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Repeated generalized seizures can produce calcified cardiac lesions in DBA/1 mice

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ABSTRACT

Studies suggest that cardiorespiratory dysfunction likely contributes to sudden unexpected death in epilepsy (SUDEP). Seizures result in autonomic and respiratory dysfunction, leading to sympathetic hyperactivity and respiratory distress, including apnea. While the heart is vulnerable to catecholamine surges and hypoxia, it remains unknown if repetitive generalized seizures lead to cardiac damage. DBA/1 mice exhibit seizure-induced respiratory arrest (S-IRA) following generalized audiogenic seizures (AGS), which can be resuscitated using a rodent ventilator. In the current study, we induced different numbers of S-IRA episodes in DBA/1 mice and determined the association of repeated S-IRA induction with cardiac damage using histology. After repetitive induction of 18 S-IRA, calcified lesions, as revealed by calcium (Ca^{2+})-specific alizarin red staining, were observed in the ventricular myocardium in 61.5% of DBA/1 mice, which was higher compared to mice with 5 S-IRA and 1 S-IRA as well as age-matched untested control mice. The incidence of lesions in mice with 9 S-IRA was only higher than that of control mice. Only 1–2, small lesions were observed in mice with 5 S-IRA and 1 S-IRA and in control mice. Larger lesions (>2500 μm^2) were observed in mice with 9 and 18 S-IRA. The incidence of larger lesions was higher in mice with 18 S-IRA (53.8%) as compared to mice with 5 S-IRA and 1 S-IRA as well as with control mice, and the incidence of larger lesions in mice with 9 S-IRA was only higher than that of control mice. Repeated induction of S-IRA in DBA/1 mice can result in calcified necrotic lesions in the ventricles of the heart, and their incidence and size are dependent on the total number of S-IRA.

1. Introduction

Sudden unexpected death in epilepsy (SUDEP) ranks second in public health burden among common neurological disorders [1]. The risk of sudden death in patients with epilepsy is 24- to 28-fold higher than that in the general population [2–4]. The mechanisms of SUDEP are still elusive, although cardiac and respiratory dysfunctions as well as cerebral “shutdown” have received considerable attention [5–9].

The heart is highly regulated by autonomic system (both sympathetic and parasympathetic) [10]. Seizures impact the heart by shifting autonomic balance toward sympathetic dominance [11–14], and this cardiac disturbance poses a potential risk of lethal arrhythmia [11]. Seizures also cause electrocardiogram (ECG) changes [11,13], *e.g.*, ST-segment depression and T-wave inversion [15,16]. Seizures evoke ictal and/or postictal respiratory distress [17–22], including apnea that can last more than 60 s in humans [17]. Seizure-induced catecholamine (norepinephrine and epinephrine) release due to sympathetic hyperactivity and hypoxia due to respiratory distress in other models are associated with extensive calcium (Ca^{2+})-mediated pathophysiological changes in the heart [23–25], which can lead to cardiac structural damage. Consistent with this, microscopic cardiac pathological changes such as fibrosis are observed postmortem in SUDEP patients [26–29]. Therefore, we hypothesized that repeated induction of generalized seizures in DBA/1 mice would produce Ca^{2+}-mediated cardiac structural damage.

2. Material and methods

2.1. Animals

The DBA/1 mouse strain was maintained in the Massachusetts General Hospital Center for Comparative Medicine animal facility.

Abbreviations: AGS, Audiogenic seizures; PND, Postnatal day; ROS, Reactive oxygen species; S-IRA, Seizure-induced respiratory arrest; SUDEP, Sudden unexpected death in epilepsy; TWA, T-wave alternans.

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Rodent food and water were available ad libitum. All experiments performed in the current study were approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital. All measures were taken to minimize the stress of the mice during experiments and the number of animals used in the study.

2.2. Induction of different numbers of generalized seizures in DBA/1 mice

A DBA/1 mouse was placed in a plexiglass chamber in a sound-proof room, subjected to acoustic stimulation by an electric bell (96 dB SPL, UC4-150, Zhejiang People's Electronics, China) [30] starting from postnatal day (PND) 28. DBA/1 mice started to exhibit generalized audiogenic seizures (AGS) followed by seizure-induced respiratory arrest (S-IRA) from PND 30–32, which could be resuscitated using a rodent ventilator (Harvard Apparatus 680, Holliston, MA, USA) (180 strokes/min at a volume of 1 ml) [31]. Five groups of DBA/1 mice with different numbers of generalized AGS/S-IRA were used in the current study. The mice in the age-matched control group were never exposed to acoustic stimulation. The mice in the second group experienced 1 S-IRA, those in the third group experienced 5 S-IRA, and those in the fourth group experienced 9 S-IRA. The mice in the fifth group were subjected to acoustic stimulation and induction of AGS over two months. Behaviors of the AGS were digitally recorded, and the duration of AGS and the incidence of S-IRA were quantified visually offline.

2.3. Histology, staining, and characterization of lesions

At PND 88 (two months after first acoustic simulation in experimental groups), mice in the five groups were deeply anesthetized (overdose with ketamine/xylazine), and hearts were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (Boston Bioproducts, Ashland, MA, USA). A heart was processed with a series of concentrations of ethanol and xylene and blocked into two halves, which were embedded in paraffin collaterally. Serial 5-μm paraffin four-chamber sections (32 intact sections per heart) were collected on slides for hematoxylin and eosin (H&E), Ca^{2+}-specific alizarin red, and fibrosis-specific picrosirius red staining [32,33].

A slide was observed under a Nikon Eclipse 80i microscope (Nikon Instruments, Melville, NY, USA) for pathological changes such as necrosis, calcific nodules, and fibrosis in all structures of a four-chamber section, including both atrial and ventricular walls. The lesions were characterized into three categories based on the calculated maximal lesion area (μm^2): grade I (>0 and ≤400), grade II (>400 and ≤2500) and grade III (>2500). The incidence of DBA/1 mice with one or more lesions and the incidence of mice with grade I, grade II, or grade III lesions in five groups of mice were calculated.

2.4. Statistical analysis

Statistical analyses were performed using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Lesion occurrence and size (grade I, II, or III) among the control group and the experimental groups with different numbers of S-IRA were compared using Chi-square test. Comparisons of the duration of seizures and the number of S-IRA between mice with and without lesions were performed using unpaired Student *t*-test. Statistical significance was inferred if $p < 0.05$.

3. Results

After 2–4 days of daily acoustic stimulation, DBA/1 mice displayed AGS composed of wild running episodes and generalized tonic–clonic seizures followed by S-IRA that could be mechanically resuscitated [9].

In one group of mice, acoustic stimulation was applied daily or at an interval of 2–3 days over two months. After ~9 daily S-IRA and resuscitation, most DBA/1 mice became refractory to daily generalized seizure induction (no seizures or only wild running seizures). These mice were then given acoustic stimulation every 2–3 days. The average number of S-IRA was 18.4 ± 2.0 (mean ± S.E.M.) in this treatment group ($n = 13$) (labeled as 18-S-IRA in the figures and the table). The H&E staining in this group of mice showed that lesions occurred in the cardiac ventricular walls in 61.5% of DBA/1 mice (Fig. 1), which was significantly higher than that in the mice with 5 S-IRA (14.3%, $n = 7$) and 1 S-IRA (20%, $n = 10$) as well as in control mice (18.2%, $n = 11$) ($p < 0.05$) (Figs. 2, 3). To determine the extent to which total seizure burden contributes to cardiac lesions, an accumulated duration of seizures was calculated by adding the duration of individual seizures (including wild running and tonic–clonic seizures) together in a mouse in each group. Theaccumulated duration of seizures in mice with lesions was 315.3 ± 19.9 s ($n = 8$), which was not significantly different from that in mice without lesions (278.7 ± 20.3 s, $n = 5$) in the 18-S-IRA group. The mean number of S-IRA in mice with lesions (19.3 ± 2.9) in 18-S-IRA group was not significantly different from that in mice without lesions (17 ± 2.2). The incidence of lesions in the mice with 9 S-IRA (60%, $n = 10$) was significantly higher than that in control mice ($p < 0.05$) but was not significantly different from that in the mice with 5 S-IRA and 1 S-IRA (Figs. 2, 3). In the 9-S-IRA group, the accumulated duration of seizures in mice with lesions (103.6 ± 8.5 s, $n = 6$) was not significantly different from that in mice without lesions (88.4 ± 9.5 s, $n = 4$).

Only grade I and grade II lesions (≤2500 μm^2) were observed in 5-S-IRA, 1-S-IRA and control groups. Grade III lesions (>2500 μm^2) were observed in 9-S-IRA and 18-S-IRA groups. The incidence of grade III lesions was significantly higher in the 18-S-IRA group (53.8%) as compared with that in the control group as well as that in mice with 1 S-IRA and 5 S-IRA ($p < 0.05$). The incidence of grade III lesions was significantly higher in the 9-S-IRA group (30%) as compared with that in the control group ($p < 0.05$) but was not significantly different from that in mice with 1 S-IRA and 5 S-IRA (Fig. 4).

The number of lesions, regardless of lesion size, in each DBA/1 mouse varied. Most of the mice in the five experimental/control groups had 1–2 lesions. Interestingly, three mice in the 18-S-IRA group each had 5–10 lesions, which was not seen in other experimental/control groups (Table 1).

The lesions observed histologically were calcified, as demonstrated by alizarin red staining (Fig. 5), a screening test for Ca^{2+}. Although these lesions likely began as necrotic cardiac myocytes, no purely necrotic foci, without calcification, were noted. Fibrosis-specific picrosirius red staining revealed no obvious and quantifiable fibrosis in DBA/1 mice with generalized AGS (1-S-IRA, 5-S-IRA, 9-S-IRA, and 18-S-IRA) and control DBA/1 mice.

4. Discussion

In the current study, we demonstrate that repeated induction of S-IRA in DBA/1 mice produces necrotic lesions in the ventricles of the heart. The incidence and size of the lesions are clearly dependent on the number of S-IRA. The calcification suggests that the cardiac lesions are initiated by local derangement of Ca^{2+} homeostasis, supporting our hypothesis.

The precise mechanisms underlying seizure-induced formation of calcified lesions in the heart of the DBA/1 mouse remain unknown. Seizures result in sympathetic hyperactivity [11,13], and sustained elevation of catecholamines under other circumstances can lead to Ca^{2+} overload and oxidative stress, which trigger mitochondrial dysfunction by generating reactive oxygen species (ROS) and cardiomyocyte death [34]. Notably, as another example of episodic surges of catecholamine, pheochromocytoma is characterized by catecholamine-mediated myocarditis, focal and diffuse myocardial fibrosis, and myocardial dysfunction [35]. Also, seizures induce respiratory distress and apnea [17–21], and the hypoxia resulting from seizure-induced respiratory dysfunction can substantially elevate ROS [36]; ROS regulates cellular function through redox modification of proteins, including L-type Ca^{2+} channels

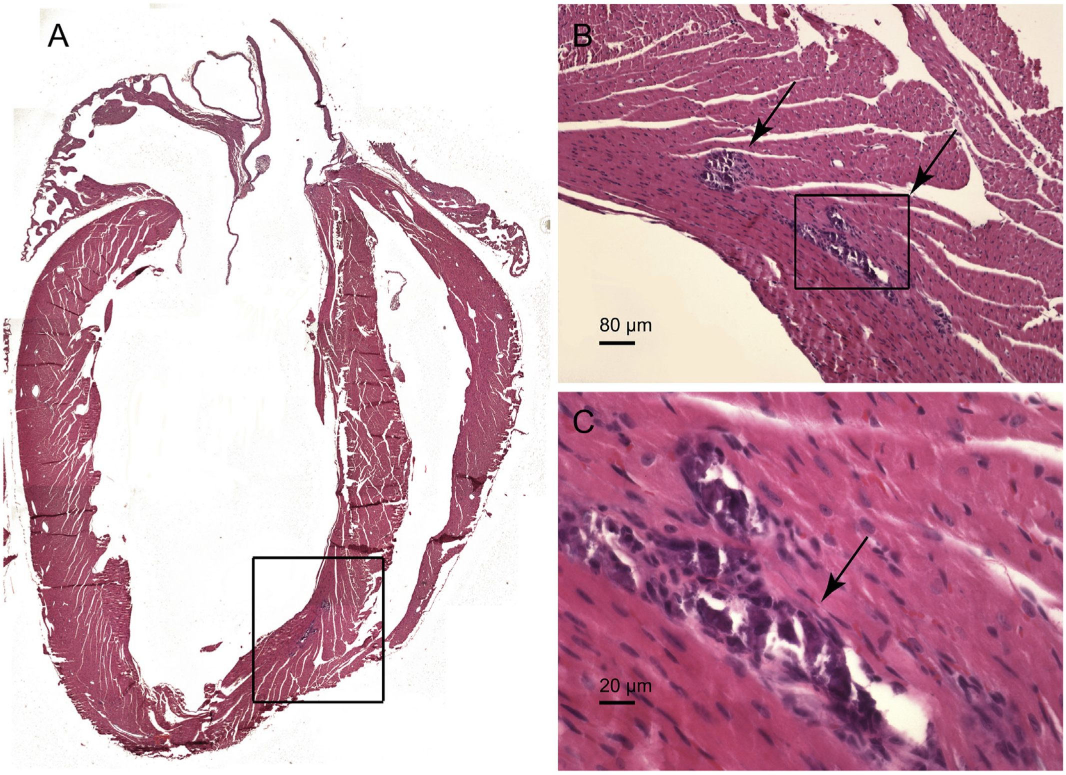

Fig. 1. Repetitive induction of S-IRA can produce necrotic lesions in the ventricles of the heart in DBA/1 mice. A, a reconstructed image of a four-chamber section of the heart in a DBA/1 mouse that underwent 18 S-IRA in a two-month period prior to histological study. The H&E staining revealed necrotic lesions in the left ventricular wall of the heart (inside the square). B, C, amplified images showing the detail of the necrotic lesions, indicated by arrows. Lesions in the square in A were expanded in B, and those in the square in B were expanded in C. Amplifications: B, 10×; C, 40×.

[37]. All of these factors can alter Ca^{2+} homeostasis and engender oxidative stress, leading to cardiac injury with calcification. In addition, DBA/1 mice may be genetically susceptible to formation of calcified necrotic lesions after cardiac injury, as the DBA/2 mouse, another strain of the DBA mouse, is reported to be genetically prone to calcified cardiac lesions [38,39]. In the current study, we observed that the size of calcified lesions is bigger in the 18-S-IRA group. This may be due to the cumulative effects of repeated catecholamine surges and/or hypoxia (and thus repeated Ca^{2+} release). While efforts were made to limit respiratory distress by applying respiratory resuscitation, further studies are

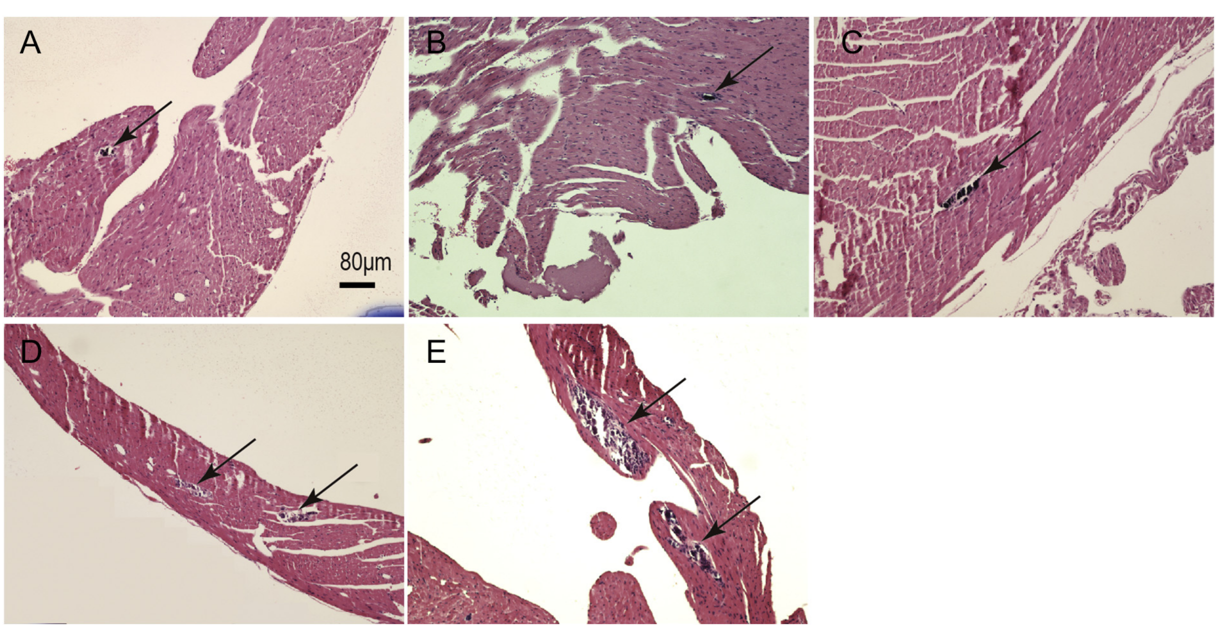

Fig. 2. Occurrence of cardiac necrotic lesions in DBA/1 mice with different numbers of S-IRA. An example of cardiac lesion(s) in control DBA/1 mice (A), DBA/1 mice with 1 S-IRA (B), DBA/1 mice with 5 S-IRA (C), DBA/1 mice with 9 S-IRA (D) and DBA/1 mice with 18 S-IRA (E). Amplifications: 10×.

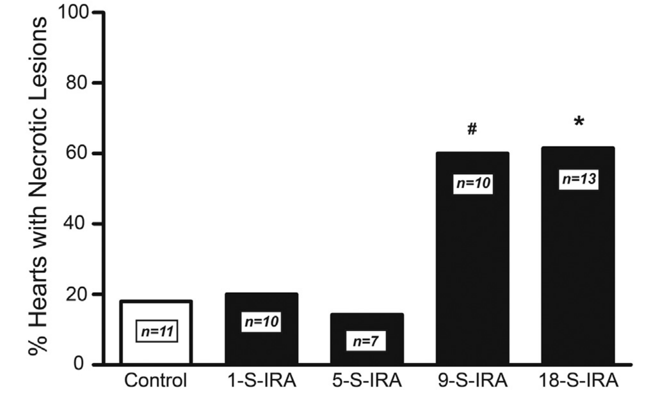

Fig. 3. The incidence of hearts with necrotic lesions is dependent on the number of S-IRA in DBA/1 mice. The incidence of necrotic lesions was significantly higher in mice with 18 S-IRA as compared with that in the control group as well as in the mice with 1 S-IRA and 5 S-IRA. The incidence of necrotic lesions in mice with 9 S-IRA was significantly higher than that in the control group but was not significantly different from that in the mice with 1 S-IRA and 5 S-IRA. * $p < 0.05$: Significantly different from the control group, 1-S-IRA group or 5-S-IRA group; # $p < 0.05$: Significantly different from the control group.

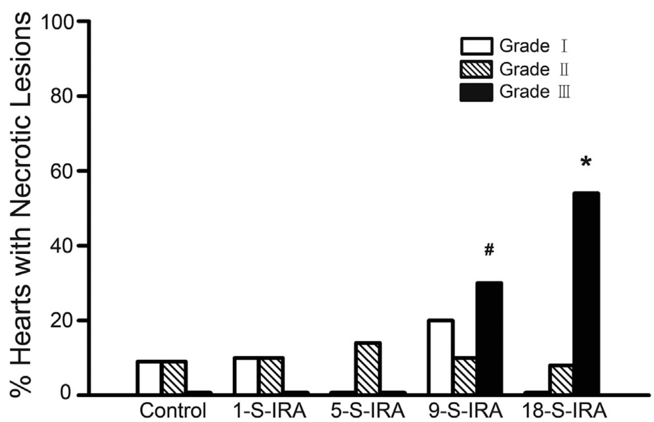

Fig. 4. The severity of necrotic lesions is dependent on the number of S-IRA in DBA/1 mice. Grade III lesions were only observed in mice with 9 and 18 S-IRA. The incidence of grade III lesions was significantly higher in mice with 18 S-IRA as compared with that in control mice as well as that in the mice with 1 S-IRA and 5 S-IRA. The incidence of grade III lesions was significantly higher in mice with 9 S-IRA as compared with that in control mice but was not significantly different from that in the mice with 1 S-IRA and 5 S-IRA. The incidence of grade I or grade II lesions was not significantly different among the treatment and control groups. * $p < 0.05$: Significantly different from the control group, 1-S-IRA group or 5-S-IRA group with grade III lesions; # $p < 0.05$: Significantly different from the control group.

needed to determine whether sympathetic hyperactivity or respiratory-induced hypoxia or both contribute to the calcified lesions observed in this model.

Table 1
The number of necrotic lesions (H&E staining) in the ventricular walls.

Seizures group (2 months)	Lesions number			
	0 lesion	1–2 lesions	3–4 lesions	5–10 lesions
Control (n = 11)	9	2	0	0
1-S-IRA (n = 10)	8	2	0	0
5-S-IRA (n = 7)	6	1	0	0
9-S-IRA (n = 10)	4	6	0	0
18-S-IRA (n = 13)	5	5	0	3

Seizure-induced cardiac injury is not uncommon. It was reported that status epilepticus evokes cardiac structural changes in rodents [40,41] and in patients with epilepsy [42]. In addition, significant elevations of high-sensitive troponin T are seen following generalized tonic–clonic seizures in as many as 26% of patients [43]. Though microscopic cardiac structural damage such as fibrosis has been observed postmortem in patients who died from SUDEP [14,26–28,44], not all studies agree [27], and the potential relationship of cardiac fibrosis to SUDEP is unknown, though it could conceivably involve susceptibility to sympathetically-mediated tachyarrhythmias [14]. In the current study, we only observed small calcified lesions in a proportion of DBA/1 mice with multiple episodes of S-IRA. Interestingly, in both 9-S-IRA and 18-S-IRA groups, the accumulated duration of seizures in mice with lesions was not significantly different from that in mice without lesions, suggesting that seizure duration is unlikely a critical factor for lesion formation. It remains unknown what the major contributing factors to cardiac lesions are. Finally, and surprisingly, repeated induction of S-IRA barely induced fibrosis in DBA/1 mice in the current study. It is likely that the lesions were too small to result in a significant amount of fibrosis.

Cardiac structural damage is often associated with cardiac functional changes [41,45], but whether the cardiac injury observed in the current study may cause cardiac functional abnormalities in DBA/1 mice remains to be investigated. Given that most of lesions occur in the ventricular wall in the current study, we speculate that these lesions may contribute to ventricular arrhythmia during seizures. Seizures produce sympathetic overdrive and in patients primarily increase the heart rate [15,46,47], up to 200 beats per minute [48], and notably generalized seizure-evoked ventricular tachycardia has been associated with SUDEP or near-SUDEP in patients [49,50]. These lesions may also compromise the mechanical function of ventricles, and depressed ventricular function is a risk marker for sudden cardiac death [51]. Previous studies suggest that patients with chronic, drug-resistant epilepsy may have cardiac electrical instability, and in particular elevated T-wave alternans (TWA) in interictal electrocardiographic recordings, a finding that is associated with sudden cardiac death in populations without epilepsy [52–56].

4.1. Limitations to the present study

In retrospect, several features of this study were not optimized to precisely determine the size and direct functional effects of the cardiac lesions, and the relationship of these lesions to SUDEP. The individual and collective total volume of the myocardial lesions could not be quantified. Although many sections of each heart were examined, they were not sequential, and it is therefore possible that an apparently small lesion in one plane of sectioning could be larger in another, and thus would have a more substantial volume than could be estimated. Although cardiac lesions comprised a small volume fraction of the ventricular walls, their functional consequences and the effects of intervention to prevent the cardiac lesions were not evaluated. Therefore, more studies are needed to investigate if repeated generalized seizure-evoked cardiac structural damage in our model is associated with measurable functional disturbances that mimic the relationship of TWA with cardiac electrical instability, and whether these functional disturbances increase the risk of lethal cardiac arrhythmia, whether ictal or interictal.

5. Conclusion

Our data demonstrate that repeated induction of S-IRA produce calcified cardiac lesions in DBA/1 mice and that the severity of cardiac injury is associated with the total number of S-IRA. Further studies are needed to determine if this Ca^{2+}-mediated cardiac structural damage increases vulnerability to potentially fatal cardiac arrhythmia as a possible model for cardiac-related SUDEP and whether sympatholytic, parasympathomimetic (such as vagal nerve stimulation [55]), and/or

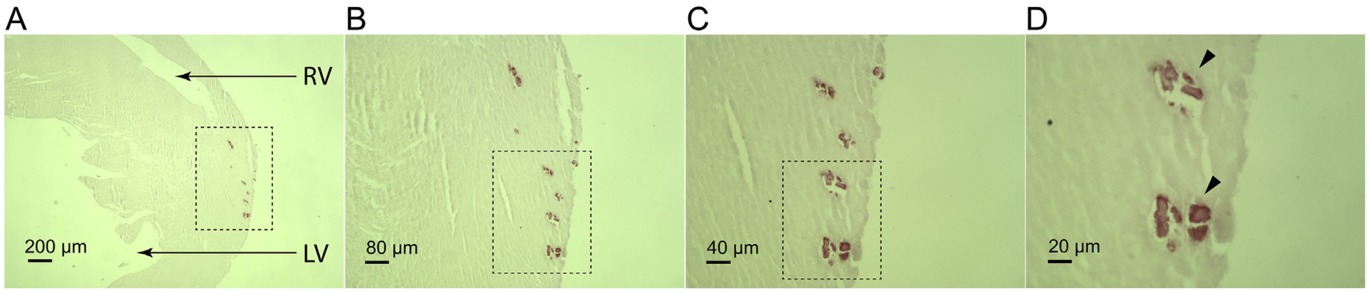

Fig. 5. Repeated generalized seizure-induced necrotic foci in the heart of DBA/1 mice are calcified lesions. An example of alizarin red staining in the mice with 18 S-IRA, showing the calcification of the necrotic lesions. Lesions in dotted rectangle in A were expanded in B, B in C, and C in D, indicated by arrow heads. Amplifications: A, 4×; B, 10×; C, 20×; D, 40×.

respiratory support therapies may decrease the number or severity of cardiac lesions.

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Conflicts of interest

None of the authors has any conflict of interest to disclose.

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