

ORIGINAL ARTICLE

Adiponectin Ameliorated Pancreatic Islet Injury Induced by Chronic Intermittent Hypoxia through Inhibiting the Imbalance in Mitochondrial Fusion and Division

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Key words: obstructive sleep apnea hypopnea syndrome; chronic intermittent hypoxia; adiponectin; mitochondrial fusion and division; pancreatic islet

Objective This study aimed to assess the protective value of adiponectin (APN) in pancreatic islet injury induced by chronic intermittent hypoxia (CIH).

Methods Sixty rats were randomly divided into three groups: normal control (NC) group, CIH group, and CIH with APN supplement (CIH+APN) group. After 5 weeks of CIH exposure, we conducted oral glucose tolerance tests (OGTT) and insulin released test (IRT), examined and compared the adenosine triphosphate (ATP) levels, mitochondrial membrane potential (MMP) levels, reactive oxygen species (ROS) levels, enzymes gene expression levels of *Ant1*, *Cs*, *Hmox1*, and *Cox4i1* which represented mitochondrial tricarboxylic acid cycle function, the protein and gene expression levels of DRP1, FIS1, MFN1, and OPA1 which represented mitochondrial fusion and division, and the protein expression levels of BAX, BCL-2, cleaved Caspase-3, and cleaved PARP which represented mitochondrial associated apoptosis pathway of pancreatic islet.

Results OGTT and IRT showed blood glucose and insulin levels had no differences among the NC, CIH and CIH+APN groups (both $P>0.05$) at 0 min, 20 min, 30 min, 60 min, 120 min. However, we found that compared to NC group, CIH increased the ROS level, reduced ATP level and MMP level. The islets of CIH exposed rats showed reduced gene expression levels of *Ant1*, *Cs*, *Hmox1*, and *Cox4i1*, decreased protein and gene expression levels of MFN1 and OPA1, increased protein and gene expression levels of DRP1 and FIS1, increased protein expression levels of cleaved Caspase-3 and cleaved PARP, with lower ratio of BCL-2/BAX at protein expression level. All the differences among three groups were statistically significant. APN treated CIH rats showed mitigated

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changes in the above measurements associated with islet injuries.

Conclusion APN may ameliorate the pancreatic islet injury induced by CIH via inhibiting the imbalance in mitochondrial fusion and division.

OBSTRUCTIVE sleep apnea hypopnea syndrome (OSAHS), as a common clinical disease, has a morbidity of 2% – 25% in China.^[1] Chronic intermittent hypoxia (CIH) is the most important pathophysiological characteristic and also a major pathogenetic factor of OSAHS.^[2] OSAHS is associated with cardiovascular diseases and diabetes.^[3-5] Saad *et al.* reported higher prevalence of OSA in Jordanian patients with type 2 diabetes mellitus (T2DM) than in the general population.^[6] Sherwain *et al.* found CIH induced the pancreatic islet β cell dysfunction in diabetic mice.^[7] Wang *et al.* reported that CIH induced inflammation and led to pancreatic cell apoptosis in rat.^[8] However, the mechanisms of which CIH causes pancreatic tissue injury need to be further investigated.

Adiponectin (APN) is a cytokine secreted by adipose tissue, which has protective roles *in vivo*, including anti-inflammation, anti-insulin-resistance, and anti-hypertension.^[9,10] Experiments *in vitro* and *in vivo* have shown that the plasma APN levels decrease in OSAHS patients, and APN protects islet cell against apoptosis.^[11-13] However, further experiments are needed to determine the exact protective mechanisms of APN on pancreatic islets. In this study, we assess the mechanism of pancreatic islet injury induced by CIH, and explore the potential protective effects of APN using CIH animal model.

MATERIALS AND METHODS

Animals and protocol

This study was approved by the Experimental Animal Ethics Committee of Southeast University. The performances followed the regulations of experimental animals. A total of 60 male adult Sprague Dawley rats (specific pathogen free) which weighted about 220 g were bought from Shanghai Silake Ltd Inc. Rats were randomly divided into three groups: normal control (NC) group, CIH group, and CIH supplemented with APN (CIH+APN) group. Rats in the CIH and CIH+APN groups received intermittent hypoxia exposures, while rats in NC group exposed to room air only.

The rats were housed in special cage with a gas control system which can regulate nitrogen and air in

the cage. The intermittent hypoxia events persisted 8 hours a day and lasted 5 weeks. Firstly, the gas control system infuses nitrogen into the cage to decrease the fraction of inspired oxygen (FiO_2) in the cage from 21% to 5%, which lasted for 15 sec during 1 min. In the subsequent 1 min period, the FiO_2 recovered to the level of room air by the controlling system. Rats in the CIH+APN group received intravenous injection of 10 μg APN (Biovision, Milpitas, USA, Catalog No: 4593) twice a week for 5 weeks, while rats in NC group and CIH group received intravenous injection of saline (0.5 mL per time) at the same frequency.

Oral glucose tolerance test and insulin released test

The oral glucose tolerance test (OGTT) and insulin released test (IRT) were used to evaluate the pancreatic islet function. After fasting for 12 hours, the rats received irrigation glucose (2g/kg body weight dextrose in phosphate buffer saline [PBS]). Then tail vein blood samples were obtained at 0 min, 20 min, 30 min, 60 min, 120 min to examine blood glucose levels by Roche device and serum insulin levels using insulin kit (Roche life Science, Basel, Switzerland).

Islet isolation

After anesthesia, open abdominal wall and inject 10 mL Hank's buffered saline solution (HBSS) (Beyotime, Shanghai, China) with collagens V (1 mg/mL) (Sigma, San Francisco, USA) into common bile duct of rats. The swelling pancreas was excised and infused into the HBSS and incubated for 15 min at 37 °C with shaking. Then we added 40 mL HBSS containing 2% bovine serum albumin (BSA), filtered the suspension by a 0.5 mm metal mesh to obtain digested suspension, centrifuged at 300 g at 4 °C for 2 min, followed by adding 4% HBSS, repeat this step. The islets were obtained by using discontinuous Ficoll density gradient centrifugation (density: 1.100, 1.077) (Sigma). A total of 469 ± 192 islets / rat were obtained.

Reverse transcription-quantitative PCR (RT-qPCR)

The total RNA of 1000 islets was obtained through using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's specifications.

Then 1 μ g total RNA was reversely transcribed to complementary DNA (cDNA) with a Transcription First Strand cDNA Synthesis Kit (Roche life Science, Basel, Switzerland) according to the manufacture's specifications. The Primers of adenine nucleotide translocator 1 (*Ant1*), citrate synthase (*Cs*), heme oxygenase 1 (*Hmox1*), and cytochrome oxidase subunit IV isoform 1 (*Cox4i1*), mitofusin1 (*Mfn1*), optic atrophy 1 (*Opa1*), dynamin related protein (*Drp1*), and mitochondrial fission protein 1 (*Fis1*) (**Table 1**) were brought from Invitrogen. The Primer was mixed with the Power SYBR Green QPCR Master Mix (Applied Biosystems, Foster City, California, USA). The quantitative RT-PCR was performed using ABI7900 fast real-time PCR System (Applied Biosystems, MA, USA) with cycling parameters of 95 °C for 30s, 95 °C for 30s and 40 cycles, and 60 °C for 30s. All PCR assays were done in triplicate. Comparative and relative quantifications of these gene products normalized to *Gapdh* and the control group were calculated by the $2^{-\Delta\Delta CT}$ method.

Mitochondrial function assessment

The adenosine-triphosphate (ATP) kit (Beyotime, Shanghai, China) and JC-1 kit (Beyotime, Shanghai, China) were used to detect ATP level and mitochondrial membrane potential (MMP) of islet as the instructions. Briefly, add 500 μ L lysis buffer to isolated islets and vortex for few seconds, then centrifuge at 12000 g at 4 °C for 5 min, and collect the supernatant. Using bicinchoninic acid kit (Beyotime, Shanghai, China) to detect the protein concentration for eliminating the error caused by different protein concentration. Add 100 μ L ATP working solution into colorimetric dish for 5 min,

then add 20 μ L supernatant and standard product into colorimetric dish with blending. The luminometer was used to detect the ATP level, based on which we calculated ATP content per 1 mg protein.

After centrifuging the pancreatic islet at 500 g for 5 min, the precipitate was collected and resuspended with 0.5 mL cell culture medium. Then 0.5 mL JC-1 solution was used to treat the islet at 37 °C for 20 min, followed by centrifuging for 4 min at 600 g. Collect the precipitate and wash twice using JC-1 staining buffer. Add 1mL JC-1 staining buffer to resuspend the precipitate. We used fluorescence spectrophotometer (Thermo Fisher Scientific, MA, USA) to detect the red and green fluorescence intensity, and the ratio of red/green fluorescence intensity to represent the MMP level.

Reactive oxygen species

We used dihydroethidium (DHE) Kit (Beyotime, Shanghai, China) to detect ROS level of islet following its performing instructions. Briefly, place the extracted islets on the slide, then treat the islets with dihydroethidium solution for 30 min at 37 °C. Fluorescence microscope (Olympus, Tokyo, Japan) was used to detect the ROS level, which was represented by the red fluorescence intensity.

Evaluation of protein expressions in mitochondria fuse and divide

The islet was treated with lysis buffer (Keygen Biotech, Jiangsu, China) and protease inhibition (Keygen Biotech, Jiangsu, China) on ice. After centrifugation at 12,000 g at 4 °C for 15 min, the supernatant was collected for further analysis. The total protein

Table 1. The primer sequences of *Drp1*, *Fis1*, *Mfn1*, *Opa1*, *Hmox1*, *Cs*, *Ant1*, *Cox4i1* and *Gapdh*

Primer	Forward	Reverse
<i>Drp1</i>	5'- ACTGGCCCCGTCAGCTTT -3'	5'- AGCTCCACACAGCGGAGGCT -3'
<i>Fis1</i>	5'- GCACGCAGTTTGAATACGCC -3'	5'- CTGCTCCTCTTTGCTACCTTTGG -3'
<i>Mfn1</i>	5'- CCTTGATCATCGATTCTGGGTTC-3'	5'- CCTGGGCTGCATTATCTGGTG -3'
<i>Opa1</i>	5'- CCTTGATCATCGATTCTGGGTTC -3'	5'- CCTGGGCTGCATTATCTGGTG -3'
<i>Hmox1</i>	5'- GTCAAGCACAGGGTGACAGA -3'	5'- CTGCAGCTCCTCAACAGC -3'
<i>Cs</i>	5'- GCACGCCAGTGCTTCTTC -3'	5'- CATGCTGCTGTCTGAAGGTC -3'
<i>Ant1</i>	5'- GTAGGATGATGATGCAGTCTGG -3'	5'- CGTCCTTCATCTTTTGAATC -3'
<i>Cox4i1</i>	5'- CACTGCGCTTGCTGCTGAT -3'	5'- CGATCAAAGGTATGAGGGATG -3'
<i>Gapdh</i>	5'- GCCCTCACCAGTGGCCTTG -3'	5'- TGCAGCCCAGGGCCTTTGAC -3'

Drp1, dynamin related protein; *Fis1*, mitochondrial fission protein 1; *Mfn1*, mitofusin1; *Opa1*, optic atrophy 1; *Hmox1*, heme oxygenase 1; *Cs*, citrate synthase; *Ant1*, adenine nucleotide translocator 1; *Cox4i1*, cytochrome oxidase subunit IV isoform 1; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

concentration of islets was measured by bicinchoninic acid kit (Thermo Fisher Scientific, MA, USA). 20 μ g islet protein was subjected to electrophoresis on 10% sodium dodecyl sulfate Polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes (Roche life Science, Basel, Switzerland) afterward. The 5% bovine serum albumin in tris-buffered saline (TBS) with 0.1% Tween-20 (TBSA) at pH 7.6 was used to blot the PVDF membranes for 1 h at room temperature. Then the PVDF membranes were incubated with the primary antibodies diluted in TBSA at 4 °C with gentle shaking overnight, followed by incubation with a peroxidase-labeled secondary antibody diluted in TBSA at 37 °C for 1 h. The PVDF membranes were infused in enhanced chemiluminescence solution. Then the protein blots were detected by using the digital imaging system (Molecular Imager[®] ChemiDocTM XRS+ System). The intensity of each protein band was normalized to β -actin. The HRP-linked secondary antibody (1:2000, Anti-rabbit IgG, cat. no. 7074; 1:2000, Anti-mouse IgG, cat. no. 7076) and the primary antibodies of DRP1 (1:1000, cat. no. 8570), OPA1 (1:1000, cat. no. 80471), BAX (1:1000, cat. no. 2772), cleaved-Caspase 3 (1:1000, cat. no. 9664), cleaved PARP (1:1000, cat. no. 94885), and β -actin (1:1000, cat. no. 4970) were bought from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies of FIS1 (1:500, cat. no. sc-376447), MFN1 (1:500, cat. no. sc-166644), and BCL-2 (1:500, cat. no. sc-7382) were bought from Santa Cruz Biotechnology (Dallas, TX, USA).

Statistical analysis

Values of three independent experiments are described as means $\pm$ SD. Differences among the three groups

were computed by one-way analysis of variance using the Tukey's post-hoc test for multiple group comparisons. Statistical difference was accepted at $P < 0.05$.

RESULTS

OGTT and insulin assessment

The OGTT and IRT showed there were no differences in the levels of blood glucose and insulin at 0 min (glucose: $F=0.68$, $P>0.05$; insulin: $F=0.11$, $P>0.05$), 20 min (glucose: $F=0.59$, $P>0.05$; insulin: $F=1.42$, $P>0.05$), 30 min (glucose: $F=0.49$, $P>0.05$; insulin: $F=1.78$, $P>0.05$), 60 min (glucose: $F=0.14$, $P>0.05$; insulin: $F=1.66$, $P>0.05$), and 120 min (glucose: $F=0.54$, $P>0.05$; insulin: $F=0.70$, $P>0.05$) among the three groups (**Figure 1**).

Mitochondria fusion and division

The protein and gene levels of FIS1 and DRP1 were significantly higher in the CIH group than those in the NC group; however, of the CIH+APN group, these protein and gene levels were lower than those in the CIH group, but were still higher than in the NC group. The differences of these indicators among the three groups were all significant (protein FIS1: $F=17.62$, $P<0.01$; DRP1: $F=21.62$, $P<0.01$; gene *Fis1*: $F=59.94$, $P<0.001$; *Drp1*: $F=24.04$, $P<0.01$).

The protein and gene levels of MFN1 and OPA1 in CIH+APN group were found to be higher than those in the CIH group and lower than those in the NC group; the differences among the three groups were significant (protein MFN1: $F=45.17$, $P=0.001$; OPA1: $F=30.89$, $P=0.001$; gene *Mfn1*: $F=18.05$, $P<0.01$; *Opa1*: $F=69.68$, $P<0.001$) (**Figure 2**).

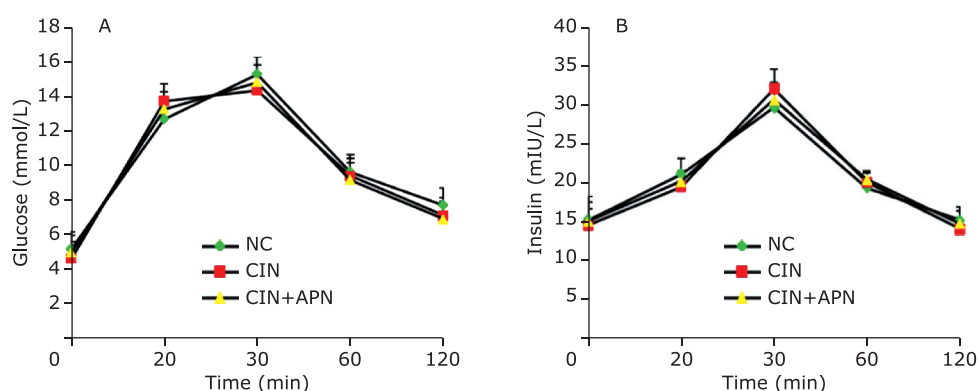


Figure 1. Results of oral glucose tolerance tests (OGTT) and insulin released test (IRT) of rats in NC, CIH, and CIH+APN group (n=20) (A) Dynamic changes in blood glucose of NC, CIH, and CIH+APN group. (B) Dynamic changes in insulin of NC, CIH, and CIH+APN group. NC, normal control; CIH, chronic intermittent hypoxia; APN, adiponectin.

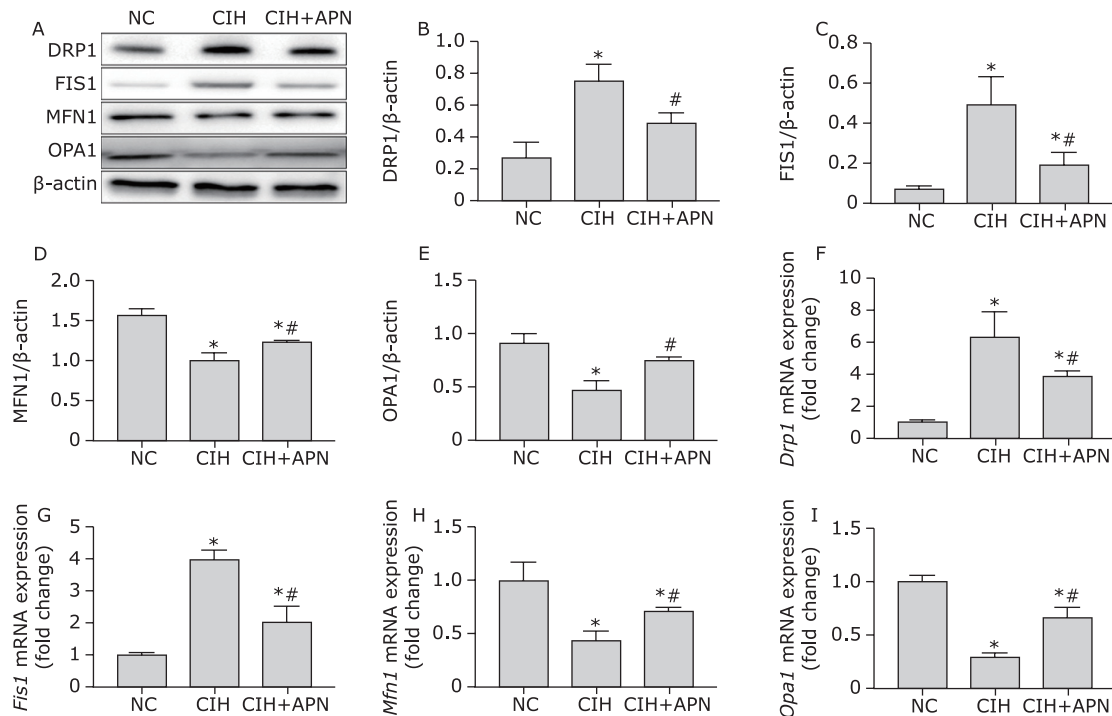


Figure 2. Mitochondrial fusion and division of the NC, CIH, and CIH+APN group

(A) The protein levels of DRP 1, FIS1, MFN1, and OPA1 of NC, CIH, and CIH+APN group. (B, C, D, E) The densitometric evaluations by the independent western blot of DRP1, FIS1, MFN1, and OPA1. (F, G, H, I) The mRNA expression levels of *Drp1*, *Fis1*, *Mfn1*, and *Opa1* of the NC, CIH, and CIH + APN group. $n=3$. * $P < 0.05$ compared to NC group; # $P < 0.05$ compared to CIH group.

Mitochondrial dysfunction

The ATP and MMP levels of islets were found to be lower in the CIH group than those in the NC group. The ATP and MMP levels in the CIH+APN group were remarkably higher than in the CIH group but were lower than in the NC group (**Table 2**).

The mRNA levels of *Ant1*, *Cs*, *Hmox1*, and *Cox4i1* were lower in the CIH group than in the NC group (**Figure 3**). These gene expression levels in the CIH + APN group were higher than in the CIH group, but were lower than in the NC group ($P < 0.05$). All the differences were significant (*Ant1*: $F=13.27$, $P < 0.01$; *Cs*: $F=225.20$, $P < 0.001$; *Hmox1*: $F=77.33$, $P < 0.001$; *Cox4i1*: $F=53.74$, $P < 0.001$).

Oxidative stress

The ROS level of islet, as represented by fluorescence

intensity in **Figure 4**, was found to be higher in the CIH group than in the CIH+APN group, and the latter was higher than that in the NC group. The differences among the three groups were significant ($F=113.40$; $P < 0.001$).

Mitochondria-associated apoptosis pathway

The protein levels of BAX, BCL-2, cleaved Caspase-3, and cleaved PARP were used to access the mitochondria-associated apoptosis pathway of islets (**Figure 5**). The protein levels of both cleaved Caspase-3 and cleaved PARP were the highest in the CIH group, and the lowest in the NC group, with that of CIH+APN group in between ($F=44.57$, $P < 0.01$; $F=69.85$, $P < 0.001$; respectively). The protein level ratio of BCL-2/BAX in the CIH+APN group was higher than that of the CIH group and lower than that of the NC group ($F=37.12$, $P < 0.001$).

Table 2. ATP level and MMP level of islets in NC, CIH, and CIH+APN group[§]

Measurements	NC group	CIH group	CIH+APN group	F	P value
ATP (nmol/mg)	15.13±1.60	7.83±0.78*	11.37±1.41*#	23.38	0.0015
MMP levels	25.96±1.33	13.22±1.03*	19.75±1.19*#	86.04	<0.0001

§ plus and minus represent mean±SD. MMP was measured by the ratio of red/green fluorescence intensity. $n=3$. ATP, adenosine triphosphate; MMP, mitochondrial membrane potential; NC, normal control; CIH, chronic intermittent hypoxia; APN, adiponectin. * $P < 0.05$ compared to NC group; # $P < 0.05$ compared to CIH group.

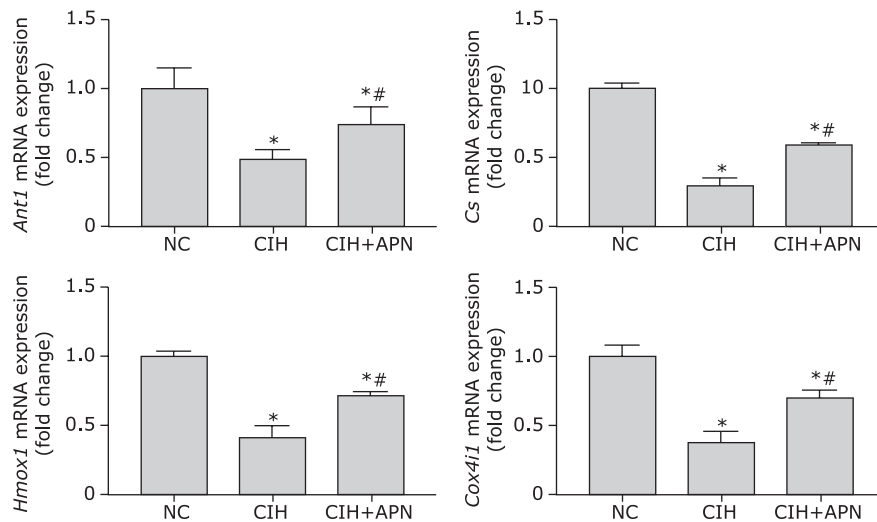


Figure 3. Comparison of mitochondrial tricarboxylic acid cycle function among NC, CIH, and CIH+APN group. The mRNA levels of *Ant1*, *Cs*, *Hmox1*, *Cox4i1* which implicate mitochondrial tricarboxylic acid cycle function of islets were significantly different among the three groups. $n=3$. * $P < 0.05$ compared to NC group; # $P < 0.05$ compared to CIH group.

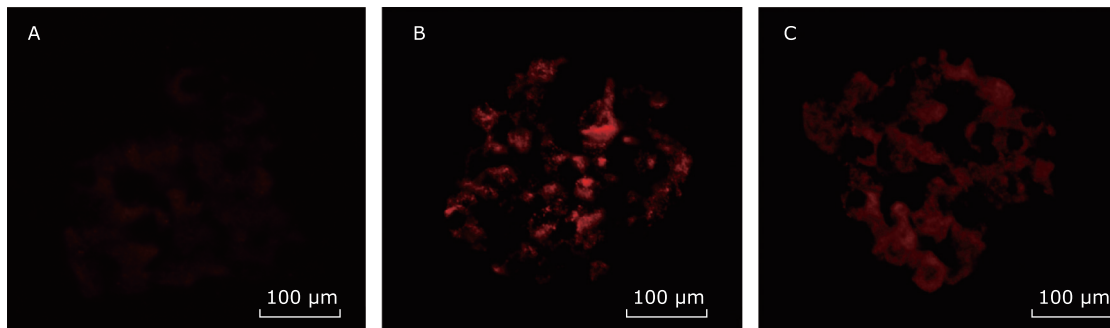


Figure 4. The reactive oxygen species (ROS) levels (Mean Fluorescence Intensity) of islet detected by dihydroethidium staining (A) of NC group; (B) of CIH group; (C) of CIH+APN group. The intensity of red fluorescence indicates ROS level. $n=3$.

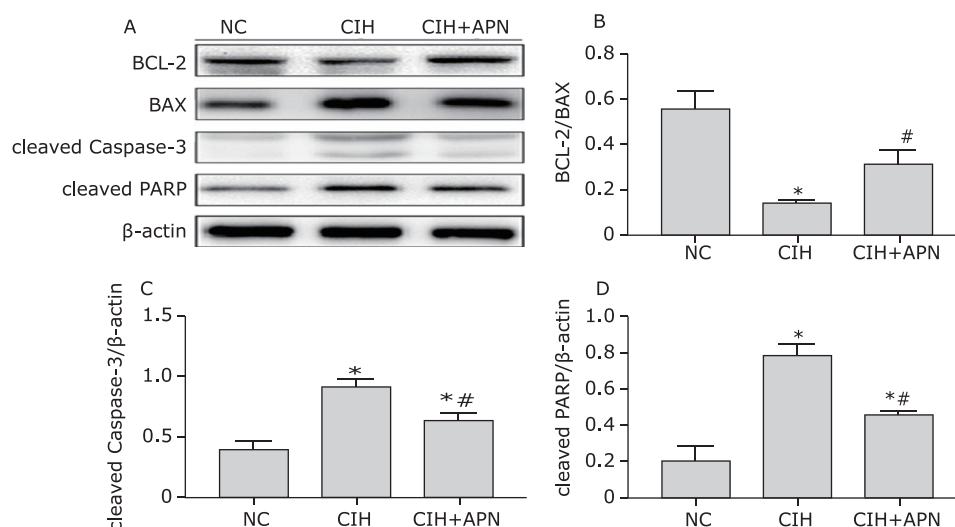


Figure 5. The expressions of proteins in mitochondria associated apoptosis pathway by western blot in NC, CIH, and CIH + APN group (A) The protein expressions of BAX, BCL-2, cleaved Caspase-3, and cleaved PARP by western blot. (B, C, D) The densitometric evaluation and comparison on BCL-2/BAX, cleaved Caspase-3, and cleaved PARP of the three groups. $n=3$. * $P < 0.05$ compared to NC group; # $P < 0.05$ compared to CIH group.

DISCUSSION

In this study, we investigated the effects of APN on the pancreatic islets after CIH exposure. Although CIH did not induce an obvious dysfunction in the pancreatic islets, cell injury was observed at molecular level. In this study, we found that CIH reduced ATP and MMP levels, generated oxidative stress, created an imbalance in mitochondrial fusion and division, and activated the mitochondria-associated apoptosis pathway in pancreatic islets. However, treatment with APN mitigated the injuries of pancreatic islets.

It is well known that OSAHS is associated with diabetes, and approximately 40% of patients with OSAHS suffer from T2DM.^[14,15] Li *et al.* found that CIH raised the fasting blood glucose level, induced the development of insulin resistance in T2DM rats, with increased glucose cultured INS-1 cells.^[16] A recent study reported that decrease in number of pancreatic islet β cells and its dysfunction was associated with the progression of T2DM.^[14] Taken together, we speculated that CIH might damage the pancreatic islets. In order to verify our hypothesis, we established an animal model mimicking the CIH conditions in OSAHS patient. In this study, we found that there were no differences in glucose and insulin levels at different time points after CIH exposure; however, we observed the mitochondrial injury after CIH exposure.

Mitochondria are important organelles, which produce energy for cell function. Researchers have found that the mitochondria are constantly in a state of fusion and division, and these dynamic processes are essential to maintain the cell homeostasis and its function.^[17] MFN1 and OPA1 are the main mitochondrial fusion-regulatory protein, and DRP1 and FIS1 are the main mitochondrial division-regulatory protein.^[18,19] Under normal conditions, mitochondrial fusion and division was in balance; however, when certain stimulators act on cells, the balance is disrupted.^[20,21] Liu *et al.* found that the imbalance in mitochondrial fusion and division, including low expression of OPA1 and high expression of DRP1 during hypoxia-reoxygenation stress, is similar to intermittent hypoxia.^[22] In this study, the imbalance in mitochondrial fusion and division in pancreatic islets was found to be associated with the reduced protein and gene expresses of MFN1 and OPA1 and the increased expression of DRP1 and FIS1 after CIH exposure.

It was reported that the imbalance in mitochon-

drial fusion and division induced the mitochondrial dysfunction.^[23] Chen *et al.* found the absence of MFN1 gene or inhibition of *Opa1* expression induced a decrease in MMP level.^[24] Nunnari *et al.* found that the imbalance in mitochondrial fusion and division could induce the mitochondrial dysfunction and oxidative stress.^[25] Consistent with these studies, our study found that after pancreatic islets exposed to CIH, the ATP, MMP and the mRNA levels of enzymes associated with mitochondrial tricarboxylic acid cycle reduced, while the ROS level increased.

We also found that the protein level of BCL-2 reduced while the protein levels of BAX, cleaved Caspase-3, and cleaved PARP which represent the mitochondria-associated apoptosis increased. It is well known that the BCL-2 family is associated with cellular apoptosis.^[26] Cleaved-Caspase 3 is the downstream of BCL-2 family which could activate the PARP, and then lead to cell apoptosis.^[27] So, the results of this study suggest that the mitochondria-associated islet cell apoptosis is activated by the imbalance in mitochondrial fusion and division. These data reveal that CIH might lead to pancreatic islet injury through interrupting the balance in mitochondrial fusion and division in pancreatic islets, further inducing the mitochondrial dysfunction and activate pathway of mitochondria-associated islet cell apoptosis.

APN is a protective cytokine that shows anti-inflammatory, anti-insulin-resistance, and anti-hypertension activities *in vivo*.^[9,10] In this study, APN supplementation ameliorated CIH induced high ROS level, low MMP level, and the imbalance in mitochondrial fusion and division, reduced the protein expression of BAX, cleaved Caspase-3, and cleaved PARP, and increased the BCL-2 protein level in pancreatic islets. These results are consistent with study by Hu *et al.* who found that APN reversed mitochondrial dysfunction under diabetes condition *in vivo and in vitro*.^[12] Koentges *et al.* reported that deficiency of APN could lead to a reduction in ATP level and mitochondrial dysfunction.^[28] Kim found that APN protected the hepatocytes against cell death in an *in vitro* experiment.^[29] Therefore, we speculated that APN reduced the pancreatic islet injury though regulating the imbalance in mitochondrial fusion and division, further ameliorating mitochondrial dysfunction and reducing mitochondria-associated pancreatic cell apoptosis under the CIH condition.

In conclusion, this study shows that CIH induces imbalance in mitochondrial fusion and division, causes

mitochondrial dysfunction, increases ROS level, and mitochondria-associated apoptosis pathway. APN ameliorated the pancreatic islet mitochondrial injury induced by CIH through inhibiting the imbalance in mitochondrial fusion and division. This study suggests the value of adiponectin in the treatment of obstructive sleep apnea syndromes. The current results need validation with cellular experiments, which is the major limitation of this study and need to be conducted in future.

Conflict of interests

The authors disclosed no conflicting interests.

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