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Florfenicol alleviated lipopolysaccharide (LPS)-induced inflammatory responses in *Ctenopharyngodon idella* through inhibiting toll / NF-κB signaling pathways

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ABSTRACT

The present study was conducted to evaluate the anti-inflammatory activity of florfenicol (FFC) against lipopolysaccharide (LPS)-induced inflammatory responses in *Ctenopharyngodon idella* in vivo and in vitro. Head-kidney (HK) macrophages were pre-treated with 10 μg/mL LPS and then exposed to different concentrations of FFC to determine its in vitro anti-inflammatory activity. Inhibitory effect of FFC on inflammatory mediators TNF-α, IL-6 and IL-1β, as well as LPS-induced nitric oxide (NO) and prostaglandin E 2 (PGE 2) production were assayed by ELISA. The expression level of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were investigated by RT-PCR. Expression level of TLR-related genes (TLR1, TLR2, TLR4, TLR7, TLR8) expression, tumor necrosis factor receptor-associated factor 6 (TRAF6), transforming growth factor-b-activated kinase 1 (TAK1), Myeloid differentiation factor 88 (MyD88), nucleus p65, NF-κBα (IκBα) were measured by RT-PCR after grass carp were treated with 50, 100 and 200 mg FFC/kg body weight for 3 days. Results from in vitro tests demonstrated that FFC dose-dependently inhibited LPS-induced inflammatory cytokines TNF-α, IL-6 and IL-1β, inflammatory factors NO and PGE 2 production in macrophages. In addition, iNOS and COX-2 expression levels decreased significantly as compared with LPS treated group. In vivo test demonstrated that treatment with FFC prevented the LPS-induced upregulation of TNF-α, IL-6, IL-1β, NO and PGE 2. The expression level of iNOS, and COX-2 in FFC-treated grass carp were also downregulated as compared with LPS treated fish. Besides, FFC blocked the expression of Toll-like receptor 2 (TLR2) and then suppressed the phosphorylation of nuclear transcription factor-kappa B (NF-κB) p65 and degradation inhibitor of IκBα. Furthermore, administration of FFC inhibited the up-regulation of IRAK4, TRAF6 and TAK1 induced by LPS. These results suggest that the anti-inflammatory properties of FFC might be the results from the inhibition of iNOS, COX-2, IL-6, IL-1β, and TNF-α expressions through the down-regulation of Toll/NF-κB signaling pathways.

1. Introduction

Inflammation is a localized protective response elicited by injury or destruction of tissues that serves to destroy, dilute, or sequester both the injurious agent and injured tissue [1]. Macrophages are major immune cells in the innate immune system. The activation of macrophages plays an important role in the inflammatory response of pathogen infection. Macrophages can kill pathogens directly through phagocytosis or indirectly by secreting various pro-inflammatory mediators such as bioactive lipids, reactive oxygen and nitrogen species, cycloxygenase-2 (COX-2), and pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) [2,3]. However, excess inflammatory mediators produced by activated macrophages are associated with tissue injuries, vascular abnormalities in gill, gut barrier failure et al. The imbalance between pro-inflammatory and anti-inflammatory cytokine expression could lead to host immunologic injury [4].

More and more research have been proved that LPS can bind to toll-like receptors (TLRs) to initiate the intracellular signaling [5]. TLRs, as a cluster of pattern recognition receptors, is a key recognition constitute of microbial components, and its cytoplasmic domain can activate downstream signaling molecules such as MyD88 [6]. MyD88, as the first identified TIR domain containing adaptor protein, was recruited by all TLRs except TLR3 in mammals [7]. Principal functional domains of MyD88 consisted of C-terminal Toll-like/IL-1 receptor (TIR) domain and N-terminal death domain [8]. The TIR domain interacted with the TIR domain of activated TLRs, and the death domain was involved in

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recruiting downstream molecule IL-1 receptor-associated protein kinases (IRAKs) [9]. Subsequently, the MyD88-IRAK complex induced the auto-ubiquitination of TRAF6, which is the only member of TRAFs family that participates in the MyD88-dependent pathway [10,11]. Then, it activated the NF-κB and activator protein (AP-1), and inflammatory cytokines were produced and eventually eliminate pathogens [12].

Extensive evidence suggest that antibiotics possess anti-inflammatory/immunomodulatory properties which are separate and distinct from their antibiotic actions [13,14]. These anti-inflammatory/immunomodulatory properties of antibiotics are widely used for inflammatory disease [15]. Florfenicol (FFC), an antibiotic used to treat infection, has previously been shown to modulate inflammatory responses in endotoxemia and acute lung injury [16,17]. Shiry et al. [18] reported that orally administrated florfenicol in rainbow trout showed greatly immunomodulatory effects. However, whether FFC has anti-inflammatory effect in addition to exerting its antibacterial activity in fish have not been demonstrated. The purpose of the work presented here was to examine FFCs on inflammatory responses in vitro and in vivo, the mechanisms and signaling pathways underlying this property was also determined.

2. Material and methods

2.1. Fish

Health grass carp (32.4 ± 5.6 g) with consistent specifications, smooth body surface and complete fin scales were pursed from a local fish farm (Guangzhou, China) and were acclimatized to laboratory conditions for 2 weeks in 500-L plastic tanks at 25 ± 1 °C. Before the experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney and spleen. Fish were also randomly sampled for PCR assay to make sure they were free from GCRV, the parasites on fish surface were check by micro examination. No bacteria, parasite and virus were detected from any of the examined tissues of the sampled fish. The fish culturing conditions are: 16 h/8 h of light/dark cycle, about 6.0 mg L^{-1} of dissolved oxygen, 0.01 mg L^{-1} of nitrites, 0.15 mg L^{-1} of ammonia, and 7.5 to 8.0 of pH value. In order to keep the water quality, 30% water in each tank was changed with aerated tap water every day. Care of animals was in compliance with the guidelines of the Animal Experiment Committee, Zhejiang Institute of Freshwater Fishery.

2.2. In vitro study

2.2.1. Culture of Head-kidney (HK) macrophages

HK macrophages was prepared according to the method by Giri et al. (2016) [19]. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 IU streptomycin sulfate/mL and 100 IU penicillin/mL. Cultures were maintained in an incubator at 26 ± 0.5 °C in 5% CO_2 and cells were subjected to analysis at 60–80% confluence.

2.2.2. In vitro effect of florfenicol (FFC) against LPS-induced inflammatory in HK macrophages

HK macrophages (1×10^5 cells/mL) were seeded in 6-well plates and then incubated with different concentrations of FFC, after 2 h incubation were then treated with 10 μg/mL LPS (isolated from *Vibrio alginolyticus*) for 24 h. In vitro test were conducted in 5 groups: Group I was control group treated with no chemical, Group II only treated with 10 μg/mL LPS, Group III, Group IV and Group V pretreated with 50, 100 and 200 μg/mL FFC for 2 h and then treatment with 10 μg/mL LPS for 24 h. The supernatants were collected, and the levels of TNF-α, IL-6 and IL-1β were measured by using a fish specific ELISA kit according to the manufacturer's instructions (MyBioSource, San Diego, USA.). Each of group was replicated three times.

2.2.3. Measurements of nitric oxide and prostaglandin E 2 (PGE 2)

Nitric oxide (NO) production was determined by measuring the nitrite concentration using the Griess assay as described previously [16]. PGE 2 synthesis was analyzed using a commercially available PGE 2 ELISA kit (Cayman, MI, USA) following the manufacturer's instructions.

2.2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to evaluate the mRNA expressions of iNOS and COX-2. Macrophages were stimulated with FFC for 2 h followed by LPS treatment for 24 h. After washing twice with PBS, total cellular RNA was extracted with TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The reaction mixture included 10 μL of THUNDERBIRD SYBR qPCR Mix, 1 μL of forward and reverse primer (10 mM) and 1 μL of cDNA, and was then filled up with ultra-pure water to a final total volume of 20 μL. All PCRs were performed at least three times. Data were analyzed by the stratagene MxPro software (stratagene mx3005p, USA).

2.3. In vivo experiment

2.3.1. Feed and experimental design

To further observe the effects of FFC against LPS-induced inflammatory response, we conducted the in vivo test. 150 *C. idella* were randomly chosen and placed into 15 cycling-filtered plastic tanks, each tank placed 10 fish, all fish were divided into 5 groups: group I and Group II were set as control group which were fed with control (basic) diet throughout the feeding trial. Group II, III, IV and V was given with an intraperitoneal injection with 2.5 mg/kg LPS administration on day 1 and day 2, group I received an equal volume of PBS instead of LPS. And then group III, IV and V were fed 50, 100 and 200 mg FFC/kg body weight, respectively. Fish were fed twice a day for 3 days. Kidney tissue from grass carp were sampled on day 4. Prior to all sample collection, fish anesthetized in a MSS-222 (Sigma-Aldrich, USA) bath (200 mg L^{-1}).

2.3.2. ELISA assays of inflammation

The kidney tissues were homogenized in cold PBS at a 1/10 (w/v) ratio, and centrifuged at 5000 g for 20 min at 4 °C, and then the supernatants were obtained for TNF-α, IL-1β and IL-6, assays using fish specific ELISA kit as mentioned in 2.2.2. NO production and PGE 2 were assayed as mentioned in 2.1.4.

2.3.3. RT-PCR

RT-PCR for the detection of the mRNA expressions of TLR-related genes expression (TLR1, TLR2, TLR4, TLR7, TLR8), NF-κB, MyD88,IRAK4, TRAF6, TAK1, nucleus p65, IκBα were performed. Briefly, the kidney tissues were homogenized with PBS on ice. The kidney cells were then collected. The total RNA was isolated from the tissues and cells using TRIzol and reverse transcribed into cDNA. Each transcript was identified using specific forward and reverse primers as per the manufacturer' instructions (Promega, Madison, WI, USA). GAPDH expression was included as an internal, housekeeping gene control. Primer sequences were listed in Table 1.

2.4. Data analyses

All data of RT-PCR were presented as means ± SD and checked for homogeneity of variances and normality. Statistical analysis was performed using SPSS 19.0 probit procedure, a P-value < 0.05 was considered to be statistically significant.

3. Results

3.1. In vitro study

3.1.1. LPS-induced inflammatory response in C. idella Head-kidney macrophages in vitro

The effects of LPS on inflammatory cytokines TNF-α, IL-6 and IL-1β production were detected by ELISA. The results showed that LPS stimulation remarkably increased the production of TNF-α, IL-6 and IL-1β

Table 1
Primers used for the analysis of mRNA expression by RT-PCR.

Genes	Forward (5′-3′)	Reverse (5′-3′)
COX-2	ATCCTTACTCACTACAAAGG	GCTGGTCCTTTCATGAAGTC
iNOS	GGAGGTACGTCTGCGAGGAGGCT	CCAGCGCTGCAAACCTATCATCCA
NF-kB	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG
TLR1	TGTGCCACCGTTTGGATA	TTCAGGGCGAACTTGTCG
TLR2	CTATCAAGTGCTCCTCAAA	CCTCACCCATGTAGTATGT
TLR4	CCACCTATTCATCTTTGCCT	GTCTTCCCCTCTTCCACATC
TLR7	GAGCATACAGTTGAGTAAACGCAC	TCTCCAAGAATATCAGGACGATAA
TLR8	TCACATCGCTTCCAGGTCTC	ACGGTGAAATAATGGGGGTT
NF-κB p65	AACCAAGAACCAGCCATACAAG	AACCAAGAACCAGCCATACAAG
IκB	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA
MyD88	GACTGTCGCCGAAATGA	TGCCTTCTCGCTCCTGT
IRAK4	CTCCACACTGAGAGCTTTATC	ATGTGCAGCTGTGTGTATCT
TRAF6	TCACTCACTGTCAGATGTC	TGTTGGCTCTTGTGTTCA
TAK1	AGACAGGACAGACACCAAT	CATCTTACAGTGCTGCTCAA
GAPDH	AGGGGCTCAGTATGTTGTGG	CTCTCTTGGCACCACCCTTA

as compared with control group. After treated with 10 μg/mL LPS for 24 h, LPS-induced nitric oxide (NO) and prostaglandin E 2 (PGE 2) production were also significantly augmented as compared with the group treated with no LPS (Fig. 1).

The mRNA expressions of iNOS and COX-2 were also determined by RT-PCR after HK macrophages treated with 10 μg/mL LPS for 24 h (Fig. 2), our results demonstrated that transcription level of iNOS and COX-2 genes were augmented. Above results indicated that activation of HK macrophages by LPS could induce the production of inflammatory mediators NO and PGE 2, and inflammatory cytokines TNF-α, IL-6 and IL-1β production, as well as the transcription of iNOS and COX-2 genes.

3.1.2. In vitro effect of FFC against LPS-induced inflammatory in HK macrophage

Protective effect of FFC against LPS-induced inflammatory in HK macrophage were shown in Fig. 1. As shown in Fig. 1, untreated macrophages produced basal levels of TNF-α, IL-6 and IL-1β production, while pre-treatment of FFC significantly suppressed LPS-induced inflammatory cytokines TNF-α, IL-6 and IL-1β production in a concentration-dependent manner. (Fig. 1). The expression level of TNF-α, IL-6 and IL-1β in the highest concentration group (group V) returned to basal level.

It was demonstrated that LPS stimulation induced PGE 2 and NO production (Fig. 1). However, FFC decreased the production of LPS-induced PGE2 and NO in a concentration-dependent manner, but only Group V showed the statistical difference ($P < 0.01$).

The transcriptional changes of iNOS and COX-2 genes in HK macrophage were shown in (Fig. 2). The two test genes transcription were markedly down-regulated after treated with FFC as compared with LPS-induced group ($p < 0.05$).

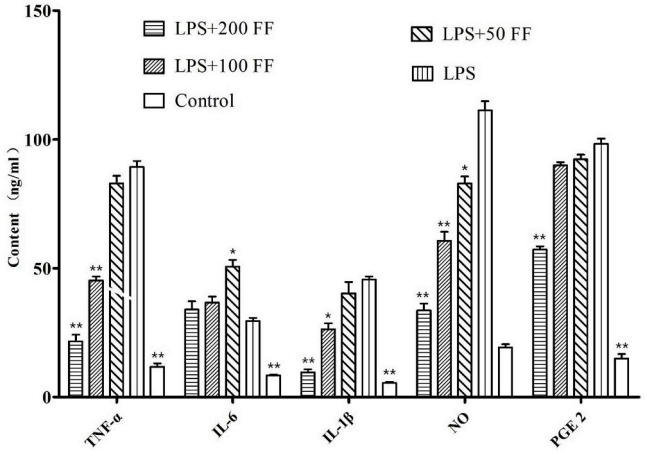

Fig. 1. In vitro effect of FFC on the levels of inflammatory cytokine in head-kidney macrophages. Data are means ± SD from three independent experiments performed in triplicate. **P < 0.01; *P < 0.05. All the group were only compared with LPS-induced group.

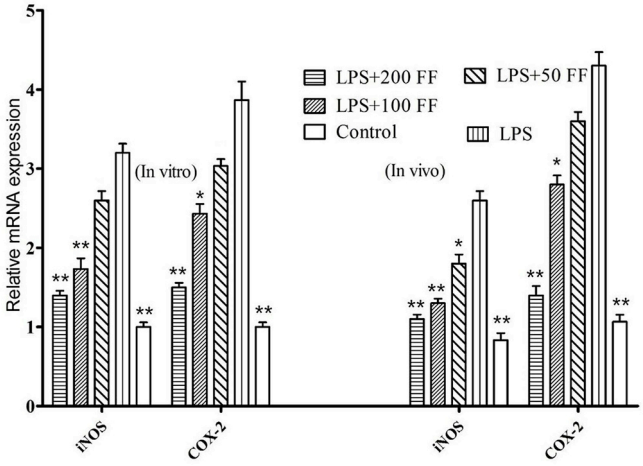

Fig. 2. RT-PCR analysis of the expression of iNOS and COX-2 expression in vitro and in vivo. Three replicates were set for the tests, with ten fish per replicate. Data are means for three assays and presented as the means ± SE. **P < 0.01; *P < 0.05. All the group were only compared with LPS-induced group.

3.2. In vivo study

3.2.1. In vivo effects of FFC on cytokine production

The effects of FFC on expression of TNF-α, IL-1β and IL-6 in the kidney of grass carp following LPS exposure were presented in Fig. 3. As showed in Fig. 3. Production of TNF-α, IL-1β and IL-6 in group IV and V were significantly suppressed as compared with LPS-induced group ($P < 0.05$), in group V with 200 mg/kg FFC the three cytokine production bring down to base level (control group treated with no chemical). However, in low concentration group (50 mg/kg) it showed no difference with control.

3.2.2. In vivo effects of FFC on the production of inflammatory mediators

The effects of FFC on the production of inflammatory mediators, NO and PGE 2 were presented in Fig. 3. LPS induced an activation on the production of NO and PGE 2, however, group III, IV and V treated with FFC lead to dose-dependent suppression of the production of NO and PGE 2($P < 0.05$). Corresponding to this result, the transcription iNOS and COX-2 genes were also down-regulated due to the administration of FFC (Fig. 3).

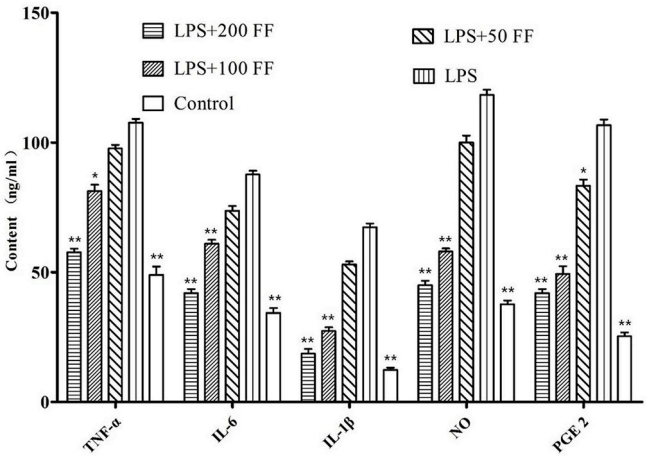

Fig. 3. In vivo effect of FFC on the levels of inflammatory cytokine in grass carp after treated with different concentrations of FFC. Data are means ± SD from three independent experiments performed in triplicate. **P < 0.01; *P < 0.05. All the group were only compared with LPS-induced group.

3.2.3. FFC inhibits the activation of Toll/NF-KB signal pathways

The results of TLR-related genes expression after treated with FFC were shown in Fig. 4. As shown in Fig. 4 the expression of TLR1, TLR2, TLR7, TLR8 were all significantly up-regulated when fish treated with LPS, however, all those genes expression except TLR1 were significantly down-regulated after fed with FFC. The expression of TLR4 showed no difference to control group and LPS-induced group, which means that LPS did not induce the TLR4 expression.

As shown in Fig. 5, the levels of IRAK4, TRAF6, p65, TAK1, IκBα and MyD88 in group II (LPS treated but no FFC treated) were all significantly up-regulated as compared with the control group. However, administration of FFC inhibited the up-regulation of IRAK4, TRAF6, p65, TAK1, IκBα and MyD88as compared with the group II (Fig. 5).

4. Discussion

Macrophages actively participate in inflammatory responses by releasing pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, and inflammatory factors, such as NO and PGE 2, that recruit additional immune cells to sites of infection or tissue injury [4]. Various studies have shown that the concentration of inflammatory cytokines produced by

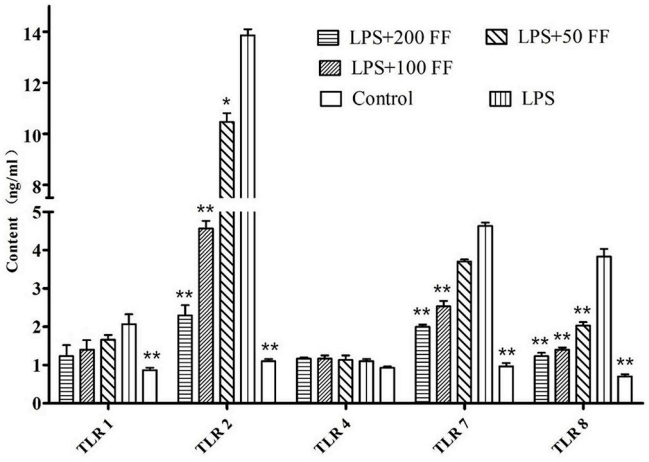

Fig. 4. RT-PCR analysis of the expression of TLR related genes expression in grass carp after treated with FFC. Three replicates were set for the tests, with ten fish per replicate. Data are means for three assays and presented as the means ± SE. **P < 0.01; *P < 0.05. All the group were only compared with LPS-induced group.

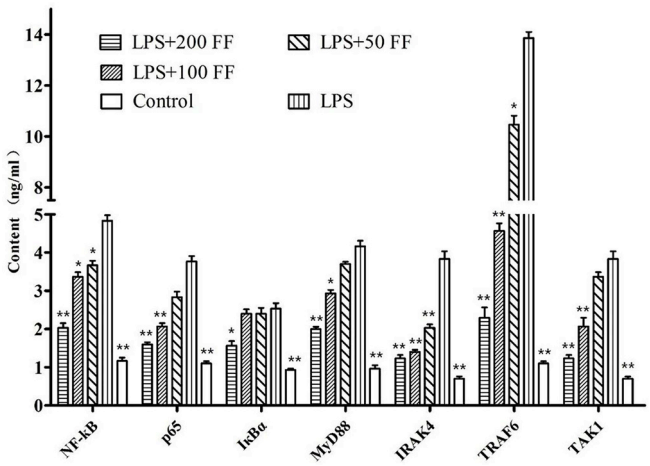

Fig. 5. In vivo effect of FFC on the TLR4 and NF-κB pathway genes expression in LPS induced grass carp. The values presented are mean ± SEM. (n = 10 in each group). P* < 0.05, P** < 0.01 vs. LPS group (only).

LPS stimulation is a reliable indicator of the extent of inflammation [7]. Therefore, inhibitors of these inflammatory molecules have been considered as a candidate of an anti-inflammatory drug.

LPS-stimulated macrophages could activate the expression of genes responsible for synthesis of inflammatory mediators, such as reactive oxygen and nitrogen species (NO, O_2^-, H_2O_2, and peroxynitrite). NO is a free radical. It plays a key role in survival and death of cells by regulating the NF-κB pathway and it plays an important role in the pro-inflammatory effects of many cell types [3]. Extensive evidence suggest that high levels or excess production of NO generated by iNOS may resulted in tissue injuries, vascular abnormalities in gill, gut barrier failure, neurotoxicity and lipids oxidation [20–23]. In the present study, we found that LPS could significantly induced the NO production (5.8 folds than control group), however, after treated with FFC the increased NO production which was induced by LPS were effectively decreased and recovered to normal level after 3 days.

Prostaglandins are small lipid molecules that play an important role in regulating fish immunity and related immune functions, mainly through immunosuppression and anti-inflammatory effects [24]. Secombes et al. [25] founded that PGE 2 could inhibited the proliferation of rainbow trout leucocytes. After added exogenous PGE 2 they found that the expression level of interleukin-6 and interleukin-10 (IL-10) were increased but the expression of TNF-α and INF-γ were inhibited [26]. Overproduction of PGE 2 were also suppressed the expression of the major histocompatibility complex II in trout head kidney leucocytes [27]. In the present study, after treated with LPS, the expression of IL-6 were significantly increased while the TNF-α production were suppressed which is consistent with the result of Seconbes et al. [25] indicated that the overproduction of PGE2 may increase the expression of IL-6 and inhibited the expression of TNF-α. But, in vivo test showed the inconsistent result that the expression of IL-6 were not increased but significantly reduced as TNF-α production. This may because in vivo test was more complex and contain more regulator system. In mammals, prostaglandin E2 is an inflammatory mediator generated by cox-2 at inflammatory sites and was involved in the development of various chronic inflammatory diseases [28,29]. Therefore, drugs could inhibit the COX-2 activity may be beneficial in preventing or treating these diseases [29–31]. Results from the present study demonstrated that FFC significantly inhibited the LPS-induced up-regulation of PGE 2 by reducing the COX-2 expression. Above results suggested that FFC could inhibited the expression of NO and PGE 2 production via suppressing the production of iNOS and COX-2.

Bacterial infection often leads to an inflammatory response mediated by cytokines in fish, and pro-inflammatory cytokines are indicators of increased inflammation and tissue damage [32]. Recent research showed that fish can also reduce tissue damage by producing anti-inflammatory cytokines to resist the pro-inflammatory cytokines. TNF-α,

IL-1β and IL-6 are the most important cytokines. They are the signal of various kinds of interaction between cells, play important roles in the process of host defense and infection and inflammation pathological development [33]. TNF-α is defined as an “early” cytokine, mainly produced by monocytes/macrophages, can elicit the inflammatory cascade, cause a tissue damage in fish [33–35]. IL-1β is an important pro-inflammatory cytokine that plays a vital part in regulating chronic disease processes and host immune responses [36–38]. IL-6, another crucial pro-inflammatory cytokine, play an important role in regulating genes expression involved in cell cycle progression and suppression of apoptosis. In the present study, we found that the levels of TNF-α, IL-1β, and IL-6 were increased evidently in grass carp after LPS exposure and that FFC pretreatment significantly decreased the levels of TNF-α, IL-1β, and IL-6. These results indicated that FFC reduced inflammatory responses may be attributed to the inhibition of inflammatory cytokines TNF-α, IL-1β and IL-6.

The transcription factor NF-κB is responsible for many vital biological processes such as immune response, cell growth and apoptosis [39–41]. It is well known that NF-κB is an important regulator of inflammatory mediators during inflammation and the expressions of several cytokine genes, including TNF-α, IL-1β and IL-6, are associated with NF-κB activation [42]. Therefore, modulation of NF-κB activation is considered to be a well method to control inflammation response. In the present study, we found that FFC inhibited LPS-induced NF-κB activation in HK macrophages which indicated that FFC could alleviated LPS-induced inflammatory response. To further explore the potential molecular mechanism of FFC suppressing the production of pro-inflammatory cytokines, we examined the activation of NF-κB p65 subunit and IκBα in the NF-κB signaling pathway. Our data showed that the phosphorylation of IκBα and (NF-κB) p65 were increased by LPS, but FFC inhibited p65activation and IκBα degradation. as we all known, p65 can regulate the expression of genes such as TNF-α, IL-1β,IL-6, IL8, β-defensin, iNOS and IFN-β [43]. So, this results explain the regulation of FFC on the expression of TNF-α, IL-1β, IL-6, iNOS and COX-2 in vitro and in vivo. Therefore, the results indicated that anti-inflammatory effect of FFC correlated with the inhibition of NF-κB activation.

Broadly speaking, LPS stimulation can initiate the intracellular signaling by binding to toll-like receptors (TLRs) [5] and then to trigger the release of inflammatory factors, free radicals, and cysteinyl aspartate specific proteinases, ultimately resulting in apoptosis [44,45]. MyD88, previously described as a myeloid differentiation marker, is an adaptor protein involved in TLRs regulation [46]. MyD88 can recruit the downstream signaling molecules such as IRAK4 [47], whereas deletion of MyD88 can abolish the response to LPS stimulation [48]. In the present study, we measured the expression levels of 5 TLRs, IRAK4 and MyD88 both in vivo. The results showed that among the 5 TLRS only TLR2 were induced by LPS, and were then reduced by oral administration of FFC. Which may due to the fact that TLR4 in teleost could not recognize LPS unlike TLR4 in mammals [49]. In order to investigate the downstream of TLR2, we performed LPS challenge experiment in vivo, the results demonstrated that MyD88, IRAK4, TRAF6 mRNA expression in LPS challenge group were increased while after treated with those gene expression were down-regulated. These results indicated that FFC can abrogate the LPS-induced MyD88, IRAK4, TRAF6 mRNA expression via TLR2.

In conclusion, our results demonstrated that pretreatment with FFC develops a potent protective effect on LPS-induced inflammatory response which may be associated with its inhibition of Toll/NF-κB signaling pathways activation.

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