T.R.
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY

THE EFFECTS OF CYPERMETHRIN ON PRIMARY HEPATOCYTE CULTURE OF VAN FISH (*Alburnus tarichi* Güldenstädt, 1814)

M. Sc. THESIS

PREPARED BY: Aso Hemn OMAR
SUPERVISOR: Assoc. Prof. Dr. Ahmet Regaib OĞUZ

VAN-2018

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ACCEPTANCE AND APPROVAL PAGE

This thesis entitled **"The Effects of Cypermethrin on Primary Hepatocyte Culture of Van Fish (*Alburnus tarichi* Güldenstädt, 1814)"** presented by Aso Hemn OMAR under supervision of Assoc. Prof. Dr. Ahmet Regaib OĞUZ in the Department of General Biology has been accepted as a M.Sc. thesis according to Legislations of Graduate Higher Education on 17/01/2018 with unanimity members of jury.

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This thesis has been approved by the committee of The Institute of Natural and Applied Science on ...../...../..... with decision number.....

Signature

Prof. Dr. Suat ŞENSOY

Director of Institute

THESIS STATEMENT

All information presented in the thesis that ethical behavior and academic rules were obtained in the frame, as well as all kinds of work that does not belong to me in this statement prepared in accordance with the rules of writing theses and reports that I referred to the complete information source.

Signature

Aso Hemn OMAR

ABSTRACT

THE EFFECTS OF CYPERMETHRIN ON PRIMARY HEPATOCYTE CULTURE OF LAKE VAN FISH (*Alburnus tarichi* Güldenstädt, 1814)

OMAR, Aso Hemn
M.Sc. Thesis, Department of Biology
Supervisor: Assoc. Prof. Dr. Ahmet Regaib OĞUZ
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Cypermethrin is a synthetic insecticide widely used in the fight against harmful insects in animal husbandry and agriculture. Although used for combat against insects, it also has negative effects on other harmless animals. Cypermethrin is used in livestock and agriculture fields in Van Lake. Congestion can also cause accumulation in lakes and aquatic areas. In this study, we aimed to determine the effects of cypermethrin on the primary hepatocyte culture of Van fish (*Alburnus tarichi*). Different concentrations of cypermethrin (1×10^{-7}, 1×10^{-6} and 1×10^{-5} M) were applied to the liver cells isolated from Van bean for 48 hours. Changes in Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Lactate dehydrogenase (LDH) levels at 24 and 48 hours of cell culture were determined in the culture medium. We also tried to determine the effects of cypermethrin on total oxidant (TOS) and antioxidant levels (TAS), malondialdehyde levels (MDA) and DNA damage. As a result of the experiments, invert light microscopy revealed that the number of hepatocytes significantly decreased in the groups treated with cypermethrin. Although the levels of AST, ALT and LDH frequently used in liver damage were increased due to the doses of cypermethrin, this increase was low or statistically insignificant compared to the control group. MDA levels increased at the end of the incubation depending on the dose. There was no difference in TAS and TOS levels when compared to the control group. DNA damage was found to increase with time of typhoon and time. As a result, it has been clearly demonstrated that the administered cypermethrin has high toxic effects on the primary hepatocyte culture of Van fish.

Keywords: *Alburnus tarichi*, Cypermethrin, Primary hepatocyte culture, Van Fish

ÖZET

SİPERMETRİNİN VAN BALIĞI (*Alburnus tarichi* Güldenstädt, 1814) PRİMER KARACİĞER KÜLTÜRÜ ÜZERİNE ETKİLERİ

OMAR, Aso Hemn
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Tez Danışmanı: Doç. Dr. Ahmet Regaib OĞUZ
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Sipermetrin hayvancılık ve ziraat alanlarında zararlı böceklerle mücadelede yaygın olarak kullanılan sentetik bir insektisittir. Her ne kadar böceklerle mücade için kullanılsa da zararsız diğer canlılar üzerine de olumsuz etkileri mevcuttur. Sipermetrin, Van Gölü havzasında hayvancılık ve zirai alanlarda kullanılmaktadır. Yoğun kullanım göl ve sucul alanlarda da birikime neden olabilmektedir. Bu çalışmada, sipermetrin'in Van balığı (*Alburnus tarichi*) primer hepatosit kültürü üzerine etkilerinin belirlenmesi amaçlamıştır. Van Balığından izole edilen karaciğer hücrelerine sipermetrinin farklı konsantrasyonları (1×10^{-7}, 1×10^{-6} ve 1×10^{-5} M) 48 saat süresince uygulanmıştır. Hücre kültürünün 24 ve 48 saatlerinde Aspartat Aminotransferaz (AST), Alanin Aminotransferaz (ALT) ve Laktat dehidrogenaz (LDH) seviyelerindeki değişim hücre kültür ortamında belirlenmiştir. Ayrıca sipermetrinin total oksidan (TOS) ve antioksidan seviyeleri (TAS), malondialdehit seviyeleri (MDA) ve DNA hasarı üzerine etkileri belirlenmeye çalışılmıştır. Deneyler sonucunda hepatosit sayısının sipermetrin uygulanan gruplarda önemli ölçüde azaldığı invert ışık mikroskobunda belirlendi. Karaciğer hasarında sıklıkla kullanılan AST, ALT ve LDH seviyeleri sipermetrin dozlarına bağlı olarak artış göstermesine rağmen bu artış kontrol grubuna göre düşük veya istatistiksel olarak önemli değildi. MDA seviyeleri, inkübasyon sonunda doza bağlı olarak artış gösterdi. TAS ve TOS seviyeleri, kontrol grubu ile karşılaştırıldığında herhangi bir farkın olmadığı gözlendi. DNA hasarının ise doza ve zamana bağlı olarak artış gösterdiği belirlendi. Sonuç olarak, uygulanan sipermetrinin Van Balığı primer hepatosit kültürü üzerine yüksek toksik etkilerinin olduğu net bir biçimde ortaya konulmuştur.

Anahtar kelimeler: *Alburnus tarichi*, Primer hepatosit kültür, Sipermetrin, Van Balığı

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2018

Aso Hemn OMAR

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SYMBOLS AND ABBREVIATIONS

Some symbols and abbreviations used in this study were presented below, Along with descriptions.

Symbols	**Description**
%	Percentage
°C	Centigrade degree
h	Hour
L	Liter
M	Molarity
mg	Milligram
Min	Minute
mL	Mililiter
mm	Milimoler
UV	UltraViolet
µL	Microliter

Abbreviations	**Description**
8-OHdG	8-hydroxydeoxyguanosine
BHT	Butylated hydroxytoluene
$CaCl_2$	Calcium chloride
EDTA	Ethylenediaminetetraacetic acid
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
L-15	Leibovitz-15
LDH	Lactate Dehydrogenase

MDA	Malondialdehyde
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaH_2CO_3	Sodium hydrogen carbonate
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
$NaHPO_4$	Sodium phosphate
PBS	Phosphate buffer saline
pH	Potential Hydrogen
rpm	Round per minute
TAS	Total antioxidant status
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TOS	Total oxidant status

1. INTRODUCTION

Van Fish (*Alburnus taritch*) is an endemic carp species on Lake Van, and it is a kind of *Cyprinidae* family. Lake Van is the largest lake in Turkey. The third biggest closed lake, and the largest soda lake of the world that situated in eastern Turkey (Figure 1.1). The surface area of the lake is 3522 km^2, the volume is 576 km^3, and surface height is 1648m (Reimer et al., 2009). The saltiness of Lake is 2.2%, pH 9.8 and alkalinity 153 $mEqxl^{-1}$, just a few animals are available, and one type of vertebrate species and a few invertebrate species inhabit the lake. The only vertebrate living in the Lake is Van fish (Danulat and Kempe, 1992; Oğuz, 2015). It's a food source with high and an important protein content for local people from ancient times to the present day (Çetinkaya, 1993).

Figure 1.1. Lake Van Basin.

The liver is a vital organ and it is necessary for life due to the various functions, it is classified into two glands, an endocrine and exocrine. Endocrine function is to release IFG (Insulin like Growth Factors) that is needed for skeletal growth. Exocrine function is secretion of bile through biliary tract. The synthesis of bile, which is transported by ducts to the duodenum and in fish to the anterior intestine. One of the functions of the liver is that it acts as a storage center for many substances, mainly glycogen and to a lesser degree lipids. Furthermore, the liver plays another vital role in the coupling and chemical decomposition of toxins and the deamination of amino acids (Takashima and Hibiya, 1995). The liver is composed of the stroma (connective tissue, trabeculae, and a reticular network) and the parenchyma (hepatocytes, blood vessels, and bile ducts).

Hepatocytes make up 70-80% of the livers mass and therefore they are the major cells of the parenchymal tissue of the liver. A typical hepatocyte is a polygonal plate-like shape with a size of 20-30 μm, they are found stacked on top of each other in layers; this proximity between them facilitates certain functions performed by hepatocytes, they are in close contact with blood-filled sinusoids and lie adjacent to canaliculi into which bile is secreted. Hepatocytes in combination with other organs play a vital role in metabolism. Some of these functions include detoxification of various metabolites, synthesis of proteins, production of biochemical necessary for digestion, synthesis and breakdown of small and complex molecules and emulsification of lipids.

Cell culture is a technique which is used to maintain and grow cells *in vitro* outside of their original environments. This technique has been used extensively as a model system for biology, disease and therapeutics. It is applied across the fields of genetics immunology, cancer, medicine, vaccine production, tissue engineering and innumerable other applications.

Cypermethrin is one of the synthetic insecticides that has been considered among the safest pesticides that are available and is used worldwide (Igbedioh, 1991). Pyrethroids are artificial forms of pyrethrins (insecticides derived from Chrysanthemum plants extracts) (Soderlund et al., 2002). Synthetic pyrethroids have become an environmentally and economically significant collection of insecticides (Kapoor et al., 1988). They are also highly effective against a broad spectrum of insects and have low mammalian toxicity (Bhunya and Pati, 1988).

In general, the chemically induced liver harm can be easily investigated in tissue culture more than in the whole animals. Cypermethrin is used for the control of a large several of veterinary and agricultural insects. It is used for crack, crevice and spot treatment and for the control of insects and pests because of its low toxicity to mammals and cypermethrin is one of the most popular contaminants in the freshwater aquatic system (Carriquiriborde et al., 2007).

There is not any scientific research about *in vivo* and *in vitro* that effects of cypermethrin on *Alburnus tarichi*. Therefore, the aim of this study is to determine the effects of cypermethrin on primary hepatocyte culture in Van fish (*Alburnus tarichi*).

2. LITERATURE REVIEW

2.1. Cypermethrin and Effects on Animals

Cypermethrin pesticides are materials that used for protecting and controlling plagues, including vectors of human or animal illness. They are used to control undesirable types of plants or animals causing damage through, or otherwise interfering with, the production, processing, store, or transfer or marketing of food (WHO, 2002).

The term of pesticide includes all chemicals that are used to kill or control pests. In marine cage culture of Atlantic salmon, *Salmo salar* (Richards, 1983; Roth et al., 1993; Hart et al., 1997; Boxaspen and Holm, 2001; Treasurer and Wadsworth, 2004). Fish sensitivity to pyrethroids may be explained by their relatively slow metabolism and elimination of these compounds. Fish are the most often tested aquatic organisms because they are the most conspicuous as predominant and are economically important to man because they are linked to the food chain.

Cypermethrin is a tacky semi-solid, yellowish brown colored and odorless chemical with a molecular form: $C_{22}H_{19}C_{l2}NO_3$ (Figure 2.1). It is a synthetic pyrethroid pesticide having three chiral centers, giving a racemic mixture of all eight possible chiral isomeric comprising four disaster isomeric pairs. It is a structure which is based on pyrethrum that having higher biological action and stability than its naturalist model (WHO, 1989). Cypermethrin, (RS)-α-cyano-3phenoxy benzyl (IRS)- cis, trans-3-(2, 2-dichlorovinyl)-2, 2-dimethyl cyclopropane carboxylate, belongs to type II pyrethroid and possess α- cyano group. Its degradation products are 3-(2, 3-dichlorovinyl)-2, 2-dimethyl cyclopropane carboxylic acid (cis + trans isomer) and 3-phenoxy benzoic acid. It is photostable and possesses high insecticidal activity.

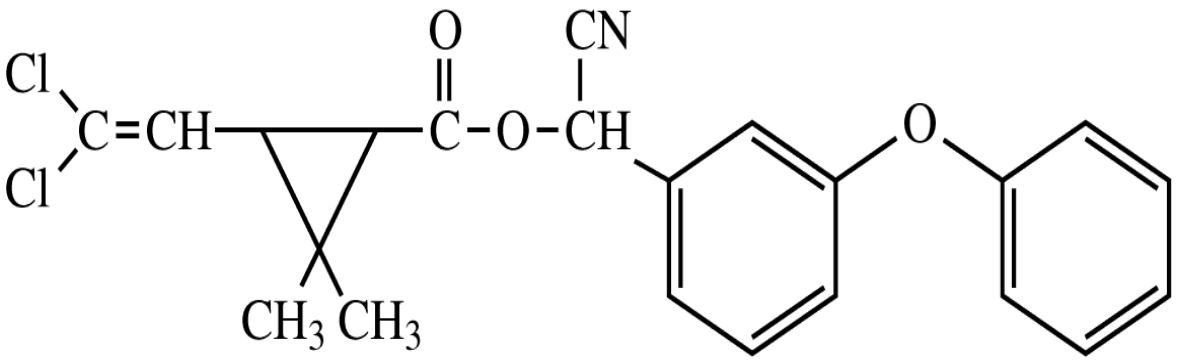

Figure 2.1. Molecular structure of cypermethrin.

Cypermethrin enters into the environment through its application as an agricultural pesticide, during its industry, transmit and storage spinal tract and may also be absorbed by inhalation of spray mist and only simply through the intact skin. Due to its lipophilic nature, cypermethrin has been found to collect in body fat, skin, liver, kidneys, adrenal glands, ovaries and brain (Tao et al., 2008).

Pyrethroids are shown to produce toxic effects on biochemical and hematology of various aquatic animals (Saxena and Seth, 2002; Saxena and Gupta, 2003). Cypermethrin is very toxic to fish and bees, humans are indirectly affected as humans consume fish in their food and also honey which is made by the bees. Thus humans can be exposed to cypermethrin toxicity directly through spraying and contact methods or indirectly through consumption the pesticide contaminated products such as fish and honey (Meister, 1992).

Cypermethrin is a broad-spectrum insecticide. In addition to kill the insects that are the target of a particular treatment, it can also reduce populations of insects and other arthropods that are economically desirable because they prey on unwanted insects or are useful pollinators.

Pyrethroids can be classified into two large groups. The kind I pyrethroids do not contain a cyano group in their molecules and include allethrin, tetramethrin, permethrin, and phenothrin. Kind II pyrethroids contain a cyano group at the carbon position and include modern compounds, such as deltamethrin, cyphenothrin, cypermethrin, and fenvalerate (Norman, 1992).

Type II pyrethroid has been widely used to control ectoparasites including moth pests, cockroaches, fleas and termites of cotton, fruit and vegetable (Meister et al., 1992). Cypermethrin is the most efficient means of pest eradication, but their use has reached at horrifying average due to a number of unwinnable effects on non-target organisms including human beings. Cypermethrin worked by raising the open time of sodium canals resulting prolonged membrane depolarization, releasing enhance the level of the neurotransmitter (outcomes in various nerve impulses instead of the usual single one) and repetitive neuronal activity; in the end leading to depletion of the neurotransmitter (Vijverberg and Bercken, 1990). It slowdowns the activity of an aminobutyric acid receptor, following excitability and convulsions (Ramadan et al., 1988).

In addition, it also blocks voltage-sensitive chloride channels (Miyamoto et al., 1995) and stops monoamine oxidase, an enzyme which breaks down neurotransmitters (Rao and Rao, 1993). As a result, a wide range of disorder related to the use of cypermethrin has been reported on insect, animal, birds and fishes even human. Acute toxicity including abnormal facial sensations, dizziness, headache, nausea, anorexia and fatigue, vomiting and increased stomach secretion irritant to skin and eye persistently muscular twitching, comate and convulsive attacks. It affects various systems in our body including hematological, biochemical, dermatological, muscular, urinary, central nervous system and digestive system (Temple and Smith, 1996).

Fish are particularly susceptible to cypermethrin, the fish can't break down cypermethrin as mammals and birds because fish nervous systems are particularly sensitive to cypermethrin. They block the sodium channels of nerve filaments, thereby lengthening their depolarization phase. Moreover, they affect the GABA receptors in the nerve filaments (Bradbury and Coats, 1989).

Cypermethrin is increased risk of food being contaminated with the insecticide, which may harm humans and domesticated animals. Insecticides from insecticides contaminated feed can be transported to young embryos through eggs and thus can cause teratological abnormalities, organ dysfunction and mortality in the young embryos hence affects the growth. Cypermethrin produces drastic effects on both the invertebrates (Gowlan et al., 2002) and vertebrates (Das and Mukherjee, 2003). In vertebrates, for

example in amphibians, it induces apoptosis in the telencephalon of *Physalaemus biligonigerus* tadpoles (Anura, Leptodactylidae) (Izaguirre et al., 2000). In fish, cypermethrin inhibits trypsin, lipase and carboxypeptidase A activity in Carp (*Cyprinus carpio*) and causes a slight increase in alpha-chymotrypsin activity (Simon et al., 1999). Cypermethrin is known to cause decrease in glycogen, pyruvate and lactate dehydrogenase and phosphorylase B activity and increase in lactate level, phosphorylase A and aldolase activities (Reddy and Yellamma, 1991). Sheela and Muniandi (1992) noticed decreases in protein, RNA and glycogen in muscle and liver of fish following cypermethrin treatment. Das and Mukherjee (2003) observed cypermethrin induced changes in DNA, RNA, LDH and ATPase of muscle, liver, brain and kidney of Indian major carp, Labeo rohita. Sheela (2000) observed that young rats are more sensitive to the toxic effects of cypermethrin than old rats because of their less developed metabolic capacity. Cypermethrin is known to affect the blood and immune system, e.g. Santoni et al. (1997) noted the cypermethrin induced increases in peripheral natural killer cell and antibody dependent cytotoxic activity in rats and Institoris et al. (1999) found that cypermethrin decreases delayed type hypersensitivity reaction, increases the number of numerical chromosome aberrations of the bone marrow cells, decreases mean cell volume of the RBCs and hematocrit value and reduces the white blood cell count in the peripheral blood of male Wistar rats. Recently, Haratym-Maj (2002) observed an increase in the number of leukocytes in peripheral blood and inhibition and mobilization of hemopoietic system in female mice following cypermethrin administration. Toxicity of cypermethrin has also been evaluated in muscular and nervous system of fish and mammals. Tonini et al., (1989) observed that cypermethrin affects the electrically evoked contractions in the muscles of guinea pig. Cypermethrin effects of Neurotoxic it has been studied in rats by Eells et al., (1992) who observed the release of acetylcholinesterase from rat brain synaptosomes with cypermethrin and its effects on the voltage sensitive sodium channel. Later on, these authors noted the cypermethrin induced depolarizing responses in rat and trout synaptosomes (Eells et al., 1993). Although a lot of work has been done on the toxicity of cypermethrin on fish and mammals, but a little work has been carried out on chicks (Kapoor et al., 1988).

Pyrethroid insecticides have been used for more than 40 years and account for 25% of the worldwide insecticide market (Shafer et al., 2005). Toxicity of pyrethroids to fish has been shown to be 1000 fold greater than to mammals and birds at comparable concentrations (Bradbury and Coats, 1989). The sensitivity of fish to aqueous pyrethroid exposure is due partly to a high rate of gill absorption and rather slow hydrolytic detoxification, but principally to the hypersensitivity of the piscine nervous system to these pesticides (Aydin et al., 2005; Viran et al., 2003). The primary target sites for pyrethroids are the voltage gated sodium channels (VGSCs) (Casida et al., 1983). Pyrethroids exert neurotoxicity by binding to and delaying the inactivation of the sodium channels, resulting in convulsions, prostration and ultimately death (Werner and Moran, 2008).

Pesticides are among the major contributor of aquatic pollution. According to Latif et al. (2013), there are more than 200 types of organic pesticides, being used in thousands of different products. These pesticides contain a number of heavy metals such as iron, chromium, cadmium, nickel, copper, lead, zinc and manganese etc. These elements ultimately reach the water bodies, and adversely affect the growth, reproduction, physiology and even survival of the nontarget aquatic organisms including fish (Hayat et al., 2007). Liver, gills and brain are the most important organs of all vertebrates, as these controls and maintain the most important life activities such as metabolism, detoxification, excretion and respiration etc. The chemical pollutants including heavy metals and pesticides adversely affected the morphology and functions of these organs and disturb the normal physiology of all animals (Atamanalp et al., 2008).

Toxic chemicals such as pesticides have been found in soil, air, water and even in animal and human tissues. These pesticides render injurious effects and even mortalities. Annually about 3 million cases of pesticide toxicity and 220,000 casualties all over the world have been reported (Naz et al., 2010). Pyrethroid insecticides were used preferably over organochlorines, organophosphates and carbamates due to their high effectiveness against a wide range of insects, low toxicity to non-target organisms (mammals) and easy biodegradability. Recently, the toxicity of pyrethroid insecticides to mammals received much attention because animals exposed to these insecticides showed changes in their

physiological activities, reproductive performance in addition to change in the enzymes activities, biochemical and hematological parameters (Yousef et al., 2003: Yousef, 2010).

Water pollution is any chemical, physical or biological changes in the quality of water that has a harmful effect on any living thing that drinks, uses or lives in (Lenntech, 1998). However, there is overwhelming evidence that agricultural use of pesticides has a major impact on water quality and leads to serious environmental consequences. Appreciation of fisheries and aquatic systems has been accompanied by increasing concern about the effects of growing human populations and human activities on aquatic life and water quality. Pesticides are one group of toxic compounds linked to human use that have a profound effect on aquatic life and water quality (Virginia, 2009). Fish and other aquatic biota may be harmed by pesticides contaminated water. Pesticides surface runoffs into the rivers and streams can be highly lethal to aquatic life, sometimes killing all the fish in a particular stream (Toughill, 1999).

2.2. The Liver Morphology

The liver is the largest and most complex gland of the body. It is the main detoxifying organ in mammals, with large amounts of phase 1 and phase 2 metabolic enzymes and it is responsible for large parts of lipid and cholesterol metabolism, the production of hormones, phagocytosis of debris and bacteria as well as participating in iron metabolism. Additionally, it has an important role in many vital functions of the body, like the production of bile, the processing and storage of nutrients and Vitamin A and the synthesis of blood proteins including albumin, lipoproteins, transferrin, growth factors and coagulation factors (LaBrecque, 1994; Kevresan et al., 2007).

In vertebrates, the liver is divided into four lobes, with each containing thousands of equally built lobules, and is served by two distinct blood supplies. The blood flows out of the liver via the hepatic vein in the direction of the inferior vena cava. Thereby, xenobiotics absorbed by ingestion have to pass the hepatocytes, the predominant cell type in liver, and can be taken up, metabolized and/or detoxified. The metabolites are excreted partly, depending on their chemical properties, into the bile canaliculi or via the venous blood into

the urine. Hepatocytes, the liver parenchymal cells, account for about 70-80% of liver mass and 65% of cell number of a normal liver, Non-parenchymal cells like Kupffer cells (15%), endothelial cells, hepatic stellate cells or pit cells make up the remaining mass (Widmann et al., 1972; Blouin et al., 1977).

In a hexagonal shaped liver lobule, the central vein is surrounded by 4-6 portal areas (Matsumoto and Kawakami, 1982) and hepatocytes are arranged in cords radiating from the central vein (Figure 2.2). Hepatic endothelial cells from the walls of the sinusoidal, the capillaries between the cords of hepatocytes. Unlike other endothelial cells, they lack a basement membrane and the endothelial structures possess pores called fenestrae, allowing the blood to flow directly around the hepatocytes. They express several adhesion molecules facilitating inflammatory cell migration, usually a response to activation by Kupffer cell signaling following liver damage (Scoazec and Feldmann, 1994; Ohira et al., 2003). The Kupffer cells, resident macrophages in the liver, represent the second largest cell population of the liver. They are located in the hepatic sinusoids, in between or on top of endothelial cells, but they also make contact to the hepatocytes through their extensions. They exhibit several important functions, such as endocytosis of foreign material and bacteria, antigen presentation and secretion of biologically active products (e.g. nitric oxide and cytokines) and play an important role in immune and inflammatory responses involving cytokine-signaling (Winwood and Arthur, 1993). Stellate cells are the fat storing cells of the liver where they reside in the space of Disse between hepatocytes and endothelial cells. They store Vitamin A in lipid droplets, synthesize extracellular matrix proteins and it has been suggested that they contribute to liver fibrosis and immune response (Ogata et al., 1991; Friedman, 1997).

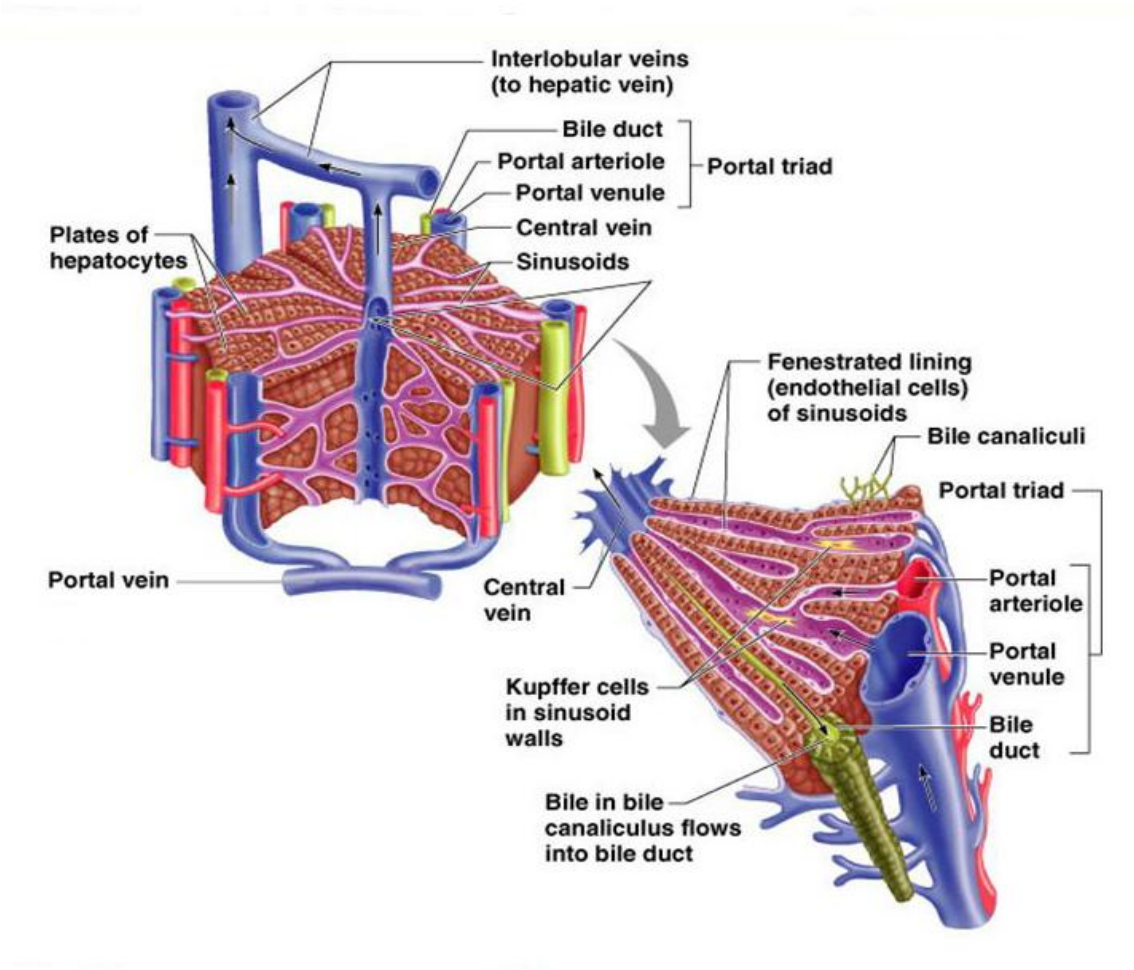

Figure 2.2. Microscopic anatomy of the liver.

2.3. Hepatotoxicity

Hepatotoxicity is a major issue in pharmaceutical drug development (Ballet, 1997). Drug-induced liver injury is the major reason for attrition in clinical studies (Wysowski and Swartz, 2005) and hepatotoxic side effects are the main reason for drug withdrawals from the market (31%). A broad variety of liver pathophysiologies have been reported, including steatosis (fatty liver), cholestasis (obstruction of bile secretion), fibrosis (increased production and deposition of extracellular matrix components), hepatitis (inflammation), necrosis (cell death) or the formation of liver tumors. These pathological findings may arise from diseases affecting the liver, but also from xenobiotics, alcohol abuse or undesired

drug-drug interactions. The pathological symptoms of certain liver diseases allow conclusions about the affected intracellular organelles. Although different histological changes can appear, a compound class often displays a typical clinical or pathological appearance. Xenobiotics administered orally first pass through the liver before entering the general blood circulation. Because the liver has multiple functions for the homeostasis of the whole body, drug-induced liver toxicity can have severe consequences. Thirty to fifty percent of acute liver failures and fifteen percent of liver transplantations are related to chemical induced hepatotoxicity (Kaplowitz, 2001; Lewis, 2002; Andrade et al., 2004).There is often a lack of reasonable understanding of the general molecular mechanisms of most drug-induced hepatoxicities (Jaeschke et al., 2002; Boelsterli, 2003; Lee, 2003). The inhibition of mitochondrial function, disruption of intracellular calcium homeostasis, activation of apoptosis, oxidative stress, inhibition of specific enzymes or transporters and the formation of reactive metabolites that cause direct toxicity or immunogenic responses are some mechanisms that have to be considered.

The drug development process comprises a variety of steps to assess whether a test compound has adequate efficacy, appropriate physicochemical properties, metabolic stability, safety and bioreactivity in humans. Hepatotoxicity in humans has a poor correlation with regulatory animal toxicity tests (Olson et al., 1998; Olson et al., 2000). However, if assays identified a compound as a human liver-toxicant, there is more than 80% correlation to the corresponding findings in animals (Xu et al., 2004). While *in vivo* models, limited by animal welfare/ethical concerns, are used to investigate systemic influences, cell culture models provide systems that can investigate specific mechanisms in a precisely controlled environment (Ulrich et al., 2003). Although there are ways to analyses the many toxicological parameters individually *in vitro*, most have low predictive value for the detection of human hepatotoxicity. The poor predictivity and sensitivity of standard *in vitro* cytotoxicity assays is due to several reasons, including strong inter-species variation, the lack of a true physiological environment of *in vitro* experiments or the insufficient culturing conditions, resulting in a loss of metabolic capabilities (Olson et al., 1998). The *in vitro* assays usually measure lethal events in late stages of toxicity, but toxicity may not always be lethal. Cytotoxicity may take several days to appear (Olson et

al., 1998; Slaughter et al., 2002; Schoonen et al., 2005), demanding repeated drug administration. In contrast to directly active compounds (primary toxins), some compounds elicit their toxic potential only as a metabolite (secondary toxins) and usually cause damage in the organ where they are produced. It raises the need for metabolically active long-term *in vitro* models that facilitate extended exposure times. Several models have been used for the detection of acute toxicity, but sub-chronic and chronic toxicities have not been addressed so far. Furthermore, standard tests generally investigate only one parameter whereas hepatotoxicity can develop via many different mechanisms and is considered a multifactorial process. In order to improve sensitivity it will be necessary to analyze several morphological, biochemical and functional endpoints in parallel. Finally, tests should be performed not only with high concentrations, causing acute toxicity, but also with *in vivo* pharmacological concentrations.

2.4. *In Vitro* Liver Models

In vitro tests have the advantage of allowing multiple testing of different compounds, doses and/ or time points simultaneously under well-defined conditions. The simplicity of some *in vitro* systems, besides saving time, money and animals used for experimentation, provides the ability to specifically manipulate and analyze a small number of well-defined parameters. The most commonly used test systems include, the isolated perfused liver, liver slices, primary hepatocytes in suspension or culture, cell lines, transgenic cells and sub-cellular fractions such as microsomes, supersomes or cytosol. The reduction in the complexity of the system and the increase in throughput offer the ability to study specific parameters more closely but create inherent constraints for each model. However, this limits their widespread use and acceptance by the regulatory authorities as an alternative for *in vivo* testing (Brandon et al., 2003). Although studies have shown that *in vitro* cytotoxicity data can be used to identify appropriate doses for *in vivo* studies (Scholz et al., 1999).

2.5. Cultures of Isolated Primary Liver Cells

Freshly isolated hepatocytes are often used for toxicological research. Although these are mostly mono-factorial systems which do not take into account the interactions between cell types or even whole organs in the body, cultures of primary rat and human hepatocytes are used in a variety of pharmacological and toxicological experiments, for example the evaluation of hepatic drug uptake and metabolism, drug interactions and hepatotoxicity (Cross and Bayliss, 2000; Brandon et al., 2003; Gebhardt et al., 2003).

Fresh liver cells can be obtained by different procedures, all of which involve perfusion of the liver with Ca^{+2}-free buffers combined with enzymes/proteases which disintegrate the extracellular matrix, leading to the separation of the cells from each other (Howard et al., 1967; Seglen, 1976). The isolation of liver cells is routinely performed for many species used in toxicity testing, but also with tissue from partial liver resections and non-transplantable whole livers from human donors (Richert et al., 2004; LeCluyse et al., 2005). In suspension, the survival of cells is short-lived, normally not longer than 6 hours. Although the system is relatively high throughput, easy to use and preserves most of the metabolizing enzymes at *in vivo* levels for a short time, it is only usable for acute toxicology or metabolism studies because the loss of contact to surrounding cells and the (ECM) environment has severe influence on the defined cell polarization and shape (Gebhardt et al., 2003).

The survival time in culture can be increased if hepatocytes are cultured on adhesive surfaces, for example, tissue culture dishes coated with extracellular matrix (ECM) components. During the perfusion procedure the cells are already primed for proliferation and can easily be forced to proliferate by the addition of mitogenic compounds, for example epidermal growth factor (EGF), allowing longer culturing (Etienne et al., 1988). However, this causes a down-regulation of metabolic enzymes and thereby induces dedifferentiation associated with a loss of many liver-specific functions and defined cell polarity (Skett and Bayliss, 1996; Luttringer et al., 2002; Paine and Andreakos, 2004). Additionally, it is known that the typical phenotypic change of hepatocytes in monolayer culture, the spreading of the cells, has a negative effect on liver-specific gene expression

(Miranti, 2002). Intracellular signaling is closely connected to the interaction between extracellular matrix (ECM), cell-adhesion molecules and the cytoskeleton and therefore has a major impact on gene expression and the metabolic capacity of the cells. Altogether, this processes lead to a loss of up to 80-90% of phase 1 and about 50% of phase 2 metabolic activity during the first 24 hours in culture (Rodriguez-Antona et al., 2002; Wilkening, et al., 2003). Culturing hepatocytes in a sandwich configuration, embedded between two layers of gelled ECM proteins (e.g., collagen I or Matrigel), has prolonged the time in culture displaying hepatocyte-specific functions dramatically (Dunn et al., 1991; Dunn et al., 1989; Richert et al., 2002; LeCluyse et al., 2000). Cells adapted and maintained their physiologically occurring polygonal shape and bile canalicular-like structures could be observed for up to 14 days in culture (Tuschl and Müller, 2006). The development of long-term primary hepatocyte cultures is an essential step towards the study of chronic effects *in vitro*. Another factor greatly influencing the morphological development and cell survival of hepatocytes in culture is the medium formulation and the addition/omission of serum, specified hormone mixtures or other supplements (Sidhu et al., 2004; Pascussi et al., 2000; Turncliff et al., 2004). Among the most frequently used basal media, Dulbecco's modified Eagle medium (DMEM), modified Chee's medium (MCM) and Williams' medium E (WME), the DMEM/F12 mix seems most appropriate to maintain liver-specific functions and to help rebuild bile canaliculi (Turncliff et al., 2006). In culture, the addition of the glucocorticoid dexamethasone (DEX), at nanomolar concentrations, is essential for the long-term preservation of hepatocyte-specific functions like polygonal hepatocyte morphology, structural integrity of cytoplasmic membranes, bile canaliculi-like structures and by maintaining the expression of liver specific transcription factors. Insulin enhances the glucose uptake of cells and contributes to maintaining liver specific gene expression. Selenium, a structural component of the enzyme glutathione peroxidase, which plays an essential role in the neutralization of metabolically generated peroxides, has also been shown to be beneficial when added to the medium (Yamada et al., 1980; Müller and Pallauf, 2003). Since it is well known that serum enhances the surface attachment ability of hepatocytes (Williams et al., 1977), cells are generally seeded in medium containing fetal calf serum, regardless of the subsequent culture conditions.

2.6. Hepatocyte Cultures

Hepatocytes are the main parts of the liver. However, liver toxicity may not always originate from these cells. Therefore, co-cultures of hepatocytes with other non-parenchymal liver cells, such as endothelial, Kupffer, or stellate cells and also stable cell lines or fibroblasts can be applied to reflect a more physiological situation. For example, the excretion of TNFα or nitric oxide by Kupffer cells can lead to inflammatory reactions or apoptosis (El-Bahay et al., 1999; Kmieć, 2001). Spheroids (spherical multicellular aggregates) will form if a crude liver cell suspension is prevented from adherence to the surface by continuous shaking. Cell-cell contacts are re-established, hepatocytes are located in the inside, non-parenchymal cells on the outside and the deposition of ECM is seen throughout the spheroids. Alginate or other materials can be added to make up the internal structure of the spheres. Several studies showed the positive effect of this culture method on the expression of hepatotypic genes and the maintenance of metabolic capacity (Landry et al., 1985; Guigoz et al., 1987). The maintenance of prolonged functional activity has been related to the restoration and stability of cell polarity and close cell-to-cell contacts (Lu et al., 2005). However, the formation of these spheroids leads to hypoxic and necrotic cells dying at their center. Additional problems arise from the accumulation of bile in the center of spheroids. Another skillful attempt to mimic a liver-like environment *in vitro* is the bioartificial liver system (3D-bioreactors). Their major advantage is the re-establishment of the 3D liver cytoarchitecture with cell-cell contacts and a three-dimensional ECM environment, combined with continuous medium perfusion, providing a constant supply of oxygen and nutrients. Today a variety of culture systems are being used for bioreactor setups (Powers et al., 2002). Different studies have shown an improvement in some hepatocyte-specific functions in co-culture with other cell types, in spheroids and in 3D-bioreactors (Sivaraman et al., 2005). They co-cultured primary hepatocytes and microvascular endothelial cells by seeding them into a decellularized porcine jejunal segment with preserved vascular structures. The supply with nutrients was accomplished by perfusion of the blood vessels with culture medium. Biochemical testing showed metabolic and morphological stability for up to three weeks. However, the preparation of these

cultures is quite elaborate, therefore their use as a high throughput tool for toxicological screening tests is unlikely.

Table 2.1. Advantages and disadvantages of primary hepatocyte culture.

	Advantages	**Disadvantages**
Primary Hepatocyte Culture	- Reasonably high throughput. - Viability and differentiation preserved for up to 2 weeks. - Potential for use of long-term cultures in chronic toxicity. - Analysis of human samples possible. - Functional drug metabolizing enzymes, transporters and bile canaliculi. - Co-culture with other liver cells possible.	- Culture may need special supplements in media. - Survival, differentiation status and function depends on culture conditions. - No culture system is able to preserve all the different liver specific functions *in vitro.* - Difficult to regain cells for fluorescence-activated cell sorting (FACS) analysis.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

Fish were caught by using the cover net in the freshwater through the Lake Van. They were feed with commercial trout feed and maintain in a light/dark cycle of 12:12 hrs. At 24ºC static condition with filter tap water (Figure 3.1).

Figure 3.1. Van Fish (*Alburnus tarichi*).

3.1.2. Reagents and chemicals

Cypermethrin that molecular weight is 416.3 g/mol, a density is 1.14 g/ml, and a purity is 91%. Leibovitz-15 (L-15) medium (1 X) with L-glutamine, liquid, sterile-filtered was from Sigma. Total antioxidants status (TAS) kit and Total oxidant status (TOS) kit purchase from Rel assay diagnostics (Turkey). 8-Hydroxydeoxyguanosine (8-OHdG) kit purchase from Bioassay technology laboratory (China). All other Labware was purchased from Sigma-Aldrich (Germany).

3.1.3. Devices and materials

- Adjustable Automotive Pipettes (Thermo Scientific, Germany)
- Aspirator (Biosan, Lativa)
- Automated Pipette Tip
- Beaker (Germany)
- Cell Culture Plates (Greiner, Malaysia)
- Cobas 6000 (Japan)
- Cooled Centrifuge (Unive, Germany)
- Erlenmeyer Flask (Germany)
- Glass Pipette (Italy)
- Hot Plate Stirrers (Korea)
- Incubator (BINDER, UK)
- Lab Bottle (Germany)
- Laboratory Hood (ESCO, UK)
- Microscope (DM 6000 Leica Microsystems, Wetzlar, Germany)
- Oven (SANYO, Germany)
- Overhead Stirrers (inoLab, Germany)
- pH-meter (inoLab, Germany)
- Refrigerator (-80ºC) (Thermo Scientific, Germany)
- Sensitive balance (precise, USA)
- Shaker (biosan, Lativa)
- Spectrophotometer (SHIMADZU UV-1800, Japan)
- Spectrophotometer cuvette (Sigma-Aldrich, Germany)
- Stopwatch
- Thermostated Water Bath
- Vortex (SciLOGEX, USA)

3.2. Methods

3.2.1. Hepatocyte isolation

After the fish were anesthetized with amino benzonate methanesulfonate (MS 222, 100 mg/L), they were sterilized by immersion in 70% ethanol. Later, the fish were dissected in laminar flow under sterile conditions, were open the abdomen of the fish (*Alburnus tarichi*), then the liver carefully excised and put into a watch glass, the fat tissue on the liver was removed. Then, the liver tissue was divided by using sterile stainless-steel and forceps into small pieces and kept in sterile buffer (Hepatocyte Buffer: 0.145 M NaCl, 5.4 mM KCl, 5 mM EDTA, 1.1 mM KH_2PO_4, 12 mM NaH_2CO_3, 3 mM $NaHPO_4$ and pH 7.5) at room temperature. This process was repeated several times in 30 minutes. Then, the tissue was transferred into hepatocyte buffer containing 2.5 mM $CaCl_2$ without EDTA. So the tissue cleaned from the blood was incubated in L-15 containing collagenase (0.5 mg/mL) for 30 minutes. After the incubation, the cells were mechanically dissociated by triturating with pipette tips. The cells that were obtained at the end of this process were centrifuged a few times in 90 X*g* for 5 minutes until the supernatant in the suspension got clear, the L-15 was put instead of the supernatant. The cell pellet was suspended by mechanical agitation. Antibiotic-antimycotic solution 10 ml/L and 5 mM $NaHCO_3$ were added to the medium to prevent contamination and pH optimization. Isolate cells were stained with trypan blue (4%) and live and dead cell counts were performed by using Thomas slide. The rate of the live cells in the culture was remarked to be over 90%. The erythrocytes were observed in culture after the process of isolation. However, the number of erythrocytes have never exceeded 10%.

3.2.2. Cell culture

Cypermethrin was prepared, the stock solution was used to prepare different concentrations (1×10^{-5}, 1×10^{-6} and 1×10^{-7} M). Then we began the culture, isolate hepatocytes were seeded in 24-cell culture plates that were previously coated with poly-L-

Lysine (P4526, Sigma) one hour later washed by distillate water. Cypermethrin in different concentrations was dissolved in L-15. In each concentration, the culture was introduced. Totally, 1 mL medium was added to each well and then the cultures were incubated at 24ºC in the humid atmosphere. Morphological changes and all the changes were investigated by the inverted microscope (Leica DM 6000), the effect of toxicity on the culture was recorded in each concentration at an interval of 24 up to 48 hours and photographs were taken. The culture medium and extracted cell were collected from plates and kept in deep freeze at -80ºC.

3.2.3. Preparation of cell extraction

Hepatocytes were exposesd to different concentration of cypermethrin (1×10^{-5}, 1×10^{-6} and 1×10^{-7} M) for 48 hours. After 24 and 48 hours cells were trypsined and centrifuged for 2000 *g* for 5 min. cell pellet was washed with PBS. Cell were homogenase and cell pellet were centrifuged and supernatant were collected.

3.2.4. Determination of enzyme assays

Aspartate Aminotransferase (AST) activity was measured by (Cobas 6000, Japan). That conversion of aspartic acid and ketoglutamic acid to glutamic and oxaloacetic acids. Alanine Aminotransferase (ALT) activity was measured by conversion of alanine and ketoglutamic acid to glutamic and pyruvic acids. The enzyme processes were coupled with nicotinamide adenine dinucleotide by converting the reduced form (NADH) to the oxidized form (NAD).

3.2.5. Determination of lactate dehydrogenase (LDH)

Firstly, washed each well twice, each time with 2 ml PBS. Then added 10 µl of 10% Triton X-100 in 1 ml potassium phosphate buffer to each well to lyse hepatocytes. After that scrape cells and transferred to a 1 ml microcentrifuge tube. Then incubated on ice for

30 minutes. Then added 50 µl of cell lysate to 2.85 ml of reaction buffer (0.1 M phosphate buffer, pH 7.4 and 5 mM NADH). Then initiate the reaction by adding 100 µl of 20 mM sodium pyruvate solution, and activity was measured by (Cobas 6000, Japan).

3.2.6. Determination of malondialdehyde (MDA)

After the cell was incubated, 200 µl from the sample was taken and put into 1 tube. 800 µl phosphate buffer, 25 µl BHT solution, and 500 µl of 30% TCA were added. The tubes were stirred with vortex and kept on the refrigerator (-80ºC) for 30 minutes. Then centrifuged at 2000 rpm for 15 minutes. 1ml from the supernatant was taken and transferred to other tubes. Then 75 µl of EDTA and 250 µl of TBA were added. Tubes were mixed in the vortex and kept in a boiling water bath (90^{o}C) for 15 minutes. Later, they were brought to room temperature and their absorbance was read by spectrophotometer at 532 nm.

3.2.7. Determination of total antioxidants status (TAS)

Total Antioxidant Status (TAS) was determined with assay kits by Rel assay diagnostics.

- Placed 500 µl reagent 1 in cell and added 30 µl standard (or sample). Read the initial absorbance at 660 nm for the first absorbance point.
- Added 75 µl reagent 2 to the cell and incubated 10 min at room temperature or 5 minutes at 37 ^{o}C. Read the absorbance a second time at 660nm.

Calculating the results

$$\text{Result} = \frac{[\ (\Delta Abs\ Std1) - (\Delta Abs\ Sample)\ ]}{[\ (\Delta Abs\ Std1) - (\Delta Abs\ Std2)\ ]}$$

Δ Absorbance Standard1= (Second Absorbance of Std1- First Absorbance of Std1)

Δ Absorbance Standard2 = (Second Absorbance of Std2- First Absorbance of Std2)

Δ Sample Absorbance = (Second Absorbance of Sample- First Absorbance of Sample)

3.2.8. Determination of total oxidant status (TOS)

Total Oxidant Status (TOS) was determined with assay kit by Rel assay diagnostics. Prepare working standard solution, is diluted 40,000 times with deionized water. A liquid of 50 ml is added to 10 ml deionized water and vortexed (The first step dilution). A liquid of 50 ml of the prepared solution is added to 10 ml deionized water and vortexed.

- Placed 500 µl reagent 1 in cell and added 75 µl the prepared standard (or sample). Read the initial absorbance at 530 nm for the first absorbance point.
- Added 25 µl reagent 2 to the cell and incubated 10 min at room temperature or 5 min at 37^{o}C. Read the absorbance a second time at 530 nm.

Calculating the results

$$\text{Result} = (\Delta AbsSample \;/\; \Delta AbsStandard2)\; X\; 10$$

Δ Sample Absorbance = (Second Absorbance of Sample - First Absorbance of Sample)
Δ Absorbance Standard 2 = (Second Absorbance of Std 2 - First Absorbance of Std 2)
Standart 2 Value = 10 µmol H_2O_2 Equiv. /L

3.2.9. Determination of 8-Hydroxydeoxyguanosine (8-OHdG)

All reagents, standard solutions and samples were prepared as instructed (Figure 3.2). All reagents were allowed to become similar with room temperature before use. The assay was performed at room temperature. Number of strips required for the assay was determined. Strips in the frames were inserted for use. The unused strips were stored at 2-8°C. 50 µl standard was added to standard well. 40 µl sample was added to sample wells and then 10 µl anti-8-OHdG antibody was added to sample wells, then 50 µl streptavidin-HRP was added to sample wells and standard wells（Not blank control well). After appropriate mixing plates were covered with a sealer. After 60 minutes of incubation at

37°C the sealer was removed and plate was washed 5 times with wash buffer. Wells were soaked with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, all wells were aspirated and washed 5 times with wash buffer, overfilling wells with wash buffer. The plate was blotted onto paper towels or other absorbent material. 50 µl substrate solution A was added to each well and then 50 µl substrate solution B was added to each well. Plate covered with a new sealer was incubated for 10 minutes at 37°C in the dark. 50 µl stop solution was added to each well, and observing color change from blue to yellow immediately. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 30 min after adding the stop solution.

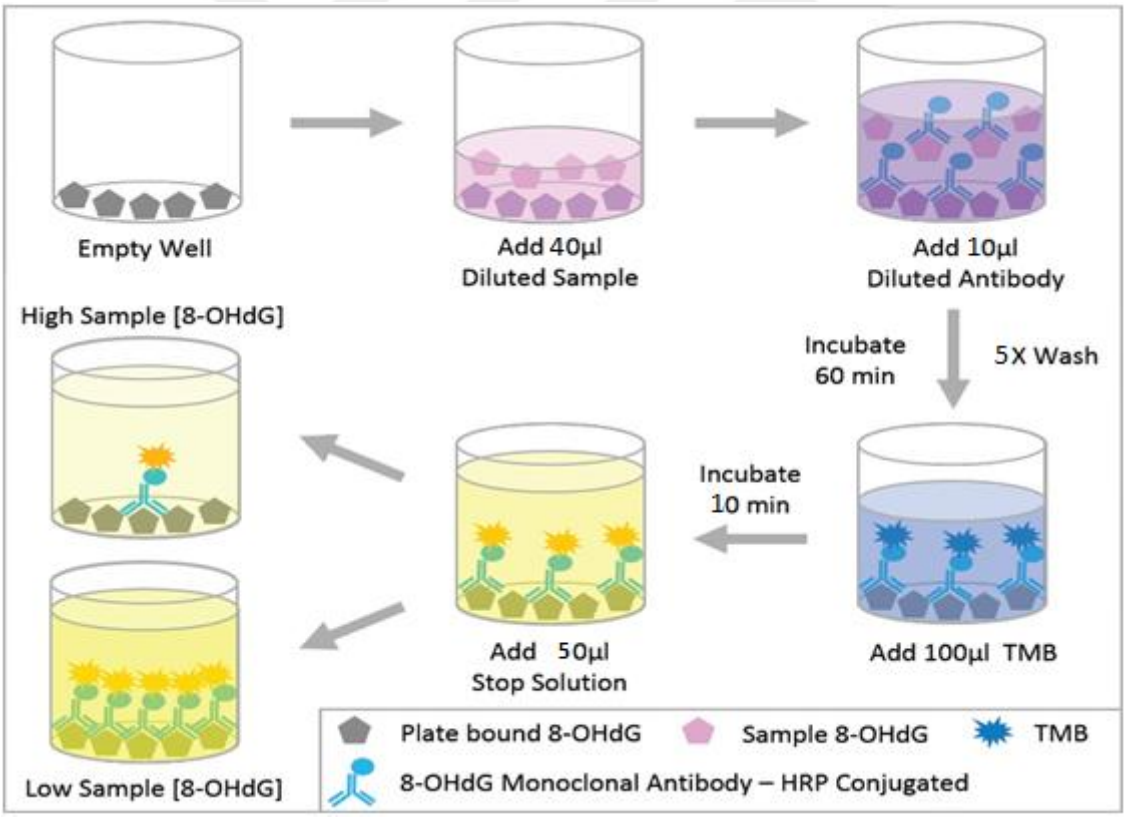

Figure 3.2. Schematic of the 8-Hydroxydeoxyguanosine competitive ELISA.

3.2.10. Protein assay

Total protein content was determined by Bradford protein assay (Bradford, 1976). At first prepared Bradford reagent. Used 200 mg of Coomassie Blue G250 dye and dissolved it in 50 ml of 95% ethanol. Then mixed this solution with 100 ml of concentrated (85%) phosphoric acid. After that made the final volume of the solution to 1 liter by adding distilled water. Later filtered the reagent through Whatman filter paper. After that transfer the filtrate in an amber colored bottle and stored at room temperature. Then prepared Protein standard by using 5 mg ovalbumin. Then dissolved it in 5 ml distilled water; this gave a protein stock solution of 1 mg/ml concentration. Later, stored the protein standard at -20°C. After that pipetted out (10 µl, 20 µl, 30 µl, 40 µl, 50 µl, 60 µl, 70 µl, 80 µl, 90 µl and 100 µl) ovalbumin standard in the glass tubes labeled 1–10. Then added distilled water to make the final volume 100 µl in each of the tubes. Then took 100 µl of each of the unknown protein dilutions in the tubes. After that added 5 ml of Bradford reagent in each of the tubes and mixed well by inversion or gentle vortex mixing. At last the absorbance was read at 595 nm.

3.3. Statistical Analysis

All values obtained at the end of analysis of samples were expressed as mean ± standard error of mean. The difference between control and cypermethrin groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test. The differences between the groups were considered significant at $P < 0.05$.

4. RESULTS

4.1. Fish

Van fish were used to cell isolation, the total weight and the total length of them were about 76-120 g and 18-20 cm. All expremental procedures performed on fish was carried out in a ccordance with animal study protocols approved by the Animal Researchers Local Ethics committee of Van Yüzüncü Yıl University. Turkey (2017/11).

4.2. Cell Culture

During experimental period cell culture was checked by inverted microscope. Isolated cells were attached with the poly-L-Lysine coat cell plate (Figure 4.1). However, there were not dead cells (Figure 4.2). At the end of cell isolation, hepatocyte cells were seen roundly. After 48 hours the 10^{-5} M groups at the end of the culture, cells were gradually decreased in all cypermethrin groups and control groups (Figure 4.3). While contamination and pH changes were not observed.

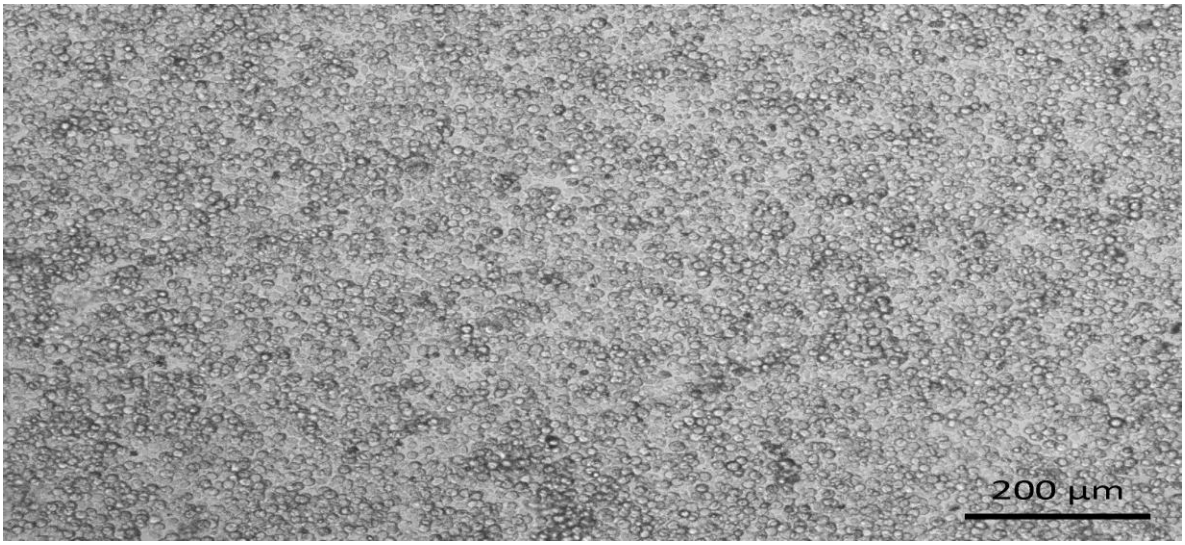

Figure 4.1. Hepatocytes culture on poly-L- lysine-coated dish after cell isolation.

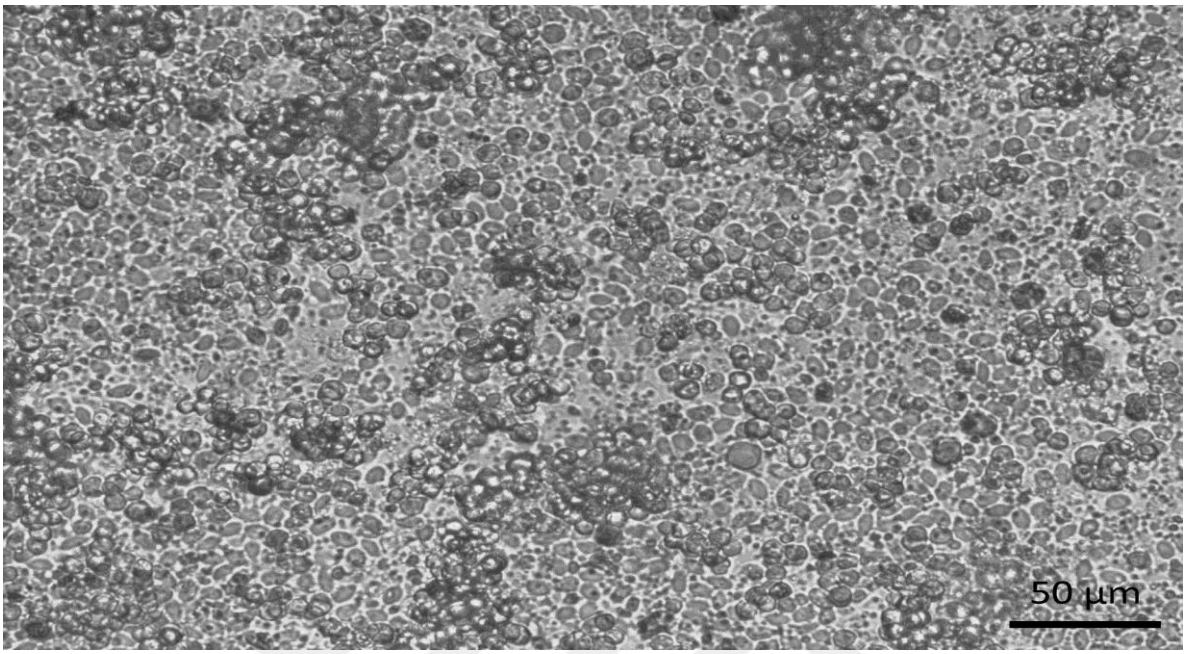

Figure 4.2. Cypermetrin (10^{-5} M) applied hepatocytes culture on poly-L- lysine-coated dish after 24 hours of cell isolation.

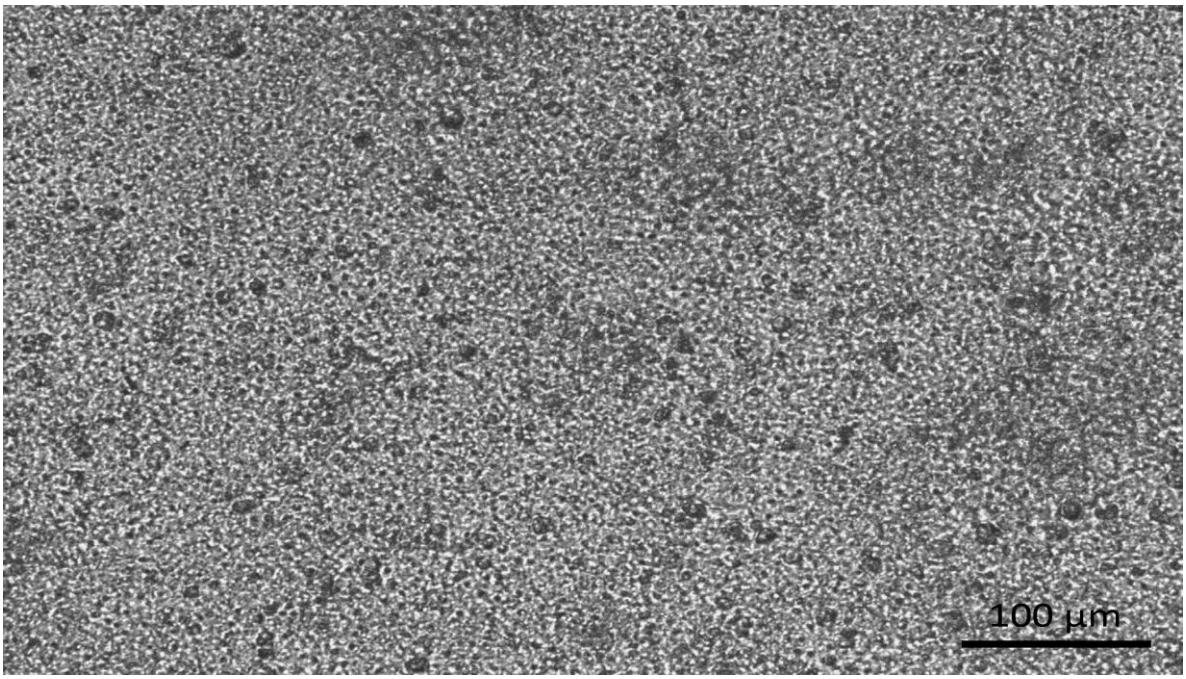

Figure 4.3. Cypermetrin (10^{-5} M) applied hepatocytes culture on poly-L- lysine-coated dish after 48 hours of cell isolation.

4.3. Aspartate Aminotransferase (AST)

Hepatocytes culture were treated with different concentration of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M), after 24 and 48 hours of incubation. Investigate the effect of our chemical on culture was compared with control doses. The result showed AST levels increased after 24 hours of incubation as shown in (Table 4.1). Therefore after 48 hours of incubation, the AST levels was not changed, as displayed in (Figure 4.4).

Table 4.1. AST levels in the culture medium.

Concentration	**24 Hours**	**48 Hours**
Control	81.44 ± 8.13939^{de}	51.06 ± 0.39573^{ab}
10^{-7} M	61.2 ± 2.37971^{bc}	53.14 ± 1.33963^{ab}
10^{-6} M	72.38 ± 6.37585^{cd}	54.3 ± 0.80436^{ab}
10^{-5} M	86.44 ± 5.31513^{e}	45.98 ± 1.58316^{a}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

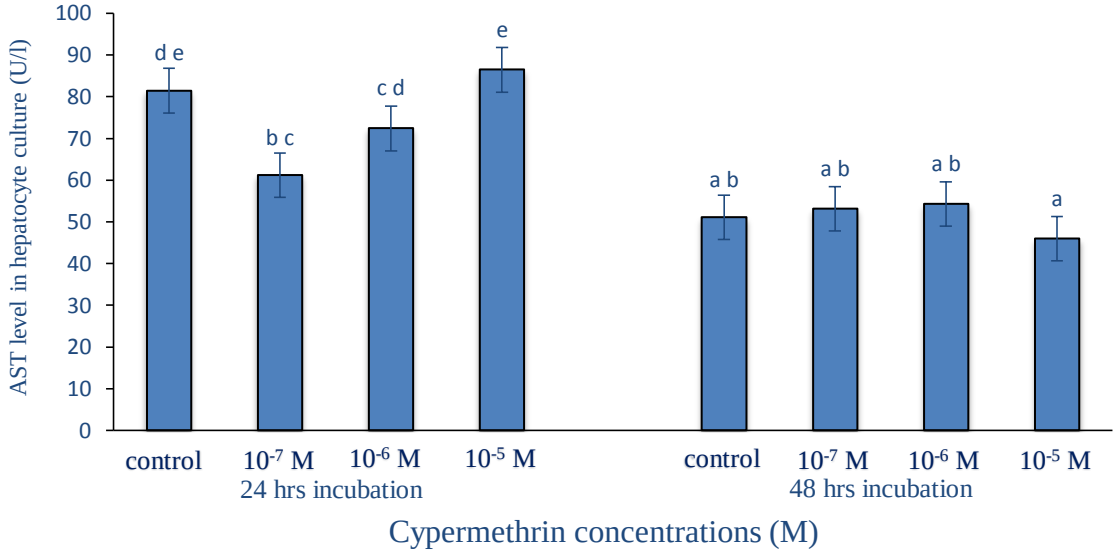

Figure 4.4. The effect of cypermethrin on the synthesis of AST in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.4. Alanine Aminotransferase (ALT)

Hepatocyte cultures were treated with different concentrations of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M). After 24 hours of incubation, ALT level of cypermethrin groups were increased but this increase was not significant as shown in (Table 4.2). Therefore after 48 hours of incubation ALT level of cypermethrin groups decreased when compared with the control group. But in the cypermethrin groups, the ranges were increased as seen in (Figure 4.5).

Table 4.2. ALT levels in the culture medium.

Concentration	**24 Hours**	**48 Hours**
Control	17.64 ± 1.76227^{de}	21.04 ± 1.37862^{ef}
10^{-7} M	19.92 ± 0.56160^{ef}	11.28 ± 0.42708^{a}
10^{-6} M	22.68 ± 0.56427^{e}	13.4 ± 0.54406^{ab}
10^{-5} M	16.42 ± 0.67483^{cd}	14.14 ± 0.32187^{bc}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

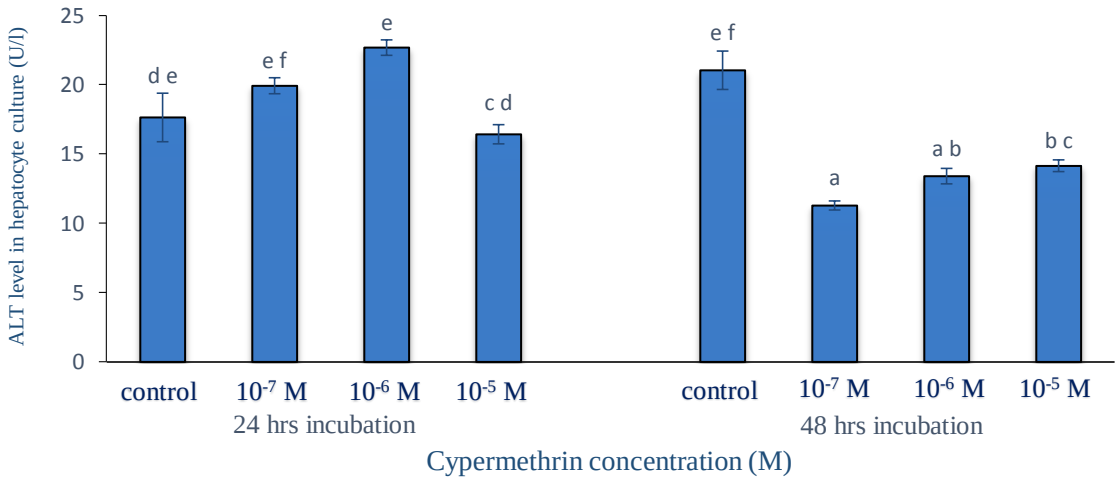

Figure 4.5. The effect of cypermethrin on the synthesis of ALT in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.5. Lactate Dehydrogenase (LDH)

The LDH measurement of hepatocyte extracted that exposed in different dose of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M). The LDH results, as shown in (Table 4.3), levels were increased in the doses and times manner, but this increase was not significant. Cypermethrin groups significantly decreased and they were compared with control groups as illustrated in Figure 4.6.

Table 4.3. LDH levels in the culture medium.

Concentration	24 Hours	48 Hours
Control	295 ± 55.87307^{bc}	428.6 ± 28.60524^{bc}
10^{-7} M	171 ± 21.28849^{ab}	61.4 ± 12.58014^{a}
10^{-6} M	292 ± 50.71548^{bc}	77.4 ± 16.09534^{a}
10^{-5} M	342.8 ± 49.16442^{bc}	87.6 ± 8.90281^{a}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

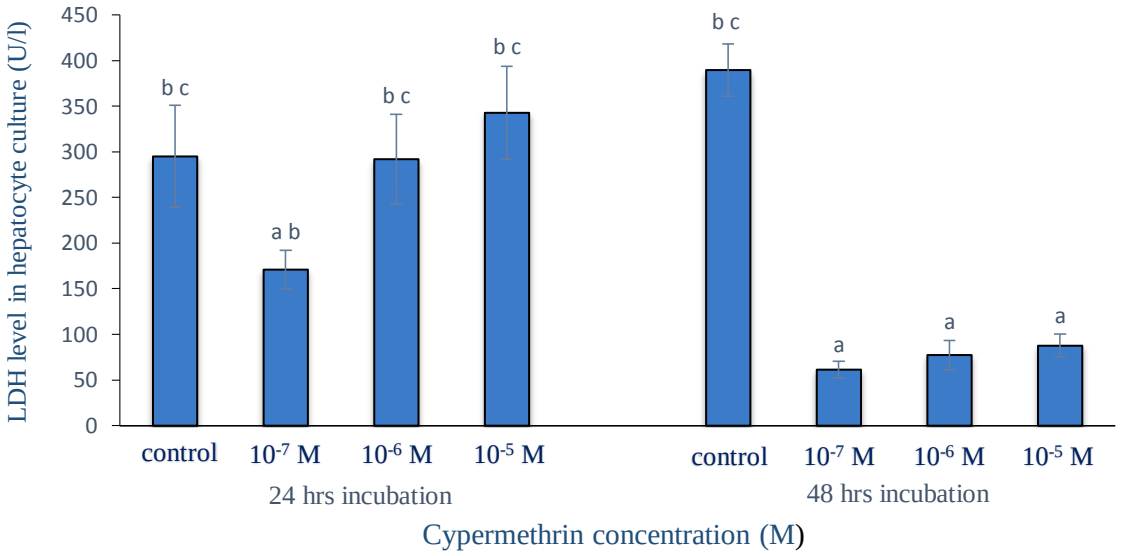

Figure 4.6. The effect of cypermethrin on the synthesis of LDH in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.6. Malondialdehyde (MDA)

At the end of incubation, MDA levels increased in the cypermethrin groups after 24 and 48 hours of incubation as observed in (Table 4.4). But when they compared with control groups the range was decreased as seen in (Figure 4.7).

Table 4.4. MDA levels in the culture medium.

Concentration	24 Hours	48 Hours
Control	0.3226 ± 0.02795^{d}	0.2130 ± 0.01342^{bc}
10^{-7} M	0.2244 ± 0.01780^{bc}	0.1520 ± 0.0812^{a}
10^{-6} M	0.2476 ± 0.01698^{c}	0.1702 ± 0.01770^{ab}
10^{-5} M	0.2602 ± 0.02365^{c}	0.1982 ± 0.02297^{abc}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

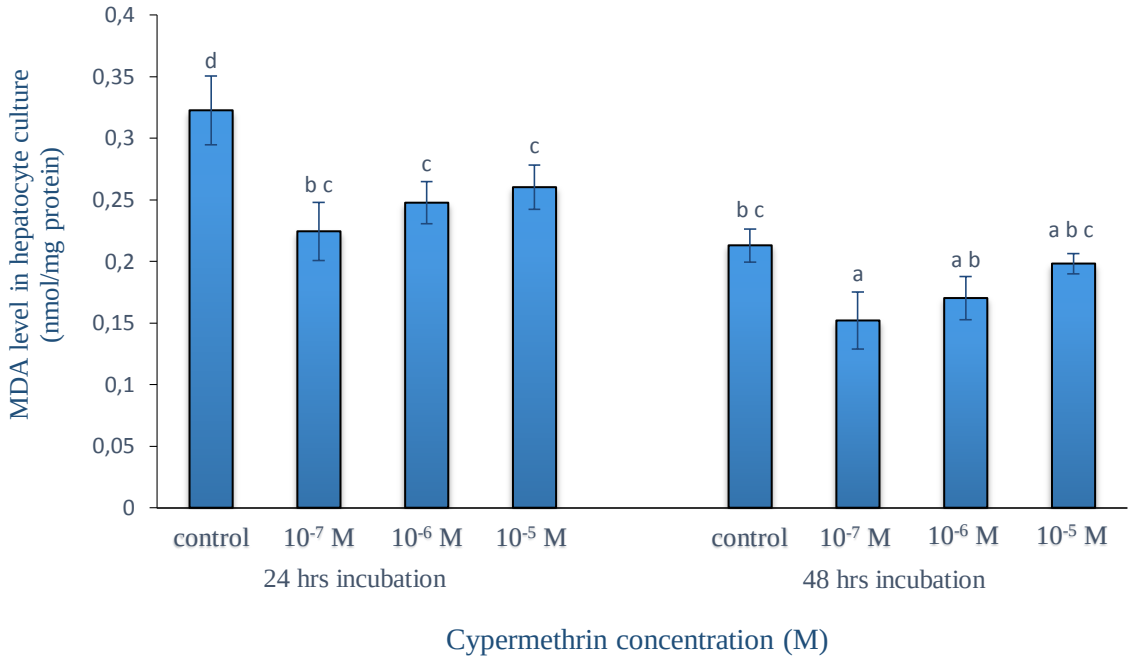

Figure 4.7. The effect of cypermethrin on the synthesis of MDA in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.7. Total Antioxidant Status (TAS)

Hepatocyte culture was treated with different concentration of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M) during 24 and 48 hours. The TAS levels, when they compared with the control groups, was not significant. The results of the analysis were displayed in Table 4.5 and Figure 4.5.

Table 4.5. TAS levels in the culture medium.

Concentration	24 Hours	48 Hours
Control	0.6542 ± 0.18918^{a}	0.5110 ± 0.11704^{a}
10^{-7} M	0.5703 ± 0.20701^{a}	0.8600 ± 0.14735^{a}
10^{-6} M	0.6234 ± 0.17441^{a}	0.3012 ± 0.09703^{a}
10^{-5} M	0.7036 ± 0.22135^{a}	0.5703 ± 0.12845^{a}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

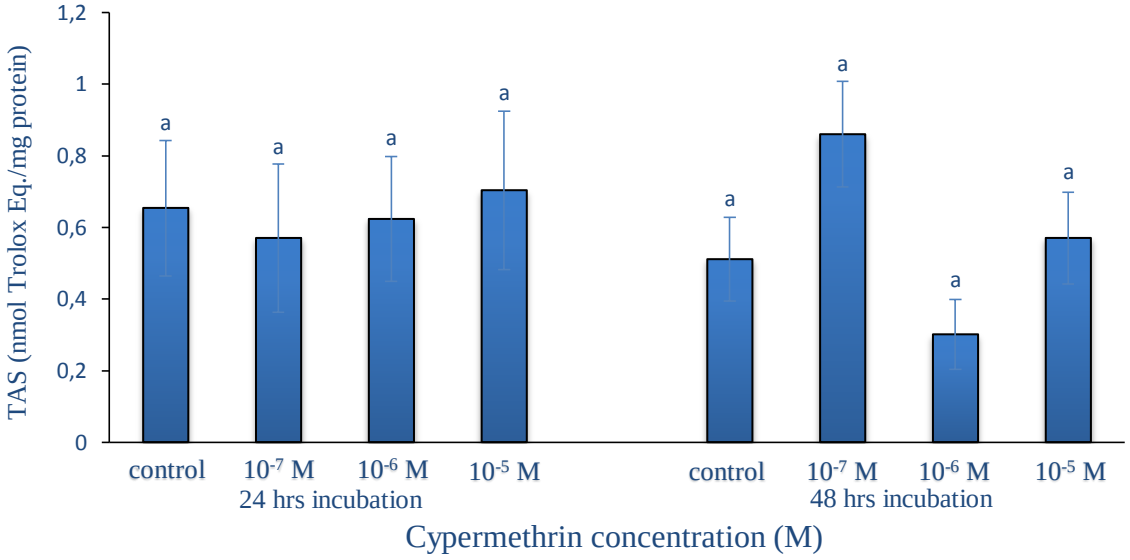

Figure 4.8. The effect of cypermethrin on the synthesis of TAS in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.8. Total Oxidative Status (TOS)

TOS level was measured by using spectrophotometer. Three different concentrations of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M) treated on hepatocyte cells. After 24 hours of incubation the values of TOS decreased when compared with the control group as shown in (Table 4.6). However, in the end of 48 hours of incubation, the range was increased significantly as illustrated in Figure 4.9.

Table 4.6. TOS levels in the culture medium.

Concentration	24 Hours	48 Hours
Control	19.3818 ± 0.83059^{cd}	8.0190 ± 0.47231^{a}
10^{-7} M	13.4356 ± 0.53533^{bc}	9.0190 ± 0.69894^{a}
10^{-6} M	13.6498 ± 0.64288^{ab}	9.7660 ± 0.41183^{a}
10^{-5} M	13.8605 ± 1.7388^{bc}	9.5073 ± 0.37557^{a}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

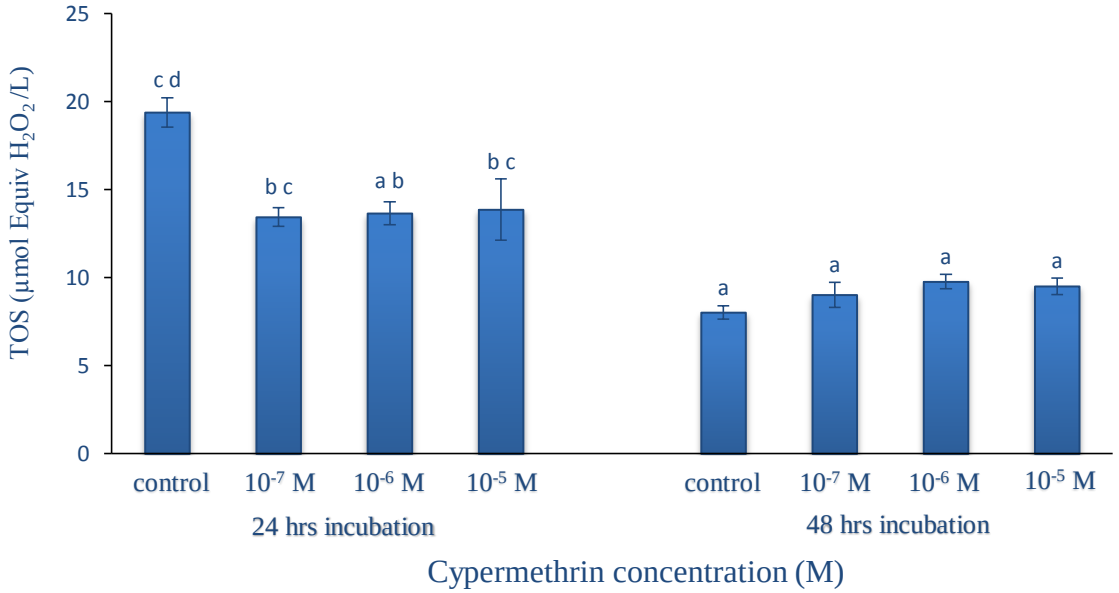

Figure 4.9. The effect of cypermethrin on the synthesis of TOS in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.9. 8-Hydroxydeoxyguanosine (8-OHdG)

The 8-OHdG levels were measured by using ELISA kit. Three different concentrations of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M) were treated on hepatocyte cells. The values of 8-OHdG decreased significantly in 10^{-7} M more than 10^{-6} M and 10^{-5} M when they compared with the control group. The results of the analysis were displayed in Table 4.7 and Figure 4.11.

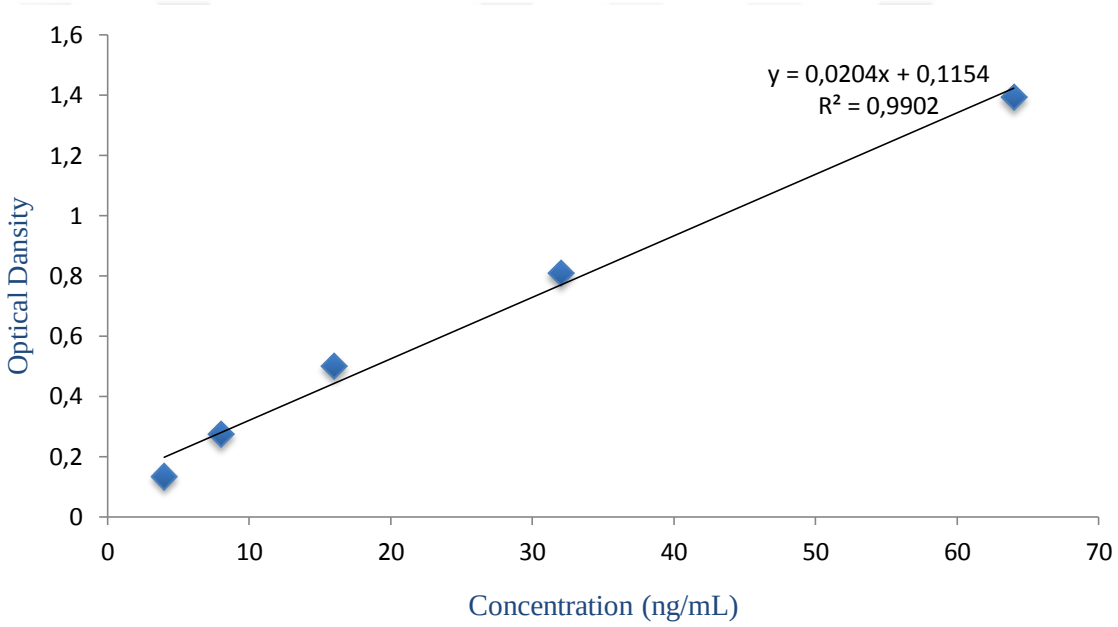

Figure 4.10. Standard curve of 8-OHdG assay in the hepatocyte culture.

Table 4.7. 8-OHdG levels in the culture medium.

Concentration	24 Hours	48 Hours
Control	16.2181 ± 1.21867^{a}	20.2747 ± 1.30813^{bc}
10^{-7} M	20.2377 ± 2.11896^{ab}	22.9804 ± 0.65993^{bc}
10^{-6} M	20.8668 ± 0.92706^{ab}	27.6176 ± 1.88795^{cd}
10^{-5} M	23.0074 ± 1.46299^{ab}	26.6569 ± 0.69192^{cd}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

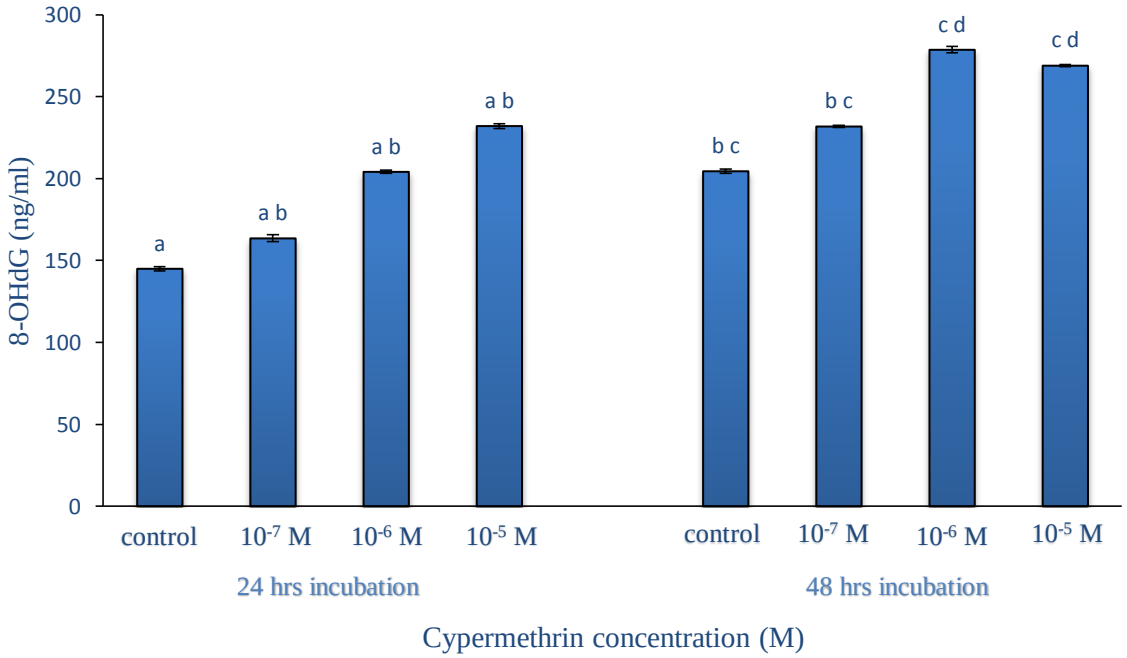

Figure 4.11. The effect of cypermethrin on the synthesis of 8-OHdG in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

4.10. Protein Assay

Amount of protein in the primary hepatocyte was determined by Bradford method and three different concentrations of cypermethrin (10^{-7}, 10^{-6} and 10^{-5} M) were treated on hepatocyte cells during 24 and 48 hours. The values of protein were decreased in 10^{-7} M more than 10^{-6} M and 10^{-5} M when they compared with the control group. The results of the analysis were displayed in Table 4.8 and Figure 4.13.

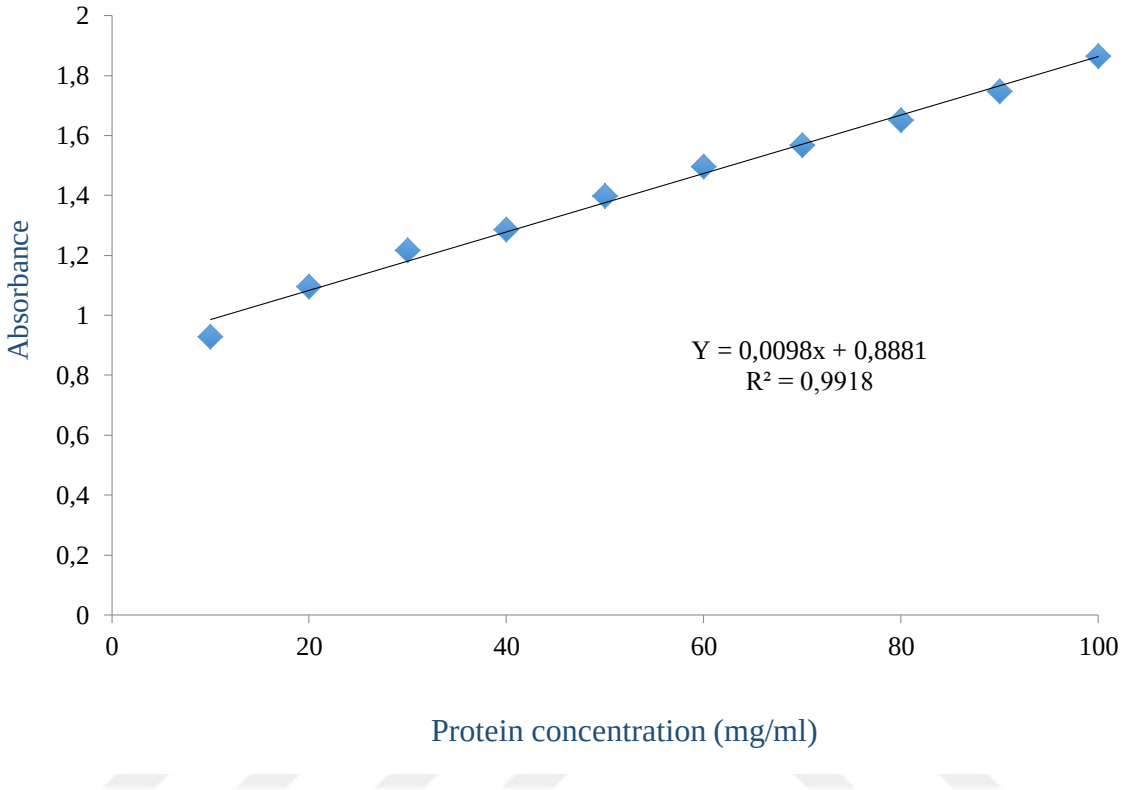

Figure 4.12. Standard curve of protein assay in the hepatocyte culture.

Table 4.8. Protein levels in the culture medium.

Concentration	**24 Hours**	**48 Hours**
Control	53.5357 ± 12.68489^{c}	44.0102 ± 8.98855^{bc}
10^{-7} M	21.8061 ± 2.57406^{a}	30.6224 ± 6.40093^{ab}
10^{-6} M	26.7857 ± 2.60366^{ab}	25.3214 ± 5.12479^{a}
10^{-5} M	40.1122 ± 6.35464^{ab}	21.2142 ± 4.11681^{a}

Values mean ± mean of standard error, and groups with different alphabetic superscripts differ significantly at $P < 0.05$.

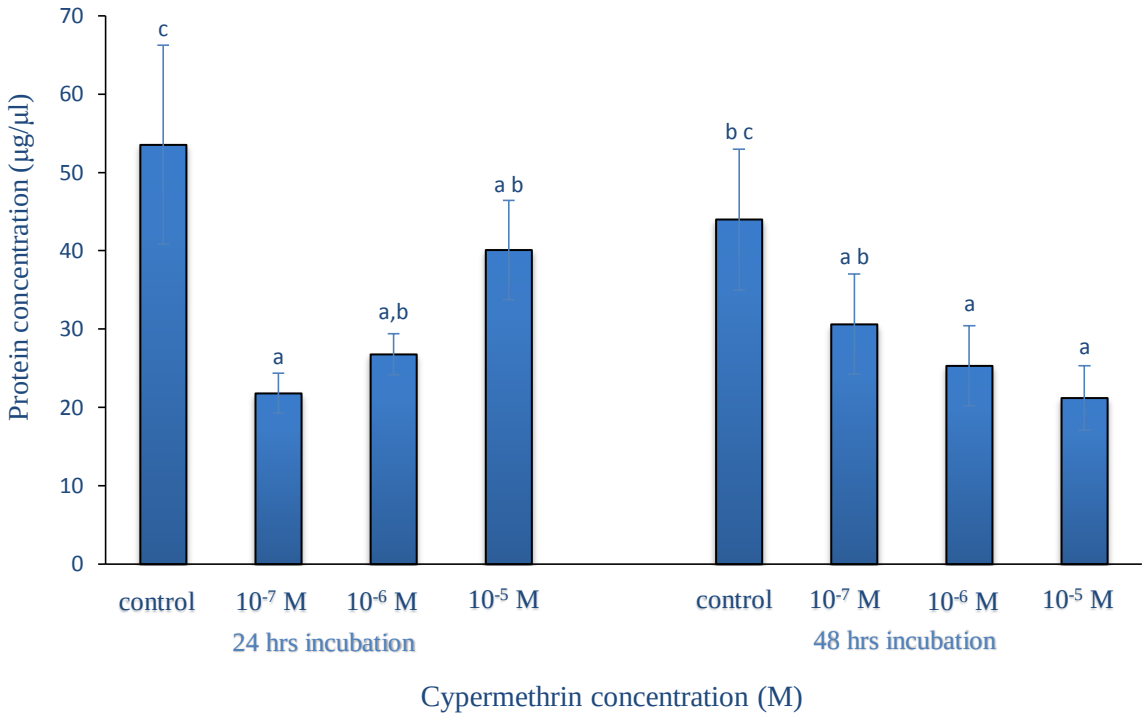

Figure 4.13. Protein concentration in primary hepatocyte culture. Results are expressed as Mean SEM (n = 5). Different letters denote significant difference ($P < 0.05$).

5. DISCUSSION AND CONCLUSION

Cypermethrin is commonly used in the agriculture sector that continuously pollutes the inland fishery water. It has also been widely used as a chemotherapeutic agent for the control of ectoparasite infestations in freshwater aquaculture. In this study it is revealed the toxic effect of different doses cypermethrin (1×10^{-7}, 1×10^{-6} and 1×10^{-5} M) on the primary hepatocyte culture of Van fish was assessed by Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Lactate dehydrogenase (LDH), Malondialdehyde (MDA), Total antioxidant status (TAS), Total oxidative status (TOS), 8-Hydroxydeoxyguanosine (8-OHdG) and Protein Assay.

Cell viability of Van fish hepatocytes reduced during experimental period and dose of cypermethrin. Several studies *in vitro* have shown severe toxic effects of the liver such as hypertrophy of hepatocytes and nuclei (Shakoori et al., 1988) and focal necrosis severe vacuolation (Monir et al., 2016) and depletion of polysaccharide content in hepatocytes (Nagarjuna and Doss, 2010). Also, similar results were shown in vitro studies (El-Tawil and Abdel-Rahman, 2001).

AST, ALT and LDH are widely use to determining cellular damage, both *in vivo* and *in vitro* studies. Therefore, the effects of harmful chemicals on liver toxicity are determine by these parameters. In this study, AST, ALT and LDH were used to determine the effects of cypermethrin on Van fish primary hepatocyte culture. Levels of AST, ALT and LDH increased with the doses of cypermethrin (Table 4.1, 4.2 and 4.3; Figure 4.4, 4.5 and 4.6). But this increase was only important after 24 hours. At the end of 48 hours, an increase in all doses of cypermethrin was observed. The groups with different doses of cypermethrin were statistically low compared to the control group. Contrary to the literature, this suggests that cypermethrin is highly toxic to Van fish hepatocytes and leads to decrease of cell numbers in cultures. El-Tawil and Abdel-Rahman, (1997) reported that cypermethrin killed approximately 50% of rat hepatocytes within 2 hours. *In vivo* applications of cypermethrin also cause irreversible damage to the liver (Velisek et al., 2006; Monir et al., 2015). At the end of 48 hours, the cause of elevated AST, ALT and

LDH in the control group may be damage to cells caused by different enzymes and mechanical agitation during hepatocyte isolation. In addition, the total protein range in the culture medium shows a decrease in cell count due to time (Figure 4.13 and Table 4.10).

Increased levels of lipid peroxidation were observed in tissues exposed to pollutants. For this reason, lipid peroxidation is often used as a bioindicator in determining oxidative stress (Farombi et al., 2008; Tiwari et al., 2010). In this study, MDA levels increased with dose of cypermethrin (Figure 4.7 and Table 4.4). However, this increase was not statistically significant. At the end of the 24 and 48 hours, it was also lower than the control group ($P < 0.05$). This may be due to the short half-life of MDA and dead cells in cultures administered with cypermethrin. Gil et al. (2010) stated that patients with paraquet poisoning had a fluctuating MDA level and that MDA was not reliable in determining the oxidative damage due to the short half-life of MDA.

Total antioxidant and total oxidant levels are shown in (Table 4.5 and 4.6; Figure 4.8 and 4.9). TAS and TOS levels decreased at the end of 48 hours compared to 24 hours. When compared with the control group, we observed that there was no statistical difference. Oxidized substances produced by cells which they are unavoidable within certain boundaries. Along with the increase of the oxidant substances in the cell, it causes damage to the lipid, protein and DNA in the cell (Sies, 1997; Velisek et al., 2006; Birben et al., 2012).

8-OHdG is a bioindicator that is often used to determine DNA-based oxidative damage. It has been used in many species and in the determination of damage to different tissues (Honda et al., 2000; Arslan et al., 2017; Geyikoglu et al., 2017). As a result of the application of cypermethrin, the levels of 8-OHdG (Table 4.7 and Figure 4.11) increase with dose and time ($P < 0.05$). The results are clearly demonstrate the damage of cypermethrin on hepatocytes.

In conclusion, oxidative damage of cypermethrin on primary hepatocyte culture has not been clearly demonstrated with liver enzymes, TAS and TOS. This thought due to the direct lethal effect of the doses used in the study of Van fish liver cells. However, oxidative damage is clearly demonstrated by parameters such as MDA and DNA damage.

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APPENDIX

APPENDIX 1. Genişletilmiş Türkçe Özet (Extended Turkish Summary)

SİPERMETRİNİN VAN BALIĞI (*Alburnus tarichi* Güldenstädt, 1814) PRİMER KARACİĞER KÜLTÜRÜ ÜZERİNE ETKİLERİ

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Sipermetrin hayvancılık ve ziraat alanlarında zararlı böceklerle mücadelede yaygın olarak kullanılan sentetik bir insektisittir. Her ne kadar böceklerle mücade için kullanılsa da zararsız diğer canlılar üzerine de olumsuz etkileri mevcuttur. Sipermetrin, Van Gölü havzasında hayvancılık ve ziraai alanlarda kullanılmaktadır. Yoğun kullanım göl ve sucul alanlarda da birikime neden olabilmektedir. Bu çalışmada, sipermetrin'in Van balığı (*Alburnus tarichi*) primer hepatosit kültürü üzerine etkilerinin belirlemesi amaçlamıştır. Van Balığından izole edilen karaciğer hücrelerine sipermetrinin farklı konsantrasyonları (1×10^{-7}, 1×10^{-6} ve 1×10^{-5} M) 48 saat süresince uygulanmıştır. Hücre kültürünün 24 ve 48 saatlerinde Aspartat Aminotransferaz (AST), Alanin Aminotransferaz (ALT) ve Laktat dehidrogenaz (LDH) seviyelerindeki değişim kültür ortamında belirlenmiştir. Ayrıca sipermetrinin total oksidan (TOS) ve antioksidan seviyeleri (TAS), malondialdehit seviyeleri (MDA) ve DNA hasarı üzerine etkileri belirlenmeye çalışılmıştır. Deneyler sonucunda hepatosit sayısının sipermetrin uygulanan gruplarda önemli ölçüde azaldığı invert ışık mikroskobunda belirlendi. Karaciğer hasarında sıklıkla kullanılan AST, ALT ve LDH seviyeleri sipermetrin dozlarına bağlı olarak artış göstermesine rağmen bu artış kontrol grubuna göre düşük veya istatistiksel olarak önemsizdi. MDA seviyeleri, inkübasyon sonunda doza bağlı olarak artış gösterdi. TAS ve TOS seviyeleri, kontrol grubu ile karşılaştırıldığında herhangi bir farkın olmadığı gözlendi. DNA hasarının ise doza ve zamana bağlı olarak artış gösterdiği belirlendi. Sonuç olarak, uygulanan sipermetrinin Van Balığı primer hepatosit kültürü üzerine yüksek toksik etkilerinin olduğu net bir biçimde ortaya konmuştur.

1. GİRİŞ

Sipermetrin, sentetik bir piretroid olup zararlı böceklerle mücadelede dünya çapında kullanılmaktadır (Igbedioh, 1991). Piretroitler piretrinin (Kasımpatı bitkilerinden elde edilen haşere ilaçları) yapay formlarıdır (Soderlund, 2002). Veterinerlik ve tarımsal alanlarda böcek kontrolü için kullanılır (Gupta, 1990). Sipermetrin, geniş bir alanda böceklere karşı oldukça etkilidir. Fakat hedef olmayan kuş, balık ve arı gibi canlılarda da toksik etkilere sahiptirler (Bhunya ve Pati, 1988). Balıklar, kimyasalın sucul alanlarda birikimi nedeniyile fazla etkilenen canlı grubudur.

Sipermetrin Van Gölü havzasında sıklıkla kullanılan bir insektisittir. Fakat kimyasalın Van Balıklarında *in vivo* ve *in vitro* etkileri hakkında herhangi bir bilimsel araştırma bulunmamaktadır. Bu çalışmanın amacı, Van balıklarında (*Alburnus tarichi*) primer hepatosit kültürü üzerine sipermetrin'in etkilerini belirlemektir.

2. MATERYAL ve YÖNTEM

1.1. Balık

Balıklar, serpme ağlar ile göle dökülen tatlı sulardan yakalandı. Yakalanan balıklar oksijen bağlı taşıma kaplarına alınarak laboratuvar ortamına getirildi. Balıklar, ticari alabalık yemi ile beslendi ve 12:12 saat fotoperiyoda bırakıldı. Akvaryumlar için filtre edilmiş çeşme suyu kullanıldı ve su sıcaklığı hepatosit izolasyonu yapılıncaya kadar 24ºC ye ayarlandı.

1.2. Kimyasallar

Sipermetrin oda sıcaklığında ve karanlıkta muhafaza edildi. Çalışmada kullanılacak Leibovitz-15 (L-15), enzimler ve diğer kimyasallar Sigma-Aldrich (Almanya) firmasından satın alındı. Diğer bütün kimyasallar yüksek saflıkta kullanıldı.

1.3. Hepatosit İzolasyonu

Balıklar su içerisine katılan fenoksi etanol (320 mg/lt) ile anestezi edildi ve sterilizasyon için %70'lik etanole daldırıldı. Daha sonra steril koşullarda laminar akım kabini (Esco) içerisinde disekte edildi. Balıkların karaciğerleri çıkartılarak saat camı içerisine alındı. Doku etrafındaki yağ dokusu temizlendikten sonra küçük parçalara ayrılan karaciğer dokusu, steril hepatosit tamponu (Hepatosit Tamponu: 0.145 M NaCl, 5.4 mM KCl, 5 mM EDTA, 1.1 mM KH_2PO_4, 12 mM NaH_2CO_3, 3 mM $NaHPO_4$ and pH:7.6) ile oda sıcaklığında muamele edildi. Bu işlem, 30 dakikalık süre içerisinde birkaç kez tekrarlandı. Daha sonra doku 2.5 mM $CaCl_2$ içeren EDTA'sız hepatosit tamponu içerisine alındı. Böylece kandan temizlenen doku, kollejenaz (0.5 mg/ml) içeren L-15 içerisinde 30 dakika inkübe edildi. İnkübasyondan sonra doku iki defa farklı çaplara sahip mikropipet uçları ile pipetlenip insülin enjektöründen geçirilerek tiritüre edidi. Bu işlem sonucunda

elde edilen hücre süspansiyounundaki süpernatant berraklaşana kadar birkaç kez 90 X*g* de 5 dakika santrifüj edildi. Santrifüj sonunda süpernatant atılarak yerine besi ortamı (Leibovitz-15) konuldu. Besi ortamına kontaminasyonun önlenmesi amacıyla antibiyotik-antimikotik solüsyon (10 ml/L) ve pH optimizasyonu için 5 mM $NaHCO_3$ eklendi. İzole edilen hücreler trypan blue (%4) ile boyanarak canlı ve ölü hücre ayrımı yapıldı. Thoma lamı kullanılarak hücre sayımı gerçekleştirildi. Kültür içerindeki canlı hücrelerin oranının %90'dan fazla olmasına dikkat edildi.

1.4. Hücre Kültürü

İzole edilen hücreler, önceden poly-L-lysine ile kaplanmış 48 kuyucuklu kültür kaplarına 1×10^6 ml olacak şekilde ekildi. Her bir kuyucuğa toplam 500 µl besi ortamı ilave edilerek kültür süresince 24ºC'lik inkübatörde nemli ortamda inkübe edildi. Hücrelerdeki morfolojik değişimler ve kontaminasyon olup olmadığı invert mikroskopta (Leica marka DM 6000 model) incelenecek ve görüntüleri alındı. Kültür ortamındaki pH değişimleri de sürekli olarak kontrol edildi. Kültür besi ortamına farklı konsantrasyonlarda (1×10^{-7}, 1×10^{-6} ve 1×10^{-5} M) sipermetrin, kontrol grubuna ise sadece çözücü ilave edildi. Her bir doz sipermetrin ve kontrol grubu 5'er adet kuyucuğa uygulandı. Kültürden alınan ekstrakt analiz yapılıncaya kadar -80ºC'de derin dondurucuda saklandı.

1.5. Aspartat Aminotransferaz ve Alanin Aminotransferaz Seviyesinin Belirlenmesi

Aspartat aminotransferaz (AST) aktivitesi, aspartik asit ve ketoglutamik asitin glutamik ve okzaloasetik asitlere dönüştürülmesi ile ölçüldü. Alanin aminotransferaz (ALT) aktivitesi alanin ve ketoglutamik asitin glutamik ve piruvik asitlere dönüştürülmesi ile belirlendi. Enzim işlemleri, indirgenmiş formun (NADH) oksitlenmiş forma (NAD) dönüştürerek nikotinamid adenin dinükleotidi ile birleştirildi. NADH'nin oksidasyonu ile üretilen absorbans azalışı 340 nm'de ölçüldü.

1.6. Lactate Dehydrogenase (LDH) Seviyesinin Belirlenmesi

LDH seviyesi, hücre kültür ortamından alınan örneklerden belirlendi. Bu nedenle aşağıdaki prosedür uygulandı:

1. Her kuyucuk iki kez, 2 ml PBS ile yıkandı.
2. Hepatositleri lize etmek için her kuyucuğa 1 ml potasyum fosfat tamponu içeren 10 µl %10 Triton X-100 eklendi.
3. Hücreler kazınarak 1 ml'lik mikrosantrifüj tüpüne aktarıldı.
4. Tüpler, 30 dakika boyunca buz üzerinde inkübe edildi.
5. 2.85 ml reaksiyon tamponuna (0.1 M fosfat tamponu, pH 7.4 ve 5 mM NADH) 50 µl hücre lizatı eklendi.
6. 100 µl, 20 mM sodyum piruvat solüsyonu ilave ederek reaksiyon başlatıldı.
7. 340 nm'de azalan absorbans oranını ölçülerek LDH değeri hesaplandı.

1.7. Malondialdehit (MDA) Seviyesinin Belirlenmesi

Hücreler inkübe edildikten sonra, numuneden 200 µl alınır ve 1 tüp içine konulduktan sonra. 800 µl fosfat tamponu, 25 µl BHT çözeltisi ve 500 µl %30'luk TCA ilave edildi. Tüpler vorteks ile karıştırıldıktan sonra buzdolabında (-80ºC) 30 dakika tutuldu. Daha sonra 2000 rpm'de 15 dakika santrifüjlendi. Süpernatandan 1 ml alınarak diğer tüplere aktarıldı. Daha sonra 75 µl EDTA ve 250 µl TBA ilave edildi. Tüpler vorteksde karıştırıldı ve kaynar su banyosunda (90ºC) 15 dakika bekletildi. Daha sonra, absorbansları 532 nm'de UV/V spektrofotometre ile okundu.

1.8. Total Antioksidan (TAS) ve Total Oksidan (TOS) Seviyesinin Belirlenmesi

Örneklerdeki antioksidanların koyu mavi-yeşil renkli ABST radikalini renksiz forma indirgemesine dayanan TAS ölçümünde; ticari kitler (Rel Assay Diagnostics) kullanılarak kit prosedüründe belirtildiği şekilde spektrofotometrede örneklerin absorbansları 660 nm'de ölçüldü. Çalışmalarda verilen standart formüle göre örneklerin TAS düzeyleri (mmol Trolox Eq/L) hesaplandı. Örnekteki oksidanların ferröz iyon-şelatör kompleksini ferrik iyonlara okside etmesiyle ferrik iyonlar asidik ortamda kromojen madde ile renk oluşturması esasına dayanan TOS ölçümünde; ticari kit (Rel Assay Diagnostics) ve kit prosedürü kullanılarak spektrofotometrede örneklerin absorbansları (530 nm'de) ölçüldü Çalışmalarda verilen standart formüle göre örneklerin TOS düzeyleri (μmol H_2O_2 Eq./L) hesaplandı.

1.9. DNA hasarının belirlenmesi

DNA hasarı ELISA kiti prosedürüne göre yapıldı. Buna göre:

1. Numune ve Standartları sulandırma solüsyonunda hazırlandı.
2. Hazırlanan standart ve numuneler, uygun kuyucuklara üçer defa eklendi.
3. Uygun kuyucuklara 10 μL seyreltilmiş antikor solüsyonu eklendi.
4. Mikpoplakanın üzerini örtün ve 1 saat (37°C) sıcaklıkta inkübe edildi.
5. Plakayı 0.35 ml Yıkama Tamponu ile 5 kez yıkayın. 30 saniye bekledikten sonra sıvı boşaltıldı.
6. Önce her kuyucuğa 50 μL kromojen çözeltisi eklendi ve sonra her kuyuya 50 μL kromojen ekledi.
7. Plaka kapatıldı ve (37°C) 10 dakika boyunca inkübe edildi.
8. Her kuyucuğa 50 μL durdurma çözeltisi eklendi.
9. 450 nm'de bir plaka okuyucu üzerinde absorbansı ölçüldü.
10. Standart eğri çizildi ve örnek konsantrasyonu hesaplandı.

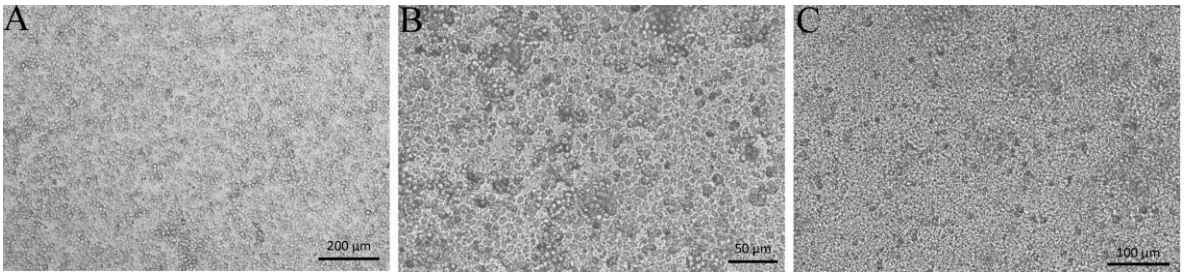
1.10. İstatistiksel Analiz

Deneyler sonunda elde edilen veriler ortlama ± Standart hata olarak verildi. Kontrol ve sipermetrin grupları arasındaki fark ANOVA (one-way analysis of variance) ile farkın derecesi ise Duncan testi ile belirlendi. Gruplar arasındaki fark $P < 0.05$'e göre verildi.

2. BULGULAR

2.1. Hücre kültürü

Kontrol ve sipermetrin uygulanan gruplar deney süresince invert ışık mikroskobu ile kontrol edildi. İzolasyon sonunda bütün hücrelerin yuvarlak şekilli olduğu gözlendi. Her ne kadar farklı işlemler uygulansa da oval şekilli eritrositlere kültür içerisinde rastlanıldı. Hücrelerin izolasyondan sonra poly-L-Lysine kaplı hücre kültürü kaplarının tabanına yapıştığı, hücre artıklarının ise medium içerisinde asılı kaldığı belirlendi. 24 saat sonunda hücrelerin gruplar oluşturarak bir araya geldiği gözlendi. Kültür sonunda gerek kontrol gerekse sipermetrin uygulanan kuyucuklarda hücre sayısında azalmanın olduğu bu azalmanın sipermetrin dozuna bağlı olarak arttığı ortaya konuldu. Kültür süresince herhangi bir kontaminasyona ve pH değişimine rastlanılmadı.



Şekil A. İzolasyon sonunda poly-L-lysine kaplı kültür kaplarındaki hepatositler.
Şekil B. Sipermetrin (10^{-5} M) uygulanan hepatositlerin 24 saat sonundaki görüntüleri.
Şekil C. Sipermetrin (10^{-5} M) uygulanan hepatositlerin 48 saat sonundaki görüntüleri.

2.2. Aspartat Aminotransferaz (AST)

AST seviyesi sipermetrin uygulanan dozlarda 24 saat sonunda artış gösterdi (Tablo 1). 48 saat sonra ise AST seviyesinde bir azalma görüldü. Bu artışlar kontrol grubu ile kıyaslandığında istatistiksel olarak önemsizdi.

Tablo 1. Kontrol ve sipermetrin uygulanan kültürlerdeki AST seviylerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	81.44 ± 8.13939^{de}	51.06 ± 0.39573^{ab}
10^{-7} M	61.2 ± 2.37971^{bc}	53.14 ± 1.33963^{ab}
10^{-6} M	72.38 ± 6.37585^{cd}	54.3 ± 0.80436^{ab}
10^{-5} M	86.44 ± 5.31513^{e}	45.98 ± 1.58316^{a}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

2.3. Alanin Aminotransferaz (ALT)

ALT seviyesi sipermetrinin artan dozlarına bağlı olarak 24 ve 48 saatler sonunda (10^{-7}, 10^{-6} ve 10^{-5} M) artış göstermiştir (Tablo 2). 24 saat sonunda sipermetrin grubu kontrol grubu ile kıyaslandığında istatistiksel olarak artış göstermiştir. 48 saat sonunda ALT seviyeleri bütün gruplarda azalma göstermiştir.

Tablo 2. Kontrol ve sipermetrin uygulanan kültürlerdeki ALT seviylerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	17.64 ± 1.76227^{de}	21.04 ± 1.37862^{ef}
10^{-7} M	19.92 ± 0.56160^{ef}	11.28 ± 0.42708^{a}
10^{-6} M	22.68 ± 0.56427^{e}	13.4 ± 0.54406^{ab}
10^{-5} M	16.42 ± 0.67483^{cd}	14.14 ± 0.32187^{bc}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

2.4. Laktat dehidrogenaz (LDH)

Laktat dehidrogenz seviyesi sipermetrin uygulanan gruplarında artış göstermiştir (Tablo 3). Fakat bu seviye 48 saat sonunda azalma göstermiştir.

Tablo 3. Kontrol ve sipermetrin uygulanan kültürlerdeki LDH seviylerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	295 ± 55.87307^{bc}	428.6 ± 28.60524^{bc}
10^{-7} M	171 ± 21.28849^{ab}	61.4 ± 12.58014^{a}
10^{-6} M	292 ± 50.71548^{bc}	77.4 ± 16.09534^{a}
10^{-5} M	342.8 ± 49.16442^{bc}	87.6 ± 8.90281^{a}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

3.5. Malondialdehyde (MDA)

MDA seviyesi sipermetrin uygulanan gruplarda doza bağlı olarak artış göstermiştir (Tablo 4). Fakat zamana bağlı olarak (48 saat) hem sipermetrin gruplarıda hem de kontrol grubunda azalma göstermiştir.

Tablo 4. Kontrol ve sipermetrin uygulanan kültürlerdeki MDA seviyelerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	0.3226 ± 0.02795^{d}	0.2130 ± 0.01342^{bc}
10^{-7} M	0.2244 ± 0.01780^{bc}	0.1520 ± 0.0812^{a}
10^{-6} M	0.2476 ± 0.01698^{c}	0.1702 ± 0.01770^{ab}
10^{-5} M	0.2602 ± 0.02365^{c}	0.1982 ± 0.02297^{abc}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

3.6. Total antioksidan (TAS) ve Total oksidan seviyesi (TOS)

Uygulama sonunda TAS seviyesinde gruplar arasında herhangi bir fark gözlenmemiştir (Tablo 5). TOS seviyesinde ise zamana bağlı olarak bir azalma belirlenmiştir (Tablo 6).

Tablo 5. Kontrol ve sipermetrin uygulanan kültürlerdeki TAS seviyelerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	0.6542 ± 0.18918^{a}	0.5110 ± 0.11704^{a}
10^{-7} M	0.5703 ± 0.20701^{a}	0.8600 ± 0.14735^{a}
10^{-6} M	0.6234 ± 0.17441^{a}	0.3012 ± 0.09703^{a}
10^{-5} M	0.7036 ± 0.22135^{a}	0.5703 ± 0.12845^{a}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

Tablo 6. Kontrol ve sipermetrin uygulanan kültürlerdeki TOS seviyelerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	19.3818 ± 0.83059^{cd}	8.0190 ± 0.47231^{a}
10^{-7} M	13.4356 ± 0.53533^{bc}	9.0190 ± 0.69894^{a}
10^{-6} M	13.6498 ± 0.64288^{ab}	9.7660 ± 0.41183^{a}
10^{-5} M	13.8605 ± 1.7388^{bc}	9.5073 ± 0.37557^{a}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

3.7. 8-Hydroksi deoksiguanozin (8-OHdG) seviyesi

8-Hydroksi deoksiguanozin (8-OHdG) seviyesi incelendiğinde hem doza hem de zamana bağlı olarak bir artışın olduğu görülmektedir. Diğer değerlerin aksine deney süresinin sonuna kadar artarak devam etmiştir (Tablo 7.).

Tablo 7. Kontrol ve sipermetrin uygulanan kültürlerdeki 8-Hydroksi deoksiguanozin seviylerindeki değişim.

Konsantrasyon	**24 Saat**	**48 Saat**
Kontrol	16.2181 ± 1.21867^{a}	20.2747 ± 1.30813^{bc}
10^{-7} M	20.2377 ± 2.11896^{ab}	22.9804 ± 0.65993^{bc}
10^{-6} M	20.8668 ± 0.92706^{ab}	27.6176 ± 1.88795^{cd}
10^{-5} M	23.0074 ± 1.46299^{ab}	26.6569 ± 0.69192^{cd}

Değerler ortalama ± standart hatanın ortalaması olarak verilmiştir. Farklı harfler gruplar arasındaki farklılıkları ifade eder $P < 0.05$.

3. TARTIŞMA ve SONUÇ

Sipermetrin, tarım sektöründe ve hayvancılıkta kullanılan bir pestisittir. Ayrıca, balık yetiştiriciliğinde ektoparazit enfeksiyonlarının kontrolü için yaygın olarak kullanılmaktadır. Bu çalışmada Van balıklarının birincil hepatosit kültüründe sipermetrin (1×10^{-7}, 1×10^{-6} ve 1×10^{-5} M) farklı dozların toksik etkileri Aspartat Aminotransferaz (AST), Alanin Aminotransferaz (ALT), Laktat dehidrogenaz (LDH), Malondialdehit (MDA), Toplam antioksidan durumu (TAS), Toplam oksidatif durum (TOS), 8-Hidroksideoksiguanozin (8-OHdG) ve Protein Analizi ile belirlendi.

Hepatosit izolasyonu ve deney süresi sonunda kontrol grubundaki hücre yapılarının literatürlere uyumlu olduğu gözlendi. Herhangi bir kimyasal uygulanmayan kontrol grubunda da hücre ölümleri gerçekleşti. Sipermetrin uygulanan gruplarda hücre sayılarının doza ve zamana bağlı olarak azaldığı mikroskobik olarak belirlendi.

Hücresel hasarın belirlenmesinde AST, ALT ve LDH hem *in vivo* hem de *in vitro* çalışmalarda yaygın bir şekilde kullanılmaktadır. Bu nedenle zararlı kimyasalların karaciğer toksisitesi üzerine etkileri bu parametreler ile belirlenmektedir. Bu çalışmada, sipermetrinin Van Balığı primer hepatosit kültürü üzerine etkilerinin belirlenmesinde AST, ALT ve LDH kullanıldı. Değerler sipermetrin dozuna bağlı olarak artış gösterdi. Fakat bu artış sadece 24 saat sonunda önemliydi. 48 saat sonunda bütün sipermetrin dozlarında azalma gözlendi. Farklı sipermetrin uygulanan gruplar, kontrol grubu ile kıyaslandığında istatistiksel olarak düşüktü. Literatüre göre zıtlık gösteren bu durum sipermetrinin Van Balığı hepatositleri için yüksek derecede toksik olduğunu ve kültürlerde hücre sayısındaki azalmaya neden olduğu söylenebilir. El-Tawil ve Ark., (1997) sipermetrinin 2 saat içerisinde rat hepatositlerininin yaklaşık %50 sini öldürdüğünü belirtmiştir. Ayrıca sipermetrinin *in vivo* uygulamaları da karaciğerde geri dönüşümsüz hasara neden olmaktadır (Velisek et al., 2006; Monir ve Ark., 2015). 48 saatin sonunda kontrol grubunda yüksek AST, ALT ve LDH'ın nedeni ise hepatosit izolasyonu sırasında farklı enzim ve mekanik ayrıştırmanın hücrelerde oluşturduğu hasar olabilir. Ayrıca, kültür ortamındaki total protein seviyeleride zamana bağlı olarak hücre sayısındaki azalmayı dolaylı olarak göstermektedir (Tablo 1-3).

Kirleticilere maruz kalan dokularda lipid peroksidasyon seviyesinde artış gözlenmektedir. Bu nedenle oksidatif stresin belirlenmesinde lipid peroksidasyonu sıklıkla kullanılan bir biyoindikatördür (Farombi et al., 2008; Tiwari et al., 2010). Bu çalışmada sipermetrinin dozlarına bağlı olarak MDA seviyeleri artış göstermiştir (Tablo 4). Fakat bu artış istatistiksel olarak önemli değildi. Ayrıca, 24 ve 48 Saatler sonunda kontrol grubuna göre de düşük seviyedeydi (P<0.05). Bu durum MDA'nın yarılanma ömürün kısa olmasından ve sipermetrin uygulanan kültürlerdeki hücre ölümlerinden kaynaklanmış olabilir. Gil ve Ark., (2010) Paraquat zehirlenmesi gösteren hastalarda MDA seviyesinin dalgalı olduğunu ve MDA'nın yarı ömrünün kısa olması nedeni ile MDA'nın oksidatif hasar belirlemesinde güvenilir olmadığını belirtmiştir.

Total antioksidan ve total oksidan seviyeleri Tablo 5 ve Tablo 6 de gösterilmiştir. TAS ve TOS seviyeleri 48 saatin sonunda 24 saate göre azalma göstermiştir. Değerler konrol grubu ile karşılaştırıldığında istatistiksel olarak bir farkın olmadığı gözlemiştir. Hücreler tarafından üretilen oksidan maddeler belirli sınırlar içerisinde kaçınılmazdır. Oksidan maddelerin hücre içerisinde artmasıyla birlikte hücre içerisindeki lipid, protein ve DNA'da hasarlara neden olmaktadır (Sies, 1997; Velisek et al., 2006; Birben et al., 2012).

8-OHdG, DNA temelli oksidatif hasarın belirlenmesinde sıklıkla kullanılan bir biyoindikatördür. Pek çok canlı türünde ve farklı dokularındaki hasarın belirlenmesinde kullanılmıştır (Honda et al., 2000; Arslan et al., 2017; Geyikoglu et al., 2017). Sipermetrin uygulaması sonucunda 8-OHdG seviyeleri (Tablo 7) doza ve zamana bağlı olarak (P<0.05) artış göstermektedir. Sonuçlar sipermetrinin hepatositler üzerindeki hasarını net bir şekilde ortaya koymuştur.

Sonuç olarak, Sipermetrinin primer hepatosit kültürü üzerine oksidatif hasarı her ne kadar karaciğer enzimleri, TAS ve TOS ile çok net olarak gösterilememiştir. Bu da çalışmada kullanılan dozların Van balığı karaciğer hücreleri için direkt öldürücü etkisinden kaynaklandığı düsünülmektedir. Fakat oksidatif hasar MDA, DNA hasarı gibi parametreler ile net bir şekilde ortaya konmuştur.

CURRICULUM VITAE

Aso Hemn OMAR was born on 9 January 1993, in sub-district of Pirmam, Erbil province of Iraq. I finished my primary, basic and high school in Pirmam. Then in the year of 2011, accepted to study Bachelor degree in Biology Department, Faculty of Science and Health at Koye University, and graduated in 2015. Began my Master of Science degree in General Biology in February, 2016 at Van Yüzüncü Yıl University in Van-Turkey.

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