Journal of the Saudi Society of Agricultural Sciences (2016) 15, 57–66

King Saud University

Journal of the Saudi Society of Agricultural Sciences

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FULL LENGTH ARTICLE

Carboxymethyl chitosan modulates the genotoxic risk and oxidative stress of perfluorooctanoic acid in Nile tilapia (*Oreochromis niloticus*)

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Received 11 October 2013; revised 17 April 2014; accepted 22 April 2014
Available online 9 May 2014

KEYWORDS

Perfluorooctanoic acid;
Chitosan;
Nile tilapia;
Fish;
Oxidative stress;
Genotoxicity

Abstract Perfluorooctanoic acid (PFOA) is one of the most commonly used perfluorinated compounds. Being a persistent environmental pollutant, it can accumulate in human tissues via various exposure routes. The aim of the current study was to evaluate the protective role of carboxymethyl chitosan (CMC) against PFOA-induced toxicity at the genetic and protein levels in Nile tilapia using the biochemistry analysis, SDS–PAGE electrophoresis, comet assay and RFLP–PCR methods. The results indicated that exposure to PFOA in water (30 mg/L) for 30 days resulted in a significant increase in ALT, AST, BUN, creatinine accompanied with a significant decrease in total protein and albumin. PFOA also increased DNA damage in electrophoresis condition and induced DNA and protein polymorphic band in comparison to control fish. CMC alone at 1% and 2% (W/W) in fish diets did not induce any alterations in the biochemical parameters, DNA or protein levels compared to the control group. Furthermore, CMC succeeded to decrease the toxicity of PFOA in a dose dependent manner. It could be concluded that PFOA induced genotoxicity and oxidative stress in fish similar to those reported in mammals. CMC is a

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Peer review under responsibility of King Saud University.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.jssas.2014.04.005

promising candidate and has a protective effect against-PFOA induced *in vivo* DNA damage and protein alteration in Nile tilapia. This effect might be attributable to its ability to decrease intracellular ROS and its antioxidant properties.

1. Introduction

Perfluorinated compounds (PFCs), such as perfluorooctanoic acid (PFOA), are remarkably stable compounds that do not undergo photolysis, hydrolysis, or biodegradation. Commercial use of PFCs for the past several decades has resulted in a broad distribution of stable precursors/metabolites in wildlife in terrestrial and aquatic environments (Yeung et al., 2006; Dai et al., 2006). Recent reviews of information about PFOA, many of which also cover other PFCs, have focused on endocrine disrupting properties (White et al., 2011); effects on the immune system (Dewitt et al., 2012); dietary exposures (D'Hollander et al., 2010; Domingo, 2012); production processes and formation from precursor compounds (Buck et al., 2011); importance in the aquatic environment (Ahrens, 2011) including drinking water sources (Murray et al., 2010); and on PFCs in biosolids applied to agricultural land (Clarke and Smith, 2011), municipal landfill leachates (Eggen et al., 2010), hazardous waste and biota (Houde et al., 2011). PFCs, including PFOA, were one of the first four groups of existing chemicals for which the USEPA Office of Pollution Prevention and Toxics recently developed Action Plans, an initial step in addressing their risks (USEPA, 2009). Although dietary intake seems to be the main source of exposure of the general population to PFOS and PFOA (Fromme et al., 2007), few studies report levels of PFOS in fish fillets. However, PFAS concentration limits in fish fillets ranging from non detectable to approximately 300 ng/g have been reported (Hoff et al., 2003).

Chitosan (COS), a cationic polysaccharide produced by the N-deacetylation of chitin under alkaline conditions, contains a linear sugar backbone of chitosan composed of β-1,4-linked glucosamine units. It exhibits a wide variety of biological activities, including antitumor activities (Suzuki et al., 1986), immunostimulating effects (Jeon and Kim, 2001), cholesterol-lowering effects (Schipper et al., 1999), antimicrobial effects (Park et al., 2004), wound healing effects (Porporatto et al., 2003), antifungal and free radical scavenging activities (Anraku et al., 2008; Park et al., 2004). Property of particular interest for this study is the antioxidant properties of chitosan (Chiang et al., 2000). Xie et al. (2001) reported that the scavenging of hydroxyl radicals by chitosan inhibits the lipid peroxidation of phosphatidylcholine and linoleate liposomes. Santhosh et al. (2006) reported that the administration of chitosan to rats prevented the oxidation of hepatotoxic lipids resulted from isoniazid or rifampicin. Similarly, chitosan inhibited glycerol-induced renal oxidative damage in rats (Yoon et al., 2008). Tomida et al. (2009) recently showed that the antioxidant properties of low MW COS are substantial, whereas high MW chitosans were much less effective in terms of antioxidant properties. The aim of the current study was to evaluate the effect of PFOA on the immune response of Nile tilapia using molecular and biochemical analyses and the possible protective effect of carboxymethyl Chitosan (CMC) against PFOA-induced toxicity.

2. Materials and methods

2.1. Chemicals, kits and reagents

Kits of Transaminase (ALT, AST), total protein (TP), albumin (Alb), Malondialdehyde (MDA), Total antioxidant capacity (TAC), Super Oxide Dismutase (SOD) and glutathione peroxidase (GPX) were obtained from Biodiagnostic (Giza, Egypt). Kits of creatinine (Cre) and blood urea nitrogen (BUN) were obtained from Quimica Clinica Aplicada (SA, Spain). Reagents for RFLP–PCR method were purchased from Invitrogen (USA). Perfluorooctanoic acid (PFOA) was purchased from Sigma-Aldrich (Munich, Germany) and Pharmaceutical grade chitosan (90% deacetylated) was obtained from the Naval Research Laboratory (Washington DC, USA). Proteinase K and RNase A were purchased from Vivantis Technologies (Malaysia). Phenol-chloroform-isoamyl alcohol was purchased from Applichem (Germany). Chloroform-isoamyl alcohol was obtained from Sigma Chemical Co. (St Louis, MO, USA). Taq polymerase was purchased from QIAGEN (Germany). *Dra*I, *Eco*RI and *Pst*I were purchased from Gibco BRL (Germany). All other reagents and chemicals were of the highest purity commercial available.

2.2. Preparation of carboxymethyl chitosan (CMC)

Two grams of low molecular chitosan (LMWC, average M_r 2.8 kDa) was alkalized in NaOH (8 g) for 12 h in a 50–50 mixture of deionized water and isopropanol (20 ml). After heating the mixture to 60 °C, monochloroacetic acid (8 g) was dissolved in isopropanol (2 ml) and slowly added to the solution over 30 min. After 6 h. the reaction was quenched by adding ethanol (50 ml) to the solution. The resulting CMC was repeatedly rinsed in ethanol and vacuum-dried until the pH of the filtered solution was neutral. The products were dissolved in water and centrifuged to separate the unreacted chitosan; the water soluble portion of the sample was removed, precipitated in ethanol and vacuum-dried. The sample was then placed in an oven at 50 °C to dry.

2.3. Fish

Nile tilapia fish (52.6 ± 5.7 g) were purchased from the El-Wafaa fish farm, Giza, Egypt and were transported in large plastic water containers supplied with battery aerators as a source of oxygen. Fish were maintained on *ad libitum* standard fish food at the Genetic of Hydrobiology Lab, National Research Centre (Dokki, Cairo, Egypt). After an acclimation period of 1 week, tilapia (n = 180) were divided separately into six experimental groups with three replicates (30 fish/group; 10/aquarium) and were placed into fish aquariums containing de-chlorinated tap water (26.7 ± 2.1 °C and pH 7.2–8.2). The

experimental protocol was approved by the National Research Center Review Committee for the use of Human or Animal Subjects.

2.4. Experimental design

Tilapia within different treatment groups were treated for 30 consecutive days as follows: group 1, untreated control; groups 2 and 3, fed fish diet supplemented with CMC at 1% (CMC1) and 2% (CMC2), respectively; group 4, fish exposed to PFOA (30 mg/L); groups 5 and 6, fish exposed to PFOA in water and fed diet supplemented with 1% and 2% of CMC, respectively. At the end of the experimental period i.e. day 31, all fish were starved and blood samples were collected from each fish for the determination of ALT, AST, TP, Alb, Cre, BUN, TAC, SOD, GPX and MDA according to the kits instructions. After the collection of blood samples, all fish were sacrificed and dissected. Liver and muscles samples from all tilapia were collected for further analysis.

2.5. Comet assay for DNA strand break determination

Isolated hepatic cells of all groups of Nile tilapia were subjected to the modified single-cell gel electrophoresis or comet assay (Fairbairn et al., 1995; Singh et al., 1988). To obtain the cells, a small piece of the liver was washed with an excess of ice-cold Hank's balanced salt solution (HBSS) and minced quickly into approximately 1 mm^3 pieces while immersed in HBSS, with a pair of stainless steel scissors. After several washings with cold phosphate-buffered saline (to remove red blood cells), the minced liver was dispersed into single cells using a pipette (Lal and Singh, 1995). In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a Zeiss fluorescence microscope axiostar plus (USA) with a green filter at 40× magnification. For each experimental condition, about 100 cells (about 25 cells per fish) were examined to determine the percentage of cells with DNA damage that appear like comets. The nonoverlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between 1 × and 2 × the nuclear diameter; and class 3 = tail longer than 2 × the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus (Collins et al., 1997; Kobayashi et al., 1995). A total damage score for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing up the values. Slides were analyzed by one observer to minimize the scoring variability.

2.6. SDS–PAGE electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE) was performed on skeletal muscles according to the method of Laemmli (1970) as modified by Studier (1973). Briefly, gradient gels, 8–16% polyacrylamide (8%; w/v) acrylamide, 375 mM Tris–Cl, pH 8.8, 0.1% (w/v) SDS, 0.0021% N,N,N′,N′-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate (APS); 16% (w/v) acrylamide 375 mM Tris–Cl, pH 8.8, 0.1% (w/v) SDS, 0.00043% TEMED, 0.05% APS and 8.5% glycerol), were made using the Ettan DALTsix gradient maker (GE Healthcare, Pittsburgh, PA, USA) and left to polymerize for 2 hours with water saturated 1-butanol laid overtop of each gel. A 5% acrylamide stacking gel was added to the top of each gel before use. After IEF, the strips were placed in SDS equilibration buffer [75 mM Tris, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS] for pH 7–11 NL strips or with 5% SDS (w/v) for pH 3–10 strips, with 1% (w/v) DTT and gently rocked for 15 min. The strips were then rinsed and placed in SDS equilibration buffer containing 2.5% (w/v) iodoacetamide and gently rocked for 15 min. The strips were then rinsed with SDS running buffer and placed on top of a polyacrylamide gel cassette and secured with 0.5% agarose solution with trace amounts of bromophenol blue. 1× SDS running buffer was placed in the bottom chamber of the DALTsix electrophoresis unit and 2× SDS running buffer was loaded into the top chamber. The protocol used for running the gels was: 1 W for 30 min, 2 W for 30 min and then 17 W × the number of gels in the DALTsix unit for 4–6 h. The protocol was terminated when the dye front moved past the bottom of the gel.

2.7. DNA extraction

Hepatic DNA was extracted using phenol-chloroform according to the method described by Sambrook et al. (1989), with some modification. Briefly, 0.2 g of liver sample was homogenized with 500 μL of extraction buffer (50 mM Tris–HCl, pH 8.0, 0.1 EDTA, 1% SDS, and 0.2 M NaCl) containing 50 μL of Proteinase K (10 mg/μl). The samples were incubated overnight at 55 °C with shaking. After incubation, the samples were treated with 30 μl of RNase A (10 mg/μl). The mixture was left at room temperature for 15–20 min, centrifuged at 13,000 rpm for 5 min then the supernatant was transferred to a clean tube. DNA was extracted twice with phenol-chloroform-isoamyl alcohol (PCI; 25:24:1), once with chloroform-isoamyl alcohol (CI; 24:1), and then precipitated twice with ethanol at −20 °C. The dried pellet was briefly re-suspended in 50–100 μl of Tris–EDTA, pH 8.0 and stored at −20 °C for further use. DNA concentration and purity were determined by measuring the absorbance of the diluted DNA solution at 260 nm and 280 nm. The quality of the DNA was determined using 0.8% agarose gel electrophoresis stained with ethidium bromide.

2.8. PCR–RFLP analysis

2.8.1. PCR primers and amplification

PCR amplification was carried out using primers of the MHC II α and MHC II β genes for tilapia. Primer design program (Primer 3, version 0.4.0) was used to build the primers for MHC genes: Forward: (MHC II α): 5′- TGA TGG AGA GGA TGT GCT GA-3′, Reverse: (MHC II α): 5′-CAT CTT CGG GTT TTG TCT GC-3′ (GenBank: AB677312.1); Forward: (MHC II β): 5′- CTG AGC TAA AGG ACA TCC AGT ACA-3′, Reverse: (MHC II β): 5′- CCT GCT CAG TAC ATC AGG ATC A-3′ (GenBank: AB677258.1). PCR

amplifications were performed using an Applied Biosystems Thermal Cycler (USA) with 35 cycles of the following steps: 94 °C for 40 s, 61 °C (MHC II α, product size: 170) or 60 °C (MHC II β, product size: 154) for 45 s and 72 °C for 1 min; preceded by initial denaturation at 95 °C for 5 min and followed by final extension for 7 min at 72 °C. PCR reactions contained a final volume of 25 µl consisting of 18.0 µl of autoclaved distilled water, 2.5 µl of 10 × PCR buffer (10 mM Tris–HCI, pH 9.1, 50 mM KCI, 0.01% Triton TM X-100, 1.5 mM $MgCl_2$), 0.08 mM dNTP mix, 0.1 µM of forward primer and corresponding reverse primer, 1.25 U of Taq polymerase and 2 µl of cDNA template. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

2.8.2. Enzyme digestion

Hepatic DNA samples (5–10 mg) were digested to completion overnight at 37 °C using 100 units of the following restriction enzymes: *Dra*I, *Eco*RI and *Pst*I. The DNA was electrophoresed on 2% TAE agarose gels.

2.9. Statistical analysis

All data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS, Version 9.1, Statsoft Inc., Tulsa, USA) followed by the Scheffé-test to assess significant differences between groups. The values were expressed as mean ± SEM. All statements of significant were based on probability of $P \leqslant 0.05$ compared to the control group.

3. Results

The results of the current study (Table 1) revealed that CMC at the two tested doses did not induce any significant effects on serum biochemical parameters. However, PFOA administration resulted in a significant increase in ALT, AST, creatinine and BUN accompanied with a significant decrease in TP and albumin.

The combined treatments of PFOA and CMC resulted in a significant improvement in all the tested biochemical parameters. This improvement was more pronounced in the group receiving a high dose of CMC and the obtained values were comparable to the control. The results also indicated that CMC exhibited antioxidant activity (Table 2). Animals treated with CMC at the two tested doses showed a significant increase in GPX, SOD and TAC in a dose dependent manner. Treatment with CMC also resulted in a significant decrease in the MDA level and a high dose of COS was more effective in the reduction of the MDA level.

The results of the comet assay for DNA strand break in nuclei from individual liver cells of *Oreochromis niloticus* fed PFOA-contaminated diet showed considerable DNA damage (Table 3). These results showed that DNA damaged cells were higher in fish exposed to PFOA compared to control fish. In addition, the DNA damaged cells categorized in class 3 were also higher in fish exposed to PFOA than all the other groups. However, the DNA damaged cells in fish treated with PFOA and CMC at the two tested doses showed a significant decrease in DNA damage compared to fish cells treated with PFOA alone. On the other hand, fish treated with CMC alone at the

Table 1 Effect of CMC on serum biochemical parameters in fish treated with PFOA.

Parameters Groups	ALT (IU/l)	AST (IU/l)	TP(g/dl)	Albumin (g/dl)	Creatinine (mg/dl)	BUN (mg/dl)
Control	28.21 ± 1.22^a	43.14 ± 2.73^a	6.33 ± 1.27^a	3.98 ± 0.25^a	0.91 ± 0.03^a	22.17 ± 1.19^a
CMC1	29.21 ± 1.41^a	33.21 ± 1.87^a	6.23 ± 2.01^a	3.78 ± 0.36^a	0.87 ± 0.25^a	20.43 ± 1.22^a
CMC2	30.63 ± 2.12^a	34.26 ± 2.22^a	6.63 ± 2.11^a	3.88 ± 1.04^a	0.87 ± 0.42^a	20.53 ± 1.63^a
PFOA	48.29 ± 3.21^b	54.93 ± 4.32^b	3.83 ± 1.82^b	1.32 ± 0.93^b	2.84 ± 0.33^b	38.74 ± 1.73^b
PFOA + CMC1	32.43 ± 1.85^c	42.74 ± 2.34^c	4.63 ± 1.04^c	2.85 ± 0.37^c	1.68 ± 0.84^c	29.84 ± 2.54^c
PFOA + CMC2	29.32 ± 1.83^a	33.94 ± 2.88^a	6.24 ± 1.97^a	3.59 ± 1.04^a	1.04 ± 0.05^a	24.73 ± 1.93^a

CMC1: Low dose of carboxymethyl Chitosan (1%).
CMC2: High dose of carboxymethyl Chitosan (2%).
PFOA: perfluorooctanoic acid.
Within each column, means superscript with different letters (a, b and c) are significantly different ($P \geqslant 0.05$) compared to the control group.

Table 2 Effect of CMC on oxidative stress markers in fish treated with PFOA.

Parameters Groups	MDA (µmol/ml)	GPX(U/ml)	SOD(U/ml)	TAC (mmol/ml)
Control	30.73 ± 2.73^a	3.62 ± 0.93^a	115.72 ± 3.86^a	99.82 ± 3.82^a
CMC1	28.72 ± 1.87^a	4.12 ± 1.12^a	117.92 ± 2.81^a	102.93 ± 3.21^a
CMC2	26.93 ± 1.56^b	4.98 ± 1.11^b	122.83 ± 2.22^b	107.32 ± 4.33^b
PFOA	47.93 ± 2.87^c	1.72 ± 0.21^c	88.83 ± 3.27^c	77.83 ± 2.11^c
PFOA + CMC1	38.94 ± 2.76^d	2.99 ± 1.13^d	102.33 ± 2.72^d	87.42 ± 3.55^d
PFOA + CMC2	33.82 ± 1.85^a	3.08 ± 0.22^a	109.32 ± 2.71^e	101.45 ± 3.83^a

CMC1: Low dose of carboxymethyl Chitosan (1%).
CMC2: High dose of carboxymethyl Chitosan (2%).
PFOA: perfluorooctanoic acid.
Within each column, means superscript with different letters (a, b and c) are significantly different ($P \geqslant 0.05$) compared to the control group.

Table 3 Visual score of DNA damage in Nile tilapia treated with PFOA and CMC.

Treatment	No. of cells analyzed	Class[Ω]				DNA damaged cells (% mean)
		0	1	2	3	
Control	100	92	5	3	0	8[c]
CMC1	100	91	5	3	1	9[c]
CMC2	100	92	4	2	2	8[c]
PFOA	100	74	7	8	11	26[a]
PFOA + CMC1	100	83	9	5	3	17[b]
PFOA + CMC2	100	89	6	3	2	11[bc]

CMC1: Low dose of carboxymethyl Chitosan (1%).
CMC2: High dose of carboxymethyl Chitosan (2%).
PFOA: perfluorooctanoic acid.
Within each column, means superscript with different letters (a, b and c) are significantly different ($P \geqslant 0.05$) compared to the control group.
[Ω] Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1× and 2× the diameter of nucleus; and 3 = tail length > 2× the diameter of nucleus.

two doses showed low DNA damage in liver cells which is similar to control fish.

To evaluate the genotoxic effect of PFOA and quantifying DNA damage in hepatic tissue, PCR–RFLP analysis was performed on DNA extracted from hepatic cells in different treatment groups. A representative example of the results obtained by RFLP analysis using MHC class II α and β primers is shown in Fig. 1 while data presented in Table (4) summarized each restriction enzyme used in the digestion analysis.

A total of 69 bands were observed (26 for *Dra*I, 21 for *Eco*RI and 22 for *Pst*I) in which 14 of these were polymorphic. Values of polymorphisms were 19.23% for *Dra*I, 19.04% for *Eco*RI and 22.72% for *Pst*I enzymes (Table 4). Most of these polymorphic bands were observed in PFOA-treaded fish (Fig. 1). However, fish treated with PFOA plus CMC1 or CMC2 showed more monomorphic bands than those treated with PFOA alone (Fig. 1). On the contrary, Nile tilapia treated with CMC1 or CMC2 showed monomorphic bands relatively similar to those in control (Fig. 1).

The electrophoretic protein pattern of Nile tilapia in different treatment groups is presented in Fig. 2 and Table 4. According to the relative front (mobility) of bands, a total of 37 bands were detected with mean of 19.6 ± 0.04. The banding patterns of the total protein fractions revealed a minor variation in different bands (22 monomorphic and 12 polymorphic bands). Moreover, the resulted protein system accounted for

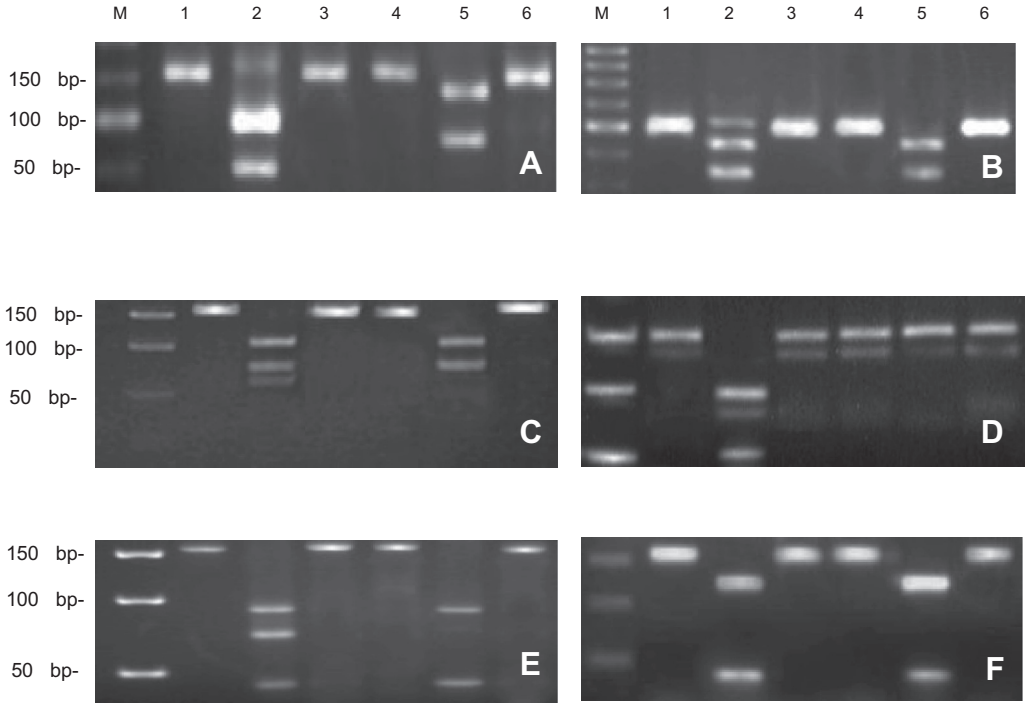

Figure 1 PCR–RFLP analysis of MHC genes class I I α (A, B and C) and class I I β (D, E and F), digested by *Dra*I (A and D), *Eco*RI (B and E) and *Pst*I (C and F) enzymes. *Lane* 1 represents control fish; *lane* 2 represents fish treated with PFOA; *lane* 3 represents fish treated with low dose of chitosan (CMC1); *lane* 4 represents fish treated with high dose of chitosan (CMC2); *lane* 5 represents fish treated with PFOA and low dose of chitosan and *lane* 6 represents fish treated with PFOA and high dose CMC.

Table 4 Detected polymorphism for each used marker system, protein and PCR–RFLP in Nile tilapia.

Marker no	Marker system		Polymorphism detected at each marker system					
	System	Marker	Monomorphic	Unique	Polymorphic	Total	Polymorphism(%)	Mean of band
1	Protein	Total protein	22	3	12	37	32.43	19.6 ± 0.04
2		*Dra*I	17	4	5	26	19.23	17.3 ± 0.06
3	PCR–RFLP	*Eco*RI	15	2	4	21	19.04	15.8 ± 0.05
4		*Pst*I	16	1	5	22	22.72	16.2 ± 0.04

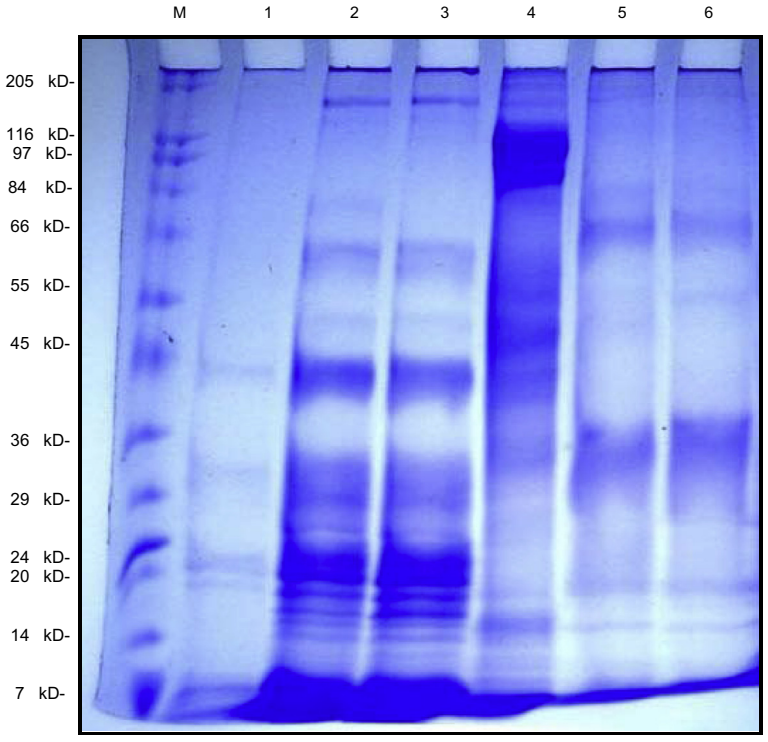

Figure 2 SDS-PAGE profile of soluble muscle proteins from Nile tilapia. *Lane* 1 represents control fish; *lane* 2 represents fish treated with low dose of chitosan (CMC1); *lane* 3 represents fish treated with high dose of chitosan (CMC2); *lane* 4 represents fish treated with PFOA; *lane* 5 represents fish treated with PFOA and low dose of chitosan; and *lane* 6 represents fish treated with PFOA and high dose of chitosan.

32.43% of polymorphism. The most polymorphism from protein electrophoretic pattern was found in tilapia treated with PFOA. Co-treatment with CMC at the two levels and PFOA decreased the polymorphism from protein patterns resulted from PFOA especially with the high level of CMC (Fig. 2). However, the protein electrophoretic pattern in fish treated with CMC alone at the two tested doses was relatively similar to the control.

4. Discussion

In the current study, we evaluated the protective role of CMC against PFOA-induced toxicity in *Nile tilapia* fish. The selected dose of PFOA was literature based (Hanson et al., 2005) however; the doses of CMC were selected based on the work of Abdel-Aziem et al. (2011). The liver was selected as a target tissue because PFOA is known to bioconcentrate and bioaccumulate in liver as demonstrated for rainbow trout (Martin et al., 2003a,b). The current results indicated that treatment with PFOA resulted in severe hepatic toxicity as indicated by the significant increase in serum ALT, AST, TP and albumin. The increase in transaminase may, in part, be due to the hepatic hypertrophic (inductive) effect of PFOA and/or may also represent borderline chronic liver toxicity (Boone et al., 2005). Moreover, the decrease in serum TP and albumin and the increase in BUN and creatinine indicated that PFOA induced protein catabolism or kidney dysfunction. In this concern, Hagenaars et al. (2008) reported that exposure of carp to PFOA resulted in liver damage however; Son et al. (2008) reported that PFOA induces hepatotoxicity but not nephrotoxicity.

The current results also indicated that PFOA treatment resulted in severe oxidative stress and ultimately led to oxidative damage as indicated by the significant increase in MDA and the significant decrease in antioxidant markers. Increased lipid peroxidation following PFOA exposure may be attributed to the induction of ROS, which enhance oxidation of polyunsaturated fatty acids and lead to lipid peroxidation (Liu

et al., 2007; Arukwe and Mortensen, 2011). It is well documented that as a result of this oxidative stress, fish, like many other vertebrates, try to reduce the damage using the antioxidant defense system (Liu et al., 2008). GSH is an important antioxidant, the content of which could reflect the antioxidant potential of the organelle, while the antioxidant enzyme GPX catalyzes the reduction of hydrogen peroxide at the expense of GSH. In the current study, PFOA induced a marked decrease in GPX and this depletion was accompanied by a significant reduction in SOD and TAC. SOD, the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion to H_2O_2 (Husain and Somani, 1998). Therefore, alteration of this parameter indicated the presence of a high level of ROS, suggesting that oxidative stress-related processes were involved in the PFOA-induced *in vivo* toxicity. In this concern, Hu and Hu (2009) and Eriksen et al. (2010) reported that exposure to PFOA resulted in the production of reactive oxygen species (ROS), dissipation of mitochondria membrane potential and apoptosis in Hep G2 *in vitro* cell system. Consequently, the observed significant reduction in SOD, GPX and TAC in PFOA group may be resulted from the high doses of PFOA as previously demonstrated (Prieto et al., 2007). In addition, the decrease in TAC may also be attributed to the excess of superoxide anion radicals resulting from the reduction in SOD activity. Indeed, previous studies indicated that high production of superoxide anion radicals inhibits TAC (Moreno et al., 2005). GPX, which functions as scavenger of hydrogen peroxide, presented a reverse trend following PFOA treatment. This distinct reaction reflected the selective effect of the two enzymes in PFOA-induced oxidative stress. Alternatively, GPX may be more potent in the liver (Kaushik and Kaur, 2003).

The current results demonstrated that PFOA induced toxicity at the genetic (DNA damage) and protein (polymorphic bands) levels in Nile tilapia hepatic and muscle tissues, respectively. Similar to these observations, Yao and Zhong (2005) reported that PFOA induced remarkable DNA strand breaks in HepG2 cells. In addition, exposure of PFOA to carp altered expression of genes mainly involved in energy metabolism and stress response in the liver (Hagenaars et al., 2008). Moreover, Shi et al. (2008) reported that exposure to PFOA resulted in cellular apoptosis and alteration of certain gene expressions related to cellular apoptosis e.g., p53 and Bax in zebrafish embryos/larvae and significantly induced the protein expression of peroxiredoxin 2, which is thought to be involved in cellular defenses against oxidative stress (Shi et al., 2009). These results suggested that oxidative damage may play an important role in the toxicity by PFOA. Previous reports suggested several mechanisms for PFOA-induced toxicity and indicated that PFOA increased intracellular reactive oxygen species (ROS), 8-hydroxydeoxyguanosine (8-OH-dG), induced DNA strand breaks and micronuclei in HepG2 cells and induction of 8-OH- dG in rat liver (Takagi et al., 1991; Yao and Zhong, 2005). Recently, Kleszczyński and Skadanowski (2011) suggested another mechanism for PFOA toxicity. These authors suggested that PFOA affected calcium homeostasis, altered plasma membrane potential, kinase phosphorylation and led to acidification. Subsequently, it changed the mitochondrial function by various but close events: protein Bad translocation, free radical generation, dissipation of mitochondrial transmembrane potential, overload with calcium ions and uncoupling of oxidative phosphorylation. These events enhance cytochrome c release, downstream activation of caspases and apoptotic DNA fragmentation (Kleszczyński and Skadanowski, 2009a,b; Kleszczyński et al., 2009).

CMC is the water-soluble form of COS and has many unique chemical, physical and biological properties such as low toxicity, biocompatibility and good ability to form films, fibers and hydrogels (Sun et al., 2008). Consequently, it has been extensively used in many biomedical fields such as a moisture-retention agent, a bactericide, in wound dressings, as artificial bone and skin, in blood anticoagulants and as a component in the drug delivery matrices (Liu et al., 2007).

Several molecular weight (MW) COS were tested as a dietary supplement (Kaats et al., 2006). High molecular weight (HMW) COS would be expected to inhibit the absorption of certain lipids and bile acids. However, low molecular weight (LMW) COS would be predicted to absorb such substances, but would also be expected to show increased antioxidant effects. Anraku et al. (2009) showed that the administration of low MW COS to human volunteers strongly inhibited the oxidation of human serum albumin and enhanced the fish immune response (Geng et al., 2011) *in vivo*. The antioxidant properties of low MW COS are substantial, whereas HMW COS was found to be much less effective in terms of antioxidant properties (Tomida et al., 2009).

According to Chiang et al. (2000), low MW COS can be absorbed from the intestinal tract and subsequently shows a number of additional bioactivities such as antitumor, cholesterol-lowering, immunostimulating, antidiabetic, antimicrobial and antioxidant effects in both the systemic circulation and the intestinal tract. In the current study, CMC was found to improve liver function, prevent DNA and protein damage and protect cells from the effects of ROS. Moreover, CMC did not only prevent oxidative injury in liver cells, but also potently interfere with apoptosis and genotoxicity due to attenuated exogenous oxidative stress. Similar, results were reported by Koo et al. (2002) who indicated that COS was able to protect against apoptosis in human astrocytoma cells induced by serum starvation. Moreover, Liu et al. (2010) stated that COS not only reversed the decrease of cell viability and proliferation activity, but ameliorated nuclear chromatin damage in H_2O_2-induced HUVECs.

In the present study, treatment with CMC alone at the two tested doses did not induce any deleterious effects. Conversely, it induced a significant improvement in the antioxidant status of the fish. This improvement was more pronounced in the group receiving a high dose of CMC. Moreover, treatment with CMC resulted in a significant reduction in all tested parameters which were increased as a result of free radicals generation produced by PFOA including serum biochemical parameters, DNA damage in liver tissues, and polymorphism of protein patterns in muscle tissues. Previous report indicated that COS enhanced the resistance to the effects of oxidative stress and increased the plasma total antioxidant radical trapping capacity (Wayner et al., 1987). Moreover, Anraku et al. (2011) reported that COS reduces the levels of pro-oxidants such as cholesterol and uremic toxins in the gastrointestinal tract. Thus, CMC has the potential ability to act as a protein antioxidant, since oxidative stress is an important pathogenic factor in PFOA toxicity. The reducing power properties of CMC are generally associated with the presence of reductions, which have been shown to exert antioxidant action by breaking the free radicals' chain by donating a hydrogen atom (Duh

et al., 1999) and/or radical scavenging mechanisms of substituting carboxymethyl group (Sun et al., 2008). Similar to the current observations, Anraku et al. (2012) concluded that the ingestion of COS resulted in a significant reduction in the levels of pro-oxidants, such as uremic toxins, in the gastrointestinal tract, thereby inhibiting the subsequent development of oxidative stress in the systemic circulation.

A more plausible mechanism of the antioxidant action of COS *in vivo* is its ability to scavenge secondary peroxyl radicals (Anraku et al., 2008). Peroxyl radicals are the products of fast reactions between dioxygen and C-centered radicals generated by the primary ROS in cell components such as proteins, lipids and DNA, and are believed to be major intermediates in the propagation of biological damage induced by the primary ROS (Willson, 1985).

5. Conclusions

The current study indicated that PFOA induced a severe oxidative stress in Nile tilapia fish including disturbances in serum biochemical parameters for liver and kidney function, oxidative stress marker and antioxidant enzymes resulted in increase in DNA damage and protein polymorphic bands similar to those reported in mammals. CMC at the two tested doses did not induce any toxic effects and enhanced the antioxidant capacity in a dose related manner. CMC also showed a potential protective effect against PFOA toxicity through its ROS scavenging properties, decreasing lipid peroxidation and increasing the antioxidant capacity of the fish. Based on these results, it may be concluded that the CMC is a nontoxic material that could be used as a suppressor of PFOA-induced genetic and protein alterations in Nile tilapia.

Acknowledgment

This work was fully supported financially by the National Research Center, Dokki, Cairo, Egypt. Project #S90402.

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