

Research Article

Could ARNI have an Effect on LV Fibrosis and Inflammatory Parameters in an Experimental Autoimmune Myocarditis Model?

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Abstract

Objectives: The aim of this study is to investigate the efficacy of sacubitril-valsartan treatment in the early period of Experimental Autoimmune Myocarditis (EAM) model, under the perspective of fibrosis and inflammatory cytokines.

Methods: The study was performed using 18 rats in three groups of EAM (n=6), EAM treatment (n=6) and control (n=6). EAM was induced by footpad injections of porcine cardiac myosin and Complete Freund's Adjuvant (CFA). The EAM group was not given any medical care. 20 mg/kg sacubitril-valsartan was given to EAM treatment (marked as treatment) group twice a day beginning from the 21st day to the 42nd day. No procedure was applied to the control group. Histopathological, biochemical and RT-PCR analyses were performed on the heart tissues taken after the 42nd day of sacrificing.

Results: Significant congestion, fibrosis, and cellular changes were observed in the EAM and treatment groups. There was 50% less severe fibrosis, which is 3rd degree, in the Treatment group compared to EAM. Severe congestion rate was lower in the treatment group compared to EAM; with the percentage of 16,6% to 50%. Though the average values of treatment group were lower than EAM group, there was obvious difference in TNF α , TGF β 1 and NT-proBNP levels (p>0.05) between the EAM and the treatment groups. There was no significant difference in IL-6 levels between the three groups.

Conclusion: In view of findings, the treatment of ARNI in acute autoimmune myocarditis may be promising on cardiac risk markers (cytokine, BNP values) and fibrosis parameters. Studies to be conducted in high-numbered groups will reveal more statistical significance.

Keywords: Experimental myocarditis, fibrosis, heart failure, sacubitril/valsartan, wistar rat

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Myocarditis is an inflammatory disease that can be due to viral or bacterial infections, toxic, drug-mediated or autoimmune causes.^[1] It may be associated with myocyte

necrosis and fibrosis. Myocarditis is an important cause of dilated cardiomyopathy (DCM).^[2] The incidence of myocarditis 22/100000 or 1.5 million in 2013 according to ICD

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diagnosis codes.^[3] Diagnosis and treatment of myocarditis are more important issues because it may cause sudden cardiac death in young and progressive heart failure.^[4]

It is well known that transforming growth factor-beta1 (TGF β 1) expression increases in response to tissue damage and plays a role in the tissue repair process and scar formation. Extracellular matrix production and tissue repair were carried out from myofibroblasts by the TGF- β 1 and signal pathways.^[5,6] TGF β 1 levels increased during hypertrophy and fibrosis. TGF- β 1 mRNA expression increase in left myocardium, also. Angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor 1 blockers (ARB) treatment decreased TGF- β 1 levels suggested that TGF β 1 induction mediates cardiomyocyte hypertrophy caused by angiotensin II.^[7,8]

An angiotensin receptor-neprilysin inhibitor (ARNI), sacubitril-valsartan, reduce the harmful effects of RAAS activation while increasing levels of potentially beneficial endogenous vasoactive peptides that are degraded by neprilysin.^[9] Neprilysin inhibitors reduce the metabolism of biologically active natriuretic peptides that stimulate natriuresis, diuresis and vasodilation. Since neprilysin also catalyses the system that metabolizes angiotensin II, neprilysin inhibitors mediate the reduction of angiotensin II levels.^[10]

Aautoreactive CD4+T lymphocytes can trigger myocarditis.^[11] It was shown that fibrosis, TGF β 1, tumor necrosis factor-alpha (TNF α) and interleukin levels are increased in rats with myocarditis.^[12,13] Sacubitril-valsartan has also been shown to have a positive effect on fibrosis in an animal study.^[14]

The aim of this study is to investigate whether the effects of sacubitril-valsartan early treatment has a positive contribution to the level of cytokines including TGF β 1, TNF α , interleukin 6 (IL 6), the areas of fibrosis, congestion and cell changes in experimental autoimmune myocarditis (EAM) model.

Methods

Animals

18 Wistar albino rats, 7 weeks old and weighing 180-200 g were used in this study. During the experiment, the rats were housed in the experimental research center, in a 12-hour night-12-hour day cycle, at room conditions with an ambient temperature of $22\pm 2^{\circ}\text{C}$. Standard feed was given ad libitum and drinking water in the light cycle for the feeding. During the dark period, bottles containing tap water were taken and replaced by bottles containing the following contents for each group. The rats were divided into 3 equal groups described as EAM, treatment and control group. Porcine cardiac myosin and Freund's Adjuvant and açık adı (PBS) were applied equally from the soles area on the 0 and 7 days to the EAM and the treatment groups. Sa-

cubitril valsartan was administered twice a day at 20 mg/kg starting from the 21st day, after immunisation by oral gavage in the treatment group. The experiment was terminated by sacrifice on the 42nd day.

Cardiac Myosin

Porcine cardiac myosin (M-0531 Sigma-Aldrich, Munich, Germany) with equal amounts of Freund's Adjuvant (25.740 MKA Thermo Fisher Scientific, USA) and PBS were administered to the footpad area on days 0 and 7 of the study period. Firstly, 0.95 mL of a phosphate-buffered solution containing 1.05 mL of calcium-activated myosin (Sigma-Aldrich, Munich, Germany) obtained from pig heart was first mixed in a tube to prepare a 4 mL suspension. Then, 2 ml of Freund's Adjuvant compound was placed in a separate tube. Liquid mixtures from both tubes were taken into a Luer lock injector. It was connected a 3-way connector and air bubbles were removed from the system. The suspension was homogenized by moving it for 10 minutes with the back and forth movement of two Luer lock injectors. The suitability of the homogeneous mixture was verified by a non-dispersible drop. The mixture was prepared fresh on the day of the application.^[15]

Evaluation of Cardiac Functions

20 mg/kg ketamine hydrochloride and 10 mg/kg xylazine were administered to all rats for echocardiographic evaluation. After rats were anesthetized, their chest areas were shaved. LVEF values of all rats were evaluated using a vivid Q cardiovascular ultrasound system device (Horten, Norway) and GE 12S (5-11 MHz) transducer on the 21st day, which is considered at the peak of the inflammation. M-Mode imaging and the Teicholtz method were used for LVEF measurements.^[16]

Collecting Tissue Samples

At the end of 42 days, animals were anesthetized by administering 35 mg/kg ketamine hydrochloride, 15 mg/kg xylazine. The cervical dislocation was performed in all rats under general anesthesia. After sacrifice, tissues were taken from all rats by thoracotomy at the end of the 42nd day. Afterward, the left ventricle of the heart was removed and taken into tubes with "DNAse / RNAse free" and placed in liquid nitrogen without waiting. In case of any negativity that can be seen in the analysis section, a backup of each tissue was taken under the same conditions and stored at -80°C .

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Gene expression levels were quantified by real-time PCR. 10-30 mg of each tissue was placed in sterile eppendorf

Table 1. LVEF Levels and p values

Biomarkers	EAM	Treatment	Control	p
21 st day LVEF%	43.6%±9.4	47.3%±6.7	74.1%±1.9	
Pro-BNP	1039.48±232.98	717.47±73.31	577.25±99.82	p<0.05*
TNF α	1.55±0.33	1.33±0.23	0.63±0.1	p<0.05*
IL 6	1.55±0.16	1.45±0.13	1.08±0.12	p>0.05
TGF β1	1.58±0.35	1.28±0.35	0.52±0.12	p<0.05*

*It means that there is a significant difference between the control group and the EAM and treatment groups. LVEF: Left Ventricular Ejection Fraction; EAM: Experimental autoimmune myocarditis; BNP: Brain natriuretic peptide; TNF α: Tumor necrosis factor α; IL 6: Interleukin 6; TGF β1: Transforming growth factor beta-1

tubes of 2 ml. Eppendorf tubes were kept in liquid nitrogen for 10 seconds. Tissue samples were homogenized in the homogenizer (Retsch MM400). Lysis buffer and mercaptoethanol were added to tubes with homogenized tissue and incubated for 1 hour at room temperature with shaking. Pure Link RNA Mini Kit (Cat No. 12183018A) was used for RNA isolation. Actions were made according to the procedure specified in the kit. NanoDrop 57 ND1000 was used to determine the purity and concentration of the obtained RNA samples. As for the purity rate, values between 1.8-2.1 were accepted from the RNAs we measured at 260/280 nm. After the total amount of RNA obtained from tissues for PCR process was equalized, synthesis was performed using the cDNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™). In the expression studies of TGF β1, TNF α and IL 6 genes with Quantitative Real-Time PCR (StepOnePlus™ Real-Time PCR System), cDNAs obtained from RNAs isolated from heart tissue were used. TaqMan (RealQ Plus 2x Master Mix, Ampliqon, Denmark) was used for the analysis of gene expression 59 levels. β-Actin (Gene Expression Assays, 250 rxns, Rn00667869) TaqMan® primer-probe was used for TGFB1 (Gene Expression Assays, 250 rxns, Rn00572010), TNF-α (Gene Expression Assays, 250 rxns, Rn99999017), IL-6 (Gene Expression Assays, 250 rxns, Rn01410330) and normalization of genes. Possible changes in gene expression levels were detected using the previously described method, RQ. The following procedure was applied to the expression of the identified genes.

Histopathological Evaluation

The heart tissue samples taken were fixed in 10% buffered formalin solution for 24 hours. Then, tissue tracing was done by cassette according to the groups and the tissues were embedded in paraffin. Sections of 4-micron 60 thickness were taken from the prepared paraffin blocks and stained with hematoxylin & eosin (H&E) with Leica ST5010 Autostainer XL in accordance with the user manual of the device and then examined with a light microscope (Olympus BX46, Japan). Vascular congestion and cellular degen-

eration were evaluated. Masson's trichrome staining was done manually to evaluate fibrosis. Scoring the groups for fibrosis, cellular changes, congestion parameters; 0: none, 1: mild, 2: moderate, 3: prominent.

Biochemical Parameters

On the 42nd day of the study, 2 ml of subclavian or intracardiac blood was collected from all rats after sacrifice. After the blood was transferred to EDTA tubes, it was centrifuged at 4000 rpm for 10 minutes. Serum samples were stored at -80°C until the analysis day. Serum samples taken for the study were kept until they reached room temperature. NT-proBNP levels were analyzed by the ELISA method (Shanghai Sunred Biological Technology, Shanghai, China).

Statistical Analysis

Statistical analysis was carried out by IBM SPSS Statistics version 22 software. The homogeneity of the obtained numerical data was first checked with the Levene test and the homogeneity of variance assumption was met. Analysis of variance (ANOVA) was used for an overall comparison between the study groups followed by Tukey as a posthoc test for pairwise comparisons. Differences were considered significant when two-sided p value <0.05.

Results

Echocardiographic Findings

Mean values of LVEF were found 43.6%±9.4 in the EAM group, 47.3%±6.7 in the treatment group, and 74.1%±1.9 in the control group (Table 1).

Biochemical Findings

NT-proBNP levels were demonstrated in Table 1. Although there was an increase in NT-proBNP levels between the EAM and the EAM-treatment groups, there was significantly difference (1039.48±232.98 and 717.47±73.31 p=0.052). There was a significant difference in NT-proBNP levels between the control group (577.25±99.82) with EAM (p=0.02) and the treatment group (p=0.002) (Fig. 1).

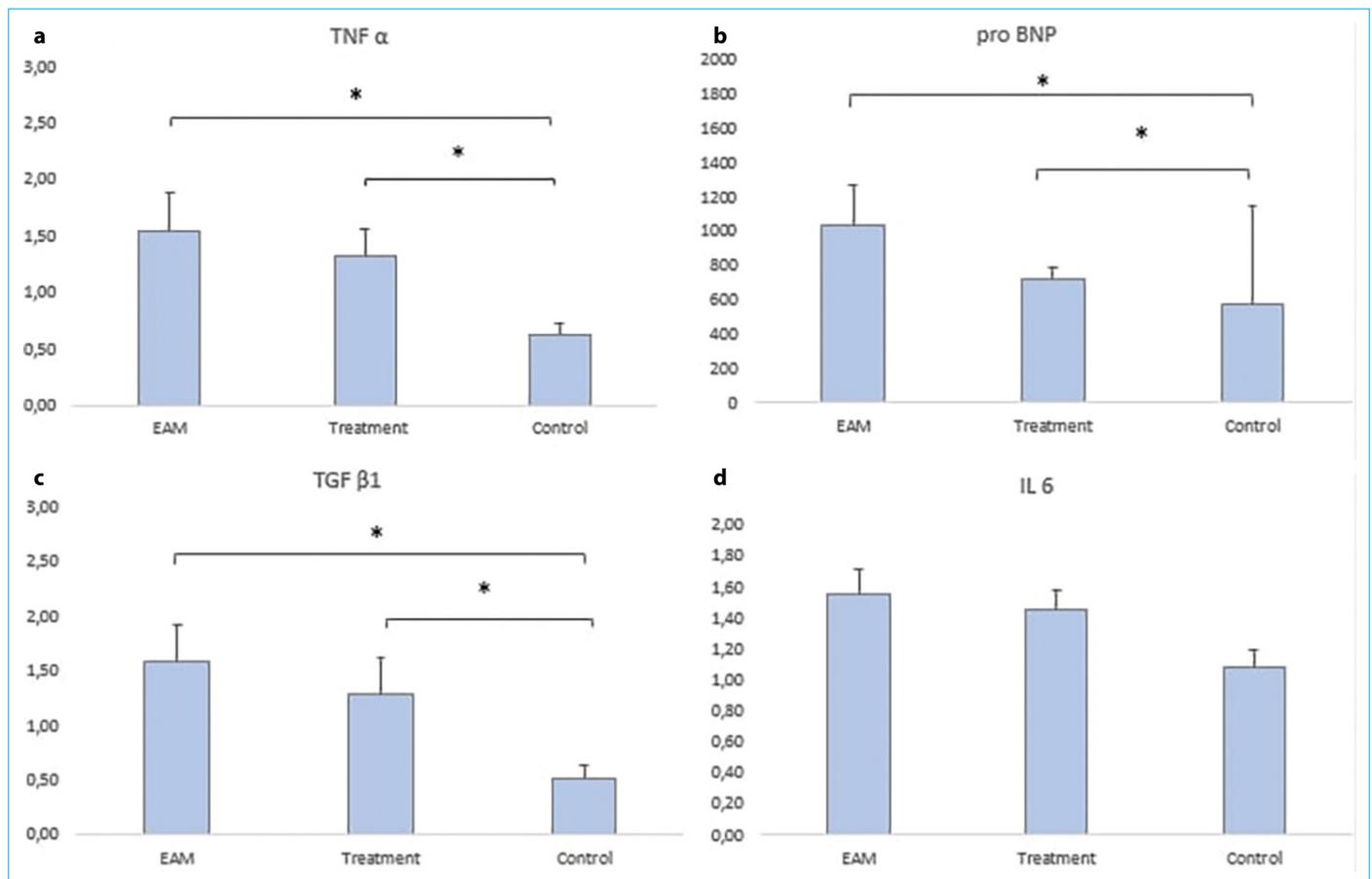


Figure 1a-d. Pro-BNP, TNF α , IL 6, TGF β 1 Levels.

BNP: Brain natriuretic peptide; TNF α : Tumor necrosis factor α ; IL 6: Interleukin-6; TGF β 1: Transforming growth factor beta-1.

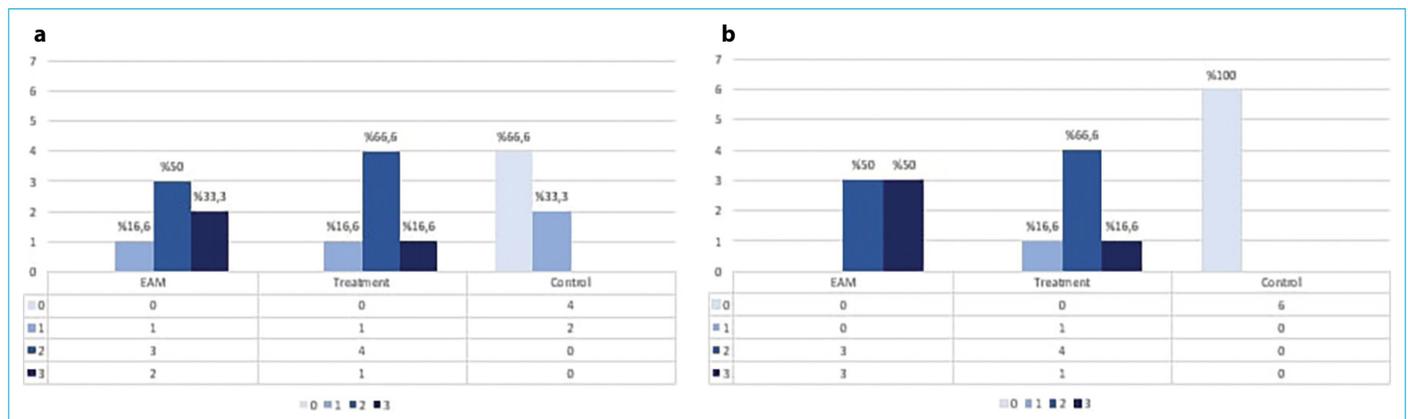


Figure 2. (a) Percentage of fibrosis in rats on scoring performed, **(b)** Percentage of congestion in rats on scoring performed.

Histopathological Findings

Statistically significant difference was observed between the EAM and the control group in terms of congestion ($p=0.02$), cell damage ($p=0.005$) and fibrosis ($p=0.007$). There was no statistical significance between the EAM group and the treatment group in terms of congestion, cell damage and fibrosis, although they were statistically significantly higher in the treatment group than in the control

group ($p=0.019$, $p=0.006$ and $p=0.001$, respectively), but there was 50% less severe fibrosis, which is 3rd degree, in the EAM and treatment group and severe congestion rate was lower in the treatment group compared to EAM; with the percentage of 16,6% to 50% (Fig. 2a, b). Histopathological photographs of H&E stained sections and Masson's trichrome stained sections belonging to the EAM, treatment and control groups are given (Figs. 3, 4).

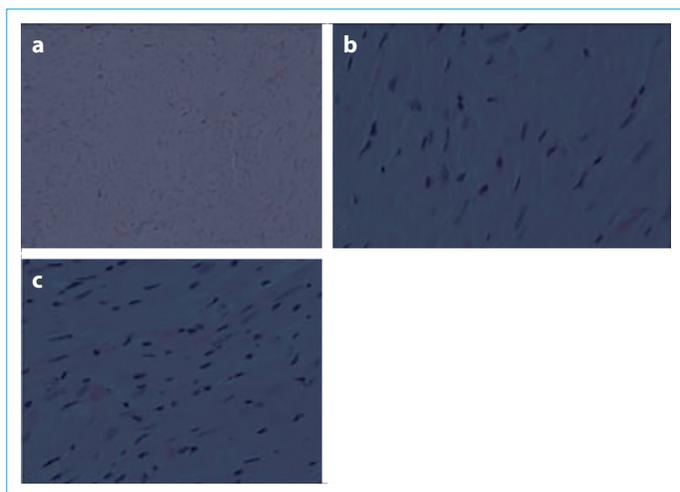


Figure 3a-c. Myocardial tissue containing degenerated nucleus in Hematoxylin Eosin (H&E) staining.

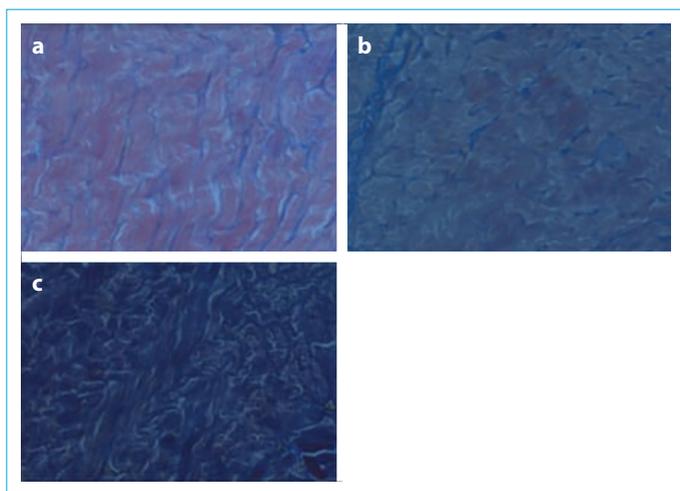


Figure 4a-c. Myocardial tissue fibrosis in Masson's Trichrome staining.

Real-Time PCR (RT-qPCR) Findings

There was a significant difference in TNF α cytokine levels between the EAM and the control groups (1.55 ± 0.33 and 0.63 ± 0.1 $p < 0.01$) and between the treatment and control groups (1.33 ± 0.23 and 0.63 ± 0.1 $p < 0.01$). TNF α cytokine levels were similar between the EAM and the treatment groups (Fig. 1). There was no significant difference in IL-6 cytokine levels between EAM, treatment and control groups (1.55 ± 0.16 , 1.45 ± 0.13 and 1.08 ± 0.12) (Fig. 1). There was a significant difference in TGF $\beta 1$ levels between EAM and control groups (1.58 ± 0.35 and 0.52 ± 0.12 $p < 0.01$). There was a significant difference in TGF $\beta 1$ levels between treatment and control groups (1.28 ± 0.35 and 0.52 ± 0.12 $p < 0.01$). TGF $\beta 1$ cytokine levels were not significantly observed between EAM and treatment groups (Fig. 1). In addition, Superman's rho correlation was found to be significant between TGF $\beta 1$ and fibrosis (0.8).

Discussion

Our results showed that there was significantly increases at NT-proBNP, TNF α , TGF $\beta 1$, IL 6 levels and fibrosis level in the early period with EAM. However, the mean values of cytokines in the treatment group were found to be lower than the EAM values.

Myocarditis is an inflammatory heart disease that may associate with the myocyte necrosis and fibrosis. EAM models have been used for many years to evaluate treatment, prognosis and survival parameters because these models resemble human myocarditis pathology. EAM models given ARB and ACEi in treatment revealed positive results about fibrosis and inflammation parameters.^[13,14,17,18] Suematsu et al.^[14] reported LVEF, cardiac output values were better and TGF β and TNF α levels were lower with ARNI treatment than valsartan treatment in C57BL /6 J rats. Another study reported in which lisinopril was given daily for treatment until sacrifice on the 21st cell necrosis rates, inflammatory cytokine levels such as TNF α and IL-6 levels were lower in the group given lisinopril in Wistar rats.^[19] As reported in these studies, ACEi and sacubitril-valsartan, which are started with immunization, have a positive effect on fibrosis, necrosis, cell changes, inflammatory cytokine levels and LVEF values. In our study, unlike the studies above, treatment was started on the 21st day of the experiment and treatment responses were evaluated 3 weeks later. Starting of treatment earlier and evaluating for the treatment response after the 4th week may affect the results. 14th-21st days are the days when the expected inflammation reaches the peak, myocarditis occurs at the cellular level, heart failure, pericardial effusion and mononuclear cell infiltration occur in EAM models.^[20,21] In the following days, EAM was established, heart failure worsened, and the level of fibrosis increased.^[22] Echocardiography is mostly performed on the 21st day that inflammation is considered the peak.^[23,24] In our study, the mean LVEF of the rats was lower in EAM groups than the control group on the 21st day.

Demonstration of fibrosis, congestion, inflammation, necrosis and cellular changes by histochemical staining method is widely used in EAMs models. Hirakawa et al.^[25] reported that the rate of fibrosis was higher in the myocarditis group than the treatment and control groups in rats. In our study, histochemical examinations performed after sacrifice on the 42nd day, a significant difference was found in the control group and the other two groups. However, EAM and treatment groups were similar. In our study 20 mg/kg/day sacubitril-valsartan treatment was used for 3 weeks in the treatment group. The reason of our insignificant difference in terms of fibrosis in our study may be explained as the drug was administered at low doses. In previous stud-

ies evaluating ACEI and ARNI treatments, these drugs were administered on the same day as immunization.^[17,18] We think that a model in which the drug is used longer and at higher doses can give better results.

NT-proBNP is an important parameter in predicting death or rehospitalization for heart failure.^[26] Ogawa et al.^[27] showed that BNP levels were lower in the group treated with ARB than the control in an experimental myocarditis model in Lewis rats. Korkusuz et al.^[28] reported that NT-proBNP levels were similar on the 1st, 7th and 21st days between experimental autoimmune myocarditis and control groups in their study using Lewis rats. In our study, no significant difference was observed between the EAM and treatment groups in NT-proBNP analysis on the 42nd day. Our results suggested that there may be an increase in NT-proBNP in the early period in sacubitril-valsartan treatment. Long-term use of sacubitril-valsartan in myocarditis may arise different results.

Increased inflammatory cytokines have been shown in EAM and inflammatory DCM.^[14,20,29,30] Smith et al.^[31] reported that TNF α was playing an effective role in the pathogenesis of myocardial inflammation in the EAM study using male A/J rats. Marina et al.^[12] reported that lower levels of TNF α in the group in higher quercetin dose than the low-dose quercetin group and the control group. In our study, TNF α levels did not show a significant difference between EAM and treatment groups. We think that starting earlier or giving a higher dose of sacubitril-valsartan in order to affect the TNF α level may change the results. It has been shown that IL 6 is increased in EAM studies. Ahmed et al.^[21] in the EAM study using Wistar rats, it was reported that the IL 6 level increased in the myocarditis group compared to the control group and decreased in the treatment with lisinopril. Considering the results of IL 6 expression in our study, although the IL 6 level increased relatively in the EAM group, there was no significant difference between the groups. Tissue samples were taken 42 days after the end of the experiment. These results show us that the level of IL 6 cytokine, which plays a role in the active inflammatory process, may decrease at the samples taken on the 42nd day or inflammation can be prevented by administering sacubitril-valsartan. Nevertheless, there are not enough studies examining the response of IL 6 level to treatment in experimental animal studies treated with sacubitril-valsartan. Therefore, the role of IL 6 in inflammation and the effect of sacubitril-valsartan on IL 6 cytokine levels are open to being investigated in the future.

TGF β signal controls the transition from myocarditis to post-inflammatory fibrous phenotype and the accumulation of pathological myofibroblasts in the heart. In EAM,

intracardiac TGF β levels gradually increase in parallel with continuously resolving cardiac infiltrates.^[32] RAAS and TGF β 1 are some of the key mediators of cardiac adaptations to hemodynamic overload and therefore play a critical role in the pathogenesis of fibrosis, cardiac hypertrophy and heart failure.^[33] Treatment with ACEi or ARB significantly reduced TGF β 1 levels in myocardial tissue, suggesting that TGF β induction in the myocardium during remodeling mediates cardiomyocyte hypertrophy caused by angiotensin II.^[8] This gives hope that an ARB molecule such as sacubitril-valsartan can reduce TGF β levels, preventing the increase in these inflammatory cytokine levels that cause fibrosis, remodeling and heart failure, and be effective in the treatment of inflammatory dilated cardiomyopathy due to myocarditis. It was reported that the TGF β antibody prevented the formation of dilated cardiomyopathy in BALB/C type rats.^[5] Kania et al.^[34] reported that myocarditis peaked 21 days after immunization and the number of cardiac fibroblasts gradually increased during follow-up in the EAM model. It was reported in the same study that anti-TGF antibody treatment prevented myocardial fibrosis in immunized mice. Likewise, in previous studies, we found fibrosis rates in the EAM and treatment groups were higher than the control group. However, the fibrosis rate was lower in the treatment group, there was no significant difference in TGF β levels between the two groups. Our results made us think that the TGF β level, which is an important marker of fibrosis, may not have been a significant difference due to the fact that sacubitril-valsartan was not initiated with immunization and/or a higher dose was not given, and the number of animals was small.

We think that the amount of drug administered, the time the drug was given, the effect of the drug and the total number of animals may affect results. There are not enough studies in experimental autoimmune myocarditis models. We think that by increasing the number of animals used in future experimental studies, the effect of sacubitril-valsartan on both fibrosis and inflammatory cytokine levels, which is a fibrosis parameter, can be revealed. It promises positive results in animal experiments to be applied by increasing the drug dose given in the studies to be conducted.

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Authorship Contributions: Concept – A.D.; Design – A.D.; Supervision – E.G., B.D.; Materials – R.O.O., Y.A.; Data collection &/or processing – A.D.; Analysis and/or interpretation – M.A.; Literature search – A.D.; Writing – A.D., E.G., B.D.; Critical review – E.G., B.D.

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