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Effects of Oxytetracycline Antibiotic on GST Enzyme Activity and Gene Expression Levels in Rainbow Trout

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***Abstract*— In this study, effects of oxytetracycline on glutathione s-transferase enzyme activity and gene expression levels in rainbow trout (*Oncorhynchus mykiss*) liver were investigated. For this purpose, liver tissues from rainbow trout exposed to oxytetracycline application were collected at third, sixth and 12th hours. The group not exposed to oxytetracycline was assigned as control. While the lowest enzyme activity of GST was at 12th, the highest enzyme activity was observed in control. While compared with the control group, enzyme activity at 12th hour was highly significant ($p < 0.001$), the third and sixth hours groups were significant ($p<0.05$). The lowest gene expression level was found in 12th group and the highest level was the third. While compared with the control group, difference at the third hour of gene expression level was highly significant ($p < 0.001$), at the sixth and 12th hours groups was not significant. As a result, there was understand that no correlation between gene expression levels and GST enzyme activity.**

***Keywords*—** *Oncorhynchus mykiss, Liver, Glutation S-Transferase, Oxytetracycline, Enzyme Activity.*

I. Introduction

Antibiotics have been used in fish health care more than 50 years. The use of tetracyclines toward to gram negative pathogenics in trout culture is an old technics [1]. Oxytetracycline is the first antibiotic which is approved by FDA [2]. It has been reported that oxytetracyclines were approved to wide using to threathments of bacterial fish diseases in many countries such as Norway, Italy and Japan [3, 4, 5, 6, 7].

Glutation S-Transferase (GST) (EC.2.5.1.18) is very much functional enzyme because of generating homeosthasis [8]. GST exists in many organisms like mammalian, insects, fish, birds and microorganisms. The most GST founding tissues are cytosoles and membranes of many organs such as liver, intestine, rena [9]. This enzyme has been categorized as GSTP1, GSTM1 and GSTT1 according to its biochemical, immunological and structural characteristics. The GSTs are tissue specific expressed enzymes [10, 11], and has many substrates. GST eliminates food based toxics. Isomerization of prostaglandin transfers nonsubstrate ligands such as hem, bilirubin, bile salts and fatty acids by bounding with GSH [12].

The research aimed to clarify effects of oxytetracycline on GST expression and also enzyme activity.

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II. Material and Methods

A. Chemicals

Oxytetracycline were obtained from BAYER (Leverkusen, Germany). All of the other chemicals used for analytical grade were purchased from Merck (Darmstadt, Germany)

B. Fish Husbandry and Maintenance

A total of 28 rainbow trout (*Onchorynchus mykiss* Walbaum) were used for this experiment. The fish were provided from Ataturk University, Fisheries Faculty. All fish were one-year-old, and its main weight was 230±20 g. The trout were kept cylindrical tanks in freshwater with water flow of 1.5 L/min, 10±1°C average water temperature, 9 ppm dissolved oxygen, pH 7.6±0.2, total hardness was 102 mg $CaCO_3$ with natural dark/light cycle. The fish were fed twice a day with a commercial trout diet (Sürsan AquaMax, Sürsan inc.). All fish were starved two days before antibiotic administration. Fish treatments were conducted according to Applied Research Ethics National Association (2002)

C. Experimental Design

The fish were randomly divided into two groups. Group 1 was the control (n=7), and group 2 was orally oxytetracycline administrated for 100 mg/kg body weight (n=21). After antibiotic administration, 7 fish were killed at 3rd, 6th and 12th hours, and liver tissue samples were collected, washed with ice-cold saline two times and immediately transferred in liquid nitrogen and stored at -80°C.

D. GST Enzyme Activity Determination

Liver tissues homogenized in a homogenizer, in 50 mM Tris/HCl buffer, pH 7.6, containing 1 mM DTT, 1 mM EDTA and 1 mM PMSF of 1/5 (w/v). Homogenate was centrifuged at 10000 rpm for 30 min and supernatant was used for enzymatic activity determination. All steps were performed in +4°C. GST (EC.2.5.1.18) enzyme activity was determined as described by Habig et al. (1974). One unit conjugates 1.0 □mol of 1-chloro-2,4-dinitrobenzene with reduced glutathione per min at pH 6.5 at 25°C. In a 1 ml reaction mix, final concentrations are 20 mM K-Phosphate, 0.68 mM EDTA, pH: 7.6, 2 mM GSH and 2.5 mM CDNB. Protein concentrations in the supernatants were determined using the Bradford assay with bovine serum albumin as the standard (13).

E. RNA isolation and cDNA synthesis

Total RNA was isolated from 50 mg frozen muscle, liver and kidney tissues with a RNeasy Lipid Tissue Mini Kit (Qiagen, cat. no. 74804) using the Qiacube robot (Qiagen, Hilden, Germany). RNA was treated with DNase I in order to avoid genomic contamination. RNA concentrations and quality were verified by means of nanodrop spectrophotometer (µDrop™ Plate, Thermo Scientific, cat. no. 12391) and RNA gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen, cat. no. 11146-016) according to the manufacturer's protocol. All cDNA was stored at − 20 °C until use.

F. TaqMan probe and primer design

Primers and TaqMan probes were designed in Primer3 software (www.bioinfo.ut.ee/primer3), checked by Primer-BLAST software and purchased from Metabion International (Martinsried, Germany). β-Actin was used as reference gene, since it was not affected by any of the treatments. In order to perform Real Time PCR, TaqMan probe of the target and reference gene were conjugated with FAM/TAMRA and CY5/BQ2 respectively. The primer and probe sequences, amplification length and genbank accession number for real-time PCR are provided in Table 1.

G. Quantitative real time PCR

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler Qiagen Rotor-Gene. The real-time PCR was carried out in a reaction volume of 50 µl containing template DNA, 900 nM of both target and reference forward and reverse primers, 250 nM both target and reference TaqMan probes, and 25 µl FastStart TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase (UNG), dNTP with dUTP, and optimized buffer component. The amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: 50 °C for 2 min for activation of optical AmpErase UNG enzyme, 95 °C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Real-Time PCR data were analysed using the efficiency $(e)^{(-\Delta Ct)}$ method, which is used to determine mRNA levels in gene expression against control group and housekeeping genes (β-Actin). Analytical sensitivity was confirmed by running standard curves. Amplification efficiency (e) was calculated based on the slopes of the curves (slope) using the formula $e = 10^{(-1/slope)}$ [14], and the slope value via Rotor-Gene software.

TABLE I. THE PRIMER AND PROBE SEQUENCES, AMPLIFICATION LENGTH AND GENBANK ACCESSION NUMBER

Gene	Primer and Probe	Sequence	Len. (bp)	Acc. Num.
GST	Forward	TGGCTGACGTTATTGTCTTCC	112	NM_001160559.1
	Reverse	CTGGGTCTGTCCTTCACCATA		
	Prob	FAM-CGGCGCGTTACCCCAAACTG-AMRA		
β-Actin	Forward.	TGGCCGTACCACCGGTAT	79	AF254414
	Reverse	GCAGAGCGTAGTCCTCGTAGATG		
	Prob	Cy5- CTCCGGTGACGGCGTGACCC -BQ2		

H. Statistical analyses

All experiments were triplicated. One-way ANOVA and Duncan's multiple range test (SPSS 22.0) was used to determine the effects of oxytetracycline. P-values below 0.05 were considered statistically significant.

III. Results and discussion

While GST activity of control group was 12.8±0.6 EU/mg, those of oxytetracycline treated groups at 3, 6 and 12h were found as respectively 10.5±0.7 EU/mg, 11.2±0.5 EU/mg, 9.5±1.1 EU/mg. The lowest enzyme activity was 12 h group, and the highest one was the control. Compared with control, the 12h group had extremely significant ($p<0.001$), the 6h and the 3h group had significant decrease ($p<0.05$). When the experimental groups were compared, differences between the sixth and third hours to 12 hours were found statistically significant ($p<0.05$), but the difference between three and six hours were not significant (Table 2).

GST plays a primary role in detoxification of different chemicals and endogenously derives reactive compounds [15]. Many endogenous GST substrates are formed as a consequence of modification of macromolecules by reactive oxygen species, and the transferases are therefore considered to serve an antioxidant function [16, 17]. GST catalyzes the conjugation of reduced glutathione (GSH) [18, 19]. Additionally several GST isoenzymes exhibit other GSH-dependent catalytic activities including the reduction of organic hydroperoxides [20] and isomerization of various unsaturated compounds [21, 22, 23].

It is reported that, in both liver and pancreas, the higher dose of tetracycline (200 mg/kg), caused significant decrease in GST activity. [24]. Depending on the degree of oxidative stress and resulting tissue damage, the antioxidant enzyme levels progressively decrease [25].

After oral administration of oxytetracycline from the third hour to the next, serum oxytetracycline concentration reaches peak level [26]. As can be seen in Figure 1, from the third hour after the oxytetracycline application, GST activity level has decreased. This is thought to cause intense deplete GSH in the liver.

TABLE II. GST ENZYME ACTIVITIES

Group	Specific Enzyme Activity (EU/mg)
Control	$12,8±0,6^{a}$
3h	$10,5±0,7^{bc}$
6h	$11,2±0,5^{bc}$
12h	$9,5±1,1^{c}$

The average for each parameter are marked with different letters to indicate groups that are different from each other, $p<0.05$, n=7

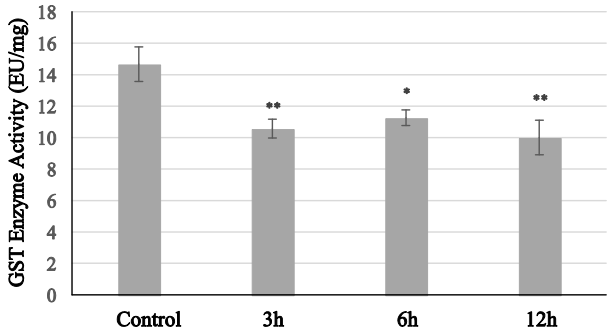

Figure 1. Comparison for GST enzyme activities of control and treatment groups.

GST gene expression levels of Oxytetracycline administrated groups (3h, 6h and 12h) were respectively 11.6±2.3 ng/ml, 2.4±1.7 ng/ml, 2.3±1.5 ng/ml (Table 3.). The highest expression level among experimental groups was 12h, and the lowest was 3h. Compared with control group there was extremely significant increase at 3h group ($p<0.001$), and differences of others were not significant.

As seen in Figure 2, at the third hour oxytetracycline reached peak serum levels, and GST gene expression has reached the highest level. After this stage, the expression level was down to normal level, because serum peak level time of oxytetracycline is at the 3-h, and half-life of it is at the 12-h [26].

There is no report about effect of oxytetracycline on GST gene expression, but relationship between several substance and GST gene expression are available.

Increased liver GST mRNA expression level in Coho salmon (*Oncorhynchus kisutch*), which exposed to cadmium and tilapia (*Oreochromis niloticus*), which exposed to cylindrospermopsin, were reported [27, 28]. Similar to these results, after oxytetracycline administration, GST gene expression value increased synchronously serum oxytetracycline level (Figure 2).

TABLE III. GST GENE EXPRESSION LEVELS RELATIVE TO β ACTIN

	Gen expression level
Control	1 ± 0^{b}
3h	$11{,}635\pm2{,}30^{a}$
6h	$2{,}460\pm1{,}77^{b}$
12h	$2{,}321\pm1{,}50^{b}$

The average for each parameter are marked with different letters to indicate groups that are different from each other, $p<0.05$, n=7

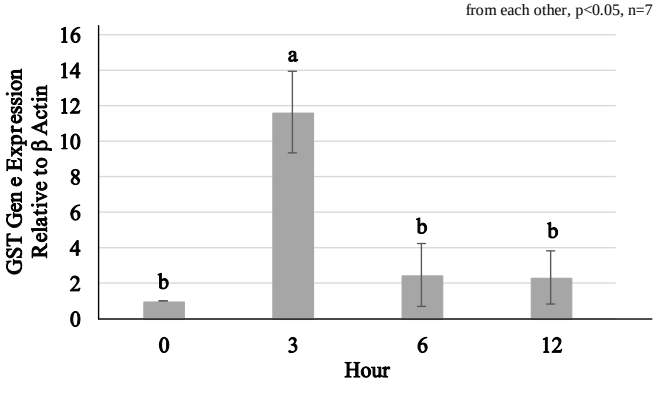

Figure 2. GST mRNA levels of oxytetracycline treated trout liver tissue at different times.

Different authors reported that there was no correlation between mRNA level and enzyme activity [29, 30, 31]. We also found similar results compatible with previous reports.

Oxytetracycline stimulated GST gene expression at the 3h period, which is the peak serum concentration. In subsequent hours, expression levels of GST genes decreased. It was considered that this originated from lowering in GST enzyme activity depending on excessive GSH consumption.

This study showed that oxytetracycline administration induced oxidative stress by reducing GST enzyme activity in liver. But there was no correlation between GST enzyme activity and gene expression level.

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