Enhancement of central norepinephrinergic neurotransmission contributes to inhibition of seizure-induced respiratory arrest by targeting the beta-1 adrenergic receptor (β1-AR) locating in the cardiomyocytes in the DBA/1 mouse SUDEP model

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Abstract

Sudden unexpected death of epilepsy (SUDEP) is the key cause of of death in patients with epilepsy. Due to the complicated pathogenesis of SUDEP, however, the exact mechanism of SUDEP remains elusive. Currently, although it is recognized that the seizure-induced respiratory arrest (S-IRA) may be a main cause for SUDEP, other factors resulting in SUDEP can not be excluded e.g arrhythmias. Our previous findings indicated that the incidence of seizure-induced respiratory arrest S-IRA and SUDEP evoked by acoustic stimulation or pentetrazol (PTZ) injection was significantly reduced by atomoxetine, a norepinephrine reuptake inhibitor (NRI), suggesting that noradrenergic neurotransmission modulates S-IRA and SUDEP. Given that norepinephrine acts on the central and peripheral target to modulate respiratory and circulation function by targeting adrenergic receptor α and beta (a-AR and β-AR) and the arrhythmias can be contributed to SUDEP. Meanwhile, to further test whether cardiac factors are implicated in S-IRA and SUDEP, we choose esmolol hydrochloride, a selective antagonist of beta-1 adrenergic receptor (β1-AR) to test it in our models. Our findings demonstrated that the lower incidence of S-IRA and SUDEP evoked by acoustic stimulation in DBA/1 mice by administration with atomoxetine was significantly reversed by intraperitoneal (IP) of esmolol hydrochloride. Importantly, the data of electrocardiogram (ECG) showed that the cardiac arrhythmia evoked by acoustic stimulation including the ventricular tachycardia, ventricular premature beat

and atrioventricular block and administration of atomoxetine significantly reduced theses arrhythmias and the incidence of S-IRA and SUDEP in our models. Thus, the dysfunction of respiratory and circulation may be implicated in the pathogenesis of S-IRA and SUDEP hand in hand and enhancing central norepinephrinergic neurotransmission contributes to inhibition of seizure-induced respiratory arrest by targeting β1-AR locating in the cardiomyocytes. Our findings will show a new light on decoding the pathogenesis of SUDEP.

Keywords: sudden unexpected death in epilepsy (SUDEP); seizure-induced respiratory arrest S-IRA); esmolol hydrochloride (Esmolol); Electrocardiogram (ECG); locus coeruleus (LC); cardiac arrhythmia

1. INTRODUCTION

It had been recognized that sudden unexpected death in epilepsy (SUDEP) is the key cause leading to the morality of epilepsy patients, especially those who had undergone antiepileptic drug resistance [1-4]. Although some important advancements concerning the pathogenesis of SUDEP had been obtained, to our certain knowledge, decoding SUDEP seems to be a huge challenge in views of the complicated mechanism of SUDEP. Indeed, seizure-induced respiratory arrest (S-IRA) is an important cause resulting in the death of epilepsy including our previous findings [2-6]. However, more and more evidence hinted that the mechanisms of SUDEP are likely to be heterogeneous [5]. Some data showed that the dysfunction in circulation may be a contributor to SUDEP. In fact, both of the dysfunction of respiratory and circulation was in implicated in S-IRA and SUDEP. Previous findings showed that the cardiac and respiratory function were simultaneously suppressed to induce the S-IRA and SUDEP evoked by acoustic stimulation in DBA/1 mice, suggesting that the autonomic nerve including the sympathetic and parasympathetic nerve were involved in the course of S-IRA and SUDEP.

Our previous data unveiled that the incidence of S-IRA and SUDEP evoked by acoustic or PTZ can be significantly reduced by IP with atomoxetine, a norepinephrine reuptake inhibitor (NRI) [6]. Considering that the central and peripheral norepinephrine transmission was associated closely with the activity of the

sympathetic nerve. It was well known that norepinephrine transmission modulates the respiratory and circulation function by targeting the adrenergic α1, α2, β1, and β2 receptors. The findings from our and other laboratories showed α1 and α2 receptors were implicated in the S-IRA and SUDEP medicated by atomoxetine in the DBA/1 mice models [7-8]. However, whether both of β1 and β2 receptors, especially β1 receptor located in the cardiomyocytes that play a key role in modulating the cardiac function seems to essentially be explored based on our and other findings. Therefore, we choose esmolol hydrochloride, a selective antagonist of beta-1 adrenergic receptor (β1-AR) to test whether the β1-AR was implicated in the atomoxetine mediated S-IRA and SUDEP. Our findings showed that the lower incidence of S-IRA and SUDEP evoked by acoustic stimulation medicated by atomoxetine was significantly reversed by Esmolol. Thus, our data further demonstrated that the cardiac factors implicated in the reduction of the incidence of S-IRA and SUDEP by atomoxetine can't be excluded and further contribute to explaining the possibility of heterogeneous factors leading to SUDEP.

2. MATERIALS AND METHODS

2.1 Animals

All experimental procedures were the agreement with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Advisory Committee of Zhejiang University. DBA/1 mice were housed and bred in the Animal Center of Zhejiang University School of Medicine and provided with rodent food and water ad libitum. DBA/1 mice of either sex were used in this experiment, as previous studies have shown, gender is not a variable that affects the S-IRA of DBA/1 mice. Aiming at acoustic stimulation murine model, DBA/1 mice were "primed" starting from postnatal day 26-28 to establish the consistent susceptibility to audiogenic seizures and S-IRA as previously described.

2.2 Seizure induction and resuscitation

S-IRA was established by acoustic stimulation as we previously described [5-12]. For the acoustic stimulation model, each mouse was placed in a cylindrical plexiglass chamber in a sound-isolated room, and audiogenic seizures (AGSZs) were evoked by

using an electric bell (96 dB SPL, Zhejiang People's Electronics, China). Acoustic stimulation was given for a maximum duration of 60 s or until the onset of tonic seizures and S-IRA in most mice in each group. Mice with S-IRA have resuscitated within 5 s post the final respiratory gasp by a rodent respirator (YuYan Instruments, Shanghai, China).

2.3 Effects of intraperitoneal (IP) administration of Esmolol on the atomoxetine-mediated suppression of S-IRA evoked by acoustic stimulation

The susceptibility of DBA/1 mice to S-IRA was confirmed 24 hours before atomoxetine or vehicle administration. To IP administration, atomoxetine and esmolol were dissolved in saline. Different dosages of esmolol were applied to a vehicle control group and treatment groups, in which DBA/1 mice would be acoustically stimulated later. Atomoxetine (15 mg/kg, Ca # Y0001586; Sigma-Aldrich) or vehicle was intraperitoneal (IP) injection in DBA/1 mice 120min prior to acoustic stimulation, 115min after infusion of atomoxetine (or vehicle), IP injection of esmolol (25mg/kg, 50mg/kg, H19991058; Qilu Pharmaceutical Co., Ltd.) or vehicle 5min before acoustic stimulation (n=5-7/per group). The incidence of S-IRA, latency to AGSZs, duration of wild running, clonic seizures, tonic-clonic seizures, and seizure scores were videotaped for offline analysis. DBA/1 mice from the different pre-treatment groups, the monitoring of ECG was performed before acoustic stimulation and post-S-IRA. To exclude the effects of esmolol on the incidence of the morality of DBA/1 mice, the group, subjected to confirmation of S-IRA before 24 hours to experiment, was pre-treated with the esmolol (50mg/kg, i.p) without administration of atomoxetine and acoustic stimulation in the same manner to observe the incidence of the morality of DBA/1 mice(n= 6).

2.6 Data analysis

Statistical analyses were performed using SPSS 23(SPSS Software Inc., USA). The incidence of S-IRA was compared in different groups using Wilcoxon Signed Rank test, as these data are nonparametric. The data on seizure scores and the latency to AGSZs, the duration of wild running, clonic seizures, and tonic-clonic seizures

were evaluated using the one-way ANOVA tests, which were presented as the mean ± SEM. Statistical significance was inferred if $p < 0.05$.

3. Results

3.1 Administration of Esmolol significantly reversed the atomoxetine-mediated suppression of S-IRA evoked by acoustic stimulation

To investigate the effects of Esmolol on the incidence of S-IRA and SUDEP medicated by atomoxetine based on our previous findings. The following experiments were performed. The incidence of S-IRA evoked by acoustic stimulation in primed DBA/1 mice was obviously reduced by atomoxetine(15 mg/kg,i.p)compared with the vehicle group(n=6, n=7, $p < 0.001$). There was no significant difference between the vehicle group and the group pre-treated with esmolol (50 mg/kg,i.p) and with vehicle (n=7,n=6, $p > 0.05$). However, the incidence of -S-IRA in the group pre-treated with atomoxetine(15 mg/kg,i.p) and esmolol(50 mg/kg,i.p) was significantly increased than the group pre-treated with atomoxetine(15 mg/kg,i.p) and the vehicle(n=6, n=7, $p < 0.05$). The incidence of -S-IRA in the group pre-treated with atomoxetine(15 mg/kg,i.p) and the vehicle was significantly decreased than in the group pre-treated with the vehicle and with Esmolol(50 mg/kg,i.p) (n=6, n=6, $p < 0.001$). The difference of latencies to AGSZs, durations of wild running, clonic seizures, tonic-clonic seizures, and seizure scores in the treatment group showed administration of esmolol significantly reversed the atomoxetine-mediated suppression of S-IRA evoked by acoustic stimulation without sensitivity to seizures. These data showed the atomoxetine-mediated suppression of S-IRA evoked by acoustic stimulation can be significantly reversed by esmolol, which means that administration of atomoxetine reduced the incidence of S-IRA by targeting the β1-AR locating in the cardiomyocytes in our models. (FIG 1)

3.2 Administration of Esmolol specifically reversed the atomoxetine-mediated suppression of S-IRA by targeting the β1-AR locating in the cardiomyocytes

To exclude whether the dose of Esmolol (50 mg/kg,i.p) produces the morality of DBA/1 mice to affect the specificity to reverse the reversed atomoxetine-mediated suppression of S-IRA in our models. The following experiments were performed in

different groups. There was no significant difference in the morality of DBA/1 mice between the group pre-treated with the vehicle and the group pre-treated with Esmolol (50 mg/kg,i.p), suggesting that the atomoxetine-mediated suppression of S-IRA can be specifically reversed by esmolol by targeting the β1-AR locating in the cardiomyocytes. (FIG 2)

3.3 Obvious cardiac arrhythmia immediately occurred with tonic seizures and S-IRA evoked by acoustic stimulation

To observe the changing of ECG in the different treatment groups to analyze the role of atomoxetine and/or esmolol in cardiac electrophysiology. We observed that the ventricular tachycardia, ventricular premature beat, and atrioventricular block frequently occurred immediately following tonic seizures and S-IRA evoked by acoustic stimulation in DBA/1 mice in a group pre-treated with the vehicle and with the vehicle. However, Compared with the vehicle group, the arrhythmia was significantly decreased in the group pre-treated with atomoxetine (15 mg/kg,i.p) and vehicle. We also observed the arrhythmia frequently occurred in the group pre-treated with esmolol and the vehicle and the group pre-treated with atomoxetine (15 mg/kg,i.p) and esmolol, suggesting that Esmolol can specifically reverse the atomoxetine-mediated suppression of S-IRA by targeting the β1-AR locating in the cardiomyocytes without resulting in extra arrhythmia. (FIG 3-4)

4. DISCUSSION

There are about 70 million people in the world who suffer from epilepsy, and 2.4 million have been diagnosed with epilepsy every year, nearly 90% occurred in developing countries[1]. The latest epidemiological studies of the American Epilepsy Society show that the incidence of SUDEP in western developed countries is about 1.11 per 1000 people per year[2], while the results of the multi-center epidemiological survey in China show that the incidence of SUDEP in China is about 2.3 per 1000 people per year[3], and the number of domestic epilepsy patients dying from SUDEP is more than 20,000 per year. More importantly, SUDEP is the main factor that leads to the death in young patients with epilepsy with an incidence two to three times that of other diseases that can lead to sudden death in young adults, and children's SUDEP

incidence is also close to adults[4]. Japan's latest epidemiological survey shows that the incidence of SUDEP in pregnant women is significantly higher than the incidence of non-pregnant women[5]. However, due to the complexity of its pathogenesis and the difficulty to obtain the real-time monitoring data of SUDEP in patients with epilepsy, the understanding of the SUDEP mechanism is still limited, and it is difficult to develop a prevention strategy. Many previous studies have shown that the pathophysiology of SUDEP is complex, including the disfunction of heart, autonomic nerves, respiratory, brain structural obstacles, and polygenesis.

In the current study, we firstly reported that the suppressant effects of S-IRA and SUDEP in DBA/1 mice by atomoxetine can be significantly reversed by administration of esmolol, a selective β1 receptor blocker. Our previous findings showed that administration of atomoxetine inhibited S-IRA and SUDEP in DBA/1 mice independently of seizure induction methods, and its inhibition of S-IRA induced by acoustic stimulation was dose-dependent[6]. Given the current clinical reports that atomoxetine can produce the effects of peripheral circulation. For example, after oral administration of atomoxetine, ADHD (Attention-Deficit/ Hyperactivity Disorder) patients showed symptoms such as increased heart rate and other symptoms[7], considering that atomoxetine may play a role in SUDEP passing through the central - peripheral - cardiac sympathetic mechanisms by affecting β1 receptors as dominate receptors in the heart. In the present study, we used the β1 receptor blocker esmolol to block the β1 receptor which locates preferentially in the cardiomyocytes of the heart to test whether the lower incidence of S-IRA by atomoxetine can be reversed by Esmolol. It turned out that the suppressant effects of S-IRA by atomoxetine were markedly reversed by esmolol, suggesting that administration of atomoxetine significantly reduced the incidence of S-IRA by enhancing the concentration of norepinephrine of the cleft between the sympathetic synapse and cardiomyocytes of the heart to combine with β1 receptors of cardiomyocytes. Of course, atomoxetine medicating the reduction of S-IRA by enhancing the concentration of norepinephrine to target the adrenergic receptor α1 and α2 in the brain was the main pathway as well. To be exact, atomoxetine medicates the reduction of S-IRA by interacting between α1,

α2, and β1 receptors located in central and peripheral sympathetic nerves. In fact, although S-IRA was the key cause leading to SUDEP, other factors are involved in the course can't be excluded as well. A previous study showed that S-IRA evoked by acoustic stimulation in DBA/1mice was characterized by the simultaneous suppression of respiration and circulation though the respiratory arrest preceded the cardiac arrest. What's more, the recent study showed that the tonic phase apnea evoked by acoustic stimulation is not sufficient for seizure-induced death in a $W/+^{Emx1\text{-}Cre}$ mouse. Therefore, the factors resulting in the S-IRA and SUDEP including respiration and circulation may be heterogeneous.

The previous research has shown that the main risk factors of SUDEP are generalized tonic-clonus and nocturnal seizures. The neuronal network of generalized tonic-clonic seizures may lead to hypoventilation, apnea, and cardiovascular failure by inhibiting brainstem respiration or autonomic nerve control centers, leading to death [10, 11]. Since the most sudden death cases occur at night or in sleep, with no witnesses, leaving many questions unanswered. In recent years, more and more researches have shown that apnea and heart failure are important potential mechanisms in SUDEP. Studies in animal models and SUDEP patients have shown that seizure-induced respiratory arrest (S-IRA) is one of the main induced factors that lead to death in many cases[12-14]. However, a respiratory arrest is not the only lethal cause, tonic-clonic seizures caused sudden, simultaneous respiratory and cardiac depression in mice, and the time of recovery of respiration and heart rate during resuscitation is also closely matched[15]. Therefore, the heart also plays an important role in the mechanism of SUDEP.

Seizure-associated arrhythmias are common and have been considered as the potential pathogenesis of SUDEP, such as tachycardia, bradycardia, and cardiac arrest after the seizures, resulting in cardiac repolarization, changes in electrolyte levels, blood pH, and catecholamine release in patients with epilepsy, which latter three factors may promote arrhythmias and lead to death by affecting cardiac excitability[16]. Tachycardia and several factors that increase susceptibility to tachyarrhythmias, known as pathological cardiac repolarization, are considered as risk factors for sudden

cardiac death (SCD) in healthy individuals and patients with epilepsy which, in this particular setting, are important factors in triggering SUDEP events[17, 18]. However, the exact mechanism of SUDEP and cardiac arrest induced by seizures remains unclear. Seizures may interfere with the normal functioning of the heart by causing autonomic disturbances [19], leading to fatal arrhythmias. Studies have found that cardiac electrical instability and autonomic dystonia are caused by cumulative cardiac damage during repeated attacks[20]. Studies also have shown that catecholamine (norepinephrine and adrenaline) released from seizures is related to a wide range of calcium (Ca2+)-mediated physiological changes, which can lead to damage to the heart structure. Our previous studies found that repeated S-IRA in DBA / 1 mice will result in necrotic damage in the ventricle which is caused by the local Ca2 + homeostasis disturbances, and the incidence and severity of injury depend on the total number of S-IRA[21]. Relevant clinical data have found that paroxysmal arrhythmias (including arrest, atrioventricular block, and rare atrial and ventricular fibrillation) usually occur after convulsive episodes and are often associated with (near)SUDEP[22]. Studies have shown that chronic epilepsy can lead to hypoxemia and the increase of catecholamine, causing damage of the heart and vascular, resulting in cardiac electrical, and mechanical dysfunction[23,24]. Some fatal incentives may be the acidosis caused by persistent hypoxemia and hypercapnia, leading to bradycardia or cardiac arrest, while in others it may be the seizures due to malignant arrhythmias[25]. This suggests that SUDEP is inextricably linked to arrhythmia.

In the present study, the ECG of DBA/ 1 mice with administration of esmolol (50 mg/kg, i.p) that can significantly reverse the lower incidence of S-IRA evoked by acoustic stimulation by administration of atomoxetine showed the mixture of sinus bradycardia, atrioventricular block, ventricular premature beat, and ventricular tachycardia in order. In the meantime, the ECG of DBA/1 mice suffered from the S-IRA evoked by acoustic stimulation characterized by the mixture of sinus bradycardia, atrioventricular block, ventricular premature beat, and ventricular tachycardia as well in our model. However, no obvious arrhythmia appeared in the DBA/1 mice without suffering from S-IRA other than the sinus bradycardia and no

apparent mortality occurred in the group of DBA/1 mice with pre-treatment with the dose of esmolol 50 mg/kg (i.p). Furthermore, the arrhythmias can be significantly reduced by atomoxetine in our models. Thus, the lower incidence of S-IRA by acoustic stimulation can be significantly as well as especially reversed by esmolol by blocking the β1-AR located in cardiomyocytes to combine the norepinephrine released by cardiac sympathetic nerve synaptic terminal in our models. Additionally, it is possible that the locus coeruleus (LC), as the largest nuclei to synthesize and release norepinephrine in the brain, may be implicated in the course of administration of esmolol to reverse the lower incidence of S-IRA and SUDEP and plays a key role in modulating S-IRA and SUDEP by regulating the respiratory and circulation function in our models. In future experiments, we will further explore it.

5. Conclusions

The current finding in our models suggested that enhancing central norepinephrinergic neurotransmission contributes to inhibition of seizure-induced respiratory arrest by targeting β1-AR locating in the cardiomyocytes and the pathway of norepinephrinergic neurotransmission-β1-AR between brain and heart may be a potential and specific target to prevent SUDEP.

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7. DISCLOSURE

All authors declare no competing interests. We had confirmed that we have read the Journal's position on issues involved in ethical publication and affirm that this study was in accordance with those guidelines.

8. Figure legends

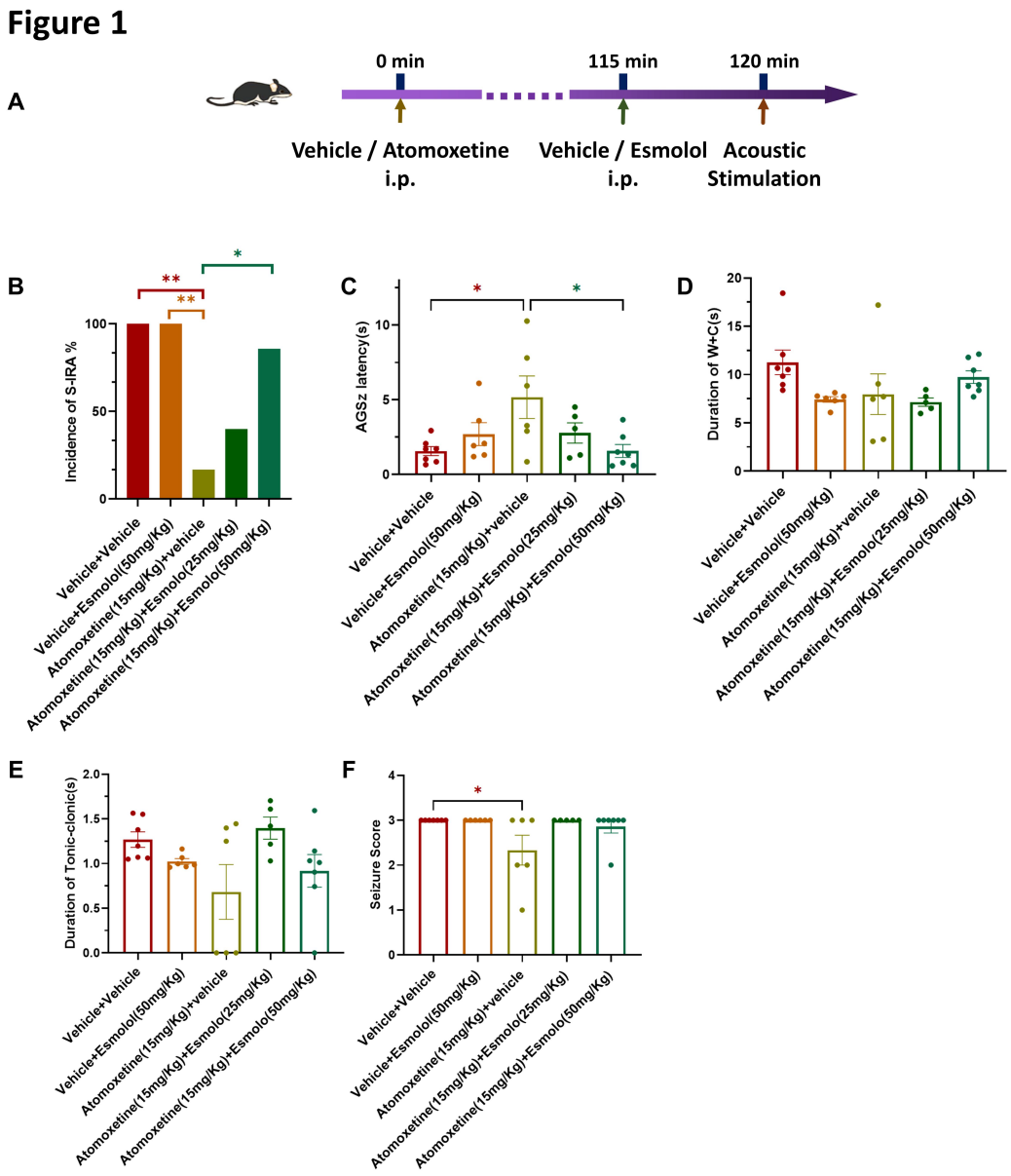

Figure 1 Administration of Esmolol significantly reversed the atomoxetine-mediated suppression of S-IRA evoked by acoustic stimulation

A. The protocol to explore the influence of administration of esmolol on atomoxetine-mediated reduction of S-IRA evoked by acoustic stimulation. **B.** Compared to the group treated with vehicle (n = 7) or vehicle and esmolol (n = 6), S-IRA evoked by acoustic stimulation was markedly lower in groups treated with i.p. atomoxetine (n = 6, **p < 0.01) at 15mg/Kg. However, the protective effect of atomoxetine in primed DBA/1 mice was significantly reversed by esmolol doses of 50mg/kg (n = 7, *p < 0.05). **C.** Furthermore, compared with the group treated with atomoxetine and vehicle (n = 6), the latencies to AGSZs in the control group (n = 7)

or the group treated with atomoxetine and esmolol (n = 7) was significantly increased ($p < 0.05$). **D-E.** There were no intergroup differences in durations of wild running plus clonic seizures (W+C), tonic-clonic seizures ($p > 0.05$). **F.** Compared to the control group (n = 7) , the seizure score was lower in the group treated with atomoxetine and vehicle (n = 6, *$p < 0.05$).

Figure 2

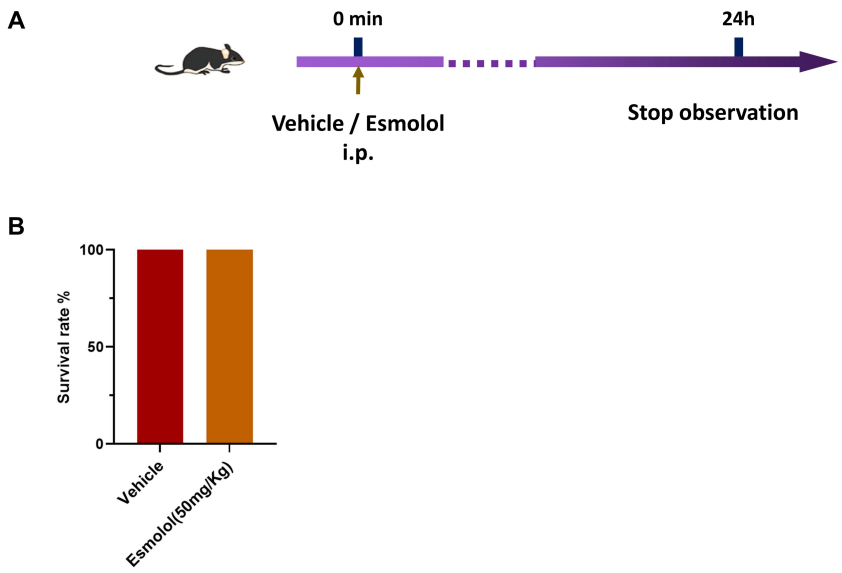

Figure 2 The dose of Esmolol (50 mg/kg,i.p.) does not produce the incidence of morality of DBA/1 mice.

A. The protocol to explore the dose of esmolol (50 mg/kg,i.p.) whether produce the morality of DBA/1 mice. **B.** There was no significant difference in the morality of DBA/1 mice between the group pre-treated with the vehicle and the group pre-treated with esmolol(50 mg/kg, i.p, n=7) ($p > 0.05$)

Figure 3

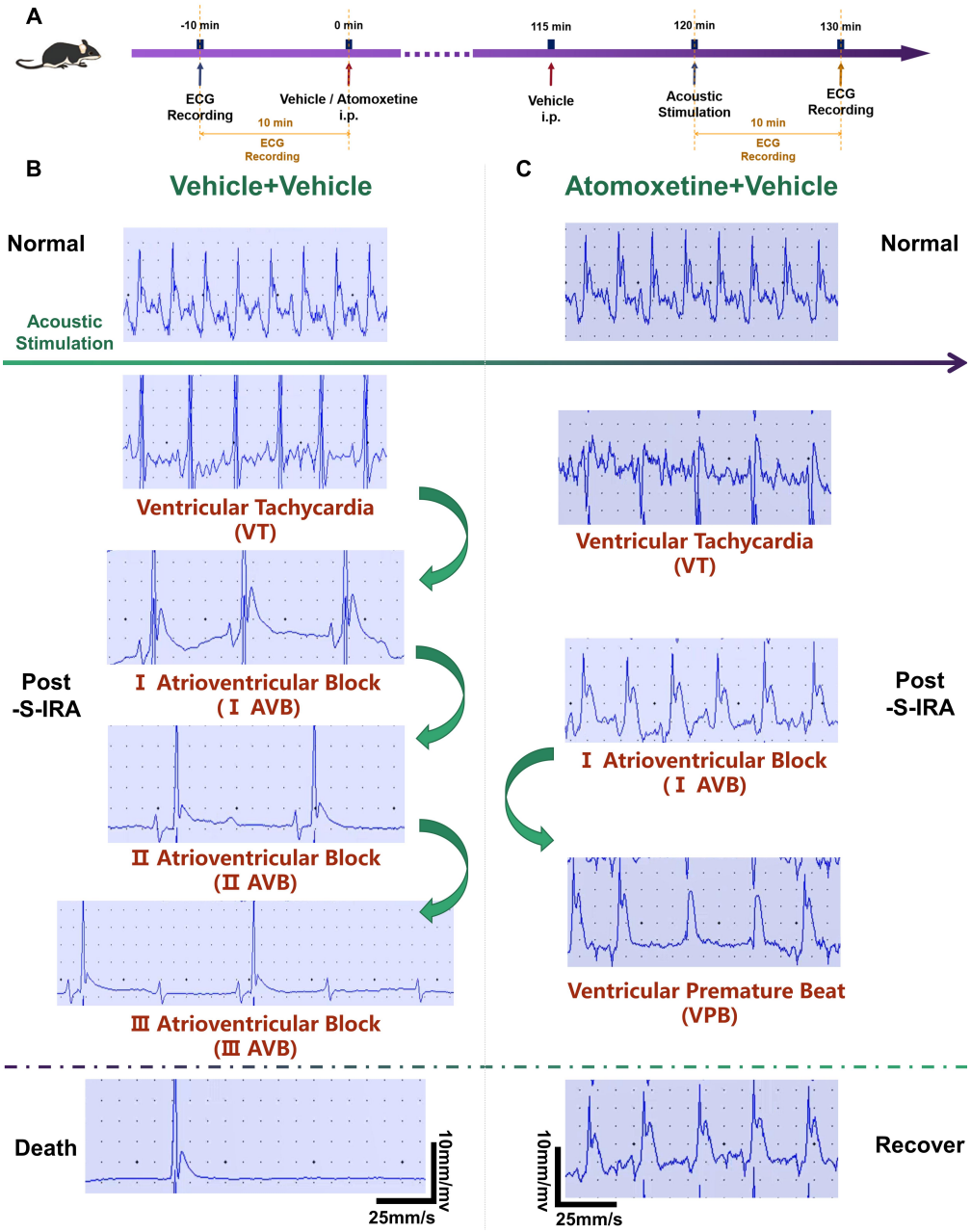

Figure 3 The effects imposed by tonic seizures on the changing of ECG in both groups treated with vehicle or atomoxetine

A. The protocol to explore the changes of electrocardiogram on atomoxetine-mediated reduction of S-IRA evoked by acoustic stimulation. **B.** The ventricular tachycardia and atrioventricular block occurred immediately following tonic seizures and S-IRA evoked by acoustic stimulation in DBA/1 mice in the control group. **C.** What's more, the group pre-treated with the atomoxetine (15mg/Kg) was observed the ventricular tachycardia, atrioventricular block, and ventricular premature beat after acoustic stimulation, and the DBA/1 mice were then recovered.

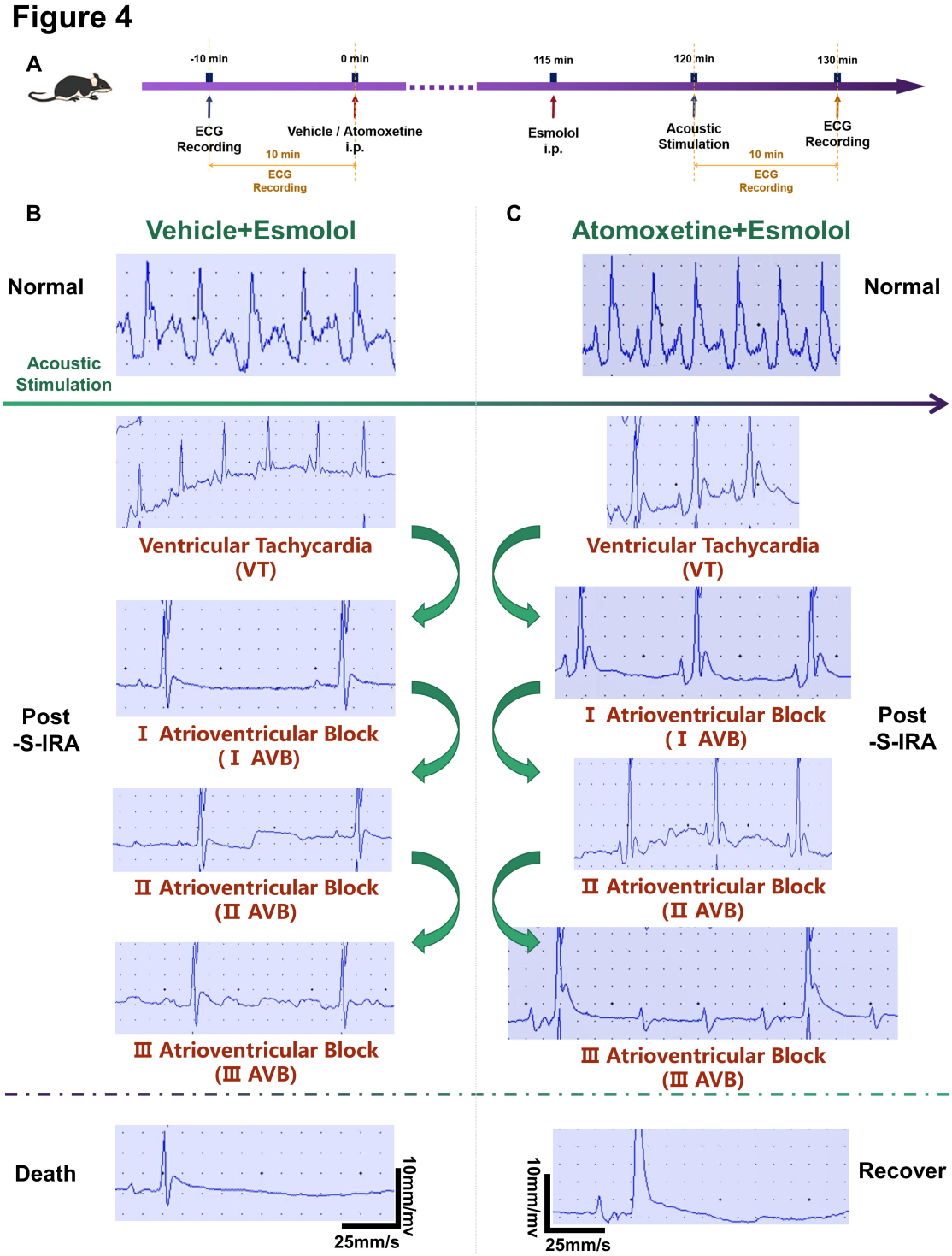

Figure 4 The effects imposed by tonic seizures on the changing of ECG in both groups treated with atomoxetine or esmolol

A. The protocol to explore the changes of electrocardiogram on the administration of esmolol reversed the atomoxetine-mediated reduction of S-IRA evoked by acoustic stimulation. **B.** The ventricular tachycardia and atrioventricular block occurred

immediately following tonic seizures and S-IRA evoked by acoustic stimulation in DBA/1 mice in the group treated with vehicle and esmolol (50mg/Kg). **C.** The group pre-treated with the atomoxetine(15mg/Kg) and treated with esmolol (50mg/Kg) was observed the ventricular tachycardia and atrioventricular block after acoustic stimulation.

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