Oxidative Stress and Calcium Dyshomeostasis Mediated CPF-Induces EPC Cell Apoptosis and Necroptosis

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Research Article

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Abstract

Chlorpyrifos (CPF) is a broadly used organophosphate insecticide, and as environmental contamination has been confirmed to be harmful to the health of humans and animals, the effects of CPF on fish are still unclear. We aim to detect whether CPF has effects on the common carps, and identify the mechanisms of the effects. In accordance with the IC_{50} of CPF for epithelioma papulosum cyprini (EPC) cells was 8.945 μ M, 0, 3, 5 and 7 μ M CPF were marked as C, L, M and H group. The results indicated that CPF exposure led to oxidative stress in EPC cells. Moreover, Calcium ion (Ca^{2+}) imaging indicated that CPF triggers Ca^{2+} dyshomeostasis, as induced ER Ca^{2+} fluxed into cytoplasm and mitochondrial, and mediates endoplasmic reticulum (ER) stress and mitochondrial Ca^{2+} overload, which have further confirmed with the increased transcription of ER stress related genes. Meanwhile, CPF induced collapse of mitochondrial membrane potential (MMP), enhancement of ROS generation and depletion of ATP level in both cytoplasm and mitochondrial, substantiated mitochondrial dysfunction has been evoked. Besides, the AO/EB staining and flow cytometry analysis ascertained that CPF induces apoptosis and necroptosis in EPC cells. Hence, we further verified that via determining the expression of apoptotic pathway genes and necroptotic pathway genes. Consequently, we illuminated the mechanisms of CPF effects on the common carps from the perspective of Ca^{2+} homeostasis, and provided a novel concept for investigating the toxicity of CPF as environmental pollution on aquatic organisms.

Highlights

- CPF caused EPC cell damage
- CPF induced EPC cell apoptosis and necroptosis.
- CPF triggered oxidative stress and Ca^{2+} dyshomeostasis in EPC cells.
- ER stress and mitochondrial dysfunction mediated CPF-induced EPC cell death.

Introduction

Chlorpyrifos, [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate] (CPF), as an organophosphorus pesticide (Greene et al.) is widely used in the agricultural sector to control a broad-spectrum of insects of economically dominant crops. Mohan (2017) has reported that approximately 5 to 10 million pounds of CPF are consumed annually in the United States. A similar amount of CPF has been consumed in Japan annually as 105,263 tons (approximately 23 million pounds) (Derbalah et al. 2019). And as estimated over 200,000 tons (approximate 44 million pounds) CPF were consumed in 2015 worldwide, and annual growth in global demand for CPF and consequent wide distribution in the environment (Huang et al. 2020). High consumption of CPF results in excessive residuals accumulated in soils and ground water (Liu et al. 2019). The surface water polluted by CPF via precipitation runoff, discharged stormwater from agricultural land and irrigation water runoff (Ensminger et al. 2011). A nationwide assessment indicated that CPF was contained in 9 % of samples which were sampled from over ten thousand (> 10,000) streams in U.S., and declared maximum CPF level arriving 3.96 μ g/L (Williams et al. 2014), which obviously exceeded the

benchmark concentration of CPF (0.57µg/L) to freshwater fish suggested by U.S. Environmental Protection Agency (EPA 2000), which means CPF has posed human and animal health risks. The main toxicity of CPF is neurotoxicity as the result of acetylcholinesterase inhibition. Additionally, genotoxicity (Rahman et al. 2002), reproductive toxicity, hepatotoxicity (Kammon et al. 2010), nephrotoxicity (Chen et al. 2011) and lung (Yazdinezhad et al. 2017) toxicity of CPF have been gradually verified. Park et al. (2013) have illuminated that CPF induced apoptosis and autophagy via a decrease of cleaved Caspase3 and P62, with an increase of LC3-II in SH-SY5Y cells. CPF mediated neuroapoptosis via suppressed mitophagy in SH-SY5Y cells (Dai et al. 2015). The generation of ROS and phosphorylation of AMP-activated protein kinase (AMPK) contributed to testicular-cell apoptosis induced by CPF has been evidenced by Chen et al. (2018). The up-regulated BCL2 associated X (BAX), and Caspase3 (CASP3) protein and mRNA expression and down-regulated BCL-2 protein and mRNA expression as the consequences of CPF-treated rats, and indicating that CPF induces apoptosis in testicular tissue (Albasher et al. 2020).

Calcium ion as a utility and ubiquitous intracellular signal regulates various fundamental cellular processes (Berridge et al. 2003), such as proliferation, differentiation, metabolism and cell death (Carreras-Sureda et al. 2018). The endoplasmic reticulum (ER) as the crucial intracellular organelle of protein synthesis, folding and main storage site of intracellular Ca^{2+} (Abhari et al. 2019). Hence, ER Ca^{2+} homeostasis plays a critical role in cell function and survival. Numerous studies have reported that palmitate induced high oxidative conditions trigger Ca^{2+} release from ER and in turn deplete ER Ca^{2+} storage (Cnop et al. 2010, Xu et al. 2015). Due to high calcium concentration is necessary for the activity of ER resident chaperones demanded by protein folding, the resulting deficiency of ER Ca^{2+} triggers ER stress, thereby subsequently aggravating oxidative stress (Eizirik et al. 2008, Ly et al. 2017). As elucidated by Li et al. (2018), ER Ca^{2+} influx to cytoplasm and mitochondria as the results of selenium S silenced mouse hepatoma cells, and suggested potential relationship with IP3R channel. Due to the plasmalemmal ion channels operating consistent Ca^{2+} influx, the cytosolic and mitochondrial Ca^{2+} homeostasis are further impaired, induces mitochondrial overload (Ly, Xu, Choi, Ha, Thoudam, Cha, Wiederkehr, Wollheim, Lee and Park 2017). Previous study has demonstrated that mitochondrial Ca^{2+} overload as the characteristics of apoptosis and necrosis (Danese et al. 2017). Mitochondrial Ca^{2+} overload results in the swelling of mitochondria with disturbance and fracture of the outer membrane and liberates mitochondrial apoptotic factors into cytoplasm as a result of apoptotic signals response, including oxidative stress, ER stress (Giorgi et al. 2012). Sustained higher mitochondrial Ca^{2+} concentration priority to mitochondrial permeability transition pore (MPTP) opening has been proved in cardiomyocytes from overexpressing calmodulin-dependent protein kinase II (CaMKII) inhibitory protein transgenic mice, and concludes Ca^{2+} promote MPTP opening and myocardial apoptosis via CaMKII activation (Joiner et al. 2012, Stoll et al. 2019). Kolar et al. (2011) found that fermentable fiber synergistically mediates mitochondrial Ca^{2+} overload and triggers Ca^{2+} -dependent apoptotic in colonocytes. In addition, mitochondrial Ca^{2+} overload, PTP opening and apoptosis due to the accumulation of the GM1-ganglioside have been identified in the human GM1-gangliosidosis mouse model (Sano et al. 2009). Furthermore, mitochondrial Ca^{2+} overload is also known as harmful to cardiomyocytes and triggers necroptosis. As reported by Luongo et al. (2017), the deletion of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the heart of adult mouse model induces myocardial

dysfunction and death, which associates with mitochondrial Ca^{2+} overload, superoxide generation enhancement, MPTP opening and necrotic cell death. Ca^{2+} overload/ROS/MPTP opening axis involved in RIPK3 promoted ER stress-triggered necroptosis in cardiac ischaemia-reperfusion injury (Zhu et al. 2018). Recently, Zheng et al. (2020) have evidenced that di-(2-ethyl hexyl) phthalate (DEHP) induced chicken cardiomyocytes necroptosis via Ca^{2+} overload.

Taken together, CPF as water contamination has threatened human and animal health. The common carps (*Cyprinus carpio* L.) as worldwide economically important freshwater fish species, has been considered as a bioindicator species for toxication studies (Altun et al. 2017). However, the effects of CPF on the common carps and the mechanisms of effects were still unclear. Under the fundamentals of the half maximal inhibitory concentration (IC_{50}) results, and EPC cells were applied as study objects, 0, 3, 5, 7 μM CPF exposure models were established in this study. After incubated 24 h, commercial kits were used to determine the levels of oxidative stress, ATP, ROS, and changes of mitochondrial membrane potential (MMP). Then, fluorescence staining was applied to image Ca^{2+} flux. Also, flow cytometry analysis, AO/EB staining, quantitative real-time PCR (qRT-PCR) and Western blot analysis were applied to identify the express of ER stress-related genes, apoptotic and necroptotic pathway-related genes. The arm of this study was to clarify the effects and mechanism of CPF on EPC cells associated with apoptotic and necroptotic pathway from the perspective of Ca^{2+} homeostasis, and provides a new concept for studying the effects of CPF as environmental pollution on aquatic organisms.

Materials And Methods

Cell resuscitation and Incubation

The frozen epithelioma papulosum cyprini (EPC) cells were stored in liquid nitrogen cell storage container at the College of Veterinary Medicine. The cryopreserved tubes were immersed into a 37 °C sterile water bath immediately, and shaken tubes to accelerating melting rate in succession. The melted liquid was transferred into medium mixture which containing 10% fetal bovine serum (Biological Industries, Beit HaEmek, Israel) and 1% double antibody (Sigma-Aldrich, St. MO, USA) of M199 cell culture medium. Transferred cell culture medium into the 25 cm^2 culture flasks which placed into automatic incubator at 27 °C.

Cytotoxicity Test

In the present study, the cytotoxicity of CPF was determined by using the Cell Counting Kit-8 assay (CCK-8 MedChemExpress, Monmouth Junction, NJ, USA). CPF (purity 99%) was purchased from Aladdin (C109843, China) and prepared in dimethyl sulfoxide (DMSO) (purity 99%). DMSO were applied to dissolve the CPF, and culture medium was added to estimate target CPF concentration. The final concentration of DMSO in the culture media was maintained below 0.05 % in all experiments. The cells were transferred into 96-well plates (NEST, Wu Xi, China) by using trypsin digestion solution (0.25%), and treated respectively with the different concentrations of CPF (0, 0.5, 1, 3, 5, 7 or 9 μM). After 24 h incubation, and CCK-8 assay

were processed to detect cell viability. The culture medium was substituted with 10% CCK-8 working solution into each well. After incubation for 1 h, Cytation™ 5 cell Imaging Multi-Mode Reader (Biotek Instrument, Vermont, USA) was operated to measure the absorption (450 nm). In accordance with the cell viability determinations, three concentrations of CPF as 3, 5 and 7 μM were applied into consequent cells incubation.

Determination of Antioxidant Parameters

In present study, commercial kits were used in the present study to detect whether CPF treatment can cause oxidative stress in EPC. Total antioxidative capacity (T-AOC, product No.A015-1-2), catalase (CAT, product No.A007-1-1), total superoxide dismutase (SOD, product No.A001-3) and malondialdehyde (MDA, product No.A003-1-1) were detected with assay kits (Nanjing Jianchang Bioengineering Research Institute, Nanjing, China) and all experimental operations were in accordance with instructions of kits.

AO/EB Staining

The cells of all groups were collected and resuspended with PBS. Based on the operation protocol, the AO/EB staining work solution (meilunbio, Dalian, China) were loaded to ultimate concentration as 10 $\mu\text{L}/\text{mL}$ for 5 min. The live, apoptotic and necrotic cells were photographed by fluorescence microscope (Thermo Fisher Scientific, USA). The cells were analyzed by using Image J software (National Institutes of Health).

Flow Cytometry Analysis

Each group cells were gathered respectively and twice rinsed with PBS, and suspended in PBS. The Annexin V-FITC/PI apoptosis kit (product No.KGA106, keygen BioTECH, China) was applied to cell death quantification. Based on the operation protocols, EPC cells were staining with the working solution which containing 5 μL of Annexin V-FITC, 5 μL of PI and 490 μL of binding buffer. The live, apoptotic and necrotic cells were quantified by flow cytometry (BD FACS Aria™ II, BD Biosciences, USA). In addition, flow cytometry was applied to determine the effects of CPF on MMP in EPC cells. JC-1 assay kit (product No.C2006, Shanghai Beyotime Biotechnology, Shanghai, China) was performed based on the manufacturer's instructions, and flow cytometry were subsequently used to assay the cells in all group which were loaded with JC-1 probe.

Ca²⁺ Imaging

The Ca²⁺ level was detected with three different Ca²⁺-fluorescent probe, Fluo-4 acetoxymethyl ester (AM) (product No. F14201, 5 μM , Invitrogen, Thermo Fisher Scientific, USA) were applied to determine the cytoplasmic Ca²⁺ level, Rhod-2 AM (product No.R1244, 10 μM , Invitrogen, Thermo Fisher Scientific, USA) were used for determine the mitochondria Ca²⁺ level, and Mag-Fluo-4 AM (product No.M14206, 5 μM , Invitrogen, Thermo Fisher Scientific, USA) were demanded for the ER Ca²⁺ level. The cell of each groups was loaded respective Ca²⁺ probes for 30–60 min at 27 °C, then the mitochondria and ER were labeled with Mito-Tracker Green FM (product No.M7514, 5 μM , Invitrogen, Thermo Fisher Scientific, USA) and ER-Tracker Red (product No.C1041, 1 μM , Shanghai Beyotime Biotechnology, Shanghai, China) respectively.

Fluorescence microscope (Thermo Fisher Scientific, USA) were performed to the image acquisition and Image J software (National Institutes of Health) were applied to quantitated fluorescence intensity.

MMP Assay

In order to investigate whether CPF can negatively affect mitochondrion, we measured MMP with JC-1 assay kit (product No.C2006, Shanghai Beyotime Biotechnology, Shanghai, China). Base on the instructions, cells were incubated at 27 °C for 20 min after loaded with JC-1 solution (10µg/mL), then washed twice with JC-1 buffer solution. The fluorescence microscope (Thermo Fisher Scientific, USA) was applied to detecting the monomeric JC-1 green emission and aggregative JC-1 red emission. The MMP assessed as the fluorescence scale of red emission normalizing to green emission was quantified with flow cytometry.

Mitochondria Isolation

The cells of each groups were obtained and respectively used to isolate mitochondria for later experiments. The cells were firstly homogenized, and then were centrifugated at 4 °C at 2000 rpm for 10 min. The supernatant was obtained and was put into a new EP tube. The EP tube was centrifugated at 4 °C at 12000 rpm for 10 min. The mitochondrial of EPC cells were isolated by using cell mitochondria isolation kit (product No.C3601, Shanghai Beyotime Biotechnology, Shanghai, China). The obtained sediment was the isolated mitochondria was used for subsequent experiments.

Detection of Intracellular and Mitochondrial ROS

To observe and determine the effects of CPF on the oxidative stress levels, we detected intracellular ROS level and mitochondrial ROS (mito-ROS) level by using ROS assay kit (product No.E004-1-1, Nanjing Jianchang Bioengineering Research Institute, Nanjing, China). The cells were staining with 2,7-dichlorofuresc in diacetate (DCFH-DA, 10 µM) for 45 min. Fluorescence microscope (Thermo Fisher Scientific, USA) were applied to observe the cells, and ROS levels were quantified by fluorospectrophotometer.

ATP Measurement

The cells were collected and incubated in 6-well plates. And intracellular and mitochondrial ATP levels were measured with ATP assay kit (product No.A095-1-1, Nanjing Jianchang Bioengineering Research Institute, Nanjing, China) base on the manufacturer's instructions.

RNA Extraction and Quantitative Real-time PCR (qRT-PCR)

Total RNA from EPC cells were extracted respectively with TRIzol reagent (product No.15596-018, Life Technologies, California, USA) base on the manufacturer's instructions, and were reverse transcript into mRNA respectively with the BioRT Master HiSensi cDNA First Strand Synthesis kit (product No.BSB40M1, Bioer Technology, Hangzhou, China). RT-PCR was used the BioRT Real Time RT-PCR kit (product No.BSB33S1, Bioer Technology, Hangzhou, China) and the QuantStudio 3 (Thermo Fisher Scientific, USA). The primers (Table 1) of mRNA used for qPCR were synthesized by Shanghai Sangon Biotechnology Co.

Ltd, (Shanghai, China). β -actin was used as reference. Each PCR product performed only one peak in melting curve. The relative abundances of mRNA were calculated base on the $2^{-\Delta\Delta Ct}$ method.

Table 1
The primer sequences used for qRT-PCR.

Genes	Sequence (5' – 3')	
CAPN1	Forward:AAGGACCGCAGGAAGAAGAGGAG	Reverse:CCCGCTTCAGATGAACACCAGAC
ATF4	Forward:GCTCGTGGTCTCCCTCTTCCTC	Reverse:CATCGCCTGCTGTGCTCTGC
eIF2 α	Forward:TCCAGGCGACGAATCCGATCC	Reverse:CTTATCCACCCGAATGACCACCAC
BIP	Forward:GGCATTGAGACTGTTGGAGGAGTG	Reverse:GGTTGTGGAAGCAGTGGAGAAG
CHOP	Forward:GATCCGCCCGTTACCAATCG	Reverse:CACCAACACCACCTTCGCTGAG
PERK	Forward:AGACGGAGAAGGCTCACAGGTG	Reverse:CGTGGTGGCAGCGATACAGAAG
DRP1	Forward:TCCAGCTTCCTCAGATCGTAGTCG	Reverse:AGTCCCGTCCAACCAAACCTCTCC
MFN1	Forward:TCACCTCTTCCTCTGCTCCCTTG	Reverse:CCACAGAACGCCACACCACTC
BCL-2	Forward:ATGCGTGAATAAGGAGATGA	Reverse:AGACCGAAGACCGTTACT
BAX	Forward:TCCACTCTTCAACCAACTC	Reverse:GCCAATAGTCTGCCATGT
Cyt-c	Forward:ACAGCCAAGACAGTCGTTACACAG	Reverse:AGACTTGTTGAGCGTGAAGCAGAC
CASP3	Forward:GCTGTGCTTCGTTAGTGT	Reverse:GAACCAAGAACCGCTCAT
CASP9	Forward:AGGCAACTGGTGACAGACTTAGAGA	Reverse:GACAGAAGGAGGCAGACGAACAC
CASP8	Forward:GCTGGTTACAGATGGTGCTGGTC	Reverse:GTCCTGGCCTTGTTCACTTCCTTC
FADD	Forward:GACGGCATAACAGGAGAAGCACAG	Reverse:CAGTCCCGCAGAGCTTTGATGAG
RIPK1	Forward:AAGATGCGGGAGGGTCTGATGG	Reverse:CAGGAGGGCGTTGGGTTTGATG
RIPK3	Forward:TACAGCGGTGTTCAGTCCAGTC	Reverse:AGCTCATCCAGTCCTTCGGTCTC
MLKL	Forward:GGAACCGCTGTCACATGACTGTC	Reverse:GTCCACCAACACTCCAGCAGAAG
β -actin	Froward:GGCTCTCTTCCAGCCTTCCT	Reverse:AGCACGGTGTGTCATACAG

Protein Extraction and Western Blot Analysis

The protein of cells was extracted by using PMSF. 100 μ L of cell lysis buffer (product No.P0013, Shanghai Beyotime Biotechnology, Shanghai, China) and 1 μ L of PMSF (product No.ST506, Beyotime Biotechnology, Shanghai, China) were added per 1×10^6 cells, and swing cell on ice for 30 min. And centrifugated at 4°C (12000 rpm, 25 min) to obtain supernatants, then added one quarter of SDS, boiling mixture for 10 min and reposit at -20°C. SDS-polyacrylamide gel electrophoresis were applied to detect the total protein. And then the gels were transferred to PVDF membranes at 200 mA in Tris-glycine buffer containing 20 %

methanol for 60–110 min. After being blocked with 5% skim milk for 120 min at 37 °C, the diluted primary antibodies were added on membranes overnight at 4°C, the dilution factor and sources of primary antibody are summarized in Table 2. Then, the membranes were washed and continuously dip in secondary antibodies against rabbit IgG (1:8000, Immunoway Suzhou, China). The protein bands were detected with chemiluminescence system (Applygen Tchnologies, Beijing, China) and X-ray films (TransGern Biotech, Beijing, China). The relative protein abundances were normalized as β -actin, and the band intensity was quantified by using Image J software (National Institutes of Health).

Table 2
Antibodies used for Western blot.

Antibodies	Dilution ratio	KD	Resource
Caspase 3	1: 500	30	Wanleibio, China
Caspase 9	1:500	35	Wanleibio, China
Caspase 8	1:500	55	Wanleibio, China
RIPK1	1:2000	76	ABclonal Biotechnology
RIPK3	1:2000	57	Laboratory made
MLKL	1:2000	55	Laboratory made
FADD	1:2000	26	ABclonal Biotechnology
Cyt-c	1:500	19	Wanleibio, China
β -actin	1:1500	42	ABclonal Biotechnology

Statistical Analysis

The data were analyzed by using GraphPad Prism (version 8.2, Graph Pad Software Inc., San Diego, CA, USA), t Student’s *t*-test, and one-way or two-way ANOVA with Turkey’s multiple comparison test. The data are present into the mean $\pm$ standard deviation, and statistically significant differences at *P*< 0.05.

Results

CPF Attenuates EPC Cell Viability

CCK-8 assay was performed to determine CPF cytotoxicity, and as results shown in Fig. 1A. The cell viability at 1, 3, 5, 7, and 9 μ M of CPF was 92.74%, 80.45%, 72.91%, 61.25%, and 44.67%, respectively. The cell viability was significantly (*P*< 0.05) reduced by 3, 5, 7, 9 μ M of CPF compared with C group and significantly (*P*< 0.05) reduced with the increase of CPF exposure. The viability curve identified that the IC₅₀ of CPF for EPC cells was 8.945 μ M, and the 95% confidence limits was 0.8834 to 1.075. The CPF concentration of 3, 5 and 7 μ M were chosen for consequent experiments as over 80%, 70%, and 60% of the cells were viable with the respective CPF treatment, and marked as L group, M group and H group.

CPF Triggers Oxidative Stress

To illustrate the level of oxidative stress in cells, T-AOC level, SOD and CAT activity, and MDA were detected in this study. As displayed in Fig. 1B, the level of T-AOC and activity of SOD and CAT were concentration-dependent and expressed significant ($P < 0.05$) decreasing tendency, the lowest activities were represented in H group. Meanwhile, MDA accumulations were proportional to the CPF concentration have been verified, as the MDA levels significant ($P < 0.05$) increasing with the enhancement of CPF exposure, and the maximum content were perceived in H group. The detection of T-AOC, SOD, CAT and MDA levels illuminated CPF induced antioxidant capacity reduction, which contributes to cell damage.

CPF Induces cell Apoptosis and Necroptosis

To observe CPF effects on EPC cells, AO/EB staining was used in this study as shown in Fig. 1C. necroptotic cells were stained as red, live cells were dyed as green and apoptotic cells were labeled as orange. The abundant red and orange cells were found in CPF-exposed groups, and became dominant with the increasing of CPF exposure. The portion of apoptotic and necroptotic cells were highest in the H group, and respectively performed significant ($P < 0.05$) differences with C group. To quantitative evaluate CPF-induced cell death, AO/EB staining and Annexin V/PI assay with flow cytometry analysis have been applied in this study. As shown in Fig. 1D, only 3.00% (Q2 + Q3) and 1.47% (Q1) of cells had apoptosis and necrosis in C group. Both proportions of apoptotic cells and necrotic cells were distinct aggrandized in the CPF-treated groups, and reached the maximum percentages in the H group as 20.39% (Q2 + Q3) and 6.78%, and over 6 times that of C group. This consequence demonstrated that CPF triggered apoptotic and necroptotic cell death in EPC cells.

To verify the CPF effect on EPC cell apoptosis, the mRNA and protein expressions of apoptosis-related factors have detected in the present study (Fig. 2A). In comparison with C group, the expression of proapoptotic genes, including BAX, Cytochrome *c* (Cyt-*c*), CASP3 and Caspase9(CASP9), displayed increasing tendency with the enhancement of CPF exposure, and BCL-2 shown significant ($P < 0.05$) decreasing with the rising of CPF concentration. Especially, the mRNA and protein expression of all apoptotic pathway factors increased significantly in H group which was compared with C group ($P < 0.05$), revealing that apoptosis has been triggered. We found that the lowest level of BCL-2 presents in the H group which performed highest expression of BAX, Cyt-*c*, CASP9 and CASP3. The expression of all apoptosis-related factors except CASP9, significant increased ($P < 0.05$) in the H group compare to in the M group, indicating that one of the main types of cell death induced by CPF at 7 μ M is apoptosis.

Evaluating the CPF effect on EPC cell necroptosis, the mRNA and protein expressions of the necroptosis-related genes, including Caspase8 (CASP8), Fas associated via death domain (Lai et al.), receptor interacting serine/threonine kinase (RIPK)1, RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL), were tested as shown in Fig. 2B. We found that the expression of CASP8 shown significant attenuating expression in CPF-treated group relative to C group ($P < 0.05$), and continuous decreasing with the heightened concentration of CPF treatment. The mRNA expressions of RIPK1 and FADD performed significant increasing tendency with enhancement of CPF treatment. Consistently, RIPK3 and MLKL

expressed significantly ($P < 0.05$) enhanced with denser CPF exposure, which indicating that CPF induced cell occurred necroptotic cell death.

CPF Causes Ca^{2+} Dyshomeostasis

To further consideration about the effect of CPF on Ca^{2+} homeostasis, we monitored Ca^{2+} levels of ER, cytoplasm and mitochondrial respectively. ER Ca^{2+} level was imaged with Mag-fluo-4 AM (green) and co-labeled by ER-Tracker Red (red) (Fig. 3A), and found that ER Ca^{2+} levels were significant ($P < 0.05$) depleted with increasing of CPF concentration, which corresponding to the ER expression no difference. In addition, cytoplasmic Ca^{2+} was labeled with Fluo-4 AM (Fig. 3B), and results display that cytosolic Ca^{2+} concentrations were significantly ($P < 0.05$) increased with CPF exposure aggravation. Consequently, CPF exposure resulting in ER Ca^{2+} outflow and accumulation in cytoplasm. Furthermore, Rhod-2 AM (red) was used to image mitochondrial Ca^{2+} level (Fig. 3C) and MitoTracker Green FM (green). The images shown that mitochondrial Ca^{2+} levels were significant ($P < 0.05$) overloaded associate to the enhancement of CPF exposure, which corresponding to no difference mitochondrial expression. As a result, CPF induces Ca^{2+} outflow from ER into cytoplasm and mitochondria, and trigger mitochondrial Ca^{2+} overload.

Ca^{2+} Dyshomeostasis Mediates ER Stress

To elucidate whether Ca^{2+} dyshomeostasis induced ER stress, the expression of intracellular ER stress-related factors, including activating transcription factor 4 (ATF4), eukaryotic translation initiation factor 2 subunit alpha (eIF2 α), ER luminal binding protein precursor (BIP), DNA damage inducible transcript 3 (CHOP), and eukaryotic translation initiation factor 2-alpha kinase 1 (PERK), were detected in this study (Fig. 3D). The mRNA expression of ER stress-related genes shown increasing tendency with the reinforcement of CPF exposure, and significant ($P < 0.05$) upregulated in H group compare with C group. And coincident results were identified in protein expression as the levels of ATF4, CHOP and PERK shown significant ($P < 0.05$) increasing associate with the elevated CPF exposure, and the peak expression were reached in H group. Thus, CPF exposure induces ER stress in EPC cells.

Ca^{2+} Dyshomeostasis Evokes Mitochondrial Dysfunction

To demonstrated the effect of ER Ca^{2+} depletion induced mitochondrial Ca^{2+} overload on mitochondrial function state, the change of MMP was tested by assessing $\Delta\Psi\text{m}$ with JC-1 assay kit. The fluorescence images elucidated that CPF exposure induced collapse of $\Delta\Psi\text{m}$ (Fig. 4A). And flow cytometer results shown $\Delta\Psi\text{m}$ obvious suppressed by CPF treatment as shown in Fig. 4B. We found that both red and green emission from the stained C group cells were intensive, which illuminated the present of both mitochondrial JC-1 aggregates and cytoplasmic JC-1 monomer. And in consistent with staining imagines, JC-1 aggregates were eliminated with increasing CPF concentration, as reduced intensity of the red emission signal.

Additionally, both cytoplasmic ROS staining and quantitative ROS levels in the cytoplasm and mitochondria confirmed that ROS generation exerted a significant stimulative effect by CPF (Fig. 4C). and as shown in Fig. 4D, the ATP levels in cytoplasm and mitochondrial were depleted with increasing

concentration of CPF, and the lowest ATP level were detected in H group. So, the ATP levels were significantly depleted by CPF.

As Dynamin related protein 1 (DRP1) and mitofusin 1 (MFN1) are vital for mitochondrial dynamics (Song et al. 2017), to confirm whether mitochondrial fusion/fission has impacted by CPF, we assess the expression of DRP1 and MFN1 in CPF exposed cells. As results shown in Fig. 4E, the mRNA level of DRP1 was significantly ($P < 0.05$) increased with CPF exposure increasing. In contrast, the MFN1 expression in mRNA was significantly ($P < 0.05$) decreased, which presenting mitochondrial fission/fusion disorder under CPF-exposed cells, and consequent imbalanced mitochondrial morphology. Therefore, CPF mediates mitochondrial Ca^{2+} overload triggers MMP collapse, ROS generation, ATP depletion, mitochondrial fusion/fission disorder, and induces mitochondrial dysfunction.

Discussion

The toxicity of CPF as environmental contamination has been demonstrated hazardous to fish and other aquatic animals, such as inducing apoptosis of liver cells (Wang et al. 2020) and kidney cells (Zhang et al. 2019). In this study, we explored the cytotoxicity of CPF on EPC cells from the perspective of calcium homeostasis, and the results exhibited that CPF could cause oxidative stress and ER stress, mediate mitochondrial Ca^{2+} overload and MMP depletion, and active mitochondria-mediated apoptotic and necroptotic pathway, eventually induce EPC cells apoptosis and necroptosis.

Ca^{2+} homeostasis is retained by the integrated and interaction of Ca^{2+} transport molecules, buffer and sensors, regulating numerous cellular processes, such as metabolism, enzyme activity modulation, cell proliferation, differentiation and division, and cell motility (Krebs et al. 2015). Disturbance of Ca^{2+} homeostasis participates in various cytopathological process, including apoptosis (Zhao et al. 2021), necroptosis (Zhu, Hu, Jin, Li, Tian, Toan, Li, Zhou and Chen 2018) and autophagy (Liu et al. 2018). Oxidative stress enhances cytosolic Ca^{2+} content via integrated calcium pumps, exchangers, channels and binding proteins (Zima and Blatter 2006). Tang et al. (2013) reported that oxidative stress induced by tert-butyl hydroperoxide affected calcium signaling pathway in CHO cells, and triggered disruption of Ca^{2+} homeostasis. And we found that oxidative stress induced by CPF exposure has been identified in accordance with oxidative parameter activity results and ROS level, as decreasing activity of T-AOC, CAT and SOD and increasing level of MDA, and enhancing generation of ROS. Additionally, Ca^{2+} imaging by Mag-Fluo-4 AM, Fluo-4 AM and Rhod-2 AM probes were applied to exhibited Ca^{2+} flux, and we found that CPF induced Ca^{2+} depleted in ER and influx to cytoplasm and mitochondria, which results in mitochondrial Ca^{2+} overload. The mitochondrial Ca^{2+} dyshomeostasis contributed to the mitochondrial permeability transition pore (MPTP) opening (Menezes-Filho et al. 2017), mitochondrial dysfunction (Biczo et al. 2018), mitochondrial ROS generation enhancement (Sun et al. 2017) and ATP depletion (Liang et al. 2010) and induce apoptotic cell death (Rasola and Bernardi 2007). Our results were consistent with previous studies, as CPF induced MMP collapse represented MPTP sustained and/or excessive opening, the enhanced ROS level, declined ATP synthesis and imbalance of mitochondrial fusion/fission indicated abnormal

mitochondrial function, and both flow cytometry analysis and expressions results have represented cell apoptosis, have occurred in CPF-exposed EPC cells. Moreover, necroptotic cell death is also associated with Ca^{2+} dyshomeostasis. Kalogeris et al. (2012) has overviewed that the increasing of intracellular and mitochondrial Ca^{2+} levels, as the result of dysfunctional ATPase-dependent ion transport mechanisms, associates with cell death by necroptotic and apoptotic mechanisms. Additionally, the aggregate of cytosolic Ca^{2+} induces necroptosis in human neuroblastoma (Nomura et al. 2014). A recent study has proposed that the Ca^{2+} overload/ROS/MPTP pathway is involved in cardiomyocytes necroptosis (Zhu, Hu, Jin, Li, Tian, Toan, Li, Zhou and Chen 2018). We found that necroptotic cell death also occurred in CPF-exposed EPC cells as the increased expressions of RIPK1, RIPK3 and MLKL, and decreased expressions of CASP8 and FADD. As a consequence, CPF induces EPC cell apoptosis and necroptosis via regulating by Ca^{2+} homeostasis.

Conclusion

Oxidative stress triggered Ca^{2+} dyshomeostasis caused ER stress and mitochondrial Ca^{2+} accumulation, MMP collapse and mitochondrial fusion/fission disorder, eventually induced EPC cell death. Since, CPF regulates cell apoptosis and necroptosis depends on Ca^{2+} dyshomeostasis-mediated mitochondrial dysfunction. This study not only provides vital data for understanding CPF pathology, but also proposed the novel perspective to explore the effects of CPF as environmental containment on aquatic organisms.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The origin sequences data used in the qRT-PCR part during this study are available from GeneBank (NCBI) under Nucleotide (<https://www.ncbi.nlm.nih.gov/nucleotide>).

Competing interests

The authors declare that they have no competing interests.

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collection, analysis, and interpretation of data and in writing the manuscript.

Author's contributions

ZM Conceptualization, Investigation, Writing – original. JC Investigation, Data curation. MY Investigation, Writing - editing. XT Funding acquisition, Writing - editing.

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References

1. Abhari BA, McCarthy N, Le Berre M, Kilcoyne M, Joshi L, Agostinis P, Fulda S (2019) Smac mimetic suppresses tunicamycin-induced apoptosis via resolution of ER stress. *Cell Death Dis* 10(3):155
2. Albasher G, Albrahim T, Alsultan N, Alfaraj S, Alharthi MS, Kassab RB, Abdel Moneim AE (2020) Red beetroot extract mitigates chlorpyrifos-induced reprotoxicity associated with oxidative stress, inflammation, and apoptosis in rats. *Environ Sci Pollut Res Int* 27(4):3979–3991
3. Altun S, Ozdemir S, Arslan H (2017) Histopathological effects, responses of oxidative stress, inflammation, apoptosis biomarkers and alteration of gene expressions related to apoptosis, oxidative stress, and reproductive system in chlorpyrifos-exposed common carp (*Cyprinus carpio* L.). *Environ Pollut* 230:432–443
4. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4(7):517–529
5. Biczó G, Vegh ET, Shalbueva N, Mareninova OA, Elperin J, Lotshaw E, Gretler S, Lugea A, Malla SR, Dawson DR, Chala P, Whitelegge J, French SW, Wen L, Husain SZ, Gorelick FS, Hegyi P, Rakonczay Z, Jr., Gukovsky I, Gukovskaya AS (2018) Mitochondrial Dysfunction, Through Impaired Autophagy, Leads to Endoplasmic Reticulum Stress, Deregulated Lipid Metabolism, and Pancreatitis in Animal Models. *Gastroenterology*, 154(3):689–703
6. Carreras-Sureda A, Pihan P, Hetz C (2018) Calcium signaling at the endoplasmic reticulum: fine-tuning stress responses. *Cell Calcium* 70:24–31
7. Chen R, Cui Y, Zhang X, Zhang Y, Chen M, Zhou T, Lan X, Dong W, Pan C (2018) Chlorpyrifos Induction of Testicular-Cell Apoptosis through Generation of Reactive Oxygen Species and Phosphorylation of AMPK. *J Agric Food Chem* 66(47):12455–12470
8. Chen W, Jiang H, Wang M, Jin J, Feng S, Bi Y, Wang H, Mao Y, Shou Z, Chen J (2011) Effects of chlorpyrifos exposure on kidney Notch2-Jagged1 pathway of early prenatal embryo. *Birth Defects Res B Dev Reprod Toxicol* 92(2):97–101
9. Cnop M, Ladrière L, Igoillo-Esteve M, Moura RF, Cunha DA (2010) Causes and cures for endoplasmic reticulum stress in lipotoxic beta-cell dysfunction. *Diabetes Obes Metab* 12(Suppl 2):76–82

10. Dai H, Deng Y, Zhang J, Han H,Zhao M, Li YZhang CTian J, Bing G, Zhao L (2015) PINK1/Parkin-mediated mitophagy alleviates chlorpyrifos-induced apoptosis in SH-SY5Y cells. *Toxicology* 334:72–80
11. Danese A, Patergnani S, Bonora M, Wieckowski MR,Prevati M, Giorgi C,Pinton P (2017) Calcium regulates cell death in cancer: Roles of the mitochondria and mitochondria-associated membranes (MAMs). *Biochim Biophys Acta Bioenerg* 1858(8):615–627
12. Derbalah A, Chidya R, Jadoon W,Sakugawa H (2019) Temporal trends in organophosphorus pesticides use and concentrations in river water in Japan, and risk assessment. *J Environ Sci (China)* 79:135–152
13. Eizirik DL, Cardozo AK,Cnop M (2008) The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* 29(1):42–61
14. Ensminger M, Bergin R, Spurlock F,Goh KS (2011) Pesticide concentrations in water and sediment and associated invertebrate toxicity in Del Puerto and Orestimba Creeks, California, 2007–2008. *Environ Monit Assess* 175(1–4):573–587
15. EPA HR, Assessment: Chlorpyrifos. US Environ, Protection Agency. In 2000
16. Giorgi C, Baldassari F, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S,Rimessi A,Suski JM,Wieckowski MR,Pinton P (2012) Mitochondrial Ca(2+) and apoptosis. *Cell Calcium*, 52(1):36–43
17. Greene DL, Khattak AJ,Liu J, Wang X, Hopson JL,Goeltz R (2017) What is the evidence concerning the gap between on-road and Environmental Protection Agency fuel economy ratings? *Transp Policy* 53:146–160
18. Huang X, Cui H, Duan W (2020) Ecotoxicity of chlorpyrifos to aquatic organisms: A review. *Ecotoxicol Environ Saf* 200:110731
19. Joiner ML, Koval OM,Li J, He BJ,Allamargot C, Gao Z, Luczak, ED,Hall, DD,Fink BD,Chen B,Yang J,Moore SA,Scholz TD,Strack S,Mohler PJ,Sivitz WI,Song LS,Anderson ME (2012) CaMKII determines mitochondrial stress responses in heart. *Nature*, 491(7423):269–73
20. Kalogeris T, Baines CP,Krenz M, Korthuis RJ (2012) Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol* 298:229–317
21. Kammon AM, Brar RS, Banga HS, Sodhi S (2010) Patho-biochemical studies on hepatotoxicity and nephrotoxicity on exposure to chlorpyrifos and imidacloprid in layer chickens. *Vet Arhiv* 80(5):663–672
22. Kolar S, Barhoumi R, Jones CK,Wesley J, Lupton JR,Fan YY,Chapkin RS (2011) Interactive effects of fatty acid and butyrate-induced mitochondrial Ca(2+)(+) loading and apoptosis in colonocytes. *Cancer* 117(23):5294–5303
23. Krebs J, Agellon LB,Michalak M (2015) Ca(2+) homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling. *Biochem Biophys Res Commun* 460(1):114–121
24. Lai F, Sinico C, De Logu A, Zaru M, Muller RH,Fadda AM (2007) SLN as a topical delivery system for *Artemisia arborescens* essential oil: in vitro antiviral activity and skin permeation study. *Int J*

25. Li X, Chen M, Yang Z, Wang W, Lin H, Xu S (2018) Selenoprotein S silencing triggers mouse hepatoma cells apoptosis and necrosis involving in intracellular calcium imbalance and ROS-mPTP-ATP. *Biochim Biophys Acta Gen Subj* 1862(10):2113–2123
26. Liang HL, Sedlic F, Bosnjak Z, Nilakantan V (2010) SOD1 and MitoTEMPO partially prevent mitochondrial permeability transition pore opening, necrosis, and mitochondrial apoptosis after ATP depletion recovery. *Free Radic Biol Med* 49(10):1550–1560
27. Liu F, Chen J, Wang P, Li H, Zhou Y, Liu H, Liu Z, Zheng R, Wang L, Yang H, Cui Z, Wang F, Huang X, Wang J, Sha W, Xiao H, Ge B (2018) MicroRNA-27a controls the intracellular survival of *Mycobacterium tuberculosis* by regulating calcium-associated autophagy. *Nat Commun* 9(1):4295
28. Liu H, Chen J, Wu N, Xu X, Qi Y, Jiang L, Wang X, Wang Z (2019) Oxidative degradation of chlorpyrifos using ferrate(VI): Kinetics and reaction mechanism. *Ecotoxicol Environ Saf* 170:259–266
29. Luongo TS, Lambert JP, Gross P, Nwokedi M, Lombardi AA, Shanmughapriya S, Carpenter AC, Kolmetzky D, Gao E, van Berlo JH, Tsai EJ, Molkentin JD, Chen X, Madesh M, Houser SR, Elrod JW (2017) The mitochondrial Na⁽⁺⁾/Ca⁽²⁺⁾ exchanger is essential for Ca⁽²⁺⁾ homeostasis and viability. *Nature*, 545(7652):93–97
30. Ly LD, Xu S, Choi SK, Ha CM, Thoudam T, Cha SK, Wiederkehr A, Wollheim CB, Lee IK, Park KS (2017) Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes. *Exp Mol Med* 49(2):e291
31. Menezes-Filho SL, Amigo I, Prado FM, Ferreira NC, Koike MK, Pinto IFD, Miyamoto S, Montero EFS, Medeiros MHG, Kowaltowski AJ (2017) Caloric restriction protects livers from ischemia/reperfusion damage by preventing Ca⁽²⁺⁾-induced mitochondrial permeability transition. *Free Radic Biol Med* 110:219–227
32. Mohan G More restrictions announced for toxic pesticide
33. Nomura M, Ueno A, Saga K, Fukuzawa M, Kaneda Y (2014) Accumulation of cytosolic calcium induces necroptotic cell death in human neuroblastoma. *Cancer Res* 74(4):1056–1066
34. Park JH, Lee JE, Shin IC, Koh HC (2013) Autophagy regulates chlorpyrifos-induced apoptosis in SH-SY5Y cells. *Toxicol Appl Pharmacol* 268(1):55–67
35. Rahman MF, Mahboob M, Danadevi K, Saleha Banu B, Grover P (2002) Assessment of genotoxic effects of chlorpyrifos and acephate by the comet assay in mice leucocytes. *Mutat Res* 516(1–2):139–147
36. Rasola A, Bernardi P (2007) The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis* 12(5):815–833
37. Sano R, Annunziata I, Patterson A, Moshiah S, Gomero E, Opferman J, Forte M, d'Azzo A (2009) GM1-ganglioside accumulation at the mitochondria-associated ER membranes links ER stress to Ca⁽²⁺⁾-dependent mitochondrial apoptosis. *Mol Cell* 36(3):500–511
38. Song M, Franco A, Fleischer JA, Zhang L, Dorn GW 2nd (2017) Abrogating Mitochondrial Dynamics in Mouse Hearts Accelerates Mitochondrial Senescence. *Cell Metab* 26(6):872–883 e5

39. Stoll S, Xi J, Ma B, Leimena C, Behringer EJ, Qin G, Qiu H (2019) The valosin-containing protein protects the heart against pathological Ca²⁺ overload by modulating Ca²⁺ uptake proteins. *Toxicol Sci*
40. Sun W, Wu X, Gao HY, J, Zhao W, Lu JJ, Wang J, Du G, Chen X (2017) Cytosolic calcium mediates RIP1/RIP3 complex-dependent necroptosis through JNK activation and mitochondrial ROS production in human colon cancer cells. *Free Radic Biol Med* 108:433–444
41. Tang TH, Chang CT, Wang HJ, Erickson JD, Reichard RA, Martin AG, Shannon EK, Martin AL, Huang YW, Aronstam RS (2013) Oxidative stress disruption of receptor-mediated calcium signaling mechanisms. *J Biomed Sci*, 20
42. Wang L, Wang L, Shi X, Xu S (2020) Chlorpyrifos induces the apoptosis and necroptosis of L8824 cells through the ROS/PTEN/PI3K/AKT axis. *Journal of Hazardous Materials*, 398
43. Williams WM, Giddings JM, Purdy J, Solomon KR, Giesy JP (2014) Exposures of aquatic organisms to the organophosphorus insecticide, chlorpyrifos resulting from use in the United States. *Rev Environ Contam Toxicol* 231:77–117
44. Xu S, Nam SM, Kim JH, Das R, Choi SK, Nguyen TT, Quan X, Choi SJ, Chung CH, Lee EY, Lee IK, Wiederkehr A, Wollheim CB, Cha SK, Park KS (2015) Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress. *Cell Death Dis*, 6:e1976
45. Yazdinezhad A, Abbasian M, Hojjat Hosseini S, Naserzadeh P, Agh-Atabay AH, Hosseini MJ (2017) Protective effects of *Ziziphora tenuior* extract against chlorpyrifos induced liver and lung toxicity in rat: Mechanistic approaches in subchronic study. *Environ Toxicol* 32(9):2191–2202
46. Zhang Q, Zheng S, Wang S, Wang W, Xing H, Xu S (2019) Chlorpyrifos induced oxidative stress to promote apoptosis and autophagy through the regulation of miR-19a-AMPK axis in common carp. *Fish Shellfish Immunol* 93:1093–1099
47. Zhao S, Yuan C, Tuo X, Zhou C, Zhao Q, Shen T (2021) MCLR induces dysregulation of calcium homeostasis and endoplasmic reticulum stress resulting in apoptosis in Sertoli cells. *Chemosphere* 263:127868
48. Zheng Y, Shi G, Cai J, Yang J, Zhang Y, Gong Y, Liu Q, Yu D, Zhang Z (2020) Di-(2-ethyl hexyl) phthalate induces necroptosis in chicken cardiomyocytes by triggering calcium overload. *J Hazard Mater* 387:121696
49. Zhu P, Hu S, Jin Q, Li D, Tian F, Toan S, Li Y, Zhou H, Chen Y (2018) Ripk3 promotes ER stress-induced necroptosis in cardiac IR injury: A mechanism involving calcium overload/XO/ROS/mPTP pathway. *Redox Biol* 16:157–168
50. Zima AV, Blatter LA (2006) Redox regulation of cardiac calcium channels and transporters. *Cardiovasc Res* 71(2):310–321

Figures

Figure 1

CPF mediated oxidative stress and induced EPC cell apoptosis and necroptosis. A: CPF effect on the EPC cell viability. EPC cells were treated with various target CPF concentration for 24 h. CCK-8 assay were processed for cell viability evaluation of EPC cells and to determine the suitable CPF exposure concentration for subsequent study. The IC₅₀ of CPF for EPC cells is 8.945 μM as analyzed by viability curves. In accordance with results, we selected CPF concentration of 3, 5 and 7 μM, and marked as L group, M group and H group for subsequent study. B: The effects of CPF on oxidative stress parameters in EPC cells. The parameter expression in C group, L group, M group and H group were detected with kits. C: AO/EB staining assessment of CPF-exposed EPC cells. Live cells were dyed as green, apoptotic cells were

labeled as orange and necroptotic cells were stained as red. Image J software were chosen to counted cells, and calculated the proportion. D: Flow cytometry assessment was applied to analyzed the effects of CPF on EPC cell death.

Figure 2

CPF effected on the mRNA and protein expressions of apoptosis and necroptosis pathway genes in EPC cells. A: The expressions of apoptosis-related genes in mRNA and protein manners. B: The expressions of

necroptosis-related genes in mRNA and protein manners.

Figure 3

Detection the effects of CPF on Ca²⁺ homeostasis and ER stress in EPC cells. EPC cells were observed by using fluorescence microscopy after loading Ca²⁺ probes. A: ER Ca²⁺ levels were labeled with Mag-Fluo-4 AM (M14206, 5 μM, Invitrogen, Thermo Fisher Scientific, USA), and ER were dyed with ER-Tracker Red (C1041, 1 μM, Shanghai Beyotime Biotechnology, Shanghai, China). B: Cytoplasmic Ca²⁺ levels were determined by Fluo-4 AM (F14201, 5 μM, Invitrogen, Thermo Fisher Scientific, USA). C: Mitochondria Ca²⁺ levels were dyed with Mag-Fluo-4 AM (M14206, 5 μM, Invitrogen, Thermo Fisher Scientific, USA) and

mitochondria were marked with Mito-Tracker Green FM (M7514, 5 μ M, Invitrogen, Thermo Fisher Scientific, USA). Image J software (National Institutes of Health) were performed to determine the fluorescence intensity, and calculated the proportion relative to C group. D: CPF effected on the expression of ER stress pathway genes in EPC cells.

Figure 4

CPF effected on mitochondrial function in EPC cells. A: The generation of JC-1 aggregates in matrix with higher MMP, and produce red fluorescence. Contrary, JC-1 failure to aggregate at lower MMP, and exists as JC-1 monomer which produces green fluorescence. Image J software (National Institutes of Health) were performed to determine the fluorescence intensity, and calculated the ratio of the red emission relative to the green emission. B: MMP assessed as the fluorescence ratio of red emission to green emission was quantified with flow cytometry. C: CPF elevated ROS levels. Cytoplasmic ROS production stained by DCFH-DA (10 μ M). cytoplasmic ROS levels determined by ROS assay kit (E004-1-1, Nanjing Jianchang Bioengineering Research Institute, Nanjing, China). ROS level of isolated mitochondria from EPC cells. D: CPF depressed ATP levels. Cytoplasmic ATP levels determined by ATP assay kit (product No.A095-1-1, Nanjing Jianchang Bioengineering Research Institute, Nanjing, China). ATP level of isolated mitochondria from EPC cells. E: Effects of CPF exposure on mRNA expression of DRP1 and MFN1 in EPC cells.

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