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Effects of Roundup Exposure on Redox Status, Cellular Apoptosis, and Antioxidant and Osmoregulatory Enzyme Expressions in Goldfish (*Carassius auratus*)

Md Imran Noor
The University of Texas Rio Grande Valley

EFFECTS OF ROUNDUP EXPOSURE ON REDOX STATUS, CELLULAR APOPTOSIS, AND ANTIOXIDANT AND OSMOREGULATORY ENZYME EXPRESSIONS IN GOLDFISH (*CARASSIUS AURATUS*)

A Thesis

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MD IMRAN NOOR

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COMMITTEE MEMBERS

Dr. MD Saydur Rahman
Chair of Committee

Dr. Ahmed Touhami
Committee Member

Dr. Sue Anne Chew
Committee Member

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ABSTRACT

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Intense anthropogenic activities of industrialized nations dramatically increase environmental pollution. This study focused on the effects of Roundup, a glyphosate-based herbicide, exposure (low- and high-dose: 0.5 and 5 mg/L for 2 weeks) on dinitrophenyl protein (DNP), nitrotyrosine protein (NTP), superoxidase dismutase (SOD), catalase (CAT), Na^+/K^+-ATPase (NKA), and renin expressions, and cellular apoptosis in the gills and kidneys of goldfish. Histopathological analysis showed widespread tissue damage in both gills and kidneys. Immunohistochemical analysis provided insights into the expression of molecular biomarkers in tissues. Fish exposed to Roundup exhibited a significant ($P<0.05$) upregulation in DNP, NTP, SOD, and CAT expressions, and apoptotic nuclei in both tissues. Additionally, exposure to Roundup significantly increased renin expression in kidneys and decreased NKA expression in gills. Overall, our results suggest that exposure to Roundup induces oxidative/nitrative stress and cellular apoptosis and alters osmoregulatory and antioxidant systems which may lead to impaired physiological functions in goldfish.

DEDICATION

I am dedicating my dissertation to my mom, dad, sister, and wife. Mom, I would never get this far without you constantly believing in me. I wish you would see me succeed in life. Dad, I admire your ability to move mountains to support me, you are my hero. Beloved little sister, your liveliness always puts a smile on my face. My loving wife, you were there for me, believing in me in the most challenging times. I am forever grateful to you for being a part of my life and guiding me in the dark, being my beacon of hope. I would also like to dedicate my thesis to all people working on educating science in general people.

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CHAPTER I

INTRODUCTION

Environmental Pollution

Humankind gained the upper hand in civilization through the industrial revolution (Förstner & Wittmann, 2012). This milestone completed the position of homo sapiens as the apex species in the world and gave humans the capacity to use and exploit natural resources to their advantage (Hawken et al., 2013; Schwab & Davis, 2018; Stearns, 2020). Over the decades, growing population, urbanization, and heavy anthropogenic activities have increased interaction with our environment leading to the exponential exploitation of natural resources, which often results in environmental pollution (Meena et al., 2018; Manisalidis et al., 2020). Different industrial and agricultural activities frequently bombard terrestrial and aquatic environments with toxic chemicals deposited into the sediment by anthropogenic activities and natural runoffs (Hossain, 2020). One of the significant sources of noxious chemical effluent in the aquatic environment is the ever-growing classification of different pesticides used in agriculture (Edwards, 1977). Pesticides are chemically engineered toxic substances used to eliminate "Pests". Pesticides, in the broad sense, can be classified into herbicides (for weeds/unwanted plant eradicator), insecticides (targeting insects), fungicides (attacks fungi), and rodenticide (for protection of crops from mice and rats) and even includes lampricide (targeting lamprey in the

aquatic environment) (Page & Thomson, 1994; Garry et al., 1996; Liu et al., 2010). Pesticide leftovers infiltrate into different parts of our environment by direct use, spray drift, aerial spraying, washing from the atmosphere by rainfall, erosion, and effluent from farming land will release runoffs from industries and sewage. Many researchers confirmed from scientifically proven studies that runoff from farmlands is the biggest source of gradual aquatic pollution, which represents the chronic/sub-chronic sources of pesticide toxicity, whereas directly implementing water and expulsion of effluent into the marine environment causes more acute but localized pollution (Edwards, 1977; Anju et al., 2010; Tsaboula et al., 2019). However, pesticides are, without question, essential elements for the agricultural industry to fight against hunger, and ecologists and toxicologists must work together to find a balanced way of using pesticides without destroying the environment.

History of Pesticide Use in the Environment

Before the commercial production of synthetic pesticides, simple instruments and natural compounds had a sluggish, never-ending war against the relentless attack of pests on the farmland. (Costa, 1987). Although the botanically derived pesticides were environmentally friendly, many of these products' major drawbacks were their high application rates, slow action time, lack of selectivity, and phytotoxicity (Kislev et al., 2004). The popularity of synthetic pesticides started winning the market in the 1940s, discovering the efficacy of dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxyacetic (2,4-D), chlordane, captan, dieldrin, endrin, parathion, and Roundup, and so on (Delaplane, 1996). Because of the high efficiency of the modern chemical formulations, there were low pest infestation, crops, vegetables, and fruits were fresher, and there were no known reports of people injury during

application because of their "environmentally safe" use (Ansar et al., 2020). General users, producers, and environmental agencies remained unconcerned about synthetic pesticides' potential health hazards for most of the 1950s. Using biotechnological tools, genetically changed crops bred were developed for specific pesticide tolerance to create their pesticides, adding a comprehensive spectrum of pesticide products to the pest control toolbox. Herbicide-tolerant plants like soybeans, rice, canola, corn, and cotton varieties resistant to corn borer and bollworm are among them (Vats, 2015). Integrated Pest Management (IPM) systems do not support pest populations' development. These developments have changed how pest control is done and can minimize or change the agrochemicals used (Morton & Staub, 2008; Unsworth, 2020).

The history of pesticide usage is incomplete without mentioning the devastating role of DDT in the world, including in the United States. It was first synthesized chemically by Othmar Zeidler, an Austrian chemist, in 1874, supervised by Adolf von Baeyer, a Bavarian scientist (Perkins, 1978). It was first depicted in a paper by W. Bausch in 1929 and then in two subsequent distributions in 1930. In 1934, a renowned German scientist and inventor, Wolfgang von Leuthold, showed in his patent the bug-spraying properties of DDT (Brand, 1921). However, it was not until 1939 that Swiss scientist Paul Hermann Müller discovered DDT's insecticidal properties, for which he was awarded the 1948 Nobel Prize in Physiology and Medicine (Lindsten & Ringertz, 2001).

During World War II, the success of DDT as an insect repellent on the battlefield and its success as a mosquito killer on islands of the south pacific earned DDT its popularity. Consequently, DDT got approval as a free pesticide for the farming industry in 1945 (Beard & Collaboration, 2006). With the help of DDT, European and North American countries got rid of malaria temporarily (de Zulueta, 1998). However, the extensive use of DDT as a common

insecticide developed a strong resistance in insects against DDT, leading to these parasites' and pests' resurgence. Even in some places, the number of problems increased than it was before (Babers & Pratt, 1954; McCart & Buckling, 2005).

The notable works by DeWitt (1956), Barnes (1946), and Dahm and Jacobson (1956) inspired many scientists, environmentalists, and even renowned news media to protest against DDT. Although their protests were unsuccessful in banning DDT from public use, the news of protesting was heard by -then-president John. F. Kennedy. Kennedy ordered his science advisory committee to investigate the claims. The movement against DDT led to the formation of the Environmental Protection Agency (EPA) in the United States. Ten years after the campaign against DDT started in 1972, DDT was finally banned in the United States. Although DDT's popularity was short-lived, its damage to the ecosystem still prevails in our environment (Matsushima, 2018).

While DDT is long gone, the road it introduced to the public, the usage of chemicals for short terms benefit on farmlands, ignoring the long-term effects, is running in full swing. Many more chemical compounds replaced DDT, so many in numbers it is more complex than ever to regulate and control. The movement surfaced to support environmental toxicity; thanks to all the works in the '60s and '70s by environmentalists, scientists, and news media, environmental agencies have a clear idea than before about the devastating effects of such toxic chemicals. Though it is good news, science is now way more developed, and the rules and regulations are much more complex than in the 60s and 70s. However, agrochemical industries frequently introduce new chemicals to the market, ignoring the toxicity of non-target species in most products. A public report published by the United States Geological Survey (USGS) narrated that farmers use 459 chemical compounds as pesticides (Zhang et al., 2018). The original number is

higher than the report mentioned (Stone et al., 2014; Van Metre et al., 2017). Amongst the plethora of these common pesticides, several reports from EPA and USDA and scientific articles denoted glyphosate as the most used herbicide (trade name Roundup) for years to come (Cox, 1998; Grube et al., 2011; Fernandez-Cornejo et al., 2014; Benbrook, 2016; Duke, 2018).

History of Roundup

EPA sales and usage division reported in 2007 indicating a substantial increase in glyphosate usage from 1987 to 2007 compared to any other pesticide glyphosate-based herbicide (GBH) usage was 81.6–83.9 million kg in 2007, which was more than double the compared with the second most used pesticide, atrazine (approximately 34 million kg) (Myers et al., 2016). Since then, GBH has become one of the most, if not the most, used pesticides in the United States (Myers et al., 2016). National Agricultural Statistics Service (NASS) reports in 2014 said that since the producers introduced genetically changed crops in the late 90s, the application rate of glyphosate inclined 9-fold in the U.S., and statistics on worldwide usage report a 15-fold increase in GBH by 2014 (Benbrook, 2016). Usage data reported by the USDA, USGS, and EPA in a combined consensus narrated that approximately 2/3rd of the total GBH sprayed since 1974 has been applied last 10 years (Benbrook, 2016).

Adverse Impacts of Glyphosate-Based Herbicide

The cancer research division of the World Health Organization's (WHO), the international agency for research on cancer (IARC) classified glyphosate in group 2A, which refers to a probable human carcinogen in WHO's monogram for evaluating health hazards

associated with organophosphate pesticides (Pearce et al., 2015). In a public experiment by the University of California San Francisco (UCSF) in 2016, a trace amount of glyphosate was found in 93% of all urine samples collected across the United States. Even scientists have found glyphosate in a wide range of everyday groceries in the United States (Gillezeau et al., 2019). A long-term research project led by Dr. Michael Antoniou at King's College London demonstrated in their experiment that when a very low level of chronic exposure (2 years) glyphosate was administered to female rats, they developed Non-alcoholic fatty liver disease (NAFLD (Myers et al., 2016). The results from Myers et al. (2016) have significant impacts on environmental toxicology as their findings are directly related to the Roundup-associated severe health risk factors. Bayer A.G., a German multinational company, currently owns Roundup, the most popular glyphosate-based herbicide. However, its previous owner, Monsanto, popularized Roundup as an herbicide worldwide (Qaim and Traxler, 2005). Reports and claims were coming from around the world against Roundup and its toxicity.

Nevertheless, Monsanto kept fighting for its most profitable product (Broughton, 2017). However, Monsanto lost their battle in a crucial cancer trial in California and had to pay $78 million as compensation to a Californian former school groundkeeper, Dewayne Jonshon, who suffered from cancer after years of working with Roundup on his farmland. Although similar to the trial of Dewayne Jonshon, thousands of other cases are hanging and unresolved (Shiva, 2019). The relentless effort of environmental scientists on Roundup toxicity profiling revealed its devastating side effects to the public, highlighting the importance of studying the effects of glyphosate in the aquatic environment because the aquatic environment takes the direct heat from herbicide usage as they all wash up to the shores of different water bodies by the rainfall, erosion, and groundwater leaching.

Effects of Glyphosate on Aquatic Organisms

The tremendous success of glyphosate-resistant crops is causing toxicity in countless non-target species in aquatic and terrestrial environments (King & Wagner, 2010). Some chemical properties of commercially produced glyphosate-based herbicides have increased toxicity by many folds. There is a difference in the raw form of glyphosate compared with the commercially produced glyphosate-based herbicide (such as Roundup) toxicity because the added surfactants in the commercial formula make the chemical formula more potent than its active ingredient (Folmar et al., 1979). To increase glyphosate adhesion's efficacy to the leaf surface and aid movement across the waxy cuticle membrane and into the plant, different surfactants (e.g., polyethoxylated tallow amine, POEA) are added to the commercial formula of Roundup (Janssens and Stoks, 2017). Although GBHs are prohibited from using in the aquatic environment, an identifiable amount of the active surfactants with active ingredients are found in surface water (Vera et al., 2010; Jones, et al., 2011; Rissoli et al., 2016), so long as the chemical compounds are inbuilt in the herbicide to deteriorate the water quality. Acute toxicity significantly depends across the taxa, with toxicity relying on the timing, scale, and route of exposure (Annett et al., 2014).

Most herbicides comprising glyphosate are barred from any direct application in the aquatic environment. However, with the prevalent use at present, there are many routes through which progressions of toxic effects of GBH reach aquatic organisms (Alavanja et al., 2013). Substantial amounts of glyphosate quantities entering the water bodies resulting from direct runoff, direct overspray, aerial spray runoff of surface, or drift during herbicide application can cause (Solomon & Thompson, 2003). Although most common herbicides are used for only

agricultural purposes, the easy application method of Roundup received popularity for domestic use on gardens and lawns by inexperienced individuals with no safety measures for proper herbicide applications (Hanke et al., 2010). There are multiple studies on the effects of GBHs in a wide variety of aquatic organisms, amongst which fish and amphibians are the prominent phyla that are facing the hardest challenge due to commercially used glyphosate.

Taxa including fish (Folmar et al., 1979; modetso & martinez, 2010; de Menezes et al., 2011; Glusczak et al., 2011; Hued et al., 2012), amphibians (Moore et al., 2012), microorganisms (Folmar et al., 1979; Bonnet et al., 2007), and invertebrates (Pérez et al., 2007), and birds (Oliveira et al., 2007) have been investigated in previous research and literary works. It is found with scientific experiments that there are several physiological and behavioral effects on these organisms, which depend on the dosage and formulation of commercially produced GBH. Previous review papers have highlighted the ecological risk assessment for Roundup herbicide in aquatic and terrestrial ecosystems and exposure to aquatic organisms because of overwater use of glyphosate (Folmar et al., 1979; Solomon & Thompson, 2003; Glusczak et al., 2011; Mertens et al., 2018). Previous research articles also focused on Roundup herbicide's ecological effects on terrestrial and aquatic ecosystems (Giesy et al., 2000), and the occurrence of glyphosate-based compounds deposited in water (Solomon & Thompson, 2003).

Exposure to Roundup and Oxidative/Nitrative Stress

Oxidative stress is a specific intercellular event that happens when an imbalance between the creation and buildup of reactive oxygen species (ROS) inside cells and the cells' ability to detoxify developed reactive ions (Betteridge, 2000). Excess reactive ion production can deteriorate several physiological functions, including interruption of cell signaling pathways

(Zorov et al., 2014). ROS are produced as a by-product of oxygen metabolism, a natural biochemical process in an organism. Dalle-Donne et al. (2003), Dorts et al. (2009), Wang et al. (2016), and many research experiments have used 2,4-dinitrophenyl protein (DNP) as the oxidative stress biomarker. DNP is the protein expressed in cells and tissues when cells or tissues go through oxidative stress due to ROS modifying protein. In addition to oxidative stress, nitrosative stress is a vital oxygen metabolism condition responsible for many vertebrates' cardiovascular disorders (Panth et al., 2016). When oxygen metabolism disorder occurs in cells, nitric oxide and superoxide react jointly, which causes stress which is referred to as nitrosative stress (Koskenkorva-Frank et al., 2013). In addition to these damages, nitrotyrosine protein (NTP) production is a biomarker for cell death (Wang et al., 2021).

Importantly, oxidative and nitrative stress are closely related. Different ROS which is involved in oxidative damage overlap with the foraging pathway of reactive nitrogen species. Several research studies, including Saenen et al. (2017), Rahman and Rahman (2021), and Lacy et al. (2022) have used NTP as the nitrosative stress biomarker in organisms exposed to different pollutants. The free radicals become a problem when environmental stressors (i.e., pesticides, chemical fertilizers, ionizing radiations, ultraviolet rays, heavy metals, and pollutants) and xenobiotics (i.e., anticlastic drugs) contribute to increased free radical production higher than the normal level (Coutellec & Lagadic, 2006). This imbalance leads to cell and tissue damage, known as oxidative stress (Sies, 2000). For their natural or purportedly beneficial effect against oxidative several antioxidant medications or supplements have been proposed in recent years, including vitamin E, flavonoids, and polyphenols (Pizzino et al., 2017). Many scientific studies have been conducted on GBH toxicity and have concluded that oxidative stress-related damage is one of many outcomes of glyphosate toxicity (El-Shenawy, 2009). Although the pathway for

toxicity of Roundup in photosynthetic aquatic organisms such as algae is well comprehended, as they possess physiological similarities with terrestrial plants, its effect on the aquatic animal is poorly understood. The components of Roundup target the 5-enolpyruvylshikimate-3-phosphate (ESPS) enzyme, which is absent in aquatic animals (Steinrücken & Amrhein, 1984); however, the toxicity from Roundup has been observed in a wide range of aquatic organisms across phyla (Folmar et al., 1979). Oxidative biomarkers have been used as a very efficient biomarker in toxicological research.

Several detrimental effects on cells can result from the production of ROS/RNS, which is linked to a mechanism of toxicity for many GBH-associated toxicants (Bagchi et al., 1995). The ROS can start oxidative damage to proteins, lipids, and nucleic acids, as well as nitrosative reaction production of reactive nitrogen species (RNS), leading to organelle damage and, ultimately, cell death (i.e., apoptosis). As a result of ROS generation, antioxidant production—activating enzymes and releasing non-enzymatic metabolites—counteracts the free radical and reduces oxidative damage (Sies, 1997). When antioxidant production can no longer compensate and neutralizes the oxidative properties of free radicals due to excess amount of ROS production, stress-related damage takes place (Page & Thomson, 1994; Lushchak et al., 2009; (de Menezes et al., 2011; Fan et al., 2013; Lushchak et al., 2009; Ma et al., 2015; Marques et al., 2014; Modesto & Martinez, 2010; Cattaneo et al., 2011; de Menezes et al., 2011; Glusczak et al., 2011).

Exposure to Roundup and Antioxidants

An interconnected network of antioxidant enzymes shields cells and tissues against oxidative damage (Sies, 1997). Pro-oxidant agents, such as oxygen and nitrogen free radicals, are

the elements that trigger a cascade of oxidative damage reactions, causing DNA damage (e.g., DNA double-strand breaks) and protein unfolding. At the same time, antioxidants at low molecular concentrations convert the active free radicals into relatively inert molecules in oxyreductive reactions (Ammendola & d'Abusco, 2020). Cell offsets the oxidative/nitrative stress (pro-oxidants) by producing more antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione, and N-acetyl-cysteine, to neutralize the series of oxidative reactions (Rahal et al., 2014). SOD and CAT are the two most critical antioxidant enzymes (Olsvik et al., 2005). SOD first catalyzes the superoxide dismutation anion, converts the anion to hydrogen peroxide, then the peroxidase enzymes, such as catalase, remove the hydrogen peroxide. (Ho et al., 1998). In the experimental setup, how any GBH alters antioxidant enzyme production depends on experimental protocol and species. For example, silver catfish exposed to moderate concentrations of Roundup show no change in SOD or CAT activity, whereas the GST in the liver declined (de Menezes et al., 2011). In the same experiment, the SOD and CAT activity decreased during the recovery period, denoted by the researcher as a compensatory response due to toxic expos that led to incomplete recovery. Similar results were also found in the silver catfish experiment (de Freitas et al., 2018). Lushchak et al. (2009) demonstrated that short-term Roundup toxicity on goldfish showed SOD inhibition in tissues of multiple organs at low to high concentrations, whereas CAT production increased significantly at relatively lower concentrations. Paiva (*Leporinus obtusidens)* exposed to a high dose of Roundup exposure (4 days) showed increased activity of CAT (Glusczak et al., 2011). It is to be noted that a high level of antioxidant production indicates increased amounts of free radicals present in the cell; hence to fight the free radicals, the antioxidant is produced. Meanwhile, low levels of antioxidants or deficiencies in antioxidants may result in synthesizing enzymes (i.e., SOD and CAT) or

increased antioxidant utilization. In all ecotoxicological studies, antioxidants are used as a robust biomarker for cellular stress. The level of antioxidant production is statistically analyzed to determine different oxidative/nitrative stress levels.

Exposure to Roundup and Osmoregulatory Stress Biomarker

Adenosine triphosphatases (ATPases) are enzymes that import many metabolites required for cellular metabolism while pumping out toxins, waste, and solutes that can obstruct biochemical functions (Gregus, 2008). The Na^+/K^+-ATPase or sodium pump (cellular ion pump) is the protein enzyme that carries out the joined extrusion and uptake of potassium (K) and sodium (Na) ions across the plasma membranes of cells of most multicellular organisms (Morth et al., 2011). Na^+/K^+-ATPases control cation transport across the cell membrane, maintaining the cellular membrane potential (Dietz, 1985; Pałecz et al., 2005; Vijayavel et al., 2007; Almeida & Vasconcelos, 2015). The Na^+/K^+-ATPase enzyme is essential for osmoregulation and is commonly utilized as a biomarker of cellular oxidative stress in stressed settings such as toxic chemical exposure. The chief uptake route for waterborne contaminants is through the gills of fish or aquatic invertebrates like oysters and bivalves. Gills are expected to be affected by waterborne glyphosate (Ajima et al., 2021). Furthermore, researchers looked into and confirmed the potential effects of glyphosate-based herbicides on the function of the Na^+/K^+-ATPase to see if this molecule has any negative impacts on ionic/osmotic balance or cholinergic status (Oruç & Usta, 2007).

The renin-angiotensin-aldosterone system (RAAS) is a physiological mechanism for regulating blood pressure and body water-electrolyte balance, with the liver, lungs, and kidneys all contributing to its activation (Gelen et al., 2021). RAAS is a peptidergic endocrine system

that relies on hormonal activity to maintain blood pressure and regulates fluid-electrolyte balance (Peach, 1977). In addition to that, the renin enzyme has autocrine and paracrine functions (Johnston, 1990). In species exposed to a toxic chemical, the expression of renin is used as a biomarker for stress-related fluid imbalance in the kidney and altered blood pressure of Elasmobranch fishes (Peach, 1977; Leung & Chappell, 2003, Lacy & Rahman, 2022).

Exposure to Roundup and Cellular Apoptosis

The process of eliminating damaged cells using different biochemical pathways in a programmed manner is known as cellular apoptosis. It is a natural process of cells eliminating injured cells due to DNA damage (Elmore, 2007). Apoptosis is an essential molecular mechanism for removing stressed cells beyond repair (Manjo & Joris, 1995; Agnello & Roccheri, 2010). Apoptosis plays a crucial role in all organisms during their embryonic developmental stage into organ development (Agnello et al., 2015). Additionally, apoptosis is critical to preventing damaged cells from turning into cancerous cells (Watson, 2006). However, necrosis, another form of cell death which refers to as unnatural cell death, is mechanistically different from apoptosis (Brown et al., 1999). Although apoptotic and necrotic pathways differ, they may happen simultaneously in stressed cells. During necrosis, bigger cell groups are associated, and cellular materials get thrown throughout the membrane to extracellular space, leading to a vigorous inflammatory response (Arnoczky et al., 2007). In contrast, during apoptosis, the cells separate into apoptotic bodies, which allows other cells to digest the damaged cells in phagocytosis, which does not cause any inflammation (da Silva et al., 1996; Ameisen, 2002; Kanduc et al., 2002).

A wide range of model organisms of different taxa has been studied on environmental stressors accelerating programmed cells. The invertebrate sponge has shown an increase in apoptotic bodies under toxic exposure (Wagner et al., 1998). Recently, Nash et al. (2019) and Nash & Rahman (2019) demonstrated high cellular apoptotic activity in American oysters caused by heat stress. In addition, multiple stressors such as cold shock treatment, toxic stress, and heat stress also induced apoptosis in spermatogenic/sperm cells of sea urchins (Pan et al., 2006; Johnstone et al., 2019). Moreover, hypertonic stress has been confirmed to induce both necrosis and apoptosis in marine teleost fish (Hashimoto et al., 1998). Also, heavy metal poisoning enhanced apoptotic activity in marine teleost cell lines (Morcillo et al., 2016). There are also a number of studies on glyphosate and glyphosate-based herbicide toxicity causing apoptosis in aquatic organisms. Recently, Uren & Santos (2015) have demonstrated transcriptome profiling results on brown trout exposed to glyphosate-based herbicides exert oxidative stress, which leads to induce apoptosis as well as gives rise to the expression of tumor suppressor gene p53 to deal with damaged DNA. A recent study carried out by Liu et al. (2022) has shown in their study of glyphosate toxicity in zebrafish embryonic development. They have shown that different concentration of glyphosate exposure causes abnormal expression of the p53 gene and different types of caspase protein which are the primary indicator of apoptosis induction. Although, all these studies mentioned above depicted a defined relationship between different environmental stressors and cellular apoptosis. There is a lack of study on the relationship of how apoptosis responses correlate with other stress biomarkers, such as oxidative/nitrative stress and antioxidant production. In my study, I would like to build a picture of how all these stress biomarkers correspond to Roundup exposure in freshwater teleost species.

Hypothesis

This study aims to scrutinize the null hypothesis that sub-chronic (2 weeks) exposure to Roundup will cause morphological changes, induce oxidative/nitrative stress by increasing RNS/ROS production, accelerate antioxidant production, alter renin and Na^+/K^+-ATPase in gill and kidney of common goldfish, *Carassius auratus*, a model freshwater teleost species (Ota & Abe, 2016; Blanco et al., 2018).

Study Objectives

The main objectives of my research were five-fold:

(i) To identify the effects of Roundup exposure on the morphology of gill and kidney in goldfish,

(ii) To determine the effects of Roundup exposure on oxidative and nitrative stress biomarkers, DNP and NTP expressions in the gill and kidney tissues;

(iii) To determine the effects of Roundup exposure on antioxidant enzymes, SOD and CAT expressions in the gill and kidney tissues;

(iv) To determine the effects of Roundup exposure on osmoregulatory enzymes, renin and Na^+/K^+-ATPase expressions in the gill and kidney tissues; and

(v) To identify the effects of Roundup exposure on cellular apoptosis.

CHAPTER II

MATERIALS AND METHODS

Experimental Fish and Bioethics

For the Roundup exposure experiment, goldfish (Carassius auratus) were purchased (150 fish; average body weight: 1.94 ± 0.50 g; length: 5.21 ± 0.48 cm) from a local pet shop (PetSmart, Brownsville, TX) and transferred to a wet laboratory at the University of Texas Rio Grande Valley (UTRGV) in Brownsville campus. Fish were stocked in 6 glass aquariums (25 fish/aquarium, 80-liter capacity each) with ~60 liters reverse osmosis (RO) freshwater with filter and aeration systems (Tetra Spectrum Brands Pet, LLC, Blacksburg, VA, USA). The room temperature was maintained at 22 °C throughout acclimatization and experimental periods. Dissolved oxygen, water temperature, and pH were measured 3 times daily using a YSI professional plus probe (Multiprobe System, Yellow Springs, OH, USA). Physiochemical parameters such as nitrite, nitrate, and ammonia were also measured weekly using an API Freshwater Masterkit colorimetric testing kit (MARS, McLean, VA, USA). One-fourth of the water in each tank was changed every 48 hours with refreshed RO water to minimize the nitrite, nitrate, and ammonia levels (>= 0.1 ppm). Fish were fed (Omega One Goldfish Pellets, a commercially produced goldfish feed, crude fat 8.0%, crude protein 33.0%, and crude fiber

3.0%, Omega Sea LLC, Painesville, OH, USA) once every day at a rate of ~4-5% body weight. Following the acclimation period (4 weeks), fish were gradually introduced into the experimental condition. Two aquariums were assigned to each treatment group (control, no Roundup; low dose (LD: 0.5 mg/L) and high dose (HD: 5 mg/L) of Roundup, 6 tanks in total). Glyphosate-based herbicide Roundup (Land and Grass killer, Bayer Corporation, Whippany, NJ, USA) was used for the chemical exposure experiment. The carbon filter cartridge from on top hang-back filter (Top Fin® Aquarium Carbon Cartridge, Top Fin, Pet SMART, Phoenix, AZ) was removed before applying the first dose of Roundup. The concentration of Roundup dosage in this study was selected according to the previous studies on Roundup exposure to other aquatic organisms (Smith et al., 2019). The selected LD (0.5 ml/L) represented the environmentally realistic dose, and the HD (5.0 ml/L) represented the Roundup concentration in accidental spills in the aquatic environment. The concentration of Roundup was maintained constant throughout the experimental period by refilling chemicals according to the water change since Roundup is homogenously soluble in water and does not precipitate (Jiang et. al., 2012). Following 2-week of Roundup exposure, the fish were sacrificed by euthanizing via immersion in a solution of tricaine mesylate (MS222, concentration >= 250 mg/L, diluted to aquarium water). Fish were placed in MS222 solution of euthanasia and kept until the gill opercular movement ceased. Then spinal cord was severed, ensuring the death under anesthesia, and tissues from the targeted organs were collected carefully to avoid contamination. All standard laboratory work procedures and animal husbandry practices for working with live animals, approved by the UTRGV Institute of Animal Care and Use Committee (IACUC protocol# AUP-21-02), were strictly followed.

Tissue Collection and Preservation

The fish's final body weight (4.08 ± 1.2 g) and length (6.14 ± 0.9 cm) were measured using a gram (g) scale (Carolina Biological Supply Company, Burlington, NC, USA) and a standard meter scale at a deep anesthetic state before dissection. After sacrifice, gill and kidney tissue samples were collected using sterilized dissection kits and cleaned trays (cleaned with 75% alcohol solution) to avoid contamination between the samples. Gill and kidney tissue samples were collected from 10 representative fish for each treatment group and immersed in 4% paraformaldehyde solution (Acros Organics, Morris, NJ, USA) for one week at 4 °C for fixation. The remaining fish (15 fish in each treatment group) tissue samples were collected in 1.5 ml DNase/RNase-free micro-centrifuge tubes quickly preserved on dry ice and finally stored at -80 °C for later molecular analysis.

Tissue Section and Slide Preparation

Tissue samples fixed in the paraformaldehyde were immersed in a serially ascending concentration (50%, 75%, 95%, and 2X 100%) of ethanol for dehydrating the tissue samples in glass vials for 30-45 min at each concentration. Tissue samples were then treated with xylene (Fisher Scientific, Hampton, New Hampshire) twice (15-30 min each) to render them translucent. The samples were then immersed in melted paraffin (Paraplast Plus, melting point 60–65 ^{0}C, Fisher Scientific) three times (1 hr each) and embedded in paraffin with histo-cassettes and molds. Paraffin-embedded tissue samples were sectioned at 5-7 µm thickness on a rotary microtome and affixed to glass slides (SuperfrostTM Plus, Thermo Fisher, Waltham, MA, USA) using a hot water bath of 40 °C. Glass slide affixed tissues were kept at -20 °C in a freezer and ready to use for histology, immunohistochemical, and *in situ* TUNEL analyses.

Histological Analysis of Tissue Samples

Affixed slides were deparaffinized with xylene wash 3 times (5-7 min each) followed by rehydration using serially diluted ethanol solution (100% 2X, 95%, 75%, and 50%). Slides were given adequate water baths prior to H&E staining (Hematoxylin and Eosin, Millipore Sigma, St. Louis, MI, USA) according to standard histology techniques (Rahman et al., 2000). After staining, slides were washed in a water bath, dehydrated in a series of ethanol solutions (50%, 75%, 95%, and 100% 2X), and cleared in xylene. Finally, coverslips (Fisherbrand Cover Glasses, Fisher Scientific, Pittsburg, PA) were mounted on the top of staining slides with Cytoseal XYL, a xylene-based glue (Richard Allen Scientific, San Diego, CA, USA).

Fontana-Mason Silver (FMS) stain was used to detect the argentaffin granules and melanin formation in goldfish kidneys according to Bishop et al. (2012). Briefly, tissue sections were deparaffinized with xylene and rehydrated with a series of ethanol dilutions. After rehydration, 10% silver nitrate solution (Fisher Chemical, Hampton, New Hampshire) was added on slides and heated at 60 °C in a conventional oven for 1 hour. After warming, the silver nitrate solution was replaced with 0.1% gold chloride (Sigma-Aldrich, Inc., St. Louis, MO, USA) solution and 5% hypo (sodium thiosulfate) consecutively. After washing, a nuclear-fast red solution (Sigma-Aldrich, St. Louis, MO, USA) was applied to the slide for 5 min. Slides were then rewashed in a water bath, dehydrated in ethanol dilutions (50%, 75%, 95%, and 100% 2X), and cleared in xylene 3 times for 5 min each. In the final step, coverslips were affixed to the slides using Cytoseal XYL. Slides were examined using a light trinocular compound microscope (AMscope, Irvine, CA, USA) and photographs were taken directly from the microscope-mounted 5-megapixel digital camera using ISCapture software (ISCapture 3.9.0.601, AMscope, Irvine, CA, USA).

Biological Data Collection from Stained Tissue Sections

The collected photographs were then used to identify the histopathological lesions in gills and kidney tissues. In gills (5 fish per treatment group), protruding lamella length (PLL: 30 measurements per individual, 300 per treatment group), the distance between lamella and interlamellar cell mass (ILCM: 12 measurements per individual, 120 per treatment group) dynamics were identified to estimate the gill remodeling (22 measurements per individual, 220 per treatment groups) as described previously by Shuang et al. (2022). To identify changes in the bowman's capsule area, around 16 measurements were taken from individual samples and 160 measurements from each treatment group according to Bernet et al. (1999). Melanin counts (intensity of melanin pigment in FMS-stained tissue sections) were taken to identify the number of melanin pigment formations in tissue from the FMS-stained slide.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using different stress biomarker antibodies according to the protocol described previously by Nash and Rahman (2019). Briefly, slides were deparaffinized using xylene (3 times, 5 min each), rehydrated using serially diluted ethanol solutions, and washed in phosphate-buffered saline (PBS, Fisher Scientific, Waltham, MA, USA) 3 times (15 min each). Bovine serum albumin (BSA, Fisher chemicals, Hampton, New Hampshire) was added (1% dilution) to the slides and incubated for 1 h to block non-specific binding with the primary antibody. After incubation, rabbit anti-DNP (Thermo Fisher, Waltham, MA, USA), mouse anti-Renin (Novus Biologicals, Littleton, CO, USA), rabbit anti-SOD (Novus Biologicals, Littleton, CO, USA), rabbit anti-CAT (Novus Biologicals, Centennial, CO, USA), mouse anti-NTP (Santa Cruz Biotechnology, Dallas, TX, USA), or rabbit anti-Na^{+}/K^{+}-ATPase

(Millipore-Sigma, St. Louis, MI, USA) primary antibodies were added to the slides and incubated for 48 h at 4 °C. To assess the negative controls, PBS was used instead of the primary antibody. After primary antibody incubation, slides were then rewashed with PBS 3 times (15 min each time) prior to applying the secondary antibody; anti-rabbit (Southern Biotech, Birmingham, AL, USA) or anti-mouse (Cell Signaling Technology, Danvers, MA, USA). The secondary antibody incubation period was 2 h at room temperature, after which slides were washed with PBS 3 times (15 min each time). The expression of protein/enzyme was detected by applying 3,3 diaminobenzidine peroxidase (Impact DAB, Vector Laboratories, Burlingame, CA, USA) substrate on the slide in dark conditions. The slides were then washed with water and dehydrated with an increasing concentration of ethanol dilutions, followed by cleaning with xylene. Finally, the coverslips were mounted to the DAB-stained slides using Cytoseal. The immunoreactive (IR) signals of NTP, DNP, CAT, SOD, renin, and Na^+/K^+-ATPase were examined using a light microscope and photographed using a digital camera (AMscope). The IR signals were measured using ImageJ software (ImageJ, Version: 998K, National Institutes of Health, Bethesda, MD, USA).

In Situ TUNEL Analysis

For identifying apoptotic cells in tissues, the deadEnd™ colorimetric terminal deoxynucleotidyl transferase dUTP nick labeling (TUNEL) system kit (Promega, Madison, WI, USA) was used in combination with DAB solution as a staining medium, and methyl green for counterstaining according to Lacy et al. (2022). Briefly, paraffin blocks sectioned at 7 µm, affixed on the glass side, deparaffinized by xylene immersion, and rehydration by serially diluted ethanol solution. Slides were then immersed in 0.85% NaCl solution followed by repeated PBS

wash, incubated with 20 µl/mL proteinase K (Invitrogen, Carlsbad, CA, USA) for 30 min to increase the permeabilization, and washed subsequently with PBS for 30 min. Slides were then equilibrated with equilibrium buffer (Promega, Madison, WI, USA) prior to re-incubating with a solution of equilibration buffer containing rTdT (recombinant terminal deoxynucleotidyl transferase) enzyme and biotinylated nucleotide mix (98:1:1 ratio) cocktail for 1 hour. Slides were washed with 1X sodium citrate buffer (SSC buffer, Promega) followed by a PBS wash before adding streptavidin horseradish peroxidase (HRP, Promega) incubation for 30 min. Sections were then washed with PBS and stained in dark conditions for 5–10 min with DAB, followed by counterstaining with 1% methyl green-blue solution for 15 min. Afterward, slides were dehydrated by ethanol dilutions, rinsed in DI water, cleaned with xylene, and mounted with Cytoseal. Photographs of apoptotic cell formation were taken using a light microscope (AMscope) and ISCapture software. ImageJ software was used to calculate the optical density (OD) of TUNEL-positive staining.

Statistical Analysis

The experimental data were analyzed through one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. A $P<0.05$ value was used for statistical significance. GraphPad Prism software (GraphPad, San Diego, CA, USA) was used to conduct statistical analysis. All data were expressed using the means ± standard error of the means (SEM).

CHAPTER III

RESULTS

Effects of Roundup Exposure on Morphological Changes in Gills and Kidneys of Goldfish

Fish exhibited several morphological changes in gills at different dosages of Roundup exposure. Fish exposed (2 weeks) to low- (0.5 ml/L) and high-dose (5 ml/L) of Roundup groups showed loss of gill architecture, increased mucus in the gill filaments, and secondary lamellae fusion compared with control groups (no Roundup) (Fig. 1A-C). Fish exposed to Roundup significantly (P<0.05, Tukey's test) increased protruding lamellae length (PLL) in the gills around 1.13-fold in low dose (PLL: 172.9 ± 2.205 μm) and ~1.42-fold in high dose (PLL 246.3 ± 2.605 μm) compared with control (PLL 153.3 ± 2.205 μm) (Fig. 2A). In the gills, the interlamellar cell mass (ILCM) area was reduced significantly (P<0.05) around 1.75-fold in low dose (ILCM: 3490 ± 78.19 μm^2) and ~2.08-fold in high dose (ILCM: 2916 ± 89.64 μm^2) Roundup treatment groups compared with controls (ILCM: 6103 ± 174.1 μm^2) (Fig. 2B). Distance between the lamellas (DL) increased significantly (P<0.05) ~1.52-fold at a low dose (DL: 143.6 ± 1.6 μm) and ~2.00-fold at high dose (DL: 188.4±2.5 μm) of Roundup treatment groups compared with controls (DL: 94.28 ± 1.084 μm) (Fig. 2C).

Similar to gills, fish exposed to Roundup displayed several morphological changes in kidney tissues (Fig. 3A-C). Kidney tissue in the control groups (no Roundup) showed

the uniform structure of glomeruli as well as low numbers of melanocyte formation (Fig. 3A). Fish exposed to low- and high-dose groups showed an uneven distribution of bowman's space and higher accumulation of melanocytes (Fig. 3B-C). In kidneys, measuring the circumference of bowman's capsule (i.e., bowman's capsule area, [BCA]) showed a significant (P<0.05, Tukey's test) size reduction of around 1.82-fold in low dose (BCA: 125.7 ± 2.94 μm^2) and ~1.97-fold in high dose (BCA: 247.5 ± 3.705 μm^2) compared with controls (BCA: 247.5 ± 3.705 μm^2) (Fig 4A). Fish exposed to Roundup showed a high level of melanin formation in kidneys (Fig. 3A-C). Melanin accumulation (MA) significantly (P<0.05) elevated around 1.74- and ~4.94-fold in the low-dose (MA: 0.22 ± 0.003) and high-dose (MA: 0.67 ±0.01738) treatment groups compared with control groups (MA: 0.1266 ± 0.002645), respectively (Fig. 4B).

Effects of Roundup Exposure on Oxidative/Nitrative Stress Biomarker in Goldfish Tissues

2,4-dinitrophenyl protein (DNP) is an important oxidative stress biomarker (i.e., a key indicator of reactive oxygen species, ROS) in tissue and/or cells during environmental stress (Zhang et al., 2013; Nash et al., 2019; Lacy et al., 2022). To determine if Roundup exposure increases oxidative stress-related damage by ROS, DNP expression was estimated in goldfish tissues. The immunohistochemical (IHC) assay revealed a significant increase in DNP expression in gill and kidney tissues when fish were exposed to Roundup (Fig. 5A-F). In the gill tissues, DNP significantly (P<0.05) increased around 1.23-fold in low dose (optical density, OD: 0.15 ± 0.004), and ~1.66-fold in high dose (OD: 0.20 ± 006) Roundup treatment groups compared with control (OD: 0.12 ± 0.003) groups (Fig. 6A). Similarly, in the kidney tissues, immunoreactive (IR) intensity of DNP expression increased ~1.15-fold in the low dose group (P<0.05, OD: 0.20±0.003), and ~1.20-fold in the high dose group (P<0.05, OD: 0.21 ± 0.004)

compared with controls (OD: 0.17 ± 0.003) (Fig. 6B). No IR signal of DNP was detected in the negative control tissues (Fig. 6C, D).

Reactive nitrogen species (RNS) induce nitrative stress, which is directly correlated with the production of 3-nitrotyrosine protein (NTP) in cells and tissues of organisms (Torreilles and Romestand, 2001; Curtis et al., 2011). Therefore, we performed an IHC assay to estimate the expression of NTP goldfish tissues. Fish exposed to Roundup (both low- and high-dose treatment groups) upregulated NTP expression in gill and kidney tissues (Fig. 7A-F). NTP expression significantly (P<0.05) increased ~1.25-fold in the low dose (OD: 0.22 ± 0.009) group and ~1.4-fold in the high dose group (OD: 0.24 ± 0.009) compared with controls (OD: 0.17 ± 0.005) group in gill tissues of Roundup exposed goldfish (Fig. 8A). Similar to the gill tissue, kidney tissues showed significant (P<0.05) upregulation (~1.3-fold) of NTP expression in both low dose (OD: 0.16 ± 0.004) and ~1.33-fold in the high dose (OD: 0.16 ± 0.004) groups compared with controls (OD: 0.12 ± 0.003) (Fig. 8B). However, no IR signal of NTP was detected in the negative control tissues (Fig. 8C, D).

Effects of Roundup Exposure on Cellular Apoptosis in Goldfish Tissues

In situ TUNEL results exhibited the presence of apoptotic cells in the gill and kidney tissues. The TUNEL IR intensity significantly (P<0.05) increased around 1.5-fold at a low dose (OD 0.66 ± 0.010) and ~2.13-fold at a high dose (OD 0.94±0.10) compared to the gills at the control (OD 0.44 ± 0.005) groups (Fig. 10A).

A similar pattern of increased apoptosis cell formation in kidney tissues was observed in a dose-dependent manner (Fig. 9D-F). The IR intensity of apoptotic cells in kidney tissues was

significantly (P<0.05) increased by around 1.72-fold in low dose (0.56 ± 0.10) and ~1.48-fold in high dose (OD 0.49 ± 0.013) compared with control (OD 0.33 ± 0.006) groups (Fig. 10B).

Effects of Roundup Exposure on Antioxidant Enzyme Expression in Goldfish Tissues

SOD is an antioxidant enzyme that plays a vital role in protecting tissues and cells from free radicals generated from oxidative and nitrative stress (Brezniceanu et al., 2007); therefore, SOD expression was assayed in goldfish tissues using IHC analysis. In gill and kidney tissues, the high expression of SOD was detected in low- and high-dose Roundup exposure groups compared with controls (Fig. 11A-F). In gills, the SOD expression significantly (P<0.05) increased ~1.75-fold in low dose (OD: 0.22±0.003), whereas in high dose of Roundup exposure groups (OD: 0.23 ± 0.004), SOD expression increased ~1.83-fold compared with control (OD: 0.13 ± 0.004) (Fig. 12A). In kidney tissue, the estimated IR intensity showed a significant increase in SOD expression in both low- (~1.96-fold, OD: 0.19 ± 0.003) and high-dose (~2.10-fold, OD 0.20 ± 0.004) treatment of groups compared with controls (OD: 0.10 ± 0.003) group (Fig. 12B). No IR signal of SOD was detected in the negative control gill and kidney tissues (Fig. 12C, D).

Similar to SOD, CAT is also an antioxidant enzyme considered an essential biomarker for identifying cellular stress levels caused by free ROS and RNS (Pedrajas et al., 1995); therefore, CAT expression was assayed in goldfish tissues. Fish exposed to Roundup showed increased CAT expression in the gill and kidney tissues (Fig. 13A-F). IR intensity of CAT expression in gill tissues exhibited a significant (P<0.05) increase of ~1.23-fold CAT expression in low dose (OD 0.13±0.002) group and ~1.40-fold CAT expression in high dose (OD: 0.15 ± 0.002) groups compared to control (OD: 0.15 ± 0.002) group (Fig. 14A). Similar to gills, CAT

expression in the kidney tissues increased around 1.14-fold in low dose (P<0.05, OD 0.16 ± 0.002), ~1.24-fold in high dose (P<0.05, OD 0.18 ± 0.003) Roundup treatment groups compared to controls (OD 0.14 ± 0.002) (Fig. 14B). No IR signal of CAT was detected in the negative control tissues (Fig. 14C, D).

Effects of Roundup Exposure on Renin Expression in Goldfish Tissues

Renin is a central hormone enzyme produced by the kidneys and is very important in regulating blood pressure and maintaining electrolyte balance in vertebrates (Persson, 2003; Wong, 2016). Any alteration in renin enzyme balance would hamper the renin-angiotensin-aldosterone system (RAAS), which would eventually impair the kidney's glomerular filtration rate and fluid imbalance in the body (Wong, 2016); hence we estimated the renin expression in the kidney tissues of Roundup exposed fish. The renin expression in kidneys showed considerable upregulation with Roundup exposure groups (Fig. 15D-F). The IR intensity of renin significantly increased around1.73-fold in low dose (P<0.05, OD: 0.12 ± 0.003) and ~2.13-fold in high dose (P<0.05, OD: 0.14 ± 0.004) Roundup treatment groups compared with controls (OD 0.06±0.003) (Fig. 16B). No IR signal of renin was detected in the negative control of gill and kidney tissues (Fig. 16C, D).

Effects of Roundup Exposure on Na^+/K^+-ATPase Expression in Goldfish Tissues

The Na^+/K^+-ATPase, also known as ion pump/sodium ion pump, is an essential enzyme for maintaining ionic balance inside cells in vertebrates (Kaplan, 2002). The sodium ion pump maintains the sodium ion concentration, helps to regulate membrane potentials inside cells, and controls osmotic equilibrium (Mulkidjanian et al., 2012; Clausen et al., 2017); therefore, we

estimated Na^+/K^+-ATPase expression in the gill and kidney tissues of Roundup-exposed fish. Our IHC results exhibited a low expression of Na^+/K^+-ATPase in both gill and kidney tissues in a dose-dependent manner (Fig. 17A-C). The estimated IR intensity of Na^+/K^+-ATPase enzyme shows a significant decline of ~1.15-fold in low dose ($P<0.005$, OD 0.19 ± 0.005) and ~1.33-fold in high dose ($P<0.005$, OD 0.17 ± 0.005) of Roundup treatment groups compared to controls (OD: 0.22 ± 0.005) (Fig. 18A-C). However, negative control tissues demonstrated no IR signals (Fig. 18C, D).

CHAPTER IV

DISCUSSION

Scientists and experts in the ecotoxicology field have been expressing grave concern about the consequences of pesticides on the environment. One of the most used pesticides is Roundup, a glyphosate-based organophosphate herbicide (GBH), which is considered a major source of environmental pollution in the United States. Notably, the sheer amount of GBH used in the farmlands is outnumbering not only the targeted unwanted weeds but also it is having a tremendously deteriorating impact on other non-targeted species in both terrestrial and aquatic environments (Benachour et al., 2007; Cavalcante et al., 2008; Harayashiki et al., 2013; Mesnage et al., 2015; Strilbyska et al., 2022; Yadav et al., 2013). There are various studies conducted on the short-term exposure or acute toxicity of Roundup in teleost species (de Menezes et al., 2011; Fan et al., 2013; Lushchak et al., 2009; Ma et al., 2015; Marques et al., 2014; Modesto & Martinez, 2010). However, only a few studies concern long-term exposure to Roundup, leaving a colossal study gap in this field (Smith et al., 2019). Nonetheless, we attempted to reduce some of the study gaps in this study by elucidating the underlying mechanism of Roundup exposure (low dose: 0.5 µg/L and high dose: 5.00 µg/L for 2 weeks) on morphological alternation, oxidative/nitrative stress, antioxidant and osmoregulatory enzyme expressions, and cellular apoptosis in the gills and kidneys of goldfish. Our results demonstrated a detrimental cellular

alteration of morphological characteristics, increased oxidative and nitrative stress biomarkers (i.e., DNA and NTP), cellular apoptosis, antioxidant enzymes (i.e., SOD and CAT) in both gills and kidneys, and increased renin expression in the kidneys, and decreased Na^+/K^+-ATPase (NKA) expression in the gills of fish exposed to Roundup. Taken together, our results suggest that Roundup exposure initiates morphological changes and induces oxidative/nitrative stress in cells and tissues of goldfish, which can be detrimental to the fitness of fish to a great extent.

Effects of Roundup Exposure on Morphological Changes in Goldfish Tissues

The fish gill is arguably the most anatomically and physiologically diversified organ because it is directly contacted with water and any toxicants (i.e., pollutants) have to go through it to come into the blood circulation (Banaee, 2012; Badroo et al., 2020). Histological analysis revealed visible lesions in the morphology of gills in fish exposed to environmental pollutants (Bernet et al., 1999; Couillard et al., 1988). The most commonly cataloged histopathological lesions are gill lamellae fusion, loss of interlamellar cell mass, and changes in the epithelium layer such as hyperplasia, lifting, necrosis, desquamation, etc. (Mallatt, 1985). An important finding of the present study is that fish exposed to Roundup increased the fusion of secondary lamellae, mucous, epithelium uplifting, and loss of gill thickness. In addition, analysis of biological values indicated that the protruding lamellae length (PLL) was significantly increased in both low- and high-dose of Roundup treatment groups. Interestingly, a strong negative correlation between interlamellar cell mass (ILCM) and Roundup exposure was estimated in low- and high-dose treatment groups, meaning gills have potentially lost their cellular mass in areas between lamellas. Moreover, we evaluated the distance between lamellae (DLL) between two adjacent lamellas in the different treatment groups and found increased DLL value in both

low- and high-dose treatment groups, indicating that the gills have become thinner to Roundup exposure. Our experimental findings aligned with Lacy et al. (2022) study as they have confirmed that long-term exposure to high temperature and pesticide mixtures (22-32 °C, 8 pesticide mixture: metolachlor, linuron, isoproturon, tebuconazole, aclonifen, atrazine, pendimethalin, and azinphos-methyl for 4 weeks) cause similar morphological changes such as decreases in ILCM, secondary lamellae curling, fusion, clubbing, and epithelial layer modification in the gills of goldfish. Another relevant study by Jacquin et al. (2019) showed that short-term exposure to high temperature and pesticide mixture (22-32 °C, 7 pesticide mixture: S-metolachlor, isoproturon, linuron, atrazine-desethyl, aclonifen, pendimethalin, and tebuconazole, for 96 h) alter the morphology of gills (e.g., reduced ILCM, reduced epithelium surface area, lamellar fusion) in goldfish. Additionally, a similar study by Mishra and Mohanty (2008) found that chromium exposure (20-40 mg/L for 96 h) causes modifications in the gills (e.g., lamellar fusion, curling, modification of gill epithelium: necrosis, hyperplasia, lifting, and desquamation) of snakehead (*Channa punctatus*). Similar to the aforementioned studies, Badroo et al. (2020) also demonstrated almost identical damage on snakehead gills when fish were exposed to paraquat dichloride (PQ, one of the most widely used herbicides in the United States; 32.93 mg/L for 24-96 h). Information gathered from these studies and together our results suggest that fish exposed to environmental pollutants (i.e., pesticides) may cause alteration in the morphology of gills as a sign of stress response in teleost fishes.

In addition to gills, the fish kidney is also one of the most important organs for toxicological study, which exhibits moderate to extreme and irreversible changes in its morphology at pesticide/chemical exposure (Shiogiri et al., 2012). Also, as the kidney is one of the major biological filtration systems in vertebrates and receives the highest amount of post-

branchial blood flows, and therefore, kidney lesions are expected in exposure to pesticides, making it a suitable bioindicator of noxious toxic stress response studies (Ortiz et al., 2003). Moreover, many studies have been conducted on the effects of various environmental stressors in the kidney as it is one of the important organs to study toxic responses and found induction of specific alteration in numerous components in the kidney of teleost species (Karlsson-Norrgren et al., 1986; Olojo et al., 2005; Randi et al., 1996). In the present study, we observed widespread morphological damages across the kidneys, including shrinkage in the bowman's capsule area, melanocyte formation, tubular epithelium degeneration, uneven distribution of organelles, hemorrhaging, and glomerular shrinkage in both low- and high-dose Roundup treatment groups. Recently, Lacy and Rahman (2022) demonstrated compelling evidence on goldfish co-exposed to high temperature and pesticide mixtures exhibit widespread damage in different organelles of the kidney (e.g., bowman's space shrinkage, melanin accumulation, and tubular epithelium degeneration) in goldfish. A similar study by Ma et al. (2015) indicated a detrimental alteration of kidney architecture (e.g., renal tubule damage and renal parenchyma vacuolization) in common carp (*Cyprinus carpio*) exposed to sublethal glyphosate dosage (52.08-104.15 mg/L for 168 h). The work of Mishra and Mohanty (2008) on spotted snakehead noted a similar observation (e.g., reduction of the renal lumen, shrinkage of the glomerulus, and expansion in bowman's area in the kidney, hypertrophied epithelial cells of renal tubules) on hexavalent chromium, a heavy metal, exposure (20-40 mg/L for 96 h). Furthermore, Samanta et al. (2016) demonstrated that glyphosate-based herbicide (17.2 mg/L for 30 days) causes similar damages in kidney morphology (e.g., glomeruli degenerative changes, hematopoietic tissue loss, and vacuolation in the renal tubules) in catfish (*Heteropneustes fossilis*). Recently, Badroo et al. (2020) demonstrated herbicide PQ exposure (32.93 mg/L for 24- 96 h) causes morphological

alterations (e.g., necrosis, bowman's space area increase, glomeruli shrinkage, and renal tubules degeneration) of different parts of kidney in spotted snakeheads. Therefore, based on our findings and together with previous studies, it can be interpreted that exposure to environmental contaminants leads to extensive damage of kidney architecture in teleost fishes.

Effects of Roundup Exposure on Oxidative/Nitrative Stress Biomarker in Goldfish Tissues

One of the main objectives of this study is to determine whether pesticide (i.e., Roundup) exposure causes oxidative and/or nitrative damage in the gill and kidney tissues of goldfish. ROS and RNS are naturally occurring free radicals that are essential for the cell transduction process; however, excessive production of free radicals contributes to oxidative and nitrative stress in cells and tissues (Durairajanayagam, 2019; Snezhkina et al., 2019). An elevated level of oxidative stress damages cellular organelles, disrupts homeostasis, induces activation of apoptotic pathways, and reduces tumor suppressor gene expression (Galadari et al., 2017; Martins et al., 2021). Both terrestrial and aquatic organisms exposed to environmental pollutants (i.e., pesticides, heavy metals, drugs, chemical waste, etc.) showed an elevated level of ROS production, which causes homeostatic imbalances (Lushchak et al., 2009). The interruption of homeostasis in the cell can be quantified using biomarker expiration, which may elucidate specific cellular stress responses mechanism at toxic exposure in aquatic environments (Valavanidis et al., 2006). In this study, we used DNP as a biomarker to quantify the oxidative stress in fish under Roundup exposure. Immunohistochemistry (IHC) results in exhibited upregulation (approximately 1.5-fold) of DNP expression in the gills suggesting a potential toxic effect of Roundup in goldfish. We observed a similar trend in the kidney where the DNP level elevated around 1.14-fold when fish were exposed to Roundup. However, in kidneys, between

LD and HD, the difference was not found. One possible explanation is during the stress period of 2 weeks, the peak of DNP expression in kidney HD was missed. These amplified expressions of DNP can be correlated with oxidative stress-related damage and overproduction of free radicals in fish tissues/organs. Recently, Lacy et al. (2022) and Lacy and Rahman (2022) reported almost identical trends in DNP expression in the gills and kidneys when goldfish were exposed to high temperature and pesticide mixtures (22-32 °C, 8 pesticide mixture for 4 weeks). A similar study by de Menezes et al. (2011) showed that a mild dosage of Roundup (0.45 -0.95 mg/L for 8 days) induces oxidative stress as various antioxidant enzyme production spiked in the liver and brain of South American catfish (*Rhamdia quelen*). In addition, Maksymiv et al. (2015) demonstrated that goldfish exposed to high concentrations of herbicide Sencor (7.14-71.4 mg/L for 96 h) induces oxidative stress and cause hepatotoxicity in the liver of goldfish. Another study on Roundup Transorb (RDT) toxicity by Martins et al. (2021) found that silverside fish exposed to environmentally relevant dosages of RDT (2.07-3.68 mg/L for 24 h) exhibit upregulation of ROS production in erythrocytes indicating oxidative damage. With the support of other studies, the interpretation of our results suggests that pesticide induces oxidative stress in fish which have defined harmful effects on overall fitness and accelerates the progression of other physiological impairments and/or complications.

Notably, RNS are various nitric oxide-derived compounds, including nitroxyl anion, nitrosonium cation, etc. that have comparable adverse effects on cells and tissues similar to ROS, induced by environmental pollutants in both terrestrial and aquatic organisms (Johnstone et al., 2019; Koskenkorva-Frank et al., 2013; Manisalidis et al., 2020; Nash et al., 2019). Importantly, NTP is widely used as an effective biomarker to estimate the nitrative stress induced by RNS (Ahsan, 2013). In this study, we observed an upregulation of NTP expression in the gills

approximately 1.3-fold and kidneys around 1.3-fold in a dose-dependent manner suggesting that the overproduction of nitric oxide-derived compounds due to Roundup exposure may cause nitrative stress-related tissue damage. Compelling evidence supporting our data was provided by Lacy et al. (2022) and Lacy & Rahman (2022), as they have also noted a similar upregulation in NTP expression in their heat stress and pesticide exposure study in goldfish. Moreover, our findings also correspond to a relevant study by Padmini et al. (2009), where they found higher NTP levels in liver tissues of grey mullets (*Mugil cephalus*) collected from highly polluted river sites compared to fish of other less polluted areas. Similarly, Ekambaram et al. (2014) found higher NTP expression in grey mullets collected from highly polluted estuaries compared with less polluted estuaries. Recently, Taysi et al. (2021) demonstrated the induction of nitrative stress by observing several-fold increases in peroxynitrite ($ONOO^-$, an RNS) in different dosages of mercury chloride (37.75-275 µg/L for 2-7 days) in the gills of rainbow trout (*Oncorhynchus mykiss*). Considering all these studies on the effect of environmental pollutants on higher NTP expression suggests that environmental pollutants/contaminants increase nitrative stress in cells and/or tissues which may lead to increased cellular apoptosis in fish.

Effects of Roundup Exposure on Cellular Apoptosis in Goldfish

The interaction of a heterogeneous array of signaling pathways regulates the cellular stress response mechanism, and the intensity of the stress responses is widely variable depending upon different stress conditions and biological factors (Lavie, 2015; Murray-Zmijewski et al., 2008)). For instance, whereas mild oxidative stress initiates a self-repairing mechanism inside cells, a high amount of free radical production produces programmed cell death (also called apoptosis) as a last resort to eradicate damaged cells/tissues (Huang et al., 2000; Kanthasamy et

al., 2003). Much evidence has been concluded that environmental pollutants (i.e., pesticides, heavy metals, industrial chemical wastes, etc.) induce cellular apoptosis (Gazsi et al., 2021; Guo et al., 2017; Parlak, 2018 ; Panetto et al., 2019l pesticide's toxicological profiling must be evaluated to ensure the safety of non-targeted species in the ecosystem (Xu et al., 2018). One of the interesting findings in our study is that exposure to Roundup increased cellular apoptosis (*in situ* TUNEL assay estimation) in both gills (approximately 1.8-fold) and kidneys (approximately 1.6-fold) of goldfish in a dose-dependent manner hinting at a solid correlation between toxic exposure and cell death. Our findings also coincide with the results of Lacy and Rahman (2022) and Lacy et al. (2022), as their papers reported comparable outcomes when they exposed goldfish to high temperature and pesticide mixture (22-32 °C, 8 pesticide mixture, for 4 weeks); the cellular apoptosis increased several-fold in the gills and kidneys of goldfish. Recently, Lanzarin et al. (2021) demonstrated wild-type and transgenic zebrafish larvae (72 hpf) exposed (5-250 µg/L) to Roundup Flex (RF) and confirmed the increase in the apoptotic cell formations in zebrafish tissues. Another study by Ramírez-Duarte et al. (2008) identified that Roundup exposure (50-97.5 mg/L for 96 h) triggers an acceleration of apoptosis and necrosis in eosinophilic granule cells/mast cells in the pirapitinga (*Piaractus brachypomus*) as the stress response. Uren & Santos (2015) revealed in their work indications of up-regulation of several key regulatory pathways, including calcium signaling, tumor necrosis factor (TNF), and mitogen-activated protein kinase (MAPK), following exposure (0.01-10 mg/L for 14 days) to both glyphosate and Roundup in brown trout (*Salmo trutta*). These incidents of increased cellular apoptosis are strongly correlated with the overproduction of oxygenic and nitrogenic free radicals which induce oxidative stress in the liver of brown trout. All the scientific studies coinciding with our results suggest that fish exposed to pesticides upregulated cellular apoptosis

in different organs. Moreover, the overproduction of ROS/RNS initiates oxidative damage which ultimately imitates the programmed cell death mechanism. In addition, (Lei et al., 2016; Pham-Huy et al., 2008) but nevertheless, in stressful environmental conditions, increased production of these compounds is required to counteract oxidative damage, making this yet another sign of oxidative stress and a sign that the body is assertively trying to prevent cell death and damage.

Effects of Roundup Exposure on Antioxidant Enzyme Expression in Goldfish Tissues

Cells have developed an innate defense mechanism to offset the activated ROS/RNS, known as an antioxidant defense system (Wang et al., 2009). Enzymes including SOD, CAT, glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR) are the crucial components of antioxidant defense systems which play an essential role in reducing oxidative stress damages (Bokov et al., 2004; Kryston et al., 2011). These enzymes also serve as environmental stress status indicators (Lortz et al., 2000; Monteiro et al., 2010). Each of the antioxidant enzymes has its distinct biochemical functions. For example, enzymes SOD and CAT are protective protein molecules that attach themselves with free radicals and aid in preventing cellular injuries, neutralizing the harmful effects of oxidative stress, including cell and DNA damage, and increasing apoptosis cell deaths (Salvi et al., 2007; Small et al., 2012; Ishaq et al., 2014). Importantly, SOD catalyzes the dismutation of the superoxide anion radical into water and hydrogen peroxide, and CAT detoxifies the hydrogen peroxide by metabolizing it into oxygen and water (de Menezes et al., 2011). One of the key findings in this study was that exposure (2 weeks) to Roundup proportionally upregulated the expression of SOD and CAT in both gills and kidneys of goldfish. Recently, Lacy et al. (2022) and Lacy and Rahman (2022) reported a considerable increase in the immunoreactive intensity of SOD enzyme in the gills and

kidneys of goldfish exposed to long-term (30 days) high temperature (22-32 °C) and pesticide mixtures (8 different pesticides). According to Ozcan et al. (2004), a combination of pesticides, 2,4-dichlorophenoxyacetic acid and azinphosmethyl exposure (0.23 and 87 ppm for 96 h) results in a considerable dose-dependent increase in SOD in the gill, kidney, liver, and brain of Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyrinus carpio*). Similarly, we also observed an elevated SOD expression (around 1.75 to 1.83-fold) in the gill and kidney tissues under realistic environmental concentrations of Roundup. Such an increase in SOD enzyme has likely neutralized the activated ROS/RNS to prevent oxidative/nitrative stress in fish tissues during exposure to environmental contaminants.

In addition to SOD, we also observed CAT expression increased around 1.23-1.40-fold in the gill and kidney tissues of fish exposed to Roundup. These results are consistent with the research findings stated above and show a high risk of inducing oxidative stress in fish exposed to the pollutant. Our results seamlessly correspond to findings by Lacy and Rahman (2022) and Lacy et al. (2022) who demonstrated that CAT expression increases in goldfish gills and kidneys in a dose-dependent manner at heat stress and pesticide mixtures. A recent experiment by Khare et al. (2019) found synergistic intoxication from pesticide exposure (methyl parathion: 16-20 mg/L and carbaryl: 4-8 mg/L for 96 h) to Indian carp (*Catla catla*) showed around 700-, 300- and a 7-fold increase in CAT expression in the muscle, livers, and gills tissues, respectively. Additionally, Lushchak et al. (2009) showed Roundup exposure (2.5-20 mg/L for 96 h) increases CAT activity around 1.5-fold in the livers and ~1.3-fold in the kidney; however, they found SOD activity decreased in the brain, liver, and kidney tissues of goldfish. The amalgamation of these results from previously conducted studies, in concert with our findings, strongly suggests that there is a strong correlation between pro-oxidant and antioxidant homeostasis alteration to

pesticide exposure. This alteration in the antioxidant system is associated with health risks and the fitness of teleost fishes.

Effects of Roundup Exposure on Renin Expression in Goldfish Tissues

Renin is an important enzyme in the renin-angiotensin-aldosterone system (RAAS, a classic endocrine system) in vertebrates (Capelli et al., 1970; Henderson et al., 1993). It plays a crucial role in fluid-ion balance, filtration, blood pressure, hypertension, and regulation of aldosterone secretion in the kidneys of vertebrates (Nishimura & Ogawa, 1973). Only a few studies covered how pesticides alter renin expression in freshwater fish kidneys (Lacy & Rahman, 2022); however, there is no information on Roundup toxicity and renin activity in teleost species. The synergistic effect of pollution-induced oxidative stress disrupted prooxidant-antioxidant imbalance, and cell death may alter the activity/expression of renin in vertebrates (Husain et al., 2015; Kabel et al., 2020; Lacy & Rahman, 2022; Mahmood et al., 2014). However, previous studies have found pollutants induced various responses in RAAS (Aztatzi-Aguilar et al., 2015; Bourdrel et al., 2021), which further complicates our understanding as some types of environmental stressors suppress the renin expression, whereas other types of stressors may elevate the renin activity. The results of the present study tried to pin down the specific effects of a single exposure to Roundup on renin expression in fish kidneys. We estimated a significant rise in renin expression with a higher dosage of Roundup, where the renin expression was elevated ~1.73-2.13-fold in goldfish kidneys, suggesting a correlation between renin expression and pesticide exposure. We also observed a similar trend of increased renin expression in fish gills under Roundup exposure. However, in the gills HD group, renin expression was not significantly different than LD. It can be suggested that the peak of renin

expression was missed in 2 weeks exposure period. Our results are consistent with Arillo et al. (1981) as they confirmed a positive correlation between pollutant exposure (unionized ammonia 20-500 µg/L for 24-48 h) and increased renin activity in rainbow trout. Contrary to our results, Lacy and Rahman (2022) showed that exposure to heat stress and pesticides exposure (22-32 °C, 8 pesticides for 4 weeks) suppressed the renin expression in the kidney of goldfish. Similar to the findings of Lacy and Rahman (2022), Bolterman et al. (2005) found renin activity declined in captopril drug (100 mg/kg/day for 15 days) exposure to hypertensive in rat kidneys. Some early studies also showed how environmental stressor (e.g., heat) increases renin activity, as presented by Eisman and Rowell (1977) & Kosunen et al. (1976). Our findings, supported by previously published works, can interpret that toxic exposure alters the renin activity/expression and damages the kidney's basic structure. We can also suggest that alteration of renin expression hampers renal activity and deviations in prooxidant-antioxidant homeostasis in teleost kidneys.

Effects of Roundup Exposure on NKA Expression in Goldfish Tissues

NKA is a key enzyme in cellular and biochemical homeostasis as it helps maintain osmotic pressure, resting membrane potential, secondary active transport, cell volume neural activity, and signal transduction (Kaplan, 2002). NKA is a driving force in the transepithelial movement of ions in the gills of aquatic organisms (Lucu & Towle, 2003). The way the NKA enzyme alters the expression in the cells/tissues under different ionic concentrations across the cellular membrane makes it a worthy candidate for toxicology research (Mulkidjanian et al., 2012; (Oruc, 2010). Therefore, we examined the NKA expression under Roundup exposure in the present study, especially in fish gills. Our IHC analysis showed a significant reduction of NKA expression in the gills when fish were exposed to low- and high-dose of Roundup,

suggesting that pesticide exposure alters the osmoregulatory balance by disrupting the ion pump function. We also observed a steep drop in NKA expression in the goldfish kidney. Our results coincide with Lacy et al. (2022) findings as they found an attenuation (~3.4-15-fold) in NKA expression in goldfish gills exposed to heat stress and pesticide mixtures. A similar observation was also reported by Staurnes et al. (1984) when they exposed salmon (*Salmo salar*) to aluminum (200 µg/L for 4-7 days); the NKA activity showed a significant drop in the gills, which is associated with reduced plasma concentration of sodium and chloride. Furthermore, Ay et al. (1999) showed exposure to both copper and lead (20-160 g/L for 14 days) drastically reduces the activity of NKA in the gills of tilapia. Additionally, many studies have confirmed environmental pollutants (e.g., pesticides) inhibit NKA activities/expression in fish (rainbow trout, zebrafish, goldfish), leaving them immunocompromised to diseases and damages (Morris et al., 2016). Combination of the information from previous studies and our results, we suggest that fish exposed to environmental contaminants may inhibit NKA expression, which additionally may exert cellular stress (e.g., oxidative damage, apoptosis, etc.) in aquatic organisms.

CHAPTER V

CONCLUSION

To the best of our knowledge, this study established the first attempt to elucidate the underlying mechanism of how Roundup induces redox status and cell death, imbalances prooxidant-antioxidant homeostasis, and alters osmoregulatory enzyme expression in the gills and kidneys of goldfish. Notably, this study also provides compelling evidence that Roundup drastically changed/damaged the morphological, biochemical, and cellular features in tissues. Our findings clearly demonstrated that exposure to Roundup drastically alters stress biomarkers by increasing the overproduction of DNP and NTP, leading to increased apoptosis and altered SOD, CAT, renin, and NKA expressions in both gills and kidneys glands of goldfish. Taken together, this study strongly suggests that the herbicide Roundup wields extensive detrimental effects on the health and fitness of goldfish. Finally, we hope that this study will advance our knowledge of the histopathological alterations and biochemical and cellular damage caused by pesticide intoxication in the tissues of teleost species. Future studies will be required to identify how much glyphosate gets absorbed in the tissues/organs of fish exposed to Roundup as well as how much epigenetic signal is transformed by Roundup exposure in the next generation (i.e., transgenerational effects).

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APPENDIX

APPENDIX

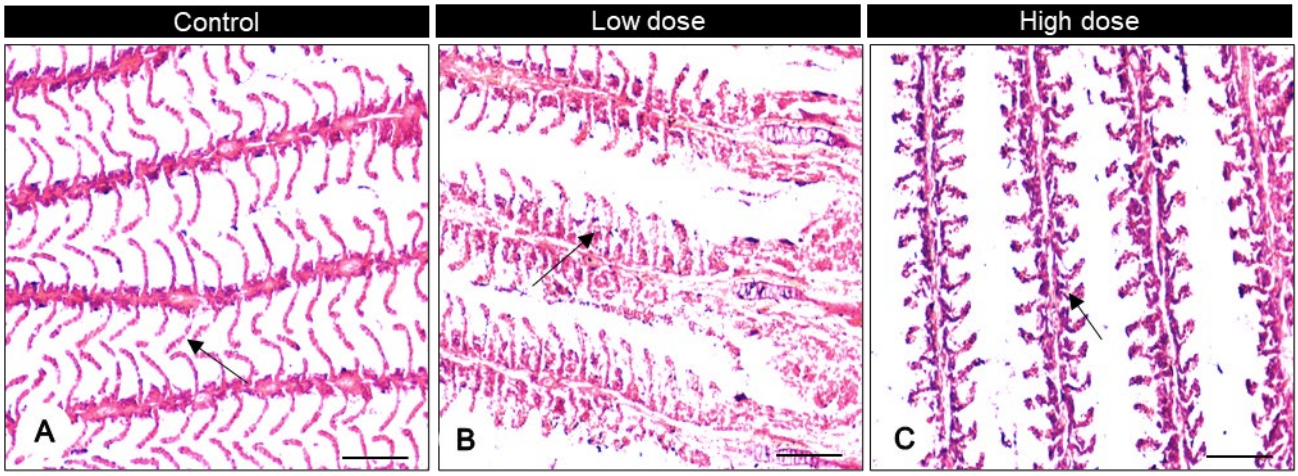

Figure 1: Effects of 2-week Roundup exposure on the morphology in gills of goldfish. (A-C) Histological appearance of representative photographs of gills in control (no Roundup) (A), low dose (B) and high dose (C). Arrow indicates some specific points (e.g., protruding lamellae length (PLL), interlamellar cell mass (ILCM) and distance between lamellae (DLL) of gill which has gone through gill remodeling in different dosage. Scale bar = 100 μm.

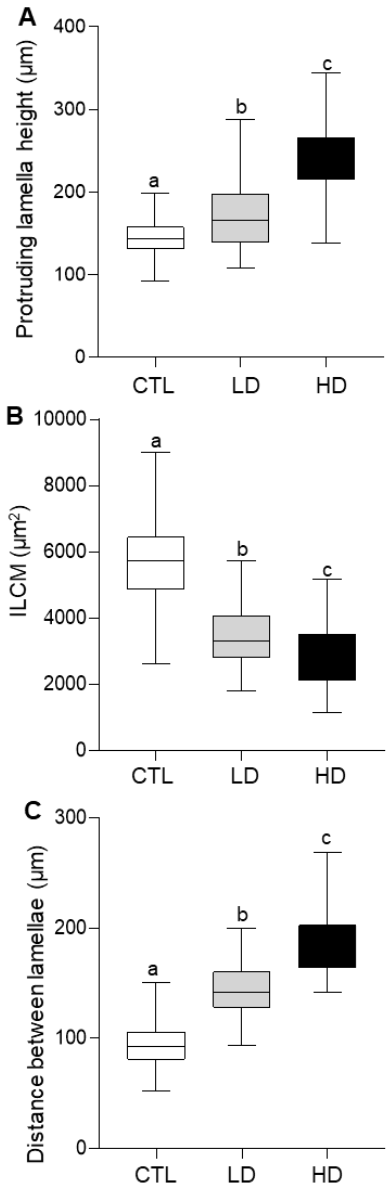

Figure 2: Effects of 2-week Roundup exposure on biological values in gills of goldfish. (A) Protruding lamellae length change value. (B) Gill interlamellar cell mass (ILCM). (C) Distance between each lamellae in gills measured. The whiskers represent maximum and minimum value for each datasets. Each value represents the mean ± SEM (N = 112-307). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$).

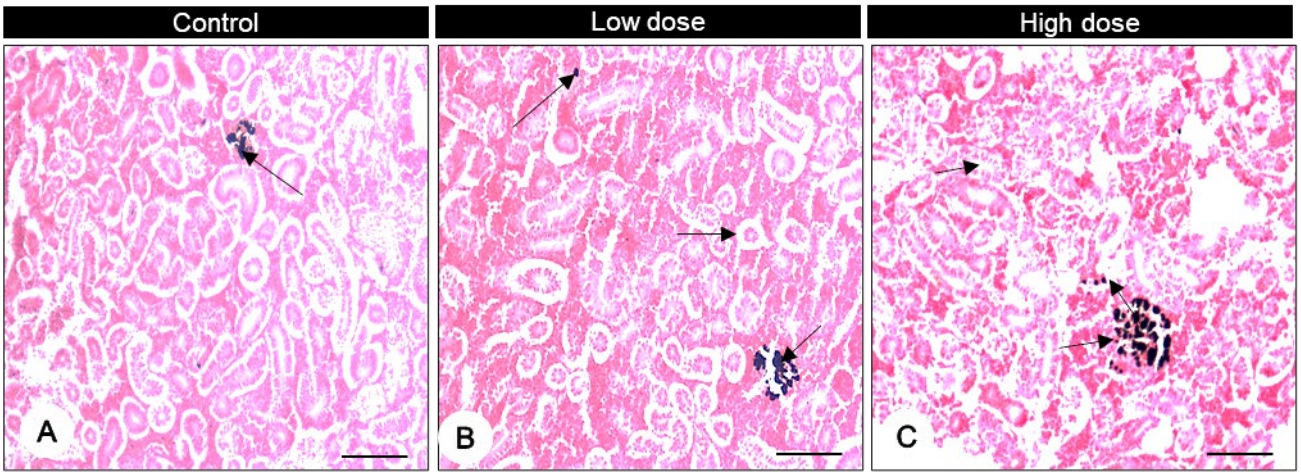

Figure 3: Effects of 2-week Roundup exposure on the morphology in kidneys of goldfish. (A-C) Histological appearance of representative photographs of kidneys in control (no Roundup) (A), low dose (B) and high dose (C). Arrow indicates some specific points (e.g., melanin pigment (MP) formed, bowman capsule (BC) area changed, etc.) in kidneys. Scale bar = 100 μm.

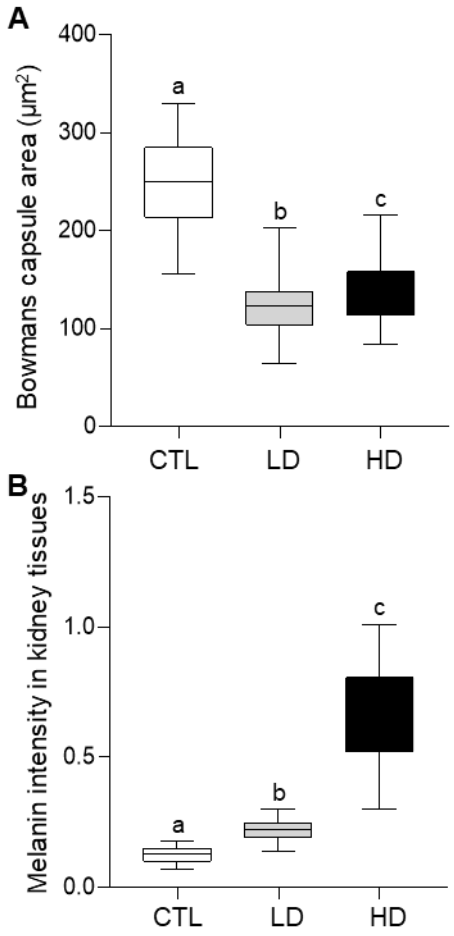

Figure 4: Effects of 2-week Roundup exposure on biological values in kidneys of goldfish. (A) Area of bowman's capsule. (B) Immunoreactive intensity of melanin formation. The whiskers represent maximum and minimum value for each dataset. Each value represents the mean ± SEM (N = 102-195). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$).

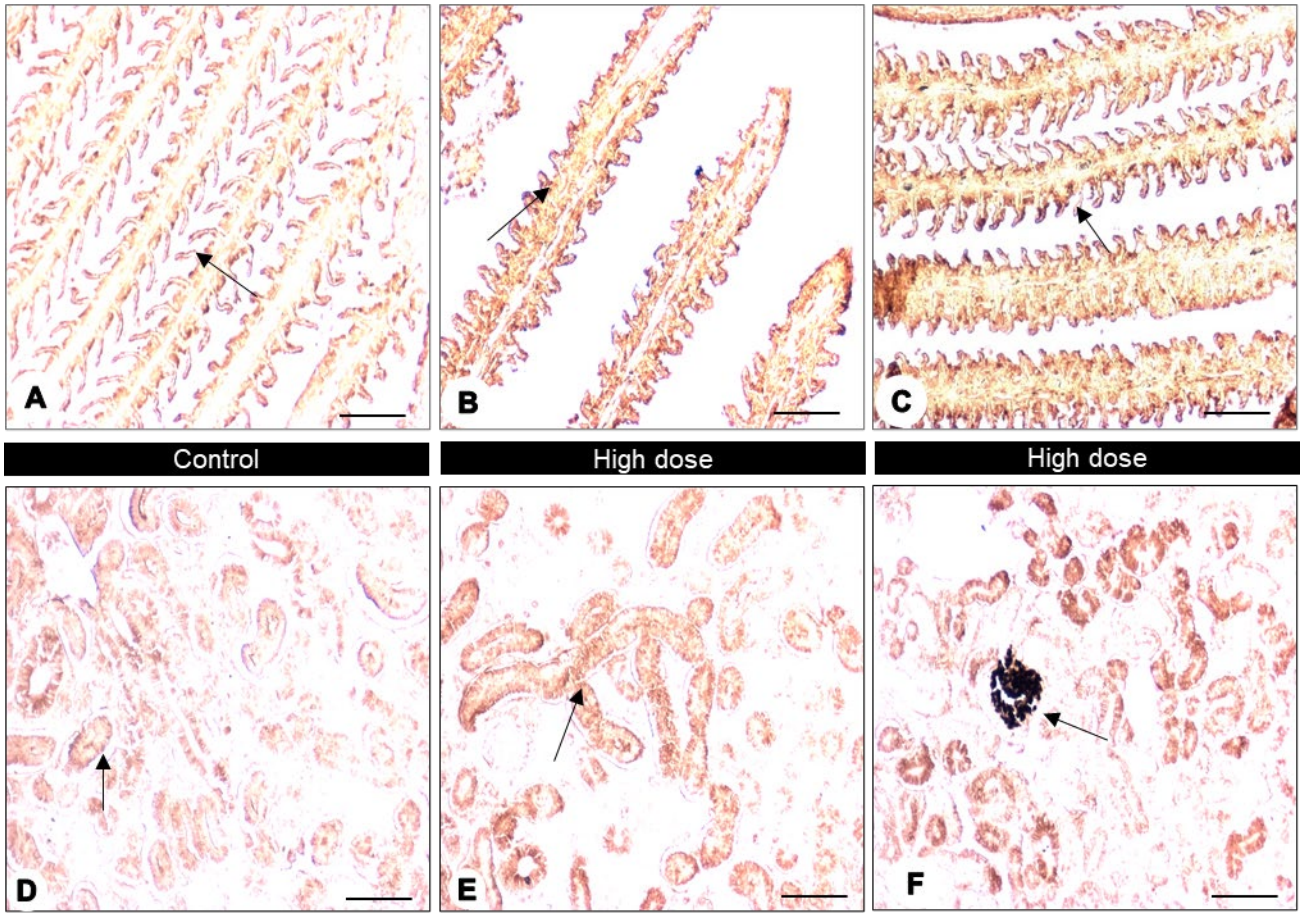

Figure 5: Effects of 2-week Roundup exposure on 2,4-dinitrophenol protein (DNP) expression in gills and kidneys of goldfish. Arrows indicate higher protein expression. DNP expression in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) DNP expression in representative photographs of kidneys in goldfish collected in CTL (A), LD (B), and HD (C) Roundup exposed groups. MP, melanin pigment. Scale bar = 100 µm.

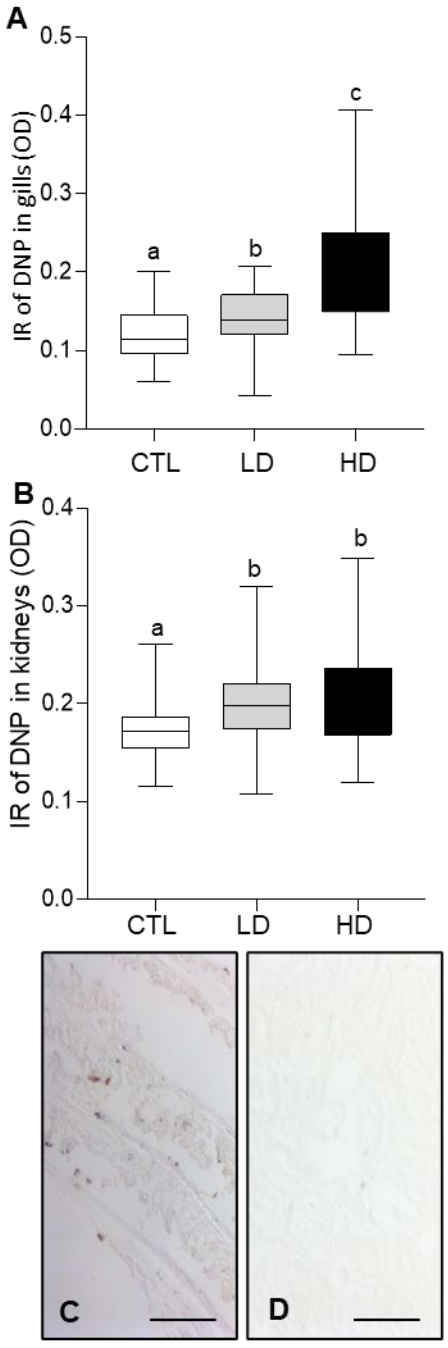

Figure 6: Effects of 2-week Roundup exposure on 2,4-dinitrophenol protein (DNP) immunoreactive (IR) intensity in (A) gills and (B) kidneys of goldfish. The whiskers represent the maximum and minimum values for each dataset. Each value represents the mean ± SEM (N = 112-307). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$). ImageJ software was used to measure the optical density (OD) values of protein expression. (C-D) Negative controls of DNP in gills (C) and kidneys (D). Scale bar = 100 µm

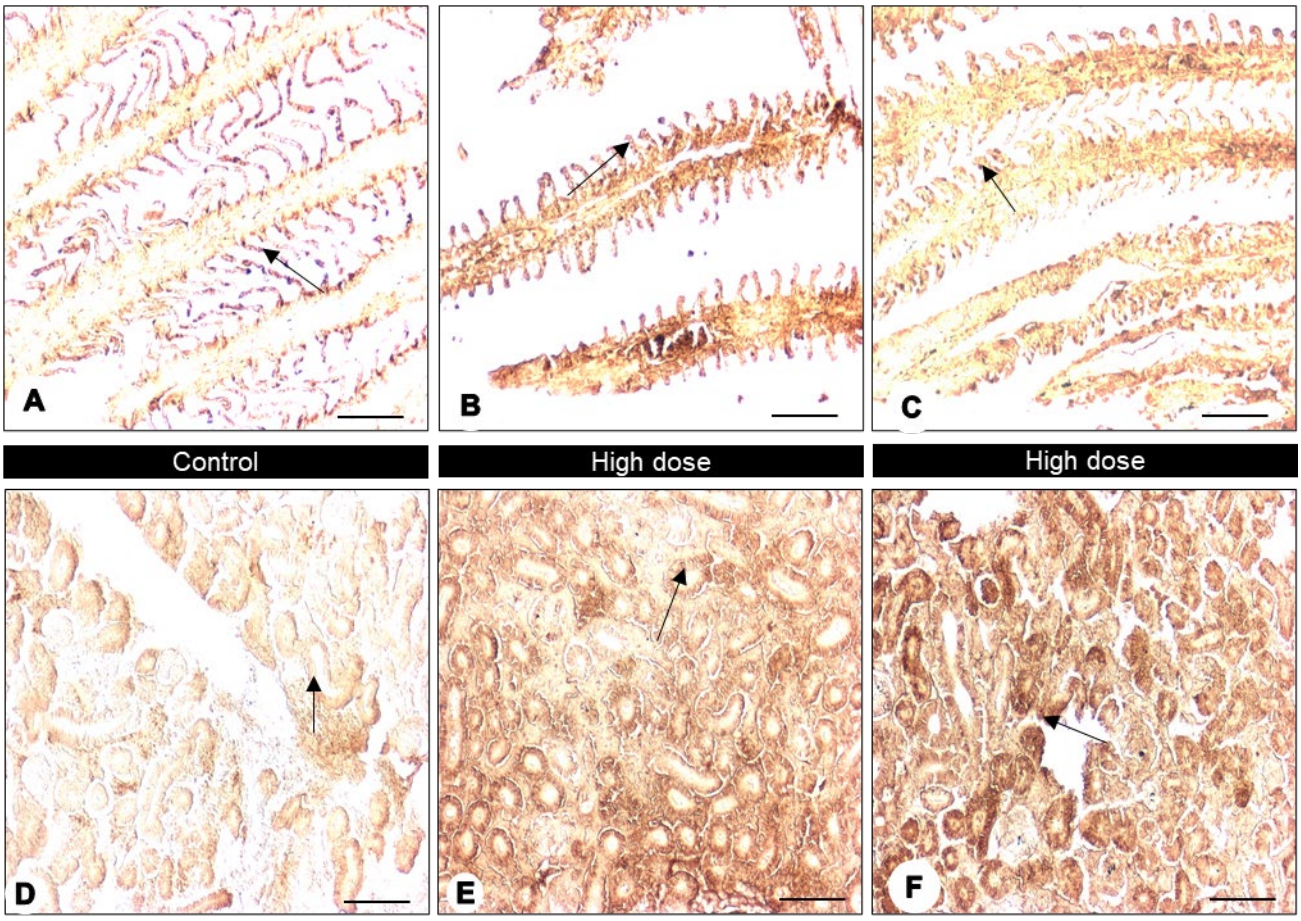

Figure 7: Effects of 2-week Roundup exposure on 3-nitrotyrosine protein (NTP) expression in gills and kidneys of goldfish. Arrows indicate higher protein expression. NTP expression in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) NTP expression in representative photographs of kidneys in goldfish collected in CTL (A), LD (B), and HD (C) Roundup exposed groups. Scale bar = 100 µm.

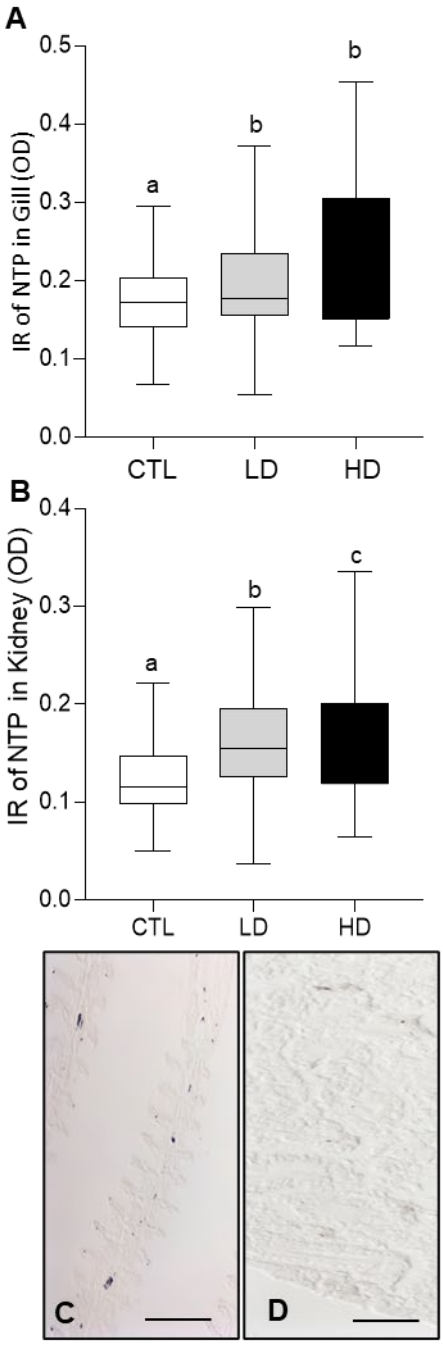

Figure 8: Effects of 2-week Roundup exposure on 3-nitrotyrosine protein (NTP) immunoreactive (IR) intensity in (A) gills and (B) kidneys of goldfish. The whiskers represent the maximum and minimum values for each dataset. Each value represents the mean ± SEM (N = 108-185). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$). ImageJ software was used to measure the optical density (OD) values of protein expression. (C-D) Negative controls of NTP in gills (C) and kidneys (D). Scale bar = 100 µm

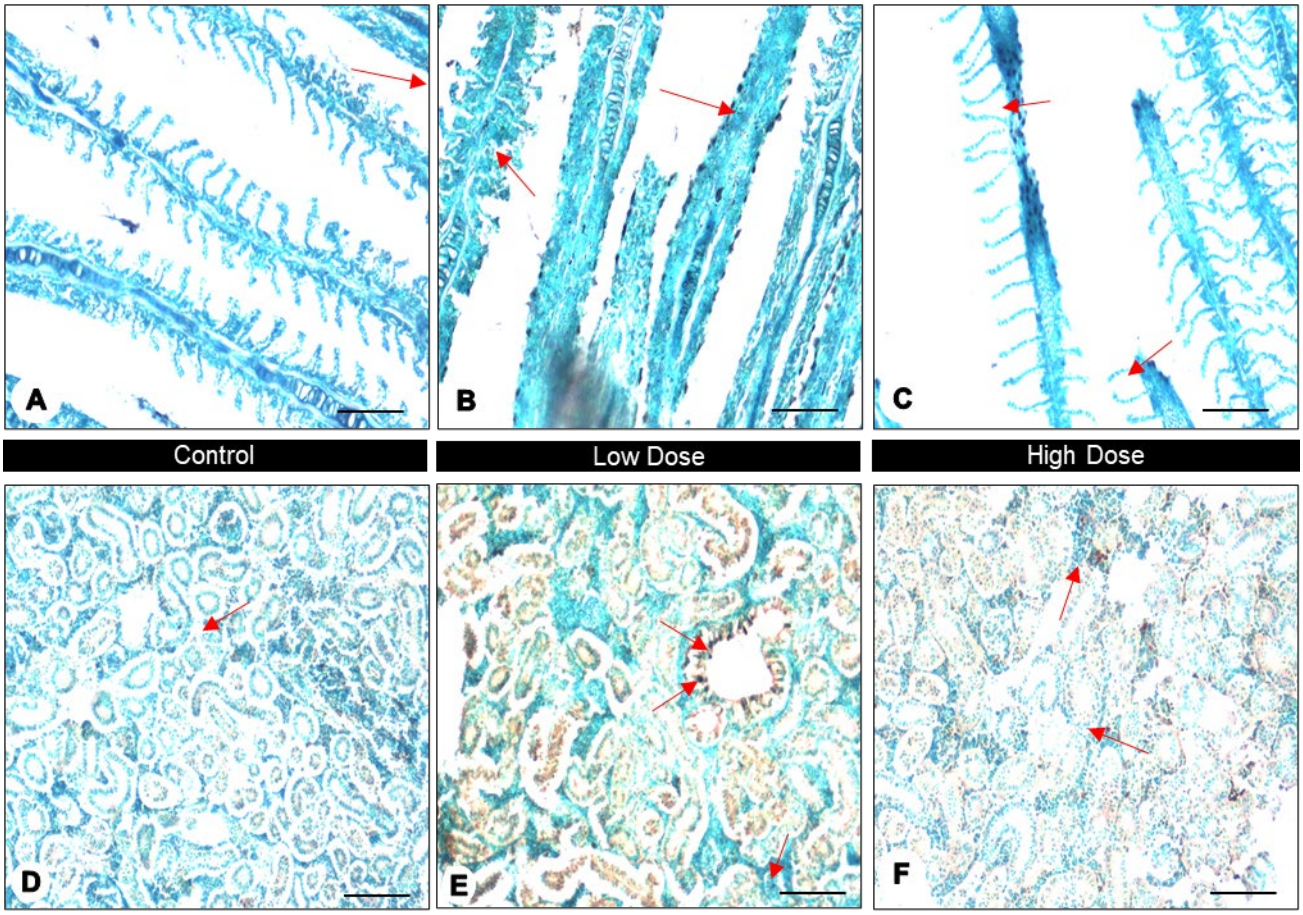

Figure 9: Effects of 2-week Roundup exposure on cellular apoptosis in gills and kidneys of goldfish. Arrows represent apoptotic nuclei. TUNNEL intensity in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) TUNNEL intensity expression in representative photographs of kidneys in goldfish collected in CTL (A), LD (B), and HD (C) Roundup exposed groups. Scale bar = 100 µm.

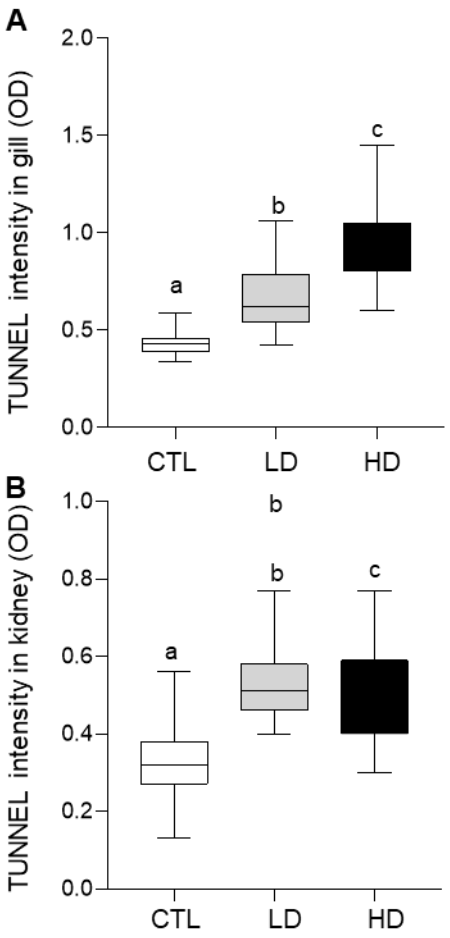

Figure 10. Effects of 2-week Roundup exposure on TUNEL immunoreactive (IR) intensity in gills and kidneys of goldfish. TUNEL (terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling) intensity in gills (A) and kidneys (B). The whiskers represent maximum and minimum value for each datasets. Each value represents the mean ± SEM (N = 182-305). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$).

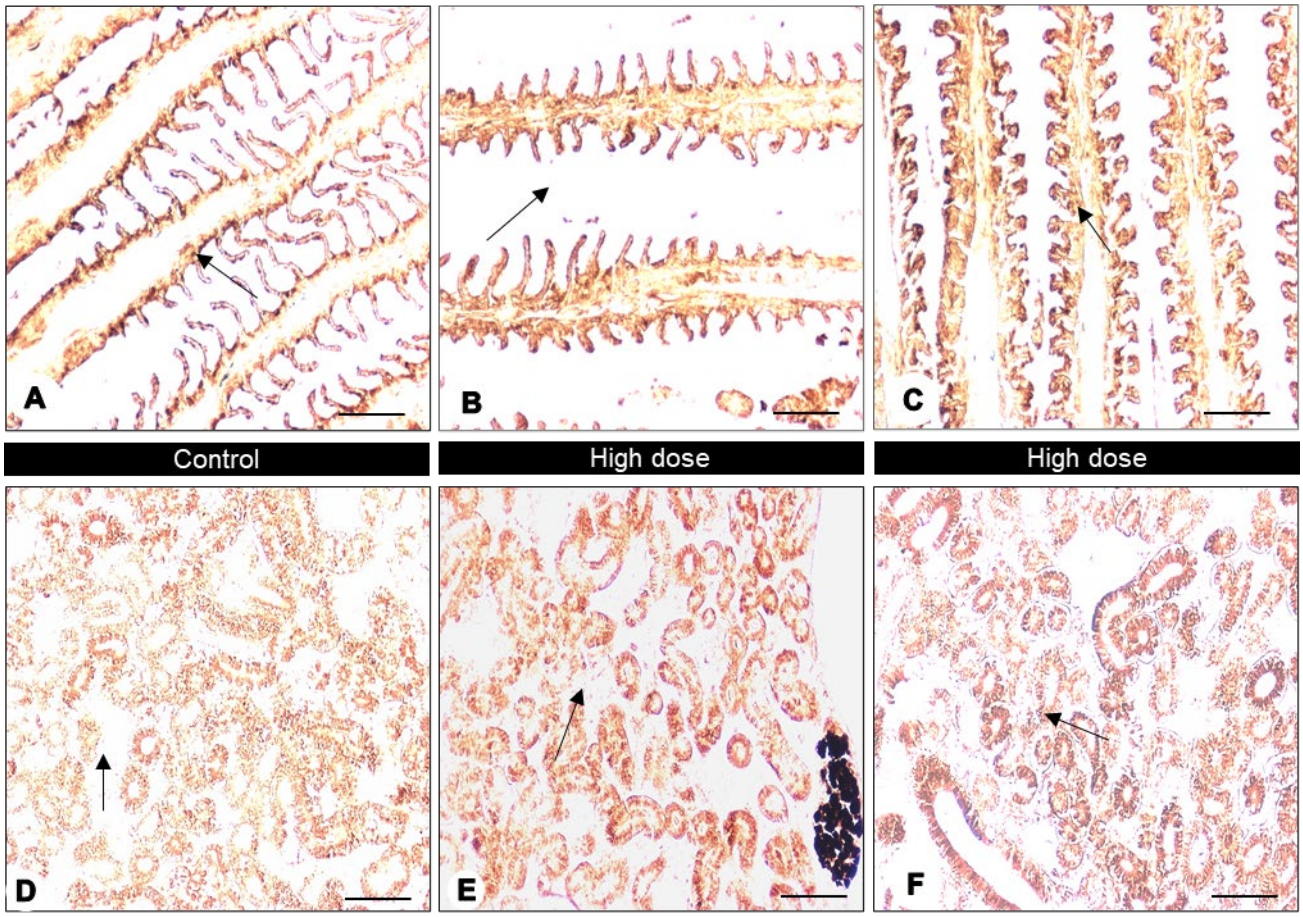

Figure 11: Effects of 2-week Roundup exposure on superoxide dismutase (SOD) enzyme expression in gills and kidneys of goldfish. Darker brown spots in tissue sections, indicated with arrows represent higher enzyme expression. SOD expression in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) SOD expression in representative photographs of kidneys in goldfish collected in CTL (A), LD (B), and HD (C) Roundup exposed groups. MP, melanin pigment. Scale bar = 100 µm.

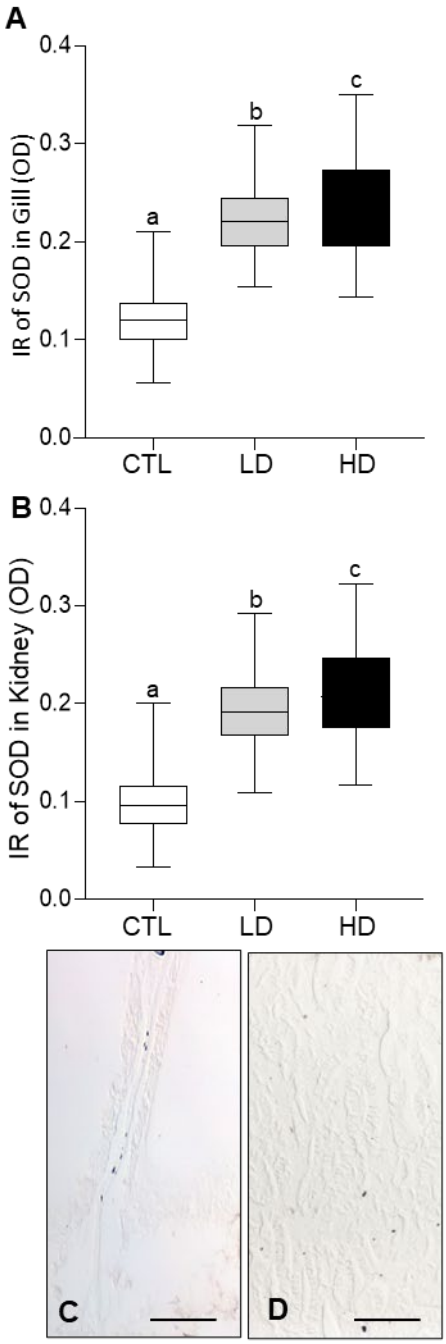

Figure 12: Effects of 2-week Roundup exposure on superoxide dismutase (SOD) enzyme immunoreactive (IR) intensity in (A) gills and (B) kidneys of goldfish. The whiskers represent the maximum and minimum values for each dataset. Each value represents the mean ± SEM (N = 128-156). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$). ImageJ software was used to measure the optical density (OD) values of enzyme expression. (C-D) Negative controls of SOD in gills (C) and kidneys (D). Scale bar = 100 µm

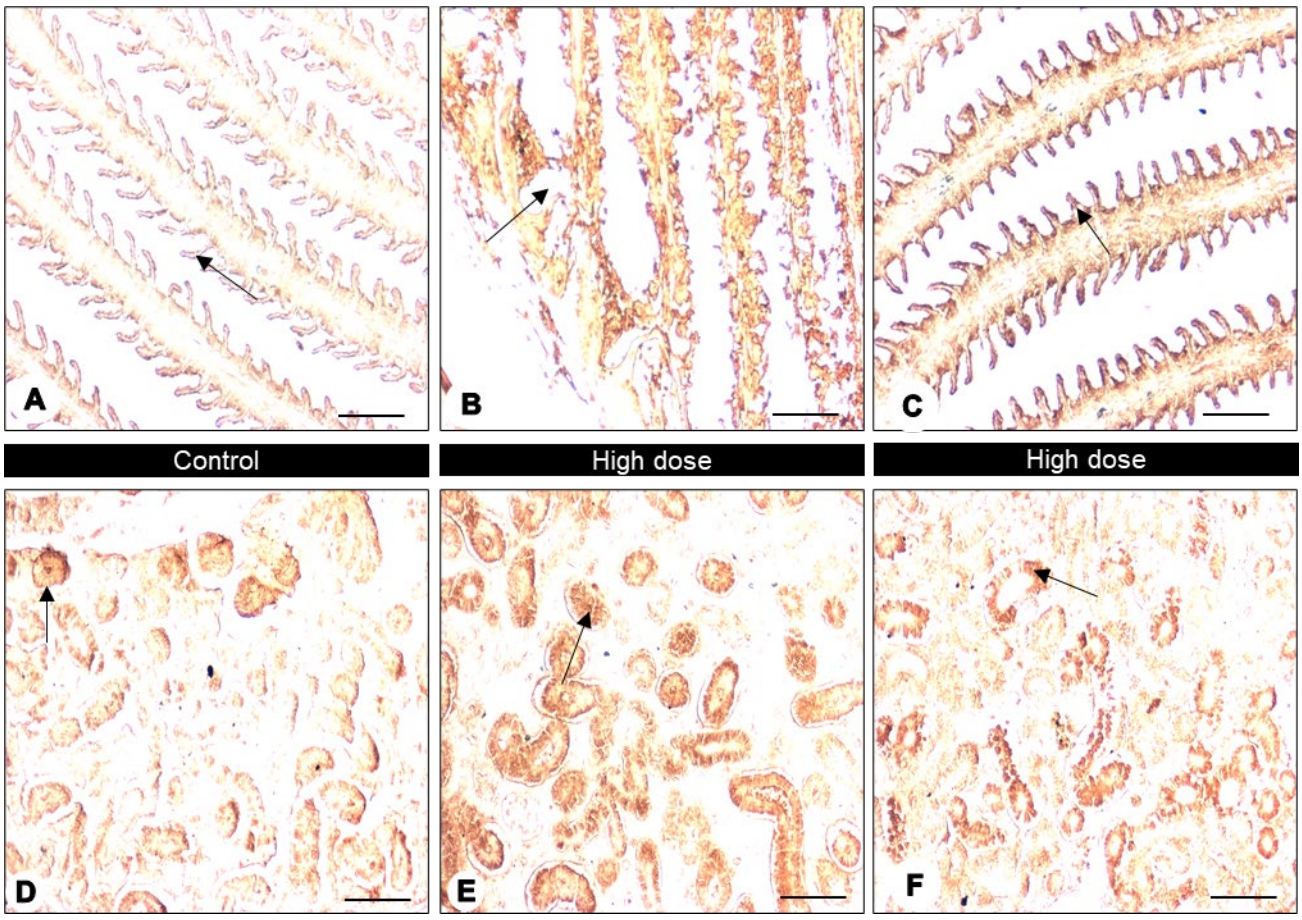

Figure 13: Effects of 2-week Roundup exposure on catalase (CAT) enzyme expression in gills and: kidneys of goldfish. Arrows indicate higher expression of CAT. (A-C) CAT expression in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) CAT expression in representative photographs of kidneys collected in CTL (A), LD (B), and HD (C) Roundup exposed groups. Scale bar = 100 μm.

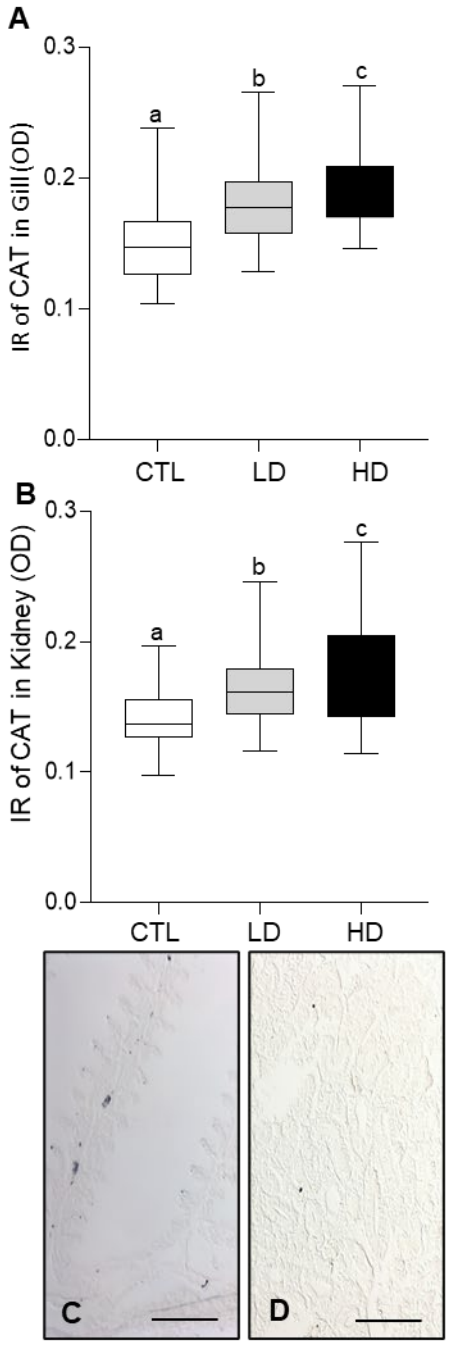

Figure 14: Effects of 2-week Roundup exposure on catalase (CAT) enzyme immunoreactive (IR) intensity in (A) gills and (B) kidneys of goldfish. The whiskers represent the maximum and minimum values for each dataset. Each value represents the mean ± SEM (N = 116-199). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$). ImageJ software was used to measure the optical density (OD) values of enzyme expression. (C-D) Negative controls of CAT in gills (C) and kidneys (D). Scale bar = 100 µm

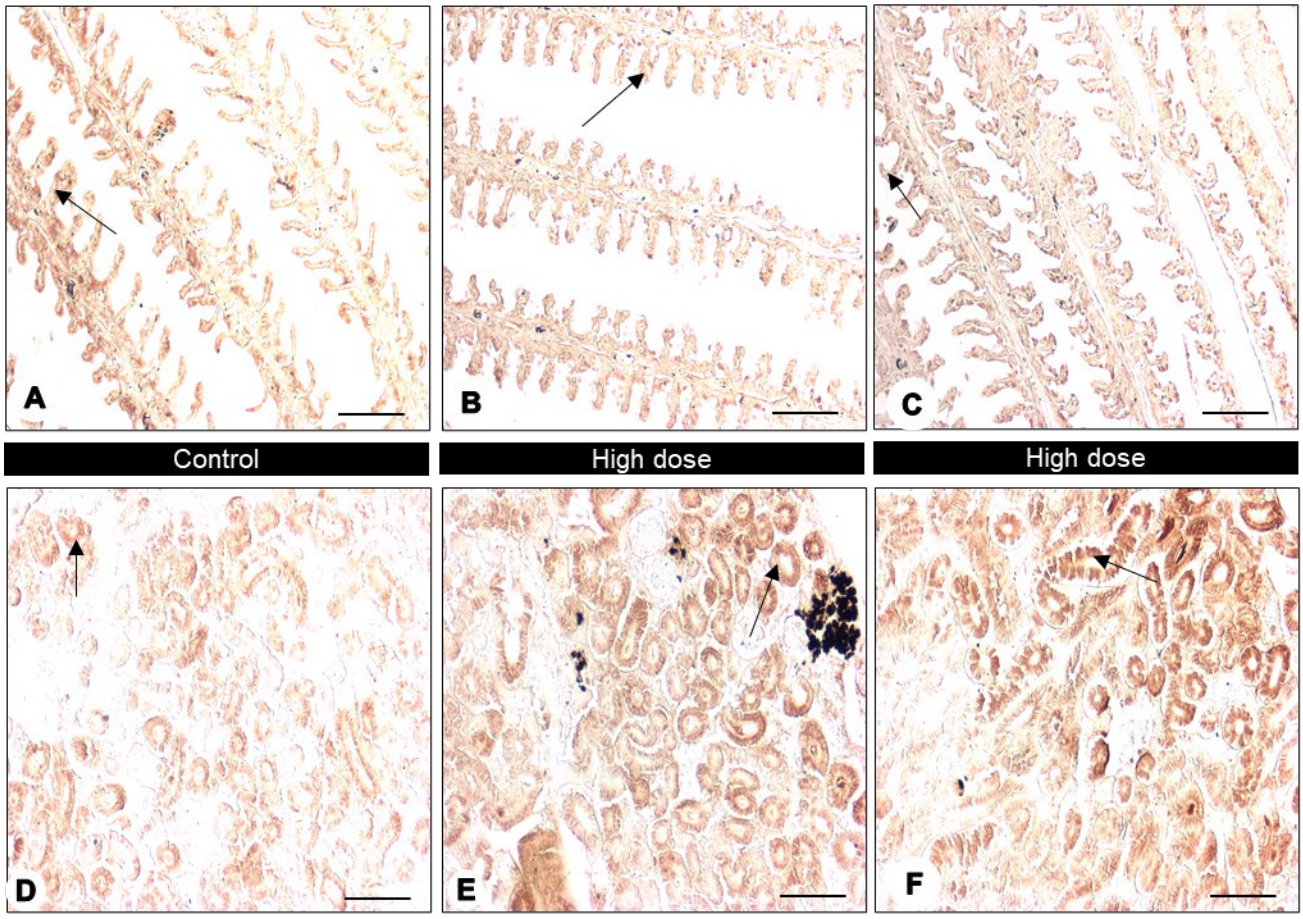

Figure 15: Effects of 2-week Roundup exposure on renin expression in gills and kidneys of goldfish. Arrows represent higher expression. Renin expression in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) Renin expression in representative photographs of kidneys in goldfish collected in CTL (A), LD (B), and HD (C) Roundup exposed groups. Scale bar = 100 μm.

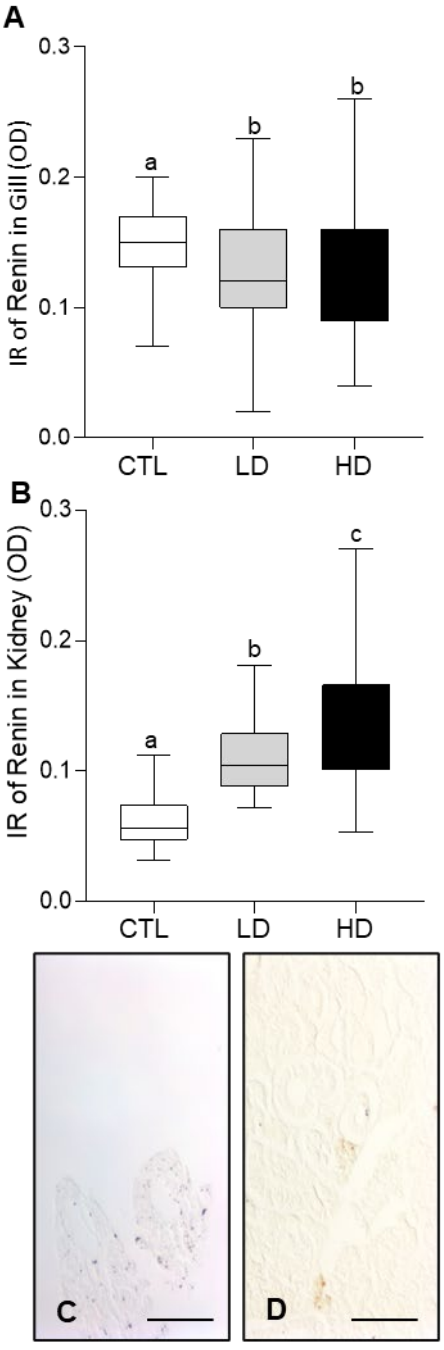

Figure 16: Effects of 2-week Roundup exposure on renin enzyme immunoreactive (IR) intensity in (A) gills and (B) kidneys of goldfish. The whiskers represent the maximum and minimum values for each dataset. Each value represents the mean ± SEM (N = 98-181). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$). ImageJ software was used to measure the optical density (OD) values of enzyme expression. (C-D) Negative controls of renin in gills (C) and kidneys (D). Scale bar = 100 µm

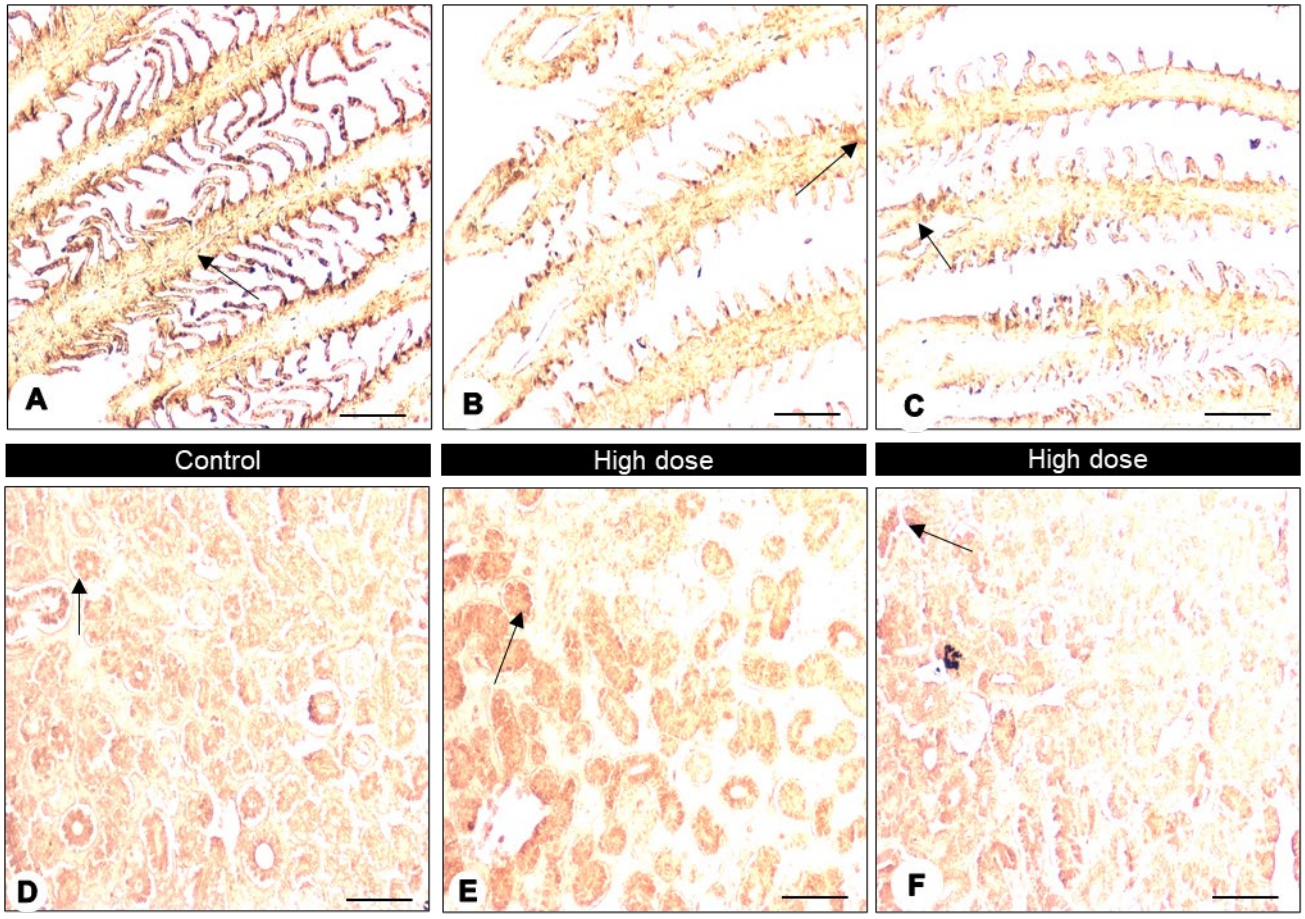

Figure 17: Effects of 2-week Roundup exposure on superoxide dismutase Na^+/K^+-ATPase (NKA) expression in gills and kidneys of goldfish. Arrows represent higher expression. NKA expression in representative photographs of gills (A) in control (no Roundup), (B) low dose (LD), (C) high dose (HD). (D-F) NKA expression in representative photographs of kidneys in goldfish collected in CTL (A), LD (B), and HD (C) Roundup exposed gropus. Scale bar = 100 μm.

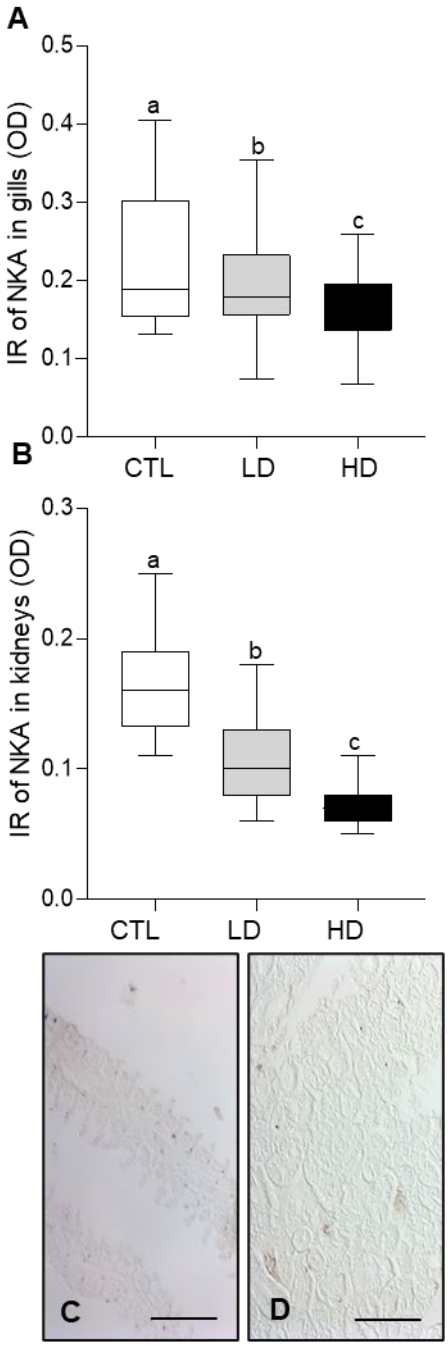

Figure 18: Effects of 2-week Roundup exposure on Na^+/K^+-ATPase (NKA) enzyme immunoreactive (IR) intensity in (A) gills and (B) kidneys of goldfish. The whiskers represent the maximum and minimum values for each dataset. Each value represents the mean ± SEM (N = 77-177). Different letters indicate significant differences (One-way ANOVA followed by Tukey's test, $P<0.05$). ImageJ software was used to measure the optical density (OD) values of enzyme expression. (C-D) Negative controls of NKA in gills (C) and kidneys (D). Scale bar = 100 µm

BIOGRAPHICAL SKETCH

Md Imran Noor earned a Bachelor of Science (B.Sc.) in Fisheries from Khulna University, Bangladesh. He worked as a Program Facilitator for the International Union of Conservation of Nature (IUCN), Bangladesh, in a dolphin conservation project funded by UNDP and GEF. In January 2021, he resumed his graduate studies in the Biochemistry and Molecular Biology Master's program at the University of Texas Rio Grande Valley (UTRGV). Noor began his study at UTRGV, supervised by Dr. MD Saydur Rahman, Associate Professor. He has been given the Presidential Graduate Research Assistantship (PGRA) at the university's prestigious award of the UTRGV President's office. He was assigned a year of Graduate Research Assistantship and a year of Graduate Teaching Assistantship as a lab instructor. Noor also took part in international, national and local conferences, including the Society for Integrative and Comparative Biology (SICB), the Texas Academy of Science (TAC), the Society of Environmental Toxicology and Chemistry (SETAC), and Engaged Scholarship and College of Science annual conference at UTRGV. He was awarded the Serafy Endowment award in the Summer of 2022. Noor earned a Master's in Biochemistry and Molecular Biology in December 2022. He intends to continue his research in any prestigious school or institution in the United States. Contact information for Md Imran Noor, email: imrannoor92@gmail.com.