Animal Nutrition 18 (2024) 119–132

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Dietary curcumin alleviates intestinal damage induced by ochratoxin A in juvenile grass carp (*Ctenopharyngodon idella*): Necroptosis and inflammatory responses

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ARTICLE INFO

Article history:
Received 10 September 2023
Received in revised form
24 November 2023
Accepted 11 April 2024
Available online 1 May 2024

Keywords:
Curcumin
Ochratoxin A
Grass carp growth performance
Mid-intestine mitochondria function
Necroptosis

ABSTRACT

Ochratoxin A (OTA) is one of the most common pollutants in aquatic feed. As a first line of defense, intestinal barriers could be utilized against OTA in order to prevent disorders. Natural product supplementation is one of the most popular strategies to alleviate toxicity induced by mycotoxins, but there is a lack of knowledge about how it functions in the teleost intestine. In this study, 720 juvenile grass carp of about 11 g were selected and four treatment groups (control group, OTA group, curcumin [Cur] group, and OTA + Cur group) were set up to conduct a 60-day growth test. After the test, the growth performance and intestinal health related indexes of grass carp were investigated. The addition of dietary Cur could have the following main results: (1) inhibit absorption and promote efflux transporters mRNA expression, reducing the residuals of OTA, (2) decrease oxidative stress by reducing oxidative damage and enhancing the expression of antioxidant enzymes, (3) promote mitochondrial fusion proteins to inhibit the expression of mitotic proteins and mitochondrial autophagy proteins and enhance mitochondrial function, (4) reduce necroptosis-related gene expression through inhibiting the tumor necrotic factor receptor-interacting protein kinase/mixed lineage kinase domain-like pathway, (5) reduce the expression of pro-inflammatory factors by inhibiting the Toll-like receptor 4/nuclear factor-κB signaling pathway to alleviate the intestinal inflammatory response. In summary, the results suggested that Cur could alleviate OTA-induced intestinal damage by enhancing antioxidant capacity and mitochondrial function as well as reducing necroptosis and inflammation in the grass carp intestine. This study provided a theoretical basis and production implications for dietary Cur that could improve growth performance and alleviate the intestinal damage induced by OTA in fish.

1. Introduction

Currently, the rapid development of the feed industry has made a huge contribution to the increase in animal production (You et al., 2022). However, in practice, the formulation of feeds based on plant-based raw materials is highly susceptible to contamination by mycotoxins, which can lead to a decline in animal production and even huge economic losses (Vardali et al., 2023). Ochratoxin A (OTA) is one of the mycotoxins that mainly contaminates corn, barley, wheat, soybean meal, and other plant-based raw materials, and its contamination level can exceed 80%

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.04.011

(Nogueira et al., 2020). Therefore, mitigating the harm of mycotoxins in feed for animals through nutritional additives has become a hot topic (Xu et al., 2022). As the first gateway organ for nutrient digestion and absorption, the function of the intestine is closely related to animal growth (Bai et al., 2022; Yang et al., 2019). Previous studies demonstrated that the dietary OTA decreased the growth associated with intestinal damage from terrestrial to aquatic dependent on dosages differently (e.g., from 1.2 mg/kg dosages in grass carp (Liu et al., 2020), 2 mg/kg in tambaqui (*Colossoma macropomum*) (Baldissera et al., 2020), 50 μg/kg to 2 mg/kg in ducks (Tong et al., 2020; Ruan et al., 2019) and 0.2 mg/kg OTA in mice (Zhang et al., 2023), etc.), thus implying the differences in sensitivity between species evaluated. However, how to mitigate the OTA-induced intestinal damage and the underlying mechanisms were not clear.

Curcumin (Cur) is a natural polyphenolic compound extracted from turmeric rhizome with anti-inflammatory, antioxidant, and anti-apoptotic properties (Kocaadam and Şanlier, 2017) and is one of the first nine natural colorants approved by (WHO, 2000) for use in the food industry in China. Currently, studies on the mitigation of organismal losses in aquatic animals caused by mycotoxins have not been reported. Induced oxidative stress is currently a well-known toxic mechanism of OTA (Guilford and Hope, 2014). The oxidative stress was inextricably linked to mitochondrial biogenesis (Huang et al., 2021), which consisted of mitochondrial antioxidant function, mitochondrial dynamics, and mitochondrial autophagy, and these three processes were regulated by peroxisome proliferators-activated receptor γ coactivator alpha/nuclear factor erythroid 2-related factor 2 (PGC-1α/NRF2), optic atrophy 1 (OPA1), dynamin-related protein 1 (DRP1) (Chen and Dorn, 2013), and Bcl-2 adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and Parkin (González-Rodríguez et al., 2022), respectively. At present, there is no relevant study on whether Cur alleviates OTA-induced oxidative stress by regulating mitochondrial biogenesis. But a study on Cur and other poisons showed that Cur may attenuate arsenic-induced damage by activating the PGC-1α/NRF2 and BNIP3/Parkin signaling pathways in duck muscle (Lan et al., 2022). Cur alleviated cisplatin-induced acute kidney injury in mice by inhibiting DRP1 and Parkin to enhance OPA1 protein expression (Ortega-Domínguez et al., 2017). The above suggests that Cur may mitigate OTA-induced intestinal toxicity by enhancing mitochondrial biogenesis, which needs to be further investigated.

In addition, the impairment of mitochondrial biological functions will further lead to programmed cell death in the organism, such as necroptosis, which further triggers inflammatory responses (Eadon et al., 2013). Necroptosis and inflammation were regulated by receptor-interacting protein/mixed-lineage kinase domain-like (RIP/MLKL) and Toll-like receptor 4/nuclear factor kappa-light-chain-enhancer of activated B cells (TLR4/NFκB) signaling pathways, respectively (Wallach et al., 2016). Although there were no studies related to whether Cur could attenuate the necroptosis and inflammatory response in the intestine caused by OTA, in chicken hepatotoxicity caused by aflatoxin, Cur could attenuate necroptosis and inflammation by inhibiting aflatoxin-activated RIP/MLKL and TLR4/NFκB signaling pathways (Li et al., 2022a). Furthermore, in duck ileum, Cur could alleviate the inflammation caused by lipopolysaccharide (LPS) by inhibiting TLR4/NFκB signaling. The above suggests that Cur may inhibit the RIP/MLKL and TLR4/NFκB signaling pathways to reduce the necroptosis and inflammatory response of the intestine induced by OTA, which is of great value and significance for further research.

Grass carp is one of the most famous freshwater species in the world and is also an important source of protein for humans (Chen and Wang, 2014). Notably, the intestinal systems of aquatic and terrestrial animals were greatly different. The fish intestine was mainly divided into primary, middle, and posterior sections (Ni and Wang, 1999). Among them, the mid-intestine (MI), which has both digestive and immune functions, is the longest intestinal segment and the main part of this study (Kong et al., 2017). Previous studies have shown that dietary soybean meal induces Atlantic salmon mainly in the midgut (Rombout et al., 2011). Previous studies have found that dietary supplementation of more than 1209 μg/kg OTA significantly inhibited growth performance and caused intestinal damage in juvenile grass carp (Liu et al., 2020), while supplementation of 400 mg/kg Cur alone significantly improved growth performance and immune and antioxidant capacity of juvenile grass carp (Ming et al., 2020). Accordingly, the objective of this research was to explore the attenuating effect of Cur on the intestinal damage in teleost model grass carp caused by OTA to lay the foundation for further research on the toxicity mechanism of OTA and for developing effective treatment strategies for OTA.

2. Materials and methods

2.1. Animal ethics statement

Experimental procedures were reviewed and approved by the Animal Care Advisory Committee of Sichuan Agricultural University (approval number: ZP-2019114003) and animal experiments complied with the ARRIVE guidelines.

2.2. Diets and experimental management

The OTA source information is in Table S1. The components of the basal diet are presented in Table S2. Grass carp were purchased from Deyang Aquatic Seeds Company (Sichuan, China). After 28 days of domestication (fed the control diet), 720 fish (initial body weight = 11.06 ± 0.05 g) were randomly placed into 6 aquaculture cages measuring 1.5 m × 0.8 m × 1.5 m in 2 ponds. According to previous studies on grass carp (Liu et al., 2020; Ming et al., 2020), OTA doses greater than or equal to 1.2 mg/kg reduced growth performance and intestinal health and 400 mg/kg Cur promoted growth performance. Therefore, 1.2 mg/kg OTA and 400 mg/kg Cur were selected for follow-up experiments. Those cages were randomly assigned into 4 groups: the control group (without OTA and Cur), the 1.2 mg/kg OTA group, the 400 mg/kg Cur group, and the 1.2 mg/kg OTA plus 400 mg/kg Cur group. Each treatment consisted of three replicates (180 fish). The pond water quality management was carried out according to the Ma et al. (2023) method. Water quality was monitored daily: dissolved oxygen >6 mg/L, water temperature 26.3 to 35.6 °C and pH 7.0 to 7.5 during the growth experimental. The fish were fed until satiated four times per day for 60 d. The method of collecting residual feed was as follows: a disc with a diameter of 80 cm was installed at the bottom of each cage and wrapped with 1-mm gauze. Feed was put into the disc when feeding. Fish were fed to satiety and 30 min later, uneaten feed was collected, dried and weighed. Feed intake was calculated on the basis of dry matter according to Cai et al. (2005). The survival rate of all grass carp was 100% during 60 days feeding experiment.

2.3. The mid-intestine collection and OTA residues detection

The fish were fasted for 24 h and then anesthetized in 50 mg/L benzocaine solutions (Wang et al., 2023). The MI sampling location is shown in Fig. S1. After removing the excluded fat in normal saline, MI was fixed in 4% paraformaldehyde for microscopic structure observation, fixed in pre-cooled 2.5% glutaraldehyde and stored at 4 °C for ultrastructure observation, stored at −20 °C for

enzyme activity and other experiments and at −80 °C for RT-PCR and Western blot tests. OTA residues were determined in MI according to the instructions (PriboFast, EKT-040).

2.4. Histological and immunofluorescence

The MI tissue (3 fish for each treatment group) was preserved in a 4% paraformaldehyde fixing solution and embedded in paraffin. The tissue was cut into 5 μm slices (40 pieces per tissue), which were stained with hematoxylin and eosin (H&E) after dewaxing. Images were captured using a Nikon TS100 light microscope (Nikon Instruments, Tokyo, Japan). For immunofluorescence staining, sections were dewaxed and rehydrated, including being put into distilled water for 20 min according to the dewaxing solution and 5 min each in 100%, 95%, 85%, and 75% alcohol, treated with 3% hydrogen peroxide, washed 3 times with 0.01 mol/L phosphate-buffered saline (PBS) for 5 min each, then put into citric acid antigen repair solution heated to boiling in the microwave oven and continued for 10 min, cooled naturally at room temperature, washed 3 times with PBS, 5 min each time, and closed with 0.5% Triton X-100 + 5% goat serum. Diluted primary antibodies against rabbit PGC-1α, OPA1, light chain 3 II (LC3II), cleaved cysteinyl aspartate specific proteinase (c-caspase 8), and interleukin 6 (IL-6) were added to different slides and incubated overnight at 4 °C in an immunofluorescent wet box. The wet cassette was removed from 4 °C and the water on the lid was dried and put into 37 °C for 1 h of rewarming, washed 3 times with PBS for 5 min each time, incubated with immunofluorescent secondary antibody for 1 h protected from light, washed 3 times with PBS for 5 min each time, stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min, washed 3 times with PBS for 5 min each time, and the slides were sealed with anti-quenching sealer. Images were captured using inverted fluorescence microscopy (each slice takes a field of view at 40×) and quantified using Image-J (NIH Image J system, Bethesda, MD). Details of the reagents, antibody dilution ratios, instrumentation, and software used above are given in Table S1.

2.5. Transmission electron microscopy (TEM)

The MI samples (3 fish for each treatment group) were fixed in 2.5% glutaraldehyde overnight. TEM sample preparation and photography were done by Chengdu Lilai Biotechnology Co., Ltd. (Chengdu, China).

2.6. Biochemical parameters analysis

The MI homogenate (6 fish for each treatment group) was prepared in 4 °C normal saline (weight:volume = 1:9), then centrifuged at 1000 × *g* at 4 °C for 10 min, and the supernatant was taken for enzyme activity detection. Then, reactive oxidative stress (ROS) and malondialdehyde (MDA), antioxidant related glutathione (GSH), total superoxide dismutase (T-SOD), copper and zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and inflammation-related (acid phosphatase [ACP] content and lysozyme [LZ] activity) enzyme activities were detected according to Dong's (Dong et al., 2017a) method. The lipid peroxidase (LPO) was detected according to Li's (Li et al., 2019) method. The immunoglobulin M (IgM) was detected according to Dong's (Dong et al., 2017b) method. The total antioxidant capacity (T-AOC) was analyzed according to the corresponding kit protocols. The information on the kits used is shown in Table S1.

2.7. Realtime PCR

The procedure (6 fish for each treatment group) was similar to our earlier investigation (Wang et al., 2015). The MI sample's total RNA was isolated by RNAiso Plus (Takara, China). The RNA quality was determined by NanoDrop 2000 (Thermo Fisher Scientific, China) and agarose gel (1.5%) electrophoresis, respectively. Then the RNA was reverse transcribed into cDNA by the PrimeScript RT reagent kit (Takara, China). The 2× SYBR Green qPCR Mix (Aidlab, China) was applied for q-PCR reactions with the internal control gene β-actin and specific primers, which were designed in accordance with the sequences of grass carp (Table S3). Beta-actin was selected as reference genes, based on their expression stability in samples evaluated using the Normfinder method (Andersen et al., 2004). The $2^{-\Delta\Delta Ct}$ method was used to assess mRNA levels.

2.8. Western blot analysis

The Western blot analysis procedure was used as previously described by Jiang et al. (2015). Briefly, tissue protein extraction and concentration determination were performed using RIPA lysis buffer (Epizyme, Shanghai, China), phenylmethanesulfonyl fluoride (Epizyme), SDS-PAGE protein sample loading buffer (Epizyme), and a BCA protein assay kit (Beyotime, Shanghai, China). The extraction of tissue nuclear protein was carried out according to the instructions of the extraction kit for nuclear protein and cytoplasmic protein (P0028, Beyotime, Shanghai, China). The protein samples (3 fish for each treatment group) were separated by SDS-PAGE, and then the protein was transferred to a PVDF membrane. Next, the PVDF membrane was blocked with 5% blocking buffer (BSA) for 2 h at room temperature and washed for 10 min three times. Then, the membranes were blocked with primary antibodies for 12 h at 4 °C. After equilibrating to room temperature for 1 h, the membranes were washed three times for 10 min and incubated with a secondary antibody solution (goat anti-rabbit horseradish peroxidase-conjugated, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The electrochemiluminescence (ECL) kit (Beyotime, P0018FM, China) was used to visualize immune complexes, and the Image J software was used for quantification. Table S4 lists the antibody information used in the Western blot analysis.

2.9. Calculating and statistical analysis

The growth performance calculation formulas are shown in Table S5. The data are expressed as the mean ± standard deviation. In IBM SPSS Statistics 25 (IBM Corp., Armonk, NY, USA), one-way ANOVA, the normal distribution test and the Duncan's multiple comparison method were performed. Two-way ANOVA and Duncan multiple comparisons were used to analyze dietary OTA levels, Cur, and their interactions. Differences were considered statistically significant when $P < 0.05$. The graphical abstract was created through BioRender.com, and the bar chart was created through GraphPad 8.0 software (GraphPad software, Inc).

3. Results

3.1. Effect of Cur on grass carp growth performance and MI histopathology with OTA treatment

Our statistical results on growth performance were presented in Table 1. Among them, final weight (FW), percent weight gain (PWG), feed intake (FI), and food coefficient rate (FCR) were affected by OTA, Cur, and OTA × Cur group interactions ($P < 0.05$). The specific growth rate (SGR) was affected by OTA and Cur

Table 1
Curcumin (Cur) supplementation improved the growth of grass carp inhibited by ochratoxin A (OTA).

Parameters	Diets				ANOVA, *P*-value > *F*-value		
	Control group	OTA	Cur	OTA + Cur	OTA	Cur	OTA × Cur
Initial weight (IW), g	11.15 ± 0.015	11.15 ± 0.0205	11.14 ± 0.0242	11.15 ± 0.0207	0.468	0.438	0.541
Final weight (FW), g	152.59 ± 05.12^{c}	93.94 ± 2.23^{a}	200.97 ± 2.79^{d}	131.22 ± 3.48^{b}	<0.001	<0.001	0.028
Percent weight gain (PWG), %	1268.31 ± 46.69^{c}	742.23 ± 21.13^{a}	1704.60 ± 25.27^{d}	1076.57 ± 31.87^{b}	<0.001	<0.001	0.027
Feed intake (FI), g	155.93 ± 0.09^{c}	115.41 ± 0.03^{a}	194.00 ± 0.05^{d}	144.95 ± 0.05^{b}	<0.001	<0.001	<0.001
Specific growth rate (SGR), %/day	4.36 ± 0.06^{c}	3.55 ± 0.04^{a}	4.82 ± 0.02^{d}	4.11 ± 0.05^{b}	<0.001	<0.001	0.095
Food coefficient rate (FCR), %	1.10 ± 0.04^{b}	1.39 ± 0.04^{d}	1.02 ± 0.01^{a}	1.21 ± 0.04^{c}	<0.001	<0.001	0.031

Values are means ± SD (n = 3 group, 60 fish/group).
The different letters a, b, c, and d represent significant differences between different groups (P < 0.05).

(P < 0.05), but not by OTA × Cur interactions (P > 0.05). The details are as follows: the addition of Cur + OTA increased percent weight gain (PWG), specific growth rate (SGR) and feed intake (FI), while decreasing Food coefficient rate (FCR) compared to the OTA-alone group (P < 0.05). Furthermore, as shown in Fig. 1B, OTA residues (23.84 ± 4.88 μg/kg) were accumulated with OTA treatment in the MI. The OTA residues decreased significantly in the OTA + Cur co-treat group (12.01 ± 2.08 μg/kg) (P < 0.05), compared with the OTA treatment group (Fig. 1B). Dietary OTA, Cur separately, and the interaction between OTA and Cur, affected the gene expression of organic anion transporters (*oat1, 3*), organic cationic transporters (*oct2*), organic anionic polypeptide transporters (*oatp1d1, 2b1*), ATP-binding cassette (ABC) transporters (*abcc2* and *abcg2*) (P < 0.05) shown in Fig. 1E. Dietary OTA, Cur separately, and the interaction between OTA and Cur, affected the gene expression of OAT1. The OTA alone treatment group significantly increased the mRNA abundances of *oat* (*1, 3*), *oct2*, and *oatp* (*1d1, 2b1*), whereas decreased the mRNA abundances of *abcc2* and *abcg2* (P < 0.05). In contrast, the OTA + Cur group significantly decreased the mRNA abundances of *oat1, oat3, oatp1d1, oatp2b1*, and *oct2*, as well as increased the mRNA abundances of *abcc2* and *abcg2* (P < 0.05) compared with the OTA group.

Excessive accumulation of mycotoxin in the gut could cause organ damage (Smith et al., 2012). As shown in our results, the OTA

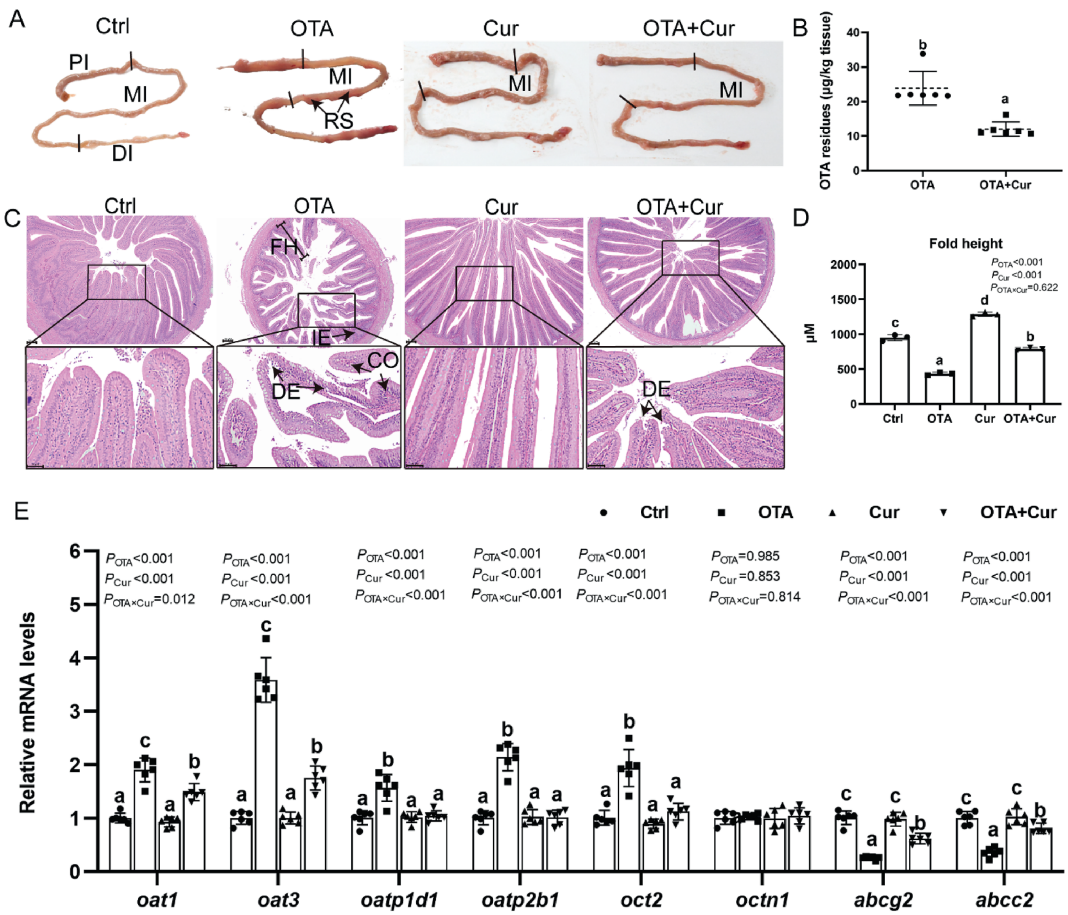

Fig. 1. Curcumin (Cur) supplementation attenuated the effects of ochratoxin A (OTA)-induced on residues, mRNA expression of different transporter and histological observation in the juvenile grass carp mid-intestine (MI). (A) A representative image of the intestinal appearance of MI, red and swollen (RS); (B) OTA residue statistics (n = 6); (C) Hematoxylin–eosin staining results (magnification 40×), n = 3 fish. FH = fold height; DE = detachment; CO = congestion. (D) Statistical results of intestinal fold height (n = 3); (E) Gene expression of transporters (*oat1, oat3, oatp1d1, oatp2b1, oct2, octn1, abcc2*, and *abcg2*). The different letters a, b, c, and d represent significant differences between different groups (P < 0.05).

treatment group showed redness and swelling in MI (Fig. 1A). In contrast, none of the other three groups had such symptoms in MI (Fig. 1A). Consistent with this, MI histopathological results also showed that the OTA treatment group showed bleeding, intestinal villi rupture, detachment, congestion, and significantly reduced intestinal fold height (Fig. 1C and D). While OTA + Cur significantly improved the above pathological phenomena and increased intestinal fold height compared with the OTA group (Fig. 1C and D). Further, the MI ultrastructure was observed by TEM, and the results are shown in Fig. 2. The control and Cur groups showed normal ultrastructure, including microvilli, tight junctions (TJ), mitochondria (MT), endoplasmic reticulum (ER), and nuclei (Fig. 2A and C). However, the OTA treatment group showed obvious necroptosis and mitophagy, including nuclear lysis, cytoplasmic lysis, membrane fragmentation, mitophagy, mitochondrial rupture, organelle enlargement and fragmentation, and nuclear fragmentation (Fig. 2B). This phenomenon was significantly improved in the OTA + Cur group, where only a small amount of nuclear fragmentation, autophagy, and mitochondrial damage were observed (Fig. 2D).

3.2. Effect of Cur on MI mitochondria antioxidant function inhibited by OTA

As shown in Fig. 3, ROS, MDA, LPO, T-AOC, GSH, T-SOD, MnSOD, GPX, and GST were affected by OTA and Cur ($P < 0.05$). CuZnSOD was affected by Cur ($P < 0.05$) but not OTA ($P > 0.05$). LPO, T-AOC, CuZnSOD, and GPX were also affected by OTA × Cur interactions ($P < 0.05$), but ROS, MDA, GSH, T-SOD, MnSOD, and GST were not affected by OTA × Cur interactions ($P > 0.05$). Compared with the control group, the OTA alone treatment group significantly increased the ROS, MDA, and LPO contents ($P < 0.05$), whereas it decreased the GSH, T-AOC, T-SOD, CuZnSOD, MnSOD, GPx and GST contents ($P < 0.05$). In contrast, the OTA + Cur group significantly decreased the ROS, MDA, and LPO contents ($P < 0.05$), whereas it increased the GSH, T-AOC, T-SOD, GPx, GST and MnSOD contents compared with the OTA group ($P < 0.05$). The effects of OTA and Cur on mitochondrial antioxidant function are shown in Fig. 4. OTA and Cur and their interactions affected gene expression of glutaredoxin 2 (*glrx2*), peroxiredoxin (*prdx (3, 5)*), thioredoxin (*trx*), and *pgc-1α*, as well as protein expression of NUCLEAR-NRF2 and PGC-1α, and immunofluorescence expression of PGC-1α ($P < 0.05$). OTA and Cur were expressed with the little sulfiredoxin (*srxn*) and sirtuins 1 (*sirt1*) genes, but are not affected by the interaction between OTA and Cur ($P > 0.05$). The OTA alone treatment group significantly decreased the mRNA abundances of *glrx2*, *srxn*, *prdx (3, 5)*, *trx*, and *pgc-1α* as well as the protein expression of NUCLEAR-NRF2 and PGC-1α compared with the control group ($P < 0.05$) (Fig. 4A–C). In contrast, the OTA + Cur group significantly increased the mRNA abundances of *glrx2, srxn, prdx (3, 5), trx, sirt1 and pgc-1α* as well as the protein expression of NUCLEAR-NRF2 and PGC-1α compared with the OTA group ($P < 0.05$) (Fig. 4A–C). The PGC-1α immunofluorescence results showed that compared with the control group, the fluorescence intensity of PGC-1α was significantly decreased in the OTA alone treatment group ($P < 0.05$), but significantly increased in the OTA + Cur co-treatment group compared with the OTA alone treatment group ($P < 0.05$) (Fig. 4D and E).

3.3. Effect of Cur on MI mitochondrial division and autophagy caused by OTA

As Fig. 5 showed, the gene expression of *opa1*, mitofusin (*mfn (1, 2)*), and sequestosome I (*p62*), *drp1, bnip3, lc3II, beclin1,* uncoordinated-51-like kinase (*ulk1*), *parkin*, and autophagy-related proteins

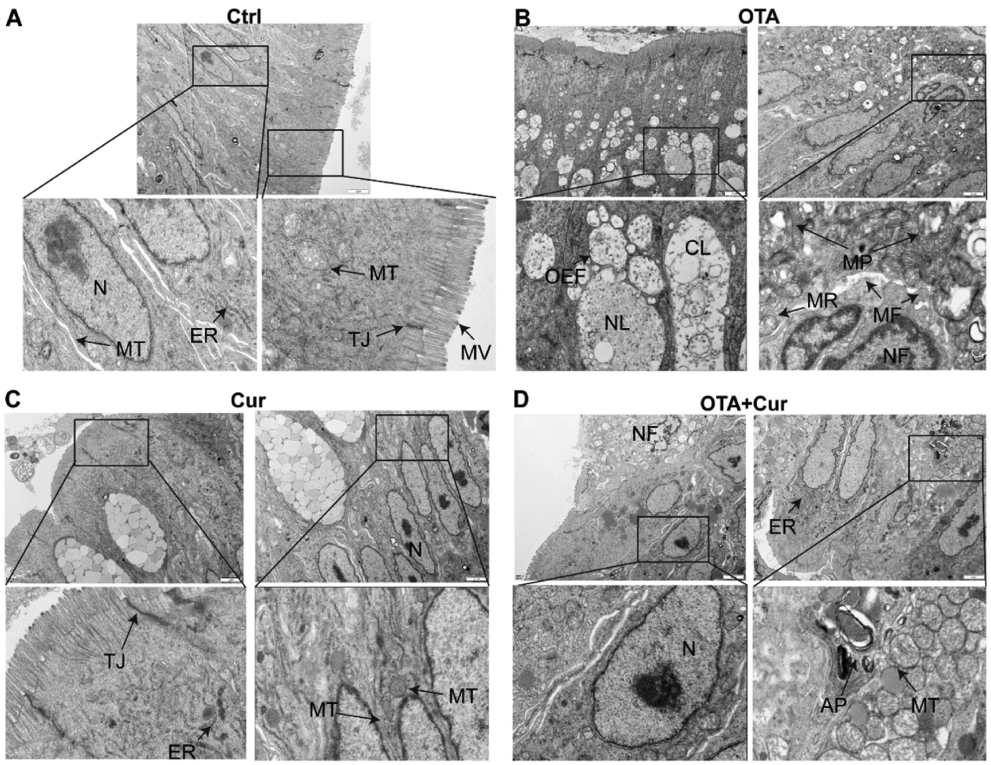

Fig. 2. Ultrastructure of the mid-intestine of juvenile grass carp was observed by transmission electron microscopy. (A) Control group; (B) OTA-treat group; (C) Cur-treat group; (D) OTA + Cur treat group, $n = 3$. Cur = curcumin; OTA = ochratoxin A; N = nucleus; MT = mitochondria; ER = endoplasmic reticulum; M = membrane; MV = microvilli; TJ = tightly junction; NL = nuclear lysis; CL = cytoplasmic lysis; MF = membrane fragmentation; MP = mitophagy; MR = mitochondrial rupture; OEF = organelle enlargement and fragmentation; NF = nuclear fragmentation; AP = autophagy.

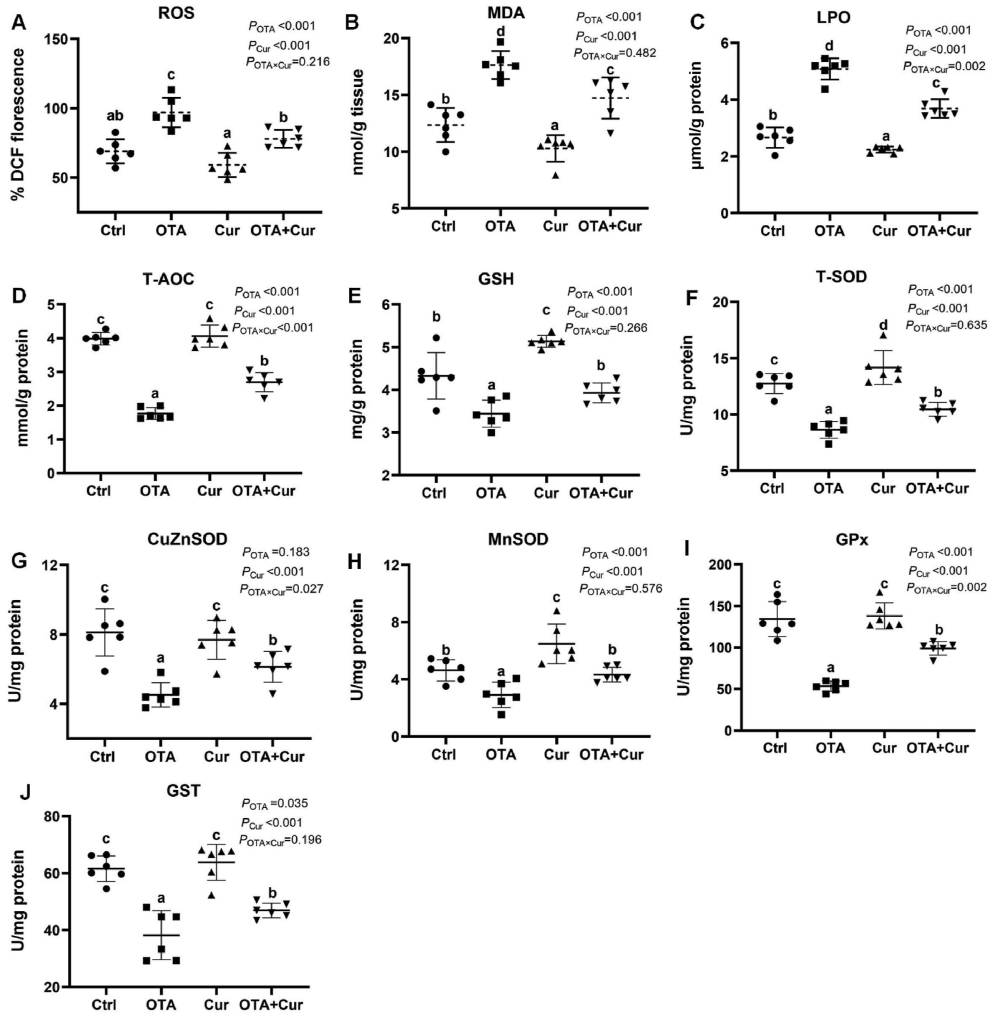

Fig. 3. Effects of dietary curcumin (Cur) supplementation attenuated the effects of ochratoxin A (OTA)-induced on ROS (A), MDA (B), LPO (C), T-AOC (D), GSH (E), T-SOD (F), CuZnSOD (G), MnSOD (H), GPx (I), and GST (J) in the mid-intestine of grass carp. The different letters a, b, c, and d represent significant differences between different groups ($P < 0.05$), $n = 6$ fish. ROS = reactive oxygen species; MDA = malondialdehyde; LPO = lipid peroxidation; T-AOC = total antioxidant capacity; GSH = glutathione; T-SOD = total superoxide dismutase; CuZnSOD = copper and zinc superoxide dismutase; MnSOD = manganese superoxide dismutase; GPx = glutathione peroxidase; GST = glutathione-S-transferase.

(*atg5, 12*) and the protein expression of OPA1, MFN (1, 2), P62, BNIP3, LC3II and PARKIN were not only affected by OTA and Cur, but also by the interaction between OTA and Cur ($P < 0.05$). DRP1 protein expression was affected by OTA and Cur, but not by their interactions ($P > 0.05$). Compared with the control group, the OTA alone treatment group significantly decreased the OPA1, MFN (1, 2), and P62 mRNA and protein abundances ($P < 0.05$), whereas it increased the *drp1, bnip3, lc3II, beclin1, ulk1, parkin, atg5*, and *atg12* mRNA abundances, as well as the DRP1, BNIP3, LC3II and PARKIN protein abundances ($P < 0.05$) (Fig. 5A–C). In contrast, the OTA + Cur group significantly increased the OPA1, MFN (1, 2), and P62 mRNA and protein abundances ($P < 0.05$), whereas it decreased the *drp1, bnip3, lc3II, beclin1, ulk1, parkin, atg5*, and *atg12* mRNA abundances as well as the DRP1, BNIP3, LC3II, and PARKIN protein abundances compared with the OTA group ($P < 0.05$) (Fig. 5A–C). As Fig. 6 showed OTA and Cur affected the immunofluorescence expression of OPA1 and LC3 ($P < 0.05$), and the interaction between OTA and Cur affected the immunosuppressive fluorescence expression of LC3 II ($P < 0.05$) but did not affect the immunofluorescence expression of OPA1 ($P > 0.05$). Consistently, the OTA alone treatment group significantly decreased the OPA1 immunofluorescence intensity while increasing the LC3 II immunofluorescence intensity compared with the control group ($P < 0.05$) (Fig. 6). In contrast, the OTA + Cur group significantly increased the OPA1 immunofluorescence intensity, whereas the LC3 II immunofluorescence intensity decreased compared with the OTA group ($P < 0.05$) (Fig. 6).

3.4. Effect of Cur on the necroptosis of MI caused by OTA

As Fig. 7 showed, OTA, Cur, and the interaction between OTA and Cur affected gene expression of the tumor necrosis (factor-α, *tnf-α*; receptor 1, *tnfr1*), TNFR1 associated death domain (DD) (*tradd*), Fas-associating protein with a novel DD (*fadd*), and *caspase-8* as well as protein expression of TNF-α, TNFR1, phosphorylated-RIP1, and phosphorylated-RIP3 and immunofluorescence expression of CLEAVED-CASPASE-8 ($P < 0.05$). Protein expression of CLEAVED-CASPASE-8 was affected by OTA and Cur ($P < 0.05$), but not by their interactions ($P > 0.05$). Protein expression of MLKL was affected by Cur ($P < 0.05$), but not by OTA and OTA × Cur interactions ($P > 0.05$). OTA alone treatment group significantly increased the *tnf-α*, *tnfr1*, *tradd*, *fadd*, and *caspase-8* mRNAs and TNF-α, TNFR1, CLEAVED-CASPASE-8, phosphorylated-RIP1, phosphorylated-RIP3, and MLKL protein abundances ($P < 0.05$). Compared with the control group. In contrast, the OTA + Cur group

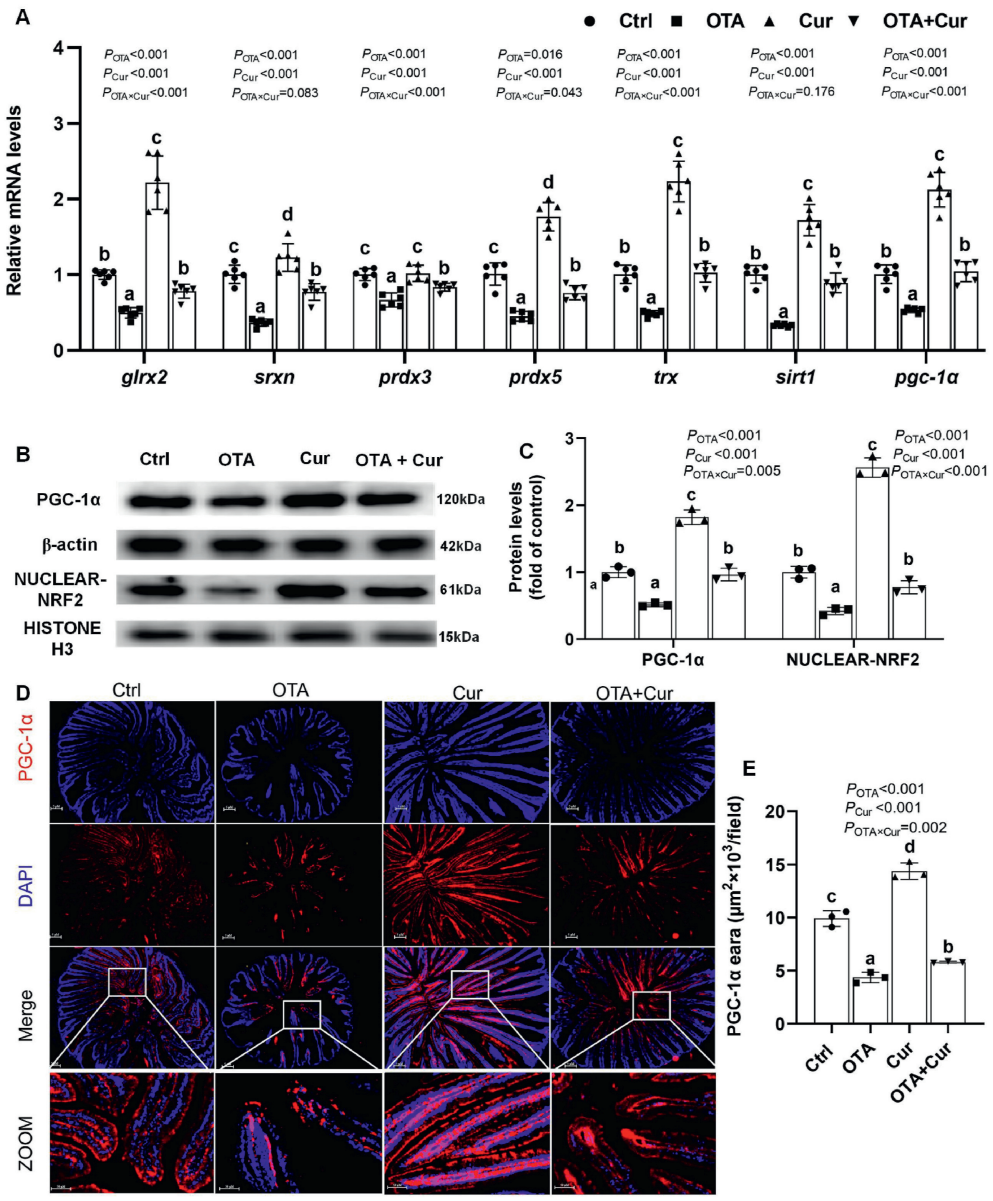

Fig. 4. Dietary curcumin (Cur) supplementation enhanced antioxidant function of mitochondria in the juvenile grass carp mid-intestine under ochratoxin A (OTA) inhibition. (A) The mRNA expression, $n = 6$. (B, C) The protein expression of NUCLEAR-NRF2 and PGC-1α, $n = 3$. (D, E) Immunofluorescence and statistical results of PGC-1α, magnification 40× ($n = 3$). The different letters a, b, c, and d represent significant differences between different groups ($P < 0.05$). NUCLEAR-NRF2 = nuclear factor erythroid 2-related factor 2; PGC-1α = peroxisome proliferators-activated receptor γ coactivator alpha.

significantly decreased the *tnf-α, tnfr1, trasdd, fadd*, and *caspase*-8 mRNAs and TNF-α, TNFR1, CLEAVED-CASPASE-8, phosphorylated-RIP1, phosphorylated-RIP3, and MLKL ($P < 0.05$) compared with the OTA group (Fig. 7A–D). The OTA treatment group significantly increased the cleaved-caspase8 immunofluorescence intensity ($P < 0.05$) compared with the control group (Fig. 7E). In contrast, the OTA + Cur group significantly decreased the cleaved-caspase-8 immunofluorescence intensity ($P < 0.05$) compared with the OTA group (Fig. 7E).

3.5. Effect of Cur on OTA-induced MI inflammation and is associated with TLR4/NFκB signaling pathway

As Fig. 8 showed, the content of acid phosphatase (ACP) activities and immunoglobulin M (IgM) contents, gene expression of inducible nitric oxide synthase (*inos*), *il-1β, il-6, il-8*, cyclooxygenase-2 (*cox2*), Toll-like receptor 4 (*tlr4*), myeloid differentiation factor 88 (*myd88*), TNF receptor associated factor 6 (*traf6*), protein expression of TLR4, phospho-NFκB (P-NFκB), phospho-inhibiting κB kinase beta (P-IKKβ), phospho-inhibitory subunit of NFκB alpha (P-IκBα) and immunofluorescence staining of IL-6 were affected by OTA, Cur and OTA interaction with Cur ($P < 0.05$). However, the activity of lysozyme (LZ) was affected by OTA and Cur ($P < 0.05$), but not by their interactions ($P > 0.05$). Protein expression of MYD88 was affected by Cur and OTA × Cur interactions ($P < 0.05$), but not by OTA ($P > 0.05$). Compared with the control group, the OTA alone treatment group significantly decreased the LZ and ACP activities as well as IgM contents ($P < 0.05$). In contrast, the OTA + Cur group significantly increased the LZ and ACP activities as well as the IgM contents ($P < 0.05$) compared with the OTA group (Fig. 8A–C). Consistently, the OTA treatment group significantly increased the proinflammatory factors and their signaling

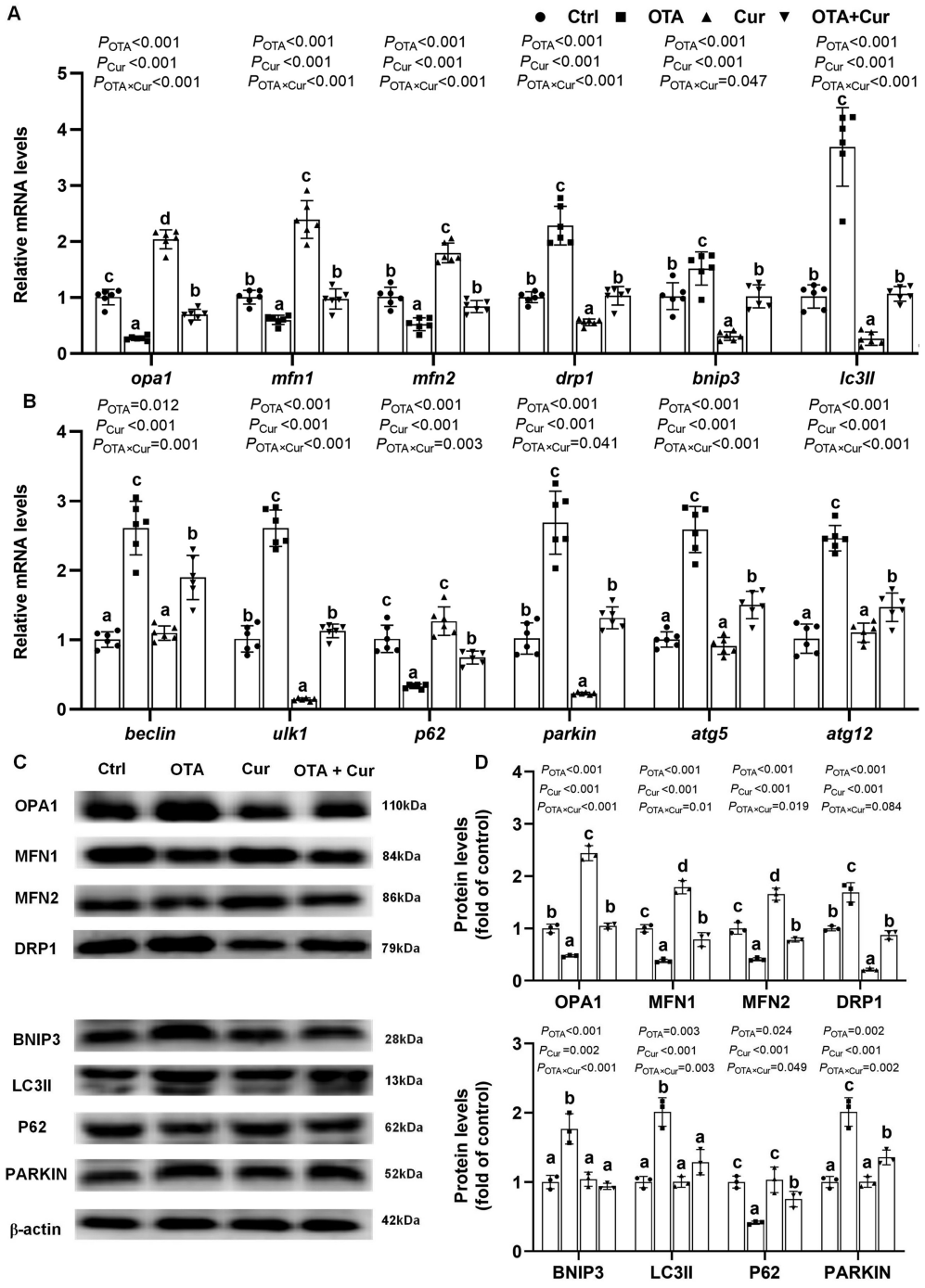

Fig. 5. Dietary curcumin (Cur) supplementation alleviated ochratoxin A (OTA)-reduced mid-intestine mitochondrial biogenesis of juvenile grass carp. (A, B) The mRNA expression, $n = 6$. (C, D) The protein expression, $n = 3$. The different letters a, b, c, and d represent significant differences between different groups ($P < 0.05$). OPA1 = optic atrophy 1; DRP1 = dynamin-related protein 1, MFN (1, 2) = mitofusin (1, 2); BNIP3 = Bcl-2 adenovirus E1B 19 kDa interacting protein 3; LC3II = light chain 3 II; P62 = sequestosome I.

regulation molecules: *inos*, *il-1β, il-6, il-8*, *cox2*, *tlr4*, *myd88*, and *traf6* mRNA abundances, as well as TLR4, MYD88, P-NFκB, and P-IKKβ protein abundances ($P < 0.05$), whereas decreased the P-IκBα protein abundances ($P < 0.05$) (Fig. 8D–F). In contrast, the OTA + Cur group significantly decreased the *inos, il-1β, il-6, il-8, cox2, tlr4, myd88*, and *traf6* mRNA abundances as well as TLR4, MYD88, P-NFκB and P-IKKβ protein abundances ($P < 0.05$), whereas increasing the P-IκBα protein abundances (Fig. 8D–F). Besides, the OTA-treatment group increased the IL-6 immunofluorescence intensity ($P < 0.05$) compared with the control group (Fig. 8G and H). In contrast, the OTA + Cur group significantly decreased the IL-6 immunofluorescence intensity ($P < 0.05$) compared with the OTA group (Fig. 8G and H).

4. Discussion

4.1. Cur improved grass carp growth and MI histopathology exposed with OTA

Cur is a feed additive with antioxidant and anti-inflammatory properties (Kocaadam and Şanlier, 2017), and previous studies showed that dietary Cur above 400 mg/kg could improve the

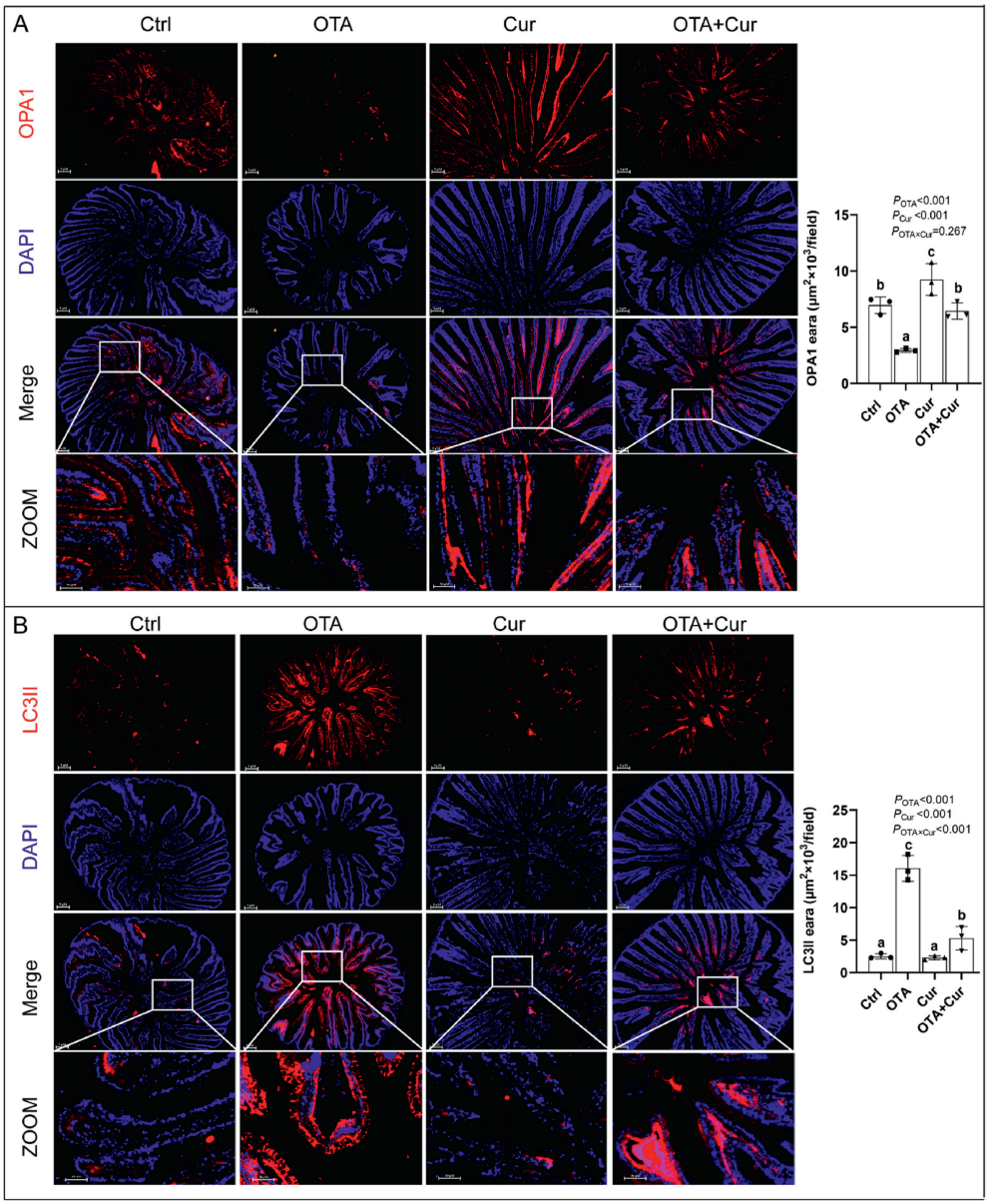

Fig. 6. Dietary curcumin (Cur) supplementation increased PGC-1α and decreased the LC3II immunofluorescence in the juvenile grass carp mid-intestine under ochratoxin A (OTA) inhibition, magnification 40× ($n = 3$). (A) Immunofluorescence and statistical results of PGC-1α; (B) Immunofluorescence and statistical results of LC3II. The different letters a, b, and c represent significant differences between different groups ($P < 0.05$). PGC-1α = peroxisome proliferators-activated receptor γ coactivator alpha; LC3II = light chain 3 II.

growth performance of grass carp (Ming et al., 2020). Studies on ducks showed that Cur could improve growth performance inhibition caused by OTA (Zhai et al., 2020). This is consistent with our study, which found that the addition of 400 mg/kg Cur alleviated the decline in growth performance-related indices WG, FI, SGR, FE, and FCR caused by 1.2 mg/kg OTA. In addition, our previous study on grass carp showed that OTA caused reduced growth performance and intestinal damage related to its accumulation in the intestine (Liu et al., 2020). In the present study, we found that, compared with the OTA alone-treatment group, the Cur + OTA co-treatment group significantly reduced the amount of residual OTA in the MI.

Furthermore, absorption and efflux transporters in the intestinal epithelium were key components of toxin accumulation, which included transport (SLC22) transporters (e.g., OAT1, 3, OCT2, and OATP1D1, 2B1) and efflux transporters (e.g., ABCG2, ABCC2) (Ohtani et al., 2008). In the present study, compared with the OTA group added alone, the Cur + OTA treatment group downregulated the gene expression abundance of *oat* (*1, 3*) and *oatp* (*1d1, 2b1*) while it upregulated *abcc2* and *abcg2*. However, Cur reduced OTA accumulation by inhibiting *oat* (*1, 3*) and *oatp* (*1d1, 2b1*) expression and promoting *abcc2* and *abcg2* expression in the grass carp MI. This may be due to the fact that Cur is a substrate for absorption and efflux transporters (Zhou et al., 2016). This was similar to the study in the cisplatin induced nephrotoxicity rat that showed Cur could reduce the gene expression of *oat* (*1, 3*), *oct2*, and *oct2*, as well as promote the gene expression of *mrp* (*2, 4*) (Sahin et al., 2015). Furthermore, we performed an analysis of intestinal morphology, as the height of intestinal folds were important indicators for measuring intestinal health (Dong et al., 2022), and found that the OTA + Cur co-treatment group improved intestinal inflammation and increased intestinal fold height compared to the OTA alone

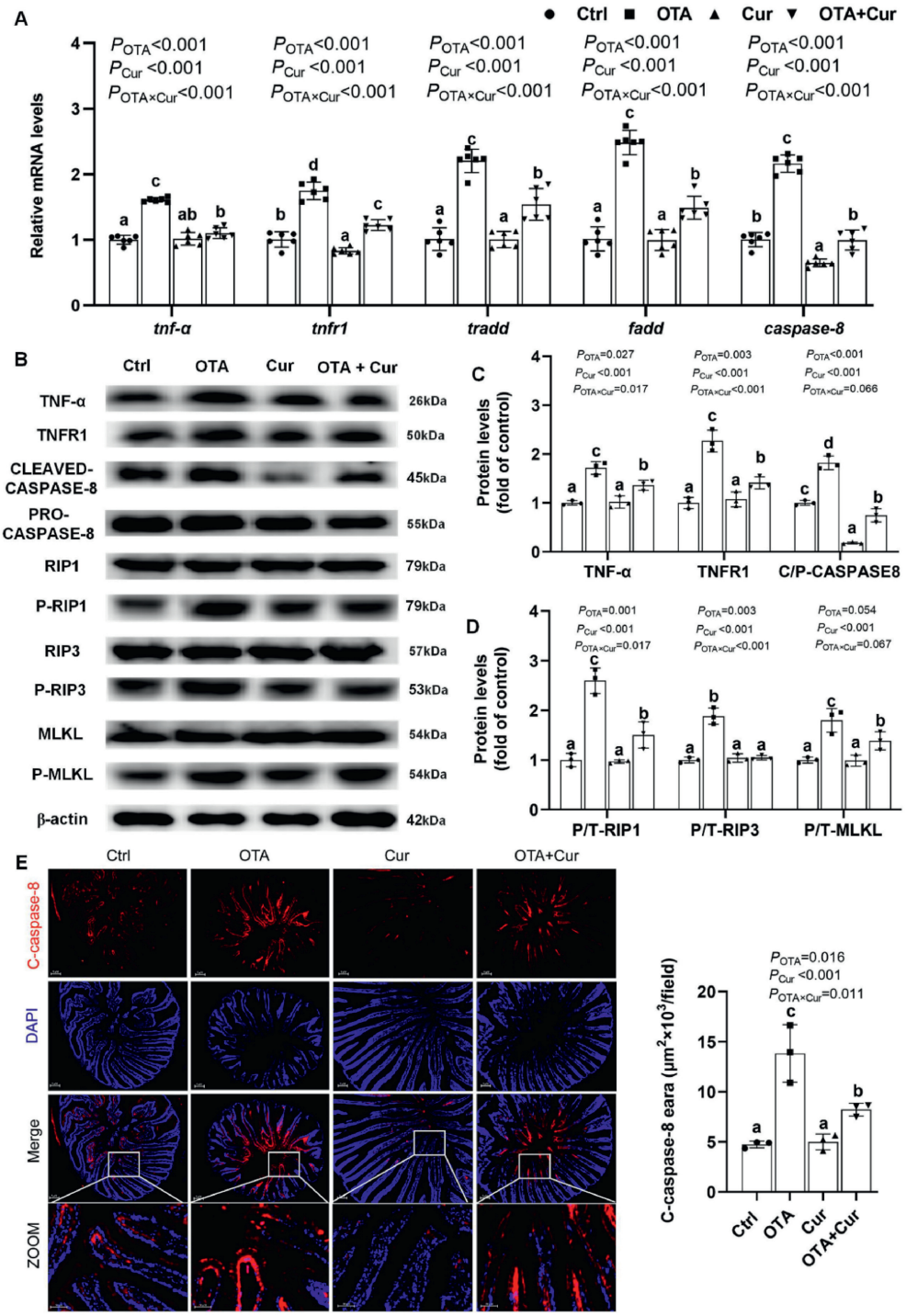

Fig. 7. Dietary curcumin (Cur) alleviated the necroptosis of the mid-intestine of grass carp caused by ochratoxin A (OTA). (A) The mRNA expression, $n = 6$. (B–D) The protein expression, $n = 3$. (E) Immunofluorescence of cleaved-caspase 8, magnification 40× ($n = 3$). The different letters a, b, c, and d represent significant differences between different groups ($P < 0.05$).

treatment group. This suggests that dietary Cur can improve OTA-damaged intestinal health. Moreover, according to the results of TEM, the Cur treatment group significantly inhibited mitochondrial autophagy and necroptosis in the intestine caused by OTA. Thus, we explored the detailed mechanism of whether Cur can improve mitochondrial function.

4.2. Cur alleviated oxidative damage caused by OTA by enhancing mitochondrial antioxidant function in grass carp MI

Mitochondrial function is mainly dependent on homeostasis, which mainly includes antioxidant function, mitochondrial biogenesis, and mitochondrial autophagy (Ghosh et al., 2019).

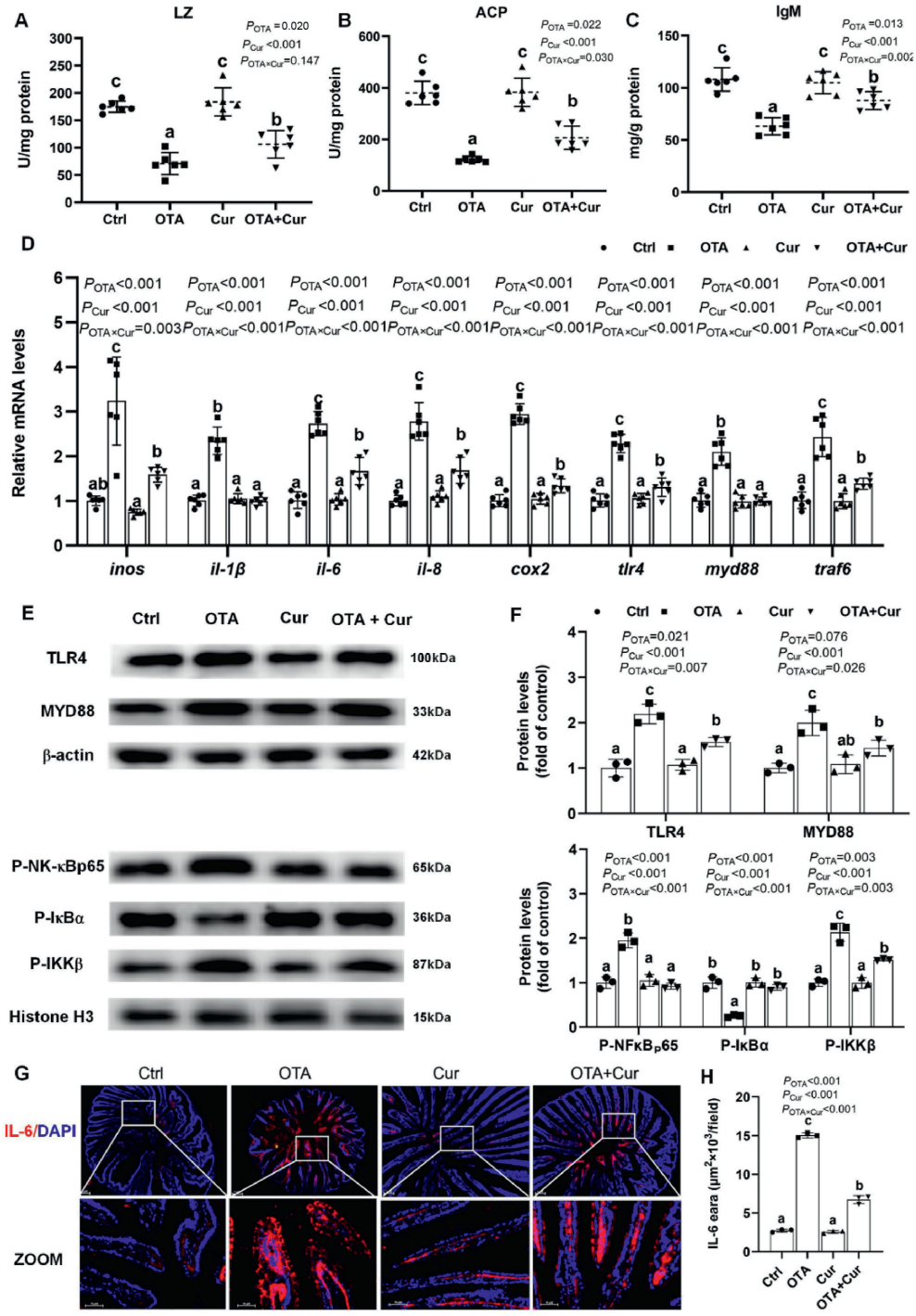

Fig. 8. Dietary curcumin (Cur) alleviated ochratoxin A (OTA)-induced mid-intestine inflammation through TLR4/NFκB signaling pathway. (A) LZ activities, $n = 6$. (B) ACP activities, $n = 6$. (C) IgM contents, $n = 6$. (D) The mRNA expression, $n = 6$. (E, F) The protein expression of TLR4, MYD88, phospho-NFκB (P-NFκB), phospho-IκBα (P-IκBα) and phospho- IKKβ (P-IKKβ), $n = 3$. (G, H) Immunofluorescence and statistical results of IL-6, magnification 40× ($n = 3$). The different letters a, b, and c represent significant differences between different groups ($P < 0.05$). TLR4 = Toll-like receptor 4; NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells; LZ = lysozyme; ACP = acid phosphatase; IgM = immunoglobulin M; MYD88 = myeloid differentiation factor 88; IL-6 = interleukin 6.

Firstly, antioxidant function of mitochondria is dependent on the related thiol antioxidant system, the GSH-Grx-MnSOD pathway (Chen et al., 2016). PGC-1α is the central stimulating element that regulates mitochondrial biogenesis, and by activating NRF2, the body produces an adaptive response to buffer the function decline of the antioxidant defense system caused by oxidative stress (Hota et al., 2012; Scarpulla et al., 2012). ROS generated by OTA is usually attributed to its bio-toxic effects on animals as the explanation for the generated ROS (Liye et al., 2016). In this study, we found that the addition of Cur significantly reduced the increase of ROS, MDA, and LPO caused by OTA and increased the activity or content of T-AOC, T-SOD, GPx, GST, MnSOD, and GSH inhibited by OTA. Consistently, the Cur-OTA co-treatment group increased the *glrx2*, *srxn*, *prdx* (*3, 5*), *trx*, *sirt1*, and *pgc-1α* as well as the protein expression of

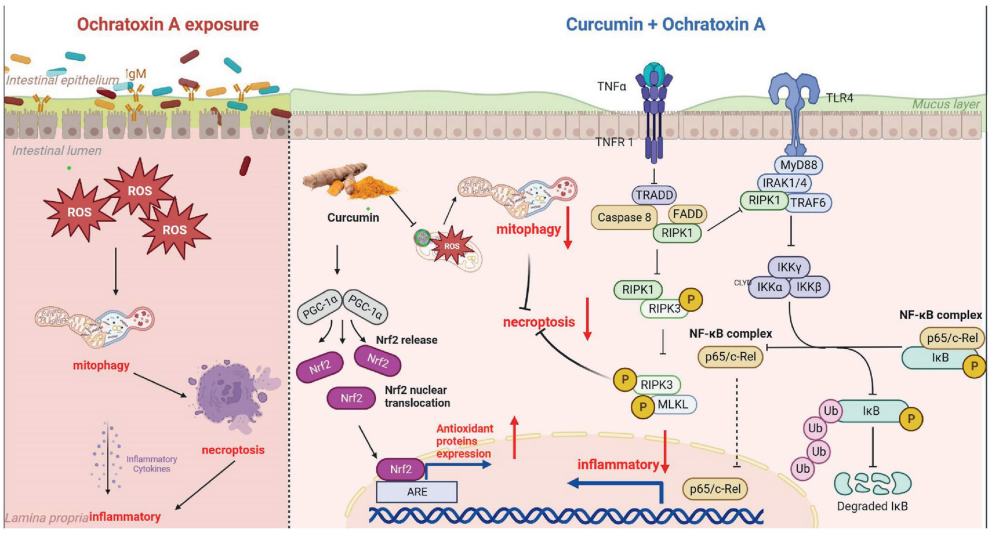

Fig. 9. Mechanism graph of curcumin alleviated intestinal damage caused by ochratoxin A in grass carp. ROS = reactive oxygen specie.

NUCLEAR-NRF2 and PGC-1α in the MI. Thus, Cur can enhance antioxidant capacities throughout the fish's mitochondria. Similarly, Cur was found to be able to activate the SIRT1/PGC-1α/NRF2 pathway, which improved isoniazid-mediated hepatic oxidative stress (Li et al., 2022b). In addition, studies on ducks showed that dietary Cur + aflatoxin B1 (AFB1) co-treatment could inhibit the generation of H_2O_2 and MDA in the liver and activate the NRF2 signaling pathway (Feng, 2021), and dietary Cur + OTA co-treatment could increase CAT activity and *nrf2* mRNAs (Zhai et al., 2020). Hence, the above results show that Cur can enhance the antioxidant function of mitochondria inhibited by OTA through the PGC1a/NRF2 signal pathway.

Secondly, it is well known that mitochondrial biogenesis and mitochondrial autophagy are closely related to maintaining mitochondrial homeostasis (Chung et al., 2019). Mitochondrial biogenesis mainly includes mitochondrial fission and fusion (Yu et al., 2020) and eliminates aging mitochondrial damage through mitochondrial autophagy (Luciani et al., 2020), thereby maintaining mitochondrial homeostasis. Mitochondrial fission and fusion are mainly related to OPA1, MFN 1 and 2, and DRP1 proteins (Wang et al., 2017), while mitochondrial autophagy is mainly carried out through the BNIP3/Parkin pathway (González-Rodríguez et al., 2022). Herein, we observed that the addition of Cur + OTA not only reduced the protein expression of OPA1 and promoted the protein expression of DRP1, but also reduced the mRNA expression of *bnip3, lc3II, beclin1, ulk1, parkin, atg5*, and *atg12*, and promoted the mRNA expression of *p62* compared with OTA alone treatment. At present, there is no effect of OTA on mitochondrial biogenesis. However, some studies have reported that Cur could promote the expression of OPA1 and MFN1 inhibited by 2-chloroethyl sulfide (CEES) in lung epithelial cells and reduce the expression of DRP1 increased by CEES (Kia et al., 2021). Cur alleviated cisplatin-induced acute kidney injury in mice by inhibiting DRP1 and Parkin to enhance OPA1 expression (Ortega-Domínguez et al., 2017). Studies in duck skeletal muscle showed that dietary Cur could reduce oxidative stress, alleviate the fission and fusion of normal mitochondria destroyed (Drp1, OPA1, MFN1/2), and induce mitochondrial autophagy (PINK1, Parkin, LC3II) induced by arsenic trioxide (ATO) (Lan et al., 2022). On the whole, Cur may indeed improve the mitochondrial function destroyed by OTA through inhibiting mitochondrial fission, promoting fusion, and inhibiting excessive autophagy.

4.3. Cur inhibited the necroptosis caused by OTA in grass carp MI

Previous research showed that mitochondrial dysfunction could lead to necroptosis (Eadon et al., 2013). In our TEM results, we found a lot of typical characteristics of necroptosis, including cell membrane fragmentation, cytoplasmic content flowing out, and organelle swelling content dissolving. Necroptosis is a kind of programmed death that follows TNFR1 stimulation by TNF (Wallach et al., 2016). In this study, we discovered that Cur decreased OTA-enhanced *tnf-α*, *tnfr1*, *tradd*, *fadd*, and *caspase-8* mRNAs. The typical feature of necroptosis is the phosphorylation of RIP1 and RIP3 to form necrotic bodies, which promotes the migration of MLKL from the cytoplasm to the cell membrane (Wallach et al., 2016). Our results also found that the OTA alone group significantly increased the expression of intestinal phosphorylated RIP1 and RIP3, while the OTA + Cur co-treatment group significantly reduced the protein expression of P-RIP1 and P-RIP3. Although there is no research on OTA-induced necroptosis and the role of Cur in it, studies in chicken liver showed that the diet supplemented with Cur could inhibit aflatoxin B1-induced cell necrosis through the TLR4/RIPK pathway (Li et al., 2022a). Additionally, our results are consistent with the study in mice. After using glucan sodium sulfate to induce colitis in C57BL/6 mice, dietary Cur could inhibit the protein expression of P-RIP1/3, indicating that Cur is an effective inhibitor targeting RIP3 (Zhong et al., 2023). These results suggest that Cur may indeed alleviate OTA-induced necroptosis by inhibiting the TNFR/RIP/MLKL signaling pathway.

4.4. Cur alleviated inflammation caused by OTA in grass carp MI

When necroptosis occurs, the cell membrane breaks and the contents flow out, which could trigger an inflammatory response (Chen et al., 2008), which is consistent with our results. Our H&E and AB-PAS staining results showed that the OTA alone treatment group presented inflammatory edema and inflammatory cell infiltration, and the number of goblet cells was significantly reduced, but the addition of Cur alleviated these phenomena. Further, the pro-inflammatory factors iNOS, IL-1β, IL-6, IL-8, and COX2 were markers of an inflammatory response (Uribe et al., 2011). Our results showed that the co-addition of Cur + OTA reduced the gene expression of proinflammatory factors (*inos, il-1β, il-6, il-8*, and *cox2*). As is well known, the TLR4/MYD88 complex can activate

NFκB to promote its entry into the nucleus and regulate downstream pro-inflammatory factor gene transcription (Zhuo et al., 2011). The activated inhibitory protein κB kinase can activate the IκBα and promote the transcription of NF-κB-IκBα into the nucleus to initiate related genes (Liu et al., 2019). In this research, our research results showed that Cur + OTA co-treatment decreased TLR4, TRAF6, MYD88, NFκB, IKKβ and increased IκBα protein expression compared with the OTA group. Previous studies in vitro and in vivo have found that OTA can trigger inflammation through the TLR4/MYD88/NFκB pathway in the porcine alveolar macrophages (PAMs) cell line (Xu et al., 2017), rat kidney, and HK-2 cells (Hou et al., 2020). At present, there is no research on Cur to alleviate OTA-induced inflammation, however, previous studies have shown that Cur decreased the protein and mRNA expression of inflammatory genes (*tlr4/myd88/nfκb*) with dietary AFB1 exposure in the chicken liver (Li et al., 2022a). The above shows that Cur may indeed inhibit the transcription of downstream pro-inflammatory factors by inhibiting the TLR4/NFκB signaling pathway, thereby alleviating the inflammatory response caused by OTA.

5. Conclusion

On the whole, this study confirms that Cur might alleviate the OTA-induced intestinal damage by improving the intestinal antioxidant function and mitochondrial function and inhibiting necroptosis and inflammatory reactions in grass carp (Fig. 9). These conclusions were supported by the following findings: dietary Cur reduced the residual OTA in the intestine, which may be related to effect of Cur on intestinal absorption and efflux of transport proteins. Furthermore, Cur might promote the gene expression of antioxidant enzymes by activating the PGC signal, thereby alleviating the intestinal oxidative stress caused by OTA. Cur also maintained intestinal health by maintaining mitochondrial homeostasis and reducing necroptosis caused by OTA. Additionally, Cur might alleviate the intestinal inflammatory reaction caused by OTA through inhibiting the nuclear entry of NFκB and the expression of downstream proinflammatory factors. The results of this study provide a theoretical basis and practical significance for aquaculture to add Cur to alleviate OTA in feed. It also confirms the feasibility of improving fish production performance and mitigating mycotoxin toxicity through the addition of natural products to feed rations in aquaculture.

Author contributions

Piao Zhao: Manuscript writing, Formal analysis; **Wei-Dan Jiang:** Data curation; **Pei Wu:** Methodology; **Yang Liu:** Resources; **Xiao-Wan Jin**, **Hong-Mei Ren:** Management; **Xiao-Qiu Zhou**, **Lin Feng:** Conceptualization, Supervision. **Xiao-Qiu Zhou** had primary responsibility for the final content of the manuscript. All authors carefully read and approved the final revision of the manuscript.

Availability of data and materials

The datasets are included in this article and available from the corresponding author on reasonable request.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This research was financially supported by the Earmarked Fund for CARS (CARS-45), National Natural Science Foundation of China for Outstanding Youth Science Foundation (31922086), the Young Top-Notch Talent Support Program, and the 111 project (D17015). The authors would like to express their sincere thanks to the personnel of these teams for their kind assistance.

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.011.

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