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# Determination of in Vitro Toxic Effects of Magnesium Oxide (MgO) and Nickel Oxide (NiO) Nanoparticles on Human Blood Tissue

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**Abstract**

*Nanotechnology is one of the fastest-growing research fields, yet the environmental and biological impacts of nanoparticles remain incompletely understood with the expansion of industry and technology, nanoparticle (NP) release into the environment has increased significantly. This study investigates the toxic effects of magnesium oxide (MgO) and nickel oxide (NiO) nanoparticles at varying doses (10, 100, and 1000 µg/mL) on human blood tissue using in vitro methods. Blood samples were divided into control (n = 6) and treatment groups (n = 6). Following NP exposure, erythrocytes and leukocytes were isolated, and antioxidant enzyme activities—superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)—and malondialdehyde (MDA) levels were measured using spectroscopic methods. DNA damage in leukocytes was analyzed using the Comet assay. The results demonstrated that increasing doses of MgO and NiO NPs elevated MDA levels and significantly reduced antioxidant enzyme activities in erythrocytes compared to the control group. DNA damage parameters, including DNA tail percentage, tail length, and tail moment, showed significant increases with higher NP doses in the treatment group. These findings indicate that MgO and NiO NPs negatively affect blood cell antioxidant defense systems and cause oxidative stress and DNA damage. In conclusion, this study highlights the toxic potential of MgO and NiO NPs at high doses in human blood tissue. The results provide valuable insights into the substructure of NP-induced toxicity, emphasizing the importance of understanding their biological and environmental effects.*

**Keywords:** *MgO, NiO, nanoparticle, comet, DNA damage*

## 1. INTRODUCTION

Factors that negatively affect the ecological balance are industrial wastes, detergents, artificial agricultural fertilizers, pesticides, inorganic salts, radioactivity and heavy metals. These substances cause great damage to nature. Most of the heavy metals are used in industry and the resulting waste materials are released into nature. In recent years, industrial developments have revealed that water resources, seas and soil are polluted by heavy metals and this pollution has a negative effect on living things. These heavy metals, which are ingested through water and nutrition, accumulate in the body of living organisms and have the effect of damaging and changing all vital activities.

Heavy metals have been found to have many carcinogenic effects. These carcinogenic metals have also been shown to cause DNA damage [1]. Symptoms such as tracheal irritation, immunologic changes, increase in alveolar macrophages, and decrease in immune suppression may occur in relation to inhalation of nickel compounds in the air [2]. When tobacco products are used, nickel is absorbed from the lung above the daily nickel intake [3]. Allergic reactions may occur in the skin as a result of skin ingestion. In studies, an increase in serum nickel was observed in cancer patients. Absorbed nickel first passes into the blood. In human body fluids, various amounts of nickel accumulation are observed in blood, urine, tissues, kidney and lung. Nickel accumulated in various diseases and stress affects metabolism [3]. Magnesium, which attracts attention with its lightness, one of the important metals that affect living things, is one of the important minerals for our body. In 1840, it was detected in an iron oxide mineral in the Vesivius region in Italy. In various studies

conducted in living organisms, it has been observed that the density of magnesium in blood plasma is highly correlated with calcium, phosphate, sodium and potassium. The majority of magnesium in plasma is free and diffusely complexed and the rest is bound to proteins [4].

Metal nanoparticles are widely used in consumer products, industrial products and especially in the field of medicine due to their easy synthesis [5]. However, recent studies have revealed the cytotoxic and genotoxic effects of nanoparticles. In many studies, an increase in DNA damage due to the increase in nanoparticle concentration has also been detected [5,6]. In a review by Hallock et al. (2009), it was stated that the toxic effects of nanoparticles depend on the basic material of the nanoparticle, i.e. which atoms it consists of, the size of the nanoparticle, whether it is coated with any coating material [7]. Metal nanoparticles, which can enter the cell and nucleus due to their small size, have been found to cause free radical formation and genetic damage by binding to DNA [5,8,9].

In pathological conditions occurring in living organisms and tumor formation, there is a balance between free radicals and antioxidants that neutralize them. The change of this balance in favor of free radicals causes oxidative stress [10]. Free radicals are substances that are formed as a result of incomplete reduction of oxygen in cells, causing changes in nucleic acids and bases in DNA, fractures in DNA, cancer formation, cell aging and death [11].

Antioxidants are chemical substances that prevent damage to cells by neutralizing free oxygen radicals in the cell [12]. It has been determined that antioxidants protect against tumor formation, aging and tissue damage caused by toxic agents by affecting free radicals [13,14].

Comet assay is a method mostly preferred in *in vitro* and *in vivo* antioxidant studies. Some enzymatic and non-enzymatic antioxidants have been found to prevent oxidative damage of DNA [15]. The toxic effect of some experimental chemicals whose genotoxic effect has not been fully determined can be determined by this method. One of the important advantages of the Comet test is the detection of DNA breaks with a very small cell sample. It also offers important advantages in terms of short time and cheapness of the chemicals used [16].

When cells with damaged DNA divide, they form mutant cells. Some chemicals can bind with DNA subunits and cause the formation of specific compounds. These compounds can be removed by the DNA repair mechanism. Some compounds bind permanently to DNA and can cause the formation of mutant cells by wrong translocation when the cell divides. Metal compounds with carcinogenic effects in humans have been identified by the International Agency for Research on Cancer (IARC). It is known that there is a link between events that cause cancer and events that cause mutations in somatic cells. It has been determined that many of the carcinogenic substances have mutagenic effects and many of the mutagenic substances have carcinogenic effects [17,18].

In this study, the cytotoxic effects of 10, 100 and 1000 µg/mL MgO and NiO NPs on human erythrocytes and leukocytes were investigated by spectrophotometer and fluorescence microscopy. Within the scope of this study, the determination of DNA damage in human blood cells as a result of the application of MgO and NiO NPs, the changes in antioxidant enzyme systems (SOD, CAT and GPx) and MDA are very important both in terms of human health and in terms of contributing to future studies on this subject.

## 2. Materials And Methods

### 2.1. Chemicals

MgO and NiO NPs were obtained from Sigma-Aldrich (Germany) and chemicals used in comet assay and biochemical analysis were obtained from Merck.

### 2.2. Preparation of Erythrocytes

In this study, 10 mL of blood samples were collected in heparinized tubes from six healthy individuals who did not smoke or drink alcohol and were not exposed to any chemical substances in the working environment. The necessary permissions for the study were obtained with the approval of the ICU Clinical Research Ethics Committee (18th meeting number and decision number 2017-KAEK-189-2019.09.25-09). Heparinized whole blood was centrifuged at 3000 rpm for 15 minutes. Leukocytes and plasma were removed, erythrocytes were washed 3 times with physiological saline solution (0.9% NaCl) and then cell suspensions were prepared with the same solution in 50% (v/v) ratio with PBS.

### 2.3. Application to Erythrocytes

Erythrocytes were divided into two groups as treatment group (n=6) and control group (n=6).

The treatment group was divided into four groups and incubated in chemicals for 1 hour. These were;

Group 1: Group in which 10 µg/mL MgO and NiO NPs were applied (n=6),

Group 2: Group in which 100 µg/mL MgO and NiO NPs were applied (n=6),

Group 3: Group in which 1000 µg/mL MgO and NiO NPs were applied (n=6),

The substances were added to the erythrocytes and incubated at 37 ºC for 1 hour. Erythrocytes were kept at -20 ºC until the study time and hemolysate was obtained by quadrupling with cold deionized water.

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) enzyme activities and malondialdehyde (MDA) content of hemolysate samples were determined by spectroscopy (Shimadzu 1800, UV/VIS Spectrophotometer, Kyoto, Japan) in comparison with the control group.

### 2.4. Isolation of Human Leukocytes and Application Groups

Peripheral blood from six healthy male volunteers (aged 25-30 years) who did not smoke or drink alcohol was collected in heparinized tubes before the test was performed. Leukocytes were isolated with Biocoll (Source BioScience, Nottingham, U.K.) separation solution [19]. Cell viability was determined by Pool-Zobel et al. (1992) trypan blue staining technique [20]. Cell viability was determined to be approximately 98%.

Leukocytes were grouped as control and experimental groups as given below:

- Control group; leukocyte hemolysate 50 µl, PBS 1.050 µl
- Application group with MgO and NiO NP; leukocyte hemolysate 50 µl, MgO and NiO NP (10, 100, 1000 µg/mL) and PBS 1.000 µl.

### 2.5. Determination of Malondialdehyde (MDA) Levels

MDA forms a pink colored complex after incubation with TBA at 90 °C under aerobic conditions. The absorbance of this complex is read at 532 nm wavelength in the spectrophotometer. Calculations and analysis were performed according to Ohkawa et al. (1979) [21]. In the test tubes, 2 mL of 0.375% TBA prepared in 15% TCA was taken and placed on 1 mL homogenate (300µl + 700µl distilled water). After vortexing, the tube was sealed and placed in a water bath at 95 °C for 30 minutes. After the water bath, the tubes were placed on ice and kept for 15 minutes, then removed from the ice and allowed to come to room temperature. After 10 minutes, the supernatant was centrifuged at 4000 rpm and the absorbance was read at 532 nm in a spectrophotometer against a blind tube. The amount of MDA, the product of lipid peroxidation, was calculated using a constant number, 1.56 x 105 M-1cm-1.

### 2.6. Determination of Antioxidant Enzyme Activities

#### *2.6.1. Determination of Superoxide Dismutase (SOD) Enzyme Activity*

The method of Marklund and Marklund was used for activity determination. For superoxide dismutase (SOD) enzyme activity determination, supernatants were centrifuged at 1000 rpm, 4 ºC for 10 min. First, different volumes of supernatant were added to the cuvettes containing Tris-EDTA buffer and enzyme source was added. Then pyrogallol was added to these mixtures and absorbance measurements were made in 440 nm spectrophotometer. As a result of the measurements, the activity was determined as U/mg Hb for blood cells [22].

#### *2.6.2. Determination of Catalase (CAT) Enzyme Activity*

Activity was determined by the Aebi method. For the determination of catalase enzyme activity, the supernatants were centrifuged at 1000 rpm for 10 minutes at 4 ºC. First, Triton X-100 was added to the supernatant to release CAT from peroxisomes. Then $H_2O_2$ was added and absorbance values at 240 nm were measured. After the calculations, the enzyme activity was given in units of U/mg Hb for blood cells [23].

#### *2.6.3 Determination of Glutathione Peroxidase (GPx) Enzyme Activity*

Paglia and Valentine's method was applied to determine GPx enzyme activity. For 20 minutes, the supernatants were centrifuged at 16,000 rpm at 4 ºC. This method is based on the principle of measuring the absorbance at 340 nm caused by the oxidation of nicotinamide-adenine-dinucleotide hydrogen phosphate (NADPH) by GR. Oxidation of NADPH to Nicotinamide-adenine-dinucleotide phosphate (NADP) causes a decrease in absorbance at 340 nm. It was also used indirectly in the determination of GPx activity. The reaction was started by adding $H_2O_2$ to this mixture and absorbance values at 340 nm

were read for 3 minutes. Enzyme activity results were given as U/mg Hb for blood cells [24].

### 2.7. Detection of DNA Damage by Comet Assay

The 1% high melting point agar was heated in a microwave oven to a liquid state. The slides soaked in liquid agar were allowed to dry at room temperature for one day. From the blood sample taken into heparinized, vacuum and sterile tubes; 15 µl of the blood sample was added into ependrof tubes containing 1000 µl of RPMI-1640 medium. 80 µl of 0.65% agarose was added to the pellet obtained by centrifugation at 2000 rpm for 5 min and pipetted. 75 µl of this mixture was poured onto the slides prepared the day before, covered and incubated at +4 °C for 30 minutes. The preparations were kept in the solubilization solution for 90 minutes and then placed in diluted electrophoresis for 40 minutes and run at 200 Volts for 4 minutes. After the end of the execution, they were kept in $dH_2O$ 3 times for 5 minutes each and left to dry. The dried preparations were prepared for microscopic examination by adding 80 µl ethidium bromide.

### 2.8. Evaluation of Data With Spss

All statistical analyzes were performed by Tukey test and one-way analysis of variance (ANOVA) using Windows SPSS 11.5 computer program. As a result, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Determination of Malondialdehyde (MDA) Level

Human blood tissue was treated with 10, 100 and 1000 µg/mL MgO and NiO NPs under in vitro conditions and MDA levels of erythrocytes were measured by spectrophotometer. The results obtained are given in Figure 3.1. When the control group was compared with the treatment groups, the increase in MDA levels was found to be significant.

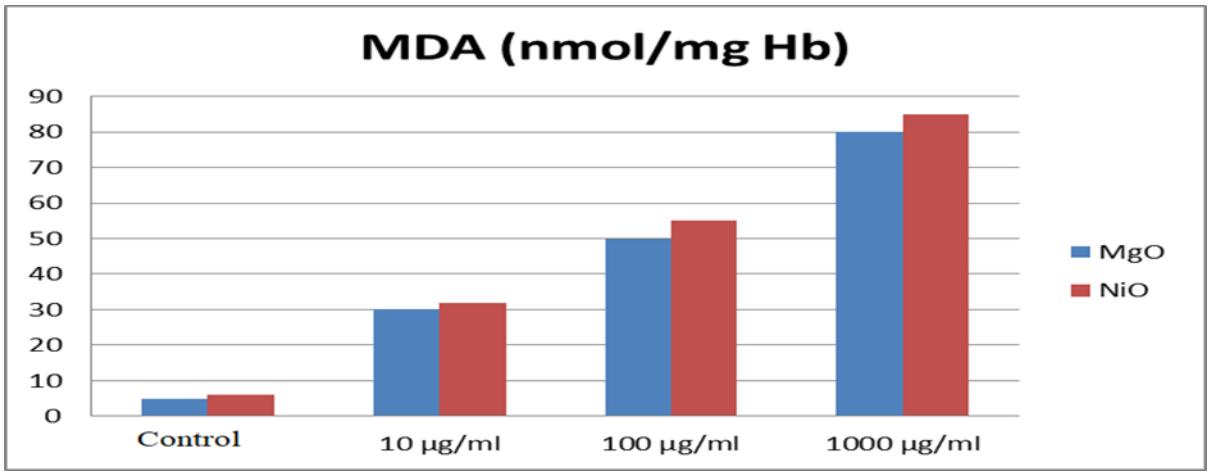


**Figure1.** *Effect of 10, 100 and 1000 µg/mL MgO and NiO NPs on MDA levels in human erythrocytes.*

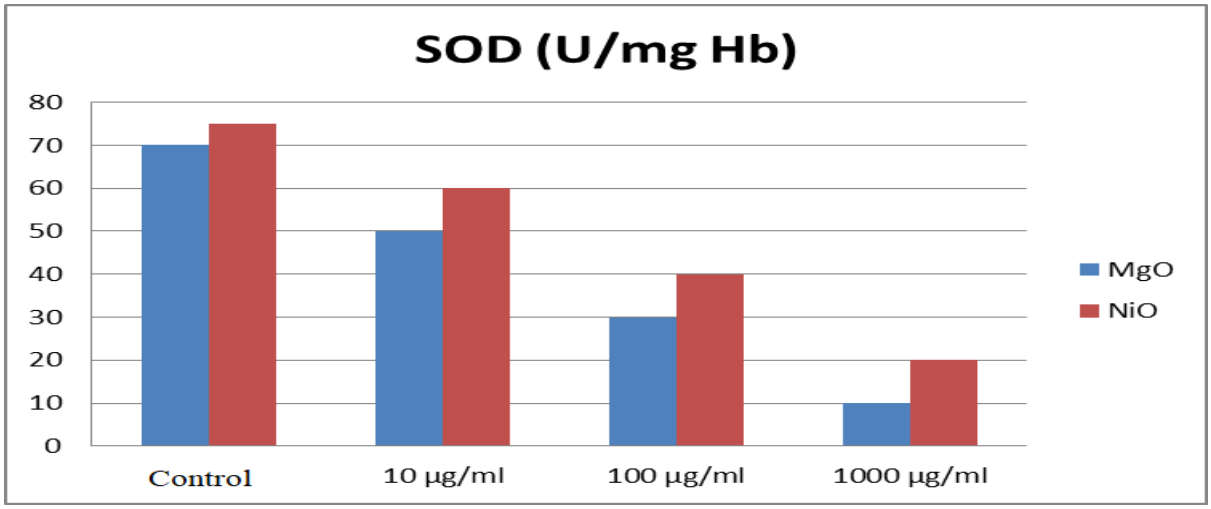


**Figure 2.** *Effect of 10, 100 and 1000 µg/mL MgO and NiO NPs on SOD enzyme activity in human erythrocytes.*

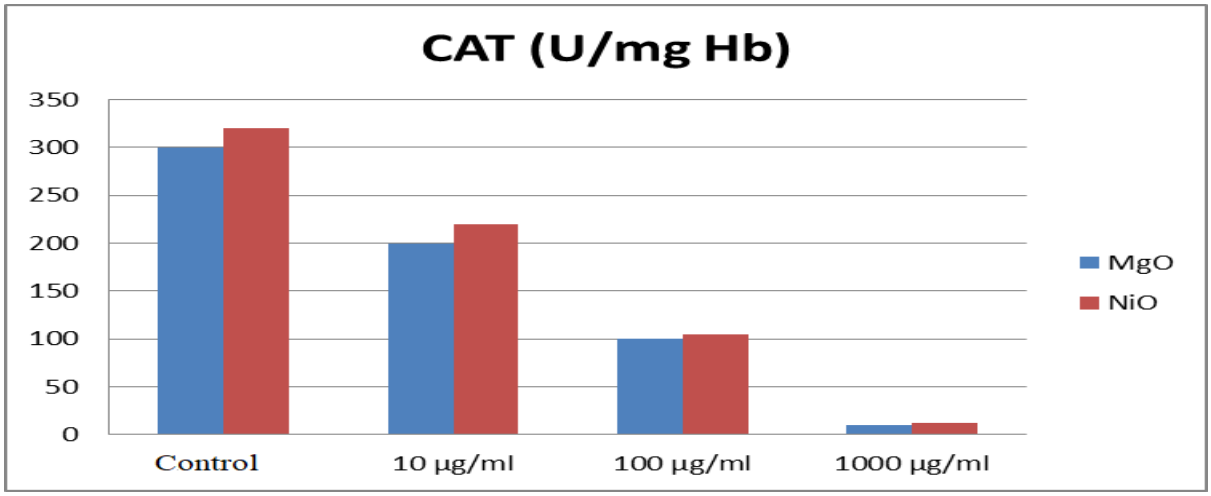


**Figure 3.** *Effects of 10, 100 and 1000 µg/mL MgO and NiO NPs on CAT enzyme activity in human erythrocytes.*

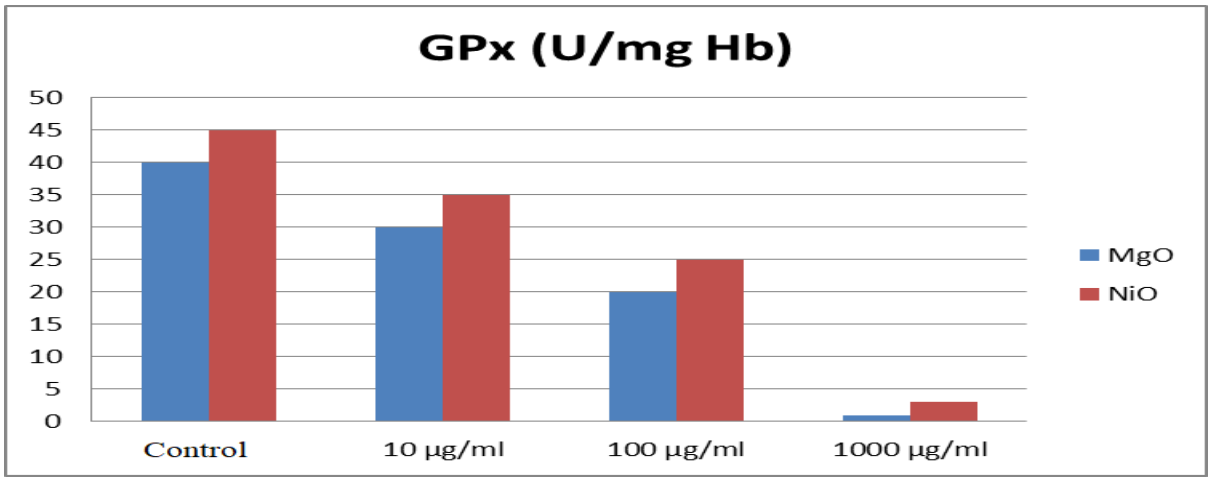


**Figure 4.** *10, 100 and 1000 µg/mL MgO and NiO applied to human erythrocytes and effect of NPs on GPx enzyme activity.*

### 3.2. Evaluation of Enzyme Activities

Human blood tissue was treated with 10, 100 and 1000 µg/mL MgO and NiO NPs under in vitro conditions and SOD, CAT and GPx enzyme activities of erythrocytes were measured by spectrophotometer. The results obtained were given in Figure 3.2-3.4.

When the control group was compared with the treatment groups, the decrease in SOD, CAT and GPx enzyme activities was found to be significant.

### 3.3. Evaluation of Comet test Results

The Comet assay includes DNA tail length, tail percentage and tail moment parameters to determinethechanges in DNA structure of leukocytes from human blood cells. Leukocytes were treated with 10, 100 and 1000 µg/mL MgO and NiO NPs under in vitro conditions and DNA tail length, tail percentage and tail moment of leukocytes were measured under fluorescence microscope and given in Table 3.1 and Figures 3.5 and 3.6. When the control and treatment groups were compared, DNA tail percentage, tail length and tail moment were significantly increas

T**able1.** *Mean values of DNA damage (±SD) tail % DNA, tail length and tail moment in control and treatment groups in blood tissue*

| Comet Parameters / Groups | Tail DNA% Mean±SD | Tail Length Mean±SD | Tail moment Mean±SD |
|---|---|---|---|
| **MgO NP** | | | |
| Control | 21.58± 1.87[a] | 14±0.25[a] | 3.02±0.04[a] |
| 10 µg/mL | 44.82 ±3.12[b] | 31±0.12[b] | 13.89±0.03[b] |
| 100 µg/mL | 75.80 ±7.25[c] | 97.41±12.35[c] | 73.83±0.89[c] |
| 1000 µg/mL | 90.78±12.25[d] | 125±19.42[d] | 113.47±2.37[d] |

| NiO NP | | | |
|---|---|---|---|
| Control | 14.52±0.66 [a] | 6.54±0.87 [a] | 0.94±0.05 [a] |
| 10 µg/mL | 36.40±1.55[b] | 26.03±2.45 [b] | 9.47±0.03 [b] |
| 100 µg/mL | 63.90±4.85[c] | 81.06±5.40 [c] | 51.79±0.26[c] |
| 1000 µg/mL | 99.86±12.20[d] | 131.42±24.21[d] | 131.23±2.95[d] |

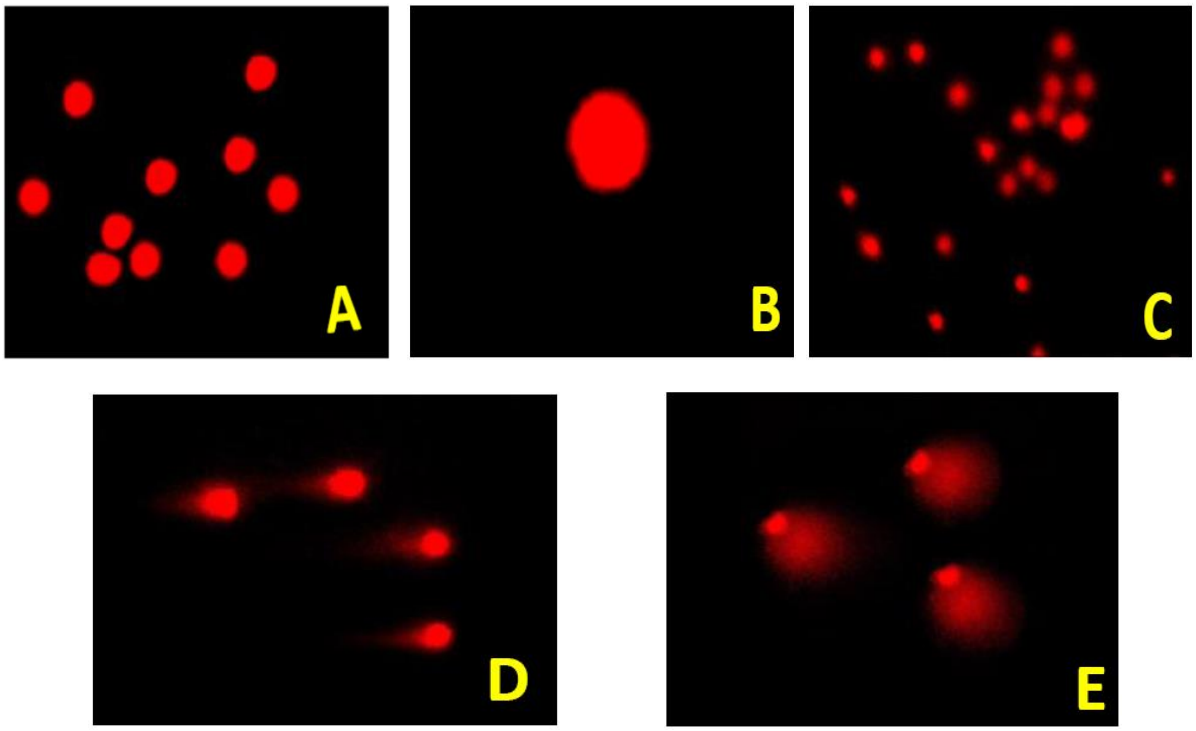


**Figure 5.** *Determination of toxic effects of increasing doses of MgO NPs on DNA structure of human blood leukocytes by comet assay. (A-B) Control group; (C) 10 µg/mL MgO NP applied group; (D) 100 µg/mL MgO NP applied group; (E) 1000 µg/mL MgO NP applied group.*

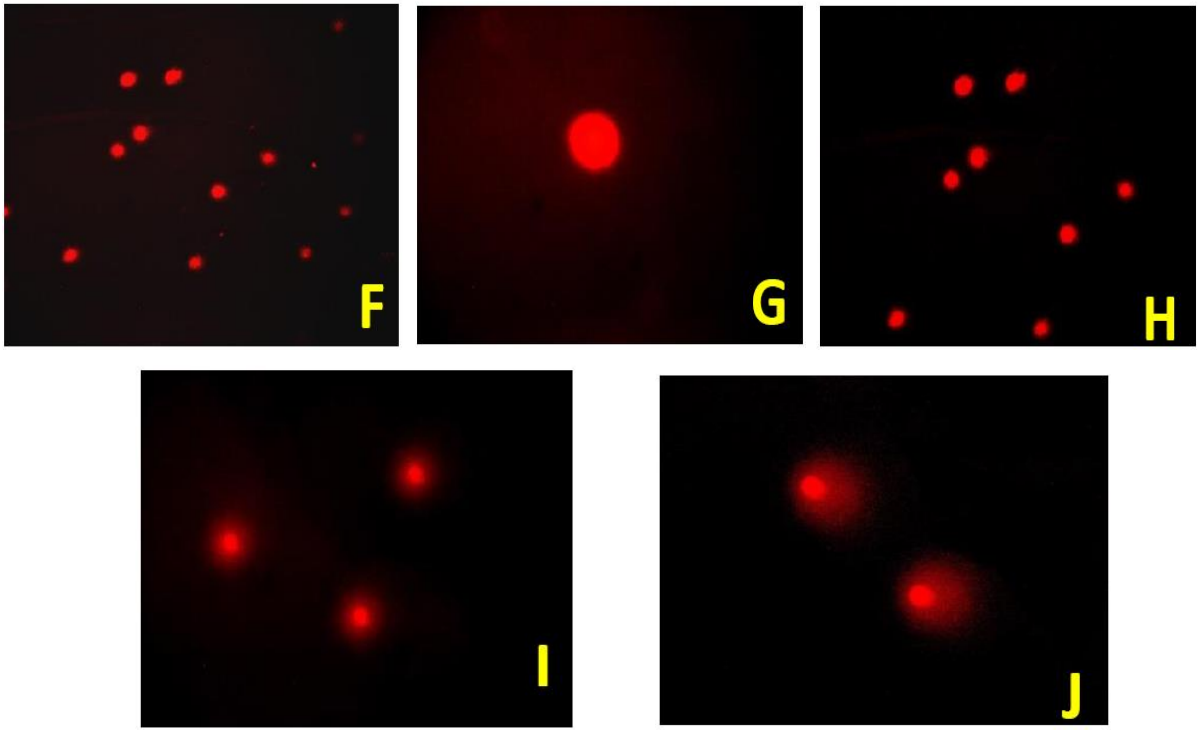


**Figure 6.** *Determination of the toxic effects of increasing doses of NiO NPs on the DNA structure of human blood leukocytes by comet assay. (F-G) Control group; (H) 10 µg/mL NiO NP applied group; (I) 100 µg/Ml NiO NP applied group; (J) 1000 µg/mL NiO NP applied group.*

## 4. Discussion and Conclusion

Nanoparticles can affect human blood cells in different ways. The most common effect is hemolysis (rupture of the erythrocyte membrane) and generation of free radicals. They can also cause erytosis or erythrocyte apoptosis [25]. Other effects on human and animal erythrocytes include morphological changes, induction of oxidative stress and alteration of enzymatic activities. In this study, 10, 100 and 1000 µg/mL MgO and NiO NPs were administered to human blood tissue under in vitro conditions and their effects on antioxidant enzyme activities of erythrocytes, MDA levels and DNA structure of leukocytes were statistically revealed by comparing control and treatment groups. Once nanoparticles enter the human body, the defense system recognizes most of these nanoparticles as foreign agents and in some cases they can activate an immune or inflammatory response. Among peripheral blood nuclear cells (PBMCs), 70-90% round nucleated lymphocytes and 10-30% monocytes are important cells of the immune system that readily emerge from peripheral blood. Recent studies have determined how the immune system responds to the presence of different NPs in the case of NP cytotoxicity of PBMCs, especially lymphocytes [26].

Martinez-Rodriguez et al. (2019) conducted in vitro studies with nickel and zinc NPs at different concentrations (50, 100 and 200 µg/mL) to evaluate NP toxicity. The toxicity of NPs was evaluated by testing red blood cell hemolysis, total cell protein content, catalase and glutathione-S-transferase activities in humans. As a result, it was determined that nickel-zinc NPs caused hemolysis and increased glutathione-S-transferase activity. In vitro studies with different NPs in viability tests in human peripheral blood mononuclear cells showed no viability [27].

In another study, Ran et al. (2015) investigated the use of suicidal death of erythrocytes as a novel predictive and prognostic parameter and the effect of Fe3O4-NPs on circulating cellular membrane structure and rheology properties of blood. As a result, although no significant changes were observed in histopathology, hematology and serum biochemistry indices in vivo, Fe3O4-NPs significantly affected hemorheology indices, including erythrocyte deformation index, erythrocyte stiffness index, red blood cell deposition index. Oxidative stress-induced calcium influx played a critical role in the enzyme activity of Fe3O4-NPs in erythrocytes. It showed that Fe3O4-NPs caused changes in the flow properties of erythrocytes and blood [28].

NPs use many pathways to enter the cell, the main ones being endocytosis or cohesion. Once inside the cell, they start to show toxic effects. The mechanism of the damages caused by NPs in the cell is not fully known and the main effects are oxidation and disintegration of the cell membrane, decrease in energy production in the cell, proliferation of reactive oxygen species and release of harmful substances [29]. The greatest toxic effect on the cell membrane structure is the oxidation of membrane proteins and lipids. In this oxidation, lipid peroxidation is used as an important parameter in scientific studies. The amount of MDA is used as a marker of the damage caused by the toxic substance to the cell [30]. In this study, MgO and NiO NPs were used at increasing doses and the amount of MDA in erythrocytes was determined. As the application doses of NPs increased, a statistical increase was observed in the MDA levels of erythrocytes.

The most common antioxidant enzymes that are activated against chemicals that cause oxidative damage are SOD, CAT and GPx [31]. These enzymes constitute the first line of the cellular defense system and their activities can increase or decrease within the cell for various reasons. SOD is an enzyme against superoxide radical and catalyzes its dismutation to $H_2O_2$, which will be used by CAT [32]. Therefore, SOD and CAT are widely used parameters in experimental studies to determine oxidative stress and toxicity. In this study, the possible effects of MgO and NiO NPs on SOD, CAT and GPx enzyme activities were determined spectrophotometrically. Antioxidant enzyme activities in the cell decreased as the amount of NPs increased. This decrease was found to be statistically significant in the control and treatment groups, and in the group where the highest dose was given, MgO and NiO disrupted the structure of the enzymes and decreased their activities.

In recent years, the comet method has been used in studies on DNA damage caused by agents such as radiation and environmental toxins. Therefore, in this study, the genotoxic effect of human blood cells exposed to MgO and NiO NPs under in vitro conditions was determined by the comet method. In this fast and sensitive method, a damaged cell takes the shape of a comet, consisting of head and

tail regions. The length and density of the tail varies according to the rate of degradation of single or double strands in the DNA. At the same time, the percentage and length of DNA in the tail provides quantitative data on damaged DNA [33].

Due to the increasing use of gold nanoparticles (Au NPs) in different fields such as medicine, biotechnology or the food sector, exposure in humans has increased significantly and toxicity assessment has become mandatory. Therefore, Avalos et al. (2018) compared the *in vivo* mutagenic and recombinogenic activity in Drosophila (SMART Test) and the potential genotoxic effects of 30, 50 and 90 nm Au NPs using the in vitro comet method. The results showed that 30, 50 and 90 nm (1-10 mg/mL) Au NPs increased DNA strand breaks in the treated groups after 24 hours of treatment [34]. (2012) evaluated the genotoxicity of Fulleren C60 NPs in vivo by using lung cells of rats administered C60 NPs using the comet method. It was determined by comet analysis that C60 NPs have genotoxic effect [35]. In this study, MgO and NiO NPs were found to damage cellular DNA under in vitro conditions by increasing their effect at increasing doses. This effect was found to be approximately three times higher in DNA tail length when the effect at 1000 µg/mL NP was compared with the effect at 10 µg/mL NP.

It has begun to be thought that nanoparticles may be among the reasons why changes are seen in many diseases, for example, the age of cancer decreasing and progressing in an aggressive structure, the age of heart attack gradually decreasing, and the clotting of the brain, which is normally seen in advanced ages, is now seen in young generations. For this reason, more scientific studies on the subject are needed [36]. In this study, it was demonstrated that the nanoparticles used in this study damage the cellular antioxidant defense system and DNA under in vitro conditions and an infrastructure was tried to be created for other scientific studies.

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