Nicorandil alleviates inflammation and oxidation in diabetic cardiomyopathy

Yi Tao1, Bing Zhou2, Jie Zou1, Yixian Yu3, Jing Zhao1, Naijia Xu1, Qiong Wang1
1Department of Endocrinology, 2Department of Acupuncture, 3Department of Neurology, Wuhan Hospital of Traditional Chinese Medicinal, Wuhan, China

*For correspondence: Email: heyk@croeg-na.com; Tel: +86-018672992705

Sent for review: 11 October 2021
Revised accepted: 18 January 2022

Abstract

Purpose: To examine the effect of nicorandil on high glucose-induced cardiomyocyte inflammation and oxidative stress.

Methods: H9C2 cardiomyocytes were divided into control group, high glucose group and nicorandil group. The survival rate of cardiomyocytes was determined using the CCK-8 method. The contents of reactive oxygen species (ROS) of cardiomyocytes were determined by flow cytometry. The contents of MDA and LDH in cell supernatant were determined by kit. Western blot and real-time PCR were used to assess oxidative stress, inflammation and apoptosis related factors in each group of cardiomyocytes. The expression levels of IL-1β were determined by immunofluorescence. Tunnel staining was used to determine the apoptosis level of each group.

Results: The expressions of SOD1 and SOD2 in the high glucose group were significantly decreased (p < 0.05). Also, the contents of MDA and LDH were significantly increased (p < 0.05). Furthermore, IL-1β, TNF-α, caspase 3 and Bax expressions were increased, while Bcl-2 expression was inhibited. IL-1β and Tunnel fluorescence also increased significantly. NF-κB and Ikkα were significantly increased, while IκB-α was inhibited. Furthermore, nicorandil inhibited oxidative stress and apoptosis, as well as NF-κB pathway and downstream factor Ikkα.

Conclusion: Nicorandil ameliorates the inflammation and oxidative damage of cardiomyocytes induced by high glucose, by inhibiting NF-κB pathway, thereby lowering apoptosis. Thus, the findings provide new insight into the development of new agents for the treatment of diabetic cardiomyopathy.

Keywords: Diabetic cardiomyopathy, H9C2, Oxidative Stress, Inflammatory, Nuclear Factor-κB

INTRODUCTION

Diabetic cardiomyopathy (DCM) is a unique cardiomyopathy associated with metabolic disorders in diabetic patients, without hypertension, coronary atherosclerosis, cardiomyocyte metabolic disorders and microvascular disease caused by heart disease [1]. In recent years, studies have shown that DCM poses a serious threat to human health and is one of the important complications of death in diabetic patients [2]. The quality of life of patients is also seriously affected. Therefore, early treatment is of great significance in reducing the mortality of DCM patients. Previous studies have shown that nicorandil has an effect on...
cardiomyocyte apoptosis in diabetic cardiomyopathy rats [3], providing a basis for the treatment of DCM.

Studies have shown that nicorandil contains a nitrate group and is an adenosine triphosphate (ATP)-sensitive potassium channel (KATP) opener [4]. On the one hand, by directly activating cGMP, the opening of the cell membrane and the mitochondrial membrane KATP is increased, which has the effect of vasodilation, thereby increasing coronary blood flow and improving vasospasm and microcirculation [5]. On the other hand, it can also promote the retention of potassium ions in the mitochondria, and play a role in inhibiting the influx of calcium ions [6]. Given that previous studies showed little effect of nicorandil on diabetic cardiomyopathy, the present study focuses on the effects of nicorandil on high glucose-induced cardiomyocyte inflammation and oxidative stress.

Inflammation is a complex pathophysiological process mediated by a variety of different signaling molecules produced by white blood cells and mast cells. It can be triggered by a variety of stimuli [7]. In inflammatory diseases, many inflammatory mediators play important roles [8]. Nuclear Factor-κB (NF-κB) is a protein involved in the regulation of DNA transcription and production of cytokines [9]. Studies have shown that NF-κB signaling pathway plays an important role in regulating inflammation and oxidative stress [10], and thus becomes a new therapeutic target for the development of inflammatory diseases.

EXPERIMENTAL

Cell culture and drug treatment

The H9C2 cardiomyocytes (Cell Culture Center, Shanghai, China) were cultured in Dulbecco’s modified Eagle medium (DMEM, Life Technology, Wuhan, China) containing 10 % fetal bovine serum (FBS, Life Technology, Wuhan, China) and 1 % penicillin/streptomycin (Life Technology, Wuhan, China). Nicorandil (Zhengkang Pharmaceutical, Taiyuan, China) was placed in a stock solution in physiological saline and stored at 4 °C. When the cells were grown to the appropriate density, the high glucose group and the nicorandil group were treated with high glucose (30 mmol/L glucose), the control group was cultured with DMEM (5 mmol/L glucose), and the nicorandil group was treated with nicorandil (50 μmol/L pre-intervention for 6 h for the nicorandil group).

Cell count Kit-8 (CCK-8) method

The optimal concentration and time of nicorandil were determined with CCK-8 (Construction, Nanjing, China). The H9C2 were cultured at a density of 2000/well for 24 h. The next day, different concentrations of nicorandil were added to the culture plate. After incubation for 1, 2, 6, and 12 h, CCK-8 solution was added to the cells, and the absorbance at 450 nm was measured.

Immunofluorescence

Paraformaldehyde (4%) was added to fix the cells. Then the goat serum was added to block at 20°C for 30 minutes. Cells were first incubated with diluted primary antibody IL-1β (Abcam, Cambridge, MA, USA, Rabbit, 1:3000) overnight at 4°C and then incubated with fluorescent secondary antibody in the dark for 1 h. The specimen was subjected to 4',6-diamidino-2-phenylindole (DAPI) (Construction, Nanjing, China) staining, incubated for 15 min in the dark, sealed with a sealing liquid, and then observed by a fluorescence microscope.

Determination of biochemical indicators

The supernatant from the medium of the cells was collected, centrifuged at 2000 rpm to obtain the supernatant, and the supernatants of each group were assayed for LDH and MDA levels using a commercial kit (Jiancheng, Nanjing, China).

Western blot assay

The cells in the culture plate were collected, total protein of each group was extracted via centrifugation after being lysed, and sample protein concentration was determined using bicinchoninic acid (BCA) kit (Camilo Biological, Nanjing, China). An equal amount of protein sample was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitro membrane (Thermo Fisher Scientific, Waltham, MA, USA). After blocking with 5 % bovine serum albumin (BSA) for 2 h, the membranes were incubated with specific antibodies overnight at 4°C.

Next day, the membranes were then incubated with the secondary antibody (goat anti-rabbit IgG antibody, Yifei Xue Biotechnology, 1:3000, Nanjing, China) for 1 h, followed by being observed via an electrochemiluminescence (ECL) system. Specific antibodies’ details were shown below: SOD1, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; SOD2, Abcam, Cambridge,

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells in each treatment group were washed with pre-cooled PBS and total RNA was extracted (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription and polymerase chain reaction were carried out, and the reaction conditions were: pre-deformation, 95 °C, 1 min, 95 °C, 15 s, 58 °C, 20 s, 72 °C, 45 s, 40 cycles. The dissolution curve was 60 – 95 °C, and the temperature was raised by 1°C every 20 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Thermo Fisher Scientific, Waltham, MA, USA) was served as an internal control. 2-ΔΔCt method was used for the quantitative analysis. All the primers are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

The supernatant of each group of cells was collected after centrifugation. According to the instructions (Jianglai, Shanghai, China), standard product wells, sample well settings, and standard product wells were added using different concentration standards, and corresponding detection antibodies were standard products added to each well and sample well, and were incubated for 1 h. After discarding the medium, the working solution was added to each well and incubated for 15 min in the dark. After termination with the stop solution, the absorbance at 450 nm was measured.

Flow cytometry

Each group of H9C2 cells was collected, and the cell concentration was adjusted by filtration using a sieve. The prepared single cell suspension was added to 2',7'-diamethyl dicyclofluorescein (DCF-DA, Kaji, Nanjing, China), and the supernatant was centrifuged, incubated with 10% FBS, and prepared again as a H9C2 single cell suspension. Flow cytometry (Becton Dickinson, Heidelberg, Germany) was used to measure the average fluorescence intensity of intracellular marker fluorescent probes.

TUNEL staining

To detect apoptosis in H9C2 cells, cells were stained with the TUNEL kit (Roche, Basel, Switzerland) according to the manufacturer's instructions, and the nuclei was stained with DAPI. Apoptosis was observed using laser scanning confocal microscopy.

Statistical analysis

All data were processed by SPSS statistical analysis software (version 26.0), and are expressed as mean ± SD (standard deviation). Differences between two groups were analyzed by Student's t-test. Comparison between multiple groups was done using one-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P < 0.05 indicated significant difference.

RESULTS

Nicorandil attenuated high glucose-induced H9C2 cell injury

The optimal concentration and optimal culture time of nicorandil for H9C2 cells were determined by CCK method (Figure 1 A). The results showed that nicorandil treated H9C2 cells had the highest cell survival rate cultured at 50 μmol/L for 6 h.

Table 1: Real time PCR primers used

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5'&gt;3')</th>
<th>Reverse (5'&gt;3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>CAGTTGAAGTTGCGACGC</td>
<td>CAGTTGAAGTTACCAGTC</td>
</tr>
<tr>
<td>SOD1</td>
<td>GGTCGACGCCGTTGCTTC</td>
<td>GGTCGACGCCGATTTTC</td>
</tr>
<tr>
<td>SOD2</td>
<td>CAGACCTGCTTACGATCGTG</td>
<td>CAGACCTGCTTACGATCGTG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GCTTCTTCTTACACGCTTC</td>
<td>GCTTCTTCTTACACGCTTC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CAGGTGGAGTTGCACTGG</td>
<td>CAGGTGGAGTTGCACTGG</td>
</tr>
<tr>
<td>Ikkα</td>
<td>GACTGTGCGGGGCTTTC</td>
<td>GACTGTGCGGGGCTTTC</td>
</tr>
<tr>
<td>IkB-α</td>
<td>GGCTGACTGACGTCCTG</td>
<td>GGCTGACTGACGTCCTG</td>
</tr>
<tr>
<td>P65</td>
<td>ACTGGGCTGGCGTTC</td>
<td>ACTGGGCTGGCGTTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACAAGTTCGCGGAAGGG</td>
<td>ACAAGTTCGCGGAAGGG</td>
</tr>
</tbody>
</table>

RT-PCR = quantitative reverse-transcription polymerase chain reaction
At the same time, the kit test results also showed that high glucose treatment promoted the expression of LDH and MDA, while nicorandil effectively inhibits the expression of LDH and MDA (Figure 1 B and C).

**Nicorandil alleviates high glucose-induced oxidative stress in H9C2 cells**

Western blot (Figure 2 A) and Real-time PCR (Figures 2 B and C) showed that the SOD1 and SOD2 expression in the high glucose group were significantly lower than in control group, while nicorandil treatment inhibited the decrease in SOD1 and SOD2 expressions. ELISA results supported the foregoing findings (Figure 2 D and E). The results of flow cytometry confirmed that the ROS level in the high glucose group increased, but in the nicorandil group, the ROS level was significantly lower (p < 0.05) Figure 2 F).

**Nicorandil alleviates high glucose-induced inflammation in H9C2 cells**

First, Western blotting results showed that high glucose induced inflammation in H9C2 cells (Figure 3 A and B). IL-1β and TNF-α expressions were significantly increased, while nicorandil treatment significantly inhibited inflammatory response. Similar results were obtained for Real-time PCR (Figure 3 C and D) and ELISA (Figures 3 E and F). Second, immunofluorescence staining results (Figure 3 G), indicate that the high glucose group H9C2 cells expression of IL-1β significantly increased, while nicorandil inhibited IL-1β expression (p < 0.05).

**Nicorandil alleviates high glucose-induced apoptosis in H9C2 cells**

Previous studies have confirmed that high glucose promoted apoptosis in H9C2 cells. Western blot results indicate that the expressions of caspase 3 and Bax in the high glucose group were significantly higher (Figure 4 A) in real-time PCR (Figure 4 B ~ D), while the expression of Bcl-2 was effectively inhibited. ELISA also
achieved similar results (Figure 4 E - G). The treatment with nicorandil significantly promoted Bcl-2 expression and inhibits Caspase3 and Bax expressions. In addition, it was observed that the proportion of positive cells in the nicorandil group significantly decreased, compared with that in high glucose group (Figure 5).

**Figure 4**: Nicorandil alleviated high glucose-induced apoptosis in H9C2 cells. (A) Nicorandil inhibited high glucose-induced Caspase3 and Bax increase and Bcl-2 decrease at protein levels. (B~D) Nicorandil inhibited high glucose-induced Caspase3 and Bax increase and Bcl-2 decrease at mRNA levels. (E~G) ELISA also showed the similar results regarding Caspase3, Bax and Bcl-2 levels. (*p < 0.05, compared with control group; #p < 0.05, compared with High glucose group)

**Figure 5**: The proportion of positive cells in the nicorandil group significantly decreased, compared with that in high glucose group via Tunnel assay (magnification: 400×)

Nicorandil inhibited activation of NF-κB pathway

First, we examined NF-κB, Ikkα and inflammatory inhibitor IκB-α expression in each group using Western blotting (Figure 6 A). The results showed that the expression of NF-κB-p65 and Ikkα increased in the high glucose group, while the expression of IκB-α was significantly inhibited. In contrast, nicorandil decreased NF-κB and Ikkα expressions and promoted IκB-α expression. Similar results were obtained for mRNA levels (Figure 6 B - D). This indicates that nicorandil inhibited the NF-κB pathway.

**Figure 6**: Nicorandil inhibited activation of the NF-κB pathway. (A) Nicorandil inhibited high glucose-induced NF-κB-p65 and Ikkα increase and IκB-α decrease at protein levels. (B~D) Nicorandil inhibited high glucose-induced NF-κB-p65 and Ikkα increase and IκB-α decrease at mRNA levels. (*p < 0.05, compared with control group; #p < 0.05, compared with High glucose group)

**DISCUSSION**

High sugar triggers a series of cardiovascular diseases such as hypertension and myocardial failure [11]. Diabetic cardiomyopathy (DCM) is an independent and specific ventricular disease, evidenced by septal thickness, ejection fraction and short axis shortening rate, which are independent of coronary heart disease and valvular disease. Myocardial disease, which exists as an independent complication in diabetic patients, is one of many causes of death [12]. The pathogenesis of DCM includes a number of very complex aspects such as myocardial cell metabolic disorders, myocardial microvascular disease, intracellular Ca²⁺ dysregulation, cytokine abnormalities, and oxygen free radical abnormalities [13].

Studies have confirmed that nicorandil has a role in regulating oxidative stress and inflammatory response [14]. Therefore, this study focused on the induction of inflammatory response and oxidative stress by the treatment of H9C2 cells with high glucose. El-Kashef DH found that nicorandil improved lung inflammation in silicotic rats [15]. A previous study found that nicorandil attenuated oxidative stress in mice with vascular calcification [16]. The results confirmed that the anti-oxidative stress factors SOD1 and SOD2 were significantly decreased after high glucose treatment of H9C2 cells, while the inflammatory
factors IL-1β and TNF-α expression were significantly increased.

In addition, caspase protein family is a key molecule regulating eukaryotic apoptosis, mainly to: 1) mitochondria-based endogenous apoptotic pathway. 2) exogenous pathways based on death receptors on cell membrane [17].

The results showed that high glucose treatment promoted apoptosis of H9C2 cells, caspase3 and Bax expression were significantly promoted, and Bcl-2 expression was effectively inhibited. These results indicate that high glucose induced inflammation in H9C2 cells, leading to redox imbalance in cells, thereby promoting apoptosis. Nicorandil significantly inhibited the inflammatory response and also inhibit the redox imbalance, thus slowing down the apoptosis.

Studies have confirmed that NF-κB is involved in the regulation of DCM [18]. NF-κB pathway is a classic inflammation and oxidative stress pathway. Studies have confirmed that NF-κB is inactive after cytoplasmic binding protein IκBα, thereby preventing NF-κB from entering the nucleus and inhibiting its activation [19]. This present research indicated that high glucose activates the NF-κB pathway, thereby promoting the expression of the downstream factor Iκkα and inhibiting IκB-α expression. At the same time, high glucose induced ROS accumulation in H9C2 cells, leading to redox imbalance and promoting the increase of LDH and MDA secretion by H9C2. The results of the current study confirm that nicorandil inhibits the NF-κB pathway, consequently inhibiting the inflammatory response and alleviating the redox imbalance.

**CONCLUSION**

The findings of this study demonstrate that nicorandil inhibits NF-κB pathway and reduces high glucose-induced oxidative stress and inflammation in H9C2 cells, thereby delaying apoptosis. Therefore, nicorandil may be of great interest as a potential drug for the treatment of DCM.

**DECLARATIONS**

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

**Open Access**

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/road), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

**REFERENCES**


Trop J Pharm Res, February 2022; 21(2): 278


