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Chlorpyrifos induced oxidative stress to promote apoptosis and autophagy through the regulation of miR-19a-AMPK axis in common carp

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ABSTRACT

Chlorpyrifos (CPF) has become a mainly pollution in water environment. Micro-RNAs (miRNAs) play an important part in the development of apoptosis and autophagy. However, the potential mechanism of CPF induced kidney toxicity and the roles of miRNAs are still unclear. To explore the underlying mechanism, the kidney of common carp exposed to different concentrations of CPF for 40 days was used as a research object. We found that CPF could damage the ultrastructure and function of kidney; and also caused antioxidant system disorder. CPF inhibited the mRNA level of miR-19a which improved AMP-activated protein kinase (AMPK). Furthermore, the detection of apoptosis and autophagy relative genes showed that the expressions of TSC complex subunit 2 (TSC2), light chain 3 (LC3), Dynein, tumor protein 53 (p53), Bcl-2 associated X protein (Bax), caspase-3 and caspase-9 were enhanced and the expressions of nechanistic target of rapamycin (mTOR), Ras homolog mTORC1 binding (Rheb) and B-cell lymphoma (Bcl-2) were reduced in dose-dependent way. Taken together, we conclude that CPF causes oxidative stress and miR-19a-AMPK axis disorder, thereby promotes apoptosis and autophagy in common carp kidney. Our study will provide theoretical basis for toxicology research and environmental protection of CPF.

1. Introduction

Chlorpyrifos (CPF), a broad-spectrum organophosphorus pesticide [1], is usually used to control different types of pests [2]. However, its negative impact on the environment, especially on the water environment, has been widely concerned by researchers in the field of environment [3]. High residual levels of CPF in water, soil, fruits and vegetables have been found in many countries [4]. Such as Ajuda, CPF has been detected in surface water at the highest concentration of 0.36 μg/L [5]. Generally, CPF can accumulate in water and get into aquatic animal via the gills and skins, affecting homeostasis negatively. These negative effects not only harm aquatic animals such as fish, but also threaten human health through the food chain. Many papers have reported the harm effect of CPF on living organisms. The exposure of CPF can cause the decreased retention of olfactory predator recognition in surgeonfish [6]. Low doses of CPF straightly affect the neurodevelopment of amphibian brains [7]. It can also damage the immunological systems [8]. Investigations in reproductive system have shown that exposure to CPF can lead to developmental toxicity in zebrafish [9]. Moreover, CPF can induce cytotoxicity via autophagy [10] and necrosis characterized by diffuse nuclear staining [11]. Generally speaking, reactive oxygen species (ROS) is the first dangerous warning of toxicity, which induces oxidative stress and is harm for bodies [12]. It is reported that CPF could cause oxidative stress in gill, liver and posterior kidney of Nile tilapia [13] and autophagy in common carp [14]. Antioxidant enzymes, such as peroxidase (POD), are important for mitigation of oxidative stress. Once antioxidant enzymes fail to turn ROS into less harmful molecules, inflammation, reproductive damage and apoptosis are activated [15–17]. Zhang et al. has shown that CPF could induce an increase in ROS, Tumor Necrosis Factor-α (TNF-α) and Interferon γ (IFN-γ) levels, which in turn, promoted inflammation and oxidative stress [18]. Similarly, reduced activity of superoxide dismutase (SOD) and induced oxidative stress were found in PC12 cell after CPF treatment [19].

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Abbreviation

ACP	Acid phosphatase
AHR	Antihydroxyl radical
AKP	Alkaline phosphatase
AMPK	AMP-activated protein kinase
ASA	Antisuperoxide anion
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma
CPF	Chlorpyrifos
IFN-γ	Interferon γ
iNOS	Induction nitric oxide synthase
LC3	Light chain 3
MiRNA	Micro-RNA
mTOR	Mechanistic target of rapamycin
NO	Nitric oxide
NOS	Nitrfic oxides synthase
p53	Tumor protein 53
POD	Peroxidase
Rheb	Ras homolog mTORC1 binding
ROS	reactive oxygen species
SOD	superoxide dismutase
TSC2	TSC complex subunit 2
TNF-α	Tumor Necrosis Factor-α

Micro-RNAs (miRNAs) are a class of non-coding single-stranded RNA molecules involved in the regulation of post-transcriptional gene expression in plants and animals [20]. MiRNAs play an important role in many biological processes, including metabolism and proliferative [21]. It has also been involved in a lot of diseases [22] such as tumor development [23] and inflammatory process [24]. MiR-17–92 cluster, a special cluster in miRNAs, can regulate development of heart and lung [25,26], and also participate in aging and cancer [27]. For example, miR-21 is the biomarker of acute kidney injury [28] and it can inhibit autophagy which targets Rab11a in renal ischemia-reperfusion [29]. MiR-34a can target Atg4B to regulate autophagic activity, which cause injury in ischemia reperfusion [30]. Mir-19 is also a significant kind of miRNA. It occupies an important position in the cluster, and has a pathogenic effect on glioma through the miR-19/RUNX3/β-catenin pathway [31]. It is reported that resveratrol can restrain miR-19 and then cause apoptosis in glioma [32]. What's more, the over-expression of miR-19b-1 can inhibit cardiac apoptosis caused by ischemia in vivo and vitro [33].

Apoptosis and autophagy are involved in the development of poisoning, and they are closely related. The formation of autophagosomes promoted by lead (II) can inhibit apoptotic in primary rat proximal tubular cells [34]. Trehalose inhibits apoptosis and promotes autophagic flux induced by cadmium [35]. B-cell lymphoma (Bcl-2), a well-established marker of apoptosis, is linked to damage under the action of poison. After 60 days treatment with CPF, a significant decrease in the protein expression level of Bcl-2, but a remarkable increase in the expression levels of Bcl-2 associated X protein (Bax), caspase-8 and caspase-9 were found in both cerebrum and cerebellum of rat [36]. Injection of carbon monoxide can induce the neuronal apoptosis in rats with a decrease of Bcl-2 and an increase in caspase-3, and hyperoxygenate hydrogen-rich saline could against the injury [37]. In addition, cell apoptosis are also involved in Nrf2 mediates CPF-induced nervous system toxicity [19]. Autophagy is always controlled by several genes, such as AMP-activated protein kinase (AMPK), Bax, Bcl-2 and mechanistic target of rapamycin (mTOR), which participates in a variety of processes such as the development and growth of living things. For example, Bax/Bcl-2 ratio are increased and autophagy are enhanced in N27 cells exposed to 10 μM CPF [38]. Xing et al. found that CPF could induce autophagy via inducing microtubule-associated protein 1 light chain 3 B (LC3B) and dynein in carp [39]. Activation of AMPK and autophagy prevented hepatocellular and mitochondrial damage induced by drug [40]. Specifically, rapamycin can decrease CPF-induced Bax and increase CPF-decreased Bcl-2 in mitochondria [10].

Up to now, few studies have demonstrated the exposure to CPF can influence the expression of miR-19a. The underlying mechanisms of CPF toxicity in the kidney and involvement of miR-19a are still unclear. In our experiment, we take the kidney of common carp exposed to CPF as the research object. We detected antioxidant enzyme activities or contents in the kidney of common carp. At the same time, we used qRT-PCR to detect the gene expression levels of miR-19a and AMPK to clarify the targeted relationship. Also, apoptosis and autophagy-related genes in the kidney of common carp were detected to illustrate the toxic effects of CPF by qRT-PCR. The experiment will provide theoretical basis for toxicology research and environmental protection of CPF.

2. Material and method

2.1. Test chemicals

CPF (purity 99%) was purchased from Aladdin China. CPF stock solution was prepared in analytical grade acetone (purity 99%). All of the working solutions of following experiments used the stock solution and the concentration of acetone in each pesticide solutions were kept at < 0.05%.

2.2. Treatment of experimental animals

All the procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Common carps (mean body length, 13 ± 1 cm; mean body weight, 190 ± 10 g) were kept in laboratory tanks (90 × 55 × 45 cm) with continuous aeration to remove chlorine gas. The pH of water was kept at 7.4 ± 0.2 and the temperature was kept at 20 ± 1 °C. All procedures followed the European Communities Council Directive (86/609/EEC) and were approved by a local ethics committee.

2.3. Toxicity test

The common carp were randomly divided into four groups as follows: high concentration group (116 μg/L CPF, named H), middle concentration group (11.6 μg/L CPF, named M), low concentration group (1.16 μg/L CPF, named L) and control group (named C). The concentrations of CPF used in the experiments were approximately 1/5, 1/50 and 1/500 of the 96 h LC_{50} [41]. In order to ensure the concentration of the poison, the water and CPF was replaced once every 1 day. After exposure to CPF for 40 d, the kidneys were quickly removed and processed as required by subsequent experiments.

2.4. Transmission electron microscope

After exposure to 116 μg/L CPF, the kidney tissues fixed in precooled 2.5% glutaraldehyde was rinsed three times with PBS for 10 min each time and then dehydrated with a graded ethanol series. The tissues were fixed with pure acetone and embedding agent (1:1) over night. After cut into pieces (thickness: 1 μm), the samples were mounted on coated copper grids, poststained with uranyl acetate for 10 min, dyed with lead citrate for 10 min and dried for observe (GEM-1200ES, Japan).

2.5. Determination of AKP and ACP

Kidney tissues were homogenized on ice in a 1:9 (w/v) ratio with

saline, and then, the homogenates were centrifuged at 2500 (r/min) for 10 min. The supernatants were used for protein assay and other determination. The determination of AKP and ACP were performed according to the kit instructions. Protein assay was detected by total protein quantitative assay kit. Absorbance was recorded at 520 nm. The detection kits were purchased from Nanjing Jiancheng Bioengineering Institute.

2.6. Determination of antioxidant enzyme, NO, NOS and iNOS

The kidney tissues in different treatment groups were used for determination of antioxidant enzyme. The activities of POD, nitrfic oxides synthase (NOS), induction nitric oxide synthase (iNOS), antisuperoxide anion (ASA) and antihydroxyl radical (AHR), and content of nitric oxide (NO) were determined with detection kits according to the manufacturer protocols. POD detection kit was purchased from Solarbio (Beijing Solarbio Science & Technology Co., Ltd.). Others were purchased from Nanjing Jiancheng Bioengineering Institute.

2.7. Quantitative real-time PCR (qRT-PCR) analysis on mRNA and miR-19a levels

Total RNA were isolated from kidney tissues by TRIzol reagent (Invitrogen, China) [42]. Reverse transcription was performed using the Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The primer sequences were designed by Oligo 6.22 in Table 1 β-Actin was used as an internal reference. QRT-PCR was conducted by Light Cycler®480 System (Roche, Basel, Switzerland). Reactions were performed in a 20-μL reaction mixture: 10 μL of 2 × TransStart TM Green qRT-PCR SuperMix, 0.4 μL of passive Reference Dye, 0.4 μL of forward primer and reverse primer, 2 μL of cDNA and 6.8 μL of PCR-grade water. The $2^{-\Delta\Delta Ct}$ method was used to analyze the transcription level of mRNA.

2.8. Statistical analysis

Statistical analysis of all data was performed using GraphPad Prism 5.0, (San Diego, CA, USA). One-way ANOVA was used for analysis of the data and Turkey method was used to compare the differences of data. Quantitative data was presented as the mean ± SD. Different letters indicate a significant difference ($P < 0.05$) between the control group and the exposure group.

3. Results

3.1. Pathological changes

The ultrastructure in the kidneys of common carp exposed to 116 μg/L of CPF was observed by transmission electron microscope. As shown in Fig. 1, there was no significant ultrastructural damage in group C. The cells were structurally intact and framework of mitochondria was complete and continuous. Conversely, we could see typical apoptosis and autophagy characteristics in group H. The cells exhibited chromatin border accumulation. The mitochondria were obviously broken into many vacuoles. We also observed formation of autophagic vacuoles. These finding suggested that exposure to CPF not only damaged the ultrastructural structure but also induced apoptosis and autophagy in the kidney of carp.

3.2. AKP and ACP activities in carp exposed to CPF

As AKP and ACP are marker indexs for evaluating renal function, we detected activities of AKP and ACP. As shown in Fig. 2, the activity of AKP in group L was higher than that in group C, and the activities in group M and H were lower than control group. The activities of ACP in group L, M and H were higher than that in group C and were dose-dependent ($P < 0.05$).

3.3. Antioxidant enzyme activities in carp exposed to CPF

Then, we detected relative antioxidant enzyme activities in four groups (Fig. 2). Compared to group C, the activities of POD, ASA and AHR in group L, M and H were lower. In addition to AHR, the activities of others were dose-dependent. Although the AHR in group M was the lowest, the activity was still declining.

3.4. NO, NOS and iNOS in carp exposed to CPF

We also detected the content of NO and the activities of NOS and iNOS in four groups (Fig. 2). After the exposure to CPF, the content of NO and the activities of NOS and iNOS were higher than that in group C, which were dose-dependent.

3.5. Exposure to CPF promoted apoptosis in common carp kidney

To explore the potential mechanism, we tested the apoptosis relative genes. As shown in Fig. 3, exposure to CPF significantly promoted the mRNA expression levels of TSC complex subunit 2 (TSC2), LC3 and Dynein. It also reduced the mRNA expression levels of mTOR and Ras homolog mTORC1 binding (Rheb). The trends were changed in a dose-dependent way. All in all, the results indicated that CPF could promote apoptosis in the kidney of common carp.

3.6. Exposure to CPF promoted autophagy in common carp kidney

As shown in Fig. 4, the mRNA expression levels of Tumor protein 53

Table 1
Gene-target primers used in qRT-PCR.

Gene	Forward Primer	Reverse primer
β-actin	5-GATGGACTCTGGTGATGGTGTGAC-3	5-TTTCTCTTTCGGCTGTGGTGGTG-3
AMPK	5-GTGGTGTTATCCTGTATGCCCTTCT-3	5-CTGTTTAAACCATTCATGCTCTCGT -3
TSC2	5-GTAACACAGAATCAGTGAATCGGA -3	5-CACACACAGAAAACACTTGAAGC-3
mTOR	5-CCACAACGCAGCCAACAA-3	5-CCCTCGTGCCACATTTCAT-3
Rheb	5-GCCAATTTGTGGACTCCTACG-3	5-CCCACCATATCCAACAATTTGC -3
LC3	5-GCAAGCAAGCTGAAGCAGAA-3	5-CTCTGCCACTGCTCCATCAC-3
Dynein	5-TCATGGGAGTAAGGCTGGTATT-3	5-TCTTCAAAGGAATACAGGGGCT-3
Bcl-2	5-TCACTCGTTCAGACCCTCAT-3	5-ACGCTTTCCACGCACAT-3
p53	5-GGGCAATCAGCGAGCAAA-3	5-ACTGACCTTCCTGAGTCTCCA-3
bax	5-GGCTATTTCAACCAGGGTTCC-3	5-TGCCAATCACCAATGCTGT-3
Caspase-3	5-CCGCTGCCCATCACTA-3	5-ATCCTTTCACGACCATCT-3
Caspase-9	5-AAATACATAGCAAGGCAACC-3	5-CACAGGGAATCAAGAAAGG-3
miR-19a	5-CAATCCTCTCAGGCTCAGTCC-3	
U6	5-CTCGCTTCGGCAGCACA-3	

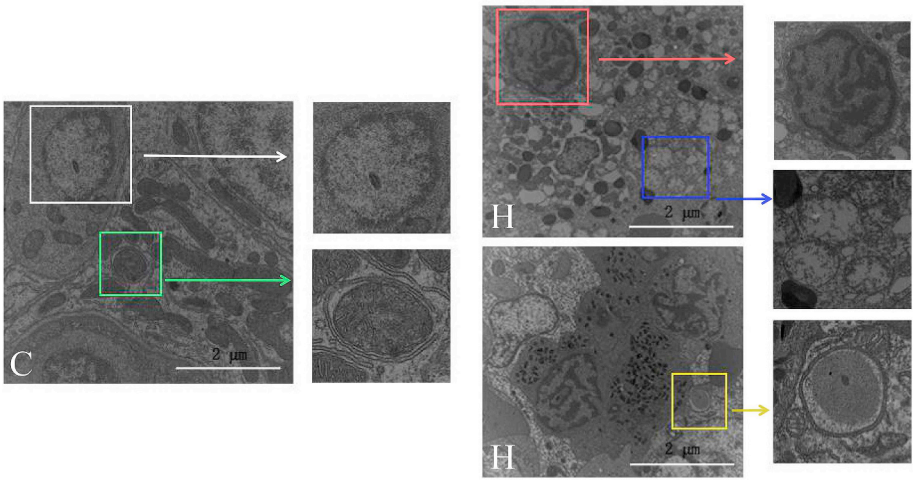

Fig. 1. The ultrastructure of kidney tissues of CPF exposed common carp. The ultrastructure of kidney tissues in group C and H were observed via transmission electron microscope. Normal cell (white square), normal mitochondrion (green square), chromatin marginalized cell (red square), mitochondrial vacuolation and fractures of mitochondrial cristae (blue square) and autophagic vacuoles (yellow square). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

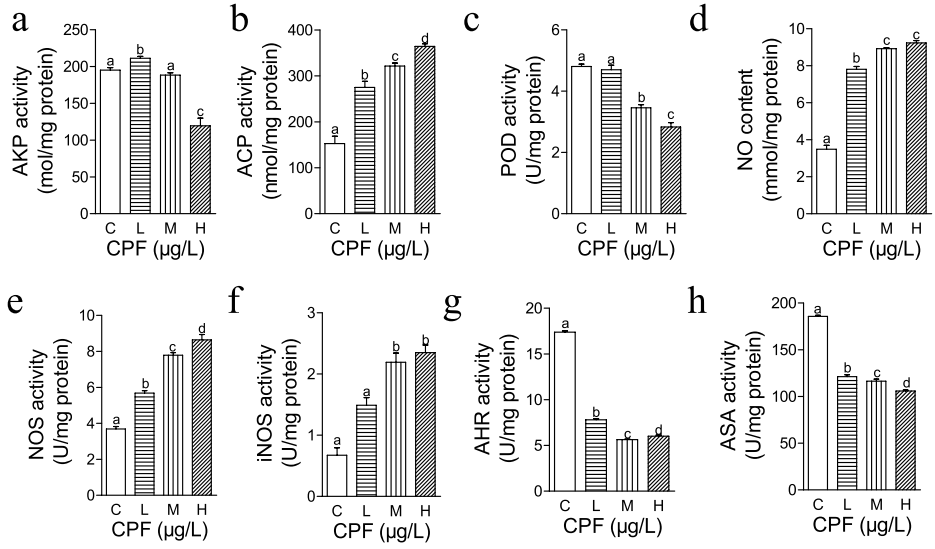

Fig. 2. Activities of AKP, ACP and antioxidant enzyme. Activities of AKP, ACP and antioxidant enzyme were detected in group C, L, M and H. (a) AKP; (b) ACP; (c) POD; (d) NO; (e) NOS; (f) iNOS; (g) AHR; (h) ASA. The data was presented as the mean ± SD (n = 3). Samples with different letters were considered as significant differences ($P < 0.05$). The samples with a common letter were not significantly different ($P > 0.05$).

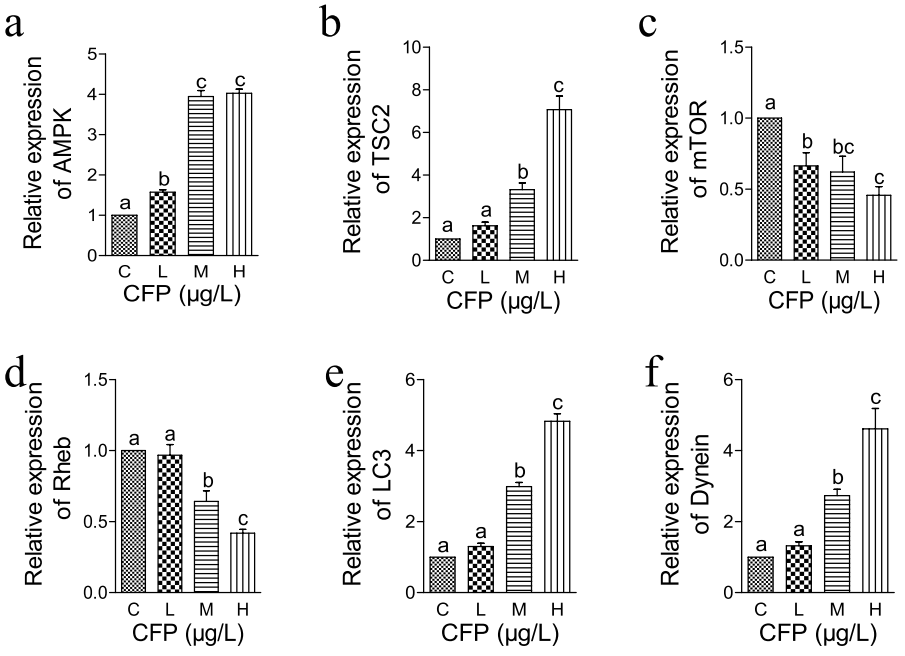

Fig. 3. MRNA expression levels of autophagy relative genes. MRNA expression levels of autophagy relative genes were detected in group C, L, M and H. (a) AMPK; (b) TSC2; (c) mTOR; (d) Rheb; (e) LC3; (f) Dynein. The data was presented as the mean ± SD (n = 3). Samples with different letters were considered as significant differences ($P < 0.05$). The samples with a common letter were not significantly different ($P > 0.05$).

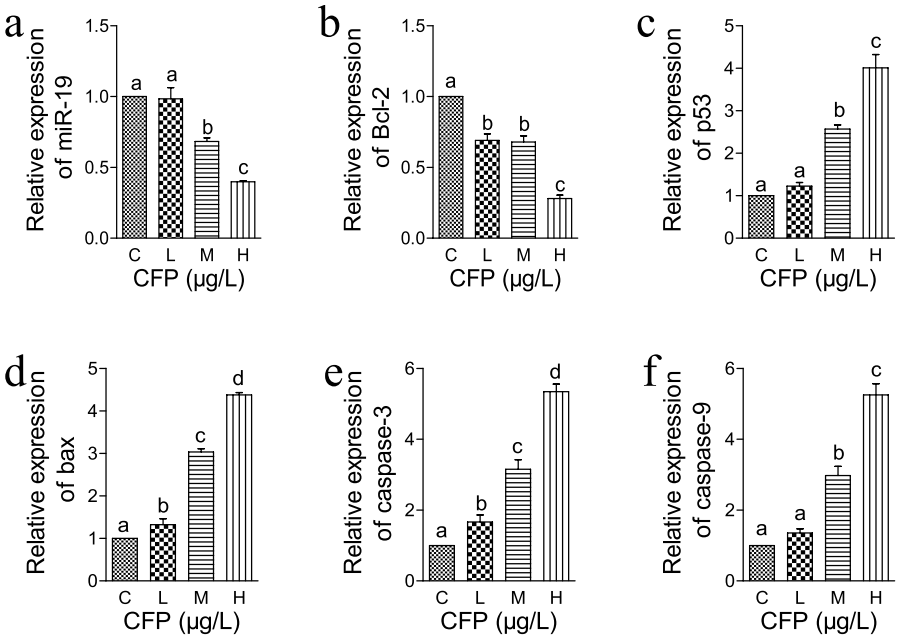

Fig. 4. MRNA expression levels of apoptosis relative genes and miR-19a. MRNA expression levels of apoptosis relative genes and miR-19a were detected in group C, L, M and H. (a) miR-19a; (b) Bcl-2; (c) p53; (d) Bax; (e) caspase-3; (f) caspase-9. The data was presented as the mean ± SD (n = 3). Samples with different letters were considered as significant differences ($P < 0.05$). The samples with a common letter were not significantly different ($P > 0.05$).

(p53), Bax, caspase-3 and caspase-9 were increased and the expression level of Bcl-2 was decreased after the exposure of CPF. Furthermore, the trends were changed in the order of concentration change. Thus, the results implied that CPF could promote autophagy in the kidney of common carp.

3.7. The toxicological effects of CPF on the expression of AMPK and miR-19a

To identify the target relationship between AMPK and miR-19a, we introduced qRT-PCR to detect the expressions of AMPK and mir-19a (Figs. 3a and 4a). After exposure to CPF, the mRNA expression of AMPK was improved. On the contrary, the expression of miR-19a was shown in the opposite trend. Furthermore, the prediction of miRNA targets website (http://www.targetscan.org/vert_72/) predicts AMPK (prkaa) can be limited by miR-19a. MiR-19a can target special 3′UTR sites in AMPK (prkaa) as shown in Fig. 5. Therefore, our results suggested that exposure to CPF regulated the relationship of miR-19a-AMPK (prkaa).

4. Discussion

CPF resided in the environment can cause disease especially in aquatic ecosystem such as immunological abnormalities and oxidative damage [43]. Xing et al. found that the levels of biotransformation enzymes and P450 were enhanced in common carp after exposure to CPF [44]. They also discovered that exposure to CPF could induce oxidative stress in brain of common carp [45]. CPF can repress the production of neutrophil extracellular traps in carp [46]. The levels of pro-inflammatory cytokines (interleukin-1β and TNF) were significantly increased in CPF-treated Wistar rats [47]. In our study, we found that CPF could damage tissue structure of kidney and promote oxidative stress. It could also downgrade the expression level of miR-19a, and furthermore, regulated the elevation of AMPK and promoted apoptosis and autophagy in the kidney.

Under normal physiological conditions, the production and elimination of ROS in organisms always remain in a relatively balanced state. However, when exogenous substances accumulate in body to a certain extent, such as pesticides and other chemical pollution, ROS and active substances will be produced in large quantities, thereby inducing lipid peroxidation. The production of such unfavorable substances can lead to changes in the antioxidant defenses, resulting in oxidative stress [48]. Many researchers have found that CPF can disturb the redox status of bodies [18,19]. POD, an important enzyme, widely presents in various organisms. POD can utilize H_2O_2 as an oxidant donor and participate in various physiological and biochemical reactions. NO plays an important role in regulating various physiological and pathophysiological mechanisms in bodies and NOS is the producer of NO. INOS is the type that produces most NO than other two types. The combination of superoxide anion radical and hydroxyl radical can cause DNA damage and harm the body function. Superoxide anion radical can be converted to H_2O_2 and O_2 in cells. The aim of the conversion is to keep cells away from the harmful effects [49]. Oxidative damage is usually caused by superoxide anion and hydroxyl radical [50]. Therefore, the activities of ASA and AHR play important roles in the antioxidant enzyme system. It has been reported that POD, ASA and AHR were decreased significantly in common carp exposed to CPF for 40 d. Besides, iNOS and NO were induced and head kidney structure was damaged [51]. Our results showed that exposure to CPF suppressed the activiey of POD, significantly induced the activities of NOS and iNOS, further induced the release of NO. What's more, ASA and AHR were reduced after the stimulation of CPF. Our results are similar to the

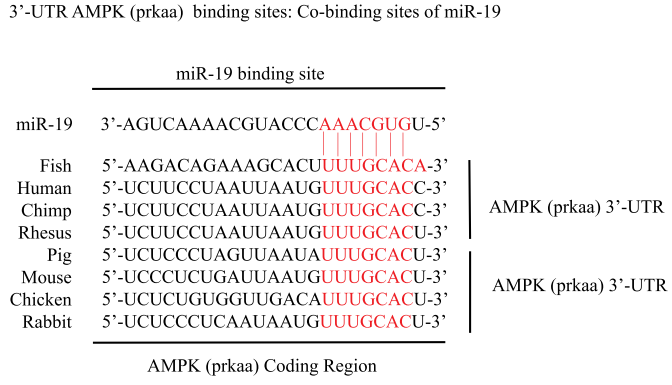

Fig. 5. TargetScan database prediction of miR-19a and AMPK (prkaa). a: mRNA expression levels of AMPK. b: level of miR-19a. c: targetScan database predict that binding sites for miRNA-19 and the targeting site in the 3′UTR of AMPK (prkaa). The data was presented as the mean ± SD (n = 3). Samples with different letters were considered as significant differences ($P < 0.05$). The samples with a common letter were not significantly different ($P > 0.05$).

previous studies, suggesting that antioxidant enzyme system were impaired and oxidative stress was triggered in the kidney of common carp exposed to CPF. Kidney is an important organ for organism to metabolic waste and poison [52]. AKP and ACP, widely distributed in various tissues and organs of fish, are important for immune protection [53] and kidney function regulation. It has been reported that exposure to CPF could significantly increase the ACP activity and reduce the ALP activity in spleen of common carp [54]. In our study, ACP levels in group L, M and H were higher than that in group C and AKP levels were opposite. All of the results suggested that kidney function was damaged with oxidative stress in the kidney of common carp exposed to CPF.

Evidences have suggested that miRNA can respond to adverse changes in the body. MiR-146a-5p could be induced in human dental pulp cells after the stimulation of LPS [55]. Exposure to Cd down-regulated the levels of miR-125a and miR-125b [56]. Our results suggested that CPF could reduce the level of miR-19a. It has been reported that the motivation of miR-19 can remit the negative effect of Tubulin Alpha 4B on breast cancer cells [57]. It is noteworthy that the knocking down expression level of miR-19–5p could increase apoptosis and reduce cell proliferation in HT29 cells [58]. AMPK has been reported as a target of gene of miR-19 [59], but the potential mechanism of the relationship between miR-19a and AMPK in fish is still indistinct. The TargetScan Web site predicts miR-19a targets AMPK in human, rabbit and chicken; by comparison, we found the same nucleotide sequence bound in fish as other involved animals. Besides, our results verified that CPF could induce the expression of AMPK and reduce the expression of miR-19a. It is the first time to clear the target relationship between miR-19a and AMPK in common carp. And it is also the first confirmation that exposure to CPF reduces miR-19a expression and disturbs miR-19a-AMPK (prkaa) axis.

The relative relationships of poison with autophagy or apoptosis have been widely reported in animals. Wang et al. has reported that atrazine can promote apoptosis and reduce the neutrophil extracellular traps in common carp [60]. He has also suggested that the mechanism of atrazine-induced apoptosis is depend on CYP450s/ROS pathway [61]. As reported, CPF toxicity had effects on cell cycle and apoptosis, and had neurotoxicity on SK-N-SH cell [62]. As regulators of mitochondrial apoptosis pathways, Bcl-2, Bax, caspase-9, caspase-3, and p53 play a center role in regulating apoptosis. CPF can induce apoptosis through the inhibition of Bcl-2 and the increase of p53, caspase-9 and caspase-3, causing damage to carp gills [63]. The mRNA levels of Bcl-2, Bax, caspase-3 and caspase-9 were found to be enhanced with the absence of selenium in germ cells of mice [64]. AMPK, TSC2, mTOR, Rheb, LC3 and Dynein are important genes in the progress of autophagy [65,66]. CPF was found to have a serious toxic effect with autophagy and oxidative reaction on BV2 cells [18]. The absence of Nrf2 can enhance the AMPK-associated autophagy induced by licochalcone A [67]. The expressions of LC3B and dynein suggested autophagy responds in the liver of carp [39]. AMPK/PI3K/mTOR pathway is involved in the PN50G-mediated apoptosis and autophagy [68]. As shown in our results, we used RT-PCR technology to further explore the specific mechanism of CPF toxicity, and found that exposure to CPF enhanced the expressions of autophagy-relative genes (TSC2, LC3 and Dynein) and apoptosis-relative genes (p53, Bax, caspase-3 and caspase-9). Besides, the expressions of autophagy-relative genes (mTOR and Rheb) and apoptosis-relative gene (Bcl-2) were reduced. Therefore, we speculated that CPF exerted toxic effects through improving both apoptosis and autophagy in the kidney of common carp.

In conclusion, CPF can induce oxidative stress and trigger miR-19a-AMPK axis deregulation to motivate apoptosis and autophagy in the kidney of common carp. Thus, our results provide new insight into the toxicological mechanism of CPF in common carp.

Date availability

Datasets generated and analyzed during the study can be obtained under reasonable request.

Declarations of interest

None.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

Shiwen Xu provided ideas for the experiment. Qiaojian Zhang completed the experiment and wrote the manuscript. Shengchen Wang and Shufang Zheng completed part of the figures. Houjuan Xing checked grammar and spelling of the manuscript. The study was supported by the “Academic Backbone” Project of Northeast Agricultural University (Project No. 700–507001).

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