Molecular cloning, immunohistochemical localization, characterization and expression analysis of caspase-9 from the purse red common carp (Cyprinus carpio) exposed to cadmium

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**A B S T R A C T**

Caspase-9, the essential initiator caspase is believed to play a central role in mitochondria-mediated apoptosis signaling. In this study, we isolated the caspase-9 gene from common carp, one of the most important industrial aquatic animals in China using rapid amplification of cDNA ends (RACE). The deduced amino acid sequence of caspase-9, composed of 436 amino acids, showed approximately 47.6% identity and 64.7% similarity to human caspase-9. It also possessed a conserved caspase-associated recruitment domain (CARD), a large subunit and a small subunit. Phylogenetic analysis clearly demonstrated that caspase-9 formed a clade with cyprinid fish caspase-9. Real-time quantitative PCR analysis revealed that caspase-9 transcripts were not significantly increased in kidney after exposure to cadmium (Cd). Whereas caspase-9 cleaved fragments were detected using Western blot analysis with the same Cd treatment condition. Furthermore, the result of immunohistochemical detection showed immunoreactivities were predominantly limited to the cytoplasm of renal tubular epithelial cells and no remarkable changes of immunopositive staining were observed after Cd treatment. Accordingly, the results signify that caspase-9 may play an essential role in Cd induced apoptosis.

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1. Introduction

Cadmium (Cd), which is frequently used in electroplating, pigments, paints, welding, and Ni–Cd batteries, is one of the major occupational and environmental toxicants in terrestrial and aquatic environments (Wang et al., 2012c). It enters the aquatic environment via natural sources such as river run-off from Cd-rich soils and anthropogenic sources like urban run-off and industrial waste dumping (Cao et al., 2012). Mounting evidence shows that Cd is a causal factor in a spectrum of biochemical and physiological dysfunctions in animal organisms. For example, acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage, anemia and osteotoxicity (Santos et al., 2004). In fish such as in common carp (Cyprinus carpio), Cd can reduce hematopoietic potential (Kondera and Witeska, 2013), and is able to induce fatty liver in Synechogobius hasta (Liu et al., 2011). At the cellular level, Cd can mediate a wide variety of cytotoxic and metabolic effects such as affecting cell proliferation and differentiation, cell cycle progression, DNA synthesis and repair, apoptosis and other cellular activities (Lee et al., 2006; Templeton and Liu, 2010).

Apoptosis is critically important for the survival of multicellular organisms as it destroys damaged or infected cells that may interfere with normal function. It is frequently observed in Cd-exposed cells and is anticipated to protect against Cd toxicity. Increasing reports have shown that Cd could induce apoptosis in various fish tissues and cells both in vivo and in vitro. For example, Cd can induce apoptosis in rainbow trout (Oncorhynchus mykiss) gills and hepatoma cell lines (Krumbschabel et al., 2010); in zebrafish gills, liver, muscles and brain (Gonzalez et al., 2006); in Atlantic salmon (Salmo salar) intestine and kidney (Berntssen et al., 2001). The central molecules of signaling pathway leading to apoptosis are the caspases. Members of the caspase family can be subdivided into initiator and effector caspases depending on their placement within the cascade of apoptosis signal transduction. Initiator caspases comprise caspase-2, -8, -9 and -10, which are capable of activating downstream caspases (executioners) after cleavage either directly through proteolysis or indirectly via a secondary messenger mechanism (Ho and Hawkins, 2005; Vidal et al., 2008; Wuerstle et al., 2012).
Among the initiator caspases, caspase-9 is required in most scenarios of apoptotic cell death, and consequently impaired caspase-9 activation has profound consequences. For example, the majority of caspase-9 knockout mice die perinatally with a markedly enlarged and malformed cerebrum caused by reduced apoptosis during brain development (Kuida et al., 1998). In cancer development and tumor progression, the expression of caspase-9 is often significantly associated with poor survival and may be an independent prognosticator in colon carcinoma (Strater et al., 2010). In freshwater crab, the activity of caspase-9 is increased in a concentration-dependent manner after Cd exposure (Wang et al., 2011). Inhibiting caspase-9 activity has the potential to block Cd-induced DNA fragmentation (Wang et al., 2012b).

Although caspase-9 may play a pivotal role in apoptosis beyond species, available information is mostly limited to its functions in respect to mammals (human, mouse and rat). In fish, caspases-9 was only characterized in very limited species such as sea bass (Dicentrarchus labrax) (Reis et al., 2007) and large yellow croaker (Pseudosciaena crocea) (Mu et al., 2010). While its roles in other species of fish remain largely unknown. Therefore, in the present study, we report the isolation of caspase-9 cDNA clones from the kidney of common carp using rapid amplification of cDNA ends (RACE) according to the cDNA sequences of caspase-9 from other fish species. Furthermore, changes in the mRNA transcripts and protein expression after Cd treatment were examined. In addition, the distribution changes of caspase-9 in the kidney tissues were also analyzed by immunohistochemical staining.

### 2. Materials and methods

#### 2.1. Cloning of caspase-9 from common carp

Total RNA was extracted using TRizol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. The cDNA sequence was amplified using a SMART PCR cDNA Synthesis Kit (Clontech, USA) according to the supplied protocol. Based on the conserved region of caspase-9 sequences in zebrafish (GenBank accession no. NM_001007404.2), white cloud mountain minnow (Tanichthys albonubes, GenBank no. EUB78545.1) and rainbow trout (GenBank accession no. NM_001124647.1), degenerate primers (Csp9F, Csp9R) were designed to obtain a partial sequence of caspase-9 with the following PCR cycling conditions: 94°C for 5 min; 30 cycles at 94°C for 30 s, 58°C for 60 s, and 72°C for 60 s; followed by 1 cycle at 72°C for 10 min. To obtain the full cDNA sequence, RACE PCR was performed using the gene-specific primers (Csp9-5-1, Csp9-5-2, Csp9-5-3, Csp9-3-1 and Csp9-3-2) and adaptor primers (UPM) with the following PCR program: 1 cycle at 94°C for 5 min; 10 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s; 25 cycles at 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s; followed by 1 cycle at 72°C for 10 min. All the purified fragments were then cloned and sequenced. The primers used in this study are listed in Table 1.

#### 2.2. Sequence and phylogenetic analysis

Protein prediction was performed using the software at the ExPaSy Molecular Biology Server (http://expasy.org). The putative coding sequences (CDSs) were analyzed for the presence of signal peptides, using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). A multiple alignment was generated using the Clustal W 1.83 program. The protein sequences of some vertebrate caspase-9 were retrieved from the GenBank database for use in the alignment. Homology analysis, including identities and similarities between deduced amino acid sequences of common carp caspase-9 and other known sequences was performed using MatGat 2.0 (Campanella et al., 2003; Wang et al., 2012a). Lastly, a phylogenetic tree was constructed using the neighbor joining method within the MEGA software package (version 5.2) and bootstrapped 1000 times. The percentage of the bootstrap values was recorded.

#### 2.3. Waterborne Cd exposure experiment

Twenty-four healthy purse red common carp specimens weighing 300 ± 50 g were obtained from a fish hatchery at the Institute of Purse Red Common Carp, Wuyuan County, China. Fish were randomly placed into 6 tanks (4 fish per group) and were kept for 7 days at 12 ± 4°C before experiments. Carp were exposed to 2.5 μM Cd²⁺ (groups A and B), 10 μM Cd²⁺ (groups C and D) and 0μM Cd²⁺ (groups E and F, as controls) by addition of Cd from a 10 μM Cd stock solution prepared in distilled water [3(CdSO₄)·8H₂O, Sigma–Aldrich, USA]. The actual concentrations of Cd in the water were determined using AA-800 type graphite furnace atomic absorption spectrophotometer (Perkin-Elmer Corp.,

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Application</th>
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<td>Upm (short)</td>
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USA). The protocol was described as follows: 100 ml water samples from different concentration Cd-treated groups were digested with nitric acid (HNO₃) and heated on a hot plate until 1 ml samples remained. Then the flask sides were washed down with deionized water and the volume of every sample was bulked to 100 ml. A 2% HNO₃ solution (v/v) was prepared by diluting 20 ml of concentrated HNO₃ (trace metal grade) in 1 L of deionized water. Then it was treated in the same way of water samples, and was used for control. A 1 mg/ml Cd standard solution was prepared by dissolving 0.5 mg of Cd with an adequate amount of concentrated HNO₃ and diluting in 500 ml of deionized water. Then a 2 μg/ml Cd working solution was prepared by dilution of the 1 mg/ml Cd standard solution in 2% HNO₃. Finally, the assay was conducted according to the following analytical conditions: wave length, 228.4 nm; spectral band width, 1.3 nm; lamp current, 7.5 mA. Calibration curve was delineated from the diluted Cd working solution. All values obtained were the means with three replicates. One-third of water in the tank was renewed every 24 h by adding fresh water containing the same concentrations as above.

After the 96 h-experiment, the fish groups A, C and E were anesthetized by MS-222 (Sandoz, Switzerland) and sacrificed. The liver, kidney and gill tissues were sampled. The samples were then divided into three parts, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C for further processing. Groups B, D and F were treated as Groups A, C and E after 168 h Cd exposure.

2.4. Ranking the stabilities of candidate reference genes and alteration of caspase-9 mRNA levels from kidney by real-time quantitative PCR

Total RNA from kidney was extracted using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer’s instructions. RNA samples were treated with RNase-free DNase (Fermentas, Lithuania) and 2 μg of the treated RNA was then reverse-transcribed with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania). The common carp caspase-9 and 5 candidate housekeeping genes (β-actin (GenBank accession no. M24113.1), GAPDH (glyceraldehyde 3-phosphate dehydrogenase, GenBank accession no. AJ870982.1), EF1α (elongation factor 1-α, GenBank accession no. AF485331.1) 18 s (18 s ribosomal RNA, GenBank accession no. FJ710826.1), ARNT2 (aryl-hydrocarbon receptor nuclear translocator 2, GenBank accession no. KF055464) cDNA fragments were generated by RT-PCR. Amplicons were gel purified, and serial 10-fold dilution used as a standard curve in each PCR. Real-time quantitative PCR was conducted on a CFX96 Touch™ real-time PCR Detection System (Bio-Rad, USA). Amplifications were carried out at a final volume of 20 μl containing 5 μl of 50-fold diluted cDNA template, 10 μl SYBR Green real-time PCR Master Mix (Takara, Japan), 0.5 μl of each primer (Table 1), and 4 μl ddH₂O, following the manufacturer’s instructions. PCR amplification was performed in triplicate using the following conditions: 3 min at 94 °C, followed by 45 cycles of 10 s at 94 °C, 15 s at 55 °C and 20 s at 72 °C. The reaction which was carried out without the cDNA sample was used as a negative control. ReFinder program (website: http://www.leonxie.com/referencegene.php), which integrates geNorm, Normfinder, BestKeeper, and the comparative ΔCT method, was used to screen the most optimal reference gene by calculating the candidate housekeeping genes ranking. The final ranking was evaluated by assigning a suitable weight to an individual gene, and the geometric mean of their weights for the overall final ranking was calculated. A lower gene Geomean of ranking value represents a higher expression stability. Eventually, the most stable candidate housekeeping gene was selected as the reference gene of caspase-9 in this study. Expression values and fold change based on the experimental groups relative to the control groups were calculated using 2−ΔΔCT method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) by DataAssist 3.0 software (Applied Biosystem, USA). The Ig 2−ΔCT values were computed for further statistical analysis.

2.5. Production of fusion proteins and polyclonal antibodies

The expression primers were designed to include an EcoRI site within the sense primer and an XHOI site within the antisense primer and used to amplify all the 436 amino acids. The primers are listed in Table 1. PCR amplifications were performed using 1 cycle at 94 °C for 5 min; 10 cycles at 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 45 s; 25 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 50 s; followed by 1 cycle at 72 °C for 10 min. The purified fragment was digested with EcoRI and XHOI, ligated to the PGEX-4T-1 expression vector (Pharosma Biotech., USA) for constructing recombinant proteins, and transformed into DH5α-competent cells. After sequencing the positive clones to ensure in-frame insertion, the PGEX-CSP9 construct was transformed into E. coli BL21 (DE3) strain for protein expression. The fusion proteins were expressed by isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and analyzed on a 12% SDS-polyacrylamide gel (SDS-PAGE). To prepare the polyclonal antibodies, IPTG was added in a final concentration of 4 mM when the culture reached OD600 = 0.6. After 4 h of culture at 37 °C, the cells were harvested by centrifugation and disrupted by sonication. The recombinant fusion proteins were purified by affinity chromatography using a glutathione-Sepharose matrix (GE Healthcare, USA). Recombinant PGEX-CSP9 fusion proteins were eluted from the resin containing 2 M urea, 50 mM KH₂PO₄ (pH 7.9), 1 mM EDTA and 50 mM NaCl.

In addition, to further estimate the recombinant proteins, In-gel digestion and protein identification was carried out on purified recombinant proteins using matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) analysis. Fusion proteins band, which was separated by SDS-PAGE, was excised from the gel after Coomassie blue staining. The gel slices were transferred to a prewashed 0.2 ml microcentrifuge tubes, then destained by adding 100 mM NH₄HCO₃, dehydrated with acetonitrile, and dried. Digestion of proteins was carried out in 50 mM NH₄HCO₃ containing 4 ng of trypsin (Promega Corp. USA) and incubated overnight at 37 °C. Proteins were identified on an ABI 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). Data obtained from tandem spectrometry which consisted of breaking into small pieces of fragments were used to search candidate proteins using Mascot (http://www.matrixscience.com) software. Parameters for all searches were as follows: MS/MS ion search as type of search, C. carpio as taxonomic category, trypsin as enzyme, carbamidomethyl cysteine residues as fixed modification, oxidation of methionine residues as variable modifications, one missing cleavage and 50 ppm as mass tolerance for the monoisotopic peptide masses. The National Center for Biotechnology Information and Swiss-Prot databases were searched on separate occasions. Results with protein scores greater than 46 are significant (P < 0.05).

To generate polyclonal antibodies, we used 4 mg recombinant proteins to immunize rabbits. The obtained polyclonal antibodies were purified by affinity chromatographic column matrix coupled with antigen. The specificity of the polyclonal antibodies was evaluated by Western blot analysis and immunohistochemical detection.

2.6. Western blot analysis

For identifying the protein expression of caspase-9 after exposure to Cd, 100 mg tissue samples from the kidney of purse red common carp were washed with phosphate-buffered saline (PBS) and then homogenized with ice-cold extraction buffer [MEB,
100 mM sodium β-glycerophosphate, 15 mM MgCl₂, 20 mM EGTA, 1 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM HEPES, 2.5 μg/ml aprotinin, and 2.5 μg/ml leupeptin; pH 7.5]. The homogenate was centrifuged at 120,000 × g for 30 min, and then the supernatant was retained. Protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology, China). Each sample, equivalent to 30 μg of total protein, was run on a 12% SDS-PAGE gel electrophoresis and subsequently transferred to a 0.45 μm PVDF membrane (Millipore, USA). Following transfer, the blotting membrane was blocked with 5% dry milk for 1 h and then incubated overnight at 4 °C in blocking with primary antibody, which was diluted 500-fold. Mouse anti-αglycoprotein monoclonal antibody (Proteintech, China) diluted 1:10,000 was used as loading control. After washing 3 times for 10 min, the membrane was further incubated for 2 h with 1:2000 diluted goat anti-rabbit IgG or goat anti-mouse IgG conjugated with peroxidase (Beyotime Biotechnology, China). After washing 3 times for 10 min in TBST (Tris Buffered Saline with Tween 20) buffer, detection was performed using the Pierce Plus enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific, USA) and X-ray film. Densitometric analysis of protein bands was performed using Gel-Pro Analyzer (Media Cybernetics, USA).

### 2.7. Immunohistochemical identification of caspase-9

Paraffin-embedded, 4 μm-thick sections of kidney samples from all groups were obtained by the standard techniques. Sections were mounted on aminopropyltriethoxysilane-treated slides. The slides were routinely deparaffinized with xylene and rehydrated with a series of ethanol washes. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in absolute methanol. After washing with PBS, the sections were subjected to antigen retrieval in boiling sodium citrate buffer (0.01 M, pH 6.0) for 10 min in a microwave oven set at 95–100 °C. After cooling for 20 min and washing 3 times with 0.1 M Tris–HCl, 5% BSA was added, and the sections were incubated for 20 min. The polyclonal antibodies were diluted 1:100 and added to slides. The slides were then maintained at 4 °C overnight, following which they were again washed 3 times. The slides were further incubated with a Polymeric-HRP-Anti-Ms/Rb IgG (Maxim, China) and washed 3 times. At room temperature, 3,3-diaminobenzidine (DAB) was added to the tissue sections, followed by incubation for 10 min without direct light. The slides were washed 3 times with reagent quality water and then counterstained with Mayer’s hematoxilin (Boster, China) for 10 min and washed under running water. Gradient ethanol was used for dehydration, and dimethylbenzene, for clearing the sections, which were then mounted in neutral resin. The slides were observed under an Olympus BX51 light microscope (Olympus Corp, Japan).

### 2.8. Statistical methods

The statistical analyses were performed using SPSS software, Version 11.5. The mRNA transcripts and protein expression were analyzed by a factorial ANOVA model, represented by 2 independent variables (time, dose) and their interaction. An F-test was done, followed by a post hoc Dunnett T3 Test. Because there were only 2 time periods, post hoc test was not required. Statistically significant difference was accepted at P < 0.05. All results are expressed as mean ± S.D. (standard deviation).

### 3. Results

#### 3.1. Molecular characterization and homology analysis of common carp caspase-9

The common carp caspase-9 mRNA is 1626 nt in length (GenBank accession no. KC676314), containing a 1311-nt CDS. The untranslated region (UTR) and 3′ UTR are 219 nt and 97 nt, respectively. The putative caspase-9 encoded a peptide of 436 amino acids, with a calculated molecular weight of 48.46 kDa and an isoelectric point of 5.9. It was predicted as a non-secretory protein without a signal peptide. The putative caspase-9 presented a vestebrate caspase-9 signature as determined by the InterProScan in the database (http://www.ebi.ac.uk/Tools/pfa/interproscan/). It exhibited a typical caspase 9 domain architecture containing a putative N-terminal CARD (residues 1–90), a caspase family p20 domain (large subunit, residues 171–303) and p10 domain (small subunit, residues 343–433) (Fig. 1). Furthermore, it suggests that Asp328 and Asp342, as the two putative cleavage aspartic acids of sea bass caspase-9 gene separate the large and small subunits (Mu et al., 2010; Reis et al., 2007). Caspase family signature histidine active-site H237 SADDCVIIHSG251, cysteine active-site K291 PKLFFIQAGG292 and caspase-9 characteristic pentapeptide active-site QACGG were located in the p20 domain (Fig. 1). The deduced amino acid sequence of common carp caspase-9 had the highest amino acid identity and similarity to that of zebrafish followed by other fish, then mammals, amphibians and crustacean, varying from among 45.8–80.5% and 62.6–89.4%, respectively (Table 2). Phylogenetic tree analysis, based on amino acid sequences alignment, revealed that common carp caspase-9 formed a cluster with zebrafish orthologous protein. Meanwhile, it was considerably nearer to that of the teleost fish than to those of other eukaryotes (Fig. 2).

#### 3.2. Determination of actual Cd concentrations in water

The measured actual concentrations of Cd in water samples from different groups were as follows: groups A and B (exposed to 2.5 μM) were 278.6435 ± 4.7982 μg/L (2.4790 ± 0.0427 μM); groups C and D (exposed to 10 μM) were 1120.1526 ± 12.6318 μg/L (9.9657 ± 0.1124 μM); groups E and F (exposed to 0 μM) were 8.6002 ± 1.3590) × 10⁻6 μM. The verified result showed that actual Cd concentrations in water were typically similar to nominal ones. For convenience, we substitute nominal concentrations (0 μM, 2.5 μM and 10 μM) for actual concentrations in the following presentations.
3.3. Identification of the optimal reference genes and effect of Cd treatment on caspase-9 mRNA expression

The stabilities of the 5 candidate reference genes were ranked within kidney samples from pure red common carp treated with Cd (Fig. 3A). The order of gene expression stability (from the most stable to the least stable) was ARNT2, 18s, EF1α, β-actin and GAPDH. Therefore, ARNT2 was used as the reference gene to normalize mRNA expression for caspase-9.

Cd exposure induced an increase in mRNA levels of caspase-9 compared with control groups (Fig. 3B). Group B (exposed to 10 μM Cd²⁺ for 96 h) have a maximum mRNA expression, which
was about 1.37-fold of the control group. However, according to the factorial ANOVA analysis, the mRNA expression of caspase-9 showed no significant effect as a function of time period between the groups exposed for 96 h and the groups exposed for 168 h ($F = 3.398, P > 0.05$). Meanwhile, the transcripts of caspase-9 did not differ significantly among fish groups exposed to different Cd concentrations ($F = 1.756, P > 0.05$). The interaction between the two factors (time and dose) was also not significant ($F = 3.161, P > 0.05$).

### 3.4. Prokaryotic expression, purification, MALDI-TOF/TOF MS analysis and antibody preparation

To generate recombinant proteins for producing polyclonal antibodies, the expression plasmid PGEX-CSP9 was transformed into the *E. coli* BL21 (DE3) strain and the PGEX-CSP9 expressed as a recombinant containing a C-terminal GST tag. After IPTG induction, the PGEX-CSP9 fusion proteins were produced and mainly expressed in precipitate (Fig. 4A).

MALDI-TOF/TOF MS analysis was used to further validate the recombinant proteins. Based on the mass spectrometry map (Fig. 4B) of the fusion proteins, the Mascot online software was used for retrieval. The result showed 6 mass peaks with value of 1196.544, 1423.635, 1495.603, 1653.704, 1953.890 and 2557.207 were exactly matched with the peptide sequences of “G33VFTQDMIDEIR384”, “G64SQAFFALECLR76”, “E77TGHALAELEQCGDVR94”, “A345LTPTPSDLVSYTFPGYVSWR170”, “D371TQAGSYWVENLDR384” and “Q417MIGSFPNLR436”, respectively, which were partial sequences of the common carp caspase-9 and had a highly convincing Mascot score of 103 ($P < 0.05$). Through in-gel digestion and identification using MALDI-TOF/TOF MS, we reached the conclusion that the expressed and purified fusion protein was structurally correct.

### 3.5. Cd activates caspase-9

To determine whether caspase-9 activation is involved in Cd-induced apoptosis of the kidney tissue, time and concentration course experiments were performed by analyzing the activation status of caspase-9 using Western blot analysis. Changes in caspase-mediated cleavage were used as a marker for apoptosis. Without Cd treatment, the levels of procaspase-9 did not differ between groups E and F (exposed for 96 h or 168 h) and no cleaved fragment was detected (Fig. 5A). After Cd treatment, an approximately 40 kDa caspase-9 cleaved fragment was detected. The protein expression of procaspase-9 was not affected by exposure time ($F = 2.845, P > 0.05$) and concentrations ($F = 0.147, P > 0.05$) (Fig. 5B), which is similar to that of the mRNA expression. In contrast, significant differences were found among groups exposed to different Cd concentrations for the expression of cleaved caspase-9 ($F = 7.315, P < 0.05$). The following Dunnett T3 Test indicated that the protein expression of cleaved caspase-9 in different conditions with
Fig. 4. Dissolvability analysis (A) and MALDI-TOF/TOF MS analysis of fusion proteins (B). (A) Common carp caspase-9 clones expressed in E. coli. Lane M, protein molecular standard; lane 1, supernate of unpurified recombinant PGEX-CSP9; lane 2, supernate of purified recombinant PGEX-CSP9; lane 3, precipitate of unpurified recombinant PGEX-CSP9; lane 4, precipitate of purified recombinant PGEX-CSP9. (B) MALDI-TOF/TOF peptide map of the tryptically digested recombinant PGEX-CSP9. The vertical axis represents the abundance of different peptides, and the horizontal axis shows the ratio between m (molecular weight) and z (charges) of distinct peptides.

Fig. 5. Protein expression analysis performed by western blotting after Cd treatment. (A) Western blots showing pro-caspase-9 and cleaved caspase-9 (arrows) after Cd treatment. GAPDH was used as a loading control. (B) Statistical significance (*P < 0.05) was analyzed using a factorial ANOVA followed by Dunnett T3 Test post hoc. Protein band density was analyzed with the Gel-Pro Analyzer. Each value is expressed as the ratio of pro-caspase-9 and caspase-9 activation levels to GAPDH level, which represents the mean ± S.D. of 4 independent samples performed in triplicate.
Cd treatment followed the hierarchical pattern: groups exposed to 2.5 μM Cd^{2+} > control groups (P<0.05) (Fig. 5B). The levels of cleaved caspase-9 did not differ between groups exposed for 96 h and 168 h (F = 1.722, P > 0.05). In addition, the interaction between the two factors (time and dose) was not significant (F = 0.688; P > 0.05). Overall, these results indicate that Cd can induce apoptosis effectively.

3.6. Immunohistological localization of caspase-9 in kidney tissues

The result of immunohistochemical detection revealed that negative control sections from which the primary antibody was replaced with non-immune serum showed a complete absence of positive, colored reaction product for caspase-9 [Fig. 6(A–C and G–I)].

For Cd-treated groups [Fig. 6(E, F, K and L)] and their control groups [Fig. 6(D and J)], moderate to strong positive labeling was observed. The immunoreactivity was mainly localized on the tubular epithelial cells, including the collecting duct epithelium, proximal and distal tubular epithelium. Glomeruli epithelial cells occasionally revealed weak immunonegative staining. Caspase-9 immunostaining was present in the cytoplasm of these cells. Hardly any staining was found in the nuclei of these cells. All groups exhibited a similar immunostaining pattern.

4. Discussion

The present study is the first to describe the initiator caspase gene caspase-9 cDNA sequence of common carp. The common carp caspase-9, which was isolated from liver using RT-PCR and RACE-PCR techniques, appears homologous nearly identical to the known vertebrates, especially for fish caspase-9 based on the overall amino acid sequence, identity, similarity and phylogenetic analysis. Furthermore, comparison of the amino acid sequence with caspase-9 sequences from other vertebrates revealed a conserved and typical caspase 9 domain architecture including a putative CARD followed by caspase family p20 and p10 domain (large and small subunits). During the process of apoptosis, the interaction between the CARD of Apaf-1 (apoptotic protease-activating factor) and the CARD of procaspase-9 in the mitochondria-mediated apoptotic intrinsic pathway is essential for the recruitment of caspase-9 into the apoptosome and its subsequent activation (Palacios-Rodriguez et al., 2011). The characteristic caspase-9 pentapeptide active-site QACGG is conserved in common carp sequence and also located at the end of the large subunit (Reis et al., 2007). The putative cleavage site separating the large and small subunits is conserved in fish and occurs in DQMD328 and DLQD342. Whereas it occurs in PEPD315 and DQLD330 in mammals, which reveals some differences between fish and mammals. The potential locations of large and small subunits are conserved in all species analyzed. The two p20-p10 heterodimers are formed after the activation cleavage of the procaspase and can cause release of the prodomain (Lavrik et al., 2005).

Apoptosis is one of major types of cell death, previous studies demonstrated that Cd could induce different types of cell apoptosis including caspase-dependent and caspase-independent mechanisms, depending on the mode of delivery, concentration, and cell types. In the case of the intrinsic pathway, although caspase-9 has a prominent role triggering the mitochondrial apoptotic cascade, studies assessing alterations in or correlating Cd-induced cytotoxicity with expression levels of caspase-9 in fish tissue are scarce compared with those in mammals. Here, we have clearly demonstrated a dose-dependent activation of procaspase-9 to caspase-9 cleavage forms following the Cd exposure of carp. In the exposure experiment, lower concentrations of Cd (2.5 μM) maximally activated caspase-9, which is similar to a previous report (Hossain et al., 2009). Higher Cd concentrations could cause necrosis (Lopez et al., 2003), which is the possible reason why groups exposed to 10 μM Cd^{2+} did not induce more caspase-9 cleavage forms compared with those exposed to 2.5 μM Cd^{2+} in the present study. However, it is important to consider that differences in cell culture and animals treatment conditions, including a shorter time of exposure results in much lower uptake of cadmium, and consequently cellular.
toxicity (Hossain et al., 2009). In the end, activated initiator caspase-9 cleaves and activates downstream effector caspases, which then cleave various target substrates and result in the biochemical and morphological characteristics associated with apoptotic cell death (Shelton et al., 2010). Therefore, the result indicates that the apoptosis mechanism induced by Cd in carp might occur, at least in part, through a caspase-dependent pathway. However, further study will be needed to determine mitochondrial alterations, the release of cytochrome c and how Cd affects the translocation of the apoptosis-inducing factor (Kim et al., 2008).

Real-time RT-PCR offers a rapid, high sensitive and automated method for the detection of mRNA expression (Schmittgen and Livak, 2008). It is widely used for different applications, such as disease diagnostic and tissue-specific gene expression analysis. To avoid bias, the expression magnitude is often normalized to a reference gene. The housekeeping gene ideally has a stable expression in all tissues at different developmental stage and is not influenced by various experimental conditions (Tan et al., 2012; Van Hiel et al., 2009). In practice, however, no one housekeeping gene is universal under all experimental conditions (McCurlley and Callard, 2008). Therefore, in this study, RefFinder program was employed to screen the most suitable housekeeping gene for mRNA expression analysis. Eventually, ARNT2 was validated as the most stable reference gene, which was consistent with the report of zebrafish exposed to ultraviolet radiation (Behrendt et al., 2010).

In mammals, it is uncommon to evaluate the occurrence of apoptosis through detecting caspases mRNA expression (Vaculova and Zhirovsky, 2008). Whereas in fish, the dynamic expression of caspase mRNA has been used as a marker at an earlier point in the apoptotic cascade. Limited evidence suggests that caspase mRNA levels are increased in fish after heavy metals or other apoptotic stimuli treatment (Jin et al., 2013; Luzio et al., 2013). While in our present study, the mRNA expression of caspase-9 did not differ significantly between Cd-treated groups and control groups, although the visible caspase-9 cleavage forms indicated the occurrence of apoptosis. The possible reason is that we used a small sample size and set up a few exposure time points. In addition, the exposure concentrations were not most optimized. However, previous studies show that mRNA levels of caspases can mainly reflect the amount of procaspases. It does not reflect the level of biologically active caspases (Pelullo et al., 2005). The similar result was demonstrated in our present study that no remarkable change of procaspase-9 protein level was found after Cd treatment. More researchers have realized that the detection of mRNA differences can only indicate a biological significance if protein expression and activity can confirm the results (Vandaele et al., 2008). Different apoptotic markers (active caspase, TUNEL and general nuclear morphology) cannot always be detected at the same time, very little is known on the exact timing of the successive steps in the apoptotic pathways, so it is crucial to employ two or more distinct assays to confirm that cell death is occurring via apoptosis (Elmore, 2007).

To date, no study has been carried out about caspase-9 immunolocalisation in fish, and only immunohistochemical staining of caspase-3 was determined in past several limited studies for Cd exposure in fish (Brunelli et al., 2011; Zarnescu, 2009). The present study revealed that caspase-9 was present at moderate to strong levels in kidney sections in all groups. It seems that Cd exposure did not remarkably influence the labeling intensity of caspase-9. According to the result of Western blot analysis, the polyclonal rabbit antibody that we produced can detect procaspase-9 and their cleaved fragments. So the staining was possibly the mixed effect of caspase-9 proenzyme form and its activated forms. In future, when we explore the immunoreactivity of caspase-9, it is important to develop primary antibodies which only identify its cleaved forms.

5. Conclusions

The present results suggest that the cloned caspase-9 gene is homologous to that of other vertebrates. The transcripts did not vary among groups exposed to Cd and control group, which shows the mRNA level of caspase-9 cannot be used the major marker at an earlier point in the apoptotic cascade. The caspase-9 cleavage forms were detected after Cd exposure, which confirmed the occurrence of apoptosis. Meanwhile, immunopositive staining was mainly observed in the cytoplasm of the kidney specimens. All these characters suggest that the apoptosis mechanism induced by Cd in carp might occur through a caspase-dependent pathway, and the role of caspase-9 in apoptosis merits in-depth investigation.

Acknowledgments

This work was supported by a grant (No. 31001128) from National Natural Science Foundation of China, a grant (No. 2010GQ0209) from Natural Science Foundation of Jiangxi Province, and a grant (No. ZZ10090030) from Technology Pedestal and Society Development Project of Jiangxi Province and a grant (No. GJ11053) from the Educational Department of Jiangxi Province.

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