Microcystin-leucine-arginine Modulates the Expression Patterns of Proinflammatory Cytokines and an Apoptotic Gene in Chicken Liver

Rania A. Elgawish[1,2], Yukinori Yoshimura[1] and Naoki Isobe[1]

[1] Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8528, Japan
[2] Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt

Microcystins (MCs) are included in drinking water and a family of cyclic heptapeptide hepatotoxins that have been implicated in the impairment of liver function in various animals. There is scarce information on the effect of MCs on cytokines and apoptotic gene expression and on whether MCs can induce inflammation and apoptosis in avian hepatic tissue. This study investigated the expression of genes related to proinflammatory interleukins, apoptosis, and antioxidant function in chicken liver tissues cultured in the presence of different doses of microcystin-leucine-arginine (MC-LR). Livers were collected from five hens and liver slices were placed in sterile tubes containing Dulbecco's medium supplemented with 0, 1, 10, or 100 ng/mL of MC-LR. After 6 h of cultivation, total RNA was extracted and quantitative PCR analysis was performed for interleukin genes (*IL-1β*, *IL-6*, and *IL-8*), *TNF sf15*, an apoptotic gene (*caspase-3*), and genes involved in antioxidant function ([catalase [*CAT*], glutathione peroxidase [*GSH-PX*], and superoxide dismutase [*SOD*]). Liver tissues in each group were fixed for histopathology. MC-LR downregulated the mRNA levels of *IL-1β, IL-8*, and *TNF sf15* as compared to the control (0 ng/mL) in dose-dependent patterns; however, the differences were not significant. The expression of *IL-6* in liver tissues exposed to 100 ng/mL of MC-LR was significantly ($P < 0.05$) lower than that in tissues exposed to 1 ng/mL. In contrast, MC-LR upregulated the mRNA expression of *caspase-3* and genes involved in antioxidant function in the liver tissues after 6 h, without the difference reaching statistical significance. Hepatocytes showed vacuolar degeneration and focal necrosis according to the dose of MC-LR. This study highlighted the risk of low doses of MC-LR in chicken liver. Moreover, MC-LR could modulate the transcriptional patterns of at least *IL-6* in liver-tissue culture of chicken after 6 h of exposure.

Key words: chicken, immune function, liver culture, microcystin-leucine-arginine

Introduction

In recent years, cyanobacterial blooms occur frequently in many parts of the world. Several cyanobacterial species are known to produce a wide variety of potent toxins, leading to dangerous cyanotoxin contamination, which is harmful to the safety of public drinking water (Li *et al*., 2011; Merel *et al*., 2013; Boopathi and Ki, 2014). Because of their high toxicity, microcystins (MCs) are the most extensively studied cyanotoxins (Campos and Vasconcelos, 2010; Puddick *et al*., 2014). Furthermore, MCs contain two variable residues, allowing differentiation between variants of MCs. These two variable elements are always standard L-amino acids. In MC-LR, these are leucine and arginine (Campos and Vasconcelos,

2010). MCs have constant chemical properties, and thus, it is difficult to remove them from water by ordinary water treatment processes (de la Cruz *et al*., 2011).

A number of studies have confirmed that liver is the target organ for MC-LR, which induces hepatotoxicity (Sun *et al*., 2011; Li and Han, 2012; Chen *et al*., 2013; Li *et al*., 2015). MCs are potent inhibitors of serine/threonine-specific protein phosphatases 1 and 2A (MacKintosh *et al*., 1990). MCs exert their toxic activity through general over-phosphorylation of cellular proteins, thus affecting a variety of basic cellular processes and expression regulation of various genes (Campos and Vasconcelos, 2010).

Macrophages can be stimulated in response to toxicants to produce a number of cytokines for the protection of the host (Kawagishi *et al*., 2001). Activated macrophages secrete interleukins such as, IL-1, IL-6, IL-8, IL-12, and TNF-*α* (Duque and Descoteaux, 2014). These cytokines are needed for the inflammatory response to toxicants (Liew, 2003). TNF-*α* is an important component in innate immunity and

inflammatory responses. MC-LR can modulate the inflammatory responses or other immune-related processes through inhibition of TNF-α (Wei *et al*., 2009).

On the other hand, MC-LR can induce cell death in various reproductive cell lines through different apoptotic signaling pathways, which finally lead to caspase activation (Chen *et al*., 2016). Moreover, the metabolism of MC-LR frequently leads to the formation of reactive oxygen species (ROS) (Weng *et al*., 2007). The ROS generated in tissues can be effectively eliminated by antioxidant and glutathione-related enzymes, such as glutathione peroxidase (*GSH-PX*), superoxide dismutase (*SOD*), and catalase (*CAT*), which protect the cells from the harmful effects of oxidative stress (Bieczynski *et al*., 2013). Excessive amounts of ROS are damaging to the cells of the immune system (De La Fuente and Victor, 2000).

The severity of avian death due to MC toxicity ranges from a few individuals to several thousands per incident. In the USA, high mortality at the Salton Sea has been linked to MCs (Carmichael and Li, 2006). In Japan, approximately 20 ducks died at a pond containing a bloom of *Microcystis aeruginosa* (Matsunaga *et al*., 1999). To date, knowledge on the effects of MC-LR on the liver of chicken *in vitro* is very limited, and whether it can induce inflammation and apoptosis in avian hepatic tissue has not been fully investigated. The present study was undertaken to elucidate the gene expression of different interleukins and genes involved in apoptosis and antioxidant function as well as histopathological alterations in the liver tissue of chicken after 6 h of exposure to different doses of pure MC-LR *in vitro*.

Materials and Methods

Birds

In total, five White Leghorn hens, approximately 200 days old, were used. The birds were euthanized with sodium pentobarbital (Somnopentyl; Kyoritsu Pharmaceutical Co., Tokyo, Japan) and immediately after that, the liver was collected, cut into cuboidal blocks of $4 \times 4 \times 2$ mm, and liver slices were made. This study was carried out in accordance with the guidelines of Animal Experimentation, Hiroshima University, Japan.

Tissue Preparation and Culture

Liver was collected and washed with sterile PBS containing 10 U/mL penicillin and 10 μg/mL streptomycin. Liver slices (approximately 60 mg$\pm$10 mg) were placed in sterile tubes for culture in Dulbecco's Modified Eagle Medium F-12 (Sigma, St. Louis, MO, USA) supplemented with 0, 1, 10, and 100 ng/mL of pure MC-LR (Wako pure chemical industries Ltd., Osaka, Japan). Livers were incubated in a CO_2 incubator for 6 h at 37°C under 5% CO_2 and 95% air. The culture was repeated five times using five different birds within the study.

RNA Extraction and Reverse Transcription

After 6 h of cultivation, total RNA was extracted from liver slices by Sepasol (Nacalai Tesque Inc., Kyoto, Japan). The extracted RNA was dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). For each sample, RNA concentration was estimated using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). The RNA was reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). The reaction mixture (10 μL) contained 1 μg of total RNA, $1 \times$ RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor, 0.5 μg of oligo (dT) 20 primer, and 50 U ReverTra Ace. Reverse transcription was carried out at 42°C for 30 min, followed by heat inactivation at 99°C for 5 min using an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster City, CA, USA)

Real-time PCR Analysis

Real-time PCR was carried out for interleukins (*IL-1β*, *IL-6*, and *IL-8*), tumor necrosis factor (*TNF sf15*), an apoptotic gene (*caspase-3*), and genes involved in antioxidant function ([catalase [*CAT*], glutathione peroxidase [*GSH-PX*], and superoxide dismutase [*SOD*]) using the Roche Light Cycler Nano system (Roche Applied Science, Indianapolis, IN, USA). Specific primers were used for each gene (Table 1). The reaction mixture (10 μL) contained 5 μL of Thunderbird SYBR qPCR mix (Toyobo Co., Ltd., Japan), 0.5 μL of each of the forward and reverse primers (Table 1), 1.5 μL of cDNA, and 2.5 μL of DNase-free water. After initial denaturation at 95°C for 30 s (except for *caspase-3*, the initial denaturation was 95°C for 3 min), PCR was carried out with a thermal cycling protocol of 95°C for 10 s and different numbers of PCR cycles with different annealing temperatures for each gene (Table 1). The data were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of each gene using *RPS17* as internal control gene for normalization. The results were expressed as fold change obtained from the ratio of the expression levels of the samples to the standard sample.

Histopathology

A part of the liver tissues in MC-LR-treated samples were fixed with 10% buffered formalin solution and embedded in paraffin. Paraffin sections (4 μm in thickness) were prepared and stained with hematoxylin and eosin (H&E).

Statistical Analysis

The significance of differences in gene expression among liver tissue groups was examined by one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant when the P value was <0.05.

Results

MC-LR downregulated the mRNA levels of *IL-1β*, *IL-8*, and *TNF sf15* as compared to 0 ng/mL MC-LR in dose-dependent patterns; however, the differences were not significant. The expression of *IL-6* in the cultured liver tissues exposed to 100 ng/mL of MC-LR was significantly ($P < 0.05$) lower than that in tissues exposed to 1 ng/mL (Fig. 1). After 6 h of cultivation, the expression of *caspase-3* and genes involved in antioxidant function tended to increase with increasing concentration of MC-LR; however, the differences did not reach statistical significance (Fig. 2).

Mild vacuolar changes along with degeneration and focal necrosis of some hepatocytes were seen in the livers of chickens exposed to a low dose (1 ng/mL) of MC-LR. In liver

Table 1. **Primers used in real-time PCR**

Symbol	Sequence (5′ → 3′)		Reference	Annealing temp. and cycles
	Forward	Reverse		
IL-1β	GGGCATCAAGGGCTACAA	CTGTCCAGGCGGTAGAAGA	NM_204524	63℃ for 30 sec.
			Nii *et al*. (2011)	45 cycles
IL-6	AGAAATCCCTCCTCGCCAAT	AAATAGCGAACGGCCCTCA	NM_204628.1	62℃ for 30 sec.
			Nii *et al*. (2011)	45 cycles
IL-8	GGCTTGCTAGGGGAAATGA	AGCTGACTCTGACTAGGAAACTGT	AJ009800	60℃ for 30 sec.
			Hong *et al*. (2006)	45 cycles
TNFSF15	CCTGAGTTATTCCAGCAACGCA	ATCCACGAGCTTGATGTCACTAAC	NM_001024578	64℃ for 30 sec.
			Woods *et al*. (2009)	45 cycles
Caspase-3	TTCAGGCACGGATGCAGATG	TTCCTGGCGTGTTCCTTCAG	NM_204725.1	55℃ for 30 sec.
			Mehaisen *et al*. (2016)	40 cycles
CAT	GGGGAGCTGTTTACTGCAAG	TTTCCATTGGCTATGGCATT		60℃ for 60 sec.
			Yarru *et al*. (2009)	
GSH-PX	TGTAAACATCAGGGGCAAA	TGGGCCAAGATCTTTCTGTAA		45 cycles
SOD	AGGGGGTCATCCACTTCC	CCCATTTGTGTTGTCTCCAA		
RPS17	AAGCTGCAGGAGGAGGAGAGG	GGTTGGACAGGCTGCCGAAGT	NM_204217	64℃ for 30 sec.
			Nii *et al*. (2011)	45 cycles

The PCR protocol was carried out as denaturation at 95℃ for 15 sec. and extension at 72℃ for 30 sec.
IL: Interleukin, *TNFSF15*: Tumor Necrosis Factor SF15   *CAT*: Catalase, *GSH-PX*: Glutathione Peroxidase, *SOD*: Superoxide Dismutase, *RPS17*: Ribosomal Protein RPS17

tissues exposed to 10 ng/mL of MC-LR, necrotic changes of hepatocytes with pyknosis of their nuclei were observed. Liver tissues exposed to 100 ng/mL of MC-LR showed massive coagulative necrosis in most of hepatic cords, with nuclear pyknosis and lysis (Fig. 3).

Discussion

With respect to the effect of MC on mammalian immunity, there is a general lack of studies focusing on the effects of MC-LR on immune cells of the chicken liver, and in particular, studies dealing with low MC-LR concentrations. The doses of MC-LR used in the current study were based on previously published reports. The MC-LR concentration of 1 ng/mL is equipotential to the provisional guideline value for MC of 1 μg/L in drinking water (WHO, 1998). In water bodies worldwide, concentrations of MCs are higher than the safety level for MC-LR (1 μg/L) (Gupta *et al*., 2003; Codd *et al*., 2005). Increased cyanotoxin levels in surface waters have been noted in Japan, China, and Portugal (concentrations above 10 μg/L, Funari and Testai, 2008). MC concentrations as high as 25000 μg/L have been found in cyanobacterial scum samples in Germany (Funari and Testai, 2008).

Our results showed that low doses of MC-LR were relevant for liver inflammation in chicken. The expression of *IL-1β*, *TNF sf15*, and *IL-8* tended to lower in a dose-dependent manner, without the differences reaching statistical significance, however. While no studies on the effect of MC-LR on proinflammatory gene expression in the liver of chicken were available, Chen *et al*. (2004) found that MC-LR had remarkable dose- dependent (1, 10, 100, 1000 nmol/L) inhibitory effects on *IL-1β* and *TNF-α* in mouse

macrophages, even though at low MC-LR concentrations. Why the low dose of MC-LR induced higher gene expression of cytokines than the high dose was not investigated in this study; however, Yuan *et al*. (2012) speculated that the increase in cytokines at 12 h post-injection of a low dose (12.5 μg/kg) of MC-LR in rabbit may be due to the irritative effects of MC-LR. Moreover, the decreased cytokine production after treatment with a high dose (50 μg/kg) of MC-LR might be owing to its immunotoxic effects (Yuan *et al*., 2012).

Our findings revealed that MC-LR affected the expression of *IL-6* in a dose-dependent manner. MC-LR at the lowest dose (1 ng/mL) increased the mRNA expression of *IL-6*, whereas the highest dose markedly diminished the expression of this cytokine. In contrast, the production of *IL-6* in macrophages (RAW 264.7 cell line) was only slightly induced at the highest concentration (1000 nM) of MC-LR (Adamovsky *et al*., 2015). The evidence of disturbance of *IL-6* potentially indicates the negative effect of MC-LR on the inflammatory reaction after a 6-h treatment.

In an attempt to elucidate the reasons behind the decline in cytokine production after treatment with a high dose of MC-LR, Lankoff *et al*. (2004) hypothesized that protein-phosphatase inhibition and free-radical production by MCs may be responsible for the changes in production of *IL-6*, and the dose of MCs is a significant factor in inducing these changes. Additionally, the authors reported dose-dependent increases in MC-LR-induced apoptosis and necrosis in chicken and human lymphocytes (Lankoff *et al*., 2004). Moreover, MC-LR antagonizes the proliferation and function of macrophages, which reduces the transcription of cytokines (Chen *et al*., 2004).

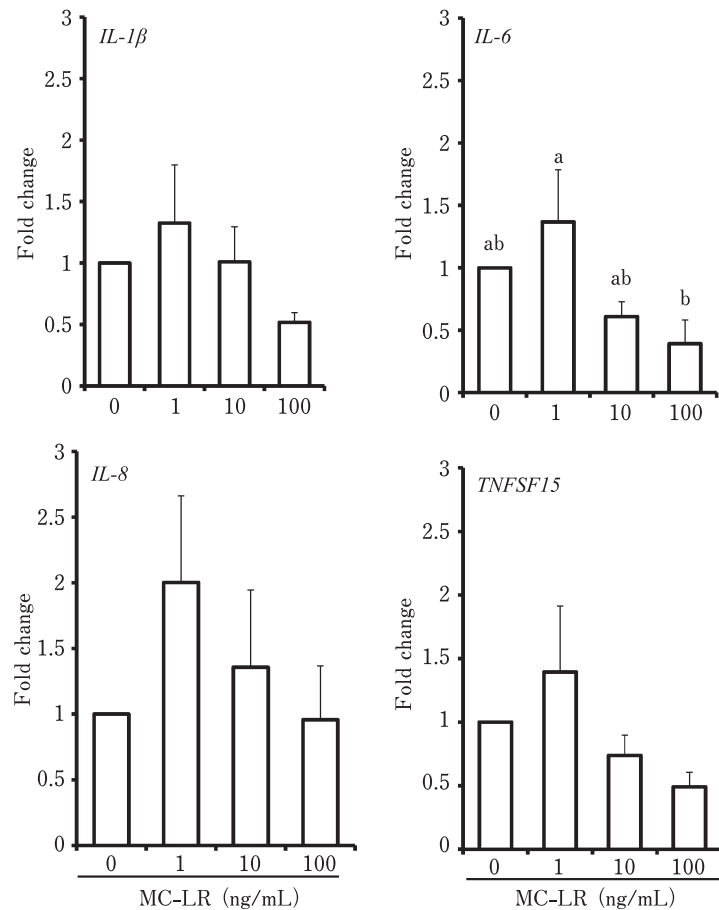Our results indicated that MC-LR did not significantly

Fig. 1. **Expression of interleukin genes and *TNF sf15* after 6 h of exposure to the indicated doses of pure MC-LR (ng/mL).** Means with different superscripts differ at $P < 0.05$.

modulate the gene expression of *caspase-3* and genes related to antioxidant activity. However, these genes tended to be upregulated in a dose-dependent manner after MC-LR exposure. Rymuszka and Adaszek (2013) confirmed the involvement of caspases in MC-induced cell death in carp. Substantial and concentration-dependent rises in caspase 3/7 activities in blood cells of carp were observed after exposure to extracts containing MCs at 0.1, 0.5, or 1 mg/mL. Similarly, Zhang *et al.* (2006) demonstrated that even a low concentration (1 μg/L) of MC-LR induced apoptosis in isolated lymphocytes of crucian carp. However, no significant increases in caspase 3/7 enzyme activities were detected in head kidney cells exposed to MCs at concentrations of 0.01 or 0.1 mg/mL (Rymuszka and Adaszek, 2013). An extract containing MCs at a high concentration significantly repressed ROS production in both blood and head kidney phagocytes stimulated with phorbol myristate acetate. On the other hand, extracts containing low concentrations of MCs had a stimulatory effect on ROS in the same cells (Rymuszka and Adaszek, 2013).

Gene expression levels of *SOD* and *CAT* were upregulated

in the liver of crucian carp after intraperitoneal injection of MC (Sun *et al.*, 2008). Furthermore, *CAT* and *SOD* activities were enhanced, but no distinct effects were observed in *GSH-PX* activity in the liver of Nile tilapia exposed to MC-LR (Prieto *et al.*, 2006). In mice exposed to two doses (0.5 LD$_{50}$ [38.31 μg/kg] and 1 LD$_{50}$ [76.62 μg/kg]) of MC-LR through intraperitoneal injection, the gene expression levels of antioxidant enzymes poorly correlated with their activity levels, and the gene expression of some antioxidant enzymes did not show any significant difference as compared to the control (Jayaraj *et al.*, 2006). The activities of *SOD*, *CAT*, and *GSH-PX* increased significantly after 6-h exposure of hepatocytes of common carp to 10 μg/L of MC-LR (Li *et al.*, 2003). It is believed that antioxidant enzymes, such as *SOD*, *CAT*, and *GSH-PX*, might play important roles in eliminating the excessive ROS after MC-LR exposure. The fact that we did not observe significant changes in gene expression of antioxidant functions may be due to the route, dose, and time of exposure to MC-LR.

In the current study, high standard errors (SEs) in some gene expression data (Fig. 1 and 2) were observed, although
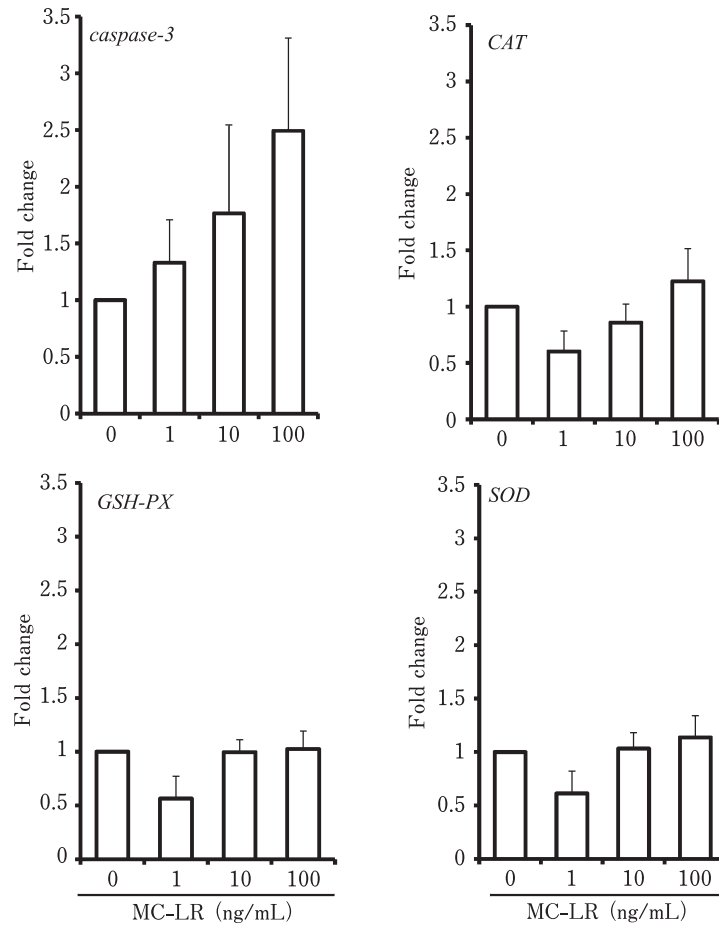
Fig. 2.　**Expression of genes related to apoptosis and antioxidant function after 6 h of exposure to different doses of pure MC-LR (ng/mL).**

the culture media and incubation time were identical in the current experiment, and the PCR analysis was carried out more than once for each gene. The variation in SEs might be explained by the variable nature of chicken liver immune cells; each chicken might have a different immune response in the liver to the doses of the MC toxin. However, while the numerical values of gene expression were different from one trial to another, which led to the SE variations, the overall gene expression patterns were similar in all trials.

In the present study, the livers of chickens showed different pathological changes according to the dose of MC-LR. The most prominent changes were degeneration and focal necrosis of some hepatocytes, which were seen in the livers of chickens exposed to a low dose (1 ng/mL) of MC-LR. In contrast, liver tissues exposed to 100 ng/mL of MC-LR showed massive coagulative necrosis in most of hepatic cords, with nuclear pyknosis and lysis. Kral *et al*. (2012) found that histopathological liver damage was MC-LR-dose-dependent. The range of histopathological alterations in livers of birds administered 7.5 mL of cyanobacterial biomass suspension containing increasing MCs quantities of 2500,

5000, 10000, and 20000 μg/kg varied from mild vacuolar dystrophy to focal liver necrosis. The most severe damage was observed in birds treated with high-dose MCs. Adamovsky *et al*. (2013) reported that in rats fed a commercial diet containing 700 and 5000 μg of MCs per kg feed, most of the liver parenchyma showed focal or diffuse fatty degeneration, often associated with loss of the trabecular structure. Moreover, some liver samples exhibited necrotic hepatic lesions. The differences in pathological findings may be owing to the dose and timing of MC administration in the organism or organ.

In conclusion, this study highlighted the important risk of MC-LR in chicken liver tissue culture, even at very low doses. Environmentally relevant low doses of MC-LR that might be commonly present in drinking water could induce inflammatory effects in the liver cells of chicken after 6 h of exposure to MC-LR.

Acknowledgments
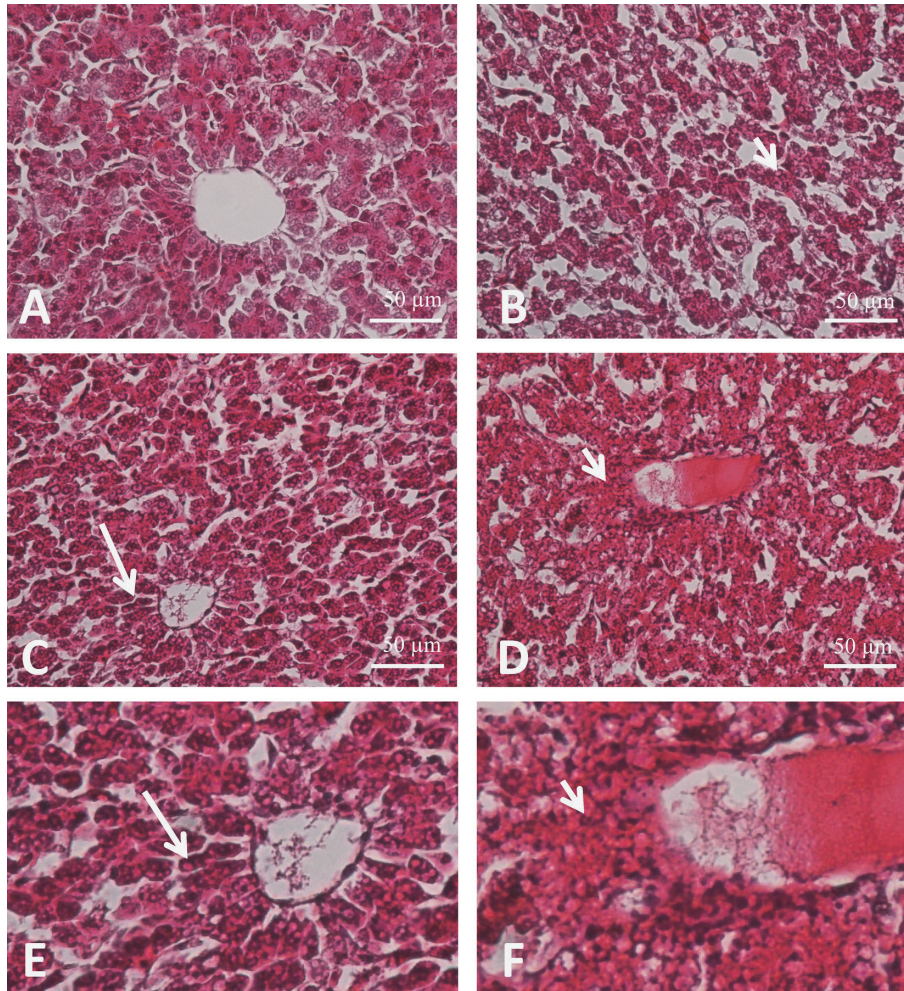
Fig. 3. **Effects of different doses of MC-LR on hepatic tissue.** (A) Liver of chicken exposed to 0 ng/mL of MC-LR showing normal hepatic architecture with abundant cytoplasm and normal nuclei. (B) Mild vacuolar changes (arrowhead) along with degeneration and focal necrosis of some hepatocytes were seen in the liver of a chicken exposed to a low dose (1 ng/mL) of MC-LR. (C) Necrotic changes of hepatocytes (arrow) with pyknosis of their nuclei were observed in liver tissue exposed to 10 ng/mL of MC-LR. (D) Liver tissue exposed to 100 ng/mL of MC-LR showed massive coagulative necrosis in most of hepatic cords (arrowhead) with pyknosis and lysis of their nuclei. (E) Liver tissue exposed to 10 ng/mL of MC-LR was magnified to show the necrotic changes of hepatocytes (arrow). (F) Liver tissue exposed to 100 ng/mL of MC-LR was magnified to show the massive coagulative necrosis (arrowhead).

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