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Size-dependent toxicological effects comparison of Aluminum oxide nanoparticles (Al_2O_3 NPs)

Alüminyum oksit nanopartiküllerinin (Al_2O_3 NP'leri) boyuta bağlı toksikolojik etkilerinin karşılaştırılması

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ABSTRACT

Aim: Modification of nanomaterials with different synthesis methods can affect their biological response, as well as their use as nanotherapeutics. It is necessary to address and understand the safety issue of these particles through toxicological evaluation with an underlying mechanism of interaction. With the fast entry of aluminum-based nanoparticles into the industry, their potential exposure has also increased significantly. Aluminum oxide nanoparticles (Al_2O_3 NPs) are among the priority materials by international organizations. Studies have not yet elucidated the toxic response of Al_2O_3 NPs depending on their size range.

Materials and Methods: Therefore, this study aimed to investigate toxicological effects of Al_2O_3 NPs depending on size range on MCF-10 and MCF-7 cells by WST-1 test, hemolytic activity on red blood cells and irritation effects by HET-CAM test.

Results: As a result of tests, all size ranges of Al_2O_3 NPs didn't show any cytotoxic effects on MCF-10 and MCF-7 cells, also none of sizes of Al_2O_3 NPs were caused hemolysis (<2%). It was observed that there was no irritating effect in all size ranges on HET-CAM test.

Conclusion: In conclusion, risk assessments in terms of characteristic features as the size of Al_2O_3 NPs showed that they have the potential to provide safe use in drug delivery systems and immobilization studies.

Keywords: Aluminum oxide nanoparticles, Size-depended toxicity, Biocompatibility, HET-CAM.

ÖZ

Amaç: *Farklı sentez yöntemleri kullanılarak nanomateryallerin modifiye edilmesi, biyolojik yanıtlarını ve nanoterapötik olarak kullanımlarını etkileyebilir. Bu parçacıkların güvenliği konusunu, altta yatan etkileşim mekanizması ile toksikolojik değerlendirme yoluyla ele almak ve anlamak gerekmektedir. Alüminyum bazlı nano parçacıkların endüstriye hızlı bir şekilde girmesiyle, potansiyel maruziyetleri de önemli ölçüde arttı. Alüminyum oksit nano parçacıkları (Al2O3 NPs) uluslararası kuruluşlar tarafından öncelikli malzemeler arasında yer almaktadır. Araştırmalarda, Al2O3 NP'lerin boyut aralığına bağlı olarak toksik yanıtı henüz ortaya konulmamıştır.*

Gereç ve Yöntem: *Bu nedenle bu çalışmada, Al2O3 NP'lerinin boyut aralığına bağlı olarak MCF-10 ve MCF-7 hücreleri üzerindeki toksikolojik etkilerinin WST-1 testi ile, kırmızı kan hücreleri üzerindeki hemolitik aktivitesinin ve HET-CAM testi ile tahriş etkilerinin araştırılması amaçlanmıştır.*

Bulgular: *Test sonuçlarında, Al2O3 NP'lerin tüm boyut aralıklarında MCF-10 ve MCF-7 hücreleri üzerinde herhangi bir sitotoksik etki göstermediği, ayrıca Al2O3 NP'ler hiçbir boyutunda hemolize neden olmamıştır (<2%).*

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HET-CAM testinde tüm boyut aralıklarında tahriş edici bir etki olmadığı gözlemlendi.

Sonuç: *Sonuç olarak, Al_2O_3 NP'lerinin boyut olarak karakteristik özellikleri açısından yapılan risk değerlendirmesinde, ilaç taşıma sistemlerinde ve immobilizasyon çalışmalarında güvenli kullanım sağlama potansiyeline sahip olduklarını göstermiştir.*

Anahtar Sözcükler: *Alüminyum oksit nanopartiküller, boyuta bağlı toksisite, biyouyumluluk, HET-CAM.*

INTRODUCTION

The physico-chemical features of nanoparticles as size, shape, surface area, surface charge as well as route and frequency of exposure are among the factors that affect their behavior in biological systems and their toxicity. There is a need to investigate these nanomaterials depending on their application areas and their potential effects on the environment and human health. Particularly metallic nanoparticles attract attention with their wide usage areas. Metal oxide nanoparticles have recently been produced at an industrial level and have widespread applications in water purification, medicine, cosmetics, and engineering (1,2). One of these materials is aluminum oxide nanoparticles that have been the subject of research due to their unique properties. The Organization for Economic Cooperation and Development (OECD) has presented 14 nanomaterials including aluminum oxide nanoparticles (Al_2O_3 NPs) as priority materials for the investigations (3). Al_2O_3 NPs are used in many fields such as enzyme immobilization, drug delivery, biosensors, wastewater management with produced approximately 20% of the 2005 world market of nanoparticles (4). This shows that their use in application areas will grow over the years with increased synthesis types of these materials. However, despite the widespread use of such a priority material, there is limited information available on size-depend potential hazards. It is necessary to determine the potential effects of the physico-chemical properties as a result of increased exposure and also bring the deficiencies into the literature.

In this study, the cellular response after exposure to different sizes of aluminum oxide nanoparticles (Al_2O_3 NPs) was discussed in order to determine the effect of size, which is an important characteristic parameter in the toxicity and effectiveness of nanomaterials.

MATERIAL AND METHODS

Materials

Aluminum oxide nanoparticles with primary sizes (Al_2O_3-48nm; Al_2O_3-78nm; Al_2O_3-100nm) were obtained from *Nanografi* Company with characteristic features data as spherical morphology and 99.95% purity. All other chemicals were obtained from Sigma-Aldrich (USA).

Characterization analyses

In order to determine the size distribution of Al_2O_3 NPs in different pH conditions (pH 4, pH 7.5 and pH 10) were determined with ZETASizer (Malvern ZETA ZS, England).

In vitro studies

Cell Culture and Cytotoxicity Assay

MCF-10 (Human mammary epithelial cell line) and MCF-7 (Human breast cancer cell line) were kindly provided by Dr. Balcan (Manisa Celal Bayar University) and Dr. Karataş (Erzurum Technical University). The cell lines were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/ streptomycin in T75-cm^2 culture flasks. The cells were incubated at 37°C in 5% CO_2. The culture was changed every 2 days until cells were confluent.

The WST-1 assay, which gauges mitochondrial reductase activity, was used to conduct the cytotoxicity test (5). 2×10^4 cells/mL were seeded in 96-well plates and then, different treatment concentrations were applied to the cultivated cells (6.25 µg -500 µg /ml) of Al_2O_3 NPs for 24h at 37 ºC. Using an inverted light microscope, the cells in each treatment group were inspected for morphological changes, and THERMO microplate reader was used to assess the optical density of each plate at 450 nm. The percentage representation of the relative viability was calculated using untreated cells as the negative control. The formula below was used to determine the viability (%):

(%) Viable cells= ([(the absorbance of the treated cells)-(the absorbance of the blank)])/([(the absorbance of the control)-(the absorbance of the blank)) x100

Hemolysis assay

The blood sample obtained from New Zealand Albino rabbit with the approval of Ege University Animal Experiments Local Ethics Committee (EÜHADYEK- July 27, 2022/no. 2022-055) to be used in the study. Test was performed according to Bakan (2020) protocols (5) and ASTM standards (6). First, 6.0 mL of phosphate buffered saline (PBS) was added to the 3 mL of blood and centrifuged at 3000 g for 10 minutes. Then, the pellet containing red blood cells (RBC) was separated and washed 3 times with an equal volume of PBS. The remaining volume after washing was diluted 1:1 with PBS. The test material was applied in different dose ranges (20-160 µg/ml) depending on the therapeutic dose. Subsequently, 0.8 mL of test samples at the applicable concentrations were combined with 0.2 mL of RBC suspension, and the tubes were incubated for 3 hours at 37 °C in a water bath, with 30 minutes mixing intervals. PBS was utilized as the negative control and Triton X-100 (1%) as the positive control. After incubation, the tubes were centrifuged at 3000 g for 4 minutes and the absorbance values of all sample supernatants were taken at 540 nm. According to the ASTM standard, <2% is considered as not hemolytic; 2%–5% slightly hemolytic; and>5% was accepted as hemolytic.

The percentage of hemolysis was calculated with the following formula;

Hemolysis %=(A sample -A0)/(A100-A0) X 100

A100 represents the absorbance of fully lysed red blood cells, A0 represents the absorbance of non-blood samples, and A sample is the absorbance value of the sample.

Hen's egg test on chorio-allantoic membrane (HET-CAM) test

The test is an alternative test developed by EURL-ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) to determine the degree of irritation. It allows to see possible effects of substances by observing alterations in the egg's chorio-allantoic membrane following exposure to the test sample. Test was carried out in accordance with ICCVAM (7) on fertilized chicken eggs (50-60g) with three independent replicates and egg were incubated at 37±0.5°C'de, 70% humidity for 7 days. On the 7th day, an area (2x2cm) was opened at the equator of the eggs and 300 µL of each test material was dropped directly onto the CAM surface and left in contact for 0.5, 2 and 5 minutes. 0.9% NaCl solution was used as a negative control and 0.1N NaOH was used as a positive control. After exposure of each sample, the membrane was examined for vascular damage and the elapsed time was recorded. The possible irritation degree score (IS) was calculated as follows;

IS = [(301 − tH) × 5]/ 300 + (301 − tL) × 7]/ 300 + (301 − tC) × 9]/ 300

where tH, tL, and tC are the corresponding (in seconds) timespans for hemolysis, lysis, and coagulation. Formulations can be categorized as non-irritating (IS < 1), slightly irritating (1 ≤ IS < 5), moderately irritating (5 ≤ IS < 10), or extremely irritating (IS > 10), based on their IS values.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software, LLC, Boston, MA, USA) and results compared with the control group using ONE-WAY analysis of variance (ANOVA). All values are expressed as means ± standard deviation (SD). Statistical significance was set to $p<0.05$.

RESULTS

Characterization analyses results

The sizes of Al_2O_3 NPs in different pH conditions were performed with ZETAsizer to observe their behaviour in diverse environments. As seen in all other types of nanoparticles, Al_2O_3 NPs tend to agglomerate at all dimension in different pH environments as presented in Figure-1.

Cytotoxicity test results

In the test results, there is no significant cytotoxic effects of all size range of Al_2O_3 NPs at applied doses on both of cell lines after 24h exposure as presented in Figure-2 and Figure-3.

Hemolysis test results

According to hemolysis test results, none of Al_2O_3 NPs showed hemolytic activity at applied doses (20, 40, 80 and 160 µg/ml) on erythrocytes as presented in Figure-4.

HET-CAM test results

The test, which was carried out to reveal the irritation effect of different sizes Al_2O_3 NPs were evaluated in terms of hemorrhage, lysis and coagulation parameters, and no irritant effects were observed at all size ranges of Al_2O_3 NPs as presented in Figure-5.

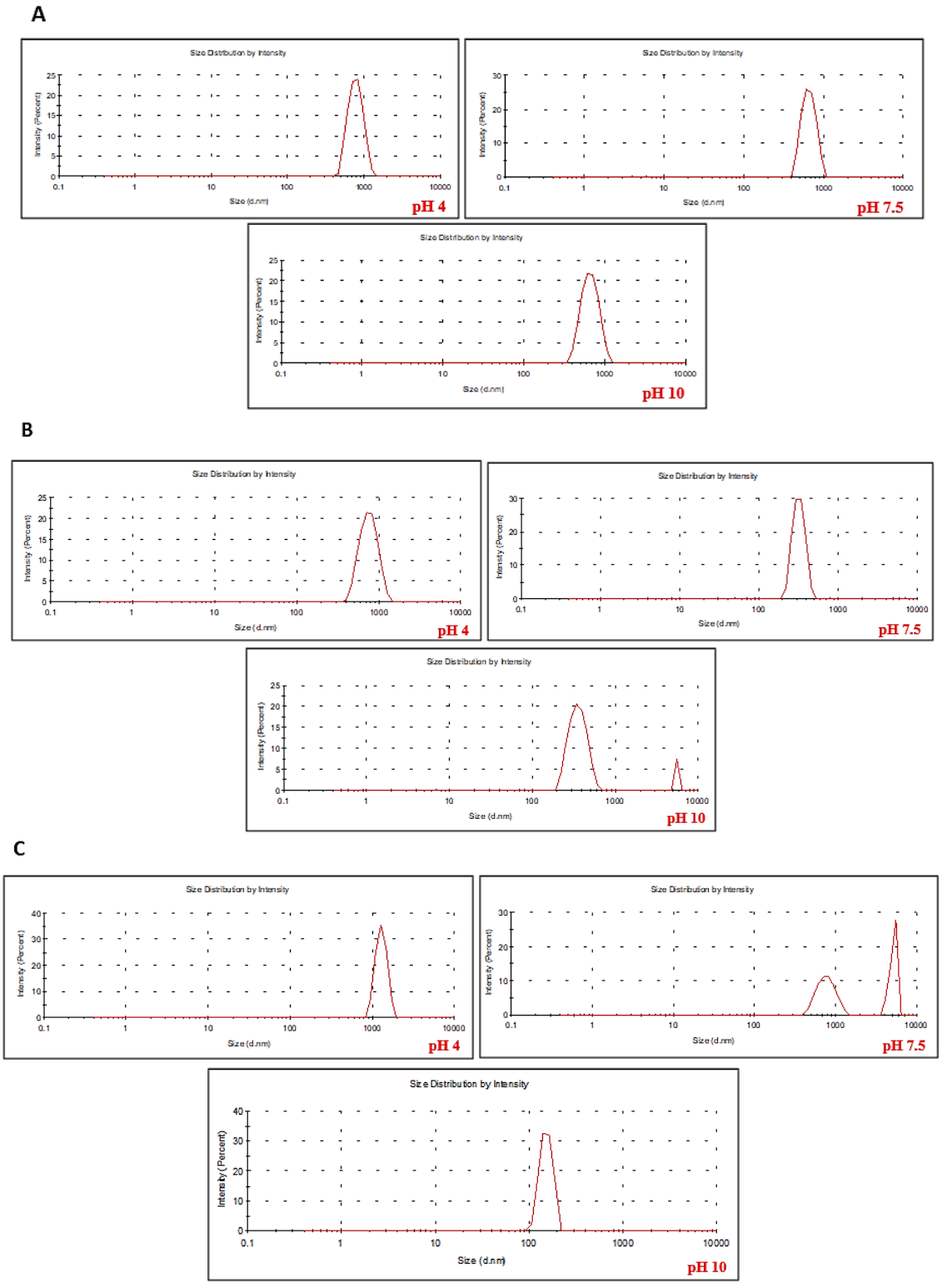

Figure-1. Size distribution of Al_2O_3 NPs (A. Al_2O_3-48nm; B. Al_2O_3-78nm; C. Al_2O_3-100nm) at different pH conditions (pH4, pH7.5 and pH10) by ZETASizer analyses.

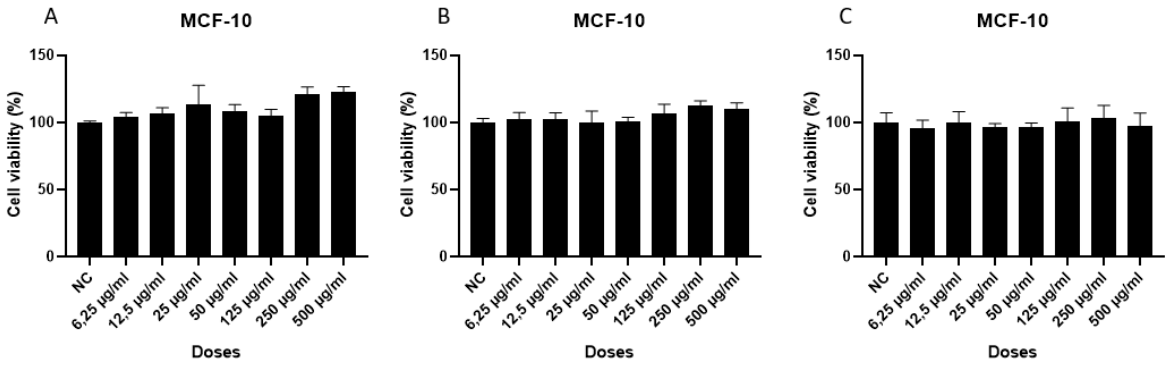

Figure-2. Cytotoxicity test results of Al_2O_3 NPs on MCF-10 cell line after 24h exposure time by WST-1 assay; **A.** Al_2O_3-48nm; **B.** Al_2O_3-78nm; **C.** Al_2O_3-100nm. All values are expressed as means ± SD with three independents repeated.

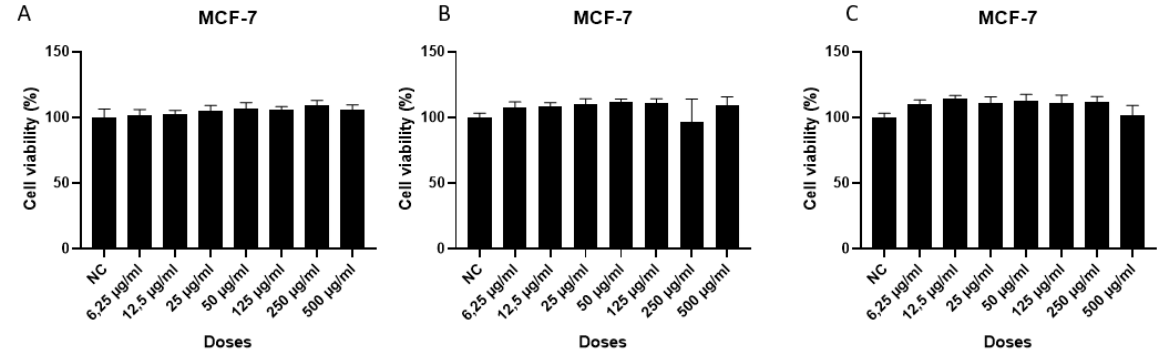

Figure-3. Cytotoxicity test results of Al_2O_3 NPs on MCF-7 cell line after 24h by WST-1 assay; **A.** Al_2O_3-48nm; **B.** Al_2O_3-78nm; **C.** Al_2O_3-100nm. All values are expressed as means ± SD with three independents repeated.

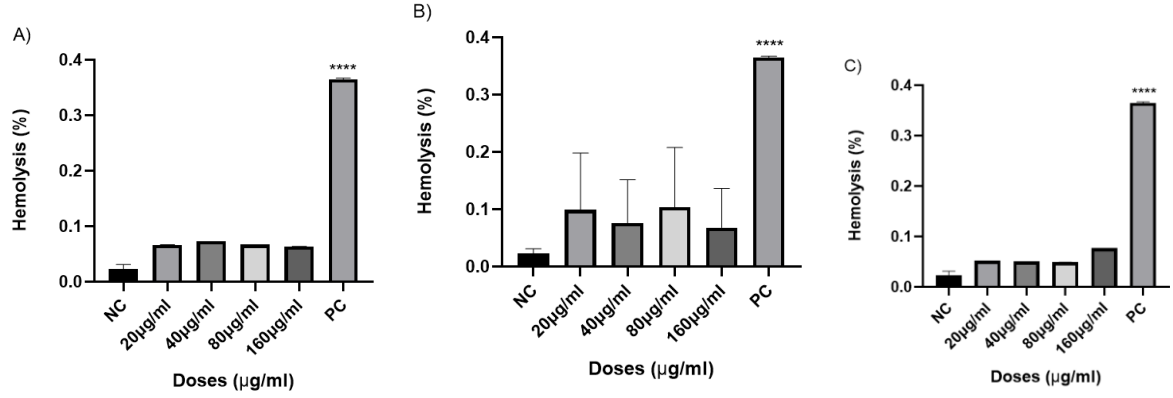

Figure-4. Hemolysis rates of Al_2O_3 NPs with different size range as **A.** Al_2O_3-48nm, **B.** Al_2O_3-78nm, **C.** Al_2O_3-100nm. Data are presented as mean ± SD from three repeats. PC, positive control; NC, negative control.

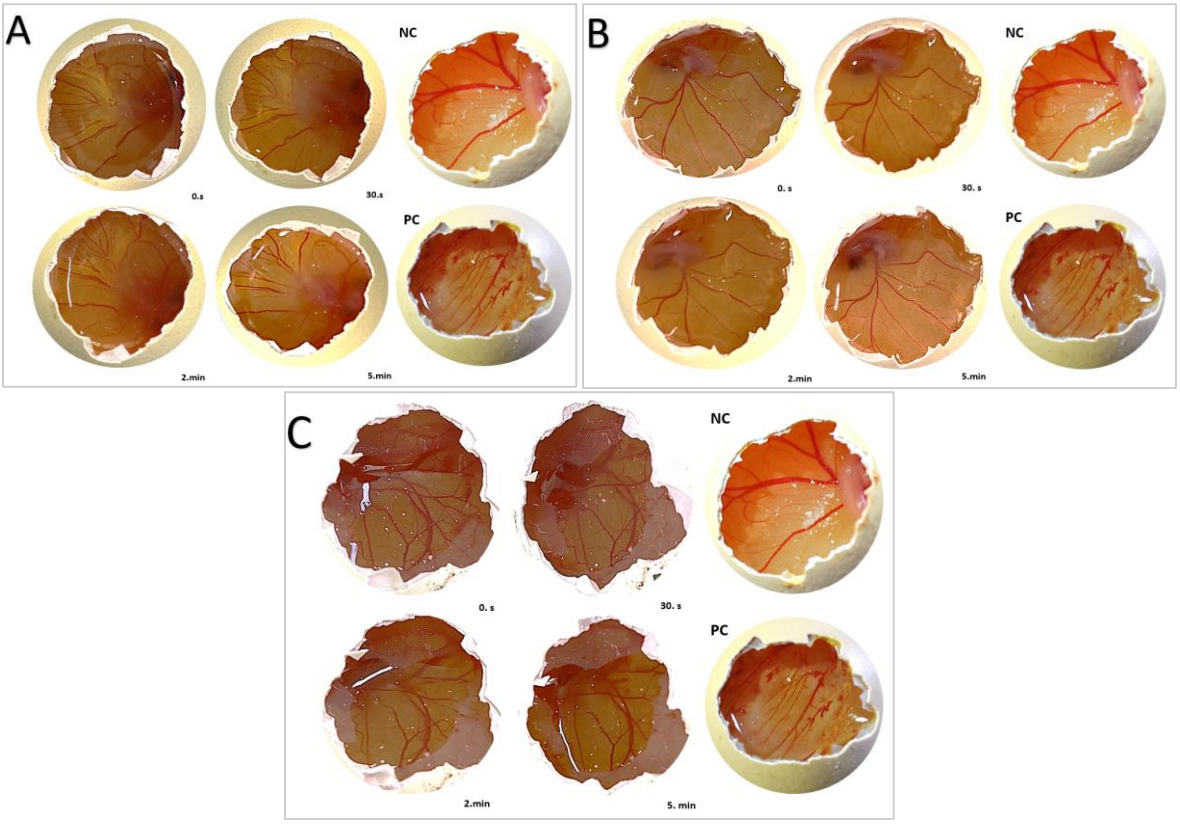

Figure-5. HET-CAM test results at different time points after Al_2O_3-48nm exposure (A); Al_2O_3-78nm (B); Al_2O_3-100nm (C). 0.9% NaCl was used as a negative control and 0.1 N NaOH was used as a positive control.

DISCUSSION

Nowadays, exposure to NPs have been increasing in many areas due to their widely use. These nanoparticles' potential toxicity is dictated by their physicochemical characteristics, including size, surface charge, and surface area, as well as the dosage, frequency of exposure, and mode of administration. In the presence of these parameters, post-exposure toxicity of nanoparticles can affect any tissue type in the body. In previous studies have shown that to exposed nanoparticles in different ways, may behave differently depending on their physicochemical properties. Size is one of these parameters which has a major impact on the toxicity of nanoparticles. As the size of NPs decreases, the surface area increases, leading to complex biophysiochemical interactions under the various environment conditions (8). Changes in the physicochemical and structural characteristics of NPs might impact their biological response, resulting in altered cytotoxicity, ROS generation, and genotoxicity (9). While the oxidative stress induced by NPs is generally caused by non-cellular factors such as particle size, surface, structure or the presence of metals in their structure, on the other hand, it is also responsible for ROS-mediated damage through biological reactions such immune cell activation and NP-cell interaction (9).

The industrial sector's adoption of nanoparticles based on aluminum has also greatly expanded their potential exposure, which is why the OECD listed Al_2O_3 NPs as high priority groups in its program on the safety of produced nanomaterials in 2007 (10). These such kind of engineered oxide nanoparticles (NPs) have the potential to exposed in many ways. In a conclusion that, these materials concern about their potential toxic effects in humans (11). Leading cause of cancer death due to breast cancer in women was increased in recently. Therefore, it was planned to study these particles in breast cells, due to their frequently potential exposure route. In addition, considering the studies conducted, there is limited in vitro studies focusing on the effects of Al_2O_3 NPs on breast cell lines, and in vivo activity as well. When NPs can enter the bloodstream and interact with blood components, they associate with blood components such as

red blood cells (RBCs) and hemoglobin (Hb). It is extremely important to investigate size-dependent toxic effects for their safety use especially in drug delivery systems. Physicochemical properties and behavior of nanoparticles in different environment conditions can be responsible to immune response, biodistribution, accumulation and clearance as well at the biological systems (12). The size distribution analysis used in the study to examine the impact of various pH levels on particle size and agglomeration revealed that the particles tended to group together. This alteration in physical properties may increase translocate through the endothelial liner and enter the circulatory system (13). Studies have shown that serum proteins have major effect on particle toxicity, probably due to agglomeration or changes in the surface chemistry (14). At the same time, the morphology feature is also may be effective on the tendency to agglomerate of the nanoparticles. In the study of Al-Gebory and Mengüç, (2018) (15), they investigate different pH conditions effects on TiO_2 NP_S and they showed that pH may have significant effect on the particle agglomeration behavior. Choi et al. (2011) (16) emphasized in their study that size and surface area of silver nanoparticles have effect on hemolytic activity. Al_2O_3 NPs have been reported by certain researchers to be less hazardous than other metal-based nanoparticles (17). On the contrary, some researches were asserted that pure aluminum has long been known to be a potential neurotoxin (18). In their study of Kim et al. (2018) showed that after 28-day repeated dose exposure of Al_2O_3 NPs as an inhaler, aluminum contents were determined at the highest level in lung tissues and there was a dose-dependent relationship in the exposure groups (19). In our study, it was revealed that Al_2O_3 NPs with different sizes (48nm, 78nm and 100nm) did not show any hemolytic activity in the applied dose range.

HET-CAM test is an ideal alternative test that can be used as an intermediate step between *in vitro* and *in vivo* preclinical evaluation. It can be used to screen the anti-inflammatory potential, irritation properties and ocular toxicity of compounds, especially on nanoparticles for fast and reliable response. HET-CAM assay provides data not only on the effectiveness of nanoparticles but also on membrane irritation-vascular bleeding, lysis and coagulation with toxicity (5). Especially, in the literature, studies on the irritation effect in particle toxicity evaluations are very limited and there isn't any study that has been found in particular where the size-dependent irritation effect of Al_2O_3 NPs. In our study, for the first time, irritation effects of Al_2O_3 NPs were evaluated with an alternative test-HET-CAM assay. The study's findings showed that there was no irritant impact in any of the three size ranges. This shows that the particle does not have any toxic effect in all three size ranges on different type of cells. However, further tests as genotoxicity and in vivo evaluation are needed to confirm that the particle is biocompatible across the entire size range.

CONCLUSION

Aluminum nanoparticles (Al_2O_3 NPs) have different characteristics under varied physiological conditions. It needs to be tested on erythrocytes, taking into account morphology and aggregation tendency, and the mechanisms that may cause hemolysis and even cytotoxicity need to be fully elucidated. Based on the tests performed within the scope of the study, it was observed that Al_2O_3 NPs in all sizes were exhibit biocompatibility. However, further studies are needed to reveal how different physico-chemical properties as other than size of Al_2O_3 NPs affect their biological properties. Also, supporting *in vitro* data with *in vivo* experiments will contribute to the risk assessment profile and safety use of these materials.

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Conflict of interest: The author(s) declare that no conflict of interest.

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