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Original Article

Melatonin suppresses the brain injury after cerebral ischemia/reperfusion in hyperglycaemic rats

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Abstract

Background and purpose: Diabetes mellitus is a disorder accompanied by oxidative and inflammatory responses, that might exacerbate vascular complications. The purpose of this study was to investigate the potential antioxidant and anti-inflammatory effects of melatonin (MLN) on streptozotocin (STZ)-induced diabetic rats subjected to middle cerebral artery occlusion followed by reperfusion (MCAO/Re).

Experimental approach: Diabetes was induced in rats by a single injection of STZ (55 mg/kg; i.p.). The cerebral injury was then induced by MCAO/Re after six weeks. After 24 h of MCAO/Re the MLN (10 mg/kg) was administered orally for 14 days. Serum and tissue samples were extracted to determine malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO), interleukin-1β (IL-1β), and the tumor necrosis factor-α (TNF-α). Part of the brain tissue was kept in formalin for pathological and immunohistochemical studies to determine nuclear factor kappa B (NF-kB) and cyclooxygenase-2 (COX-2) immune reactivity.

Findings/Results: MCAO/Re in STZ-induced hyperglycaemic rats caused a decrease in brain GSH, an increase in brain MDA, and NO was increased in both serum and brain tissue. Rats showed a prominent increase in the serum and brain inflammatory markers *viz.* IL-1β and TNF-α. Oral treatment with MLN (10 mg/kg) for two weeks reduced the brain levels of MDA, NO, IL-1β, and TNF-α. Impressive amelioration in pathological findings, as well as a significant decrease in NF-kB and COX2 immune stained cells of the cerebral cortex, hippocampus, and cerebellum, occurred after treatment with MLN. It also succeeded to suppress the exacerbation of damage in the brain of hyperglycaemic rats.

Conclusion and implications: Daily intake of MLN attenuates the exacerbation of cerebral ischemic injury in a diabetic state.

Keywords: Brain injury; Cerebral ischemia/reperfusion; Diabetes; Melatonin; Rats.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by an increase in blood glucose level and accompanied by an excessive production of reactive oxygen species (ROS) (1) which leads to many diabetic complications as alteration of the extracellular matrix, apoptosis of endothelial cells (2) as well as the alteration in the vascular tone. Alteration in the vascular tone occurred as a result of reduced bioavailability of nitric oxide (NO) due to its rapid oxidative inactivation (3).

In the current study, the diabetic complication induced in the rat model was exacerbated by cerebral artery occlusion and reperfusion (MCAO/Re). Reperfusion conducted after long occlusion of the external carotid artery led to a prominent increase in the production of ROS which is the underlying cause for cell apoptosis due to oxidative damage for lipid, DNA, and proteins at the ischemic region (4). Cerebral damage occurs after ischemia and reperfusion will also lead to inflammatory neurodegeneration (5).

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ROS activate the nuclear factor-Kappa B (NF-κB) which in turn will enhance the release of tumour necrosis factor (TNF)-α and interleukin-1β (IL-1β) leading to inflammatory responses (6). Furthermore, the activated NF-κB and cyclooxygenase-2 (COX-2) which are prominent markers of inflammation will generate ROS leading to further neurodegeneration in the ischemic region (7). All the above showed that diabetes associated with ischemia and reperfusion enhanced both oxidative stress and inflammatory response thereby aggravation of the cerebral injury.

Melatonin (MLN) is a neurohormone released from the pineal gland (8). It was proved in previous research to exhibit a potent antioxidant activity (9). MLN can scavenge different ROS effectively; it also has an excellent penetrating ability to cross nearly all the physiological barriers as a result it can inhibit tissue damage. (10). In a previous study, MLN decreased the oxidative stress significantly in the retinal of streptozotocin (STZ)-induced diabetic rats (11). Melatonin is a well-known neuroprotection agent; many novel studies are still discussing this issue to reveal the mechanism of action behind this property and numerous recent studies have investigated its effect on diabetic complications including diabetic neuropathy and cardiac remodelling (12-13). In another study conducted by Metwally *et al*. authors declared that although the MLN treatment did not prevent the development of STZ-induced diabetes mellitus and it had no effect on the blood glucose level of the diabetic rats, it significantly ameliorated the diabetes-induced oxidative stress and neurodegeneration. Their results also showed that MLN exerted a potent therapeutic effect against the neurological complications of hyperglycaemia, therefore it can be used to treat diabetic neuropathy (14). This study has been designed to examine the ameliorative effect of MLN against neurodegeneration in brain tissue induced by ischemia followed by reperfusion in STZ-induced diabetic rats by determining its effect on oxidative stress e.g. glutathione (GSH), malondialdehyde (MDA), and nitric oxide (NO) levels as well as inflammatory mediators e.g. IL-1β and TNF-α using ELISA Technique.

The immune reactivity for NF-kB and COX-2 was assessed and immunohistochemical technique. Ischemic neuronal damage was assessed by histopathological examination.

MATERIALS AND METHODS

Animals

Forty male Wistar rats (250-350 g) were purchased from the animal breeding unit at the National Research Centre, Dokki, Giza, Egypt. Animals were housed in cages with water and food *ad libitum*, and the animal room temperature was kept at a constant temperature of 20 ± 1 °C on a 12/12-h light/dark cycle. Adequate measures were taken to minimize pain or discomfort of the animals. The experimental protocol was done according to the regulations established by the Ethics and Animal Care Committee of the National Research Centre, Giza, Egypt which are also in consistent with the National Institutes of Health guide for Care and Use of Laboratory Animals (Publication No. 85-23).

Drugs

STZ and MLN were purchased from Sigma, St. Louis, the USA. All other chemicals used in the experiment were of the highest available grade.

Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of STZ at a dose of 55 mg/kg, dissolved in distilled water. Twenty-four h after induction, hyperglycaemia in the experimental groups was confirmed by determining serum glucose levels. Rats with glucose levels of 250 mg/dL were considered diabetic (15). On the other hand, rats that were not hyperglycemic were excluded from the study (About 10%).

Cerebral artery occlusion and reperfusion

Six weeks after STZ treatment, the cerebral injury was induced by MCAO/Re (16). The rats were anesthetized with halothane (4% for induction and 1.5% for maintenance) under spontaneous respiration. The right common carotid artery was isolated under an operating microscope after a midline incision on the neck.

All branches of the external carotid artery were ligated. The tip of a 4-0 surgical nylon monofilament rounded by flame heating was inserted through the internal carotid artery and advanced to occlude the origin of MCA. Two h later, the filament was withdrawn to enable reperfusion. The distance from the bifurcation of the common carotid artery to the tip of the suture was approximately 20 mm in all rats. Then, the rats were allowed to recover from anesthesia at room temperature. MCAO/Re mortality rate was about 25% of the total number of rats subjected to the operation.

Experimental protocol

Fifty rats were divided randomly into two sets; the first set served as a normal control group (n = 10), and the second set served as an STZ-induced diabetic group (n = 40). Hyperglycaemic rats were then divided into three groups as follows: STZ group (n = 10), STZ group subjected to MCAO/Re after 6 weeks of STZ injection (n = 10), and STZ group subjected to MCAO/Re after 6 weeks of STZ injection, and after 24 h were treated with MLN (10 mg/kg) for 2 consecutive weeks (n = 10) (11,17).

Determination of serum glucose level

Whole blood was extracted from the retroorbital plexus of all the rats. Serum was separated using a centrifuge (ALC Centrifugette 4206, Milano, Italy) at 1500 *g* for 5 min. The serum was then placed in labeled plastic tubes and stored at -20 °C until testing. Glucose serum levels were determined using the Trinder method (Glucose GOP-PAP) (18).

Determination of oxidative and nitrosative stress biomarkers

Reduced GSH, NO, and MDA were determined according to the method described respectively (19-21). All markers were assessed in brain tissue. NO was also determined in serum.

Determination of inflammatory cytokines

TNF was quantified using a rat TNF kit (Invitrogen, USA), and IL-1β was assessed using a rat IL-1β kit (R&D Systems, USA). The optical density (OD) of all samples at 450 nm was measured using a Spectramax (M2, Molecular Devices, USA). All markers were assessed in both serum and brain tissue.

Histopathological examination and assessment of ischemic neuronal damage

At the end of the experiment, following the scarification of rats, brains were sectioned and routinely processed. The processed tissues were cut into 5 μm thick sections and stained with hematoxylin and eosin (H&E) for routine histopathological examination. For assessment of ischemic neuronal damage, the number of viable neurons was counted in the cerebral cortex and CA1 hippocampal subdivision in five random high-power fields, according to the method of Dave *et al.* (22). Additionally, vascular congestion and gliosis were assessed in the brains of different groups.

Immunohistochemical analysis

All immunohistochemical staining procedures were performed as described previously (23). Briefly, the tissue sections were incubated with 3% hydrogen peroxide to inhibit endogenous peroxidase. After that, the slices were incubated with rabbit polyclonal anti-NF-kB (Abcam, Egypt) and rabbit polyclonal anti-COX2 (Abcam, Egypt) antibodies overnight at 4 °C. Diaminobenzidine was used for the demonstration of immune reaction. The immune reactivity for NF-kB and COX-2 was Semi quantitatively assessed in ten random high-power fields (HPF), according to the percentage of positive cells in the high-power field, as described by Hassan *et al*. (24). A semi-quantitative scale graded from 0 to 3 was used in which 0, no staining; 1, positive staining in < 30% of cells per HPF; 2, positive staining in 30-70% of cells per HPF; or 3, positive staining in > 70% of cells per HPF.

Statistical analysis

Data were presented as mean ± SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons. *P < 0.05 was statistically considered significant. On the other hand, NF-kB and COX2 immunohistochemical staining assessment were analyzed by performing the Kruskal-Wallis non-parametric ANOVA test

followed by the Mann-Whitney *U* test. In all cases, *P* < 0.05 was assumed to denote statistical significance.

RESULTS

Serum glucose levels induced by streptozotocin injection

Twenty-four h after the last dose of MLN, serum glucose levels were determined and rats showed a marked increase in serum glucose levels in both STZ and STZ + MCAO/Re groups as compared to the normal control group indicating a prominent hyperglycaemic state. Treatment of STZ + MCAO/Re rats with MLN for 14 consecutive days did not show any alteration in the serum glucose level as represented in Table 1.

Effect of melatonin on oxidative and nitrosative stress biomarkers

Reduced GSH showed a significant decrease in both STZ and STZ + MCAO/Re groups when compared to normal non-diabetic rats. Treatment with an oral dose of MLN for 14 days to diabetic rats with brain injury induced by MCAO/Re showed a significant increase in GSH level when compared to STZ group and

STZ + MCAO/Re as shown in Fig. 1A. On the other hand, MDA was found to increase significantly in the STZ group and STZ + MCAO/Re as compared to the non-diabetic control group. It was also observed that the STZ + MCAO/Re group showed a significant increase in brain MDA as compared to the STZ group. Treatment of diabetic rats with brain injury with MLN reduced brain MDA when compared to the STZ group and to STZ + MCAO/Re as shown in Fig. 1B. Brain NO showed a significant increase in STZ and STZ + MCAO/Re groups when compared to the control group. Treatment of STZ + MCAO/Re group with MLN reduced tissue NO when compared to STZ + MCAO/Re as shown in Fig. 1C. Serum NO was found to increase significantly in STZ and STZ + MCAO/Re groups as compared to the control group. It was also observed that brain injury induced by MCAO/Re in diabetic rats showed a significant increase in brain NO as compared to the STZ group. Treatment of diabetic rats with brain injury by the administration of MLN reduced serum NO when compared to STZ + MCAO/Re as shown in Fig. 2. All results obtained showed that treatment with MLN restored GSH, MDA, and NO levels to the normal state.

Table 1. Effect of melatonin on serum glucose level in diabetic rats with cerebral injury induced by MCAO/Re. All data represent mean ± SEM. *$P < 0.05$ Indicates significant differences compared to the normal control group, n = 10.

Groups	Serum glucose (mg/dL)
Normal Control	109.82 ± 4.68
STZ	312.75 ± 28.16*
STZ + MCAO/Re	294.50 ± 20.54*
STZ + MCAO/Re +MLN	353.67 ± 23.09*

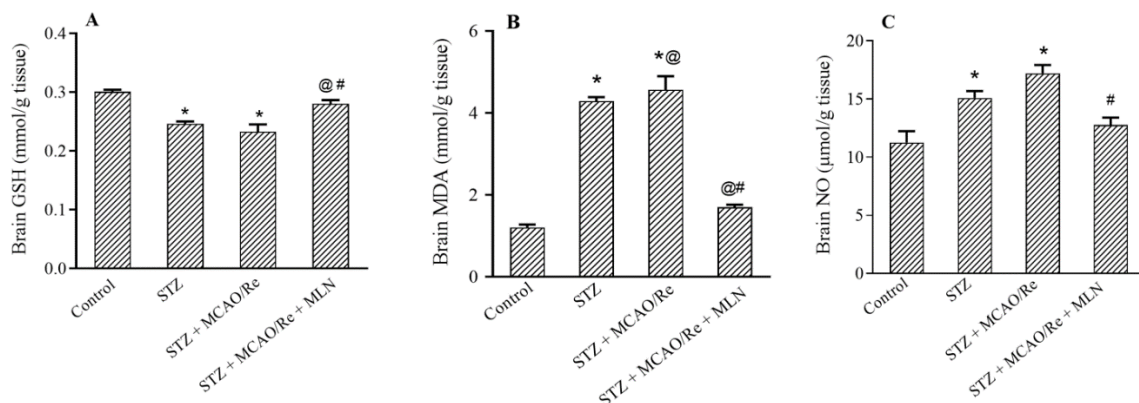MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin.

Fig. 1. Effect of melatonin on (A) GSH, (B) MDA, (C) brain NO in diabetic rats. All data are presented as mean ± SEM. In all cases, a *P* value of < 0.05 was assumed to denote statistical significance. *$P < 0.05$ Compared to the normal control group, $^@P < 0.05$ *vs* STZ group, $^#P < 0.05$ against STZ + MCAO/Re group. MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin; GSH, glutathione; MDA, malondialdehyde; NO, nitric oxide.
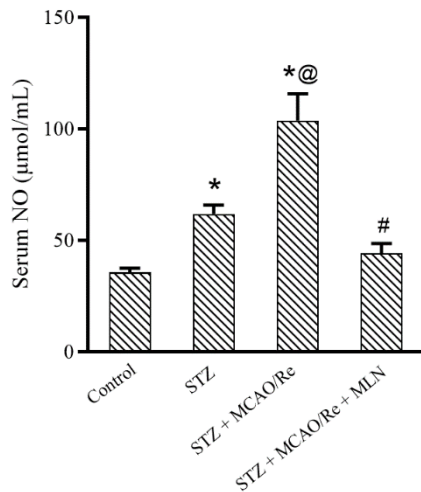
Fig. 2. Effect of melatonin on serum NO in diabetic rats. All data are presented as mean ± SEM. In all cases, a $P < 0.05$ was assumed statistically significant. * $P < 0.05$ compared to the normal control group, @$P < 0.05$ against STZ group, #$P < 0.05$ vs STZ + MCAO/Re group. MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin; NO, nitric oxide.

Effect of melatonin on inflammatory cytokines

Induction of diabetes mellitus by single injection with STZ significantly increased the cytokines TNFα and IL-1β in both brain tissue and serum when compared to the control (non-diabetic rats). Brain injury-induced in diabetic rats by MCAO/Re also significantly increased both inflammatory cytokines in brain tissue and serum when compared to the control of non-diabetic rats and when compared to diabetic rats. Treatment of diabetic rats with brain injury by oral administration of MLN for 14 days significantly reduced the level of TNFα and IL-1β in both brain tissue and serum when compared to diabetic rats and diabetic rats with brain injury as presented in Table 2.

Effect of melatonin on histopathological alterations

Histopathological assessment of ischemic neuronal damage recorded in the brains of different groups is illustrated in Table 3.

The normal histological structure of the cerebral cortex, hippocampus, and cerebellum was demonstrated in the brain of normal control rats, with no definite damage. The cerebral cortex showed normal neurons and glial cells as well as normal cerebral cortical blood vessels (Fig. 3A). Similarly, normal hippocampal neurons were demonstrated (Fig. 3B).

Table 2. Effect of melatonin on cytokine markers in diabetic rats with cerebral injury induced by MCAO/Re. All data represented as mean ± SEM. *$P < 0.05$ Indicates significant differences compared to normal control group, @$P < 0.05$ vs STZ group, and #$P < 0.05$ against STZ + MCAO/Re group, n = 10.

Groups	Tumor necrosis factor-α		Interleukin-1β	
	Brain tissue (pg/g tissue)	Serum (pg/mL)	Brain tissue (pg/g tissue)	Serum (pg/mL)
Normal Control	12.3 ± 0.33	25.14 ± 0.67	21.74 ± 0.58	10.65 ± 0.28
STZ	27.61 ± 0.38*	93.5 ± 1.61*	101.08 ± 1.34*	22.82 ± 0.48*
STZ + MCAO/Re	51.44 ± 1.23*@	124.14 ± 3.39*@	139.65 ± 3.66*@	42.42 ± 0.97*@
STZ + MCAO/Re + MLN	20.53 ± 0.54*@#	43.7 ± 1.16*@#	38.87 ± 1.0*@#	18.12 ± 0.48*@#

MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin.

Table 3. Effect of melatonin on the histopathological assessment of ischemic neuronal damage recorded in the brains in diabetic rats with cerebral injury induced by MCAO/Re. All data represent the mean ± SEM. *$P < 0.05$ Indicate the significant differences compared to the normal control group, @$P < 0.05$ vs STZ group, #$P < 0.05$ compared to STZ + MCAO/Re group, n = 10.

Groups	Number of viable neurons (count/HPF)		Congestion, oedema, and haemorrhage	Gliosis
	Cerebral cortex	Hippocampus		
Normal Control	53.33 ± 3.38	57.00 ± 2.30	Nil	Nil
STZ	27.00 ± 2.64*	30.33 ± 2.90*	Moderate	Mild
STZ + MCAO/Re	18.00 ± 1.52*@	16.33 ± 5.84*@	Diffuse	Moderate
STZ + MCAO/Re + MLN	43.00 ± 3.51*@#	43.33 ± 3.48*@#	Mild	Mild

MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin. HPF, high power field.

The cerebellum of the normal group showed a normal outer molecular layer and inner granular cell layer with normal intermediate Purkinje cell (Fig. 3C). In contrast, the degeneration of cerebral cortical neurons associated with a decreased number of viable ones was demonstrated in the STZ group (Fig. 3D). Additionally, the degeneration of pyramidal neurons of the hippocampus was also demonstrated (Fig. 3E). Necrotic changes were demonstrated in Purkinje cells of the cerebellum which appeared intensely eosinophilic with shrunken pyknotic nuclei (Fig. 3F). More severe and pronounced histopathological alterations were observed in the STZ + MCAO/Re group. Vascular congestion associated with oedema and hemorrhage, ischemic neuronal damage, and gliosis was frequently demonstrated in this group, concurrently with a massive reduction of surviving neurons (Table 3). Widespread neuronal degeneration associated with vascular congestion and oedema and proliferation of glial cells were characteristically demonstrated in the cerebral cortex, hippocampus, and cerebellum of this group (Fig. 3G-I). Treatment with MLN significantly ameliorated the histopathological changes compared to other groups. An increased number of surviving neurons and reduction of degenerated neurons were recorded in the cerebral cortex and hippocampus (Fig. 3J and K), haemorrhage, and oedema were also diminished. Similarly, Purkinje cells of the cerebellum appeared normal in most examined sections, with only sparse necrotic cells (Fig. 3L).

Effect of melatonin on immunohistochemical staining

The result of NF-kB and COX-2 immunohistochemical staining demonstrated in the brain of different treated groups is summarized in Table 4.
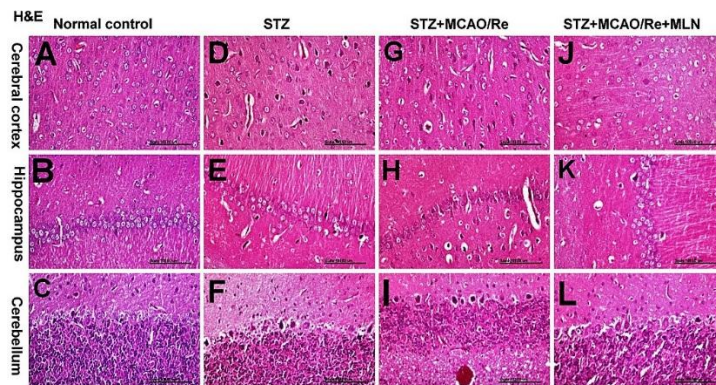

Fig. 3. Photomicrograph of the brain (cerebral cortex, hippocampus, and cerebellum) stained with hematoxylin and eosin (H&E). (A-C) Normal control rats, (D-F) STZ group, (G-I) STZ + MCAO/Re group, and (J-L) STZ + MCAO/Re + MLN group; scale bar = 100 μm. MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin.

Table 4. Effect of melatonin on brain NF-kB and COX-2 immunohistochemical staining in diabetic rats with cerebral injury induced by MCAO/Re. All non-parametric data are represented as the median. A semi-quantitative scale graded from 0 to 3 was used in which 0, no staining; ≥ 1 to < 2, positive staining in $< 30\%$ of cells per HPF; ≥ 2 to <3, positive staining in 30-70% of cells per HPF; or ≥ 3, positive staining in > 70% of cells per HPF.

Groups	NF-kB immune staining (% of positive cells/HPF)			COX-2 immune staining (% of positive cells/HPF)		
	Cerebral cortex	Hippocampus	Cerebellum	Cerebral cortex	Hippocampus	Cerebellum
Normal Control	0	0	0	0	0	0
STZ	2.5	2	2	2	1.5	2
STZ + MCAO/Re	3	3	3	3	2	3
STZ + MCAO/Re + MLN	2	1	1	1	1	1

NF-kB, nuclear factor kappa; COX-2; cyclooxygenase-2; MCAO/Re, middle cerebral artery occlusion followed by reperfusion; HPF, high power field; STZ, streptozotocin; MLN, melatonin.
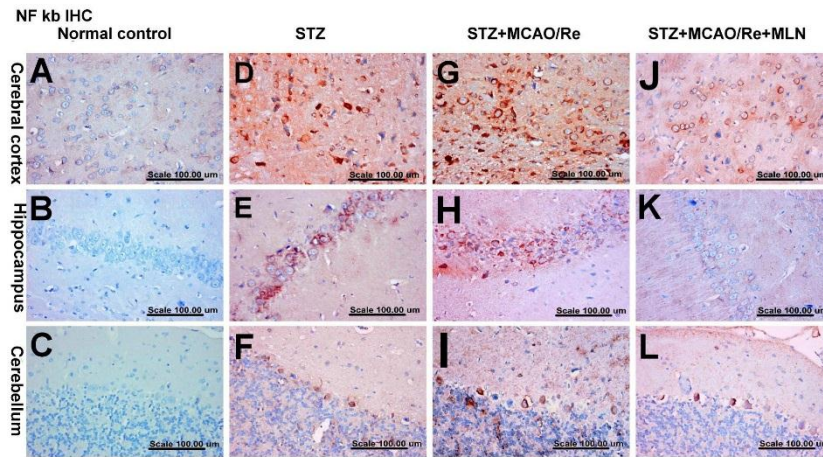
Fig. 4. Evaluation of melatonin effect on nuclear factor kappa B in the brain (cerebral cortex, hippocampus, and cerebellum) of (A-C) normal control rats, (D-F) STZ group, (G-I) STZ + MCAO/Re group, and (J-L) STZ + MCAO/Re + MLN group hematoxylin and eosin (H&E) method has been used for staining; scale bar = 100 μm. MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin.

Fig. 5. Evaluation of melatonin effect on cyclooxygenase-2 in the brain (cerebral cortex, hippocampus, and cerebellum) of (A-C) normal control rats, (D-F) STZ group, (G-I) STZ + MCAO/Re group, and (J-L) STZ + MCAO/Re + MLN group hematoxylin and eosin (H&E) method has been used for staining; scale bar = 100 μm. MCAO/Re, middle cerebral artery occlusion followed by reperfusion; STZ, streptozotocin; MLN, melatonin.
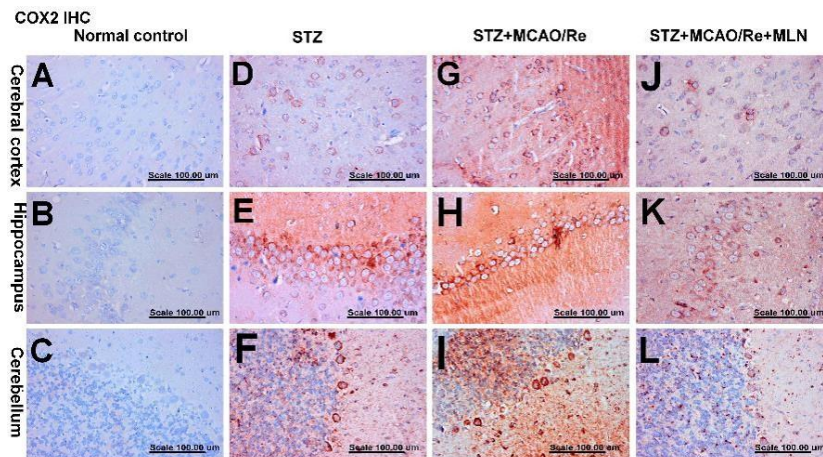
The cerebral cortex, hippocampus, and cerebellum of normal control rats revealed no evidence of NF-kB immunohistochemical staining (Fig 4A-C). Meanwhile, a significant increase in the percentage of NF-kB-immune stained cells was recorded in the STZ group. The immune reactivity was mainly demonstrated in the nuclei of neurons and glial cells of the cerebral cortex (Fig. 4D), hippocampal neurons (Fig. 4E), and Purkinje cells of the cerebellum (Fig. 4F). On the other side, a significant difference was recorded in the brains of STZ + MCAO/Re group, with a pronounced increase of % of positively stained cells with intense nuclear staining in the cerebral cortex (Fig. 4G), hippocampal neurons

(Fig. 4H) and cerebellar Purkinje cells (Fig. 4I). Conversely, the cerebral cortex, hippocampus, and cerebellum of the STZ + MCAO/Re + MLN group revealed a pronounced reduction of positively stained (Fig. 4J-L, respectively), and this is significantly different from STZ and STZ + MCAO/Re groups.

Negative COX-2 immune staining was demonstrated in the cerebral cortex (Fig. 5A), CA1 hippocampal subdivision (Fig. 5B), and cerebellum (Fig. 5C). On the other hand, an increased percentage of positively stained cells, which revealed weak cytoplasmic staining, was recorded in the cerebral cortex and hippocampus of the STZ group (Fig. 5D and E, respectively). But intense cytoplasmic staining

was demonstrated in cerebellar Purkinje cells of this group (Fig. 5F). A dramatic and significant increase in the percentage of COX-2 immune staining with strong cytoplasmic staining was recorded in the cerebral cortex (Fig. 5G), hippocampus (Fig. 5H), and cerebellum (Fig. 5I) of STZ + MCAO/Re group. Impressive amelioration with a significant decrease in the percentage of COX-2 immune stained cells was recorded in the cerebral cortex, hippocampus, and cerebellum of STZ + MCAO/Re + MLN group (Fig. 5J-L, respectively), and this was significantly different from other groups.

DISCUSSION

Cerebral ischemia; one of the major complications of diabetes, is caused by the massive production of ROS creating a state of oxidative stress. Therefore, the goal of the present study was to examine the ameliorative effect of MLN against neurodegeneration in brain tissue induced by ischemia followed by reperfusion in STZ-induced diabetic rats. The same experimental protocol was conducted previously (25). To achieve this goal, diabetes was induced then the ischemia/reperfusion was performed to emphasize the concept that diabetic complication induced in the rat model was exacerbated by MCAO/Re. Then this exacerbated complication has been furtherly treated with MLN.

The current study revealed that brain injury induced by MCAO/Re in diabetic rats showed an increase in MDA, NO, and on the other hand, a reduction in GSH when compared to diabetic rats. In a previous study, brain injury was induced in non-diabetic rats and showed a significant increase in MDA, NO, and a significant decrease in GSH as compared to normal rats (26). Although the antioxidant effect of MLN was confirmed in previous studies, the aim of the present study was to determine the impact of MLN; a potent antioxidant and anti-inflammatory hormone, in ameliorating the neurodegeneration in diabetic rats subjected to ischemia followed by reperfusion.

Administration of MLN in the current study restored GSH, MDA, and NO levels to the

normal state indicating that MLN had a powerful antioxidant effect. MLN is an effective antioxidant in different *in vivo* and *in vitro* models of neurodegenerative diseases by scavenging of free radicals as well as increasing of gene expression of many antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (27).

It has been also observed that diabetic rats exhibited significant increases in serum and brain tissue levels of TNF and IL-1β that have been exacerbated with cerebral injury induced by MCAO/Re. The same results had been also recorded in a research conducted by Iwata *et al.* (25) who revealed that TNF and IL-1β had been upregulated in diabetic rats and were remarkably enhanced following reperfusion after ischemia.

Treatment of diabetic rats with induced ischemia followed by reperfusion with MLN; in the current study, at a dose of 10 mg/kg for two consecutive weeks as it is the most effective dose used in previous similar studies (diabetes and ischemia) performed on retina, myocardium, kidney, and neurons showing significant antioxidant, antiapoptotic, and anti-inflammatory effects as well as an ameliorative effect at histopathological studies. Therefore, we used the same dose to see the possible ameliorative effect on diabetic ischemic rats (28-30). MLN had shown an impressive reduction in the level of both inflammatory cytokines in both serum and brain tissues with no significant reduction in serum glucose levels. This indicates that these effects due to its anti-inflammatory properties and not due to its anti-hyperglycemic effect that may counteract the detrimental effect of diabetes on brain ischemic injury. Our results were identical to those of a previous study which had shown that MLN ameliorated ischemic damage by reducing cytokines release in brain ischemic injury due to its ability to inhibit the synthesis of inflammatory cytokines such as TNF and IL-1β (25).

The immunohistochemical study recorded a significant increase of NF-κβ in the brains of the STZ + MCAO/Re group, with a pronounced increase in the percentage of positively stained cells with intense nuclear staining in the cerebral cortex, hippocampal neurons, and

cerebellar Purkinje cells. Besides the increase of NF-κβ in brain tissue, a significant increase in brain and serum TNF and IL-1β have been observed. Therefore, our study confirmed the causal relationship between the increase of NF-κβ and the increase of TNF and IL-1β. The same result was also had been given in the previous study (31). Besides the increase of NF-κβ, a dramatic increase in the percentage of COX-2 immune staining with strong cytoplasmic staining was in the cerebral cortex, hippocampus, and cerebellum of STZ + MCAO/Re group were detected. In a previous study, it had been shown a significant increase in the COX-2 immunoreaction in the diabetic and diabetic groups with MCAO/Re in comparison to the control group (32).

Treatment with MLN to STZ + MCAO/Re group revealed an impressive amelioration with significant a decrease in the percentage of NF-κβ and COX-2 immune stained cells was recorded in the cerebral cortex, hippocampus, and cerebellum of STZ + MCAO/Re + MLN group. In a close study of our research, previous results showed that MLN reduced the increased levels of proinflammatory cytokines COX-2 as well as reducing the expression of NF-κβ in diabetic rats (33).

In the present study, diabetes showed degeneration of cerebral cortical neurons associated with a decreased number of viable ones, degeneration of pyramidal neurons of the hippocampus, and necrotic changes were demonstrated in Purkinje cells of the cerebellum which appeared intensely eosinophilic with shrunken pyknotic nuclei. In a previous study, the STZ group showed shrunken Purkinje cells with pyknotic nuclei, revealing apoptosis. Also, the number of Purkinje cells in the cerebellum decreased in number when compared to the control group (34).

Induction of ischemia followed by reperfusion to diabetic rats showed more severe and pronounced characteristic neuronal degeneration associated with vascular congestion and oedema as well as the proliferation of glial cells in the cerebral cortex, hippocampus, and cerebellum. These findings were in accordance with those obtained by Zeng *et al.* which reported an exacerbation of histopathological findings in diabetic rats subjected to ischemia followed by reperfusion when compared to diabetic rats (35).

Treatment with MLN led to an increment of surviving neurons and reduction of degenerated neurons in the cerebral cortex and hippocampus, as well as diminished hemorrhage and oedema. Purkinje cells of the cerebellum were restored to normal structure in most examined sections. In a previous study, MLN given to diabetic rats reversed most of the histopathological changes caused by diabetes in the cerebral cortex (14).

CONCLUSION

Treatment of diabetic rats suffering from the ischemia induced by MCAO/Re restored all redox homeostasis biomarkers to the nearly the normal level and significantly decreased inflammatory cytokine release. It also ameliorated the immune reactivity for NF-kB and COX-2 different brain portions. Finally, it can be concluded that daily intake of MLN attenuates the exacerbation of cerebral ischemic injury in a diabetic state, which may be attributed to antioxidant and anti-inflammatory effects in the brain. Our findings showed that MLN exerts a powerful antioxidant property evidenced by inhibition of oxidative stress and restoring GSH and NO levels in brain tissue. In addition, MLN exerted a strong anti-inflammatory effect mainly by inhibiting inflammasome activity that activates the highly pro-inflammatory cytokines which significantly appeared in the results obtained.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST STATEMENT

The authors declared that there is no conflict of interest in this study.

AUTHORS' CONTRIBUTION

The pharmacological study had been divided equally among the authors; D.O. Saleh, G.A. Abdel Jaleel, S.W. Al-Awdan. and G.F. Asaad. Histopathological and immunohistochemical studies had been conducted by A. Hassan.

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