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BOLU ABANT İZZET BAYSAL UNIVERSITY
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Department of Chemistry

THE EFFECT OF FLUVOXAMINE ON THE XENOBIOTIC METABOLIZING ENZYME ACTIVITIES AND ANTIOXIDANT SYSTEM IN RAINBOW TROUT (ONCORHYNCHUS MYKISS)

MASTER OF SCIENCE

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APPROVAL OF THE THESIS

THE EFFECT OF FLUVOXAMINE ON THE XENOBIOTIC METABOLIZING ENZYME ACTIVITIES AND ANTIOXIDANT SYSTEM IN RAINBOW TROUT (ONCORHYNCHUS MYKISS) submitted by **Avin Abdulrahim Ibrahim IBRAHIM** and defended before the Examining Committee Members listed below in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Chemistry, Institute of Graduate Studies of Bolu Abant Izzet Baysal University in 2.01.2023** by

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Avin Abdulrahim Ibrahim IBRAHIM

ABSTRACT

THE EFFECT OF FLUVOXAMINE ON THE XENOBIOTIC METABOLIZING ENZYME ACTIVITIES AND ANTIOXIDANT SYSTEM IN RAINBOW TROUT (ONCORHYNCHUS MYKISS)
MSC THESIS
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BOLU, JANUARY 2023
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Fluvoxamine is a drug used to treat depression and anxiety disorders. It is present in aquatic systems due to widespread usage. But, the effect of fluvoxamine on the aquatic organism is not well known. In this study, we aimed to determine the effect of fluvoxamine on CYP1A-associated 7-ethoxyresorufin O-deethylase (EROD), CYP3A-associated erythromycin N-demethylase (ERND), glutathione S-transferase (GST) and antioxidant enzymes, catalase (CAT) and glutathione reductase (GR) activities in rainbow trout (*Oncorhynchus mykiss*). For this purpose, 24 fish were divided into three groups as control group, 10 microgram/L fluvoxamine administration group, and 50 microgram/L fluvoxamine administration group. Fish were treated with indicated doses of fluvoxamine for 96 hours. At the end of the experimental period, fish were killed and livers were taken. Microsomes and cytosols were prepared and enzyme activities were measured in these fractions. The EROD activity results of the 10 microgram/L fluvoxamine administration group and the EROD activity results of the 50 microgram/L fluvoxamine administration group were found to be significantly different from the EROD activity results of the control group. The GST activity results of the 50 microgram/L fluvoxamine administration group were found to be significantly different from the GST activity results of the control group. The GR activity of the 50 microgram/L fluvoxamine administration group was found to be significantly different from the GR activity results of the control group. No difference was found in ERND and CAT activities between the groups. The results of this study showed that fluvoxamine administration affected some of the enzyme activities in rainbow trout.

KEYWORDS: Antioxidant system, Cytochrome P450, Fish, Fluvoxamine, Liver, Xenobiotic

ÖZET

FLUVOKSAMİN VERİLMESİNİN GÖKKUŞAĞI ALABALIĞINDA (ONCORHYNCHUS MYKİSS) KSENOBİYOTİK METABOLİZE EDEN ENZİM AKTİVİTELERİ VE ANTIOKSİDAN SİSTEM ÜZERİNE ETKİSİ
YÜKSEK LISANS TEZI
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Fluvoksamin, depresyon ve anksiyete bozukluklarının tedavisinde kullanılan ilaçlardan biridir. Yaygın kullanımı nedeniyle sucul ortamlarda bulunmaktadır. Ancak fluvoksaminin suda yaşayan organizmalar üzerindeki etkisi iyi bilinmemektedir. Bu çalışmada amacımız, fluvoksaminin CYP1A ile ilişkili 7-etoksiresorufin O-deetilaz (EROD), CYP3A ile ilişkili eritromisin N-demetilaz (ERND), glutatyon S-transferaz (GST) ve antioksidan enzimler olan katalaz (CAT) ve glutatyon redüktaz (GR) enzim aktiviteleri üzerindeki etkisini gökkuşağı alabalığında (*Oncorhynchus mykiss*) belirlemektir. Bu amaçla 24 balık kontrol grubu, 10 mikrogram/L fluvoksamin uygulanan grubu ve 50 mikrogram/L fluvoksamin uygulama grubu olmak üzere üç gruba ayrılmıştır. Balıklar 96 saat boyunca belirtilen dozlarda fluvoksamin ile muamele edilmiştir. Muamele süresi sonunda balıklar öldürülmüş ve karaciğerleri alınmıştır. Mikrozomlar ve sitozoller hazırlanmıştır ve bu fraksiyonlarda enzim aktiviteleri ölçülmüştür. 10 mikrogram/L fluvoksamin uygulanan grubun EROD aktivite sonuçları ve 50 mikrogram/L fluvoksamin uygulanan grubun EROD aktivite sonuçları kontrol grubunun EROD aktivite sonuçlarından anlamlı olarak farklı bulunmuştur. 50 mikrogram/L fluvoksamin uygulanan grubun GST aktivite sonuçları, kontrol grubunun GST aktivite sonuçlarından anlamlı derecede farklı bulunmuştur. 50 mikrogram/L fluvoksamin uygulanan grubun GR aktivitesi, kontrol grubunun GR aktivitesi sonuçlarından anlamlı ölçüde farklı bulunmuştur. Gruplar arasında ERND ve CAT aktivitelerinde fark bulunmamıştır. Bu çalışmanın sonuçları, fluvoksamin uygulamasının gökkuşağı alabalığında bazı enzim aktivitelerini etkilediğini göstermiştir.

ANAHTAR KELİMELER: Antioksidan sistem, Sitokrom P450, Balık, Fluvoksamin, Karaciğer, Ksenobiyotik

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LIST OF ABBREVIATIONS AND SYMBOLS

ε-ACA : ε-Amino caproic acid
BSA : bovine serum albumin
CYP : cytochrome P450
CAT : catalase
DMSO : dimethyl sulfoxide
DNA : deoxyribonucleic acid
ERND : erythromycin N-demethylase
EROD : 7-ethoxyresorufin O-deethylase activities
EDTA : ethylenediaminetetraacetic acid disodium salt dihydrate
FDA : food and drug administration
FAK : focal adhesion kinase
GSSG : glutathione disulfide (oxidized glutathione)
GR : glutathione reductase
GSTs : glutathione S-transferases
GBM : glioblastoma multiforme
GSH : glutathione
5-HT : 5-hydroxytryptamine
HEPES : N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic Acid
MDA : malondialdehyde
MPO : myeloperoxidase
NO : nitric oxide
NADP+ : β-nicotinamide adenine dinucleotide phosphate
NADPH : reduced nicotinamide adenine dinucleotide phosphate
KPi : potassium phosphate buffer
PMSF : phenylmethanesulfonyl fluoride
ROS : reactive oxygen species
SEM : standard error of mean
SSRI : selective serotonin reuptake inhibitor

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1 INTRODUCTION

In various ways, marine pollution is a worldwide issue. It has an impact on the condition of the oceans, seas, lakes, and rivers. Both developed and developing nations are affected, and all governments have responsibilities on this issue and must focus on its importance and solution of it (1).

Pharmaceuticals are one of the most important classes of reasons for pollution in aquatic environments due to their widespread usage, chemico-physical characteristics, and unknown actions in aquatic creatures. Many medications and the byproducts of their transformation are only partially retained in wastewater treatment plants after administration. Then they are transferred into the aquatic environment. Thousands of tons of drugs are used annually to treat illnesses and livestock and aquaculture (2). Their effects on aquatic organisms are not well known.

1.1 Fluvoxamine

Fluvoxamine is also called 5-methoxy-1-[4-(trifluoromethyl)-phenyl]-1-pentanone-O-(2-aminoethyl)-oxime. It is a drug from the selective serotonin reuptake inhibitor (SSRI) group that is commonly used to treat serious depression and other anxiety disorders (3). Its structure is given in Figure 1.1. Molar mass is 318.33 g/mol. It has a 120–122 °C melting point, and 1 g/L water solubility (4).

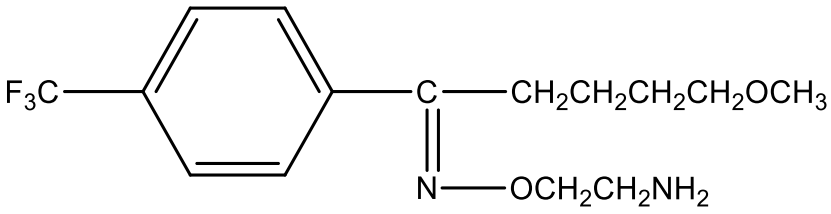

Figure 1.1 Chemical structure of fluvoxamine (3).

Fluvoxamine is structurally distinct from other SSRIs. It is the only monocyclic SSRI and belongs to the 2-aminoethyloximethers of aralkylketones (5–7).

Fluvoxamine is a novel antidepressant that inhibits serotonin reuptake in neurons powerfully and selectively (8). It appears that its antidepressant action derives from reuptake inhibition-induced facilitation of serotoninergic neurotransmission. Its therapeutic efficacy can be comparable to imipramine and clomipramine (9).

There is evidence that fluvoxamine helps depressed patients sleep better. Fluvoxamine considerably and more quickly improved depressed patients' sleep quality than fluoxetine did, according to a recent double-blind trial comparing the two medications (10). According to reports, fluvoxamine works to increase resting metabolic rate, which prevents weight gain (11). Its impact on cardiovascular disease has been studied and it has been shown that it has no effect on cardiovascular function and is safe for patients suffering from this disease (12).

The time required to eliminate half of it is 12 to 15 hours, when compared to other SSRIs, this value is low (13). Fluvoxamine has a modest protein-binding characteristic (about 81% in plasma). Plasma proteins-fluvoxamine interaction can impact its pharmacokinetics, including its tissue distribution and clearance (14).

Side effects of fluvoxamine can be listed as cardiovascular side effects (hypotension, hypertension), dermatological side effects (sweating), gastrointestinal side effects (constipation, dry mouth, nausea, vomiting, weight loss), and neuropsychiatric side effects (anxiety, tremor, and dizziness) (15).

1.1.1 Environmental Monitoring

Fluvoxamine can be eliminated from the body without being metabolized and as a result of this elimination, it can reach aquatic environments. In a study conducted in the USA, fluvoxamine has been measured as 0.7–4.6 ng/L in surface water (16).

In another study conducted in the hospital sewage system in Greece, the maximum amount of fluvoxamine has been measured as 511 ng/L (17). In addition, fluvoxamine has been detected in organisms living in aquatic systems. In a study conducted in the USA, fluvoxamine has been measured as 0.83 ± 0.04 ng/L in shark plasma (18).

1.1.2 The Other Studies on Fluvoxamine

Fluvoxamine can also be used in the treatment of cancer. Studies have shown that it strongly inhibits actin polymerization. It is a necessary process for cancer cell migration and invasion (19). Fluvoxamine successfully prevents the development of focal adhesions and invasion of human Glioblastoma multiforme (GBM) cells. Additionally, fluvoxamine reduced focal adhesion kinase (FAK) and phosphatidylinositol-3-kinase protein kinase B. Also, daily fluvoxamine therapy in mice with hGICs prevents tumor cell invasion and increases mouse life. Their

results imply that it can be used for anti-invasion therapy of these cells and can be used safely for a long time (19).

Fluvoxamine's ability to treat ulcers has been studied in rats. According to reports, fluvoxamine possesses antiulcer qualities (20). Indomethacin harms the stomach by preventing the production of cytoprotective PG as well as oxidative and antioxidant responses such as MDA, MPO, GSH, and NO. Fluvoxamine appears to have antiulcer action in stomach tissues treated with indomethacin. It achieves this action by activation of the antioxidant system (20).

In another study, the beneficial role of fluvoxamine on monoamine levels has been demonstrated in particular brain regions (21). It produces its effect by preventing the production and secretion of monoamines and neurotransmitters such as serotonin and dopamine. This finding is important since many neurodegenerative diseases are related to the instability of dopamine and serotonin levels in the brain. Thus, early-stage Parkinson's disease patients may benefit from using fluvoxamine to prevent serotonergic/dopaminergic disruption, which worsens parkinsonism (21).

Fluvoxamine is a widely available and inexpensive drug. The effect of fluvoxamine has been tested on corona (COVID-19) virus infection. It has been shown that it decreases the symptoms of patients getting COVID-19, and fluvoxamine has also an anti-inflammatory effect since it is an agonist for the sigma-1 receptor. It reduces platelet aggregation and mast cell numbers in patients suffering from the coronavirus (8).

1.1.3 Metabolism of Fluvoxamine

The main way of fluvoxamine metabolism includes oxidative demethylation (which accounts for 42 to 50% of total fluvoxamine metabolism), oxidative deamination (15 to 23 percent), =N-O bond breakage (12%), and N-acetylation (8%). Hepatic cytochrome P450 (CYP) is responsible for mediating the first three of these metabolic processes (13).

As a result, following oral administration of [^{14}C] fluvoxamine, the majority of the fluvoxamine (94%) is recovered in the form of metabolites in the urine, with just trace levels of the original molecule (<4%) (13,22). Nine of the eleven urine metabolites form 85 percent of total urinary radioactivity (22).

The isoenzymes, CYP2D6 and CYP1A2 metabolize fluvoxamine. It doesn't have any pharmacologically significant active metabolites (23). Fluvoxamine inhibits CYP1A2, CYP2C19, CYP3A4, and CYP2D6 (24).

In humans, fluvoxamine is primarily metabolized to fluvoxamino acid. It is produced by oxidative demethylation of the aliphatic methoxy group from fluvoxamino alcohol (Figure 1.2) (25).

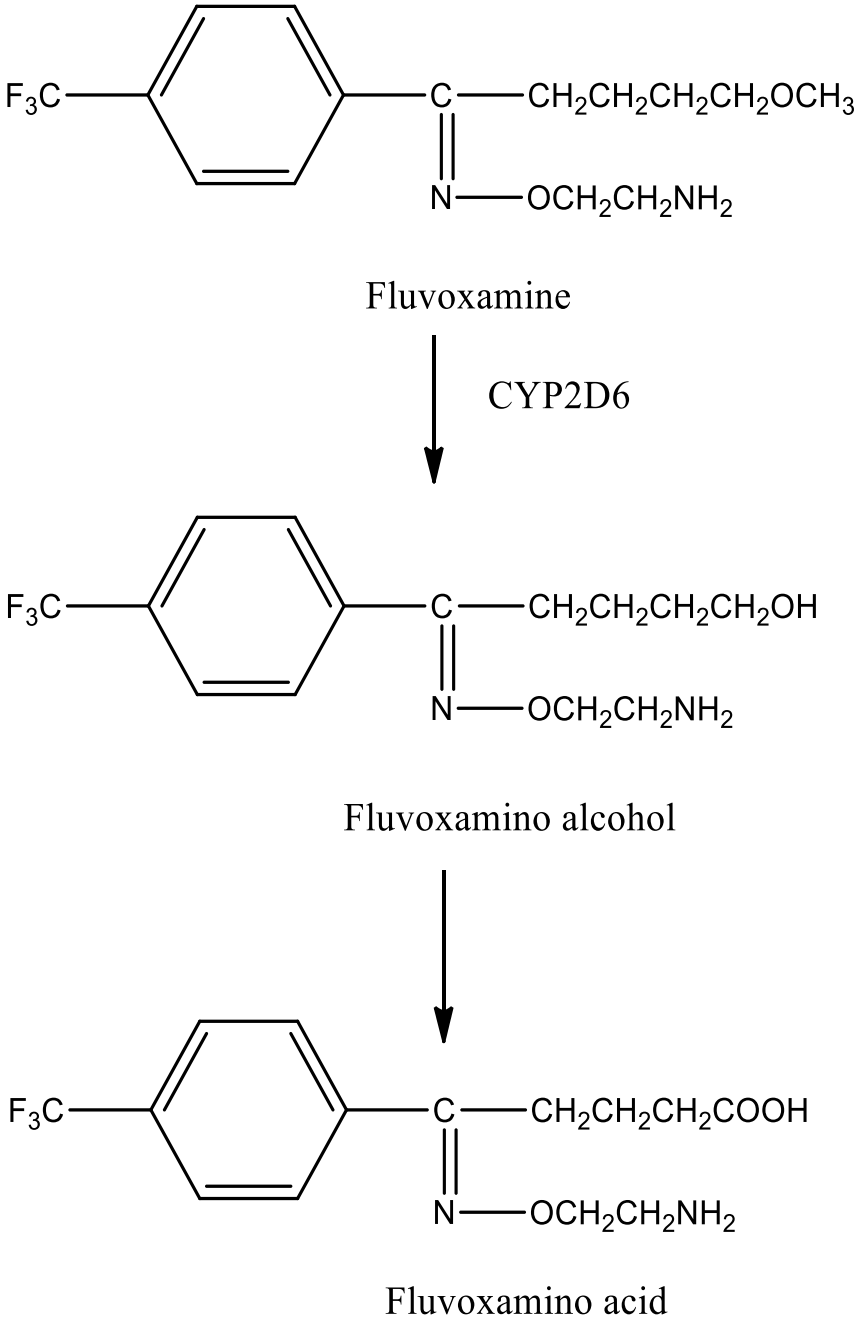

Figure 1.2 Two metabolites of fluvoxamine (26).

1.2 Biomarkers

Almost any parameter that reflects a biological system's contact with a possible danger, which could be chemical, physical, or biological can be considered as a biomarker. The measured parameter can be a physiological parameter, cellular parameter, biochemical parameter, or molecular parameter (27).

Biomarkers are used in medication development, clinical trials, and therapy assessment procedures for a variety of reasons. Biomarkers can help in the selection of candidate molecules for clinical trials, as well as the understanding of their pharmacology and the characterization of the disease subtypes for which therapeutic intervention is most appropriate (28).

Biomarkers give information about exposure to complex mixtures of contaminants in the environment. They express the synergistic, cumulative, or antagonistic effects of chemicals on biological systems (29). Any influential factor's spatial and temporal variations are also taken into account. Pollutant-organism interactions are represented pharmacologically and toxicologically by biomarkers. Furthermore, the negative consequences of both the original chemical and its metabolites are evaluated by biomarkers (29).

Furthermore, biomarkers exhibit a rapid reaction and great sensitivity to environmental impact, suggesting that they could be employed as an "early warning system". Finally, they provide critical information about biological responses to contamination in the environment. This cannot be accomplished just using traditional environmental monitoring techniques. Biomarkers can help to build bridge in the gap between these traditional methods by correlating cause (pollution) and effect (biological response) (30).

The other advantage of using biomarkers includes the identification of the impact of not only parent chemicals but also their metabolites and their total effects on the organism, tissue, and cell (31).

1.2.1 Biomarkers in Fish

Physiological, biochemical, and molecular effects of chemicals are the parameters used in the evaluation of the effects of chemicals on fish. These effects can be followed in the level of enzyme activity, protein level, and gene expression in fish (32).

Each fish biomarker is assessed for its possible application in environmental risk assessment (ERA) programs. Phase I enzymes such as hepatic 7-

ethoxyresorufin-O-deethylase (EROD) activity, biotransformation products such as biliary polycyclic aromatic hydrocarbons metabolites, reproductive system indicators such as plasma vitellogenin level (VTG), and genotoxic indicators such as hepatic DNA adducts are accepted as the most useful fish biomarkers for ERA (33).

Fish have been employed as a bioindicator in recent years due to their sensitive reactions to pollution in the aquatic environment. These fish reactions are now quantifiable because of biomarkers. It is well recognized that the cytochrome P450 system is crucial to the biotransformation of xenobiotics, or their oxidative metabolism, which triggers reactions in fish. CYP1, CYP2, and CYP3 are a few of the cytochrome P450 families involved in the metabolism of xenobiotics (34).

One of the most important aspects of using biomarkers to assess environmental health is correctly identifying the stress agent. Changes in biomarker measures can be caused by environmental toxins, but they also frequently reflect other factors such as organism health and seasonal fluctuations. The robustness of the biomarkers selected is evaluated with many parameters (e.g., reliability, environmental relevance, and repeatability) (Figure 1.3) (35).

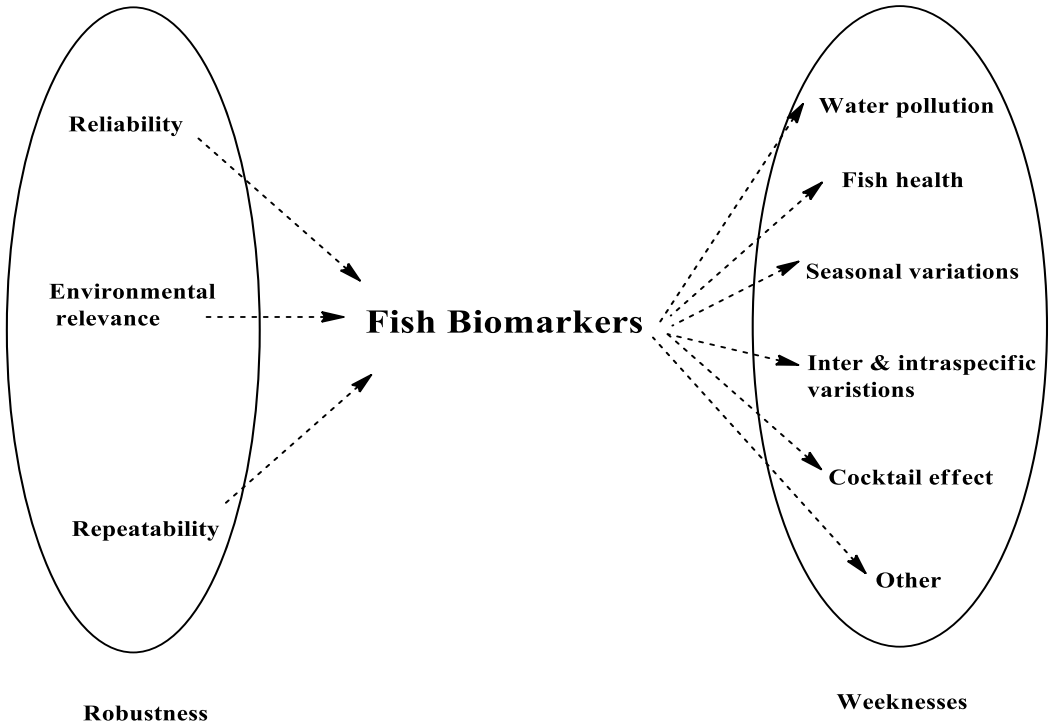

Figure 1.3 The reliability and restrictions of applying fish biomarkers to assess water toxicity (35).

1.3 Biotransformation

Biotransformation is the enzymatic conversion of a lipid-soluble xenobiotic substance into polar and less lipid-soluble metabolites that can be excreted. Both "detoxication" and "toxication" processes can occur during biotransformation (36). The liver is the primary organ involved in biotransformation, although it can occur in many other tissues, including the skin, kidneys, lungs, and gut (37).

Almost all vertebrate groups have enzyme systems related to biotransformation, however, mammals have the most comprehensive records of these systems. Early research on fish detoxication systems seemed to suggest that they were unable to undergo microsomal oxidations (38) or conjugations (39).

Today, it has been well known that fishes contain mixed-function oxidase systems, which often appear to have lower specific activity than analogous mammalian systems (40).

In the first stage of biotransformation, xenobiotics are metabolized by the enzymes that reside in the cell's microsomal region and are activated by reduced nicotineamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (41,42). Many xenobiotics are known to be inducers of mixed-function oxidase activity in addition to being substrates for those enzymes (5).

Phase I and Phase II biotransformation processes are the two types of biotransformation reactions catalyzed by enzymes (Table 1.1). The majority of oxidative phase I reactions are catalyzed by monooxygenases. Most of these reactions are cytochrome P450 (CYP) dependent. Endogenous molecules including fatty acids, some hormones, and retinoids, and exogenous molecules including drugs, solvents, industrial chemicals, and environmental contaminants are oxidatively metabolized by the CYPs. Phase II reactions include conjugation reactions of xenobiotics (43).

Table 1.1 Examples of Phase I and Phase II reactions (44).

Action	Enzyme	Substrate/Cofactor
Phase I		
Oxidation	Cytochrome-p-450	O_2
Hydroxylation		
Sulphoxidation		
Dealkylation		
Azoreduction	Cytochrome p-450 reductase	H^+
Nitroreduction		
Co-oxidation	Peroxidase	H_2O_2
	Catalases	
Hydrolysis	Esterases	H_2O
	Epoxide hydrolases	
Hydration	Carbonic anhydrase	$CO_2 + H_2O$
PhaseII		
Sulphation	Sulphotransferase	PAPS + ATP
Glucuronidation	Glucuronyl transferase	UDP-GA + ATP
Acetylation	Acetyl transferase	Acetyl-CoA + ATP
Methylation	Methyl transferase	SAM + ATP
Glutathione Conjugation	Glutathion transferase	Glutathione + ATP

1.3.1 Cytochrome P450s

Cytochrome P450-dependent monooxygenases are found mostly in the endoplasmatic reticulum (ER) and mitochondria of all organs in all vertebrates including fish (45). They belong to the phase I enzyme family. Their main function is to open or add functional groups for phase II conjugation reactions (Figure 1.4). Lipophilic xenobiotics are generally converted to more water-soluble molecules in Phase I reactions, which is the initial step for the excretion of chemicals. However, some of the metabolites produced in these reactions are very reactive, which can lead to increased toxicity and carcinogenicity at the end (46).

Figure 1.4 General cytochrome P450 catalyzed reaction (47).

The Soret peak, created by light absorption at wavelengths near 450 nm, is a distinctive property of cytochrome P450s, the name cytochrome P450 comes from the fact that it is a pigment with a 450 nm absorbance when it is reduced with sodium dithionate and gassed with CO (48).

The cytochromes P450 are a large multigene family of heme thiolate proteins that catalyze xenobiotic molecules and endogenous molecules (49). Different in vitro techniques can be used to follow activity changes in the cytochrome P450s (50).

More than 20000 P450 sequences have been found in the superfamily of heme-containing proteins known as cytochrome P450 (51). The human genome carries 57 cytochrome P450 genes and there are 18 mammalian CYP families. Xenobiotics are mostly catalyzed by the CYP1A, CYP2B, CYP3A, CYP2C, and CYP2E subfamilies of cytochrome P450s (52,53).

Selective serotonin reuptake inhibitors (SSRIs), one of the more recent antidepressants, is a powerful inhibitor of numerous isozymes of CYPs. Because of the burgeoning and frequently confusing literature on antidepressant-induced inhibition of CYPs, it is critical to put real and perceived distinctions and similarities between each pharmaceutical treatment into a proper context (54).

There are polymorphic cytochrome P450s in human populations that are connected to variations in enzyme activity (55). It has been proposed that genotyping of CYP enzymes may increase the effect of various medication treatments by adjusting drug doses according to individual genetic profiles. Variability in the drug metabolism rate can change the amounts of active chemicals (56).

At its most basic level, cytochrome P450s comprise heme in the ferric (Fe^{+3}) form (57). The cytochrome P450 enzymes add one oxygen atom to the molecule using NADPH as an electron donor (Figure 1.5). Electrons are transferred to cytochrome P450 from NADPH by cytochrome P450 reductase (57). Ferric iron is converted into ferrous form and one atom of oxygen is reduced to water the other is transferred to substrate in a cytochrome P450 catalyzed reaction (57).

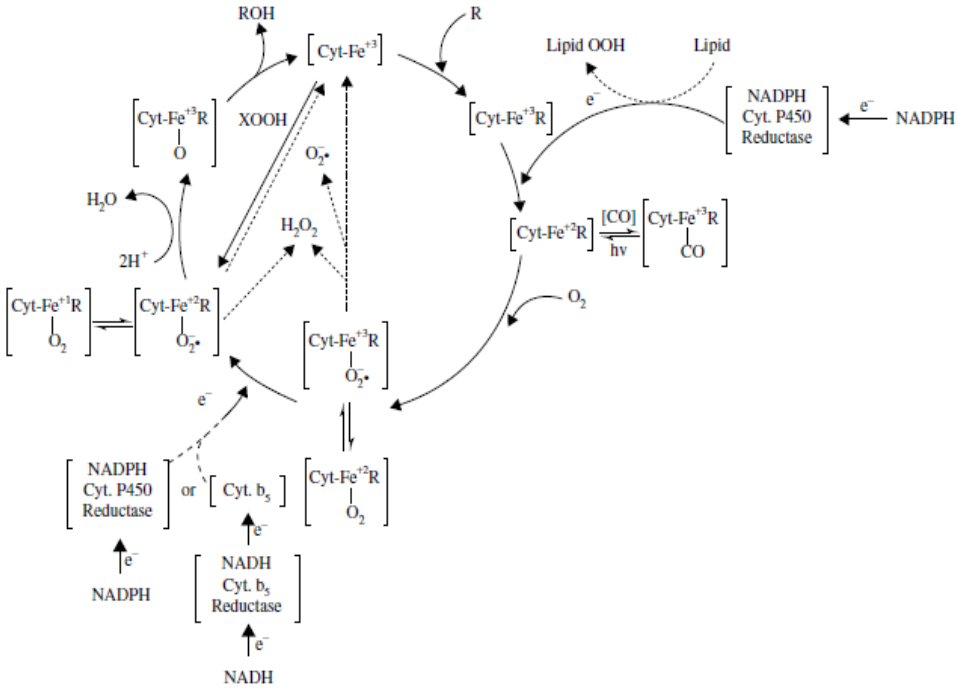

Figure 1.5 The sequence of events for P450s (58).

1.3.1.1 Cytochrome P4501A

Cytochrome P450 1A (CYP1A) is one of the cytochromes P450 subfamilies in humans and fish responsible for the bioactivation reactions of some molecules. CYP1A1 and CYP1A2 are the two functional genes found in this subfamily, and both are well-conserved across species (59). The hydroxylation and oxidation of aromatic substances are catalyzed by CYP1A (60).

About 9% of therapeutic medications, such as antidepressants, antipyretics, antipsychotics, analgesics, and anti-inflammatory pharmaceuticals are bio-transformed by CYP1A (60,61).

The Ah receptor is the receptor that regulates the expression of the CYP1A gene (45,62–65). Induction is started when a particular xenobiotic binds to a protein complex that contains the Ah receptor and the heat-shock protein 90 (HSP 90). After that, the Ah receptor attaches to another protein called aryl hydrocarbon receptor nuclear translocator (ARNT), which allows it to be moved to the cell nucleus, where ARNT binds to a particular region of the DNA called the xenobiotic regulatory element. Transcription factors have now access to the CYP1A gene's promoter region and allow messenger RNA production (45,65).

Many organic compounds, such as PAHs, PCBs, and dioxins are catalyzed by CYP1A. In the presence of these substances, the amount of CYP1A increases, which can be seen in both enzyme activity levels and the amount of protein. The spectrofluorometric assessment of 7-ethoxyresorufin O-deethylase (EROD) activity catalyzed by this enzyme determines the level of cytochrome P4501A (66).

1.3.1.2 Cytochrome P4503A

CYP3A isozymes have a role in the metabolism of 45–60% of commonly used medications and other substances such as steroid hormones, poisons, and carcinogens (67). CYP3A4, 3A5, and 3A7 are members of CYP3A in humans. There are many structurally varied xenobiotics and endogenous molecules metabolized by CYP3A. Many medications are metabolically eliminated by the human CYP3A subfamily than any other biotransformation enzyme. Because the CYP3A enzyme is found in the liver and small intestine, it plays a role in first-pass and systemic metabolism. The expression of CYP3A in the liver and small intestinal tissues varies by up to 40-fold (68).

Since they have extremely broad substrate specificities for both endogenous and exogenous substrates, including steroids, bile acids, eicosanoids, retinoids, xenobiotics like medicines, and procarcinogens, CYP3A enzymes are among the most functionally flexible types of CYPs (69–73). According to certain theories, the topologies of all CYP enzymes are similar, particularly in terms of structurally conserved sections like the heme-binding domain, oxygen-binding region, and key locations connected to redox interactions (74).

The CYP3 family is divided into four subfamilies: CYP3A, CYP3B, CYP3C, and CYP3D in fish (75). CYP3A27 and CYP3A45 are two types of CYP3A found in rainbow trout (76–78).

1.3.2 Glutathione S-Transferase

The glutathione S-transferases (EC 2.5.1.18) are a family of proteins whose enzymic function is to combine reduced glutathione (GSH) with a variety of electrophilic chemicals that are frequently metabolized to mercapturates and eliminated in bile or urine (Figure 1.6) (79).

These dimeric enzymes are widely dispersed and make up roughly 2–4% of the liver's total cytosolic proteins (80).

Although several mammalian GST isoforms can be induced, the regulation of these proteins is complicated, and their expression is tissue- and

developmentally-specific (e.g., some are expressed in the brain and testis only). More than 100 xenobiotic substances have been demonstrated to operate as inducers, and several GSTs are susceptible to hormonal influences (growth hormone, thyroxine, and insulin) (81).

GSTs are crucial cellular proteins that guard against electrophilic poisons and have roles in several physiological processes such as hormone production and tyrosine catabolism (82). GST crystal structures have demonstrated that catalytically competent substrate binding to the active site is necessary for enzyme activity. Due to their distinctive active sites, GST enzymes from various subfamilies frequently allow the prediction of substrate preference. Although specificity mechanisms have not yet been fully understood, crystal structure studies have aided to understand how GSTs catalyze various types of reactions (82).

Glutathione S-transferases (GST) have considerable role in phase-II reactions of endogenous and exogenous molecules. GST function and regulation have an impact on cell development, oxidative stress, disease progression, and prevention (83). They also involve in the metabolism and detoxification of a broad range of xenobiotics, including medicines (84). Glutathione S-transferases are enzymes performing the conjugation process of chemicals in the presence of glutathione (Figure 1.6) (82).

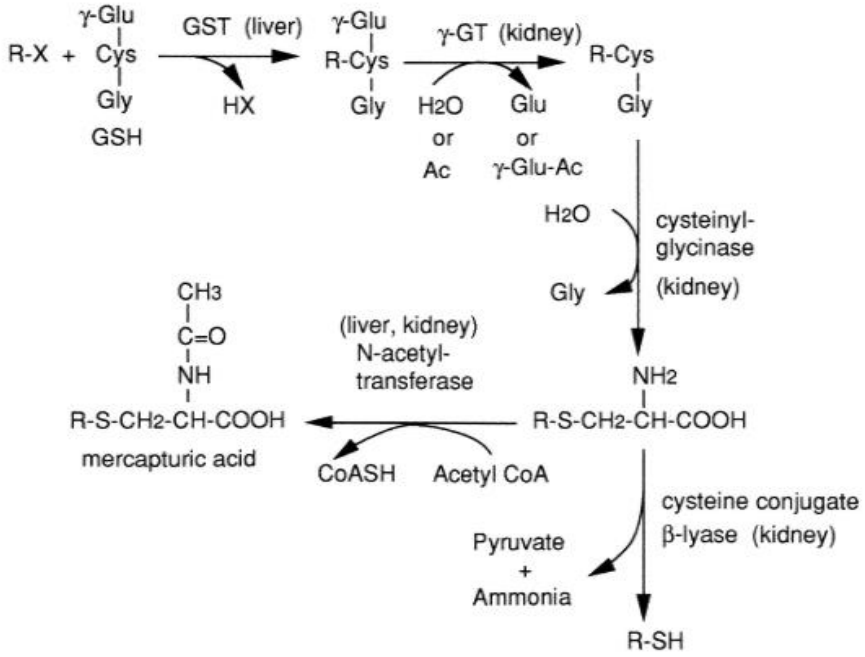

Figure 1.6 Conjugation reaction catalyzed by glutathione S-transferase (85).

1.4 The Oxidative Stress and Antioxidant Enzymes

Oxidative stress is defined as an increase in the generation of reactive oxygen species (ROS) and disruption in the redox balance of the cell. The superoxide anion radical, alkoxyl, and hydroxyl radicals are examples of reactive oxygen species (86). Oxidative stress is characterized by a diminished capacity of endogenous systems to fight against oxidative attack as a result of increased ROS formation or a decrease in antioxidant protective function. Its severity has been connected to several diseases, including cardiovascular diseases, cancer, and aging (87).

Reactive oxygen species are generated by both endogenous and exogenous factors including alcohol, tobacco smoking, pollutants, industrial solvents, polyaromatic hydrocarbons, drugs, pesticides, and radiation (88).

An antioxidant is a compound that significantly reduces or prevents the oxidation of an oxidizable substrate (89). During biotransformation events, oxygen radicals can develop. The antioxidant system neutralizes the effects of these radicals. While antioxidant compounds such as vitamins C, vitamin E and glutathione play a part in eliminating the effects of oxygen radicals, antioxidant enzyme activities also participate in the removal of radicals. Catalase, glutathione reductase, and glutathione peroxidase enzyme activities are among them) (Figure 1.7 (90,91).

Many pollutants (or their metabolites) have the potential to induce oxidative stress-related damage (92). Increased incidence of idiopathic lesions and neoplasia among fish living in polluted habitats, for example, has been linked to increased oxidative stress caused by pollution exposure (92). The superoxide anion radical (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are the reduction products of molecular oxygen (O_2), which are extremely powerful oxidants capable of reacting with essential biological macromolecules, potentially causing to enzyme deactivation, lipid peroxidation (LPOX), DNA damage, and cell death (92).

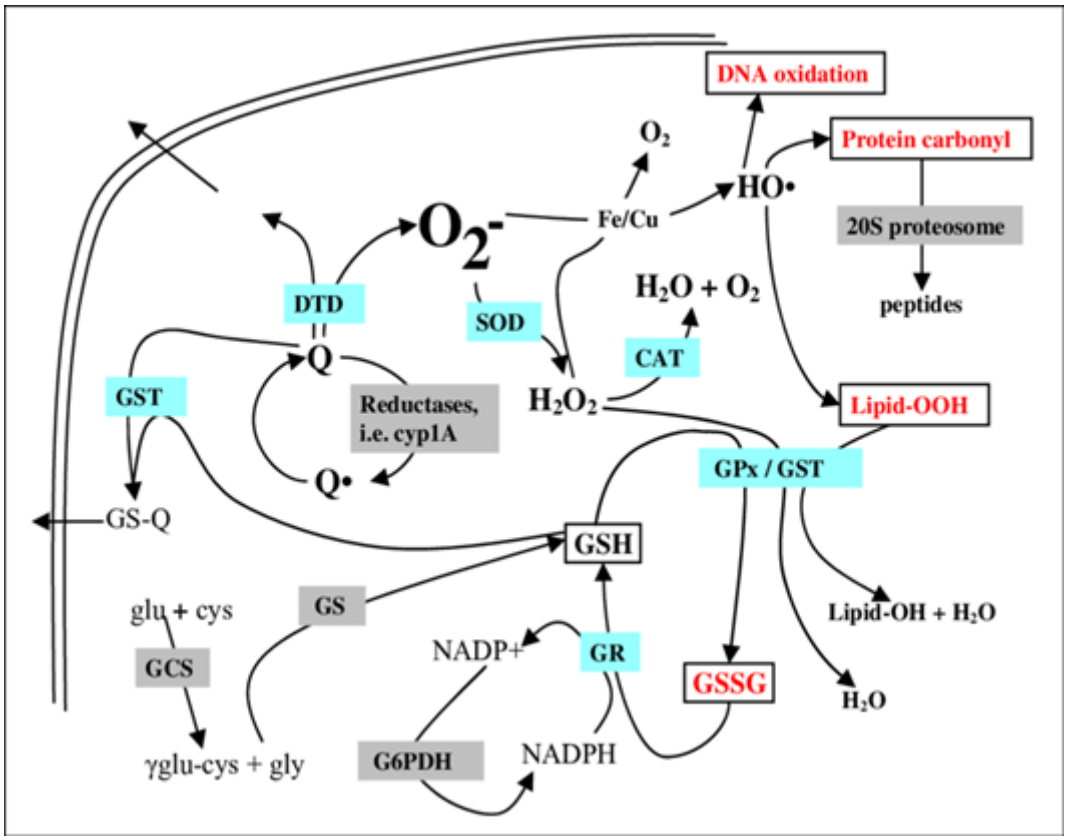

Figure 1.7 Enzymes found in oxidative stress and their role (93).

1.4.1 Catalase

Catalase (CAT) is one of the antioxidant enzymes. It contains a heme group. It preserves cells from the harmful impact of hydrogen peroxide. It has been linked to several physiological and pathological disorders in humans (94). Catalase has the greatest turnover rate and catalyzes more than a million molecules of hydrogen peroxide every second (95,96).

Catalase is a tetrameric protein containing more than 500 amino acids in each subunit. It can interact with hydrogen peroxide with porphyrin heme (iron) groups. Its optimum pH range is between 4 and 11 (97–100), which is a rather wide range. The peroxisome is a cellular organelle with a bipolar environment where catalase is typically found (101). Catalase has been used in various industries, including paper, food, textile, pharmaceutical, and, as one of its newest fields of application, bioremediation because it is primarily present in all species (aerobic and anaerobic) (102–105).

Many mammalian and non-mammalian cells include catalase. The liver and kidney organs, as well as mammalian erythrocytes, contain the highest quantities of it (106–108).

The enzyme catalase is the primary control mechanism for hydrogen peroxide metabolism (109,110). High levels of it in red blood cells give protection against high amounts of hydrogen peroxide (H_2O_2) in these cells. The activities of catalase are poor in the other tissues like the pancreas and heart (111).

Catalase is an oxidoreductase that regulates intracellular hydrogen peroxide levels by converting harmful hydrogen peroxide to water. When catalase is present, hydrogen peroxide is broken down by two reaction series. One H_2O_2 molecule oxidizes heme to an oxyferryl species (Figure 1.8). This results in a porphyrin cation radical by removing one oxidation equivalent from the iron and one from the porphyrin ring (Por). Then, the second hydrogen peroxide interacts with compound I. Compound I is oxidized to produce free enzymes again, water, and oxygen. Two molecules of water and one molecule of oxygen are produced as a result of a catalase-catalyzed reaction (112,113).

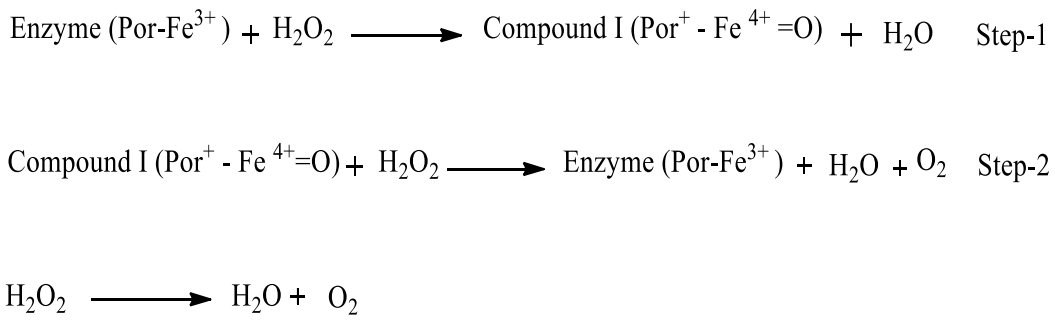

Figure 1.8 General reaction steps of catalase (113).

1.4.2 Glutathione Reductase

Glutathione reductase (EC 1.6.4.2) is a protein with two identical subunits. It has a flavin adenine dinucleotide group in its structure as a redox-active prosthetic group, which transfers reducing equivalents from NADPH to the protein's disulfide group, which then reduces the substrate GSSG (114). It catalyzes the reduction of glutathione disulfide by NADPH. The reaction catalyzed by this enzyme is given in Figure 1.9 together with its role in the antioxidant defense system. In both eukaryotes and prokaryotes, glutathione reductase is a highly active and widely distributed enzyme. It has been linked to some metabolic activities that are crucial to the cell's survival (115).

Glutathione reductase is a homodimeric flavoprotein with a prosthetic group of two FAD molecules that can be reduced by NADPH. The dimeric enzyme's molecular weight is predicted to be between 100 and 120 kDa (116).

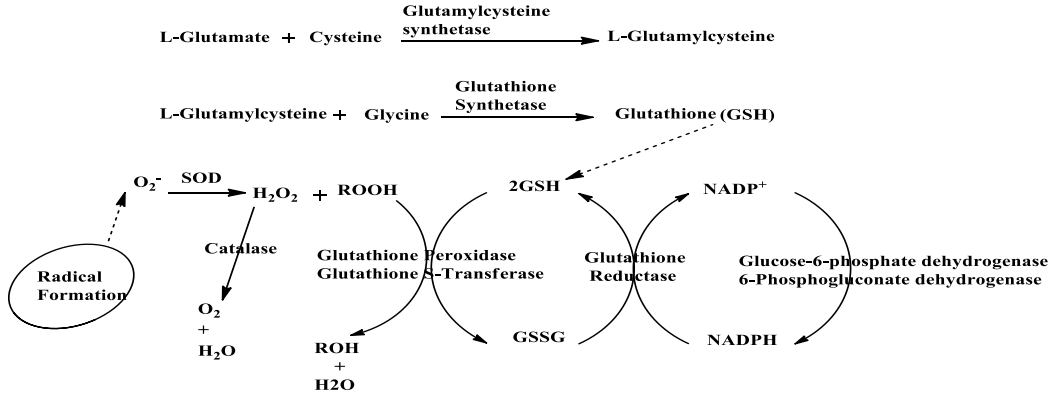

Figure 1.9 Role of glutathione reductase in the antioxidant system (116).

There are two phases in GR catalytic cycle: a reductive half-reaction and an oxidative half-reaction. FAD, GR's prosthetic group, is reduced by NADPH during the reductive half-reaction, and reducing equivalents are transferred to a redox-active disulfide. The resultant dithiol combines with glutathione disulfide in the oxidative half-reaction, reducing the final electron acceptor GSSG to two GSH at the active site of GR (117).

Glutathione reductase has been related to several diseases and ailments, including the development of anxiety (118), and is well-recognized to play a significant part in the response to oxidative stress in both plants and mammals (119). Glutathione reductase is an enzyme that was purified and characterized by a variety of plants, fish, and creatures (120,121).

Maintaining the level of reduced cellular GSH is dependent on the glutathione redox cycle and GR is a necessary protein for that cycle. For the reduction of oxidized glutathione, more than one step is necessary. After GR is reduced by NADPH, it reacts with GSSG to create GSH and GRred-SG complex under the exchange in the disulfide bond. A second GSH molecule is created and the GRred-SG complex undergoes some electron reformation, reverting to its oxidized state (Figure 1.10) (114,122).

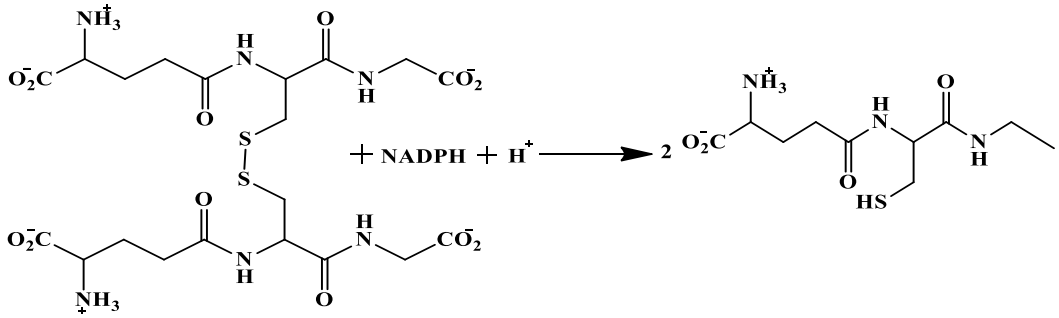

Figure 1.10 General reaction of glutathione reductase (114).

1.5 Rainbow Trout (*Oncorhynchus mykiss*)

The rainbow trout (*Oncorhynchus mykiss*) is a bony fish native to North America. It was later distributed to other countries since it is an economically important fish. It is a member of the Salmonidae family. Rainbow trout have very high environmental adaption potential (123). Rainbow trout can endure a wide range of water temperatures and other environmental factors such as water quality, but they prefer well-oxygenated water and temperatures between 13 and 18 degrees Celsius (124).

There are several cold freshwater sources where rainbow trout can be raised, and it is relatively simple to modify the environment for breeding, marketing, and other purposes (125). Its advantages include adaptability to the farming environment, reproducibility, and resistance to diseases (126). Based on their physiological requirements, their gastrointestinal tract (GI) can quickly adapt to environmental changes (127).

In addition to its economic significance, rainbow trout have been widely used as a model organism for research in fields as varied as cancer studies, toxicology studies, immunology studies, fish physiology studies and the studies related with fish development and evolution (128). Several cell rainbow trout cell lines behave in developed and used in genetic studies (129–132). Rainbow trout is a suitable alternative model for molecular and biochemical investigations requiring larger tissues since it has big body size compared to the other model fish species like zebrafish or medaka (128).

Several immunochemical, catalytic and nucleic acid hybridization techniques have been used in fish, and methods have been used to confirm that more than 30 fish species contain the gene CYP1A (133). The two members of the CYP1A subfamily have been found in rainbow trout (*Oncorhynchus mykiss*) and designated as CYP1A3 and CYP1A1, respectively (134). The amino acid sequence for CYP1A in rainbow trout has been found to resemble their mammalian equivalent by 60% (135,136)

2 AIM AND SCOPE OF THE STUDY

Fluvoxamine is a drug that is commonly used to treat depression. The detection of this chemical in aquatic ecosystems today shows that sewers are used to transfer it to water sources. In recent years, fish have been exposed to numerous drugs used to treat people. The effects of these chemicals on fish are among the issues that have been discussed in recent years. Due to the absence of certain enzymes or changes in their activity, fish might react with substances in different ways.

In this study our aim was:

1. to study the effect of fluvoxamine on Phase I enzyme activities, CYP1A and CYP3A.
2. to study the effect of fluvoxamine on Phase II enzyme activity, glutathione S-transferase.
3. to study the effect of fluvoxamine on antioxidant enzyme activities, catalase and glutathione reductase in rainbow trout liver.

3 MATERIALS AND METHODS

3.1 Materials

Dimethyl sulfoxide (DMSO; $(CH_3)_2SO$; 116743), dipotassium hydrogen phosphate (K_2HPO_4:105101), ethanol (C_2H_5OH; 100983), formaldehyde (CH_2O; 344198), glycerol(1.04092.2500),), glacial acetic acid (CH_3COOH; 100056), hydrogen peroxide (H_2O_2; 108600), potassium dihydrogen phosphate (KH_2PO_4; 104871), potassium chloride (KCl; 104936) and triton X- 100 ($C_{14}H_{21}(C_2H_4O)nOH$; 112298) were bought from Merck KGaA, Darmstadt, Germany. Ammonium acetate ($CH_3CO_2NH_4$; A1542), acetylacetone ($CH_3COCH_2COCH_3$; 00900), ε-amino caproic acid (ε-ACA; $C_6H_{13}NO_2$ A2504), bovine serum albumin (BSA; A7511 or A7888),), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; C7631), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; $C_{10}H_{14}N_2Na_{2O8}.2H_2O$; E5134), erythromycin ($C_{37}H_{67}NO_{13}$; E0774), Folin-Ciocalteu's phenol reagent (F9252), glucose-6-phosphate dehydrogenase (G5885), L-glutathione reduced (GSH; G6013), L-glutathione oxidized disodium salt (GSSG; G4626), N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES; $C_8H_{18}N_2O_4S$; H3375), phenylmethanesulfonyl fluoride (PMSF; $C_7H_7FO_2S$; P7626), perchloric acid ($HClO_4$; 30755) and resorufin ethyl ether ($C_{14}H_{11}NO_3$; E3763) were bought from Sigma-Aldrich, Saint Louis, Missouri, The USA.

Fluvoxamine maleate ($C_{19}H_{25}F_3N_2O_6$; SF8780) was bought from Solarbio Life Sciences (Beijing, China).

Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH; $C_{21}H_{26}N_7Na_4O_{17}P_3$; A1395) and disodium salt of β-nicotinamide adenine dinucleotide phosphate ($NADP^+$; $C_{21}H_{27}N_7NaO_{17}P_3$; A1394) were bought from Applichem (Darmstadt, Germany).

The highest grade of purity was preferred for the other chemicals and obtained from commercial sources.

3.2 Methods

3.2.1 Animals And Treatment

The effect of fluvoxamine on fish was studied in this study. Samples of rainbow trout (*Oncorhynchus mykiss*) were taken from a local fish farm near Bolu (Turkey). Fish samples were divided into 3 groups (Table 3.1). The average gram weight of fish (average weight ± SEM) was 266 g ± 6 for 24 rainbow trout

(Oncorhynchus mykiss). The fish was kept in a 200 L tank (the tank's total capacity was 250 liters). Aeration equipment was used to keep the tanks aired at all times (air pump and stone). A fluorescent lamp and a timer were used in the laboratory to create a 12-hour light/12-hour dark-light cycle. Fluvoxamine was given to the fish in two dosages. During the chemical application period (96 hours), all fish was left hungry to eliminate any differences that may occur due to feeding, taking into account changes in eating patterns such as underfeeding and overfeeding. Every 12 hours, the aquarium water was changed and the chemical application was repeated. The first group was the control group. The second group was named the 10 µg/L of fluvoxamine administration group. The third group, which was given fluvoxamine at a concentration of 50 µg/L was named 50 µg/L of the fluvoxamine administration group. The Animal Experiments Local Ethics Committee of Bolu Abant Izzet Baysal University Medical School granted ethical permission for animal-based studies involving their treatment and care (Process number: 2021/12**)**. Liver tissues were taken at the end of the 96 hours. The tissues were preserved in the -80 ºC deep freezer after they were labeled and packaged until the microsomes and cytosol preparation stages.

Table 3.1 Animal groups

Groups		
Group-1 Control 8 Fish	**Group-2** 10 µg/L fluvoxamine 8 Fish	**Group-3** 50 µg/L fluvoxamine 8 Fish

3.2.2 Preparation of Microsomes and Cytosols from Rainbow Trout Liver

The rainbow trout liver microsomes and cytosols were prepared using the procedure described by Arınç and Sen (137). Tissues were taken from the deep freezer and washed with distilled water to remove any extra blood from the livers, then with 1.15 % KCl. All of the following steps were done in an ice bath at a

temperature of 0-4 °C. The liver tissues were cut with surgical scissors into little pieces.

It was then homogenized in a homogenization solution containing 1.15 % KCl, 0.25 mM of Ɛ-ACA, 0.1 mM of PMSF, and 1 mM EDTA. A Potter-Elvehjem glass homogenizer and the drill were used in the homogenization of tissues. The tissue suspension was centrifuged at 10000×g for 20 minutes at 4°C using a Sigma 3-30K Refrigerated Centrifuge with a 12156 rotor (Saint Louis, Missouri, USA). The upper phase was taken and filtered through cheesecloth. The pellet was discarded because it contained unwanted organelles. The collected tissue fraction was centrifuged for 60 minutes at 45000 rpm (105000×g) using a Beckman Optima L- 90K ultracentrifuge with a 70.1 Ti rotor (Beckman Coulter Inc., Fullerton, California, USA).

After centrifugation, the upper phase was cytosol and taken into Eppendorf tubes and stored in a deep freezer at -80ºC until used. The pellet containing microsomes was washed with 1.15 % KCl solution containing 1.0 mM EDTA, pH 7.4. Then centrifugation step was repeated at the same speed for 50 minutes. At the end of the centrifugation period, the upper phase was discarded. The collapsed part was microsome and manually homogenized in resuspension solution (1.0 mL of 25 % glycerol containing 1.0 mM EDTA pH 7.4 solution for each gram of fish tissues) using a Potter-Elvehjem glass homogenizer. Microsomes were separated into Eppendorf tubes after homogenization and stored in a -80 ºC deep freezer after being gassed with nitrogen.

3.2.3 Measurement of Protein Concentration

Concentrations of microsomal and cytosolic proteins were measured by using the Lowry method (138). Liver cytosols were re-centrifuged at 20000 rpm for 30 minutes at 4°C with Sigma 3-30K Refrigerated Centrifuge before the determination of protein content. The upper phase was taken and used for the determination of cytosolic protein. The centrifuged cytosols were kept at -80 °C until the measurement of cytosolic enzyme activities. Rainbow trout fish cytosolic and microsomal samples were diluted 75 times. Five different concentrations of standards (0.020, 0.050, 0.100, 0.150, and 0.200 mg/mL BSA) were prepared from stock (1 mg/mL stock) standard solution. After that, 2.5 mL of alkaline copper reagent including 2% copper sulfate, 2% sodium potassium tartrate, and 20% Na_2CO_3 in 0.1 N NaOH was added into all tubes. All the tubes were incubated for

10 min at room temperature. At the end of the incubation period, Folin-Ciocalteu's phenol reagent (0.25 mL of 1.0 N) was added and tubes were mixed and kept at room temperature for 30 minutes. The color intensity was measured by using a spectrophotometer (Hitachi U-2900 UV-Vis Double Beam Spectrophotometer) at 660 nm. The total protein concentration was calculated from the BSA standard calibration curve.

3.2.4 Measurement of 7-Ethoxyresorufin-O-Deethylase Activity

The activity of the 7-ethoxyresorufin-O-deethylase connected with cytochrome P4501A in fish liver microsomes was measured using Burke and Mayer's (1974) method with some changes (139). In this method, the production of 7-hydroxyresorufin from 7-ethoxyresorufin is measured in the presence of the enzyme and NADPH (Figure 3.1) (140). The applied method was optimized for rainbow trout. The 7-ethoxyresorufin solution was used as the substrate and the resorufin solution was used as a standard.

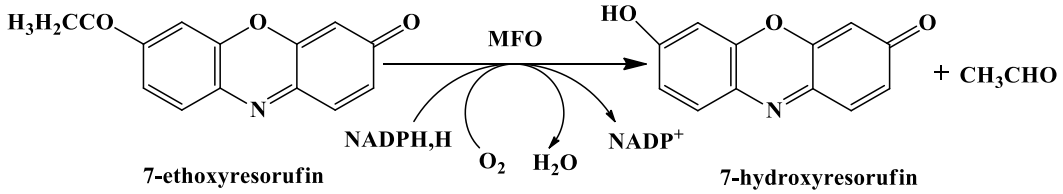

Figure 3.1 7-Ethoxyresorufin O-deethylase reaction (139).

7-ethoxyresorufin stock solution (0.5 mM) was made ready for daily use in DMSO and then 45 μM daily solution was made ready in 0.2 M KPi buffer pH 7.8 containing 0.2 M NaCl. 0.5 mM NADPH generating system was made ready from 2.5 mM magnesium chloride, 14.6 mM HEPES, pH 7.8, 2.5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, and 0.5 mM $NADP^+$. In the preparation of it, the tube content was kept at 37°C for 5 minutes.

The reaction tube contained 100 mM KPi at pH 7.8 containing 100 mM NaCl, 1.2 mg/mL BSA, 0.5 mM NADPH generating system, and 200 μg fish liver microsome. The reaction was started with the addition of 6.5 μM 7-ethoxyresorufin and monitored for 5 minutes in a spectrofluorometer at 535 nm (excitation) and 585 nm (emission) wavelengths (Hitachi F-4500).

Reading the resorufin's fluorescence units allowed us to quantify the enzyme activity. The reaction rate was quantified using a resorufin standard calibration curve.

3.2.5 Measurement of Erythromycin N-Demethylase Activity

Erythromycin N-demethylase activity is typically catalyzed by the CYP3A enzyme. The method described by Cochin and Axelrod (1959) was used to measure the activity of erythromycin N-demethylase (141). The Nash method was used to measure the quantity of formaldehyde (142). In this method, formaldehyde is formed as a product. Most drugs are metabolized by cytochrome P4503A. This method depends on the formation of formaldehyde from erythromycin in the presence of an enzyme and NADPH (Figure 3.2).

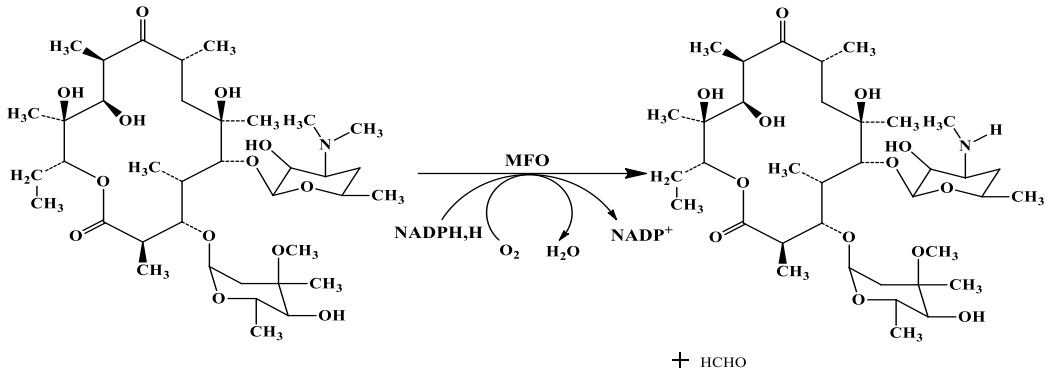

Figure 3.2 Erythromycin N-demethylase reaction(141).

The reaction tube contained 100 mM HEPES buffer pH 7.8, 0.5 mM NADPH generating system, 1.0 mM erythromycin and 4.0 mg microsomal protein in a final volume of 0.250 mL. For all samples, zero-time blank tubes were prepared by adding 0.250 mL of 0.75 N perchloric acid solution before the addition of the NADPH.

Enzyme activity was started with the addition of 0.0375 mL of NADPH generating system to tubes including zero time blank tubes. The tubes were kept at 25°C for 15 minutes in a shaker containing water. Then, 0.250 mL of 0.75 N perchloric acid was added to stop the enzyme activity. All the tube content was transferred to Eppendorf tubes. Tubes were centrifuged at 15000xg for 20 minutes at 4°C by Sigma 3-30K Refrigerated Centrifuge.

Formaldehyde standards were prepared from 0.5 mM formaldehyde solution at four different concentrations (0.012, 0.025, 0.050, and 0.100 mM). NASH reagent was prepared daily. 4.62 g ammonium acetate was dissolved in water and 0.06 mL of acetylacetone was added and the solution was completed to15.0 mL by the addition of water and 0.09 mL of glacial acetic acid was added to get NASH reagent. 0.250 mL standards and samples were incubated with 0.188

mL Nash reagent at 50°C for 10 minutes. The yellow color was developed and its intensities in each tube were measured at 412 nm using a spectrophotometer (Jasco V-530 UV/VIS Spectrophotometer). Known formaldehyde concentrations were used as standards in the calculation of enzyme activities.

3.2.6 Measurement of Total Glutathione S-Transferase Activity

Total glutathione S-transferase activity in fish liver cytosols was determined by the spectrophotometric method of Habig *et al.* (1974) (143). The method is based on the formation of a 1-glutathione-2,4-dinitrobenzene conjugate with reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) and in the presence of an enzyme (Figure 3.3).

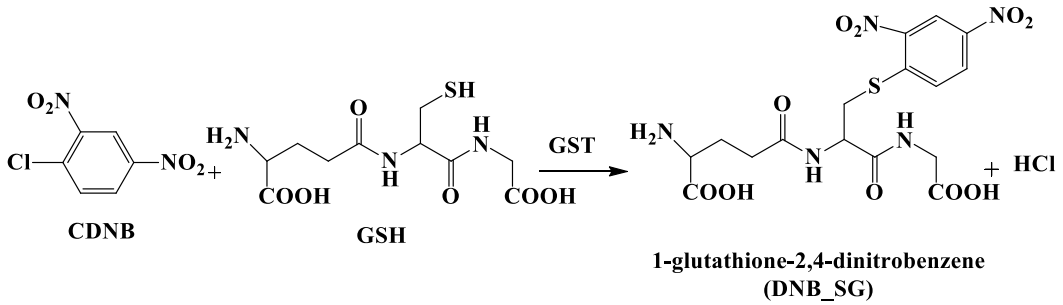

Figure 3.3 Total glutathione S-transferase reaction (144).

In this method, 100 mM KPi pH 7.4, 3 mM GSH, and 0.15 mL diluted enzyme (25 times with 10 mM KPi pH 7.4 phosphate buffer) were used. 1 mM CDNB was added as substrate and the reaction was started with the addition of substrate. The blank tube contained all the content except the enzyme. In place of the enzyme, the same amount of KPi, pH 7.4 was added. The rate of reaction was followed by measuring the product at 340 nm for 2 minutes with a spectrophotometer (Jasco V-530 UV/VIS). Activities were calculated using 9.6 $mM^{-1}cm^{-1}$ as an extinction coefficient (ε340).

3.2.7 Measurement of Catalase Activity

The spectrophotometric method was used in the determination of catalase enzyme activity in fish liver cytosols (145). The presence of oxidative stress can be determined by measuring catalase activity. The method's basis is based on the decomposition of H_2O_2 (Figure 3.4).

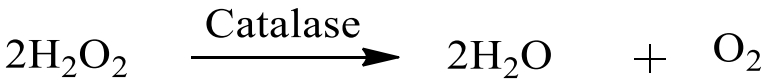

Figure 3.4 Catalase reaction (146).

Rainbow trout cytosols were treated with 1 percent Triton X-100 (10 times) for 10 minutes to assess catalase enzyme activity. Before the experiment, the treated samples were further diluted 25 times with 50 mM KPi pH 7.0.

Reaction tubes contained 2.0 mL of diluted rainbow trout liver cytosol and 150 mM H_2O_2 in a final volume of 3.0 mL. H_2O_2 addition started the reaction. A blank tube was prepared from diluted enzyme and phosphate buffer pH 7.0. Activities were measured at 240 nm for 1 minute by a Hitachi U-2900 spectrophotometer. In enzyme activity calculations, 0.0364 $mM^{-1}cm^{-1}$ was used as the extinction coefficient (ε240).

3.2.8 Measurement of Glutathione Reductase Activity

Glutathione reductase activities were determined in rainbow trout liver cytosol according to the method of Carlberg and Mannervick (121). The reduction of oxidized glutathione to its reduced form is catalyzed by GR and is carried out in the presence of NADPH. One molecule of NADPH is consumed to reduce one molecule of oxidized glutathione according to the equation given in Figure 3.5.

$$ROH + GSSG + NADPH + H^+ \xrightarrow{GR} ROOH + 2GSH + NADP^+$$

Figure 3.5 Conversion of GSSG to GSH (116).

Consumption of NADPH was measured in a typical assay medium (100 mM KPi, pH 7.0, 50 µL of enzyme source, 0.1 mM reduced nicotineamide adenine dinucleotide, 0.5 mM EDTA, pH 7.0, and distilled water at a final volume of 2 mL) at 340 nm in this method. 1 mM glutathione disulfide was used to initiate the reaction. The reaction rate was followed for 5 minutes spectrophotometrically (Hitachi U-2900 UV-Vis Double Beam Spectrophotometer). In the calculation of enzyme activities, 6.22 $mM^{-1}cm^{-1}$ value was used as the extinction coefficient (ε 340).

3.2.9 Statistical Analysis

Enzyme activities were expressed throughout the thesis as mean enzyme activity ± SEM. The LSD (for parametric data) and Mann-Whitney U (for nonparametric data) tests were used in the statistical analysis. SPSS Statistics 21.0 program was used in these analysis. $p \leq 0.05$ was the accepted statistical significance level.

4 RESULTS

The effect of fluvoxamine (two different doses) was determined on CYP1A, CYP3A, glutathione S-transferase, catalase, and glutathione reductase in the liver of rainbow trout. 24 fish were separated into three groups as control, 10 micrograms/L, and 50 microgram/L fluvoxamine administration groups. After 96 hours experimental period, liver tissues were taken. Microsomes and cytosols were prepared and protein concentrations were determined. CYP1A and CYP3A associated 7-ethoxyresorufin O-deethylase (EROD) and erythromycin N-demethylase (ERND) activities were determined in microsomes. The other activities were determined in cytosols. Duplicates and sometimes more measurements were done in all enzyme activities.

4.1 7-Ethoxyresorufin O-Deethylase Activities

Tables 1-3 give the results of protein concentrations of microsomes and 7-ethoxyresorufin O-deethylase (EROD) activities. The average EROD activity of the control group was 13.86±1.74 pmol/min/mg protein, the average EROD activity of 10 microgram/L fluvoxamine administration group was 32.64±9.22 pmol/min/mg protein and the average EROD activity of 50 microgram/L fluvoxamine administration group was 38.67±7.78 pmol/min/mg protein. When the statistical analysis was performed, the EROD activity results of the 10 microgram/L fluvoxamine administration group and the EROD activity results of the 50 microgram/L fluvoxamine administration group were found to be significantly different from the control group (Figure 4.1).

Table 4.1 Protein concentrations of microsomes and EROD activities in the control group.

Sample Number	Protein Concentrations (mg/L)	Average Activity (pmol/min/mg protein)
1	11.75	13.64
2	10.96	10.06
3	10.23	20.48
4	7.15	21.06
5	14.27	15.43
6	9.34	12.53
7	12.31	10.41
8	10.03	7.31
Average Activity±SEM N=8		13.86±1.74

Table 4.2 Protein concentrations of microsomes and EROD activities in 10 microgram/L fluvoxamine administration group.

Sample Number	Protein Concentrations (mg/L)	Average Activity (pmol/min/mg protein)
1	11.67	20.11
2	8.38	14.00
3	10.92	28.44
4	10.45	89.75
5	11.38	51.60
6	10.11	22.76
7	12.52	12.52
8	10.13	21.94
Average Activity±SEM N=8		32.64±9.22

Table 4.3 Protein concentrations of microsomes and EROD activities in 50 microgram/L fluvoxamine administration group.

Sample Number	Protein Concentrations (mg/L)	Average Activity (pmol/min/mg protein)
1	15.00	74.78
2	11.21	35.36
3	10.64	19.06
4	11.09	21.64
5	8.96	24.74
6	13.42	57.52
7	13.46	37.60
Average Activity±SEM N=7		38.67±7.78

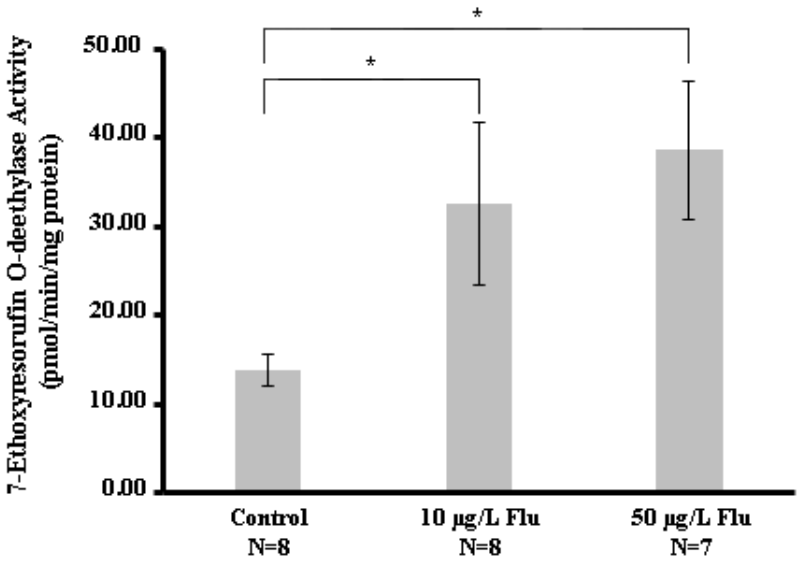

Figure 4.1 EROD activity results.

4.2 Erythromycin N-demethylase Activities

Tables 4-6 give erythromycin N-demethylase (ERND) activity results of rainbow trout obtained from the control and administration groups. The average ERND activity of fish in the control group was 0.094±0.059 pmol/min/mg protein, the average ERND activity of fish in the 10 microgram/L fluvoxamine administration group was 0.032±0.015 pmol/min/mg protein and the average ERND activity of fish in 50 microgram/L fluvoxamine administration group was

0.070±0.014 pmol/min/mg protein. When the statistical analysis was performed, no difference was observed between the groups (Figure 4.2).

Table 4.4 ERND activities in the control group.

Sample Number	Average Activity (pmol/min/mg protein)
1	0.048
2	0.012
3	0.000
4	0.002
5	0.092
6	0.060
7	0.039
8	0.500
Average Activity±SEM N=8	0.094±0.059

Table 4.5 ERND activities in 10 microgram/L fluvoxamine administration group.

Sample Number	Average Activity (pmol/min/mg protein)
1	0.045
2	0.015
3	0.010
4	0.000
5	0.003
6	0.130
7	0.015
8	0.035
Average Activity±SEM N=8	0.032±0.015

Table 4.6 ERND activities in 50 microgram/L fluvoxamine administration group.

Sample Number	Average Activity (pmol/min/mg protein)
1	0.112
2	0.027
3	0.088
4	0.067
5	0.012
6	0.116
7	0.044
8	0.095
Average Activity±SEM N=8	0.070±0.014

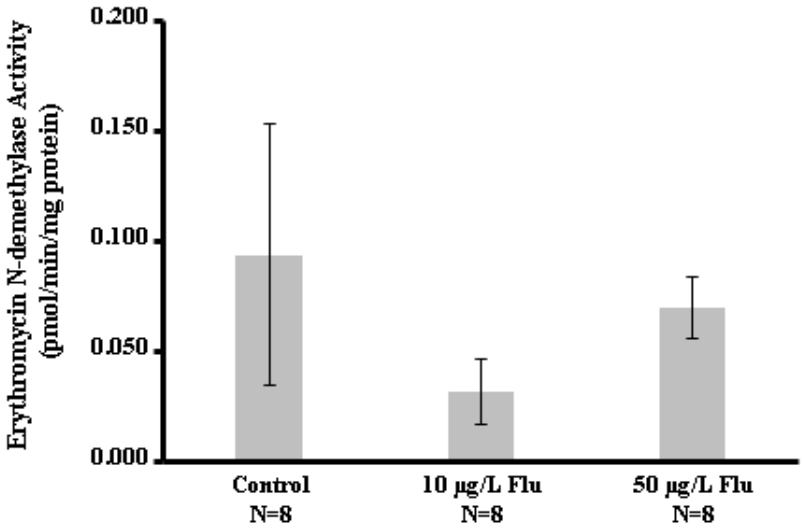

Figure 4.2 ERND activity results.

4.3 Total Glutathione S-Transferase Activities

Tables 7-9 give the results of glutathione S-transferase (GST) activity measurements obtained in rainbow trout liver cytosols. The average GST activity in the control group was 1186±70 nmol/min/mg protein, the average GST activity in the 10 microgram/L fluvoxamine administration group was 1424±120 nmol/min/mg protein and the average GST activity in 50 microgram/L fluvoxamine

administration group was 1564±89 nmol/min/mg protein. When the statistical analysis was performed, the GST activity results of the 50 microgram/L fluvoxamine administration group were found to be significantly different from the control group (Figure 4.3).

Table 4.7 Protein concentrations of cytosols and GST activities in the control group.

Sample Number	Protein Concentrations (mg/L)	Average Activity (nmol/min/mg protein)
1	11.93	1247
2	11.35	1241
3	6.66	1422
4	11.49	1198
5	12.35	1296
6	7.55	947
7	9.85	831
8	7.02	1308
Average Activity±SEM N=8		1186±70

Table 4.8 Protein concentrations of cytosols and GST activities in 10 microgram/L fluvoxamine administration group.

Sample Number	Protein Concentrations (mg/L)	Average Activity (nmol/min/mg protein)
1	13.40	913
2	6.23	1564
3	9.04	1170
4	11.47	1738
5	7.93	1786
6	11.14	1434
7	9.73	1734
8	12.07	1054
Average Activity±SEM N=8		1424±120

Table 4.9 Protein concentrations of cytosols and GST activities in 50 microgram/L fluvoxamine administration group.

Sample Number	Protein Concentrations (mg/L)	Average Activity (nmol/min/mg protein)
1	11.65	1153
2	9.49	1804
3	8.48	1844
4	11.66	1661
5	12.46	1718
6	8.39	1541
7	9.81	1558
8	11.47	1237
Average Activity±SEM N=8		1564±89

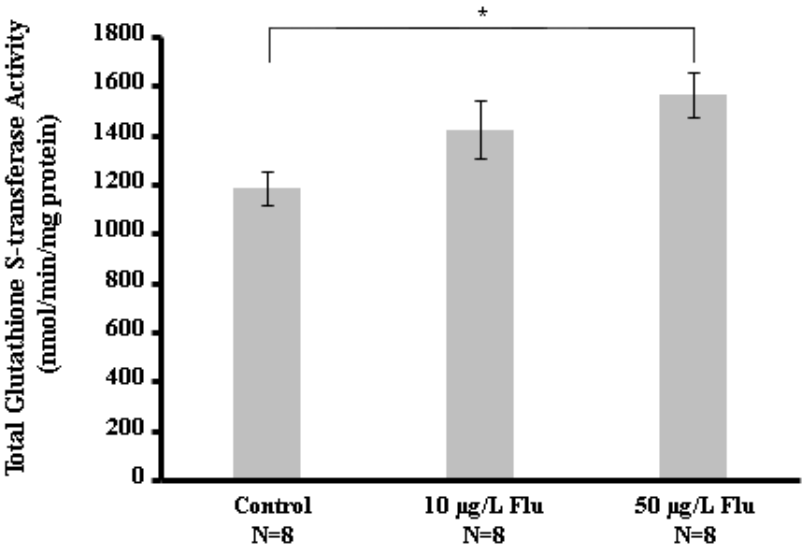

Figure 4.3 GST activity results.

4.4 Catalase Activities

Tables 10-12 give the catalase (CAT) activity measurements obtained in rainbow trout liver cytosols. The average catalase activity in the control group was 274±23 µmol/min./mg protein, the average catalase activity in the 10 microgram/L fluvoxamine administration group was 362±38 µmol/min./mg protein and the average catalase activity in the 50 microgram/L fluvoxamine administration group

was 359±59 µmol/min/mg protein. When the statistical analysis was performed, no difference was found between the groups (Figure 4.4).

Table 4.10 CAT activities in the control group

Sample Number	Average Activity (µmol/min/mg protein)
1	331
2	298
3	176
4	212
5	371
6	292
7	297
8	216
Average Activity±SEM N=8	274±23

Table 4.11 CAT activities in 10 microgram/L fluvoxamine administration group.

Sample Number	Average Activity (µmol/min/mg protein)
1	372
2	477
3	195
4	420
5	243
6	325
7	500
8	368
Average Activity±SEM N=8	362±38

Table 4.12 CAT activities in 50 microgram/L fluvoxamine administration group.

Sample Number	Average Activity (µmol/min/mg protein)
1	465
2	375
3	659
4	248
5	465
6	266
7	255
8	137
Average Activity±SEM N=8	359±59

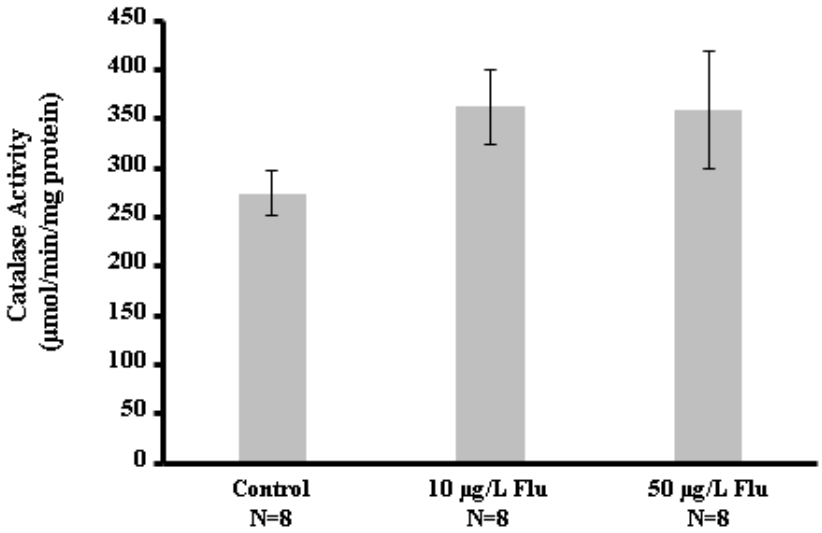

Figure 4.4 CAT activity results.

4.5 Glutathione Reductase Activities

Tables 13-15 give the results of glutathione reductase (GR) activity measurements obtained in rainbow trout liver cytosols. The average GR activity in the control group was 20.97±1.30 nmol/min/mg protein, and the average GR activity in the 10 microgram/L fluvoxamine administration group was 21.04±2.53 nmol/min/mg protein and the average GR activity in the 50 microgram/L

fluvoxamine administration group was 17.01±1.47 nmol/min/mg protein. When the statistical analysis of the obtained data was performed, the GR activity of the 50 microgram/L fluvoxamine administration group was found to be significantly different from the control group (Figure 4.5).

Table 4.13 GR activities in the control group

Sample Number	Average Activity (nmol/min/mg protein)
1	20.84
2	16.03
3	24.70
4	22.54
5	25.52
6	15.58
7	19.98
8	22.59
Average Activity±SEM N=8	20.97±1.30

Table 4.14 GR activities in 10 microgram/L fluvoxamine administration group.

Sample Number	Average Activity (nmol/min/mg protein)
1	13.60
2	15.90
3	16.84
4	19.30
5	31.16
6	16.50
7	22.64
8	32.41
Average Activity±SEM N=8	21.04±2.53

Table 4.15 GR activities in 50 microgram/L fluvoxamine administration group.

Sample Number	Average Activity (nmol/min/mg protein)
1	17.19
2	13.37
3	15.26
4	15.38
5	14.46
6	26.26
7	14.89
8	19.30
Average Activity±SEM N=8	17.01±1.47

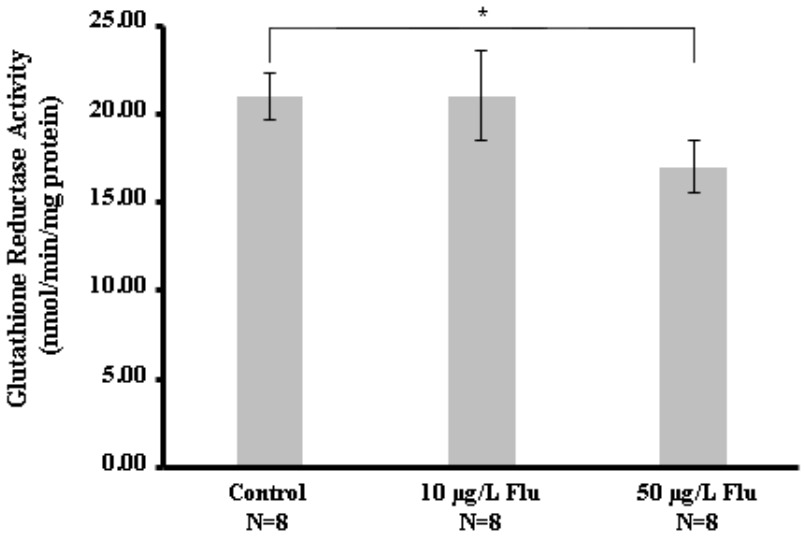

Figure 4.5 GR activity results.

5 DISCUSSION

Living organisms are exposed to a wide variety of xenobiotics including drugs in their lives. Organisms do not always completely metabolize many human drugs and residues of them can enter waterways. These residues may alter the physiology and reproductive behavior of aquatic creatures, among other harmful impacts. It is generally recognized that both hazardous and non-toxic substances can have harmful effects by affecting enzyme catalyzed processes.

One of several recently created drugs, fluvoxamine particularly inhibits the neuronal reuptake of serotonin (5-hydroxytryptamine) in humans (3). Fluvoxamine is quickly absorbed by the digestive system and dispersed throughout the body (147). Fluvoxamine is subjected to significant oxidative metabolism in the liver to produce nine identified metabolites (22). But these metabolites are not pharmacological activity. Fluvoxamine has a low plasma protein binding (77%) relative to other SSRIs (13). Fluvoxamine has significant drug interaction potential and inhibits particularly CYP1A2 and the other cytochrome P450s including CYP3A4 and CYP2D6 (24).

Xenobiotic metabolizing enzymes within the body metabolize xenobiotics. These reactions are often classified as Phase I and Phase II reactions. Cytochrome P450s (CYPs) catalyze most of the oxidative reactions of phase I. The CYPs are a protein superfamily that is involved in the metabolism of both endogenous compounds and exogenous compounds including drugs (60,148–152). In the xenobiotic metabolism, phase II biotransformation processes are conjugation reactions. Xenobiotic conjugation reactions include amino acid conjugation, glucuronidation, methylation, sulfonation, acetylation, and conjugation with glutathione. As a result of these reactions, the hydrophilicity of chemicals increases, and the rate of elimination increases. Glutathione S-transferase with reduced glutathione catalyzes the conjugation of many xenobiotics including drugs, epoxides, and phase I metabolites (82,153,154).

In this study, the impact of fluvoxamine was determined on CYP1A, CYP3A, GST, glutathione reductase, and catalase in the liver of rainbow trout. CYP1A is an important cytochrome P450 enzyme since it activates non-toxic molecules to toxic forms. In addition, it catalyzes the biotransformation of about

9% of therapeutic drugs, including antidepressants, antipyretics, antipsychotics, analgesics, and anti-inflammatory pharmaceuticals (60,61).

The spectrofluorometric assessment of 7-ethoxyresorufin O-deethylase (EROD) activity in the liver is commonly used to assess CYP1A induction (155). In this study, the administration of fluvoxamine elevated EROD activities in both of the administration groups. The EROD activity results of the 10 microgram/L fluvoxamine administration group and the 50 microgram/L fluvoxamine administration group were found to be significantly different from the control group activities. It has been shown in one of the in vitro studies that fluvoxamine is a strong inhibitor of CYP1A in carp liver (156). The inhibitory effect of fluvoxamine on CYP1A has been shown in another in vitro study in the human liver microsome and human placenta (156,157).

The most prevalent cytochrome P450 in the liver is CYP3A, which is crucial for drug metabolism (158,159). The erythromycin N-demethylase enzyme is used to evaluate the activity of the CYP3A enzyme. The average ERND activities of the treatment groups (10 g/L and 50 g/L) were less than the control in this study. But there was no statistically significant difference in ERND activities between the groups. In one of the in vitro studies, it has been shown that fluvoxamine inhibits the CYP3A-associated activity in the carp liver (156). In another study, the inhibitory effect of fluvoxamine has been shown on CYP3A in humans (160). Inhibition of CYP3A activity with fluvoxamine affects not only the metabolism of chemicals in living organisms but also endogenous compounds like hormones.

Glutathione S-transferases are a multifunctional protein superfamily found in practically all eukaryotic and prokaryotic cells that can detoxify against endogenous and external harmful chemicals (161,162). They are involved in the conjugation of reduced glutathione with xenobiotics including phase I products, carcinogen chemicals, and epoxides (82,153,154). The GST activity results of the 50 microgram/L fluvoxamine administration group were found to be significantly different from the control group activities.

Reactive oxygen species are generated in living organisms. They cause oxidative stress. They are highly reactive molecules and harmful to living organisms. ROS regulates the activity of Ca-ATPase and disrupts lipid, protein, and DNA structures (163). ROS are clinically related to diabetes, autoimmune illness, inflammatory immunization injury, and blood loss-related organizational damage,

as well as cancer (164). Superoxide dismutase, glutathione reductase, catalase, and glutathione peroxidase are antioxidant enzymes that neutralize the harmful impacts of reactive oxygen species (165). The studies have indicated that fluvoxamine reduces oxidative stress in mice (166). In this study, the activities of glutathione reductase and catalase enzymes were measured in rainbow trout liver cytosols. Catalase, which contains heme, protects cells from the damaging effects of hydrogen peroxide. It has been connected to numerous physiological and pathological conditions in people (94). In this study, there were no statistically significant differences in the catalase activities of the treatment groups (10 g/L and 50 g/L) from the control group. In one of the studies about fluvoxamine, ulcer in the stomach decreases catalase activity in rats, and pretreatment of rats with fluvoxamine keeps the catalase level closer to the control level (167). In another study, catalase activity decreases in arthritic rats, fluvoxamine restores this activity (168).

The other antioxidant enzyme activity, glutathione reductase is a dimeric protein having two identical subunits. Flavin adenine dinucleotide, a component of the protein that is redox-active, facilitates the transfer of electrons from NADPH to the protein's disulfide group, which reduces the substrate GSSG (169). In this study, the GR activity of the 50 microgram/L fluvoxamine administration group was found to be significantly different from the control group activities. GR activity decreased when fish were treated with 50 microgram/L of fluvoxamine. The impact of fluvoxamine on glutathione reductase has not been studied in any organism yet. The inhibitory effect of another SSRI, fluoxetine has been shown on baker’s yeast glutathione reductase (170). However, the other antidepressant molecules increase the mRNA expression of glutathione reductase in human monocytic U-937 cells (171).

The results clearly showed that fluvoxamine modified xenobiotic metabolizing enzyme activities such as CYP1A and GST. In addition, the antioxidant enzyme, glutathione reductase was also affected by the fluvoxamine administration.

6 CONCLUSIONS AND RECOMMENDATIONS

In this study, CYP1A-associated EROD, CYP3A-associated ERND activities, GST, and antioxidant enzyme activities, CAT, and GR were determined in rainbow trout treated with two different doses of fluvoxamine. EROD activities of 10 micrograms/L and 50 microgram/L fluvoxamine administration groups were different from the EROD activity results of the control group. CYP1A is an enzyme activity found in the transformation of toxic chemicals into more toxic ones. An increase in this activity with fluvoxamine may affect the metabolism of the other compounds that fish are exposed to. Among the activities examined in our study, the GST activities in the 50 microgram/L fluvoxamine administration group were different from the results of the control group. GSTs are generally found in the elimination of drugs and chemicals. An increase in this enzyme activity with fluvoxamine may increase the elimination of some chemicals including toxic chemicals from the fish. In addition, the GR activities in the 50 microgram/L fluvoxamine administration group were significantly different from the activities of the control group. The results showed that the antioxidant enzyme activity system was also affected by fluvoxamine administration.

In our study, some of the activities were modified especially in the high-dose fluvoxamine administration group. If higher doses of fluvoxamine are used, the effect of fluvoxamine may be more. Higher doses can be given for a longer period to see the effect of fluvoxamine on these enzyme activities and the others.

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