Int J Clin Exp Med 2017;10(8):11759-11768
www.ijcem.com /ISSN:1940-5901/IJCEM0052217

Original Article

Inhibition of IK1 caused by KCNJ2 interference restrains ventricular rate in rat model of III° atrioventricular block

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Received March 5, 2017; Accepted March 31, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Purpose: Biological pacemaker pacing activity is affected by the inward rectifier potassium current (IK1) and the inward rectifier potassium channel (Kir2) is encoded by KCNJ2 gene. The purpose of this study is to investigate the effect of KCNJ2 gene and Kir2.1 protein on the ventricular rate, so as to provide a new effective idea for the study of biological pacemaker. Methods: The double-stranded DNA oligo which can cause most RNA interference effect to KCNJ2 was made. The small hairpin RNA (shRNA) was linearly connected to the pLVTHM vectors. The recombinant lentivirus was harvested from 293T cells. Rat III° atrioventricular block (AVB) model was constructed and the optimal virus titer was confirmed. Rats with III° AVB were transfected with virus and the ventricular rate was observed by ECG. The expression levels of KCNJ2 gene and Kir2.1 protein were analyzed with PCR, Western blot and immunity histochemistry. Results: The shRNA of KCNJ2 gene was successfully inserted into lentiviral vector. The titer of the virus was 1.07×10^9 TU/mL. The optimal virus titer to lentiviral vector transfection in rat was 1×10^9 TU/mL. After transfection, the expression of KCNJ2 gene and Kir2.1 protein was suppressed and then the ventricular rate of rats was increased. Conclusion: Inhibition of the expression of KCNJ2 gene and Kir2.1 protein can increase the ventricular rate in model rats.

Keywords: Lentiviral vector, III° AVB, KCNJ2, Kir2.1, IK1, ventricular rate

Introduction

Since the first artificial cardiac pacemaker was implanted in the human body in 1958, it had gradually become the preferred treatment of slow arrhythmia such as sick sinus syndrome and severe atrioventricular block [1]. However, some defects during its application have gradually exposed such as limited battery life, infections, reoperations and intravenous thrombosis [2-5]. Furthermore, for children, with the continuous growth and development, the pacemaker implanted earlier will gradually unable to meet the needs of the body. Then they will need to be reimplanted the pacemaker. This defect limits its use in these children.

With the progress of molecular biology, researchers try to take advantage of life science and technology research to develop a biological pacemaker to replace artificial cardiac pacemaker in order to overcome the above defects. At present, there are two major strategies in developing biological pacemaker: gene therapy and cell therapy. Cell therapy is a method of applying stem cells (embryonic stem cells and mesenchymal stem cells) and sinus node cells. But there are lots of unsolved problems such as immunological rejection, tumorigenicity and ethical issue, etc. However, these defects can be effectively avoided by gene therapy. Building biological pacemaker by gene therapy has received more and more attentions. Gene therapy is the application of genetic engineering to create human biological pacemakers. Developing biological pacemaker by gene therapy is rooted on three strategies: (1) over-expressing the neurohormone receptors to increase the atria electric activity [6, 7]; (2) over-expressing the HCN2 gene in diastolic phase [8-10]; (3) suppressing the inward-rectifier potassium current (IK1) to break the balance of the potassium current inside the ventricular cells [11, 12]. Researchers have found that it is the effect of

IK1 that keeps the resting membrane potential of adult ventricular myocytes at a negative potential level, thereby inhibiting the potential pacing activity [13]. Silva *et al.* [14-16] found that after suppression IK1 by 81%, the ventricular myocytes will generate a spontaneous action potential; pacing rates of ventricular myocytes will be increased with the increase of IK1 inhibition rate.

IK1 is a kind of potassium channel on cardiomyocytes [17]. It has a major effect on resting membrane potential and it is currently the most promising approach to biological pacemakers [18]. Kir2.1, which is encoded by KCNJ2 gene, is the channel protein of IK1. It is abundant in atrial and ventricular myocytes while sinus node cell is lack of this kind of channel. The overexpression of Kir2.1 is the main cause of myocardial weakness [19]. Researchers have found that overexpression of Kir2.1 in embryonic stem cell-derived cardiomyocytes alters their electrical phenotypes and lowers the risk of ventricular tachyarrhythmias [20]. Miake *et al*. [11] found that myocardial excitability was increased by inhibiting the expression of Kir2.1 in rat ventricular myocytes. Thereby further led to an increased frequency of ventricular beat. They also found that the ventricular IK1 current of guinea pigs was inhibited through the negative dominant. Guinea pigs occurred ventricular premature beats according to ECG.

In our previous study [21], we have researched the effect of KCNJ2 gene expression inhibition on the beating frequency with rat ventricular myocytes in vitro. The result showed that the beating frequency was significantly increased in rat ventricular myocytes. In the present research, we constructed III° AVB rat models and performed in vivo study with model rats. Through inhibiting IK1 by suppressing the expression of KCNJ2 gene and Kir2.1 protein with RNA interference technology, we explored whether the ventricular rate of model rats could be improved. The research may provide a new effective idea for the study of biological pacemaker.

Materials and methods

Materials

Healthy adult rats (Wistar, clean grade, average weight: 248 ± 12 g) were offered by animal center of Medicine School of Shandong University. Applications of the animal were in accordance with the specification for laboratory animal use of the Ministry of Health of the People's Republic of China.

Construction of lentiviral vector and determination of virus titer

According to our previous research, the double-stranded DNA oligo which can cause most RNA interference effect to KCNJ2 was made. The small hairpin RNA (shRNA) sequences were designed according to the optimal interference sites. Then the shRNA was linearly connected to the pLVTHM vectors (Shanghai Bion Biomedical Technology Co., Ltd, China) after annealed. The insertion sites are MluI and ClaI after the H1 promoter. The recombinant plasmids were transformed into competent cells (Invitrogen company, American) and then identified by digestion after collected. Digestion products were extracted for gel electrophoresis. The fragment of pLVTHM was 280 bp and positive siRNA clone was 340 bp.

Four plasmid DNA (pMDLg-pRRE, pRsv-REV, pMD2G, and interfering plasmids) were extracted with plasmid extraction kit (Axygen Corporation, American). The recombinant lentivirus was harvested from 293T cells (ATCC company, American) when they were cotransfected with lentiviral packaging system after 72 h. The virus particles were collected and transfected with Hela cells which were seeded in 6-well plates. The expression of GFP was observed under fluorescence microscope 4 days after transfection. The virus titer (TU/mL) was determined by gradient dilution method.

Establishment of rat III° AVB model

Healthy adult rats were fed for 1 week. Rats were fasted for 12 hours before surgery. Water was prohibited for 4 hours before surgery.

The rats were anesthetized with 3% sodium pentobarbital by intraperitoneal injection (dose: 30 mg/kg). Then they were placed in the supine position. Before surgery, ECG of these rats was recorded. The pericardium was incised along the longitudinal axis in order to expose the heart. A 50 μL needle was completely penetrated into the myocardium. The needle insertion was as **Figure 1A** and the blocking site of III°

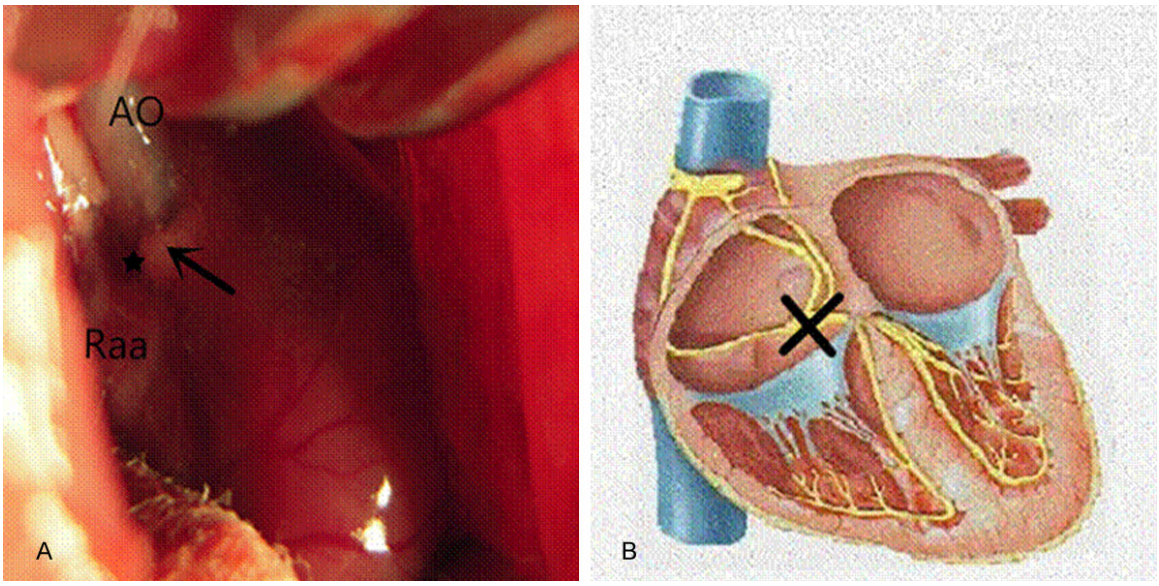

Figure 1. The injection site of the needle. A: Right atrial appendage (Raa); aorta (Ao); needle point (★); fat pad (↖). B: Blocking site of III° AVB rat models.

AVB rat models was as **Figure 1B**. Then 25 μL 70% ethanol was injected to destroy the atrioventricular node [22]. The changes of ECG were observed. If rats occurred III° AVB, the ECG was observed for another 30 min. In order to ensure the models were successfully constructed, another 72 h was observed for rats with III° AVB. The rats were treated appropriately after surgery and they were continued to be fed.

Determination of optimal titer

12 normal rats were divided into two groups: group A and group B. The rats in each group were conducted to the same surgery. With the apical surface of the heart as the center, 30 μL negative lentiviral vector was injected at 6 points (group A: 1×10^8 TU/mL; group B: 1×10^9 TU/mL). The diameter of the 6 points was about 1 cm around the apical surface of the heart. In order to collect myocardial tissues, 2 rats in each group were euthanized after anesthesia on the 4th day, 7th day and 10th day after transfection. The myocardial tissues were prepared into frozen sections before fluorescence detection. The transfection rate was detected by immunofluorescence microscopy and then the optimal virus titer was confirmed.

Grouping and viral transfection of rat models

The III° AVB rat models were divided into three groups: (1) Interference group: transfected with lentiviral vector (lentiviral vector with shRNA); (2) Negative control group: transfected with negative lentiviral vector (lentiviral vector without shRNA); (3) Control group: operated without transfection. ECG was recorded 30 minutes before surgery. With the apical surface of the heart as the center, transfection of each group was performed at 6 points. The diameter of the 6 points was about 1 cm around the apical surface of the heart. Each point was injected with 5 μL. ECG was recorded 30 minutes after transfection. On the 7th day, 14th day and 28th day after transfection, the ventricular rate was also evaluated by ECG.

Detection of KCNJ2 gene after transfection

RNA was extracted from myocardial tissues of rats in each group and the reverse transcription was performed. The RNA expression of KCNJ2 gene in the myocardium of experimental rats was examined by RT-PCR and Q-PCR. Three-step cycling of RT-PCR was performed as follows: denaturation for 30 s at 94°C, annealing for 30 s at 57°C and extension for 30 s at 72°C. The 3-step cycle was repeated for 34 cycles and followed by a final extension at 72°C for 8 minutes. Three-step cycling of Q-PCR was performed in 40 cycles of 94°C for 30 s, 57°C for 15 s and 72°C for 45 s. The fragment length of β-actin primer gene sequence was

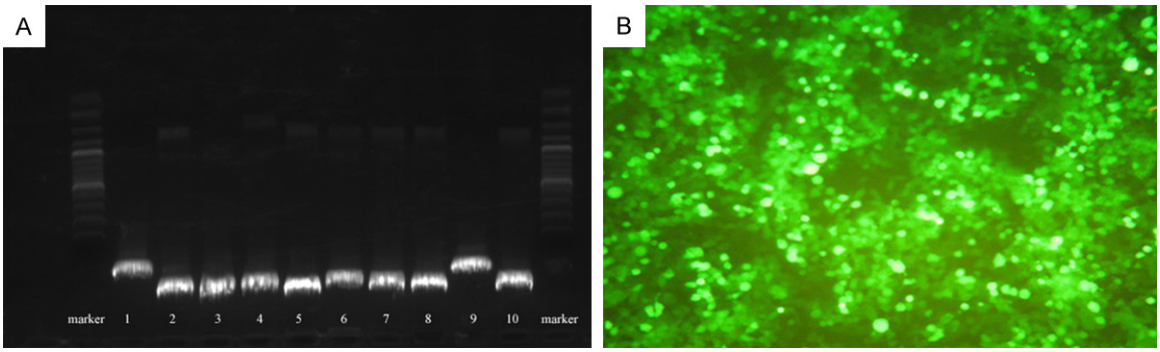

Figure 2. A. Double enzyme digestion of the expression vector. Marker fragments were 3000, 2500, 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 in an order from large to small. 2, 5, 6, 7, 8, 10 were about 330 bp, which belonged to positive clone. 4 was 250 bp, which belonged to negative clones. B. Transfected 293T cells under fluorescent microscopy. The transfection efficiency was above 90%.

243 bp. The sequence of KCNJ2 primer gene was as follows: upstream 5'-TTAGAAGCATCCAGATCCCCG-3'; downstream 5'-GCGGTGGATGCTGGTAATCTT-3'. Its fragment length was 373 bp.

Detection of the expression of Kir2.1 protein after transfection

Detection by immunohistochemistry: The myocardial tissue was fixed in formaldehyde solution for 24 h before it was embedded in paraffin at 60°C. Then the paraffin block was made into paraffin sections with the thickness of 20 μm. After dewaxed with xylene and dehydrated with alcohol, the sections were placed in the citrate antigen retrieval solution for antigen retrieval at 98°C for 15 min. Then the sections were incubated overnight at 4°C with rabbit anti-Kir2.1 (1:100, Abgent company, American), followed by incubation with biotinylated secondary antibody (1:200, Beijing Zhong Shan Jinqiao Biological Company, China) at 37°C for 30 min. DAB chromogenic reagent (Beijing Zhong Shan Jinqiao Biological Company, China) was used to staining for 10 minutes before using 10% hematoxylin to staining for 3 minutes. The color of positive staining material was brown.

Detection by western blot: The protein sample solution was obtained from the myocardial tissue sections. Then 20 μL protein sample solution was separated by SDS-PAGE and transferred onto PVDF membranes at 100 mA for 120 min. The membrane was blocked with blocking solution. Then it was incubated overnight at 4°C with rabbit anti-Kir2.1 (1:100), followed by incubation with biotinylated secondary antibody (1:200) at room temperature for 12 h. Chemiluminescence was detected using the ECL Western Blotting Detection Kit (Ferments Company, Canadian).

Statistical analysis

The data were analyzed with SPSS 17.0. All data were expressed as mean ± standard deviation (SD). Differences between groups were compared using one-way ANOVA and unpaired t-test. The $P < 0.05$ was considered statistically significant.

Results

Construction lentiviral vector and determination of virus titer

According to our previous research, the double-stranded DNA oligo which can cause most RNA interference effect to KCNJ2 is as follows: 5'-CGCGTCCCCgcgtgtgtgtctgaggtcaTTCAAGAGAtgacctcagacacacacgcTTTTTGGAAA3'; 5'-CGATTTCCAAAAAgcgtgtgtgtctgaggtcaTCTCTTGAAtgacctcagacacacacgcGGGGA-3'. shRNA was designed according to the optimal interference sites. The lentiviral vector also contains the EF1-alpha promoter, which regulates the expression of GFP.

The target gene which was obtained after double enzyme digestion reaction of the constructed lentiviral vectors was conducted to gene amplification and the amplification products was detected by agarose gel electrophoresis as is shown in **Figure 2A**, indicating that the con-

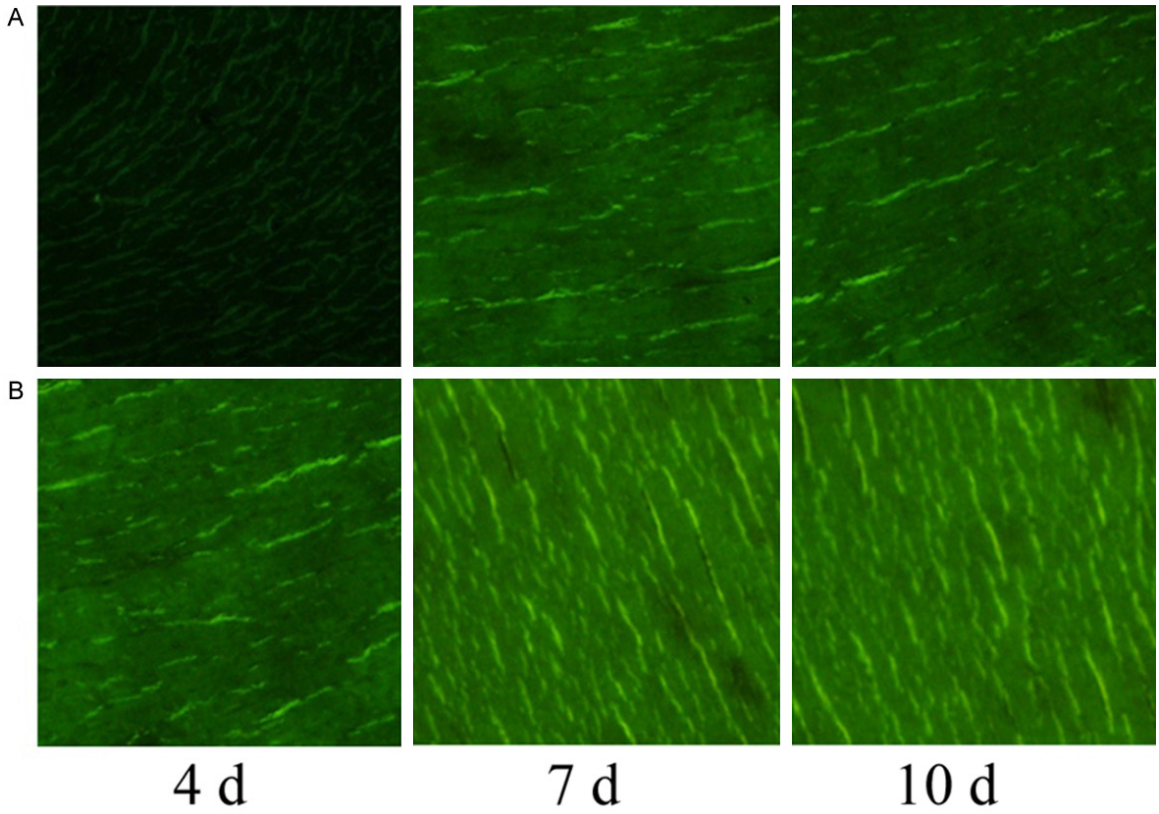

Figure 3. Fluorescence microscopy photos of lentiviral vector-transfected myocardial tissue at different time points in different virus titers. (virus titer: group A 1 × 10^8 TU/mL, group B 1 × 10^9 TU/mL).

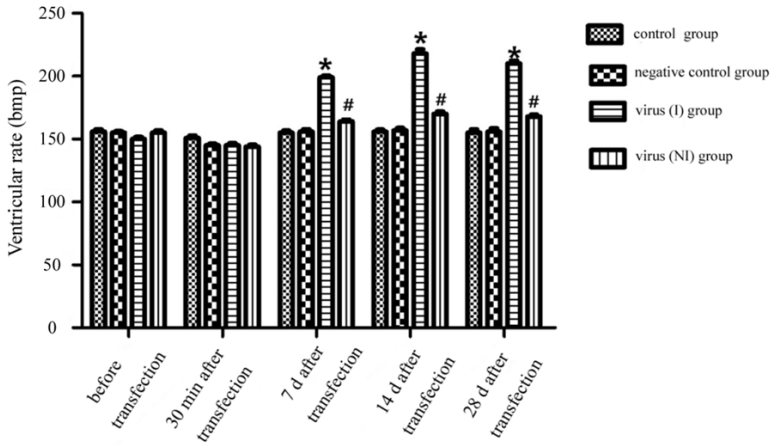

Figure 4. The ventricular rate of each group at different time points after transfection. *There was statistically significant difference among virus (I) group, control group and negative control group ($P < 0.05$). #No statistically significant difference was found among virus (NI) group and control group and negative control group.

struction of lentiviral vector with interference fragment was achieved. The 293T cells which were transfected with the packaged virus solution for 48 h were observed under fluorescent microscope and the transfection efficiencies was above 90% (**Figure 2B**), indicating that the packaging of lentivirus was achieved and the virus was of good quality. The virus titer was 1.07 × 10^9 TU/mL according to gradient dilution method.

Establishment of rat III° AVB model

Compared to normal rats (456 ± 24 times/min), the heart rate of model rats (156 ± 6 times/min) was significantly decreased. Forty eight rats were successfully modeled according to ECG. Rats of III° AVB model were treated with virus transfection. The number of rats in each group was as follows: control group (n = 12); negative control group (n = 14); virus intervention group (n = 22).

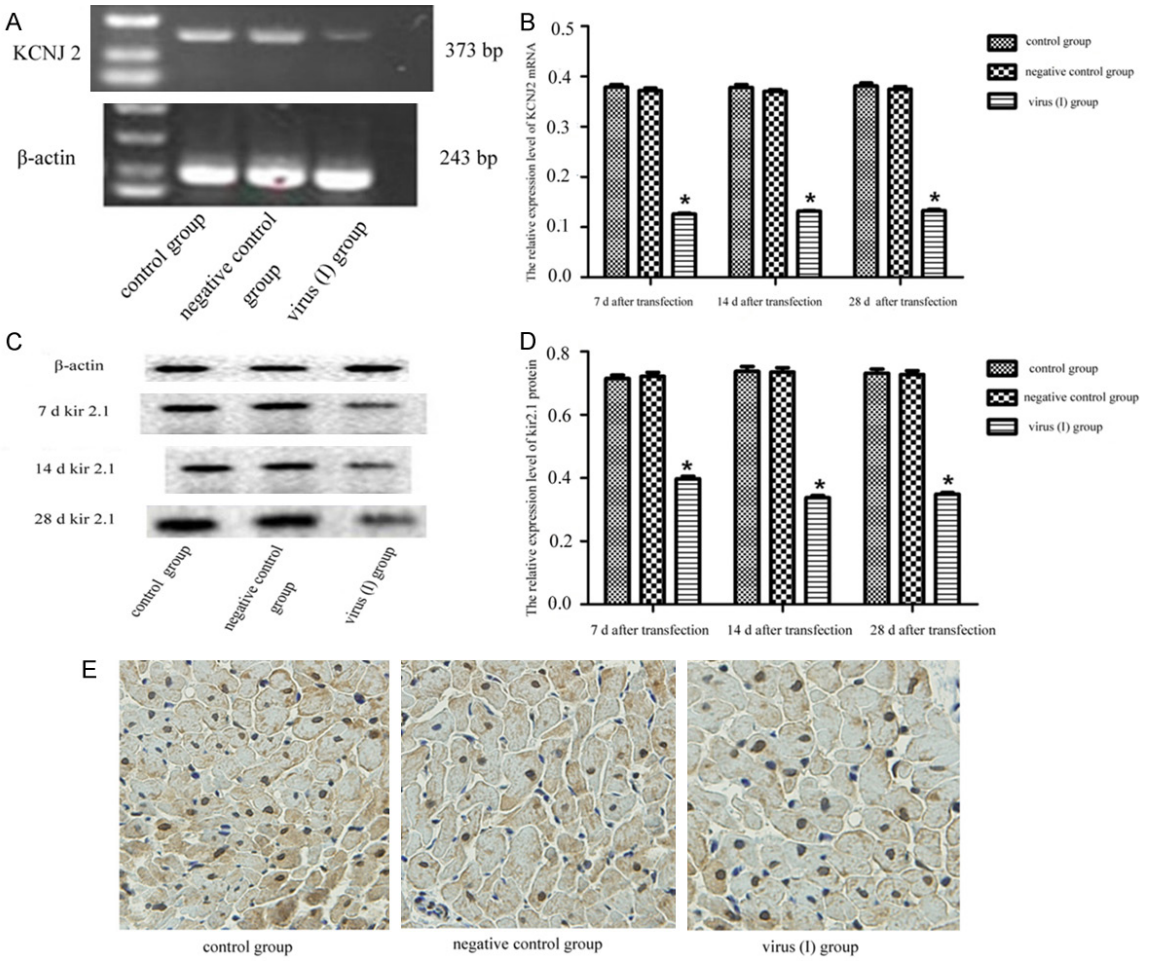

Figure 5. The expression of KCNJ2 gene and Kir2.1 protein after transfection. A. Expression level changes of KCNJ2 mRNA in rat ventricular myocytes 7 days after virus infection; B. The relative expression level of KCNJ2 mRNA in rat ventricular myocytes at different time points after virus transfection. *There was statistically significant difference among the virus (I) group and the negative control group and the control group ($P < 0.01$). C. The expression of Kir2.1 protein in rat ventricular myocytes at different time points after virus transfection; D. The relative expression level of Kir2.1 protein in rat ventricular myocytes at different time points after virus transfection. *There was statistically significant difference among the virus (I) group and the negative control group and the control group ($P < 0.01$). E. Kir2.1 protein expression of rats detected by immunohistochemistry 7 days after transfection. The color of positive staining material was brown. It was expressed both in cytoplasm and cell membrane. The expression of virus (I) group decreased significantly compared with the former two groups.

Titer determination of lentivirus transfecting myocardial tissue

Myocardial tissue frozen sections of two group rats (group A and group B) was obtained at different time points and was observed under fluorescent microscopy (**Figure 3**). The transfection efficiency was higher on the 7th day than that on the 4th day of the two groups (virus titer: group A 1×10^8 TU/mL; group B 1×10^9 TU/mL). There was no significant difference in transfection efficiency between the 10th day after transfection and the 7th day after transfection. The results indicated that the transfection efficiency was relatively stable after 7 days and the transfection efficiency was higher in group B. So we selected the virus titer of 1×10^9 TU/mL to transfect myocardium tissue.

The changes of ventricular rate after virus transfection

The ventricular rate of 12 rats of the virus intervention group was increased 7 days after transfection. Then the 12 rats were named as virus (I) group and the remaining rats of the virus intervention group named as virus (NI) group. The ventricular rate was gradually increased

after transfection in rats of the virus (I) group (**Figure 4**). On 14 days after transfection, it reached the fastest. Statistical analysis showed that, compared with control group and negative control group, the ventricular rate was significantly increased in virus (I) group after transfection ($P < 0.05$). The increasing rate was about 134.8%. No significant difference was found between control group and negative control group.

The changes of KCNJ2 gene after transfection

The amplified fragment length of KCNJ2 mRNA in rat myocardial tissue was 343 bp. The electrophoresis band location of RT-PCR product was consistent with the theoretical value (**Figure 5A**). The results of Q-PCR detection showed that the relative expression of KCNJ2 mRNA in the ventricular muscle tissue of virus (I) group was significantly lower than that in the control group and negative control group ($P < 0.01$) (**Figure 5B**). The relative expression of KCNJ2 mRNA was the lowest on the 7th day after transfection (0.126 ± 0.002) and there was no significant difference when it was compared with 14 days after transfection (0.132 ± 0.001) and 28 days after transfection (0.133 ± 0.002), indicating that the expression of KCNJ2 mRNA in the ventricular muscle tissue of the virus (I) group was stable from the 7th day after transfection. Namely, the interference of lentivirus to KCNJ2 gene was stable 7 days after transfection and the inhibition rate was about 77%.

The expression of Kir2.1 protein after transfection

Testing results of western blot: The relative expression of Kir2.1 protein in the virus (I) group was significantly lower than that in the control group and negative control group ($P < 0.01$) at the time points of the 7th day (0.398 ± 0.008), 14th day (0.338 ± 0.006) and 28th day (0.349 ± 0.005) after transfection. Furthermore, the relative expression of Kir2.1 protein of the virus (I) group was the lowest (0.338 ± 0.006) on 14th day after transfection and the inhibition rate of protein expression was about 55%. No significant difference was found between negative control group and control group (**Figure 5C**, **5D**).

Testing results of immunochemistry: The color of positive staining material was brown. The expression localization of Kir2.1 protein in virus (I) group and negative control group was consistent with that of control group (**Figure 5E**). Semi-quantitative results showed that the expression of Kir2.1 protein in virus (I) group at different time points was significantly lower than that in the control group ($P < 0.01$). The expression level of Kir2.1 protein of virus (I) group gradually became stable from the 7th day after transfection.

Discussion

In this study, the interference sequences were constructed by using the optimal interference sites of KCNJ2 gene which was confirmed in the early stage of research. The construction of lentiviral vector with interference fragment was achieved. During the rat modeling process, in order to facilitate the depth of the needle toward the nodal tissue, the needle had been prepared by making a 90° bend in the shaft 3 mm from the tip. Thus the needle could only be inserted into the myocardium up to a maximum of 3 mm from the epicardial surface [23, 24]. The success rate of modeling was about 71% and the model rats could be maintained long-term stability. The optimal virus titer to transfect myocardium tissue was 1×10^9 TU/mL by gradient dilution method. Compared with other ways, gradient dilution method was more accurate and reliable [5, 24-27]. Transfection of rats with III° AVB model was performed with lentiviral vector. The ventricular rate of the virus (I) group was significantly increased when compared with control group and negative control group. The increasing rate was about 134.8%. The PCR result showed that the expression of KCNJ2 gene decreased significantly in the virus (I) group and the highest inhibition rate was about 77%. The expression of Kir2.1 protein in rat ventricular myocardium was detected by Western Blot and immunohistochemistry. The expression of Kir2.1 protein in the virus (I) group was significantly lower than that in the control group and the negative control group. The highest inhibition rate was about 55%, which was consistent with the decrease of KCNJ2 gene expression in ventricular myocardium. It was confirmed that the ventricular rate of rats with III° AVB model could be improved through inhibition IK1 by silencing KCNJ2 gene and Kir2.1 protein.

IK1, which is encoded by KCNJ2 gene, is widely distributed in myocardial tissue. It has strong inward rectification effect [28, 29]. IK1 belongs

to the Kir2x family. The number of Kir2.1 in the myocardium accounts for about 80% of the total amount of IK1 [30, 31]. Researchers have found that IK1 dysfunction was related to a variety of arrhythmias [32-35]. Overexpression of Kir2.1 in transgenic mice may be associated with arrhythmias such as slow heart rate, ventricular premature beats, atrioventricular block, atrial fibrillation and ventricular fibrillation [36-41]. Silva and Rudy also believed that inhibition of ventricular IK1 current was the ideal way to construct a biological pacemaker [14]. In 2007, Channels et al. found that inhibition of IK1 currents in ventricular myocytes could induce ventricular myocytes to restore pacing activity [42]. This result further provides experimental evidence for the construction of biological pacemaker by inhibiting ventricular IK1 current. IK1 is highly expressed in ventricular and atrial myocytes. It has the ability of keeping the resting potential at a negative level and contributes significantly to repolarizing current during the terminal phase of the action potential in ventricular myocytes. Kir2.1 assemble to form tetrameric inward rectifier potassium channels in many cell types, including cardiac myocytes [28, 43]. It is the inward rectifier potassium channel and is essential to the generation of IK1. Ventricular myocytes will presumably be converted to pacemaker cells if IK1 is inhibited [44]. We presumed that the presence of a large amount of IK1 will presumably inhibit the pacing ability of myocardial cells. In this research, the expression of Kir2.1 protein in rat ventricular myocardium was inhibited through silencing KCNJ2 gene and then IK1 was further suppressed. The results indicated that the ventricular rate of the virus (I) group was significantly increased. This results were consistent with the previous results and verified the above speculation. It provided a certain experimental basis for the treatment of slow arrhythmia by constructing biological pacemaker in vivo.

Of course, there were also limitations. Why did the change in the ventricular rate of the model rats in the virus (NI) group be insignificant and whether it was related to viral transfection process or other interfering factors. These questions still need to be further explored. In addition, the increase rate of ventricular rate of the virus (I) group was still limited compared with the normal rats. Whether cardiac function and activity of model rats can be improved and how effective it is still need to be further studied. Moreover, whether it can further improve the ventricular rate of the rat model by improving the viral vector to further increase the inhibition rate of KCNJ2 gene in the animal model also need to continue to study.

In conclusion, this study established a stable rat III° AVB model and found that there was a negative correlation between changes of ventricular rate and the expression of KCNJ2 gene and Kir2.1 protein. It was confirmed that the increase of beating frequency of ventricular myocytes in rats with III° AVB was caused by inhibiting the IK1 through suppressing the expression of KCNJ2 gene and Kir2.1 protein in myocardium. This proved that it is feasible to increase the ventricular rate of rat by inhibiting the KCNJ2 gene to inhibit IK1 in the rat ventricular myocytes. The study provides a valuable experimental basis for in vivo study of biological pacemakers.

Acknowledgements

This article is supported by China Shandong Provincial Natural Science Foundation (ZR20-13HM056).

Disclosure of conflict of interest

None.

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