

# Characterization of atrial histopathological and electrophysiological changes in a mouse model of aging

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**Abstract.** The detailed mechanisms of age-related atrial structural and electrophysiological changes remain elusive. Small animal models have recently been used for the investigation of atrial tachyarrhythmia. In this study, we investigated the hypothesis that atrial structural and electrical characterization with aging provides a substrate for atrial fibrillation using a mouse model of aging. Male Kunming mice aged 2 (young), 12 (middle-aged) and 24 months (aged) were used in this study. A surface electrocardiogram and sinus node recovery time (SNRT) were recorded at baseline. Atrial fibrillation (AF) inducibility and duration were measured by a transesophageal electrode catheter. Collagen content was assessed by the collagen volume fraction. Whole cell configuration using the patch clamp technique was performed for the transient outward potassium ( $I_{to}$ ) and ultra-rapid delayed rectifier potassium ( $I_{kur}$ ) currents. P-wave duration, SNRT and rate-corrected SNRT were longer in the aged group than in the remaining 2 groups, paralleled by inducibility significantly being increased in the aged group. The right atrium had significantly higher levels of fibrosis than the left atrium in all the groups ( $P < 0.05$ ), whereas the extent of fibrosis in the left atrium had a higher positive correlation with age relative to the right atrium ( $P < 0.05$ ). Moreover, in old age, the dispersion of left relative to right atrium repolarization and augmented  $I_{to}$  currents contributed to vulnerability to AF. Nevertheless,  $I_{kur}$  currents in the atrial myocytes showed no age-related changes. The present study demonstrates that in addition to the structural alterations, aging can also cause integrative and cellular electrophysiological changes in a mouse model of aging, facilitating AF initiation and maintenance.

## Introduction

Atrial fibrillation (AF), as a rhythm disorder characterized by chaotic electrical activity of the atria, is a frequent cause of cardiovascular morbidity and mortality worldwide. Its prevalence is 0.4 to 1.0% in the general population and increases with age (1). The mechanisms underlying AF remain elusive, and AF is thought to be maintained either via ectopic foci, multiple wavelets, or fibrillatory conduction emanating from a small number of stable rotors (2). Electrical and structural remodeling have emerged as crucial components in the persistence of AF (3). Electrical remodeling, such as changes in major repolarized ion channels, leads to the shortening of the action potential duration (APD) and the loss of APD rate-dependent adaptation, while structural remodeling (fibrosis) leads to an increase in atrial conduction slowing, re-entry, and thereby inducible AF (3).

Studies using large animal models (rabbits, dogs, canines and goats) have demonstrated a possible link between age-related changes in histopathological and electrophysiological properties and the vulnerability to AF (4-8). However, time-saving and low-cost animal models, such as rats and mice, have not been fully characterized to date. Rodent models for AF have been reported in a limited number of studies, but there are insufficient data for young vs. old rats (9), young vs. middle-aged rats (10) or mice (11). Moreover, small animal models, in particular mouse models, provide powerful tools in the investigation of basic structural and electrical mechanisms of cardiac arrhythmias. It is believed that sustained fibrillatory activity does not exclusively depend on critical myocardial mass or that critical mass is smaller than the mouse atrial surface of  $< 35 \text{ mm}^2$  (12). The inducibility of AF in the whole mouse heart *in vivo* has previously been evaluated using transvenous atrial stimulation, isolated stimulation and vagal activation by pharmacological intervention (29). Transesophageal stimulation is a minimally invasive method for atrial stimulation without direct atrial manipulation or mechanical changes during cardiac surgery or catheterization. Moreover, serial examinations of the same animal under different experimental conditions can be easily performed. These can facilitate the characterization of age-dependent electrophysiological properties in an individual animal.

Therefore, in the present study, adopting 3 sequential time-points (young, middle-aged and aged) for further investigation in a mouse model, we aimed to evaluate dynamic alterations

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associated with atrial fibrosis, and to determine atrial electrical properties related to vulnerability to AF.

## Materials and methods

**Animals.** For our study, male healthy Kunming mice aged 2 (young), 12 (middle-aged), and 24 months (aged) were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised in 1996). We chose these ages of mice based on previous study (13). The ages of 2, 12 and 24 months are equivalent to the human age of ~20, 40 and 70 years, respectively. The characteristics of the mice are shown in Table I.

***In vivo global electrophysiological analysis.*** Sinus cycle length (SCL) was determined by averaging 3 consecutive R-R intervals. P-wave duration (PWD) was measured by determining the earliest onset and latest offset of atrial deflection from 3 simultaneously recorded surface leads.

For the transesophageal electrophysiological study, a 2-French octapolar mouse electrophysiological catheter [8 0.5 mm circular electrodes; electrode pair spacing, 0.5 mm (Ciber Mouse; NuMed Inc., New York, NY)] was used for recording cardiac electrograms as well as for pacing the heart using consecutive electrode pairs. This catheter was inserted into the esophagus with a depth of 3–4 mm and unipolar recordings were obtained from each ring electrode. The threshold of pacing was examined by a 1 msec pulse width and a pacing rate 10 beats/min faster than normal. The electrode catheter was adaptively positioned to the site closest to the left atrium to ensure that constant atrial capture and correspondence to the minimum threshold (<1.5 mA) were attained. Sinus node recovery time (SNRT) was measured after a 30-sec pacing train with a basic cycle length (BCL) of 100 msec, a stimulus amplitude of 2-fold diastolic capture threshold and a stimulus duration of 1 msec. The SNRT was defined as the interval between the last stimulus in the pacing train and the onset of the first sinus return beat. Rate-corrected SNRT (CSNRT) was defined as the SCL subtracted from the SNRT.

Inducibility of atrial tachycardia and fibrillation (AT/F) was examined by applying 15-sec bursts (25 msec BCL, 2-fold diastolic capture threshold and 1 msec duration). This series of bursts was repeated 10 times. AF was defined as a period of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 sec (14). AT was defined as rapid and regular rhythm lasting for at least 30 sec.

***Histopathological analysis.*** After *in vivo* electrophysiological analysis, the mice were sacrificed and prepared for histopathological analysis. The aortas were then perfused via the common iliac bifurcation with pre-cooled PBS for 5 min. The left and right auricles were quickly cut and snap-frozen in liquid nitrogen for 1 min and stored at -80°C. Sections (5 µm in thickness) of the paraffin-embedded tissue were stained with picrosirius red (Direct Red 80; Sigma Aldrich) (15). Briefly, tissue sections were rinsed in distilled water and incubated in saturated 0.1% picrosirius red for 90 min. The sections were

Table I. Age and weight of the mice in the 3 groups.

	Young	Middle-aged	Aged
Age (months)	2	12	24
Weight (g)	35.4±4.2	52.1±5.4	60.5±7.2

Weight is presented as mean ± SD.

then rinsed twice with 0.01 N HCl for 1 min, dehydrated with an ethanol gradient and prepared for collagen analysis. Images were captured by a digital charge-coupled device (CCD) camera (Pro 150ES; Pixera Corporation, Los Gatos, CA), and connected to a polarized microscope (E600POL; Nikon, Tokyo, Japan) equipped and modified with a commercially available circular polarizer (Kenko, Tokyo, Japan). The collagen volume fraction (CVF) was calculated as described previously (15).

***Patch-clamp experiments.*** To isolate high-quality mouse atrial myocytes, we adopted a Langendorff-perfused method based on modified intubation skills (16). In brief, mice were anesthetized using an intraperitoneal injection of 0.4% pentobarbital sodium solution at a dose of 50 mg/kg body weight. The inferior vena cava was exposed under direct vision following an abdominal median incision. While 4°C heparinized saline at a dose of 4–5 ml (100 U/ml) was administered into the inferior vena cava, the abdominal aorta was dissected for bloodletting. Subsequently, the thoracic aorta, isolated through thymus dissection following a left thoracotomy between the second and fourth intercostal space, was slightly intubated by a self-made catheter (7 F paracentetic needle with blunt tip, ring-shaped notching 0.5 mm from the distal ending) at a position of 2 mm distance from the root of the aorta. A syringe with 3 ml saline was connected to the end of the catheter for excluding the internal air. The successful intubation was fixed to the aorta by sealing with 5-0 silk. The heart was then quickly excised and removed to the Langendorff apparatus for immediate retrograde perfusion. Atrial myocytes were prepared using enzymatic digestion and then separated from Kraft-Brühe (KB) solution by centrifuging at 600 rpm for 3 min. The cells were maintained at room temperature for the experiments that followed and used within 10 h.

Myocytes were transferred into an experimental chamber (~3 ml) mounted on the stage of an inverted microscope (TE2000-S; Nikon) and allowed to adhere to the glass bottom of the chamber. Cells were perfused with extracellular solution. A ruptured-patch whole cell voltage clamp was used to measure transient outward potassium ( $I_{to}$ ) and ultra-rapid delayed rectifier potassium ( $I_{kur}$ ) currents, and membrane capacitance ( $C_m$ ), while a current clamp was used for measuring a single cell action potential (AP). For  $I_{to}$  and  $I_{kur}$  measurements, the internal solution contained (in mmol/l) 110 K-aspartate, 20 KCl, 1 MgCl<sub>2</sub>, 5 Mg-ATP, 10 HEPES, 10 EGTA and 5 Na<sub>2</sub>-creatine phosphate, pH 7.3–7.4 (NaOH). To block Ca<sup>2+</sup>-activated chloride currents, 0.01 mmol/l niflumic acid was added to the pipette solution. The external

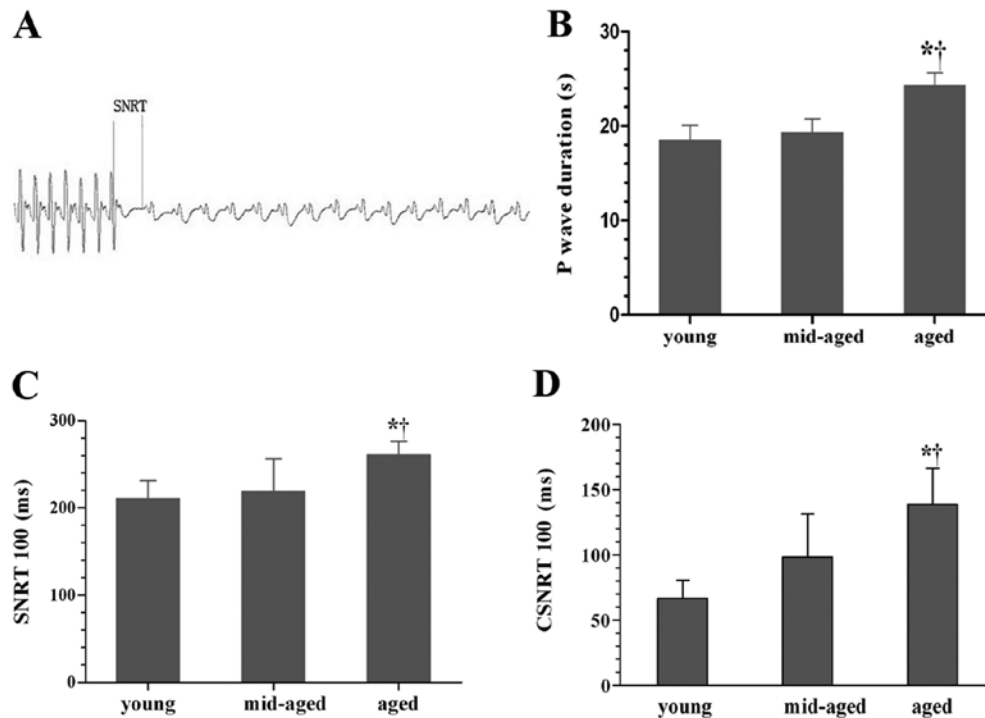


Figure 1. Global electrophysiology. (A) Typical representation of SNRT. (B) Comparison of P-wave duration (PWD) among the 3 groups. (C) Comparison of SNRT at a cycle length of 100 msec in the different groups. (D) Comparison of CSNRT at a cycle length of 100 msec in the different groups. A value of  $P < 0.05$  was considered to indicate a statistically significant difference. \*PWD in the aged vs. the young group ( $P < 0.05$ ); †PWD in the aged vs. the middle-aged group ( $P < 0.05$ ); SNRT, sinus node recovery period; CSNRT, corrected sinus node recovery period.

solution contained (in mmol/l) 137 NaCl, 5.4 KCl, 1.8  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 0.33  $\text{NaH}_2\text{PO}_4$ , 5 HEPES, 10 D-glucose and 4-aminopyridine, pH 7.3-7.4 (NaOH).  $\text{Ca}^{2+}$  channels were blocked with 0.05 mmol/l  $\text{CdCl}_2$ . Atropine of 1  $\mu\text{mol/l}$  was used to prevent muscarinic receptor activation, and 5  $\mu\text{mol/l}$  E-4031 was added to the external solution to block the rapid delayed rectifier current ( $I_{kr}$ ). In addition, EGTA was used for chelating free  $\text{Ca}^{2+}$ , thereby blocking  $I_{to2}$  and  $I_{Na-Ca}$ . For AP measurements, the internal solution contained (in mmol/l) 110 K-aspartate, 20 KCl, 1.8  $\text{MgCl}_2$ , 10 HEPES, 5 Mg-ATP, 0.05 EGTA and 5  $\text{Na}_2$ -creatine phosphate (pH 7.3-7.4, KOH). The external solution contained (in mmol/l) 137 NaCl, 5.4 KCl, 1  $\text{MgCl}_2$ , 1.8  $\text{CaCl}_2$ , 10 D-glucose, 0.33  $\text{NaH}_2\text{PO}_4$  and 5 HEPES (pH 7.3-7.4, NaOH). Signals were filtered with 2.9 and 10 kHz Bessel filters and recorded by an Axopatch700 A amplifier with the Digidata1320A-pClamp 8.2 Data Acquisition System (Axon Instruments, Foster City, CA). All experiments were conducted at room temperature ( $25 \pm 1^\circ\text{C}$ ).

**Statistical analyses.** Values are presented as the means  $\pm$  standard deviation. Surface ECG and esophageal electrogram were measured by 3 independent observers and compiled for statistical interpretation. Differences between groups were analyzed using a two-tailed Student's t-test, analysis of variance (ANOVA), Kruskal-Wallis H test, Mann-Whitney U test or Chi-square test, where appropriate. A two-tailed  $P < 0.05$  was considered to indicate a statistically significant difference. All statistical analyses were performed with SPSS software.

## Results

**In vivo global electrophysiology.** A total of 32 mice had the transesophageal rapid pacing procedure performed. No complications due to anesthesia or surgical preparation of the esophagus were observed in the young group and middle-aged group. Two out of 10 mice in the aged group died from either sinus bradycardia or complete atrioventricular block by rapid atrial pacing and had to be excluded from the analysis.

The threshold of pacing ( $0.95 \pm 0.38$  mA) in the young group was almost compatible with the middle-aged group ( $1.36 \pm 0.44$  mA) and the aged group ( $1.22 \pm 0.21$  mA). There was no significant difference in SCL among the 3 groups [ $112.73 \pm 17.78$  sec (young),  $110.42 \pm 11.64$  sec (middle-aged) and  $117.34 \pm 23.73$  sec (aged)]. PWD in the aged group had a significantly higher prolongation than the young and middle-aged group (Fig. 1B) ( $P < 0.05$ ). A typical representation of SNRT measurement is shown in Fig. 1A. SNRT and CSNRT at a S1S1 stimulation cycle length of 100 msec in the aged group had a significantly higher prolongation relative to the young and middle-aged group ( $P < 0.05$ ); however, there was no significant difference between the young and middle-aged group (Fig. 1C and D) ( $P > 0.05$ ). The inducibility of AT/F among the 3 groups is shown in Table II. Although the duration of each AF episode was not significantly different among the groups, there was an overall induced rate of AT/F that increased significantly with age ( $P < 0.05$ ). Vulnerability of the atrial myocardium was defined as when AT/F was successfully induced in the mouse model.

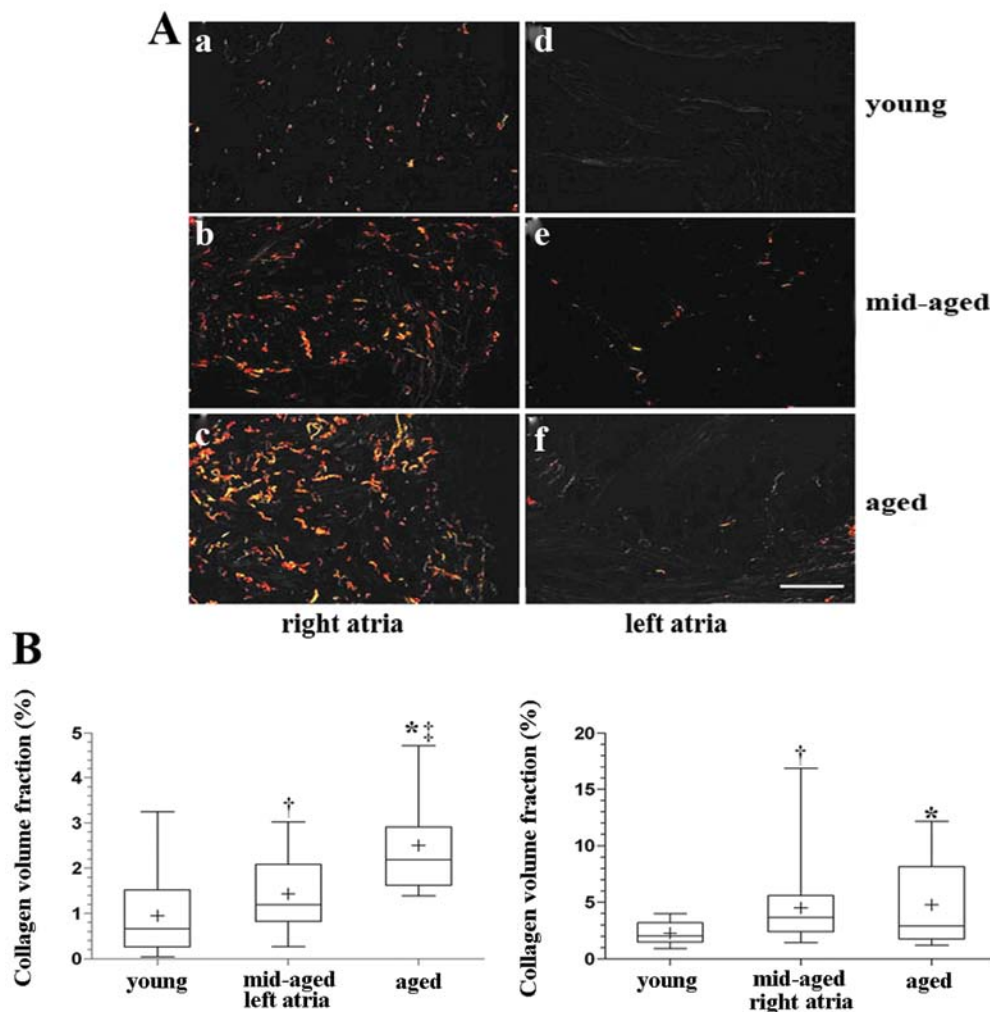


Figure 2. Collagen deposition in the left and right atrium. (A) Representative images of collagen deposition (x100-fold; scale bar, 100  $\mu$ m). (B) Statistical comparisons shown by box-whisker plots. The data are separated into quartiles; the top of the box shows the 75th percentile, the line within the box shows the median, and the bottom of the box shows the 25th percentile. The top of the whisker denotes the maximum value, and the bottom of the whisker denotes the minimum value. +, median. \* $P < 0.05$  represents the aged vs. the young group; † $P < 0.05$  represents the aged vs. the middle-aged group; ‡ $P < 0.05$  represents the middle-aged vs. the young group.

*Age-related dynamic changes in the atrial CVF.* Collagen fibers were much thicker in the right atrium than the left atrium, and the CVF was higher in the right atrium among the 3 groups (Fig. 2A). Spearman rank correlation analysis showed that the CVF in the left atrium significantly correlated with age ( $P < 0.01$ ,  $r = 0.592$ ) (Fig. 2B). The CVF in the right atrium was higher in the aged and middle-aged groups than in the young group; however, but there was no significant difference between the aged and middle-aged groups. Spearman rank correlation analysis showed that the CVF in the right atrium correlated with age ( $r = 0.326$ ,  $P < 0.01$ ); however, this did not achieve the level of the left atrium (Fig. 2B).

Overall, the right atrium showed significantly higher fibrosis than the left atrium in all the groups, and the extent of fibrosis in the left atrium had a higher positive correlation with age relative to the right atrium.

#### Patch clamp experiments

*Age-related changes in Aps and Cm.* In the current clamp mode, APs were elicited by square current pulses of 400–600 pA amplitude and 3 msec duration. A steady-state

AP was considered as the last of a train of 20 at the same stimulation rate. The APD at 20, 50 and 90% repolarization (APD<sub>20</sub>, APD<sub>50</sub>, APD<sub>90</sub>, respectively) has a tendency towards prolonging followed by shortening in regard to the age development, whereas the APD<sub>90</sub> in the left relative to the right atrium demonstrated a significant difference in the aged group ( $P < 0.05$ ) (Table III).

In the whole voltage clamp mode, single atrial myocyte Cm was elicited by a square pulse of depolarization. Mean left atrial Cm was  $34.2 \pm 1.4$  pF (young) ( $n = 33$ ),  $39.3 \pm 1.9$  pF (middle-aged) ( $n = 33$ ) and  $46.7 \pm 2.4$  pF (aged) ( $n = 19$ ), respectively, while mean right atrial Cm was  $30.0 \pm 1.4$  pF ( $n = 33$ ),  $32.8 \pm 1.9$  pF ( $n = 33$ ) and  $40.1 \pm 2.6$  pF ( $n = 19$ ), respectively. These results demonstrate that Cm of the left and right atrium increases with advancing age, and there is a stronger tendency towards changes in left atrial Cm with age relative to right atrial Cm (Fig. 3).

*Age-related changes of  $I_{to}$ .* Fig. 4A shows voltage-dependent  $I_{to}$  elicited by voltage steps of 100 msec to between -40 and +50 from -50 mV. The holding potential of -50 mV was used to exclude possible  $I_{Na}$  and  $I_{Ca,T}$  contamination during

Table II. Comparisons of the inducibility of AT/F among the 3 groups.

	Young	Middle-aged	Aged
Induction rate of AF (%)	27.3	63.6 <sup>a</sup>	62.5 <sup>b</sup>
Median duration for each AF (S)	3.04±1.32	2.54±0.86	2.66±0.22
Induction rate of AT (%)	0	9.1 <sup>a</sup>	25 <sup>b</sup>
Vulnerability to AT/F (%)	27.3	72.7 <sup>a</sup>	87.5 <sup>b</sup>

<sup>a</sup>The middle-aged vs. the young group (P<0.05); <sup>b</sup>the aged vs. the young group (P<0.05). Median duration for each AF was presented as mean ± SD. AT/F, atrial tachycardia and fibrillation; AF, atrial fibrillation.

Table III. Comparison of APDs between the left and right atrium.

Group	No.	APD <sub>20</sub> (msec)		APD <sub>50</sub> (msec)		APD <sub>90</sub> (msec)	
		Left	Right	Left	Right	Left	Right
Young	7	3.9±0.2	4.2±0.3	5.0±0.6	5.2±0.4	22.1±2.6	22.5±2.4
Middle-aged	7	5.5±0.5	5.9±0.4	9.3±1.0	9.5±0.9	31.9±3.2	32.9±3.3
Aged	7	4.9±0.4	5.4±0.6	6.7±0.6	7.5±0.9	22.8±2.7	27.6±2.8 <sup>a</sup>

<sup>a</sup>P<0.05 vs. the left atrium. Data are presented as the means ± SD. APD, action potential duration.

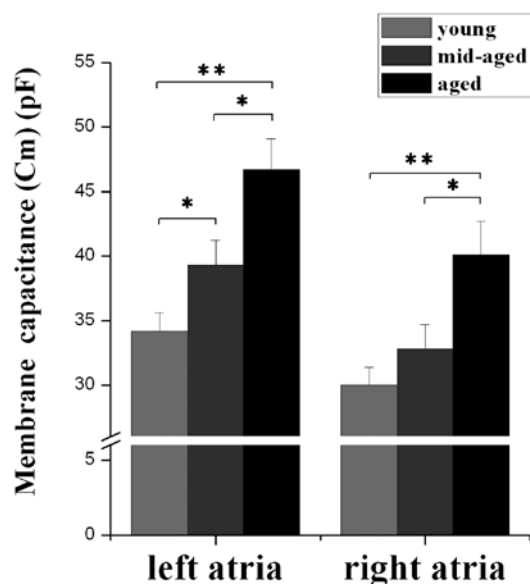


Figure 3. Membrane capacitance (Cm). Comparison of atrial Cm among the 3 groups. \*P<0.05, \*\*P<0.01.

current recording. We found that  $I_{to}$  was activated rapidly and achieved peak current within minutes after delivering testing potentials, followed by a rapid decrease in the steady state baseline current level.  $I_{to}$  increased with testing potentials in a typically voltage-dependent manner. When 4 mM 4-AP was added to the external solution,  $I_{to}$  was blocked by >80% (Fig. 4A).

As shown in Fig. 4B,  $I_{to}$  was activated at -30 mV and current density was not significantly different among the 3 groups.

The current density of  $I_{to}$  in the aged group was larger than that in the young and middle-aged groups at -10 mV (P<0.05), and this was even more significant when the testing potential was increased. At a test potential of +50 mV, the current density was 7.8±0.9 pA/pF (n=6), 10.0±1.0 pA/pF (n=6) and 12.0±1.4 pA/pF (n=5) in the young, middle-aged and aged groups, respectively, indicating that current density increased with age (P<0.05).

Fig. 4C shows the activation curve of  $I_{to}$  by depolarization to +50 from -50 mV. With age, the voltage-dependent  $I_{to}$  activation curve slightly shifted in a negative direction.  $V_{1/2}$  and  $K$  were 12.8±0.9 and 9.4±0.9 mV (n=6), 11.5±0.6 and 9.5±0.6 mV (n=6), and 10.9±0.8 and 9.2±0.7 mV (n=6) in the young, middle-aged and aged groups, respectively, indicating that there were no significant changes in  $V_{1/2}$  and  $K$  with age (P>0.05).

The voltage dependence of the steady state inactivation relationship was investigated using a standard 2-pulse protocol: a 2,000 msec preconditioning pulse ranging from -80 to 0 mV followed by a 140 msec test pulse to +50 mV.  $V_{1/2}$  and  $K$  were -49.7±2.1 and 8.1±1.9 mV (n=6), -48.8±1.6 and 7.3±1.5 mV (n=6), and -48.7±1.5 and 7.0±1.4 mV (n=6) in the young, middle-aged and aged groups, respectively, indicating that there were no significant changes in  $V_{1/2}$  and  $K$  with age (P>0.05) (Fig. 4D).

Age-related changes of  $I_{kur}$ .  $I_{kur}$  were elicited by a 80-msec prepulse to +30 mV to inactivate  $I_{to}$ , followed by 140-msec test pulses between -40 and +50 mV after a 10-msec interval from -50 mV at 0.2 Hz. At a test potential of +50 mV, the current density was 4.1±0.7 pA/pF (n=6), 4.2±1.0 pA/pF (n=6) and 4.2±0.8 pA/pF (n=5) in the young, middle-aged and aged group, respectively, indicating that there was no significant difference among the groups (P>0.05) (Fig. 5B).

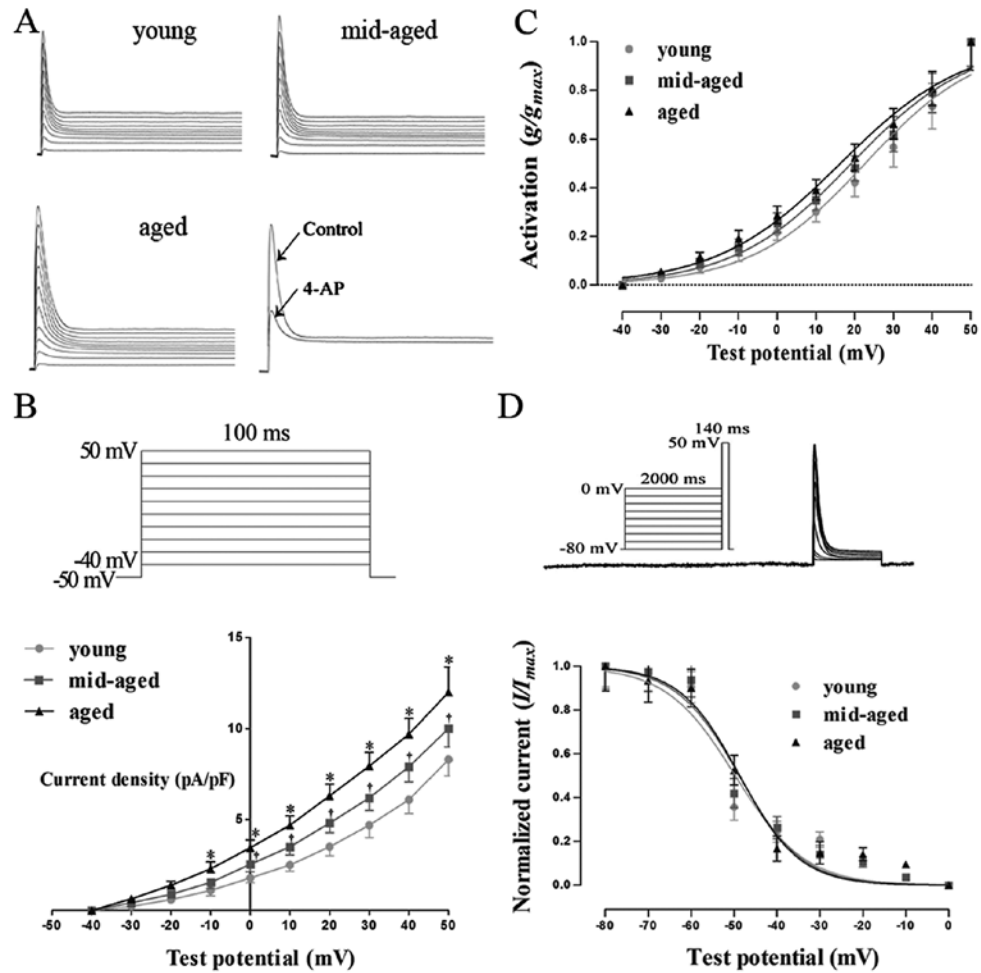


Figure 4. Analyses of  $I_{to}$  currents. (A) Original tracings of  $I_{to}$  in atrial myocytes in the different groups (young, middle-aged and aged). Original tracings of  $I_{to}$  before and after addition of 4-AP are shown. (B) I-V relationship curve of  $I_{to}$  in the different groups. The stimulation protocol of  $I_{to}$  is shown. (C) Activation curves of  $I_{to}$  from atrial myocytes in the different groups. (D) Inactivation curves of  $I_{to}$ . Typical original tracings of inactivation curves are shown. \* $P < 0.05$  represents the aged vs. the middle-aged and young groups; † $P < 0.05$  represents the middle-aged vs. the young groups.

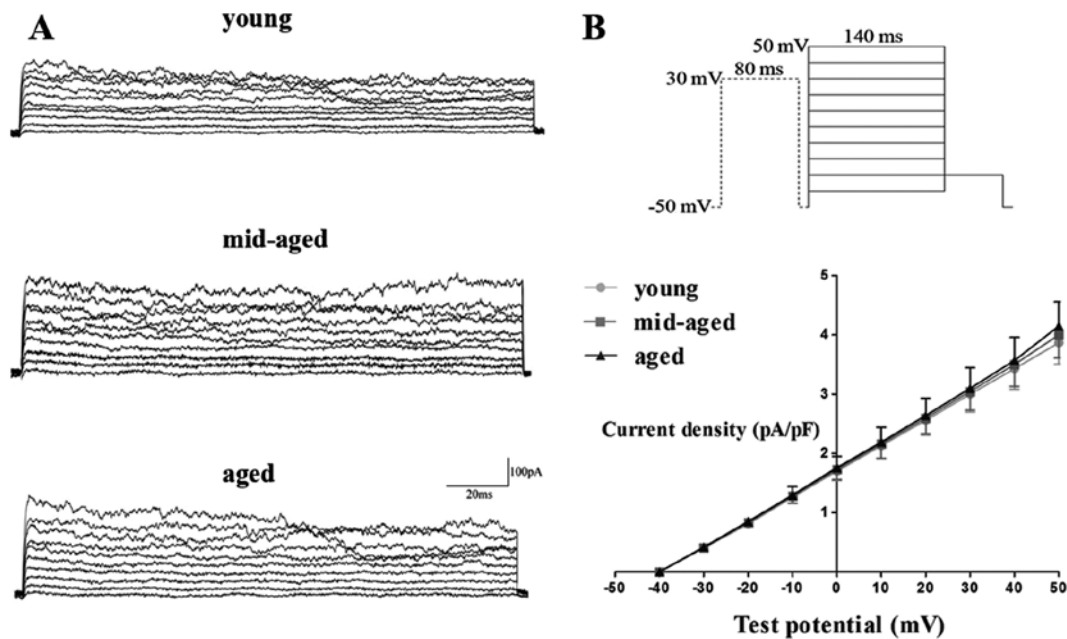


Figure 5. Analyses of  $I_{kur}$  currents. (A) Original tracings of  $I_{kur}$  in atrial myocytes in the different groups (young, middle-aged and aged). (B) Stimulation protocol of  $I_{kur}$ . The I-V relationship curves of  $I_{kur}$  from atrial myocytes in the different groups are shown.

## Discussion

**Main findings.** In the present study, we performed measurements of *in vivo* global electrical properties by the transesophageal atrial rapid pacing method and found the following: (i) Compared with the other 2 groups, PWD, as well as the SNRT and CSNRT at a cycle length of 100 msec were longer in the aged group, indicating the slowing of intra-atrial conduction and sinus nodal dysfunction. ii) Although the duration of each AF episode was not significantly different among the groups, the total inducibility, represented as the key point for evaluating vulnerability to AT/F, was significantly increased with aging. As regards changes in collagen in the left and right atrium, we found that the right atrium showed significantly higher fibrosis relative to the left atrium in all the groups, whereas the extent of fibrosis in the left atrium had a higher positive correlation with age compared to the right atrium. Furthermore, from the cellular electrophysiological experiments, the results showed that age-related augmented  $I_{to}$  currents and no age-related changed  $I_{kur}$  currents, as well as the subsequent AP discrepancy in old age, contributed to the dispersion of repolarization, which further promoted AF.

**Age-related abnormal pulse initiation.** Abnormal pulse initiation due to the sinoatrial node dysfunction is associated with advancing age (17). If the sinus node fails to fire, then there is a significant tendency towards manifestation of abnormal impulse initiation in atrial cells. In the present study, measurements of SNRT and CSNRT at a cycle length of 100 msec showed that aged mice had a longer SNRT than the young and middle-aged mice. Recently, Stiles *et al* (18) demonstrated that the prolongation of SNRT, regarded as sinus node dysfunction, is a key contributor which facilitates the progression of paroxysmal AF to permanent AF. Surprisingly, it has been previously found that although the SCL increases with age, the SNRT decreases with age (19), which is in contrast to our results. The likely mechanism for these observations is that conduction in the sinus node worsens with age and limits the ability of an extra-stimulus to overdrive and suppress sinus node automaticity. A previous study found enlargement of the sinus node, hypertrophy of sinus nodal cells, and remodeling of the extracellular matrix in aged rats (20). Therefore, they concluded that the age-dependent decrease in sinus nodal function is due to structural remodeling of the sinus node. Consequently, decreased automaticity and conductivity of the sinus node are associated with shortening of atrial refractoriness and increasing the atrial effective refractory period (ERP) dispersion, which in turn creates a substrate for AF initiation and perpetuation (21).

**Age-related abnormal pulse conduction.** Abnormal pulse conduction in the aged atrium is a contributor to the maintenance of AF. Hence, it is important to comprehend the nature of both the electrical and structural remodeling that occurs with age. In the current study, surface ECG showed that PWD was significantly longer in the aged group than in the young group and had a prolongation tendency in the middle-aged group, which indicated that the intra-atrial pulse conduction time was prolonged with increasing age. It has been reported that the PWD significantly correlates with age in animals (6) and

humans (22), and depends mainly on intra-atrial conduction time and atrial size. The slowing of intra-atrial conduction, one of the most important requirements for the initiation of re-entry, is more frequently observed in patients with paroxysmal AF than in normal subjects (23). Moreover, atrial dilatation increases the amount of atrial tissue that can accommodate multiple re-entry circuits (3). Therefore, P-wave prolongation and atrial dilatation possibly reflect age-related atrial remodeling that is favorable for AF; thus, aged mice, compared to young mice, possess a substrate that is more susceptible to AF.

It is well known that the profound structural remodeling that occurs with age affects the ability of a premature beat to penetrate the substrate and initiate propagation. In the present study, we observed a significant correlation between the degree of interstitial fibrosis and aging in the left and right atrium. In a previous study, Burkauskienė *et al* (24) demonstrated that the diameter and area of collagen fibers positively correlate with aging, whereas the amount of collagen fibers does not alter with age. This indicates that changes in the collagen network may be a progressively developing process. Another study reported that spatial distribution of the collagen network was significantly different in patients with ischemic cardiomyopathy compared with those without heart disease (25). Taken together, these findings, including ours, suggest that atrial interstitial fibrosis, which is increased with age and heart disease, possibly enhances the heterogeneous slowing of atrial conduction, which may be responsible for the age-related increase in AF vulnerability. In general, there is a close correlation between the onset of AF and structural remodeling in the left atrium (26). However, Nakajima *et al* (27) found that although similar levels of cardiomyocyte apoptosis were present in the right and left atria of MHC-TGF $\alpha$  hearts, the extent of fibrosis was more pronounced in the right atrium than in the left atrium. They also revealed cardiomyocyte cell activity in left atrial cardiomyocytes, but not in right atrial cardiomyocytes by tritiated thymidine incorporation studies. These data support the notion that cardiomyocyte cell cycle induction can antagonize fibrosis in the myocardium. In agreement with previous results, the present study found that the collagen volume of the right atrium was higher than that in the left atrium in all the groups. Although we found that the volume of fibers increased with age in the left and right atrium, less fibrosis was present in the left atrium, which may be due to faster cell cycle activity in the left atrium.

**Age and predisposition to AF.** In our study, we compared the inducibility of atrial AT/F to evaluate vulnerability to arrhythmias. Our results showed that the inducibility of atrial AT/F significantly increased with age, indicating that aging is a key contributor to AF episodes. In addition, inducible AF was also observed in the young and middle-aged group, in contrast to the negative results presented in the study by Guzadhur *et al* (28). A possible reason is that the susceptibility of the mouse atrium to the induction of AF appears to be increased when transesophageal pacing instead of isolated pacing (28) or transvenous pacing (29) is performed. It is possible that relative to isolated or transvenous stimulation, unipolar transesophageal atrial stimulation affects a larger area of the atrium, which may involve the vagal nerve. This may result in a more global inho-

mogeneous and abnormally delayed activation of the atrium, which is believed to be the basic mechanism of induction of atrial arrhythmia by premature depolarization. Moreover, the atrial stretch is known to enhance atrial vulnerability and may be augmented in this setting of transesophageal stimulation. Of note, the present study showed that PWD was only prolonged in aged group relative to other 2 groups, not reconciling the high incidence of tachyarrhythmia in the middle-aged and aged group. The discrepancy may be due to more electrophysiological remodeling in the middle-aged group, while structural remodeling was predominant in the aged group.

*Age-related cellular electrophysiological changes conducive to AF.* AP changes with age are affected by different types of inward and outward ion currents.  $I_{to}$  and  $I_{kur}$ , which are the major ion currents involved in the repolarization process in mice, contribute to age-related AP changes and AF initiation and maintenance. In the present study, a  $Ca^{2+}$ -independent transient outward current ( $I_{to1}$ ) was recorded by the addition of  $CdCl_2$  to the external solution to block the L-type  $Ca^{2+}$  current and the addition of a  $Ca^{2+}$  chelating agent (EGTA) to the internal solution. Our results showed that  $I_{to}$  was suppressed by >80% after the addition of 4-AP. Dun *et al* (30) found that  $I_{to}$  current densities of canine right atria were significantly increased in the aged group compared with the adult group, whereas inactivation slowing and steady state inactivation curves showed a trend to shift toward depolarization. Mansourati *et al* (31) also found that  $I_{to}$  was decreased in adult patients with heart diseases; however, the results did not reflect the normal electrophysiological properties of humans due to the existence of pathological factors. We found that  $I_{to}$  current densities increased with age, which is in agreement with the results presented in the study by Crumb *et al* (32), whereas the activation and inactivation properties of  $I_{to}$  were not different among the 3 groups. As such, the augmentation of  $I_{to}$  may have contributed to the shortening of atrial APs in the aged group. Following the inactivation of  $I_{to1}$ , a slowly inactivating current remains, termed  $I_{sus}$  or  $I_{kur}$ . There is a general consensus that the  $K_v$  1.5 subunit is largely responsible for  $I_{kur}$  in human atrial myocytes, similar to other species, such as the mouse and dog. Since this subunit is much less abundant in the ventricle than in the atrial myocardium, it appears to be quite specific to atrial myocytes.  $I_{kur}$  densities are significantly increased in aged canine right atrial cells compared with adult cells (30). However, KN-93, a specific CaMKII inhibitor, has been shown to dramatically inhibit  $I_{kur}$  in aged right atrial cells compared with adult right atrial cells, indicating that increased  $I_{kur}$  in aged RA cells is most likely due to CaMKII upregulation (33). To the best of our knowledge, no age-related changes in  $I_{kur}$  have thus far been observed in human atrial myocytes. In alignment with human atrial data, the present study using mice also demonstrated that there were no age-related changes in  $I_{kur}$  among the different groups, which may be due to stability after birth.

The abnormal shape of the AP contour derived from a single isolated cell is conducive to AF. Anyukhovskiy *et al* (34) found that  $APD_{90}$  averaged across all regions was significantly longer in the aged compared with adult tissues. Notably, the range of  $APD_{90}$  values was wider in the aged vs. the adult group as a result of an increase in duration, occurring mainly in right

atrial fibers (34). However, clear-cut evidence of age-related changes in APs in normal human atria is lacking, as no age-related changes in APD and ERP have been obtained (35). In the present study, we found that  $APD_{90}$  in the left and right atrium was prolonged with age, followed by a decrease in the aged group, particularly in the left atrium. In agreement with our report, Brorson *et al* (36) used 4 age groups to examine right atrial ERPs and monophasic APs in healthy male humans *in vivo*. They found that the middle-aged group showed a significantly longer right atrial ERP than the other groups. However, there was no progressive trend in APD with age. Thus, these results do not support the traditional view that APDs are prolonged with age. In general, AP is prolonged followed by shortening with age may due to a decrease in the calcium current in aged atrial tissue (6).

*Limitations.* The transesophageal stimulation technique used in the current study includes difficulties in atrial electrogram detection during AF, and therefore, the evaluation of the atrial AF heart rate is not always possible. Additionally, due to the limitations of experimental devices and technologies, measurements of atrial ERP and intra-atrial conduction velocity were not conducted for further insight into the electrophysiological mechanisms. Finally, it should be acknowledged that the pathophysiological characteristics of AF in mice may be different from those in humans.

In conclusion, the results from our study demonstrate that aging causes structural remodeling, as well as integrative and cellular electrophysiological changes, facilitating increased dispersion of repolarization and re-entry formation. This in turn creates a substrate for AF initiation and perpetuation. Furthermore, there may be an association between structural and electrical remodeling, which can exert vicious cycle effects on the development of AF.

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