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ETBR overexpression attenuates experimental autoimmune myocarditis in rats by inhibiting ICAM-1

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Background: An experimental autoimmune myocarditis rat model was established by subcutaneous injection of PCM. The effect of ETBR overexpression on autoimmune myocarditis was observed via tail vein injection of ETBR overexpression lentivirus in rats. We further investigated the mechanisms involved in the regulation of autoimmune myocarditis by ETBR overexpression. **Methods:** Six rats were randomly selected from 24 male Lewis rats as the NC group, and the remaining 18 rats were injected with porcine PCM on day 0 and day 7, respectively, to establish the EAM rat model. The 18 rats initially immunized were randomly divided into 3 groups: EAM group, ETBR-oe group, and GFP group. On day 21 after the initial immunization of rats, cardiac echocardiography and serum BNP concentration were performed to evaluate cardiac function, myocardial tissue HE staining was performed to assess myocardial tissue inflammatory infiltration and myocarditis score, and mRNA expression of IFN-γ, IL-12, and IL-17 were detected by QRT-PCR. Subsequently, immunohistochemical analysis was performed to detect the localization and expression of ETBR and ICAM-1 proteins, and the expression of ETBR and ICAM-1 was verified by QRT-PCR. **Results**: On day 21 after initial immunization, LVEDd, LVEDs, and serum BNP concentrations increased in the hearts of rats in the EAM group compared with the NC group ($P < 0.01$), and EF and FS decreased compared with the NC group ($P < 0.01$). LVEDd, LVEDs, and serum BNP concentrations decreased in the ETBR-oe group compared with the EAM group, while EF and FS increased significantly ($P < 0.01$). HE staining showed that a large number of inflammatory cell infiltrates, mainly lymphocytes, were seen in the EAM group, and the myocarditis score was significantly higher than that of the NC group ($P < 0.01$). Compared with the EAM group, myocardial tissue inflammatory cell infiltration was significantly reduced in the ETBR-oe group, and myocarditis scores were significantly lower ($P < 0.01$). The mRNAs of inflammatory factors IFN-γ, IL-12 and IL-17 in myocardial tissue of rats in the EAM group exhibited elevated levels compared

with the NC group ($P < 0.01$) while the mRNAs of IFN-γ, IL-12 and IL-17 were significantly decreased in the ETBR-oe group compared with the EAM group ($P < 0.01$). Immunohistochemistry showed that the staining depth of ETBR protein in myocardial tissue was higher in the EAM group than in the NC group, and significantly higher in the ETBR-oe group than in the EAM group, while the staining depth of ICAM-1 was significantly higher in the EAM group than in the NC group, and significantly lower in the ETBR-oe group compared with the EAM group. The mRNA expression level of ICAM-1 was significantly higher in the EAM group than in the NC group ($P < 0.01$), and was significantly lower in the ETBR-oe group compared with the EAM group ($P < 0.01$).

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Abstract

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Results: On day 21 after initial immunization, LVEDd, LVEDs, and serum BNP concentrations increased in the hearts of rats in the EAM group compared with the NC group ($P < 0.01$), and EF and FS decreased compared with the NC group ($P < 0.01$). LVEDd, LVEDs, and serum BNP concentrations decreased in the ETBR-oe group compared with the EAM group, while EF and FS increased significantly ($P < 0.01$). HE staining showed that a large number of inflammatory cell infiltrates, mainly lymphocytes, were seen in the EAM group, and the myocarditis score was significantly higher than that of the NC group ($P < 0.01$). Compared with the EAM group, myocardial tissue inflammatory cell infiltration was significantly reduced in the ETBR-oe group, and myocarditis scores were significantly lower ($P < 0.01$). The mRNAs of inflammatory factors IFN-γ, IL-12 and IL-17 in myocardial tissue of rats in the EAM group exhibited elevated levels compared with the NC group ($P < 0.01$) while the mRNAs of IFN-γ, IL-12 and IL-17 were significantly decreased in the ETBR-oe group compared with the EAM group ($P < 0.01$). Immunohistochemistry showed that the staining depth of ETBR protein in myocardial tissue was higher in the EAM group than in the NC group, and significantly higher in the ETBR-oe group than in the EAM group, while the staining depth of ICAM-1 was significantly higher in the EAM group than in the NC group, and significantly lower in the ETBR-oe group compared with the EAM group. The mRNA expression level of ICAM-1 was significantly higher in the EAM group than in the NC group ($P < 0.01$), and was significantly lower in the ETBR-oe group compared with the EAM group ($P < 0.01$).

Key words: ETBR, autoimmune myocarditis, ICAM-1

Introduction

Myocarditis is an inflammatory disease of the heart muscle caused by various etiologies, with viral infections being the most common[1]. The pathogenesis of viral myocarditis includes direct damage to the myocardium by viral infection and indirect damage caused by the host immune response[2]. There is increasing evidence that overactive inflammatory and autoimmune responses triggered by viral infections, rather than direct viral infections, are the main cause of the development of myocarditis[3-5]. Approximately 40-66% of patients with myocarditis are reported to recover completely on their own within the first 4-12 weeks, but approximately half of patients with acute myocarditis with autoimmune myocardial injury exhibit significant signs of heart failure, arrhythmias and sudden cardiac death[6]. Unfortunately, the mechanisms of myocarditis have not been fully elucidated and there are no effective treatment strategies for myocarditis, which makes myocarditis a serious cardiovascular health problem.

Endothelin-1 (ET-1) is a 21-amino acid active peptide, which is the main vasoconstrictor secreted by endothelial cells and has pro-oxidant and inflammatory effects[7]. ET-1 mediates its action through two types of receptors including ET-1 receptor type A (ETAR) and ET-1 receptor type B (ETBR). Under physiological conditions, ETAR expresses in smooth muscle cells and can mediate vasoconstriction, whereas ETBR is mainly found in endothelial cells and mediates vasorelaxation[8]. Current studies have shown that the endothelin system is widely involved in cardiovascular diseases and ETBR is closely associated with pulmonary hypertension, heart failure, artery hypertension, atherosclerosis, and chronic kidney disease[9-13]. However, there are fewer studies related to ETBR and myocarditis, and the mechanism of ETBR in myocarditis has not been elucidated.

ICAM-1 is a cell surface glycoprotein and adhesion receptor that regulates the recruitment of leukocytes from the circulation to inflammatory sites[14]. ICAM-1 is mainly expressed in immune cells, endothelial cells and epithelial cells, and a variety of inflammatory factors highly induce ICAM-1 expression, suggesting that ICAM-1 plays an important role in mediating immune and inflammatory responses[15]. Myocarditis is an inflammatory disease of the myocardium associated with immune damage. Similar to other immune disorders and pathologic inflammatory responses, innate and adaptive immune responses are involved in the development and pathogenesis of myocarditis[16]. Liu et al. found that curcumin attenuated LPS-induced myocarditis in vitro by inhibiting ICAM1/CD40/NF-κB[17]. Previous studies have found that ICAM-1 expression is also regulated by some microRNAs. MicroRNA-141 was found to downregulate ICAM-1 in endothelial cells, thereby reducing leukocyte adhesion and attenuating myocardial ischemia-reperfusion injury[18]. Furthermore, microRNA-27 can target ICAM-1 and protect against LPS-mediated inflammatory injury in H9c2 cells by inhibiting ICAM1 expression[24]. Therefore, inhibition of ICAM-1 production and release may be a potential

therapeutic strategy for myocarditis. However, the mechanism of ICAM-1 inhibition to protect myocardium from inflammatory damage has not been elucidated. There was a study reporting that increased ETBR activity in the tumor endothelium leads to a decrease in T lymphocytes homing to the tumor and to the failure of immunotherapy[19]. Hence, we hypothesized that ETBR overexpression may play a protective role in myocarditis by suppressing ICAM-1 expression Based on the above, we first observed the effects of ETBR on cardiac function and inflammation in rats with myocarditis using a PCM-induced EAM rat model. The expression level of ICAM-1 in the ETBR overexpressed EAM model was further examined to verify the regulation of ETBR on ICAM-1 expression. Finally, we clarified the effect of ICAM-1 knockdown on myocarditis by RNAi technology to investigate the molecular mechanism of ETBR overexpression protecting the heart from immune injury in myocarditis.

Materials & Methods

Animals

Twenty-four male Lewis rats (age, 6-7 weeks), weighing 180-200g, were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). Animals were kept under standard conditions with a mean temperature of 21°C ± 2°C, a mean relative humidity of 50% ±20 and a defined day-and-night-cycle of 12 h light and 12 h dark. No control diet was required in this experiment, and all diets were standardized. All animals need to be euthanized before the end of the experimental program. The details of the method are described below. Rats were anesthetized with an intravenous overdose of pentobarbital (100-150 mg/kg) and killed by cervical dislocation under deep anesthesia. At the scene of execution, other animals should not be present, and the carcass should be properly disposed only after the death of the animal is confirmed. The experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the United States National Institute of Health (Publication No. 85-23, revised in 1996), and all the performed experiments were approved by the Ethics Committee of the Animal Care and Use Committee of The Affiliated Hospital of Nanchang University, Nanchang, China (IACUC Issue No:202205QR004)

Construction of the recombinant lentiviral vector

The gene sequence of rat ETBR (NM_017333) was first obtained by searching the NCBI website, and the target gene sequence (Forward: 5'- GGAATTGGGGTTCCAAAATG-3'; Reverse: 5 '- CCTTATAGTCCTTATCATCGTC -3') was synthesized by chemical synthesis. The recombinant positive clones were selected for PCR and sequencing, and the lentiviral vector containing ETBR-shRNA was obtained by ligation with GV492 vector. The instrumental vector plasmid carrying ETBR gene sequence and the viral packaging helper plasmid were co-

transfected with 293T cells, and the virus was harvested, concentrated and purified at 48-72 h after the completion of transfection to finally obtain the completed ETBR gene overexpression lentiviral vector. The ETBR protein was detected by Western Blot to confirm whether the ETBR gene overexpression lentiviral vector was successfully packaged. The negative control virus was set as a green fluorescent protein (GFP) null-loaded lentivirus. Viral titer was determined based on the expression of GFP, and the titer of the viral strain was routinely 1×10^8 TU/ml.

Establishment of EAM rat models

The EAM rat model was constructed as our previous study[20]. Briefly, purified porcine myocardial myosin (PCM, Sigma Aldrich, USA) was dissolved in 0.15 mol/L Phosphate Buffered Saline (PBS) and the final concentration was adjusted to 2 g/L. Then the myosin was emulsified with Complete Freud's Adjuvance (CFA) (Sigma Aldrich, USA) in a 1:1 ratio. Each rat in the experimental group was injected subcutaneously with 200 μL PCM-CFA emulsion in the inguinal region, foot pad region, and axilla on day 1 and day 7, respectively. Rats in the control group were injected subcutaneously with CFA 100 μL on day 0 and day 7, respectively.

Experimental Study Design and Grouping

Six normal rats were randomly selected as the normal control group (NC group), and the remaining 18 rats were injected with porcine cardiac myosin (PCM) on days 0 and 7 to establish the EAM rat model. The 18 rats were randomly divided into 3 groups: EAM group, ETBR-oe group and GFP group. 1×10^8TU lentivirus/rat (200 μL) was injected into the tail vein of rats in the ETBR-oe group on the day of initial immunization, while the rats in the GFP group were injected with the same dose of empty lentivirus and the rats in the NC and EAM groups were injected with the same dose of saline.

Assessment of cardiac function by echocardiography

On day 21 after the initial immunization, cardiac echocardiography was performed in each group of rats to evaluate cardiac structure and cardiac function. The anesthesia machine and ultrasound system were connected, and the concentration of anesthetic gas isoflurane was adjusted to a suitable value to confirm that the rats were well anesthetized. Parasternal left ventricular long-axis views were taken, and M-section images were obtained at the level of the mitral tendon cords, and the left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVEDs), left ventricular fractional shortening (FS), and left ventricular ejection fraction (EF) were measured. A minimum of 5 cardiac cycles were measured in each rat.

Histopathological Analysis

Myocardial tissue was fixed in 10% formaldehyde solution and embedded in solid paraffin at 65-70°C. The thickness of the sections was fixed at 2-3 um and stained with hematoxylin and eosin (HE). The pathological changes of myocardial tissue such as cardiomyocytes, myocardial interstitium, and inflammatory cells were observed under light microscope, and five high magnification fields were randomly selected for each section, and the percentage of the area of inflammatory cell infiltration and necrotic area of myocardial tissue in each field of view to the whole field of view was calculated, and its average value was used for myocarditis scores. And myocarditis scores were exactly as following: 0, no inflammation; 1, <25% of the involved heart portion; 2, 25-50%; 3, 50-75%; and 4, >75%[21]. Data were analyzed by an observer who was blinded to the treatment in rats.

Immunohistochemical Analysis

To detect the expression and distribution of ETBR and ICAM-1 in myocardial tissue, we performed immunohistochemical analysis on prepared paraffin sections of myocardial tissue. Paraffin sections were baked at 65°C for 2 hours. After dewaxing and rehydration, antigen repair was performed on tissue sections in citrate buffer at pH 6.0. PBS was used to wash the sections and then endogenous peroxidase was removed with 3% H_2O_2 for 30 min. 50 ul of normal goat serum was added to each section for closure and incubated for 10 min at room temperature. The protein primary antibody was incubated in a wet box overnight at 4°C in the refrigerator, followed by washing the sections and incubating the corresponding protein secondary antibody for 50 min at 37°C. The signal was amplified with an acid-biotin-horseradish peroxidase procedure, using diaminobenzidine as a color developer. The semi-quantitative levels and localization of ETBR and ICAM-1 proteins were observed microscopically.

Enzyme-linked immunosorbent assay (ELISA) for serum brain natriuretic peptide (BNP)

After echocardiography, blood was collected from the inferior vena cava of the rats. The venous blood was centrifuged for 10 min and rat serum was obtained. The serum BNP concentration was measured by BNP assay kit according to ELISA kit specification. BNP assay kit was purchased from Abcam Trading Co. (Shanghai, China).

Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR)

RNA was extracted and isolated from rat myocardial tissue by homogenization, centrifugation and dissolution. A PCR tube was taken and a solution containing more than 100ng of RNA was added as a template for reverse transcription, and 1μl of reverse transcription primers were added for reverse transcription. This process was performed according to the instructions of RevertAid First Strand cDNA Synthesis Kit from Transgenbiotech Co. (Beijing, China). QRT-PCR analysis was then performed using the ABI Prism 7000 system (Abcam, CA, USA) to detect the mRNA expression. The relative mRNA expression levels of each molecule were normalized by subtracting the corresponding GAPDH threshold cycle (CT), which was done by using the $\Delta\Delta C_T$ comparison method. A list of real-time PCR primer sequences is presented in **Table 1**.

Western blot analysis

Total proteins from myocardial tissue were extracted by radio-immunoprecipitation assay (RIPA) lysis buffer (Solarbio, Beijing, China). Protein was quantified using the BCA protein assay kit (Beyotime, Shanghai, China). Proteins (40ug) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, CA, USA) and transferred to nitrocellulose filter membranes (Millipore, Massachusetts, USA). Membranes were blocked with 5% nonfat dry milk and 3% bovine serum albumin (Sigma, St Louis, USA) in TBS-T (20 mM/l Tris, pH 7.6, 137 mM/l NaCl, 0.05% Tween). Diluted anti-EABR I antibody (Abcam, CA, USA) were added and incubated overnight at 4°C. Then, diluted anti-EABR II antibody (Abcam, CA, USA) was added and incubated at room temperature for 2h, followed by development using enhanced chemiluminescence reagent (Beyotime, Shanghai, China). The exposed protein bands were converted to data using Image software (Bio-Rad, CA, USA) to obtain grayscale values for result analysis.

Statistical Analysis

SPSS version 20.0 software(IBM Corporation, NY, USA) was used to analyze the statistical data obtained from the experiments, and the data were presented as the mean ± SEM, and GraphPad Prism version 8.0.1 software (GraphPad Software, Inc, CA, USA) was used to produce statistical graphs. One-way ANOVA and LSD test were used for comparison between groups, and $P < 0.05$ was considered statistically significant.(*P＜0.05,**P＜0.01)

Results

Measurement of ETBR expression in myocardial tissues by QRT-PCR and western blot

To explore the effect of ETBR overexpression on autoimmune myocarditis, we first infected EAM rats with lentivirus carrying ETBR gene. QRT-PCR detected the relative mRNA level of ETBR, and the results showed that the ETBR mRNA expression level in the ETBR-oe group was significantly higher than that in the EAM and NC groups ($P< 0.01$) (**Fig1a**). Moreover, western blot results showed that the protein expression level of ETBR was significantly higher in the ETBR-oe group compared to the EAM and GFP groups(**Fig1b**), indicating that the lentivirus infection was successful.

Effects of ETBR overexpression on cardiac function in EAM rats

Echocardiography was performed on day 21 after pcm immunization in rats to assess the effect of ETBR overexpression on cardiac function. Compared with the NC group, LVEDd and LVEDs were significantly increased while EF and FS were significantly decreased in the EAM group rats ($P< 0.01$). There was no significant difference in LVEDd, LVEDs, EF and FS of rats in the GFP group compared with the EAM group ($P > 0.05$). However, compared with the EAM group, LVEDd and LVEDs of rats in the ETBR-oe group were significantly decreased while EF and FS were significantly increased ($P< 0.01$), suggesting that ETBR gene overexpression could alleviate ventricular dilation and improve cardiac function in EAM rats (**Table 2**).

Effects of ETBR overexpression on inflammatory cell infiltration in EAM rats

On the day 21 after the initial immunization, HE staining showed that the cardiomyocytes in the NC group were neatly arranged and morphologically normal, and no inflammatory cell infiltration was seen in the interstitium. In contrast, in the EAM group, myocardial cells were disorganized, some of them were lysed, and inflammatory cells (mainly lymphocytes) were evidently found in the interstitial part, and the myocarditis score was significantly higher than that of the NC group ($P < 0.05$). In the GFP group, cardiomyocytes were disorganized with some degree of inflammatory cell infiltration, and the difference in myocarditis score was not statistically significant compared with the EAM group ($P > 0.05$). In the ETBR-oe group, only a small amount of cardiomyocytes were disorganized and swollen, and inflammatory cell

infiltration was significantly reduced compared with the EAM group, and the myocarditis score was significantly lower than that of the EAM group ($P < 0.05$)(**Fig.2a and Fig.2b).** Therefore, ETBR overexpression alleviates inflammatory cell infiltration in EAM rats.

Effects of ETBR overexpression on serum BNP concentration

The relative serum BNP concentration was significantly higher in the EAM group compared with the NC group on day 21 after the initial immunization ($P < 0.05$). The relative serum BNP concentration in the GFP group were not statistically significant compared with the EAM group ($P > 0.05$). However, relative serum BNP concentration were significantly lower in the ETBR-oe group compared with the EAM group ($P < 0.05$)(**Figure 3**).

Effects of ETBR overexpression on expression of inflammatory cytokines IFN-γ, IL-12 ,and IL-17

The relative mRNA levels of inflammatory factors IFN-γ, IL-12 and IL-17 in the myocardial tissue of rats in the EAM group showed elevated levels compared with the NC group, and the differences were statistically significant ($P < 0.01$). The relative mRNA levels of IFN-γ, IL-12 and IL-17 were significantly decreased in the ETBR-oe group compared with the EAM group ($P < 0.05$) (**Figure 4**).

Distribution and expression of ETBR and ICAM-1 proteins in myocardial tissue by immunohistochemical assay

To investigate the relationship between ETBR and ICAM-1, we first reviewed and collected paraffin sections of myocardial tissues from each group of rats, and then used immunohistochemistry to detect the distribution of ETBR and ICAM-1 in the myocardial tissues of EAM rats. Immunohistochemical results showed that ETBR protein was mainly localized in the myocardial cell membrane and cell plasma. Moreover, compared with the EAM group, ETBR protein stained significantly more deeply in myocardial tissues in the ETBR-oe group than in the EAM group, whereas ICAM-1 stained significantly less deeply in myocardial tissues in the ETBR-oe group. These data suggest that both ETBR and ICAM-1 were expressed in myocardial tissues and that ETBR overexpression may inhibit the expression of ICAM-1 in the EAM model, which needs to be further verified using western blot method (**Figure 5**).

Effects of ETBR overexpression on ICAM-1 expression in EAM rats

To confirm the effect of ETBR overexpression on ICAM-1 expression in EAM rats models, we detected the relative expression levels of ICAM-1 mRNA in the myocardial tissue of each group by QRT-PCR method. On day 21 of immunization, the protein expression level of ICAM-1 in myocardial tissues of the EAM group was significantly higher than that of the NC group ($P < 0.05$). There was no significant difference in the protein expression level of ICAM-1 between the GFP and EAM groups ($P > 0.05$). However, the protein expression levels of ICAM-1 in myocardial tissues were significantly lower in the ETBR-oe group compared with the EAM group ($P < 0.01$) (**Figure 6**).

Discussion

Myocarditis is an inflammatory disease of the heart muscle caused by various etiologies, mainly viral infection and post-infection autoimmunity, often developing into dilated cardiomyopathy and heart failure[22]. However, the mechanism by which immune damage leads to cardiac dysfunction and heart failure remains unclear. EAM is an animal model that mimics myocarditis and has become an important tool for understanding the mechanisms of immune damage in myocarditis. In this experiment, the EAM model was induced by subcutaneous injection of PCM, and the mRNA and protein expression levels of ETBR were found to be significantly increased in the myocardial tissue of rats in the EAM model group. Therefore, ETBR may play a key role in the immune damage of autoimmune myocarditis.

To clarify the effect of ETBR overexpression on EAM, we first performed the preparation of ETBR overexpression lentivirus and then intervened in autoimmune myocarditis by injecting ETBR overexpression lentivirus. We found that LVEDd and LVEDs decreased, while EF and FS increased significantly in the ETBR-oe group compared with the EAM group, indicating an improvement in cardiac function. Serum BNP concentration is a key biomarker for the diagnosis of cardiac insufficiency with good sensitivity[23]. We detected BNP concentration in serum by ELISA and found that the results of serological examination and echocardiography were consistent. Serum BNP was also significantly lower in rats in the ETBR-oe group compared to the EAM group. Moreover, this experiment further explored the effect of ETBR overexpression on inflammation levels in EAM rats. We found that after ETBR overexpression intervention in the EAM model, myocardial tissue showed only a small amount of myocardial cell arrangement disorder and cell swelling, inflammatory cell infiltration was significantly reduced, and myocarditis score was significantly decreased, suggesting that ETBR overexpression can reduce the inflammatory pathological damage of autoimmune myocarditis.

Autoimmune myocarditis is considered immunologically to be a CD4+ T lymphocyte-mediated immune damaging disease[24]. CD4+ T cells can be classified into different effector subpopulations based on their biological functions and specific cytokine production, mainly including Th1, Th2, Th17[25]. Th1 cells, which secrete mainly IL-2, IFN-γ and IL-12, and Th17

cells, which secrete mainly IL-17, exhibit pro-inflammatory properties in myocarditis[26, 27]. Cooperation of Th1 and Th17 cells determines the transition from autoimmune myocarditis to dilated cardiomyopathy[28]. Zhang et al.(2015) found that Apigenin attenuated experimental autoimmune myocarditis in mice by reducing Th1-related inflammatory cytokines (IFN-γ and IL-2) [29]. Moreover, Su et al. (2012) found that inhibition of IL-17 secreted by Th17 cell has been shown to reduce the severity of myocarditis and improve the cardiac pathological changes of myocarditis[30]. We examined IFN-γ, IL-12, and IL-17 cytokines and found that the mRNA of the inflammatory factors IFN-γ, IL-12 and IL-17 in myocardial tissue of rats in the EAM group showed elevated levels compared with NC group. The results suggested that Th1 and Thl7 cells were active in EAM rats. We subsequently intervened in EAM rats by injecting ETBR overexpression lentivirus, and the results suggested that ETBR overexpression decreased mRNA expression levels of Th1 and Th17-related cytokines.

Previous studies have shown that ICAM-1 plays an important role in mediating immune and inflammatory responses, however, the role of ICAM-1 in myocarditis has not been clarified. We found that both ETBR and ICAM-1 were expressed in myocardial tissue, and ICAM-1 levels were significantly decreased after ETBR overexpression intervention in EAM rats by immunohistochemical analysis and QRT-PCR, which indicated that ETBR overexpression inhibited ICAM-1 expression in the EAM model

In summary, our study first demonstrate that ETBR overexpression may downregulate inflammation and improve cardiac function in EAM rats by inhibiting ICAM-1. However, our study has several limitations. We does not validate the interaction between ETBR and the ICAM-1 by immunoprecipitation method, and have not detected the expression of ETBR and ICAM-1 in myocarditis patients. Previous animal studies have shown that EAM is an autoimmune disease mainly mediated by CD4+ T lymphocytes but not CD8+ T lymphocytes[31]. CD4+ T lymphocytes homing is considered as the initiating event in autoimmune myocarditis, which was highly dependent on ICAM-1[32, 33]. Further studies are warranted to investigate the role of ICAM-1 in cardiac homing of CD4+ T lymphocytes during the pathological process of autoimmune myocarditis.

Conclusion

ETBR overexpression significantly reduced inflammatory levels and pathological changes and significantly increased cardiac function in EAM rats, probably due to the inhibition of ICAM-1. Thus, ETBR may be a promising novel therapeutic target for myocarditis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no competing interests exist.

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Figure 1

Measurement of ETBR expression in myocardial tissues after lentiviral transfection

Fig.1a: QRT-PCR detected the relative mRNA level of ETBR; Fig.1b:western blot detected the protein expression level of ETBR.(*P□0.05, **P□0.01)

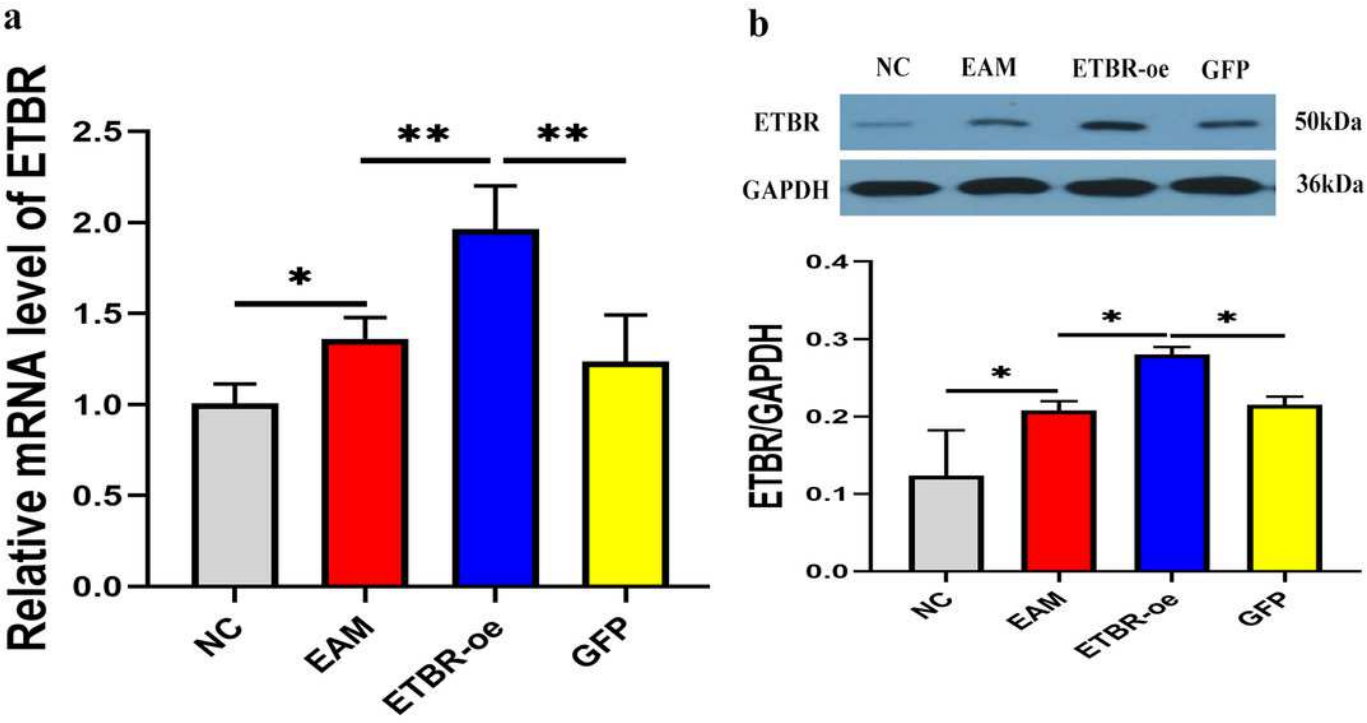

Figure 2

Effects of ETBR overexpression on inflammatory cell infiltration in EAM rats

Fig.2a: HE staining for detection of inflammatory cell infiltration in myocardial tissue; Fig.2b: Myocarditis score for detecting inflammatory levels in myocardial tissue. (**P□0.01)

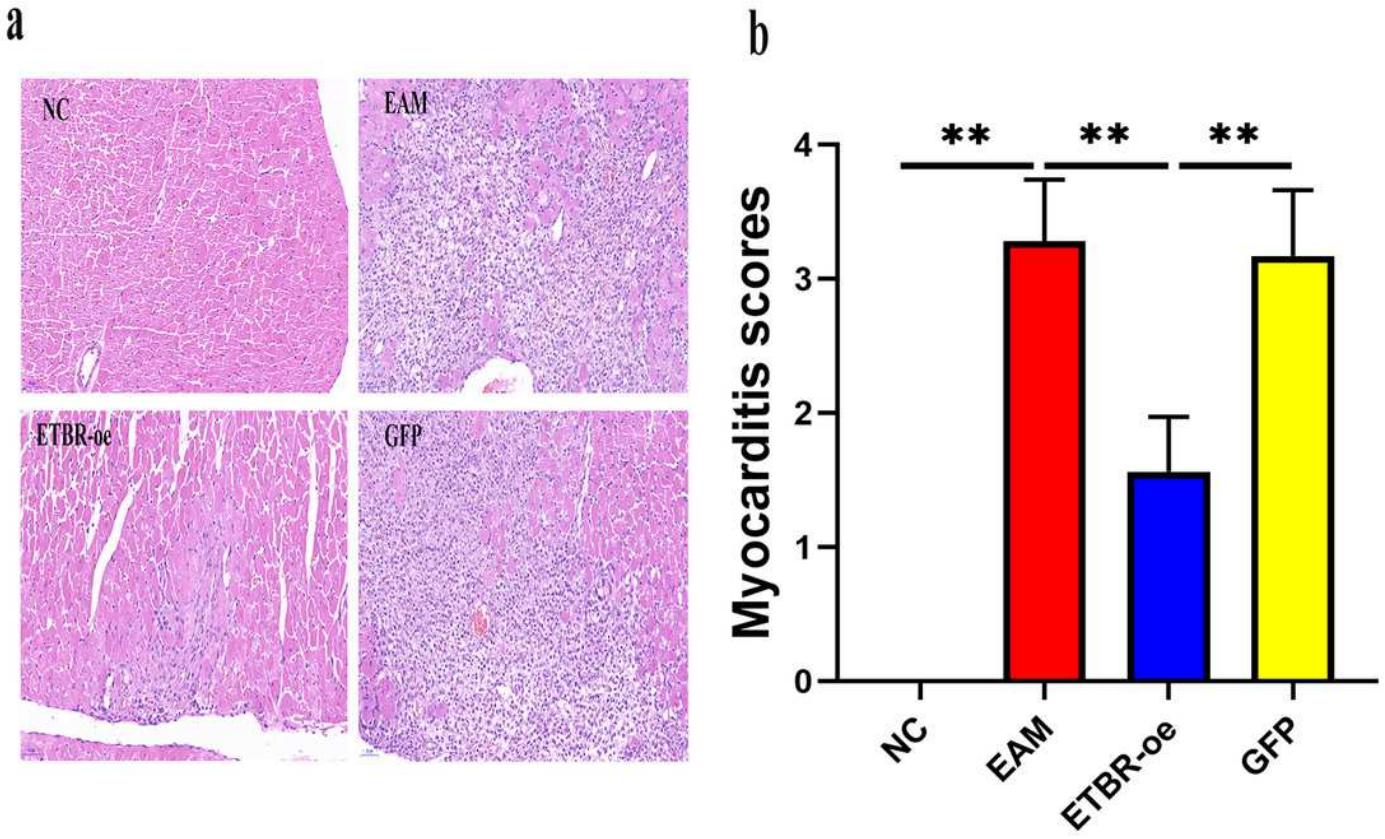

Figure 3

Effects of ETBR overexpression on serum BNP concentration

The relative serum BNP concentration was detected by ELISA analysis(**P□0.01)

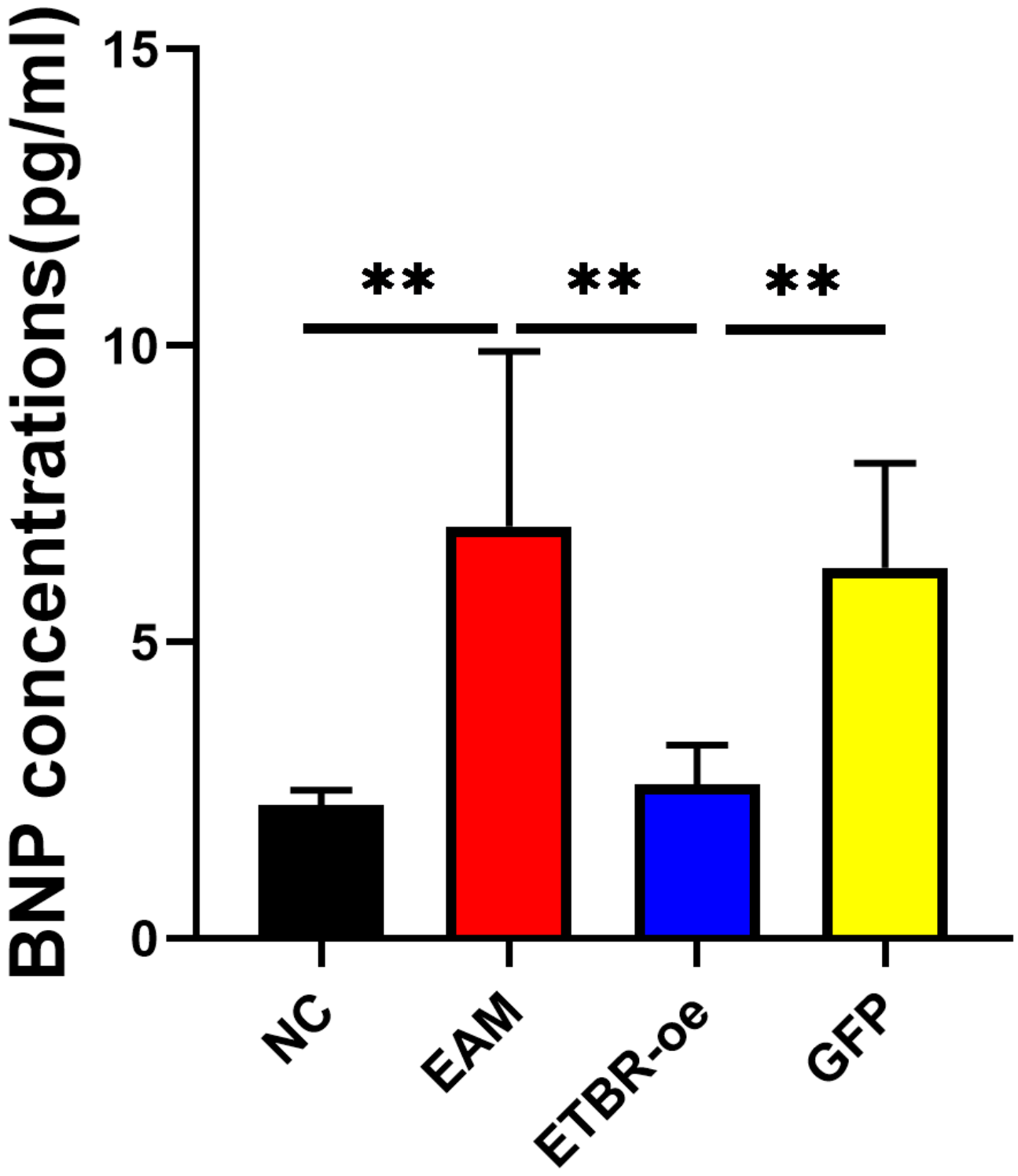
BNP concentrations(pg/ml)
15
10
5
0
**
**
**
NC
EAM
ETBR-oe
GFP

Figure 4

Effects of ETBR overexpression on expression of inflammatory cytokines IFN-γ, IL-12 and IL-17

QRT-PCR detected the relative mRNA level of IFN-γ, IL-12 and IL-17. (*P□0.05, **P□0.01)

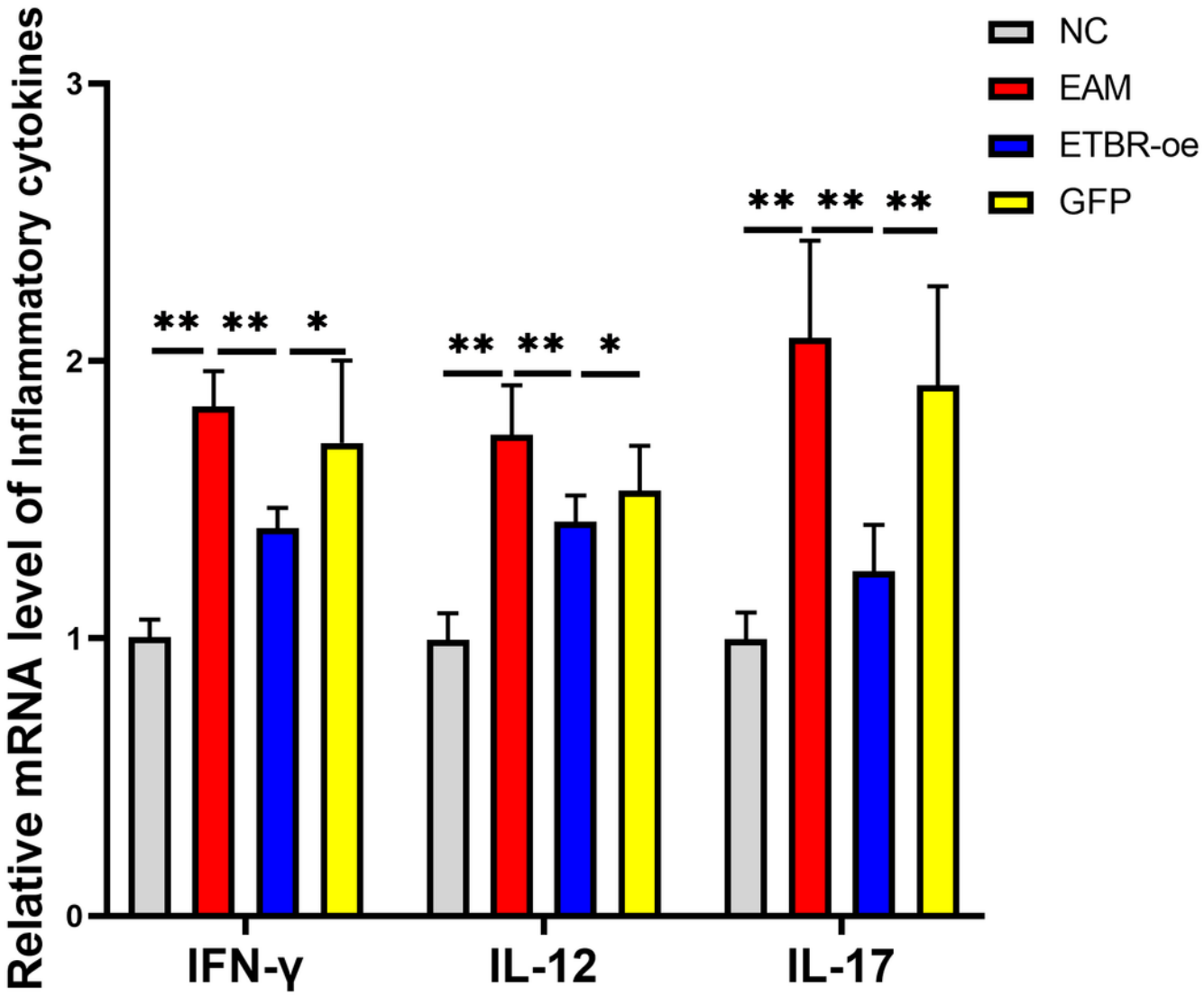

Figure 5

Distribution of ETBR and ICAM-1 proteins in myocardial tissue by immunohistochemical assay

The brownish-yellow particles are ETBR and ICAM-1 proteins, and the blue color is the nucleus. The more specific proteins are expressed, the darker the brownish-yellow particles are.

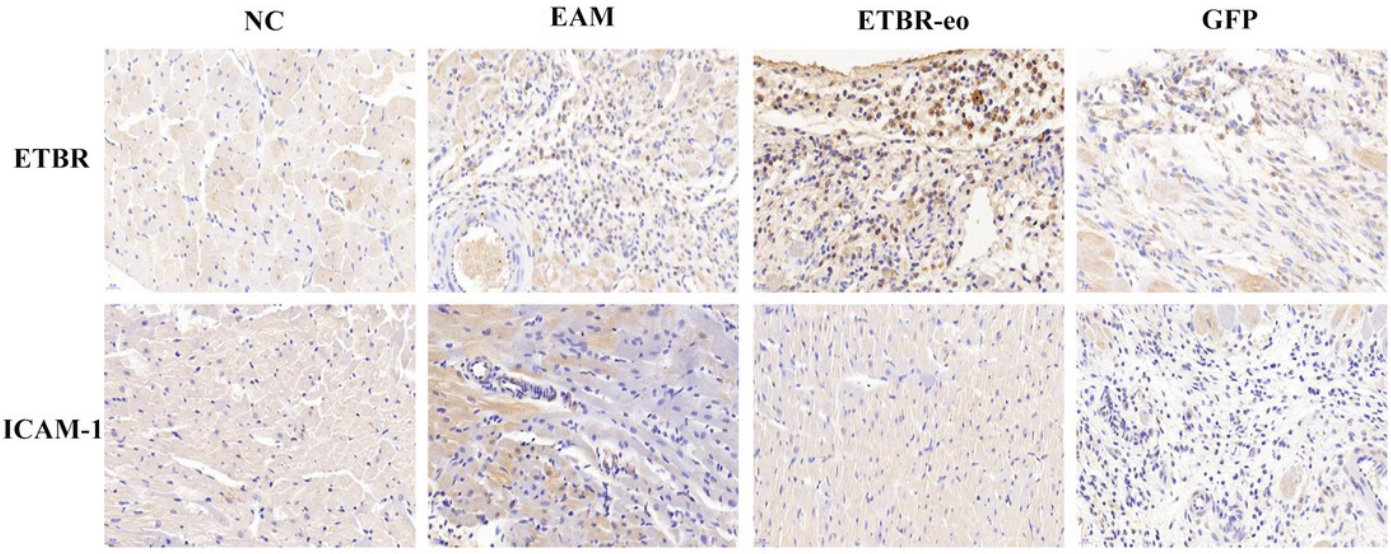

Figure 6

Effects of ETBR overexpression on ICAM-1 expression in EAM rats

QRT-PCR detected the relative mRNA level of ICAM-1. (**P□0.01)

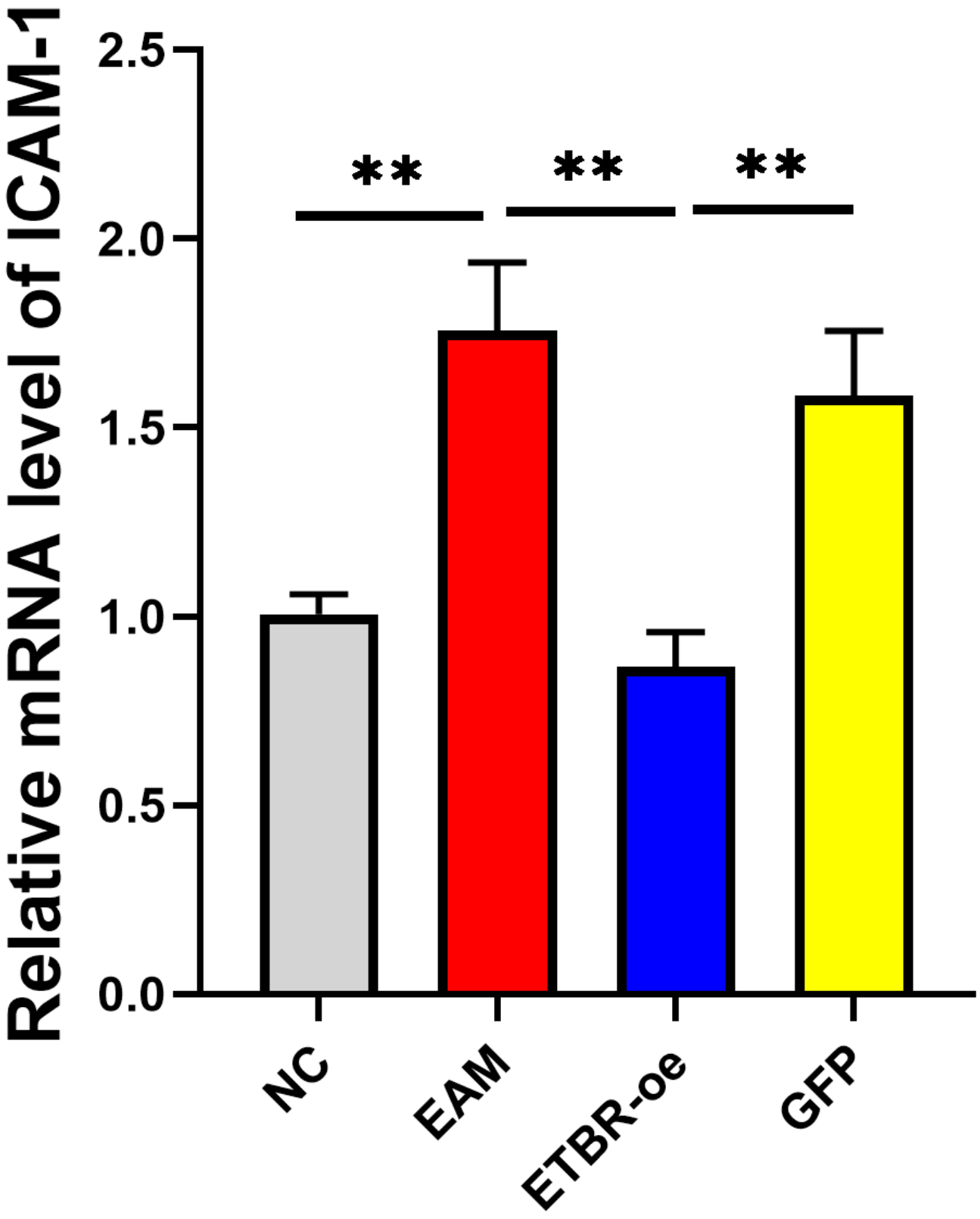
Relative mRNA level of ICAM-1
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
NC
EAM
ETBR-oe
GFP

Table 1(on next page)

Primers used for QRT-PCR

Primers used for QRT-PCR

Table 1 Primers used for QRT-PCR

Primer name	Primer sequences(5'-3')
ETBR-F	CCTTTTGTCCGAGCCAGAGC
ETBR-R	GGATTGGAAGCACCAGGAGAA
ICAM-1-F	CTGTCGGTGCTCAGGTATCC
ICAM-1-R	TGTCTTCCCCAATGTCGCTC
GAPDH-F	CAAGTTCAACGGCACAGTCAAG
GAPDH-R	ACATACTCAGCACCAGCATCAC
IFNγ-F	TCCTCTTTGACCAATCATTCTTTCT
IFNγ-R	ATTCCTCTGGTCAGCAGCAC
IL-12-F	TGACATGTGGACGAGCATCT
IL-12-R	CAGTTCAATGGGCAGGGTCT
IL-17-F	AAACGCCGAGGCCAATAACT
IL-17-R	GGTTGAGGTAGTCTGAGGGC

Table 2(on next page)

Evaluation of heart function in each group by echocardiography

Evaluation of heart function in each group by echocardiography

Table 2 Evaluation of heart function in each group by echocardiography

Group	LVEDd(mm)	LVEDs(mm)	EF(%)	FS(%)
NC	**5.55±0.18**	**2.88±0.46**	**88.73±5.91**	**53.02±7.80**
EAM	**6.95±0.35**$^{\#\#}$	**4.17±0.33**$^{\#\#}$	**72.65±5.35**$^{\#\#}$	**35.35±4.02**$^{\#\#}$
ETBR-oe	**5.48±0.59****	**2.72±0.46****	**87.57±3.86****	**50.65±5.23****
GFP	**6.71±0.13**$^{\Delta}$	**3.72±0.31**$^{\Delta}$	**75.47±3.48**$^{\Delta}$	**37.82±2.70**$^{\Delta}$

** P<0.01 vs EAM group and GFP group; # #P <0.01 vs Control group; **$^{\Delta}$P ＞0.05 vs** EAM group