Effect of RGOs on the Efficacy of Nkx2.5 Transfected Bone Marrow Mesenchymal Stem Cells Transplantation in Treatment Heart Failure in Rats

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Research

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Abstract

Background: Heart failure (HF) is one of the major serious diseases to do harm to human health. Drugs, interventional treatment and heart transplantation are currently the main methods to treat HF. However, they fail to improve the patient's condition. Mesenchymal stem cells (MSCs) can regenerate functional cardiomyocytes and are promising to become a new therapeutic measure to treat heart failure. We assumed that *Rehmannia glutinosa* oligosaccharide (RGOs) has the synergistic effect with Nkx2.5 transfected MSCs (Nkx2.5) transplantation in treatment heart failure.

Methods: MSCs and Nkx2.5 were preconditioned by RGOs. The apoptosis rate was detected by flow cytometry and the expressions of cardiac specific genes were analyzed with quantitative real-time PCR and Western blot *in vitro*. Heart failure models were duplicated by injecting doxorubicin (total dose of 15mg/kg) intraperitoneally in male SD rats. When the models were prepared, rats were randomly divided into 6 groups: Control (CON), HF, MSCs, Nkx2.5, RGOs and RGOs combined with Nkx2.5 (RGOs+Nkx2.5) group. Echocardiography was used to detect cardiac function in rats. HE staining was used to observe the pathological changes of myocardium, and Masson staining was used to calculate the collagen volume fraction to detect the degree of myocardial fibrosis. The total mRNA was extracted to detect the following genes including cTnI, CX43, TGF- β 1, Collagen I, MEF2 and GATA4 by Q-PCR. Protein of myocardial tissue was extracted to detect the expression of cTnI, CX43, MEF2 and GATA4, by western blot.

Results: RGOs could not enhance cardiac specific gene expressions including CK, α -MHC, however it improved the survival of Nkx2.5 induced by H_2O_2 *in vitro*. In rat heart failure models, RGOs alone improved the heart pumping function and decreased collagen volume fraction (CVF), TGF- β 1 and collagen I expression, and increased MEF2 and GATA4 mRNA expression. Moreover, RGOs cooperated with Nkx2.5 in improving left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV). Furthermore, RGOs and Nkx2.5 combination also increased CX43 expression, whereas decreased CVF and collagen I expression.

Conclusion: RGOs has the synergistic effect with Nkx2.5 gene transfected MSCs transplantation in treatment with heart failure through decreasing myocardial fibrosis, inhibiting ventricular remodeling, and increasing the expressions of GATA4, MEF2.

Background

Heart failure (HF) is a clinical syndrome of breathlessness, lower extremity swelling, fatigue, and exercise intolerance affecting a large portion of the population worldwide [1]. It is a consequence of various cardiovascular diseases, including, but not limited to, myocardial infarction, hypertension, heart valve disease, and myocarditis [2]. Cardiac function of these patients is increasingly compromised with the progression of adverse ventricular remodeling and cardiac fibrosis, and many eventually develop fatal end-stage heart failure. Heart failure is associated with high rehospitalization rate and poor prognosis,

which imposes enormous burden on health and economy globally [3]. Current medical and intervention therapies have limited efficacy for heart failure, and heart transplantation is the only radical treatment but is problematic because of donor shortage [4].

Stem cell transplantation is a new technology developed in recent years. Cell differentiation and paracrine can be used to replace and repair damaged myocardium and reverse ventricular remodeling to improve the cardiac function and improve the quality of life of patients with heart failure [5]. Based on the preclinical and clinical data available to date, mesenchymal stem cells (MSCs) are considered a promising cell type for the treatment of heart failure. MSCs are nonhematopoietic, multipotent self-renewing cells with the ability to differentiate into multiple mesodermal lineage cell types and to transdifferentiate into ectodermal and endodermal cells [6]. However, stem cell therapy suffers from low rates of retention, survival, and differentiation at the target site [7]. Therefore, it is very important to find a safe method for inducing differentiation of MSCs into cardiomyocyte.

Nkx2.5 gene, also known as CsX gene, is a homeobox transcription factor that is required for the formation of the heart and vessels during development, with significant postnatal down-regulation and reactivation in disease states, characterized by vascular remodeling, and also with the effect of maintaining a series of cardiac related genes, including GATA4, TBX5, and MEF2C, and the occurrence of normal cardiac morphology [8]. In our previous cell experiments, we found that Nkx2.5 improved cardiac function, promoted the differentiation of transplanted MSCs into cardiomyocyte-like cells, decreased fibrosis formation [9]. These considerations have focused our interests on enhancing the therapeutic effect of Nkx2.5 gene transfection.

Rehmannia glutinosa Oligosaccharides (RGOs), a low molecular weight polysaccharide component extracted from Chinese Traditional Medicine, which can protect ischemic myocardium by scavenging oxygen free radicals and reducing cell apoptosis [10]. The previous studies revealed that RGOs played an important role in cell regeneration [22]. So we hypothesized that RGOs could assist to enhance the efficacy of Nkx2.5 transfected bone marrow mesenchymal stem cells transplantation in treatment heart failure. During our experiment, the effect of RGOs combined with Nkx2.5 gene transfection on MSCs in the treatment of heart failure was studied and the synergistic effect of RGOs and Nkx2.5 gene transfection on MSCs transplantation was analyzed to support the hypothesis.

Methods

Bone marrow mesenchymal stem cells (MSCs)

The isolation, culture, identification and gene transfection of MSCs were prepared as described previously [8,9,11]. Briefly, MSCs were isolated from the lower limb bone of 4-week-old male rats (supplied by the Experimental Animal Center of PLA General Hospital), and cultured in culture medium (α -MEM+10% fetal bovine serum+100U/ml of penicillin, and 100mg/ml of streptomycin). Then they were identified using fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD29, CD44, CD11b (ebioscience, Inc., San Diego, CA, USA), CD34 (Novus Bio-logicals, LLC., Littleton, CO, USA),

analyzed by fluorescence-activated cell sorting (Epics XL cytometer, Beckman Coulter). MSCs were transfected with the recombinant virus vector containing Nkx2.5 according to the previously described methods[12]. The Nkx2.5 was transfected by the recombinant virus at a multiplicity of infection of 26.4.

Apoptosis detection

Annexin V/PI double staining treatment groups were induced apoptosis by H₂O₂. When the cells grew to 85-95% fusion, H₂O₂ solution was added to make the final concentration 100μmol/L, and the cells acted in the incubator for 24h. After that the cells were collected and centrifuged for 5 min at 2000 r/min. The supernatant was discarded and then washed twice with PBS buffer. The cells were resuspended in 300 μL of binding buffer, and FITC-labeled Annexin V (20 μg/mL) and PI (50 μg/mL) (BD PharMingen, USA) were added, 5μL each. The reaction proceeded in the dark for 15 min, and cell apoptosis was detected by flow cytometry (BD FACS ARIA II, SORP, USA) [13].

Heart failure models establishment

To establish Heart failure model in rats, we followed the methods of Deng et al 2017 [9]. After the rat heart failure model was induced, 60 rats were randomly divided into 6 groups: The control group, heart failure group (HF), MSCs group, Nkx2.5 group, RGOs group, and RGOs+Nkx2.5 group. The MSCs group, Nkx2.5 group and RGOs+Nkx2.5 group injected MSCs or MSCs-Nkx2.5 (0.5 ml per rat, cell suspension 2.0× 10⁷/ml) via caudal vein. The control group, HF group and RGOs group received Low sugar DMEM medium (0.5 ml per rat). Then the RGOs and RGOs +Nkx2.5 group were given RGOs by intraperitoneal injection for 10 days (200mg/kg/d). Other groups were injected with normal saline 2ml/kg/d for 10 days.

Echocardiography

Cardiac function was assessed at 4 weeks after cell transplantation by transthoracic echocardiography, which was performed by an investigator who blinded to treatment allocation [15]. Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 7.5-MHz transducer. End diastole was measured at the time of the apparent maximal LV diastolic dimension, and end systole was measured at the time of the most anterior systolic excursion of the posterior wall, according to the American Society for Echocardiology leading-edge method from at least 3 consecutive cardiac cycles.

Histological and immunohistochemical analysis

Ten weeks after cell transplantation and RGOs treatment, the rats were euthanized with a pentobarbital (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) overdose (200mg/kg). The hearts were quickly excised and fresh frozen in Tissue Tek O.C.T. freezing medium (Sakura, Torrance, California). 5-μm sections were made and stained with Masson's trichrome. Four slides, equally distributed through the injured area, were taken from each heart according to the methods suggested by Deng et al and Kim et al,

respectively [9,16]. Collagen volume fraction (CVF) was measured with SPOT 3.5.1 software (Diagnostic Instruments, Sterling Heights, Michigan).

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from 50 to 100 mg of the frozen heart tissues by 1 ml TRIzol (Invitrogen), One microgram of total extracted RNA was reverse transcribed to cDNA via Prime Script RT reagent kit (Takara Bio Inc.) following the manufacturer's protocols, and quantitative real-time PCR was performed in duplicate in a total reaction volume of 25µl using SYBR-Green master mix (Applied Biosystems, Foster City, CA) using the conventional protocols. The primer sequences for real-time PCR were listed in Table 1. The stability of 36β4 expression among different groups verified the application of this gene for an appropriate endogenous control for normalization. Quantitative data was calculated using the comparative CT method.

Western blotting

Tissue or cellular proteins were prepared were extracted and the protein preparations were loaded on 12 % Bis–Tris polyacrylamide gels for electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). After blocked with 5 % nonfat milk in PBST (PBS plus 0.05 % Tween 20) for 1 h, the PVDF membranes were incubated with mouse monoclonal antibody for GAPDH in PBST containing 5 % nonfat milk at 4°C overnight. The density of the bands was scanned and analyzed with NIH Image J software. The density of the CX43, cTNI, MEF2 and GATA4 band was normalized to the density of GAPDH band in each sample.

Statistical analysis

Statistical analysis was performed with SPSS17.0 software. Group data were presented as mean ± SEM. Differences between two groups were evaluated by Student's t-test. Differences among groups were determined by one-way analysis of variance, followed by the Scheffe multiple-comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

RGOs improved the survival rate of MSC-Nkx2.5 induced by H2O2 in vitro

In order to study the effects of RGOs on apoptosis of MSC-Nkx2.5, we used H₂O₂ to induce apoptosis. Results showed that the apoptosis rate of MSCs transfected with Nkx2.5 gene was reduced to 51.7 ± 8.4%. RGOs treatment reduced the apoptosis rate to 38.9 ± 3.2%, which was lower than Nkx2.5 group. Even RGOs combined Nkx2.5 reduced the apoptosis to 36.1 ± 2.8% (Fig. 1A). However, this increase in survival was not due to increased conversion to cardiomyocytes. Our results showed that CK mRNA expression in RGOs group was slightly higher than MSCs group, while α-MHC mRNA expression in RGOs group was a little bit lower than MSCs group. Compared with MSCs group, CK mRNA and α-MHC mRNA

expression in RGOs + Nkx2.5 combined group increased by 3.7 ± 0.3 times and 10.6 ± 0.6 times respectively. And Compared with Nkx2.5 group, CK mRNA and α -MHC mRNA expression in the combined group showed an increased trend, but there was no statistical significance (Fig. 1B and 1C).

The effect of RGOs on the efficacy of Nkx2.5 in improving left ventricular function

We measured body weight (BW), wet weight (WW) and heart-to-body-weight ratio (WW/BW) after 10 weeks treatment. We found that heart-to-body-weight ratio (WW/BW) in heart failure (HF) group was significant increased to 3.12 ± 0.25 mg/g. While WW/BW of RGOs group and RGOs + Nkx2.5 combined group reduced to 2.93 ± 0.13 mg/g and 2.85 ± 0.17 mg/g respectively. But compared with Nkx2.5 group, WW/BW of RGOs + Nkx2.5 combined group showed a downward trend but no statistical significance (Fig. 2A). Echocardiography was used to study the effect of RGOs on cardiac function in rats with heart failure. The left ventricular internal dimension at systolic (LVID-s) and left ventricular end-systolic volume (LVESV) in the RGOs group significantly decreased compared with HF group. Moreover, the reduction of LVID-s, LVEDV and LVESV in the RGOs + Nkx2.5 combined group was even more pronounced than that in Nkx2.5 group and RGOs group separately (Table 2). Therefore, the left ventricular function of RGOs group increased normally to 67.53% in LVEF and 39.6% in LVFS compared with HF group. Similarly, the left ventricular function of RGOs + Nkx2.5 combined group was significant higher than RGOs group. And compared with Nkx2.5 group, LVEF and LVFS in combined group showed an upward trend but no statistically significant difference (Fig. 2B and 2C).

RGOs increased CX43 mRNA expression in rat models of heart failure

In order to investigate the effect of RGOs on the transformation of stem cells into myocardium, we detected the expression changes of myocardial specific genes CX43 and cTnI. Compared with control group, CX43 mRNA and protein expression were significantly decreased in HF group. On the contrary, CX43 mRNA in RGOs group was increased to 5-fold higher than HF group. And CX43 mRNA in RGOs + Nkx2.5 combined group was even increased to 9.0-fold higher than HF group, which was obviously higher than RGOs group. Compared with Nkx2.5, CX43 mRNA in RGOs + Nkx2.5 combined group showed an increasing trend without statistical difference. Moreover, CX43 mRNA and protein expression in RGOs and combined group had the consistent changes trend. The increase of CX43 protein expression in RGOs + Nkx2.5 combined group was higher than RGOs group and Nkx2.5 group respectively (Fig. 3A). The mRNA expression of cTnI in Nkx2.5 group, RGO group and RGO + Nkx2.5 combined group showed a trend of gradual increase, but this trend was not obvious in the protein expression of cTnI. cTnI protein expression in RGO group and the combined group were similar, slightly higher than that of the Nkx2.5 group, but no statistical difference was found (Fig. 3B).

RGOs enhanced the efficacy of Nkx2.5 in reducing myocardial fibrosis in rat models of heart failure

The degree of left ventricular fibrosis was detected by Masson staining. In control group, the myocardial fibers were arranged neatly with a small amount of collagen fibers. But in HF group, the number of blue collagen fibers increased significantly (Fig. 4A). Collagen volume fraction (CVF) was calculated to

evaluate the degree of myocardial fibrosis. CVF in HF group was significantly higher than the control group (6.74 ± 0.87 vs. 2.73 ± 0.59). CVF of RGOs group was lower than that of HF group. The decrease of CVF in RGOs + Nkx2.5 group was more obvious than that in Nkx2.5 group and RGOs separately (Fig. 4B). TGF- β 1 and Collagen α mRNA expression in HF group increased dozens of times more than control group. However, TGF- β 1 and Collagen α mRNA expression significantly decreased in RGOs group, even Collagen I mRNA expression in RGOs + Nkx2.5 combined group decreased more than Nkx2.5 group and RGOs group separately. While TGF- β 1 in RGOs + Nkx2.5 combined group showed a downward trend but no statistical significance compared with the Nkx2.5 group (Fig. 4C and 4D).

Effects of RGOs and Nkx2.5 on MEF2 and GATA4 expression

The expression of MEF2 mRNA and protein in HF group were significantly lower than those in the control group. Compared with HF group, the expression of MEF2 mRNA and protein were significantly increased in MSCs group. The increase of Nkx2.5 group was more obvious than that of MSCs group. The expression of MEF2 mRNA and protein in RGOs group were higher than those in HF group. Similarly, the expression of MEF2 mRNA and protein in RGOs + Nkx2.5 group was also higher than those in RGOs group. But there was no significant difference between RGOs + Nkx2.5 group and Nkx2.5 group (Fig. 5A and 5B). Compared with the control group, the mRNA expression of GATA4 decreased by 40.09 times, and the protein expression of GATA4 decreased by 3.72 times in HF group. The expression of GATA4 mRNA and proteins were higher in Nkx2.5 group and RGOs group than that in HF group, moreover the degree of increase in Nkx2.5 group was significantly higher than that in RGOs group, but there was no significant difference between the RGOs + Nkx2.5 group and Nkx2.5 group (Fig. 5C and 5D).

Discussion

Our results mainly documented the effect of RGOs on the efficacy of Nkx2.5 transfected bone marrow mesenchymal stem cells transplantation in treatment heart failure in rats. First, we found that RGOs and RGOs + Nkx2.5 combined group had a strong protective effect on H₂O₂-induced apoptosis, and the apoptosis rate was significantly reduced *in vitro*. Second, RGOs and RGOs + Nkx2.5 combination treatment effectively improved left ventricular function and CX43 expression, inhibited myocardial fibrosis, and reduced the expression of TGF- β 1 and Collagen α . Third, RGOs and combination treatment enhanced the expression of GATA4 and MEF2, and probably played a protective role through transcriptional regulation.

Stem cell therapy represents a promising treatment for cardiac disorders, including heart failure. Recent reports have suggested the greater therapeutic effects of the anti-inflammatory, trophic, paracrine and immune-modulatory functions associated with these cells, which induce them to restore normal healing and tissue regeneration by modulating immune reactions, regulating inflammation, and suppressing fibrosis [43]. Some clinical trials showed that stem cell therapy significantly improved the cardiac function, and it has been used successfully in small studies in ischemic, dilated and restrictive cardiomyopathies [17, 18]. An important advantage of MSCs is that they do not express major histocompatibility complex

class II antigens, therefore they are non-immunogenic which makes them good candidates for allogeneic therapies [39]. Results of published investigations describing MSCs safety and efficacy in a variety of injury and disease models demonstrate the unique qualities of this reparative cell population in adapting to the specific requirements of the damaged tissue in which the cells integrate [44]. Thus, more studies are needed to investigate new strategies to improve the efficacy of MSCs therapy for heart diseases. In our previous research, we have found that Nkx2.5 enhanced the efficacy of MSCs transplantation in treatment adriamycin-induced heart failure in rats. Both MSCs and MSCs-Nkx2.5 improved cardiac function, promoted the differentiation of transplanted MSCs into cardiomyocyte-like cells, decreased fibrosis formation and collagen volume fraction in adriamycin-induced rat heart failure models. Moreover, the effect was much more remarkable in MSCs-Nkx2.5 group [9]. In this study, we further found RGOs has the synergistic effect with Nkx2.5 gene transfected MSCs transplantation in treatment with heart failure.

RGOs is a popular traditional Chinese medicine extracted from *Rehmannia glutinosa* [19]. Being an antioxidant, RGOs inhibits the progression of experimental thrombosis and atherosclerosis [20, 21]. Studies revealed that RGOs was already reported to play a role in cell regeneration [22]. In our experiment, we documented that RGOs has a strong protective effect on H₂O₂-induced apoptosis, reducing the apoptosis rate from 66.9–38.9% which significantly improved stem cell survival. Similarly, Zhang Y et al found that RGOs decreased H₂O₂-induced apoptosis in adipose-derived mesenchymal stem cells. The research also showed that RGOs promoted adipose-derived MSC proliferation through VEGF and HGF paracrine signaling, which may reduce H₂O₂-induced MSCs apoptosis [25]. RGOs not only reduce the apoptosis, but also promote the differentiation of stem cells. Prior research by Wang XH et al suggested that RGOs induced MSCs to differentiate into cardiomyocyte-like cells *in vitro* [23]. Conversely, our *in vitro* experiment didn't find an increase in CK and α -MHC expression, and it seemed that RGOs did not affect cell differentiation. Bhang SH et al found that the administration of additional exogenous growth factors or cytokines as co-inducers in combination with 5-azacytidine may be required to promote differentiation of MSCs into cardiomyocyte-like cells [41]. Thus, RGOs could not induce differentiated cardiomyocytes because it could not play a paracrine role to produce cytokines *in vitro* to produce differentiated effects. Therefore, *in vivo* studies are needed to further elucidate the promoting and enhancing effects of RGOs.

Left ventricular remodeling including increased LV mass and cardiac fibrosis causes left ventricular diastolic and systolic dysfunction, which may consequently lead to clinical complications such as heart failure, atrial fibrillation, ischemic heart disease, and other vascular events [40]. Our *In vivo* studies found that RGOs improved heart systolic and pumping function. RGOs and RGOs combined Nkx2.5 significantly reduced body weight ratio. In echocardiography, our data further confirmed that RGOs combined with Nkx2.5 could significantly reduce the left ventricular systolic and diastolic volumes. Therefore, RGOs has a synergistic effect with Nkx2.5, and enhances the inhibitory effect of Nkx2.5 gene transfection on ventricular remodeling. Disease-related left ventricular remodeling is a complex process involving cardiac myocyte growth and death, vascular rarefaction, fibrosis, inflammation, and electrophysiological remodeling. Myocardial fibrosis is characterized by an excessive deposition of extracellular matrix proteins, especially collagens, leading to cardiac remodeling, with increased stiffness, hypertrophy and

eventually HF [28]. Transforming growth factor- β 1 (TGF- β 1) plays an important role in the pathogenesis of cardiac remodeling and fibrosis [29, 30]. For an angiogenesis effect, TGF- β 1 displays a biphasic role. Low concentrations of TGF- β 1 synergistically enhance, whereas high concentrations decrease the vascular invasion of cultured endothelial cells induced by angiogenic factors. High doses of TGF- β 1 may have a cytotoxic effect that lead to programmed cell death and senescence, which could result in a low differentiated rate [32]. This study found that RGOs not only strongly inhibited TGF- β 1 and Collagen I mRNA expression, but also collaborated with Nkx2.5 to reduce the expression of Collagen I mRNA and the percentage of CVF. It suggested that RGOs could play a synergistic effect with Nkx2.5 transfected MSCs to improve myocardial fibrosis and inhibit ventricular remodeling.

It was obvious that RGOs and Nkx2.5 improved cardiac function, which may due to the effect by promoting the differentiation of cardiomyocytes. We studied the effect of RGOs and RGOs combine with Nkx2.5 on expression of specific cardiac genes *in vivo*. cTnI and CX43 are important myocardial structural proteins with myocardial specificity. CX43 plays an important role in the electro-mechanical activities of the myocardium. The changes in the amount and function of CX43 will lead to blocked cardiac electrical conduction, leading to cardiac desynchrony and arrhythmia [24]. In our *in vivo* study, CX43 expression of rats in the heart failure group was significantly decreased, while CX43 expression was increased after stem cell transplantation, was further increased after Nkx2.5 gene transfection, and greatly increased after RGOs combined Nkx2.5 treatment. RGOs alone and in collaboration with Nkx2.5 induce MSCs to exert their cardiac myocyte properties, thus improving ventricular remodeling. Troponin is a regulatory protein tightly attached to contraction fibers and an inhibitory subunit of actin. cTnI has myocardial specificity, and is also known to be an early marker of myogenic differentiation. Our experiment documented that there was no statistically significant difference in cTnI between each group, which may be related to the high level of basic expression of cTnI protein in original cardiomyocytes and the limited number of metastatic surviving stem cells.

Cardiac muscle transcription factors [Nkx2.5, myocyte enhancer factor 2(MEF2), GATA binding protein 4 (GATA4)] is known to regulate cardiac differentiation and development [31, 33, 34]. Jamali M and his colleagues have shown that the inability to form cardiac muscle because of the loss of Nkx2.5 activity is also accompanied by the loss of endogenous expression of cardio myoblast genes, such as GATA4 and MEF2 [10]. The MEF2 transcription factors are key regulators of cardiac myogenesis and morphogenesis. In this study, the induction of HF led to a significant decline in the cardiac expression of MEF2, which is consistent with the results of the study by Razeghi and colleagues, who reported downregulation of MEF2 expression in diabetic patients with HF [35]. RGOs and combination treatment could increase the expression of MEF2. Optimal Nkx2.5 activity requires physical interactions with other cardiac-restricted factors, such as GATA4 and serum response factor. In cardiac myocytes, MEF2 proteins are recruited by the cardiac-specific GATA4 transcription factors to target promoters and functionally synergize with this family of tissue-restricted zinc finger proteins. Our research also found that RGOs increased GATA4 expression in rat heart failure model. Bisping et al demonstrated that GATA4 regulated the pattern of cardiomyocyte hypertrophy and protected the heart from load-induced failure [37]. The enhanced expression of GATA4 helps in the acceleration of heart development and remarkably increases the

number of cardiac cells. GATA4 is not only an important transcription factor, but also an important regulator of apoptosis. The expression of GATA4 can inhibit apoptosis of myocardial cells [34, 36]. The antiapoptotic effects of RGOs and the combined group in our study may also be caused by up-regulation of GATA4. Morin et al demonstrated that MEF2 and GATA4 physically interact *in vivo* and *in vitro* and that MEF2 regulates gene expression through protein-protein interaction with GATA4 [42]. Abdelmonem and colleagues demonstrated that augmented MEF2 and GATA4 expression after preconditioning reveals the ability of NaHS to enhance the differentiation of MSCs into cardiomyocytes, which is a critical step in the treatment of HF [38]. GATA4 administration reduces postinfarct ventricular fibrosis and improves ventricular function in a rat coronary ligation model. Inagawa K et al found that a combination of 3 cardiac transcription factors, GATA4, MEF2c and TBX5 (GMT), reprograms fibroblasts directly into functional cardiomyocytes in vitro [26]. Remarkably, inhibition of pro-fibrotic signaling using GMT (GATA4, MEF2c, TBX5) or GHMT (GATA4, HAND2, MEF2c, TBX5) that target the transforming growth factor- β or Rho-associated kinase pathways converts embryonic fibroblasts into functional cardiomyocyte-like cells, with the efficiency up to 60% [27]. Our finding highlights RGOs could enhance the expression of GATA4 and MEF2, and RGOs combined with Nkx2.5 showed an increasing trend of GATA4 and MEF2 mRNA expression, which could be declared that the synergistic effect of RGO might be generated by increasing the expression of GATA4 and MEF2 genes to enhance the transformation of cardiomyocytes.

Conclusion

In conclusion, this study provides better understanding of RGO's enhancement effect on Nkx2.5-MSCs in treatment heart failure. RGOs not only reduced the apoptosis rate *in vitro*, but also RGOs effectively improved left ventricular function *in vivo*. RGOs increased CX43 expression to display the characteristics of cardiomyocytes. Furthermore, RGOs also enhanced the efficacy of MSCs-Nkx2.5 in decreasing fibrosis formation and collagen volume fraction while increasing the expressions of GATA4 and MEF2. Therefore, RGOs has the synergistic effect with Nkx2.5 transfected MSCs transplantation in treatment heart failure, and may be considered as a useful drug for clinical applications.

Abbreviations

HF: Heart failure; MSCs: Mesenchymal stem cells; RGOs: Rehmannia glutinosa oligosaccharide; Nkx2.5: Nkx2.5 transfected MSCs; CVF: Collagen volume fraction; LVEDV: Left ventricular end-diastolic volume; LVESV: Left ventricular end-systolic volume; LVID-s: Left ventricular internal dimension at systolic; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fractional shortening; CI: Cardiac index; PVDF: polyvinylidene difluoride; BW: body weight; WW: wet weight; TGF- β 1: Transforming growth factor- β 1; MEF2: myocyte enhancer factor 2; GATA4: GATA binding protein 4

Declarations

Ethics approval and consent to participate

The study had the approval of Animal Care and Use Committee of PLA general hospital.

Consent for publication

Not applicable

Availability of supporting data

All materials are commercially available, and data are as presented in this article open for access.

Competing interests

The authors declare that there are no competing interests.

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Author's contributions

LW and BD did experiments including heart failure models establishment, cell isolation and treatment, immunohistochemical analysis, real-time PCR analysis, western blotting. LW also undertook grant writing, implementation data interpretation, manuscript writing. XXH took part in apoptosis detection and echocardiology examination. RZ produced the data table and figure for the manuscript and executed part of the data analysis. YL participated in the design of this research and the manuscript revision. QLZ is the chief of this project. He took part in the whole course of experiments, data analysis, manuscript preparation and confirmed the final version of this manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 & 2 tif are only available as a download in the Supplemental Files section.

Figures

Figure 1

RGOs increased the survival rate without enhancing cardiac specific gene expression in vitro. (A) Effect of RGOs on apoptosis rate induced by H₂O₂, (B) and (C) Q-PCR analysis of CK mRNA and α-MHC mRNA expression. Values were expressed as mean ± SEM, n=8. *P<0.05 versus H₂O₂ group, ▲P<0.05 versus Nkx2.5 group, #P<0.05 versus MSCs group.

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The effect of RGOs on the efficacy of Nkx2.5 in improving left ventricular function. A. WW/BW in rat models of heart failure; B. LVEDV and LVESV in rat models of heart failure; C. LVEF and LVFS in rat models of heart failure. Values were expressed as mean $\pm$ SEM, n=8. * P<0.05 versus control group; ▲P<0.05 versus HF group; #P<0.05 versus RGOs group; & P<0.05 versus Nkx2.5 group

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Figure 4

RGOs enhanced the efficacy of Nkx2.5 in reducing myocardial fibrosis in rat models of heart failure. A Masson's trichrome staining on left ventricle, B Collagen volume fraction (CVF%) was measured with SPOT 3.5.1 software, C TGF-β1 mRNA expression significantly decreased in RGOs group, D Collagen I mRNA expression significantly decreased in RGOs group and combined group. Values were expressed as

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