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Changes in the levels of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine are involved in perfluorooctanoic acid induced developmental cardiotoxicity in chicken embryo

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ABSTRACT

Perfluorooctanoic acid (PFOA), a persistent organic pollutant, is associated with developmental toxicity. This study investigated the mechanism of PFOA-induced developmental cardiotoxicity in chicken embryo, focusing on the interactions between developmental exposure to PFOA and the levels of L-carnitine (LC), acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC) in the heart. To evaluate the developmental cardiotoxicity, fertile chicken eggs were exposed to 0.1, 0.5, 1, 2 or 5 mg/kg PFOA via air cell injection. Furthermore, exposure to 2 mg/kg PFOA, with or without 100 mg/kg LC were applied to investigate the effects of LC supplement. The results of functional and morphological assessments confirmed PFOA induced developmental cardiotoxicity in chicken embryo, which could be alleviated by co-exposure to LC. LC–MS/MS results also revealed remarkable decrease in LC, ALC and PLC levels in embryonic day six (ED6) chicken embryo hearts as well as LC level in embryonic day fifteen (ED15) chicken embryo hearts following developmental exposure to 2 mg/kg PFOA. Meanwhile, co-exposure to 100 mg/kg LC significantly elevated the levels of LC, ALC and PLC in chicken embryo hearts. Significantly elevated expression level of carnitine acetyltransferase (CRAT) in PFOA-exposed ED6 chicken embryo hearts was observed via western blotting, while LC co-exposure counteracted such changes. In conclusion, changes in the levels of LC, ALC and PLC in early embryonic stages are associated with PFOA induced developmental cardiotoxicity in chicken embryos.

1. Introduction

Perfluorooctanoic acid (PFOA), a member of the perfluoroalkyl acids (PFAAs) family, is commonly used in the production of many polymers. The most well-known example of polymer involved the use of PFOA is polytetrafluoroethene (PTFE), which is then widely used in many products, such as non-stick coatings, water repellent coatings and fire retardants (Begley et al., 2005). Since the last decade, in vitro and in vivo animal studies has established association between PFOA exposure and endocrine disruption, immune toxicity, carcinogenicity and developmental toxicity (Biegel et al., 2001; Dewitt et al., 2008; Vested et al., 2013; Wolf et al., 2007); moreover, epidemiological studies on PFOA-exposed populations have also suggested increased risks of kidney and testicular cancer, pregnancy-induced hypertension, thyroid disease, hypercholesterolemia, ulcerative colitis, rheumatoid arthritis and elevation of alanine aminotransferase (Barry et al., 2013; Darrow et al., 2013, 2016; Steenland et al., 2013, 2015; Winquist and Steenland, 2014a, 2014b).

Since transient disruption in developing organism could potentially induce persistent effects in the adulthood (Goh et al., 2011), developmental toxicity has drawn particular attention among the health risks associated with PFOA. In animal models, retarded development and higher mortality were reported following PFOA exposure, and peroxisome proliferator-activated receptor alpha (PPAR alpha) activation was identified as a potential mechanism of toxicity (Abbott et al., 2007). However, PPAR alpha independent effects were elucidated as well (Buhrke et al., 2015). Many still remain unknown in PFOA induced developmental toxicity.

In our previous studies, PFOA-induced developmental cardiotoxicity in chicken embryo was reported (Jiang et al., 2012), and

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the potential molecular and cellular mechanisms were explored (Jiang et al., 2013, 2015). However, further study is still needed to fully appreciate the mechanism of toxicity towards this endpoint.

It is generally well-accepted that PFOA could act as a PPAR alpha agonist (Post et al., 2012). PPAR alpha plays a crucial role in regulation of fatty acid beta oxidation (Grygiel-Gorniak, 2014). In laboratory models, PFOA exposure was associated with elevation of beta-oxidation related gene products, such as fatty acid binding protein (FABP) and acyl-CoA oxidase (Petrescu et al., 2013). Thus, it is possible that PFOA exposure could affect other cellular components in the process of fatty acid beta oxidation. In the current study, a potent PPAR alpha antagonist, GW6471, was utilized to investigate the role of PPAR alpha in PFOA induced developmental cardiotoxicity in chicken embryo.

L-Carnitine (LC) is an amino acid derivative and nutrient that participates in fatty acid beta oxidation. Particularly, LC plays a crucial role in the carnitine shuttle, in which it serves as a carrier, transports long chain fatty acids into mitochondria and short chain acetyl/propionyl groups from mitochondria to cytoplasm (Reuter and Evans, 2012). One study found that PFOA exposure decreased endogenous LC level in hepatic cells by promoting its transformation to acetyl-L-carnitine (ALC) in liver cell line (Peng et al., 2013), while LC deficiency in myocardium could actually lead to cardiomyopathy and cardiac remodeling (Kilic et al., 2012). These findings, along with the fact that myocardium heavily relies on fatty acid oxidation as a major energy resource (Olpin, 2013), suggest that the mechanism of PFOA-induced developmental cardiotoxicity might involve changes in the levels of LC, ALC and propionyl-L-Carnitine (PLC) in the heart.

In this study, we utilized the chicken embryo model to assess the effects of PFOA developmental exposure on endogenous LC, ALC and PLC levels. Extragenous LC supplement were also applied to further assess the role of LC, ALC and PLC in PFOA induced cardiotoxicity. Additionally, the expression level of carnitine acetyltransferase (CRAT) in embryonic day six (ED6) chicken embryo hearts were assessed, since CRAT is the crucial enzyme that handles the conversion of LC to its short-chain derivatives, ALC and PLC. Our data provided experimental evidence for the mechanism of PFOA-induced developmental cardiotoxicity and potentially general mechanism of toxicity for PFOA.

2. Materials and methods

2.1. Animals

Fertile chicken eggs were purchased from Lanke poultry center (Jimo, China). Upon arrival, eggs were cleaned with 20% povidine iodine and carefully dipped dry with paper towel. Eggs were then candled in a dark room and the air cells were marked with pencils. Eggs were then weighed, numbered and evenly assigned into different treatment groups based on weight.

2.2. Materials

Perfluorooctanoic acid (77262, CAS 3825-26-1), L-carnitine (C0158, CAS 541-15-1), acetyl-L-Carnitine (A6706, CAS 5080-50-2), propionyl-L-carnitine (42602, CAS 20064-19-1), GW6471 (G5045, CAS880635-03-0) and other chemical supplies (if not otherwise mentioned) were purchased from Sigma Aldrich (St. Louis, MO, US). Monoclonal antibody against CRAT (bs-15397R) was purchased from Bioss (Beijing, China). Monoclonal antibody against GAPDH (TA-08) was purchased from ZSGB-BIO (Beijing, China). General laboratory supplies were all of highest grade obtainable.

2.3. Air cell injection

Air cell injection was performed as described in Jiang et al. (2012) with slight modifications. Three batches of studies were performed, the first batch was designed to evaluate the developmental cardiotoxicity in an expanded dose rangep; second batch was utilized to investigate the effects of LC or GW6471 co-treatment, while the aim of the third was to evaluate the effects of PFOA and/or LC treatment on LC, ALC and PLC levels in chicken embryo heart.

For the first batch, different PFOA dosing suspensions in sunflower oil were used (1, 5, 10, 20 and 50 mg/ml) so that the desired PFOA doses (0.1, 0.5, 1, 2 and 5 mg/kg egg weight) could be achieved at 0.1 μg/g (egg weight) injection. For the second batch, dosing suspensions in oil (PFOA 20 mg/ml, LC 1000 mg/ml and GW6471 12.5 mg/ml) were prepared so that the desired doses (PFOA 2 mg/kg egg weight, PFOA 2 mg/kg egg weight + GW6471 1.25 mg/kg egg weight, PFOA 2 mg/kg egg weight + LC 100 mg/kg egg weight and LC 100 mg/kg egg weight) could be achieved at 0.1 μl/g (egg weight) injection. The LC and PFOA + LC preparations included 20% distilled water and 10% ethanol to improve the solubility and stability of LC in suspension (oil volume was correspondingly decreased to ensure identical total volume). PFOA 20 mg/ml and/or LC 1000 mg/ml dosing suspension identical to those used in the second batch were used in the third batch.

After brief cleaning with 70% ethanol, a metal probe was used to drill a hole (approximately 0.7–1 mm in diameter) in the center of marked air cell area. After well-suspension with vertex, desired volume of dosing suspension were injected into air cell. The hole was then sealed with melted paraffin.

2.4. Egg incubation

Injected eggs were incubated in an Keyu egg incubator (Dezhou, China). Temperature, humidity and turning frequency were preprogrammed by the incubator. The incubation temperature started at 37.9° Celsius and gradually decreased to 37.1° Celsius, while humidity started at 50% and gradually increased to 70% as incubation continued. Turning was stopped during the last two days of incubation. One day before hatching, eggs were individually placed in labeled small containers to hatch. Hatchling chickens were moved to a warmed plastic box with water supplement until experiment. All procedures followed national institutes of health guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University.

2.5. Electrocardiography

Hatchling chickens (12–24 h post hatch) were anesthetized with 33 mg/kg pentobarbital via intraperitoneal injection. Two electrodes were embedded subcutaneously on both sides of the chest and connected to the Taimeng BL-420E+ multifunctional biosensor (Sichuan, China). Electrocardiography were recorded, R–R intervals were measured and heart rates were calculated with the equation: Heart rate (beats per minute) = 60/R–R interval (second).

2.6. Histological assessment of heart tissue

For the PFOA evaluation group, ED19 chicken embryos were removed from eggshell and quickly decapitated with sharpened scissors. For the PFOA + LC group, anesthetized hatchling chickens were quickly decapitated with sharpened scissors. Heart and liver weights were recorded, hearts were fixed in 10% formaldehyde for 24 h, and then embedded in paraffin. Embedded tissues were sectioned with a Leica RM2016 microtome at six μm. Sections were then stained with hematoxylin and eosin (HE) kit (Beyotime, Bejing,

China) following manufacturer provided protocol. Sections were visualized with an Olympus microscope (SXZ9, Tokyo, Japan). Photos were analyzed with ImageJ (NIH, US). A standard ruler was used for calibration. Right ventricular wall thickness was measured as described in Jiang et al. (2012).

2.7. LC–MS/MS measurement of LC, ALC and PLC

After developmental exposure to vehicle, PFOA 2 mg/kg or PFOA 2 mg/kg + LC 100 mg/kg, ED6, 15, 19 chicken embryo hearts and D1 hatchling chicken hearts were carefully isolated with fine forceps and scissors and stored in −80 freezer until further use. ED6 was selected to represent early stage of heart development, while ED15 represented middle stage, in which oxygen consumption significantly increased (Tazawa et al., 2011). ED19 and D1 post-hatch were chosen to represent late stage heart development and to compare before and after hatching process. Before the measurement, tissues were grinded with liquid nitrogen. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) was performed by Beijing Mass Spectrometry Medical Research Co., Ltd. (Beijing, China). Briefly, appropriate amount of water were added and mixed thoroughly, the resulting suspensions were centrifuged at 12000 rpm, 10 min. Supernatants were then collected, 50 μl supernatant was mixed with 200 μl acetonitrile thoroughly, centrifuged again, and the resulting supernatants were subjected to LC–MS/MS (Ultimate3000 – API 3200 Q TRAP). For the detailed parameters used in the LC–MS/MS measurement and the quality controls, please refer to Supplementary materials.

2.8. Western blotting

ED6 chicken embryo hearts were homogenized in radio immunoprecipitation assay (RIPA) buffer (Beyotime, Beijing, China) supplemented with 2 μg/ml aprotinin (Solarbio, Beijing, China) and 1:100 PMSF (Solarbio, Beijing, China). Protein concentrations were determined with BCA (Beyotime, Bejing, China). Even amount of proteins were mixed with SDS sampling buffer (Beyotime, Beijing, China), denatured by heating to 90° Celsius for 10 min and electrophoresis was performed in 10% acrymide gels. Samples were then transferred to polyvinylidene fluoride (PVDF) membrane and probed with CRAT antibody (1:1000) or GAPDH antibody (1:2000). The blots were visualized with ECL chemoluminscence kit (Solarbio, Beijing, China) and UVP 810 gel imaging system (UVP, Upland, CA, US). Semi-quantification was performed with ImageJ (NIH, US), the relative CRAT expression levels were first normalized to corresponding GAPDH levels, and then normalized to the control samples on the same gel. Sample size was three from three independent samples per group.

2.9. Statistical analysis

Statistical analysis was performed with SPSS 17.0. Homogeneity of variance test (Levene's test) was utilized to ensure homogeneity among groups. If inhomogeneity was detected, logarithmic transformation was performed. Then one way analysis of variance (ANOVA) was performed. When ANOVA returned positive results, post-hoc least significant difference (LSD) tests were used to determine differences among groups. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. General parameters of ED19 chicken embryos and hatchling chickens treated with PFOA

The general parameters of ED19 chicken embryos (for the histological assessment) and hatchling chickens (for the functional assessment) were reported in Fig. 1, including the slim body weight, liver index, heart index and hatchability (calculated by total number hatched/total eggs). No statistical significance was detected among the groups.

3.2. Evaluation of PFOA-induced developmental cardiotoxicity

In the histological assessment of PFOA exposed ED19 chicken embryo hearts, 2 and 5 mg/kg (egg weight) PFOA exposure induced significant thinning of the right ventricular wall in ED19 chicken embryo hearts (Fig. 2A). In the functional assay, 0.5, 1, 2 and 5 mg/kg (egg weight) PFOA exposure induced significant increase of heart rate in hatchling chickens (Fig. 2B).

3.3. General parameters of hatchling chickens treated with PFOA, LC and/or GW6471

The general parameters of hatchling chickens used in the co-treatment study, including the slim body weight, liver index, h

eart index and hatchability (calculated by total number hatched/total eggs) were reported in Fig. 3. No statistical significance was detected among the groups.

3.4. Morphological changes of hatchling chicken hearts exposed to PFOA, LC and/or GW6471

Remarkably decreased right ventricular wall thickness was detected in the hatchling chickens developmentally exposed to 2 mg/kg (egg weight) PFOA relative to control group animals. In contrast, treatment of 100 mg/kg LC along with 2 mg/kg PFOA significantly elevated the right ventricular wall thickness relative to 2 mg/kg PFOA group and had no statistical significance relative to control group. Additionally, co-treatment of GW6471 along with PFOA did not induce significant morphological changes comparing to control group. Solo LC treatment had no remarkable effects (Fig. 4A,B).

3.5. Heart rate of hatchling chickens developmentally exposed to PFOA, LC and/or GW6471

As shown in Fig. 4C, the heart rate of hatchling chicken developmentally exposed to 2 mg/kg (egg weight) PFOA was significantly elevated comparing to that of control chicken. Meanwhile, co-exposure to 100 mg/kg (egg weight) LC reverted such changes, significantly decreased the heart rate comparing to PFOA group, while had no significant difference relative to control group. On the other hand, co-exposure of GW6471 along with PFOA still significantly elevated the heart rate relative to control group. Additionally, exposure to LC without PFOA had no significant impact on the heart rate.

3.6. Levels of LC, ALC and PLC in ED6, 15, 19 chicken embryo hearts and D1 hatchling chicken hearts

In ED6 chicken embryo hearts, LC–MS/MS revealed significant decrease in LC, ALC and PLC levels in 2 mg/kg PFOA exposed embryos comparing to control group (Table 1A).

In ED15 chicken embryo hearts, significant decrease in LC was still observed in PFOA treated animals (Table 1B), while no remark-

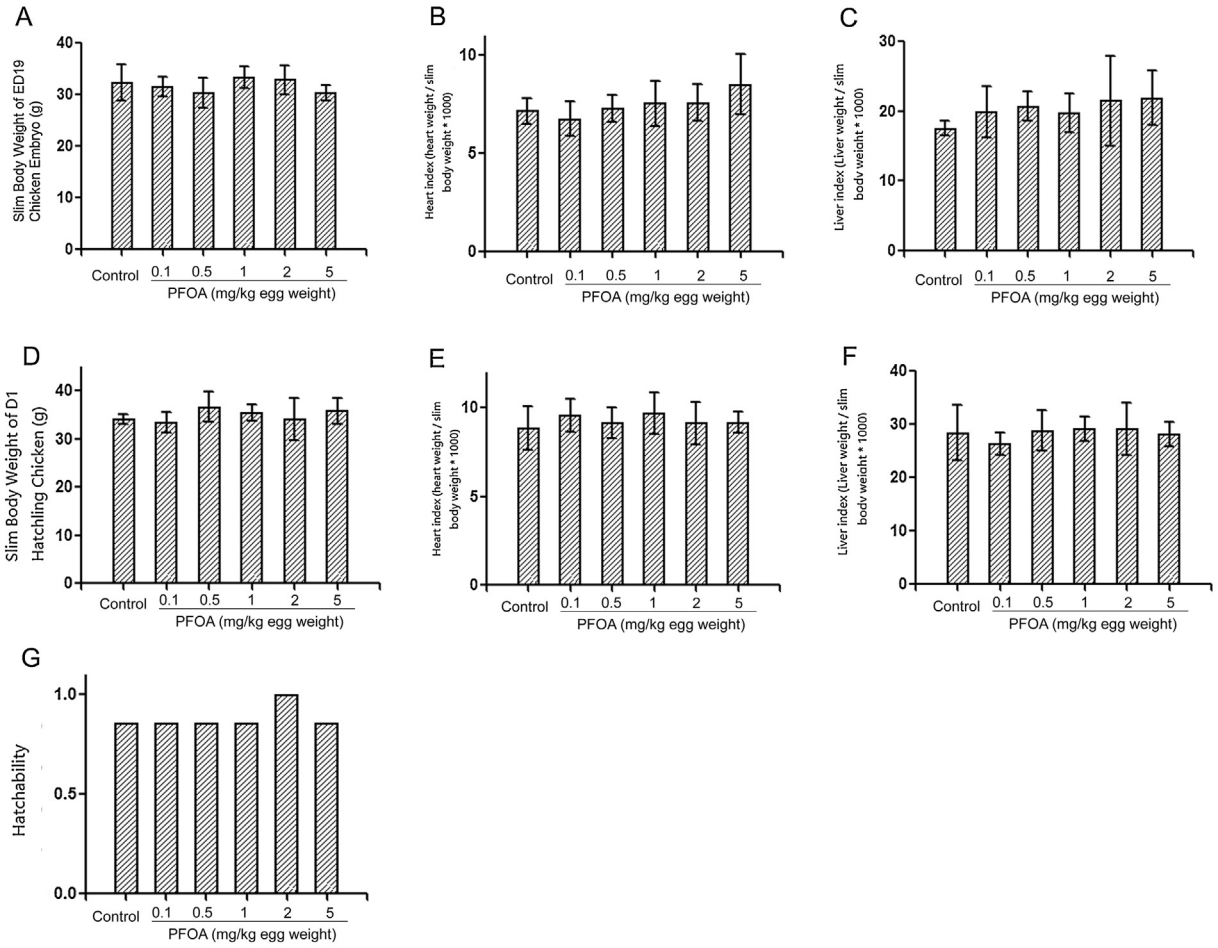

Fig. 1. General toxicity parameters of the ED19 chicken embryo and hatchling chickens from PFOA-induced developmental cardiotoxicity evaluation study.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), PFOA 0.1, 0.5, 1, 2 or 5 mg/kg (egg weight) and incubated at 37.9–37.1 ◦ celesus, 50–70% humidity until ED19 or hatch. Slim body weight (whole body weight − yolk weight), heart index (heart weight/slim body weight * 1000), liver index (liver weight/slim body weight * 1000) and hatchability for the hatchling chickens (number of chickens hatched/total egg numbers) were recorded. Data were presented as mean + standard derivation. For the ED19 chicken embryos, N = 4–5 per group. For the hatchling chickens, N = 4–6 per group. (Note: Actual number of animals per group is at least 5, but a few yolk weight were not recorded thus those animals were excluded from this figure.)

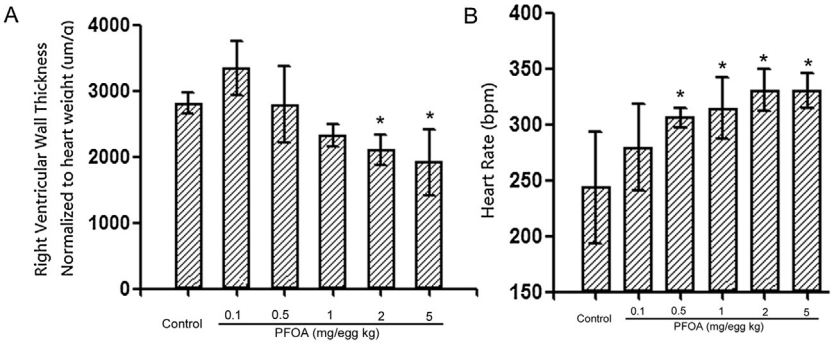

Fig. 2. Evaluation of PFOA-induced developmental cardiotoxicity.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), PFOA 0.1, 0.5, 1, 2 or 5 mg/kg (egg eight) and incubated at 37.9–37.1 ◦ celesus, 50%–70% humidity until ED19 or hatch. ED19 chicken embryo hearts were processed for histologial assessment and ECG was used to assess cardiac function in hatchling chickens. Data were presented as mean + standard derivation.
N = 5 per group.
*: statistically different from vehicle control group ($P < 0.05$).
A. Quantification of the ED19 chicken embryo heart right ventricular wall thickness normalized to heart weight.
B. Quantification of hatchling chicken heart rate.

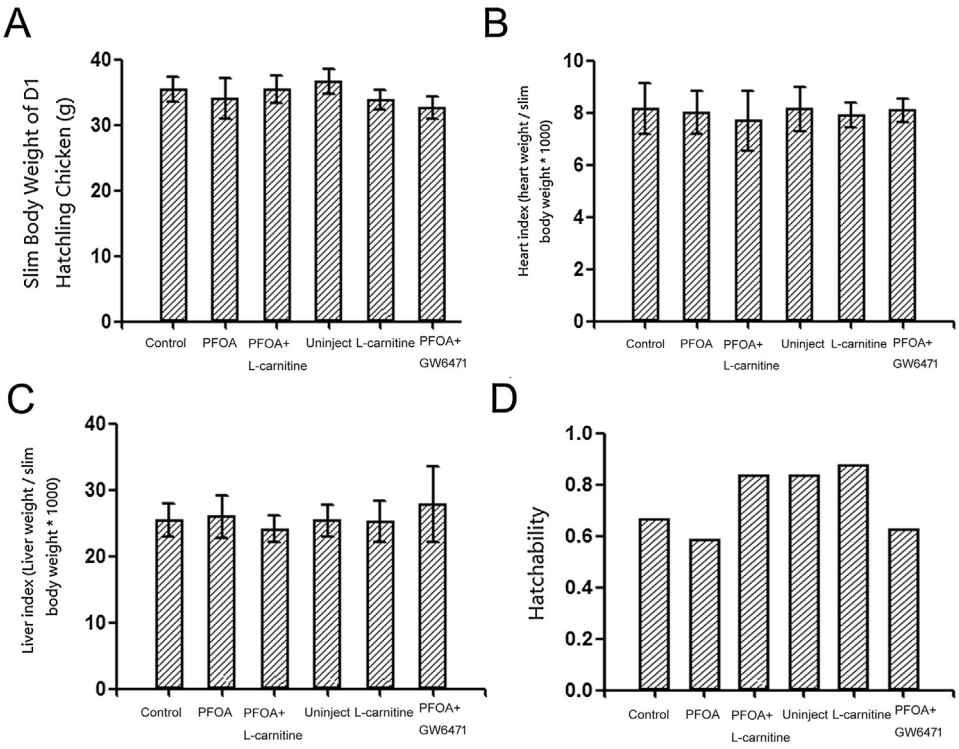

Fig. 3. General toxicity parameters of the hatchling chickens treated with PFOA and/or L-carnitine.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), PFOA 2 mg/kg, L-carnitine 100 mg/kg, PFOA 2 mg/kg + L-carnitine 100 mg/kg or PFOA 2 mg/kg + GW6471 1.25 mg/kg and incubated at 37.9–37.1 ◦ celesus, 50%–70% humidity until hatch. Hatchling chickens were euthanasized, slim body weight (whole body weight − yolk weight), heart index (heart weight/slim body weight * 1000), liver index (liver weight/slim body weight * 1000) and hatchability (number of chickens hatched/total egg numbers) were recorded. Data were presented as mean + standard derivation. N = 5–10 per group.

Table 1
L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine levels in ED6, 15, 19 chicken embryo and hatchling chicken hearts treated with PFOA and/or L-carnitine.

A. L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine levels in ED6 chicken embryo hearts

	ED6 chicken embryo heart LC level (ng/mg)	ED6 chicken embryo heart ALC level (ng/mg)	ED6 chicken embryo heart PLC level (ng/mg)
Vehicle	0.74 ± 0.17	13.36 ± 3.34	0.57 ± 0.18
PFOA 2 mg/kg	0.48 ± 0.18[a]	7.92 ± 2.97[a]	0.35 ± 0.16[a]
PFOA 2 mg/kg + LC 100 mg/kg	8.24 ± 1.12[a,b]	89.09 ± 13.38[a,b]	3.91 ± 0.74[a,b]

B. L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine levels in ED15 chicken embryo hearts

	ED15 chicken embryo heart LC level (ng/mg)	ED15 chicken embryo heart ALC level (ng/mg)	ED15 chicken embryo heart PLC level (ng/mg)
Vehicle	0.84 ± 0.15	1.38 ± 0.26	0.17 ± 0.03
PFOA 2 mg/kg	0.58 ± 0.15[a]	1.24 ± 0.32	0.14 ± 0.04
PFOA 2 mg/kg + LC 100 mg/kg	4.48 ± 0.67[a,b]	11.46 ± 1.74[a,b]	0.97 ± 0.24[a,b]

C. L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine levels in ED19 chicken embryo hearts

	ED19 chicken embryo heart LC level (ng/mg)	ED19 chicken embryo heart ALC level (ng/mg)	ED19 chicken embryo heart PLC level (ng/mg)
Vehicle	1.82 ± 0.34	1.38 ± 0.26	0.29 ± 0.05
PFOA 2 mg/kg	1.74 ± 0.18	1.24 ± 0.32	0.25 ± 0.03
PFOA 2 mg/kg + LC 100 mg/kg	5.73 ± 0.69[a,b]	11.43 ± 1.52[a,b]	0.96 ± 0.32[a,b]

D. L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine levels in D1 hatchling chicken hearts

	D1 hatchling chicken heart LC level (ng/mg)	D1 hatchling chicken heart ALC level (ng/mg)	D1 hatchling chicken heart PLC level (ng/mg)
Vehicle	2.52 ± 0.32	1.93 ± 0.66	0.45 ± 0.09
PFOA 2 mg/kg	3.02 ± 0.40[a]	2.28 ± 0.27	0.43 ± 0.04
PFOA 2 mg/kg + LC 100 mg/kg	8.00 ± 0.98[a,b]	10.16 ± 2.04[a,b]	1.92 ± 0.57[a,b]

Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), PFOA 2 mg/kg or PFOA 2 mg/kg + L-carnitine 100 mg/kg and incubated at 37.9–37.1 ◦ celesus, 50%–70% humidity until 6, 15, 19 days or hatch. Hearts were isolated and subjected to LC–MS/MS for the levels of L-carnitine (LC), acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC). Data are expressed as mean ± standard derivation. N = 6 per group.

[a] Statistically different from vehicle group within same time point for the same endpoint (P < 0.05).
[b] Statistically different from PFOA 2 mg/kg group within same time point for the same endpoint (P < 0.05).

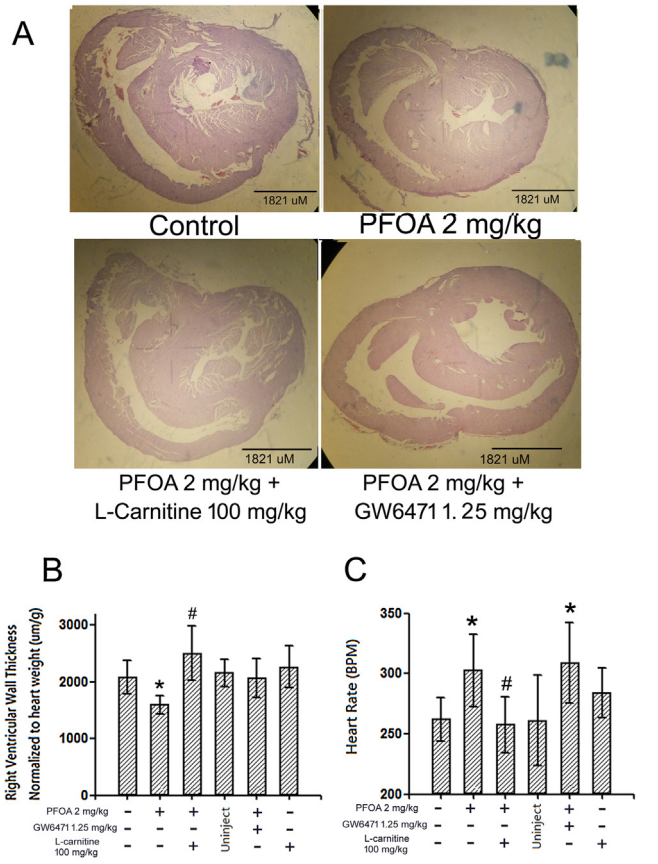

Fig. 4. Morphological and functional changes in hatchling chickens treated with PFOA and/or L-carnitine.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), PFOA 2 mg/kg, L-carnitine 100 mg/kg, PFOA 2 mg/kg + L-carnitine 100 mg/kg or PFOA 2 mg/kg + GW6471 1.25 mg/kg and incubated at 37.9–37.1 ° celesus, 50%–70% humidity until hatch. Hatchling chickens were anesthetized with 33 mg/kg pentobarbital, electrocardiography was recorded with BL-420E+ (Taimeng, Chengdu, China) and heart rates were calculated. Chickens were then sacrificed, hearts were collected and processed for histological assessment.
*: statistically different from vehicle control group ($P < 0.05$).
#: statistically different from PFOA 2 mg/kg group ($P < 0.05$).
A. Representative pictures of hatchling chicken heart histology pictures.
B. Quantification of the hatchling chicken right ventricular wall thickness normalized to heart weight (N = 4–5 per group).
C. Quantification of hatchling chicken heart rate (N = 5–6 per group).

able changes in ALC and PLC levels were observed (Table 1B). In ED19 chicken embryo hearts, developmental exposure to PFOA did not significantly affect LC, ALC or PLC levels (Table 1C). In D1 hatchling chicken hearts, the PFOA in ovo exposure had no remarkable impact on ALC and PLC levels (Table 1D), but induced a small yet significant increase in LC level (Table 1D). On the other hand, extragenous LC supplement very effectively increased the level of LC, ALC and PLC at all time points tested (Table 1A–D).

3.7. Western blotting for CRAT in D6 chicken embryo hearts

Western blotting results indicated that the CRAT protein expression level was significantly elevated in ED6 chicken embryo hearts following developmental exposure to 2 mg/kg (egg weight) PFOA relative to control, while co-treatment with extragenous LC reversed such change, returning the level close to control level (Fig. 5).

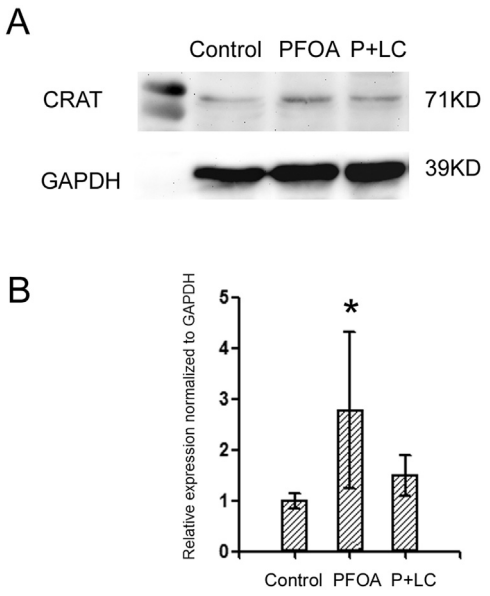

Fig. 5. Western blotting for CRAT expression in D6 chicken embryo hearts treated with PFOA and/or L-carnitine.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), PFOA 2 mg/kg or PFOA 2 mg/kg + L-carnitine 100 mg/kg and incubated at 37.9–37.1 ° celesus, 50%–70% humidity until D6. Embryo hearts were isolated, protein samples were extracted with radio immunoprecipitation assay (RIPA) buffer and subjected to western blotting for CRAT. N = 3 per group.
*: statistically different from vehicle control group ($P < 0.05$).
A. Representative western blotting picture for CRAT.
B. Quantification for CRAT expression normalized to GAPDH.

4. Discussion

4.1. PFOA-induced developmental cardiotoxicity

PFOA, although had been phased out in US in 2015 (Bjerregaard-Olesen et al., 2016), remains to be an important persistent organic pollutant (POP), and is still being produced in other parts of the world (Li et al., 2015). Given the extreme stability of PFOA in both environment and biota, the exposure to human and animals is likely to continue (Fromel and Knepper, 2010). PFOA induced developmental toxicity is among the major health concerns associated with it. Wolf et al. (2007) and Abbott et al. (2007) demonstrated the retarded development and increased embryo loss/offspring mortality, and activation of PPAR alpha was associated with such developmental toxicity (Abbott et al., 2007). PFOA induced developmental cardiotoxicity was a relatively recently discovered effect (Jiang et al., 2012), which potentially adds PFOA to the list of POPs that induce developmental cardiotoxicity, such as dioxin and PCBs (Borlak and Thum, 2002; Fujisawa et al., 2014). Furthermore, as reported in Jiang et al. (2012), LC–MS/MS revealed PFOA concentration of 5670 ng/ml in 2 mg/kg (egg weight) PFOA injected hatchling chicken serum, which was higher than normal population value (approximately 3–4 ng/ml) (Post et al., 2012), but not too far away from occupational exposed population values, in which a mean serum concentration of 83.0 ng/ml and a high value of 17556.6 ng/ml was reported (Steenland et al., 2009). These findings highlight the needs to further assess this potential risk associated with the developmental cardiotoxicity observed in chicken. In current study, PFOA-induced developmental cardiotoxicity in chicken embryo was evaluated with the dose range expanded from 0.5–2 mg/kg in Jiang et al. (2012) to 0.1–5 mg/kg. PFOA dose selection was based on previous study (Jiang et al., 2012) and human occupational exposure data (Steenland et al., 2009). The inclusion of 0.1 mg/kg doses was aimed to evaluate the potential

effects at the doses close to mean human occupational exposure levels (83 ng/ml in Steenland et al., 2009), while the inclusion of 5 mg/kg was designed to demonstrate rare conditions which simulate the extremely high exposure cases (such as the high value of 17556.6 ng/ml in Steenland et al., 2009). Consistent with previous study by Jiang et al. (2012), morphological changes were observed in ED19 chicken embryo hearts at 2 and 5 mg/kg PFOA exposure, while PFOA doses equal or higher than 0.5 mg/kg resulted in functional changes. Further study used the PPAR alpha antagonist, GW6471 to appreciate the role of PPAR alpha in these endpoints. While GW6471 did not effectively prevent the heart rate elevation, it indeed had some protective effects against the thinning of right ventricular wall. These results suggest that the mechanism of PFOA induced developmental cardiotoxicity has mixed nature, both PPAR alpha dependent and PPAR alpha independent mechanism are involved.

4.2. LC and heart development

LC is an amino acid derivative nutrient participating in fatty acid metabolism, which helps to maintain the normal, balanced fatty acid metabolism and protects mitochondria functionality (Guarnieri, 2015). Meanwhile, myocardium was known to utilize fatty acid as one of the major energy resource (Ajith and Jayakumar, 2016), making LC an important nutrient for myocardium. LC deficiency had been associated with children cardiomyopathy, suggesting the critical role of LC in heart development/homeostasis (Fu et al., 2013). On the other hand, PFOA is known as a PPAR alpha agonist, the latter is generally accepted to be associated with fatty acid beta oxidation (Post et al., 2012), a process in which LC plays a critical role. In current study, co-exposure of 100 mg/kg LC with 2 mg/kg (egg weight) PFOA effectively abolished PFOA induced cardiac effects, suggesting that PFOA-induced developmental cardiotoxicity is associated with LC level in the heart. Our results support the claims that l-carnitine supplement exert cardioprotective effects (Tousson et al., 2016). The dose selection of LC was based on FDA's dosing information on marketed LC preparations for human consumption (50–100 mg/kg/day for infants and children) (USFDA, 2015). Additionally, no remarked changes in heart rate or heart morphology were observed following LC only treatment, indicating relative safety as well as specificity of LC against PFOA-induced effects instead of general cardiac effects. This is the first demonstration that LC supplement could counteract PFOA induced developmental cardiotoxicity in chicken embryo.

4.3. The effects of PFOA exposure to LC content in chicken embryo heart

LC induced protection against PFOA-induced developmental cardiotoxicity suggested the role of LC in such toxicity. Adequate LC level in myocardium is essential for the heart to maintain its normal morphology and functionality. A mutation in carnitine transporter, OCTN1 resulted in severe LC deficiency, and subsequently cardiomyopathy, and extragenous LC supplement alleviated symptoms (Kilic et al., 2012). Although no existing data have showed that environmental pollutant could affect LC levels in organism, multiple studies associated environmental factors, such as diet or disease with such effects (Berry-Kravis et al., 2001; Conlon et al., 2016). PFOA was correlated with decreased LC in human liver cells (Peng et al., 2013), but no study focused on PFOA's effect on LC in developing heart. In the current study, LC–MS/MS was utilized to measure the endogenous levels of LC and its short chain derivatives ALC and PLC. Interestingly, our findings revealed significantly decreased levels of LC, ALC and PLC in PFOA exposed ED6 chicken embryo hearts relative to control, and the percentage changes were actually more prominent in ALC and PLC levels (35.0%, 40.7% and 39.2% decrease for LC, ALC and PLC relative to control, respectively). These data suggest that developmental exposure to PFOA could affect the levels of LC and its derivatives in early chicken embryo hearts. The further decrease of ALC and PLC levels suggests that PFOA impaired the ability of developing heart to convert free LC to ALC and PLC. LC and ALC are essential in maintaining mitochondria function, preventing free acetyl group building up (Stephens et al., 2007). ALC was known to be able to modulate energy metabolism and exert neuroprotective effects (Kocsis et al., 2016). On the other hand, PLC was known to be associated with sports performance (Andreozzi et al., 2008). Considering the observed cardiac functional changes in chicken embryo heart following developmental exposure to PFOA, changes in the levels of LC, ALC and PLC might be contributing to PFOA induced developmental cardiotoxicity. On the other hand, extragenous LC supplement very effectively elevated LC, ALC and PLC levels, which might help to explain the protection against PFOA induced cardiotoxicity by extragenous LC supplement.

As the embryo develops, however, in ED15 chicken embryo hearts, only a significant change (31.5%) in LC level were observed and no significant changes were observed in ALC or PLC levels. In D19 chicken embryo hearts, no significant differences were observed between control and PFOA group hearts regarding to LC, ALC or PLC levels, which may attribute to the maturation of liver and kidney, the organs capable of synthesizing endogenous LC (El-Hattab and Scaglia, 2015) and compensating for the initial decrease caused by PFOA. Interestingly, in D1 hatchling chickens, a slight but significant increase of LC levels in heart was observed, which seems to be a little over-compensation. This phenomenon is consistent with previous studies in which early life environment impacts induce long-lasting health risk in later adult life (Langley-Evans, 2006). Our findings associated developmental PFOA exposure to decreased cardiac LC, ALC and PLC levels in early embryo. Although these changes disappeared in later embryo stages, they might be contributing to the developmental cardiotoxicity induced by PFOA. Since primary carnitine deficiency (PCD) patients could have lower than normal circulating free LC (Sarafoglou et al., 2010), they might be at increased risk of such effects, which guarantees further study.

4.4. Role of CRAT in PFOA induced developmental cardiotoxicity

Carnitine acetyltransferase (CRAT, or CAT1) is an enzyme mainly expressed in skeletal muscle and heart (Muoio et al., 2012; Seiler et al., 2015), which is responsible for converting free LC to ALC or PLC. Alterations of CRAT expression levels and/or activities were associated with muscle fatigue, muscle metabolism and endothelial nitric oxide signaling (Muoio et al., 2012). Since the levels of ALC and PLC in ED6 embryo heart were decreased following developmental exposure to PFOA, CRAT levels in ED6 chicken embryo hearts were investigated in the current study. Interestingly, opposite to the expectation, the CRAT expression was actually significantly enhanced relative to control group. While this result is consistent with reports that PPAR agonist fenofibrate induced CRAT expression (Kienesberger et al., 2014), enhanced CRAT expression technically should elevate the levels of ALC and PLC in the heart, which was contradictive with the LC–MS/MS results that the decrease in ALC and PLC levels actually were slightly more prominent than decrease in LC levels. A possible explanation is that PFOA exposure decreased LC/ALC/PLC via currently unknown, PPAR alpha independent mechanism, and the increased CRAT expression actually served as protective compensation against decreased level of ALC and PLC, which seems to be still insufficient to offset the decrease of ALC and PLC. This hypothesis is supported by the results of extragenous LC supplement treatment, in which the CRAT expression level was returned to control level. However, further study is needed to confirm the primary target of PFOA in the

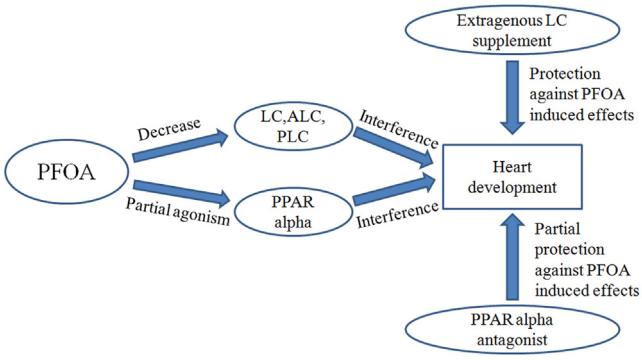

Fig. 6. Summary of the current study.
In this study, effects on the levels of endogenous l-carnitine (LC), acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC) were associated with PFOA-induced developmental cardiotoxicity, which is further confirmed by the extragenous LC supplement protection. The role of peroxisome proliferator-activated receptor alpha (PPAR alpha) was also investigated, which was found to be involved, but the PPAR alpha antagonist, GW6471 could only exert partial protection.

LC/ALC/PLC and CRAT system and the ultimate effects in hearts and potentially other organisms.

5. Conclusion

As summarized in Fig. 6, extragenous LC supplement antagonized PFOA-induced developmental cardiotoxicity in chicken embryo, while PPAR alpha antagonist GW6471 had limited effects. Developmental PFOA exposure was associated with decreased LC, ALC and PLC levels and elevated CRAT expression levels in ED6 chicken embryo heart. Effects on the levels of endogenous LC and its derivatives might be contributing to PFOA-induced developmental cardiotoxicity in chicken embryo.

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Conflict of interest

There are none.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etap.2016.10.017.

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