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Left atrial endocardial fibrosis and intra-atrial thrombosis – landmarks of left atrial remodeling in rats with spontaneous atrial tachyarrhythmias

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Abstract

Introduction: Histological abnormalities are common findings in the left atria (LA) of atrial fibrillation (AF) patients. We aimed to assess LA histological abnormalities in our model of spontaneous atrial tachyarrhythmias in rats. *Materials and Methods*: LA sampling was performed in 12 spontaneously hypertensive rats (SHRs) and eight age-matched Wistar–Kyoto (WKY) rats. Tissue sections were stained with Masson's trichrome and Hematoxylin–Eosin–Safran and examined with a light microscope. A 0 to 3 scoring system was used to quantify the severity of LA structural abnormalities. LA von Willebrand factor (vWF) content was also assessed using immunohistochemical staining. *Results*: In six of the eight SHRs, LA fibrosis, inflammatory infiltrates, and myocyte necrosis of varying grades of severity were observed. The most frequent feature was endocardial fibrosis, which was observed in six SHRs and in none of the WKY rats. Intra-atrial thrombosis was found in three SHRs and in none of the WKY rats. The intensity of vWF-related fluorescence was higher in the atrial endocardium of SHRs compared to age-matched WKY rats. *Conclusions*: Our findings reinforce the role of LA structural abnormalities in atrial arrhythmogenicity. However, two SHRs did not present LA histological abnormalities despite the presence of arrhythmias. This finding suggests that the LA remodeling-atrial tachyarrhythmia relationship could be highly nonlinear and that atrial fibrosis is more likely to be a facilitator of atrial arrhythmogenicity, rather than a prerequisite. We also provide evidence that intra-atrial thrombosis accompanies LA structural remodeling in arrhythmic rats. Increased endocardial platelet adhesion molecule vWF could contribute to this increased thrombogenicity.

Keywords: atrial fibrillation, structural remodeling, fibrosis, thrombosis.

Introduction

Histological abnormalities are a common finding in left atrial samples of patients that present atrial arrhythmias such as atrial fibrillation [1–3]. Cardiac remodeling, and particularly left atrial structural abnormalities, has been shown to precede the onset of atrial arrhythmias, since it emanates from cardiac damage due to coronary artery disease, hemodynamic overload from valve disease or hypertension [4]. The resulting atrial remodeling involves changes in the structure and the function of the atria, modifications of atrial electrical and contractile functions, changes in atrial extracellular matrix [5]. Together, these alterations create an arrhythmogenic substrate, essential for the onset and particularly for the persistence of atrial arrhythmias. Accumulating data suggest that atrial structural abnormalities are not only the cause, but also the consequence of atrial fibrillation, participating in the maintenance of a vicious circle, with atrial fibrillation-induced atrial structural remodeling favoring atrial fibrillation persistence [6].

Experimental studies suggest that the components and the severity of atrial structural remodeling vary depending particularly on coexisting cardiac conditions and specie [5, 7, 8]. Comparable structural and ultra-structural abnormalities with those encountered in patients with atrial fibrillation have been described in various animal models of atrial arrhythmia [5, 9]. Nevertheless, more severe and extensive lesions have been reported in humans with atrial fibrillation than in animal models of atrial arrhythmia. Particularly, atrial interstitial fibrosis is a common feature of atrial fibrillation in humans [3], while, according to light microscopy studies, both intercellular and inter-bundle interstitial fibrosis seem to be less important in animal models of atrial arrhythmia [4]. However, left atrial structural abnormalities have never been assessed in an animal model of spontaneous atrial arrhythmias. All previous data were obtained using models in which arrhythmia was triggered by interventional means, such as programmed stimulation protocols or pharmacologic interventions [10–12]. A number of essential features for atrial fibrillation occurrence, including structural remodeling, could have been artificially induced in those models.

We recently described the first experimental model of unprovoked atrial tachyarrhythmias in aging spontaneously hypertensive rats (SHRs) [13]. In order to document the

structural changes associated with this increased atrial arrhythmogenicity in SHRs, we aimed to assess the presence, the components, and the severity of left atrial histological abnormalities in our rat model of spontaneous atrial tachyarrhythmias, using standard histological examination.

Materials and Methods

Animals

Twelve 55-week-old male SHRs (471±5 g) and eight age-matched normotensive Wistar-Kyoto (WKY) rats (576±11 g) were purchased from Elevage Janvier (Le Genest Saint Isle, France).

All animals were housed in a climate-controlled room (21 to 22^{0}C), with a 12 hours light/dark cycle (on 7 AM / off 7 PM), in an accredited animal facility. The rats were housed in groups of two to three rats per cage and fed standard rat pellets and tap water *ad libitum*.

All experiments were performed in accordance with the guidelines of the French Ministry of Agriculture for animal experimentation and were approved by the local Animal Ethics Committee.

Heart sampling

All animals were weighted and then euthanized with an intraperitoneal injection of a terminal dose of sodium pentobarbital (>100 mg/kg). The hearts were excised, weighted, and underwent immediate (within two minutes after removal) fixation in 10% buffered formalin and were processed for paraffin histology.

Histological examination

Histological examination was performed on eight SHRs and four WKY rats, by an independent anatomo-pathologist who was unaware of the rat's blood pressure status (hypertensive or normotensive) or cardiac rhythm (presence or absence of atrial arrhythmias).

Deparaffinized tissue sections 5 μm thick were stained with Masson's trichrome or Hematoxylin–Eosin–Safran and examined with an Axiolab (Carl Zeiss MicroImaging GmbH, Germany) light microscope. The region examined was the lateral wall of the left atrium. Characteristic areas were photographed using the Zeiss AxioCam ICc1 digital camera system (Carl Zeiss MicroImaging GmbH) and AxioVision Release 4.8.2. software (Carl Zeiss MicroImaging GmbH) was used for image processing.

A scoring system was used to quantify the degree of fibrosis, myocyte necrosis, and inflammatory cell infiltration in left atrial samples from hypertensive and normotensive rats. According to this scoring system, "0" corresponded to the absence of abnormalities, "1" defined mild abnormalities, "2" defined moderate abnormalities, and "3" defined severe abnormalities. Left ventricular wall thickness was measured for each heart sample at 2.5× magnification.

von Willebrand factor immunohistochemical staining

Left atrial endocardium of the remaining SHRs (n=4) and WKY rats (n=4) was evaluated using monoclonal antibodies directed towards von Willebrand factor. Immunohistochemical analysis was performed by an independent investigator, who was unaware of the rat's blood pressure status (hypertensive or normotensive) or cardiac rhythm (presence or absence of atrial arrhythmias).

Paraffin sections were prepared with standard procedures from euthanized animals. Sections were deparaffinized in methylcyclohexane (two baths for 10 minutes) and rehydrated through graded series of ethanol to water. Endogenous peroxidase was blocked with a 10 minutes incubation of 3% hydrogen peroxide. Antigens were unmasked by boiling at 97^{0}C for 40 minutes in sodium citrate buffer (pH 7.3) after deparaffinization. The slides were then incubated for one hour with the primary antibody in a humidified, dark, chamber, at room temperature. Rabbit anti-von Willebrand factor (ab6994; Abcam, Cambridge, MA; 1:1000) was used as primary antibody. After three five minutes washes with PBS, sections were incubated at room temperature for one hour with secondary Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Invitrogen, Carlsbad, CA; 1:1000) and a second series of PBS washes was performed. All sections were counterstained with DAPI for 10 minutes, PBS washed, and then aqueous mounted.

Sections were examined with a confocal laser scanning microscope (Leica TCS SP5; Leica Microsystems GmbH, Mannheim, Germany).

Three serial sections were examined for each sample. von Willebrand factor staining intensity was assessed by visual examination.

Statistics

Data are expressed as the mean ± SEM or median and range, as appropriate. Between-group comparisons were performed using the unpaired t-test or the Mann–Whitney U-test, as appropriate.

Correlations were ascertained with Spearman's rank correlation method. A two-tailed p-value of less than 0.05 was considered statistically significant.

Statistical analyses were undertaken using GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

Left ventricular hypertrophy indexes

Heart weight-to-body weight ratios, left ventricular wall thickness, and ventricular myocyte diameter were measured in order to assess the presence and the severity of left ventricular hypertrophy in SHRs compared to WKY rats.

In SHRs, heart weight-to-body weight ratios were 56±3% higher than in age-matched WKY rats (p=0.01).

Left ventricular wall thickness was 67±2% greater (p<0.01) in SHRs (5513±201 μm) than in age-matched WKY rats (3300±238 μm). Similarly, myocyte diameter was significantly greater in SHRs (Figure 1A) compared to age-matched WKY rats (Figure 1B).

Taken together, these indexes confirmed the presence of significant left ventricular hypertrophy in hypertensive rats.

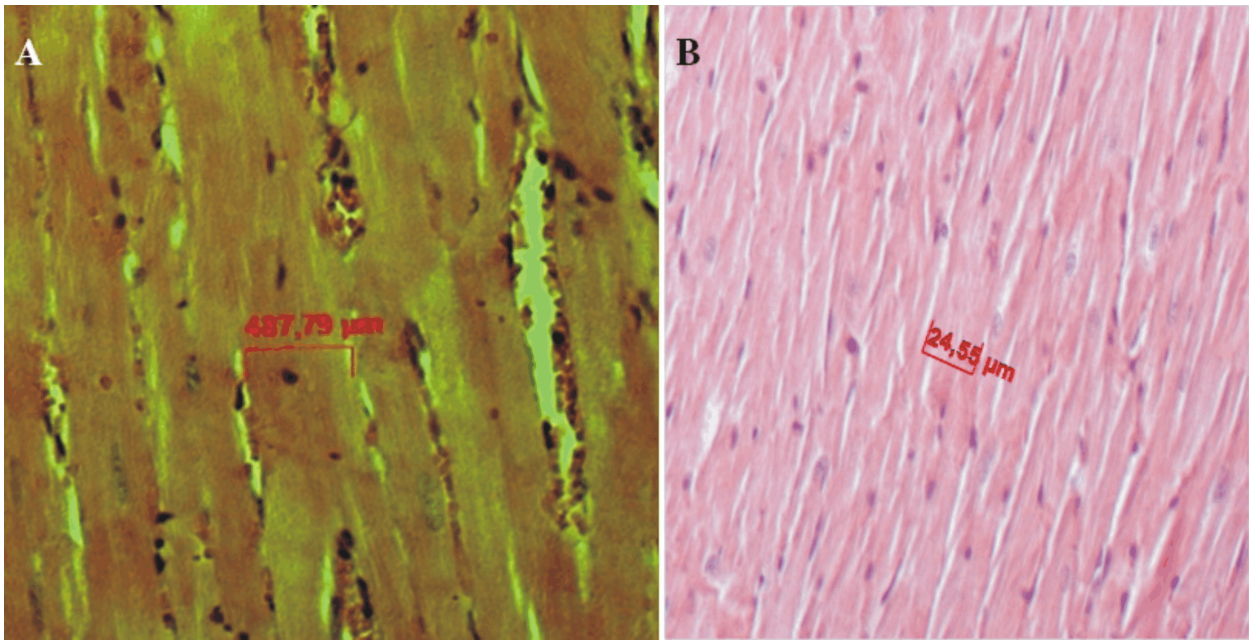

Figure 1 – ***Left ventricular samples (Hematoxylin–Eosin–Safran staining) from SHRs (A) and WKY rats (B) showing increased myocyte diameter in SHRs compared to WKY rats.***

Left atrial structural remodeling

In six of the eight SHRs, left atrial endocardial and interstitial fibrosis, inflammatory infiltrates, and myocyte necrosis of varying grades of severity were observed. The most frequent feature was endocardial fibrosis (median score 1.5, range 0–3), which was observed in six SHRs (Figure 2, A and B). None of the WKY rats presented endocardial fibrosis. In four SHRs, several aspects of early-stage, progressive, endocardial fibrosis could be identified on the same sample (Figure 2A).

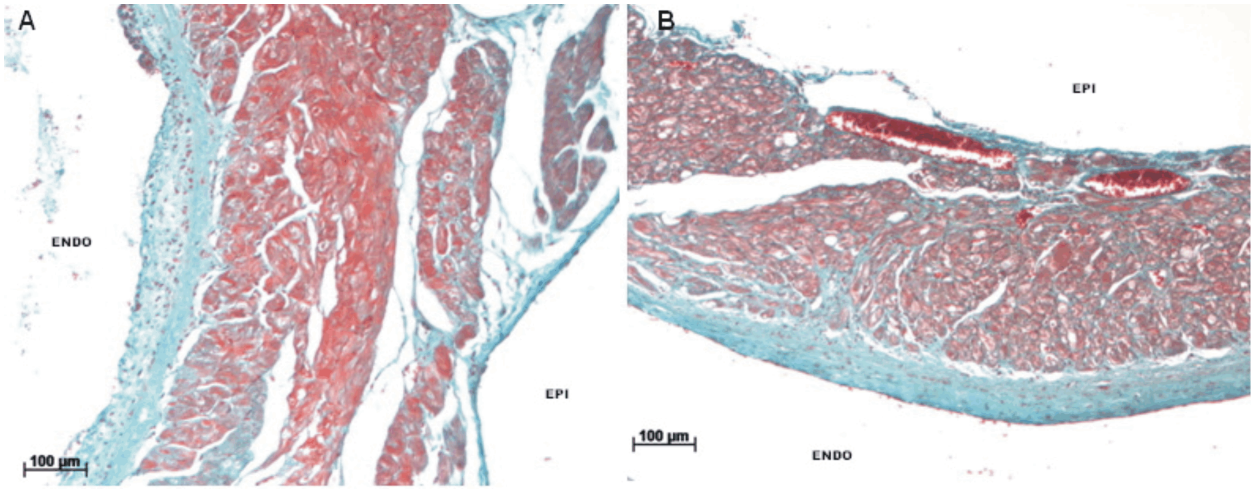

Figure 2 – ***Left atrial samples (Masson's trichrome staining) from SHRs showing – (A) progressive, stratified endocardial fibrosis, and (B) stable, evolved endocardial fibrosis. ENDO – endocardial and EPI – epicardial borders of the left atrial wall.***

Left atrial interstitial fibrosis was observed in three of the eight SHRs (median score 0, range 0–2). Myocyte necrosis was present in four of the eight SHRs (median score 0.5, range 0–3), while only two of the eight SHRs presented inflammatory infiltrates (median score 0, range 0–3). Only one of the four WKY rats presented mild interstitial fibrosis, myocyte necrosis, and inflammatory infiltrates.

In two of the eight SHRs histological examination of the left atria did not reveal any structural abnormality. In the other SHRs, the severity of structural lesions was highly variable among individuals and heterogeneous within the same sample.

In three SHRs histological examination identified the presence of intra-atrial thrombosis (Figure 3). None of the WKY rats presented intracardiac thrombi. There was no significant difference regarding any of the histological abnormalities (*i.e.*, endocardial or interstitial fibrosis, myocyte necrosis, or inflammatory infiltrates) between the SHRs that presented intra-atrial thrombosis and the SHRs that did not. Also, we found no significant correlation between the presence of intra-atrial thrombosis and any of the other histological abnormalities (all $p>0.05$).

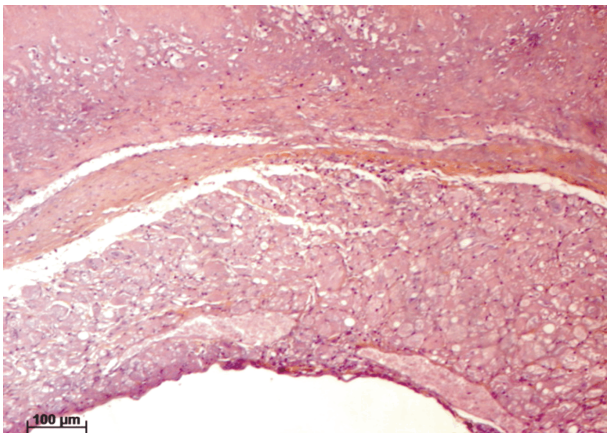

Figure 3 – ***Intra-atrial thrombosis in an SHR (Hematoxylin–Eosin–Safran staining).***

Left atrial von Willebrand factor content

In WKY rats, the immunoreactivity for von Willebrand factor in both the endocardium and the endothelium of intra-myocardial vessels varied widely among individual atrial samples, whereas apparent immunoreactivity for von Willebrand factor was consistently seen in the atrial endocardium and the endothelium of intra-myocardial vessels of SHRs.

In the WKY group, only focal or little immunoreactivity for von Willebrand factor was seen in the atrial endocardium (Figure 4A). On the other hand, apparent immunoreactivity for von Willebrand factor was extensively seen in the endocardium of the left atria of all SHRs (Figure 4B).

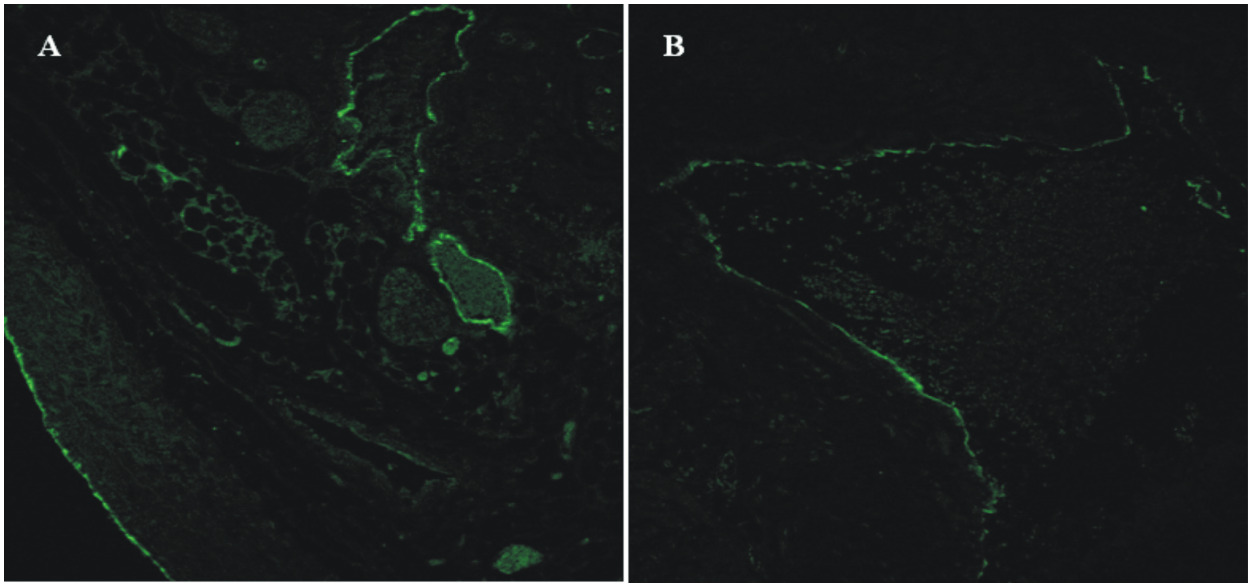

Figure 4 – ***Immunostaining for von Willebrand factor (fluorescent green) showing higher intensity of staining in SHRs (A) compared to WKY rats (B).***

The intensity of von Willebrand factor-related fluorescence was significantly higher in both the atrial endocardium and the endothelium of intra-myocardial vessels of SHRs (Figure 4A) than in those from age-matched WKY rats (Figure 4B).

Discussion

The main findings of the present study were (i) left atrial histological abnormalities were present in six of the eight SHRs; (ii) in SHRs, left atrial endocardial fibrosis was the most frequent feature, while in age-matched WKY rats endocardial fibrosis was completely absent; (iii) in two of the eight SHRs histological examination did not reveal any left atrial structural abnormality; (iv) three of the eight SHRs presented intra-atrial thrombosis, while none of the WKY rats did so; and (v) SHRs presented significantly higher intensity of von Willebrand factor-related fluorescence in both the atrial endocardium and the endothelium of intra-myocardial vessels compared to age-matched WKY rats.

Atrial fibrosis as arrhythmia substrate

Atrial remodeling can occur as response to cardiac damage due to coronary artery disease, aging, hemodynamic overload from valve disease or hypertension [4]. Changes in structure and function of the atria, modifications of atrial electrical and contractile functions, and changes in atrial extracellular matrix, accompany cardiac remodeling and create an arrhythmogenic substrate essential for the occurrence of atrial arrhythmias [10, 14–16]. Several experimental studies have provided evidence of an association between altered atrial structure and increased inducibility of atrial tachyarrhythmias [10, 14–16]. In dogs, ventricular tachypacing induced heart failure [17] and produced atrial interstitial fibrosis comparable to that previously reported in atrial fibrillation patients [3]. In that model, atrial fibrosis was associated with localized regions of conduction slowing, and increased conduction heterogeneity [17]. In the same vein, using a rat model, Hayashi H *et al.* demonstrated increased susceptibility to atrial fibrillation in aged rats and suggested that heterogeneous atrial interstitial fibrosis and atrial cell hypertrophy could contribute to aging-related atrial conduction slowing, conduction block, and inducible atrial fibrillation [18]. Atrial fibrosis has been shown to increase atrial fibrillation vulnerability in several other animal models [17, 19] and in a transgenic mouse model for selective atrial fibrosis [20].

Recent studies in both humans [1] and in animal models of aging [18, 21] demonstrated a strong correlation between aging and atrial fibrosis. Furthermore, in the presence of hypertension, the remodeling process seems to be accelerated [22], probably due to a combination of factors such as earlier development and more severe diastolic dysfunction, impaired plasma volume control, intensified neurohormonal activation, and atrial myopathy secondary to oxidative stress and lipoapoptosis.

Our results reinforce the role of aging and hypertension in left atrial remodeling and atrial arrhythmogenesis. Indeed, six of the eight SHRs presented varying degrees of endocardial fibrosis, while no such abnormality was observed in WKY rats. However, left atrial histological abnormalities observed in SHRs that presented atrial arrhythmias were significantly less severe than those previously reported in atrial fibrillation patients. Moreover, only mild to moderate left atrial interstitial fibrosis was present in no more than three SHRs, whilst this feature is a constant finding in left atrial samples of patients with atrial fibrillation [2, 3]. However, studies in humans are limited to left atrial samples obtained from patients that undergo cardiac surgery or explantation. Concomitant serious heart conditions such as coronary artery disease, valve disease or congenital heart defects in these patients could be at least partially responsible for the more extensive structural changes observed in human left atrial samples. Thus, one could conclude that left atrial structural abnormalities of most atrial fibrillation patients could actually be much less severe than those reported so far.

Interestingly, some of the rats that presented atrial arrhythmias did not present endocardial fibrosis, or any other left atrial structural abnormality. This finding suggested that the left atrial remodeling/fibrosis-atrial tachyarrhythmia relationship could be in fact highly nonlinear, and that atrial fibrosis is more likely to be a facilitator of atrial arrhythmogenicity in our model, rather than a prerequisite. Abnormal autonomic tone or molecular abnormalities could play critical roles in such settings were little or no anatomic abnormalities are present [23–25].

It remains to be established if targeting prevention or reversal of structural atrial remodeling will result in the reduction of the major impact that atrial fibrillation represents for the public health and to determine the best time to intervene to lower the risk of occurrence and/or recurrence of atrial arrhythmias.

Increased atrial thrombogenicity in arrhythmic rats

In three of the eight SHRs that presented atrial arrhythmias, histological examination revealed the presence of intra-atrial thrombi, while no such abnormality was observed in age-matched WKY rats. These results are in accordance with previous clinical data that link atrial fibrillation to a high-risk of morbidity and mortality from thromboembolism and stroke [26].

The pathophysiology of thrombosis relies on Virchow's triad of coagulation, which includes abnormalities in blood flow, vessel wall and blood constituents [27]. It is largely accepted that blood stagnation in the left atrium is an important thrombogenic factor in atrial fibrillation patients [28]. However, the coexistence of other conditions, such as hypertension, aging, or diabetes, increases the risk of stroke even more [28]. Moreover, the left atrium, particularly the left atrial appendage, is the most frequent location of embolic thrombi, even when the right atrium undergoes the same pathological process [29], and despite the fact that blood stasis in the left and the right atrial appendage is similar [30]. Taken together, these observations suggest that although left atrial stasis may be one source of thromboembolism in atrial fibrillation patients, this does not provide a complete explanation.

Abnormal haemostatic constituents indicative of thrombogenesis have been demonstrated in atrial fibrillation patients [31–33]. Recent data support a critical role of endothelial dysfunction in the atrial fibrillation-related thromboembolic risk [27].

Accordingly, we thought to assess the presence and the extent of atrial endocardial impairment in SHRs compared to age-matched normotensive rats by performing immunohistochemical staining of von Willebrand factor, a marker of both increased thrombogenicity and endothelial dysfunction. It has been well established that von Willebrand factor is required for platelet adherence to the de-endothelialized vessel wall [34–36] and its role as a key element in the thrombotic process makes von Willebrand factor a reliable marker of pro-thrombotic risk.

In the present study, immunohistochemical staining of von Willebrand factor revealed significantly higher intensity of von Willebrand factor-related fluorescence in the atrial endocardium of SHRs compared to age-matched WKY rats. These findings indicate that atrial endocardial impairment is significantly more severe in SHRs than in age-matched WKY rats. This alteration could contribute to the increased atrial thrombogenicity observed in arrhythmic SHRs. These results also suggest a potential role of increased endocardial platelet adhesion molecule von Willebrand factor in left atrial thrombogenesis in the presence of atrial tachyarrhythmias such as atrial fibrillation.

Potential limitations

Histological and immunohistochemical examinations performed in this study provided only semiquantitative results. The histological protocol used in this study cannot exclude the presence of microfibrosis in the left atrial samples of studied animals. Additional studies are required to precisely quantify left atrial structural abnormalities and particularly left atrial fibrosis, as well as the intensity of von Willebrand factor immunostaining in this model.

Conclusions

Our findings reinforce the role of left atrial structural abnormalities and particularly left atrial fibrosis in atrial electrical instability. However, the fact that two of the eight SHRs did not present left atrial histological abnormalities despite the presence of arrhythmias, suggests that the left atrial remodeling/fibrosis-atrial

tachyarrhythmia relationship could be in fact highly nonlinear, and that atrial fibrosis is more likely to be a facilitator of atrial arrhythmogenicity in our model, rather than a prerequisite. We also provide evidence that intra-atrial thrombosis accompanies left atrial structural remodeling in arrhythmic rats and that increased endocardial platelet adhesion molecule von Willebrand factor could contribute to this increased atrial thrombogenicity.

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