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Ethoxyquin attenuate oxidant stress, inflammatory response and apoptosis in liver of *Channa argus* fed with high-fat dietary

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ABSTRACT

Lipid is an important nutrient in fish dietary, while fatty liver disease induced by feeding high-fat dietary (HFD) is usual disease in aquaculture industry and also make more economic loss. *Channa argus* is a kind of economic fish species and also affected by fatty liver in farming. To explore effect of dietary supplemented ethoxyquin (EQ) alleviate liver injure induced by feeding HFD, *C. argus* fed with HFD diet and HFD supplemented with EQ for 90 days, and antioxidative status, inflammation related biochemical parameters and genes were determined. EQ supplementation could diminish the increased triglyceride (TG) and total cholesterol (TC) levels in liver induced by feeding HFD. Moreover, aminotransferases and pro-inflammation factors levels in serum indicated that feeding HFD could led to liver injure. Feeding HFD could induce high malondialdehyde (MDA) level and decreased levels of anti-oxidant enzymes to impair anti-oxidant capacity of *C.argus*. Whereas, EQ supplementation could regulate nuclear erythroid 2-related factor 2 (Nrf2) for improving anti-oxidant capacity of *C. argus* to alleviate oxidant stress induced by lipid accumulation. EQ could attenuate up-regulated nuclear factor κB (NF-κB) induced by feeding HFD in liver for restraint of pro-inflammatory cytokine genes expression. In conclusion, EQ supplementation in dietary could diminish lipid accumulation and alleviate oxidant stress and inflammation response.

1. Introduction

Channa argus is a kind of freshwater perciform fish family *Channidae*, due to their wide-range diet, parental care, rapid colonization and spread, they are economically important freshwater fishes (Jiang et al., 2016; Jia et al., 2008). Commercial feeds for *C. argus* have been developed based on many reports and studies, fish fed dietary with 12% fat or 42% protein could increase growth performance and liver lipid level (Sagada et al., 2017), high-fat dietary (HFD) were used for more economic benefits in many aquatic farms. As previous study reported fatty liver could be induced by feeding HFD for 10 weeks and it is a normal disease in aquaculture industry (Luo et al., 2010). In previous studies, fatty liver could induce oxidant stress and apoptosis (Kim et al., 2006; Tanaka et al., 2009). Lipid accumulation is a key factor for inducing oxidant stress and liver injure. Oxidant stress would be induced by exceed reactive oxygen species (ROS) which could damage proteins, DNA and lipid in organisms (Morais et al., 2007; Boujard et al., 2004). To eliminate free radical, anti-oxidant enzymes are important, such as catalase (CAT), superoxide dismutase (SOD) and glutathione-related enzymes (Cao et al., 2019). Moreover, nuclear erythroid 2-related factor 2 (Nrf2) is a key regulator of anti-oxidant stress (H. Zhao et al., 2019; W.W. Zhao et al., 2019; Qiang et al., 2019). Previous studies reported that HFD could upregulated kelch-like ECH-associated protein-1 (Keap1) which could inhibit Nrf2 activated, and impair anti-oxidant ability (Tanaka et al., 2009). Activated Nrf2 would be translocated into nucleus and increase anti-oxidant function genes expression, including glutathione S-transferase (GST), heme oxygenase 1 (HMOX-1), SOD and NAD(P)H dehydrogenase quinone (NQO-1), etc. which could protect the liver damaged by oxidant stress fed with HFD (Limbu et al., 2019; Dai et al., 2019). Inflammatory also was induced by fed HFD, and nuclear factor κB (NF-κB) would be activated (Tanaka et al., 2009). Moreover, TLR1 and TLR2 were upregulated to increase inflammatory gene expression in *Oreochromis niloticus* and non-alcoholic fatty liver disease (Mota et al., 2016; Jia et al., 2020).

Ethoxyquin (EQ) is a synthetic component which could treat liver injure induced by oxidant stress and pro-inflammatory response

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(Iskusnykh et al., 2021; Blaszczyk et al., 2013). Previous studies have reported that dietary supplemented with EQ could improve liver anti-oxidant function through modulation GST activity (Iskusnykh et al., 2021; Bohne et al., 2007). In addition, studies have shown that EQ could decrease the levels of inflammatory biochemical parameters in liver (Lin et al., 2019; Miyazawa et al., 1985). However, there is rare report about the effects of dietary supplemented EQ in HFD-induced fatty liver. Therefore, the aim off this study was to explore EQ attenuates oxidant stress and inflammatory response in *C. argus* fed with HFD.

2. Materials and methods

2.1. Experimental diet

The control diet was contained 9% fat level and 40% crude protein (CK). Compared with the control diet, HFD was contained 22% fat level and 40% crude protein (HFD). Based on the HFD, 200 mg EQ (Sigma, Germen, 97.5% purify) were supplemented in per kg dietary contained 22% fat (EQ). The diet was uniformly mixed in a micromixer at room temperature, and dried under aseptic conditions, pelleted and stored at − 4 °C until used. Moreover, the formulation and nutrient content of the diets were shown in Table S1.

2.2. Experimental procedures

Juvenile *C. argus* (aged 12 weeks; weight 38 ± 2 g) were acclimated to lab condition in a recirculation system (28 ± 2 °C; dissolved oxygen > 6 mg/L; pH 7.2–8.1) for 2 weeks and fed on the control diet twice per day prior to the experiment. After acclimation, *C. argus* were randomly divided into nine tanks (3 tanks per group, 50 fish per tank). Moreover, each dietary treatment group were fed at approximately 4% of their body weight and twice per day for 90 days, and kept at experimental condition as previous described.

2.3. Sample collection

At day 30, 60 and 90 of experiment, 10 fish were randomly collected from each group and anesthetized with tricaine methane sulfonate (MS-222, 100 mg/mL, Darmstadt, Germany). Blood was collected from caudal veins of sample fish, and serum was separated by centrifugation (4500 rpm, 4 °C and 12 min). And the liver tissue of sample fish was gathered and flash-frozen in liquid nitrogen for measurement of enzymatic activity, gene relation expression and biochemical parameters. All of the experimental fish were used in accordance with the NIH Guide for the Care and all experimental protocols for this research were approved (21 July, 2018) by the Regulations for Animal Experimentation of Jilin Agricultural University (JLAU08201409).

2.4. Biochemical parameters analysis

Serum GPT, GOT, triglyceride (TG) and total cholesterol (TC) were measured with commercial assay kits according to manufacturer's introduction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and IL-8 were detected by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instruction (Jingmei, Jiangsu, China). The malondialdehyde (MDA) levels, glutathione (GSH) levels and activities of superoxide dismutase (SOD) and catalase (CAT) were determined in serum and liver using commercial assay kits according to manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Quantitative Real-time PCR

Simply P total RNA kit (Bioflux-Bioer, Hangzhou, China) was used for total RNA extraction from the liver tissues. The concentration of RNA samples was examined using Nanodrop 2000c (Thermo Fisher Scientific, Foster city, CA, USA), and the complementary DNA (cDNA) was synthesized using PrimeScript™ RT reagent kit with gDNA eraser (Takara, Dalian, China). The quantitative real-time PCR was performed with Applied Biosystems® 7500 Real-Time PCR systems (Thermo Fisher Scientific, Foster city, CA, USA), using SYBR Green Master Mix (Takara, Dalian, China). The primers of the immune-related genes studied and the β-actin are shown in Table 1.

2.6. Data analysis

Results were presented as means standard deviation (SD). Statistical analysis was performed using SPSS v16.0 software and GraphPad PRISMM v7.0. All data were subjected to a one-way analysis of variance. In all cases, significant differences were considered as $P < 0.05$.

3. Results

3.1. Liver injure

The levels of TC and TG in serum and liver were determined, the results showed in Fig. 1A and B, after feeding HFD 90 days, the levels of TC and TG in serum and liver were significantly increased ($P < 0.05$). Otherwhile, the levels of TG and TC in serum and liver of *C. argus* fed diet supplemented EQ were significantly decreased than HFD group ($P < 0.05$).

To evaluate the liver injury degree, the levels of GPT, GOT, IL-1β, IL-

Table 1
Primer sequences and annealing temperature for qPCR.

Target gene	Sequence (5′-3′)	Annealing temp. (°C)	Putative/ Reference
TNF-α Forward	ACAATACCACCCCAGGTCCCA	61	Li et al. (2020)
TNF-α Reverse	ACGCAGCATCCTCTCATCCAT		
IL-8 Forward	GAGTCTGAGCAGCCTGGGAGT	61	Li et al. (2020)
IL-8 Reverse	CTGTTCGCCGGTTTTCAGTG		
IL-1β Forward	GTTTACCTGAACATGTCGGCTTACG	59	Li et al. (2020)
IL-1β Reverse	AGGGTGCTGATGTTCAGCCCA		
IκBα Forward	AAAGTTGACTTGTGGACGAT	59	Putative
IκBα Reverse	ATTGACAGCAACTTTCCCAC		
NF-κB Forward	TCTGCTCCATGCTTATTGC	55	Putative
NF-κB Reverse	CCTCACCTTCAGGCACTT		
Nqo1Forward	TAGATGGTGTCCCGTTGT	58	Putative
Nqo1 Reverse	TCACTGTCCTCCCTTGGT		
Hmox1 Forward	TAGTGGCTGCTGGGACAA	55	Putative
Hmox1 Reverse	CCAGGCTTTGGAGGAAGA		
GST Forward	GAGGGAGAGCGACACGAC	62	Putative
GST Reverse	TCACATAACCCAAAACGG		
Keap1 Forward	ACACTAATCGCTGGTCCTTT	55	Putative
Keap1 Reverse	TGTTTGTATCGGGCTGGT		
Nrf2 Forward	GGGTCTGGAGGGTGAGTT	60	Putative
Nrf2 Reverse	CTGCTGGAGTGAGTAGTTGG		
Bax Forward	AGGAAGACACGCTGAGAC	55	Putative
Bax Reverse	TTGGAAACCTGAGGGAAA		
Bcl2 Forward	GAGACATCTCCTGCTCAATG	59	Putative
Bcl2 Reverse	TCGAAATAGGTGGGCTTT		
Cas3 Forward	CAGGCTACTACTCAT	55	Putative
Cas3 Reverse	ATCCAGGTAAAGTG		
Cas8 Forward	CACTGAGGTGGCA	53	Putative
Cas8 Reverse	GCTCGACGGATAG		
β-actin Forward	CACTGTGCCCATCTACGAG	57	Li et al. (2020)
β-actin Reverse	CCATCTCCTGCTCGAAGTC		

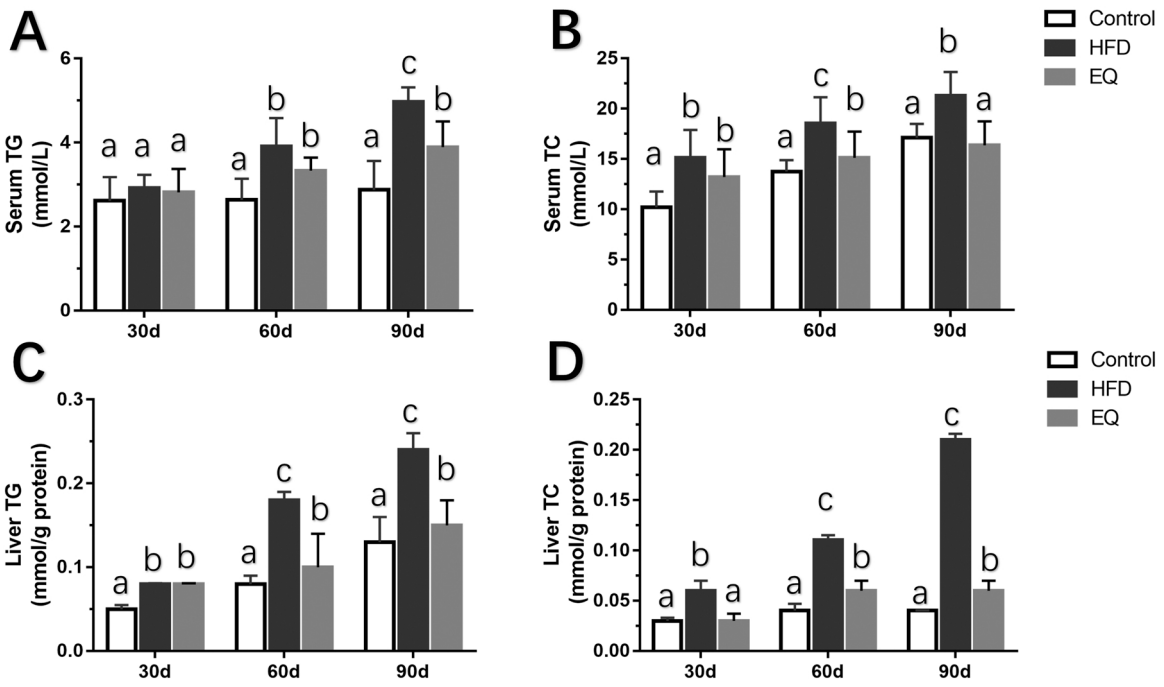

Fig. 1. Biochemical parameters in serum and liver of *C. argus* fed with control diet, HFD and EQ supplemented dietary for 90d. (A) TG level in serum of *C. argus*; (B) TC level in serum of *C. argus*; (C) TG level in liver of *C. argus*; (D) TC level in liver of *C. argus*; The values are normalized to control values and expressed as means ± S. D. (n = 10). Values with different letters denotes significant differences between groups at the $P < 0.05$.

8 and TNF-α in serum were detected. The results showed that serum GPT and GOT levels of HFD group at 90 day (Fig. 2A and B) were significantly higher than CK group ($P < 0.05$). Moreover, the levels of GOT and GPT of EQ group were significantly decreased than HFD group ($P < 0.05$). Pro-inflammatory cytokines levels were similar with GPT and GOT results, IL-8, IL-1β and TNF-α levels in serum of EQ group were also significantly lower than HFD group ($P < 0.05$; Fig. 2C and E).

3.2. Biochemical parameters related anti-oxidant ability

The levels of anti-oxidant enzyme activity and MDA were determined in serum of each group *C. argus*. MDA levels in serum and liver of fish fed with HFD were significantly increased than CK group ($P < 0.05$; Fig. 3A and E), and there was no significant difference between EQ supplemented group and CK group ($P > 0.05$). Moreover, the levels of anti-oxidant enzymes (GST, SOD and CAT) in serum of *C. argus* fed with HFD were significantly decreased at 90 days ($P < 0.05$; Fig. 3B–D), and GST and SOD levels in EQ supplemented group were significantly higher than HFD group ($P < 0.05$). Similarly, those anti-oxidant enzymes in liver were determined as shown in Fig. 3F–H, GST and CAT levels in EQ supplemented group were significantly higher than CK group and HFD group at 90 days ($P < 0.05$).

3.3. The mRNA expression of genes related Nrf2 pathway

Nr-f2 gene plays a key function to regulate many genes related anti-

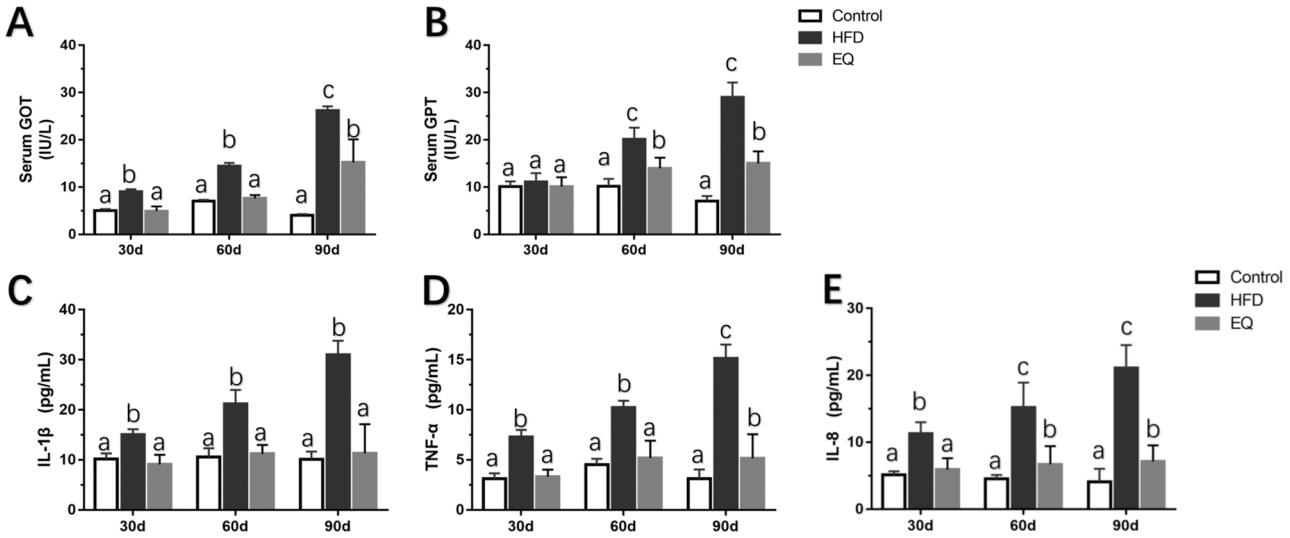

Fig. 2. Biochemical parameters related with liver injure of *C. argus* fed with control diet, HFD and EQ supplemented dietary for 90d. (A) GOT level in serum of *C. argus*;(B) GPT level in serum of *C. argus*; (C) IL-1β level in serum of *C. argus*; (D)TNF-α level in serum of *C. argus*; (E) IL-8 level in serum of *C. argus*. The values are normalized to control values and expressed as means ± S.D. (n = 10). Values with different letters denotes significant differences between groups at the $P < 0.05$.

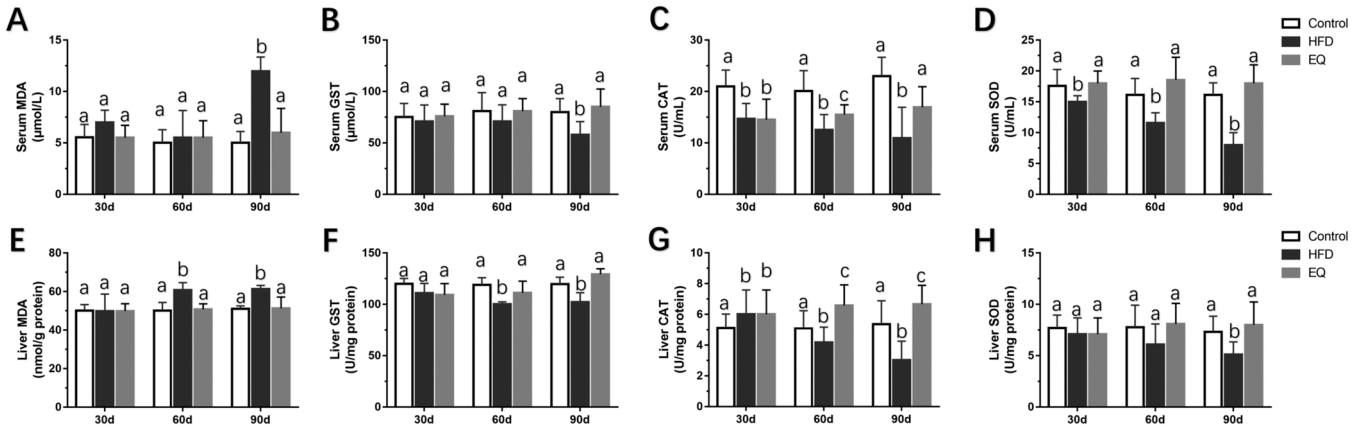

Fig. 3. Anti-oxidant statues in serum and liver of *C. argus* control diet, HFD and EQ supplemented dietary for 90d. (A–D) Effects of anti-oxidant statues in serum of *C. argus*; (E–H) Effects of anti-oxidant statues in liver of *C.argus*. The values are normalized to control values and expressed as means ± S.D. (n = 10). Values with different letters denotes significant differences between groups at the $P < 0.05$.

oxidant capacity, such as Hmox-1, Nqo-1 and GST. Therefore, the expression of those genes related Nr-f2 pathway in liver were determined by qPCR, as shown in Fig. 4A–E. Nr-f2 expression in liver of *C. argus* fed with HFD was significantly lower than CK group at 30, 60 and 90 days ($P < 0.05$), while the gene expression of Keap1 related repressing Nrf2 expression was significantly increased in HFD group than CK group ($P < 0.05$). Otherwise, the expression of Nr-f2 in liver of *C.argus* fed with EQ supplement was significantly increased than CK group and HFD group ($P < 0.05$), and Keap1 expression in EQ group was also significantly decreased ($P < 0.05$). Similarly, the expression of GST, Hmox-1 and Nqo-1 genes, downstream of Nr-f2, were also constrained in *C.argus* liver of HFD group than CK group at 90 days, while those genes expression were significantly increased in liver of *C.argus* of EQ group at 90 days ($P < 0.05$).

3.4. The mRNA expression of inflammatory genes

NF-κB, activated by isolation from its binding protein IκB, is the important proinflammation regulator, its downstream genes, such as IL-1β, IL-8 and TNF-α, were determined by qPCR, the results were as shown in Fig. 5A–E. Compared with the control group, expression level of NF-κB in *C.argus* liver fed with HFD was significantly increased at 90 days ($P < 0.05$). Otherwhile, feeding with EQ supplemented dietary could significantly alleviate the increase of NF-κB expression induced by feeding HFD at 90 days ($P < 0.05$). On the contrary of NF-κB expression, the expression level of IκB in liver of HFD group was significantly lower than the control group ($P < 0.05$), while this gene expression level in EQ supplemented group was significantly increased than HFD group at 90 days ($P < 0.05$). NF-κB downstream genes, including IL-8, IL-1β and TNF-α, were down-regulated by feeding with HFD, and those genes expression levels in *C.argus* liver fed with HFD were significantly higher than the control group at 90 days ($P < 0.05$). Moreover, the expression levels of IL-8, IL-1β and TNF-α in liver of *C.argus* fed with EQ supplemented dietary were decreased than HFD group at 90 days ($P < 0.05$).

3.5. The mRNA expression of genes related apoptosis

To explore the effect of EQ supplementation alleviate apoptosis

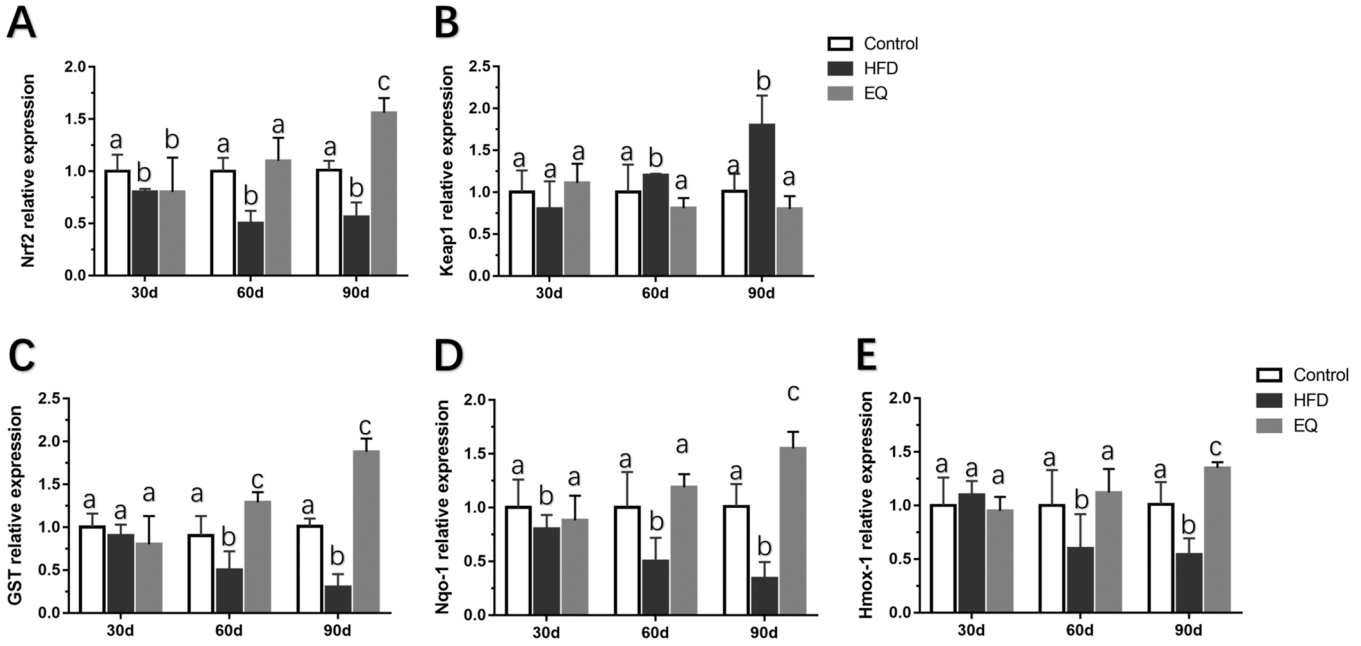

Fig. 4. The mRNA expression of genes related Nr-f2 pathway in liver of *C. argus* control diet, HFD and EQ supplemented dietary for 90d. The values are normalized to control values and expressed as means ± S.D. (n = 10). Values with different letters denotes significant differences between groups at the $P < 0.05$.

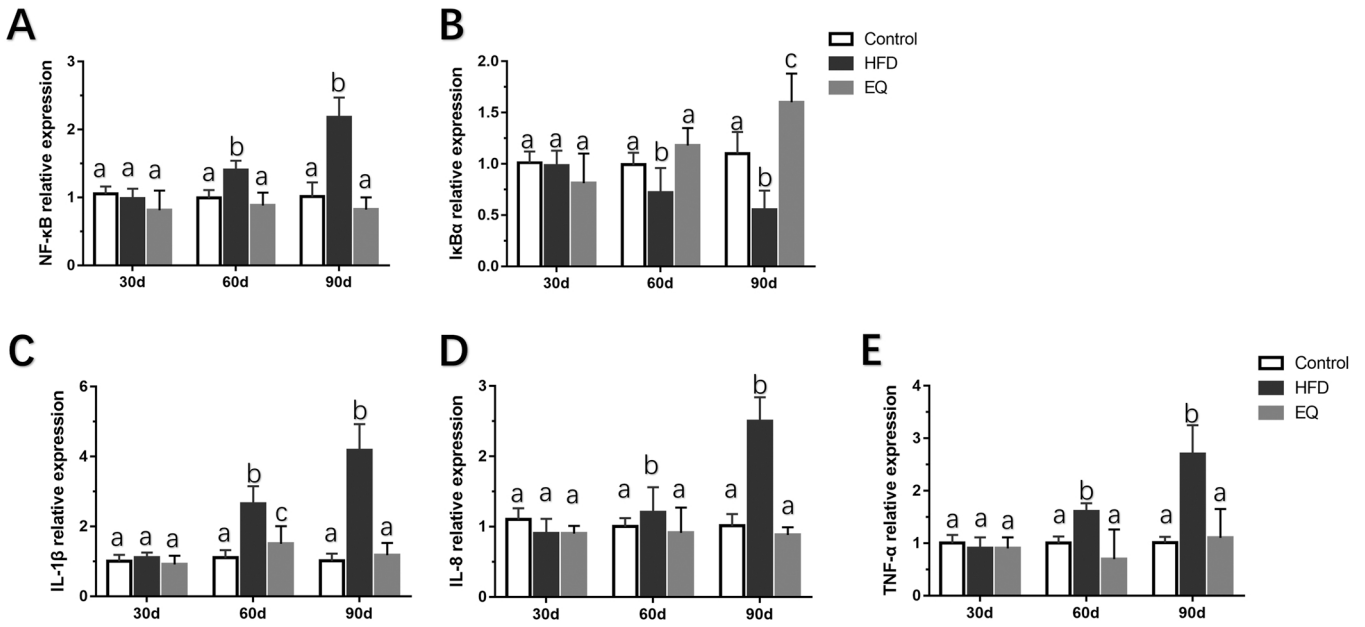

Fig. 5. The mRNA expression of genes related inflammatory cytokines in liver of *C. argus* control diet, HFD and EQ supplemented dietary for 90d. The values are normalized to control values and expressed as means ± S.D. (n = 10). Values with different letters denotes significant differences between groups at the $P < 0.05$.

induced by feeding HFD in *C.argus*, the mRNA expression levels of genes related apoptosis were determined. As shown in Fig. 6, after 90 days of feeding experiment, the mRNA expression levels of Caspase-3 (Cas3) and Cas8 were significantly increased in liver of *C.argus* fed with HFD ($P < 0.05$; Fig. 6C and D). Moreover, the expression of Bcl-2 that is as inhibitor of apoptosis was significantly down-regulated in liver of *C. argus* fed with dietary supplemented EQ ($P < 0.05$; Fig. 6A). However, Bax gene is also regulator of apoptosis, and EQ supplementation could attenuate increased expression level of Bax in liver induced by feed HFD ($P < 0.05$; Fig. 6B).

4. Discussion

To gain more benefit, more fish were fed with HFD in the intensive farming. Moreover, fatty liver induced by feeding HFD is a normal disease in aquaculture and led to more economic lost. In the previous study, feeding HFD could led decreased anti-oxidant capacity and increased inflammation responses (Jia et al., 2020, 2019; Spahis et al., 2017). EQ is a stronger anti-oxidant to prevent the oxidation of animal dietary. *C. argus* used as experimental animal, EQ was supplemented in dietary to relieve oxidant stress induced by feeding HFD, which has not yet been reported (Iskusnykh et al., 2021; Blaszczyk et al., 2013). In this study, *C. argus* were fed with HFD and HFD supplemented EQ, biochemical parameters related with anti-oxidant statue and inflammation were determined to evaluate alleviation effects for fatty liver of EQ.

Lipid accumulation was induced by feeding HFD (Tao et al., 2018). In this study, feeding HFD could increase the levels of TG and TC in serum and liver. Moreover, EQ supplementation dietary could alleviate increased levels of TG and TC, together with GOT and GPT. GOT and GPT are the indicators to evaluate liver statue (Jia et al., 2020), those results could indicate that EQ supplement dietary may alleviate liver injure induced by feeding HFD. MDA is an important biochemical parameter for evaluating oxidant stress statue (Li et al., 2020). Feeding HFD could also led to decreased anti-oxidant capacity (Jia et al., 2020; Chen et al., 2016). EQ is as anti-oxidant supplemented in dietary to prevent oxidation (Blaszczyk et al., 2013). Through determining the levels of biochemical parameters, decreased MDA level in serum and liver of *C.argus* fed with EQ supplementation dietary suggested that EQ supplementation dietary could alleviate oxidant stress of *C.argus* induced by feeding HFD. Moreover, the levels of anti-oxidant enzymes were also increased in serum and liver of *C.argus* fed with EQ supplementation dietary, those results suggested that EQ could improve anti-oxidant capacity of *C.argus*. Nr-f2 is an important gene to regulate anti-oxidant response (Ma, 2013; Li et al., 2018) Many studies reported HFD could down-regulate Nr-f2 expression level to impair anti-oxidant defense to induce liver injure (Limbu et al., 2019; Jia et al., 2019; Tao et al., 2018). Similarly, down-regulated expression of Nr-f2 in liver was determined in HFD group, and EQ supplementation could activate Nr-f2 and increase expression levels of its down-stream genes, such as GST and SOD (Narasimhan et al., 2014). Previous studies have reported that EQ could improve anti-oxidant capacity to alleviate liver injure (Bohne et al., 2007).

Inflammation response is also induced by feeding HFD in liver (Spahis et al., 2017). Many studies have reported that feeding HFD could

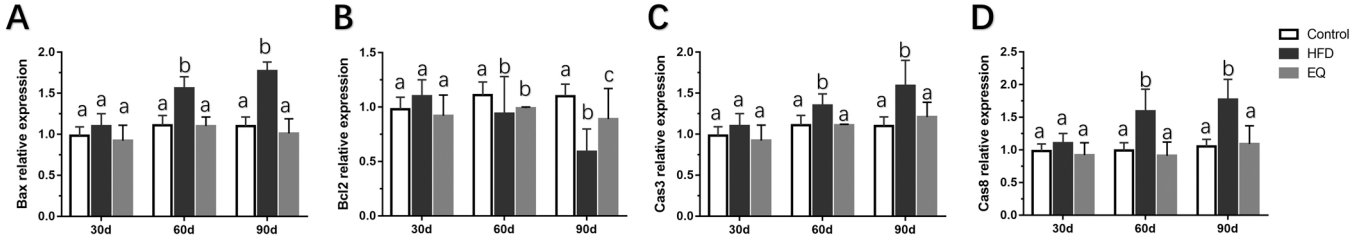

Fig. 6. The mRNA expression of genes related apoptosis in liver of *C. argus* control diet, HFD and EQ supplemented dietary for 90d. The values are normalized to control values and expressed as means ± S.D. (n = 10). Values with different letters denotes significant differences between groups at the $P < 0.05$.

up-regulate expression of NF-κB to enhance inflammation response (Zhao et al., 2015; Jin et al., 2019). In this study, feeding HFD could activate NF-κB through restraint expression of IκB. Moreover, EQ supplementation cloud down-regulate NF-κB expression level to attenuate inflammation response. IL-1β and TNF-α, as a marker of liver injure, would be released in the resident macrophages of the liver (Liu et al., 2016; H. Zhao et al., 2019; W.W. Zhao et al., 2019). In this study, feeding HFD could activate NF-κB expression and increase expression of its down-stream genes in liver to induce liver injure. Corresponding to HFD group, the expression level of NF-κB was significantly decreased in liver of *C.argus* fed with EQ supplementation dietary. EQ supplementation could decrease expression of IL-1β and TNF-α, and alleviated inflammation response via NF-κB pathway. Similarly, EQ reduced the expression of pro-inflammatory cytokine genes (Iskusnykh et al., 2021). It is generally considered that Bcl-2 family protein could regulated apoptosis, including Bcl-2 and Bax (Alkhouri et al., 2011). Previous studies showed that HFD could led to hepatocyte apoptosis (Jia et al., 2020; Lu et al., 2017). In this study, EQ supplementation could attenuate increased expression of genes, including Bax, Cas3 and Cas8, related with pro-apoptosis genes, and up-regulate Bcl-2 gene expression to inhibit apoptosis. As previous studies reported, inflammation response and oxidant stress are key reason for liver injure induced by feeding HFD, and inflammatory cytokines also regulated apoptosis in fish. In this study, those results indicated that EQ supplementation could alleviate inflammation response induced by feeding HFD through NF-κB pathway, and also regulate apoptosis via Bcl-2 family genes.

As previous described, feeding HFD could lead to liver injure through impairing anti-oxidant capacity and inflammation response. EQ supplementation could decrease expression of NF-κB for restraint of pro-inflammatory cytokine genes. Moreover, EQ supplantation could improve decreased anti-oxidant capacity induced by feeding HFD through activating Nr-f2 pathway. Therefore, EQ supplementation could alleviate live injure induced by feeding HFD through activating Nr-f2 and suppressing NF-κB.

5. Conclusion

In summary, this study demonstrated that EQ supplemented in dietary could alleviate decreased anti-oxidant capacity induced by feeding HFD via Nr-f2 pathway. Moreover, EQ supplemented in dietary could diminish expression of genes related inflammatory cytokines for restraint of inflammation response through NF-κB pathway. EQ supplementation could also regulate apoptosis through up-regulating Bcl-2 gene expression and inhibiting Bax gene expression.

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CRediT authorship contribution statement

Tian Jiaxin: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Tao Qingyan:** Conceptualization, Investigation, Visualization. **Li Ying:** Methodology, Writing – original draft. **Wang Guiqin:** Methodology, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2021.100889.

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