Fish and Shellfish Immunology 84 (2019) 627–638

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Chronic exposure to the ionic liquid [C_8mim]Br induces inflammation in silver carp spleen: Involvement of oxidative stress-mediated p38MAPK/NF-κB signalling and microRNAs

Junguo Ma, Xi Chen, Guangyuan Xin, Xiaoyu Li*

College of Life Science, Henan Normal University, Xinxiang, Henan, 453007, China

ARTICLE INFO

Keywords:
Ionic liquids
Silver carp
Oxidative stress
p38MAPK/NF-κB
microRNAs
Inflammatory response

ABSTRACT

The present study aimed to determine the chronic toxicity of 1-methyl-3-octylimidazolium bromide ([C_8mim]Br) on the silver carp to further reveal the toxicological mechanisms of ionic liquids. Chronic exposure of silver carp to [C_8mim]Br at concentrations of 1.095 and 4.380 mg/L for 60 d was conducted under laboratory conditions. The results revealed that chronic exposure to [C_8mim]Br inhibited the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and reduced glutathione (GSH) levels while markedly increasing malondialdehyde (MDA) and protein carbonyl (PC) levels in fish spleen, indicating that [C_8mim]Br treatment induced oxidative stress. Additionally, long-term exposure to [C_8mim]Br markedly upregulated the expressions of nuclear factor-κB (NF-κB), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ); altered the levels of transforming growth factor-β (TGF-β); and increased the mRNA levels of p38MAPK, c-fos, c-jun, and c-myc, suggesting that long-term exposure to [C_8mim]Br might promote the inflammatory response in fish spleen and that p38MAPK/NF-κB signalling may potentially be involved in this process. Moreover, [C_8mim]Br-exposure altered lysozyme activity and complement 3 (C3) and immunoglobulin M (IgM) content, indicating that chronic [C_8mim]Br exposure also has immunotoxic effects on silver carp. Furthermore, we also found that [C_8mim]Br exposure reduced miR-125b levels, altered miR-143 levels, and upregulated miR-155 and miR-21 levels, suggesting that these miRNAs may be involved in the [C_8mim]Br-induced inflammatory response in fish spleen. In summary, the present study indicates that chronic exposure to [C_8mim]Br induces inflammation in fish spleen and that oxidative stress-mediated p38MAPK/NF-κB signalling and miRNAs may play a key role in this process.

1. Introduction

Ionic liquids (ILs) are organic salts that consist entirely of ions with a melting point lower than 100 °C [1] that have been widely considered to be (and extensively used as) environmentally safe “green solvents” since the 1990s due to their unique physiochemical properties, such as insignificant vapor pressure, non-combustibility, superior solubility, and exceptional thermal and chemical stability [2]. However, ILs are not always “eco-friendly” or “green”; because they fail to satisfy all the conditions of green chemistry [3]; this been confirmed by ecotoxicity testing showing that they are toxic to many different cells and organisms, including human cells [4], algae [5], plants [6], invertebrates [7], and vertebrates [8]. Moreover, increasing evidence has demonstrated that the toxicity of some ILs is equal to or even greater than that of conventional organic solvents [9]. In addition, the environmental degradability [10] and biodegradability [11] of ILs are usually poor, giving ILs the potential to stay in ecosystems for a long time after their entry through inevitable effluents and accidental spills with the extensive use [12]. Therefore, ILs have a long residence time in the environment and pose a potential threat to aquatic organisms and human, and the long-term ecotoxicity of ILs remains challenging to evaluate. However, the effects of long-term and low-level exposures to ILs have not been studied for any organisms until now [13].

Fish play a crucial role in keeping the aquatic ecosystem balanced [14], and they are also frequently used as a toxicological model to evaluate the toxicity of chemicals in aquatic ecosystems [15,16]. Currently, there are many reports regarding IL toxicity in fish, including reports on acute toxicity in zebrafish [17] and goldfish [18]; biochemical toxicity in goldfish [18] and zebrafish [19]; genotoxicity in zebrafish [20]; developmental toxicity in goldfish [21]; and IL-induced

* Corresponding author.
E-mail address: lixiaoyu65@263.net (X. Li).

https://doi.org/10.1016/j.fsi.2018.09.052
Received 12 June 2018; Received in revised form 17 September 2018; Accepted 18 September 2018
Available online 19 October 2018

histological alterations in goldfish [22]. In addition, IL exposure also alters the enzymatic and metabolic parameters of silver carp [8]. However, information on the effects of ILs on the immune system of fish is scarce [23].

The fish immune system plays an important role in protecting fish from pathogen infection and external toxicants [24], and it responds rapidly to stress from xenobiotics in aquatic ecosystems by changing the levels or activity of components of the immune index, which may be biomarkers enabling early warnings of aquatic environmental toxicant stress [25]. Although no data have yet confirmed the presence of ILs in the environment, which might be the detection of ILs in water is often limited by detection limits and the cost of analysis [26], however, safety information should be provided for ILs according to current legislation because of their large-scale production and extensive use [27]. Additionally, in order to provide a better future for ILs and avoid making the same mistakes of polychlorinated biphenyls (PCBs), the toxicity test and potential risk should be evaluated before the ILs was found in the environmental waters [28]. In this study, we aimed to determine the effects of long-term exposure to a widely utilized and investigated imidazolium-based IL [7,29], [C_8mim]Br, on silver carp (*Hypophthalmichthys molitrix*) to evaluate the immunological effects of low doses ILs on fish and to understand IL-related environmental and human health risks.

2. Materials and methods

2.1. Chemicals, commercial kits, and fish

[C_8mim]Br (CAS No. 61545-99-1) (purity > 99%) was purchased from Hubei Hengshuo Chemical Co., Ltd., China.

Kits for the determination of oxidative stress parameters and iNOS activity were obtained from the Nanjing Jiancheng Bioengineering Institute (China). ELISA kits for the determination of NF-κB, cytokine, IgM, and C3 levels were purchased from Wuhan ColorfulGene Biological Technology Co., LTD, China.

Healthy juvenile silver carp (weight: 43.72 ± 2.17 g, length: 17.72 ± 1.32 cm) were purchased from an aquarium fishery in China. The fish were maintained in a 200-L tank with aerated and dechlorinated tap water at 25 ± 1 °C with natural light/dark photoperiods under laboratory conditions for 2 weeks prior to the experiment. The domesticated conditions of the fish were as previously described [8], and the physico-chemical parameters of the water used (GB5479–2006) were as follows: temperature 25 ± 1 °C, pH 7.4 ± 0.2, dissolved oxygen 6.7 ± 0.5 mg L^{-1}, hardness 18 ± 1.5 mg L^{-1} $CaCO_3$, turbidity 1.5 NTU. The fish were fed twice daily to satiation. The present study was conducted according to the China Law for Animal Experimentation (ethics approval no. SCXK (YU) 2005–0001).

2.2. [C_8mim]Br exposure and sampling

After acclimatization, 54 fish were randomly divided into 3 groups (18 fish in each group): two [C_8mim]Br-treated groups and one aerated and dechlorinated tap water control group. The toxicity testing of [C_8mim]Br was performed using two concentrations of 1.095 and 4.380 mg/L, which were chosen to represent 1/200 and 1/50 of the 72 h-LC_{50} in silver carp, which was previously determined by Li et al. [8] The silver carp were exposed to the [C_8mim]Br solution for 60 d, and a daily food ration of 1% of the fish body weight was provided. The water and [C_8mim]Br solution were completely changed every day, and no fish mortality was observed during the test. The experiment was repeated three times.

Six fish were randomly taken from each of the groups after 15, 30, and 60 d of exposure. The fish were immediately anaesthetized with Tricaine (100 mg/L) and dissected, and their spleens were collected and washed with ice-cold phosphate-buffered saline (PBS). The spleens were stored at −80 °C for later biochemical and molecular assay.

2.3. Determination of oxidative stress indicators

The spleen samples were homogenized at a 1/10 (w/v) ratio in cold PBS, and centrifuged at 3000 g for 10 min at 4 °C, and then the supernatants were obtained for biochemical assays. Assays for SOD, CAT, GSH and MDA in fish spleen were carried out using commercial kits following the methods described previously [30]. The GPx activity and PC content were measured using diagnostic reagent kits following the manufacturer's protocols. The GPx activity is presented as units of enzymatic activity per mg protein (U/mg protein), and the PC content is presented as nmol per mg protein. The total protein content of the spleen samples was determined as described by Lowry et al. [31].

2.4. NF-κB level assay

Fish spleen NF-κB levels were assayed using ELISA kits (# DRE96222) following the manufacturer's instructions, and the absorbance was recorded at 450 nm with a Thermo Scientific Multiskan Spectrum spectrophotometer (Thermo Scientific, Shanghai, China).

2.5. iNOS activity assay

iNOS activity in fish spleen was measured with a NOS typed assay kit (# A014-1) according to the kit's protocols. The absorbance was monitored at 530 nm iNOS activity was calculated from the nitrite standard curve and is expressed as U per mg protein. The total protein content of the spleens was determined as described by Lowry et al. [31].

2.6. Cytokine content assay

Fish spleens were homogenized in cold PBS at a 1/10 (w/v) ratio, and centrifuged at 8000 g for 15 min at 4 °C, and then the supernatants were obtained for IL-1β, IL-6, TNF-α, IFN-γ, and TGF-β assays using diagnostic reagent kits (#DRE96017, #DRE96191, #DRE96155, #DRE96157, and #DRE96210, respectively) according to the manufacturer's instructions.

2.7. Lysozyme (LYZ) activity assay

LYZ activity in silver carp spleen was assayed according to the methods of Binuramesh and Michael [32] as previously described [33]. LYZ activity (U) was defined as the amount of enzyme that caused a decreased in OD 520 of 0.001 per min.

2.8. Contents of C3 and IgM

The C3 and IgM levels in fish spleen were measured using appropriate kits (# DRE96085 and #DRE96050, respectively) following the method previously described [33].

2.9. RNA isolation and quantitative real-time PCR (qPCR)

Total RNA isolation from fish spleen, first-strand cDNA synthesis, and qPCR were performed with a TRIzon Reagent Kit, a HiFi-MMLV cDNA Kit, and an UItraSYBR Mixture (with ROX) (CoWin Biosciences, Beijing, China), respectively, as previously described [30]. The qPCR primers for p38MAPK, c-fos, c-jun, c-myc, and β-actin, are listed in Table 1. The amount of target mRNA was normalized to the mRNA level of the reference gene β-actin, as computed by formula $2^{-\triangle\triangle Ct}$ [34].

miRNA isolation from fish spleen, first-strand cDNA synthesis, and qPCR were performed with a miRNA Purification Kit, a miRNA cDNA Synthesis Kit, and a miRNA qPCR Assay Kit (CoWin Biosciences, Beijing, China), respectively, using methods described previously [35]. The upstream primers, which were miRNA-target specific primers, were

Table 1
Specific primers used for qPCR in the present study.

Primers	Sequences
p38MAPK	Forward: 5′-GAAGGTCGCCGTGAAGAA-3′
	Reverse: 5′-TCCAGGGAGGTGGCAGGTGT-3′
c-fos	Forward: 5′-TCTTTGCACGCCTGTCGT-3′
	Reverse: 5′-AGTCAGTTCCATAGCCCTGC-3′
c-jun	Forward: 5′-TCTGTGTCCCAAGAACGTG-3′
	Reverse: 5′-AGTATTCAGGTCCGCGTACA-3′
c-myc	Forward: 5′-GAGACGGAGGTGTCGGATT-3′
	Reverse: 5′-TGTGAGTTCTGCGTTTGTCATT-3′
β-actin	Forward: 5′- CTTTTCCAGCCATCCTTCCT -3′
	Reverse: 5′- GGTCAGCAATGCCAGGGTA -3′
miR-125b	Forward: 5′-TCCCTGAGACCCTAACTTGTGA-3′
miR-143	Forward: 5′-TGAGATGAAGCACTGTAGCTC-3′
miR-155	Forward: 5′-TTAATGCTAATCGTGATAGGGGT-3′
miR-21	Forward: 5′-GCTTATCAGACTGGTGTTGG-3′
U6	Forward: 5′-GCTTCGGCAGCACATATACTAA-3′
	Reverse: 5′-GCTTCACGAATTTGCGTGTCAT-3′

designed based on sequences from miRNA RNA-seq analysis in silver carp (data not shown) (Table 1). The downstream primers were purchased with the miRNA qPCR Assay Kit. The amount of target miRNA was normalized to U6 levels and was computed by formula $2^{-\triangle\triangle Ct}$ [34]. All qPCR reactions were performed in accordance with the MIQE guidelines [36].

2.10. Statistical analysis

All data were analysed using SPSS 23.0 software (SPSS, Chicago, IL, USA). The *p* values were determined by one-way ANOVA and post-hoc pairwise comparisons among all groups using the Duncan's test. $p < 0.05$ was considered to be statistically significant (* < 0.05 and ** < 0.01).

3. Results

3.1. Oxidative stress and antioxidant response in the silver carp spleen following [C_8mim]Br exposure

The changes in SOD, CAT, and GPx activity, and GSH, MDA, and PC level are presented in Table 2. Compared to that of controls, the SOD activity in the spleen of fish exposure to 1.095 mg/L [C_8mim]Br was significantly upregulated, while at the higher exposure concentration (4.380 mg/L), SOD activity in the spleen was markedly decreased at 30 and 60 d. The CAT activity in fish spleen was markedly increased after exposure to 1.095 mg/L [C_8mim]Br at 30 d compared to that of controls. However, in the spleen of fish exposed to 4.380 mg/L [C_8mim]Br, CAT activity was clearly increased at 15 d, but was decreased at 30 and 60 d compared to that of controls. In addition, GPx activity was significantly increased after 15–30 d of [C_8mim]Br exposure (except in the 4.380 mg/L group at 30 d) but was markedly inhibited at 60 d (Table 2). Similarly, GSH levels in fish spleen showed a tendency resembling the pattern of GPx changes after 60 d of [C_8mim]Br exposure (Table 2). The MDA content was not noticeably changed in the spleen after 1.095 mg/L [C_8mim]Br exposure but was markedly increased after 30 and 60 d of 4.380 mg/L [C_8mim]Br treatment. In addition, the PC content was significantly increased except in the 1.095 mg/L group at 15 and 30 d with respect to control levels (Table 2).

3.2. Expression of NF-κB in fish spleen after [C_8mim]Br-exposure

The results of ELISA showed that compared with that of the control groups, there was a significant increase in NF-κB content in the spleen of fish exposed to [C_8mim]Br for 60 d, but no significant changes were observed in the 1.059 mg/L group at 15 and 30 d (Fig. 1 A).

3.3. Effect of [C_8mim]Br on iNOS activity

The changes in iNOS activity are shown in Fig. 1 B. Overall, [C_8mim]Br exposure significantly promoted iNOS activity in the fish spleen, although no significant changes in activity were observed at 15 d in the treated groups compared with the activity in the controls.

3.4. Cytokine contents

The effects of [C_8mim]Br on IL-1β, IL-6, TNF-α, IFN-γ, and TGF-β levels in silver carp spleen are shown in Fig. 2. The IL-1β content was markedly decreased in the [C_8mim]Br-treated groups at 15 d but was significantly increased at 30 and 60 d (except in the 1.095 mg/L group at 30 d) compared with that of the control groups (Fig. 2 A). In addition, the IL-6 and TNF-α levels in [C_8mim]Br-treated fish were significantly upregulated at 30 and 60 d compared to control levels (Fig. 2 B, C). Additionally, the splenic IFN-γ content in [C_8mim]Br-treated fish exhibited a tendency similar to the pattern forIL-1β (Fig. 2 D). However, the TGF-β content was markedly upregulated after 15–30 d of [C_8mim]Br exposure and significantly downregulated at 60 d compared to that of controls (Fig. 2 E).

Table 2
Antioxidant status in the spleen of silver carp after [C_8mim]Br exposure for 60 d.

Antioxidant system	Exposure time (d)	Control	1.095 mg/L	4.380 mg/L
SOD (U/mg protein)	15	30.833 ± 0.967	38.233 ± 0.616*	29.552 ± 1.042
	30	29.233 ± 0.620	46.883 ± 1.083**	22.708 ± 0617*
	60	28.195 ± 0.724	39.803 ± 0.876*	21.124 ± 1.096*
CAT (U/mg protein)	15	12.007 ± 0.274	12.545 ± 0.578	17.912 ± 0.315**
	30	12.550 ± 0.924	18.049 ± 0.267**	9.802 ± 0.475*
	60	12.028 ± 0.373	11.076 ± 0.597	7.901 ± 0.253**
GPx (U/mg protein)	15	49.523 ± 1.886	58.539 ± 2.384*	60.317 ± 2.866*
	30	47.424 ± 1.423	58.430 ± 1.395*	40.462 ± 1.784*
	60	47.037 ± 2.059	40.469 ± 1.731*	36.007 ± 2.053**
GSH (nmol/mg protein)	15	4.202 ± 0.088	4.796 ± 0.089*	5.100 ± 0.087*
	30	4.240 ± 0.055	5.526 ± 0.121**	3.297 ± 0.074*
	60	4.011 ± 0.081	4.003 ± 0.107	3.039 ± 0.096*
MDA (nmol/mg protein)	15	2.846 ± 0.071	2.847 ± 0.054	3.193 ± 0.066
	30	3.055 ± 0.068	2.993 ± 0.028	4.196 ± 0.101**
	60	3.052 ± 0.080	3.174 ± 0.039	4.832 ± 0.056**
PC (nmol/mg protein)	15	3.032 ± 0.108	2.835 ± 0.062	3.625 ± 0.095*
	30	2.867 ± 0.052	3.057 ± 0.058	3.741 ± 0.077*
	60	3.200 ± 0.096	3.908 ± 0.118*	4.720 ± 0.174**

Notes: Asterisks denote a response that is significantly different from the control (*$p < 0.05$, **$p < 0.01$).

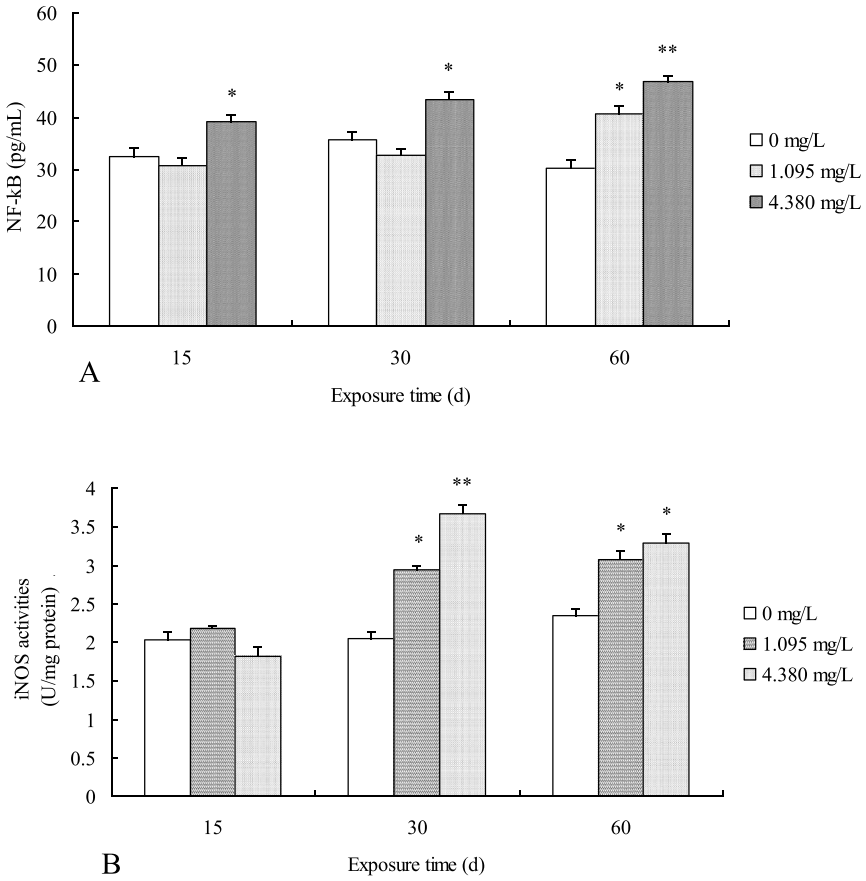


Fig. 1. Effects of [C_8mim]Br on NF-κB content and iNOS activity in the spleen of silver carp.
Data are means ± SD from three independent experiments performed in triplicate. Asterisks represent significantly different compared with the control (*$p < 0.05$, **$p < 0.01$). (A) The content of NF-κB. (B) The activity of iNOS.

3.5. Transcription levels of p38MAPK, c-fos, c-jun, and c-myc

After 60 d of [C_8mim]Br exposure, the p38MAPK transcription levels in the treatment groups were significantly upregulated compared with those of the control groups (Fig. 3 A). The transcript levels of c-fos, c-jun, and c-myc mostly increased in the [C_8mim]Br-treated groups during the exposure period, although the c-myc levels were not significantly increased in the 1.095 mg/L group at 30 and 60 d, as shown in Fig. 3.

3.6. LYZ activity

As shown in Fig. 4 A, LYZ activity was upregulated after 15 or 30 d of [C_8mim]Br treatment, but it was inhibited at the end of the test (60 d) except in the 1.095 mg/L group.

3.7. IgM and C3 content

The changes in the levels of IgM and C3 in the spleen of silver carp after [C_8mim]Br treatment are shown in Fig. 4; the IgM contents was mostly upregulated after [C_8mim]Br exposure, although it was downregulated in the 4.380 mg/L group at 60 d compared to that of the control group (Fig. 4 B). Furthermore, there were no significant changes in C3 content in 1.095 mg/L [C_8mim]Br-treated fish during the test; however, the C3 content in fish spleen was markedly increased after 30 d and significantly decreased after 60 d of 4.380 mg/L [C_8mim]Br exposure (Fig. 4 C).

3.8. miRNAs

The effects of [C_8mim]Br on miRNA expression in silver carp spleen were determined by qPCR. As shown in Fig. 5, there was no significant change in miR-125b expression in [C_8mim]Br-treated fish at 15 d, but there was a general decrease in expression at 30 and 60 d compared to that of controls (Fig. 5 A). The miR-143 levels in fish spleen were generally downregulated at 15 and 30 d but were markedly upregulated at 60 d compared to those of controls (Fig. 5 B). In addition, the miR-155 levels were markedly upregulated in the spleens of [C_8mim]Br-treated fish, although the change was not significant in the 1.095 mg/L group at 15 d (Fig. 5 C). The levels of miR-21 were also markedly upregulated in the [C_8mim]Br-treated fish in comparison with those of the controls (Fig. 5 D).

4. Discussion

Exposure to environmental pollutants may cause toxicity related to enhanced ROS production, leading to a deficiency in the antioxidant defence system and thereby resulting in an imbalance between the production of free radicals and their elimination by antioxidants and detoxifying systems [30,37] that can then cause oxidative stress and even oxidative damage in fish [38]. Previous studies have indicated that dysregulation of cellular redox balance and induced oxidative stress are the primary causes of the acute toxicity of ILs [7,18,38]. However, relatively little is known about whether oxidative stress is involved in the chronic toxicity of ILs on organisms [13].

Oxidative stress occurs when ROS generation exceeds the antioxidant capacity of tissues [39]. Antioxidant enzymes play an

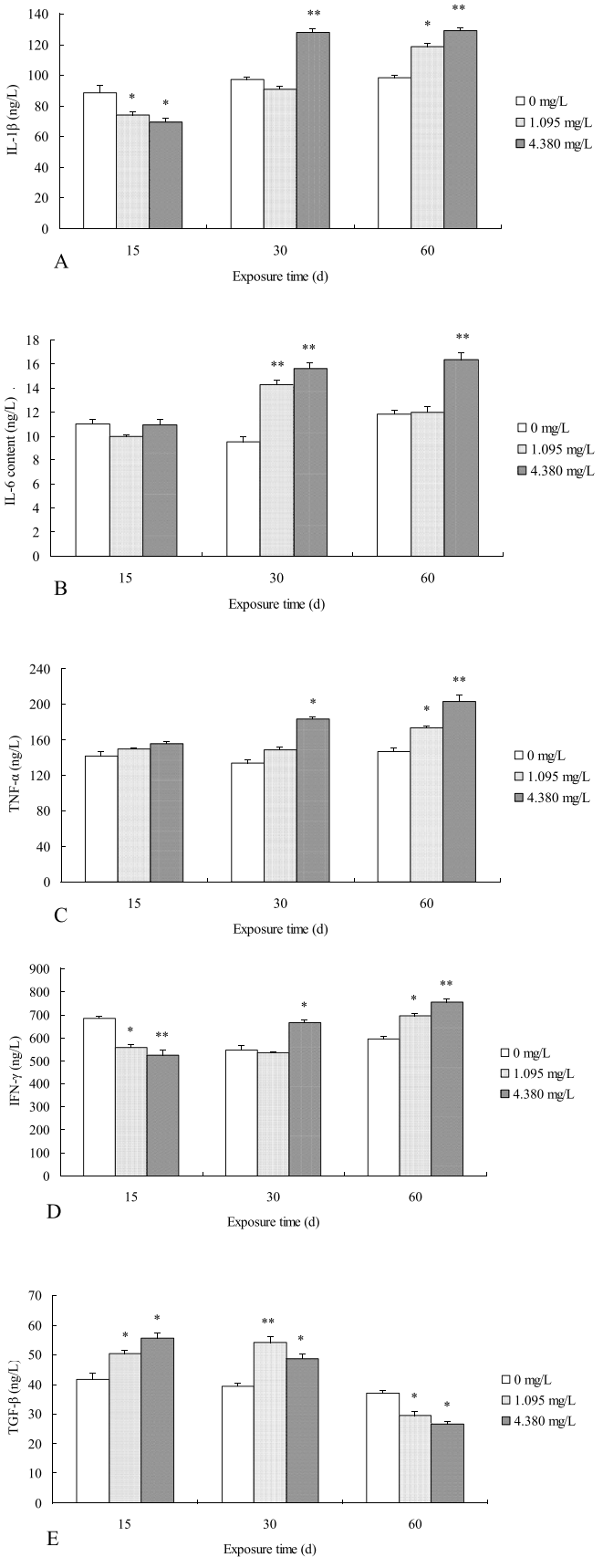


Fig. 2. Effect of [C_8mim]Br on the levels of inflammatory cytokine in the spleen of silver carp.

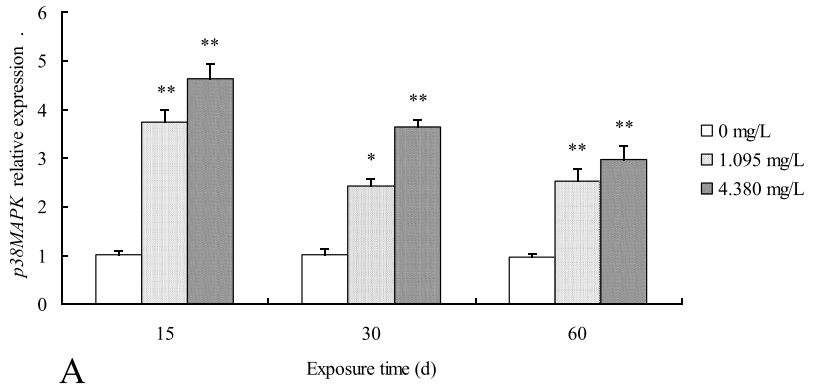
Data are means ± SD from three independent experiments. Asterisks represent significantly different compared with the control (*$p < 0.05$, **$p < 0.01$). (A) The content of IL-1β. (B) IL-6. (C) TNF-α. (D) IFN-γ. (E) TGF-β.

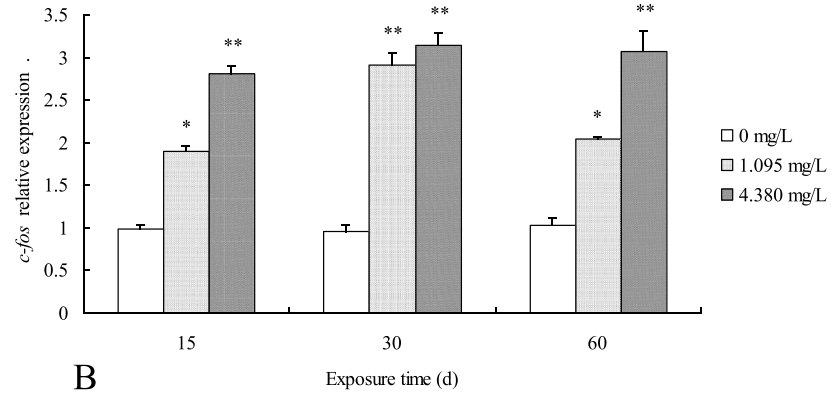
important role in maintaining the dynamic balance between ROS production and elimination during the early stages of the cellular defence mechanism [40]. Among these enzymes, SOD, CAT, and GPx are the first line of defence against oxidative stress; SOD catalyses superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2), and then CAT and GPx enhance the rate of which H_2O_2 is dismutated to form water (H_2O) and molecular oxygen (O_2) [41]. In the present study, we observed that exposure to a low concentration of [C_8mim]Br generally increased the activity of SOD, CAT, and GPx, while exposure to a high concentration of [C_8mim]Br or long-term (30 or 60 d) exposure significantly decreased their activity in the spleen of the silver carp (Table 2). This finding may be explained as follow: increased SOD activity is probably a response to the stress caused by [C_8mim]Br, which might generate oxyradicals and lead to the transformation of O_2^- into H_2O_2, and subsequently, H_2O_2 may be metabolized to H_2O and O_2 by the increased CAT and GPx [41], which may help subdue the impact of increased ROS generation [42]; however, with long-term exposure to [C_8mim]Br, when the production of ROS exceeds the scavenging abilities of SOD, CAT, and GPx, ROS may inhibit SOD, CAT, and GPx activity by oxidizing cysteine or other amino acids in the enzymes [43].

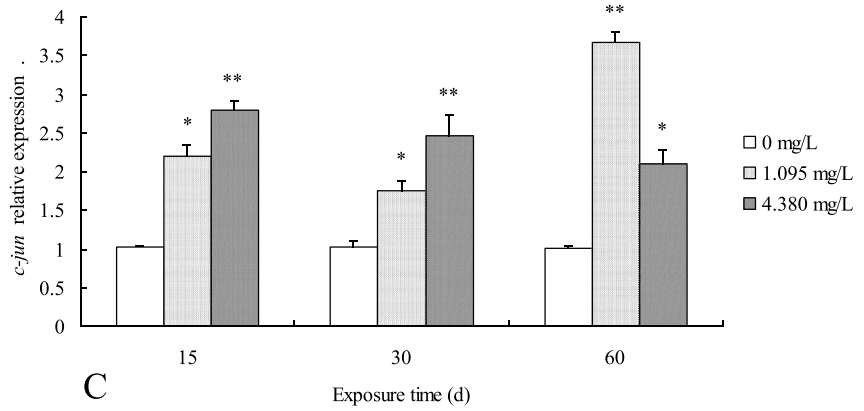
The non-enzymatic antioxidant GSH is one of the scavengers that participates directly in detoxification reactions as accumulated xenobiotics are eliminated from the body and plays a critical role in protecting cells from free radical damage [44]. In the present study, significant increases in GSH levels were noted following exposure to 1.095 mg/L [C_8mim]Br for 15 and 30 d and 4.380 mg/L [C_8mim]Br for 15 d, which is consistent with the results of a previous study on [C_8mim]Br in *Daphnia magna* [45], suggesting that the increased GSH might protect against further oxidative stress caused by [C_8mim]Br. However, the GSH levels were drastically declined in the 4.380 mg/L [C_8mim]Br-treated group at 30 and 60 d (Table 2), which would likely prompt an accumulation of superfluous free radicals in fish spleen that may damage membrane lipids and cause lipid peroxidation [46].

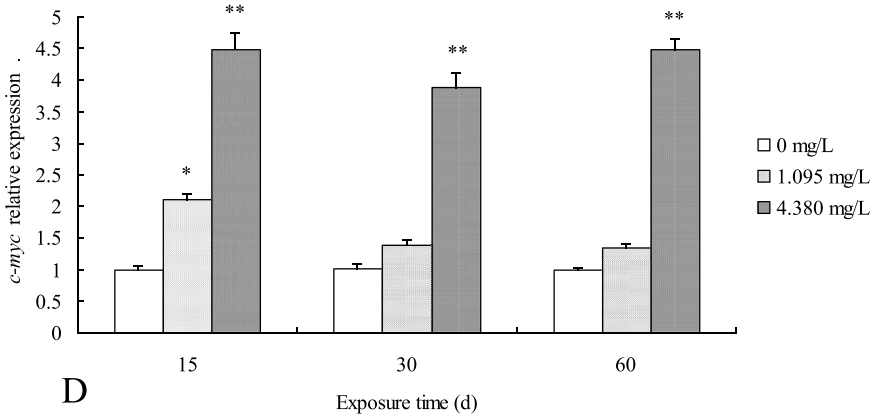
Lipid peroxidation is known as the key index of oxidative injury of the cellular components [47]. MDA is a main end product of lipid peroxidation and can be considered a valuable indicator of lipid peroxidation [48]. Li et al. [18] reported that acute exposure to [C_8mim]Br causes significant increases in MDA levels in the hepatopancreas of goldfish and suggested that alterations in MDA content can be indicator of IL toxicity in fish. In the current study, MDA levels in fish spleen did not vary significantly after 1.095 mg/L [C_8mim]Br exposure, which might indicate a resistance to oxidative stress via the antioxidant defence system conferred mainly by the transient induction of SOD, CAT, and GPx activity and the transient increase in GSH levels in the fish spleen (Table 2). However, a significant upregulation in the MDA content was found in the spleens of 4.380 mg/L [C_8mim]Br-treated fish at 30 and 60 d compared to that of the control group (Table 2), which is similar to the results of a study by Zhang et al. [38] that examined the responses of biochemical markers in the livers of zebrafish exposed to [C_6mim]Br, suggesting that excess free radical production and lipid peroxidation may also participate in the chronic toxicity mechanism of [C_8mim]Br and that long-term exposure to [C_8mim]Br may cause oxidative damage in silver carp spleen. Oxidative damage includes not only lipid peroxidation but also protein and DNA oxidation and enzyme inactivation [49]. PC has frequently been used as a reliable biomarker of oxidative damage in proteins [50]. Many reports have indicated that exposure to xenobiotics can elevate the levels of PC [51,52]; however, there have been no reports regarding the adverse effects of ILs on the expression of PC in fish. In this study, [C_8mim]Br exposure significantly upregulated PC levels in fish spleen, although the change was insignificant in the 1.095 mg/L group at 15 and 30 d with respect to control levels (Table 2), suggesting that [C_8mim]Br exposure also induces oxidative damage in proteins in silver carp spleen.

Oxidative stress and inflammation are closely related; ROS can trigger inflammation through immune recognition of molecules released from dying cells, and activated immunocytes, such as









(caption on next page)

Fig. 3. Transcription levels of p38MAPK, c-fos, c-jun, and c-myc in the spleen of silver carp after 1.095 and 4.380 mg/L [C_8mim]Br exposure for 60 d.
Data are means ± SD from three independent experiments. Asterisks represent significantly different compared with the control (*$p < 0.05$, **$p < 0.01$). (A) p38MAPK level. (B) c-fos. (C) c-jun. (D) c-myc.

neutrophils, granulocytes, and macrophages, can also release ROS [53]. ROS can also function as signalling molecules for NF-κB, which is a quickly inducible stress-responsive transcription factor and is very sensitive to cellular oxidative stress [54]; the activated NF-κB can in turn promote the production of pro-inflammatory cytokines, thus inducing inflammatory responses [55]. However, no previous studies have examined the effects of ILs on the levels of NF-κB and inflammatory factors in fish. In the present study, [C_8mim]Br exposure promoted an increase in splenic NF-κB content in the silver carp after 60 d (Fig. 1 A), which might be due in part to the superfluous ROS accumulation in the spleen caused by [C_8mim]Br.

Studies have shown that pro-inflammatory-related molecules such

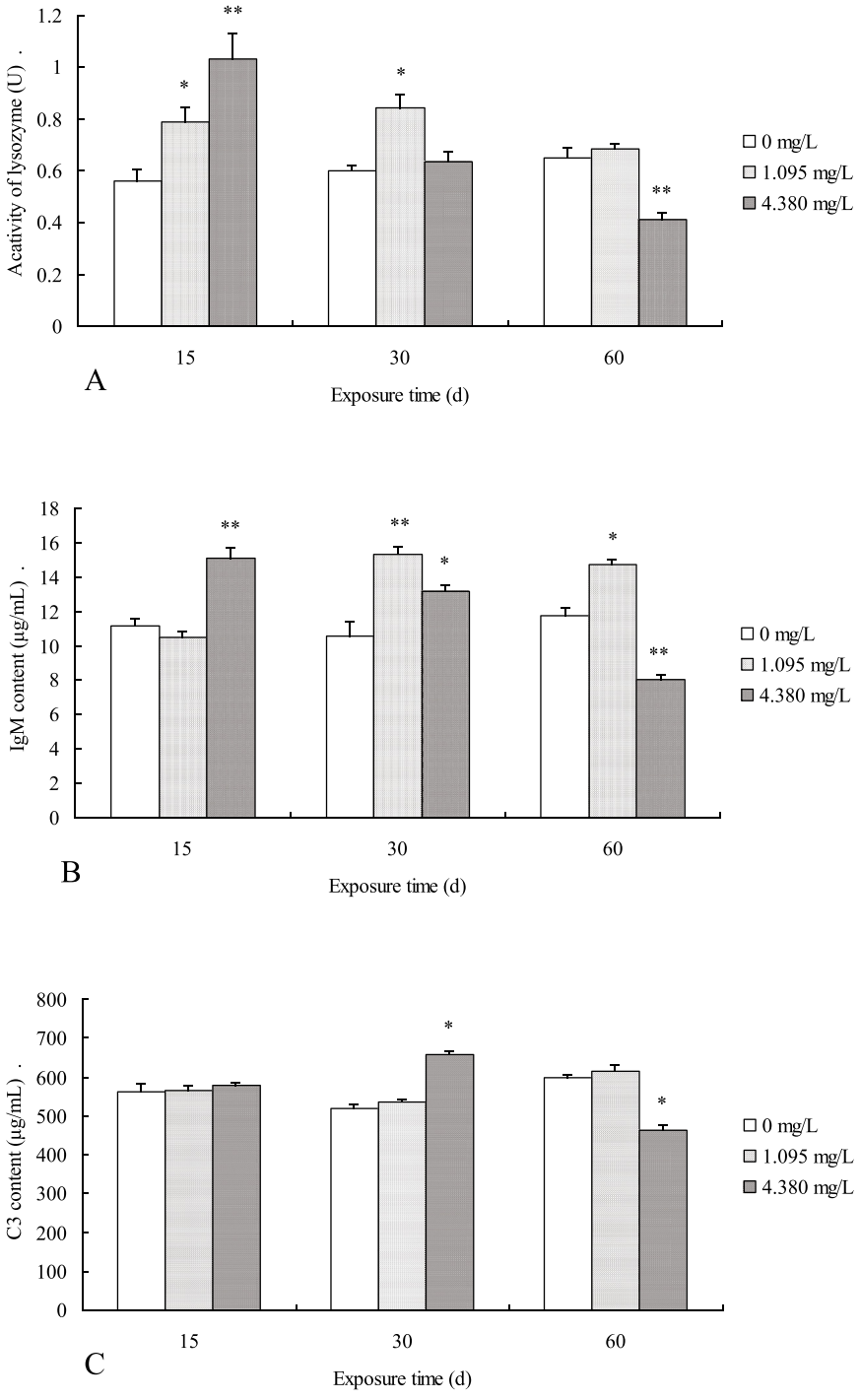
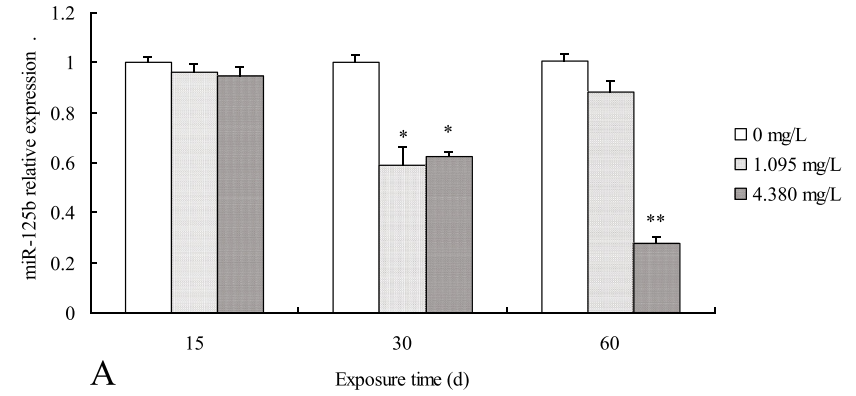
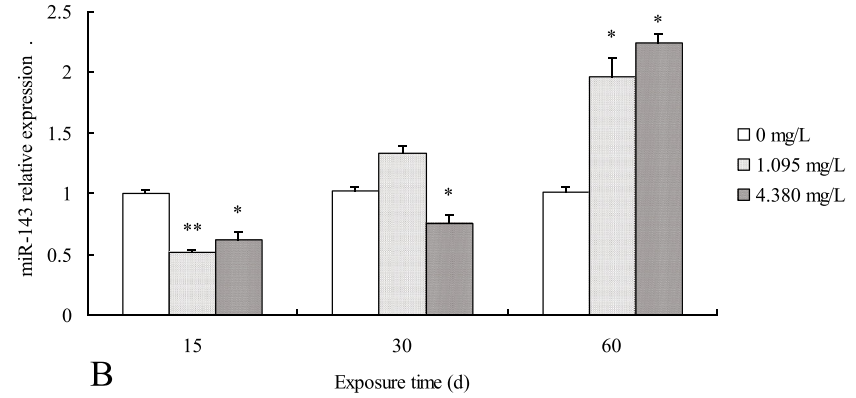
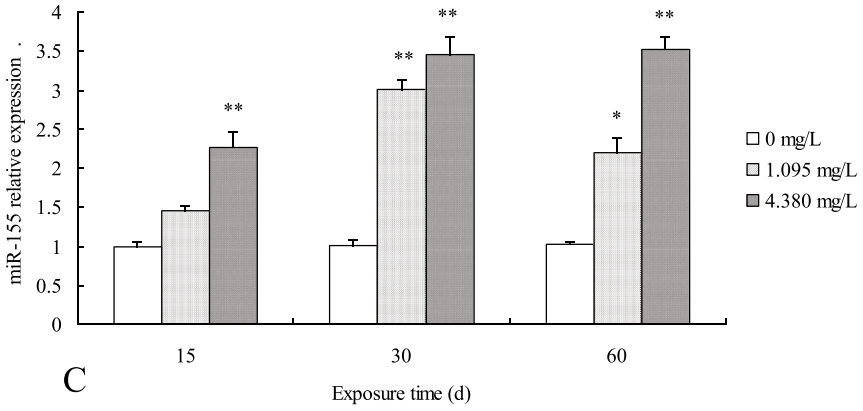
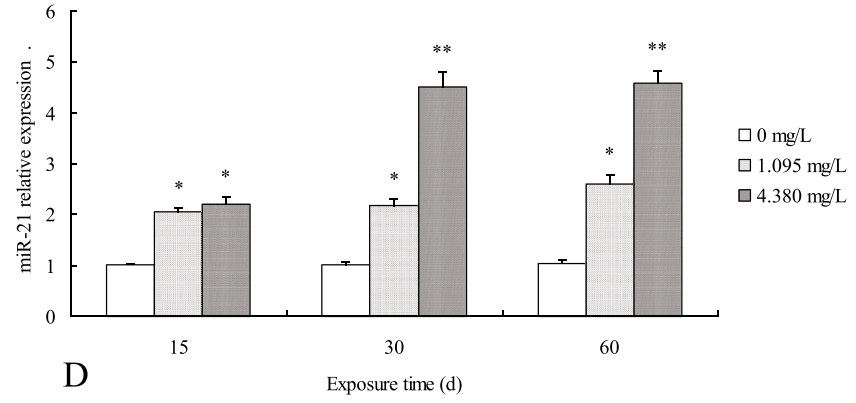


Fig. 4. Altered of the LYZ activity and C3 and IgM contents in the spleen of silver carp exposed to [C_8mim]Br for 60 d.
Data are means ± SD from three independent experiments. Asterisks represent significantly different compared with the control (*$p < 0.05$, **$p < 0.01$). (A) The activity of LYZ. (B) The content of IgM. (C) C3.









(caption on next page)

Fig. 5. Effects of [C_8mim]Br on miRNAs expressions in the spleen of silver carp.
Data are means ± SD from three independent experiments performed in triplicate. Asterisks represent significantly different compared with the control (*$p < 0.05$, **$p < 0.01$). (A) miR-125b mRNA level. (B) miR-143. (C) miR-155. (D) miR-21.

as iNOS, IL-1β, IL-6, and TNF-α can be activated by NF-κB signalling in fish [56]. In the present study, iNOS activity in spleen was significantly increased by [C_8mim]Br exposure (Fig. 1 B), suggesting that [C_8mim]Br exposure may induce inflammatory reactions in silver carp spleen, as iNOS is the product of an early response gene in the inflammatory process and is frequently used as an inflammatory and oxidative stress marker [57]. IL-1β and IL-6 are important pro-inflammatory cytokines that are considered to be indicators of the inflammatory response [58], and TNF-α and IFN-γ also play a crucial role in the development and maintenance of inflammatory reactions [59,60]; IL-1β and TNF-α are regarded as the most prominent "first-line" pro-inflammatory cytokines. Upregulation of these cytokines is often related to the inflammatory response. In the current study, for the first time, we revealed that long-term exposure to [C_8mim]Br increases the content of IL-1β, IL-6, TNF-α, and IFN-γ in the silver carp spleen (Fig. 2 A, B, C, D), a phenomenon that can also be observed in HepG2 cells after microcystin-LR-exposure [61], suggesting that an inflammatory reaction is elicited at the site of [C_8mim]Br-induced spleen damage and that the upregulation of pro-inflammatory cytokines may be related to ROS and NF-κB signalling caused by [C_8mim]Br in fish spleen. TGF-β is an anti-inflammatory cytokine that plays a crucial role in limiting the inflammatory response and initiating processes of tissue recovery by blocking the expression of pro-inflammatory cytokines [62]. In the present study, for the first time, we found that TGF-β levels are significantly upregulated in the spleen of [C_8mim]Br-treated fish after 15 and 30 d but are downregulated after 60 d (Fig. 2 E), indicating that the increased TGF-β content might be beneficial neutralizing the production of pro-inflammatory cytokines caused by [C_8mim]Br exposure, and that decreased TGF-β might fail to limit or ultimately terminate the overexpression of pro-inflammatory cytokines. Overall, the upregulation of pro-inflammatory cytokines including IL-β, IL-6, TNF-α, and IFN-γ and the downregulation of the anti-inflammatory cytokine TGF-β might promote the inflammatory response in fish spleen [63].

Additionally, many studies have demonstrated that the production of pro-inflammatory cytokines is also associated with the activation of MAPKs, and the c-jun N-terminal kinases and p38MAPK pathways are preferentially stimulated by pro-inflammatory cytokines or extracellular stress [64]. Importantly, inflammatory cytokines including IL-1β, IL-6, and TNF-α can activate the p38MAPK pathway, and activated p38MAPK also plays a key role in regulating pro-inflammatory cytokine biosynthesis and transcription [65]. Ma et al. [66] found that p38MAPK can be activated by CCl_4 and heavily participates in inflammatory processes in the mouse liver. The current study revealed for the first time that [C_8mim]Br exposure markedly promotes the expression of p38MAPK in the fish spleen (Fig. 3 A). Liu et al. [67] showed that the p38 MAPK has been implicated in the activation of NF-κB and that ROS can activate the MAPK pathways, further stimulating several inflammatory cytokines [64]. Based on these results, we speculate that the p38MAPK pathway may play an important role in inflammatory processes in [C_8mim]Br-stimulated fish spleen. *c-fos*, *c-jun*, and *c-myc* are main members of the proto-oncogene family and play a key role in tumour promotion and carcinogenesis. Recently, accumulating evidence has revealed that *c-fos*, *c-jun*, and *c-myc* are involved in inflammatory diseases and toxicity induced by pollutants [68,69]. The activated MAPK can activate the downstream transcription factors *c-fos*, *c-jun*, and *c-myc* [70]. In the present study, for the first time, we found that chronic [C_8mim]Br exposure markedly upregulates the transcript levels of *c-fos*, *c-jun*, and *c-myc* in silver carp spleen, indicating that p38 MAPK-mediated *c-fos*, *c-jun*, and *c-myc* expression may play a crucial role in the inflammation promoting activity of [C_8mim]Br.

To further verify whether [C_8mim]Br exposure induces inflammatory reactions in fish, we further investigated the expression of immune-related parameters in the spleens of [C_8mim]Br-treated fish to understand the relationship between [C_8mim]Br and immune reactions. LYZ is known to be a key defence molecule in fish non-specific immunity, which is crucial in mediating protection against xenobiotics, and it is also a conventional terminal index of fish immunotoxicity. Li et al. [23] discovered that acute exposure of brocarded carp to 100 mg/L [C_8mim]Br first increased and then decreased the LYZ activity in the spleen, while 300 mg/L [C_8mim]Br treatment always inhibited the spleen LYZ activity, suggesting that acute [C_8mim]Br exposure might induce immunotoxicity in fish. In the current study, chronic [C_8mim]Br treatment generally upregulated the LYZ activity in spleen at 15 and 30 d and then inhibited its activity by 60 d (Fig. 4 A), indicating that chronic exposure to [C_8mim]Br might stimulated the fish immune system and hence activate LYZ at the earlier time points, while subsequent greatly increased stress and even injury might inhibit LYZ activity at the late stage of [C_8mim]Br exposure. As a natural antibody, IgM is thought to be associated with a strong non-specific immune response in teleosts, and plays a crucial role in resistance to xenobiotics in fish [71]. In the present study, the IgM content was significantly increased after [C_8mim]Br exposure, although it was reduced in the 4.380 mg/L [C_8mim]Br-treated group at 60 d (Fig. 4 B), which is similar to the results of a study in brocarded carp treated with [C_8mim]Br, suggesting that chronic IL exposure also inhibits the fish immune system. Several studies have indicated that C3 is another important component of the fish immune system and that environmental toxicants might affect C3 levels and disturb immune function in fish [15,72]. In the present study, 4.380 mg/L [C_8mim]Br significantly promoted fish spleen C3 content after 30 d but markedly inhibited C3 content after 60 d (Fig. 4 C), suggesting that IL exposure may disturb the immune system of silver carp by affecting C3 content [23].

miRNAs are a class of evolutionarily conserved endogenous small single-stranded noncoding RNAs that are widespread in eukaryotes [73] and participate in a wide range of biological processes, including proliferation, differentiation, metabolism, and immunity [74]. Currently, miRNAs have attracted great interest in toxicology [75–77] and increasing evidence has indicated that they may play a particularly crucial role in cellular responses to toxicants exposure [35]. miRNAs such as miR-146, miR-155, and miR-21 have been found to be ubiquitously upregulated in response to immune-related signalling pathways activated by xenobiotic or environmental chemicals [78]. However, there have been no reports regarding the effects of ILs on the expression of miRNAs. Among miRNAs, miR-125b is an important regulator of immune processes, and the downregulation of miR-125b is a necessary step to permit TNF-α production, because this miRNA targets TNF-α transcripts to regulate inflammation [79]. The current study revealed that the mRNA levels of miR-125b in silver carp spleen were markedly lower than those of controls after 60 d of [C_8mim]Br exposure (Fig. 5 A), while the TNF-α levels were significantly increased (Fig. 2 C), indicating that miR-125b may be involved in IL induced immune responses. As a multi-functional miRNA, miR-143 can also influence inflammatory responses by regulating TGF-β, TNF-α and NF-κB pathways [80,81]. In this study, [C_8mim]Br treatment generally reduced the miR-143 levels after 15 and 30 d and subsequently promoted miR-143 expression after 60 d in fish spleen (Fig. 5 B), suggesting that miR-143 is also involved in immunomodulation in the fish spleen caused by [C_8mim]Br. However, further research is needed to address this possibility.

miR-155 has also been identified as a typical multi-functional miRNA and is a central promoter that regulates inflammation and immune cell functions in a variety of contexts [82]. miR-155 can be

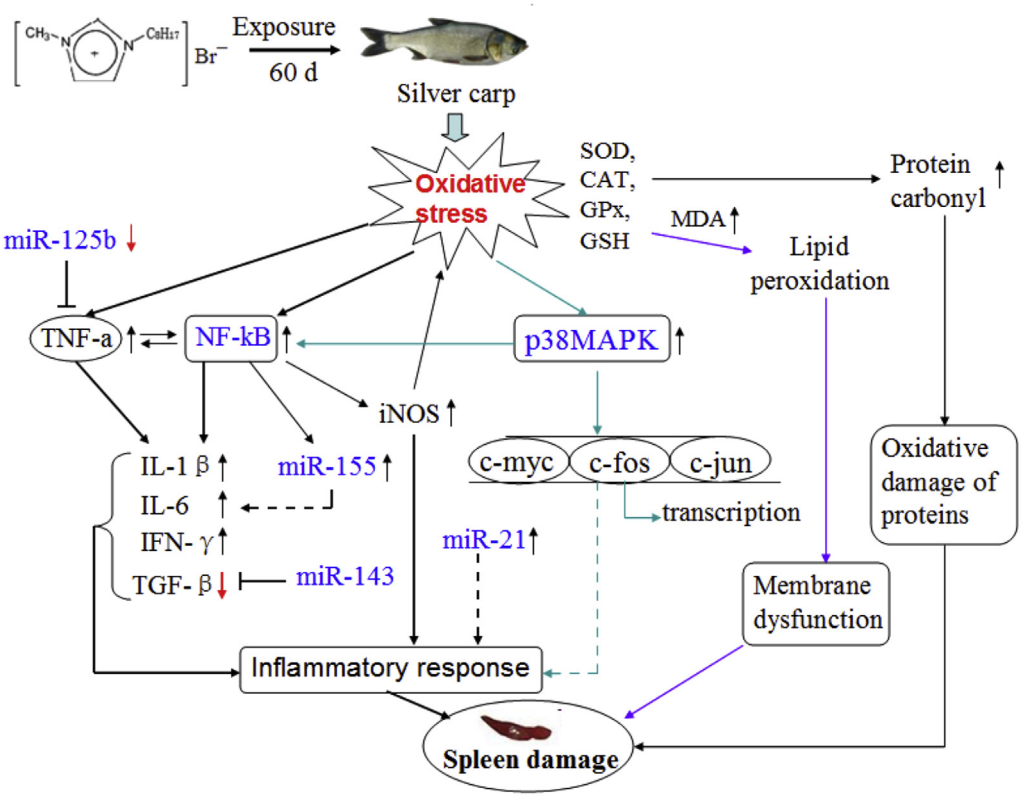


Fig. 6. Suppositional pathways of [C_8mim]Br-induced inflammation in silver carp spleen.
(Short arrow upward or downward symbolizes the up-regulated or down-regulated mRNA or protein content, respectively).

rapidly induced during the inflammatory response, and the induction of miR-155 promotes or stabilizes the expression of pro-inflammatory cytokines [83], which is regulated by NF-κB activation [82]. Tili et al. [79] showed that LPS exposure upregulates miR-155 levels, which may directly or indirectly increase the rate of translation of TNF-α transcripts while enhancing TNF-α production. In the current study, [C_8mim]Br treatment markedly increased the levels of miR-155 (Fig. 5 C), and [C_8mim]Br exposure was found to promote the immune inflammatory response of silver carp spleen (Fig. 2), indicating that the upregulation of miR-155 may participate in the IL induced NF-κB-mediated inflammatory response in fish. Recently, increasing numbers of studies have proven that miR-21 is an onco-microRNA that also plays a crucial role in the inflammatory response. Roy and Sen [84] established that miR-21 can target the tumour suppressor PDCD4, a pro-inflammatory protein that promotes the activation of NF-κB and suppresses IL-10, and that miR-21 also targets PTEN, an inhibitor of AKT, which in turn activates NF-κB [85]. Interestingly, expression of the miR-21 precursor transcript is promoted by NF-κB [86], indicating the interplay of miR-21 and NF-κB in the inflammatory response. In this study, miR-21 levels were markedly elevated in fish spleen following [C_8mim]Br exposure (Fig. 5 D), indicating that increased levels of miR-21 might also participate in IL induced inflammation in fish and that miR-21 could be considered a new biomarker of inflammation [87].

5. Conclusion

In conclusion, the present study reveals that the oxidative stress and inflammatory reaction induced by [C_8mim]Br exposure in fish spleen are possibly the main biochemical and molecular mechanisms underlying the chronic toxicity of ILs, as summarized in Fig. 6. Furthermore, within these mechanisms, oxidative stress-mediated p38MAPK/NF-κB signalling and miRNAs may play a crucial role. The present study may be helpful to illuminate the chronic toxicity mechanisms of the ILs as well as how to safely use them in the future.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Nos. 31702349 and 31472285) and the Key Subjects of Biology in Henan Province, China.

References

[1] J.S. Wilkes, A short history of ionic liquids-from molten salts to neoteric solvents, Green Chem. 4 (2002) 73–80.
[2] A. Bado-Nilles, A.O. Diallo, G. Marlair, P. Pandard, L. Chabot, A. Geffard, C. Len, J.M. Porcher, W. Sanchez, Coupling of OECD standardized test and immunomarkers to select the most environmentally benign ionic liquids option–towards an innovative "safety by design" approach, J. Hazard Mater. 283 (2015) 202–210.
[3] P.T. Anastas, M.M. Kirchhoff, Origins, current status, and future challenges of green chemistry, Acc. Chem. Res. 35 (2002) 686–694.
[4] X. Li, J. Ma, J. Wang, Cytotoxicity, oxidative stress, and apoptosis in HepG2 cells induced by ionic liquid 1-methyl-3-octylimidazolium bromide, Ecotoxicol. Environ. Safe. 120 (2015) 342–348.
[5] H. Liu, J. Wu, X. Zhang, Y. Xia, Y. Li, S. Du, Enantioselective oxidative stress caused by chiral ionic liquids forms of 1-alkyl-3-methyl imidazolium tartrate on *Scenedesmus obliquus*, Sci. Total Environ. 595 (2017) 819–827.
[6] R. Biczak, Quaternary ammonium salts with tetrafluoroborate anion: phytotoxicity and oxidative stress in terrestrial plants, J. Hazard Mater. 304 (2016) 173–185.
[7] J. Ma, X. Dong, Q. Fang, X. Li, J. Wang, Toxicity of imidazolium-based ionic liquids on *Physa acuta* and the snail antioxidant stress response, J. Biochem. Mol. Toxicol. 28 (2014) 69–75.
[8] X. Li, J. Ma, W. Lei, J. Li, Y. Zhang, Y. Li, Cloning of cytochrome P450 3A137 complementary DNA in silver carp and expression induction by ionic liquid, Chemosphere 92 (2013) 1238–1244.
[9] V. Tsarpali, S. Dailianis, Toxicity of two imidazolium ionic liquids, [bmim][BF_4] and [omim][BF_4], to standard aquatic test organisms: role of acetone in the induced toxicity, Ecotox. Environ. Safe 117 (2015) 62–71.

[10] A. Modelli, A. Sali, P. Galletti, C. Samorì, Biodegradation of oxygenated and non-oxygenated imidazolium-based ionic liquids in soil, Chemosphere 73 (2008) 1322–1327.
[11] A. Romero, A. Santos, J. Tojo, A. Rodríguez, Toxicity and biodegradability of imidazolium ionic liquids, J. Hazard Mater. 151 (2008) 268–273.
[12] T.P.T. Pham, C.W. Cho, Y.S. Yun, Environmental fate and toxicity of ionic liquids: a review, Water Res. 44 (2010) 352–372.
[13] M.E. Heckenbach, F.N. Romero, M.D. Green, R.U. Halden, Meta-analysis of ionic liquid literature and toxicology, Chemosphere 150 (2016) 266–274.
[14] C. Faggio, A. Torre, E. Pelle, F. Raffa, V. Villari, F. Trischitta, Cell volume regulation following hypotonic shock in hepatocytes isolated from *Sparus aurata*, Comp. Biochem. Physiol. A 158 (2011) 143–149.
[15] J. Ma, X. Li, Alteration in the cytokine levels and histopathological damage in common carp induced by glyphosate, Chemosphere 128 (2015) 293–298.
[16] V. Aliko, M. Qirjo, E. Sula, V. Morina, C. Faggio, Antioxidant defense system, immune response and erythron profile modulation in gold fish, *Carassius auratus*, after acute manganese treatment, Fish Shellfish Immun. 76 (2018) 101–109.
[17] C. Pretti, C. Chiappe, D. Pieraccini, M. Gregori, F. Abramo, G. Monni, L. Intorre, Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*), Green Chem. 8 (2006) 238–240.
[18] X.Y. Li, S.H. Zeng, X.Y. Dong, J.G. Ma, J.J. Wang, Acute toxicity and responses of antioxidant systems to 1-methyl-3-octylimidazolium bromide at different developmental stages of goldfish, Ecotoxicology 21 (2012) 253–259.
[19] T. Liu, Y. Guo, J. Wang, J. Wang, L. Zhu, J. Zhang, C. Zhang, Assessing toxic effects of [Omim]Cl and [Omim]BF_4 in zebrafish adults using a biomarker approach, Environ. Sci. Pollut. Res. 23 (2016) 7360–7368.
[20] Z. Du, L. Zhu, M. Dong, J. Wang, J. Wang, H. Xie, T. Liu, Y. Guo, Oxidative stress and genotoxicity of the ionic liquid 1-octyl-3-methylimidazolium bromide in zebrafish (*Danio rerio*), Arch. Environ. Contam. Toxicol. 67 (2014) 261–269.
[21] S.H. Wang, P.P. Huang, X.Y. Li, C.Y. Wang, W.H. Zhang, J.J. Wang, Embryonic and developmental toxicity of the ionic liquid 1-methyl-3-octylimidazolium bromide on goldfish, Environ. Toxicol. 25 (2010) 243–250.
[22] X.Y. Li, S.H. Zeng, W.H. Zhang, L. Liu, S. Ma, J.J. Wang, Acute toxicity and superficial damage to goldfish from the ionic liquid 1-methyl-3-octylimidazolium bromide, Environ. Toxicol. 28 (2013) 207–214.
[23] X.Y. Li, X.Q. Miao, L.F. Zhang, J.J. Wang, Immunotoxicity of 1-methyl-3-octylimidazolium bromide on brocarded carp (*Cyprinus carpio* L.), Ecotoxicol. Environ. Safe. 75 (2012) 180–186.
[24] M. Burgos-Aceves, A. Cohen, Y. Smith, C. Faggio, Estrogen regulation of gene expression in the teleost fish immune system, Fish Shellfish Immun. 58 (2016) 42–49.
[25] E. Fiorino, P. Sehonova, L. Plhalova, J. Blahova, Z. Svobodova, C. Faggio, Effects of glyphosate on early life stages: comparison between *Cyprinus carpio* and *Danio rerio*, Environ. Sci. Pollut. Res. 25 (2018) 8542–8549.
[26] A. Piotrowska, A. Syguda, B. Wyrwas, L. Chrzanowski, T. Luckenbach, H.J. Heipieper, Effects of ammonium-based ionic liquids and 2,4-dichlorophenol on the phospholipid fatty acid composition of zebrafish embryos, PloS One 13 (2018) e0190779.
[27] Regulation (EC) No 1907, Of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, 2006.
[28] G. Chatel, E. Naffrechoux, M. Draye, Avoid the PCB mistakes: a more sustainable future for ionic liquids, J. Hazard Mater. 324 (2017) 773–780.
[29] A. García-Lorenzo, E. Tojo, J. Tojo, M. Teijeira, F.J. Rodríguez-Berrocal, M.P. González, V.S. Martínez-Zorzano, Cytotoxicity of selected imidazolium-derived ionic liquids in the human Caco-2 cell line. Sub-structural toxicological interpretation through a QSAR study, Green Chem. 10 (2008) 508–516.
[30] J. Ma, Y. Liu, D. Niu, X. Li, Effects of chlorpyrifos on the transcription of CYP3A cDNA, activity of acetylcholinesterase, and oxidative stress response of goldfish (*Carassius auratus*), Environ. Toxicol. 30 (2015) 422–429.
[31] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[32] C. Binuramesh, R.D. Michael, Diel variations in the selected serum immune parameters in *Oreochromis mossambicus*, Fish Shellfish Immun 30 (2011) 824–829.
[33] J. Ma, Y. Li, D. Niu, Y. Li, X. Li, Immunological effects of paraquat on common carp, *Cyprinus carpio* L, Fish Shellfish immun 37 (2014) 166–172.
[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle Ct}$ method, Methods 25 (2001) 402–408.
[35] J. Ma, Y. Li, L. Yao, X. Li, Analysis of microRNA expression profiling involved in MC-LR-induced cytotoxicity by high-throughput sequencing, Toxins 9 (2017) 23.
[36] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2015) 611–622.
[37] M.A. Burgos-Aceves, A. Cohen, Y. Smith, C. Faggio, MicroRNAs and their role on fish oxidative stress during xenobiotic environmental exposures, Ecotoxicol. Environ. Safe. 148 (2018) 995–1000.
[38] C. Zhang, J. Wang, M. Dong, J. Wang, Z. Du, B. Li, L. Zhu, Effect of 1-methyl-3-hexylimidazolium bromide on zebrafish (*Danio rerio*), Chemosphere 192 (2018) 348–353.
[39] I. Celik, M. Turker, Y. Tuluce, Abcisic acid and gibberellic acid cause increased lipid peroxidation and fluctuated antioxidant defense systems of various tissues in rats, J. Hazard Mater. 148 (2007) 623–629.
[40] X. Li, Y. Liu, L. Song, J. Liu, Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR, Toxicon 42 (2003) 85–89.
[41] H. Kappus, Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance, in: H. Sies (Ed.), Oxidative Stress; Oxidants and Antioxidants, Academic Press, London, 1985, pp. 273–310.
[42] S. John, M. Kale, N. Rathore, D. Bhatnagar, Protective effect of vitamin E in dimethoate and malathion induced oxidative stress in rat erythrocytes, J. Nutr. Biochem. 12 (2001) 500–504.
[43] F. Geret, A. Serafim, L. Barreira, M.J. Bebianno, Effect of cadmium on antioxidant enzyme activities and lipid peroxidation in the gills of the clam *Ruditapes decussates*, Biomarkers 7 (2002) 242–256.
[44] M.M. Gehringer, E.G. Shephard, T.G. Downing, C. Wiegand, B.A. Neilan, An investigation into the detoxification of microcystin-LR by the glutathione pathway in Balb/c mice, Int. J. Biochem. Cell Biol. 36 (2004) 931–941.
[45] M. Yu, S.H. Wang, Y.R. Luo, Y.W. Han, X.Y. Li, B.J. Zhang, J.J. Wang, Effects of the 1-alkyl-3-methylimidazolium bromide ionic liquids on the antioxidant defense system of *Daphnia magna*, Ecotoxicol. Environ. Safe. 72 (2009) 1798–1804.
[46] R.S. Verma, A. Mehta, N. Srivastava, Comparative studies on chlorpyrifos and methyl parathion induced oxidative stress in different parts of rat brain: attenuation by antioxidant vitamins, Pestic. Biochem. Physiol. 95 (2009) 152–158.
[47] J.M. Gutteridge, Lipid peroxidation and antioxidants as biomarkers of tissue damage, Clin. Chem. 41 (1995) 1819–1828.
[48] H.H. Draper, M. Hadley, Malondialdehyde determination as index of lipid peroxidation, Methods Enzymol. 186 (1990) 421–431.
[49] C. Faggio, V. Tsarpali, S. Dailianis, Mussel digestive gland as a model for assessing xenobiotics: an overview, Sci. Total Environ. 613 (2018) 220–229.
[50] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, R. Colombo, Protein carbonyl groups as biomarkers of oxidative stress, Clin. Chim. Acta 329 (2003) 23–38.
[51] S. Parvez, S. Raisuddin, Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch), Environ. Toxicol. Pharmacol. 20 (2005) 112–117.
[52] M.N. Ajima, P.K. Pandey, K. Kumar, N. Poojary, Alteration in DNA structure, molecular responses and Na^+-K^+-ATPase activities in the gill of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) in response to sub-lethal verapamil, Ecotoxicol. Environ. Saf. 147 (2018) 809–816.
[53] F.H. Epstein, S.J. Weiss, Tissue destruction by neutrophils, N. Engl. J. Med. 320 (1989) 365–376.
[54] A.S. Baldwin Jr., The NF-κB and IκB proteins: new discoveries and insights, Annu. Rev. Immunol. 14 (1996) 649–681.
[55] G. Cheng, Y. Zhao, H. Li, Y. Wu, X. Li, Q. Han, C. Dai, Y. Li, Forsythiaside attenuates lipopolysaccharide–induced inflammatory responses in the bursa of Fabricius of chickens by downregulating the NF-κB signaling pathway, Exp. Ther. Med. 7 (2014) 179–184.
[56] A. Rebl, T. Goldammer, H. Seyfert, Toll-like receptor signaling in bony fish, Vet. Immunol. Immunopathol. 134 (2010) 139–150.
[57] P. Ramyaa, R. Krishnaswamy, V.V. Padma, Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells–upregulation of Nrf2 expression and down regulation of NF-κB and COX-2, BBA-Gen. Subjects 1840 (2013) 681–692.
[58] K.A. Papadakis, S.R. Targan, Role of cytokines in the pathogenesis of inflammatory bowel disease, Annu. Rev. Med. 51 (2000) 289–298.
[59] M. Nakamura, T. Manser, G.D. Pearson, M.J. Daley, M.L. Gefter, Effect of IFN-γ on the immune response in vivo and on gene expression in vitro, Nature 307 (1984) 381–382.
[60] K.J. Tracey, A. Cerami, Tumor necrosis factor, other cytokines and disease, Annu. Rev. Cell Biol. 9 (1993) 317–343.
[61] J. Ma, Y. Li, H. Duan, R. Sivakumar, X. Li, Chronic exposure of nanomolar MC-LR caused oxidative stress and inflammatory responses in HepG2 cells, Chemosphere 192 (2018) 305–317.
[62] C.J. Secombes, J. Zou, K. Laing, G.D. Daniels, C. Cunningham, Cytokine genes in fish, Aquaculture 172 (1999) 93–102.
[63] A. Rymuszka, Ł. Adaszek, Pro-and anti-inflammatory cytokine expression in carp blood and head kidney leukocytes exposed to cyanotoxin stress-An in vitro study, Fish Shellfish Immunol. 33 (2012) 382–388.
[64] Y. Ikeda, A. Murakami, H. Ohigashi, Ursolic acid promotesthe release of macrophag emigration inhibitory factor via ERK2 activation in resting mouse macrophages, Biochem. Pharmacol. 70 (2005) 1497–1505.
[65] J.J. Haddad, The involvement of L-γ-glutamyl-L-cysteinyl-glycine (glutathione/GSH) in the mechanism of redox signaling mediating MAPK p38-dependent regulation of pro-inflammatory cytokine production, Biochem. Pharmacol. 63 (2002) 305–320.
[66] J.Q. Ma, J. Ding, L. Zhang, C.M. Liu, Ursolic acid protects mouse liver against CCl_4-induced oxidative stress and inflammation by the MAPK/NF-κB pathway, Environ. Toxicol. Pharmacol. 37 (2014) 975–983.
[67] H.S. Liu, C.E. Pan, Q.G. Liu, W. Yang, X.M. Liu, Effect of NF-κB and p38 MAPK in activated monocytes/macrophages on pro-inflammatory cytokines of rats with acute pancreatitis, World J. Gastroenterol. 9 (2003) 2513–2518.
[68] H.Y. Li, P. Xie, G.Y. Li, L. Hao, Q. Xiong, In vivo study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (c-fos, c-jun and c-myc) in liver, kidney and testis of male Wistar rats injected i.v. with toxins, Toxicon 53 (2009) 169–175.
[69] Y. Li, J. Ma, Q. Fang, X. Li, C-fos and c-jun expression in the liver of silver carp and the effect of microcystins, J. Biochem. Mol. Toxicol. 28 (2014) 157–166.
[70] P. Joseph, T.K. Muchnok, M.L. Klishis, J.R. Roberts, J.M. Antonini, W.Z. Whong, T.M. Ong, Cadmium-induced cell transformation and tumorigenesis are associated with transcriptional activation of c-fos, c-jun, and c-myc proto-oncogenes: role of cellular calcium and reactive oxygen species, Toxicol. Sci. 61 (2001) 295–303.
[71] Y. Gao, Y.Y. Yi, H.Z. Wu, Q.Y. Wang, J.B. Qu, Y.X. Zhang, Molecular cloning and

characterization of secretory and membrane-bound IgM of turbot, Fish Shellfish Immunol. 40 (2014) 354–361.
[72] B. Magnadóttir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151.
[73] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[74] I.W. Boss, R. Renne, Viral miRNAs and immune evasion, BBA-Gene Regul. Mech. 1809 (2011) 708–714.
[75] T. Gant, E. Marcxylo, miRNAs in toxicology: the link to epigenetic effects, Toxicol. Lett. 238 (2015) S24.
[76] J. Ma, X. Li, MicroRNAs are involved in the toxicity of microcystins, Toxin Rev. 36 (2017) 165–175.
[77] J. Ma, Y. Li, M. Wu, C. Zhang, Y. Che, W. Li, X. Li, Serum immune responses in common carp (*Cyprinus carpio* L.) to paraquat exposure: the traditional parameters and circulating microRNAs, Fish Shellfish Immunol. 76 (2018) 133–142.
[78] Z. Gao, Y. Dou, Y. Chen, Y. Zheng, MicroRNA roles in the NF-κB signaling pathway during viral infections, BioMed Res. Int. 2014 (2014) p436097.
[79] E. Tili, J.J. Michaille, A. Cimino, S. Costinean, C.D. Dumitru, B. Adair, M. Fabbri, H. Alder, C.G. Liu, G.A. Calin, C.M. Croce, Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-α stimulation and their possible roles in regulating the response to endotoxin shock, J. Immunol. 179 (2007) 5082–5089.
[80] J.R. Pekow, U. Dougherty, R. Mustafi, H. Zhu, M. Kocherginsky, D.T. Rubin, S.B. Hanauer, J. Hart, E.B. Chang, A. Fichera, L.J. Joseph, M. Bissonnette, miR-143 and miR-145 are downregulated in ulcerative colitis: putative regulators of inflammation and protooncogenes, Inflamm. Bowel Dis. 18 (2012) 94–100.
[81] B. Hoesel, J.A. Schmid, The complexity of NF-κB signaling in inflammation and cancer, Mol. Canc. 12 (2013) 86.
[82] R.M. O'Connell, K.D. Taganov, M.P. Boldin, G. Cheng, D. Baltimore, MicroRNA-155 is induced during the macrophage inflammatory response, Proc. Natl. Acad. Sci. Unit. States Am. 104 (2007) 1604–1609.
[83] K.A. Jablonski, A.D. Gaudet, S.A. Amici, P.G. Popovich, M. Guerau-de-Arellano, Control of the inflammatory macrophage transcriptional signature by miR-155, PloS One 11 (2016) e0159724.
[84] S. Roy, C.K. Sen, MiRNA in innate immune responses: novel players in wound inflammation, Physiol. Genom. 43 (2011) 557–565.
[85] K.D. Taganov, M.P. Boldin, K.J. Chang, D. Baltimore, NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses, Proc. Natl. Acad. Sci. Unit. States Am. 103 (2006) 12481–12486.
[86] R.T. Marquez, E. Wendlandt, C.S. Galle, K. Keck, A.P. McCaffrey, MicroRNA-21 is upregulated during the proliferative phase of liver regeneration, targets Pellino-1, and inhibits NF-κB signaling, Am. J. Physiol.-Gastr. L. 298 (2010) G535–G541.
[87] F. Olivieri, L. Spazzafumo, G. Santini, R. Lazzarini, M.C. Albertini, M.R. Rippo, R. Galeazzi, A.M. Abbatecola, F. Marcheselli, D. Monti, R. Ostan, E. Cevenini, R. Antonicelli, C. Franceschi, A.D. Procopio, Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging, Mech. Ageing Dev. 133 (2012) 675–685.