Long Term Perinatal Deltamethrin Exposure Alters Electrophysiological Properties of Embryonic Ventricular Cardiomyocyte*

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Summary: Increased use of pyrethroids and the exposure to pyrethroids for pregnant women and children have raised the concerns over the potential effect of pyrethroids on developmental cardiotoxicity and other abnormalities. The purpose of this study was to investigate whether long term perinatal deltamethrin exposure altered embryonic cardiac electrophysiology in mice. Pregnant mice were administered with 0 or 3 mg/kg of deltamethrin by gavage daily from gestational day (gd) 10.5 to gd 17. 5. Whole cell patch-clamp technique was used in electrophysiological study, and real time RT-PCR was applied to analyze the molecular changes for the electrophysiological properties. Deltamethrin exposure resulted in increased mortality of pregnant mice and decreased viability of embryos. Moreover, deltamethrin slowed the maximum depolarization velocity (Vmax), prolonged the action potential duration (APD) and depolarized the maximum diastolic potential (MDP) of embryonic cardiomyocytes. Additionally, perinatal deltamethrin exposure decreased the mRNA expression of Na^+ channel regulatory subunit Navβ1, inward rectifier K^+ channel subunit Kir2.1, and delayed rectifier K^+ channel subunit MERG while the L-type Ca^{2+} channel subunit, Cav1.2 expression was increased. On the contrary, deltamethrin administration did not significantly alter the regulation of β-adrenergic or muscarinic receptor on embryonic cardiomyocytes. In conclusion, deltamethrin exposure at perinatal stage significantly alters mRNA expression of embryonic cardiac ion channels and therefore influences embryonic cardiac electrophysiological properties. This highlights the need to understand the persistent effects of pyrethroid exposure on cardiac function during embryonic development due to potential for cardiac arrhythmogenicity.

Key words: pyrethroid; deltamethrin; embryonic cardiomyocytes; action potential; developmental cardiotoxicity

Pyrethroid insecticides have been used for more than 40 years and account for more than 30% of the worldwide insecticide sales[1]. Type Ⅰ and type Ⅱ pyrethroids cause paralysis and ultimately death of the organism by keeping sodium channels open in the neuronal membranes. Pyrethroids are generally considered to be a safer alternative to other insecticides because they exhibit low mammalian toxicity and low environmental persistence[2–4]. However, metabolic detoxification mechanisms are not fully elucidated in very young mammals, and susceptibility to pyrethroids might be potentially increased in this population[5]. Type Ⅱ pyrethroids, such as deltamethrin, is known to induce long lasting inhibition of voltage-activated sodium channels[4, 6]. Significant levels of pyrethroid metabolites, including those of deltamethrin, have been

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found in the urine of pregnant women and children[7, 8], in umbilical cord blood and sera[9], in meconium[10] and in the breast milk of lactating women[11]. This poses questions regarding the safety of deltamethrin, so developmental cardiotoxicity, neurotoxicity and other adverse developmental effects are currently being studied in these susceptible populations[6].

Indeed accumulating evidence has shown that early-life environmental exposure is probably the most important component in the etiology of some diseases including cancer, metabolic and cardiovascular diseases[12–16]. Pyrethroid compounds can cross the placental barrier and are known to interfere with hormonal and neurological development, the immune system and other physiological functions[17–20]. Pyrethroids have also shown greater toxicity in neonatal than in adult rats, possibly due to incomplete development of detoxifying enzymes[21].

To date, limited studies have examined the effects of synthetic pyrethroids on developmental outcomes. A study by Bell *et al* reported an increased risk of fetal death due to congenital anomalies when synthetic pyrethroids were used in the same township, range, or section during the 3rd to 8th week of pregnancy[22]. Similarly, Hanke *et al* found a significant reduction in birth weight among the offspring of mothers potentially exposed to synthetic pyrethroids during the three months prior to conception and the first trimester of pregnancy[23]. In laboratory animals, one study showed that cypermethrin was able to induce oxidative stress and apoptosis through the involvement of caspases in zebrafish embryos[24]. Another study demonstrated that deltamethrin increased early embryonic deaths and caused growth retardation in rats[25]. Since global use of pyrethroids has increased, it demands a more complete understanding of their mode of action and effect on development. In this study, we aimed to investigate the impact of type Ⅱ pyrethroid deltamethrin on electrophysiological properties of embryonic hearts.

1 MATERIALS AND METHODS

1.1 Chemicals

Deltamethrin was purchased from Sigma Chemical Co. (USA). All other reagents were from Sigma or as indicated in the specified methods.

1.2 Animals and Deltamethrin Administration

Eight- to ten-week-old female KUNMING mice were mated with male mice. On the next day morning, if a vaginal plug appeared, the day was designated as gestational day (gd) 0.5. Pregnant female mice were orally administered either 0 or 3 mg/kg bodyweight deltamethrin, dissolved in corn oil daily from gd 10.5 to gd 17.5 by gavage[26, 27]. All procedures were conducted in accordance with the U.S. National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Tongji Medical College of Huazhong University of Science and Technology.

1.3 Single Cell Preparation

The pregnant mice were sacrificed on gd 18.5 by cervical dislocation and the embryonic hearts were harvested. Ventricles were enzymatically dissociated into single cells as we previously described[28]. The single cells were plated onto sterile gelatin-coated glass coverslips, cultured in Dulbecco'S Modified Eagle's Medium (DMEM, Gibco, USA) containing 20% fetal bovine serum (FBS, Gibco, USA) and kept in the incubator for 24–36 h for later use. Spontaneously beating cardiomyocytes were used for functional studies.

1.4 Electrophysiological Recordings

Action potentials (APs) were recorded using Axopatch 200A amplifier in whole-cell patch-clamp technique and a Digital 1200 interface controlled by pCLAMP 9.0 software. Data were analyzed using Clampfit software (Axon Instruments, USA). Patch pipettes (2–3 MΩ tip resistance) were fabricated from borosilicate capillaries using an electrode puller (700C, Japan). All patch-clamp experiments were performed at 37°C. Cardiomyocytes were superfused with the normal Tyrode's solution containing (mmol/L): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, D-glucose 10 (pH 7.4, adjusted with NaOH). Patch pipettes were filled with the internal solution (mmol/L): KCl 50, K-asparate 80, EGTA 10, HEPES 10, Na_2ATP 3.0, $MgCl_2$ 1.0 (pH 7.4, adjusted with KOH).

1.5 Quantitative RT-PCR

Total RNA was extracted from embryonic ventricles using TRIzol (Invitrogen, USA). Revere transcription was performed on 2 ng of total RNA. Briefly, 2 ng of total RNA was reversely transcribed to cDNA. First strand cDNA was synthesized by using M-MLV reverse transcriptase (Invitrogen, USA) with dT (18) oligo (Ribo, China).

For real-time quantitative RT-PCR, the SYBR-Green PCR MasterMix (TOYOBO CO., LTD, Japan) was used with gene-specific primers listed in table 1. Real-time quantitative PCR was performed using the first strand cDNA (1.6 μL), 0.4 μL 10 mmol/L forward and reverse primers, 10 μL SYBR-Green PCR MasterMix to total volume of 20 μL. The PCR process consisted of 40 cycles of 10 s denaturation at 95°C, 20 s annealing at 60°C, and 15 s extension at 72°C. All reactions were run in triplicate. Reaction and signal detection were performed by Mx3000P real-time PCR system (Agilent Stratagene, USA). The CT values of each gene were converted into absolute copy numbers using a standard curve for GAPDH.

1.6 Data and Statistical Analysis

For the analysis of individual electrophysiological experiments, APs were analyzed by averaging using

Table 1 Primers used for real-time PCR

Gene		Primer sequence (5′-3′)
Nav1.5	Forward	ATGGTCATTGGCAACCTTGTGGT
	Reverse	CTGATCTCTTCATCCTCTTC
Navβ1	Forward	CGCTATGAGAATGAGGTGCTG
	Reverse	CGTAGTCGCCAGAGTGGTTG
Cav1.2	Forward	CCTGGCCATGCAGCACTAT
	Reverse	GCTCCCAATGACGATGAGGA
Kir2.1	Forward	TCTCACTTGCTTCGGCTCAT
	Reverse	ACTTGTCCTGTTGCTGGTACA
MERG	Forward	GCCTTAGGCCAATCAGGCT
	Reverse	ACAGGACCTCCTGGGC
GAPDH	Forward	AACTTTGGCATTGTGGAAGG
	Reverse	GGATGCAGGGATGATGTTCT

AP analysis software programmed by Dr. Philipp Sasse (University of Bonn, Germany). Data are presented as mean±SEM, with *n* representing the number of experiments or cells for analysis. Student's paired or unpaired *t* test was used where applicable. A value of *P* of less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Deltamethrin Increased the Mortality Rate of Pregnant Mice and Decreased Embryo Viability

Intragastric administration of deltamethrin decreased both the livability of pregnant mice and the number of fetuses. In the control group, all the 20 pregnant mice (100%) survived. Five days of deltamethrin administration resulted in the death of 42.8% pregnant mice [6 out of the total of 14 (fig. 1A)]. As shown in fig. 1B, the control group pregnant mice had a significantly higher average number of embryos (12±0.6/mice) than the deltamethrin group (5±0.8/mice) (P<0.01).

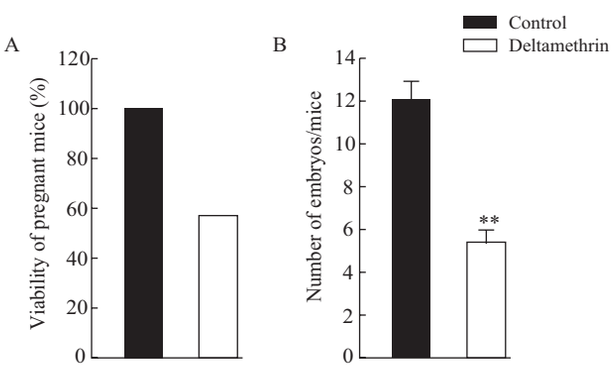

Fig. 1 Deltamethrin increased the mortality rate of pregnant mice and decreased embryo viability
A: All the 20 control pregnant mice (100%) survived, while 5 days of deltamethrin administration resulted in the death of 42.8% pregnant mice (6 out of the total of 14). B: The control group pregnant mice had a significantly higher average number of embryos (12±0.6/mice) than the deltamethrin group (5±0.8/mice). $^{**}P$<0.01 *vs.* control

2.2 Effects of Deltamethrin on APs and Expression of Ion Channels in Embryonic Ventricular Myocytes

By recording the APs, deltamethrin was found to significantly alter the AP parameters: the maximum diastolic potential (MDP) depolarized from –58.5±1.2 mV to –53.9±0.6 mV, the maximum depolarization velocity (Vmax) decreased from 53.5±6.3 V/s to 34.9±5.9 V/s, and the action potential duration at 90% of repolarization (APD90) was significantly prolonged from 105.3±9.0 ms to 148.7±14.7 ms (P<0.05, n=25) (fig. 2A, table 2). These changes suggested alteration of ion channels such as Na^+ ion channel, L type Ca^{2+} channel and K^+ channels. Thus, we investigated the expression pattern of Nav1.5, Navβ1, Cav1.2, Kir2.1 and MERG by real-time RT-PCR. The results demonstrated that there was no change in Nav1.5, however, Cav1.2 was upregulated while Navβ1, Kir2.1 and MERG were downregulated (fig. 2B and 2C). We postulate that these findings in ion channels may be responsible for the effect of deltamethrin on APs.

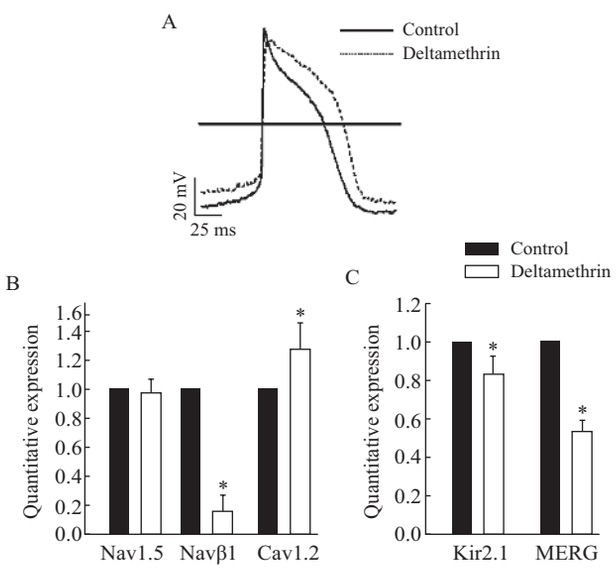

Fig. 2 Effect of deltamethrin on APs and expression of ion channels in embryonic ventricular myocytes
A: Deltamethrin significantly altered the AP of fetal ventricular myocytes by depolarizing the maximum diastolic potential (MDP), slowing the maximum depolarization velocity (Vmax) while prolonging the AP duration at 90% of repolarization (APD90). B, C: Administration of deltamethrin significantly upregulated ion channel subunit of Cav1.2, and downregulated Navβ1, Kir2.1 and MERG transcripts. $^*P$<0.05 *vs.* control

Table 2 Effects of deltamethrin on AP parameters

Groups	Amplitude (mV)	MDP (mV)	Vmax (V/s)	APD90 (ms)
C	106.8±4.1	–58.5±1.2	53.5±6.3	105.3±9.0
D	97.5±2.9*	–53.9±0.6*	34.9±5.9*	148.7±14.7*

APD90, action potential duration at 90% of repolarization; MDP, maximum diastolic potential; Vmax, maximum depolarization velocity of phase 0. *P<0.05 *vs.* control

2.3 Deltamethrin Did Not Alter the β-adrenergic or Muscarinic-regulation on AP Frequency of Embryonic Ventricular Myocytes

Pyrethroid compounds can cross the placental barrier and are known to interfere with hormonal and neurological development[17–20]. Accordingly, we evaluated the impact of deltamethrin on hormonal regulation in mouse embryonic ventricular cardiomyocytes. Application of isoproterenol (ISO) led to a significantly typical and comparable increase of the AP frequency (n=14, P<0.05) (fig. 3A). On the other hand, application of carbachol (CCh, 1 μmol/L) led to a significant negative regulation on AP frequency of the mouse embryonic cardiomyocytes (n=16, P<0.05) (fig. 3B). Similar responses to both ISO and CCh were found in the control group as well as the deltamethrin groups (P>0.05, fig. 3C and fig. 3D), indicating that deltamethrin had no effect on either β-adrenergic or muscarinic receptors signaling in embryonic cardiomyocytes.

3 DISCUSSION

Recently, attention has focused on the potential for developmental pyrethroid exposure to contribute to cardiovascular neurobehavioral dysfunction[29]. Although there have been several reports on the potential developmental neurotoxicity of pyrethroids in animals, the data on cardiotoxic effects are limited. The present study investigated the electrophysiological effects of the type Ⅱ pyrethroid, deltamethrin, on murine embryonic ventricular myocytes. The novel findings were that deltamethrin significantly changed the electrophysiological properties of ventricular cardiomyocytes by altering the ion channel expression pattern. Meanwhile deltamethrin did not change the β adrenergic or muscarinic regulation on APs recorded in embryonic ventricular cardiomyocytes. To the best of our knowledge, this is the first study to report deltamethrin-induced changes in cardiac ionic channel expression especially the cardiac ionic channel expression changes during embryonic development.

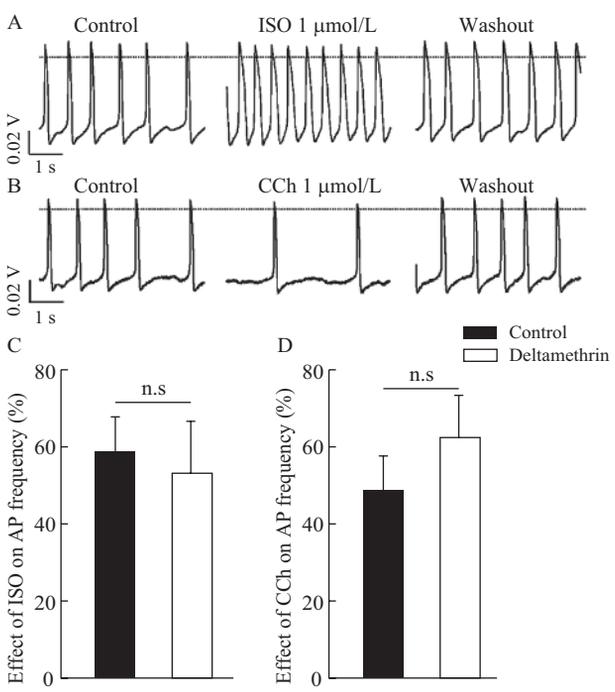

Fig. 3 Deltamethrin did not alter the β-adrenergic or Muscarinic regulation of embryonic ventricular myocytes
A: original recordings of the effect of isoproterenol (ISO) on embryonic ventricular AP in the deltamethrin-treated groups; B: original recordings of the effect of carbachol (CCh) on embryonic ventricular AP in the deltamethrin-treated groups. C, D: Statistical analysis showed that deltamethrin did not significantly change the β-adrenergic (C) or muscarinic (D) receptor signaling activities in the embryonic hearts. n.s, not significant

The classical well-known mode of action of deltamethrin is related to its preferential binding to sodium channels[30, 31], and more precisely associated with a particular amino acid sequence in the intracellular linker connecting domains Ⅱ and Ⅲ of cockroach sodium channel[32]. Our data are an addition to previous research findings which have reported that pyrethroids modify gating characteristics of voltage sensitive sodium channels by downregulating the regulatory subunit Navβ1 in embryonic myocytes. A number of studies[33–36] found that co-expression of Navβ1 subunit with the Nav1.5 subunit increased the Na^+ current density, accelerated the rate of recovery from inactivation, and caused a negative shift in steady-state inactivation with no effect on voltage-dependent activation, as well as hastening the rate of inactivation. Therefore, the decrease in Navβ1 by deltamethrin would theoretically decrease the Na^+ current, which in turn would decrease the maximum depolarization velocity of phase 0 in embryonic ventricular myocytes.

Previous reports have demonstrated that pyrethroids can prolong the APD and provoke sporadic early after-depolarizations (EADs) in cardiac myocytes[37]. EADs are significant for exacerbating the dispersion of myocardial action potential duration, with a possible direct link to arrhythmias[38, 39]. This arrhythmogenic effect has been explained by increased INa persistence because these agents shift Na^+ channel activation to more negative potentials and can slow the rate of voltage-dependent inactivation and/or deactivation[3, 40, 41]. The decrease in Navβ1 in our study could also explain the persistent INa increase and the prolonged APD. Furthermore, we observed increase in Cav1.2 ionic channel transcript and a decrease in Kir2.1 and MERG ionic channel transcripts. Therefore, it is plausible that a sustained increase in inward Ca^{2+}current and a decrease in K^+ current during repolarization may be responsible for not only the prolongation of the

ADP[42, 43], but may also explain the arrhythmogenic effect of deltamethrin on the heart[44, 45], because it can induce Q-T prolongation of ECG and ventricular arrhythmias of the torsade-de-pointes type.

The decrease in Kir2.1 ionic channel transcript may explain the mechanism behind the decrease of MDP in our study. Our data also showed the lack of significant differences in the response of the cells to isoproterenol (a beta adrenergic agonist) or carbachol (a muscarinic agonist) in both control and deltamethrin groups. This may suggest that deltamethrin acts directly on cardiac ionic channels and probably at the molecular level to alter cardiac activity rather than alter hormonal or autonomic nervous activity of the heart.

It has been reported that children, particularly neonates can be biologically more sensitive to the same toxicant exposure on a body weight basis than adults[21, 46]. Although pyrethroids are considered to be safer than other insecticides, rodent studies suggest that early-life and pubertal pyrethroid exposures alter neurobehavioral functioning[6, 47, 48]. Therefore care should be taken to minimize exposure to pyrethroids for children, pregnant women and animals owing to their popularity and increased usage in pest control. In our study, there was increased mortality of pregnant mice and decreased viability of embryos at 3 mg/kg deltamethrin dosage compared to some previous studies[49–51]. This could be due to the fact that our study administered deltamethrin daily to the pregnant mice compared to other studies that administered every 3 days during gestation and lactation. This also suggested less dose of deltamethrin should be adopted in our future work. However, our results are in agreement with some previous studies which reported that deltamethrin caused various adverse effects in epidemiological and experimental studies including retardation of growth, hypoplasia of the lungs, dilation of the renal pelvis and increase in placental weight[25].

The main limitation of this study is that we did not draw dose-response curve of deltamethrin on the pregnant mice and therefore we could not conclusively state the dose at which lethality occurred. So far the direct correlation of blood concentration with the gavage dosage is unknown; it is difficult to declare that our observation could be evidenced by the accumulation for food or environmental contamination. Nevertheless, our study demonstrated novel findings regarding the effect of deltamethrin on embryonic cardiac electrophysiology that add knowledge to the toxic effects of pyrethroids on animals and humans.

Pyrethroid type Ⅱ, deltamethrin, affected the basic electrophysiological property of embryonic cardiomyocytes by changing the expression pattern of ion channels. This implies the need to avoid the persistent exposure to pyrethroid during development.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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