

Effect of intermittent hypoxia on rat INS-1 cells and the protective effect of melatonin

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Abstract. – OBJECTIVES: This study aimed to observe the influence of intermittent hypoxia on rat INS-1 cells and the protective effect of melatonin (MT).

MATERIALS AND METHODS: Intermittent hypoxia condition was induced in rat INS-1 cells. The supernatants were used to detect oxidative stress indicators, and the cells were used to detect JNK1 mRNA and JNK1/2 protein. After different dose-dependent interventions of MT, the cells were harvested to observe corresponding oxidative stress indicators and JNK1/2 protein change.

RESULTS: With prolonged exposure time, malondialdehyde (MDA) increased in cultured supernatants whereas superoxide dismutase (SOD) activity decreased. Cells with intermittent hypoxia showed significantly increased JNK1 mRNA expression, whereas phosphorylated JNK1 was highly expressed on the third day. With increased MT dose, MDA in cultured supernatants decreased whereas SOD activity increased. In the group dosed with 100 μ M MT, phosphorylated JNK1 protein expression significantly decreased.

CONCLUSIONS: Intermittent hypoxia can cause oxidative damage to INS-1 cells possibly by increasing the JNK1 transcription level and protein activation. A high dose of MT (100 μ M) can protect INS-1 cells from oxidative damage induced by intermittent hypoxia.

Key Words:

Obstructive sleep apnea hypopnea syndrome, Intermittent hypoxia, Oxidative stress, Pancreatic β cells, Melatonin.

Abbreviations

MT = Melatonin; IH = Intermittent hypoxia; OSAS = Obstructive sleep apnea syndrome; INS-1 cells = Insulinoma cell line; JNK $\frac{1}{2}$ = c-Jun N-terminal kinase $\frac{1}{2}$; DMSO = Dimethyl sulfoxide; PDX-1 = Pancreatic and duodenal homeobox 1 (also known as insulin promoter factor 1); RIPA = Radio Immunoprecipitation Assay; BCA = Bicinchoninic acid; ECL = Enhanced chemiluminescence; UVP = Ultra-violet products; MnSOD = Manganese superoxide dismutase; GPX1 = glutathione peroxidase 1.

Introduction

A large number of epidemiological and clinical studies¹ have confirmed that obstructive sleep apnea syndrome (OSAS) is closely related to type-2 diabetes, and this relationship is independent of confounding factors such as obesity and family history. OSAS causes and aggravates type-2 diabetes, and the mechanisms are enhanced by sympathetic activity, intermittent hypoxia (IH), hypothalamic-pituitary-adrenal dysfunction, systemic inflammatory response, adipocytokine change, and sleep deprivation. Thus, the development of type-2 diabetes from OSAS is a complex process involving various factors.

During sleep, the repeated upper airway collapse in OSAS patients can cause serious recurrent apnea, leading to characteristic IH. Thus, patients are exposed to an alternation of low oxygen tension and normal oxygen tension. IH is similar to the oxygen abnormality in the ischemic/reperfusion process and can initiate the oxidative stress. At present, OSAS is considered an oxidative stress-induced disease².

In the field of endocrine disease research, oxidative stress has been proven to be an important mechanism of insulin resistance, β -cell dysfunction, and diabetes triggering³. Insulin resistance and β -cell dysfunction are the two main pathophysiological defects in type-2 diabetes mellitus. Recent studies have confirmed that IH can cause insulin resistance independent of autonomic nervous system excitement and obesity factors⁴. However, the effect of IH on β -cell dysfunction is seldom reported.

Many antioxidants can reduce oxidative damage to the body. Melatonin (MT) is one of the major hormones synthesised and secreted in the pineal gland in mammals. In addition to regulat-

ing the circadian rhythm, sleep, and incretion, MT has an anti-aging function. The physiological concentration of MT can scavenge active oxygen in the body, exhibiting antioxidant effect. Research shows that MT can affect the *in vitro* and *in vivo* secretion of insulin in pancreatic β -cells, as well as reduce metabolic disorder and oxidative stress in diabetes⁵.

Our previous studies⁶ have suggested that IH can cause oxidative damage to mouse pancreatic tissue, and MT can prevent oxidative stress caused by IH. As a characteristic of OSAS, IH also induces pathophysiological changes as initiating factors, such as enhanced sympathetic activity⁷ and hypothalamic-pituitary-adrenal dysfunction⁸. However, for IH-induced oxidative damage to islet cells, the direct role of IH or its indirect effects such as functional changes of the sympathetic nervous system or HPA axis that affect islet cell function is unknown. To eliminate the indirect effect of the complex nerve-endocrine network, the direct effect of IH on rat INS-1 cells must be determined to observe the impact on β -cells and the protective effects of MT, as well as to explore the underlying mechanisms.

Materials and Methods

Cell Culture and Treatment

INS-1 cells (provided by the China Center for Type Culture Collection, Wuhan, China) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS (fetal bovine serum) (Gibco BRL, Life Technologies, London, UK), 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 50 μ mol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μ g/mL streptomycin) at 37°C in a 5% CO₂ incubator for conventional culture.

The programme in the IH chamber (Oxycycler Model C42; BioSpherix Instruments, Redfield, NY, USA) was as follows: 10% oxygen for 30 s, 21% oxygen for 60 s, and 260 s for each run, 14 times per hour, 8 hours a day for 3 days at 5% CO₂ (Figure 1).

INS-1 cells were digested by 0.25% trypsin, centrifuged, and then suspended in RPMI-1640 culture medium. The cell suspension (2×10^5 /ml) was seeded onto a 60 mm² plate for 12 h of culture, and the medium was changed after 12 h to remove residual trypsin. After 24 h, the plates were placed in an intermittent hypoxic chamber.

IH

Normoxic (CON; 1, 2, and 3 days) and IH (1, 2, and 3 days) groups included three plates of cells ($n = 3$). The CON group was cultured under normoxic conditions, whereas the IH group was placed in an IH chamber 8 h/day. After daily exposure, the cell culture supernatants were collected to detect superoxide dismutase (SOD) activity and malondialdehyde (MDA) content and maintained at -70°C. Fresh medium was then added. After exposure for 1, 2, and 3 days, the cells were collected to measure JNK1 mRNA, phosphorylated JNK1/2, and total JNK1/2 protein expression and then frozen at -70°C.

Blocking of JNK Protein Activity

The samples for the blocking of JNK protein activity were grouped as follows: CON+0.1% dimethylsulfoxide (DMSO), IH+0.1% DMSO, and Sp600125 (Sigma-Aldrich Corp., Saint Louis, MO, USA) 10 μ M ($n = 3$). The CON group was cultured under normoxic conditions, whereas the IH and SP600125 groups were placed in the IH chamber for 8 h/day. After daily exposure, the medium was changed. Three days later, the cells were collected to detect PDX-1 mRNA and protein expression.

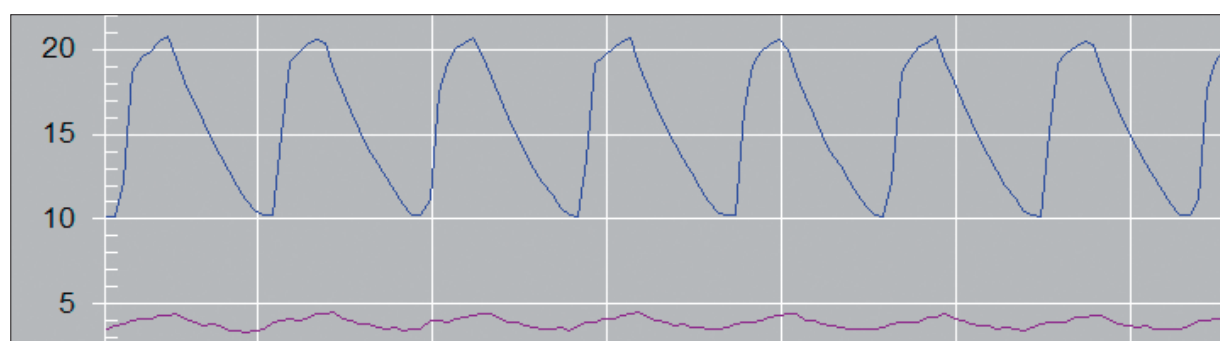


Figure 1. The oxygen concentration-time curve diagram in cell intermittent hypoxic cabin (blue curve was O₂ concentration changes, the purple curve was CO₂ concentration changes).

Effect of MT

The samples for the determination of the effect of MT were grouped as follows: CON+0.1% DMSO, IH+0.1% DMSO, MT (M7250, Sigma-Aldrich Corp., Saint Louis, MO, USA) 10 nM, MT 1 μ M, MT 100 μ M, and NAC (A7250, Sigma-Aldrich Corp., St Louis, MO, USA) 100 μ M ($n = 3$).

The CON group was cultured under normoxic conditions, whereas the IH, MT, and NAC groups were placed in the IH chamber 8 h/day. After daily exposure, the medium was changed. Three days later, the cultured supernatants were collected to detect oxidative stress indicators, and cells were harvested for the detection of JNK1/2 protein.

Oxidative Stress Indicators

The cell culture supernatants were taken for measurements of total superoxide dismutase (SOD) (T-SOD) and MDA by colorimetric determination (Jiangcheng Biotechnology, Nanjing, China).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was used to detect the JNK1 mRNA and PDX-1 mRNA levels. A sequence of the specific primers used were as follows: β -actin (Gene ID: 81822), forward primer (5'-TGTCACCAACTGGGACGATA-3'), reverse primer (5'-ACCCTCATAGATGGG

CACAG-3'), 181 bp; JNK (Gene, ID: 116554), forward primer (5'-TGATGACGCCTTACGTGGTA-3'), reverse primer (5'-GGCAAACCATTCTCCATA-3'), 120 bp; and PDX-1 (Gene ID: 29535), forward primer (5'-AAACGCCACACACAAGGAGAA-3'), reverse primer (5'-AGACCTGGCGGTTCA-CATG-3'), 150 bp. The total RNA of the cells in each group was extracted by PrimeScript RT Reagent Kit with gDNA Eraser (DRR047, Takara Biotechnology Co., Ltd., Dalian, China), and first-strand cDNA was synthesised. SYBR Premix Ex Taq II (DRR081, Takara Biotechnology Co., Ltd., Dalian, China) was added for amplification. The reaction system (G6000 quantitative PCR instrument) had a volume of 20 μ l. The PCR programme was as follows: initial denaturation at 95°C for 30 s, denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 20 s for a total of 45 cycles. The qPCR results were automatically analysed with a fluorescence quantitative instrument and recorded as CT values. The amount of gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Western Blot Analysis

The stimulated cells were collected from the freezer, and 50 μ l of Radio Immunoprecipitation Assay (RIPA) lysate was added. Ice cracking lasted for 10 min, and $12\ 000 \times g$ was centrifuged for 3 min to 5 min. The supernatants were used to detect protein concentration using a Bicinchoninic Acid (BCA) kit. Then, 80 μ g protein was taken for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The concentration of the separation gel was 12%, and the concentration of the stacking gel was 5%. The proteins were transferred from the gel onto a Polyvinylidene fluoride (PVDF) membrane with the BIO RAD system (Hercules, CA, USA). Skim milk was used for blocking. The primary antibody (1:1000; p-JNK antibody, #9251, Cell Signaling Technology, Inc., Danvers, MA, USA) was added at 4°C for overnight incubation. The secondary antibody (1:25,000) was added for 2 h of incubation at room temperature. After ECL-PLUS, UVP scan imaging was used to calculate the quantitative optical density values.

Given that phosphorylated protein and total protein were at the same location, strip buffer (15 g glycine, 1 g SDS, and 10 ml Tween 20 in a total volume of 1000 ml; pH 2.2) was used to wash the p-JNK antibody. After reclosure, a new primary antibody, t-JNK antibody (1:1000; #9252, Cell Signaling Technology, Inc., Danvers, MA, USA), was added at 4°C for overnight incubation followed by the addition of a secondary antibody and ECL-PLUS scan. Finally, a strip solution was used to wash the t-JNK antibody, and β -actin was detected with β -actin Antibody (Zhongshan Golden Bridge Biological Engineering Co., Ltd.). Given that the β -actin molecular weight is 42 kDa and that of JNK1 is 46 kDa, the values were too close and thus easily lost during cutting, so duplicate detection was conducted after washing. PDX-1 protein detection with PDX-1 antibody (# 2437, Cell Signaling Technology, Inc., Danvers, MA, USA) was repeated using the above steps. The molecular weight was also 42 kDa; thus, duplicate detection was conducted after washing.

Statistical Analysis

All data were analysed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were expressed as the mean \pm standard deviation (SD). The differences among the various indicators in the groups were analysed by non-parametric single factor ANOVA, with $p \leq 0.05$ as the statistically significant level.

Results

Effects of IH on Oxidative Stress Indicators

MDA in cell culture supernatants with different time programmes showed no significant change ($p > 0.05$) (Figure 2A). The IH group compared with the CON group under the same time programmes showed no statistically significant difference ($p < 0.01$). With prolonged IH exposure time, the MDA content progressively increased in the IH group, and the difference of the 1 day group from the 2 and 3 day groups was significant ($p < 0.01$). However, no significant difference was found between the 2 and 3 day groups ($p > 0.05$).

Under a normoxia culture, supernatant SOD activity had no significant difference in different periods ($p > 0.05$) (Figure 2B). The IH group compared with the CON group in the corresponding period showed a significant difference ($p < 0.01$). With prolonged exposure time, SOD

vitality progressively decreased in the IH group and the groups showed significant differences ($p < 0.01$).

Effects of IH on JNK1

CON groups with different time programmes showed no significant changes in JNK1 mRNA (Figure 3A). In the corresponding time program, JNK1 mRNA expression increased in the IH group ($p < 0.05$). IH groups with different time programmes exhibited decreased JNK1 mRNA expression with extended time. The IH 3 day and IH 1 day groups showed a significant difference ($p < 0.05$).

The CON 1 day and 2 day groups were compared with the IH1 day and 2 day groups (Figures 3B and 3C). For t-JNK1/ β -actin, t-JNK2/ β -actin, p-JNK2/t-JNK2, and p-JNK1/t-JNK1, no significant differences were found among the groups. Between the CON 3 day and IH 3 day groups, only p-JNK1/t-JNK1 showed a significant difference ($p < 0.05$).

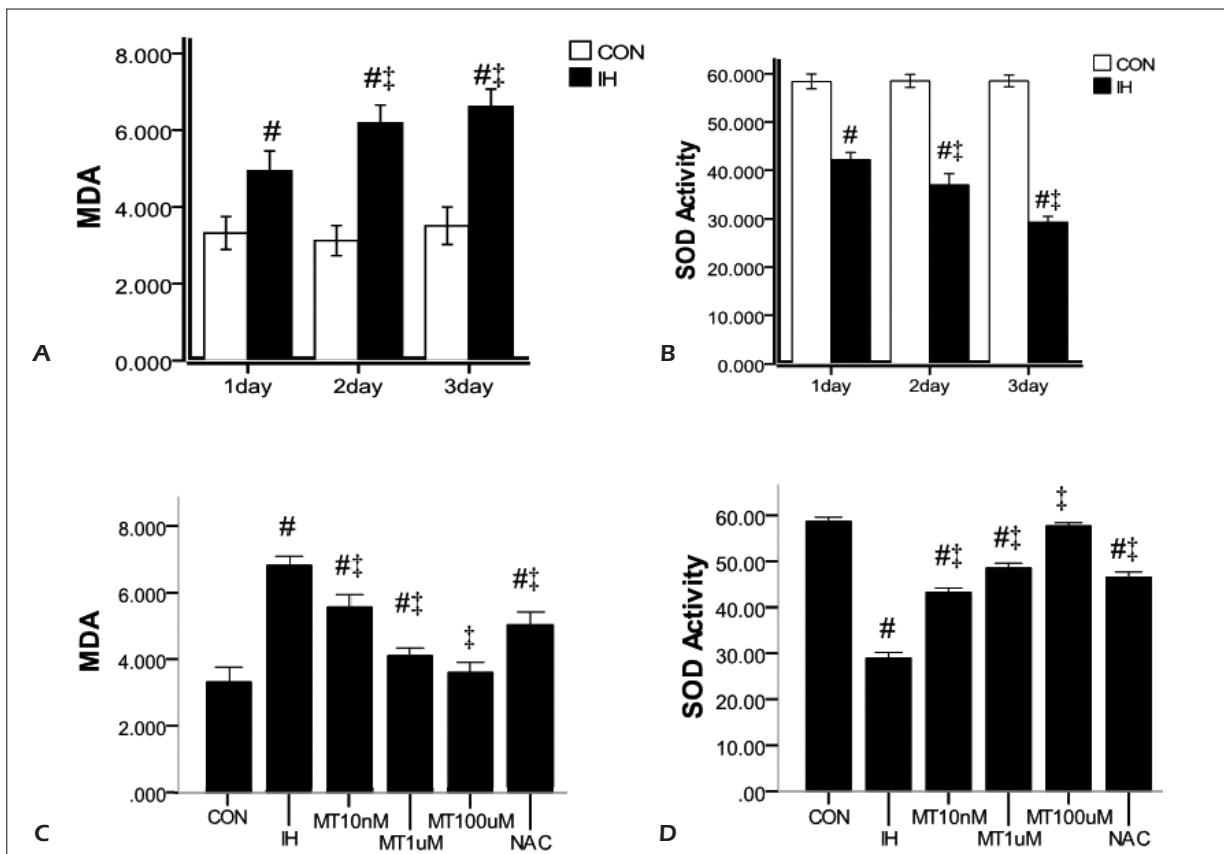


Figure 2. A-B, The different impacts of intermittent hypoxia with different time programs on MDA, SOD in INS-1 cell culture supernatants, IH and CON group, * $p < 0.05$, # $p < 0.01$; compared with IH1 day group, † $p < 0.05$, ‡ $p < 0.01$. C-D, The effects of antioxidant intervention on MDA and SOD vitality in INS-1 cells cultured supernatants under intermittent hypoxia conditions, compared with CON group, * $p < 0.05$, # $p < 0.01$; compared with IH group, † $p < 0.05$, ‡ $p < 0.01$.

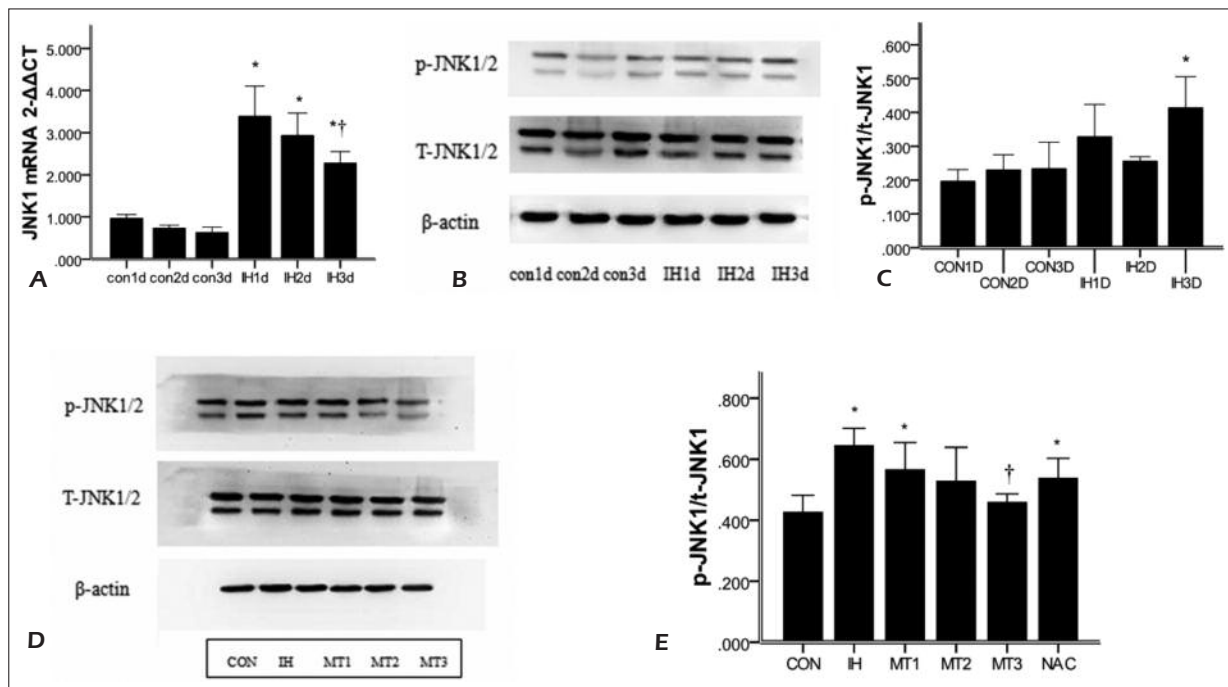


Figure 3. **A**, The different impacts of intermittent hypoxia with different time programs on JNK1 mRNA of INS-1 cells, IH and CON group, * $p < 0.05$, # $p < 0.01$; compared with IH1 day group, † $p < 0.05$, ‡ $p < 0.01$. **B-C**, The different impacts of intermittent hypoxia with different time programs on JNK protein of INS-1 cells, IH and CON group, * $p < 0.05$, # $p < 0.01$; compared with IH1 day group, † $p < 0.05$, ‡ $p < 0.01$. **D-E**, The impacts of anti-oxidant intervention on phosphorylated JNK1 in INS-1 cells under intermittent hypoxia conditions, compared with CON group, * $p < 0.05$, # $p < 0.01$; comparison with IH group, † $p < 0.05$, ‡ $p < 0.01$.

PDX-1

In the PDX-1 mRNA comparisons among groups (Figure 4A), the expression of PDX-1 mRNA in the IH group was significantly lower than that in the CON group ($p < 0.05$). After the blocking of JNK protein activity (SP600125), PDX-1 mRNA significantly increased compared with the IH group ($p < 0.05$).

The comparison of PDX-1 protein among groups (Figures 4B and 4C) showed that among

the IH, CON, and SP600125 treatment groups, PDX-1 protein expression of the IH group significantly decreased ($p < 0.05$), but no difference was observed between the CON and SP600125 treatment groups.

Effects of Antioxidant on Oxidative Stress Indicators

MDA in the cultured supernatants decreased with increased MT dose (Figure 2C). Compared

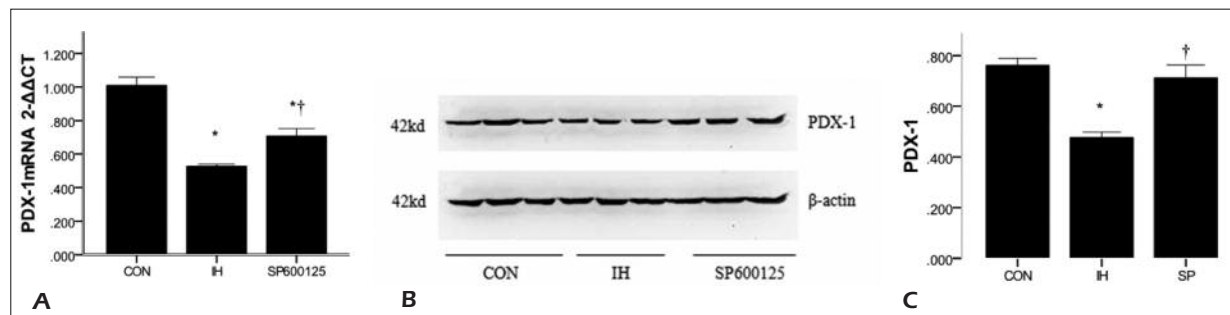


Figure 4. **A**, After the blocking of JNK protein activity the impacts of PDX-1mRNA in INS-1 cells under intermittent hypoxia conditions. **B-C**, After the blocking of JNK protein activity the impacts of PDX-1 protein in INS-1 cells under intermittent hypoxia conditions. Compared with CON group, * $p < 0.05$, # $p < 0.01$; compared with IH group, † $p < 0.05$, ‡ $p < 0.01$.

with IH, MT and NAC reduced MDA in culture supernatants ($p < 0.01$). In the MT 100 μM group, MDA was reduced to the level of the CON group ($p > 0.05$). With increased MT dose, SOD activity in cultured supernatants increased (Figure 2D). Compared with the IH group, MT and NAC increased the SOD activity ($p < 0.01$). In the MT 100 μM group, SOD activity was enhanced to the level of the CON group ($p > 0.05$).

Effects of Antioxidant on Phosphorylated JNK1

Non-parametric test statistical analysis of data showed significant differences between the CON and IH/MT 10 nM/NAC groups ($p < 0.05$) (Figures 3D and 3E). The IH and CON/MT 100 μM groups showed significant differences ($p < 0.05$). In the MT 100 μM group, phosphorylated JNK1 expression significantly decreased, and no significant difference was found in the CON group. Moreover, no difference was found between the NAC and IH groups ($p > 0.05$).

Discussion

IH is a characteristic feature of OSAS. Recurrent IH can lead to abnormal oxygen supply similar to ischemia/reperfusion. Excessive reactive oxygen triggers the body's oxidative stress. Thus, OSAS is also an oxidative stress disorder² and a multi-systemic disease⁹.

Oxidative stress-induced pancreatic β -cell damage is an important mechanism of diabetes mellitus. Studies have shown that the content of the antioxidant enzyme system is less in islet β -cells than in other cells, making them vulnerable to oxidative damage to easily become apoptotic cells¹⁰. Moreover, many free radicals induce β -cell injury and diabetes¹¹. OSAS, being an oxidative stress disease, may be a necessary link between chronic IH and β -cell damage.

In this study, rat INS-1 cells were cultured *in vitro*, and the change in β -cell function caused by IH (single factor) was observed. Results indicated that MDA in cell culture supernatants was higher in the IH group, and SOD activity significantly decreased. A progressive increase in oxidative damage appeared over time. IH also induced enhanced excitability of sympathetic nerve, hypothalamic-pituitary-adrenal axis changes, and systemic inflammatory response, which were excluded. By contrast, the single factor (IH) can cause oxidative damage to cells.

This finding was consistent with the result of a recent study¹² that IH can significantly reduce the secretion of insulin in pancreatic β -cells and isolated islets. The JNK1 mRNA and protein detection of cells with different IH time programmes showed that IH can enhance JNK1 mRNA expression and phosphorylated JNK1 proteins compared with the normal control group. JNK belongs to mitogen activated protein kinase family and, thus, plays an important role in cell proliferation, differentiation, development, and apoptosis. IH-induced oxidative stress can induce neuronal apoptosis in the hippocampus by activating JNK¹³.

Xu et al¹⁴ found that IH can induce the apoptosis of β -cells. The oxidative stress induced by JNK activation can reduce insulin gene expression, whereas the inhibition of the JNK pathway can protect islet β -cells from oxidative stress injury¹⁵. JNK is expressed in the cytoplasm under normal circumstances. When high glucose and hyperlipidemia-induced oxidative stress stimulates JNK, it transfers to the nucleus for activation to weaken PDX-1-DNA binding activity and insulin gene transcription, and then it reduces PDX-1 expression. These insulin synthesis disorders affect the function of islet cells¹⁶. Research¹⁷ has shown that the islet cells of JNK1-deficient mice after glucose-stimulation secrete more insulin than normal mice and JNK2 gene-deficient mice and were more resistance to cytokine-induced apoptosis. This finding indicated that JNK1 plays an important role in the process of islet cell dysfunction, whereas JNK1 antisense oligonucleotide inhibits phosphorylated JNK1 expression and β -cell apoptosis¹⁸. Thus, a close relationship exists between JNK1 and islet cell dysfunction.

According to our experimental results, IH stimulated cells to produce oxidative stress, thereby activating JNK1-mediated cell dysfunction. To test this hypothesis, SP600125 was given under IH to block the activity of JNK protein. Subsequently, PDX-1 mRNA and proteins were detected. We found that in the IH group with no blocking factor PDX-1, mRNA and protein expression in INS-1 cells decreased, whereas in the group with blocking factors PDX-1, the mRNA level slightly recovered. Compared with the IH group, IH-induced inhibition significantly affected protein expression. All the above findings indicated that phosphorylated JNK1 proteins exerted their effect through protein-protein interactions.

However, SP600125 is a non-specific inhibitor of JNK. SP600125 can inhibit JNK1/2/3, and specific JNK1 inhibitors can prove the role of JNK1 in IH. In our next study, siRNA-mediated RNAi technology will be used to suppress JNK1 gene to further clarify the role of JNK1 in IH-induced oxidative stress.

The relationship between MT and type-2 diabetes have been the subject of much debate in the field of endocrine research¹⁹. Some researchers have concluded that MT can inhibit insulin secretion, whereas other scholars have proven that MT secretion decreases under the diabetic state²⁰. Kemp et al²¹ suggested that the overnight co-incubation of MT and INS-1 cells can cause increased insulin secretion, although different viewpoints exist. MT, as a free radical scavenger, has a certain protective role in alloxan and streptozotocin-induced diabetes²². Endogenous MT, the main secretory product of the pineal gland, participates in many physiological functions due to its efficacy as a free radical scavenger and indirect antioxidant. The radical scavenging ability of MT is four times that of glutathione, 14 times that of mannitol, and two times that of vitamin E. Exogenous MT exhibited protective action against cadmium induced oxidative stress related neurotoxicity in rats²³, and it could decrease the adriamycin-induced oxidative stress and cardiotoxicity²⁴. The MT secretion pattern of OSAS patients is abnormal, and no nocturnal peak secretion is observed²⁵. In the afternoon, MT level is high, which is the reason for the daytime sleepiness of OSAS patients. MT can increase the transcription of antioxidant enzymes²⁶ and reduce the generation of β -amyloid peptide²⁷ to weaken IH-induced rat hippocampal damage, reduce IH-induced microvascular injury, and insulin resistance²⁸.

In this study, INS-1 cells were disrupted by different doses of MT (10 nM, 1 μ M, and 100 μ M) to observe the protective effect of MT on INS-1 cells under IH. The results showed that MDA in cultured supernatants decreased with increased MT, SOD activity increased in the MT 100 μ M group, and MDA decreased but SOD increased to a considerable level in the CON group. The phosphorylated JNK1 level in the MT 100 μ M group significantly decreased compared with the IH group, but no significant difference from the CON group existed. NAC can also reduce the expression of phosphorylated proteins, but no significant difference among the IH groups was found.

The preliminary findings of our group⁶ have also confirmed that MT can enhance MnSOD and GPX1 mRNA expression in mouse pancreatic tissue under IH, which reflected the radical scavenging capacity of IH and its role in enhancing the antioxidant enzyme activity. This report demonstrated that MT can suppress IH-induced phosphorylated JNK1 protein expression.

In addition, given the various physiological functions of MT such as the regulation of circadian rhythms, sleep, and endocrine, as well as anti-aging, we removed these physiological roles in the body. The simple antioxidant and its protective effect on IH-induced β -cellular oxidative damage were observed *in vitro*. The results were consistent with our expectation that MT can directly protect cell function through the antioxidant effect. Therefore, IH (single factor) can increase the MDA content in cell culture supernatants, decrease SOD activity, and increase the expression of JNK1 mRNA and phosphorylated proteins in INS-1 cells. Intervention with MT can mitigate oxidative damage, which verified the direct oxidative damage of IH on cells and the antioxidant effect of MT. This finding was consistent with the result of previous studies^{26,27} that MT can be used as an antioxidant to protect the hippocampus.

Conclusions

IH may directly result in the oxidative injury of INS-1 cells. With prolonged exposure time, this injury was aggravated. Oxidative stress resulted from the induction of JNK1 transcription and protein activation. The blocking of JNK protein activity mitigated the IH-induced inhibition of PDX-1 mRNA and protein expression. MT can reduce IH-induced oxidative damage to INS-1 cells, which may be achieved by inhibiting JNK1 transcription and protein activation.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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