ARC Journal of Nutrition and Growth
Volume 8, Issue 1, 2022, PP 9-18
ISSN No. (Online) 2455-2550
DOI: http://dx.doi.org/10.20431/2455-2550.0801002
www.arcjournals.org

Investigation of Toxic Effects of Bendiocarb on L929 Cell Line

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Abstract: *In this study, the determination of bendiocarb-induced oxidative stress and toxic effects on L929 fibroblast cells was completed using the Antioxidant test, MTT test, Comet test and Apoptosis test. The LC_{50} dose, viability and cytotoxicity of bendiocarb-treated L929 cells were evaluated using the MTT assay. LC_{50} dose was determined as 90 µg/mL. SOD, CAT, GPx and MDA tests were used to evaluate the antioxidant activity and MDA levels were determined by ELISA. In the study, DNA tail lengths, tail moment and tail percentage were analyzed using the Comet test to evaluate whether bendiocarb caused DNA damage. In addition, to measure the apoptosis caused by Bendiocarb, L929 cells, which were applied to increasing doses of bendiocarb using fluorescent dyes, were stained with Acridine Orange-Ethidium Bromide and their images were taken by a fluorescent microscope. In the findings, it was determined that L929 cells inhibited proliferation after 90 µg/mL. These results were confirmed by the Comet test and the Apoptosis test. Treatment of bendiocarb significantly decreased its antioxidant activity and increased MDA formation. It showed a strong enhancing effect on oxidative stress induced by bendiocarb in L929 cells and a reducing effect on fibroblast viability. This reveals the potentially harmful effect of bendiocarb-induced oxidative stress, cytotoxicity and DNA damage in determined concentrations.*

Key Words: *L929, Bendiocarb, MTT, Oxidative Stress, DNA Damage, Apoptosis.*

1. INTRODUCTION

Many products are used in agriculture to protect agricultural chemicals, crops and animals from unwanted living species. Such substances are also insecticides [1]. One of the insecticide groups frequently preferred for household and agricultural purposes belongs to carbamates. This group of carbamates shows neurotoxicity in mammals and insects and they are biodegradable [2]. Carbamate pesticides are organic compounds, esters of carbamic acid, which is an unstable compound [3]. Vertebrates and invertebrates are highly sensitive to the acute toxicity of insecticides [4]. Carbamate insecticides reversibly inhibit the acetylcholine enzyme. In humans, carbamate compounds are absorbed in the body by multiple exposure routes, especially dermal absorption is quite rapid. Carbamate insecticides are rapidly eliminated from the body and do not accumulate in mammalian tissue [5].

Bendiocarb is a broad-spectrum insecticide on living organisms. It is used to deal with unwanted household and agricultural pests rather than disease-carrying animals such as flies and mosquitoes [6]. Bendiocarb, a carbamate insecticide, inhibits the enzyme cholinesterase, which plays a role in neurotransmission. When bendiocarb exposure ends, cholinesterase inhibition and its effects recover quickly [5,7]. Bendiocarb is responsible for the toxic effect on production of ROS, besides acetylcholine esterase inhibition. Exposure to bendiocarb long-term, enzyme inhibition results in the accumulation of many acetylcholine enzymes at the receptor sites, resulting in continuous stimulation of the nervous systems. [8,9]. Symptoms observed in organisms exposed to bendiocarb include blurred vision, weakness, abdominal pain, headache, nausea, chest discomfort, muscle tremors, sweating, and decreased heart rate. Symptoms such as heart irregularities and loss of reflexes may also be experienced. The causes of death are cessation of breathing, paralysis of the respiratory system muscles and intense narrowing of the lung openings [10].

L929 cell line is subcutaneous connective tissue fibroblast cells obtained from *Mus musculus*. Fibroblasts are widely used in oxidative stress and tumor research, are also preferred for measurement of necrosis factor-induced

cytotoxicity, DNA damage. Continuous cell lines such as L929 mouse fibroblasts are a suitable cell lines for testing cytotoxic properties due to their reproducible growth rate and biological response [11,12].

Oxidative stress is responsible for many diseases such as diabetes, cardiovascular and neurological diseases and inflammatory disorders, especially cancer [13]. Reactive oxygen species (ROS) are free radicals that are atoms or groups of atoms containing at least one unpaired electron. Among the reactive oxygen species, the most physiologically produced and active ones are superoxide anion (O_2^-), hydroxyl radical (OH^-) and a non-radical compound hydrogen peroxide (H_2O_2) [14]. With the increase of the amount of ROS in the cell, free radicals attack important intracellular and extracellular structures. As a result, the disruption of the functions of the cell and the oxidative balance of ROS is called oxidative stress [15,16]. Antioxidants are substances that are formed as a result of metabolic and physiological processes in the organism, which can cause many diseases and can neutralize highly reactive free radicals [17,18]. The antioxidant enzyme is one of the most important parts of the antioxidant defense system and plays an important role in sweeping free radicals [19]. Superoxide dismutase (SOD) enzyme is responsible for the elimination of superoxide, which catalyzes the dismutation of superoxide molecule into oxygen and hydrogen peroxide. Without the SOD activity, this free radical leads to the formation of hydroxyl radicals (OH^-) [20]. Catalase (CAT) and glutathione peroxidase (GPx) are produced in abundance in the mitochondrial matrix during the reduction process of H_2O_2 and O_2 radicals. The CAT enzyme breaks it down into oxygen and water when the amount of H_2O_2 increases. The GPx enzyme removes H_2O_2 from the perimeter in the presence of reduced glutathione [21]. The amount of malondialdehyde (MDA) produced in the cell is widely used to measure lipid peroxidation [22]. Free radicals in the cell membrane structure initiate the lipid peroxidation, a chain reaction that produces substances such as MDA. Lipid peroxidation changes the fluidity of cell membranes and reduces their capacity [23,24].

The term of cytotoxicity is used to show toxicity in cells, it expresses the state of causing cell death. Cytotoxicity is defined as the damage to cells at different levels depending on the exposure time and dose of the investigated substance, and as a result, the toxic capacity of the investigated substance appears. The cells may die or lose their ability to reproduce as a result of these events. The basis of the methods for determining cell viability is to determine the amount of live/dead cells at the end of the study [25]. Commonly used cytotoxicity test is the MTT test, which also provides information about cell proliferation, enables the determination of toxicity both morphologically and spectrophotometrically according to the formation of formazan crystals and solution density [26].

Cell damage is expressed as the deterioration of cell metabolism and functions when exposed to excessive stress as a result of internal and external factors. There are of two types of cell damage, reversible damage and irreversible damage. Reversible damage is a variety of stress changes that a cell undergoes due to both external and internal environmental changes. Depending on the extent of the damage, a cellular response occurs and homeostasis is restored. Irreversible damage occurs when the cell exceeds its ability to repair itself [27,28]. Genotoxic damage is detected with the comet test. Broken DNA, stretches moving away from the nucleus to the anode by electrophoresis are called comets. The amount of DNA in the tail is proportional to the number of thread breaks and provides a reliable rapid damage test [29,30,31].

Death receptors belong to the TNF receptor superfamily. They initiate a signaling pathway that results in cell survival or death, and cells die by apoptosis or necrosis. Necrosis; is a regulated cellular response to stress. It is a diverse response of cells to stress, depending on cell type and extracellular conditions, the type and intensity of stressful stimuli, and other factors. Apoptosis is the programmed cell death of unnecessary or potentially harmful cells in the body. It is an energy-dependent process that triggers cell death through the division of certain proteins in the cytoplasm and nucleus, mediated by proteolytic enzymes called caspases. Dying cells shrink and condense into apoptotic bodies [32]. Among the most common applications of fluorescent microscopy for cell death studies, acridine orange/ethidium bromide (AO/EB) staining identifies apoptotic and necrotic cells [25].

Based on these information, the study aims to reveal the toxicity of bendiocarb on the L929 cell line and the oxidative stress it causes *in*

vitro, by studying the MTT test, comet test, apoptosis test, antioxidant enzymes and MDA levels, genetically and cytotoxicity biochemical, spectrophotometric and microscopic analyzes.

2. Material and Methods

During the study, in order to keep the cells under investigation away from factors such as stress, the solution/solvent added to the cells was sterilized by passage and incubation at 37 °C, in the appropriate medium (RPMI 1640), in %5 CO_2, and centrifugation steps were performed for 5 min. It was treated at a low speed of 1000 rpm for a period of time. In this study, while investigating the toxic effects of bendiocarb on fibroblast cells, L929 mouse fibroblast cell line was chosen as the cell line. The cells were obtained from the American Type Culture Collection (ATCC). While preparing bendiocarb material, a 250 µg/mL stock was prepared by dissolving the dry matter (Bayer Ficam W 15 g insecticide) in dH_2O. Insecticide doses of 45,90,180 µg/mL were prepared by diluting the stock with RPMI 1640 medium.

2.1. MTT Test

Using a 96 well plate, the cells were distributed evenly in 3 repetitions with 10^3 cells in each well and incubated for 24 hours to allow the cells to adhere to the surface. It was completed and left to incubation for 24 hours for the doses to take effect. 10 µl of yellow colored MTT solution was added to all wells including the control group. After 4 hours, PBS buffer was added and measurements were taken at 570 nm absorbance in the spectrophotometer [26].

2.2. Determination of SOD, CAT and GPx Enzyme Activities

Enzyme activities were measured using the Bioassay Technology Laboratory ELISA Kit. 25 cm^3 flasks were set to have 10^6 cells per well, including control, were seeded into each one and incubated for 24 hours to adhere to the surface. Bendiocarb doses are added as control, 45, 90, 180 µg/mL. It was added sequentially as mL and the medium was added to make the final volume of 5 mL and incubated for 24 hours. The cells were taken into microcentrifuge tubes using trypsin-EDTA solution and centrifuged. In the following procedures, the kit protocol was applied. Finally, measurements were taken at 420 nm absorbance in the spectrophotometer [20].

2.3. Determination of Malondialdehyde Amount

MDA level were measured using the Bioassay Technology Laboratory ELISA Kit. 25 cm^3 flasks were set to have 10^6 cells per well, including control, were seeded into each one and incubated for 24 hours to adhere to the surface. Bendiocarb doses were added as control, 45, 90, 180 µg/mL. It was added sequentially as and the medium was added to make the final volume of 5 mL and incubated for 24 hours. The cells were taken into microcentrifuge tubes using trypsin-EDTA solution and centrifuged. In the following procedures, the kit protocol was applied. Finally, measurements were taken at 420 nm absorbance in the spectrophotometer.

2.4. Comet Test

For the comet test, 6 plates were used, $2x10^5$ cells were evenly distributed in each well. The cells were left to incubate for 24 hours to adhere to the surface. Bendiocarb doses were added sequentially as 45, 90, 180 µg/mL and the control group was formed. It was then removed with trypsin-EDTA solution and centrifuged. Low melting point agarose (LMA) was pipetted directly into the cells and spread onto previously coated agar slides and covered with a coverslip. The slides were incubated for 30 minutes at +4 °C. The cells in the frozen agarose were subjected to lysis, pre-electrophoresis, electrophoresis and dH_2O processes, after a 15-minute drying process, stained with EtBr and examined under a fluorescent microscope, their photographs were taken [31].

2.5. Apoptosis Test

A 96-well plate was used for the apoptosis test. The cells were seeded at 10^5 per well and incubated for 24 hours for the cells to adhere. Bendiocarb doses were added 45, 90, 180 µg/mL and a control group consisted of 4 groups. The cells were taken into eppendorf tubes using of tripsin-EDTA solution. The cells were pipetted in a 1:1 ratio with the dye prepared with acridine orange (AO) and ethidium bromide (EtBr) by pipetting into the ependorf, and spread on the slides, covered with a coverslip, and analyzed under fluorescent microscope for photography [25].

3. Results

3.1. MTT Test

When the treatment groups were compared to the control, a significant color change in the plate was observed at the doses of 90 and 180 µg/mL of bendiocarb and greatly reduced cell viability. Wells treated with bendiocarb doses of 45 µg/mL were showed values and color same

to the control group. Therefore, 45, 90, 180 µg/mL bendiocarb doses were studied in subsequent studies. Formazan crystals, which give a purple solution, can only be produced by living cells, in this case we can say that there is no vitality in the wells that do not give color. As a result, Bendiocarb showed a cytotoxic effect on the L929 cell line with the increasing application doses [figs 1-2].

Figure1. *Photograph of L929 cells attached to the flask surface, taken with a light microscope.*

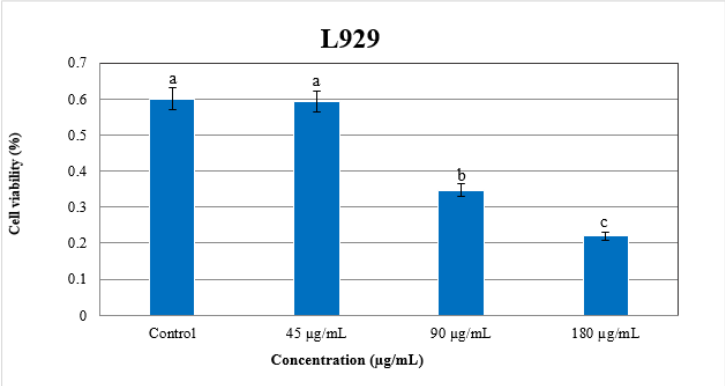

Figure2. *Effects of Bendiocarb L929 on cell viability (Groups that do not have the same letters on the columns express the difference between each other (P<0.05).*

3.2. Determination of SOD, CAT and Gpx Enzyme Activities and Amount of Malondialdehyde

When bendiocarb application groups at increasing doses were compared with the control group in terms of SOD, CAT, GPx enzyme activities, the dose-related increase in oxidative stress was detected with a decrease in the enzyme activity examined. It was determined that the increase in the amount of MDA was significant when the bendiocarb administration groups were compared with the control at doses with increasing MDA levels. The results were shown graphically in Figs. 3-6.

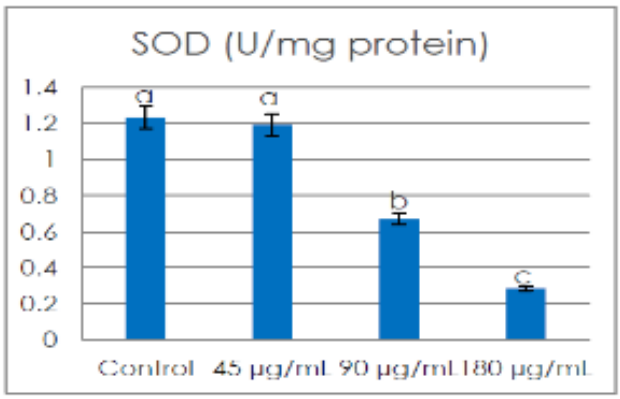

Figure 3. *Comparison of SOD enzyme activities of control and treatment groups. (Groups that do not have the same letters on the columns represent the difference (P<0.05)).*

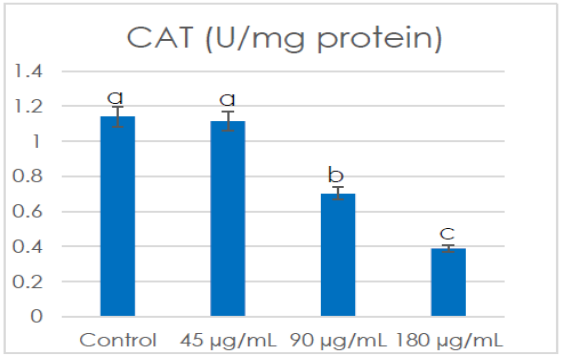

Figure 4. *Comparison of CAT enzyme activities of control and treatment groups. (Groups that do not have the same letters on the columns represent the difference (P<0.05).)*

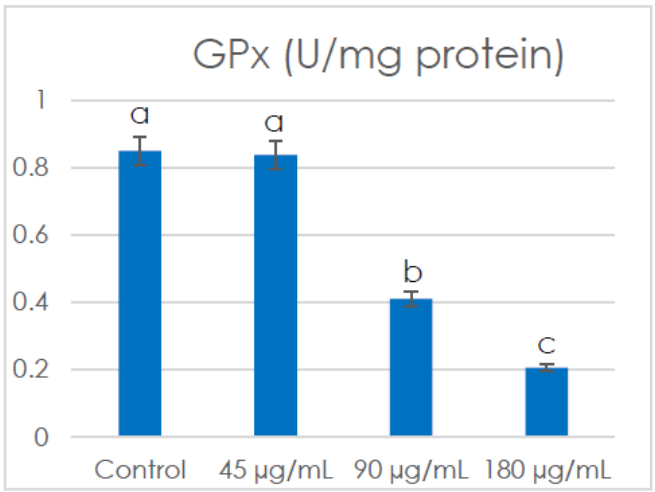

Figure 5. *Comparison of GPx enzyme activities of control and treatment groups. (Groups that do not have the same letters on the columns represent the difference (P<0.05).)*

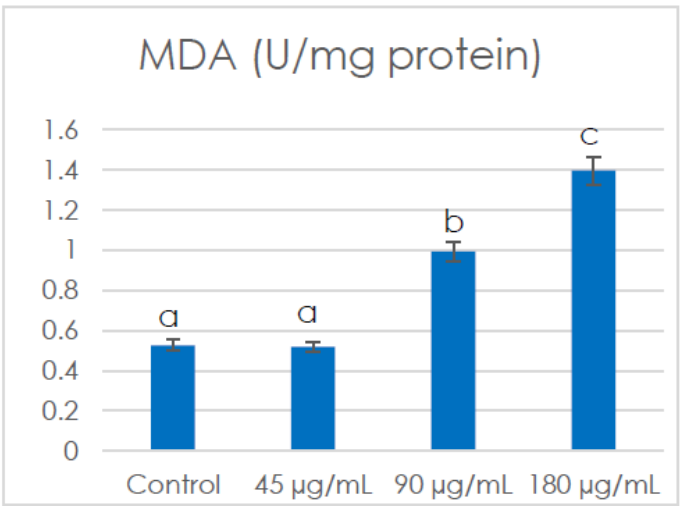

Figure 6. *MDA levels of control and treatment groups. (Groups that do not have the same letters on the columns indicate the difference between each other (P<0.05)).*

3.3. Determation DNA Damage of Bendiocarb with Comet Test

In our thesis study, genotoxicity measurements were made by the comet test method of preparations obtained from L929 cell lines exposed to three concentrations of bendiocarb (45 µM, 90 µM, 180 µM). The images obtained under the fluorescent microscope as a result of the concentrations studied are shown in Figure 7. When the concentration increase was compared with the control group, it was determined that there was an increase in DNA damage.

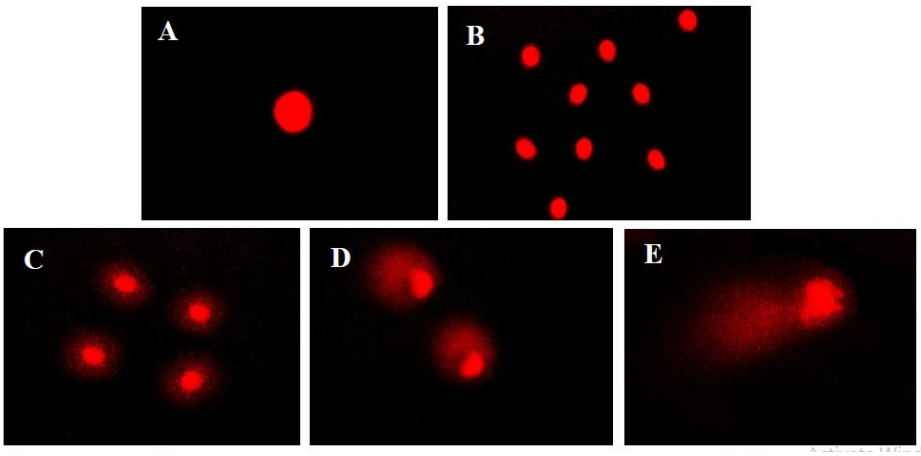

Figure7. *Comet images of L929 cells taken under a fluorescent microscope. Control group (A, B), 45 µg/mL bendiocarb treatment group (C), 90 µg/mL bendiocarb treatment group (D) , 180 µg/mL bendiocarb treatment group (E).*

3.4. Determation Cell Death with Apoptosis/Necrosis Test

In our study, cytotoxicity measurements were made by the apoptosis test method of the preparations obtained from L929 cell lines exposed to three concentrations of bendiocarb (45 µM, 90 µM, 180 µM). When the concentration increase was compared with the control group, it was determined that there was a decrease in cell viability. While the cells in the control group were seen as green, it was observed that the yellow, orange and red color increases were higher as the dose increased in the application groups. Green color indicates live cells, yellow-orange color indicates apoptosis, red color indicates necrotic dead cells.

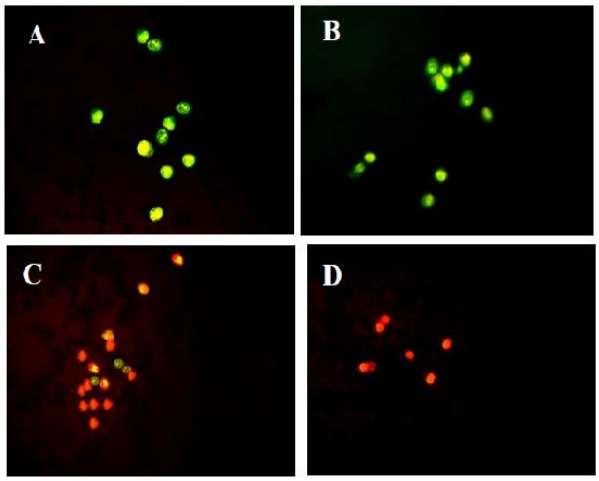

Figure8. *Apoptosis and necrosis images of L929 cells taken under a fluorescent microscope. Control group (A), 45 µg/mL bendiocarb treatment group (B), 90 µg/mL bendiocarb treatment group (C), 180 µg/mL bendiocarb treatment group (D).*

4. DISCUSSION

Since the toxic reflections of pesticides can be especially effective on the endocrine systems and adipose tissue, they cause the induction of diseases such as cancer. Many studies on the carcinogenic effects on living things and their effects on the emergence of other diseases draw attention to and support the toxic effects of pesticides on living things [33, 34]. In this study, the toxicity of bendiocarb was investigated together with oxidative stress, genotoxic effect and apoptotic effect in L929 cells treated with 45, 90 and 180 µg/mL concentrations and bendiocarb-induced oxidative stress on L929 mouse fibroblast cell line was determined by measuring SOD, CAT, GPx enzyme activities and MDA levels. Genetic damage and cell viability were tried to be determined by using comet, apoptosis and MTT analysis tests. The relationship between cell damage, apoptotic effect and oxidative stress with bendiocarb, a carbamate insecticide, was determined. This suggests that pesticides may be associated with various diseases such as cancer, asthma and leukemia. The risk of health hazards due to

pesticide exposure depends on the level of exposure as well as the toxic effect of the ingredients. It is estimated that %95 of applied pesticides has the potential to affect non-target organisms and degrade widely in the environment [35]. It has been reported that exposure to pesticides has harmful effects on the environment and human health, and literature studies have shown that it generally causes genotoxicity, mutagenicity, carcinogenicity, teratogenicity, neurotoxicity, endocrine disruption, reproductive development and immunological effects [36,37,24]. Because of pesticides are often applied in mixtures to crops, their residues can be found in food and drinking water. If streams and groundwater are used as a source of drinking water and previous treatment fails to remove pesticide residues, humans may be exposed to pesticide mixtures and their degradation [38]. Human exposure to low-dose pesticide mixtures can originate from foods and drinking water, and can also have long-term, adverse health effects, some of are associated with an increase in neurodevelopmental diseases along with chronic degenerative diseases [34]. Although the general population is exposed to pesticides, workers and farmers in the agrochemical industry represent a high-risk group due to occupational and environmental exposure. Occupational exposure to pesticides in agricultural workplaces occurs during mixing, loading preparation and spraying applications of pesticides [39]. It is difficult to determine the individual effects of pesticides, as complex mixtures are often used in the presence of additives in commercial forms. The effects of long-term occupational exposure at low pesticide doses are difficult to diagnose because they include temporary and non-specific health consequences [40]. It has been suggested that carbamates can induce oxidative damage through the generation of free radicals and the replacement of antioxidant enzymes or free radical sweeping enzymes [41]. Seth et al. found that CAT activity was significantly reduced in both pesticide-applied and non-pesticide-applied groups [42]. Again, a similar study shows that enzymatic changes, changes in oxidative balance, and genotoxic damage occur in workers exposed directly or indirectly to pesticides under experimental design conditions [43]. In addition, during the inhibition of the acetylcholine enzyme of these carbamate compounds, high consumption of ATP, coupled with the inhibition of oxidative phosphorylation, can produce excessive amounts of ROS and RNS in the cell by disrupting energy levels [44]. Studies have shown that pesticides can accumulate in living tissues even if they are used unconsciously and carelessly. It has been shown that insecticides increase chromosomal abnormalities and micronucleus formation in human lymphocytes [45]. In subjects exposed to pesticides, Valencia et al., (2021) clearly demonstrated higher levels of primary DNA damage, confirming the toxic effects of pesticide exposure as we noted in our study [46]. C. Lopez et al., (2016) showed that occupational exposure to a pesticide mixture caused a significant increase in the level of DNA damage. Exposed workers showed significantly longer tail lengths than unexposed individuals [47]. Using comet analysis in peripheral blood, Benedetti et al., (2013) shows that exposure to pesticides induces DNA damage and is a result of oxidative damage from exposure to pesticide mixtures [48]. Continuous exposure and persistence of unrepaired genotoxic damage caused by the generation of pesticides and free radicals can lead to higher levels of cytogenetic changes [46]. Superoxide anion is a highly reactive free radical that can attack a wide variety of cellular targets, including lipids, proteins, and DNA bases. [49]. In their study, Rosa et al. In 2006, they showed that superoxide anions cause apoptosis [50]. The data obtained from the apoptosis test, which was studied together with the antioxidant tests, were in agreement with this opinion. Esch et al., (2002) reported the caused mutations in MDA cells with lipid peroxidation [51]. The MTT test, an *in vitro* cytotoxicity test, is based on the idea that toxic chemicals affect the basic functions of cells common to all cells and that toxicity can be measured by assessing cellular damage [52]. This study is compatible with the results of other literature and bendiocarb showed a dose-dependent reducing effect in the direction of cell proliferation compared to the control in L929 cells.

5. Conclusion

The concentrations used in dose determination for analysis of toxicity in this study were 45 µg/mL,90 µg/mL,180 µg/mL and the mean dose killing %50 of cells as a result of the MTT test (LC_{50} value) was calculated 90 µg/mL for 24 hours. It was determined by the MTT test that increasing doses of bendiocarb reduced cell proliferation. Bendiocarb was studied at

increasing doses in this study, compared to the control group, there was no significant difference in the SOD, CAT, GPx enzyme activity of the 45 µg/mL treatment group, but a decrease by half in the 90 µg/mL and 180 µg/mL treatment groups compared to the control. Along the Bendiocarb treated cells as 180 µg/mL increased the MDA level considerably, the LC_{50} dose showed an increase in the amount of MDA by half compared to the control. Bendiocarb applied to L929 cells at increasing doses was observed to increase the tail at doses of 90 µg/mL and 180 µg/mL due to DNA damage in the cells. Smaller amounts of tail were observed when compared to the control and 45 µg/mL dose. In this study, a few live cells were observed at the LC_{50} dose of 90 µg/mL bendiocarb applied to determine apoptosis, while dead cells were observed intensely in the results of 180 µg/mL bendiocarb applied, while at the 45 µg/mL dose, the cells were found to be close to the control.

As a result, toxic effective doses of bendiocarb were determined by the MTT method, it was determined by comparing the amount of tail that bendiocarb caused DNA damage at high concentrations, decreased viable cells and increased bendiocarb concentrations with the comet method. When the effects of SOD, CAT, GPx enzymes were examined, it was determined that the activity of SOD, CAT, GPx decreased considerably and increased the MDA level in the perimeter.

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Citation: *Derya GUL1 & Dilek PANDIR "Investigation of Toxic Effects of Bendiocarb on L929 Cell Line" ARC Journal of Nutrition and Growth (AJNG) 8, no. 1, 2022, pp. 9-18. DOI: http://dx.doi.org/10.20431/2455-2550.0801002*