RESEARCH ARTICLE

Effect of water temperature and microcystins on tissue distribution and elimination of epigallocatechin gallate (EGCG) in snakehead fish (*Ophiocephalus argus Cantor*)

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Epigallocatechin gallate (EGCG), as the maximum content component of catechin from tea, has many function activities such as lowering cholesterol, resisting stress, and improving immunity, and is widely used in livestock breeding. This study was conducted to evaluate the effect of water temperatures and intraperitoneal administration of microcystins (MCs) on the tissue distribution and elimination of EGCG in snakehead fish (*Ophiocephalus argus Cantor*) by RP-HPLC method. The elimination mechanism of EGCG was also investigated in this study. The results showed that EGCG was eliminated more faster at 20°C than that at 10°C in tissues. Then the elimination lifetimes at 20°C were also determined in this experiment after intraperitoneal injection of MCs (MC-LR) at the dose of 50 μg/kg for 7 days. The detected results showed that EGCG was significantly eliminated slower in MCs-treated group than it in control group. Besides, the mRNA levels of carboxylesterase and cytochrome P450 genes in snakehead fish liver were determined. The results showed that the expression levels of carboxylesterase and cytochrome P450 genes were significantly inhibited by low water temperature and MCs toxin. Therefore, the elimination of EGCG was significantly affected by water temperature and MCs. The effect mechanism also included the expression levels difference of carboxylesterase and cytochrome P450 genes except for hemodynamics change.

Keywords: snakehead fish (*Ophiocephalus argus Cantor*); epigallocatechin gallate (EGCG); tissue elimination; water temperature; microcystins.

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Introduction

China is one of the biggest aquaculture countries in the world. Recent years, the fish diseases have become more and more serious in some aquaculture farms because of intensive farming and aquatic water polluting. Some published literatures have reported that fish growth rate has been significantly increased by using of high-fatty feed and the level of lipoprotein lipase in fish liver was markedly increased [1]. With the usage of high-fatty feeds for long time, some fatty liver disease in fish is becoming a serious problem [2]. Besides, with the chemical industry developing, a large number of pollutants were inlet into the aquatic water and accumulated in fish tissues. These polluting chemicals could also cause fish diseases [3, 4]. Therefore, poor disease resistance and high susceptibility to some pathogens have occurred in farming fish because of fish liver injury in recent years. Microcystins (MCs) is a kind of cyanobacterial toxin that often

appears in eutrophic water and does great damage to the liver of aquatic animals [5]. The impact of MCs on the pollution of aquatic products is related to the health of people. Some chemical feed additives or fish medicines were very easy to remain in fish tissues after usage [6, 7]. Accordingly, fish feed additive extracted from natural raw material should be developed and used to increase the disease resistance. The pharmacokinetic characteristics of natural products in fish are the key step in the development of feed additive.

Green tea has been the most widely consumed non-alcoholic beverage in the world. A group of active compounds named as polyphenolic substances were found in tea, which account for 18-30% of the dried leaves weight [8, 9]. The major constituent of these substances was epigallocatechin gallate (EGCG), which accounts for nearly 50% of polyphenolic chemicals. In recent years, a number of studies showed that EGCG exhibited some biological activities such as anticancer, anti-nutagenicity, prevention of cardiovascular disease, regulation of endocrine and immune system, reduction of liver inflammation, and carbon tetrachloride–induced hepatic fibrosis in rodents [10-15]. Besides, EGCG significantly inhibits the absorption of cholesterol in intestinal tract [16]. Thus, it was also used to treat hyperlipemia and protect fatty liver injury in rodents [17, 18]. In recent research, tea polyphenolic substances with mainly component of EGCG have been used for food additives of livestock farming because of its strong antioxidation and free radical scavenging function [19]. Therefore, the effect of water temperature and MCs intake on EGCG pharmacokinetics were needed to study.

Snakehead fish (*Ophiocephalus argus Cantor*) belongs to omnivorous and carnivorous fish. This fish is commonly cultured in China, which is well-known for their convenience in farming, strong disease resistance, and easy food processing. In this research, snakehead fish was used to study the effect of different water temperatures, microcystins (MCs), acetylation modification on the EGCG pharmacokinetics, and tissue distribution. The objectives of this study were to analyze the disposing process of EGCG at different water temperatures and environmental toxin intervention, which lastly served as reference for the amount of aquaculture application.

Materials and methods

Animals

Two hundred and ten (210) healthy snakehead fishes (mean body 430±20 g) were obtained from YUETENG pisciculture (Hangzhou, Zhejiang, China) and kept in flow through glass tank and acclimatized for one week before the experiment. These snakehead fishes were randomly divided into three groups and each group had seventy (70) fishes. A certain amount of microcystins (Express Technology Co., Ltd, Hangzhou, Zhejiang, China) was accurately weighed and dissolved in 0.9% saline to prepare microcystins injection. Fishes in the first group and the second group were used to study EGCG distribution and elimination in different water temperatures (20°C and 10°C). The fishes in the third group were maintained at 20±1°C and was intraperitoneally administered of EGCG (Hetian Bio-technology Co., Ltd, Hangzhou, Zhejiang, China) prepared by dissolving in methanol to a concentration of 50 mg/mL as stock solution, after intraperitoneal injection of microcystins (MC-LR) at 50 μg/kg. The water quality in tanks was tested daily. The pH was approximately 7.0 and the oxygen level was more than 10 mg/L due to the inflation pump. The water temperature was controlled at 10±1°C and 20±1°C with heating bars in two groups, which was used to evaluate the effect of water temperature on the EGCG tissue distribution and elimination. All the fishes were supplied with EGCG-free commercial diet before administration. On the day before study, the fishes were not fed.

Reversed phase high performance liquid chromatography (RP-HPLC) analysis of EGCG in tissue sample

The HPLC system used in this research was SHIMADZU-20AT series equipment (SHIMADZU, Kyoto, Japan), with a UV detector and a Zhida N2000 instrument workstation. EGCG separation was achieved on a Hypersil BDS C18 reversed-phase column (5 µm, 250 mm × 4.6 mm). The mobile phase was composed of acetonitrile and 0.1% citric acid solution (10:90, V/V) (Milk Chemical Instrument Co., Ltd, Hangzhou, Zhejiang, China) at a flowrate of 1.0 mL/min. The column temperature was kept at 35°C and the detector wavelength was set at 278 nm. The analysis of tissue samples (liver, kidney, spleen, muscle, and gonad) was carried out. 0.6 g of homogenized tissue sample was transferred to a 10 mL tapered glass tube with a screw cap and vortex-mixed for 1 min. Then, 3 mL of ethyl acetate was added and vortex-mixed for 3 mins. After centrifugation at 6,000 rpm for 5 mins, the organic phase was transferred to a glass test-tube. This extraction process was repeated twice. All the organic phases were combined and evaporated to dry at 45°C under a stream of nitrogen gas. Then, 0.2 mL acetonitrile and water (1:4, V/V) was added to the tube followed by adding 1.0 mL of n-hexane. After centrifugation at 6,000 rpm for 3 mins, all the n-hexane was discarded. This extraction was repeated twice. After final centrifugation at 18,000 rpm for 5 mins, 20 µL of sample was injected directly into the RP-HPLC system.

Calibration curve

EGCG standard solution was prepared with methanol. Eight concentrations of EGCG for the calibration curve were prepared by dilution from the stock solution (50 mg/mL), which were 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 200.0, 500.0 mg/L, respectively. The serial diluted solution (0.6 mL of each concentration) was evaporated to dry at 45°C under a stream of nitrogen gas. Then, 0.6 mL of EGCG-free tissue homogenates was added. The calibration range was 0.2-500 mg of EGCG per milliliter for all the tissues. Calibration standards were extracted and assayed according to the method mentioned above. The calibration curve was constructed based on peak-area of EGCG (Y-axis) vs concentrations of spiked EGCG (X-axis).

Extraction recovery and precision

Blank tissue homogenates (tissue homogenates without EGCG) were spiked with the standards of EGCG at low (0.5 mg/L), medium (5.0 mg/L), and high (50.0 mg/L) concentrations, which were used as quality control samples (QC) later within the calibration curve. Recovery of EGCG with extraction method above was determined by comparing observed peak-area ratios in extracted tissue samples to those of non-processed standard solutions. Precision of the RP-HPLC method were assessed by assaying five replicate QC samples above, respectively. Intra-day precision was evaluated at different times during the same day. Inter-day precision was determined over five different days.

Tissues distribution of EGCG

Fishes in the first and second groups were given a single dose of 150 mg/kg body weight of a solution containing EGCG at 50mg/mL. The fishes in the third group were intraperitoneally administered EGCG at a single dose of 150 mg/kg body weight after they were treated with intraperitoneal administration of MC-LR at 50 µg/kg for a consecutive week. After administration of EGCG, five fishes were sampled at each time point including 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 96 h, 120 h, and 144 h. All the samples were prepared by the same method described above. The concentration of EGCG in each tissue samples was calculated using the calibration curve. The parameters of tissue distribution and elimination were calculated by the method of linear regression.

Transcription levels of carboxylesterase and cytochrome P450 genes

According to our previous studies, the metabolism of EGCG closely related to the carboxylesterase and cytochrome P450 activities in mammals [20]. It was speculated that EGCG metabolism and elimination should also closely relate to carboxylesterase and cytochrome P450 in snakehead fish. The carboxylesterase and

cytochrome P450 genes of Snakehead fish had been cloned in our lab and both sequences had been submitted to GeneBank (accession number: KJ192368 and KJ192371). The gene cloning primer sequences of carboxylesterase were 5'-TCCTCCGGCCGAACCACTGT-3' (forward) and 5'-GCAGTCCTCCTGGCCCATCA-3' (reverse), while the primers for cytochrome P450 gene were 5'-GACAAGGATGAGCCCATAGAG-3' (forward) and 5'-GATCTGAGGGGTTGTTGAGTGA-3' (reverse). The tissue total RNAs were extracted by using Tri-reagent (HAOGENE BIOTECH, Hangzhou, Zhejiang, China) and quantitated by spectrophotometer. Reverse transcription was performed by using the Superscript^{TM}II transcriptase and RNasin (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's direction. Quantitative real time PCR (qRT-PCR) was performed with Brilliant SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA). The 18S rRNA was used as internal reference gene with the forward and reverse primers of (5'-CGGACACGGAAAGGATTGACAGA-3' and 5'-CGCTCCACCAACTAAGAACG-3'), respectively. All the oligonucleotide primers were synthesized by Shanghai Sangon Biotech (Shanghai, China). The total volume of PCR reaction was 25 µL with 1.0 µL cDNA template, 0.5 µL of each primer, 12.5 µL SYBR Premix ex Taq^{TM} (2×) and 10.5 µL ddH_2O. The PCR reaction condition consisted of pre-degeneration at 95°C for 1 min followed by 40 cycles of 95°C for 10 s and 63°C for 25 s. All values were calculated by using the delta C_t method and expressed as the change relative to the 18S rRNA.

Statistics

All data were expressed as mean ± standard deviation (SD). A probability of $P < 0.05$ was considered statistically significant using paired samples T test of SPSS (18.0) (Statistical Product and Service Solutions, Shanghai, China)

Results

Specificity of chromatograms

The RP-HPLC chromatogram peak of EGCG assayed by the standard solution was 14.932 min (Figure 1A). No significant interfering peak appeared for normal tissue samples (Figure 1B). Blank tissue samples spiked with EGCG also yielded chromatogram peaks identical to that by the standard solution (Figure 1C). A chromatogram of a tissue sample obtained after EGCG administration showed that identifiable EGCG peak was clear and strong without any endogenous substances at the corresponding time (Figure 1D).

Calibration and validation of the method

Evaluation of the method was performed with a calibration curve over the concentration range 0.2-500.0 mg/kg for tissues EGCG. The slope and intercept of the calibration graph were calculated by weighted least squares linear regression. The regression equation of the curve and correlation coefficients (*r*) were calculated as followed. A liner relationship between peak-area and concentrations was good (Table 1).

Table 1. Calibration curve of EGCG in tissues.

Tissue types	Curve equation	Correlation
Liver	$Y = 6005.6X + 540.54$	r =0.9998
Kidney	$Y = 6030.7X + 369.62$	r =0.9999
Spleen	$Y = 5986.4X - 132.8$	r =0.9998
Muscle	$Y = 6019.1X + 150.9$	r =0.9998
Gonad	$Y = 5998.6X - 865.97$	r =0.9998

The lower limit of quantification (LLOQ) was found to be 0.2 mg/kg in tissues. The intra-day and inter-day precisions were less than 10.0% for all three concentrations. Extraction recovery of EGCG at concentrations of 0.5, 5.0, and 50.0 mg/kg in tissues were more than 85.0%, respectively. All the datum of EGCG proved good precision and extraction recovery of the RP-HPLC method developed (Table 2).

Tissues distribution of EGCG

After intraperitoneal injection of EGCG at 150 mg/kg dose, EGCG was determined at a series of time points within 144 h in tissues at two temperatures (10°C and 20°C). The EGCG concentrations in snakehead fish tissues with a

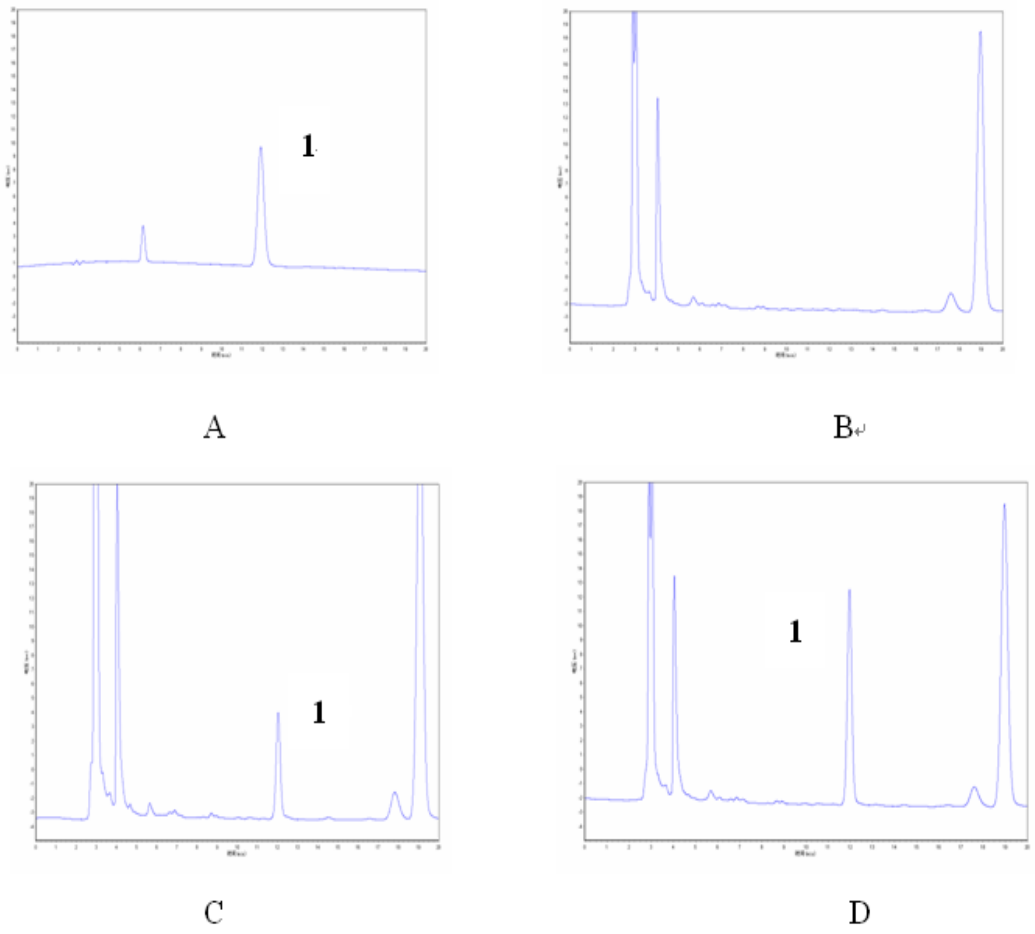

Figure 1. Specificity of chromatograms. A: EGCG standard solution; B: blank liver tissue; C: blank liver tissue spiked with EGCG; D: liver tissue after administration of EGCG. (Peak 1: EGCG).

Table 2. Extraction recovery and precision of EGCG in tissue homogenates (n=5).

Sample	Spiked concentration (mg/kg)	Recovery (%)	Precision（%）	
			Intra day	Inter day
Liver	0.5	85.81±8.28	6.83	9.65
	5.0	90.78±7.12	5.66	7.84
	50.0	92.36±6.68	4.29	7.23
Kidney	0.5	87.41±8.29	6.82	9.49
	5.0	91.86±7.05	5.88	7.67
	50.0	94.36±6.27	6.64	7.86
Spleen	0.5	86.21±8.16	6.21	9.47
	5.0	91.86±7.05	5.88	7.67
	50.0	94.36±6.27	6.64	7.86
Muscle	0.5	84.38±7.11	8.43	9.27
	5.0	90.41±8.91	8.75	9.86
	50.0	90.67±8.23	7.52	9.08
Gonad	0.5	86.42±7.58	8.71	9.83
	5.0	91.31±9.11	6.28	9.98
	50.0	93.36±7.27	3.28	7.79

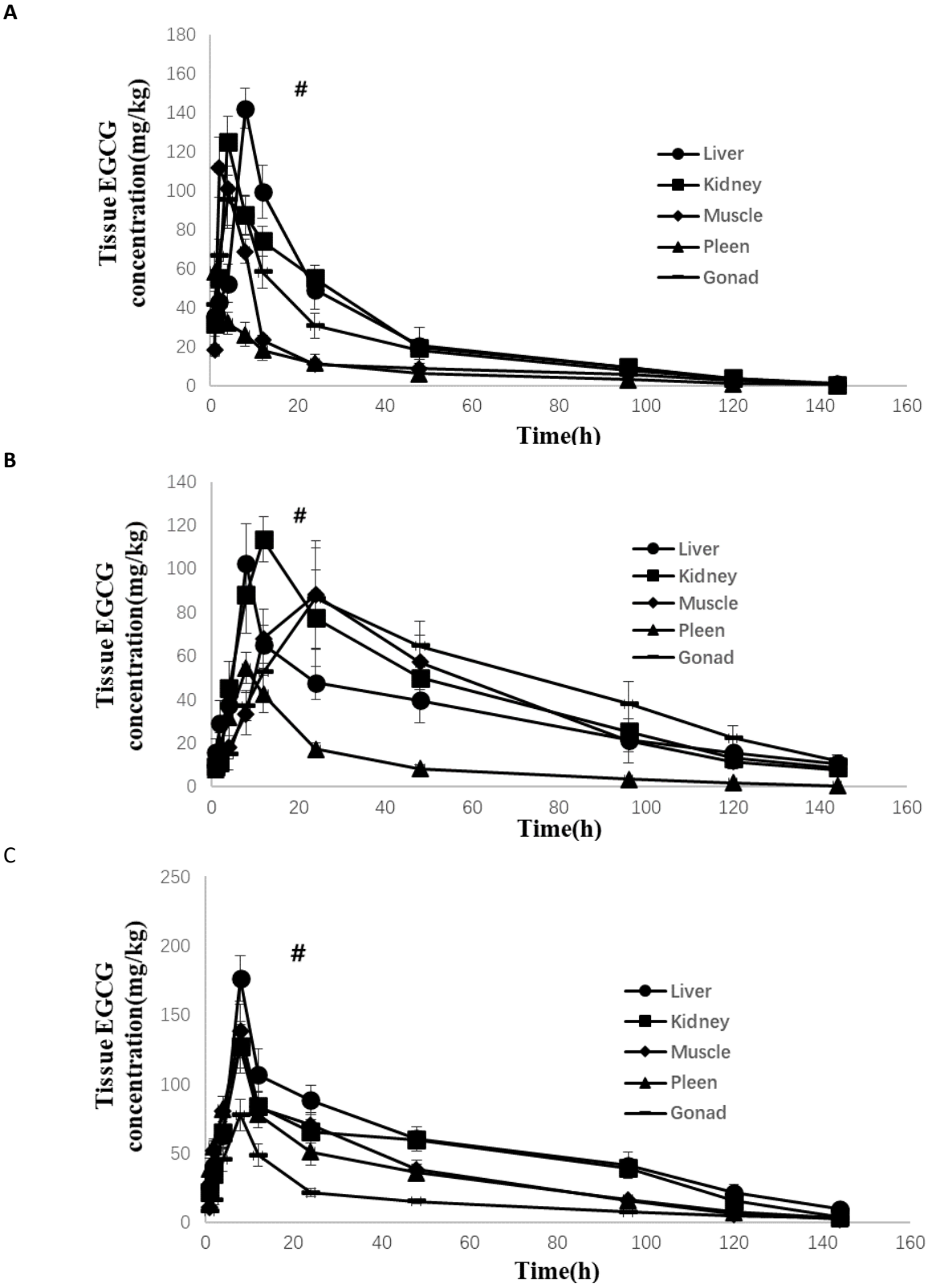

Figure 2. EGCG concentrations in different tissues at different experimental groups. **A**: high temperature; **B**: low temperature; **C**: MC-LR treatment. #: $p<0.05$.

pretreatment of MC-LR were also determined. At two water temperatures, the distribution concentrations of EGCG in tissues were still above the detection limit at 144 h. The maximum concentrations of EGCG in the tissues were higher at 20°C than it at 10°C, while the maximum liver concentration was the highest in MC-LR pretreated group. The elimination rate at 20°C was faster than it at 10°C. All the concentrations in liver and kidney were given in Figure 2. In high and low temperature groups, EGCG concentrations in liver and kidney were found to be higher than that in the other tissues, while the EGCG concentrations in liver and kidney were detected to be the highest in MC-LR pretreatment group. The elimination lifetimes of EGCG in different tissues were calculated and listed in Table 3.

Table 3. Elimination half-time of EGCG in different tissues.

Tissues	20°C (h)	10°C (h)	MC-LR treatment (h)
Liver	20.63	52.11	39.60
Kidney	21.93	38.93	34.82
Spleen	26.35	35.54	29.24
Muscle	28.76	46.51	25.86
Gonad	25.11	70.71	33.97

Transcription levels of carboxylesterase and cytochrome P450 genes

According to the primer of carboxylesterase gene, the transcription level of carboxylesterase and cytochrome P450 gene in snakehead fish liver was detected by real-time PCR method. The results showed that the gene transcription levels were higher at 20 ºC than at 10 ºC, while the transcription level was significantly decreased by MCs treatment. All the results were showed in Figure 3. It was also suggested that transcription level of carboxylesterase and cytochrome P450 gene in snakehead fish liver was significantly affected by water temperature and MCs, which was consistent with elimination trend of EGCG in different groups.

Discussion

Recent years, some reports on the metabolism and elimination of EGCG were also published in mammals, such as mouse, rat, beagle dog, and rabbit [21-25]. However, no research on the pharmacokinetics of EGCG was performed in snakehead fish until now. Moreover, there was no studies reported about the influence of MCs and water temperature on the EGCG tissue distribution and elimination in snakehead fish.

Fish is cold-blooded animal, their physiological characteristics are deeply influenced by the water temperature, such as blood circulation, metabolism, and immunity system [26-28]. That is why their physiological characteristics and resistance to disease vary a lot in different seasons. This study performed experiments at different water temperatures, and from the results we found that EGCG distribution and elimination were slower at a lower temperature (10°C). While EGCG distribution and elimination were faster at a higher temperature (20°C), the EGCG distribution to peripheral tissues after absorption and the peak concentrations of the tissues were detected in the shortest time, which made it much easier for EGCG to penetrate into snakehead fish other organs.

Now, as environmental pollution intensifies, the eutrophication of aquaculture waters is becoming more and more serious, which has contributed to freshwater algae bloom due to plenty of algae toxins in aquaculture waters was produced. MCs is a kind of cyanobacteria toxin, which can enter animals through many ways and seriously threaten the health of animals [29-31]. Recent years, many studies showed that MCs exhibited multiple organ toxicity, such as liver and kidney [32, 33]. In this experiment, MCs was injected intraperitoneally to study the MCs effects on EGCG distribution and elimination in snakehead fish. The results showed that the elimination characteristics of EGCG were significantly affected by MCs pretreatment with very low EGCG elimination. It was suggested that

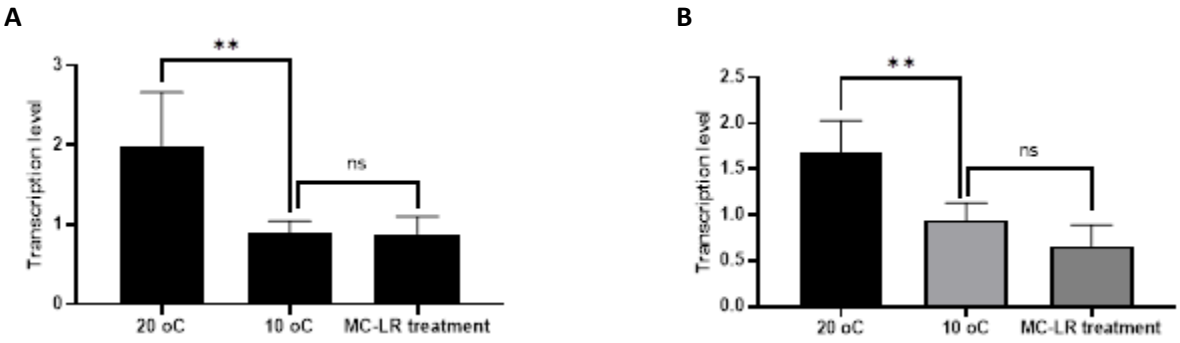

Figure 3. Transcription levels of carboxylesterase and cytochrome P450 genes in different experimental groups. **A**: carboxylesterase; **B**: cytochrome P450.

blood vessel and liver of snakehead fish had been damaged by MCs, which was in accordance with aquatic toxicity induced by MCs.

In the future, the application of catechins to farming aquaculture should be with a consideration for the season effect on the physiological characteristics of fish. In the case of the elimination of xenobiotic compounds in animals, the slower elimination of EGCG in fish would be attributed to slow excretion in urine and low metabolism in liver. Therefore, the transcription levels of carboxylesterase and cytochrome P450 genes were studied here in snakehead fish liver, which suggested that those two genes expression was significantly inhibited by low temperature and MC-LR. That is to say, the EGCG distribution and elimination progress was markedly affected by the water temperature and MCs toxin. This study would provide a scientific basis for scientific breeding of snakehead in future.

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