater infarcts in global ischemia (89). Furthermore, in experimental stroke in rats receiving rat-derived pineal gland allografts displayed reduced cerebral infarction than stroke rats that did not receive the transplants (90). In a model of global ischemia, a single post-ischemic melatonin infusion led to improvements in behavior and infarction volumes at 120 days (91). However, these re-

sults were disputed by investigators who used different models of global ischemia, where no significant differences in outcomes at 140 days after melatonin treatment (92).

The findings of this study were supported by some authors who described the long-lasting anti-inflammatory effects of a single intraperitoneal injection of melatonin following 1 hour of middle cerebral artery occlusion (MCAO) plus 2-3 days of reperfusion (93). Also, it was reported that melatonin treatment (continuous i.v. infusion of 10 mg/ kg / h) during the immediate six hours following a 15min global cerebral ischemia episode, resulted in a significant long term preservation of pyramidal neurons in the hippocampus as compared to neuron population of control animals when measured 90 days after the induction of global ischemia in rats (91). In addition, it was found that exogenous melatonin administration prevented the increases in cerebral and cerebellar cortical NO production after I/R injury in transient BCCAO in adult Mongolian gerbils (94).

The binding of melatonin to its receptors improved membrane fluidity

and reduced PMN cell infiltration into damaged tissue via limiting the adhesion of leukocytes to endothelial cells, thus increasing blood flow and reducing edema (95). Decreased formation of oxidatively modified proteins and the upregulation of oxidative stress-responsive genes, such as Hsp70, used as indicator of astroglial stress, were consistent with a protective effect of melatonin (36). Melatonin could exert a wide range of antiapoptotic effects, mainly targeting mitochondria, thus enhancing cell survival pathways leading to cell rescue (95). It might be possible to treat neurodegenerative disorders by inhibiting mitochondrial cell death pathways and promoting mitochondrial homeostasis (96).

Melatonin had been demonstrated to reduce the expression of GFAP, whose accumulation was related to the creation of new astrocytic processes and reactive gliosis (97, 98). In contrast, it was found that melatonin treatment did not influence I/R-induced GFAP response and might not mediate neuroprotective actions following I/R via astrocytic mechanisms (37). Moreover, several studies suggested that melatonin may be of therapeutic value in ameli-

orating hypoxic-ischemic (H/I) damage of myelinated nerve fibers through normalization of the myelination process (98,99).

The improvement observed with melatonin administration a neuroprotective drug are related to its bioavailability, its potential for exerting wide-spread neuroprotective actions, further amplified and prolonged by its metabolites (100), its ability to reduce inflammation, its capability to protect cytoskeleton organization, and its anti-apoptotic actions. Moreover, melatonin does not interfere with the thrombolytic and neuroprotective actions of other drugs (36). Infarct volume, amount of edema, frequency of apoptosis, degree of glial activation, quantity of oxidized macromolecules, degree of neurological deficits, molecular alterations have all been shown to be improved if the animals are given melatonin at the time or shortly after induction of transient ischemia and subsequent reperfusion (101). Melatonin also promotes neurogenesis by suppressing inflammation and more extensive studies are required to examine these points. Overall, melatonin treatment showed long term benefits in experimental I/R studies as a re-

sult of better preserved brain tissues in the acute stage ⁽¹⁰²⁾.

Conclusion

On the basis of the current study, it can be concluded that melatonin administration in I/R could ameliorate the associated histological and immunohistochemical changes in rat cerebellar cortex with long term benefits.

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دراسة هستولوجية وهستوكيميائية مناعية لتأثير الميلا تونين على إصابة قشرة

المخيخ فى الفأر بعد ربط مؤقت للشريان السباتي المشترك فى الناحيتين

يشير نقص تروية المخ إلى عدم كفاية تدفق الدم لتلبية احتياجاته من التمثيل الغذائى مما يؤدي إلى العديد من التغيرات الوظيفية والتركيبية ويعتبر المخيخ أحد المناطق المعرضة لنقص هذه التروية. يتميز الميلا تونين والذي يفرز من الغدة الصنوبرية بتأثيراته المضادة للأكسدة، والالتهابات وأيضاً للموت المبرمج للخلايا. لذلك أجريت الدراسة الحالية لتقييم أثر إعطاء الميلا تونين على الإصابة المستحدثة من التدفق بعد الإحتباس الدموي فى قشرة المخيخ للفئران. وقد استخدم فى هذه الدراسة ٣٠ من ذكور الفئران البيضاء البالغة وقسموا إلى ثلاث مجموعات (اشتملت كل مجموعة على ١٠ فئران). مثلت المجموعة (أ) الفئران الضابطة ، في حين اشتملت المجموعة (ب) على الفئران التى تعرضت لأنقطاع عابر للدم عن طريق ربط الشريان السباتي المشترك فى الناحيتين في نفس الوقت لمدة ١٠ دقائق وتم تقسيمها إلى ٢ مجموعة فرعية: (ب١) بعد ٣ أيام من الإصابة) و(ب٢) (بعد شهر واحد من الإصابة). أما المجموعة (ج) فقد اشتملت على الفئران اللتي تم تعريضها للإصابة كما فى المجموعة (ب) مع حقنها بثلاث جرعات من الميلا تونين فى التجويف البريتونى (بجرعة ٥ ملليجرام / كجم) ٣٠ دقيقة قبل نقص التروية , واحدة وثلاث ساعات بعد إعادة سريان الدم على التوالى وأيضاً تم تقسيمها إلى ٢ مجموعة فرعية: (ج١) (بعد ٣ أيام من الإصابة مع إعطاء الميلا تونين) و(ج٢) (بعد شهر واحد من الإصابة مع إعطاء الميلا تونين). تم تشريح المخيخ لإعداده لإعداده لصبغة الهيماتوكسلين والإيوسين. وتم أيضاً إجراء دراسة هستوكيميائية مناعية للكشف عن الخلايا الإيجابية لكل من (ب١) و(ب٢) والبروتين الغروى الليفى الحمضى. وقد أظهرت عينات المجموعة الفرعية (ب١) انكماش خلايا بركنجى والموت المبرمج للخلايا الحبيبية والتي زادت فى العدد بشكل ملحوظ فى المجموعة الفرعية (ب٢) . وقد لوحظ تحسن فى هذه التغيرات النسيجية فى عينات المجموعة الفرعية (ج) مع إعطاء الميلا تونين. كما بدا الهيكل النسيجي من قشرة المخيخ من المجموعة الفرعية (ج٢) أكثر أو أقل مماثلة مع المجموعة الضابطة. وقد لوحظ عدد قليل من الخلايا الإيجابية لكل من (ب١) و(ب٢) البروتين الغروى الليفى الحمضى في قشرة المخيخ لفئران المجموعة الفرعية (ب١) مع زيادة ملحوظة فى المجموعة الفرعية (ب٢) . وقد لوحظ أيضاً في عينات المجموعتين الفرعيتين (ج١) و(ج٢) انخفاضاً واضحاً فى عدد الخلايا الإيجابية لكل من الوصمتين المناعتين السابقتين بالمقارنة مع المجموعات الفرعية المقابلة لهما فى المجموعة (ب). ويمكن أن نخلص إلى أن حقن الميلا تونين يمكن أن يخفف من التغيرات المصاحبة لأنقطاع الدم ثم سريانه فى قشرة المخيخ للفئران من خلال دوره الوقائى العصبى.