THE PROTECTIVE ROLE OF PROTOCATECHUIC ACID (PCA) AND ALPHA TOCOPHEROL (VITAMIN E) COMBINATION ON ALUMINUM-INDUCED LIVER TOXICITY IN RATS

PhD Dissertation

Mojahed Mohamed Albadawi ALSAFI

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PhD Dissertation

Department of Biology

Programme in Molecular Biology

Supervisor: Assoc. Prof. Gözde AYDOĞAN KILIÇ

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FINAL APPROVAL FOR THESIS

This thesis titled THE PROTECTIVE ROLE OF PROTOCATECHUIC ACID (PCA) AND ALPHA TOCOPHEROL (VITAMIN E) COMBINATION ON ALUMINUM- INDUCED LIVER TOXICITY IN RATS has been prepared and submitted by Mojahed Mohamed Albadawi ALSAFI in partial fulfillment of the requirements in "Anadolu University Directive on Graduate Education and Examination" for the Degree of PhD in Biology Department has been examined and approved on 28/10/2022.

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SUPERVISOR APPROVAL

PhD student Mojahed Mohamed Albadawi ALSAFI, whom I supervise, has completed this thesis titled THE PROTECTIVE ROLE OF PROTOCATECHUIC ACID (PCA) AND ALPHA TOCOPHEROL (VITAMIN E) COMBINATION ON ALUMINUM- INDUCED LIVER TOXICITY IN RATS. According to my inspections, the work is scientifically and ethically appropriate for the student to the thesis defense exam.

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ABSTRACT

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Supervisor: Assoc. Prof. Gözde AYDOĞAN KILIÇ

Aluminum (Al) is the most extensively dispersed metal in the environment, which can be transmitted to animals and humans through food and water consumption. Prolonged exposure to Al damages the liver and is thought to be the primary cause of many liver disorders. Protocatechuic acid (PCA) and Vitamin E (Vit E) are polyphenol structured substances with well-known antioxidant, anti-inflammatory, metal chelating properties. The aim of this study is to prevent liver disease and oxidative stress caused by Al by combining PCA and vitamin E antioxidants. SOD, CAT, and GST enzymes were measured as oxidative stress markers. Total GSH levels and malondialdehyde levels, which is a marker of lipid peroxidation were determined. To detect DNA damage, a single cell gel electrophoresis (SCGE) assay was performed. Proliferating cell nuclear antigen (PCNA), a marker of hepatocyte proliferation, BRCA1 to evaluate DNA repair and 8-OHdG as a DNA oxidation marker, were measured using immunohistochemistry labeling. The findings of this study revealed that all applications of PCA and Vit E, either separately or in combination, were significantly effective in reducing Al-induced liver damages. Combined antioxidant therapy, which represents the core of the present study, has been shown to have a significant regulatory effect. The findings of the study will make an important contribution to the literature for the development of new therapeutic designs based on PCA and Vit E combination and pave the way for future studies that will highlight how the combined applications of these two molecules work at the molecular level.

Keywords: Aluminum, Protocatechuic acid, Vitamin E, Oxidative stress.

ÖZET

PROTOKATEŞUIK ASIT VE ALFA-TOKOFEROL KOMBINASYONUNUN ALÜMINYUM İLE İNDÜKLENEN KARACIĞER TOKSISITESINI DÜZENLEYICI ETKILERININ ARAŞTIRILMASI

Mojahed Mohamed Albadawi ALSAFI

Biyoloji Anabilim Dalı

Moleküler Biyoloji Bilim Dalı

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Danışman: Doç. Dr. Gözde AYDOĞAN KILIÇ

Alüminyum (Al), gıda ve su tüketimi yoluyla hayvanlara ve insanlara bulaşabilen, çevrede en yaygın olarak bulunan metaldir. Uzun süre Al'a maruz kalmak karaciğer toksisitesine neden olmakta ve çeşitli karaciğer hastalıklarının gelişimine zemin hazırlamaktadır. Protocatechuic asit (PCA) ve Vitamin E (Vit E), iyi bilinen antioksidan, anti-inflamatuar, metal şelatlama özelliklerine sahip polifenol yapılı bileşiklerdir. Bu çalışmada PCA ve Vit E kombinasyonuyla çoklu ve güçlü antioksidan etki ve DNA hasarının oluşumunu engelleyici etki oluşturularak, Al kaynaklı oksidatif stresin ve karaciğer patolojisinin baskılanması amaçlanmaktadır. Oksidatif stres belirteçleri olarak SOD, CAT ve GST enzimleri ölçümlenmiştir. Total GSH düzeyleri ile lipid peroksidasyonunun bir belirteci olan malondialdehit düzeyleri belirlenmiştir. DNA hasarını saptamak için tek hücreli jel elektroforezi (SCGE) testi yapılmıştır. Hepatosit proliferasyonunun bir belirteci olan çoğalan hücre nükleer antijeni (PCNA), DNA oksidatif hasar belirteci olarak 8- OHdG, ve DNA onarımının yorumlanabilmesi amacıyla da BRCA1 düzeyleri immünohistokimya etiketlemesi kullanılarak değerlendirilmiştir. Bu tez çalışmasının bulguları, PCA ve Vit E'nin ayrı ayrı veya kombinasyon halindeki tüm uygulamalarının Al kaynaklı karaciğer hasarını azaltmada önemli ölçüde etkili olduğunu ortaya koymuştur. Çalışmanın bulgularının PCA ve Vit E kombinasyonuna dayalı yeni tedavi tasarımlarının geliştirilmesi için literatüre önemli bir katkı sağlayacağı düşünülmektedir Ayrıca, bu iki molekülün birleşik uygulamalarının moleküler düzeyde nasıl çalıştığının mekanizmalarının araştırılmasına ışık tutacak olan gelecekteki çalışmaların önünü açacağı düşünülmektedir.

Anahtar Sözcükler: Alüminyum, Protokateşik asit, Vitamin E, Oksidatif stres.

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Mojahed Mohamed Albadawi ALSAFI

28/10/2022

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is an original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all the data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with "scientific plagiarism detection program" used by Eskişehir Technical University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

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GLOSSARY OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
8-OdHG	:	8- hydroxydeoxyguanosine
Al	:	Aluminum
ALP	:	Alkaline Phosphatase
ALT	:	Alanine Transaminase
AP-1	:	Activator Protein 1
ARE	:	Antioxidant Response Element
AST	:	Aspartate Transaminase
ATP	:	Adinosine Triphosphate
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
EDTA	:	Ethylenediamine tetraacetic acid
g	:	Gram
GPx	:	Glutathione Peroxidase
GR	:	Glutathione Reductase
GSH	:	Glutathione
GSSG	:	Glutathione disulfate
GST	:	Glutathione S Transferase
i.p	:	Intraperitoneal
i.v	:	Intravenous
IL	:	Interleukin
Kg	:	Kilogram
LDL	:	Low Density Lipoprotein
LPO	:	Lipid Peroxidation
LPS	:	Lipopolysaccharides
MDA	:	Malondialdehyde
MEPP	:	Mitochondrial Energy Production Pathway
Mg	:	Milligram

Min	:	Minute
mL	:	Milliliter
mM	:	Millimolar
NADPH	:	Nicotinamide Adenine Dinucleotide Phosphate
NF-KB	:	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NO	:	Nitic Oxide
Nrf2	:	Nuclear Factor E2- related Factor 2
OTM	:	Olive Tail Moment
OXPHOS	:	Oxidative Phosphorylation
PBS	:	Phosphate Buffer Saline
PCA	:	Protocatechuic acid
PCNA	:	Proliferating cell nuclear antigen
PUFA	:	Polyunsaturated fatty acid
ROS	:	Reactive oxygen species
rpm	:	Round per minite
SCGE	:	Single Cell Gell Electrophoresis
SOD	:	Superoxide Dismutase
TCA	:	Tricarboxylic acid
TNF- α	:	Tumor Necrosis Factor- Alpha
v.v	:	Volume: Volume
Vit E	:	Vitamin E
μM	:	Micromolar

1. INTRODUCTION

Aluminum (Al) is a metal that forms 8% of the earth's crust that is used in a variety of ways in our daily lives. Food, air, drinking water, cosmetics, deodorants, certain pharmaceuticals, and immunizations are all sources of aluminum in humans (Greger, J., 1992). Although aluminum is the most abundant element in nature, it has no known biological function (Becaria, A et al., 2002). Aluminum can be found in various forms in our environment. These include aluminum sulfate, aluminum hydroxide, aluminum fluoride, monomeric organic and inorganic forms. These Al species, which we encounter at many stages of life, can be accumulated and cause toxic effects on various organ systems (brain, liver, bone, kidney, and blood) in the human body. For many years, it was assumed that Al accumulates in the liver at very low doses that does not result in a significant toxic effect. However, studies indicated that administration of a single dose of 50 mg/kg/day i.v. caused Al accumulation in rat liver at levels that could produce a toxic effect (Bogdanovic, M et al., 2008). Due to the excess of lifetime exposure sources and the potential for chronic exposure above this dose, Al-induced oxidative stress is at the forefront causative agent of many diseases such as Alzheimer's, Parkinson's, kidney, and liver damage diseases (García-Sánchez, A et al., 2020; Inan, E and Ayaz, A. A., 2018).

The liver is an important organ in the human body that performs a variety of functions including metabolism, immunity, digestion, detoxification, and vitamin storage (Ozougwu, J. C., 2017). The liver is a novel organ that accounts for about 2% of an adult's total body weight and it receives 75% of its blood supply from the portal vein and 25% from the hepatic artery. Among the many important functions of the liver, it has been demonstrated that it is the organ where bile is produced, fat-soluble vitamins are stored, and drug metabolism occurs. One more significant capability of the liver is the detoxification of xenobiotics. The liver essentially utilizes xenobiotics by changing over them from a lipophilic to a hydrophilic structure by means of stage I and stage II responses that happen principally in the smooth endoplasmic reticulum of hepatocytes (Kalra, A et al., 2018).

When laboratory animals are exposed to toxic substances, their reactions are more pronounced in the liver than in other organs. This is due to the liver's high metabolic capacity and portal blood supply. Toxins affect the liver more than other organs because it is directly exposed to them as they enter the bloodstream via the intestine and portal vein. The liver is a key organ in the conversion of exogenous chemicals into toxic

metabolites. Toxins that require metabolic activation are less sensitive to other organs that lack such metabolic capacity. Despite the fact that many animal species are used in research, rats and mice are the most commonly used. The main purpose of researching the toxic responses that can occur in the livers of these experimental animals is to develop a good model for determining the effects of these substances on animals and humans. (Haschek, W. M., and Rousseaux, C. G., 1998).

One of the major challenges in interpreting studies attempting to demonstrate a benefit from antioxidant therapy for liver disease is that the precise mechanisms of action of certain compounds referred to as "antioxidants" are unknown (Singal, A. K et al., 2011). Many plant compounds exhibit antioxidant capacity in vitro and even in vivo, but these effects may not be sufficient to explain their biological activity (Kasote, D. M et al., 2015). There is strong evidence that several antioxidants primarily attribute their effects to changes in the cell's ROS and redox state. Vitamin E, for example, functions as an antioxidant by forming a complex with unpaired electrons, thereby stabilizing free radical compounds, and preventing lipid peroxidation (Napolitano, G et al., 2019).

Polyphenols are a class of physiologically active substances found in plant-based foods such as fruits, vegetables, cereals, and coffee that are consumed by humans. Polyphenols have been related to the protection of degenerative diseases and preventing oxidative changes in low-density lipoprotein, which are the major cause of atherosclerosis endothelial lesions (Abbas, M et al., 2017). Polyphenols are antioxidant chemicals with a wide range of bioactive properties, including the ability to scavenge free radicals and reduce radical generation to prevent lipid peroxidation (Reitzer, F et al., 2018). They've been attributed to have a protective effect on heart disease, cancer, inflammation, aging, microbiological and neurological problems, renal and lung diseases, UV damage, and osteoporosis. (Herbello-Hermelo, P et al., 2018).

Previous research has shown that protocatechuic acid (PCA), a polyphenol found in over 500 plant species mainly in green tea, olives, brown rice, and white grape wine, has antioxidant, anti-inflammatory, and anti-apoptotic properties (Kakkar, S and Bais. S., 2014; Liu, C. L et al., 2002). Other polyphenolic compounds, particularly those in the anthocyanin group, which are difficult to absorb by the stomach and intestine, are converted to PCA as a metabolic end product by bacteria in the intestine (Hu, R et al., 2020; Tian, L et al., 2019). In a culture of rat hepatocytes, several characteristics of PCA were investigated for their protective benefits against oxidative breakdown caused by

prooxidants. PCA has been reported to protect hepatocytes from cytotoxicity and genotoxicity by scavenging free radicals (Khan, A. K et al., 2015).

α-tocopherol (Vit E) is a fat-soluble antioxidant present in the phospholipid bilayer of cell membranes that helps to prevent free radical production and mitigate their detrimental effects (Khalaf, M. A et al. 2017). By scavenging oxidative free radicals, vitamin E protects polyunsaturated fatty acids (PUFAs), low-density lipoprotein (LDL), and other components of the cell membrane against oxidation (Pillai, A. and Gupta, S., 2005). The biological action of vitamin E extends beyond its antioxidant capabilities. Vitamin E is involved in the control of the inflammatory response, gene expression, membrane-associated enzymes, cellular signaling modulation, and cell proliferation (El Hadi, H et al., 2018). Vitamin E, with its strong antioxidant capacity, has been found to be useful in avoiding hepatocyte damage and lowering liver toxicity, particularly when paired with other antioxidant substances (Yao, Z et al., 2015; Kamal-Eldin, A. 2019).

The combination of PCA and Vit E is thought to substantially minimize liver inflammation, hepatocyte damage, DNA damage from Al exposure, and cell proliferation by successfully decreasing oxidative stress. Therefore, this study aims to determine the protective effect of the combined administration of protocatechuic acid (PCA) and alpha-tocopherol (vitamin E) on aluminum-induced liver toxicity in Sprague Dawley rats. Al was administrated to experimental animals for 42 days. During the first 28 days, the experimental groups containing Al were treated with Al, while the other groups were treated with physiological saline. After the 28th day, PCA and vit E were administrated to the experimental groups following the protocols. The experimental animals were dissected under anesthesia at the end of the administration period and the liver tissues were examined. Superoxide dismutase (SOD), catalase (CAT), and glutathione S transferase (GST) enzymes were measured as oxidative stress markers. Total glutathione (GSH) levels as well as malondialdehyde levels, which is a marker of lipid peroxidation (LP) were determined. To detect DNA damage, a single cell gel electrophoresis (SCGE) assay was performed. Proliferating cell nuclear antigen (PCNA), a marker of hepatocyte proliferation, BRCA1 for the evaluation of DNA damage repair and 8-oxo guanine as a DNA oxidation damage marker, were evaluated using immunohistochemistry labeling.

1.1. Heavy Metals And The Mechanism Of Toxicity

Heavy metals are chemical molecules with a high atomic mass and density that occur normally in nature. Their widespread presence in the environment originates from

their wide range of industrial, residential, agricultural, medical, and biotechnological applications (Tchounwou, P. B., 2012). The formation of reactive oxygen derivatives and oxidative stress reactions are two of the most important causes of heavy metal toxicity (Flora, S. J. S et al., 2008). In biological systems, metals can initiate reactions that result in polyunsaturated fatty acids oxidation, and interact with nuclear proteins and DNA, causing oxidative damage to biological intracellular molecules (Juan, C. A., et al., 2021). Endogenous antioxidants are the initial line of defense against cellular damage caused by reactive oxygen derivatives. This system's main components are superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT), and glutathione peroxidase (GSH-Px). They are considered central antioxidants in encountering free radicals, especially against the superoxide anion radical ($O_2^{\bullet}$), which is generated continuously in normal human metabolism via the mitochondrial energy production pathway (MEPP) (Ighodaro, O. M and Akinloye, O. A., 2018). In the body, SOD breaks down superoxide anions ($O_2\bullet$) into hydrogen peroxide (H_2O_2) and individual oxygen radicals. GSH-Px then converts the H_2O_2 into H2O and O_2 (Birben. E et al., 2012). GSH is utilized during the GSH-Px reaction, and glutathione reductase (GR) transforms it into the reduced form "active form." However, when the production rate of reactive oxygen intermediates exceeds the antioxidant systems' capacity to eradicate them, oxidative stress occurs (Galván-Arzate, S., et al., 2005).

1.2. General Characteristics And Sources Of Aluminum

Aluminum (Al) which constitutes around 8% of the earth's crust; is considered the third most prevalent element after oxygen and silicon, which living organisms get into contact with it in a variety of ways. Aluminum is a chemical element with an atomic number 13 and an atomic weight of 26.98 u. Along with gallium, boron, indium, and thallium, it is part of the 13th group of the periodic table (Krupińska, I et al., 2019). Elemental aluminum does not exist in its pure condition since it is extremely reactive and does not exist as a free metal in nature. It occurs in nature in stable combinations with other materials, including aluminum hydroxide, aluminum sulfate, aluminum fluoride, monomeric organic, and inorganic forms. Aluminum chloride is the most soluble in water and one of the most toxic forms. It creates toxic effects by reaching plants, animals, and humans through drinking water or the food chain (Shimizu, S., et al 2022; Sjögren, B., et al., 2015). Aluminum-containing compounds were assumed to be safe until recently, which is why they are used in so many food storage goods like cans, pots, and aluminum

foil. Processed foods, cooking and storage containers, various medical practices, and occupational exposure are important causes of Al accumulation in humans. Foods containing the most Al; are those that contain high levels of food additives such as processed cheeses, baking powders, and ready-made cake mixes (Dordevic. D et al., 2019). In addition, metal release from cooking utensils, foods stored for a long time in boxes containing Al, and drinking water are considered among other sources of oral exposure to aluminum (Kaizer. R et al., 2010). Al is added to drugs and water purification processes, that's why city waters contain high levels of Al ions (Goma, A. A. E. M., and Tohamy, H. 2016). Like many metals, soluble forms of Al show better absorption and distribution in target organs. In all of its chemical forms, Al has significant toxicokinetic and toxicodynamic effects (Yokel, R. A et al., 2006; Sjögren, B et al., 2015).

1.3. Effects Of Aluminum On Human Health

Aluminum has been linked to several numbers of diseases, including aluminum bone disease and encephalopathy. For decades, scientists have hypothesized a relationship between Al and neurodegenerative disorders like Alzheimer's disease (Kawahara, M., 2005). Recently, it has been identified as a cause of several diseases, including Alzheimer's disease, Parkinson's disease, and liver cholestasis (Becaria, A et al., 2003). Although Al is not a vital element, it is toxic to many vital organs such as the brain, bones, kidneys, liver, and blood (Kutlubay, R et al., 2007). The toxic effects of Al are a key player in triggering oxidative stress, genotoxicity, immunological modulations, inflammation, enzymatic dysfunction, apoptosis, necrosis, iron dyshomeostasis, and dysplasia (Igbokwe, I. O., et al., 2019). Al has several distinct chemical properties. Al only has one oxidation state: Al^{3+}. Negatively charged oxygen-donor ligands are preferred by Al^{3+}. As a result, Al^{3+} attaches to DNA or RNA phosphate groups, altering DNA architecture and gene transcription (Anitha, S., and Rao, K. S. J. 2002). Al also has been shown to exert its effects by disrupting membrane lipid fluidity and magnesium (Mg^{+2}) and calcium (Ca^{+2}) homeostasis and then triggering the production of reactive oxygen/ nitrogen species “free radicals" that cause oxidative stress (Mailloux, R. J et al., 2011). Mitochondrial metabolism is the most important site for the toxicological effects of Al. Fe-dependent and redox-sensitive enzymes of the citric acid cycle (TCA) and oxidative phosphorylation (OXPHOS) are significantly reduced by Al exposure (Mailloux, R. J et al., 2006). Furthermore, increased intracellular lipid aggregation due to enhanced lipogenesis and a reduction in -oxidation of fatty acids have been linked to Al toxicity

(Mailloux. R. J et al., 2011). Many studies have shown that OR has a role in inducing apoptosis, increasing lipid peroxidation, and modifying antioxidant enzyme function (SOD and CAT) (Razo-Estrada, A. C et al., 2013; Abubakar, M. G et al., 2003). A study of aluminum toxicity demonstrated that increased MDA levels and decreased activities of superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) were detected in liver samples of rats treated with aluminum chloride ($AlCl_3$). In addition, the hepatotoxic effect of Al was evidenced by higher ALT, AST, ALP, and LDH activities in plasma of $AlCl_3$-administrated rats compared with the low total protein control (Cheraghi, E., and Roshanaei, K., 2019).

1.4. Free Radicals And Oxidative Stress

A free radical is a molecule or atom that has one or more single unpaired electrons. Unpaired electrons are frequently very reactive. As a result, free radicals can cause organ and tissue damage by disrupting the structure of proteins, nucleic acids, and lipids (Di Meo, S. and Venditti, P., 2020). Small amounts of reactive oxygen species (ROS) like hydroxyl radical (OH•), superoxide ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) are generated during oxygen metabolism under normal conditions. The mitochondrial electron transport chain and oxidase enzymes such as NAD(P)H oxidases, xanthine oxidases, lipoxygenases, cyclooxygenases, cytochrome P450 enzymes, and uncoupled nitric oxide synthases are the principal sources of reactive oxygen and nitrogen species (V Goncharov, N., et al., 2015). ROS is involved in several biological processes, including the regulation of transcription factors, signaling pathways, and direct interactions with a range of intracellular molecules (Vaziri, N. D., and Rodríguez-Iturbe, B. 2006). Furthermore, reactive oxygen species (ROS) produced by active leukocytes and macrophages are potent antibacterial agents (Papayannopoulos, V and Zychlinsky, A., 2009). Under normal conditions, the antioxidant defense system, which consists of several endogenous enzymes and dietary antioxidant compounds, safely neutralizes ROS formed during metabolism. However, in many pathological circumstances, the ROS formation rate exceeds the antioxidant defense system's ability to neutralize it, resulting in oxidative stress (Rao, P. S et al., 2011). Under these conditions, ROS attacks biomolecules and alters redox-sensitive signal transduction pathways and transcription factors, causing several damages and dysfunctions (Trachootham, D., et al 2008). It is increasingly recognized that elevated levels of ROS may be a key factor in the pathogenesis of several chronic diseases including cardiovascular disease,

atherosclerosis, cancer, diabetes, Parkinson's, Alzheimer's disease, and liver-related dysfunctions (Shankar, K, and Mehendale. H. M., 2014).

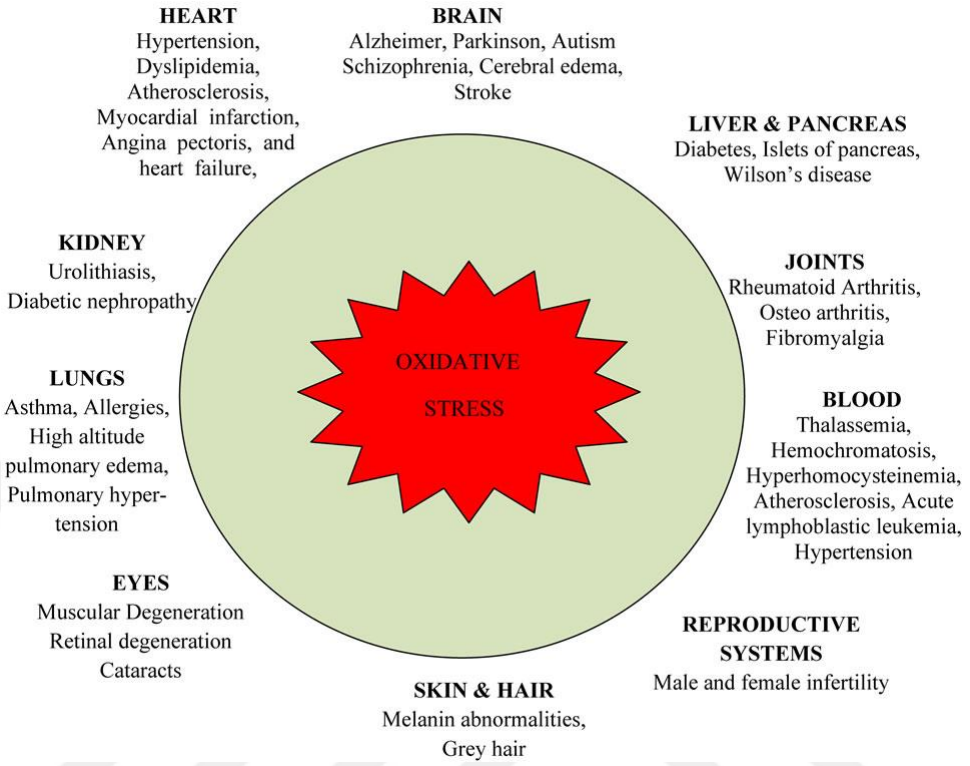

Figure 1.1. *Oxidative stress-induced diseases in humans' organs (Rahman, T et al., 2012).*

1.5. Proliferating Cell Nuclear Antigen (PCNA)

Is a nuclear protein of 36 kDa related to the cell cycle (Hall. P.A et al., 1990). This protein plays a crucial role in DNA replication by working primarily with polymerases to replicate the entire genome with high fidelity. DNA polymerase, which is required for DNA synthesis during replication and DNA repair, depends on PCNA as a processivity factor (Strzalka, W., & Ziemienowicz, A., 2011). PCNA functions extend beyond DNA replication to also regulate cell cycle, chromatin remodeling, DNA repair, and other critical cellular processes (Mjelle, R et al., 2015). Studies have revealed that PCNA is essential for the advancement of the cell cycle as well as DNA synthesis and repair. Proliferating cell nuclear antigen (PCNA), a part of the replication and repair process, is essential for nucleic acid metabolism. Throughout the cell cycle, PCNA levels change. Its level rises progressively and significantly in the S phase prior to DNA synthesis. In the G2 and M stages, its concentration declines (Cardano, M., et al., 2020). This characteristic makes PCNA useful as a proliferation marker (Kurki, P. et al., 1986). PCNA labeled immunohistochemically demonstrates continuous DNA replication in

addition to DNA damage that leads to carcinogenesis. The presence of PCNA is a marker for uncontrolled cell growth (Dietrich, D. R., 1993). In recent years, it has become clear that PCNA interacts with proteins necessary for cell cycle progression but unrelated to the DNA polymerase machinery.

1.6. BRCA1

The BRCA1 gene is a tumor suppressor and growth-restricting gene that is found in the 17q21 region of the long arm of the 17th chromosome (Chisholm, K. M. et al., 2008). The BRCA1 breast cancer susceptibility gene has been related to a wide range of biological processes, and multiple DNA repair mechanisms, including nucleotide excision repair (NER). The protein produced by this gene is widely distributed and essential for controlling cell development and differentiation (Katagiri. T et al., 1996). This gene essentially cooperate at various phases of the DNA damage response (DDR) and DNA repair, particularly in double-strand breaks, to protect the integrity of the genome (Roy. R et al., 2012). BRCA1 is a pleiotropic DNA damage response protein that functions in checkpoint activation, apoptosis, and DNA repair (Majidinia, M et al., 2017). When DNA double-strand breaks (DSB) resulting from endogenous or external factors are detected by the cell's damage-sensing machinery, the cell cycle is postponed while the repair mechanisms attempt to fix the damage (Tutt, A., & Ashworth, A., 2002). DNA double strands linked with replication make up the majority of BRCA1. It is well known that they work in harmony during homologous recombination (HR), a crucial DNA repair procedure that uses the sister chromatid that has not been damaged to carry out high-quality repair of breaks (Trenner, A., & Sartori, A. A., 2019). BRCA1 serves as a bridge between effectors that carry out the proper DSB repair during HR and sensors that detect DNA damage at cell cycle checkpoints (Deng, C. X., 2006).

1.7. Antioxidant Defense Systems/ Mechanisms

Organisms that need oxygen for energy production (Aerobic organisms) have developed antioxidant defense systems that comprise enzymatic and non-enzymatic antioxidants that effectively limit the detrimental effects of ROS (Birben, E. et al., 2012). Maintenance of sufficient antioxidant levels and localization of antioxidant enzymes are all part of antioxidant capacity regulation (Sies, H., 1993). Major antioxidant enzymes against superoxide radicals include SOD, CAT, and GSH-Px (Reddy, R. D. and Yao, J. K., 1996). These enzymes collaboratively act to prevent superoxide and hydroxyl radicals from oxidizing lipids SOD converts superoxide to hydrogen peroxide, which is then

destroyed by CAT into water and oxygen, avoiding the formation of hydroxyl radicals. In addition to CAT, GSH-Px is a well-known intracellular antioxidant enzyme that oxidizes reduced GSH to glutathione disulfide, converting peroxides and hydroxyl radicals into non-toxic forms (GSSG). The enzyme glutathione reductase (GR) converts GSSG to GSH (Wu, J. Q., 2013).

1.7.1. Enzymatic Endogenous Defense Systems

1.7.1.1. Superoxide dismutase (SOD)

Superoxide dismutase is a metal protein that was discovered in 1968. Superoxide dismutases (SODs) are common enzymes that catalyze the disproportionation of superoxide to molecular oxygen and peroxide, guarding the cell against harmful consequences of aerobic respiration (Perry, J. J et al., 2010). Based on the metal co-factor they carry, SODs can be divided into four groups (FeSOD), (MnSOD), (Cu/ZnSOD), and (NiSOD) (Sen, S., and Chakraborty, R. 2011). FeSOD and NiSOD are both bacterial enzymes, with FeSOD being found in chloroplasts. CuZnSODs are found in eukaryotes' extracellular matrix and cytoplasm. MnSOD is found in all bacteria and eukaryotes. MnSOD is located in the mitochondrial matrix of eukaryotes, an organelle compartment that produces a high rate of superoxide radical (Azadmanesh, J and Borgstahl, G. E., 2018). The most prevalent type of superoxide dismutase is (Cu/Zn-SOD) which is located in the cytoplasm and has a molecular weight of 32 kDa. Although Cu/Zn-SOD activity may be found in a variety of tissues, it is most commonly found in the liver and kidney, which play an active metabolic role (Okado-Matsumoto, A., and Fridovich, I. 2001).

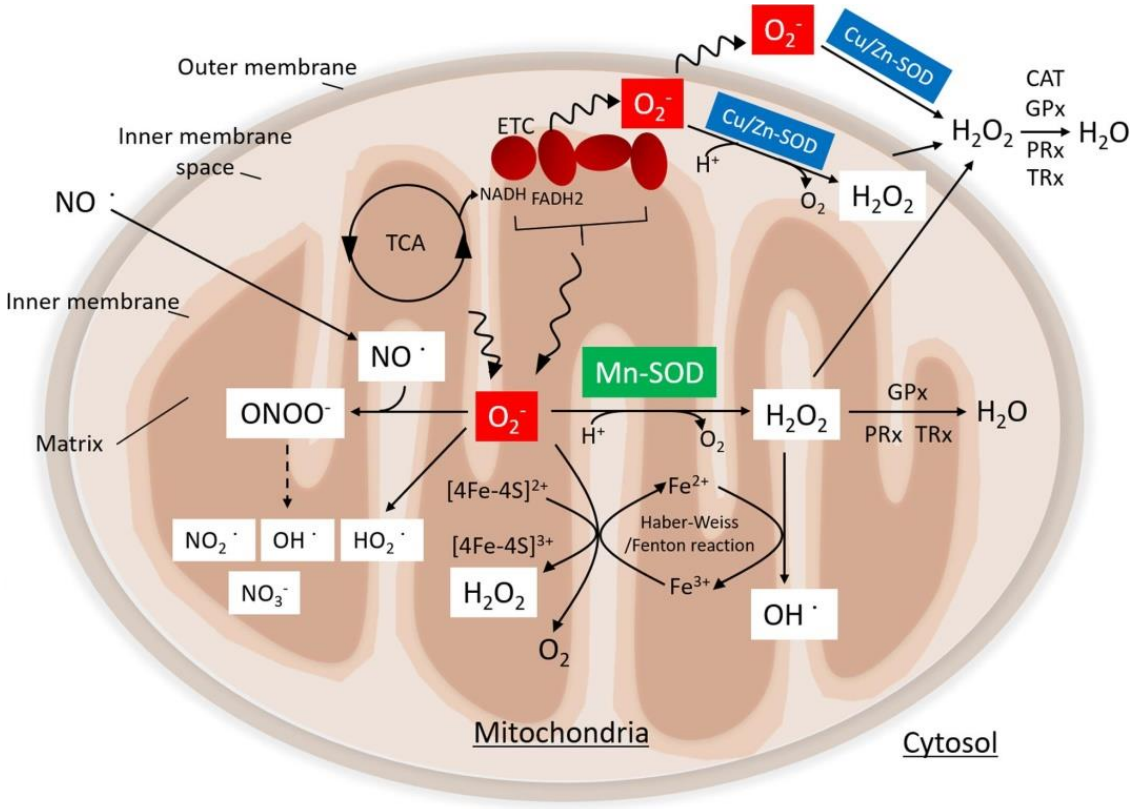

Figure 1.2. *SOD enzyme mechanism of action (Kitada, M. et al., 2020).*

1.7.1.2. Catalase (CAT)

Catalase is a hemoprotein with four subunits, each of which has a heme group and an NADPH molecule (Majumder, D. et al., 2017). Because of its fascinating function in neutralizing lipid metabolic byproducts, it is frequently located in intracellular organelles like peroxisomes. Because liver cells have more peroxisomes than other organs, hydrogen peroxide produced during lipid metabolism is converted by catalase to H_2O and O_2. Hydrogen peroxide is not a reactive radical and does not react with biological substances. However, it is very important because it causes the formation of hydroxyl radical (OH), which is the most reactive form of oxygen species, by using it as the precursor of the Fenton reaction under the catalysis of Fe and Cu ions (Gutteridge, J. M. 1994).

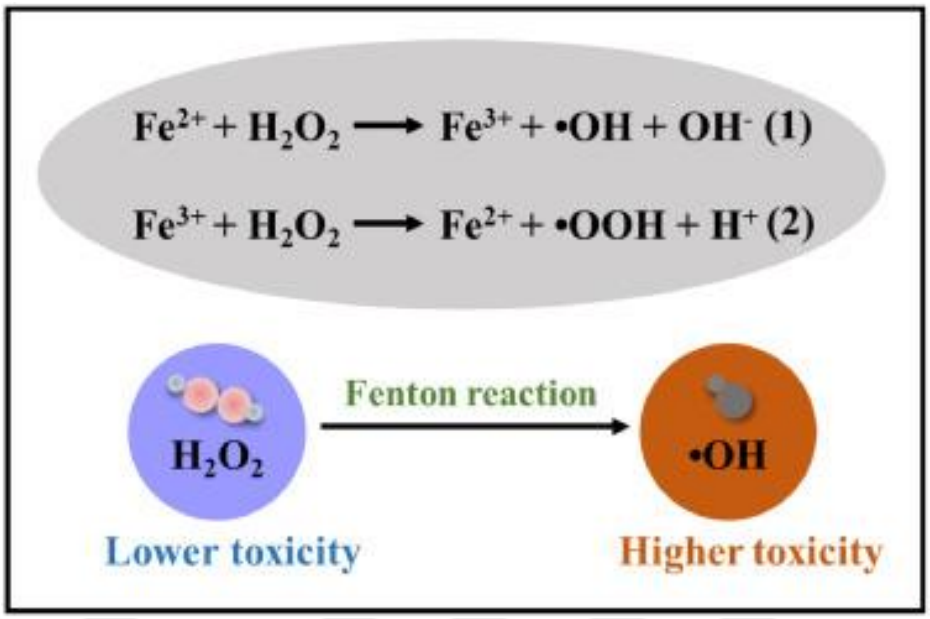

Figure 1.3. *Fenton reaction (Meng, X., et al., 2020).*

1.7.1.3. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) was initially discovered in 1957 as an erythrocytic enzyme that protects hemoglobin from oxidative destruction. GPx is a crucial endogenous enzyme that provides the conversion of H2O2 into water (Lubos, E., et al., 2011). The enzyme is efficient in terminating the lipid peroxidation reactions preventing cells from OS (Ighodaro, O. M., and Akinloye, O. A., 2018). There are four main selenium-dependent GPx identified as isozymes in mammalian tissues: GPx1, GPx2, GPx3, and GPx4 which are mainly located in the liver, gastrointestinal, plasma, and kidneys respectively (Margis, R et al., 2008). The insulin signaling pathway is regulated by GPx1. GPx2 plays a double role in the cancer formation process, relying on the mechanism of initiation and stage of cancer; GPx3 is membrane-associated, which might explain its peroxidative activity despite low plasma GSH levels; and GPx4 controls apoptosis (Brigelius-Flohé, R., and Maiorino, M., 2013). Glutathione peroxidases catalyze the reduction of hydroperoxides (ROOH) by glutathione peroxidases (GSH). The final products are water, alcohol (ROH), and glutathione disulfide (GSSG). The enzyme glutathione reductase is responsible for the cell's GSH regeneration from GSSG (Mannervik, B., 1985).

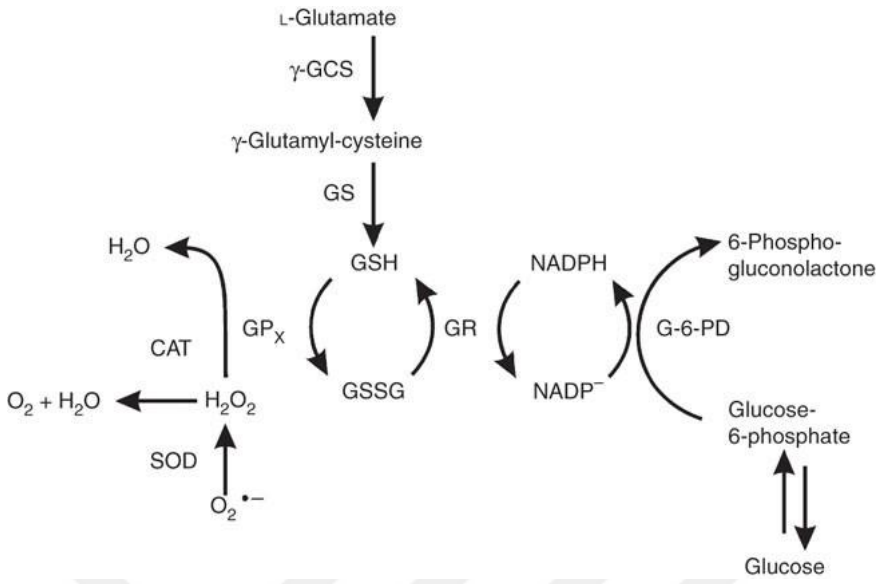

Figure 1.4. *Schematic view of antioxidant enzyme mechanism of action (Weydert, C. J., and Cullen, J. J.2010).*

1.7.1.4. Glutathione S transferase (GST)

Soluble enzymes and microsomal enzymes are two supergene groups that encode glutathione S-transferase (GST)-active proteins. These two GST families are known to be important in cell defense against oxidative stress and xenobiotics. They b a variety of cellular compounds, including oxidized lipids and DNA products resulting from intracellular damage caused by reactive oxygen species (Hayes, J. D., and Strange, R. C. 2000).

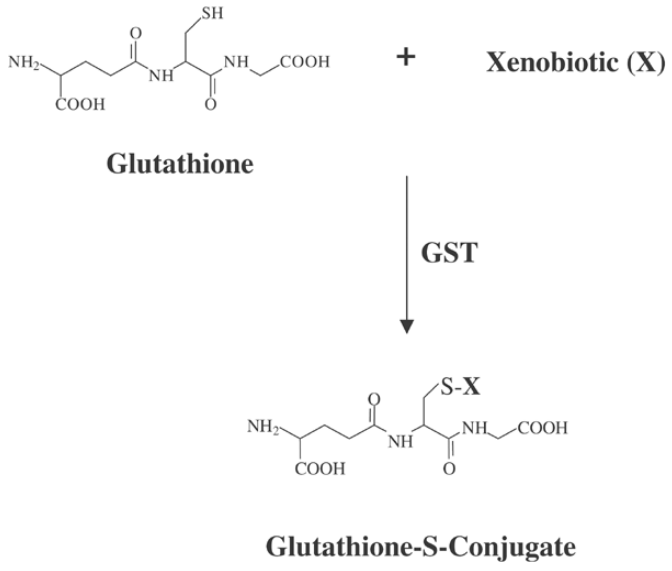

Figure 1.5. *Glutathione S transferase mechanism of action (Townsend, D. M., and Tew, K. D. 2003).*

The many GST enzymes have long been thought to be part of a cell's defense against a variety of toxic substances produced both internally and externally from the environment. The addition of GSH to electrophiles with a wide variety of chemical configurations is the typical reaction of GST enzymes. Although the physiological role of each GST protein is unknown, a large body of in vitro evidence implies that these proteins function as dimeric enzymes (Strange, R. C. et al., 2001).

1.7.2. Non-Enzymatic Defense Systems

1.7.2.1. Glutathione (GSH)

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-cysteine-glycine) that functions as an endogenous antioxidant in mammals. In human cells, GSH is the most abundant low molecular weight antioxidant redox recycling thiol, and it plays a critical role in cellular defense against oxidative damage. It serves as a radical scavenger as well as a key component of the catalytic activity of many antioxidant enzymes (Zhu, R. et al., 2012). GSH is a powerful antioxidant that maintains redox balance, cell survival, growth, and death, and participates in several metabolic activities (Marengo, B., et al., 2016). GSH also plays a role in cell signaling and protein interactions, all of which help the body fight oxidants. GSH is also thought to have a function in intracellular copper transport and detoxification. GSH reduces the potentially harmful effects of potentially hazardous redox interactions between metals and oxygen during the detoxification process (Fenton reaction). Finally, GSH might be used to store cysteine, which can be hazardous in excessive concentrations (Filomeni, G., et al., 2002). Glutathione forms conjugates with xenobiotics, damaging superoxides, or antineoplastic medicines through processes mediated by glutathione-S-transferases (GSTs). GST is required for phase II detoxification, which involves the separation and elimination of phase I metabolic products (Nakamura, Y et al., 2003). Glutathione also detoxifies peroxides produced by oxygen radicals by lowering oxidized centers on DNA, proteins, and other macromolecules via trans-hydrogenase (Srivastava, S. et al., 2017).

1.7.2.2. Vitamin E (α - tocopherol)

α tocopherol (Vit E) is a fat-soluble antioxidant located in the phospholipid bilayer of cell membranes to prevent the production of free radicals and limit their effects (Khalaf, M. A et al. 2017). Although vitamin E may be found in nearly all cell membranes, the inner mitochondrial membrane, which is the main compartment of the electron transport system, is the principal repository of membrane-bound vitamin E

(Evans W. J., 2000). Vitamin E insufficiency can lead to neurological problems, myopathies, and decreased erythrocyte lifetime. Vitamin E is the most important lipid-soluble antioxidant, preventing lipid peroxidation in cellular membranes and thereby protecting cell integrity (Cervantes, B., and Ulatowski L. M., 2017). Vitamin E is a chain-breaking antioxidant that inhibits the proliferation of free radical reactions. Although vitamin E has been recognized as a vital supplement for reproduction since 1922, the physiological processes underlying its functions remain unclear (Brigelius-Flohé, R., and Traber, M. G. 1999). Tocopherols and tocotrienols are all forms of Vitamin E, with α -tocopherol being the most prevalent and physiologically active form. The significance of α -tocopherol in the prevention of chronic diseases has been widely explored due to the high antioxidant capacities of tocopherols. The biological action of vitamin E extends beyond its antioxidant capabilities. Vitamin E is found to be involved in the control of inflammatory responses, gene expression, membrane-associated enzymes, cellular signaling, and cell proliferation (El Hadi, H et al., 2018). Vitamin E administration is also expected to decrease hepatic steatosis, inflammation, hepatic stellate cell activation, and collagen mRNA expression, as well as mitigate fibrosis (Phung, N et al., 2009). Vitamin E may be a prooxidant as well as an antioxidant, and it may have non-antioxidant roles as a signaling molecule, a gene expression regulator, and perhaps in the prevention of cancer and atherosclerosis. Individual isomers have various affinities to these new unusual activities since vitamin E comprises eight structurally similar tocopherols and tocotrienols (Schneider, C., 2005).

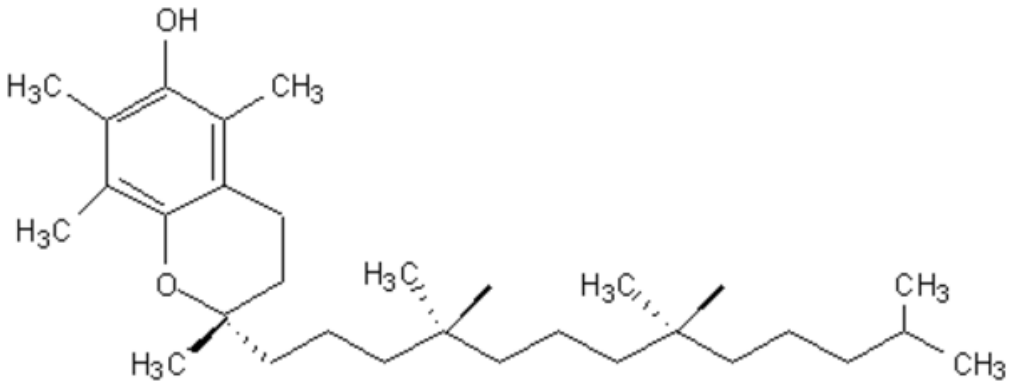

Figure 1.6. *Chemical structure of alpha-tocopherol (Vitamin E) (Shanmugam, A. K. et al., 2010).*

1.7.3. Other Exogenous Antioxidants

Polyphenols are natural antioxidant agents found in fruits, vegetables, essential oils, cereals, tea, and beverages (Zhang, H and Tsao, R., 2016). Plants have been shown to have around 8000 isolated and identified phenolic chemical compounds (Singh, B et al., 2017). Polyphenol consumption has been linked to a lower risk of heart disease, cancer, atherosclerosis, diabetes, osteoporosis, and neurological diseases (Scalbert, A., et al., 2005). Although it is difficult to anticipate the effects of polyphenol consumption on disease prevention in humans, the most commonly recognized mechanism is that polyphenols serve as antioxidants by scavenging or restricting the generation of free radicals (Chiva-Blanch, G., and Visioli, F., 2012). Polyphenols are powerful antioxidants that neutralize reactive species by donating an electron or hydrogen atom. Polyphenols limit the level of oxidation by preventing or deactivating the formation of free radical precursors. In lipid peroxidation chain reactions, they generally operate as direct radical scavengers (chain breakers), providing free radicals an electron, neutralizing and converting them to stable "less reactive" radicals, therefore terminating the chain processes (Tsao, R., 2010).

1.7.3.1. Protocatechuic acid (PCA)

Protocatechuic Acid (PCA) is a natural phenolic acid that is found in many plants and is used in several traditional Chinese herbal treatments (Abdelmageed, M. E et al., 2021; Li, X et al., 2011). PCA has been demonstrated to inhibit H_2O_2-induced apoptosis and possesses a wide range of biological actions, including antibacterial, anti-inflammatory, antihyperglycemic, antiapoptotic, and antiproliferative properties (Rashmi, H. B., and Negi, P. S., 2020; Guan, S et al., 2006). An increasing amount of research suggests that PCA can operate on a range of molecular sites to produce a variety of biological consequences. PCA has been illustrated to have anti-inflammatory characteristics in addition to its neuroprotective properties. PCA also appears to have chemopreventive properties, since it suppresses chemical carcinogenesis in vitro and has pro-apoptotic and anti-proliferative effects in many tissues (Masella, R et al., 2012). It has been determined that high levels of superoxide ions and hydrogen peroxide are reduced with PCA application, and this decrease is simultaneous with the decrease in catalase (CAT) and superoxide dismutase (SOD) activities (Li, X et al., 2011). It has been demonstrated that PCA protects rats against menadione-induced oxidative stress by

boosting antioxidant capacity and phase II enzymes by upregulating the expression of Nrf-2 (Ibitoye, O. B., and Ajiboye, T. O., 2020).

Because of the number of phenolic hydroxyl groups in the PCA molecule (Figure 1.6), the molecule exhibits significant antioxidant and anti-radical properties (Sroka, Z., and Cisowski, W. 2003). PCA esters, on the other hand, show stronger antioxidant activity than PCA, probably due to their increased lipid solubility, which is a key element in lipid peroxidation stabilization (Siquet, C et al., 2006). When exposed to a xenobiotic, antioxidant enzyme activity is found to reduce significantly. PCA can provide the capacity to restore the equilibrium of the endogenous antioxidant system by enhancing GPx, SOD, and CAT activities, and lowering or terminating the lipid peroxidation cascade (An, L. J et al., 2006). PCA has been discovered to reduce TNFα-induced cell death in human umbilical vein endothelial cells, indicating that it possesses an anti-apoptotic effect. PCA also boosted cell proliferation while significantly reducing mitochondrial dysfunction by decreasing mitochondrial membrane potential loss, ROS production, GSH depletion, caspase-3 activation, and Bcl-2 down-regulation (Zhou-Stache, J et al., 2002).

In vitro and in vivo studies have revealed that many phytochemicals, such as phenolic compounds, have anti-inflammatory properties. Many studies show that polyphenols may act as signal transduction pathway modifiers to provide favorable effects (Santangelo, C et al., 2007). The idea of using these food components to ameliorate the progression of diseases associated with chronic inflammation may be a useful strategy; PCA seems to be a suitable candidate, as recent studies have shown its anti-inflammatory properties (Hsu, C et al., 2009). PCA is thought to suppress the inflammatory cytokine IL-6 and chemokine IL-8 in LPS-stimulated human gingival fibroblasts (HGFs), resulting in anti-inflammatory actions. Also, PCA has been found to suppress the LPS-induced inflammatory response through modulating NF-B activation (Wang, Y et al., 2015).

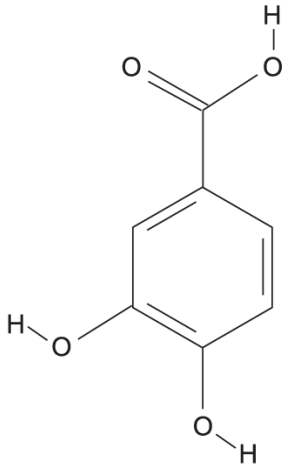

Figure 1.7. *Chemical structure of protocatechuic acid (Khan, A. K., et al., 2015).*

1.8. Antioxidant Therapy

ROS can produce several potentially hazardous oxidative reactions. ROS generation causes lipid peroxidation, inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative damages to critical proteins. All of these responses are thought to have a role in the pathophysiology of inflammation, shock, and ischemia/reperfusion. (Cuzzocrea, S., et al., 2001).

The association between free radicals and a variety of diseases has been well documented. The generation of oxidants from endogenous sources is after the initial disease process in most human diseases, and oxidative damage exacerbates the underlying lesion. Increased oxidative stress is linked to three key problems that occur during this kind of tissue injury: increased endogenous superoxide radical formation, delocalization of heme proteins or metalloproteins, and antiradical defense deficiencies (Henke, S. L. 1999). Excessive quantities of superoxide radicals are produced in chronic inflammatory diseases. When a significant number of phagocytes are activated in a small region, tissue injury can occur (Halliwell, B. 1992).

Medical experts are skeptical of the idea that antioxidants might be used as a medicine to cure diseases, and They seek for controlled long-term, randomized experimental trials with a sample size of thousands of patients to prove that antioxidant therapy is effective for a specific medical condition. Nonetheless, the fact is that the number of controlled scientific research confirming the importance of antioxidant treatment in diseases is rising (Núñez-Sellés, A. J. 2005). It has been shown that taking

1,000 mg of vitamin C and 400 IU of vitamin E on a daily basis can effectively reduce the risk of pre-eclampsia in women, and that is due to improvements in placental biochemical markers and oxidative stress, demonstrating the rationale for prophylactic antioxidant use (Traber, M. G., and Stevens, J. F. 2011).

1.9. Liver Structure

The liver is the organ with the largest volume in the body, where many different functions are performed, and the connection between these functions is provided. With a weight of about 1500 grams, it constitutes about 2% of the body weight. Hepatocytes (parenchymal cells), known as liver cells, are cells arranged in rows of 25-40 μm in diameter. Hepatocytes make up about 67% of the cells in the liver. Apart from this, there are different cell types in liver tissue including Ito cells, bile duct epithelial cells, fibroblasts, and Kupffer cells. The sinusoidal surface of the hepatocyte contains microvilli extending towards the disse space, which are in direct contact with the blood to fulfill their high absorption and secretion function. Perisinusoidal cells are located in the disse space, endothelial cells, Kupffer cells, and liver-associated lymphocytes are located in the sinusoidal space (Zaefarian, F et al., 2019). Endothelial cells are longitudinally structured, flat cells with large pores between them. While keeping hepatocytes away from large molecules, it provides nutrition with small-molecule nutrients. Ito cells, on the other hand, are located under the endothelial cell layer, in the disseal space. They are adjacent to endothelial cells or embedded in the microvilli of hepatocytes. They are fat-storing cells that provide the connection between hepatocytes (McCuskey, R. C., and Eddie Wisse, E. 2010). The main function of Kupffer cells is to perform phagocytosis. Macrophages are responsible for the clearance of immune complexes, endotoxins, and lesioned erythrocytes. Perisinusoidal cells, on the other hand, are cells rich in vitamin A, located in the disse range, and a source of hepatocyte growth (Naito, M et al., 2004).

The functional unit of the liver in cylindrical form with a diameter of about 2 mm is called a "lobule". There are approximately 100,000 lobules in the human liver. The lobules are in the form of polyhedral prisms in the liver tissue. At each corner of the hexagonal structure, there is a three-element canal system known as the "portal triad", consisting of the hepatic artery, portal vein, and bile duct. The central vein is located at the center of the lobule (Lin, Q et al., 2019).

The liver has blood flow through two different veins. One of them is the hepatic artery, which carries oxygenated blood leaving the aorta (20%). The other is the portal

vein, which carries nutrient-dense blood collected from the spleen and capillaries of the digestive system (intestine). While 1450 milliliters of blood are transported to the liver in one minute from the two arteries mentioned, hormones from other organs such as the pancreas also come to the liver through this vein. The hepatic vein carries blood from the liver to the heart (Eipel, C et al., 2010).

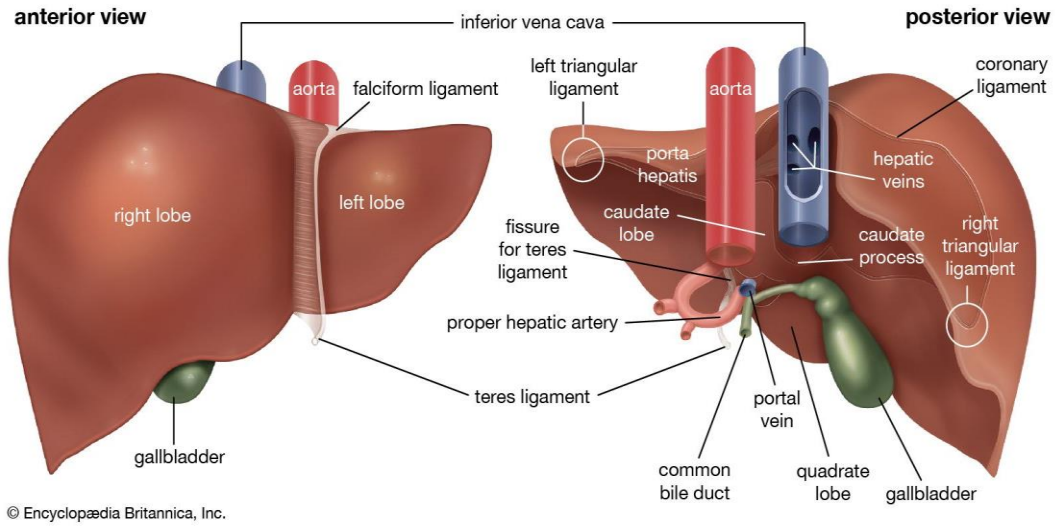

Figure 1.8. *Liver structure (Britannica, T. Editors of Encyclopaedia., 2020).*

1.10. Liver Pathology

The names and meanings of liver necrosis are unique. "Focal necrosis" refers to the death of a group of hepatocytes. Lymphocytes and macrophages can also be seen in these regions. Because necrotic hepatocytes are quickly eliminated, they are rarely visible in the necrosis region (Ferrell, L. 2000). Necrosis that affects large groups of hepatocytes is known as "bridging necrosis." "Massive necrosis" is the more severe form of bridging necrosis; Submassive when it almost completely involves several acini, the term massive is used when there is almost no intact parenchyma. Centrilobular necrosis is a kind of zonal necrosis that affects a particular area of the acini (Krishna, M. 2017). Degeneration is another reaction that happens in the liver in response to many detrimental events. Ballooning degeneration, or cellular swelling, is an indication of reversible cell injury. This appearance is frequent in hepatocytes next to other types of necrosis zones and can be caused by a variety of harmful events. Hepatocytes exposed to bile suffer from "hairy degeneration," which is characterized by smaller cytoplasmic vacuoles (Yip, W. W., and Burt, A. D. 2006). Acidophilic bodies are cytoplasmic globules in the liver that are

smaller than normal hepatocytes and usually are cells undergoing apoptosis. The production of collagen in the tissue is defined by "fibrosis," which occurs as a natural consequence of lesion healing. Therefore, the connection between hepatocytes and blood may be considerably diminished since it happens in the Disse area. Hepatocytes are cells with a strong capacity for regeneration that is defined as a natural reaction to overcome the massive loss or damage of hepatocytes in the liver tissue (Lee, U. E., and Friedman, S. L., 2011).

1.11. The Relationship Between Oxidative Stress And Liver Diseases

The redox state is a key player in many liver diseases, including metabolic, inflammatory, and proliferative disorders. The involvement of oxidative stress in the genesis of a variety of degenerative disorders is well understood (Arauz, J. et al., 2016). Severe exposure to high amounts of ROS, such as in hepatic ischemia, can cause substantial damage to the human body. In addition to their detrimental effect, ROS are potential secondary messengers that are formed in response to hormones, growth factors, cytokines, and ATP. As a result, the role of oxidants in cells is complicated and dependent on the oxidant and antioxidant enzymes homeostasis (Cichoż-Lach, H., and Michalak, A. 2014).

Mitochondria are the primary generator of ROS in mammalian cells. During the oxidative phosphorylation process, oxygen metabolites are generated. Synthesized oxygen free radicals are engaged in signal transmission, gene expression, and pathogen defense agents, and they play a beneficial role in cell physiology. Interfering with electron transport, on the other hand, can result in the elevation of superoxide generation which is harmful to cells (Zorov, D. B et al., 2014).

Increased OS is nearly always linked to chronic liver disease. Several investigations have discovered that oxidative stress alters protein expression patterns in mammalian cells. This modulation is caused by the activation of redox-sensitive transcription factors such as NF-kappaB, AP-1, and G proteins (Cichoż-Lach, H., and Michalak, A. 2014). In addition, cellular kinases, which are remarkable members of the mitogen-activated protein kinase family, have been found to play a crucial role in altering protein expressions (Zima, T., and Kalousová, M., 2005). The relevance of oxidative-dependent cellular signaling pathways is shown by changes in protein expression. This pathogenic chain reaction causes hepatocyte death by exposing the liver to high oxidative stress. The mechanism behind this process is yet unknown, and research is continuing.

The liver is particularly vulnerable to oxidative stress due to its metabolic activities. To counteract ROS, the liver has a unique defensive mechanism in which nuclear factor E2-related factor 2 (Nrf2) plays a key role. When ROS and electrophile levels are high, Nrf2 is released from its sequestration and translocated to the nucleus, where it stimulates the transcription of cellular defense genes (Galicia-Moreno, M et al., 2020). Oxidative stress activates the antioxidant response element (ARE), which activates a set of antioxidant genes as a protective mechanism (Cicho-Lach, H., and Michalak, A. 2014). By controlling ARE-containing gene expression, Nrf2 regulates the enzymes involved in GSH homeostasis, NAD(P)H quinone oxidoreductase 1, and UDP-glucosyltransferase. Cells become more responsive to OS when ARE-containing genes are expressed improperly (Ma Q., 2013). Several studies have shown the role of Nrf2 in the progression of liver disorders. By increasing gene expression, the Nrf2-ARE loop appears to protect against liver disease including viral hepatitis, fibrosis, and cancer in animal models. Furthermore, this loop aids liver regeneration. Nrf2 deletion induces liver damage in response to pollutants and a high-fat diet, resulting in increased mitochondrial ROS production. The discovery of Nrf2 has enhanced our understanding of oxidative stress and might lead to the development of innovative therapies for redox-related liver disorders (Mann, G. E., et al., 2007; Cicho-Lach, H., and Michalak, A. 2014).

ROS are unquestionably essential in the production and progression of a variety of chronic liver disorders. Oxidative stress, which plays a role in the liver fibrogenic response, is at the root of viral and alcoholic liver disorders. Hepatocytes, stellate cells, endothelial cells, and Kupffer cells all play a role in the injury's pathophysiology, contributing to ischemia/regeneration, necrosis, and apoptosis. All these modifications alter gene expression leading to drastic liver damage (Ramos-Tovar, E. and Muriel, P., 2020).

2. MATERIALS AND METHODS

2.1. Experimental Animals and Experimental Groups

In this work, all studies were carried out on laboratory animals at Anadolu University. The guidelines of the Ethics Committee for Experimental Animals were followed (Ethics Committee Decision No: 2018-52). Forty-eight male Sprague Dawley rats weighing between 200 and 225 g were used for the experiments. The duration of the experiment was set at 6 weeks (42 days). At the end of the 42nd day, the animals were dissected. The rats were dissected after anesthesia with 80 mg/kg ketamine, and part of the liver tissue was processed for histological tissue examination and single-cell gel electrophoresis. The remaining part was frozen in liquid nitrogen and stored at -860C for biochemical examinations (Green, C. J et al. 1981).

Before starting the experiments, the animals were acclimatized to laboratory conditions for 5 days, with 6 animals in each cage, at a temperature of 24°C, 55% humidity, and 12 hours of light-darkness (8:00 am and 8:00 pm), by offering normal food and water. $AlCl_3$ was administrated intraperitoneally (i.p.) at a dose of 70 mg/kg/day for 28 days (Ali A. A et al., 2016). PCA and Vit E (water-soluble D-alpha-tocopherol form) were administrated from day 28 for 14 days. PCA 5 mg/kg/day i.p.; Vit E administrated i.p. at a dose of 50 mg/kg/day (Thenmozhi, A. J. et al., 2015; Karageorgos, N. et al., 2006; Shi, G. F. et al., 2006).

The experimental and control groups were planned as indicated below (n=6). According to the literature, administrating 70 mg/kg/day of Al over 28 days promotes neuronal degeneration (Ali, A. A et al., 2016). As a result, day 28, when neuronal degeneration first manifests in AD, was chosen as the start date for PCA and Vit E administration. Because the liver is immediately exposed to toxins when they enter the circulation via the intestine and portal vein, and toxic responses are more prevalent in the liver than in other organs, we hypothesized that the liver was injured prior to the onset of Alzheimer's disease.

Experimental Groups

Group I (Control): Physiological saline (0.9%) i.p. for 42 days. The second physiological saline (0.9%) i.p. was administrated for 14 days from the 28th day.

Group II (Al): $AlCl_3$ was administrated intraperitoneally (i.p.) at a dose of 70 mg/kg/day for 42 days, and physiological saline (0.9%) i.p. was administrated for 14 days starting from the 28th day.

Group III (Al+Vit E): $AlCl_3$ was administrated intraperitoneally (i.p.) at a dose of 70 mg/kg/day for 42 days, and Vit E was administrated at a dose of 50 mg/kg/day i.p. for 14 days starting from the 28th day.

Group IV (Al+PCA): $AlCl_3$ was administrated intraperitoneally (i.p.) at a dose of 70 mg/kg/day for 42 days, and PCA was administrated at a dose of 5 mg/kg/day i.p. for 14 days from the 28th day. has been applied.

Group V (Al+PCA+Vit E): $AlCl_3$ was administrated intraperitoneally (i.p.) at a dose of 70 mg/kg/day for 42 days, and PCA and Vit E were administrated together for 14 days from the 28th day. PCA was administrated at a dose of 5 mg/kg/day and Vit E was administrated at a dose of 50 mg/kg/day as i.p.

Group VI (Vit E): Physiological saline (0.9%) was administrated to the animals for 42 days, and Vit E was administrated i.p. at a dose of 50 mg/kg/day for 14 days starting from the 28th day.

Group VII (PCA): Physiological saline (0.9%) was administrated to the animals for 42 days, and PCA was administrated at a dose of 5 mg/kg/day for 14 days from the 28th day.

Group VIII (PCA+Vit E): Physiological saline (0.9%) was administrated to the animals for 42 days, and PCA was administrated at a dose of 5 mg/kg/day and Vit E at a dose of 50 mg/kg/day i.p. for 14 days from the 28th day.

2.2. Biochemical Analysis

2.2.1. Superoxide dismutase (SOD) measurement

Superoxide dismutase activity (SOD) in liver samples was measured by using the commercial kit (Cayman Chemical Company Ann Arbor, MI. USA) Item No. 706002, which measures all three types of SOD (Cu/Zn, Mn, and FeSOD). Cayman's Superoxide Dismutase Assay uses a tetrazolium salt for the detection of xanthine oxidase and superoxide radicals produced by hypoxanthine. One unit of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. The assay provides a simple, reproducible, and rapid tool for testing SOD activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates.

1 unit enzyme: It is the enzyme activity that provides the autoxidation of 1 micromole 5,6,6a,11b-tetrahydro-3,9,10,-trihydroxybenzo [c] fluorene (BXT-01050) in 1 minute at 37 oC and pH 8.8.

2.2.2. Catalase (CAT) activity measurement

The CAT activity measurement was carried out according to the method of Beers R.F., Sizer I.W., (1952). This method is based on the fact that the absorbance value of hydrogen peroxide at a wavelength of 240 nm decreases during the reaction catalyzed by the enzyme CAT and this decrease is monitored spectrophotometrically. The rate of decrease of absorbance per minute is directly proportional to the enzyme activity.

Principle: CAT activity was determined by following the decomposition of hydrogen peroxide at 240 nm (De240nm = 40M-1 cm-1).

1 unit of enzyme is defined as the amount of enzyme required to decompose 1.0 micromole of hydrogen peroxide in 1 minute at 25^{o}C and pH 7.0.

2.2.3. Total glutathione (GSH)

Total glutathione (GSH) was performed using the Cayman glutathione assay kit (Item No. 703002). Cayman's GSH Assay uses a carefully optimized enzymatic recycling method using glutathione reductase to quantify GSH. The sulfhydryl group of 10-12 GSH reacts with DTNB (5,5'-dithio-bis-2-(nitrobenzoic acid) and produces yellow 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide produced together is GSTNB (between GSH and TNB) recycles GSH by reduction by glutathione reductase and produces more TNB production is directly proportional to this recycling reaction, which is directly proportional to the concentration of GSH in the sample. Measurement of its absorbance provides an accurate estimate of GSH in the sample. GSH is readily oxidized to the disulfide dimer GSSG. GSSG is produced during the reduction of hydroperoxides by glutathione peroxidase. GSSG is reduced to GSH by glutathione reductase and is its reduced form found primarily in biological systems. Because the Cayman GSH test uses glutathione reductase, both GSH and GSSG are measured, and the test reflects total glutathione.

2.2.4. Glutathione S transferase (GST)

Glutathione S-transferase enzyme activity is determined according to the method of Xie. C et al. (2001). The principle of the method is based on the measurement of the compound formed as a result of the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and GSH at a wavelength of 340 nm in the spectrophotometer. 1 unit of enzyme is the amount of enzyme that catalyzes the formation of 1 micromole of CDNB-GSH conjugate at 25^{o} C in 1 minute.

2.2.5. Determination of lipid peroxidation in tissues by determination of malondialdehyde (MDA)

The MDA determination was carried out according to the method of Ohkawa H, Ohishi N,Yagi K. (1979).

Principle: Malondialdehyde, which can be measured with thiobarbituric acid, is formed by the peroxidation of fatty acids with 3 or more double bonds. Thiobarbituric acid and malondialdehyde form the pink compound that can be measured at 532 nm in the spectrophotometer.

The tissue samples were homogenized at 10% (w:v) in 1.15% KCl. The mixture was centrifuged at 4000 rpm for 10 minutes. After centrifugation, the supernatant was utilized for MDA determination. 200 µl of an 8.1% sodium dodecyl sulfate solution, 1500 µl of a 20% acid buffer, 1500 µl of a 0.8% thiobarbituric acid solution, and 700 µl of distilled water were added to 500 µl of the supernatant. After cooling, the samples were stored in the incubator for 1 hour at 95°C, and then the clear portion of the samples was collected without additional mixing. The supernatant was then centrifuged at 4000 rpm for 10 minutes. The mixture was then mixed with 1 mL distilled water and 5 mL butanol/pyridine combination 15/1 (v:v). It was then combined and centrifuged at 4000 rpm. The absorbance against the blank was measured at 532 nm after the top liquid was removed.

2.2.6. Bradford protein assay

Total protein levels were determined according to the Bradford, M. M. (1976) method. This method is based on the formation of color by organic dyes (Coomassie Brilliant Blue G-250) by interacting with the acidic and basic groups of proteins. The amino acid composition of the protein (especially basic amino acids such as arginine and aromatic amino acids) is important in the formation of the blue color. In the method, the protein gives maximum absorbance at 595 nm wavelength. Bradford stain, bovine serum albumin (BSA), and distilled water were used for protein determination. A standard curve was formed by forming various concentrations of BSA. These concentrations are 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml. 200 µl of Bradford dye was added to the standards and tissue samples for protein determination. 200 µl of Bradford dye and 10 µl of H_2O were mixed and added to the plate well as a blank. The prepared samples and standards were placed in the spectrophotometer after waiting for 5 minutes. Reading was done by setting the absorbance value to 595 nm.

2.3. Histopathological Examinations

The liver tissue obtained after dissection was placed in a fixative solution containing 4% paraformaldehyde (pH 7.2-7.3). The tissues, which were divided into 1mm3 pieces, were kept in the fixative solution at +4 °C for 24 hours. The material was washed with 0.1 M sodium phosphate buffer (pH 7.4). The tissues were passed through a graded series of alcohols to remove water. It was kept in a LR white/alcohol (100%) (2/1) (v:v) mixture. The tissues were treated overnight with pure LR White. After embedding, 700 nm thick sections were taken from the blocks prepared in the ESTU research laboratory using the Leica UC6 ultra-microtome and diamond blade. Samples stained with toluidine blue were examined histopathologically under the Leica DM 6000B light microscope 63 and 100 x magnifications (Bancroft, J. D., and Gamble, M., 2008)..

2.4. PCNA, BRCA, And Oxo-G Immunofluorescence

3µm thick sections from tissue blocks prepared for histological examination were subjected to indirect immunofluorescence. They are individually labeled with a rabbit monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Cell Signaling Technology 2586S), a rabbit polyclonal BRCA 1 antibody (Cell Signaling Technology 9010S), and a mouse 8- oxo-guanine (8- OHdG) antibody (SantaCruzz biotechnology, INC. sc-516176). The tissue sections were incubated in a blocking solution for 30 minutes at room temperature according to the immunofluorescence protocol. After washing with PBS for 3x5 minutes, tissues were incubated with the appropriate dilution of primary antibody prepared with 1% BSA in PBS buffer for 1 night at 4°C. After 3x5 minutes of washing with PBS, the samples were incubated with the appropriate dilution of the Alexaflor 488 secondary antibody prepared with 1% BSA in PBS buffer for 1 hour at room temperature in the dark. After washing with PBS for 3x5 min, immunofluorescence examinations were performed under a Leica DM 6000B fluorescence microscope at 63× magnification. Fluorescence intensity was digitized by analyzing randomly selected areas on at least 20 images of the preparations of the experimental animals from each group using the Image J program (Bancroft, J. D., and Gamble, M., 2008).

2.5. Single Cell Gel Electrophoresis (SCGE) / Comet Assay Method

0.1 g liver tissue was comminuted using a Potter Elvehjem homogenizer with Merchant EDTA buffer. The samples homogenized by comminution were used for the comet assay. 8 µl of sample and 73 µl of 0.1% low melting temperature agarose were mixed with agarose, poured onto a slide covered with agarose, and covered with a

coverslip. The samples were stored at +4oC for 5 minutes. Then the coverslip was removed, and the samples were stored in lysis buffer (2.5M NaCl, 100 mM EDTA, 10mM Tris, 1% Triton X-100, pH 10) at +4ºC for 1 hour. Samples were added to the alkaline buffer (1 mM EDTA, 300 mM NaOH, pH 13) and kept at +4ºC for 40 minutes. All samples were electrophoresed at 25 V and 300 mA for 30 minutes. Neutralization was performed 3 times for 5 minutes after electrophoresis. All preparations were stained with SYBR green (1ul/10mL) and analyzed under a Leica DM 6000B fluorescence microscope. An average of 100 cells was counted from each preparation. BS 200 ProP, BAB Imaging System was used for comet analysis (Kilic. V., 2019).

2.6. Statistical Analysis

The SPSS Statistics V26 2019 program was used for the statistical analysis. After conducting the normality tests, the data were analyzed using the One-Way ANOVA test. For further analysis of the data, Dunnett's T3 test, one of the post hoc tests, was used. When $p<0.05$, differences between groups were considered statistically significant. All values are given as mean ± standard error.

3. RESULTS

3.1. Results Of The Histological Analysis

The following figures show light microscopic observations of liver tissues obtained from the different experimental groups. The control group showed normal histo-architecture and normal liver cells in the histological sections of the liver (Figure 3.1-3.4). In the Al- administrated liver tissue sections, damaged hepatocytes, damaged and congested sinusoids were observed accompanied by apoptotic and necrotic hepatocytes, presence of inflammatory cells, and steatosis (Figure 3.5-3.8). In the Al+PCA group of animals, there were no observable changes in the pattern of hepatocytes and sinusoidal canals (Figure 3.9-3.13). However, mild microvesicular fatty changes and few occurrences of apoptotic events were observed. The Al+Vit E group showed normal general structure and normal hepatocytes, with limited inflammatory and apoptotic cells and the presence of microvesicular fatty changes. No necrotic cells were detected (Figure 3.14-3.16). Al+PCA+Vit E (Figure 3.17-3.20) showed normal liver tissue architecture, normal hepatocytes, and normal sinusoids with rare apoptotic events and infiltration of inflammatory cells. PCA, PCA+Vit E, and Vit E (Figure 3.21-3.23, respectively) groups showed normal tissue structure and no histological abnormalities were detected.

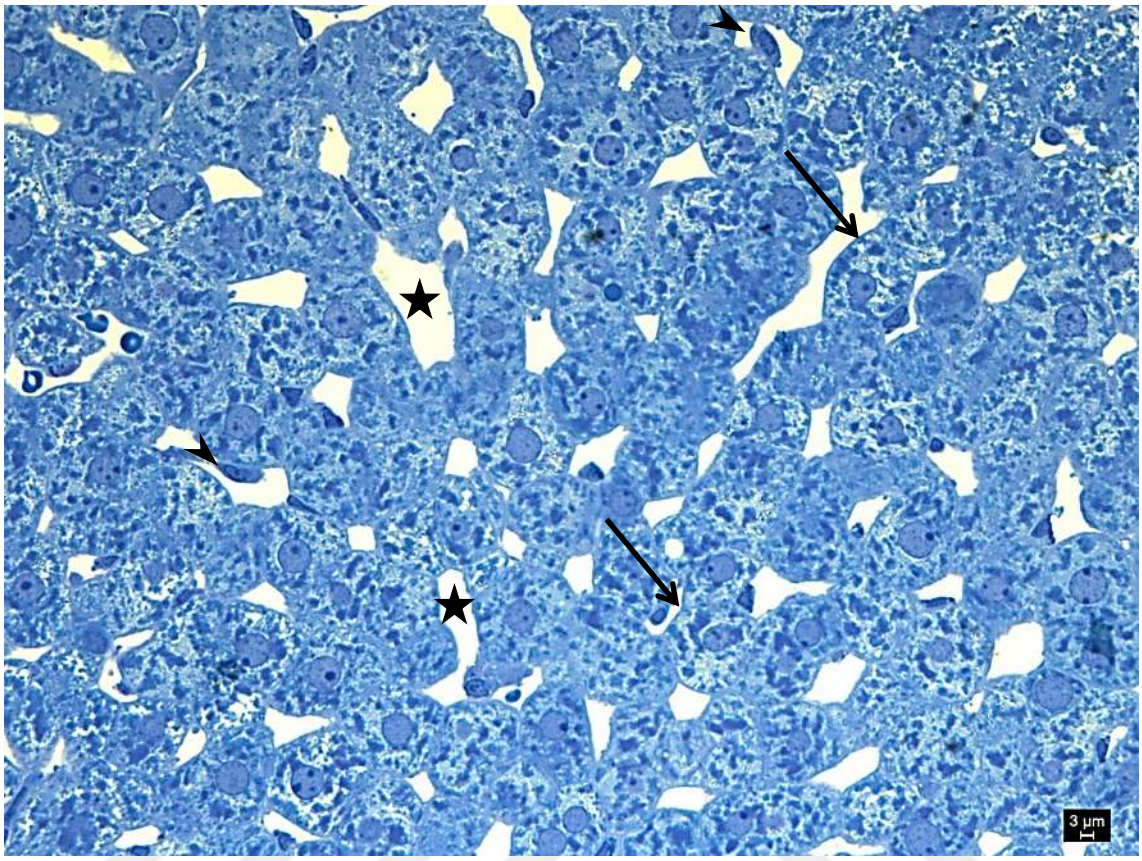

Figure 3.1. *Control liver section shows normal liver tissue structure with normal hepatocytes (arrows), normal sinusoidal canals (stars), and normal Kupffer cells (arrowheads) X63*

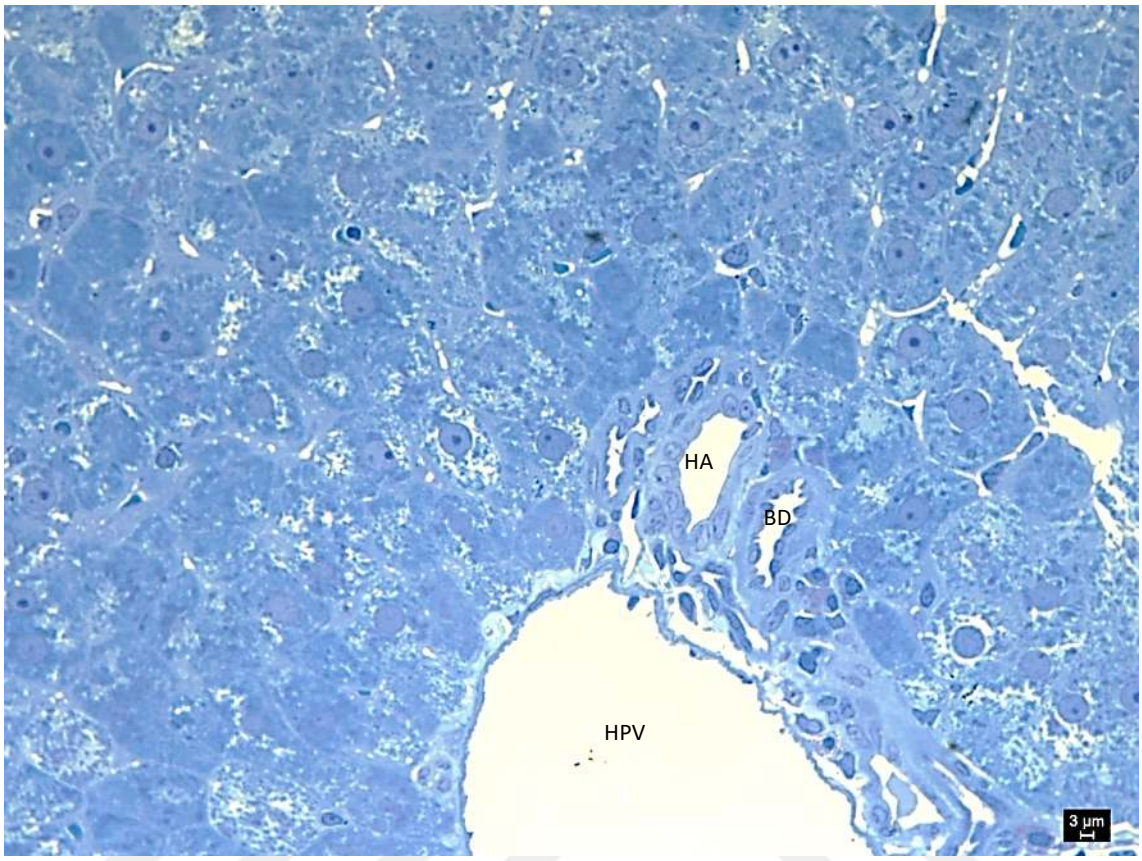

Figure 3.2. *Control liver section shows normal hepatic portal vein (HPV), hepatic artery (HA), and bile duct (BD). X63*

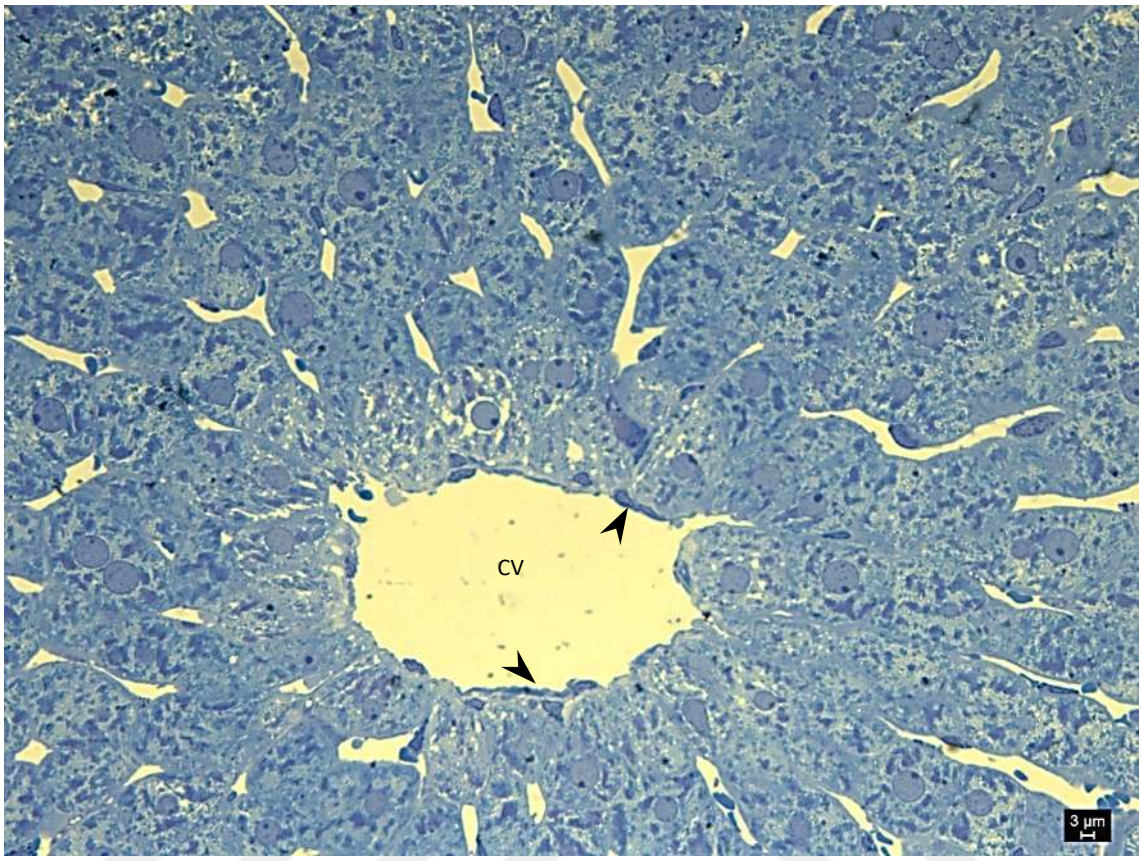

Figure 3.3. *Control liver section shows a normal central vein (CV) associated with normal epithelial cells (arrowheads). X63*

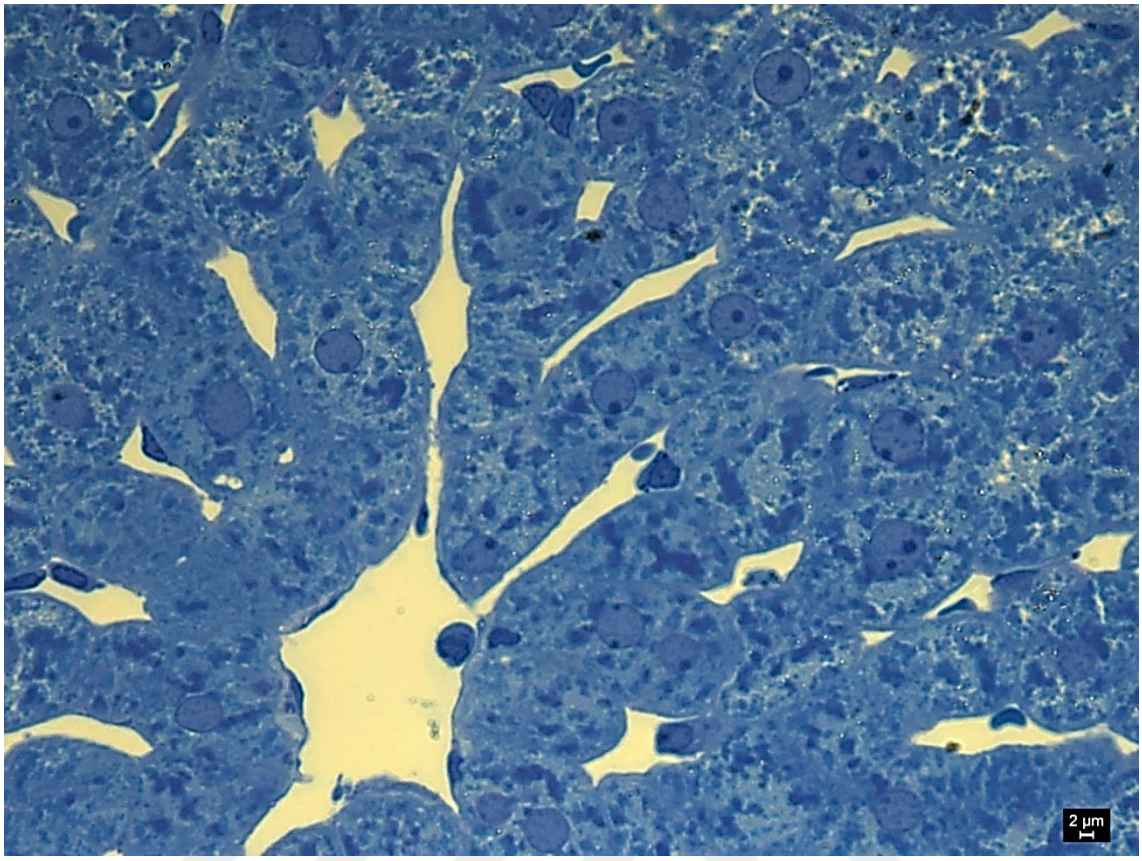

Figure 3.4. *Control liver section show normal histoarchitecture, normal sinusoidal canals, and normal hepatocytes. X100*

Figure 3.1-3.4 control group (normal saline was injected intraperitoneally (i.p.)). Sections show normal sinusoids normal hepatocytes and normal histoarchitecture.

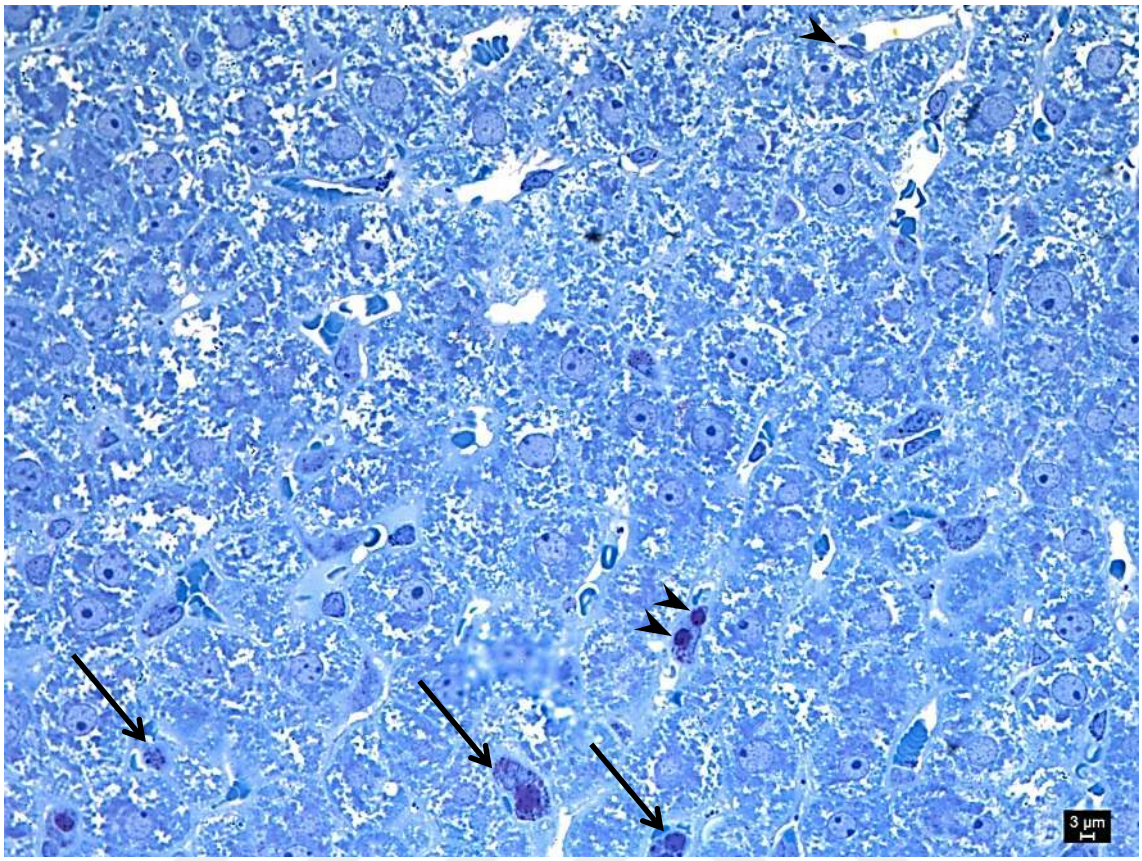

Figure 3.5. *Al-administrated liver section, shows several irregularities combined with a loss of sinusoidal space, apoptotic hepatocytes (arrowheads), and inflammatory cells infiltrations (arrows) x63.*

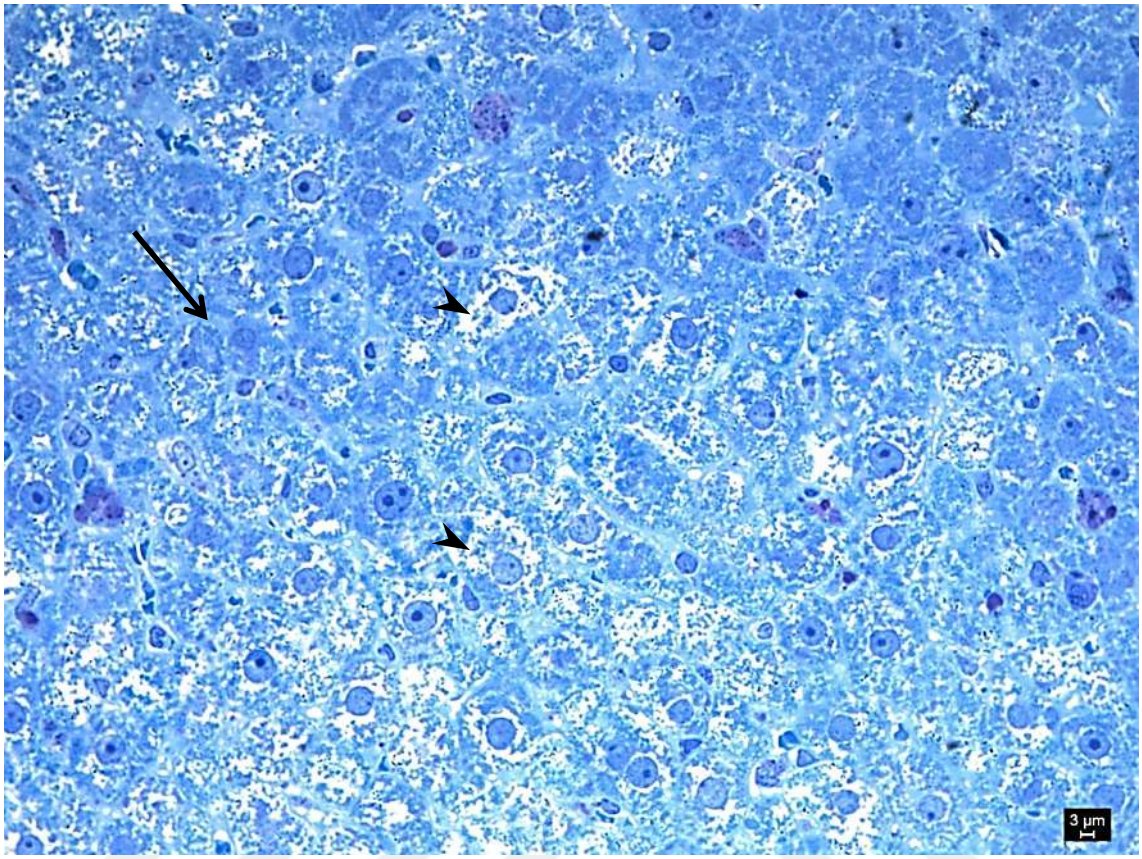

Figure 3.6. *Al-administrated liver section shows impaired sinusoids, shrunken hepatocytes (arrow) with empty intracellular content (arrowheads) x63*

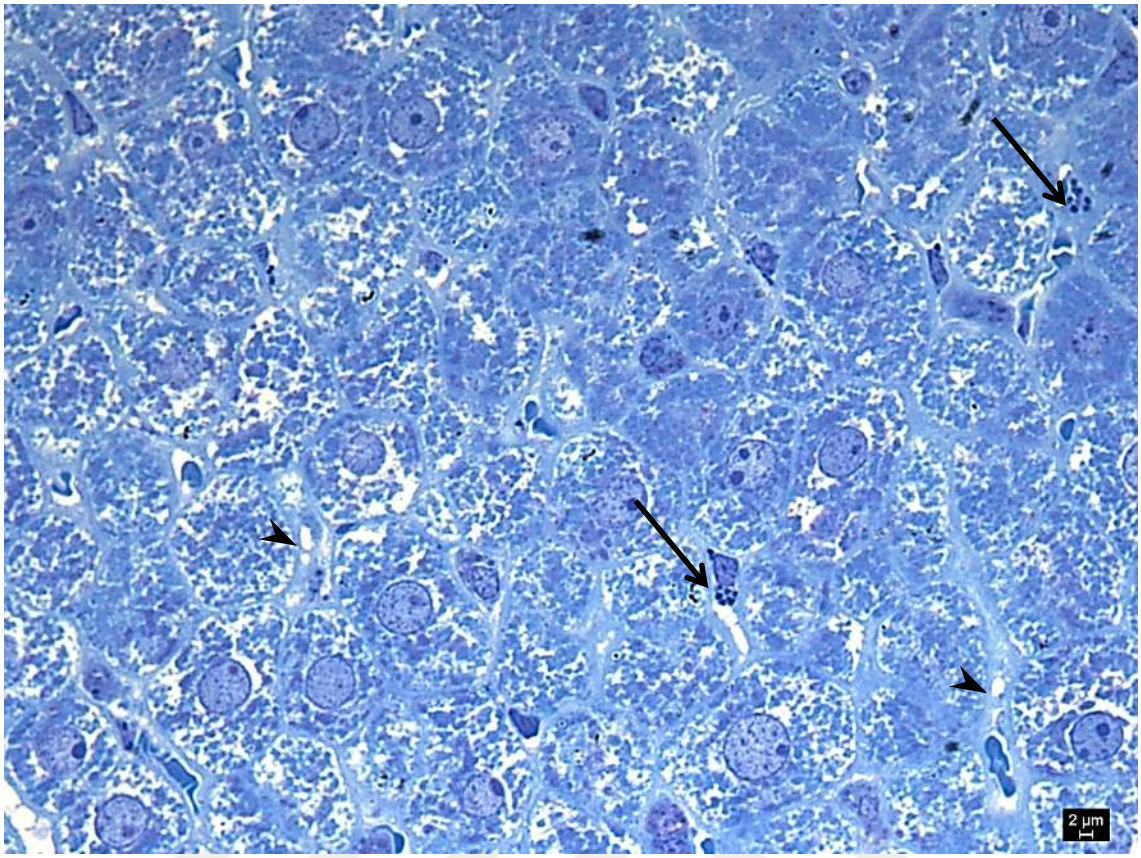

Figure 3.7. *Al-administrated liver section shows steatosis (arrowheads) and Kupffer cells with inflammatory cells' infiltrations (arrows) x100*

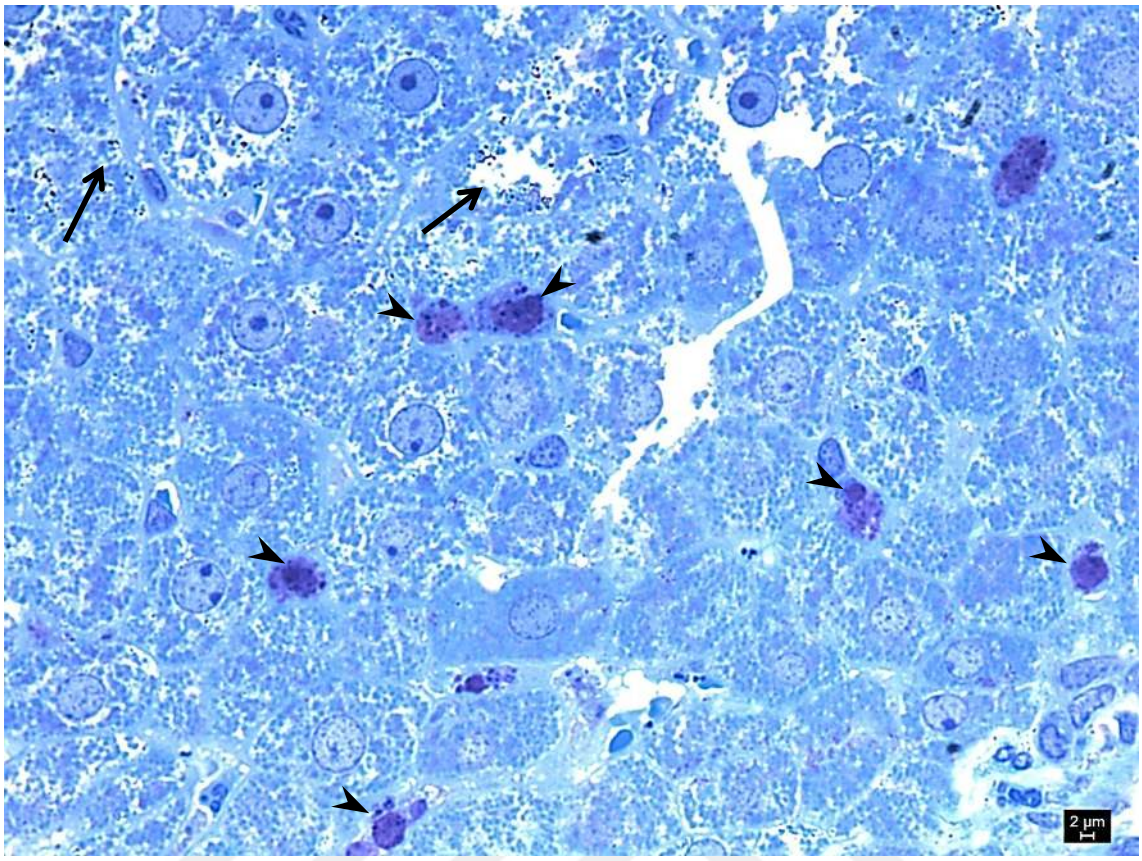

Figure 3.8. *Al-administrated liver section shows spotty necrotic hepatocytes accompanied by eosinophilic inflammatory celL infiltrations (Arrows) and apoptotic acidophil bodies (arrowheads).X100.*

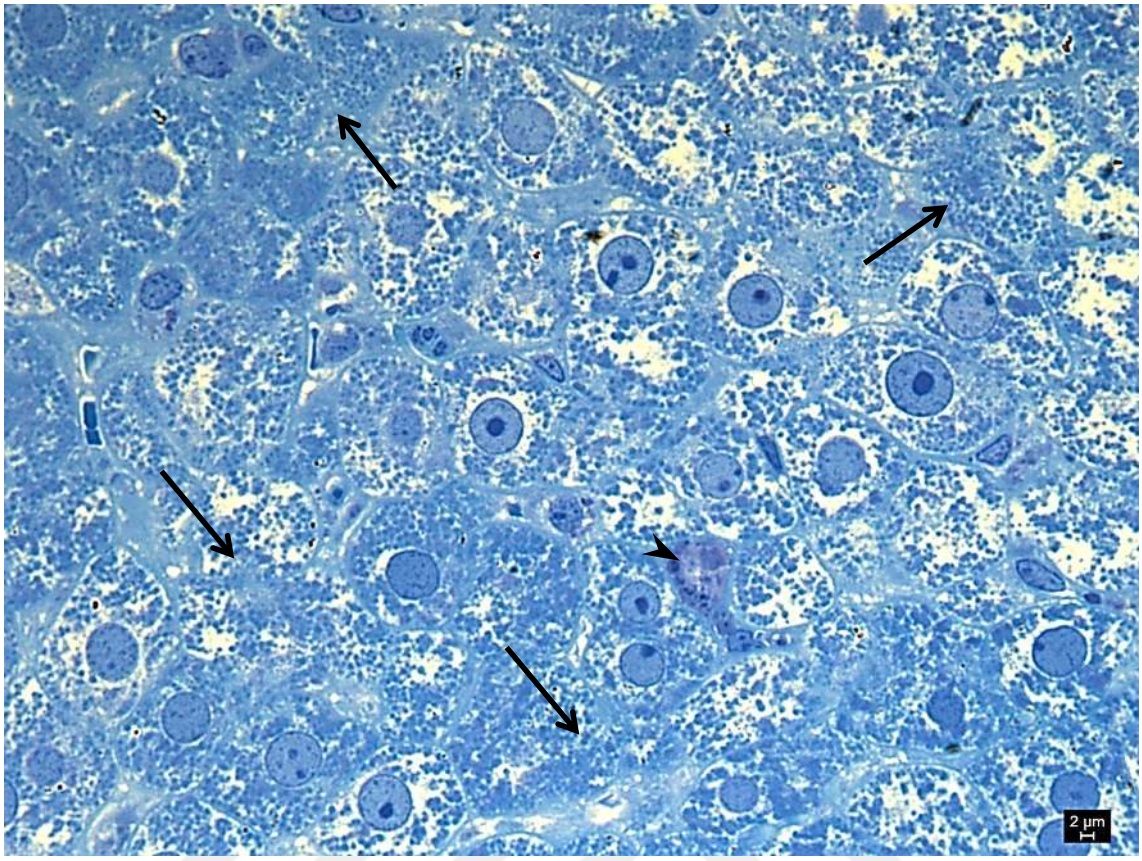

Figure 3.9. *Al-administrated liver section shows possible necrotic cells (Arrows) and apoptotic hepatocytes associated with inflammatory cells infiltration (arrowhead). X100*

Figure 3.5-3.9 Al group ($AlCl_3$ administrated at a dose of 70 mg/kg intraperitoneally (i.p.)). Sections show damaged sinusoids and steatosis with discernible apoptotic and necrotic appearance in individual hepatocytes.

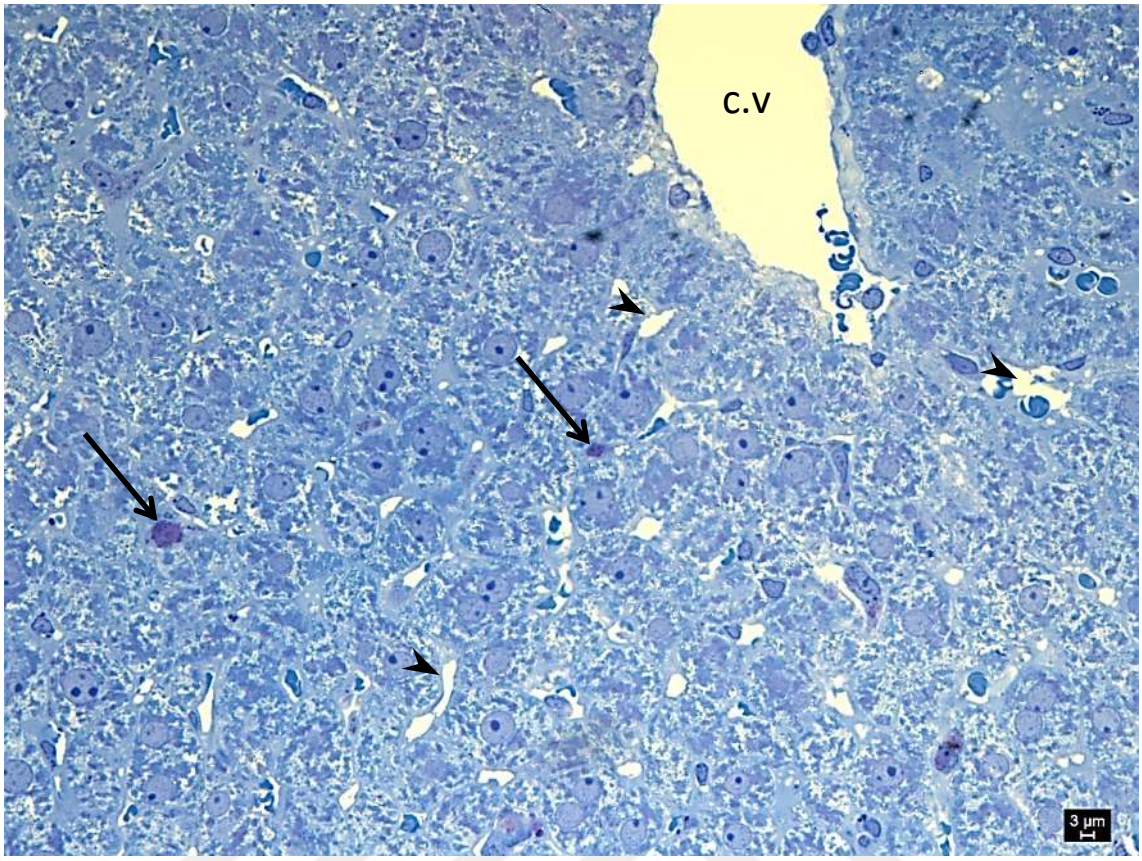

Figure 3.10. *Al+PCA administrated liver section shows improvement in the general tissue architecture, enhanced sinusoidal canals (arrowheads) and hepatocytes restore their normal appearance. However deformed central vein (C.V) and apoptotic hepatocytes (arrows) were observed. X63*

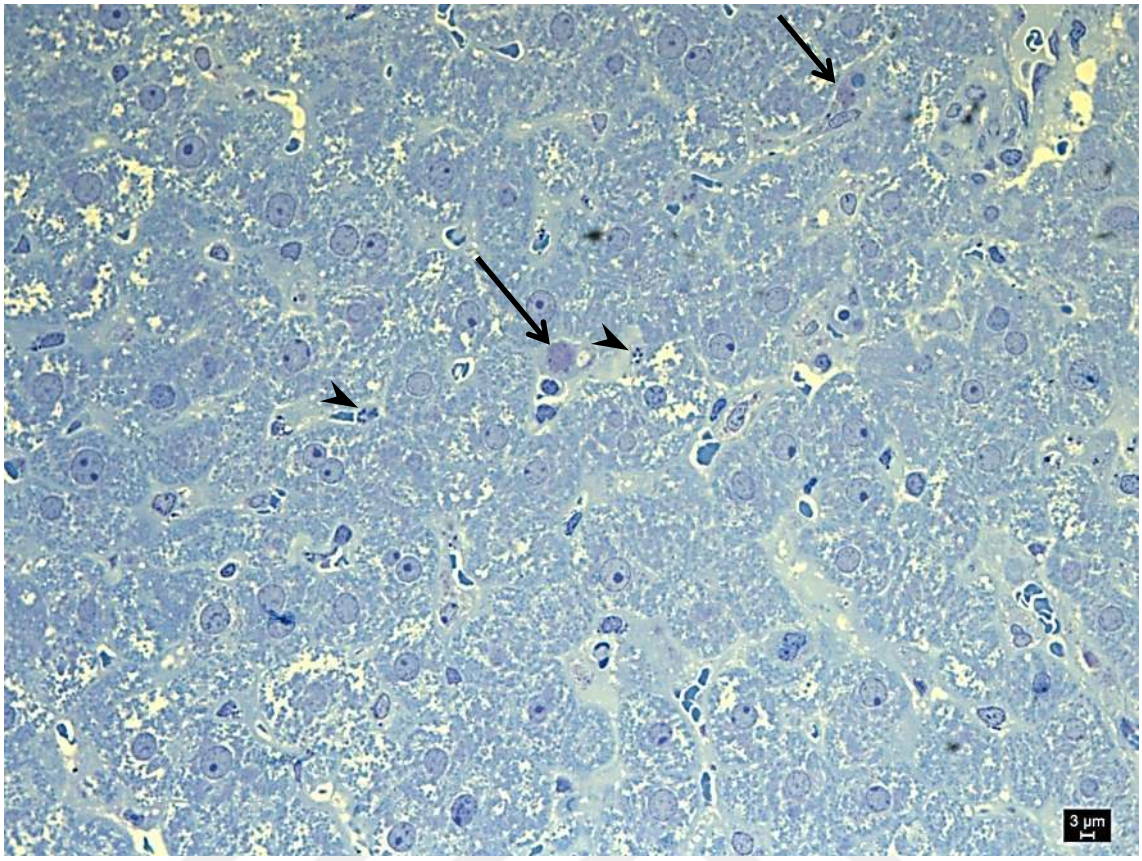

Figure 3.11. *Al+PCA administrated liver section shows inflammatory cell infiltrations (arrowheads) and apoptotic hepatocytes (arrows). X63*

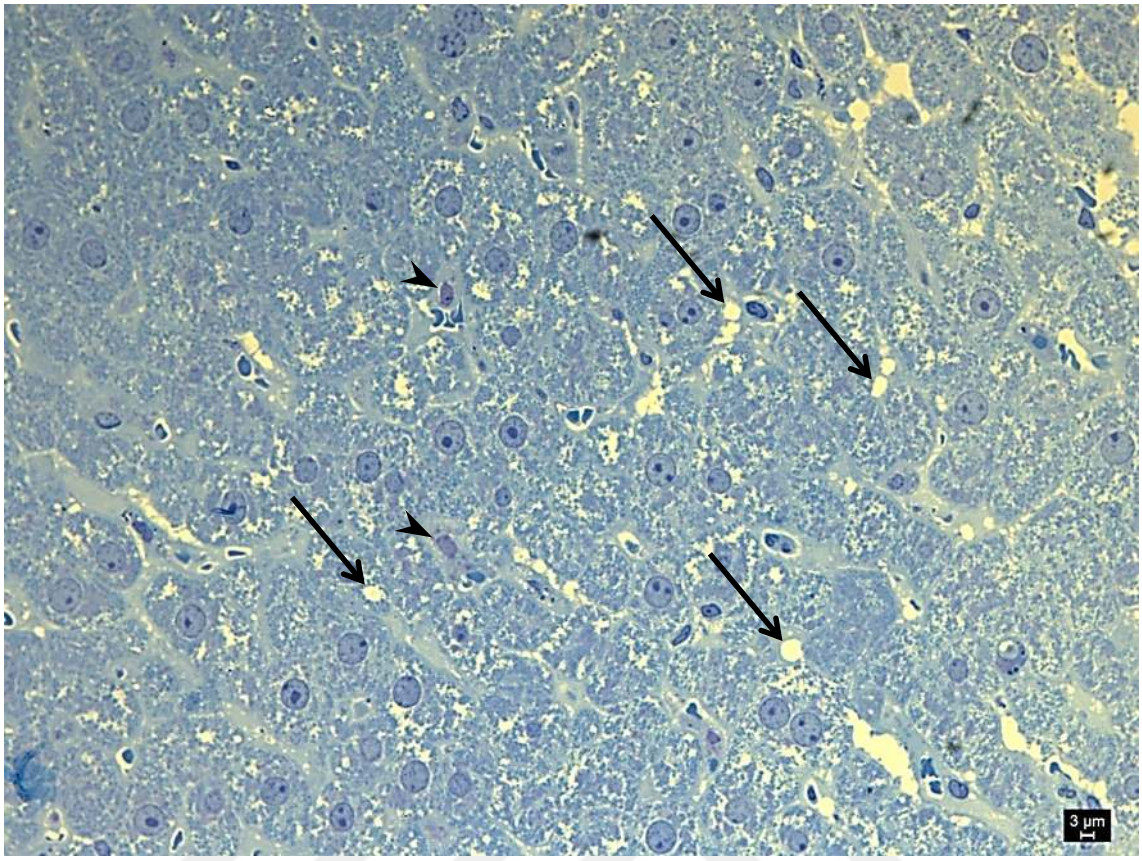

Figure 3.12. *Al+PCA administrated liver section shows fatty infiltrations (Arrows) and apoptotic hepatocytes (Arrowheads). X63*

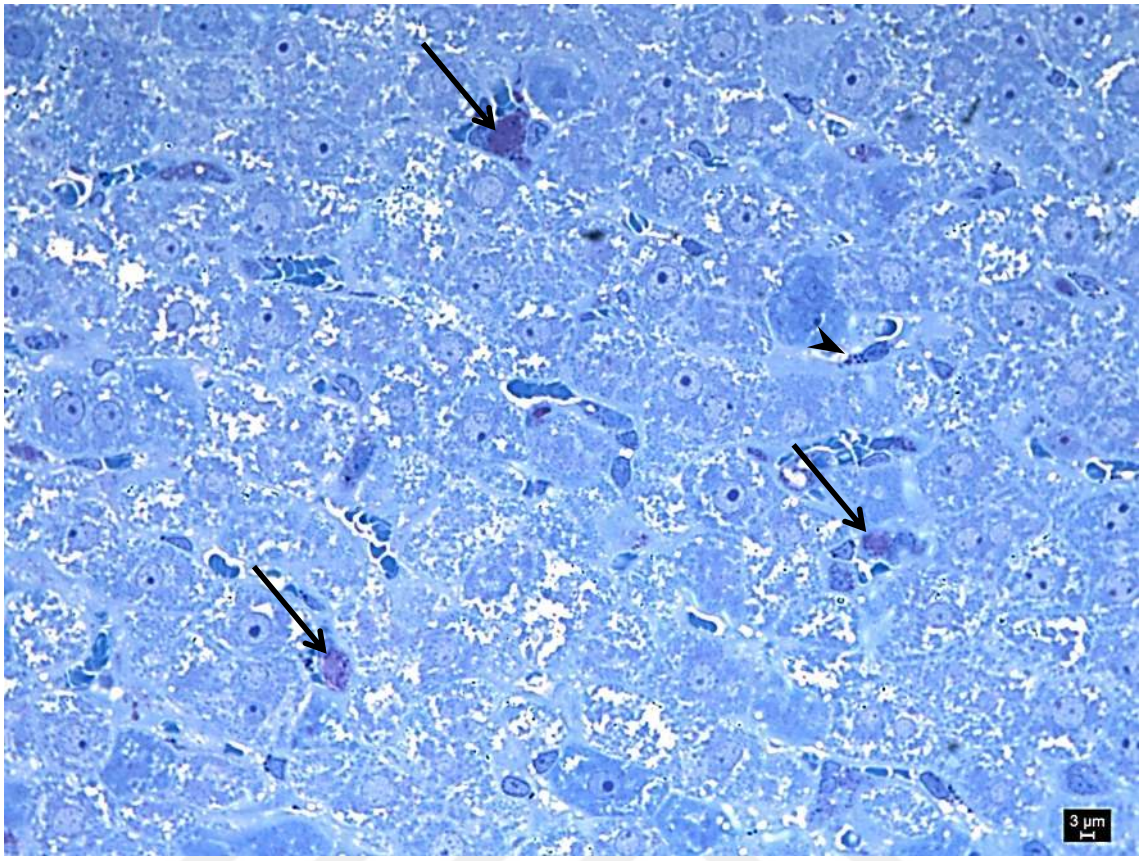

Figure 3.13. *Al+PCA administrated liver section shows acidophil apoptotic bodies (arrows) and kupffer cells with the presence of inflammatory cells (arrowhead) X6.*

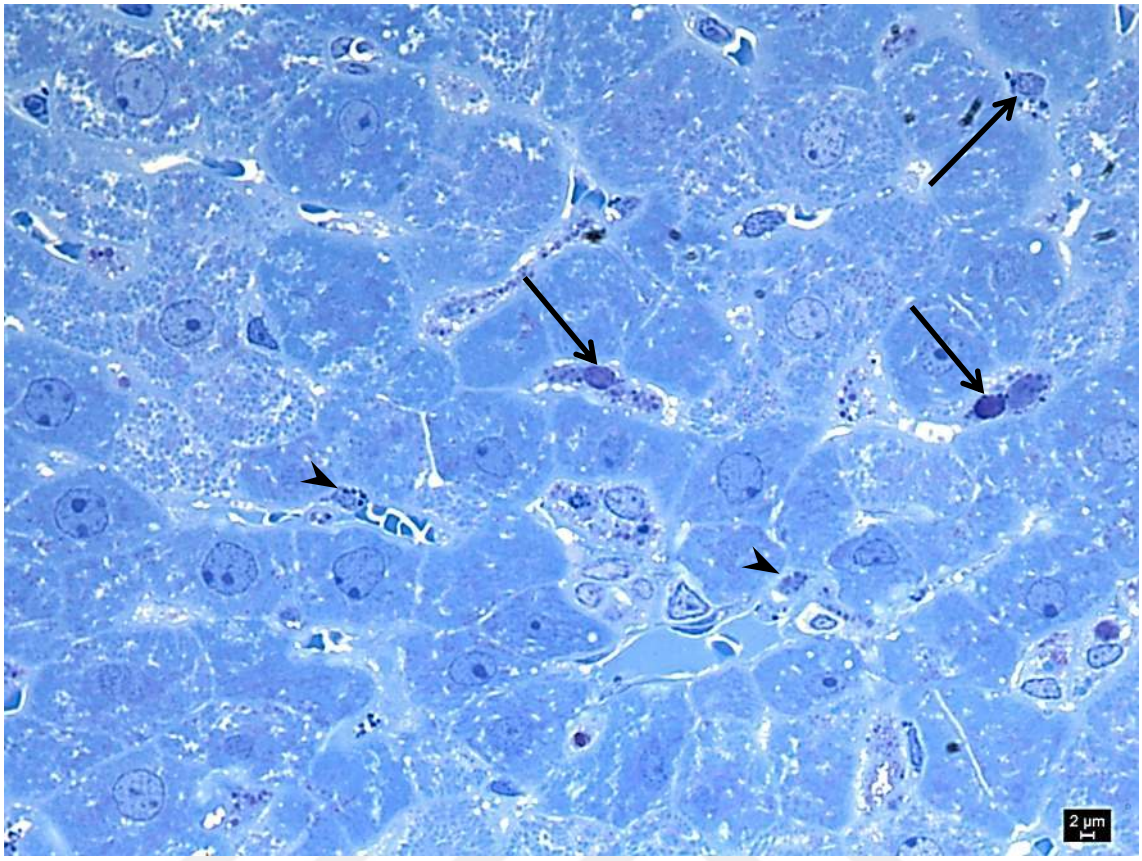

Figure 3.14. *Al+PCA administrated liver section shows congested sinusoids, inflammatory lymphatic infiltrations (arrowheads), and apoptotic hepatocytes (arrows). X100*

Figure 3.10-3.14 Al+PCA group of animals (AlCl3 was administrated at a dose of 70 mg/kg intraperitoneally (i.p.)) for 28 days then PCA was administrated for 14 days at a dose of 5mg/kg/day i.p). Liver tissue shows improvement in the general tissue structure and enhancement in the sinusoidal canals. However, steatosis, inflammatory cell infiltrations and a few apoptotic events (acidophil bodies) were detected.

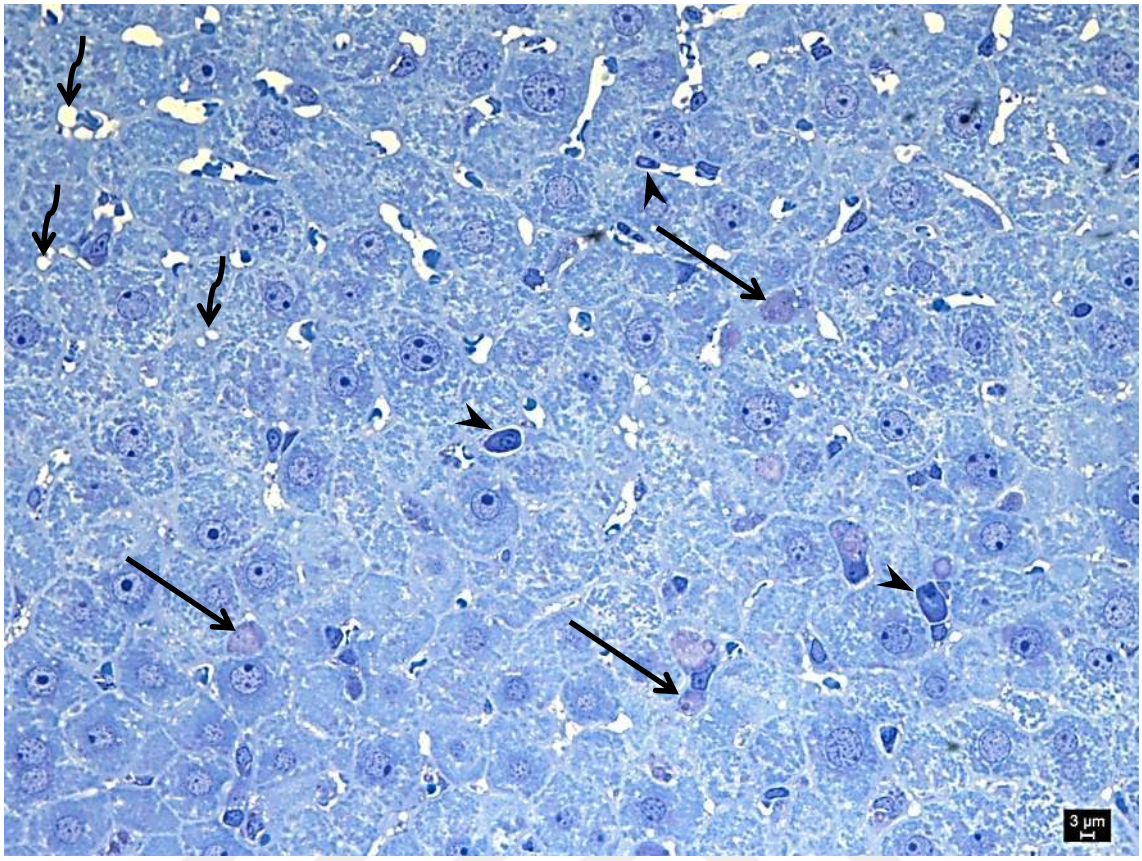

Figure 3.15. *Al+Vit E administrated liver section. Shows apoptotic hepatocytes (arrows), fatty change lipid droplets (zigzag arrow), and kupffer cells (arrowheads). X63*

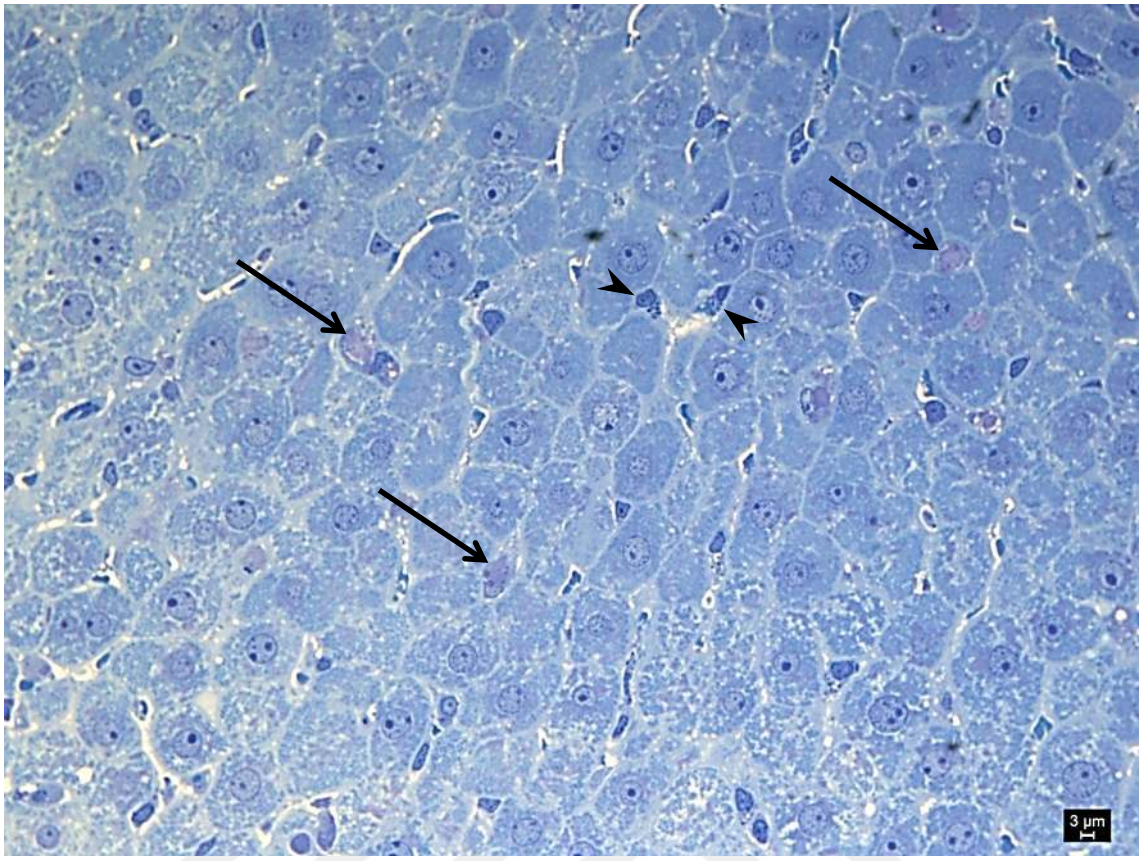

Figure 3.16. *Al+Vit E administrated liver section. Apoptotic hepatocytes (arrows) and kupffer cell associated with inflammatory cells (arrowheads). X63*

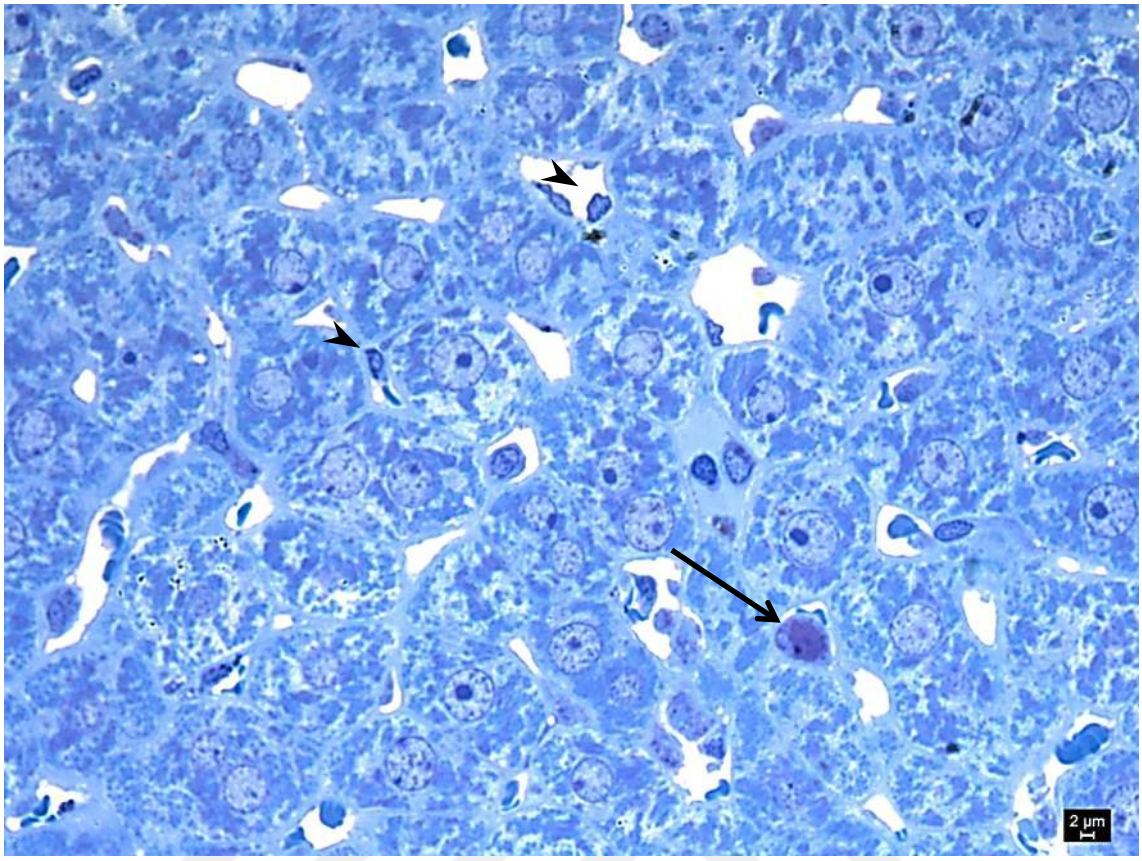

Figure 3.17. *Al+Vit E administrated liver section. Normal tissue architecture, apoptotic cell (arrow), and Kupffer cells (arrowheads). X100*

Figure 3.15-3.17 represents Al+Vit E administrated animal group. (Al was administrated at a dose of 70 mg/kg intraperitoneally (i.p.)) for 28 days then Vit E was administrated for 14 days at a dose of 50mg/kg/day i.p) This group shows intact histo-architecture of liver sections, normal shape of hepatocytes, and undamaged sinusoidal canals. Nevertheless, rare apoptosis, and mild fatty change were observed in different sections

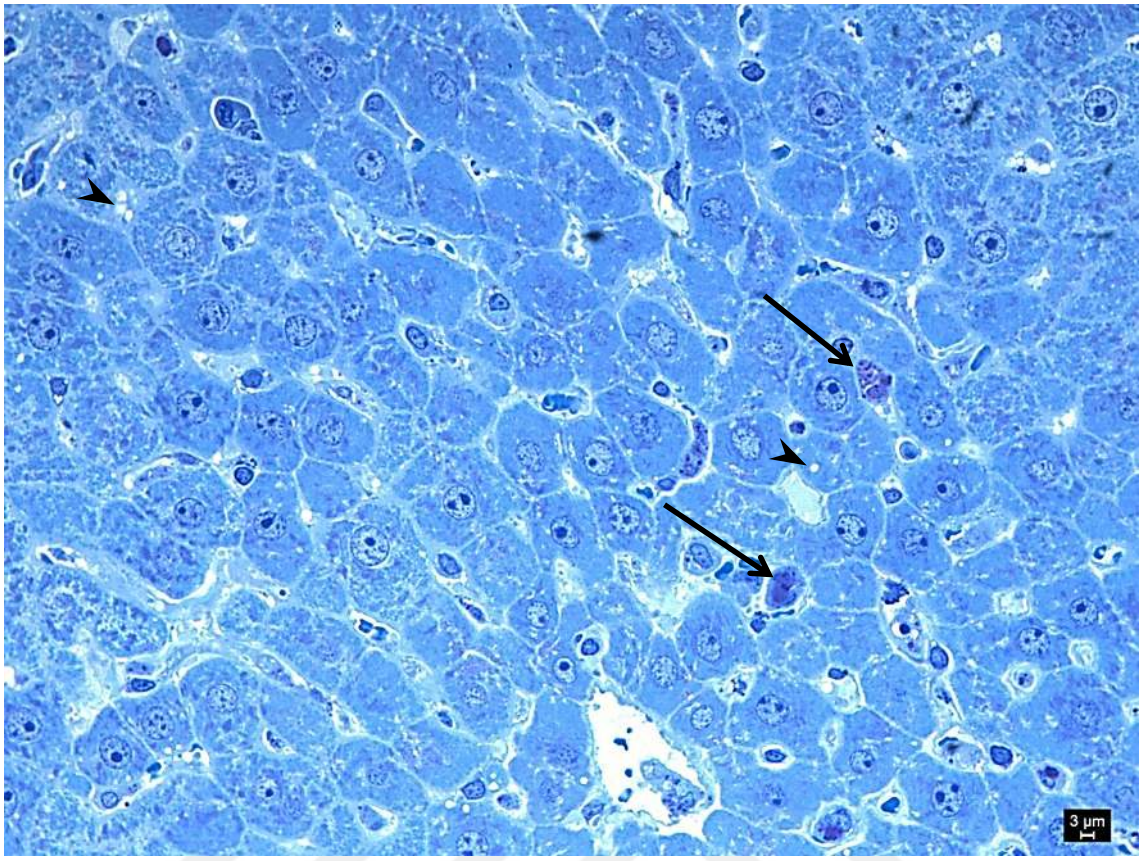

Figure 3.18. *Al+PCA+Vit E administrated liver section. Shows intact tissue structure with normal hepatocytes, apoptotic events were detected (arrows) and mild microvesicular fatty change was observed (arrowhead). X63*

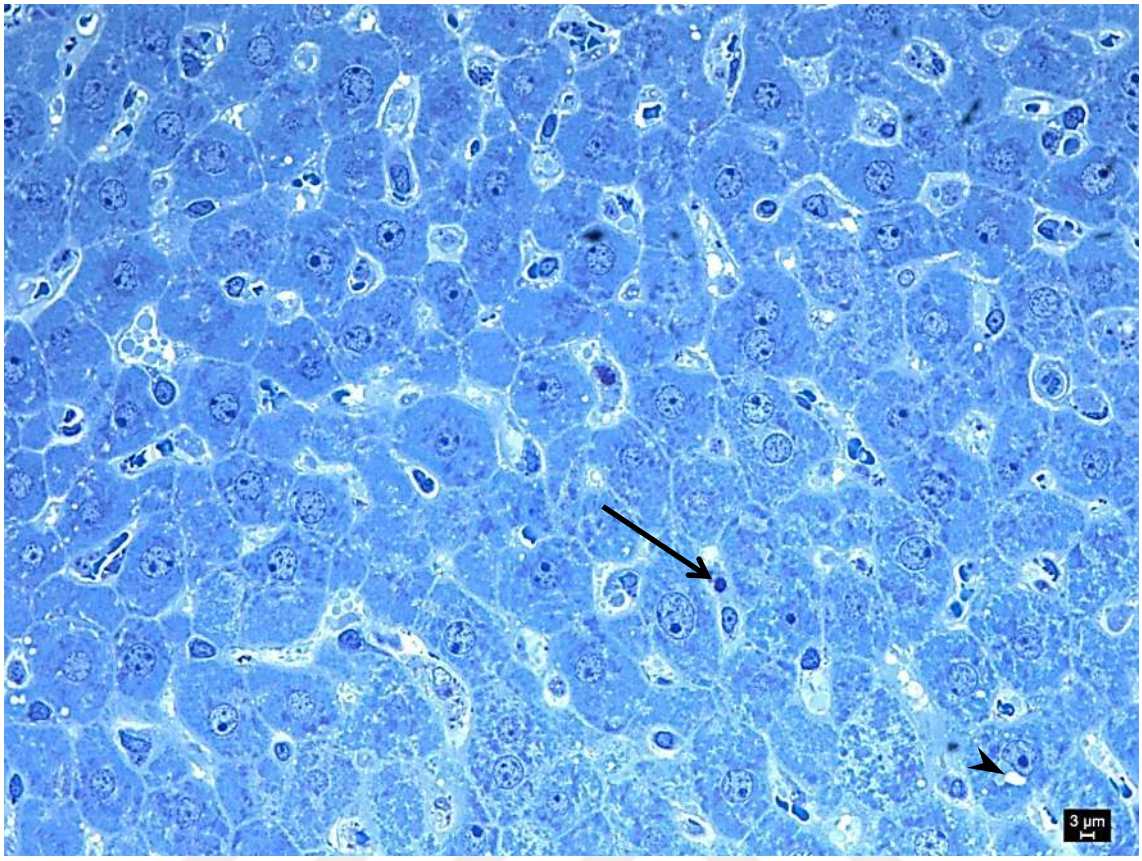

Figure 3.19. *Al+PCA+Vit E administrated liver section. Shows normal tissue structure with occasional apoptotic (arrow) and microvesicular fatty change events (arrowhead). X63*

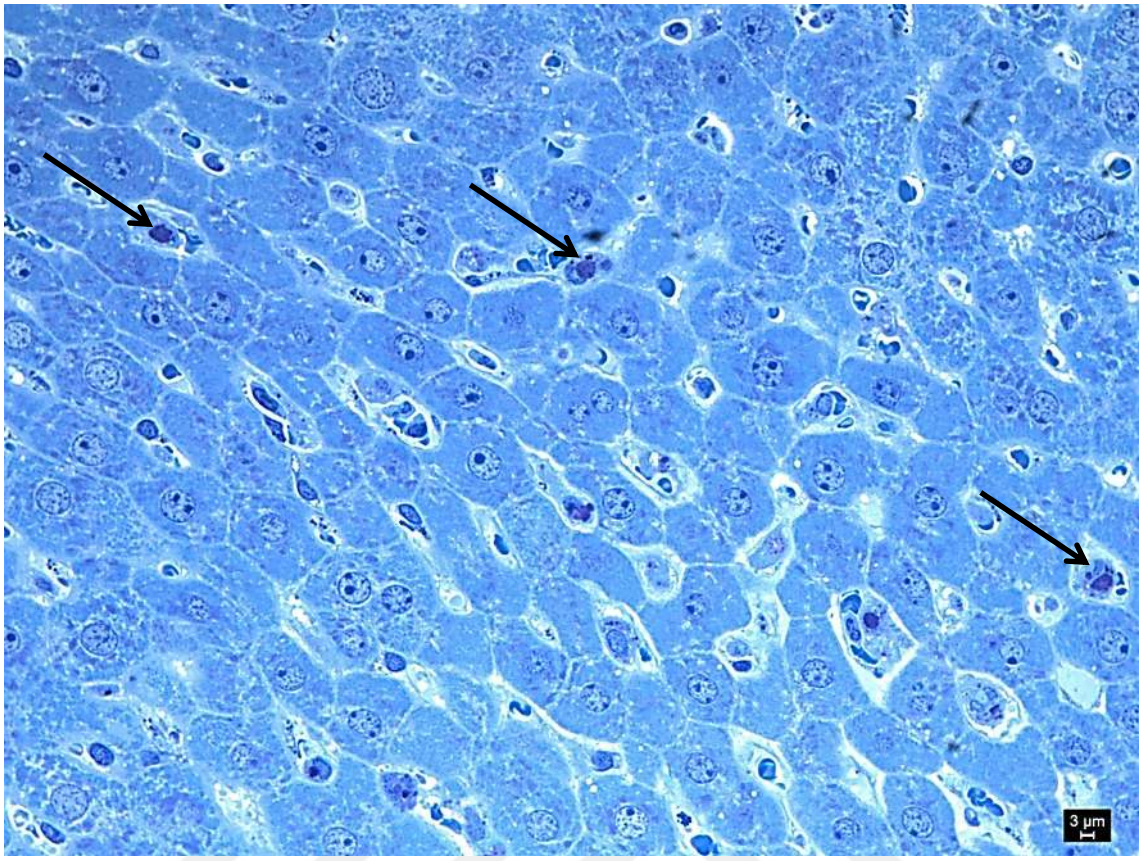

Figure 3.20. *Al+PCA+Vit E administrated liver section. Shows apoptotic cells (arrows) X63*

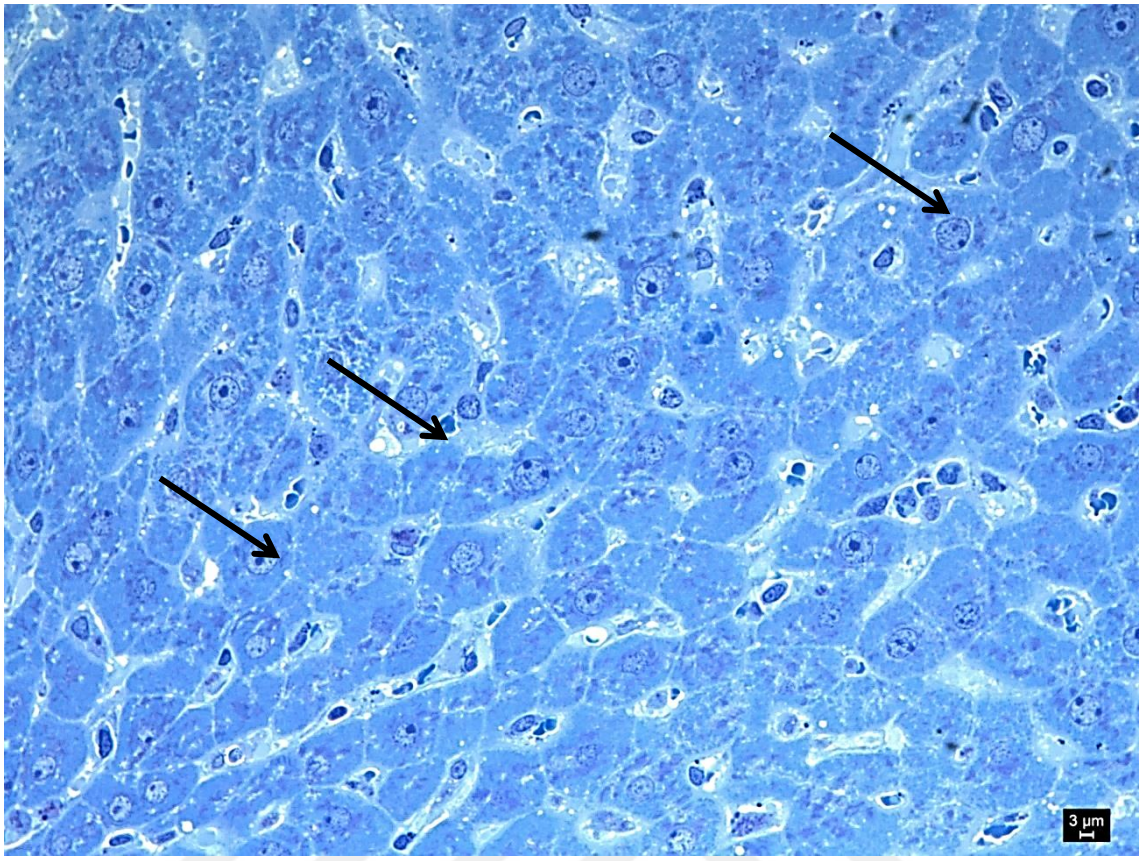

Figure 3.21. *Al+PCA+Vit E administrated liver section. Shows scattered lipid droplets (arrows). X63*

Figure 3.18-3.21 represents Al+PCA+Vit E group of animals (Al was administrated at a dose of 70mg/kg intraperitoneally for 28 days then PCA aa t dose of 5 mg/kg/day and Vit E at dose of 50mg/kg/day were administrated for 14 days i.p)). photographs show intact histological structure and normal hepatocytes with slightly congested sinusoids associated with rare apoptotic events and relatively less inflammatory cells infiltration with mild microvesicular fatty changes.

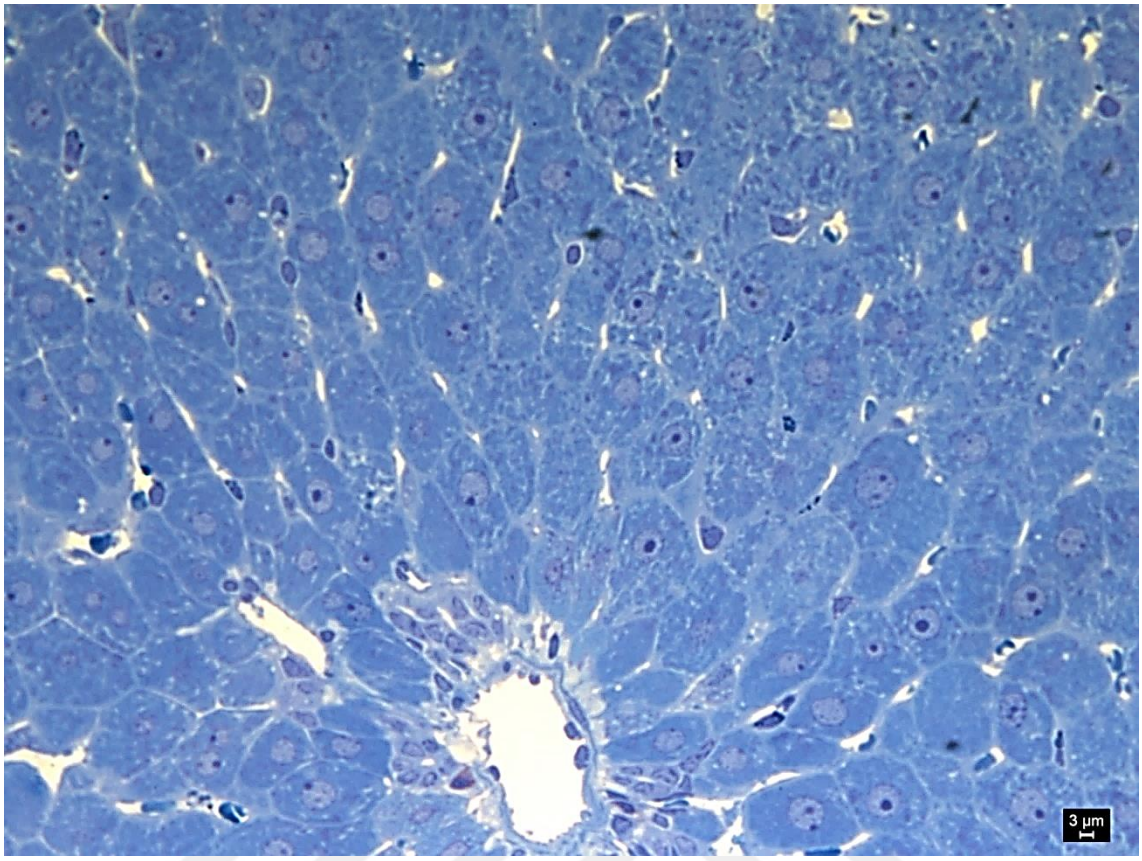

Figure 3.22. *Liver tissue section of PCA group*

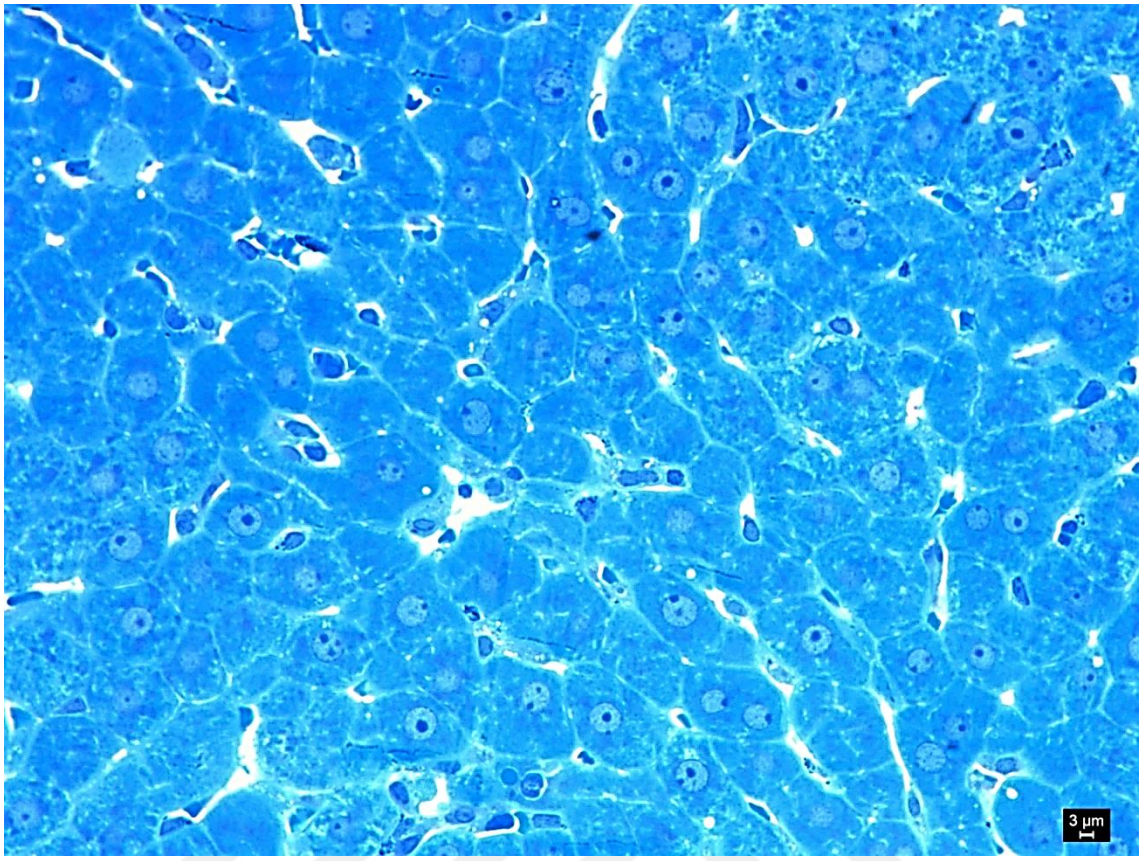

Figure 3.23. *Liver tissue section of Vit E group*

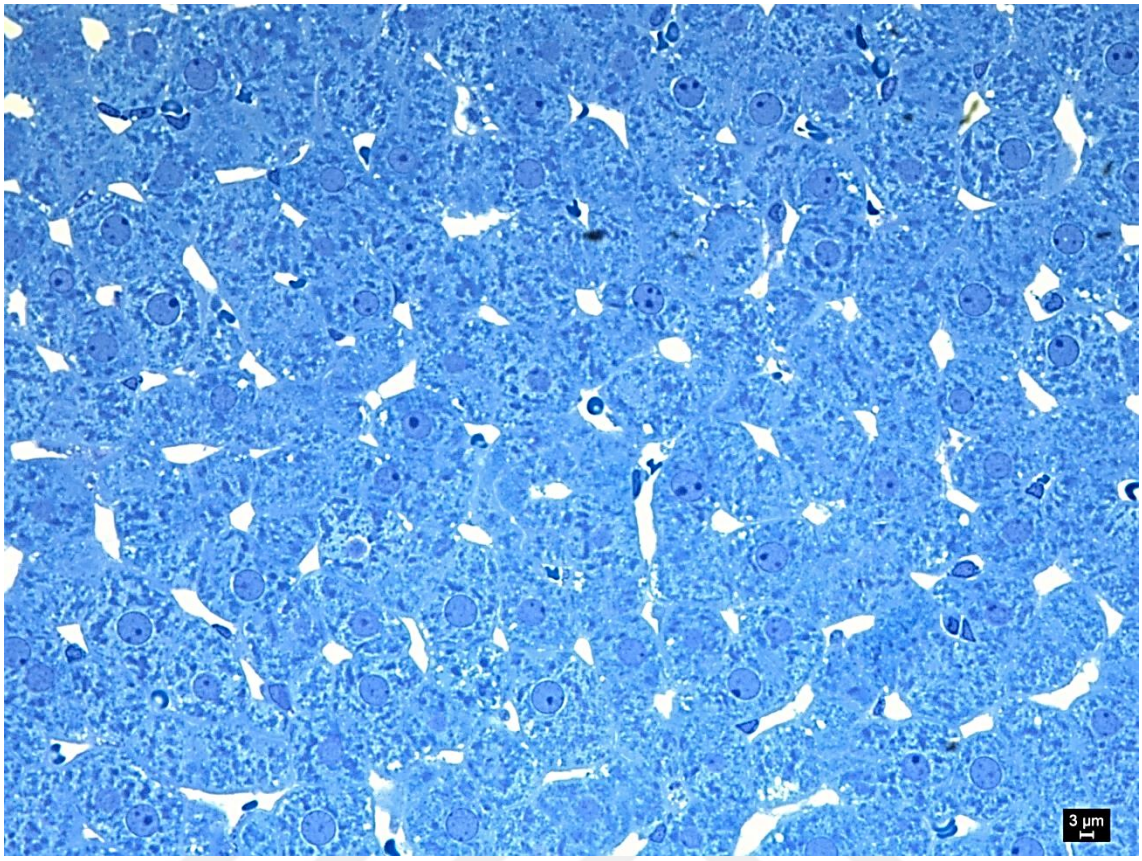

Figure 3.24. *Liver tissue section of PCA+ Vit E group*

Figure 3.22-3.24 PCA (3.22) (physiologic saline 0.9% was administrated intraperitoneally (i.p.) for 28 days then PCA was administrated for 14 days at a dose of 5mg/kg/day i.p), Vit E (3.23) (physiologic saline 0.9% was administrated intraperitoneally (i.p.) for 28 days then Vit E was administrated for 14 days aa t dose of 50mg/kg/day i.p) and PCA+Vit E (3.24) (physiologic saline 0.9% was administrated intraperitoneally (i.p.) for 28 days then PCA 5mg/kg/day and Vit E 50mg/kg/day was administrated for 14 days i.p) animal groups. Sections shows normal architecture of liver tissues, and no abnormalities were detected.

3.2. Biochemical Analysis Results

Figure 3.25-3.30 shows the results of the biochemical analysis. Administration of Al resulted in a significant decrease in the activities of SOD and CAT antioxidant enzymes by (19%) and (65.5%), respectively, compared to the control group. The activity of SOD and CAT in Al+PCA group increased by (6%) and (46.35%), respectively, compared to the Al group (Fig. 3.25&3.26). However, in the Al+Vit E group, no significant increase in the activity of SOD was observed. Nevertheless, the CAT enzyme's activity was increased about 30% in comparison with the Al group. In the Al+PCA+Vit E group there was a reduction in SOD activity by 14.5% compared to control group. However, there was an increment about 5.2% when compared to the Al group. On the other hand, in Al+PCA+Vit E group the CAT activity was remained at the same level when compared to Al group. Al administration led to approximately 2-fold decrease in the total level of GSH (49.9%). Administration of Vit E and PCA separately resulted in increase of totla glutathione by 34.12% and 43.9% respectively. Regarding Al+PCA+Vit E group, the total glutathione level found to decrease about 40.6% when compared to control and increased by 15.7% when compared to Al group. Lipid peroxidation was extremely increased to about (65.5%) in the Al group compared to the control group (Fig. 3.27). Al administration resulted in significant reduction in GST antioxidant enzyme activity by (76.6%) the compared to control group. In contrast, GST activity was increased by (60.9%) and (56.8%) in Al+PCA and Al+Vit E respectively when compared to Al group and decreased by (40.2%) and (45.9%) when compared to the control group. In Al+PCA+Vit E group, GST enzyme activity was approximately 2 folds increased compared to Al group, yet it remained lower by (54%) compared to the control group (Fig. 3.28). In contrast, lipid peroxidation was significantly reduced to about (58.5%) in Al+Vit E group compared to the Al group. Lipid peroxidation was found to be decreased by (23%) and (32.6%) in the Al+PCA and Al+PCA+Vit E groups respectively when compared to Al group, however, it remained higher by (55.25%) and (48.7%) when cared to the control group (Fig 3.29). Total protein level investigations showed no differences between different experimental groups (Fig 3.30).

Table 3.1. *SOD activity results (Mean ± Standard error)*

Experimental Group	SOD activity (U/mg prt)	
Control	26.110 ± 0.473533	
Al	21.263 ± 0.383681	(a)
Al+Vit E	22.120 ± 0.641561	(a)
Al+PCA	24.453 ± 0.369745	(b)
Al+PCA+Vit E	23.897 ± 1.074249	
PCA	26.579 ± 0.448814	(b)
Vit E	26.050 ± 0.201246	(b)
PCA+Vit E	26.333 ± 0.289444	(b)

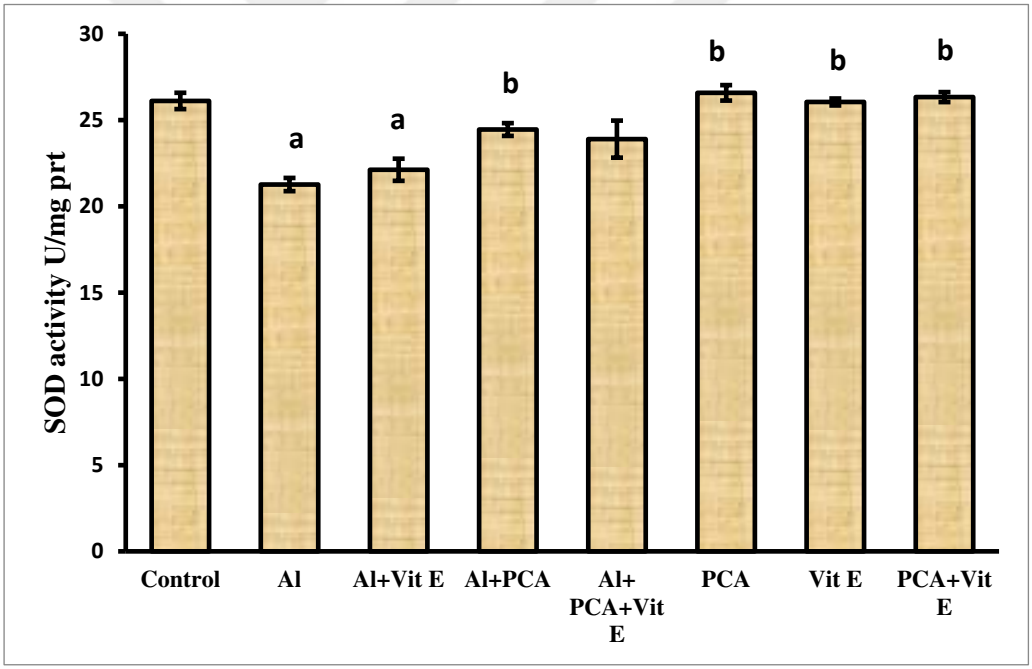

Figure 3.25. *SOD activity U/mg prt*

Table 3.2. *CAT activity results (Mean ± standard error)*

Experimental Group	CAT activity (U/mg prt)	
Control	79.7491 ± 2.670826	
Al	27.5078 ± 0.305453	(a)
Al+Vit E	39.2539 ± 2.65782	(a,b)
Al+PCA	58.3208 ± 2.528469	(a,b,c)
Al+PCA+Vit E	29.2105 ± 0.204594	(a,c,d)
PCA	80.9979 ± 2.292499	(b,c,d,e)
Vit E	80.2534 ± 2.050767	(b,c,d,e)
PCA+Vit E	80.4729 ± 0.503031	(b,c,d,e)

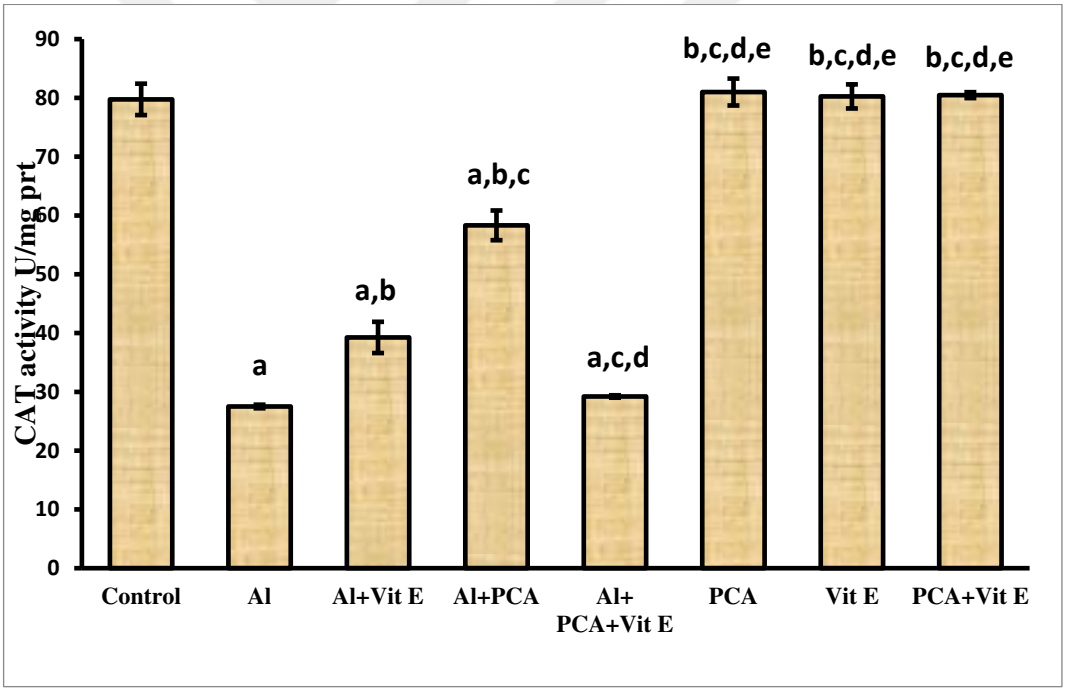

Figure 3.26. *CAT activity U/mg prt*

Table 3.3. *GSH results (Mean ± standard error)*

Experimental Group	GSH (U/mg prt)	
Control	44.834 ± 0.090208	
Al	22.422 ± 0.440922	(a)
Al+Vit E	39.980 ± 0.347349	(a,b)
Al+PCA	34.037 ± 0.280624	(a,b,c)
Al+PCA+Vit E	26.608 ± 0.21917	(a,b,c,d)
PCA	44.155 ± 0.66546	(b,c,d,e)
Vit E	43.134 ± 0.60216	(b,c,d,e)
PCA+Vit E	44.140 ± 0.26846	(b,c,d,e)

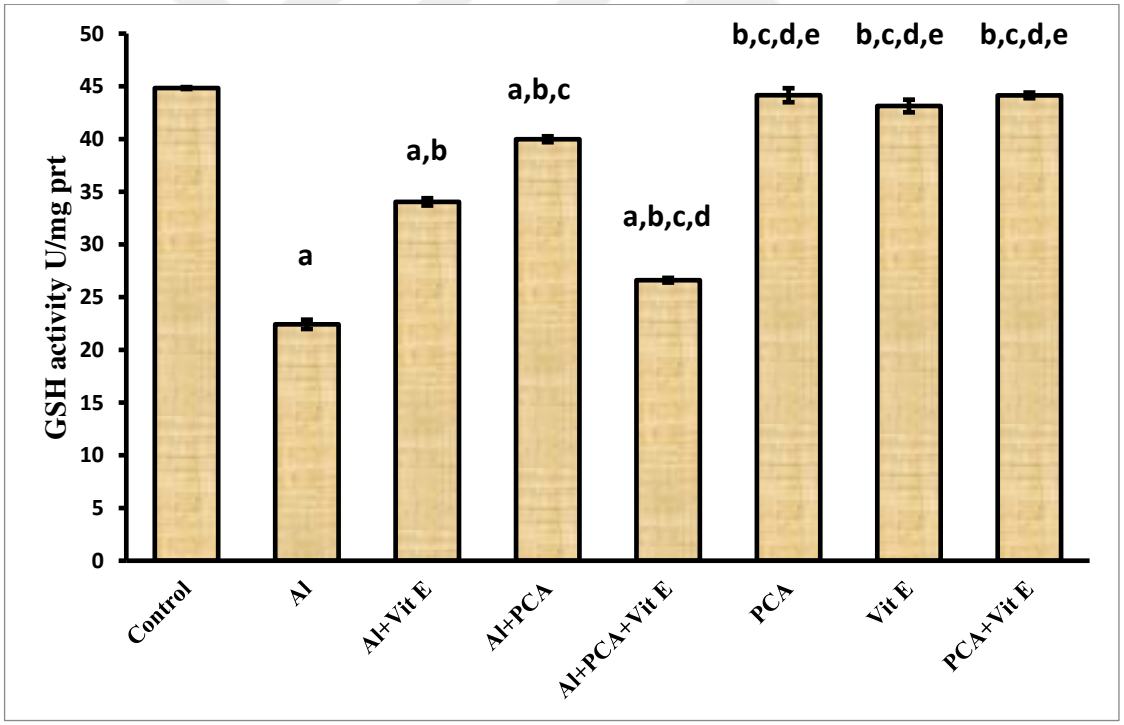

Figure 3.27. *Total GSH (U/mg prt)*

Table 3.4. *GST activity results (Mean ± standard error)*

Experimental Group	GST (U/mg prt)	
Control	29,865 ± 0.219829	
Al	6,677 ± 0.166279	(a)
Al+Vit E	17,741 ± 0.173636	(a,b)
Al+PCA	19,296 ± 0.177476	(a,b,c)
Al+PCA+Vit E	13,120 ± 0.084971	(a,b,c,d)
PCA	29,811 ± 0.172784	(b,c,d,e)
Vit E	29,356 ± 0.262331	(b,c,d,e)
PCA+Vit E	29,336 ± 0.283851	(b,c,d,e)

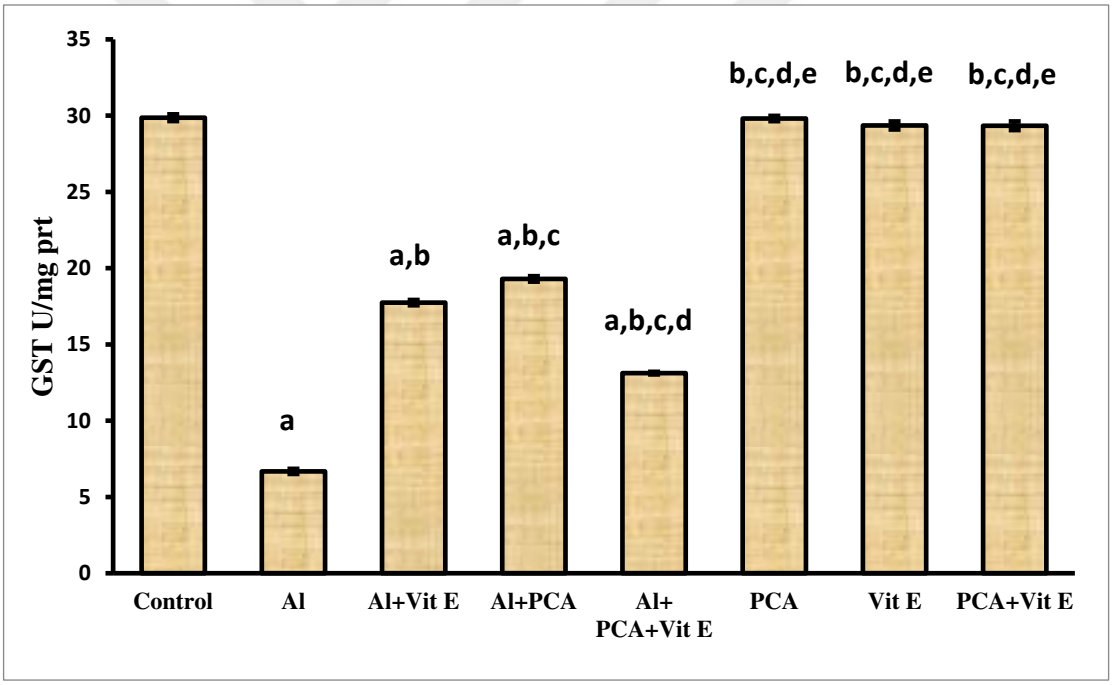

Figure 3.28. *GST activity U/mg prt*

Table 3.5. *MDA results (Mean ± standard error)*

Experimental Group	MDA (nmol/mg prt)	
Control	16.618 ± 0.00157	
Al	45.890 ± 0.00583	(a)
Al+Vit E	21.345 ± 0.00233	(a,b)
Al+PCA	36.8 ± 0.001726	(a,b,c)
Al+PCA+Vit E	32.254 ± 0.00356	(a,b,c,d)
PCA	16.2545 ± 0.0018	(b,c,d,e)
Vit E	17.3454 ± 0.0025	(b,c,d,e)
PCA+Vit E	16.0727 ± 0.0016	(b,c,d,e)

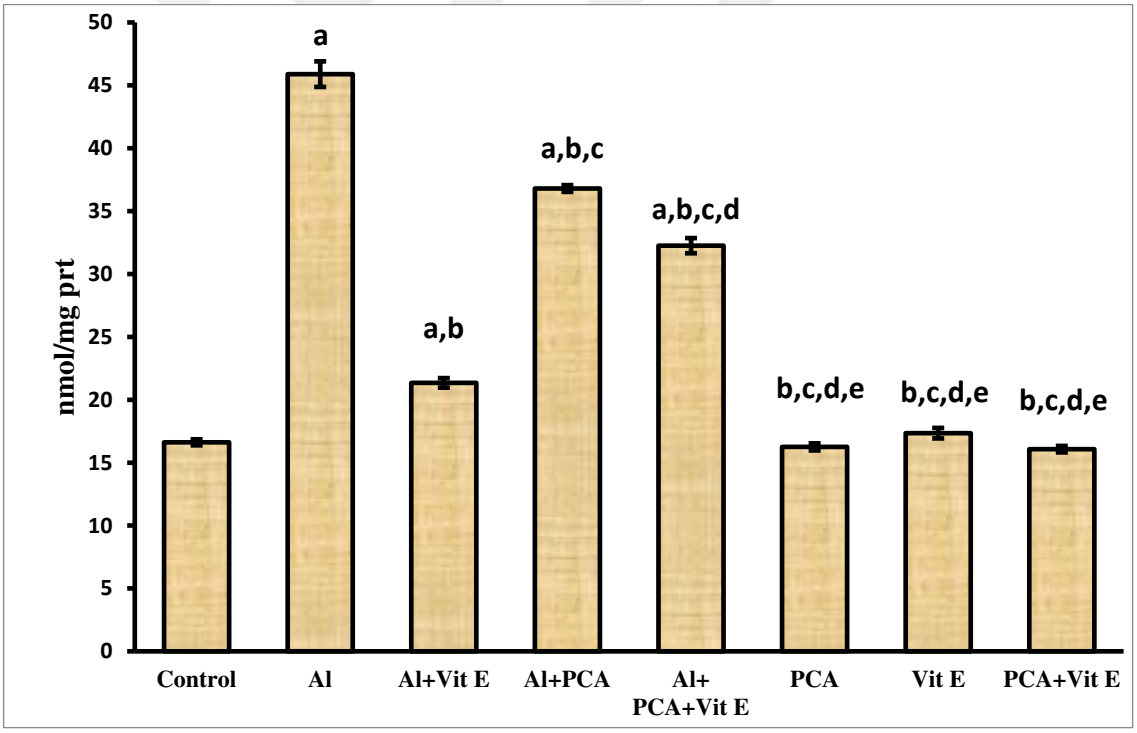

Figure 3.29. *MDA levels nmol/mg prt*

Table 3.6. *Bradford protein level results (Mean ± standard error)*

Experimental Group	Protein concentration (mg/ml)
Control	2.376865 ± 0.029232
Al	2.191058 ± 0.02821
Al+Vit E	2.234708 ± 0.057184
Al+PCA	2.009084 ± 0.044754
Al+PCA+Vit E	1.924733 ± 0.042359
PCA	2.112311 ± 0.024066
Vit E	1.989913 ± 0.035761
PCA+Vit E	2.217897 ± 0.022917

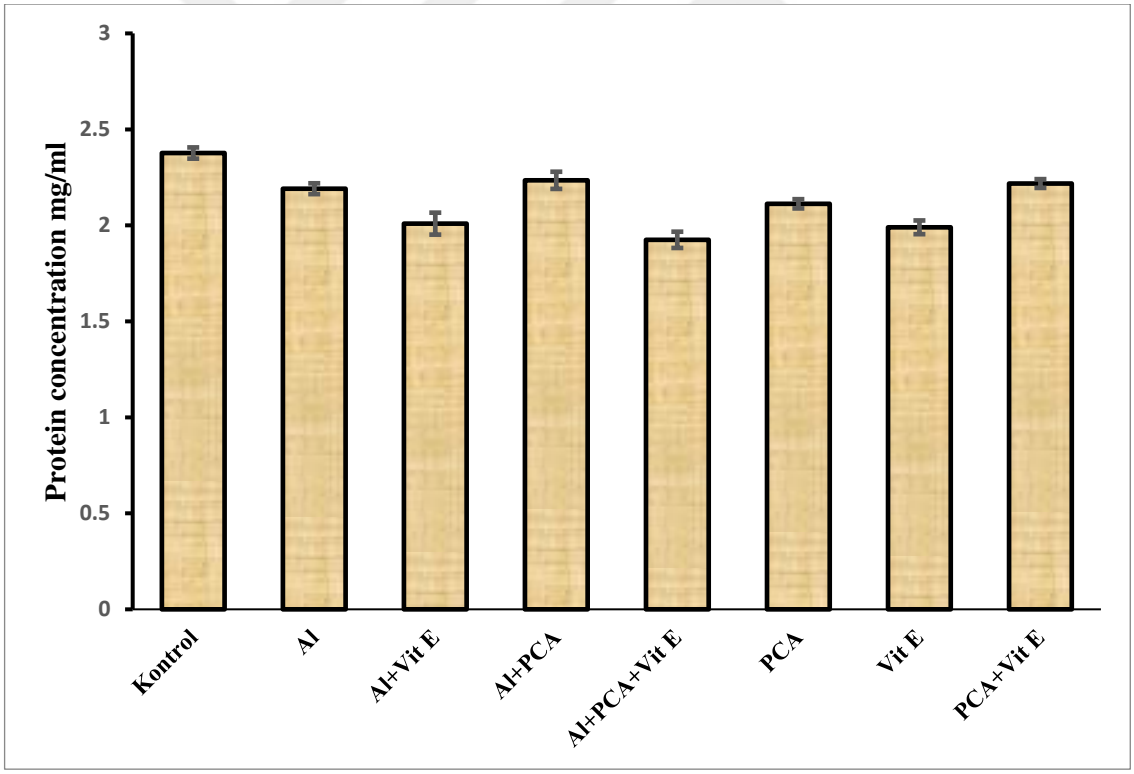

Figure 3.30. *Bradford protein levels in different experimental groups*

Figure 3.25-3.30 biochemical evaluation of liver antioxidant enzymes and lipid peroxidation levels in the different experimental groups. SOD activity (U/mg/prt) (Fig 3.25). CAT activity (CAT U/mg prt) (Fig 3.26). Total GSH (U/mg prt) (Fig 3.27). GST activity (Fig 3.28). MDA level (nmol/mg/prt) (Fig 3.29). Bradford protein level (nm/mg) (Fig 3.30). Control (0.9% saline i.p.); Al: Al administrated animals (70 mg/kg/day for 28 days i.p.); Al+PCA: Al and PCA administrated animals (70 mg/kg/day for 28 days i.p from day 28 for 14 days PCA 5mg/kg/day was administrated i.p.); Al+Vit E: Al (dose of 70 mg/kg/day for 28 days was administrated i.p.) from day 28 for 14 days Vit E with dose of 50 mg/kg/day was administrated i.p., Al+PCA+Vit E (Al 70 mg/kg/day for 28 days i.p from day 28 for 14 days PCA 5mg/kg/day and Vit E with dose of 50 mg/kg/day); was administrated to the animals in the group. a significant difference compared to control group ($P< 0.05$); b Significant differences compared to the Al group ($P< 0.05$); c Significant differences compared to the Al+Vit E group ($P< 0.05$). d Significant differences compared to Al+PCA ($P<0.05$). e Significant differences compared to Al+PCA+Vit E ($P< 0.05$). All data were expressed as mean ± SE. Statistical analysis were performed with SPSS package program and using one-way ANOVA followed by Dunnett T3 test as post hoc test.

3.3. Hepatic DNA Damage Evaluation (Comet Assay)

Illustrative photographs of the comet assay results showing DNA damage in hepatocytes of the experimental animal groups represent head and tail moments (Figure 3.31-3.38). Control group shows normal hepatocytes (Figure 3.31). Significant DNA damage was observed in Al-treated animals compared to the control group (Figure 3.32). DNA damage was reduced by approximately 58.7% in the Al+Vit E treated animals compared to the Al treated group. In the Al+PCA+Vit E group, DNA damage was also significantly reduced compared to the Al group (49.30%) (Figure 3.33). A significant difference was observed compared to the Al+Vit E groups. However, no significant difference was observed compared to the Al+PCA group. Following the same pattern, a significant reduction in DNA damage was observed in Al+PCA treated animals with respect to tail moment (44.27%) (Figure 3.34). Statistical analysis showed that Vit E had a greater effect on DNA damage recovery than PCA, as a significant difference was observed between the Al+Vit E and Al+PCA animal groups in tail movement in favor of the Al+Vit E group. The Al+PCA animal group showed an insignificant difference compared to Al+PCA+Vit E, while a significant difference was observed between the

Al+Vit E and Al+PCA+Vit E groups. The graph shows that the PCA, Vit E, and PCA+Vit E groups had no significant differences compared to the control group.

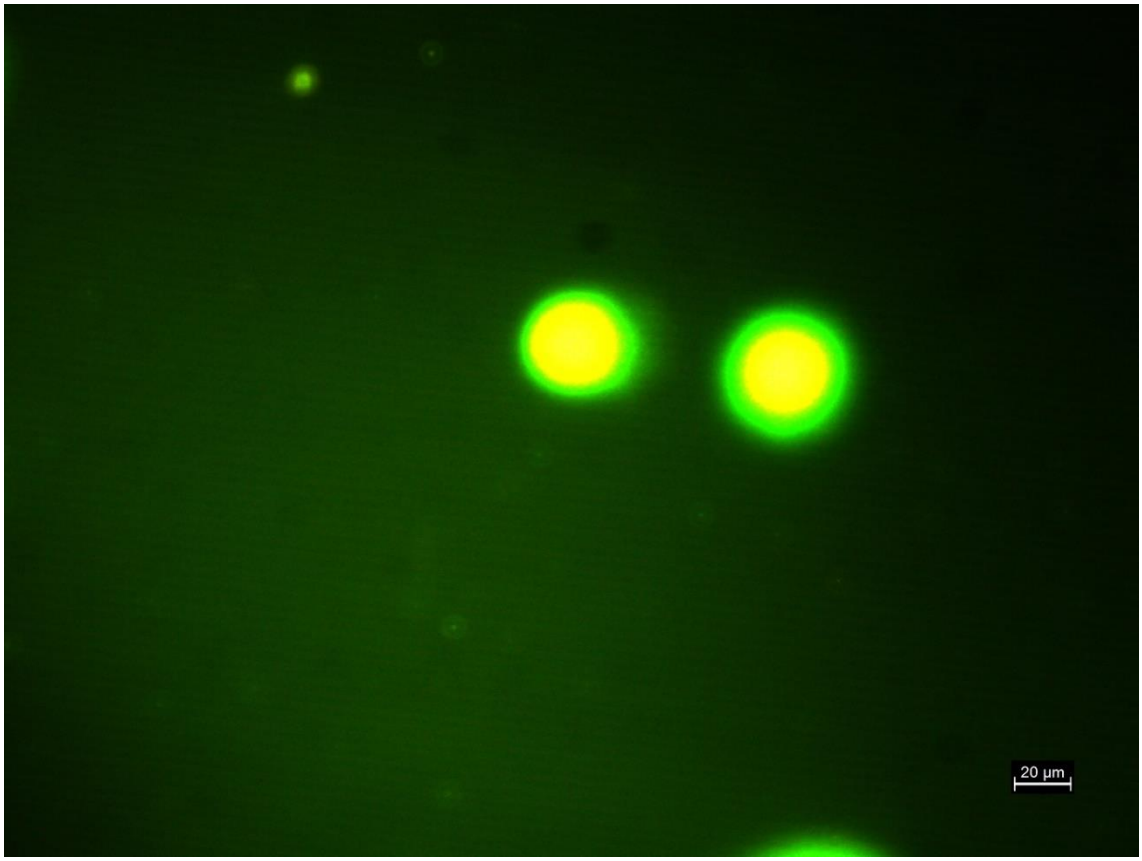

Figure 3.31. *Control group.Single-cell gel electrophoresis imaging. Scale 20 µm*

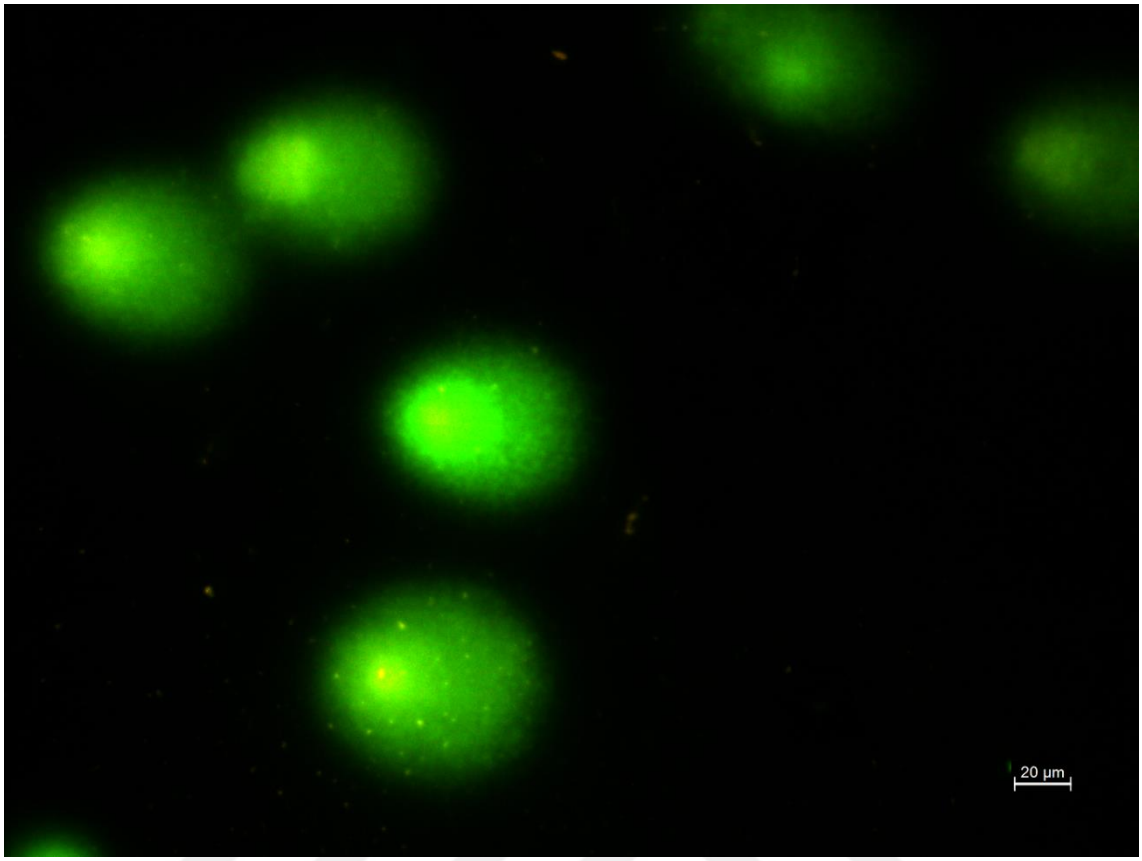

Figure 3.32. *Al group. Single-cell gel electrophoresis imaging. Scale 20 µm*

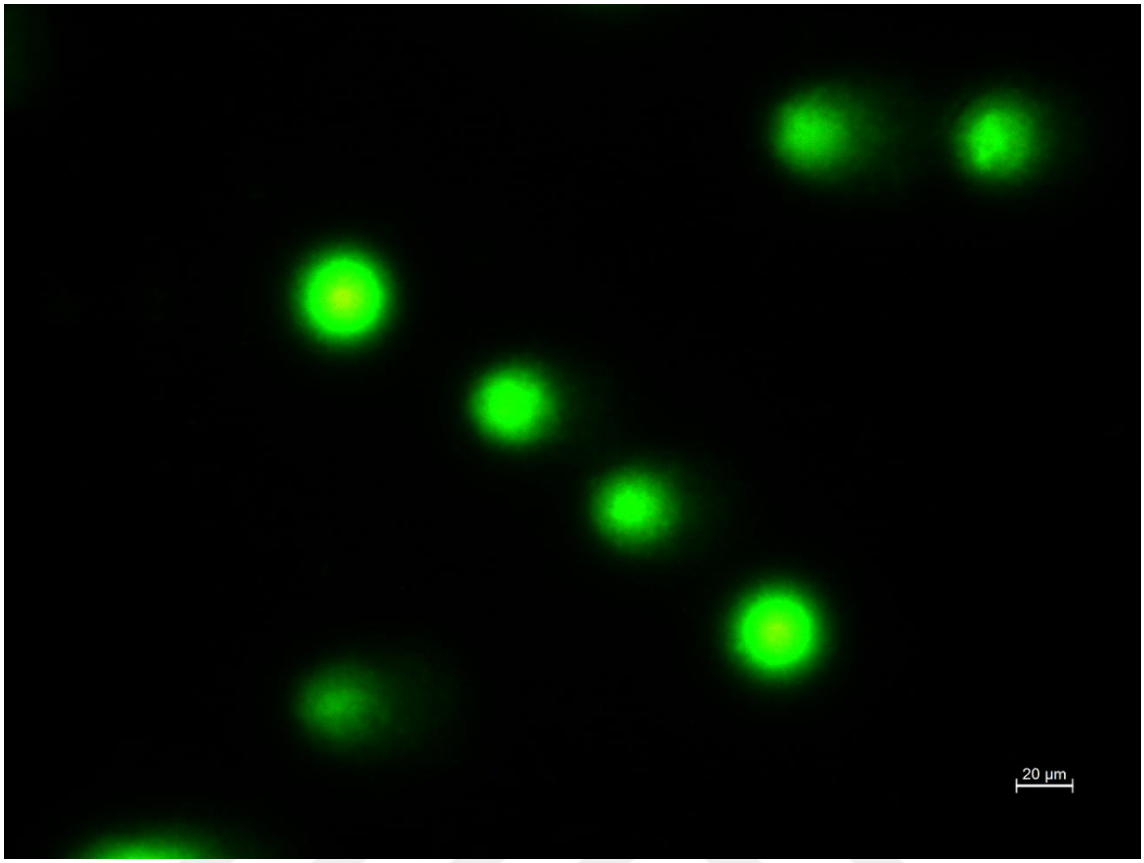

Figure 3.33. *Al+Vit E group. Single-cell gel electrophoresis imaging. Scale 20 µm*

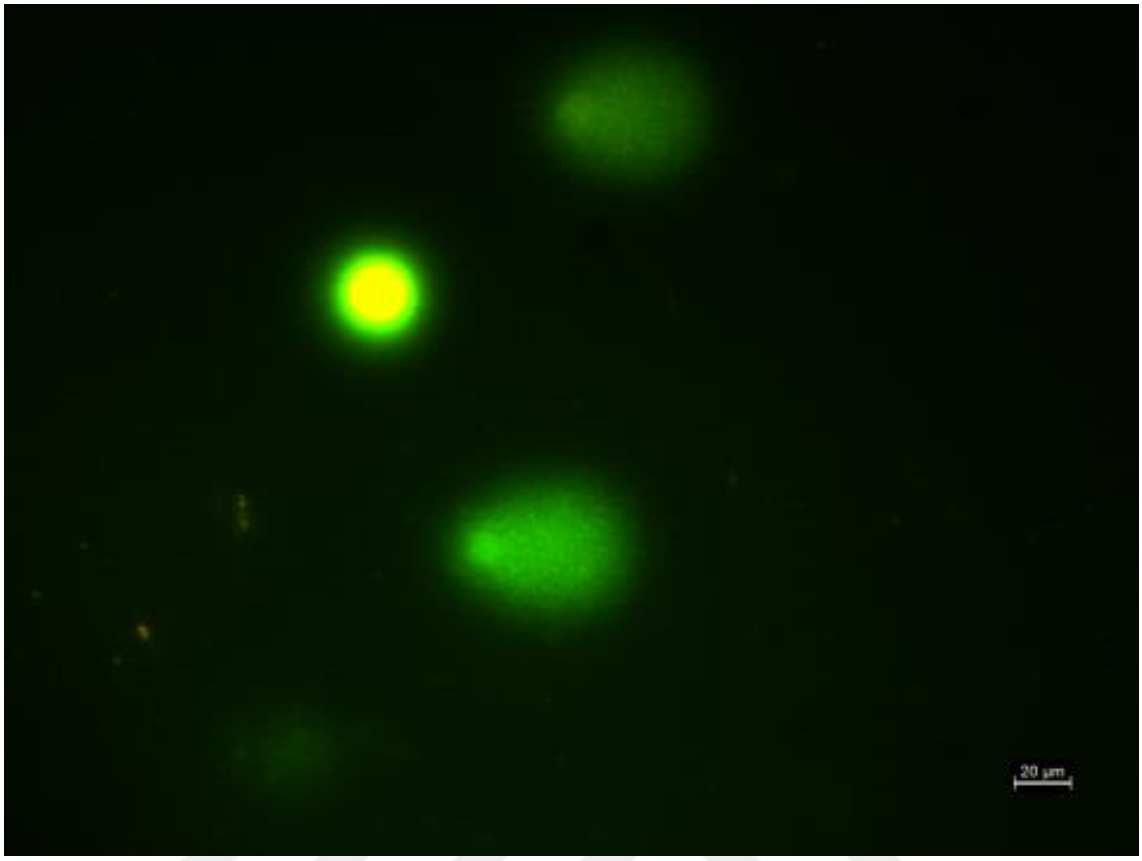

Figure 3.34. *Al+PCA group. Single-cell gel electrophoresis imaging. Scale 20 µm*

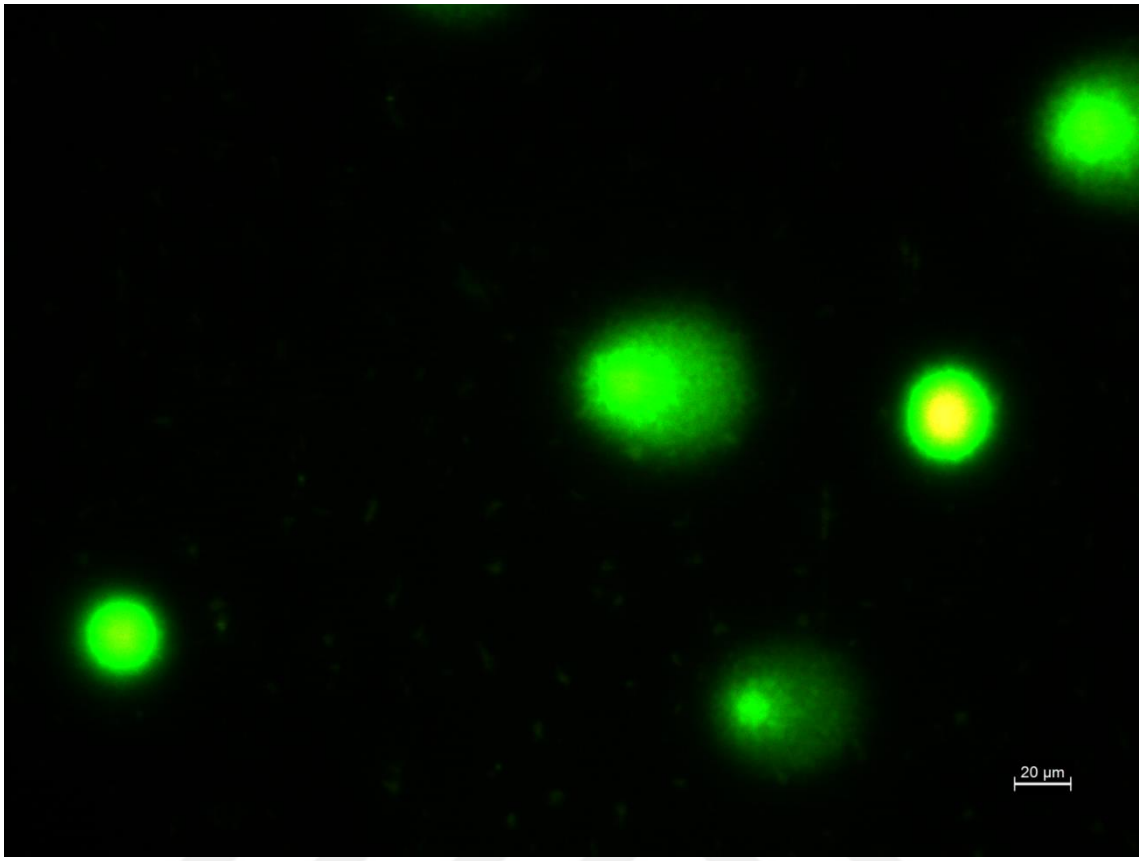

Figure 3.35. *Al+PCA+Vit E group. Single-cell gel electrophoresis imaging. Scale 20 µm*

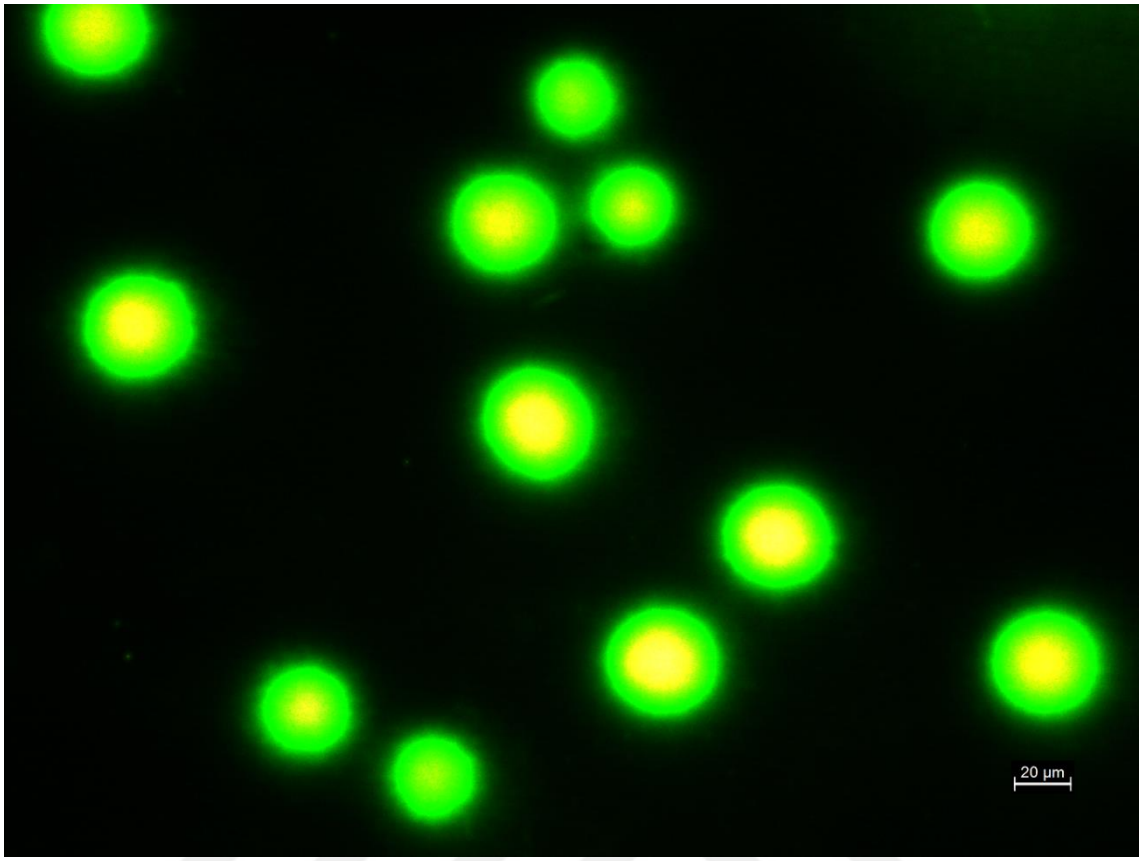

Figure 3.36. *PCA group. Single-cell gel electrophoresis imaging. Scale 20 µm*

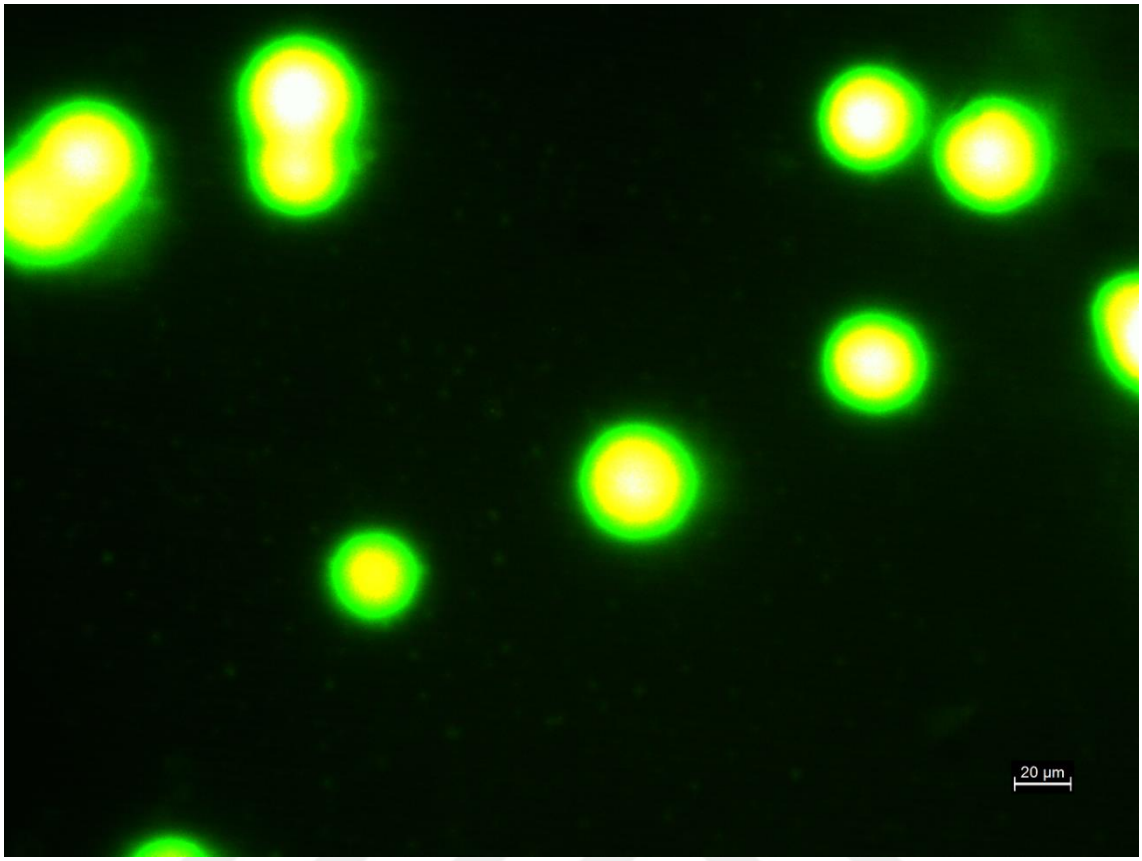

Figure 3.37. *Vit E group. Single-cell gel electrophoresis imaging. Scale 20 µm*

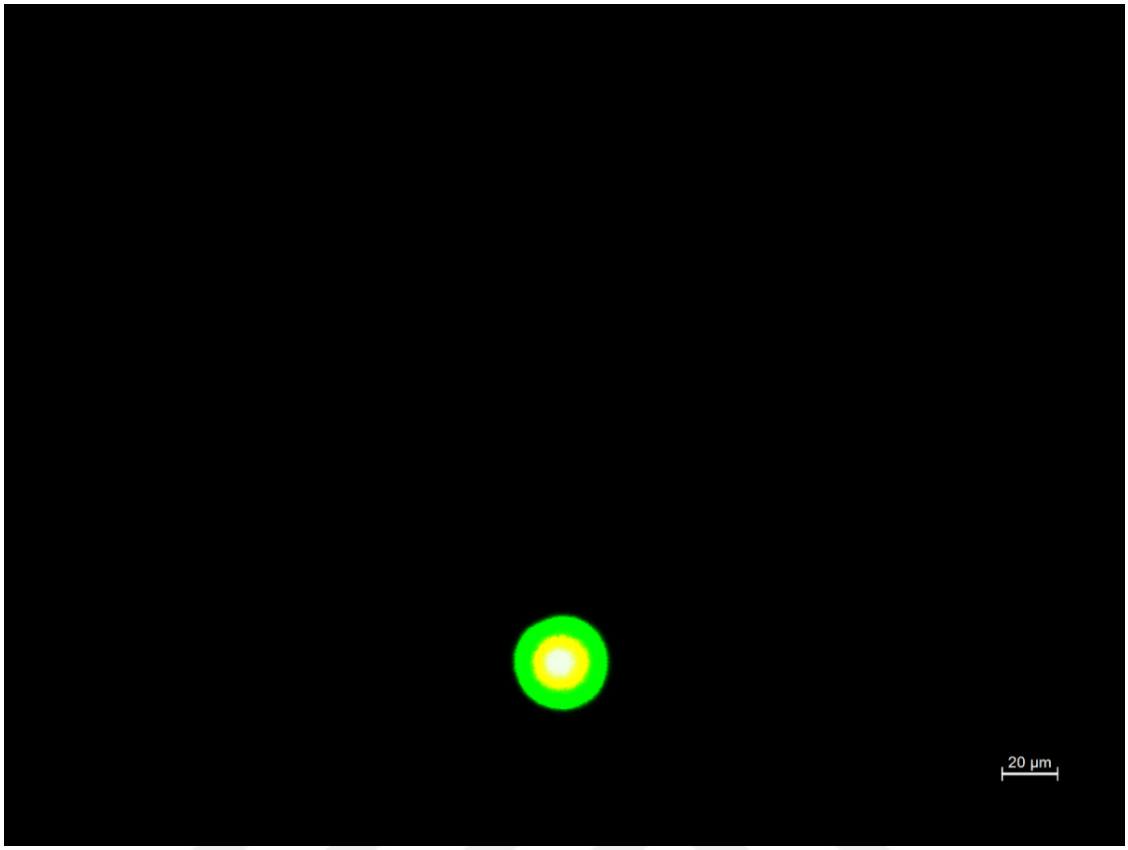

Figure 3.38. *PCA+Vit E group. Single-cell gel electrophoresis imaging. Scale 20 µm*

Figure 3.31-3.38 representative photographs of comet assay results showing DNA damage assessment in liver tissues from different groups. Control (Figure 3.31) (0.9% saline i.p.). Al- administrated animals ($AlCl_3$ 70 mg/kg/day for 28 days, then 0.9% saline i.p. administrated) (Figure 3.32). Al+Vit E administrated animals ($AlCl_3$ 70 mg/kg/day for 28 days i.p., then 50mg/kg/day Vit E was administrated i.p. for 14 days) (Figure 3.33). Al+PCA administrated animals ($AlCl_3$ 70 mg/kg/day for 28 days i.p., then 5mg/kg/day PCA was administrated i.p. for 14 days) (Figure 3.34) Al+PCA+Vit E administrated animals ($AlCl_3$ at a dose of 70 mg/kg/day was administrated i.p. for 28 days, then 5mg/kg/day PCA and 50mg/kg/day Vit E were administrated i.p. for 14 days) (Figure 3.35). PCA administrated animals (0.9% saline was administrated i.p. for 28 days, then 5mg/kg/day PCA was administrated i.p. for 14 days) (Figure 3.36). Vit E (0.9% saline was administrated for 28 days, then 50mg/kg/day Vit E was administrated i.p. for 14 days) (Figure 3.37). PCA+Vit E (0.9% saline was administrated i.p. for 28 days, then the 50mg/kg/day Vit E was administrated i.p. for 14 days) (Figure 3.38). Photographs of comets in control and experimental groups with representative head and tail moments.

Table 3.7. *Comet assay analysis represented by tail moment (Mean ± standard error)*

Experimental Group	Protein concentration (mg/ml)	
Control	1.859 ± 0.16397175	
Al	8.8088 ± 1.61538223	(a)
Al+Vit E	3.6376 ± 0.74653638	(a,b)
Al+PCA	4.9092 ± 1.26520119	(a,b,c)
Al+PCA+Vit E	4.4666 ± 0.9645293	(a,b,c,d)
PCA	1.7973 ± 0.22584817	(b,c,d,e)
Vit E	1.8279 ± 0.20781582	(b,c,d,e)
PCA+Vit E	1.7963 ± 0.23814119	(b,c,d,e)

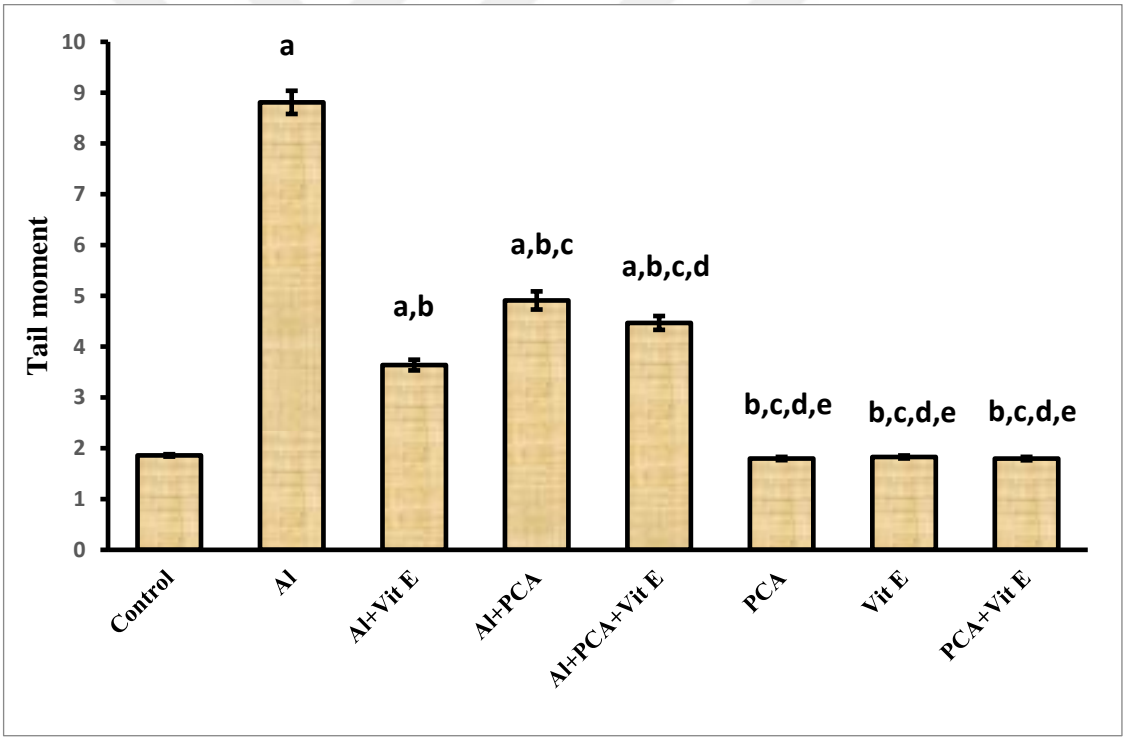

Figure 3.39. *Tail moment diagram.* ***a*** *Significant differences compared to the control group (P< 0.05);* ***b*** *differences compared to the Al group (P< 0.05);* ***c*** *Significant differences compared to Al+Vit E group (P< 0.05).* ***d*** *Significant differences compared to Al+PCA (P<0.05).* ***e*** *Significant differences compared to Al+Vit E+PCA (P< 0.05). All data were expressed as mean ± SE. Statistical analysis of groups was performed with the SPSS package program and using one way ANOVA followed by Dunnett T3 test as post hoc test.*

3.4. PCNA Immunofluorescence Evaluation

There was no observable immunofluorescence signal of PCNA in control group of animals. Fluorescence intensity was increased over three-fold in Al group of animals in the cytoplasm of Kupffer cells due to tissue damage. Al+Vit E group exhibit a significant decrease in fluorescence intensity in the cytoplasm of Kupffer cells of PCNA expression when compared with Al group (75%), however, it is still high when compared to the control group. Al+PCA group, show increased nuclear PCNA when compared to the control group, simultaneously, Kupffer cells exhibited lower fluorescence intensity when compared with Al group. In Al+PCA+Vit E group, the fluorescence signal of PCNA was higher at about 58.20% than the control group and lower than Al group of animals by 31.21%. In PCA, Vit E and PCA+Vit E groups, no nuclear PCNA fluorescence was detected.

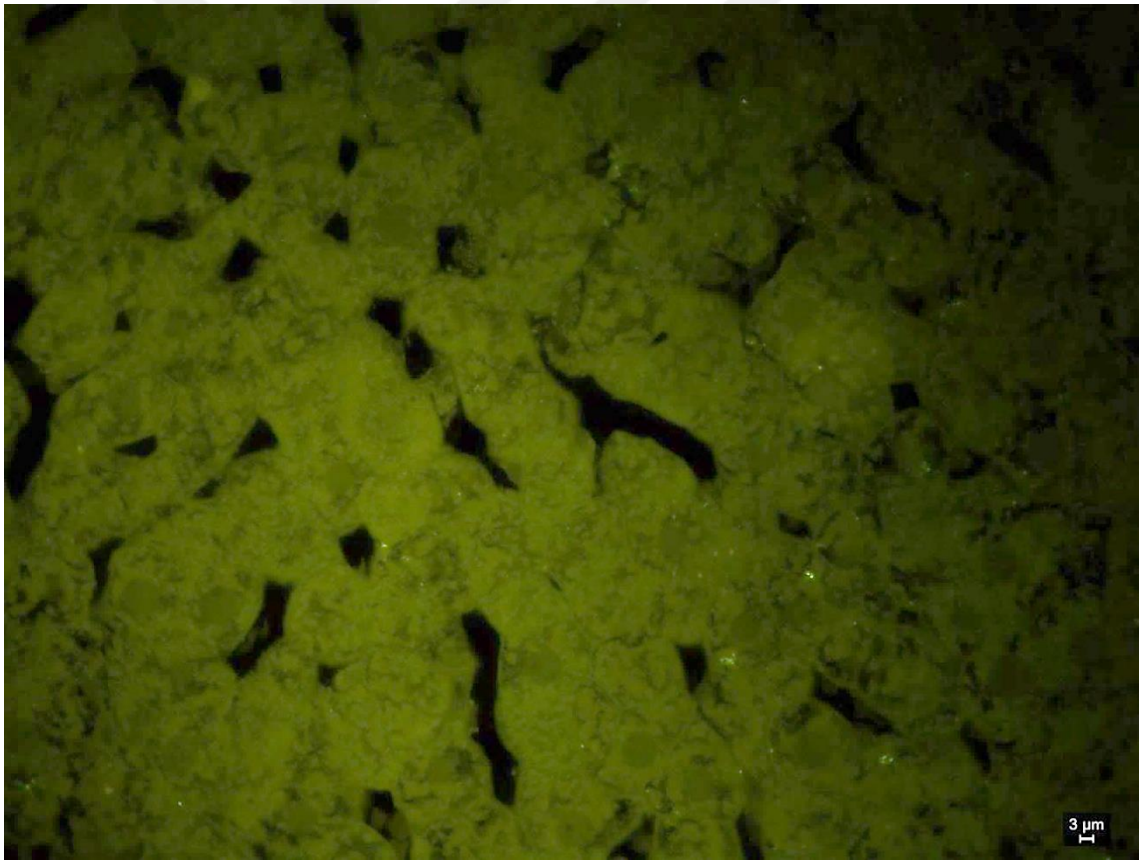

Figure 3.40. *Control group PCNA immunofluorescence labeling*

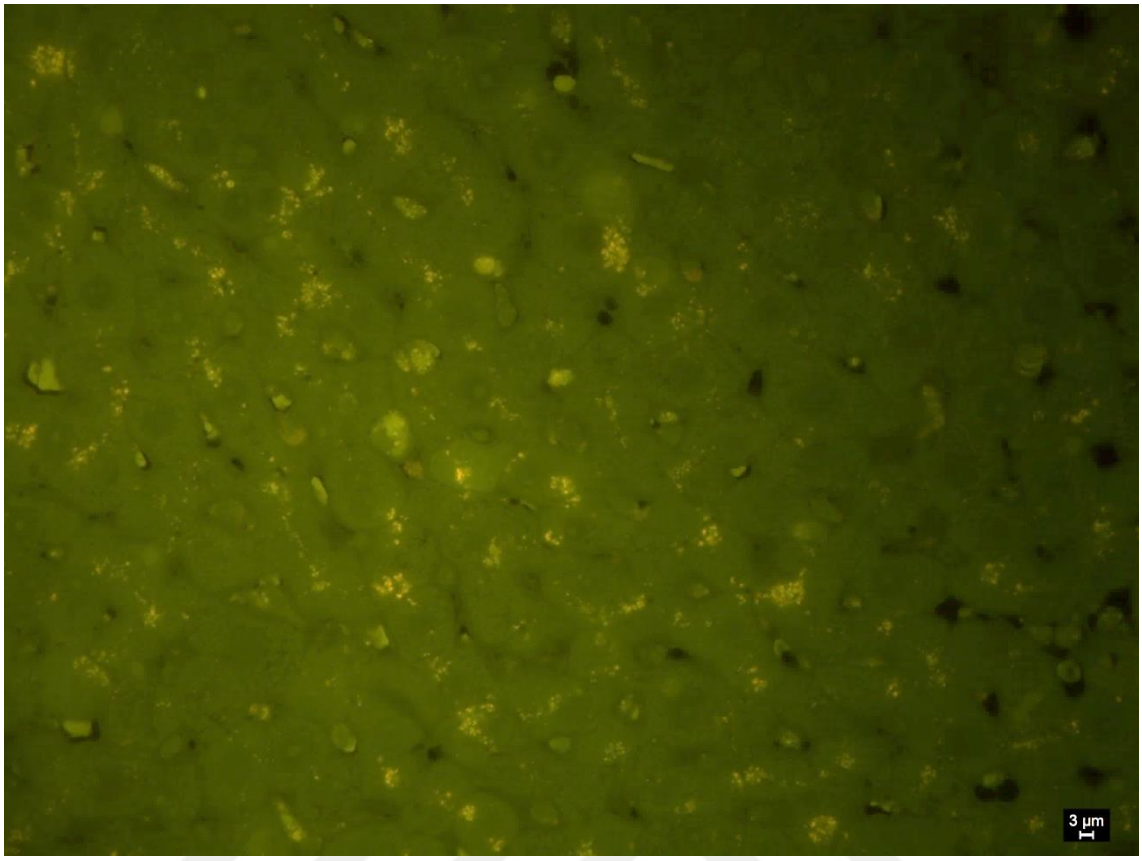

Figure 3.41. *Al-administrated group PCNA immunofluorescence labeling*

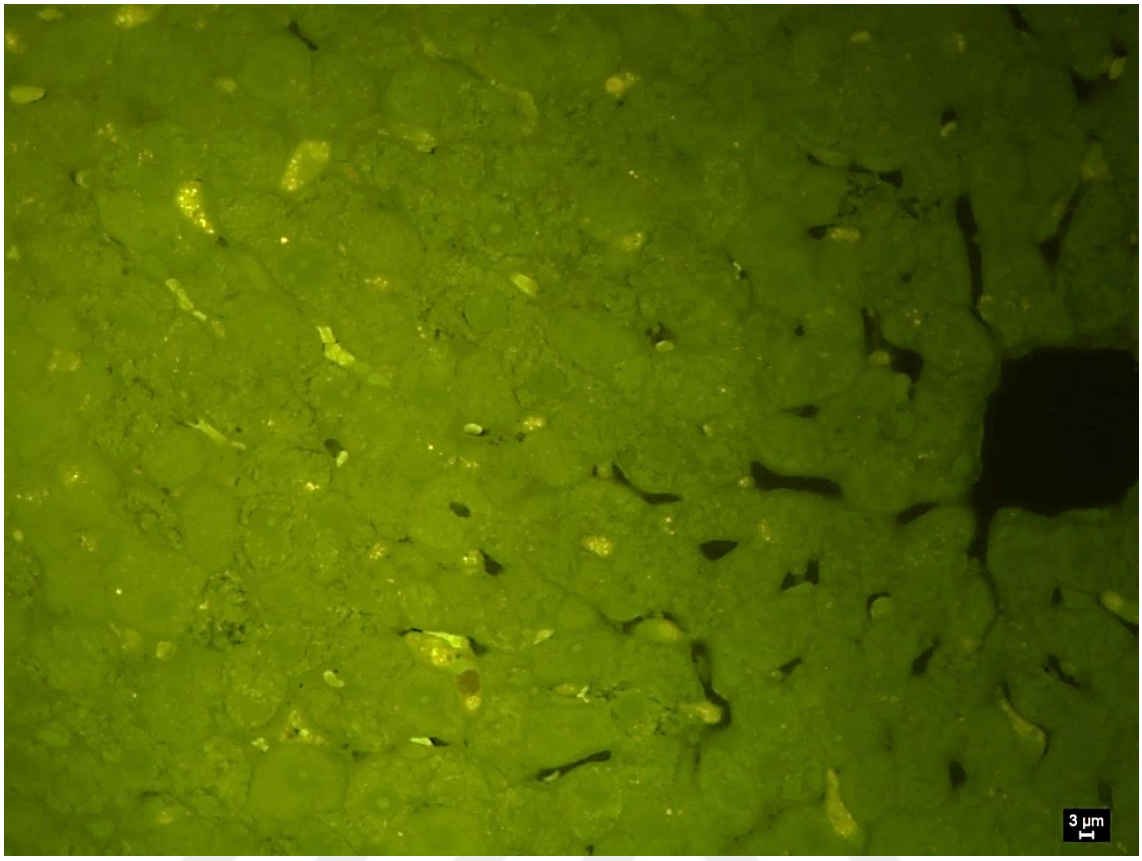

Figure 3.42. *Al+Vit E administrated group PCNA immunofluorescence labeling*

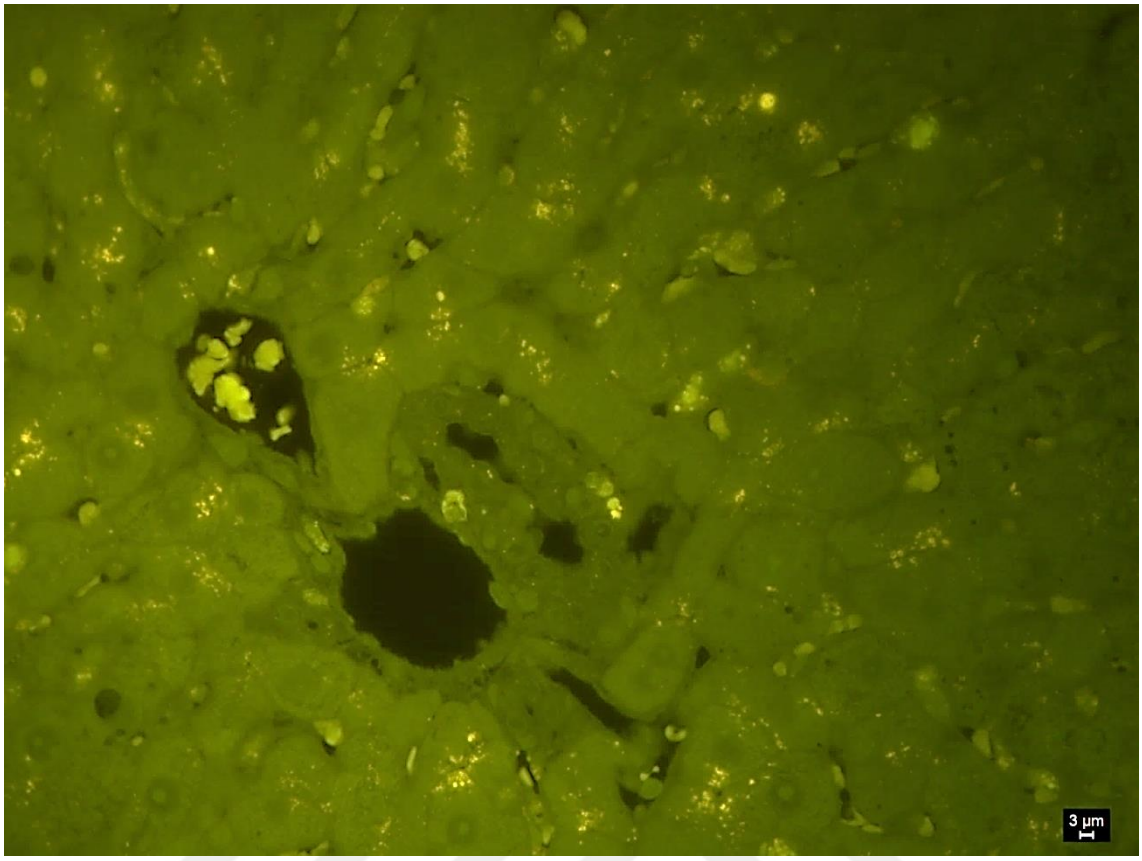

Figure 3.43. *Al+PCA administrated group PCNA immunofluorescence labeling*

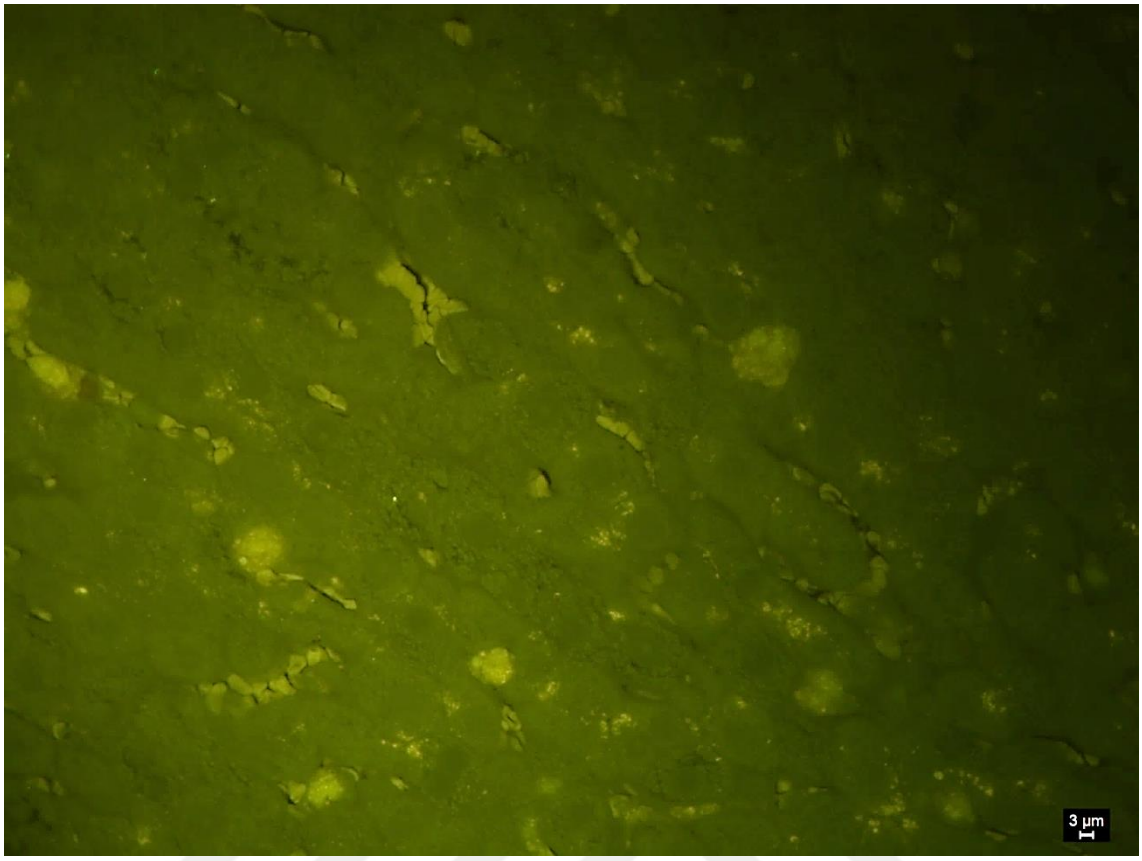

Figure 3.44. *Al+PCA+Vit E administrated group PCNA immunofluorescence labeling*

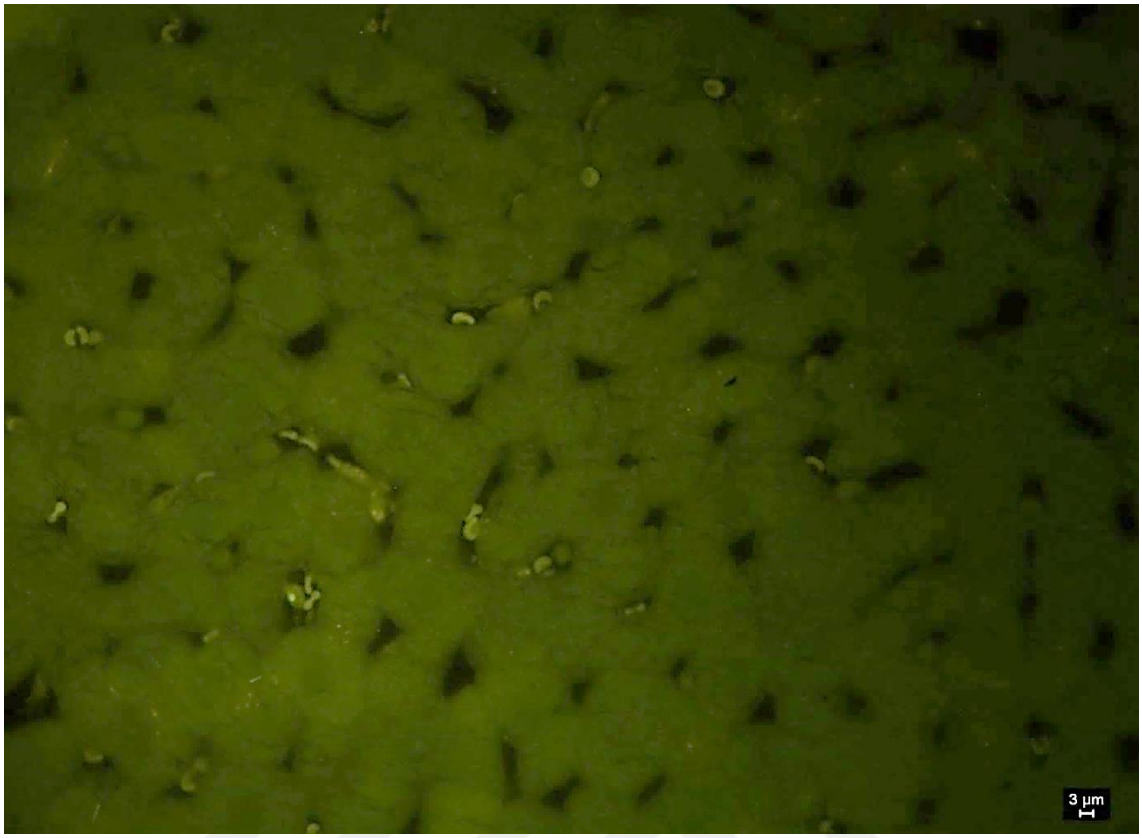

Figure 3.45. *PCA administrated group PCNA immunofluorescence labeling*

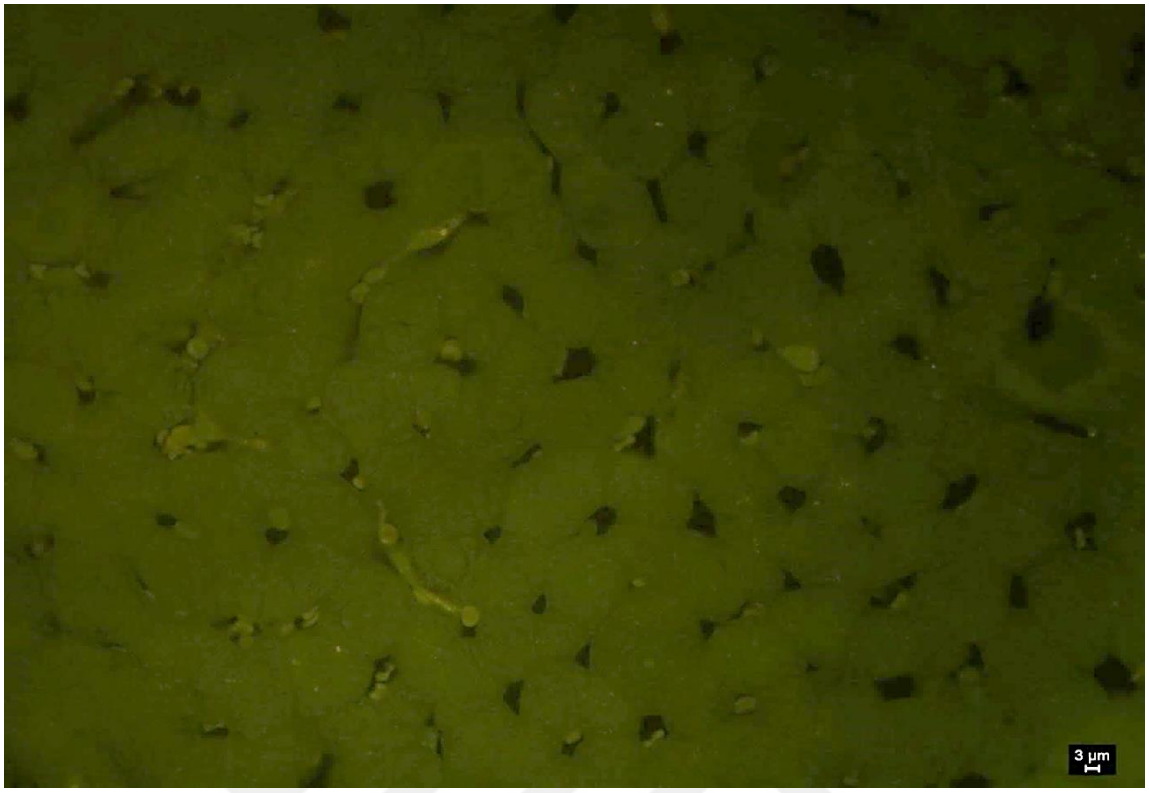

Figure 3.46. *Vit E administrated group PCNA immunofluorescence labeling*

Figure 3.47. *PCA+Vit E administrated group PCNA immunofluorescence labeling*

Figure 3.40-3.47. PCNA immunofluorescence labeling in liver tissue: Control (9% saline i.p.) liver with no observable nuclear signal of PCNA (Figure 3.40). Al administrated animals (70 mg/kg/day i.p.) showing significant increase in PCNA immunofluorescence (Figure 3.41). Al+Vit E administrated animals (70 mg/kg/day for 28 days then Vitamin E 50mg/kg/day for 14 days i.p.) significant reduction in PCNA immunofluorescence was detected (Figure 3.42). Al+PCA pre-administrated animals (70 mg/kg/day for 28 days i.p. PCA 5 mg/kg/day for 14 days i.p) showing increased PCNA immunofluorescence (Figure 3.43). Al+PCA+Vit E group (70 mg/kg/day for 28 days i.p. PCA 5 mg/kg/day and Vit E 50 mg/kg/day for 14 days i.p). characterized with reduction in PCNA immunofluorescence (Figure 3.44). PCA (0,9% saline for 28 days i.p then PCA 5mg/kg/day for 14 days i.p) (Figure 3.45), G) Vit E (0,9% saline for 28 days then Vitamin E 50mg/kg/day for 14 days i.p) (Figure 3.46) and PCA+Vit E (0,9% saline for 28 days then PCA 5mg/kg/day and 50mg/kg/day for 14 days i.p) (Figure 3.47) groups no nuclear immunofluorescence was detected.

Table 3.8. *PCNA immunofluorescence analysis represented by fluorescence intensity (Mean ± standard error)*

Experimental Group	PCNA fluorescence intensity (mg/ml)	
Control	12.4757 ± 1.292551	
Al	43.3681 ± 1.411314	(a)
Al+Vit E	24.7768 ± 1.687495	(a,b)
Al+PCA	33.9227 ± 1.764125	(a,b,c)
Al+PCA+Vit E	29.8308 ± 1.979508	(a,b,c,d)
PCA	12.5236 ± 1.0207	(b,c,d,e)
Vit E	12.6315 ± 1.884863	(b,c,d,e)
PCA+Vit E	12.9007 ± 1.735123	(b,c,d,e)

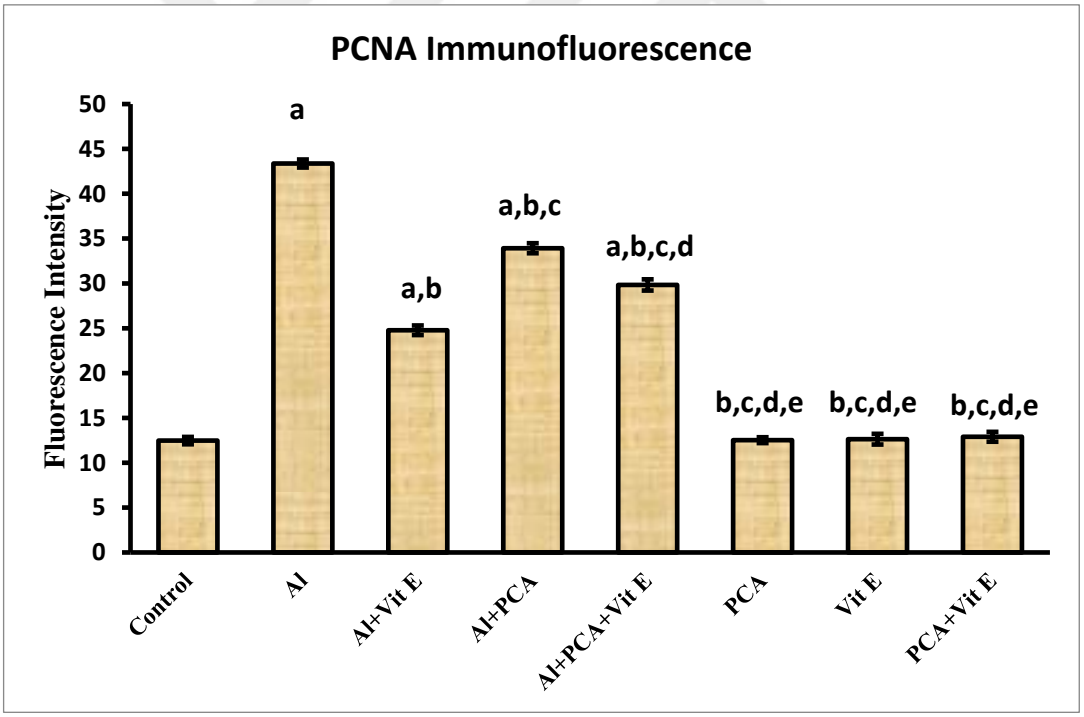

Figure 3.48. *Showing the comparison of fluorescence intensity between control and experimental groups.* ***a*** *Significant differences compared to control group (P< 0.05);* ***b*** *Significant differences compared to Al group (P< 0.05);* ***c*** *Significant differences compared to Al+Vit E group (P<0.05).* ***d*** *Significant differences compared to Al+PCA (P<0.05).* ***e*** *Significant differences compared to Al+Vit E+PCA (P< 0.05). All data were expressed as mean ± SE. Statistical analysis of groups were performed with SPSS package program and using one-way ANOVA followed by Dunnett T3 test as post hoc test.*

3.5. BRCA1 Immunofluorescence Evaluation

There was no observable nuclear signal of BRCA1 in the control group sections. Fluorescence intensity was increased over two-fold in the Al- administrated group of animals in the cytoplasm of Kupffer cells due to tissue damage. Al+Vit E group exhibited a significant decrease in fluorescence intensity of BRCA1 expression in the cytoplasm of Kupffer cells when compared with Al group (62.5%), however, it remained high by 7.5% when compared to the control group. Al+PCA group, exhibited increased nuclear BRCA1 fluorescence intensity when compared to the control group. In Al+PCA+Vit E group, the fluorescence signal of BRCA1 was higher about 26% than in the control group and lower than the Al group of animals by 28.35%. In PCA, Vit E and PCA+Vit E groups, no nuclear BRCA1 immunofluorescence was detected.

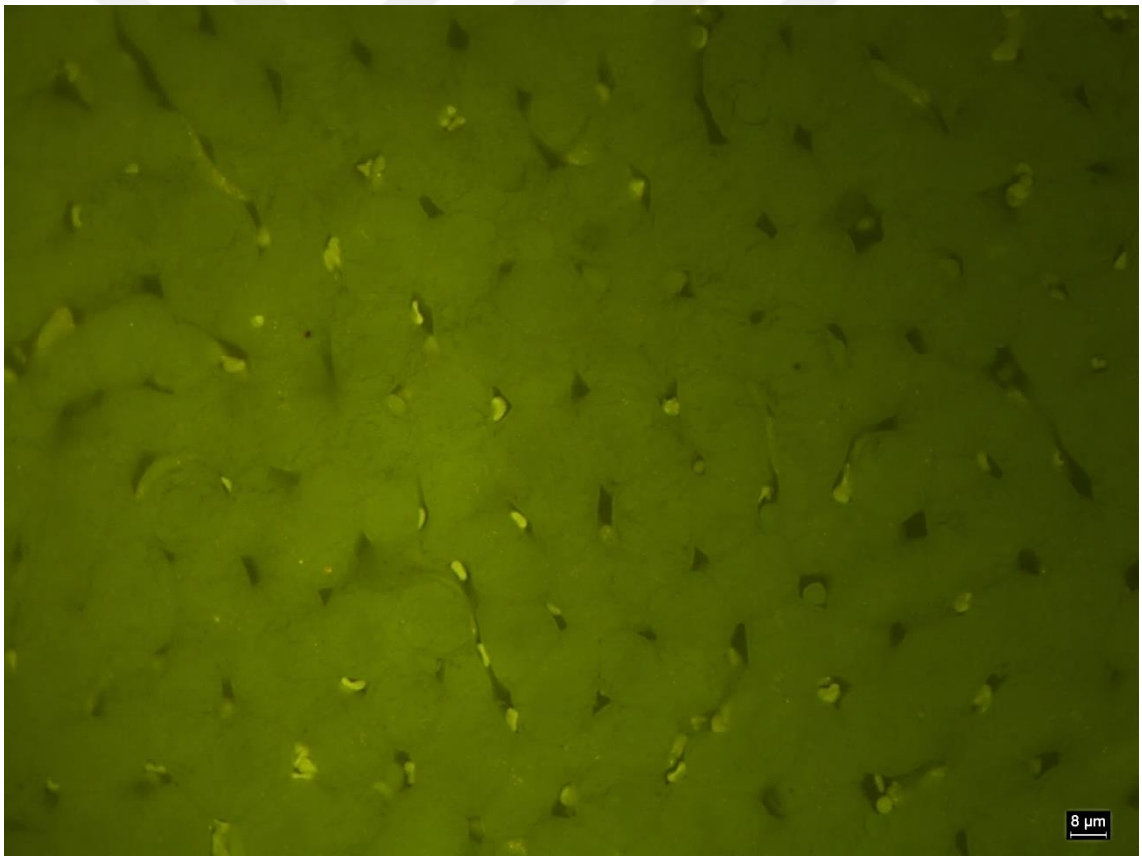

Figure 3.49. *Control group BRCA1 immunofluorescence labeling*

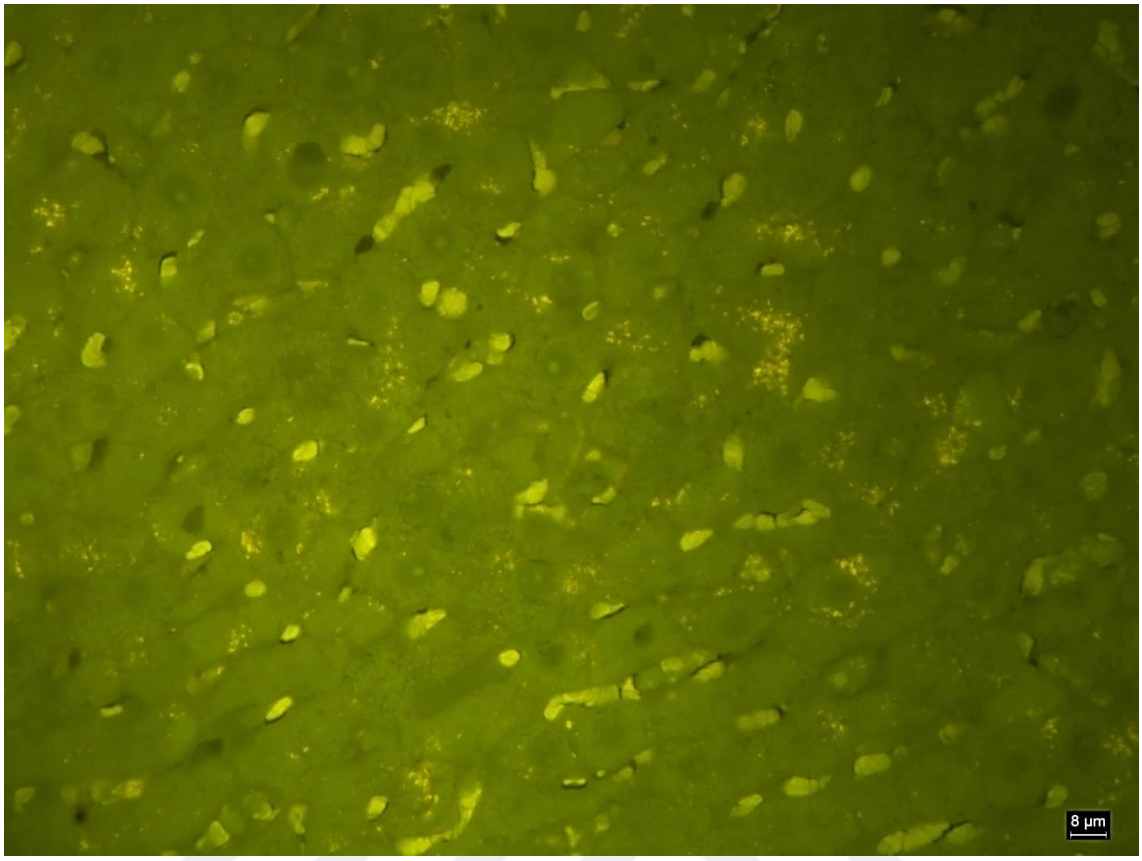

Figure 3.50. *Al-administrated group BRCA1 immunofluorescence labeling*

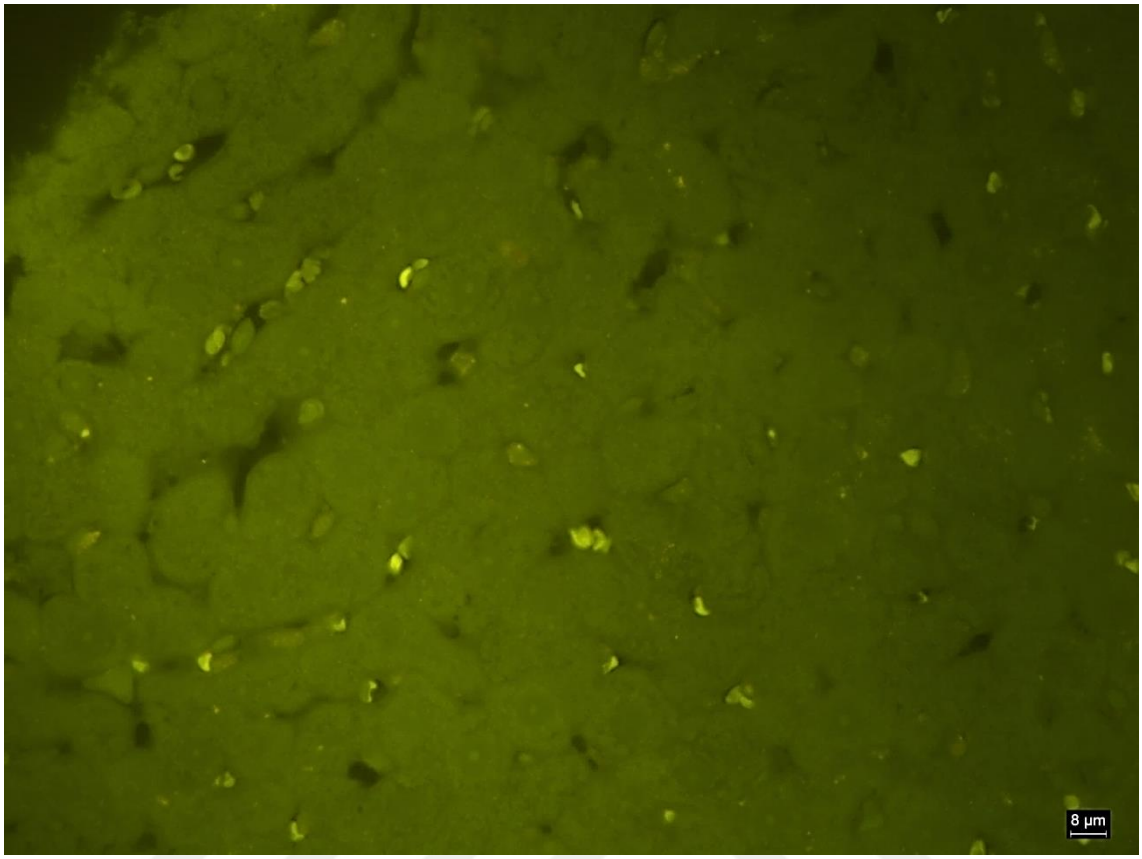

Figure 3.51. *Al+Vit E administrated group BRCA1 immunofluorescence labeling*

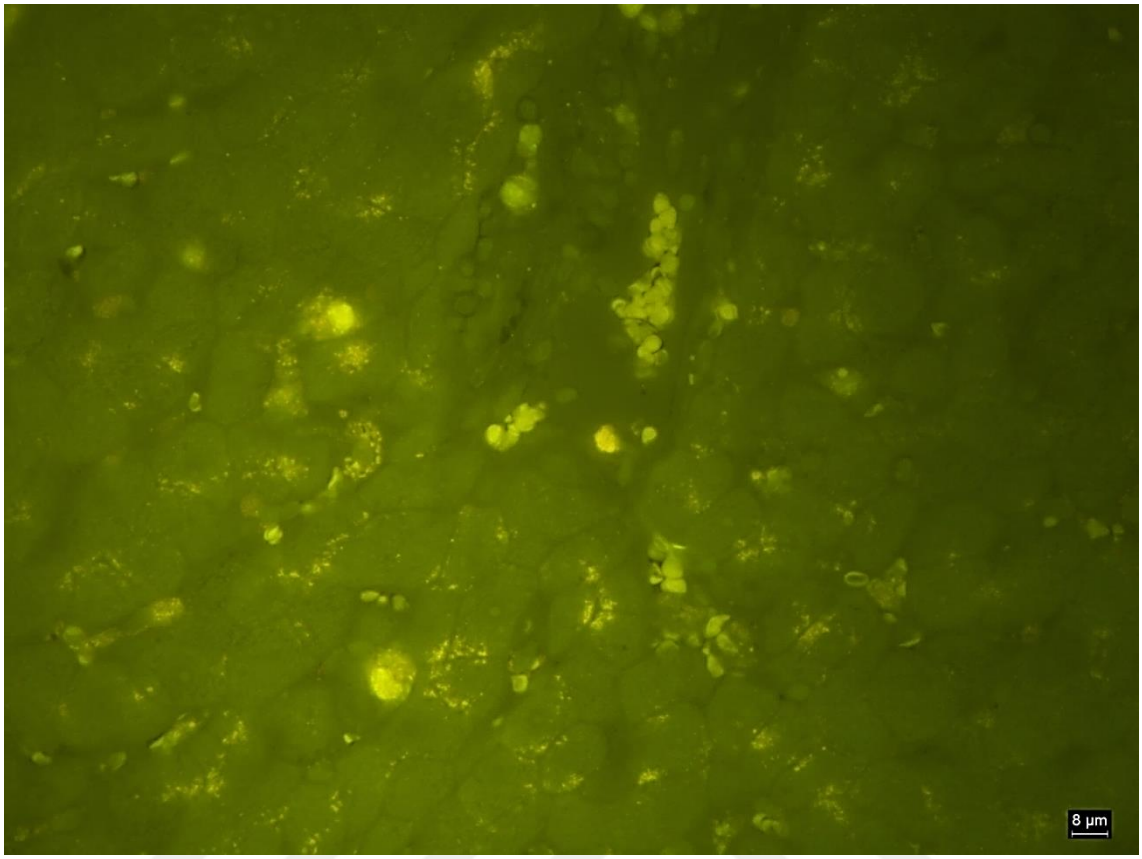

Figure 3.52. *Al+PCA administrated group BRCA1 immunofluorescence labeling*

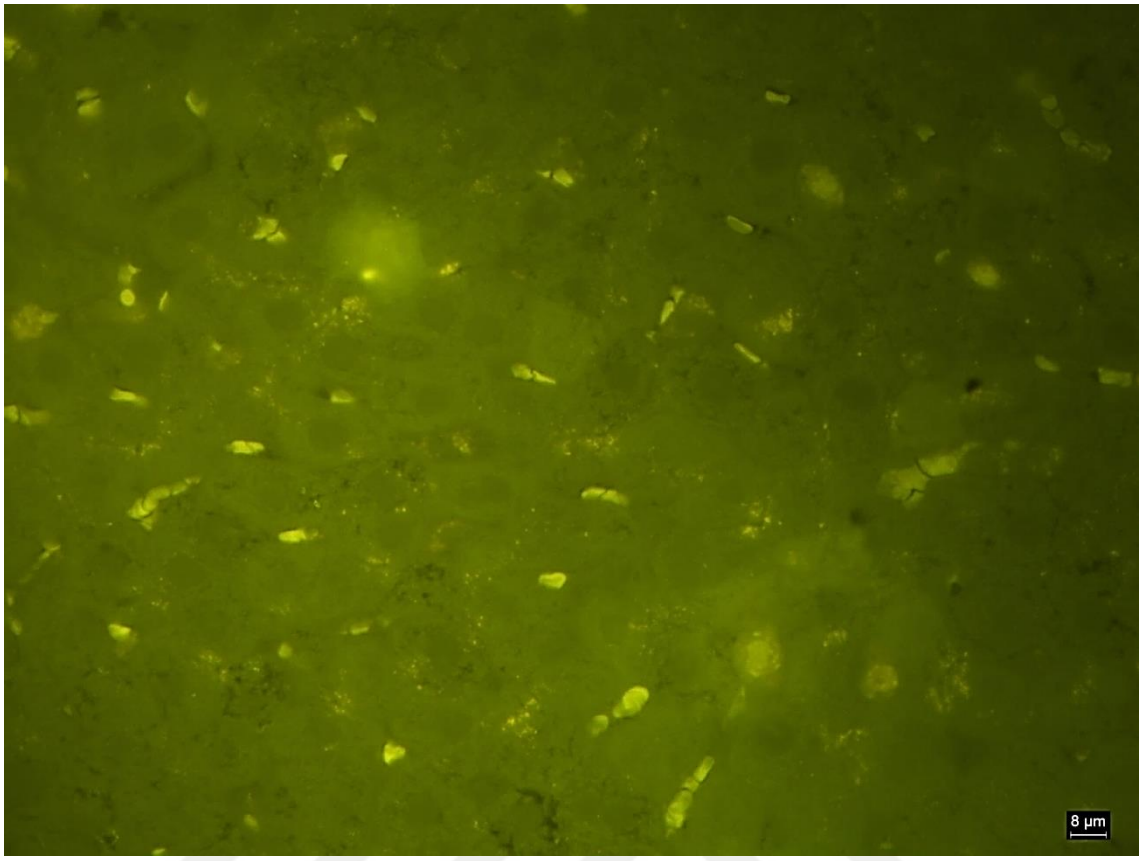

Figure 3.53. *Al+PCA+Vit E administrated group BRCA1 immunofluorescence labeling*

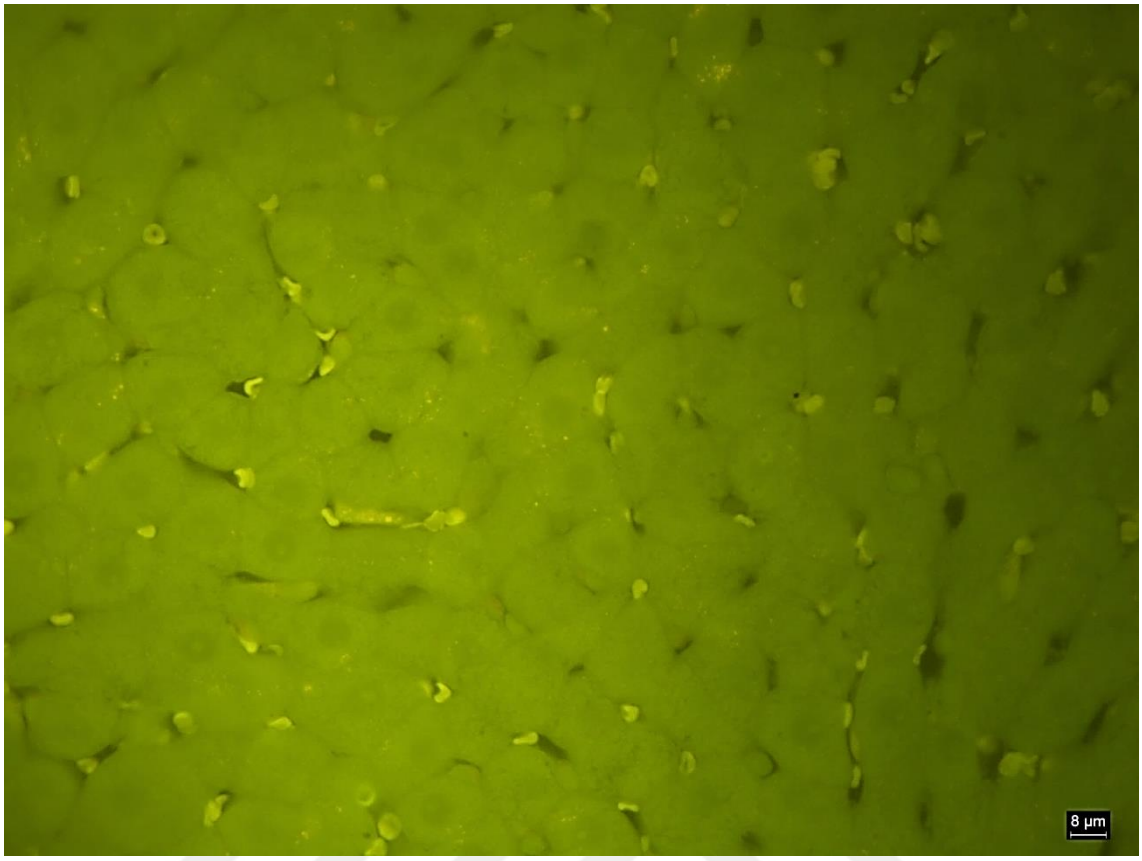

Figure 3.54. *PCA administrated group BRCA1 immunofluorescence labeling*

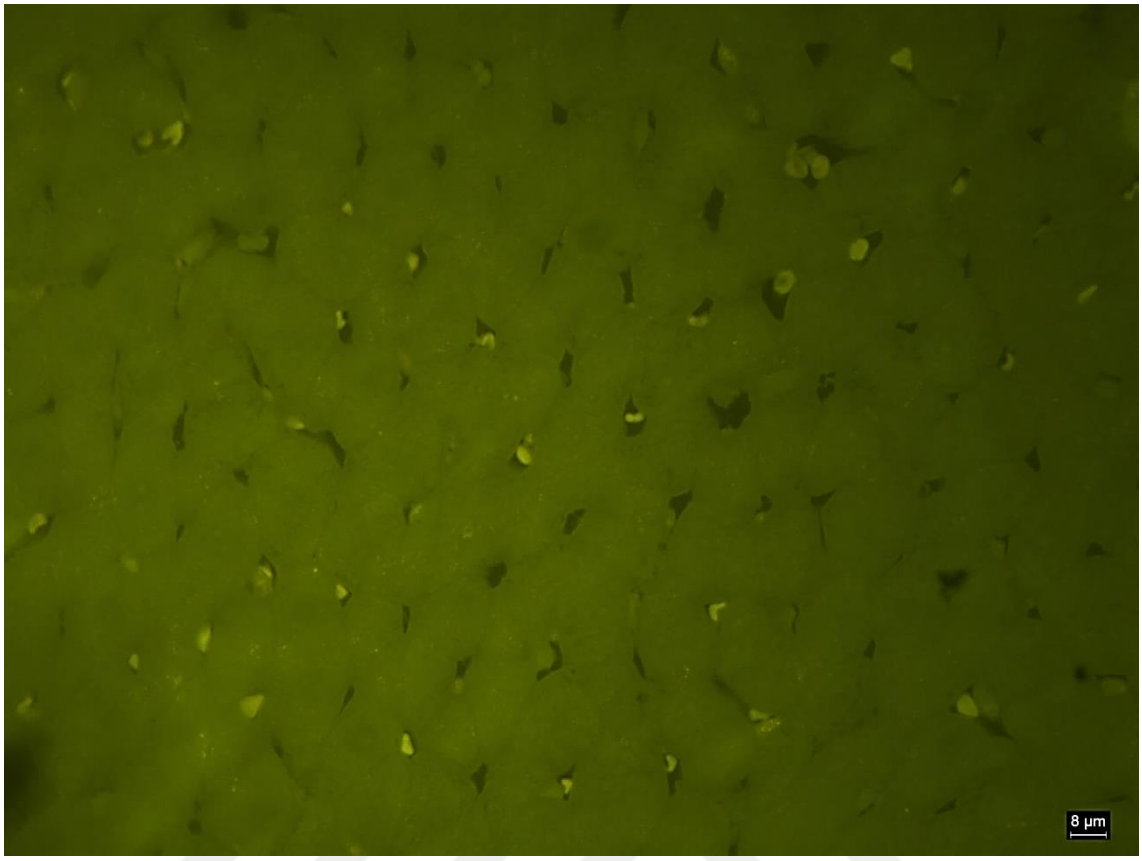

Figure 3.55. *Vit E administrated group BRCA1immunofluorescence labeling*

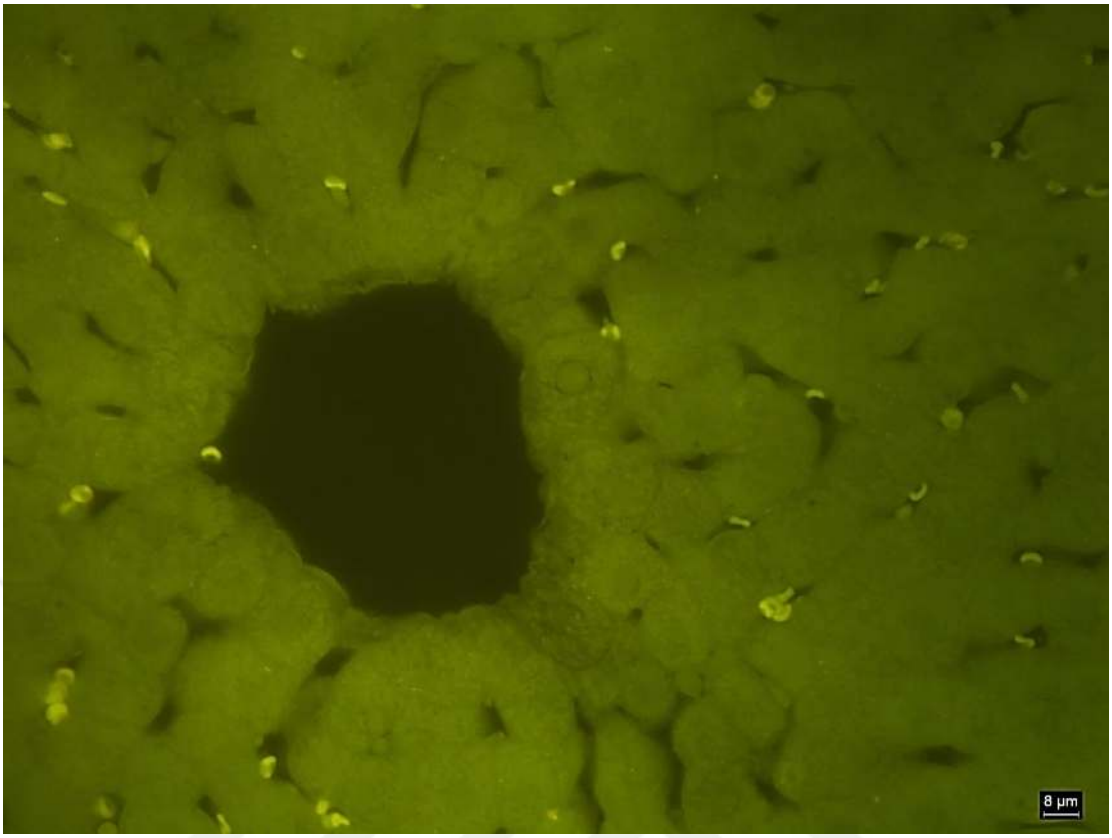

Figure 3.56. *PCA+Vit E administrated group BRCA1 immunofluorescence labeling*

Figure 3.49-3.56 BRCA1 immunofluorescence labeling in liver tissue: Control (9% saline i.p.) liver with no observable nuclear signal of BRCA1 (Figure 3.49). Al administrated animals (70 mg/kg/day i.p.) showing significant increase in BRCA1 immunofluorescence (Figure 3.50). Al+Vit E administrated animals (70 mg/kg/day for 28 days then Vitamin E 50mg/kg/day for 14 days i.p.) compared to Al group, significant reduction in BRCA1 immunofluorescence was detected (Figure 3.51). Al+PCA pre-administrated animals (70 mg/kg/day for 28 days i.p. PCA 5 mg/kg/day for 14 days i.p) increased BRCA1 immunofluorescence was observed in comparison with control group and decreased immunofluorescence when compared with the Al group (Figure 3.52). Al+PCA+Vit E group (70 mg/kg/day for 28 days i.p. PCA 5 mg/kg/day and Vit E 50 mg/kg/day for 14 days i.p) characterized with reduction in BRCA1 immunofluorescence when compared to the Al group (Figure 3.53). PCA (0,9% saline for 28 days i.p then PCA 5mg/kg/day for 14 days i.p) (Figure 3.54), Vit E (0,9% saline for 28 days then Vitamin E 50mg/kg/day for 14 days i.p) (Figure 3.55) and PCA+Vit E (0,9% saline for 28 days then PCA 5mg/kg/day and 50mg/kg/day for 14 days i.p) (Figure 3.56) groups no fluorescence signal intensity was detected.

Table 3.9. *BRCA1 immunofluorescence analysis represented by fluorescence intensity (Mean ± standard error)*

Experimental Group	BRCA1 fluorescence intensity (mg/ml)	
Control	52.0463 ± 5.112573	
Al	109.2557 ± 6.309897	(a)
Al+Vit E	63.9177 ± 5.76359	(a,b)
Al+PCA	93.1367 ± 8.066802	(a,b,c)
Al+PCA+Vit E	83.2989 ± 3.493444	(a,b,c,d)
PCA	52.3615 ± 4.347348	(b,c,d,e)
Vit E	52.3072 ± 3.064928	(b,c,d,e)
PCA+Vit E	52.5626 ± 3.962604	(b,c,d,e)

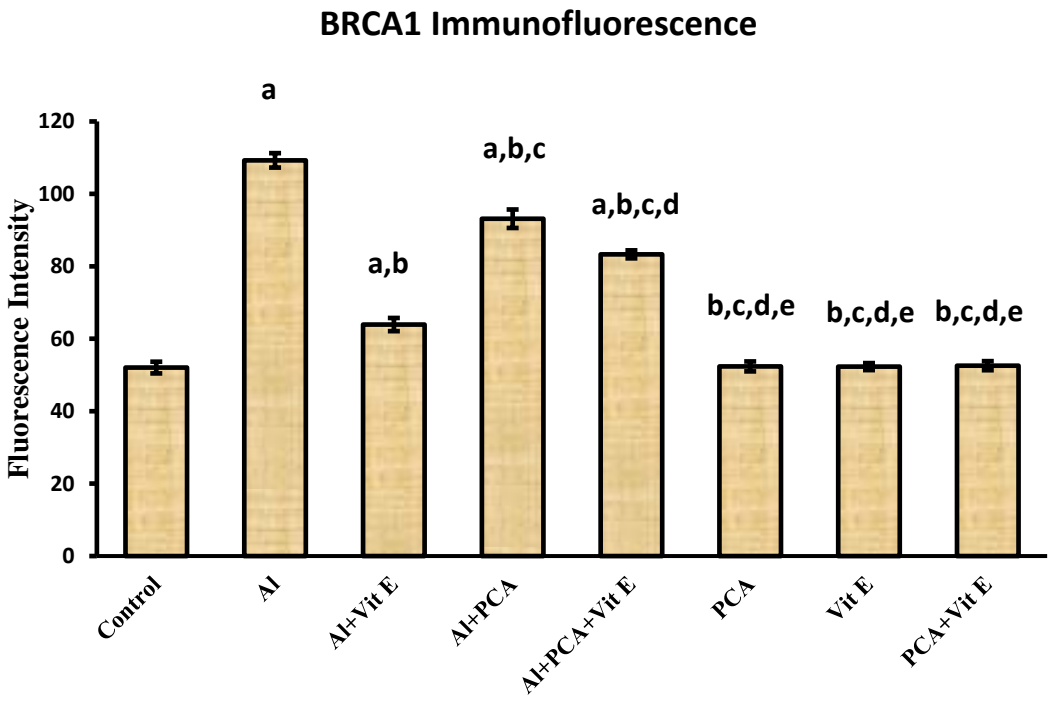

Figure 3.57. *Showing the comparison of BRCA1 fluorescence intensity between control and experimental groups.* ***a*** *significant differences compared to the control group (P<0.05); b Significant differences compared to Al group (P< 0.05); c Significant differences compared to Al+Vit E group (P< 0.05). d Significant difference compared to Al+PCA (P<0.05). e Significant differences compared to Al+VitE+PCA(P< 0.05). All data were expressed as mean ± SE. Statistical analysis of groups were performed with the SPSS package program and using one way ANOVA followed by the Dunnett T3 test as a post hoc test.*

3.6. 8-Hydroxydeoxyguanosine (8- OHdG) Immunofluorescence Evaluation

There was no observable nuclear signal of 8- OHdG in the control group sections. Immunoreactivity for 8- OHdG was frequently observed in the nucleus of hepatocytes with a fine granular pattern in the nucleus of the Al-administrated groups. The fluorescence intensity was increased about two-fold in Al group of animals. Al+Vit E group exhibited a significant decrease in fluorescence intensity in the nucleus of the hepatocytes when compared with Al group (45.5%). Instead, Al+PCA and Al+PCA+Vit E groups, showed increased immunoreactivity of 8- OHdG by approximately 27.6% for both groups when compared to control. In PCA, Vit E and PCA+Vit E groups, no nuclear 8- OHdG fluorescence was detected.

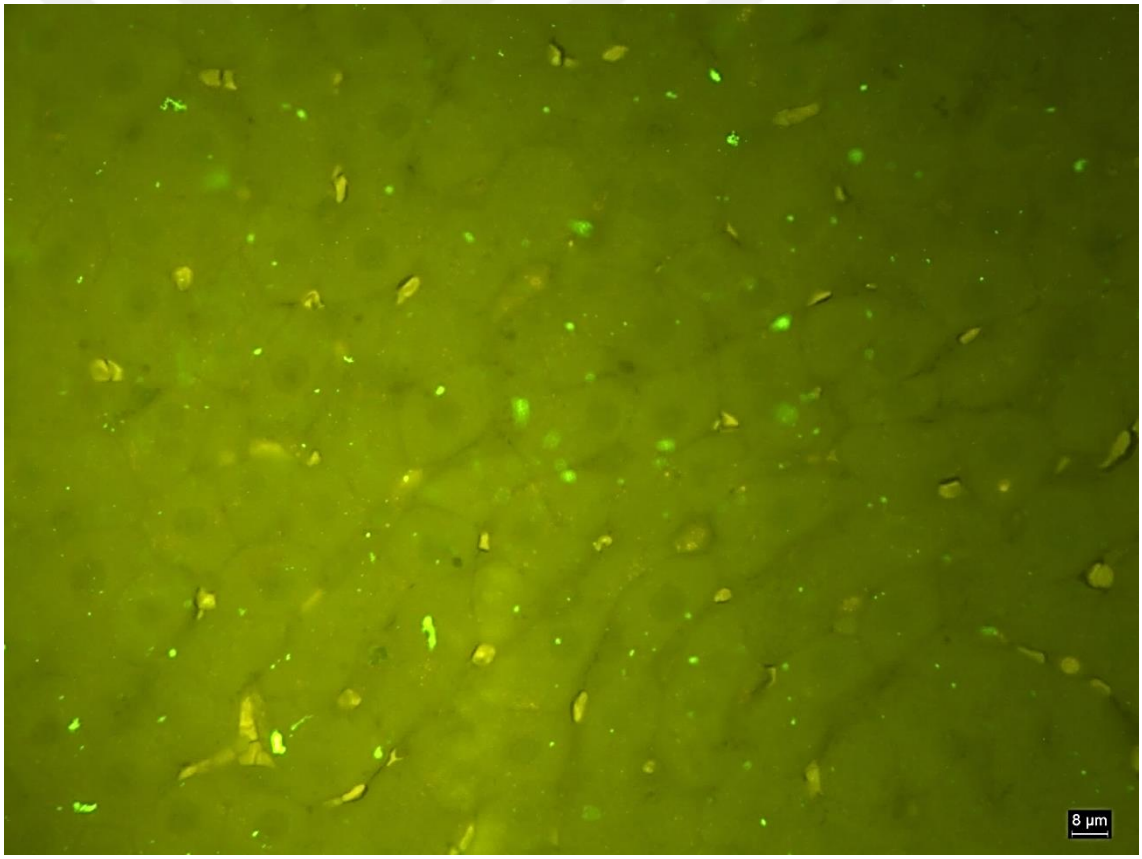

Figure 3.58. *Control group 8- OHdG immunofluorescence labeling*

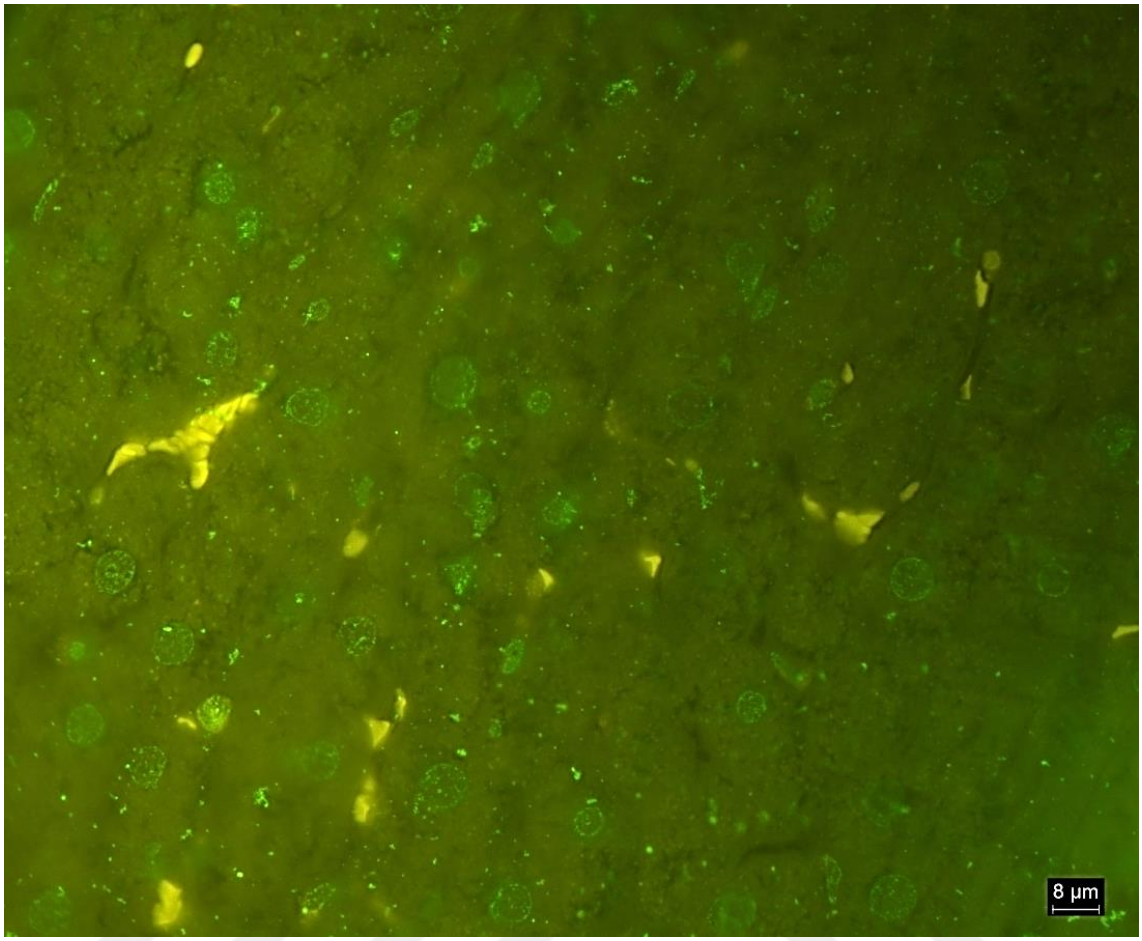

Figure 3.59. *Al-administrated group 8- OHdG immunofluorescence labeling*

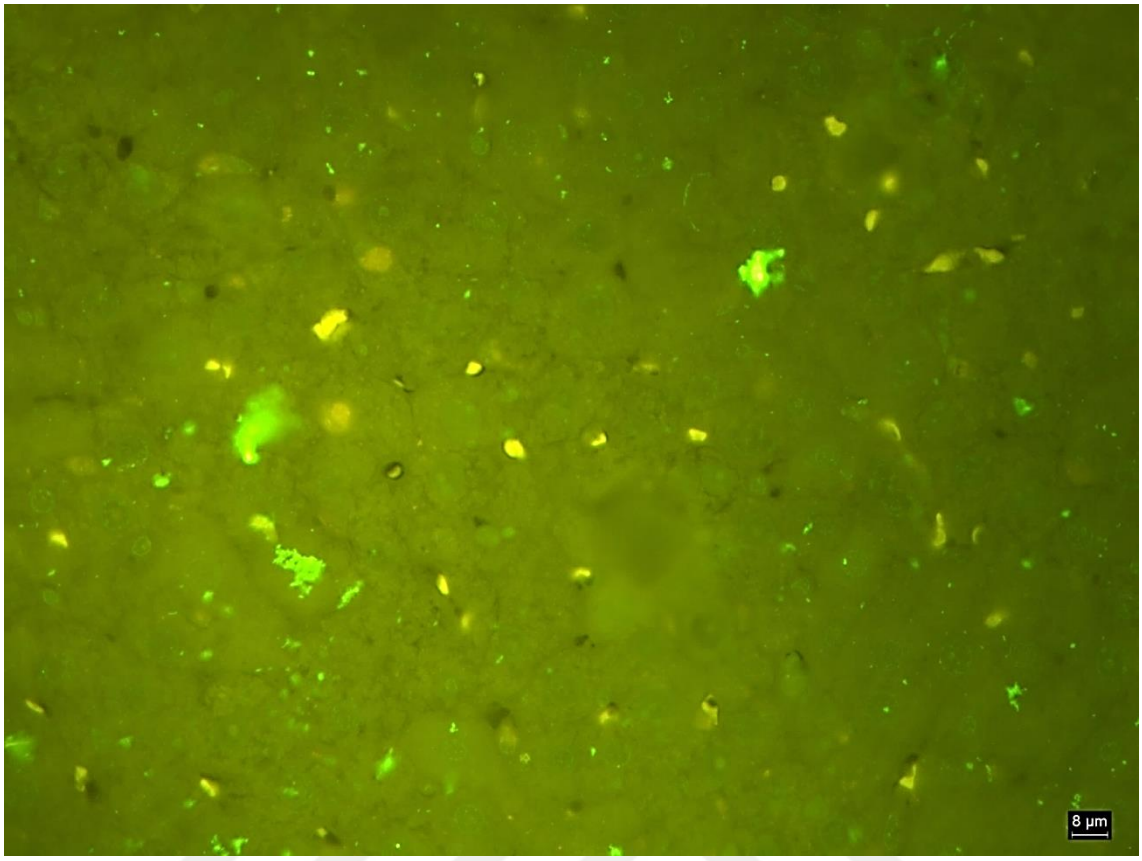

Figure 3.60. *Al+Vit E administrated group 8- OHdG immunofluorescence labeling*

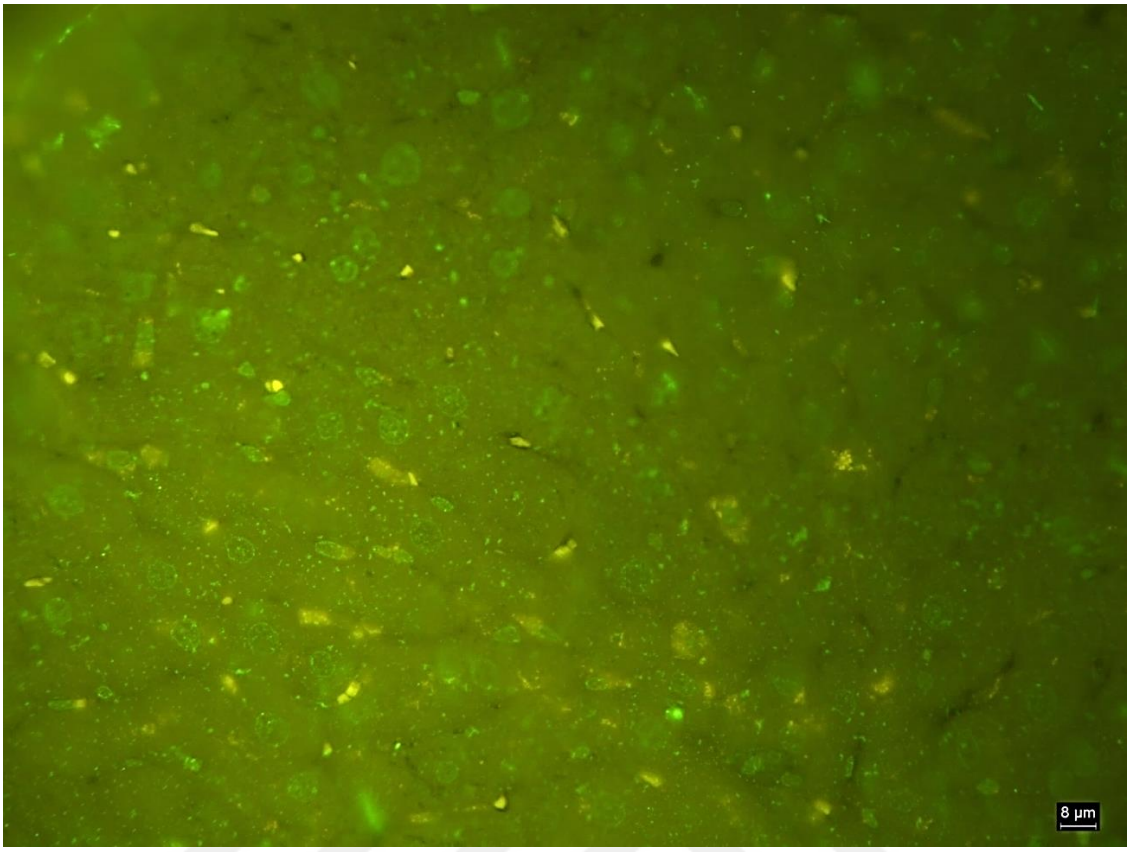

Figure 3.61. *Al+PCA administrated group 8- OHdG immunofluorescence labeling*

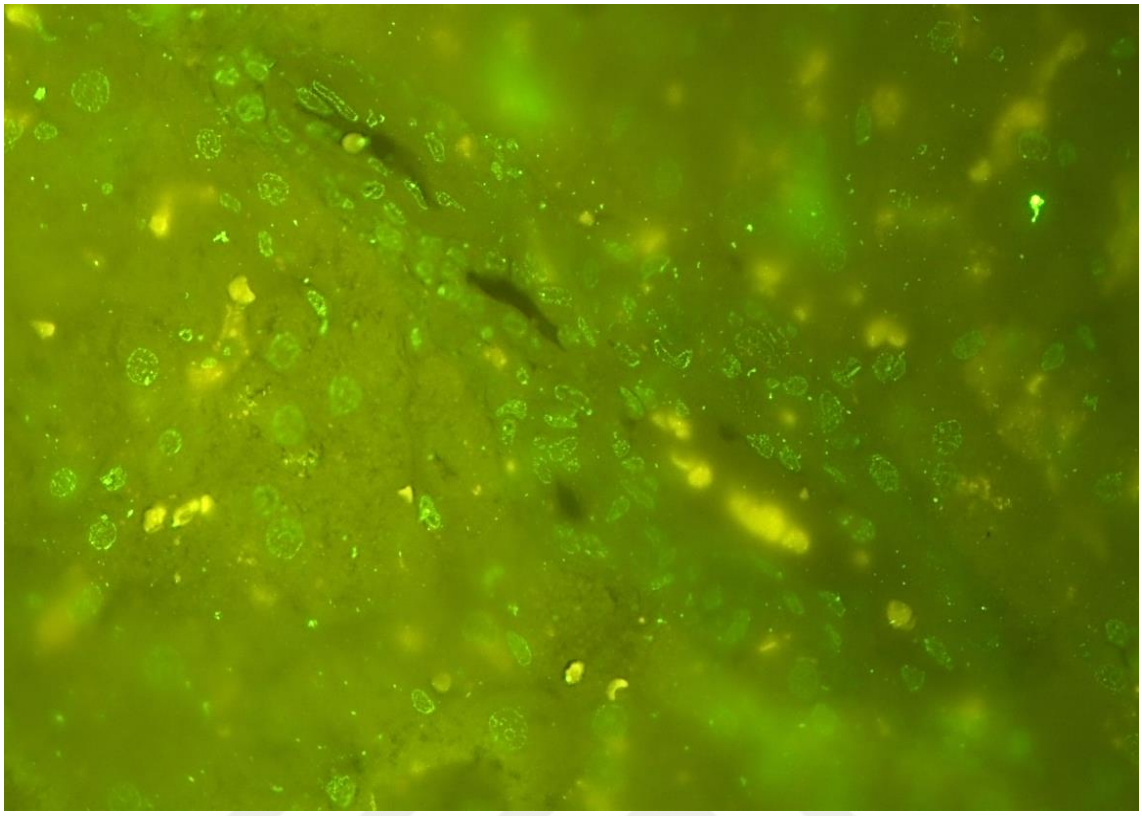

Figure 3.62. *Al+PCA+Vit E administrated group 8- OHdG immunofluorescence labeling*

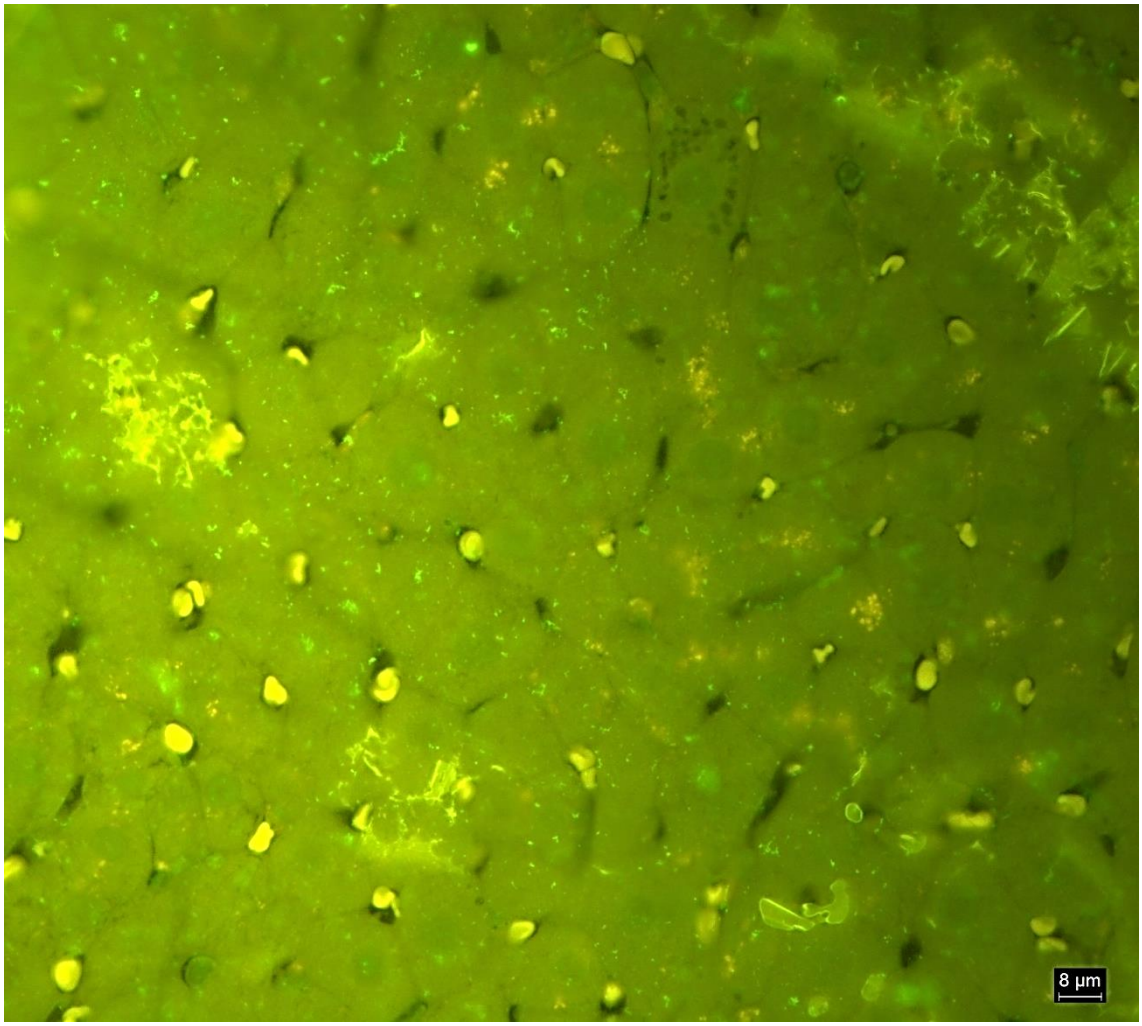

Figure 3.63. *PCA administrated group 8- OHdG immunofluorescence labeling*

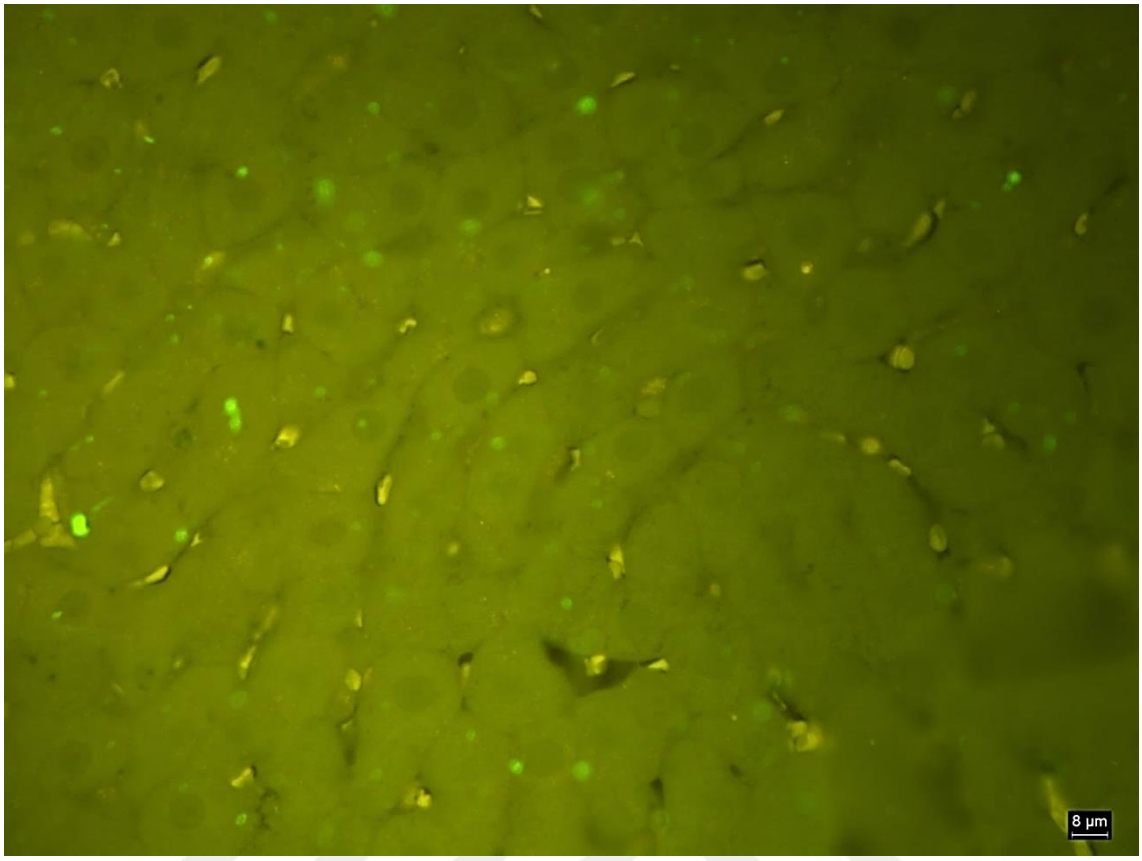

Figure 3.64. *Vit E administrated group 8- OHdG immunofluorescence labeling*

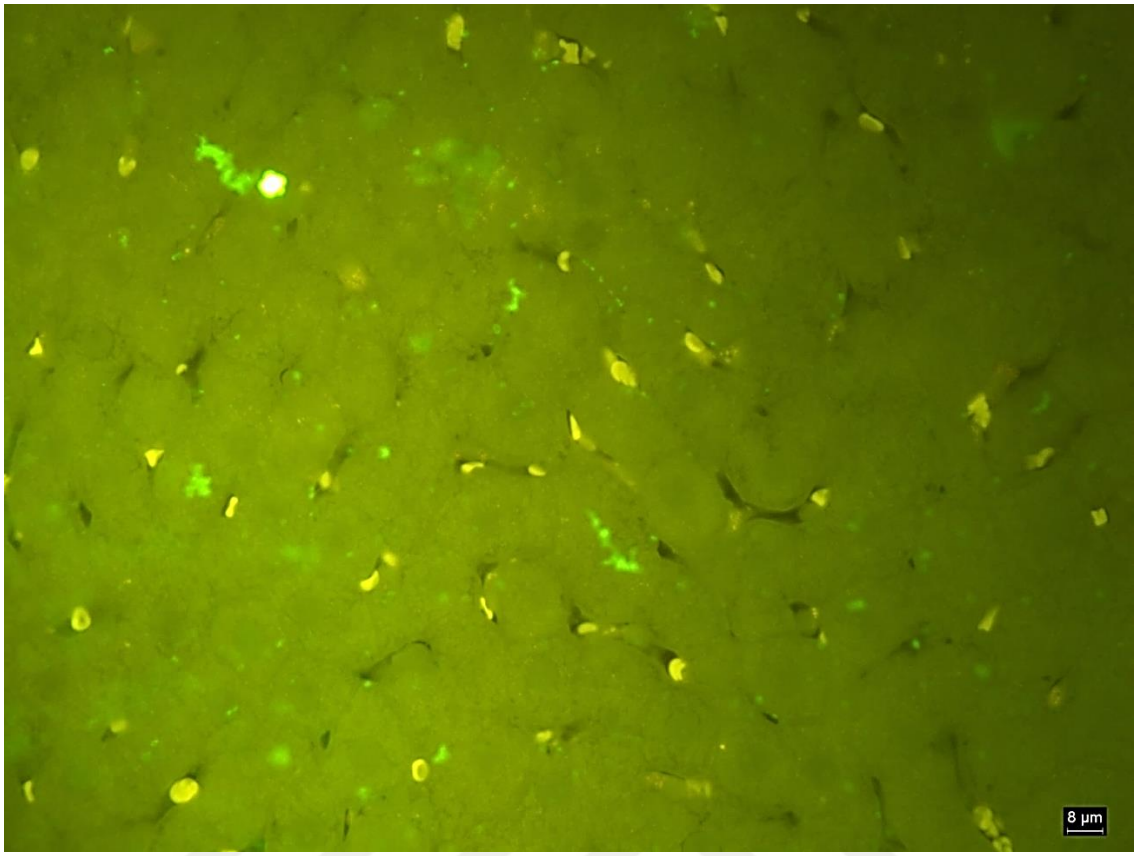

Figure 3.65. *PCA+Vit E administrated group 8- OHdG immunofluorescence labeling*

Figure 3.58-3.65 8- OHdG immunofluorescence labeling in liver tissue: Control (9% saline i.p.) liver with no observable nuclear signal of 8- OHdG (Figure 3.58). Al administrated animals (70 mg/kg/day i.p.) showing significant increase in 8- OHdG immunofluorescence (Figure 3.59). C) Al+Vit E administrated animals (70 mg/kg/day for 28 days then Vitamin E 50mg/kg/day for 14 days i.p.) compared to Al group, significant reduction in oxo G immunofluorescence was detected (Figure 3.60). Al+PCA pre-administrated animals (70 mg/kg/day for 28 days i.p. PCA 5 mg/kg/day for 14 days i.p). characterized by slight reduction in immunoreactivity when compared to Al (Figure 3.61). E) Al+PCA+Vit E group (70 mg/kg/day for 28 days i.p. PCA 5 mg/kg/day and Vit E 50 mg/kg/day for 14 days i.p) characterized with reduction in 8- OHdG immunofluorescence (Figure 3.62). PCA (0,9% saline for 28 days i.p then PCA 5mg/kg/day for 14 days i.p), (Figure 3.63), Vit E (0,9% saline for 28 days then Vitamin E 50mg/kg/day for 14 days i.p) (Figure 3.64)and PCA+Vit E (0,9% saline for 28 days then PCA 5mg/kg/day and 50mg/kg/day for 14 days i.p) (Figure 3.65) groups no fluorescence signal intensity was detected.

Table 3.10. *8- OHdG immunofluorescence analysis represented by fluorescence intensity (Mean ± standard error)*

Experimental Group	8-OHdG fluorescence intensity (mg/ml)
Control	58.0118 ± 2.378785
Al	111.410 ± 2.052792 (a)
Al+Vit E	70.179 ± 1.320238 (a,b)
Al+PCA	88.922 ± 2.231571 (a,b,c)
Al+PCA+Vit E	88.354 ± 2.214898 (a,b,c,d)
PCA	58.392 ± 2.685731 (b,c,d,e)
Vit E	58.010 ± 2.012524 (b,c,d,e)
PCA+Vit E	59.188 ± 2.025166 (b,c,d,e)

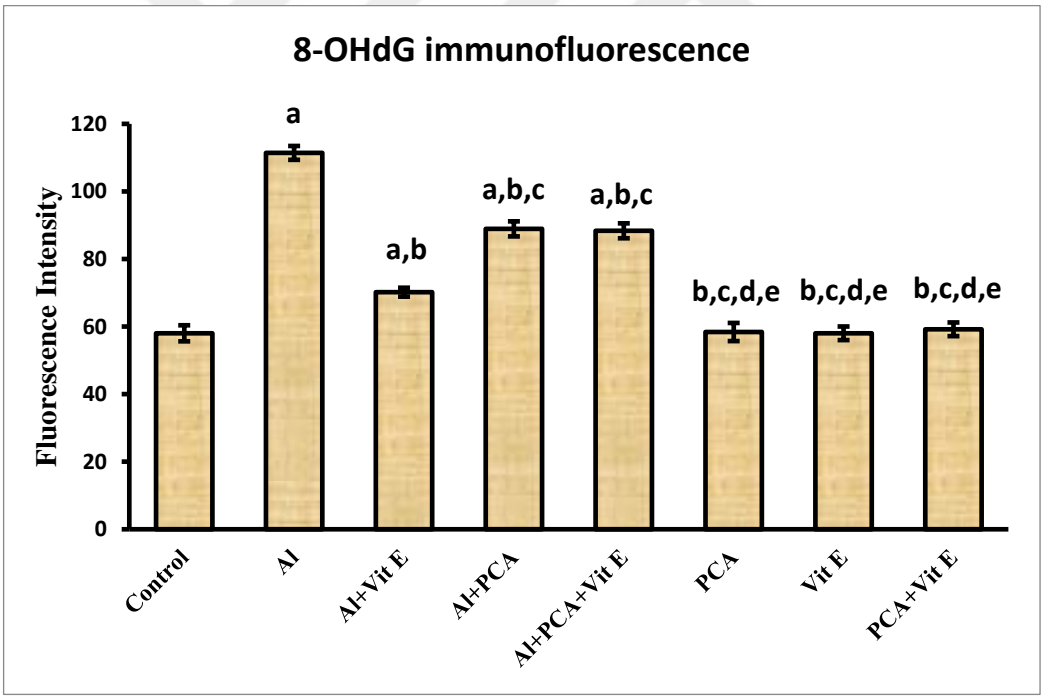

Figure 3.66. *Showing the comparison of 8- OHdG fluorescence intensity between control and experimental groups.* ***a*** *significant difference compared to the control group (P< 0.05);* ***b*** *Significant differences compared to Al group (P< 0.05);* ***c*** *Significant differences compared to Al+Vit E group (P< 0.05).* ***d*** *Significant differences compared to Al+PCA (P<0.05).* ***e*** *Significant differences compared to Al+Vit E+PCA (P< 0.05). All data were expressed as mean ± SE. Statistical analysis of groups were performed with the SPSS package program and using one-way ANOVA followed by the Dunnett T3 test as a post hoc test.*

4. DISCUSSION

Aluminum (Al) is one of the most prevalent toxic metals in the earth's crust. It has attracted a lot of attention because of its high environmental availability and detrimental impact on human health (Kumar, V., and Gill, K. D. 2014; Han, S. et al., 2013). Al can enter the biological systems in several ways, including inhalation, ingestion, and cutaneous absorption (Nayak. P., 2002; Li, X et al., 2017). Because of the presence of aluminum in many foods, oral consumption has been the most common route for this metal to enter human biological systems. The toxicity of aluminum to the brain, liver, skeletal muscles, heart, and bone marrow is widely recognized; However, the mechanism underlying its action is still unknown (Krewski. D et al., 2007; Malluche, H. H., and Faugere, M. C. 1988). Aluminum has been shown to cause severe neurotoxicity, neurodegeneration, and liver-related disorders in vitro and in vivo animal studies (Nie, J. 2018).

The failure of the hepatocytes to complete their physiological tasks has been linked to several liver disorders. Type II diabetes, obesity, and hepatic steatosis have all been linked to alterations in aerobic metabolism, mitochondrial number, and structure (Mailloux. J. R et al., 2011). The present systems biology method to assess the full metabolic disruption caused by Al toxicity has made it possible to identify major metabolic components in hepatocyte dyslipidemia (Lemire, J et al., 2011). The mitochondria are the principal target for Al-mediated toxicity due to their acidic and pro-oxidant natures, as well as Al's potential to disrupt Fe binding sites. Tricarboxylic acid cycle (TCA) activity disruption and reduced aerobic ATP generation have been directly linked to Al-mediated oxidative stress and disruption of Fe-dependent enzymes (Handy, D. E., and Loscalzo, J., 2012).

PCA is a phenolic acid that is mostly present in plants and is thought to be the primary metabolite of complex polyphenols like anthocyanins and procyanidins, which are often present in large quantities in vegetables and fruit (Masella, R et al., 2012). It has been reported that PCA has various pharmacological effects such as antioxidant, antiviral, nephroprotective, anti-hyperlipidemic, and neurological effects (Khan, A. K et al., 2015). Nonetheless, the precise mechanism underlying the antioxidant capacity of PCA has not yet been systematically investigated (Li, X. et al., 2011). Due to its hydrophobic nature, vitamin E is found in almost all cell membranes and plays a crucial role in protecting against lipid peroxidation (Howard, A.C et al., 2011). In order to preserve the oxidative

stability of the membrane-bound lipids and defend against reactive species damage, α-tocopherol and other antioxidants must be incorporated into mitochondria and other cellular compartments. This is due to the fact that one of the reactive oxygen species' primary targets is assumed to be the inner mitochondrial membrane, where the electron transport mechanism is located (Lauridsen, C., and Jensen, S. K. 2012). It is widely known that α-tocopherol has antioxidant characteristics, particularly due to its chain-breaking antioxidant action in vivo lipid peroxidation (Traber, M. G., and Atkinson, J. 2007). The liver is the organ most vulnerable to oxidative damage due to its high rate of metabolic activity, high iron (Fe) concentration, and affinity for biotransformation (Cichoż-Lach, H., and Michalak, A. 2014).

The findings of the current study are consistent with the literature's descriptions of oxidative stress and liver damage caused by Aluminum intoxication. Al has several pathways for oxidative damage. Polyunsaturated phospholipids are being attacked by reactive oxygen species (ROS) produced by aluminum toxicity, such as O_2^-, H_2O_2, OH^*, and OH^- (Su, L. J et al., 2019). Aluminum can also stimulate lipid peroxidation initiated by iron in the Fenton reaction, which causes ROS production and Fe^{3+} formation (Exley C., 2004). The lipid peroxidation findings in the present study revealed that the malondialdehyde (MDA) level as a marker of lipid peroxidation in the Al group increased approximately 3 folds when compared to the control group. However, the MDA level in the Al+PCA and Al+Vit E administrated groups was found to be reduced by (~20%) and (58.5%), respectively when compared to the Al group, where there is a ~30% reduction in the Al+PCA+Vit E group in comparison with the Al group. This confirms the effects of PCA and Vit E against oxidative stress, which have been reported in previous studies. According to the lipid peroxidation results, the combined administration of PCA and Vit E was significantly different from the effect they created separately. The sole administration of Vit E was found to be the best in attenuating lipid peroxidation. It was demonstrated by Pillai, A., and Gupta, S (2005) that Vitamin E plays an important role in stabilizing polyunsaturated fatty acids (PUFAs), low-density lipoproteins (LDL), and other components of the cell membrane against oxidation by free radicals by scavenging oxidative free radicals. Additionally, it has been documented by Lauridsen, C., and Litta, G (2022) that vitamin E is nature's most effective lipid-soluble, chain-breaking antioxidant, protecting cellular membranes from being attacked by lipid peroxyl radicals. Abdel-Daim, M. M., and Abdeen, A. (2018) also concluded that vitamin E, which has a

hydroxyl group, works as a hydrogen atom donor for $OH^{\bullet}$, generating a non-radical product, and so protects FPN-induced lipid peroxidation, as demonstrated by a considerable drop in MDA levels.

In the context of antioxidant enzymes, our study revealed that SOD activity in the Al intoxicated group was slightly reduced when compared to the control group. However, there were not any significant differences between the other groups. The functional structure of the SOD enzyme works on the superoxide radical and converts it to hydrogen peroxide. Scientists postulated that Al interacts with the $O_2^{\bullet-}$ radical to form AlO_2^{2+}, a more reactive radical molecule for biological tissues. This prevents the conversion reaction of the $O_2^{\bullet-}$radical to H_2O_2, which is catalyzed by SOD. It is thought that the lack of change in SOD activity in the groups exposed to Al toxicity may be related to this situation. Another explanation was elucidated by Das, T. K. et al., (2015), in which they stated that aluminum superoxide radical ions (AlO_2^{2+}) were produced correspondingly to the reaction of Al^{3+} with superoxide ($O2^{-}$). AlO_2^{2+} then reduces Fe^{3+} to Fe^{2+} and induces oxidative stress via the Fenton and Haber-Weiss reactions, resulting in cellular oxidative stress due to Al toxicity. Our study suggests that PCA and Vit E combined application, prevent lipid peroxidation by increasing exogenous antioxidant capacity. Instead, the only significant increment in SOD activity was found to be after the sole administration of PCA. On the other hand, after Al administration, there was a discernible reduction in CAT activity of "about 3 folds" in comparison to the control. According to some studies, exposure to Al inhibits the activity of CAT and SOD enzymes. In a study conducted by Cheraghi, E., and Roshanaei, K., (2019), $AlCl_3$ administration resulted in a substantial drop in total protein compared to control. Also, $AlCl_3$ administration significantly reduced superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels but increased malondialdehyde (MDA) level in the liver compared to control. Cell culture studies have also shown that Al suppresses the synthesis of ferritin, inhibits iron uptake into these important Fe-sequestrating proteins, causes deformation of Fe- containing proteins "such as CAT enzyme" and promotes oxidative damage (Wu, J. K. 2004). These outcomes are consistent with our findings. In contrast, different research illustrated that Al accumulates in the liver over a very long period of time and is only slowly absorbed by the digestive system. Eight weeks of oral aluminum administration led to statistically significant increases in brain and liver glutathione reductase activity but had no impact on SOD or CAT activity (Sadauskiene, I et al., 2020). It is well known that Al mostly

circulates in the blood linked to transferrin and molecules with low molecular masses. Al may thereby disrupt the homeostasis of Fe by displacing it from transferrin, causing Fe to be released into circulation (Mujika, J. I et al., 2011). According to Kim, Y., et al. (2007), intestinal Fe absorption may be impaired by Al exposure. Aluminum interferes with the brain's ability to maintain iron homeostasis through a number of pathways, including the transferrin receptor, a non-transferrin iron transporter, and ferritin. Additionally, in Chen, X et al., (2021) study, 175 mg/kg orally administrated aluminum to rats resulted in a significant increase in MDA level in the Al group, while SOD enzyme activity was suppressed. Also, in a study conducted by Cui, Y et al., (2020), it was stated that the MDA level increased considerably in the Al group, while CAT and SOD activities decreased (Cui, Y et al., 2020). Similarly, GSH and GST in the current study were found to be significantly decreased after Al administration, and PCA was found to restore the activity of the enzymes when solely administrated. According to Othman, M. S. et al., 2020 study, $AlCl_3$ toxicity increased oxidative stress as evidenced by rises in lipid peroxidation (LPO) and nitric oxide (NO) levels and falls in glutathione (GSH), GPx, SOD, CAT, and GR levels. Moreover, GST level was indicated to be significantly reduced after Al toxicity, as evidenced in El-Demerdash, F. M. 2004 study, which clearly revealed that $AlCl_3$ significantly induced lipid peroxidation and decreased the activity of glutathione S-transferase (GST).

According to the performed experiments in the current study, a respectable number of cellular damages were detected in the livers of experimental animals exposed to Aluminum. Histopathological sections of experimental groups were shown to have damaged hepatocytes, congested sinusoids, and apoptotic/necrotic events in the liver sections. Immunological cell infiltrations and nuclear vacuolation have also been observed. Mohamed, H.H. (2017) reported that the $AlCl_3$-treated groups displayed an irregular hepatocyte shape in addition to the lack of the typical hepatic cord pattern. Additionally, pyknotic, dilated sinusoids with significant fatty alterations indicated by round, pointed vacuoles in the cytoplasm of hepatocytes as well as necrosis events of hepatocytes were observed. It was also noted that experimental animals treated with $AlCl_3$ orally had steatosis, an increase in hepatocyte diameter, a dispersion in the radial distribution of hepatocytes, pyknosis, and karyokinesis as a result of Al intoxication (Agarwal, D. R., and Jain, S. 2011). In the liver of mice with acute Al toxicity, extensive injuries characterized by loss of the normal architecture of parenchymatous tissue,

congested sinusoids, infiltration of inflammatory cells, cellular degeneration with nuclear pyknosis, and the presence of necrotic areas were observed (El-Sayed, W. M et al., 2011). According to Geyikoglu, F et al., (2012), the livers in Al-treated groups revealed severe pathological damage such as congestion of the central vein, sinusoidal dilatation, and lipid accumulation. Similarly, Saleh, S. I., et al. (2017) reported that oral administration of $AlCl_3$ resulted in degeneration in the rat liver, dilated and occluded central vein, and increased Kupffer cells in blood sinusoids. Ahmed, W. et al. (2022) indicated that liver sections from rats given 70 mg/kg/day of $AlCl_3$ had sinusoidal dilatation, widespread ballooning degeneration of hepatocytes, and dilated central veins. Histological analysis of liver sections by Abdel-wahab, W. M et al. (2012) showed that there was significant hepatocyte necrosis and degeneration, centrilobular necrosis, congestion of the central vein, vacuolization of the cytoplasm, infiltration of inflammatory cells, dilatation, and congestion of the blood sinusoids. Also, hepatic dysfunction, a rise in lipid peroxidation, and a decrease in the activity of antioxidant enzymes in the liver were all brought on by oral administration of $AlCl_3$ at a dose of 20 mg/kg per day for 30 days. Moreover, the effect of Al exposure on the liver resulted in cellular disorganization, irregular cell shape, cellular disruption, and different histopathological events (Al-Hazmi M. A et al., 2021). The histological findings within the scope of this study agree with the pathological effects of Al that have been documented in the literature. Various intensities of necrotic and apoptotic events, lipid accumulations, and vacuolation were observed. Intense cellular damage and tissue pathology were observed. In all groups treated with Al, damaged hepatocytes, damaged and congested sinusoids were observed, accompanied by apoptotic and necrotic hepatocytes, presence of inflammatory cells infiltrations, and steatosis (Figure 3.5-3.9), except for the Al+Vit E group, in which Vit E seemed to restore the normal condition of the Al- pretreated section. In the Al+PCA group of animals, there were no observable changes in the pattern of hepatocytes and sinusoidal canals (Figure 3.11-3.14). However, mild microvesicular fatty changes and a few occurrences of apoptotic events were observed. The Al+Vit E group showed normal general structure and normal hepatocytes, with limited inflammatory and apoptotic cells and the presence of microvesicular fatty changes. No necrotic cells were detected (Figure 3.15-3.17). Al+PCA+Vit E (Figure 3.18-3.21) showed relatively improved liver tissue architecture and sinusoids with rare apoptotic events and infiltration of inflammatory cells. PCA, PCA+Vit E, and Vit E (Figure 3.22-3.24, respectively) groups showed normal tissue

structure and no histological abnormalities were detected. It has been shown that in the Al+Vit E group, the histological structure was generally mild and moderate, in line with the lipid peroxidation findings, and was similar to the control group, except for minor pathological formations at the cellular level. Histological findings demonstrate the regulatory and preventive effects of Vit E when administrated separately to groups exposed to Al. However, the protective and antioxidative effects of PCA and PCA+Vit E combined administration were found to be not significantly active in reducing the pathological events. Regarding the histopathological results, we can conclude that the formed steatosis, necrosis, and inflammation were prevented in the Al+Vit E group where the tissue sections seemed to return to their normal condition and the deformities were ameliorated after the administration of Vit E. Only reversible changes such as microvesicular degeneration were observed. Liver tissue and hepatocytes in Al+PCA and Al+PCA+Vit E were found to continue to harbor some structural and histological abnormalities. The remaining groups appeared normal in their histological architecture.

Al administration causes a significant amount of ROS generation, which is crucial for Al-induced genotoxicity. DNA damage, genomic instability, and cell proliferation induced by OS may induce cancer development and progression even if these circumstances return to normal (Davalli, P et al., 2018). PCNA is a DNA sliding clamp for replicative DNA polymerases, a biomarker of cell proliferation, and an important part of the eukaryotic chromosomal DNA replisome. A growing body of research has revealed its interesting capacity to interact with several molecules involved in several metabolic processes, including DNA repair, control of cell cycle, translesion DNA synthesis, Okazaki fragment processing and DNA methylation (Maga, G., and Hubscher, U. 2003). In the liver, Al was identified predominantly in the lysosomes of macrophages and Kupffer cells (Fiejka, M. et al., 1996). It has been demonstrated that reactive oxygen/nitrogen species induce the activation and proliferation of Kupffer cells and hepatic satellite cells, mediating cirrhosis, and carcinogenesis (Li, S et al., 2015; Ali, H et al., 2019). In the current study, the Al group of rats showed a considerable rise in PCNA immunofluorescence intensity in the cytoplasm of the hepatocytes and Kupffer cells, which was associated with considerably higher DNA damage. Pre-administration of vitamin E reduced DNA damage and decreased Kupffer cell growth. High immunofluorescence intensity was found in the Al+PCA and Al+PCA+Vit E groups, demonstrating that Kupffer cells had an autophagic response to certain kinds of

hepatocellular damage. Bogdanovic, M et al. (2008) elucidated that, Kupffer cell hyperplasia was observed in aluminum pretreated rats. Scientists speculate that the Kupffer cell hyperplasia occurred to mitigate and protect liver tissue from the damaging effect of Al toxicity.

Studies on Al-induced genotoxicity, illustrate that prolonged exposure to Al nanoparticles is implicated in high levels of DNA damage in hepatocytes (Yousef, M. I et al., 2019; Zhang, Q. L et al., 2011). To evaluate the genotoxicity impact of aluminum, several studies using single-cell gel electrophoresis (Comet assay) and immunofluorescence detection of 8-OHdG formation "according to DNA oxidation" have shown that aluminum induces DNA damage in a dose-dependent manner. According to Sharma, V et al., (2011), the Olive tail moment (OTM) and % tail DNA in the Comet test show that oxidative stress caused by the administration of ZnO NPs resulted in a considerable increase in DNA damage in human hepatocytes. Similarly, He, L. F., and Chen, J. G. (2006) indicate that too much fluoride intake causes oxidative stress resulting in DNA damage, apoptosis, and alterations in the cell cycle of rat hepatocytes as the rate of the comet assay greatly increased following fluoride treatment. Moreover, Lankoff, A et al., (2006) demonstrate that Al affects human peripheral blood cells in a way that is both genotoxic and cytotoxic as observed on the levels of OTM and apoptosis respectively. Additionally, 8-OHdG "which is an oxidized form of guanine, is one of the major mutagenic lesions generated under oxidative stress in mitochondrial DNA" in urine and the comet assay test showed substantially higher results in the exposed group, according to Samir, A. M., and Rashed, L. A. (2018). They claimed that potential health risks among exposed employees may result from extended exposure to aluminum, which is shown by an increase in serum aluminum. Furthermore, Xu, F et al. (2017) show that an accumulation of reactive oxygen species in mitochondria leads to higher formation levels of 8-hydroxydeoxyguanosine. Also, exposure to $AlCl_3$ stimulates oxidative stress in mitochondria, which may be a causative agent to dysfunctional mitochondrial energy metabolism and liver malfunction. Our study's SCGE results revealed that the Al group's DNA damage was almost four times more than that of the control group as indicated by the tail moment values. In the Al+PCA group and Al+VitE group, DNA damage was significantly lower than in the Al group by 44.27% and 58.7%, respectively. Additionally, the combined application of PCA and Vitamin E in the Al+PCA+VitE group showed a significant decrease compared to the Al by 49.31%. According to statistical analysis of

the obtained results in this study, Vit E had a greater effect on DNA damage recovery than PCA, as a significant difference was observed in tail movement in favor of the Al+Vit E group. Following the same pattern, the results of our study revealed an immunofluorescence detection of intracellular 8-oxoG in hepatocytes. 8-oxoG immunoreactivity is detected in the Al, Al+PCA, and Al+PCA+Vit E experimental groups. However, very little immunoreactivity was seen in the Al+Vit E group, demonstrating that Vit E supplementation alone had the greatest impact on decreasing oxidative stress-induced genotoxicity. The results in the Al+Vit E group of comet and 8-oxoG have been proven to be consistent with one another, indicating that the genotoxicity of aluminum was reduced following Vit E administration. The BRCA1 gene is a DNA repair gene that is present in the cell's cytoplasm and nucleus and is in charge of preserving the integrity of the genome (Wang, G. H. et al., 2018). BRCA1 plays a key role in the restoration of DNA double-strand breaks (Tutt, A. and Ashworth, A., 2002). Recent research suggests that the tumor suppressor BRCA1 controls oxidative stress. It has been demonstrated that BRCA1 controls Nrf2-dependent antioxidant signaling by associating physically with Nrf2 and assisting in the stabilization and activation of that protein (Gorrini, C et al., 2013). BRCA1 overexpression decreases H_2O_2-induced DNA damage and apoptosis and upregulates a number of antioxidant genes in human breast cancer cells (Saha, T et al., 2009). By inhibiting nuclear factor erythroid-derived 2 like 2, Bae, I et al. (2004) show that BRCA1's capacity to promote antioxidant response element-dependent transcription and protect cells from oxidative stress is diminished. These findings point to a unique role for BRCA1 in defending cells against oxidative stress. This function is in line with the hypothesis that BRCA1 serves as a caretaker gene to maintain genomic integrity. BRCA1 immunofluorescence results in the current investigation revealed a minimal degree of background immunoactivity in the control group. It was observed that BRCA1 expression was induced in the cytoplasm of Kupffer cells in the Al-administrated group. It is thought that BRCA 1 protein production was boosted due to the high level of OS and toxic effect. In the Al+Vit E group, a low level of non-significant fluorescence intensity is observed. In Al+PCA and Al+PCA+Vit E groups, it was observed that BRCA1 expression decreased in Kupffer cell cytoplasm due to the reparative response agaist the DNA damage in those cells. In the PCA, Vit E, and PCA+Vit E groups, a low level of non-significant fluorescence intensity was observed.

The findings in the current study are in accordance with the findings of previous studies on the protective effects and antioxidant efficiency of PCA and Vit E. Although molecular interactions can result in a change in the structure and activity of these molecules when applicated together, it is found that PCA and Vit E combination therapy was also effective to reduce the oxidative damage induced by Al in the liver. These results are thought to provide an important contribution to the literature for the future PCA and Vit E based therapy and drug design studies, in terms of the regulation of oxidative stress related tissue and organ damages (such as liver complications and neurological disorders) induced by Al.

5. CONCLUSION AND RECOMMENDATIONS

PCA and Vitamin E are considerably helpful in reducing the pathological formations brought on by aluminum exposure in rat liver tissue. However, it is important to note that when the two antioxidants are applied together, there is a contentious result. We postulate that the administration of these two antioxidants together results in an esterification reaction, in which the carboxylic acid group in PCA and the hydroxyl group in vitamin E react with one another to generate an ester as the reaction's product. Another explanation for this phenomenon is that, depending on the amount provided to the experimental animals, these antioxidants might either be pro- or antioxidants. Therefore, multiple dosages must be administrated to the experimental groups to determine the appropriate dose that modulates the effects of Al toxicity and confirm the effect of the combined application of these two antioxidants. The specific ability of vitamin E to mitigate the peroxidation of the lipids, protecting the hepatocellular membranes from toxins and free radicals, accounts for its effectiveness when administrated alone in reducing lipid peroxidation and DNA damage, as evidenced in the comet assay and 8oxo G immunofluorescence. On the other hand, we found that applying PCA alone is more effective at regulating the levels of the antioxidant enzymes SOD, CAT, and GST as well as GSH, indicating that this phenolic acid can boost the activity of antioxidant enzymes by preventing the formation of reactive oxygen/nitrogen species. According to the findings of this study, PCA and vitamin E had a reduced impact when used in combination as opposed to when used separately. However, the current study is a basic brick for innovation of novel therapeutic designs based on PCA and Vit E, as well as paving the way for future studies to highlight and underpin the mechanisms of how these two antioxidants' combined applications work at the molecular level.

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CURRICULUM VITAE

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Education

- **2017-2022**

PhD in Molecular Biology, University of Anatolia/ Eskisehir Technical University, Eskisehir, Turkey

Dissertation title "*The protective role of protocatechuic acid (PCA) and alpha tocopherol (vitamin E) combination on aluminum- induced liver toxicity in rats*". Holder of the YTP scholarship.

- **2013-2016**

Master of Science in Genetics and Molecular Biology, University of Khartoum, SUDAN.

Thesis Title "*Cytochrome Oxidase subunit one (COI) sequence variations among prostate cancer Sudanese patients*".

- **2007-2013**

Bachelor of Science (**First Class Honour)** department of Zoology, University of Khartoum, SUDAN

Experience

- **2016-2017**

Lecturer, Faculty of Science, Department of Biotechnology, Alneelain University Khartoum, SUDAN. Teaching practical courses of Invertebrate, vertebrate, embryology, and molecular genetics for First, second- and third-year students respectively.

Lecturer, Faculty of Science and Biotechnology, Department of Biotechnology, Omdurman Islamic University, Khartoum, SUDAN. Teaching Animal tissue culture theory course for 3rd year students, Department of Biotechnology.

Lecturer, Faculty of Science, Department of Zoology, University of Khartoum, Khartoum, SUDAN. Teaching practical courses of Cell biology, Molecular biology, Bioinformatics and Advanced Microbial Genetics for the master students of Genetics and molecular biology program.

- **2013-2016**

Teaching assistant, Faculty of Medicine, Department of Anatomy, University of Khartoum, SUDAN. Teaching practical course for subject of General biology (Anatomy) for the first-year students.

Teaching assistant, Faculty of Science, Department of oology, University of Khartoum, SUDAN. Teaching practical courses for subjects; Molecular biology and Biochemistry for 3rd and 4th year students.

Scholarships and Conferences

- Scholarships: I got a full scholarship to conduct a Ph.D. at Anadolu University from Turkish scholarship programs in 2017.
- Conferences: In 2017 I had the opportunity to present my master's thesis work in a conference held in Uganda organized by International Society for Computational Biology (ISCB) and African Society for Bioinformatics and Computational Biology (ASBCB). ISCB Africa ASBCB Conference on Bioinformatics 2017 from Oct 10 to 2017 Oct 13 Entebbe, Uganda. To attend this conference, I got a full travel fellowship (airplane tickets and accommodation) for the entire period of the conference from Wellcome Trust.

Technical Skills

• In vivo animal experiments

• Histopathological analysis

• Single cell gel electrophoresis (Comet assay)

• Enzyme biochemical analysis techniques

• ELISA

• Western blotting

• Immunohistochemistry

• Spectrophotometry

• TUNEL assay

Experienced in different molecular techniques including:

•DNA extraction

•PCR and gel electrophoresis

•Different bioinformatics software packages to analyse sequences to identify mutations and their probable impact on structure and function of their corresponding proteins.

• SPSS analysis.

• Light compound microscopes

Professional Activities

Professional Workshops:

• Participated in Bioinformatics in Molecular biology and genetics workshop 2013. Topics covered: Bioinformatics, genomics, proteomics sequence analysis, molecular modeling, bioinformatics SNPs analysis and next generation sequencing.

Diploma in Parasitology: Excellent. Topics covered: Theoretical and practical aspects in Parasitology.

Training Courses:

• Introduction to Bioinformatics IBT 2017. (92 contact hours). Topics covered:

• Explain the use of Bioinformatics

• Key Bioinformatics techniques and tools

• Locate important biological databases and retrieve data

• Use selected tools effectively to run specific Bioinformatics analyses

• Understand the strengths and limitations of various Bioinformatics techniques

Professional Memberships

• International Society for Computational Biology (ISCB).

•African Society for Bioinformatics and Computational Biology (ASBCB)

• H3ABioNet (Pan African Bioinformatics Network for H3Africa).

Language

•Arabic: Mother tongue.

•English: IELTS certificate

•Turkish: C1 certificate.