EXPERIMENTAL STUDY

Antiremodeling Effect of Xanthine Oxidase Inhibition in a Canine Model of Atrial Fibrillation

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Summary

In a canine rapid atrial stimulation model of atrial fibrillation (AF), we have demonstrated an increased production of reactive oxygen species (ROS) along with electrical and structural remodeling. In the present study, we hypothesized that antioxidants can suppress atrial remodeling canines with AF. We therefore evaluated the effect of febuxostat, a xanthine oxidase (XO) inhibitor and a pure antioxidant, on atrial remodeling.

AF was produced by performing a 3-week rapid atrial pacing (400 bpm) in 13 dogs divided into three groups: pacing + febuxostat group (n = 5; atrial pacing with 50 mg/day of febuxostat (administration); pacing control group (n = 5; atrial pacing without any drug administration); and non-pacing group (n = 3). Electrophysiological studies were conducted in the first 2 groups every week. Atrial tissue fibrosis was evaluated by Azan and immunofluorescent staining of fibronectin. Oxidative stress was evaluated by DHE and FCF-DA staining.

Shortening of the refractory period and increase in AF inducibility appeared gradually in the pacing control group, but such changes were suppressed in the pacing + febuxostat group (P = 0.05). The pacing control group showed increase in fibrosis, which was suppressed in the febuxostat group. In DHE and DCF-DA staining, the pacing control group showed an increase in oxidative stress, which was suppressed in the pacing + febuxostat group. The pacing control group exhibited fibronectin expression, which was suppressed in the pacing + febuxostat group.

The antioxidant effect of febuxostat may achieve an inhibition of new-onset AF in canines.
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Key words: Reactive oxygen species, Oxidative stress, Atrial remodeling, Febuxostat

Oxidative stress plays an important role in tissue degeneration in various diseases, e.g., myocardial interstitial fibrosis in myocardial infarction and atherosclerosis.1-4 Such interstitial changes can be caused by various factors, including inflammatory cytokines, angiotensin-II, sympathetic nerve activation, and oxidative stress.5-6 In atrial fibrillation (AF), atrial interstitial fibrosis promotes the construction of AF arrhythmogenic substrate by destructing the electrical connections between atrial myocytes.7,8 As a result, the conduction velocity of atrial stimulation is decreased, leading to the appearance of multiple random reentry of AF due to shortening of the re-entrant circuit wavelength.9,10 Previously, we demonstrated the suppressive effect of carvedilol, a beta-blocker with an antioxidative action, on atrial remodeling and increase in AF inducibility in a canine AF model. These results suggest an important role of reactive oxygen species (ROS) in promoting atrial tissue fibrosis and the possibility of using an antioxidant as antiremodeling therapy.11,12

In this study, we hypothesized that treatment with an antioxidant suppresses atrial remodeling in canines with AF. We therefore evaluated the effect of febuxostat, a xanthine oxidase (XO) inhibitor and a pure antioxidant, on atrial remodeling in a canine AF model.

Methods

Initial surgery: The canine AF model was set up as previously described.13 Briefly, 13 adult female beagle dogs (11.1 ± 1.2 kg body weight) were anesthetized with intravenous pentobarbital (Nembutal14; 25 mg/kg), followed by an additional dose of 2 mg/kg at the end of each hour. Preceding the intubation, the dogs were anesthetized with intramuscular butorphanol tartrate (Butorphanol15, 0.3 mg/kg) as an analgesic. Ventilation was maintained using an endotracheal tube with a mechanical ventilator (Model...
Figure 1. Schematic of the study protocol. This figure shows the schematic of the study protocol. Each dog underwent initial surgery and was allowed to recover for 1 week without pacing before the start of atrial rapid pacing (day 0). Atrial rapid pacing (400 bpm) was performed for 3 weeks in the pacing control (n = 5) and pacing + febuxostat (n = 5) groups. In the pacing + febuxostat group, febuxostat (5 mg/kg/day) was orally administered from day 14 to day 21. Atrial tissue was sampled in each dog at the end of the protocol. See text for the details.

SN-480-5; Shinano Manufacturing, Tokyo, Japan) with 100% oxygen. One pair of electrodes was sutured to the right atrial free wall and another pair to the left atrial free wall to monitor atrial electrograms. The other ends of the electrode wires were tunneled subcutaneously and exposed at the back of the neck. For continuous rapid atrial pacing, a unipolar screw-in lead (CapSureFix 5568; Medtronic Inc., Minneapolis, MN, USA) was inserted through the right external jugular vein and the distal end of the lead was screwed into the endocardial side of the right atrial appendage. The proximal end of the pacing lead was connected to a rapid pulse generator (Soletra, Medtronic Inc., Minneapolis, MN, USA), which was implanted into a subcutaneous pocket at the back of the neck. To mimic the hemodynamics in clinical cases of AF, atrioventricular block was not produced in this study.

All studies were performed in accordance with the guidelines specified by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine. The investigation conforms to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Study protocol:** To attain stable baseline conditions, each dog was allowed to recover after the initial surgical procedure for at least 1 week without pacing. The non-pacing group comprised three dogs that were not paced. Rapid atrial pacing (400 beats per minute [bpm]) was performed in 10 out of the 13 dogs, which were divided into the following two groups: the pacing control group (n = 5; dogs that underwent atrial pacing but were given no medication) and the pacing + febuxostat group (n = 5; dogs that underwent atrial pacing and were administered 50 mg/day febuxostat starting 2 weeks before the initiation of rapid pacing) (Figure 1).

**Electrophysiological study:** An electrophysiological study was performed every week in the pacing control and febuxostat groups to evaluate AF inducibility, atrial effective refractory period (AERP) at the left atrial pacing site, and atrial conduction velocity (CV) between the atria. These parameters were evaluated at each evaluation time point, i.e., every week throughout the entire study protocol. To evaluate AF inducibility, the incidence of AF induction was calculated with atrial burst pacing for 3 s at the minimal pacing cycle length that achieved 1:1 atrial capture at the pacing site at 4 times the diastolic threshold with a pulse width of 2 ms. AF was defined as a spontaneous irregular atrial rhythm lasting longer than 5 seconds. Atrial burst pacing for AF induction was delivered 5 times at the left atrial pacing site in each dog at each evaluation time point. AF inducibility was calculated as ratio of the time of AF induction and the time of burst pacing. AERP was measured with basic drive cycle lengths of 300, 200, and 150 ms at the left atrial site. The pacing output was set at twice the diastolic threshold during each evaluation at the left atrial pacing site. The coupling interval of the premature stimulus was shortened by 2-ms steps. The longest coupling interval of premature beats that failed to capture the atrium was reported as the local AERP. Because the AERP data varied among the in-
Figure 2. A: Result of the electrophysiological study of AF inducibility. This figure summarizes the data of the electrophysiological study in the pacing control (n = 5) and pacing + febuxostat (n = 5) groups. This panel shows the AF inducibility along the time course. The AF inducibility was around 30% in the pacing control group, but it was significantly lower in the pacing + febuxostat group. See text for the details. B: Result of the electrophysiological study of ΔAERP. This figure shows the ΔAERP data along the time course. The upper left panel shows the ΔAERP data at base cycle length of 300 ms. The upper right panel shows the ΔAERP data at base cycle length of 200 ms. The lower left panel shows the ΔAERP data at base cycle length of 150 ms. This figure summarizes the data of the electrophysiological study in the pacing control (n = 5) and pacing + febuxostat (n = 5) groups. The pacing control group exhibited AERP at each base cycle length shortening from day 7 and it continued until the end of the protocol. The pacing + febuxostat group tended to exhibit smaller AERP at base cycle length of 150 ms shortening than the pacing control, but the difference was significant only on day 14. See text for the details. B: Result of the electrophysiological study of Δ%CV. This figure shows the Δ%CV data along the time course. The upper left panel shows the Δ%CV data at base cycle length of 300 ms. The upper right panel shows the Δ%CV data at base cycle length of 200 ms. The lower left panel shows the Δ%CV data at base cycle length of 150 ms. This figure summarizes the data of the electrophysiological study in the pacing control (n = 5) and pacing + febuxostat (n = 5) groups. Although the pacing control group exhibited a gradual decrease in Δ%CV along the time course, such a change was almost totally negated in the pacing + febuxostat group, and significant differences were observed on day 7. However, significant differences in Δ%CV data were observed on days 14 and 21 only at base cycle length 150 ms. See text for the details.

Table 1. Echocardiographic Parameters on Day 21

|  | Pacing control (n = 5) | Pacing + febuxostat (n = 5) | P value |
|---|-----------------------|-----------------------------|---------|
| LAD (mm) | 23.7 ± 1.2 | 22.9 ± 0.8 | 0.237 |
| LAV (mm³) | 7.3 ± 1.6 | 6.3 ± 1.7 | 0.386 |
| LVDD (mm) | 29.8 ± 5.1 | 30.3 ± 3.7 | 0.852 |
| LVDs (mm) | 20.4 ± 4.5 | 20.7 ± 3.2 | 0.919 |
| LVEF (%) | 59.8 ± 7.8 | 60.4 ± 7.8 | 0.667 |
| ΔLAD (mm) | 0.09 ± 0.07 | 0.16 ± 0.07 | 0.612 |
| ΔLAV (mm³) | 0.35 ± 0.2 | 0.21 ± 0.2 | 0.325 |
| ΔLVEF (%) | −0.21 ± 0.15 | 0.14 ± 0.1 | 0.375 |

LAD, left atrial dimension; LAV, left atrial volume; LVDD, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVEF, left ventricular ejection fraction; ΔLAD, change in LAD after 3 week pacing; ΔLAV, change in LAV after 3 week pacing; and ΔLVEF, change in LVEF after 3 week pacing.
Histology of atrial tissues: histological and biochemical analyses. 

All dogs were anesthetized and ventilated mechanically as the right and left atrial free walls were rapidly excised for an alkaline battery (9 V). Subsequently, small portions of small amount of electric current directly to the heart using heart under anesthesia and euthanasia was performed in catheter in the pacing control and pacing + febuxostat cardiac output, were measured using a thermo-dilution arterial pressure, pulmonary arterial wedge pressure, and change caused by febuxostat administration, hemodynamic parameters, were calculated as

| Heart rate (bpm) | 199.6 ± 20.1 | 181.6 ± 14.9 | 0.146 |
|-----------------|--------------|--------------|-------|
| Systolic blood pressure (mmHg) | 157.2 ± 12.6 | 149.2 ± 10.1 | 0.362 |
| Diastolic blood pressure (mmHg) | 80.8 ± 10.1 | 73.4 ± 13.2 | 0.349 |
| Systolic pulmonary arterial pressure (mmHg) | 24.6 ± 6.5 | 26.2 ± 6.2 | 0.701 |
| Diastolic pulmonary arterial pressure (mmHg) | 10.6 ± 1.8 | 11.6 ± 4.5 | 0.654 |
| Pulmonary arterial wedge pressure (mmHg) | 8.2 ± 3.3 | 7.6 ± 2.9 | 0.769 |
| Cardiac output (L/minute) | 3.97 ± 0.79 | 3.54 ± 0.53 | 0.343 |

Echocardiographic study: An echocardiogram (EUB-6500 and EUP-S50, Hitachi Medical Corporation, Tokyo, Japan) was recorded before the initial surgery and at day 21, i.e., at the end of the entire protocol. In each recording, left atrial dimension (LAD), left atrial volume (LAV), left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), and left ventricular ejection fraction (LVEF) were evaluated. The changes in LAD, LAV, and LVEF after the 3-week pacing protocol were calculated as Δparameters by subtracting the values before the initial surgery from the values after 3 weeks of pacing.

Hemodynamic parameters: At the end of the protocol, all dogs were anesthetized and ventilated mechanically as previously mentioned in the initial surgery to evaluate the hemodynamic parameters. To exclude hemodynamic change caused by febuxostat administration, hemodynamic parameters, including systemic blood pressure, pulmonary arterial pressure, pulmonary arterial wedge pressure, and cardiac output, were measured using a thermo-dilution catheter in the pacing control and pacing + febuxostat groups at the end of the 3-week protocol. After the hemodynamic evaluation, the chest was opened to expose the heart under anesthesia and euthanasia was performed in all dogs, including the non-pacing group, by the following procedure: ventricular fibrillation was induced with a small amount of electric current directly to the heart using an alkaline battery (9 V). Subsequently, small portions of the right and left atrial free walls were rapidly excised for histological and biochemical analyses.

Histology of atrial tissues: At the end of the protocol, small portions of the left atrial free wall were excised for histological and biochemical analyses. Structural changes of the atrial tissue were evaluated with hematoxylin-eosin (HE) and Azan staining. The %area of fibrosis was quantified by NIH imaging of the Azan stained tissue, with the average result from 50 randomly selected views used to report the data for each individual dog. For in situ localization of reactive oxygen species (ROS), we incubated frozen sections (25 μm) of left atrial tissue with fluorophores sensitive to O2, dihydroethidium (DHE, 10 μmol/L; Sigma-Aldrich, Japan), hydrogen peroxide (H2O2), 5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, and acetyl ester (DCF-DA, 10 μmol/L; Molecular Probes). DHE specifically reacts with intracellular O2 and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctuate nuclear staining. The specificity of DHE and DCF signals for detecting O2 and H2O2 was confirmed by preincubation with polyethylene glycol-superoxide dismutase (PEG-SOD; 500 U/mL, Sigma) and PEG-catalase (350 U/mL, Sigma), respectively. The stained sections were then observed under a fluorescence microscope (LSM710; Carl Zeiss MicroImaging, USA).

Immuno-staining with antifibronectin antibody: The cardiac tissues were fixed in periodate lysine paraformaldehyde (0.1 M L-lysine/0.05 M phosphate buffer, PH 7.4; 150 mL, 8% paraformaldehyde; 50 mL, NaI04; 428 mg) for 6 hours. They were frozen after a stepwise change of sucrose. The frozen sections (25 μm) of left atrial tissue with fluorophores sensitive to O2, dihydroethidium, H2O2, or DCF-DA were incubated with primary antibodies (antifibronectin antibody; abcam®; USA) and subsequently with a secondary antibody (goat anti-mouse IgG H & L; Alexa Fluor® 594, abcam®; USA) and haloidin (antistain 488 phallloidin; Amanita phalloides; Cytoskeleton, Inc., USA). The sections were incubated in liquid DAPI/ Anti-fade Solution (DAPI; 4',6-diamino-2-phenylindole dihydrochloride, Antifade Solution; p-phenylenediamine and glycerol in PBS solution, 0.4 μg/mL DAPI suspended in Antifade; Merck Millpore, Germany). The stained sections were then observed under a fluorescence microscope (LSM710; Carl Zeiss MicroImaging, USA).

Statistical analysis: JMP 11 (SAS Japan Inc., Tokyo, Japan) statistical software package was used for performing statistical analyses. Values are presented as mean (standard deviation). Basic comparative statistics were analyzed using Friedman test (a non-parametric test) or Mann-Whitney U test. A P value of < 0.05 was considered statistically significant.

Results

Electrophysiological studies: Figure 2A, B, and C shows the time course of the results of electrophysiological studies. AF inducibility increased over time in the pacing control and pacing + febuxostat.
CONTROL GROUP. In the pacing + febuxostat group, AF inducibility also increased over time, but it was significantly lower than that in the pacing control group. AERP shortening (negative ΔAERP) was observed from a relatively early phase in the pacing control group. AERP shortening was also observed in the pacing + febuxostat group, but the degree of AERP shortening was smaller than that in the pacing control group. The Δ% of CV gradually decreased over time in the pacing control group. In contrast, in the pacing + febuxostat group, this Δ% of CV decrease was almost totally negated, and it was significantly different from that in the pacing control group at days 7 to 21.

**Figure 3.** Histology of the atrial tissues. This figure exhibits representative examples of HE and Azan staining of atrial tissues in the three groups evaluated in this study. In the HE staining, the pacing control group (middle upper panel, n = 3) showed interstitial proliferation, disalignment, and size-irregularity of the muscle fibers in comparison with the non-pacing group (left upper panel, n = 3). In contrast, in the pacing + febuxostat group (right upper panel, n = 3), such slight abnormality seems to be suppressed. In the Azan staining, interstitial fibrosis was increased in the pacing control group (middle lower panel, n = 3) in comparison with the non-pacing group (left lower panel, n = 3), but such fibrosis was obviously suppressed in the pacing + febuxostat group (right lower panel, n = 3). The graph shows the result of the quantification of the fibrotic area. The %area of fibrosis was increased in the pacing control group in comparison with the other two groups. See text for the details.
**Echocardiography:** Table I shows the echocardiography findings on day 21, with Δ data indicating changes in parameters at the end of the study from those obtained before the initial surgery. There were no significant differences in any of the parameters between the pacing control and pacing + febuxostat groups.

**Hemodynamic parameters:** Table II shows hemodynamic parameters evaluated at the end of the 3-week protocol in the pacing control and pacing + febuxostat groups. There were no significant differences between the two groups.

**Histopathology:** Figure 3 shows representative examples of the HE and Azan staining of the atrial tissues in the non-pacing, pacing control, and pacing + febuxostat groups. On HE staining, the pacing control group tissues had interstitial proliferation, disalignment, and irregularly sized muscle fibers compared with the non-pacing group. In contrast to the pacing group, such abnormalities appeared to be suppressed in the pacing + febuxostat group. On the Azan staining, interstitial fibrosis was more prominent in the pacing control group than in the non-pacing group; however, fibrosis was suppressed in the pacing + febuxostat group compared with the pacing group. In the quantification of the fibrotic area, % area of fibrosis was greater in the pacing control group than in the non-pacing group, and it was suppressed in the pacing + febuxostat group compared with the pacing group.

**Evidence of oxidative stress:** Upon DHE and DCF-DA staining, the pacing control group was observed to have significantly greater fluorescence intensity than the non-
Expression of fibronectin: On antifibronectin-1 (FN-1) antibody staining, markedly greater FN-1 expression was observed in the pacing control group compared with the non-pacing group, but FN-1 expression was suppressed in the pacing + febuxostat group. FN-1 was mainly located in the interstitial area (Figure 5). DAPI staining was seen in the nucleus, and phalloidin staining was seen in the cytoplasm.

Discussion
This study revealed several important findings. First, the 3-week rapid atrial pacing protocol caused atrial remodeling that was characterized by electrophysiological changes (increased in AF inducibility, AERP shortening, and decreased CV) and structural changes (extra-cellular matrix synthesis and interstitial fibrosis). Second, evidence of hyperoxidative stress was observed in atrial myocytes. Third, the expression of fibronectin, a mediator that promotes interstitial hyperproliferation, was observed mainly in interstitial areas of the atrial tissue. Finally, these findings were significantly suppressed by febuxostat, an XO inhibitor.
Role of oxidative stress in atrial remodeling: In our previous reports, we demonstrated a suppressive effect of several drugs on atrial remodeling.\textsuperscript{2,6,16-18} Olmesartan, atorvastatin, and carvedilol have all shown to suppress interstitial proliferation or intercellular fibrosis.\textsuperscript{2,6,19} In this study, although the intra-atrial conduction was partially suppressed, the decrease in the conduction velocity was suppressed. Those results are in accordance with the study results of enalapril or candesartan.\textsuperscript{20,21} These results also indicate that interstitial proliferation is an important mechanism in the impairment of intra-atrial conduction, an electrophysiological change resulting from atrial remodeling in AF. In previous reports, we also documented the overexpression of several mediators of interstitial proliferation, such as connective tissue growth factor and periostin.\textsuperscript{5,6} Therefore, it is reasonable to speculate that activation of networks of these mediators is the key to atrial structural remodeling in AF. Although various stimuli can activate these mediators, hyperoxidative stress plays an important role in such activation. In the report of Zheng et al., hypersynthesis of ROS was observed in parallel with vascular and myocardial damage in an ischemic heart disease model, indicating that hyperoxidative stress is likely an important factor in myocardial damage.\textsuperscript{22} In our previous study using the canine model, hyperoxidative stress in atrial tissue was suppressed by carvedilol,\textsuperscript{6} suggesting that the antioxidative effect of carvedilol may suppress atrial tissue fibrosis.\textsuperscript{12,23,24} In the present study, we chose to use febuxostat, an XO inhibitor and a pure antioxidant. Our results demonstrate that febuxostat suppresses AF inducibility, decreases atrial interstitial fibrosis, and lowers fibronectin 1 expression. These findings clearly indicate the antioxidant effect of febuxostat in suppressing AF inducibility in canines with AF.

However, Shiroshita-Takeshita et al. have reported that vitamin C, a nonspecific antioxidant, was not effective in suppressing the electrical remodeling in a similar canine AF model.\textsuperscript{25} Although the precise mechanism of this difference in results is unclear, it probably depends on the difference in the model and efficacy of the drug. In the series of our preceding studies using the same canine model, we have demonstrated the importance of oxidative stress as the inducer of structural remodeling at least in our model.\textsuperscript{5,6} Additionally, while vitamin C is a nonspecific antioxidant, febuxostat is stronger antioxidant that has shown antiremodeling effects in this study.

Mechanism of the effect of febuxostat on the atrial remodeling: Recent reports have documented that uric acid can directly injure vascular endothelium and/or renal tubular cells in hyperuricemia by damaging the uric acid transporter.\textsuperscript{26-28} These changes may cause sodium retention and vascular endothelial dysfunction, resulting in hypertension and/or renal dysfunction.\textsuperscript{24,29} It is likely that hyperuricemia induces atrial remodeling in the same way and that febuxostat may suppress atrial remodeling by decreasing the uric acid levels. However, the canines used in this study did not have hyperuricemia, indicating a different mechanism for atrial remodeling. Unlike in humans, the metabolism of uric acid in canines is not strongly dependent on the action of XO.\textsuperscript{30} The antiremodeling effect of febuxostat in this study thus appears to occur through its effect as an antioxidant. Therefore, it is conceivable that febuxostat may have a clinically useful antiremodeling effect on atrial tissues in patients with AF.

Limitations: Our study has several limitations. First, because His-bundle ablation was not performed in our animal model, progression to tachycardia-induced heart failure cannot be ruled out. However, we confirmed that the influence of this potential progression was small,\textsuperscript{5,6,16-18} because we found no significant changes in hemodynamic parameters. Second, the hemodynamic parameters were evaluated using a thermo-dilution catheter only at the end of the 3-week protocol, not every week. Third, the echocardiography was performed only twice, before initial surgery and at the end of the 3-week protocol. Pentobarbital anesthesia and mechanical ventilation were necessary for this protocol, and these procedures may promote oxidative stress, which could influence the evaluation of AF inducibility and oxidative stress markers. Finally, the tendency of the electrophysiological data was not constant in this study. This is probably because dogs are not homogenic animals; thus, the in vivo data may include variations. This should be a potential limitation of the canine model.

Conclusions
Febuxostat, an XO inhibitor and a strong antioxidant, suppressed AF inducibility in a canine model, a finding we consider to have been caused by its antioxidant effect.

Disclosures
Conflicts of interest: This study received no financial support from any commercial sources. Febuxostat bulk powder was supplied by Teijin Co. Ltd. (Tokyo).

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