Int J Clin Exp Med 2020;13(7):5267-5274
www.ijcem.com /ISSN:1940-5901/IJCEM0112175

Original Article

The effects of miR-21 on the apoptosis of endothelial cells and the expressions of inflammatory factors in a CHD rat model by targeting NLRP3

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Received April 9, 2020; Accepted May 10, 2020; Epub July 15, 2020; Published July 30, 2020

Abstract: Objective: This study was designed to explore the effects of miR-21 on the apoptosis of endothelial cells and the expressions of the inflammatory factors in a CHD rat model by targeting NLRP3. Methods: 20 healthy SD rats were randomized into a control group (CG) fed a normal diet and an AS group (ASG) fed a diet rich in fat. 3 months later, the 2 groups were examined and compared in terms of their total cholesterol (TC), triacylglycerol (TG), high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDLC), and Ca^{2+} levels. The rats' endothelial cells were isolated from their coronary arteries and incubated using homocysteine (HCY) for the CHD model. Afterward, the cells were divided into a blank control group (non-transfected cells), a negative control group (transfection of the unrelated sequences), an miR-21 mimic group (transfection of the miR-21 overexpression), an miR-21 inhibitor group (transfection of the miR-21 inhibitor), and an miR-21 inhibitor +si NLRP3 group (transfection of miR-21 and deactivation of NLRP3). A comparison study was performed among the 5 groups to determine their interleukin (IL) -1β, IL-6, and IL-18 levels, and the cell viability was tested using the MTT method. Results: (1) The TC, TG, HDLC, LDLC, and Ca^{2+} levels were lower in the CG compared with the ASG ($P<0.05$); (2) While no significant difference was found among the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group in terms of their miR-21, NLRP3, caspase-1 mRNA, NLRP3 protein, and caspase-1 protein expressions ($P>0.05$), in the miR-21 mimic group, miR-21, the NLRP3 protein, and the caspase-1 protein were expressed at high levels, and NLRP3 and the caspase-1 mRNA were expressed at low levels compared with the corresponding levels in the miR-21 inhibitor group ($P<0.05$); (3) No significant difference was found among the blank control group, the negative control group, or the miR-21 inhibitor +si NLRP3 group in terms of their IL-1β, IL-6, and IL-18 levels, but the miR-21 mimic group showed lower levels of the same compared to the miR-21 inhibitor group ($P<0.05$); (4) Among the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group, the cell viability was not significantly different ($P>0.05$), but it was significantly higher in the miR-21 mimic group compared with the level in the miR-21 inhibitor group ($P<0.05$). Conclusion: The overexpression of miR-21 can alleviate the apoptosis of endothelial cells associated with CHD and reduce the expressions of the inflammatory factors, due to its targeted inhibition of NLRP3.

Keywords: miR-21, NLRP3, CHD model, apoptosis of endothelial cells, inflammatory factor expressions

Introduction

Cardiovascular disease is characterized by its highest incidence and mortality and by affecting the largest number of patients in the world. Studies show that CHD has become the largest cause of death in developing and developed countries. CHD, also known as coronary atherosclerotic cardiopathy, develops when the vascular lumen is narrowed or blocked by the atheropathy in the coronary artery, which easily leads to myocardial ischemia, hypoxia, or necrosis. CHD patients are prone to chest pain, nausea, vomiting, and other clinical symptoms [1-3]. The pathogenesis of the disease is relatively complex. In the current clinical study, hypertension, hyperlipidemia, hyperglycemia, irritability, and fed-upness are the risk factors for CHD. When the disease first occurs, there are about 1/3 of the patients died suddenly [4,

5]. Data shows that the number of deaths due to CHD in the world is as high as 7 million every year, ranking it first as the single leading cause of death. In China, the disease incidence and mortality related to CHD require more attention. According to the literature, the morbidity of CHD in China is increasing year by year, and may reach 3.7 times of what it was in 2000 [6, 7] by 2030 if no proper measures are taken.

microRNA is a small, single stranded non-coding RNA molecule with about 22 nucleotides that participates in the regulation of gene expression in animals and plants and that has been confirmed through many studies. Its main mechanism is to affect the physiological mechanism of the body by degrading the target gene's mRNA or regulating its protein expression [8, 9]. A member of the miRNA family, miR-21 has been shown to be widely involved in the development and progression of various cardiovascular diseases. A comparison study on CHD patients and normal individuals showed a higher expression of miR-21 in the former, and the expression may increase if the patients also suffer from arrhythmia. Through analysis, miR-21 has been shown to have a close relationship to CHD arrhythmia and plays an important role in the development and progression of such diseases [10, 11]. The role of the inflammatory response in atherosclerotic lesions has been supported by a massive amount of study results. Referring to practices, the inflammatory response can promote the secretion of inflammatory factors to further aggravate the damage to the endothelial cells of the coronary artery and accelerate atherosclerosis. At present, there are many studies on the damage caused by miR-21 to the endothelial cells of CHD patients, but few focus on the specific mechanism [12, 13]. NLRP3 is an intracellular polyprotein compound mainly distributed in the intercellular matrix and cell membranes, and it can participate in a variety of immune and inflammatory reactions [14]. Through animal experiments, it was found that the overexpression of miR-21 can alleviate the apoptosis of the endogenetic cells and can reduce the expressions of the inflammatory factors in CHD patients, possibly due to its targeted inhibition of NLRP3.

Materials and methods

Experimental animals

20 healthy and pure SD rats were sourced from the Beijing Vital River Laboratory Animal Technology Co., Ltd. and divided into the CG and ASG. On average, the rats weighed (241.73±20.21) g and were (3.43±0.32) months old in the CG and weighed (240.76±19.98) g and were (3.39±0.76) months old in the ASG ($P>0.05$); All the rats were kept in an environment set to 25°C and 65% humidity, and provided with tap water and forage for their free intake. The study was approved by the Ethics Committee of our hospital and was implemented according to the *Declaration of Helsinki*.

Pathological modeling

After 1-week adaptive feeding, normal forage and water were provided to the rats in the CG, and high-fat forage (added with vitamin D3 powder at a dose of 1.25×10^6 U/kg) to the ASG. During feeding, vitamin D3 powder was intramuscularly injected into the right lower extremities of the rats at a dose of 3×10^6 U/kg (body weight) every 30 d. The whole intervention process lasted 90 d, without interruption.

Isolation, incubation, and intervention of the endothelial cells of the coronary artery

The coronary artery was isolated from each of the healthy SD rats, cut into pieces, processed, and digested using pancreatin, then added into DMEM containing 10% FCS, incubated, and iterated at 37°C with 5% CO_2, and then incubated in HCY (0.25 mmol/L) for 24 h to create the CHD model cells. Afterward, the model cells were divided into the blank control group (cells not transfected), the negative control group (transfection of unrelated sequences), the miR-21 mimic group (transfection of miR-21 overexpression), the miR-21 inhibitor group (transfection of miR-21 inhibitor), and the miR-21 inhibitor +si NLRP3 group (transfection of miR-21 and deactivation of NLRP3) for 24 h of incubation and testing.

Observation indexes and evaluation criteria

Test of the blood lipids and calcium levels: After intervention for 90 d and fasting overnight, all the rats in the 2 groups were anaesthetized to isolate the aorta ventralis and collect blood from their arteries, from which, plasma was obtained and tested to determine the TC, TG, HDLC, LDLC, and Ca^{2+} levels with a biochemical analyzer. Each index was tested three times and the average was determined.

Table 1. Comparison of the CG and ASG in terms of blood lipids and calcium levels ($\bar{x} \pm s$) (mmol/L)

Group	n	TC	TG	HDLC	LDLC	Ca^{2+}
ASG	10	14.89±2.92	4.71±0.13	2.59±0.14	6.81±0.19	3.56±0.11
CG	10	2.87±0.19	1.32±0.31	2.01±0.11	1.39±0.31	2.88±0.19
t	-	12.99	31.985	10.301	47.139	9.795
P	-	<0.001	<0.001	<0.001	<0.001	<0.001

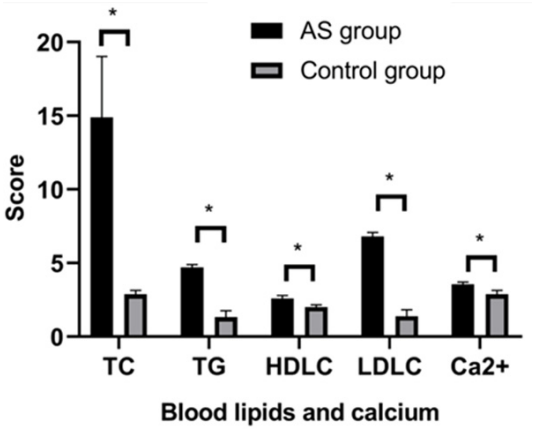

Figure 1. Comparison of the CG and the AGS in terms of their blood lipid and calcium levels. The TC, TG, HDLC, LDLC and Ca^{2+} levels were higher in the ASG compared with the CG (P<0.05). * indicates P<0.05 compared between the two groups for the same index.

Gene fragment testing: Total RNA was extracted and tested using the UV technique and using denaturing formaldehyde electrophoresis. Next, the miR-21, NLRP3, and caspase-1 mRNA expressions in the 5 groups were determined using real-time quantitative PCR. Each index was tested 3 times and the average was calculated.

Protein expressions: The proteins were extracted, including NLRP3 and caspase-1, and tested using the Western blot method with BCA kits from the Shanghai Regal Biotechnology Development Co., Ltd. The operations were carried out in strict accordance with the kit instructions. Each index was tested 3 times, and the average was calculated.

Cell viability testing: The cells in the 5 groups were processed with MTT solution, and then mixed with dimethyl sulfoxide to test the OD using an ELISA instrument. The cell viability was calculated according to the formula: cell viability = (OD of the Experiment Group-OD of the Blank Group)/OD of the Blank Group.

Inflammatory factor level testing: The supernatant was collected and tested for IL-1β, IL-6, and IL-18 using ELISA. Intergroup comparisons were performed.

Statistical analysis

The statistical analysis was performed using SPSS 22.0. In the case of numerical data expressed as $\bar{x} \pm s$, the intergroup and intragroup comparisons were carried out using independent-samples t tests. In the case of nominal data expressed as [n (%)], the intergroup and intragroup comparisons were carried out using X^2 tests. The intragroup comparisons at different points were carried out using ANVOA. For all the statistical comparisons, significance was defined as P<0.05.

Results

Comparison of the CG and ASG in their blood lipids and calcium levels

The TC, TG, HDLC, LDLC, and Ca^{2+} levels were significantly elevated in the ASG compared to the CG (P<0.05, **Table 1** and **Figure 1**).

Gene fragment test results

Through tests and comparisons, no significant differences were found among the blank control group, the negative control group, or the miR-21 inhibitor +si NLRP3 group in terms of the miR-21, NLRP3, or caspase-1 mRNA levels (P>0.05). When compared with the other groups, the miR-21 mimic group showed a high expression of miR-21 and low expressions of NLRP3 and caspase-1 mRNA (P<0.05), but in the miR-21 inhibitor group, the results were exactly the opposite (P<0.05, **Figure 2**).

The cell protein expressions in the five groups

No significant differences were found among the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group in their expressions of the NLRP3 and caspase-1 proteins after the tests and comparisons (P>0.05). The NLRP3 and caspase-1 proteins were expressed at a higher level in the miR-21 mimic group (P<0.05), and at a lower level in the miR-21 inhibitor group compared with the other groups (P<0.05, **Figure 3**).

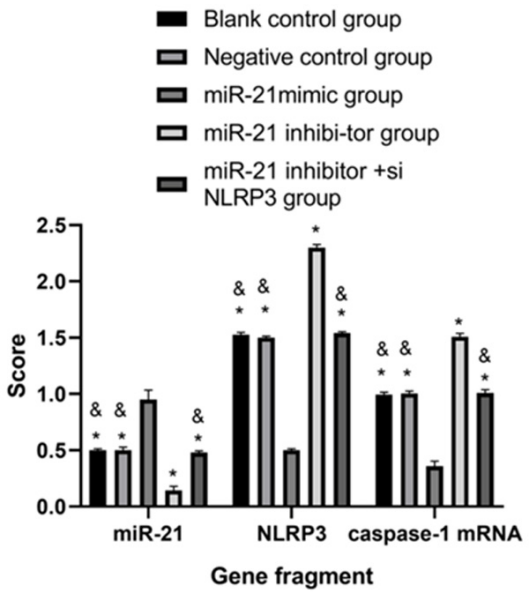

Figure 2. Comparison of the five groups in their miR-21, NLRP3, and caspase-1 mRNA levels. While no significant difference was demonstrated among the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group in their miR-21, NLRP3, caspase-1 mRNA levels (P>0.05), the highest and lowest expressions of miR-21 were observed in the miR-21 mimic group and the miR-21 inhibitor group respectively (P<0.05). For the expressions of NLRP3 and caspase-1 mRNA, the results were just the opposite (P<0.05). * indicates P<0.05 compared with the miR-21 mimic group, & indicates P<0.05 compared with the miR-21 inhibitor group.

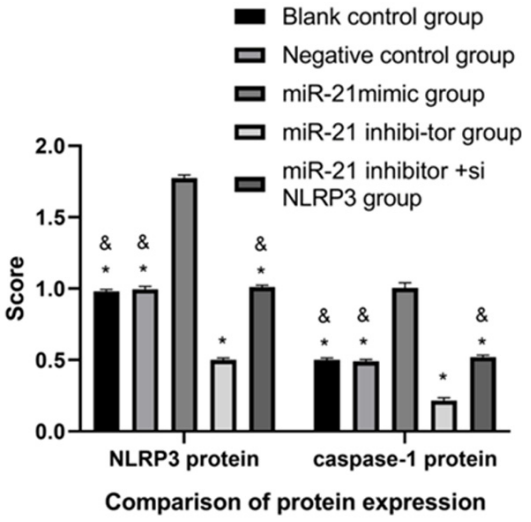

Figure 3. Comparison of the 5 Groups in their expressions of the NLRP3 and caspase-1 proteins. While no significant differences were found among the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group in their expressions of NLRP3 and caspase-1 (P>0.05), the highest and lowest expressions of NLRP3 and caspase-1 were observed in the miR-21 mimic group and the miR-21 inhibitor group respectively (P<0.05). * indicates P<0.05 compared with the miR-21 mimic group, & indicates P<0.05 compared with the miR-21 inhibitor group.

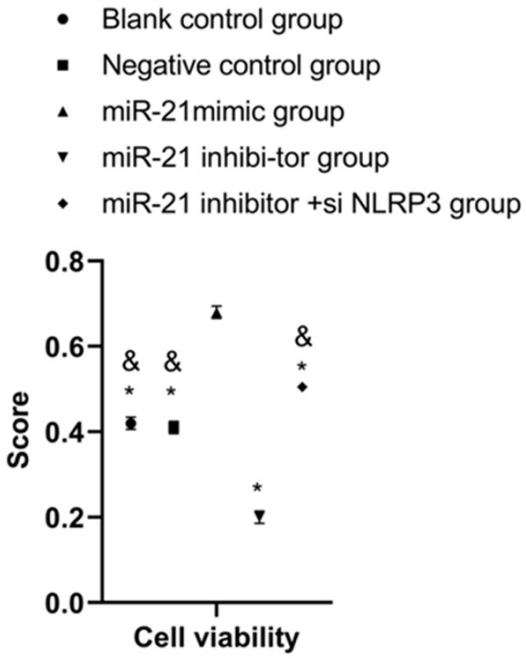

Figure 4. Comparison among the five groups in cell viability. For the cell viability, no significant differences were demonstrated among the blank control group, the negative control group, or the miR-21 inhibitor +si NLRP3 group (P>0.05), but the highest and lowest values were found in the miR-21 mimic group and the miR-21 inhibitor group respectively (P<0.05). * indicates P<0.05 compared with the miR-21 mimic group, & indicates P<0.05 compared with the miR-21 inhibitor group.

Cell viability in the five groups

MTT showed that the cell viabilities of the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group were not significantly different (P>0.05). Among the five groups, the highest and lowest cell viabilities were found in the miR-21 mimic group and the miR-21 inhibitor group respectively (P<0.05, **Figure 4**).

The IL-1β, IL-6, and IL-18 levels in the five groups

The tests and comparisons revealed no significant differences among the blank control

Table 2. Comparison of the five groups in their IL-1β, IL-6, and IL-18 levels ($\bar{x} \pm s$) (pg/ml)

Group	IL-1β	IL-6	IL-18
Blank control group	9.09±0.22	43.76±4.87	78.98±5.87
Negative control group	9.13±0.27	43.66±4.77	79.88±5.70
miR-21 mimic group	3.65±0.21*	20.98±4.81*	23.07±3.87
miR-21 inhibitor group	17.87±1.22*,#	63.89±5.98*,#	90.09±4.73
miR-21 inhibitor +si NLRP3 group	9.11±0.19	43.89±3.99	79.77±4.09

Note: compared with the blank control group, *P<0.05; compared with the miR-21 mimic group, #P<0.05.

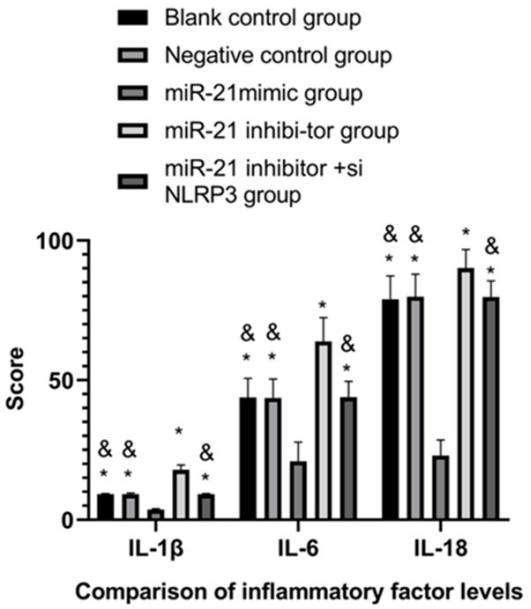

Figure 5. Comparison among the five groups' IL-1β, IL-6, and IL-18 levels. No statistical differences were observed among the blank control group, the negative control group, and the miR-21 inhibitor +si NLRP3 group in terms of their IL-1β, IL-6, and IL-18 levels (P>0.05). The three indexes reached their lowest levels in the miR-21 mimic group and their highest level in the miR-21 inhibitor group (P<0.05). * indicates P<0.05 compared with the miR-21 mimic group, & indicates P<0.05 compared with the miR-21 inhibitor group.

group, the negative control group, and the miR-21 inhibitor +si NLRP3 group in their IL-1β, IL-6, and IL-18 levels (P>0.05). The lowest and highest IL-1β, IL-6, and IL-18 levels were observed in the miR-21 mimic group and the miR-21 inhibitor group respectively (P<0.05, **Table 2** and **Figure 5**).

Discussion

In recent years, with the changes in living standards and diets of the Chinese, the case rate of cardiovascular and cerebrovascular diseases has been increasing on a yearly basis. Although a lot of manpower and material resources have been invested in their prevention, diagnosis, and treatment, and some positive results have been achieved, cardiovascular and cerebrovascular diseases are still the leading cause of death in China [15]. CHD is a complex cardiovascular disease that develops due to the influence of many factors such as environment and heredity. There are great differences in its pathogenesis and pathological mechanisms, so patients also face various clinical risks. At present, the commonly used ECG and myocardial injury markers in the clinical prediction of the above risks are somewhat limited, leading to insufficient attention to early diagnosis [16, 17]. Some studies have pointed out that the recent development of medical technology is pointing the prognoses of CHD patients in a favorable direction, but some patients still die due to high-risk cardiovascular events such as acute myocardial infarction or stroke a few months or years after their diagnoses, imposing heavy burdens on their families and on society [18]. Therefore, one of the key clinical study directions of CHD is to screen the analysis markers used to identify cardiovascular events or patients at a high risk of death, so as to improve the CHD prognosis and reduce the sudden death rate.

As a non-coding RNA with an endogenous regulatory function, micro-RNA can negatively control the target genes by degradation or translational suppression, so as to regulate the physiological mechanism [19]. Recent research has shown that there are about 1,880 micro-RNA precursors in the human genome, and they affect the expressions of 60% of the gene fragments, indicating that miRNA plays an important role in various biological processes in the human body, such as cell differentiation, proliferation, and apoptosis [20]. In addition, significant achievements have been made in the studies concerning tumor regulation and the repair of damaged cardiac muscle cells in recent years. Some studies point out that

miRNA participates in the regulation of endothelial cells, the proliferation of smooth muscle cells, and neovascularization, and affects the migration and apoptosis of malignant tumor cells at the same time, revealing the huge potential of miRNA in the prevention, differentiation, and diagnosis of cardiovascular diseases [21].

Being a member of the miRNA family, miR-21 is overexpressed in a lot of cancer tissues according to existing studies, and has a close relation with the development and progression of cancer. Furthermore, its extensive involvement in the development and progress of diversified cardiovascular diseases has also been confirmed by a number of studies [22]. However, there are few studies focusing on the effects of miR-21 on the apoptosis of endothelial cells in the coronary artery or on the expressions of the inflammatory factors. To address this shortage, animal and *in vitro* cell experiments were performed in the present study. The results revealed that, compared with the blank control group, in the negative control group and the miR-21 inhibitor +si NLRP3 group the expression of miR-21 rose while the expressions of NLRP3 and caspase-1 dropped in the miR-21 mimic group, and contrary results were observed in the miR-21 inhibitor group, indicating that the overexpression of miR-21 can inhibit the expressions of the NLRP3 gene and caspase-1 mRNA, as well as the NLRP3 and caspase-1 proteins according to a further comparison of protein expression. In some studies, the NLRP3 inflammasome was designated as a key point in the studies of cardiovascular diseases and a bridge between inflammation and immunity. It can maintain and activate inherent and adaptive immunity, and protect cells. However, the long-term existence of inflammasome in the human body will result in the overexpression of the inflammatory factors and the long-term inflammatory status, leading to inflammatory necrosis of cells and tissue damages [23]. The results of this study also show that the overexpression of miR-21 can inhibit the expressions of the inflammatory factors such as IL-1β, IL-6, and IL-18 - possibly due to NLRP3. Normally, NLRP3 is self-suppressed, but it is activated by pathological changes to raise the levels of IL-1β and IL-18 for an extensive participation in the inflammatory response [24]. As we found in this study, the significantly elevated expression of NLRP3 in the miR-21 inhibitor group indicates that a low expression of miR-21 results in the NLRP3 inflammasome being activated, leading to the elevation of various inflammatory factors and a series of waterfall reactions, worsening the inflammatory damage to the coronary artery. To further explore the effects of miR-21 on the apoptosis of endothelial cells in the coronary artery, this study compared different groups for the viability of cells after HCY. The results showed that the cells in the miR-21 mimic group were the most viable, and the cells in the miR-21 inhibitor group were the least viable [25]. In other studies, it has been shown that miR-21 is expressed at a high level in tumor tissues and extensively participates in the differentiation, apoptosis, and proliferation of cells. A high expression of miR-21 enhances the migration ability of tumor cells, and induces abnormal cell proliferation as well as an abnormal capacity against apoptosis [26]. This conclusion is also supported by the fact that the highest cell viability occurs in the miR-21 mimic group.

In conclusion, the overexpression of miR-21 alleviates the apoptosis of endothelial cells and reduces the expressions of various inflammatory factors in CHD patients, possibly due to the target suppression of the NLRP3 gene by miR-21. This mechanism is expected to generate new ideas and targets for the prevention, diagnosis, and treatment of CHD. Though this study explored the effects of miR-21 on the apoptosis of endothelial cells in the coronary artery and the expressions of inflammatory factors through animal experiments, the analysis is limited to NLRP3 and caspase-1, but it fails to be more specific at the molecular level. In addition, the results are not supported by clinical data. Future studies should focus on the participation of miR-21 in the clinical treatment of CHD and the molecular mechanisms, in order to build a more solid theoretical foundation.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Disclosure of conflict of interest

None.

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