Melatonin attenuates hippocampal neuron apoptosis and oxidative stress during chronic intermittent hypoxia via up-regulating B-cell lymphoma-2 and down-regulating B-cell lymphoma-2-associated X protein

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ABSTRACT

الأهداف: دراسة الأثر العصبي للميلاتونين ضد نقص الأوكسجين المتقطع والخصائص الفيسولوجية لمتلازمة انقطاع التنفس.

الطريقة: أجريت هذه الدراسة خلال الفترة من يناير 2011م إلى سبتمبر 2012م في مستشفى تونقي، جامعة هوزانق للعلوم والتقنية، وهان، الصين. تم تقسيم 38 فئران ويستر عشوائياً إلى 3 مجموعات (10 كل مجموعة) وشملت على مجموعة التحكم، مجموعة نقص الأوكسجين المتقطع، ومجموعة الميلاتونين. تعرضت الفئران إما إلى (تركيز أوكسجين متغير بشكل دوري من 21.78+0.65 إلى 6.57+0.57% أو معدل الدورة الهوائية (30 دورة/ساعة)، 8 ساعات/يوم لمدة 4 أسابيع.

النتائج: أن التعرض لنقص الأوكسجين المتقطع يؤدي إلى انخفاض إحصائي في أنزيم ديسموتاز ومضاد سرطان الغدة اللمفاوية في الحصين لمجموعة نقص الأوكسجين بالمقارنة مع مجموعة التحكم ومجموعة الميلاتونين. في المقابل ارتفع موت الخلايا العصبية بشكل إحصائي بالمقارنة مع ارتفاع الميثيل وبروتين موت الخلايا، والبروتين المصاحب للباكسل في مجموعة نقص الأوكسجين المتقطع بالمقارنة مع المجموعتين الأخرى. أن استخدام الميلاتونين يقلل من ارتفاع نشاط الميثيل و والبروتين المصاحب للباكسل، واستعادة نشاط ديسموتاز والبروتين واستعادة نشاط أنزيم ديسموتاز للمعدل الطبيعي.

خاتمة: تشير النتائج بأن الميلاتونين يقلل من موت الخلايا العصبية التابعة نقص الأوكسجين المتقطع بالبحث عن موت الخلايا الأوكسجين التفاعلي ينظم بروتين موت الخلايا المبرمج، ويقلل من نقص الأوكسجين المتقطع الناتج عن إصابة الإجهاد وينتج أثر عصبي.

Objectives: To investigate the neuroprotective effect of melatonin against chronic intermittent hypoxia (CIH), the major pathophysiologic features of obstructive sleep apnea syndrome.

Methods: This study was conducted between January 2011 and September 2012 in Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China. Thirty 8-week Wistar rats were randomly divided into 3 groups (10 each): a control group, a vehicle-treated CIH group; and a melatonin-treated (10 mg/kg) CIH group. Rats were exposed to either intermittent hypoxia (IH) (oxygen concentration changing periodically from 21.78 ±0.65 to 6.57±0.57%), or air-air cycling at a rate of 30 cycles/hour, 8 hour/day for 4 weeks.

Results: The CIH exposure led to a significant decrease in superoxide dismutase (SOD) activity and anti-apoptotic protein B-cell lymphoma-2 (BCL-2) expression in the hippocampus of CIH group rats compared with that of the control group and melatonin-treated CIH group. In contrast, hippocampal neuronal apoptosis increased significantly in parallel to an augment in 3,4-methylenedioxyamphetamine (MDA) content and pro-apoptotic protein Bcl-2-associated X protein (BAX) expression in CIH group than the other 2 groups. Melatonin administration abrogated the increase in MDA activity, as well as BAX expression, and restored SOD activity and BCL-2 expression to nearly their normal levels.

Conclusion: These results indicate melatonin can inhibit hippocampal neuron apoptosis following CIH by scavenging reactive oxygen species, up-regulating anti-apoptotic protein BCL-2 and down-regulating pro-apoptotic protein BAX, and thus, alleviate CIH-induced oxidative stress injury and produce neuroprotection effects.

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Obstructive sleep apnea syndrome (OSAS) is characterized by recurrent episodes of partial or complete obstruction of the upper airway, leading to intermittent hypoxia (IH) and frequent arousals from sleep. Patients with OSAS often complain of a certain degree of memory deficits and cognitive impairments, which are supposed to be typical symptoms commonly associated with Alzheimer's disease (AD), and could be induced by hippocampal neuron apoptosis resulting from repeated occurrence of hypoventilation/re-oxygenation associated oxidative stress.[1,2] Previous studies have found that 4-week IH could lead to enhanced neuronal oxidative stress injury and neuronal apoptosis, which may provide a causal link between chronic intermittent hypoxia (CIH) and neurological dysfunction of patients with OSAS.[1,3] Continuous positive airway pressure (CPAP) is the gold standard treatment for OSAS. It is the most effective currently available treatment for patients with moderate-to-severe OSAS. The CPAP treatment can cure abnormal breathing during sleep, and thus, improve sleep quality, sleepiness, as well as OSAS-related disorders. For example, it has been demonstrated that the elimination of OSAS-related nocturnal apnea episodes could improve sleep quality, as well as excessive daytime sleepiness (EDS) in patients with AD.[4] However, not all patients find this therapy ideal and will guarantee a good adherence to it, and cognitive functioning in OSAS patients seems partially improved by CPAP treatment.[5,6] Other therapeutic strategies such as pharmacotherapy are needed to be found for patients with OSAS. Melatonin (N-acetyl-5-methoxytryptamine), synthesized mainly by the pineal gland, plays an important role in several physiological functions including the process of sleep regulation, sleep/wake rhythms control, regulation of circadian rhythm, and seasonal control of reproductive and immune mechanisms. Moreover, reductions in circulating melatonin and dysfunction of melatonergic signaling are closely related to age-related diseases, such as AD.[7] In addition, as an effective antioxidant and mitochondrial function protector, it has the ability to inhibit apoptosis of neuronal cells, as well as several different cell types.[8-11] As suggested by accumulated evidence, exogenous melatonin in combination with exercise may be a more effective therapy for spinal cord injury (SCI) caused by diseases or trauma than other rehabilitation treatments mainly owing to its antioxidant effects.[11] Recently, the β-amyloid peptides generation in combination with the expression of its precursor protein β-site amyloid protein precursor (APP) cleavage enzyme (BACE) has been reported to increase after short-term IH treatment, which precedes the apoptotic cell death observed in the hippocampus, and could be abolished by daily pharmacological administration of melatonin.[12] Furthermore, melatonin has a protective effect against hippocampal oxidative stress injury of rats with short-term IH.[13,14] However, mechanisms associated with the neuroprotective effects of melatonin against long-term IH, also known as CIH, remain largely undefined.[12-14] Thus, our study tested the hypothesis that melatonin administration could relieve CIH-induced hippocampus neuron apoptosis and associated oxidative stress by establishing an animal model of OSAS, and tried to evaluate the therapeutic potential of melatonin in OSAS patients.

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Methods. This study was conducted between January 2011 and September 2012 in Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology, China. Thirty 2-month old male Sprague-Dawley (SD) rats weighing 200±5 g were purchased from the Animal Laboratory Center of Tongji Hospital Affiliated to Tongji Medical College and used in the research. Experimental procedures were conducted in strict accordance with the NIH Guiding Principles in the Care and Use of Animals.[15] The ethical approval for the study was obtained from the Huazhong University of Science and Technology.

The IH procedures. A total of 30 SD rats were randomly assigned to 3 groups: a control group (Con, n=10, sham hypoxia/re-oxygenation); a vehicle-treated CIH group (Veh+CIH, n=10); and a melatonin-treated CIH group (MT+CIH, n=10). Briefly, rats were kept in customized chambers (BioSpherix OxyClycler A84, Lacona, NY, USA), which were equipped with gas injectors and sensors for oxygen levels under specific-pathogen-free (SPF) conditions with a room temperature maintained at 22±2°C, and free access to food and water. A gas delivery system was used to regulate the flow of the room air, oxygen or nitrogen, while the oxygen concentration in cages was constantly monitored. For the IH exposure, oxygen concentration in cages were alternated periodically from 21.78±0.65 to 6.57±0.57% every 2 minutes, 8 hours/day for 4 weeks. Briefly, nitrogen was infused to reduce the oxygen concentration to approximately 6% for 60 seconds followed by oxygen injection to return the oxygen concentration back to approximately 21% for another 60 second. Desired oxygen concentrations were achieved by a mixture of nitrogen with oxygen. For the

control group, the atmosphere in cages changed every minute by inflating room air and oxygen to maintain oxygen concentration at approximately 21%. Rats were treated with daily intraperitoneal (IP) injections of either 0.9% saline solution containing 2% ethanol (vehicle), or melatonin (10 mg/kg, Sigma, St. Louis, MO, USA) 30 minutes prior to hypoxia exposure. The melatonin solution was freshly dissolved in absolute ethanol and diluted with saline to achieve the ethanol concentration of 2% before use.

Sample collection. At the end of the designated duration of IH exposures, rats from the 3 groups were anesthetized by IP injection of pentobarbital (50 mg/kg, Sigma, St. Louis, MO, USA), and perfused transcardially with 0.9% saline buffer (37°C), and then with 4% paraformaldehyde (PFA) dissolved in cold 0.1 mol/L phosphate-buffered solution (PBS, pH-7.2). The hippocampal tissues were dissected out, fixed in 4% PFA, and subsequently imbedded in paraffin. For those used for malondialdehyde (MDA) and superoxide dismutase (SOD) assays, rats were perfused only with saline, and the hippocampus were quickly removed, frozen in liquid nitrogen, and stored at -80°C freezer.

Oxidative stress assays. The hippocampal SOD activity and MDA levels were assayed using commercially available SOD kits and MDA kits according to the manufacturer's instructions (Nanjing JianCheng Bioengineering Institute, China).[3,16] The reaction product of SOD (550 nm) and MDA (532 nm) was measured spectrophotometrically. The MDA concentration (nmol) in the hippocampal tissue was normalized to the protein content (mg protein) and expressed as nmol/mg protein. While the SOD activity was normalized and expressed in U/mg protein.

Analysis of hippocampal neuron apoptosis. Hippocampal neuron apoptosis was detected by terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method using an In Situ Cell Apoptosis Detection Kit (Roche Corporation, Mannheim, Germany) similar to previous studies.[3,16] Four-μm thick of paraffin-embedded tissue sections were prepared, dewaxed, rehydrated, pre-incubated with proteinase K, and then exposed to TUNEL reaction mixture according to the manufacturer's instructions. Tissue sections were observed under a 10×10 power lens and 5 fields were randomly selected of each slice (Olympus BX51, Tokyo, Japan). The TUNEL-positive neurons were shown by pale yellow, brown, or dark brown coloration in nuclei. The TUNEL staining was quantified using Image-Pro Plus (IPP) software (version 7.0, Media Cybernetics Inc, MD, USA), and the rate of apoptosis was expressed by integrated option density (IOD).

Immunohistochemistry assays of B-cell lymphoma-2 (BCL-2) and BCL-2-associated X protein (BAX). To detect the expression of BCL-2 and BAX in the hippocampus, paraffin-embedded tissue slices were dewaxed and blocked in PBS containing 5% goat serum, 0.1% bovine serum albumin, and 0.1% Tween-20. Then, they were incubated with primary antibodies to BCL-2 (rabbit, Santa Cruz Corporation, CA, USA) and BAX (rabbit, Santa Cruz Corporation, CA, USA). After incubation with biotinylated secondary antibodies followed by streptavidin-horseradish peroxidase (HRP) (Boster Bioengineering Corporation, Wuhan, China), they were visualized using diaminobenzidine (DAB). Tissue sections were observed under a 10×20 power lens and 5 fields were randomly selected from each slice (Olympus BX51, Tokyo, Japan). The BCL-2 and BAX positive cells were stained by pale yellow, brown, and dark brown coloration in cytoplasm. The number of BCL-2 and BAX positive cells in each slice was calculated by IPP software by quantifying and averaging the IOD of the previously selected 5 fields.

Western-blot analysis of BCL-2 and BAX. Equivalent proteins (40 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (PVDF, 0.22uM, Millipore, Bedford, MA, USA). To block nonspecific binding sites, membranes were incubated in tris-buffered saline solution containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) skim milk. Membranes were washed and probed individually with primary rabbit antibodies, namely BCL-2 antibody (1:1,000; Cell Signaling Technology, Beverly, MA, USA), BAX antibody (1:1,000; Cell Signaling Technology, Beverly, MA, USA) and β-actin (1:1,000; Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight either in TBST containing 5% (w/v) BSA or 5% (w/v) skim milk. After incubation with HRP-conjugated goat anti-rabbit immunoglobulin (Ig)G (1:1,000; Cell Signaling Technology, Beverly, MA, USA) for one hour at room temperature, antibody specific bands were developed using an Enhanced Chemiluminescence Detection Kit (ECL) (Beyotime Biotech, Shanghai, China). Membranes were exposed on Kodak film for an appropriate time. Quantification of the bands was assessed by AlphaEaseFC software (Alpha Innotech, CA, USA).

Statistical analysis. All quantitative data were expressed as mean ± standard deviation, and analyzed by Statistical Package for Social Sciences version 18.0 software (SPSS Inc, Chicago, IL, USA). Statistical difference was performed by one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) (L)-test. A $p<0.05$ was considered statistically significant.

Results. *Melatonin reduced hippocampus oxidative stress injury induced by CIH (Figure 1).* The CIH led to a statistically significant rise in the hippocampal MDA levels of CIH group rats (1.70±0.17 nmol/mg protein) than that of the control group (0.68±0.07 nmol/mg protein), and melatonin-treated CIH group (1.10±0.16 nmol/mg protein) (p<0.01, n=8). As an antioxidant, the SOD activity of the hippocampal tissues was significantly lower in the CIH rats (32.29±2.98 U/mg protein) compared with that of the control rats (64.28±5.29 U/mg protein) and melatonin-treated rats (46.92±5.66 U/mg protein) (p<0.01, n=8). Although melatonin intervention had anti-oxidative effect to a certain extent, both MDA and SOD activities could not return to normal levels with statistical differences between the control group and melatonin-treated group (p<0.01).

Melatonin protected hippocampal neuron against apoptosis caused by CIH (Figure 2). Hippocampal neuron apoptosis evaluated by TUNEL was greatly enhanced in rats following CIH procedures than that receiving sham hypoxia and melatonin administration (IOD of control group - 548.33±160.18; IOD of Veh+CIH group - 2004.44±572.97; IOD of MT+CIH group - 810.59±93.73; Con versus Veh+CIH - p<0.001; Veh+CIH versus MT+CIH - p<0.001, n=6-7). Meanwhile, rats treated with melatonin plus CIH exposure showed a significant reduction in the number of TUNEL-positive neural cells compared with those receiving vehicle treatments.

Melatonin treatment up-regulated BCL-2 and down-regulated BAX (Figure 3 & Figure 4). In order to observe the changes in the expressions of anti-apoptotic protein BCL-2 and pro-apoptotic protein BAX in rat's hippocampal neurons, the study detected the BCL-2 positive neurons and BAX positive neurons by immunohistochemistry method together with content of BCL-2 and BAX by Western-blot analysis. Results showed a significant reduction in the BCL-2 positive neurons of rats hippocampus after 4-week duration of IH (IOD: 1095.14±193.78) than sham hypoxia/re-oxygenation procedure (IOD: 4875.33±754.78) (p<0.001, Con - n=6, Veh+CIH - n=7). While melatonin administration would significantly increase the number of BCL-2 positive cells (IOD: 2848±717.77, n=6) (p<0.001). At the same time, there were significant differences in the number of BAX positive neurons among the 3 experimental groups (IOD of control group - 1673±295.97; IOD of Veh+CIH group - 2859.98±301.64; IOD of MT+CIH group - 2085.44±229.76; CIH versus Con & MT+CIH - p<0.001; Con versus MT+CIH - p<0.05; n=6-7). The changing tendency of BCL-2 and BAX protein content in the hippocampus was similar to that of BCL-2 positive neurons and BAX positive neurons (p<0.05, n=3).

Discussion. The present study reveals that a 4-week IH exposure lead to more severe oxidative stress injury of rats' hippocampal neurons, in which antioxidant SOD activity is significantly attenuated and lipid peroxidation production MDA increased. Moreover, the hippocampal neuron apoptosis is significantly enhanced following CIH procedure with BCL-2 expression down-regulated and BAX expression up-regulated. While melatonin treatment could decrease the oxidative stress and neuronal cells apoptosis accompanied by reversed changes in MDA content,

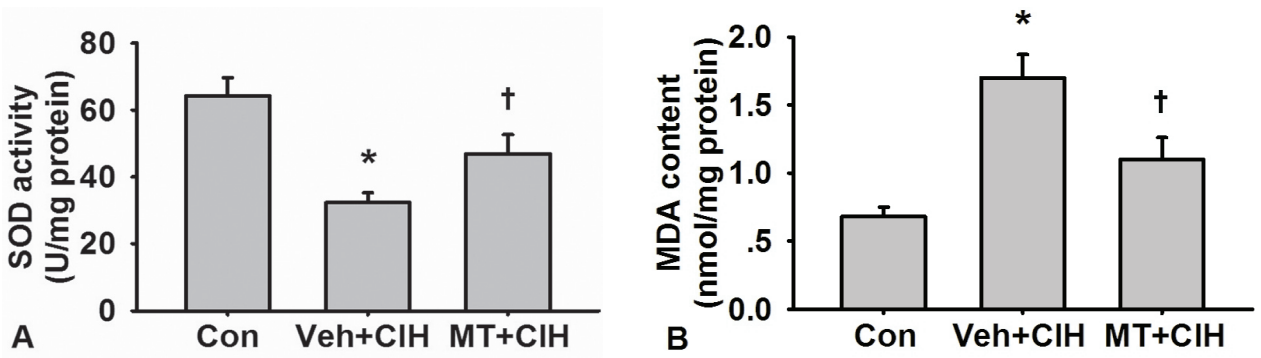

Figure 1 - Chronic intermittent hypoxia (CIH) resulted in a significant decrease in superoxide dismutase (SOD) activity and an increase in malondialdehyde (MDA) activity in hippocampal homogenates, while melatonin treatment could restore the levels of SOD and MDA activities. **A**) Activity of SOD in vehicle-treated CIH group (Veh+CIH) was significantly lower than that of the control group (Con) and melatonin-treated CIH group (MT+CIH). **B**) Content of MDA in CIH group was significantly higher than that of control and MT+CIH group. Results of SOD and MDA activity levels were expressed as units of SOD per milligram of protein (U/mg protein) and nanomole of MDA per milligram of protein (nmol/mg protein). Data were shown as mean ± standard deviation and analyzed by ANOVA (n=8). *p<0.01, versus Con group and MT+CIH group. †p<0.01 versus Con group.

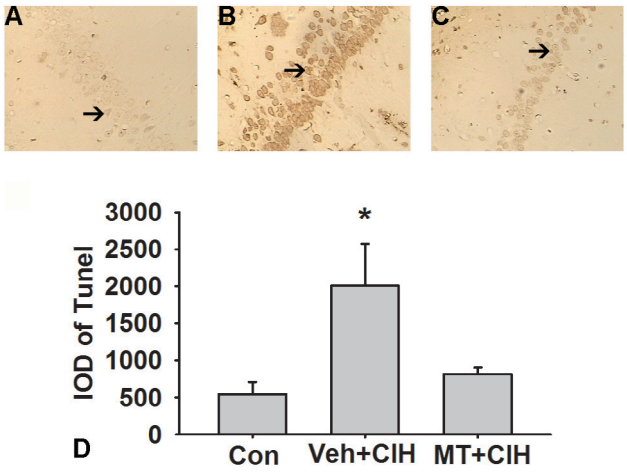

Figure 2 - Neuron apoptosis assessed by terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling (TUNEL) was significantly increased in rats hippocampus following chronic intermittent hypoxia (CIH) procedure, which could be reversed by melatonin treatment (×100). The TUNEL-positive neurons were stained by pale yellow, brown, or dark brown coloration in nuclei (arrows): **A**) the control group (Con); **B**) the vehicle-treated CIH group (Veh+CIH); **C**) the melatonin-treated CIH group (MT+CIH). **D**) Summary data from TUNEL-staining. The TUNEL staining was quantified using Image-Pro Plus software and the rate of apoptosis was expressed by integrated option density (IOD). Data were shown as mean ± standard deviation and analyzed by ANOVA followed by least significant difference (L) test (n=6-7). *p<0.001 versus Con group and MT+CIH group.

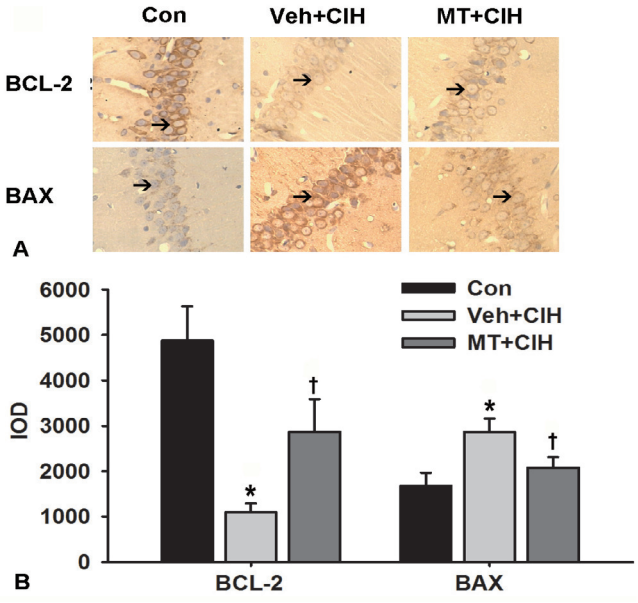

Figure 3 - Immunohistochemical assays of B-cell lymphoma-2 (BCL-2) and BCL-2-associated X protein (BAX) expression in paraffin-embedded sections from rat hippocampus (magnification ×200). **A**) (**Upper**) The BCL-2 expression of hippocampal neurons from control group (Con), vehicle-treated CIH group (Veh+CIH) and melatonin-treated CIH group (MT+CIH). (**Lower**) The BAX expression of hippocampal neurons from control group, vehicle-treated CIH group and melatonin-treated CIH group. The BCL-2 and BAX positive cells were stained by pale yellow, brown, dark brown coloration in cytoplasm (arrows). **B**) Summary data from the immunohistochemical assays of BCL-2 and BAX' The expression levels of BCL-2 and BAX were quantified using Image-Pro Plus software and expressed by integrated option density (IOD). Data were shown as mean ± standard deviation and analyzed by ANOVA followed by least significant difference (L) test (n=6-7). *p<0.001 versus Con group and MT+CIH group. †p<0.05 and p<0.001 versus Con group for BAX and BCL-2 expressions.

SOD activity, BCL-2 expression, and BAX expression levels were induced by CIH. The ability of melatonin to prevent neuronal morphological changes characteristic of apoptosis, such as the shrunken and rounded morphology resulting from condensed chromatin and fragmented DNA, as well as internucleosomal DNA degradation has been observed, and demonstrates that melatonin play a role in the neuroprotection against neurodegenerative diseases related to oxidative stress.[8] Although the neuroprotective effects of melatonin have been recognized, the cellular and molecular mechanisms associated with its role in the anti-apoptosis actions are not fully illuminated.

The results of the study suggest that melatonin can prevent hippocampal neuron apoptosis following repeated hypoxia/re-oxygenation by scavenging reactive oxygen species (ROS), up-regulating expression of anti-apoptotic protein BCL-2 and down-regulating expression of pro-apoptotic protein BAX. These findings are in accordance with those of previous studies,[8,9,11,13,17-21] which relate the anti-apoptotic effects of melatonin to its antioxidant properties and its role in modulating apoptosis-regulatory proteins. Accumulated evidence suggest that melatonin is a direct radical scavenger and can eliminate the hypochlorous acid, hydroxyl radical, peroxynitrite anion, singlet oxygen, and hydrogen peroxide except for its function in alleviating lipids, protein, and DNA oxidation.[11,12] In addition, pre-treatment with melatonin can stimulate the activities of antioxidant enzymes such as glutathione reductase (GRd), SOD, GPx, and glucose-6-phosphase dehydrogenase. It has been proposed that melatonin is more efficient in the oxygen radical elimination process than glutathione or mannitol since it can directly neutralizes the ·OH via a nonenzymatic reaction without involving a membrane receptor.[11] Apart from its various additional effects on anti-oxidative damage via antioxidant enzymes modulation and ROS elimination, melatonin can also reduce radical formation by enhancing Complex I activity to prevent excessive electron leakage as well as down-regulating inducible nitric oxide synthase (iNOS) level. At the

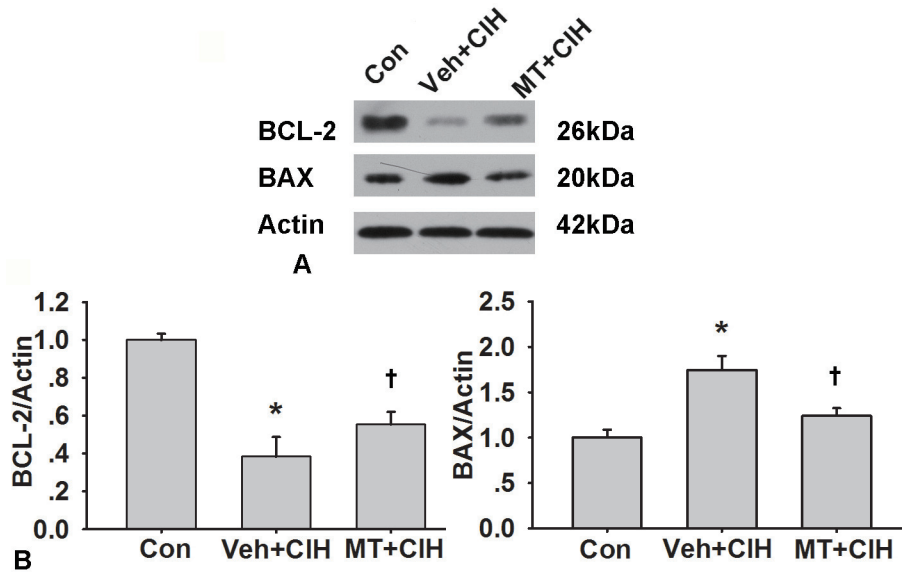

Figure 4 - Chronic intermittent hypoxia (CIH) exposure greatly enhanced the expression of pro-apoptotic B-cell lymphoma-2 (BCL-2)-associated X protein (BAX) and inhibited anti-apoptotic protein BCL-2 expression. Melatonin administration abrogated the increase in BAX expression and restored BCL-2 content to nearly their normal levels. **A**) Protein expression of BCL-2 and BAX analyzed by Western blot. **B**) Summary data from western-blot analysis of BCL-2 and BAX Protein content is normalized to actin and that of control rats is presented as 100%. Values are expressed as means ± standard deviation (n=3). Con - Control group, Veh+CIH - vehicle-treated CIH group, MT+CIH - melatonin-treated. *p<0.05 versus Con group and MT+CIH group . †p<0.05 versus control group. kDa - kiloDalton

same time, melatonin administration would maintain the mitochondrial membrane potential, and thus prevent intracellular cytochrome c release, as well as electron leakage from mitochondria but activate the autophagic process, which possibly contributes to its protective role in degenerative disorders such as AD to Parkinson's disease (PD) involving mitochondrial dysfunction as a primary or secondary cause.[10,22,23] In vivo experiments have demonstrated that melatonin treatment could restore mitochondrial function via enhancing mitochondrial glutathione levels and ATP production by stimulating Complex I and Complex IV, and by inhibition of p38 mitogen-activated protein kinase (MAPK), p53 and GSK-3β phosphorylation.[24] These effects are accompanied by inhibiting the consecutive activation of apoptotic pathway via stimulating the mRNA levels of anti-apoptotic protein BCL-2, diminishing caspase-3 and caspase-9 activity, and reducing poly-ADP-ribose polymerase (PARP) activation. Still, melatonin pre-incubation is able to inhibit the mRNA level of pro-apoptotic protein BAX, as well as NF-κB activation, the latter participating in the regulation of early response genes involved in redox status and subsequent oxidative damage.[17-19] Recent findings have shown that melatonin can activate Nrf2-ARE pathway to reduce oxidative stress damage and decrease Ca^{2+} influx through transient receptor potential melastatin-like 2 (TRPM2) channels activated by H_2O_2, thus inhibit apoptosis.[20,21] Numerous studies have linked melatonin to its function of anti-oxidative injury via the amelioration of mitochondrial dysfunction and anti-apoptotic effects. However, the exact signaling pathways in the neuroprotective functions of melatonin during CIH exposure remain largely unclear and further investigations need to be conducted.

The OSAS is characterized by repeated hypoxia/re-oxygenation, which acts as the main trigger for multiple complications. Animal models of CIH have been developed to mimic the pattern of IH throughout the night experienced by patients with OSAS. Generally, animal models are induced by changing the inspired gas from a 20-60 seconds period of hypoxia of 3-10% fraction of inspired oxygen (F_{IO2}) to 45-90 seconds period of normoxia, which continues for 5-8 hour per day over 14-35 days for CIH.[25] Previous research have demonstrated that the oscillations of the oxygen concentration resulting from IH simulate the multiple cycles of ischemia/reperfusion, and therefore increase ROS production leading to enhanced oxidative stress.[26] Oxidative damage is believed to be associated with a variety of neurodegenerative disorders ranging from AD to PD to Huntington's disease (HD) to amyotrophic lateral sclerosis (ALS).[27] Excess amount of ROS is harmful and can directly damage a variety of critical biomolecules, including proteins, lipids, carbohydrates and DNA,[27,28] and lipids are the biological targets, which

are most prone to oxidation. The MDA, recognized as a maker of lipid oxidation, has been involved in multiple pathological contexts, such as cancer etiology, pre-eclampsia, diabetes, cardiovascular diseases and even Alzheimer's type dementia. Its levels in the hippocampus of CIH rats can reflect the degree of lipid peroxidation caused by free radicals.[29] Meanwhile, the antioxidant capacities decrease as suggested by the diminished activity of SOD, which is thought to provide the first line of defense against oxidative damage to DNA, and can directly remove oxygen free radicals by converting them to hydrogen peroxide subsequently reducing to water.[30] In addition, ROS act as signaling molecules to control numerous physiological and pathological processes including programmed cell death.[28] When ROS are produced beyond the mitochondrial antioxidant capacity, the mitochondrial permeability transition (MPT) pore located on the outer membrane opens leading to the permeability of the inner mitochondrial membrane. Thereafter, several pro-apoptotic factors, such as cytochrome c is released to trigger the formation of apoptosome and the consequent apoptosis process. Moreover, BAX and MPT may work together to cause the release of cytochrome c.[27]

Apoptosis, also known as programmed cell death, is essential for the maintenance of cellular homeostasis whose deregulation will lead to cell loss accompanied by various pathologic disorders, particularly the neurodegenerative diseases.[31,32] It is an intrinsic cellular process, in which the cells kill themselves in a controlled manner. A variety of signal transduction pathways involve in the apoptosis process, of which BCL-2 family related apoptotic pathway plays a central role.[33] Currently, proteins of BCL-2 family are classified into 2 main categories, namely anti-apoptotic members (BCL-2, Bcl_{-xL}, A1, Bcl-w, Mcl-1, and Boo) and pro-apoptotic members (BAX, BAK, and BOX). The relative ratio of anti-apoptotic protein BCL-2 and pro-apoptotic protein BAX seems to determine the sensitivity or resistance of cell to apoptotic stimuli. If BCL-2 or Bcl_{-xL} fails to exert its anti-apoptotic property, BAX oligomerization takes place and apoptosis accelerates.[32] The BCL-2 family proteins participate in the modulation and execution of cell death and can preserve or disrupt mitochondrial integrity. Once activated cytosolic BAX translocates to outer mitochondrial membrane in response to death stimuli, promoting the release of pro-apoptotic regulators such as cytochrome c, second mitochondrial activator of caspases/Direct IAP protein with low pI (SMAC/DIABLO), HtrA2/Omi, endonuclease G and apoptosis-inducing factors (AIF). In addition, pathways through post-translational modifications of BCL-2 family proteins including phosphorylation and caspase-mediated proteolysis also contribute to cell apoptosis initiation.[32-34] Moreover, BCL-2 has been considered to have antioxidant effects, while BAX has the ability to trigger ROS generation.[28,35] Therefore, the anti-apoptotic and pro-apoptotic functions of BCL-2 family proteins correlated with their capacities to maintain redox homeostasis play a significant role in regulating cell death. Consistently, the present data also show that the balance between BCL-2 and BAX expressions performs a critical role in the regulation of hippocampal neuron apoptosis. Moreover the up-regulation of BCL-2 level and down-regulation of BAX level induced by melatonin treatment is quite associated with a decrease in neuron apoptosis together with oxidative stress observed in CIH exposure. Thus ,we conclude that melatonin can protect neurons against CIH-induced apoptosis via its anti-apoptotic effects of regulating apoptosis-related genes expressions, which might be closely related to its role of anti-oxidative damage.

One limitation of the present research is that we did not investigate how BCL-2 protein family and oxidative stress interacts with each other during CIH-induced hippocampal neuron apoptosis. Since BCL-2 is supposed to have anti-oxidative property either by its ability to inhibit ROS production or by direct clearance of ROS,[28,35] and ROS can cause and regulate cell apoptosis by modulating BCL-2 family proteins,[36] thus, further studies should be conducted to explore the interaction between BCL-2 family protein and oxidative stress during CIH exposure, and to elucidate the exact mechanisms associated with melatonin neuroprotective function against CIH-associated cell apoptosis.

In conclusion, CIH exposure could enhance apoptosis of hippocampal neurons. Whereas melatonin treatment would greatly attenuate oxidative stress injury and activate anti-apoptotic signal pathways, thus reduce the number of hippocampal apoptotic neurons. Since melatonin has neuroprotective effects in animal models of OSAS and is relatively nontoxic, it could be a candidate agent in the anti-oxidative treatment to protect neurons against CIH-induced increased amount of ROS. Recent studies have shown that melatonin derivatives also have remarkable protecting effect on synaptic transmission failure during hypoxia/reperfusion.[37] It is worth mentioning that the neuroprotective roles of melatonin in PD and AD have been documented and melatonin, as well as melatonin receptor agonists is suggested to be useful in preventing or delaying the disease initiation and progression.[7,8,10] Therefore, melatonin itself or its analogs should deserve more attention concerning their neuroprotective effects in CIH-associated oxidative stress damage, and may

provide an effective means of prevention and treatment of memory and cognitive impairment caused by OSAS.

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