Article

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Subcutaneous BNP Injections in Rabbits: A Novel Approach to Mitigate Myocardial Remodeling in Atrial Fibrillation

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Background: Atrial fibrillation (AF) is a prevalent cardiac arrhythmia associated with increased morbidity and mortality, highlighting the need for novel therapeutic strategies. This study aimed to evaluate the effects of B-type natriuretic peptide (BNP) on cardiac structural remodeling in a rabbit model of AF.

Methods: Rabbits were subjected to rapid pacing to induce an AF model, and BNP was delivered subcutaneously at a dose of 20 µg/kg/d twice per day for three weeks. Electrophysiological measurements were taken to assess the AF induction rate and atrial effective refractory period (AERP), while echocardiographic measurements evaluated left atrial size and function. Histological examinations included hematoxylin and eosin (H&E) staining and Masson's trichrome staining to observe myocardial tissue structure and fibrosis. The ultrastructure of myocardial tissue was observed using a transmission electron microscope.

Results: The study found that BNP treatment significantly reduced the AF induction rate ($p < 0.001$), improved AERP ($p < 0.001$), and ameliorated structural and functional changes in the left atrial ($p < 0.05$). Histological analysis demonstrated decreased myocardial fibrosis post-BNP treatment ($p < 0.05$). Results also showed that BNP attenuated the cardiomyocyte remodeling caused by AF, as evidenced by significant effects on the expression levels of transforming growth factor-β 1 (TGF-β1), tissue inhibitors of matrix metalloproteinases 1 (TIMP1), matrix metalloproteinase 9 (MMP9), and Collagen I/III ($p < 0.05$).

Conclusion: These findings suggest that subcutaneous injections of BNP may serve as an effective therapeutic agent in mitigating cardiac structural remodeling in AF, offering significant clinical implications for treating this condition.

Keywords: atrial fibrillation; BNP; myocardial remodeling; myocardial function

Introduction

Driven by the global aging process, the incidence of atrial fibrillation (AF) continues to increase [1]. Since 1989, the global prevalence of AF has more than doubled, with the current total number of patients approaching 60 million [2]. Age advancement is a pivotal risk factor for the increased incidence of atrial fibrillation; moreover, chronic conditions such as cardiovascular diseases, abnormal body weight, and hyperglycemia also significantly trigger the development of AF [3]. Current management of atrial fibrillation focuses on symptom alleviation and the prevention of complications. Pharmacological and non-pharmacological heart rate and rhythm management strategies are employed to improve symptoms, while anticoagulation therapy is central to reducing the risk of stroke [4]. However, these treatments often come with potential adverse effects and frequently fail to meet the desired therapeutic outcomes demanded by both healthcare providers and patients alike [5]. Structural remodeling, notably characterized by myocardial fibrosis, is recognized as one of the most critical contributors to the onset and perpetuation of atrial fibrillation [6]. The regulation of atrial fibrosis involves complex cellular and molecular mechanisms. Identifying novel biomarkers is crucial for understanding the pathophysiology in individual AF patients. This has significant implications for developing new methods to prevent and treat atrial fibrosis in AF.

B-type natriuretic peptide (BNP) is a hormone primarily produced in the heart, playing a crucial role in maintaining cardiovascular health and balance. Under normal physiological conditions, BNP supports cardiac function through various mechanisms, including promoting vasodilation, increasing urine output, and sodium excretion, thereby helping to regulate blood pressure and fluid balance [7]. Additionally, BNP can inhibit the renin-angiotensin-aldosterone system, reducing excessive activation of the cardiovascular system, and countering cardiac hypertrophy and fibrosis, thus protecting the heart from adverse stimuli [8].

In fact, subcutaneous injection of BNP has been shown to significantly improve cardiac and renal function in patients with asymptomatic systolic heart failure [9]. Additionally, in cases of acute myocardial infarction, patients perfused with a low dose of BNP showed significant improvements in LVEF and left ventricular end-systolic volume after one month [10]. In the BELIEVE II study, patients with acute myocardial infarction who received nesiritide had statistically significant improvements in left ventricular end-diastolic volume index and left ventricular end-systolic volume index [11].

In pathological cardiac conditions, such as during myocardial remodeling, the role of BNP becomes particularly significant. Myocardial remodeling refers to the structural and functional changes the heart undergoes in response to injury or overload, typically manifesting as cardiac hypertrophy and fibrosis. BNP can alleviate the adverse effects of cardiac remodeling and slow the progression of heart failure through its anti-fibrotic and anti-hypertrophic effects. In the study by Kapoun *et al.* [12], the effects of BNP on human cardiac fibroblasts in fibrosis, fibroblast transformation, cell proliferation, and inflammatory responses were elucidated, highlighting BNP's effect on transforming growth factor-β 1 (TGF-β1)-mediated myocardial fibrosis and suggesting its therapeutic role in myocardial remodeling.

The subcutaneous injection of BNP, a technique involving the continuous or periodic administration of BNP via subcutaneous injection, is a treatment strategy aimed at combating cardiac diseases, particularly heart failure (HF) and atrial fibrillation (AF) [13]. Compared to intravenous injections or oral administration, it offers advantages such as prolonged drug action, high patient compliance, and enhanced safety [14], thereby gradually gaining acceptance in clinical practice. However, its effectiveness in controlling myocardial remodeling caused by atrial fibrillation still requires further investigation.

This study aimed to explore the therapeutic effects of subcutaneous injection of BNP on myocardial remodeling caused by AF. A rabbit model of AF was successfully established using rapid pacing techniques, followed by subcutaneous injections of BNP. The research found that BNP treatment significantly improved the structural and functional abnormalities caused by AF, highlighting the potential clinical value of subcutaneous injection of BNP as an innovative treatment approach. This offers a new perspective and practical basis for the treatment of AF.

Materials and Methods

Induction, Treatment, and Sample Collection of Rabbit AF Model

Eighteen adult New Zealand white rabbits (body weight 3.0–3.5 kg) were purchased from Harbin Medical University Animal Research Center. They were housed under conditions of 22–24 °C with a 12-hour light/dark cycle and had access to sufficient food and water. Each animal was assigned a random number, which was then used to randomly allocate the animals into three groups: the Sham group, the AF group, and the BNP group. All rabbits were divided into three groups (N = 6 per group) as follows:

(1) Sham: Rabbits underwent thoracotomy and right atrial electrode fixation only, without rapid pacing;

(2) AF: Rabbits underwent continuous rapid atrial pacing at 600 beats/min for three weeks;

(3) BNP: Rabbits received BNP (Nuodikang Biological Pharmaceutical Company Ltd., Chengdu, China. Pharmaceutical Approval Number S20050033) injection subcutaneously at a dose of 20 µg/kg/d twice per day for three weeks and underwent continuous rapid atrial pacing concurrently [15]. All rabbits received routine electrocardiogram examinations daily. The BNP solution was prepared by dissolving 0.5 mg of BNP in 100 mL of physiological saline.

During the surgery, the rabbits were anesthetized with ketamine (35 mg/kg; K113, Sigma Aldrich, St. Louis, MO, USA) and xylazine (5 mg/kg; X1126, Sigma Aldrich). After the experiment, the animals from each group were euthanized with an overdose of sodium pentobarbital (150 mg/kg) and perfused with saline followed by 4% paraformaldehyde. For Western blot and quantitative polymerase chain reaction (qPCR) experiments, tissues were thoroughly perfused with saline, randomly sampled for myocardial tissue, and stored at –80 °C. For tissues intended for hematoxylin and eosin (H&E) and Masson staining, after perfusion with saline and 4% paraformaldehyde, myocardial tissues were obtained and immersed in 4% paraformaldehyde, then stored in a refrigerator at 4 °C.

Echocardiographic Measurements

After three weeks of rapid atrial pacing, the cardiac structure and function of all rabbits were evaluated using transthoracic echocardiography (Philips CX50, Phillips, Amsterdam, Netherlands). Measurements included left atrial (LA) diameter, left atrial volume maximum (LAVmax), left atrial volume minimum (LAVmin), and left atrial ejection fraction (LAEF).

Electrophysiological Measurements and AF Induction

During the study, an electrophysiological stimulator was utilized to employ the S1S2 pacing protocol with an incremental/decremental method. This protocol was based on basic cycle lengths (BCL) of 200 ms and 150 ms to determine the atrial effective refractory period (AERP)200ms and AERP150ms. Initially, a series of S1 stimulations were applied to the heart, with each stimulation at a pulse width of 2 ms and a voltage of twice the threshold voltage, administered consecutively eight times.

Subsequently, a premature S2 stimulation was introduced at the end of atrial diastole. The initial S1S2 interval was set at 50 ms or 150 ms and adjusted incrementally or decrementally by 10 ms with each subsequent trial. As the measurement approached the AERP, the adjustment interval was reduced to 2 ms to precisely determine the AERP. The longest S1S2 interval at which the S2 stimulation failed to provoke atrial excitation was recorded as the AERP value. To ensure experimental accuracy and stability, this measurement procedure was repeated three times, and the average of the three AERP values obtained was considered the final AERP measure.

During the experiments, a 10 Hz frequency, 2 ms pulse width S1S1 burst stimulation method was employed. Each rabbit received 10 consecutive 10-second stimulations with a 30-second interval between stimulations to allow for adequate cardiac recovery. The number of successful inductions and the duration of atrial fibrillation in each rabbit were recorded; the occurrence of either atrial fibrillation or atrial tachycardia was considered a successful induction. Each experimental group consisted of 6 rabbits, totaling 60 inductions.

The AF induction rate was calculated using the formula: AF induction rate = (total number of successful inductions of atrial fibrillation in each group/total number of inductions in the group) $\times$ 100%.

Echocardiography was performed using a probe model S5-1 with a frequency range of 3–10 MHz to obtain detailed cardiac images and assess cardiac function.

H&E Staining

The rabbits were euthanized with an overdose of sodium pentobarbital (150 mg/kg) and subsequently perfused with saline followed by formaldehyde solutions. Heart tissue samples were collected and fixed in 4% formaldehyde. After fixation, the tissues were embedded in paraffin and sectioned consecutively. The tissue sections were then dehydrated, cleared, and sequentially stained with hematoxylin and eosin (G1005, ServiceBio, Wuhan, China). Finally, the morphology and structure of the myocardial tissues were observed under a microscope (CX41, Olympus, Tokyo, Japan).

Masson's Trichrome Staining

Collagen fibers in myocardial tissue were visualized using a Masson's trichrome staining kit (G1006, ServiceBio, Wuhan, China). Briefly, paraffin sections were first dehydrated and deparaffinized. They were then treated with Solution A for 12 hours. After rinsing with tap water, the sections underwent sequential immersion in a mixture of Solutions B and C, followed by Solutions D and E. Subsequently, they were transferred directly into Solution F for staining for 20 seconds. Finally, the stained samples were removed, sealed with neutral resin, and examined under a microscope (CX41, Olympus, Tokyo, Japan) to observe the distribution of collagen fibers within the myocardial tissue. The collagen volume fraction (CVF) in different fields of view was calculated accordingly.

Table 1. Antibodies used in Western blot assay.

Name of antibodies	Company	Catalog	Dilution
Collagen I	wanleibio, Shenyang, China	WL0088	1:2000
MMP9	Bioss, Beijing, China	bs-4593R	1:2000
TIMP1	Bioss	bs-0415R	1:2000
TGF-β1	Bioss	bs-0086R	1:2000
Collagen III	Bioss	bs-0549R	1:2000
Goat anti rabbit IgG-HRP	wanleibio	WLA023	1:2000
β-actin	wanleibio	WL01372	1:5000

TGF-β1, transforming growth factor-β 1; TIMP1, tissue inhibitors of matrix metalloproteinases 1; MMP9, matrix metalloproteinase 9; IgG-HRP, immunoglobulin G antibody conjugated with Horseradish Peroxidase.

Western Blot

The left atrial myocardial tissue from rabbits (approximately 100 mg) was finely minced, and radioimmunoprecipitation assay (RIPA) lysis buffer was added for thorough homogenization. The mixture was lysed on ice for 30 minutes, followed by centrifugation to obtain the total protein lysate. After measuring the total protein concentration using a bicinchoninic acid (BCA) kit (Cat. P0012S, Beyotime, Shanghai, China), the appropriate amount of protein to load was calculated. Subsequently, 20 µg of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, transferred to a membrane, and blocked with 5% skim milk powder. The membrane was then incubated overnight with the primary antibody, washed three times with tris-borate-sodium-0.05%Tween-20 (TBST), incubated with the secondary antibody at room temperature for 1 hour, and developed for colorimetric or chemiluminescent detection. The intensity of the protein bands was quantified using Image Pro Plus software (Ver. 6.0, Media Cybernetics, Bethesda, MD, USA), with β-actin serving as an internal reference. Details of the antibodies used are listed in Table 1.

Real-Time Polymerase Chain Reaction (Real-Time PCR)

Approximately 50 mg of myocardial tissue was placed in a mortar, finely ground to a powder with liquid nitrogen, and transferred to a centrifuge tube. Total RNA was extracted from the tissue using the TRIpure method (RP1001, BioTeke, Beijing, China). The extracted total RNA was then reverse transcribed into cDNA using the BeyoRT II M-MLV Reverse Transcription Kit (D7160L, Beyotime, Shanghai, China).

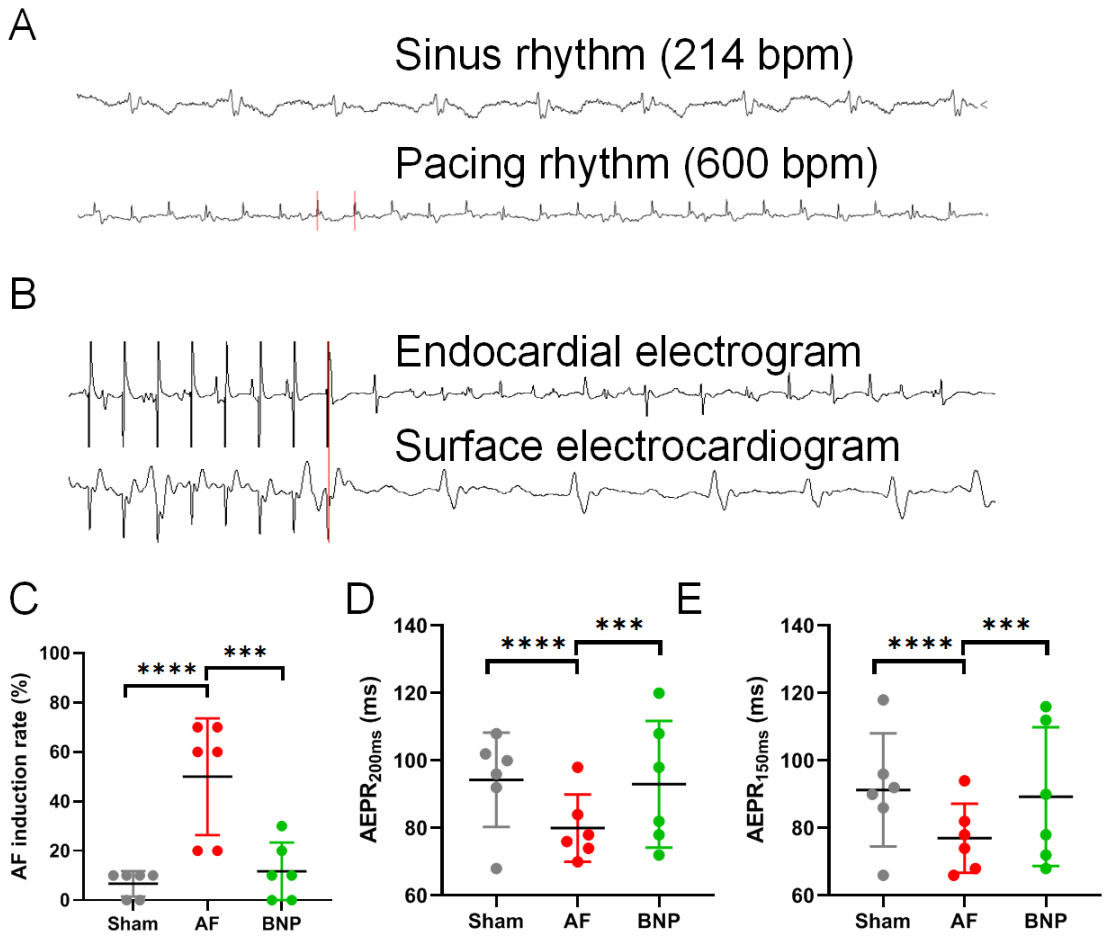

Fig. 1. Effects of rapid pacing on atrial fibrillation (AF) induction and atrial effective refractory period (AERP) in rabbits. (A) Increase in heart rate from baseline to 600 bpm demonstrated by rapid pacing. (B) Confirmation of increased heart rate via Endocardial electrogram and Surface electrocardiogram. (C) Comparison of AF induction rates between the AF model group and Sham group. (D) AERP was measured at 200 ms in the AF model vs. the Sham group. (E) AERP was measured at 150 ms in the AF model vs. Sham group. ***$p < 0.001$, ****$p < 0.0001$.

For PCR amplification, a reaction mixture containing SYBR Green (SY1020, Solarbio, Beijing, China) and primers synthesized by GenScript Biotech Corporation was prepared. Real-time PCR experiments were performed on an Exicycler 96 qPCR system (BIONEER Corporation, Daejeon, Korea). The primers used were as follows:

- β-actin: Forward 5′- CCAGGTCATCACCATCGG -3′, Reverse 5′- TGTCCACGTCGCACTTCA -3′;

- TGF-β1: Forward 5′- AGGACCTGGGCTGGAAG -3′, Reverse 5′- CGGGTTGTGCTGGTTGTA -3′.

β-actin expression served as the internal reference for normalization. The relative expression levels of TGF-β1 were calculated using the $2^{-\Delta\Delta Ct}$ method.

Transmission Electron Microscopy

After fixation with glutaraldehyde, the tissues were embedded in agarose. Subsequently, they were fixed with osmium tetroxide, dehydrated through graded alcohol and acetone treatments, and embedded in resin. Once polymerized, ultrathin sections were cut. These sections were then stained with a 2% uranyl acetate-saturated alcoholic solution, washed, and further stained with lead citrate solution. After drying on copper grids, the sections were ready for observation under a transmission electron microscope (H7650, HITACHI, Tokyo, Japan).

Data Statistics and Analysis

In the data analysis phase, all quantitative data were presented as mean ± standard deviation and visualized appropriately. One-way Analysis of Variance (ANOVA), followed by a least significant difference (LSD) post-hoc test, was used to assess overall differences between groups, with a significance level set at $p < 0.05$. Statistical analyses were conducted using SPSS 22.0 software (IBM Corp., Chicago, IL, USA). Each animal experimental group comprised six animals, and all detection experiments were independently repeated at least three times.

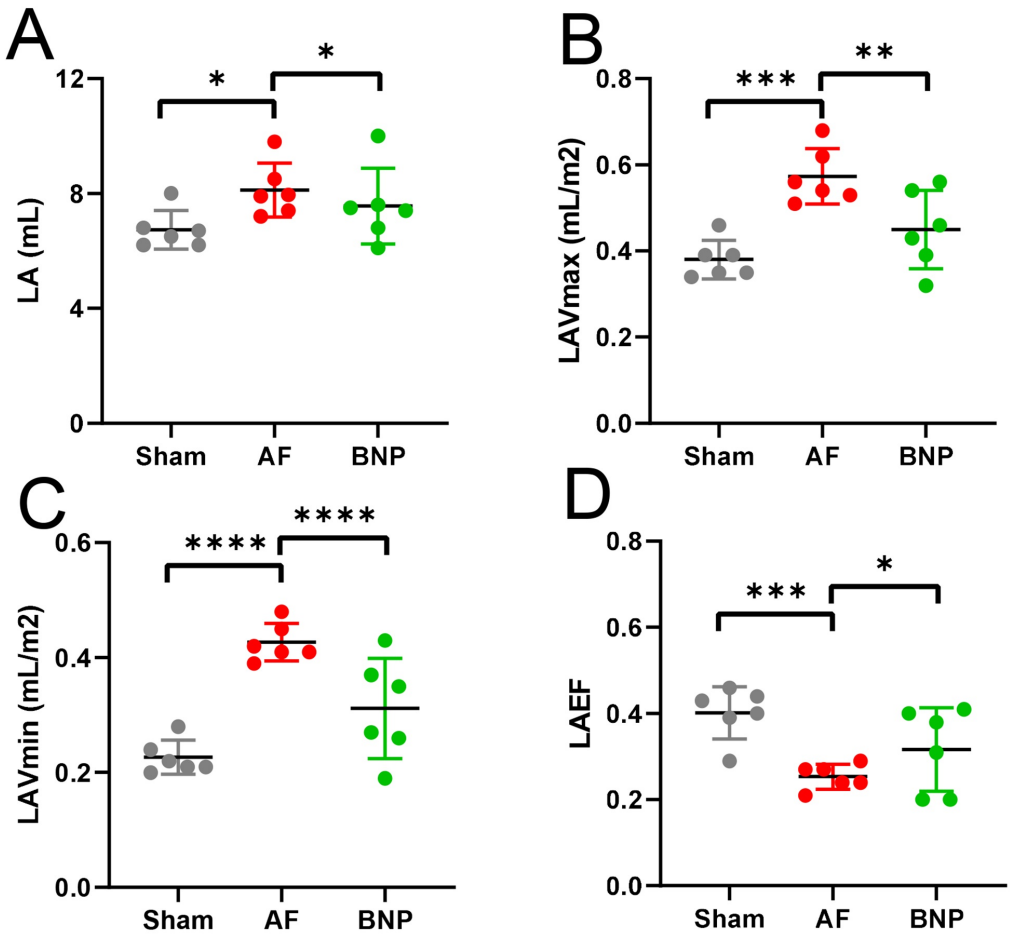

Fig. 2. Echocardiographic measurements showing the impact of B-type natriuretic peptide (BNP) treatment on left atrial (LA) size and function in AF rabbits. (A) Changes in LA size post-BNP treatment. (B) Variations in left atrial volume maximum (LAVmax) with treatment. (C) Adjustments in left atrial volume minimum (LAVmin) following BNP administration. (D) Alterations in left atrial ejection fraction (LAEF) due to BNP treatment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Results

Electrophysiological Measurements

As depicted in Fig. 1, following rapid pacing induction, the heart rate of rabbits increased from an initial 214 bpm to 600 bpm (Fig. 1A). This change was observable via the endocardial electrogram and confirmed on the surface electrocardiogram (Fig. 1B). Fig. 1C shows that compared to the control group (Sham group), there was a significant increase in the AF induction rate in the AF rabbit model group ($p < 0.0001$). However, after subcutaneous injections of BNP, there was a notable decrease in the AF induction rate in the AF rabbit model group (Fig. 1C, $p < 0.001$). Furthermore, compared to the Sham group, the atrial effective refractory period (AERP) in the AF rabbit model group was significantly shortened at basic cycle lengths of 200 ms (AERP200ms) and 150 ms (AERP150ms) ($p < 0.0001$). Nevertheless, following BNP treatment, AERP200ms and AERP150ms in the AF rabbit model group significantly recovered, and the differences with the Sham group were no longer statistically significant (Fig. 1D,E, $p < 0.001$).

Echocardiographic Measurements

As depicted in Fig. 2, compared to the control group (Sham group), the AF rabbit model group exhibited significant increases in LA (Fig. 2A), LAVmax (Fig. 2B), and LAVmin (Fig. 2C), while LAEF showed a significant decrease (Fig. 2D, $p < 0.05$). These data highlight substantial cardiac functional changes in the atrial fibrillation rabbit model.

Moreover, after subcutaneous injection of BNP in the AF model rabbits, LA, LAVmax, and LAVmin values significantly decreased, accompanied by a significant increase in LAEF (Fig. 2A–D, $p < 0.05$). These results strongly suggest the potential therapeutic value of BNP in addressing atrial structural remodeling induced by AF.

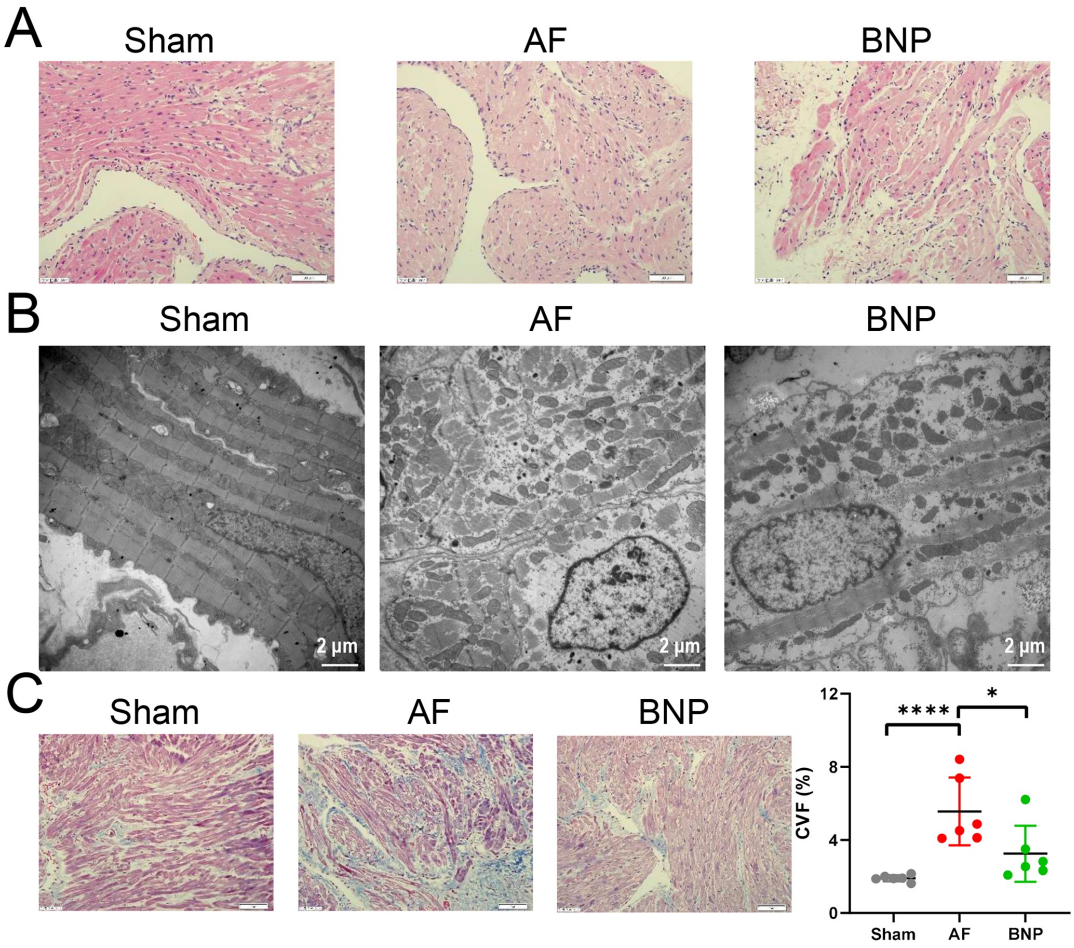

Fig. 3. Histological examination of myocardial tissue highlighting the therapeutic impact of BNP on cardiac remodeling induced by AF. (A) Hematoxylin and eosin (H&E) staining reveals changes in myocardial cell structure. Scale: 50 µm. (B) TEM was used to observe improvement in myocardial tissue structure post-BNP treatment. (C) Masson's trichrome staining depicting fibrosis levels before and after BNP administration. Scale: 50 µm. *$p < 0.05$, ****$p < 0.0001$.

The Therapeutic Effect of BNP on Cardiac Structural Remodeling Induced by AF

To validate the therapeutic efficacy of BNP against AF-induced myocardial tissue remodeling, an extensive analysis of rabbit myocardial tissue was conducted using H&E staining, Masson's trichrome staining, and transmission electron microscopy. The results, displayed in Fig. 3, revealed that rabbits in the AF model group exhibited characteristics such as increased myocardial cell volume, disordered arrangement, and blurred cell boundaries, contrasting sharply with the orderly arranged myocardial cells observed in the Sham group. However, after BNP treatment, pathological changes in myocardial tissue were significantly improved (Fig. 3A,B). Additionally, Masson's trichrome staining showed a noticeable increase in fibrosis levels in the myocardial tissue of the AF model group, indicated by an increased area of blue collagen fiber staining and a significant rise in the collagen volume percentage compared to the Sham group (Fig. 3C). Following BNP treatment, myocardial fibrosis in the AF model rabbits was significantly alleviated, with the collagen volume percentage notably decreased (Fig. 3C, $p < 0.05$).

According to the data presented in Fig. 4, compared to the Sham group, the expression of TGF-β1, matrix metalloproteinase 9 (MMP9), Collagen I, and Collagen III in the myocardial tissue of the AF model rabbits was significantly upregulated, accompanied by a significant decrease in the expression of tissue inhibitors of matrix metalloproteinases 1 (TIMP1). After subcutaneous administration of BNP, a significant inhibitory effect was observed on the expression of TGF-β1, MMP9, Collagen I, and Collagen III. Conversely, the expression of TIMP1 was positively promoted (Fig. 4A–C, $p < 0.05$).

Discussion

AF is a prevalent cardiac arrhythmia that not only diminishes patients' quality of life but is also closely linked to serious complications such as myocardial remodeling and

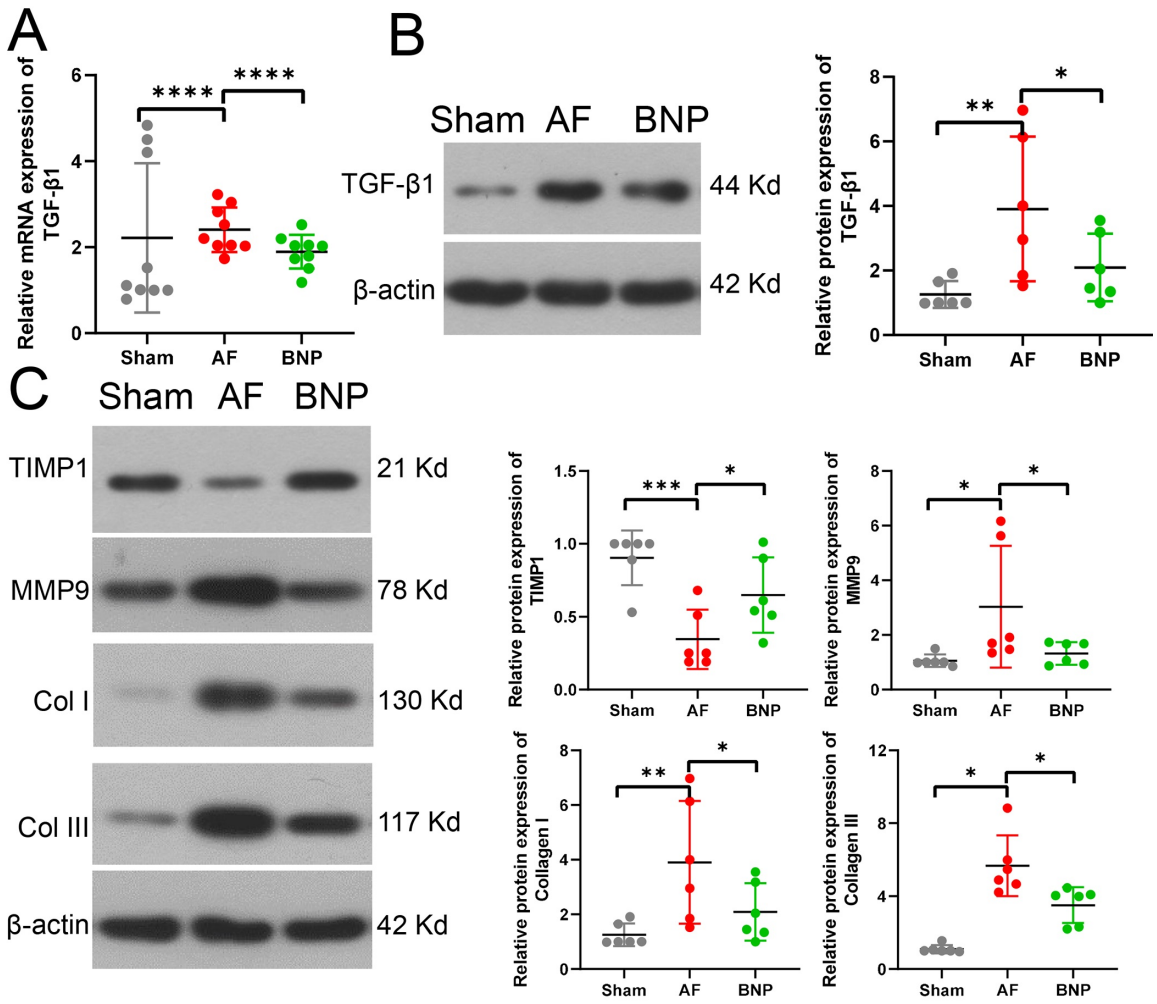

Fig. 4. Expression alteration of remodeling-related proteins in the myocardial tissue of AF rabbit model post BNP-treatment. Comparative analysis of mRNA (A), protein (B) expression of TGF-β1 in AF model before and after BNP treatment. (C) Measurement of TIMP1, MMP9, Collagen I, and Collagen III expression before and after BNP treatment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

heart failure [16]. BNP, a cardiac hormone, plays a critical role in regulating blood pressure and maintaining cardiovascular stability [17]. Previous studies have primarily examined the acute effects of BNP administration [18,19]. In contrast, this research investigates the potential therapeutic efficacy of subcutaneous BNP injections in inhibiting AF-induced myocardial remodeling. Through electrophysiological parameter measurements, echocardiographic assessment, and histological examination, this study demonstrates that subcutaneous BNP administration significantly improves cardiac structure and function in an AF rabbit model. These findings underscore the potential application of BNP in treating chronic heart diseases and emphasize its significance as a novel strategy for addressing AF-induced myocardial remodeling.

The persistent electrophysiological changes caused by AF also contribute to atrial wall thickening, leading to hemodynamic abnormalities and triggering myocardial remodeling [20]. BNP is widely recognized as a protective factor in cardiac diseases. Secreted by the ventricular walls during heart expansion or pressure, BNP promotes sodium and water excretion, dilates blood vessels to reduce blood pressure, and inhibits the renin-angiotensin system, thereby reducing cardiac load and preventing excessive myocardial remodeling [17]. Clinical observations indicate that BNP levels tend to increase in AF, reflecting the heart's response to increased cardiac load [21]. Elevated BNP levels not only aid in diagnosing and assessing the severity of cardiac diseases but also hold the potential to evaluate the risk of all-cause mortality in AF patients [22]. Clinical studies have shown that BNP infusion can improve left ventricular infarction areas in acute myocardial infarction patients, likely through its anti-fibrotic effects [7]. This infusion method has clinical potential for myocardial structural remodeling

post-acute myocardial infarction [23]. Research has further linked BNP significantly with myocardial remodeling in AF patients [24]. *In vitro* experiments demonstrate that BNP treatment inhibits fibrosis, and myofibroblast transformation, and reduces proliferation-related protein expression in cardiac fibroblasts induced by TGF-β [12]. Clinically, BNP infusion in heart failure treatment, such as with Nesiritide, has shown improvements in dyspnea compared to nitroglycerin, with lower adverse event incidences [25]. Moreover, in heart failure treatment trials like PRECEDENT, recombinant human brain natriuretic peptide (rhBNP) has shown advantages over dobutamine in controlling mortality and severe ventricular arrhythmias [26]. In this study, after BNP treatment, significant downregulation of fibrosis and proteins associated with myocardial remodeling (TGF-β1, TIMP1, MMP9, Collagen I, and Collagen III) was observed in the AF model group.

BNP has been widely applied as an effective medication for treating heart failure. Clinical trials have shown that BNP infusion can improve hemodynamics, increase urine output, and stabilize serum creatinine levels in patients recovering from cardiac surgery with myocardial suppression [27]. In experimental models of myocardial infarction, BNP infusion enhances left ventricular function and reduces post-infarction remodeling [28]. However, BNP injection in heart failure treatment may lead to hypotension, potentially increasing the risk of mortality and renal function impairment [29]. Thus, careful dosage monitoring is essential when using BNP.

Subcutaneous administration of BNP is preferred over infusion. It effectively improves left ventricular structure and function while mitigating tolerance issues associated with infusion therapy [24]. Patients with preclinical diastolic dysfunction experienced sustained improvement in cardiac diastolic function after receiving subcutaneous injections of BNP for 12 weeks [30].

Despite these benefits, the therapeutic application of BNP in atrial fibrillation requires further investigation. This study observed a significant decrease in the incidence of atrial fibrillation in the AF model group following subcutaneous BNP administration, suggesting its potential effectiveness in treating atrial fibrillation.

Conclusion

In summary, this study demonstrates that subcutaneous injections of BNP significantly enhance cardiac structure and function in a rabbit model of AF, suggesting potential applications for treating AF-induced cardiac remodeling. Additionally, BNP treatment reduces the incidence of AF, implying significant clinical implications. However, a limitation of this study is its restriction to an animal model of atrial fibrillation, underscoring the need for further investigation into the efficacy and safety of BNP treatment for AF in human subjects.

Availability of Data and Materials

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

Author Contributions

SZ and HZ designed the research study. SZ and RH performed the research. HZ provided help and advice. RH and JY analyzed the data. All authors were involved in the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study adhered to the principles of laboratory animal use issued by the National Institutes of Health and was approved by the Medical Ethics Committee of The People's Hospital of Liaoning Province (Ethics Approval No. 2022-k065).

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Conflict of Interest

The authors declare no conflict of interest.

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