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Excess Imidacloprid Exposure Causes the Heart Tube Malformation of Chick Embryos

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23 **Abstract**

24 As a neonicotinoid pesticide, imidacloprid is widely used to control sucking
25 insects on agricultural planting and fleas on domestic animals. However, the extent to
26 which imidacloprid exposure has an influence on cardiogenesis in early embryogenesis
27 is still poorly understood. In vertebrates, the heart is the first organ to be formed. In
28 this study to address whether or not imidacloprid exposure affects early heart
29 development, the early chick embryo has been used as an experimental model because
30 of the accessibility of chick embryo at its early developmental stage. The results
31 demonstrate that exposure of the early chick embryo to imidacloprid caused
32 malformation of heart tube. Furthermore, the data reveal that down-regulation of
33 GATA4, Nkx2.5 and BMP4 and up-regulation of Wnt3a led to aberrant
34 cardiomyocyte differentiation. In addition, imidacloprid exposure interfered with
35 basement membrane (BM) breakdown, E-cadherin/Laminin expression and mesoderm
36 formation during the epithelial-mesenchymal transition (EMT) in gastrula chick
37 embryos. Finally, the DiI-labeled cell migration trajectory indicated that imidacloprid
38 restricted the cell migration of cardiac progenitors to primary heart field in gastrula
39 chick embryos. A similar observation was also obtained from the cell migration assay
40 of scratch wounds *in vitro*. Additionally, imidacloprid exposure negatively affected
41 the cytoskeleton structure and expression of corresponding adhesion molecules. Taken
42 together, these results reveal that the improper EMT, cardiac progenitor migration and
43 differentiation are responsible for imidacloprid exposure-induced malformation of
44 heart tube during chick embryo development.

45

46 **Keywords:** Imidacloprid; chick embryo; heart tube; EMT; cardiac progenitor

47 migration; differentiation.

48

49 **Introduction**

50 Organogenesis requires the precise layout of multiple cell types into a specific
51 three-dimensional architecture that is essential for normal organ formation. During
52 embryonic organ development, an obligatory process is tissue fusion, such as that of
53 the optic cup, palate, heart, neural tube, eyelids and body wall ^{1, 2}. Tissue fusion
54 appears to occur in numerous organs. Our previous study demonstrated that the
55 deficiency of specific transcription factors and signaling molecules could exhibit the
56 fusion defects in many organs, for instance, in neural tube defects ³ and cardiac bifida
57 ⁴. As a model of organogenesis, cardiogenesis involves a series of morphogenetic
58 steps. In vertebrates, the heart develops from three distinct pools of cardiac
59 progenitors: the cardiac precursor in splanchnic mesoderm (primary and secondary
60 heart field), cardiac neural crest and the pro-epicardium. From the perspective of
61 morphological alteration, it is chronologically composed of primary heart tube fusion,
62 cardiac looping and accretion, cardiac septation and coronary vasculogenesis ⁵. The
63 primary heart field gives rise to the major structures of the heart, including the atrias
64 and ventricles, while the secondary heart field contributes to the cardiac outflow tracts
65 ⁶. Myocardial progenitors undergo Epithelial-Mesenchymal Transition (EMT),
66 proliferate, differentiation and migration into the primary heart field in the process of
67 heart tube formation. EMT is a morphogenetic transition process in which cells lose
68 their epithelial characteristics and gain mesenchymal properties underlying the
69 alterations of adheren junction (AJs), tight junction (TJs) and gap junction (GJs) ^{7, 8}.
70 In the formation of primary heart fields, the precardiac cells initially migrate out of

71 the anterior primitive streak at the gastrula stage and then move symmetrically into
72 crescent location ⁹⁻¹¹. Cell migration, proliferation and differentiation are guided by its
73 micro-environment ¹².

74 The morphogenesis of chick cardiac looping involves four phases: pre-looping
75 phase (HH8-9); C-shaped bend (HH9⁺-13); S-shaped heart loop (HH14-16) and
76 primitive outflow tract formation (about 4.5 days). Within days 6-14, expansion and
77 growth of the ventricular wall benefit principally from cardiomyocyte proliferation in
78 the compact myocardium. At day 14.0, cardiac neural crest cells (CNCs) give rise to
79 the adventitia of the large veins and the coronary arteries. In this context, any
80 disruption to cardiac precursor cell migration and differentiation during cardiogenesis
81 may result in congenital heart malformations.

82 Heart development is a complex process that is tightly regulated through
83 spatio-temporal gene expression and cell-cell interaction. In previous studies of heart
84 tube assembly in the chick embryo, we have reported that fibroblast growth factor
85 (FGF) signaling, through an endoderm-derived signal, is required for regulating
86 pro-cardiac mesoderm cell migration ^{10, 13}. Additionally, bone morphogenetic protein
87 2 (BMP2) is released from the anterior endoderm and Wnt antagonists are essential
88 for precardiac mesoderm cells to differentiate into mature cardiomyocytes during
89 cardiomyogenesis ¹⁴⁻¹⁶. Furthermore, transcription factors Nkx-2.5, GATA4,
90 myocardin and TBX5 have crucial roles in dictating morphogenesis and
91 differentiation of the heart ^{16, 17}. Vascular endothelial growth factor (VEGF) also plays
92 a vital role in the angiogenic expansion of the early network ¹⁸.

93 The neonicotinoid pesticide, imidacloprid,
94 1-((6-Chloro-3-pyridinyl)methyl)-N-nitroimidazolidinimine, has been extensively
95 used to control sucking insects, termites, soil insects on crops ¹⁹ and fleas on domestic
96 animals ^{20, 21}. Various products containing this chemical, including liquids, granules,
97 dusts and packages, have been sold in the US since 1994. In the EU, use of
98 imidacloprid was restricted for 2 years in 2013 because research showed a link
99 between imidacloprid and bee death (EASAC 2015, Ecosystem services, agriculture
100 and neonicotinoids). As a systemic insecticide, imidacloprid products are usually
101 sprayed on soil and leaves, and then spread to the plant's stems, leaves, fruit and
102 flowers ^{22, 23}. Imidacloprid can then penetrate into the nervous system of sucking
103 insects and combine selectively with nicotinic acetylcholine receptors (nAChR),
104 producing toxic effects ²⁴. When insects consume plants treated with imidacloprid
105 products, their nervous systems are damaged leading to death. Due to steric conditions
106 at the nAChR, imidacloprid has much lower toxicity to mammals. However, humans
107 can be exposed to imidacloprid products *via* skin/eye contact or through consumption
108 or inhalation when handling the pesticide or an animal recently exposed to
109 imidacloprid. The toxicity of imidacloprid in human adults is due to disruption of
110 nervous system signal transduction ²⁵. Once humans are exposed, imidacloprid
111 products can cross the lining of the intestine and be transported to the whole body
112 through circulation of the blood. However, little is known about its potential toxic
113 effects on early embryo development apart from a few reports on human health such
114 as reproductive ability. Currently, increasing attention is being paid to the toxic effects

115 of pesticides on embryo development, including cardiovascular system. Unfortunately,
116 as yet there is no direct evidence of toxicological effects on cardiogenesis or
117 corresponding mechanisms. In this study, a chick embryo model ²⁶ has been used to
118 investigate whether or not imidacloprid could affect cardiogenesis and, if so, to
119 elucidate the underlying cellular and molecular mechanism.

120

121 **Materials and methods**

122 *Chick manipulations*

123 Fertilized leghorn eggs were acquired from the Avian Farm of South China
124 Agriculture University (Guangzhou, China). Two approaches were employed to carry
125 out the imidacloprid exposure in this study. The imidacloprid powder was dissolved in
126 dimethyl sulfoxide (DMSO), 0.1% DMSO was used as control to observe the
127 potential effect of the solvent.

128 For imidacloprid exposure at the early embryonic stage, Hamburger-Hamilton
129 (HH) stage 0 chick embryos from fertilized eggs were incubated with either 0.1%
130 DMSO (control) or 500 μ M imidacloprid ²⁷ in early chick (EC) culture medium in a
131 humidified incubator (Yiheng Instruments, Shanghai, China) at 38°C and 70%
132 humidity until the chick embryos developed to the HH10 stage. Alternatively, 500 μ M
133 imidacloprid was directly applied to one side of the gastrula-stage embryos, with the
134 other side being exposed to 0.1% DMSO as a control.

135 For imidacloprid exposure at a later embryonic stage, HH4 chick embryos were
136 exposed to either 0.1% DMSO (control) or 500 μ M imidacloprid through injection

137 into windowed eggs *in vivo* and then further incubated for 4.5 days and 14 days. The
138 experiments were performed in triplicate with 20 eggs assigned to each group, and
139 surviving embryos were harvested for further assessment.

140 ***In situ hybridization***

141 Whole-mount *in situ* hybridization of chick embryos was performed according to
142 a standard *in situ* hybridization protocol²⁸. Briefly, digoxigenin-labeled probes were
143 synthesized for VMHC²⁹, GATA5³⁰, BMP2 and NKX2.5 (supplied by Dr. Thomas M.
144 Schultheiss). The whole-mount stained embryos were photographed and then frozen
145 sections prepared on a cryostat microtome (LeicaCM1900) at a thickness of 15–20
146 mm.

147 ***Immunofluorescent staining***

148 Chick embryos were harvested at the end of the experiment and fixed overnight
149 in 4% paraformaldehyde at 4°C. Whole-mount embryos were immunofluorescently
150 stained using MF20 (1:500, DSHB, USA), E-cadherin (1:50, BD Transduction
151 Laboratories, USA), Laminin (1:100, DSHB, USA) antibodies. Briefly, the fixed
152 embryos were incubated with these primary antibodies at 4°C overnight on a rocker.
153 Following extensive washing, the embryos were incubated with the appropriate
154 anti-mouse IgG conjugated to Alexa Fluor 488 or anti-rabbit IgG conjugated to Alexa
155 Fluor 555 (1:1000, Invitrogen, USA), overnight at 4°C on a rocker. All embryos were
156 finally counterstained with DAPI (1:1000, Invitrogen, USA) at room temperature for
157 1 hour.

158 ***RNA isolation and semiquantitative RT-PCR***

159 Total RNA was isolated from HH4, HH8 chick embryos using a Trizol kit
160 (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA
161 was synthesized to a final volume of 25µl using SuperScript RIII first-strand
162 (Invitrogen, USA). Following reverse transcription, PCR amplification of the cDNA
163 was performed as described previously. The primers used for RT-PCR are provided in
164 the Figure S3. The PCR reactions were performed on a Bio-Rad S1000TM Thermal
165 cycler (Bio-Rad, USA). The final reaction volume was 50 µl composed of 1 µl of
166 first-strand cDNA, 25 µM forward primer, 25 µM reverse primer, 10 µl
167 PrimeSTARTM Buffer (Mg²⁺ plus), 4µl dNTPs Mixture (TaKaRa, Japan), 0.5 µl
168 PrimeSTARTM HS DNA Polymerase (2.5U/µl TaKaRa, Japan) and RNase-free water.
169 The cDNA was amplified for 30 cycles. One round of amplification was performed at
170 94°C for 30 s, 30 s at 58°C, and 30 s at 72°C. The PCR products (20 µl) were resolved
171 using 1% agarose gels (Biowest, Spain) in 1× TAE buffer (0.04 M Trisacetate and
172 0.001 MEDTA) and 10,000x GeneGreen Nucleic Acid Dye (Tiangen, China) solution.
173 The resolved products were visualized using a transilluminator (Syngene, UK) and
174 photographs captured using a computer-assisted gel documentation system (Syngene).
175 The housekeeping gene GAPDH was run in parallel to confirm that equal amounts of
176 RNA were used in each reaction. The ratio between intensity of the fluorescently
177 stained bands corresponding to genes and GAPDH was calculated to quantify the
178 level of the transcripts for those genes mRNAs. The RT-PCR result was representative
179 of three independent experiments.

180 *Cell trace with DiI*

181 Carbocyanine dye 1, 1V-dioctadecyl-3, 3, 3V, 3V-tetramethylindocarbocyanine
182 perchlorate (DiI, Molecular Probes, Inc.) was used to label small groups of primitive
183 streak cells. A 2.5% stock solution of DiI was diluted in ethanol, 1:10 in 0.3 M
184 sucrose, and injected into the anterior primitive streak of HH3 chick embryo by air
185 pressure through a micropipette, which was pulled from a 1 mm glass capillary in a
186 vertical micropipette puller (WD-2, Chengdu Instrument Company). In general, each
187 labeled tissue in the anterior primitive streak contained approximately 10–30 cells.

188 *Cell lines and culture*

189 The H9c2 rat cardiac myoblast cell line was obtained from ATCC (American
190 Type Culture Collection, CLR-1446, USA). The cells were cultured in a humidified
191 incubator with 5% CO₂ at 37°C in six-well plates (1×10⁶ cells/ml) containing DMEM
192 (Gibco, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Gibco,
193 Gaithersburg, MD, USA), and exposed to imidacloprid (500µM); 0.1% DMSO was
194 used as a control. The cells were photographed using an inverted fluorescence
195 microscope (Nikon, Tokyo, Japan) with NIS-Elements F3.2 software. After 12 hours
196 incubation, immunofluorescent staining against phalloidin (F-actin, 1:1000,
197 Invitrogen, Waltham, MA, USA) and anti-Myh7 (1:100, Proteintech, USA) was
198 performed on the incubated H9c2 cells. A minimum of 5 images were assayed *per*
199 treatment group. DAPI (1:1000, Invitrogen, USA) was used as counterstain.

200 *Migration assay*

201 H9c2 cells were seeded in 6-well plates with DMEM (10% FBS) medium. At
202 confluency, a wound was induced by scratching the monolayer with a 10-µl pipette tip.

203 The cells were then washed 3 times with sterile PBS. H9c2 cells were incubated in
204 serum-free DMEM medium with 500uM or 0.1% DMSO under 5% CO₂ conditions.
205 Images were acquired at 12h and 24h post-scratching. At least 3 wells were analyzed
206 in each treatment group and the images were taken using an inverted microscope
207 (Nikon Eclipse Ti-U, Japan).

208 ***Western blot***

209 Chick embryos (HH4 and HH7) were collected and lysed with CytoBuster™
210 Protein Extraction Reagent (#71009, Novagen). The total protein concentration was
211 established using a BCA quantification kit (BCA01, DingGuo BioTECH, CHN).
212 Samples containing equal amounts of protein were resolved by SDS-PAGE and then
213 transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5%
214 Difco™ skim milk (BD) and then incubated with primary and secondary antibodies.
215 The antibodies used were TBX5 , GATA4 and GATA6 (Abcam USA),
216 HRP-conjugated anti-mouse IgG and anti-rabbit IgG (Cell Signaling Technology,
217 USA). All primary and secondary antibodies used were diluted to 1:1000 and 1:2000
218 in 5% skim milk, respectively. The protein bands of interest were visualized using an
219 ECL kit (#34079, Thermo Fischer Scientific Inc.) and GeneGnome5 (Syngene). The
220 staining intensity of the bands was determined and analyzed using Quantity One
221 software (Bio-Rad).

222 ***Photography***

223 Following immunofluorescent staining or *in situ* hybridization, the whole-mount
224 embryos were photographed using a stereo-fluorescent microscope (Olympus MVX10)

225 and associated Olympus software package Image-Pro Plus 7.0. The embryos were
226 sectioned into 14 μm -thick slices using a cryostat microtome (Leica CM1900) and the
227 sections were then photographed with an epi-fluorescent microscope (Olympus LX51,
228 Leica DM 4000B) and CN4000 FISH Olympus software package.

229 ***Data analysis***

230 The thickness of ventricular wall and trabecular muscle and the distance of
231 wound closure in wound healing experiments as well as the lengths of the long and
232 short axes were all quantified with Image-Pro Plus 6.0. The cell trace with DiI
233 experiments, Dil⁺ cells were manually counted with Image-Pro Plus 6.0. Statistical
234 analyses for all the experimental data was performed using a SPSS 13.0 statistical
235 package program for Window. The data were presented as mean \pm SD. Statistical
236 significance were determined using paired T-test, independent samples T-test or
237 one-way analysis of variance (ANOVA). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate
238 statistically significance between control and drug-treated groups. $P < 0.05$ was
239 considered to be significant.

240

241 **Results**

242 ***Imidacloprid exposure increases cardiac malformation during chick cardiogenesis***

243 The heart is the first functional organ in the developing embryo. There are three
244 crucial phases in the development of heart formation: 2-, 4.5- and 14-day (Fig. 1A).
245 To investigate the effects of excess imidacloprid exposure on heart tube formation in
246 chick embryos, we cultured the embryos as shown in Figure S1. In the first place, we

247 found that 35% (n = 28/80), 42.5% (n = 34/80) and 50% (n = 40/80) of 500 μ M
248 imidacloprid-treated chick embryos had died after 2, 4.5 and 14 days incubation,
249 respectively. Corresponding mortalities were only 5% (n = 3/60), 6.67% (n = 4/60)
250 and 8.33% (n = 5/60) in the 0.1% DMSO-treated chick embryos (Fig. 1B). Our results
251 showed that the growth of imidacloprid-treated embryos is slightly faster than 0.1%
252 DMSO-treated ones at 21h and, conversely, slightly delayed at 48h. (21h: DMSO =
253 1345 \pm 74.43 μ m, imidacloprid = 1629 \pm 82.45 μ m, P < 0.05; 48h: DMSO = 4183 \pm
254 45.57 μ m, imidacloprid = 3866 \pm 56.58 μ m, P < 0.001; n = 40 for each groups; Figs.
255 1C-C5, D).

256 The average number of somites in imidacloprid-treated group at 48h was about
257 10 pairs compared to 12 pairs in 0.1% DMSO-treated group (DMSO = 12.43 \pm 0.17,
258 imidacloprid = 10.03 \pm 0.15, n = 40 for each groups, P < 0.05; Fig. 1E). Next, E4.5
259 imidacloprid-treated whole embryos weights were obviously lower than 0.1%
260 DMSO-treated ones (DMSO = 0.26 \pm 0.03g, n = 10, imidacloprid = 0.19 \pm 0.01g, n =
261 34, P < 0.01; Fig. 1F). H&E staining revealed that the thicknesses of the ventricular
262 walls (DMSO = 47.52 \pm 0.95 μ m, n = 10, imidacloprid = 28.85 \pm 0.72 μ m, n = 14, P <
263 0.001) and the trabecular muscles were both reduced in imidacloprid-treated group
264 compared with 0.1% DMSO-treated controls (DMSO = 25.27 \pm 0.56 μ m, n = 10,
265 imidacloprid = 12.57 \pm 0.31 μ m, n = 14, P < 0.001; Figs. 1G, G1-G2, H, H1-H2; I, J).
266 Additionally, the size and weight of imidacloprid-treated hearts were smaller and
267 lighter than those of 14-day 0.1% DMSO-treated embryos (DMSO = 0.08 \pm 0.01g, n
268 = 10; imidacloprid = 0.07 \pm 0.01g, n = 16, P < 0.05; Figs. 1K, L, M). The weight of

269 whole embryo showed a similar tendency (DMSO = 7.60 ± 0.31 g, n = 10,
270 imidacloprid = 6.03 ± 0.29 g, n = 16, P < 0.01; Fig. 1N). Transverse sections (Figs.
271 1K1, L1) and histograms established that the right ventricular wall (RV) was
272 dramatically thicker (DMSO = 409.10 ± 24.73 μ m, n = 10, imidacloprid = $598.20 \pm$
273 36.10 μ m, n = 16, P < 0.001; Fig. 1O) whilst there was no significant difference in the
274 left ventricular wall (LV) and interventricular septum (ISV).

275 Some atypical C-looping heart tube was evident when imidacloprid-treated
276 embryos reached HH10. According to the phenotype features, we divided them into
277 four classifications: normal (Figs. 2A, B), mild (Figs. 2A1, B1), intermediate (Figs.
278 2A2, B2) and severe (Figs. 2A3, B3), and all were stained with MF20 antibody and
279 ventricular myosin heavy chain (VMHC) probe, respectively. In the 0.1%
280 DMSO-treated embryonic heart, the heart tubes are fully C-looped (normal = 100%, n
281 = 80/80), while abnormal morphological looping of heart tube occurred in the
282 imidacloprid-treated groups (normal = 13.6%, n = 8/59, mild = 39%, n = 23/59,
283 intermediate = 28.8%, n = 17/59, severe = 18.6%, n = 11/59; Fig. 2C). At stage HH10,
284 the C-shape loop of the heart tube has formed in control embryos (Fig. 2D) as
285 indicated by MF20 immunofluorescent-staining (Fig. 2E). The single cavity of the
286 heart tube was also evident in corresponding transverse sections for these 0.1%
287 DMSO-treated embryos (Figs. 2F, F1-F3). In contrast, some heart tubes of the HH10
288 imidacloprid-treated chick embryos presented in cardiac bifida (Fig. 2G), as shown in
289 the MF20 immunofluorescently-stained heart tubes (Fig. 2H) and corresponding
290 transverse section of the heart tubes. The two cavities were found in the transverse

291 sections of the heart tubes (Figs. 2I, I1-I3).

292 ***Imidacloprid treatment represses cardiomyocyte differentiation***

293 Figure 3A reveals the principal signaling pathways (Wnt, BMP&FGF and VEGF)
294 involved in the regulation of cardiomyocyte differentiation at cardiac crescent stages
295 (HH7-8). To explore whether imidacloprid exposure affects these crucial gene
296 expressions of cardiomyocyte formation, we firstly exposed imidacloprid to one side
297 of the embryos, using the other side as control. This approach has been previously
298 described in detail ³¹, and its advantage is in avoiding experimental artifacts due to the
299 different velocities of embryo development. *In situ* hybridization results (Fig. 3B)
300 showed that both GATA5 and Nkx2.5 expression were down-regulated on the
301 imidacloprid-treated side, while VMHC and BMP2 expression was maintained. The
302 results of RT-PCR showed that imidacloprid exposure increased Wnt3a expression;
303 dramatically inhibited GATA4, TBX5, VEGFR2 and BMP4 expression, but did not
304 affect BMP2, Fgf8 and VMHC expression (Fig. 3C). The comparisons of gene
305 expressions are shown in Figure S.2A. The results of westren-blot showed that the
306 imidacloprid exposure inhibited GATA4, GATA6 and TBX5 expression at protein
307 level (Fig. 3D-E).

308 ***Imidacloprid exposure interfered with EMT at gastrula chick embryos***

309 Cardiac progenitor cells derived from lateral plate mesoderm cells, which were
310 undergo EMT (Fig. 4A). During EMT, E-Cadherin down-regulation and N-cadherin
311 up-regulation are considered to be indispensable ³². Here, E-Cadherin in
312 DMSO-treated embryos was mainly expressed in the apical side of epiblast (Figs. 4B,

313 B1-B1'). In contrast, expression of E-Cadherin in imidacloprid-induced embryos
314 extended to epiblast, mesoderm and hypoblast (Figs. 4C, C1-C1'). RT-PCR showed
315 that imidacloprid treatment reduced expression of N-cadherin and increased that of
316 E-Cadherin.

317 During chick gastrulation, the earliest sign of EMT is the breakdown of BM at the
318 midline³³⁻³⁵. Compared to 0.1% DMSO-treated embryos (Figs. 4D, D1-D1'),
319 imidacloprid treatment shortened the midline distance (DMSO = $241.80 \pm 13.99\mu\text{m}$, n
320 = 10, imidacloprid = $170.50 \pm 7.60\mu\text{m}$, n = 10, P < 0.01; Figs. 4E, E1-E1', F),
321 implying that EMT was delayed. RT-PCR data (Fig. 4G) showed no significant
322 difference between the expression of RhoA between DMSO and imidacloprid groups.
323 Imidacloprid treatment reduced the expression of P120, β -catenin, CX43 and
324 claudin12; increased the expression of Vinculin, Par3 and occluding, but had no effect
325 on expression of AJs and TJs, including Wnt3a, Claudin-1, ZO-1 and α -actin. As a
326 result, it is concluded that imidacloprid treatment induced delayed EMT during
327 cardiogenesis in gastrula chick embryo. The comparisons of gene expressions are
328 shown in Figure S. 2B and C. The results of westren-blot showed that the expression
329 imidacloprid exposure down regulated N-cadherin, but up regulated E-cadherin at
330 protein level (Figs. 4H-I).

331 ***Imidacloprid inhibited the migration of cardiac progenitor cells***

332 Cardiac progenitor cells are the resources of the heart tube and migrate bilaterally
333 in the lateral plate mesoderm to eventually form the cardiac crescent^{9,10}. To follow the
334 migration trajectory of cardiac progenitor cells, DiI dye was injected into anterior

335 primitive streaks in HH3 chick embryos as shown in Figs. 5A, 5B. The embryos were
336 then exposed and cultured on either 0.1% DMSO (control) on both sides (Fig. 5A) or
337 with imidacloprid on one side (Fig. 5B). The photographs were taken after 9-hour and
338 20-hour incubations. The results showed that the Dil⁺ mesoderm cells in the control
339 group migrated symmetrically at bilateral sides of embryos (n = 18, P > 0.05; Figs.
340 5C-E, C1-E1, F), while many fewer Dil⁺ mesoderm cells were observed after 9- and
341 20-hour incubations at the side of imidacloprid-treatment compared to the control
342 (DMSO = 91.00 ± 1.38, imidacloprid = 43.38 ± 1.45, n = 18, P < 0.001; Figs. 5G-I,
343 G1-I1, J-K).. This difference in Dil⁺ cardiac progenitor cell migration clearly suggests
344 that imidacloprid exposure restrained the cell migration of cardiac precursors towards
345 the site of heart tube formation.

346 *Imidacloprid exposure suppressed the migration, polarization, and protrusion*
347 *formation of cardiac cells in vitro.*

348 To examine the behavior of treated cells, we used H9c2 cells cultured *in vitro* in
349 presence of imidacloprid. The scratch-wound assay showed that imidacloprid
350 exposure inhibited H9c2 cells migration, as reflected in the extent of “wound” closure
351 after 24h incubation from the 0.1% DMSO and imidacloprid-treated groups
352 respectively (12h: DMSO = 41.93 ± 1.06%, imidacloprid = 32.54 ± 2.66%, P < 0.05;
353 24h: DMSO = 61.47 ± 0.92%, imidacloprid = 46.81 ± 2.07%, P < 0.001, n = 8 for
354 each group; Figs. 6A, B, B1-B2, C, C1-C2, D). Actin and Myh7 are primary
355 cytoskeletal components and are involved in the formation of cell filopodia,
356 lamellipodia and protrusions during cell migration³⁶. F-actin and Myh7 fluorescent

357 microscopy demonstrated that compared to 0.1% DMSO exposure (Figs. 6E-F, I)
358 imidacloprid exposure (Figs. 6G-H, J) caused a loss of cell polarization. To quantify
359 this effect, the ratios of long to short axes of cells exposed to either DMSO or
360 imidacloprid were calculated.

361 Elongation of cells exposed to imidacloprid was significantly less than that of
362 0.1% DMSO-treated control cells (DMSO = $3.13 \pm 0.24\mu\text{m}$, $n = 25$, imidacloprid =
363 $2.31 \pm 0.11\mu\text{m}$, $n = 25$, $P < 0.01$; Fig. 6K). More cell protrusions occurred in the
364 majority of cells exposed to 0.1% DMSO compared to those treated with imidacloprid
365 (DMSO = 85.69 ± 3.19 , imidacloprid = 59.79 ± 2.89 , $n = 10$ for each group, $P < 0.01$;
366 Fig. 6L). In addition, the fluorescence intensities of Myh7 were determined (DMSO =
367 188.50 ± 0.94 , $n = 25$, imidacloprid = 136.60 ± 3.10 , $n = 32$, $P < 0.001$; Fig.6M).
368 RT-PCR data (Fig.6N) revealed that imidacloprid treatment reduced the expressions
369 of Vinculin, Par3, ZO-1, CX-43, Claudin-1 and α -actin, but increased the expression
370 of P120. The other tight junction gene (Claudin-12) was not affected. The
371 comparisons of gene expressions are shown in Figure S.2D. Furthermore, we also
372 detected the behavior of imidacloprid-treated chicken cardiac muscle cells³⁷. The
373 results confirmed that imidacloprid exposure could suppress the migration,
374 polarization, and protrusion formation of cardiac cells in vitro (Figure S.4).

375

376 **Discussion**

377 The toxicity of imidacloprid varies greatly across species. As a neurotoxic
378 insecticide, it has been used globally to control sucking insects in agriculture and

379 animal husbandry¹⁹. Similarly, monocrotophos, an organophosphate insecticide, also
380 has been found to greatly affect the development of zebrafish in a
381 concentration-dependent manner³⁸. It has been reported that concentrations of
382 imidacloprid in the environment was 320 µg/L near Noordwijkerhout, Netherlands,
383 exceeding European toxicity directives, while one fifth of water samples taken in
384 California were above the United States Environmental Protection Agency's level for
385 invertebrates (35 µg/L for acute toxicity and 1.05 µg/L for chronic toxicity)³⁹.
386 Accumulation of this pesticide on plants and animals will inevitably be transferred to
387 humans through close contacts and food contamination. A study on the biological
388 safety of imidacloprid products is therefore particularly important^{27,40}. In a previous
389 study, we conducted a concentration gradient to select the proper concentration. In our
390 previous study, we conducted a concentration gradient to select the proper
391 concentration. We found that mortality and ratio of malformations were both
392 increased with the increase of the concentration⁴¹. The concentration of imidacloprid
393 (500 µM) in this study was similar to that reported for earlier literature reports²⁷. We
394 considered that, for an acute toxicity experiment, the acceptable range should be less
395 than 1000 times the environmental concentrations, and the concentration we selected
396 here, 500 µM (127.8 mg/L), was within this range. $\alpha 7nAChR$ has been reported to be
397 increased during cardiac hypertrophy in the rat⁴². Our previous study also found that
398 AChR and AChE were presented in early chick embryos. We detected these
399 expressions with acetylcholinesterase and acetylcholine receptors by RT-PCR. This
400 work shows that expressions of both acetylcholinesterase and acetylcholine receptors

401 were inhibited by treatment with imidacloprid ⁴³. Pregnant women is a kind of
402 vulnerable groups, human embryonic development is likely to be affected by
403 cumulative toxic effects if pregnant women are exposed over the long-term to
404 imidacloprid. During embryogenesis, the heart is the first organ to be developed.
405 Severe developmental defects in the heart could cause embryonic death. Hence, it is
406 vital to determine whether or not exposure to this widely-used chemical could affect
407 development.

408 The chick embryo was selected to systemically investigate the potential toxic effect
409 of imidacloprid exposure on early heart tube formation in this study. Chick embryos
410 develop to HH10 for about 2days. Ventricular segment firstly bulge ventrally and then
411 flips to the right side. In this way, the heart fuses and a primitive C-shaped heart tube
412 is formed ¹⁶. At 4.5 days, the cardiac looping process is completed ⁴⁴. At 14 days, the
413 expansion and growth of the ventricular wall has ended and a mature heart is
414 produced (Fig. 1A). Our results show that imidacloprid exposure significantly
415 retarded the growth of chick embryos (Fig. 1) and increased the incidence of different
416 degrees of cardiac malformations (Fig. 2). MF20, the marker of myosin II heavy chain
417 in muscles, was exploited to outline the morphology of heart tubes, and is clearly
418 expressed in the myocardium of single and complete heart tubes in 0.1%
419 DMSO-treated control (Figs. 2F, F1-F3). In contrast, the unfused cavity marked by
420 MF20 is evident in the imidacloprid-treated group (Figs. 2I, I1-I3) implying that
421 imidacloprid exposure might result in cardia bifida. Furthermore, the development of
422 ventricular wall and trabecular muscle in 4.5 days was delayed by the imidacloprid

423 treatment. In comparison to the reduction of cardiac volume and weight in 14 days,
424 the thickness of right ventricular wall was significantly increased in compensation
425 following imidacloprid exposure (Fig. 1O). Imidacloprid exposure-induced embryonic
426 mortality in the first two days is much higher than in the other two phases (Fig. 1B).
427 This finding also further confirms that the first two days is the crucial period for heart
428 tube formation. It was this period that we addressed in this study.

429 Morphogenesis of the heart tube during embryo development relies on a precisely
430 coordinated expression of cardiac-associated genes. Crescent formation mainly
431 requires several signal factors, including Wnt, BMP and Fgf signaling, which
432 coordinately control cardiomyocyte differentiation-related genes (NKX2.5,
433 GATA4/5/6 and T-box). . Among those signal pathways, Wnt3a/ β -catenin signal is
434 deemed to be a negative regulator, the others being positive ¹⁶. In this study, we found
435 that imidacloprid exposure up-regulated Wnt3a expression and slightly
436 down-regulated the expression of BMP4, with not much change being observed in the
437 expressions of BMP2 and Fgf8. Knock-out or mutation of GATA4 and GATA5, the
438 zinc-finger transcription factors for cardiogenesis, leads to cardia bifida in mice ⁴⁵
439 whilst over-expression of GATA5 induces ectopic Nkx2.5 expression. The GATA6
440 promoter in both mouse and chick contains functionally important Nkx2.5 binding
441 sites. Similarly, the murine Nkx2.5 promoter contains GATA sites that are involved in
442 early heart field expression ⁴⁶. Likewise, the unlooped heart is associated with TBX5
443 mutation. Furthermore, VEGFR2 and its ligand VEGF are the cardiac- and endothelial
444 marker at the cardiac crescent stage ⁴⁷. It has been observed that imidacloprid

445 exposure could result in an obvious down-regulation of VEGFR2 (Fig. 3). From the
446 results of western blot we also found the down regulated of GATA4, GATA6 and
447 TBX5. All these results imply that imidacloprid-treated could significantly inhibit
448 cardiomyocyte differentiation during heart tube formation.

449 It is known that cardiac crescent cells date from myocardial precursor cells initiated
450 at the anterior primitive streak of gastrula embryo. Using the Dil⁺ migration assay, we
451 showed that the cell migration of myocardial precursor cells was suppressed by the
452 exposure to imidacloprid (Figs. 5G-I). In comparison to the 0.1% DMSO-exposed
453 side of embryos, the less migratory Dil⁺ myocardial precursor cells in the
454 imidacloprid-exposed side demonstrate that imidacloprid exposure indeed interfered
455 with precardiac cell migration toward the primary heart fields. However, the
456 possibility of an influence on cell proliferation cannot be excluded.

457 To investigate how imidacloprid affects cell migration, we employed scratch wound
458 assay and found that exposure inhibited H9c2 cells and chicken cardiac muscle cells
459 migration (Figs. 6B-B2, C-C2 and Fig.S4). It has been reported that cells migration
460 properties are related to cellular cytoskeleton modulation or to relevant adherence
461 factors ^{48, 49}. These data show that imidacloprid exposure disturbed cell internal
462 structure (Fig. 6G) and reduced the number of stress fibers (Fig. 6H). Moreover, cell
463 migration also relies on cell-cell junctions, including AJs, TJs and GJs etc (Fig. 6H).
464 Classic cadherins, including E-cadherin and N-cadherin, are crucial molecules in
465 calcium-dependent cell adhesion and supply *trans*-homophilic binding to other
466 cadherins on adjacent cells, whereas their intracellular domains firsthand interact with

467 p120-catenin. Vinculin, an actin-binding protein, connects intracellular actin filaments
468 by forming a mixture of, for example, α -catenin and β -catenin⁵⁰. TJs located at the
469 top of the lateral membranes, including the claudin family and occluding, exhibit
470 “barrier” and “fence” functions that involve binding to intracellular ZO-1⁵¹. GJs, such
471 as CX43, form multiple channels that allow the passage of small molecules and
472 electrical signals⁵². All the mentioned-above cell adhesion molecules were
473 down-regulated by imidacloprid (Fig. 6N), which suggested that this exposure
474 certainly interfered with cell migration and cardiac crescent formation during heart
475 tube formation.

476 Cardiac precursor cells derive from epiblast cells after undergoing EMT. EMT not
477 only needs to down-regulate expression of E-cadherin (required to maintain epithelial
478 cell contact) but also requires up-regulating the expression of N-cadherin, the
479 mesenchymal cell adhesion molecules. The Wnt/ β -catenin signaling pathway plays
480 regulatory role in the adhesion belt. Moreover, break-down of BM, marked by laminin
481 and the alteration of others cell-cell adhesion factors (AJs, TJs, GJs), are also very
482 important in EMT. In this research, imidacloprid treatment led to E-cadherin
483 up-regulation and N-cadherin down-regulation at mRNA and protein levels in the
484 gastrula chick embryos. This treatment also enhanced laminin expression but had little
485 influence on AJs (p120, Vinculin, Par3, β -catenin) and GJs (CX43). These data
486 indicate that imidacloprid-exposure interference with EMT is achieved through
487 altering the relevant adhesion molecules.

488 In summary, these studies reveal that imidacloprid exposure negatively influenced

489 EMT, cell migration and cell differentiation in heart tube formation. Figure 7
490 summarises schematically how imidacloprid might cause these changes. But, at
491 present, the mechanisms of cardiogenesis are only incompletely understood.

492 Furthermore, imidacloprid products are likely to flow into drinking water in poultry
493 farms, which may have impact on the quantity and quality of hatching eggs.

494 Thus, further experiments are required to explore the precise molecular
495 mechanism by which imidacloprid affects cardiogenesis, thereby contribute to
496 improve poultry industry.

497

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504

505 **Competing Financial Interest**

506 The authors have declared that no competing interests exist.

507

508 **Figure legends**

509 *Figure 1. Imidacloprid retarded development of the chick embryos and resulted in*
510 *abnormal heart formation. A:* The illustration shows the crucial points (2-, 4.5- and

511 14-day) in chick embryos heart development. **B**: Graph shows the mortality rate in
512 0.1% DMSO and 500 μ M imidacloprid-treated chick embryos at days 2, 4.5 and 14,
513 respectively. **C-C2**: Representative appearance of 0.1% DMSO-treated chick embryos
514 for 0- (C), 21- (C1) and 48- (C2) hs. **C3-C5**: Representative appearance of
515 imidacloprid-treated chick embryos for 0- (C3), 21- (C4) and 48- (C5) hs. **D**: Bar
516 chart shows the length of embryos following treatment at 0-, 21-, 48h. **E**: Bar chart
517 shows the pair numbers of somites at 48h. **F**: Bar chart shows the whole embryo
518 weight of chick embryos in E4.5. **G, G1-G2**: Representative appearance of the
519 4.5-day developing hearts in 0.1% DMSO-treated group (G), transverse section was
520 taken at the level indicated by dotted lines in F and stained with H&E stains (G1). The
521 high magnification images were taken from the sites indicated by boxed regions in G1
522 (G2). The black line and boxed region in G2 marked the ventricular wall and
523 trabecular muscle, respectively. **H, H1-H2**: The example shows the appearance of
524 4.5-day developing hearts in the imidacloprid- treated group (H), transverse section
525 was taken at the level indicated by dotted lines in H and stained with H&E stains (H1).
526 The high magnification images were taken from the sites indicated by boxed regions
527 in H1 (H2). The black line and boxed region in H2 dotted the ventricular wall and
528 trabecular muscle, respectively. **I**: Bar chart compares the ventricular wall thickness
529 of hearts. **J**: Bar chart compares the trabecular muscle layers. **K**: Representative
530 appearance of the 14-day mature hearts in 0.1% DMSO-treated group. **L**: Example
531 shows appearance of 14-day mature hearts in the imidacloprid- treated group. **K1, L1**:
532 Transverse section was taken at the levels indicated by dashed lines in K and L. **M-N**:

533 Bar chart shows the heart weight and the whole embryo weight. **O**: The bar chart
534 showing the thickness of ventricular wall in 14-day mature hearts. Abbreviations: LV,
535 left ventricle; RV, right ventricle; IVS, interventricular septum. Scale bars = 2000 μm
536 (C, C3); 1000 μm (C1-C2, C4-C5); 500 μm (G-H); 300 μm (G1-H1); 50 μm (G2-H2);
537 300 μm (K-L); 1000 μm (K1-L1).

538

539 **Figure 2. The classification of imidacloprid exposure-induced heart malformations**
540 **in gastrula chick embryos.** **A-A3**: Representative appearances of phenotypes
541 classification of hearts in gastrulating chick embryos immunofluorescently-stained
542 with MF20 antibody, including normal (A), mild (A1), intermediate (A2) and severe
543 (A3), respectively. **B-B3**: *In situ* hybridization shows VMHC expression in
544 representative appearances of phenotypes classification of hearts in gastrulating chick
545 embryos. **C**: Bar chart shows the rate of heart phenotype classification (%) in 0.1%
546 DMSO- and imidacloprid-treated group. **D-E**: Representative bright-field images of
547 0.1% DMSO-treated HH10 embryo (D) and heart tube immunofluorescently-stained
548 with MF20 antibody (E). **F, F1-F3**: F: Representative transverse sections at the levels
549 indicated by dotted white line in E. DAPI staining is used as a counterstain in F1. F2
550 is the merged image. F3 is the enlarged view of boxed region in F2. **G-H**:
551 Representative bright-field images of 0.1% DMSO-treated HH10 embryo (G) and
552 heart tube immunofluorescently-stained with MF20 antibody (H). **I, I1-I3**: I:
553 Representative transverse sections at the levels indicated by dotted white line in H.
554 DAPI staining is used as a counterstain in I1. I2 is the merged image. I3 is the

555 enlarged view of boxed region in I2. Scale bars = 150 μm (A1-A4, B1-B4, E, H); 500
556 μm (D, G); 100 μm (F, F1-F3, I, I1-I3).

557

558 **Figure 3. Imidacloprid exposure repressed the differentiation of cardiac progenitor**

559 **cells.** **A:** Overview of the signaling pathways that have been implicated into

560 cardiomyocyte formation. **B1-B4:** The embryos were incubated with 0.1% DMSO

561 (left) and imidacloprid (right) at either side until HH7 and processed for *in situ*

562 hybridization for GATA5 (B1), NKX2.5 (B2), VMHC (B3), BMP2 (B4). **B1'-B4':**

563 Representative transverse sections at the levels indicated by dotted black lines in

564 B1-B4. **C:** RT-PCR showing the expressions at HH7 chick embryos. **D:** Western-bolt

565 showing the expressions at protein level in HH7 chick embryos. **E:** The bar chart

566 showing the comparisons of gene expressions in D. Scale bars = 200 μm (B1-B4);

567 100 μm (B1'-B4').

568

569 **Figure 4. Imidacloprid exposure interfered with EMT during chick gastrulation.** **A:**

570 The illustration shows the EMT during chick gastrulation. **B:** Representative images

571 of 0.1% DMSO-treated HH4 chick embryos immunofluorescently-stained with

572 E-Cadherin. **B1-B1':** The transverse sections at the levels indicated by dotted white

573 line in B. The section was counterstained with DAPI (B1'). E-Cadherin is expressed

574 on the apical side of epiblast of 0.1% DMSO-treated embryo (white arrow in B1'). **C:**

575 Representative images of imidacloprid-treated HH4 chick embryos

576 immunofluorescently-stained with E-Cadherin. **C1-C1':** The transverse sections at

577 levels indicated by dotted white line in C. The section was counterstained with DAPI
578 (C1'). E-Cadherin expression level was enhanced on epiblast layer, and ectopic
579 expression in the mesoderm layer following imidacloprid treatment (white arrows in
580 C1'). **D**: Representative image of 0.1% DMSO-treated HH4 chick embryos
581 immunofluorescently-stained for laminin. **D1-D1'**: The transverse sections at levels
582 indicated by dotted white line in D. The section was counterstained with DAPI (D1').
583 Laminin is expressed on the BM of 0.1% DMSO-treated embryo (white dotted line
584 showing the gap in D1'). **E**: Representative image of imidacloprid-treated HH4 chick
585 embryos immunofluorescently-stained for laminin. **E1-E1'**: The transverse sections at
586 the levels indicated by dotted white line in E. The section was counterstained with
587 DAPI (E1'). Laminin is expressed on the BM of imidacloprid-treated embryo (white
588 dotted line showing the gap in E1'). **F**: Bar chart shows the gap distance of laminin
589 (μm) with 0.1% DMSO- and imidacloprid-treated HH4 chick embryos. **G**: RT-PCR
590 shows the expressions N-cadherin mRNA level in the HH4 chick embryos. **H**:
591 Western-bolt showing the expressions at protein level in HH4 chick embryos. **I**: The
592 bar chart showing the comparisons of gene expressions in H. Scale bars = 300 μm
593 (B-E); 100 μm (B1-E1, B1'-E1').

594

595 **Figure 5. Imidacloprid exposure restricted cardiac progenitor cell migration.** **A**: The
596 pattern of DiI-labeled cardiac progenitor cell migration following 0.1% DMSO
597 treatment on the both sides of embryos. **B**: The pattern of DiI-labeled cardiac
598 progenitor cell migration following 0.1% DMSO treatment at the left side and

599 imidacloprid exposure at right side of embryos. **C-E**: Fluorescence images were taken
600 at 0- (B), 9- (C) and 20- (D) hour. Note: both sides of embryos were exposed to 0.1%
601 DMSO. **C1-E1**: The merged images of bright-field and B-D respectively. **F**: Bar chart
602 shows the number of cardiac precursor cells migration based on A. **G-I**: Fluorescence
603 images were taken at 0- (G), 9- (H) and 20- (I) of incubation. The left sides of
604 embryos were exposed to 0.1% DMSO, while the right sides were exposed to
605 imidacloprid. **G1-I1**: The merged images of bright-field and G-I respectively. **J**: Bar
606 chart shows the number of cardiac precursor cells migration based on F. **K**: Bar chart
607 shows the number of embryo incidence of symmetrical migration or asymmetric
608 migration in 0.1% DMSO- and imidacloprid groups. Scale bars = 600 μ m (C-E, C1-E1,
609 G-I, G1-I1).

610

611 **Figure 6. The imidacloprid exposure suppressed H9c2 cells migration, polarization**
612 **and protrusion formation.** **A**: The sketch illustrates migration of H9c2 cells as
613 detected by the wound-healing assay. **B-C**: The representative images of H9c2 cells
614 scratch test at 0-hour incubation from 0.1% DMSO-treated (B) and
615 imidacloprid-treated (C) groups respectively. **B1-C1, B2-C2**: The representative
616 images of H9c2 cells scratch test at 12-hour (B1, C1), 24-hour (B2, C2). **D**: The bar
617 chart shows the percentage of wound closure (%) at 12-hour, 24-hour. **E-F**:
618 Representative image of actin filaments in 0.1% DMSO -treated H9c2 cells were
619 visualized by staining with F-actin (red), and cell nuclei were stained with DAPI
620 (blue). White dotted lines show the long and short axes of cells. F is the enlarged view

621 of E. (The boxed region in F shows stress fiber assay in H9c2 cells). **G-H:**
622 Representative image of actin filaments in imidacloprid-treated H9c2 cells were
623 visualized by staining with F-actin (red), and cell nuclei were stained with DAPI
624 (blue). White dotted lines show the long and short axes of cells. H is the enlarged
625 view of G. (The boxed region in H shows stress fiber assay in H9c2 cells). **I-J:**
626 Representative images of 0.1% DMSO and imidacloprid-treated H9c2 cells
627 immunofluorescently-stained with Myh7, respectively. **K:** Bar chart showing the ratio
628 of long axis to short axis. **L:** Bar chart shows cells containing stress fibers (%). **M:**
629 Bar chart shows fluorescence intensity of Myh7 (AU). **N:** RT-PCR showing the
630 expressions at mRNA level in HH7 chick embryos exposed either 0.1% DMSO or
631 imidacloprid. Scale bars = 200 μ m (B, B1-B2, C, C1-C2); 100 μ m (E- J).

632

633 ***Figure 7. Model depicting how imidacloprid exposure induced heart tube***
634 ***malformation during chick cardiogenesis.***

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