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EXPRESSION PATTERN OF IMMUNE-RELATED GENES IN THE LIVER OF ROHU, *LABEO ROHITA* (HAMILTON) EXPOSED TO CARBON TETRACHLORIDE TOXICITY

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Immune responses in the liver play a crucial role in the detoxification process and rendering protection against different diseases. The present study was targeted to analyze the expression kinetics of few immune genes (pro-inflammatory, immune receptors, anti-microbial and innate immunity-related) using qPCR in liver tissue of carbon tetrachloride (CCl_4) treated rohu (*Labeo rohita*). Rohu juveniles were injected with single intraperitoneal toxic dose of 30% CCl_4 to induce liver damage. Histopathological findings showed the presence of focal necrosis and vaccuolation of hepatocytes. Increase in the level of pro-inflammatory cytokine (interleukin-6, IL-8 and IL-15) transcripts was observed immediately after CCl_4 post-exposure (from 6 h to 120 h), thus indicating the role of enhanced level of cytokines inducing liver damage. The degree of liver damage was also reflected by reduction in expression of immune related molecules (viz., TLR 22, transferrin and hepcidin), which are mostly synthesized in liver tissue. No effect was noticed in synthesis of mitochondrial antiviral-signaling protein (MAVS) transcripts with the current level of CCl_4 exposure. These results suggest that there is a critical balance between immune molecules that may play essential roles in the orchestration of immune defense in fish during liver dysfunction.

INTRODUCTION

Liver plays a crucial role in metabolism of the toxic chemicals in both fish and mammals because of its portal location within the circulation, its anatomic and physiologic structure (Allis *et al*., 1996). Liver which is a site of xenobiotics metabolism and transformation, possesses high risk of toxic damage (Lattuca *et al*. 2009). Xenobiotics induce production of reactive oxygen species (ROS) which is detoxified by the antioxidative system of liver. When the antioxidant system is unable to cope with the excessive production of ROS, it results in oxidative stress leading to tissue damage. Carbon tetrachloride (CCl_4) is a model solvent for classical hepatotoxicity, commonly found in freshwater that contaminates both the aquatic environment and accumulate toxic substance in aquatic organisms (Statham *et al*., 1978). CCl_4 causes multifactorial damage ranging from lipid peroxidation and inflammation to apoptotic or necrosis reaction via oxidative stress pathway. Endoplasmic cytochrome P450 induces CCl_4 activation producing trichloromethyl radical. These free radicals cause lipid peroxidation and over production of inflammatory cytokines leading to damage of hepatic tissues (Boelsterli, 2003). CCl_4 has been shown to increase the levels of glutathione pyruvate transaminase (GPT), glutamate oxalate transaminase (GOT), malondialdehyde (MDA) enzymes and reduce the levels

of antioxidant enzymes viz., superoxide dismutase (SOD), and glutathione peroxidase (GPx), catalase, glutathione (GSH) and total antioxidant capacity (T-AOC) in common carp (*Cyprinus carpio* L.) intraperitoneally injected with 30% CCl_4 in arachis oil (0.5 ml/kg body weight) at 72 h post-injection (Jia *et al*., 2014). Upregulations in the gene expressions of toll-like receptor 4 (TLR - 4), cytochrome P450 2E1 (CYP2E1), nuclear factor-ƙB (NF-ƙB), inducible nitric oxide synthase (iNOS), and inflammatory cytokines like interleukin-1β (IL-1β), IL-6 and IL-12 after CCl_4 exposure are also seen (Jia *et al*., 2014). Different fish species respond differently to CCl_4 exposures. Only 8 mM concentration of CCl_4 dose causes severe damage to carp hepatocytes and liver tissue got impaired at a dose of 0.15 ml/kg body weight of carp (Jia *et al*., 2012). The dose related damage by CCl_4 to the liver in various fish species, English sole (Casillas *et al*., 1983), Nile tilapia (Chen *et al*., 2004), rainbow trout (Statham *et al*., 1978), brown trout (Krasnov *et al*., 2007), rosy barbs and amphioxus (Bhattacharya *et al*., 2008) has already been reported. The toxicity of CCl_4 has mostly been investigated in various fish species by means of measuring blood biochemical changes and histopathological examination. However, the detail cellular and molecular immune-related events by CCl_4 toxicity particularly in Indian major carps have not been investigated earlier, and remain unclear. Hence, the present study was undertaken to study the pattern of expression of several immune related genes in liver tissue of rohu for understanding immunological changes at molecular level.

MATERIALS AND METHODS

Fish

Juveniles of rohu (*Labeo rohita*) weighing 30.00 ± 10.20 g were collected from the farm of the ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India. Fish were reared in 700-L ferro-cement tanks and acclimated to the experimental conditions before two weeks of initiation of the experiment. A commercial pellet diet at 3% of their body weight was given to fish twice a day prior to the experiment. One–tenth of the tank water was exchanged with freshwater daily in order to remove waste feed and faecal materials from bottom of the tanks. Appropriate aeration was provided in the tanks to maintain optimal oxygen level in water. The basic physico-chemical parameters were maintained at their optimal level and the water temperature during the experiment varied between 25-28 °C.

Exposure of fish to carbon tetrachloride

After acclimation, 28 numbers of fish were divided randomly into two groups, control group containing 4 numbers of fish and experimental group containing 24 fish. Four fish were kept in 200-L FRP tank for each treatment with similar management conditions. Control group fish were intraperitoneally injected with olive oil, whereas, the treatment group was intraperitoneally injected with 30% (v/v) CCl_4 in olive oil at a volume of 1 µl/g body weight. The fish were sacrificed with overdose of anaesthesia (MS222, Sigma) at different time periods (6, 12, 24, 36, 48 and 120 h) post-exposure to CCl_4 and liver samples were collected, and one portion

(~ 100 mg) was kept in RNAlater (Sigma, USA). Rest part of the liver tissue was preserved in 10% neutral buffered formalin for histology following routine protocol of haematoxylin and eosin staining. Except for histological assay, all tissue samples were stored at 4 °C for 24 h and then transferred to -20 °C until RNA extraction to study the gene expression pattern.

RNA extraction and cDNA synthesis

RNA extraction was done using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction, followed by DNase I (Fermentas, Canada) treatment to remove genomic DNA contamination in addition to 1 µl (1 U/µl) of RNase inhibitor (Fermentas, Canada) to inhibit degradation of RNA. Subsequently, cDNA synthesis was carried out using M-MLV reverse transcriptase (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The quality and quantity of RNA samples were checked using NanoDrop ND 1000 (NanoDrop Technologies Inc., USA).

PCR amplification

The semi-quantitative PCR was performed for different immune relevant genes and β-actin as house-keeping gene (Table 1) to know the specificity of primers and products. All amplification reactions consisted of an initial denaturation at 95 °C for 2 min prior to 35 cycles of 95 °C denaturation for 30 s with different annealing temperatures for 45 sec and 72 °C extension for 1 min 30 sec, followed by a final 72 °C extension for 10 min using 1.5 units of *Taq* DNA polymerase and finally by cooling at 4 °C (Genie, India). The generated PCR products (8 µl) were then analyzed by electrophoresis on 1.0% agarose gel.

Real-time PCR

Real-time PCR was carried out with different primers using Light Cycler 96 SW 1.1 (Roche, Germany). Briefly, 1 µl of cDNA was used as template in a total reaction mixture of 10 µl containing 5 µl of 2X Fast Start Essential DNA Green Master (Roche, Germany), 0.5 µl (5 pmole) of each forward/revese primers (Table 1) and 3 µl of PCR grade H_2O provided in the kit. The qPCR program included a pre-denaturation at 95 ºC for 10 min and 40 cycles of amplification at 95 ºC for 10 s, at respective annealing temperatures for 10 s, and 72 ºC for 20 s followed by melt curve analysis at 95 ºC for 10 s, 65 ºC for 60 s and 97 ºC for 1 s and cooling at 37 ºC for 30 s. β-actin was used as a reference gene for the study (Robinson *et al.*, 2012). No template controls were run each time. *Tm* analysis was done to check primer specificity. The quantification cycle (Cq) values were imported in an excel file.

Table 1 : Primer details used for gene expression analysis

Primer Id	Primer Sequence (5'-3')	Amplicon size (bp)	Annealing temp.	Reference
β-actin	F-TGGCAATGAGAGGTTCAGGT R-TGGCATACAGGTCCTTACGG	139	56 °C	Robinson *et al*. (2012)
ApoAI –RT	F-TGGAGGCTGTGCGTGTA R- GCTCGCCCAGTTCATTC	164	59 °C	Mohapatra *et al*. (2014)
IL-1β	F-GTGACACTGACTGGAGGAA R-AGTTTGGGCAAGGAAGA	164	51 °C	Self-designed
IL-6	F-GGACCGCTTTGAAACTCT R-5'-GCTCCCTGTAACGCTTGT-3'	212	54 °C	Dash *et al*. (2014)
IL-8	F-AAAGGGTTCTTACTGG R-TTTAGACATCTCGGACT	170	57 °C	Self-designed
IL-15	F-ACCAACAATCTCGCTTTCG R-GTTCAACGGGCATTCCAT	160	56 °C	Das *et al*. (2015)
Hep	F-TACAGTACATCAGCTCCTC R-GATCAGAATTTGCAGCAGTA	330	50 °C	Mohapatra *et al*. (2011)
MAVS	F-CACCTCCTGTCATAAATAGC R-AAGCCAAGAAAGACACCT	160	52 °C	Self-designed
Transferrin	F-GGACTACCAGCTGTTGTGCAT R-GCCACCATCGACTGCAAT	487	48 °C	Sahoo *et al*. (2009)
TLR 22	F-TCCTACAATGCCAAAGATGAG R-CAGGAACACCAGAATCAGTACATCC	273	54 °C	Panda *et al*. (2014)

Relative expression analysis

The quantification cycle values (Cq) were calculated using Light Cycler 96 SW 1.1 and the data were exported. n-fold differential expression was calculated using the comparative Cq method (Livak and Schmittgen, 2001) by calculating the average of each Cq for the triplicate samples. Cq value for the sample of each cDNA was deducted from its respective Cq value of β-actin to get ΔCq value. The mean of each sample was done, as the samples were taken in triplicates. Further, ΔΔCq as obtained by subtracting ΔCq value of sample from ΔCq of the control. Fold difference was calculated as $2^{-\Delta\Delta Cq}$.

Statistical analysis

The average fold expression for replicate samples for each time period was calculated and presented as mean ± SE. The average fold expression of four control group fish was considered as 0 h value. Further, differences between the mean values were analyzed using one-way ANOVA followed by Duncan's multiple range tests, with values $P < 0.05$ as significantly different. All values of n-fold differential expression were plotted in a graph.

RESULTS

Quality check of isolated RNA from samples

The quality of the RNA from the samples isolated as examined by taking OD_{260} nm/OD_{280} nm was found to be in the range of 1.8 to 2.0. Further integrity of the RNA samples were checked by RT-PCR using β-actin primer pairs and strong amplification products for β-actin gene (139 bp) were found for all the samples.

Amplification of various gene products in liver cDNA samples using RT-PCR and agarose gel electrophoresis

All the primer sets for various gene products were checked by running RT-PCR using control liver RNA samples. The expected product sizes of 139 bp, 164 bp, 164 bp, 212 bp, 170 bp, 160 bp, 330 bp, 160 bp, 487 bp and 273 bp were obtained for β-actin, apolipoprotein A-I (ApoA I), interleukin (IL)-1β, IL-6, IL-8, IL-15, hepcidin, mitochondrial antiviral-signaling protein (MAVS), transferrin, and toll-like receptor 22 (TLR 22) genes, respectively.

Expression of inflammatory molecules in rohu liver following CCl_4 exposure

The expression of IL-6 transcripts increased immediately from 6 h of exposure of CCl_4 in liver tissue of rohu and its highest level was observed at 120 h among all time points taken here (Fig. 1a). Similarly, the expression of IL-8 gene showed an increase in liver tissue samples of experimental fish immediately after 6 h with a peak level of expression being detected at 48 h in comparison to control fish (Fig. 1b). However, the expression of IL-15 was slightly high at 120 h post-exposure of CCl_4 in rohu liver tissue in comparison to all other periods of exposure or control fish liver sample (Fig. 1c). On the other hand, the expression of IL-1β didn't vary up to 48 h of post-exposure of CCl_4 in comparison to control, except a mild increase at 120 h post-exposure (Fig. 1d).

Expression of innate immune molecules in rohu liver following CCl_4 exposure

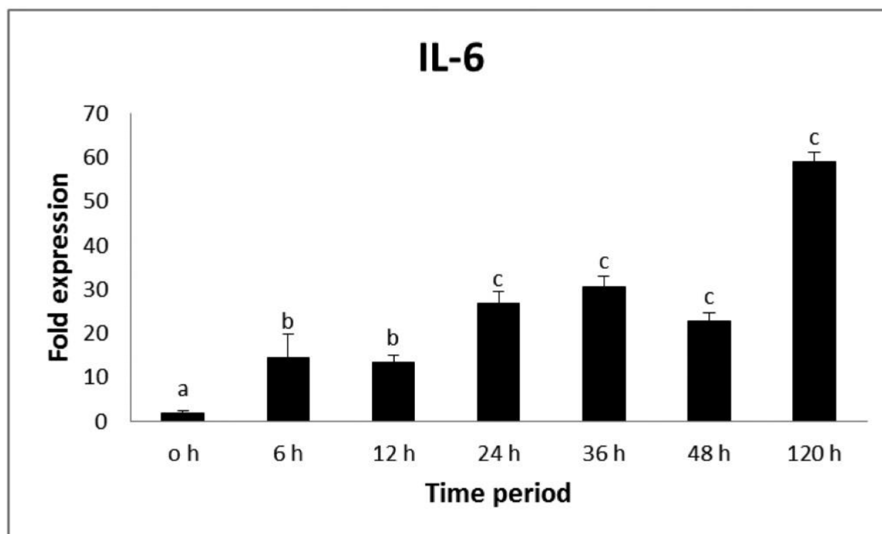
The expression of transferrin gene showed a significant decline immediately after CCl_4 exposure till the completion of the experiment, except a transient rise at 12 h post-exposure in comparison to control (Fig. 1e). Similarly, the expression of TLR 22 declined significantly post CCl_4 exposure from 6 h onwards in comparison to control except a mild transient rise at 24 h post-exposure (Fig. 1f).

Expression of anti-bacterial molecules in rohu liver following CCl_4 exposure

The expression of anti-bacterial molecules, apolipoprotein A1 showed a transient rise at 24 h post-exposure (Fig. 1g). In sharp contrast the expression of hepcidin was below detectable limit at 6 h post-exposure of CCl_4. However, its expression gradually increased 48 h of post-exposure of CCl_4 till 120 h (Fig. 1h).

Expression of anti-viral molecules in rohu liver following CCl_4 exposure

The mRNA levels of MAVS showed a variable levels of expression in the CCl_4 exposed fish in comparison to control (Fig. 1i), although the levels remained higher than the control fish throughout the period of study.



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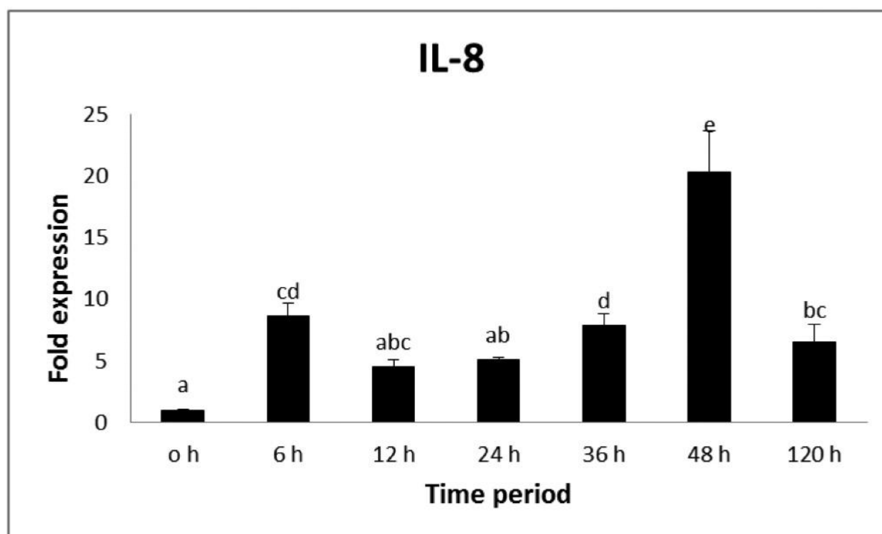

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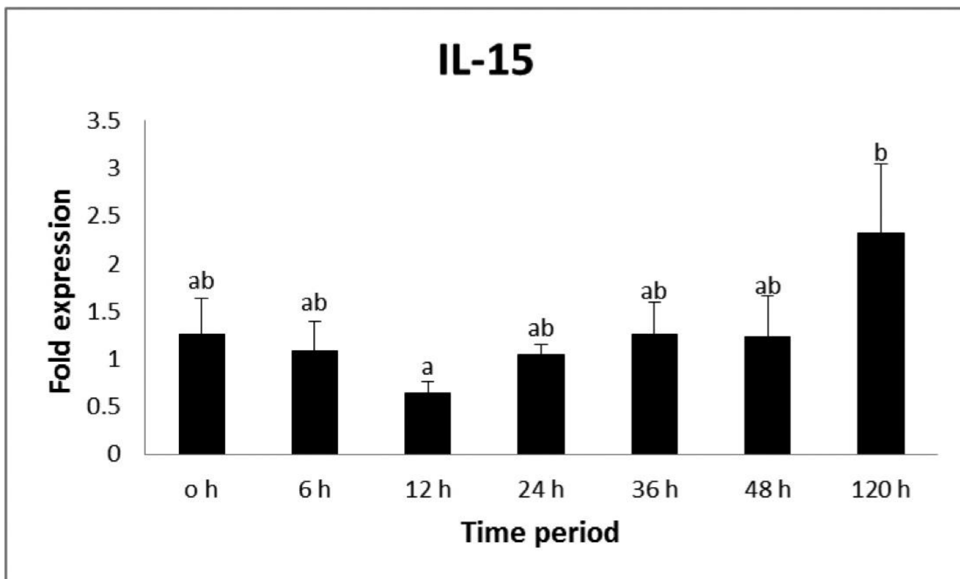

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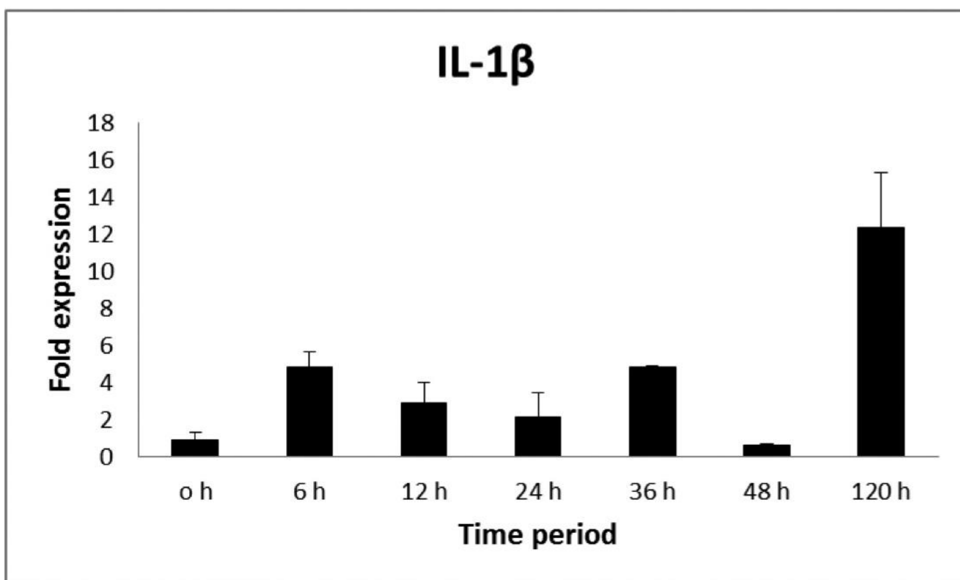

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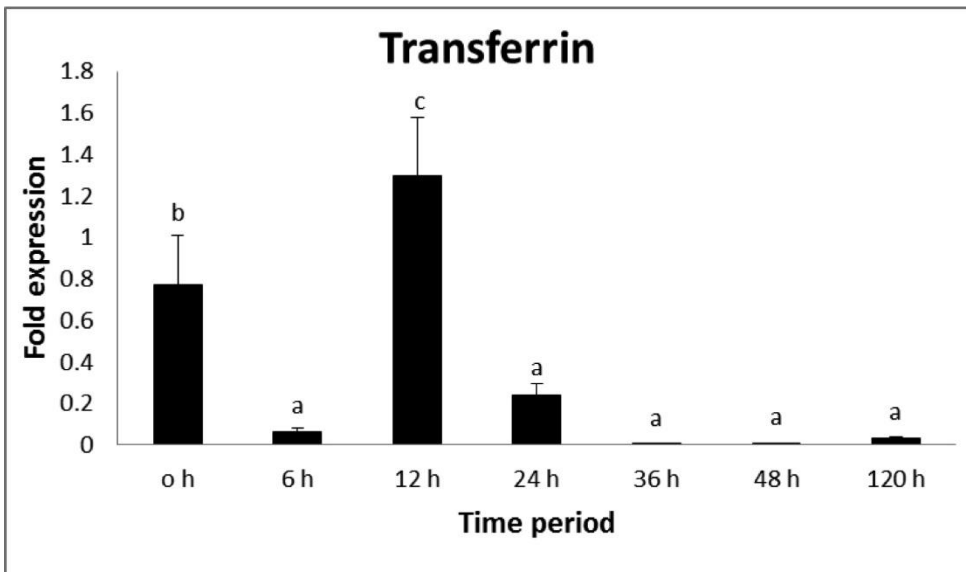

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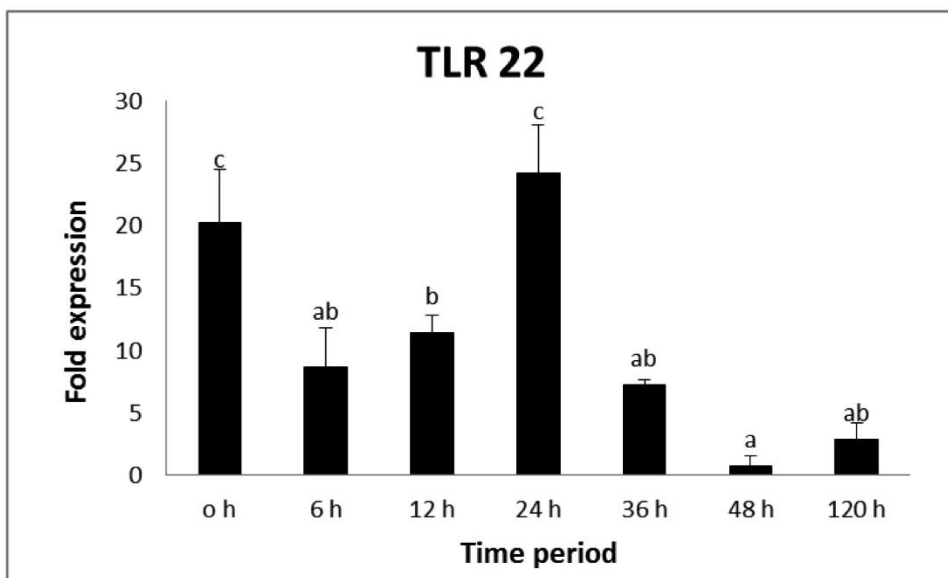

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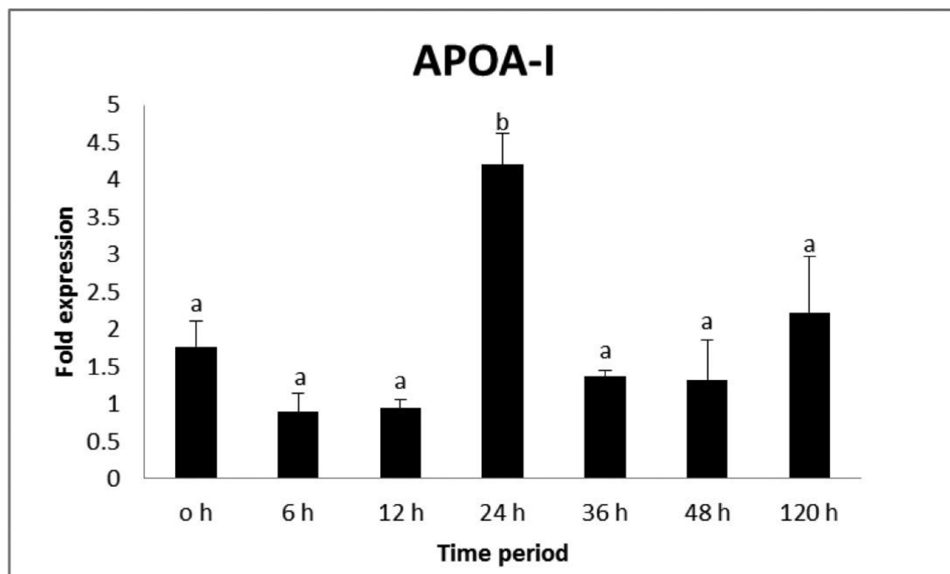

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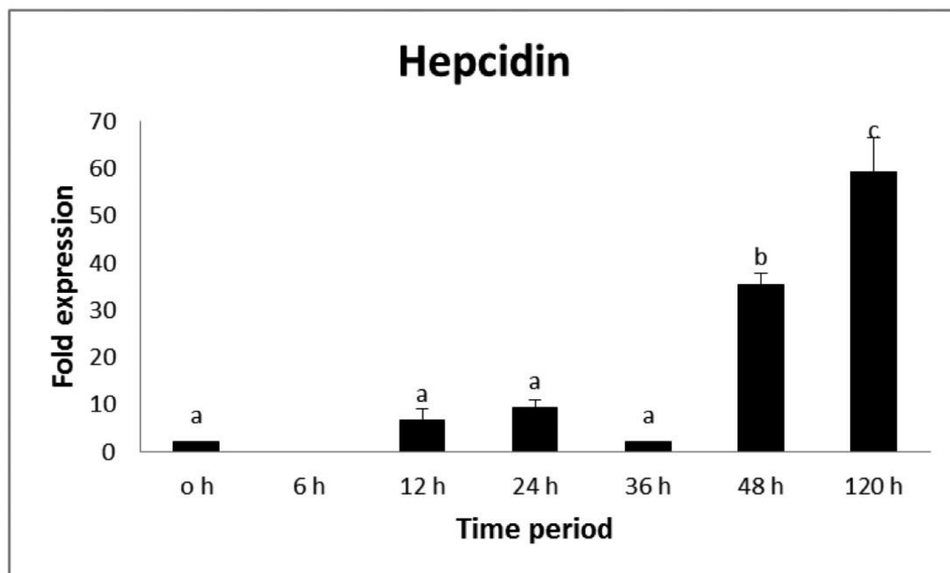

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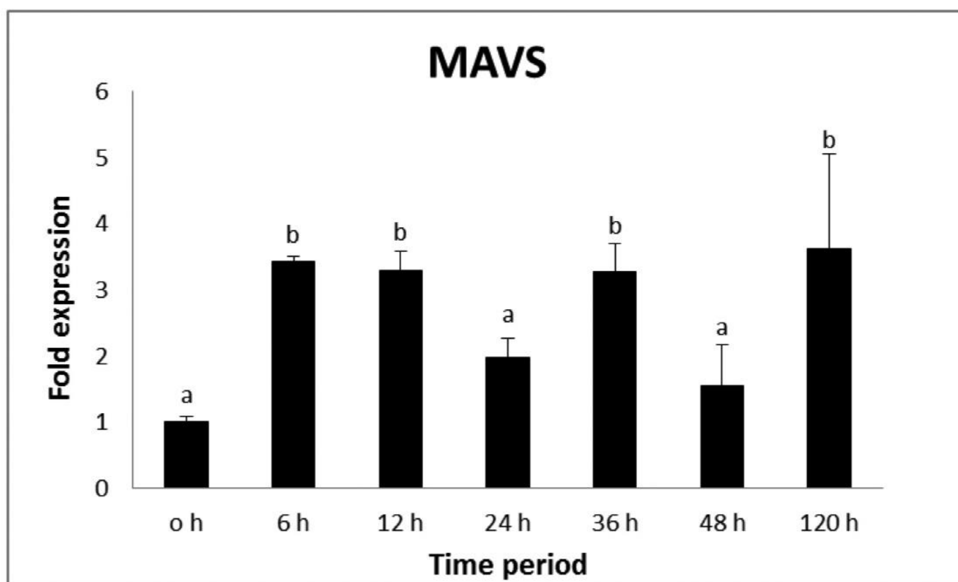

1 (i)

Fig. 1. Expression analysis of various innate immune genes; IL-6 (a), IL-8 (b), IL-15 (c), IL-1β (d), Transferrin (e), TLR 22 (f), ApoA I (g), Hepcidin (h) and MAVS (i) in the liver of rohu at different time periods of post-exposure to CCl_4. Data are presented as mean ± S.E. of three samples at each time period. Bars bearing the different letter(s) are significantly different ($P<0.05$). The fold difference was calculated as $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq$ = (ΔCq sample - ΔCq calibrator) and ΔCq = (Cq value of gene of interest - Cq value of β-actin). The control group (0 h of post-exposure) was taken as calibrator in the analysis.

Histopathology

The injected fish liver tissue revealed massive focal areas of necrosis without inflammatory reaction after 24 h – 36 h of CCl_4 exposure (Fig. 2). The hepatocytes in general revealed massive cytoplasmic vacuolation followed by nucleolysis in the affected areas.

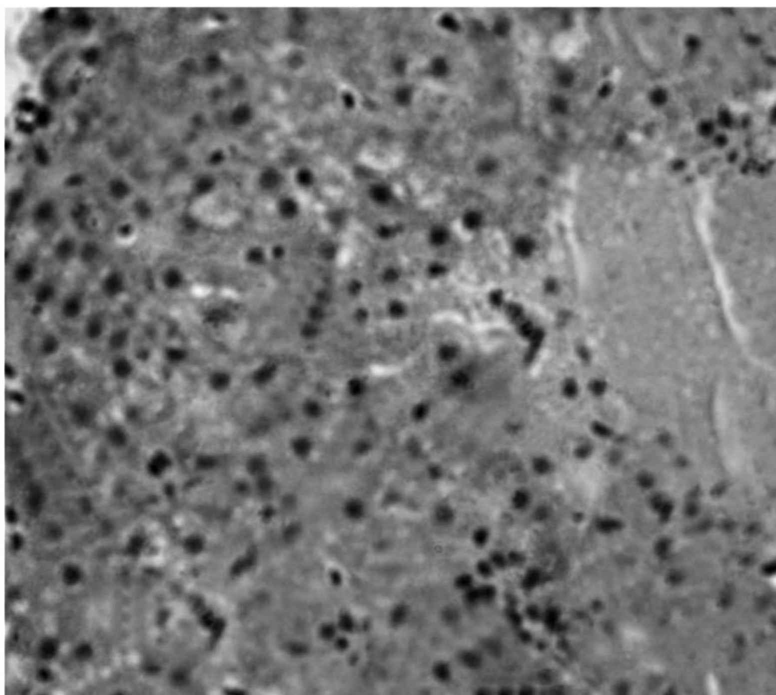

Fig. 2. Liver showing massive focal necrosis and degeneration of hepatocytes. CCl_4 - 36 h post-exposure.

DISCUSSION

The aim of the study was to investigate the possible effect of CCl_4 toxicity on immune genes in liver of rohu. CCl_4 hepatotoxicity was confirmed by the histopathological changes in the liver tissue of rohu. CCl_4 induced hepatotoxicity has also been studied in other fish species earlier (Jia *et al*., 2014; Cao *et al*., 2015; Liu *et al*., 2015). In fish, lesions commonly observed in acute toxicity by CCl_4 include areas of diffuse focal necrosis, laminar or subcapsular necrosis (Chen *et al*., 2004), cytoplasmic vacuolations and nucleolysis (Jia *et al*., 2014). The histopathological findings noticed in this study generally support earlier findings in other fish species.

It is generally believed that CCl_4 is metabolised by the liver tissue using the cytochrome P450 system (Yin *et al.*, 2011). CCl_4 causes oxidative stress and lipid peroxidation to induce liver damage. CCl_4 induced reactive oxygen species (ROS) production causes tissue damage and also initiates inflammation. The inflammatory responses were mostly mediated by non-parenchymal cells of the liver tissue; following activation by CCl_4 they release large amounts of inflammatory associated cytokines (Domitrovic *et al.*, 2011).

TLR 22 specific to teleosts is recently shown to recognize multiple pathogens associated molecular patterns (PAMP) and other products of damage tissue. It plays an important role in the regulation of innate immune system in the infectious and inflammatory diseases. Liver is one of the important sites of TLR 22 synthesis (Panda *et al.*, 2014). The down-regulation of TLR 22 noticed in our study could be due to damage to the hepatic tissue.

Geier *et al.* (2002) reported that CCl_4 rapidly induces the production of pro-inflammatory cytokines in Kuffer cells leading to liver damage. Further studies have also demonstrated that cytokine inhibition effectively reduces liver damage in mice. The elevated levels of IL-6, IL-15, and IL-8 transcripts in the liver of CCl_4 treated rohu was noticed in our study. These data indicated and further confirmed the role of cytokine-related up-regulation being an important mechanism for hepatocellular damage.

Liver is also an important site of synthesis of antimicrobial proteins viz., apolipoprotein A I and hepcidin; and iron binding proteins like transferrin. In our study, the level of transferrin transcripts was significantly low at all the time period except at 12 h post-exposure, thus indicating the degree of damage to the liver tissue. Similarly, the level of antimicrobial peptide hepcidin transcript was too low till 36 h post exposure followed by a marked rise thereafter. The increasing level of transcripts of hepcidin after 2 days of CCl_4 exposure in rohu probably indicates the stabilization of hepatocytes to CCl_4 induced damage or regeneration of new hepatocytes leading to increase in synthesis that needs further in depth study. On the contrary, the level of apolipoprotein A I transcripts did not show a major change after CCl_4 induced liver damage. It might be due to transport or migration of this transcripts from other sites of synthesis, for example intestinal tissue that might not have been damaged by CCl_4 and may be serving as a site of synthesis of ApoA I (Glickman and Green, 1977) or their slow breakdown from the system.

Mitochondrial antiviral-signaling protein (MAVS) has been identified from many fish species and it plays a major role in innate immune response against viruses (Kasthuri *et al.*, 2014). In this study an increase in level of transcripts in MAVS in CCl_4 induced liver damaged tissue at most of the time periods indicates poor effect of CCl_4 on MAVS synthesis, but this conjecture is speculative and needs to be elucidated by various experiments in teleosts.

The present study demonstrated that CCl_4 could induce hepatotoxicity in rohu within 6 h of post-exposure. An array of indication of synthesis of pro-inflammatory cytokines (increased level of IL-8, IL-6, IL-15) might be playing major role causing damage to the liver, thus leading to reduction in synthesis of liver specific innate immune molecules viz, transferrin, TLR 22 and hepcidin. The findings of the study suggest that the presence of CCl_4 in the aquatic environment might be playing important role in suppression of the immune system of fish through focal/limited damage without causing significant mortality thus increasing their susceptibility to various infections.

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REFERENCES

Allis, J. W., B. L. Brown, J. E. Simmons, G. E Hatch, A. McDonald and D. E. House, 1996. Methanol potentiation of carbon tetrachloride hepatotoxicity: the central role of cytochrome P450. *Toxicology*, **112**: 131–140.

Bhattacharya, H., S. Zhang and Q. Xiao, 2008. Comparison of histopathological alterations due to sublethal CCl_4 on rosy barb (*Puntius conchonius*) and amphioxus (*Branchiostoma belcheri*) with implications of liver ontogeny. *Toxicol. Mech. Methods*, **18**: 627–633.

Boelsterli, U. A., 2003. Mechanistic Toxicology. Taylor & Francis, New York, NY, USA.

Cao, L., W. Ding, J. Du, R. Jia, Y. Liu, C. Zhao, Y. Shen and G. Yin, 2015. Effects of curcumin on oxidative activities and cytokine production in Jian carp (*Cyprinus carpio* var. Jian) with CCl_4-induced liver damage. *Fish Shellfish Immunol.,* **43**: 150-157.

Casillas, E., M. Myers and W. E. Ames, 1983. Relationship of serum chemistry values to liver and kidney histopathology in English sole (*Parophrys vetulus*) after acute exposure to carbon tetrachloride. *Aquat. Toxicol.,* **3**: 61–78.

Chen, C. Y., G. A. Wooster and P. R Bowser, 2004. Comparative blood chemistry and histopathology of tilapia infected with *Vibrio vulnificus* or *Streptococcus iniae* or exposed to carbon tetrachloride, gentamicin, or copper sulfate. *Aquaculture*, **239**: 421–443.

Das, S., A. Mohapatra, B. Kar and P. K. Sahoo, 2015. Molecular characterization of interleukin 15 mRNA from rohu, *Labeo rohita* (Hamilton): its prominent role during parasitic infection as indicated from infection studies. *Fish Shellfish Immunol.,* **43:** 25-35.

Dash, P., P. K. Sahoo, P. K. Gupta, L. C. Garg and A. Dixit, 2014. Immune responses and protective efficacy of recombinant outer membrane protein R (rOmpR)-based vaccine of *Aeromonas hydrophila* with a modified adjuvant formulation in rohu (*Labeo rohita*). *Fish Shellfish Immunol.,* **39**: 512-523.

Domitrovic, R., H. Jakovac and G. Blagojevic, 2011. Hepatoprotective activity of berberine is mediated by inhibition of TNF-α, COX-2 and iNOS expression in CCl_4 intoxicated mice. *Toxicology,* **280**: 33-43.

Geier, A., S. K. Kim, T. Geloff, C. G. Dietrich, F. Lammer and S. J. Karpen, 2002. Hepatobiliary organic anion transporters are differentially regulated in acute toxic liver injury induced by carbon tetrachloride. *J. Hepatol.,* **37**:198-205.

Glickman, R. M. and P. H. Green, 1977. The intestine as a source of apolipoprotein A1. *Proc. Natl. Acad. Sci. U. S. A.,* **74**: 2569-2573.

Jia, R., L. Cao, J. Du, J. Wang, Y. Liu, G. Jeney, P. Xu and G. Yin, 2014. Effects of carbon tetrachloride on oxidative stress, inflammatory response and hepatocyte apoptosis in common carp (*Cyprinus carpio). Aquat. Toxicol*., **152**: 11-19.

Jia, R., L. Cao, P. Xu, G. Jeney and G. Yin, 2012. In vitro and in vivo hepatoprotective and antioxidant effects of *Astragalus* polysaccharides against carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus carpio*). *Fish Physiol. Biochem.,* **38**: 871–881.

Kasthuri, S. R., Q. Wan, I. Whanq, B. S. Lin, S. Y. Yeo, C. Y. Choi and J. Lee, 2014. Functional characterization of the evolutionary preserved mitochondrial antiviral signaling protein (MAVS) from rock bream, *Oplegnathus fasciatus*. *Fish Shellfish Immunol.,* **40**: 399-406.

Krasnov, A., S. Afanasyev and A. Oikari, 2007. Hepatic responses of gene expression in juvenile brown trout (*Salmo trutta lacustris*) exposed to three model contaminants applied singly and in combination. *Environ. Toxicol. Chem.,* **26**: 100–109.

Lattuca, M., G. Malanga, C. A. Hurtado, A. Pe´rez, J. Calvo and S. Puntarulo, 2009. Main features of the oxidative metabolism in gills and liver of *Odontesthes nigricans* Richardson (Pisces, Atherinopsidae). *Comp. Biochem. Physiol. B.,* **154:** 406–411.

Liu, Y., J. Du, L. Cao, R. Jia, Y. Shen, C. Zhao, P. Xu and G. Yin, 2015. Anti-inflammatory and hepatoprotective effects of *Ganoderma lucidum* polysaccharides on carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus Carpio* L.). *Intl. Immunopharmacol.,* **25**: 112-120.

Livak, K. J. and T. D. Schmittgen, 2001. Analysis of relative gene expression data using realtime quantitative PCR and the 2-AACT method. *Methods,* **25:** 402-408.

Mohapatra, A., B. Kar, A. Dixit, L. C. Garg and P. K. Sahoo, 2014. Cloning, sequencing and characterization of apolipoprotein A1 in *Labeo rohita*. *In*: 10th Indian Fisheries and Aquaculture Forum held at NBFGR, Lucknow during 12-15 November 2014; pp. 383.

Mohapatra, A., M. Mishra, S. Das, A. Das, M. Biswas and P. K. Sahoo, 2011. Cloning, sequencing and expression analysis of hepcidin gene of *Labeo rohita* to *Aeromonas hydrophila* infection. *In*: Eight Symposium on Diseases in Asian Aquaculture held at Mangalore, India, during November 21-25, pp. 205.

Panda, R. P., V. Chakrapani, S. K. Patra, J. N. Saha, P. Jayasankar, B. Kar, P. K. Sahoo and H. K. Barman, 2014. First evidence of comparative responses of Toll-like receptor 22 (TLR-22) to relatively resistant and susceptible Indian farmed carps to *Argulus siamensis* infection. *Dev. Comp. Immunol.,* **47**: 25-35.

Robinson, N., P. K. Sahoo, M. Baranski, K. D. Mahapatra, J. N. Saha, S. Das, Y. Mishra, P. Das, H. K. Barman and A. E. Eknath, 2012. Expressed sequences and polymorphisms in rohu carp (*Labeo rohita*, Hamilton) revealed by mRNA-seq. *Mar. Biotechnol.,* **14**: 620-633.

Sahoo, P. K., B. R. Mohanty, J. Kumari, A. Barat and N. Sarangi, 2009. Cloning, nucleotide sequence and phylogenetic analyses, and tissue-specific expression of the transferrin gene in *Cirrhinus mrigala* infected with *Aeromonas hydrophila*. *Comp. Immunol. Microbiol. Infect. Dis.,* **32**: 527-537.

Statham, C. N., W. A. Croft and J. J. Lech, 1978. Uptake, distribution, and effects of carbon tetrachloride in rainbow trout (*Salmo gairdneri*). *Toxicol. Appl. Pharmacol.,* **45**: 131–140.

Yin, G. J., L. P. Cao, P. Xu, G. Jeney, M. Nakao and C. P. Lu, 2011. Hepatoprotective and antioxidant effects of *Glycyrrhiza glabra* extract against carbon tetrachloride (CCl_4)-induced hepatocyte damage in common carp (*Cyprinus carpio*). *Fish Physiol. Biochem.,* **37**: 209-216.