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Investigation of genotoxic and apoptotic effects of zirconium oxide nanoparticles (20 nm) on L929 mouse fibroblast cell line

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ABSTRACT

In recent years, advances in nano-scale technology have led to the use of nanoparticles in almost all areas of life. Nanoparticles (NP) have been acknowledged as one of the emerging environmental threats; however, studies in different components of environment are limited. Among the available nanoparticles, zirconium oxide nanoparticles are widely used in tooth coating and cosmetic products. In this study, three different concentrations of 20 nM Zirconium Oxide nanoparticles were used to investigate the genotoxic and apoptotic effects *in vitro* conditions by single cell gel electrophoresis (Comet) and flow cytometry methods on L929 Mouse Fibroblast cell line. In this study, we aimed to decipher whether Zirconium Oxide nanoparticles had genotoxic and apoptotic effects on fibroblast cells or not. The relationship between the apoptotic effect and the genotoxic effect was examined. Three different concentrations (50 µg/ml, 100 µg/ml and 150 µg/ml) of 20 nM Zirconium Oxide nanoparticles were used on L929 cells in order to determine the relationship between apoptotic and genotoxic parameters. Our results support that Zirconium Oxide nanoparticles induced DNA damage and apoptosis at all used concentrations. *In vivo* studies are currently conducted to further evaluate the cytotoxic effect of these nanoparticles.

1. Introduction

Nowadays, nanotechnology is among the most supported and established areas of research, where large investments are made since it is considered as the most important and crucial field of work by many developed countries. Nanotechnology aims to design and manufacture materials in nanometer dimensions and to produce new devices and tools with better functions With this new technology, previously unknown and unpredictable properties of different materials have been discovered. Findings in the field, have been used in a wide range of industrial applications from developing new devices and systems to weaving high tech textile products [1,2].

Although nanotechnology brings out new opportunities for manufacturing in different industries, it comes with its possible harmful outcomes, especially those on human health. Besides their use in different fields of nano products, harmful effects on human health or environment have not been fully elucidated yet. Nanotechnological products are already found in the market, the literature on toxic side effects is rather limited [2]. There is an urgent need to study their effects on the human health in order to prevent detoriorating results. Because of this need, there has been a spike increase in the toxicology studies of these nanoparticles which has created the fields of nanotoxicology and nanogenotoxicology [3].

It has been shown that nanoparticles can penetrate through biological barriers and accumulate in different organs and may cause toxic effects, such as oxidative stress, DNA damage, cell death, and morphological changes [4–8].

Recent studies have shown that several nanoparticles, including metals, have cytotoxic and genotoxic effects on humans. In the nucleus, nanoparticles can directly interact with the DNA and cause damage [9]. Reactive oxygen species (ROS) formation on the surface of nanoparticles or metal ions released from the surface of particles can interfere with the cellular pathways and damage the cellular components as well as the DNA [10]. Nanoparticles may induce ROS production by affecting electron transport chain in the mitochondria or by stimulating cytochrome P450 enzymes as well as NADPH-oxidase [10,11].

Genotoxic effect can be measured by many different techniques and methods. Among these methods, the single cell gel electrophoresis method (Comet) is the most prevalent one and was originally discovered by Rydberg and Johanson in 1978 to measure DNA breaks and

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was later developed by Ostling and Johanson in 1984. Single chain fractures can be detected on the single-stranded DNA by application of alkaline lysis conditions and this technique was developed in 1988 by Singh et al. [12]. A landmark achievement was reached in 2016 when the organization for economic Co-operation and development adopted a comet asssay guideline for *in vivo* testing of DNA strand breaks [13]. The term 'comet assay' was officially introduced in 2000 in the Medical Subject Headings, which are used in the US National Libraries of Medicine, including PubMed. The new millennium also brought the first set of guidelines for biomonitoring studies [14] as well as the *in vitro* and *in vivo* comet assay in genetic toxicology, based on a meeting of experts at the International Workshop on Genotoxicity Test Procedures [15]. This method can accurately, precisely, rapidly, and cheaply measure single chain fractures formed on the DNA strands by various agents, oxidative stress and toxic heavy metals by using a small sample volume and is widely used in the studies on the living cells [16–18].

Flow cytometry is based on examining the cells flowing in stream of fluid through laser beams. It can measure the apoptotic effects of the chemicals. It has been a widely used technique in the clinical laboratories, in many different areas such as food toxicology and marine biology. Flow cytometry analysis can inform us about various properties of the cells such as immunophenotype, DNA content, enzyme activities, membrane potential and viability [19,20].

Metal nanoparticles and metal oxide nanoparticles have been used in many areas of the industry because of their small size and large size options, as well as their versatile surface features, such as charge, shape, conductivity, melting and freezing point differences [21]. Some examples of their utilization areas are aluminum fuel additives, explosives, golden cellular imaging photodynamic therapy, iron magnetic imaging, environmental cleaning, silver antimicrobial agents, photography, copper antibiotic treatments, conductors, saryum polishers, computer chip production, manganese catalyst pillars, nickel battery production, Dot titanium photocatalysts, zinc skin protectors, sun creams, and zirconium toothpicks [22].

In this study, we aimed to assess the possible cellular toxicity/apoptotic and genotoxicity mechanism of ZrO_2 nanoparticles. The study was designed to investigate their cytotoxicity, genotoxicity-oxidative damage and apoptosis in L929 mouse fibroblast cells exposed to ZrO_2 nanoparticles.

2. Materials and methods

2.1. Chemicals

Zirconium oxide (ZrO_2) (High purity, 99.95%, CAS Number 1314-23-4) was obtained from US Research Nanomaterials, Inc. According to the manufacturer, Zirconium Oxide Nanopowder, Crystal Phases: monoclinic, APS: 20 nm, Color: White, Morphology: shape near spherical True Density: 5.89 g/cm3.

A stock solution of ZrO_2 (500 µl) using steriled distilled water was prepared and stored at 4 °C in darkness. RPMI 1640 and fetal Calf serum were purchased from Sigma. Other chemicals or solvents used in this study were of cell culture, HPLC, or analytical grade.

2.2. Dose selection and the size of nanoparticle

Since there is no study of ZrO_2, Silica dioxide was used as reference for the concentration and particle size. In study performed by Battat et al. [23] it is found that 150 µg/ml concentration of Silica dioxide nanoparticle (20 nm and 6 nm) have genotoxic and cytotoxic effects on human peripheral blood lymphocytes, therefore; in this study, particle size and concentration were selected as 20 nm and 150 µg/ml, respectively.

Table 1
Damaged Cells (DNA Migration in Comet Assay), Distribution Among the Different Classes of Damage, and Score in Reference to the Genotoxicity Test in the L929 fibroblast cells exposed to ZrO_2 nanoparticle *in vitro*.

treatment	Repeat number	Tip 0	Tip I	Tip II	Tip III	Tip IV
50 µg/ml ZrO	1th repeat	52	34	12	2	0
	2th repeat	48	32	17	3	0
	3th repeat	44	30	22	4	0
100 µg/ml ZrO	1th repeat	40	26	22	12	0
	2th repeat	47	25	18	10	0
	3th repeat	54	24	14	8	0
150 µg/ml ZrO	1th repeat	36	46	10	4	4
	2th repeat	36	42	15	4	3
	3th repeat	34	38	20	4	2
NK	1th repeat	60	32	4	4	0
	2th repeat	66	28	3	3	0
	3th repeat	72	24	2	2	0
PK (H_2O_2)	1th repeat	42	24	12	10	12
	2th repeat	33	27	19	20	11
	3th repeat	24	30	26	10	10

NC: Negative Control; PC: Positive Control.

Table 2
Damaged cell percentages and Genetic Damage Index values in L929 fibroblast cells exposed to ZrO_2 nanoparticle *in vitro*.

Treatment	GDI ± SE	DCP ± SE
50 mg/ml	75.0 ± 11.0	20.0 ± 6.00
100 mg/ml	91.0 ± 15.0**	28.0 ± 6.00**
150 mg/ml	96.0 ± 2.00**	22.0 ± 4.00**
Negatif Kontrol	43.0 ± 9.00	6.0 ± 2.00
Pozitif kontrol	149.0 ± 21.5***	43.3 ± 8.3***

p < 0.05,*p < 0.001, NC: negative control; PC: Positive control; SE: Standard Error.

Table 3
Flow cytometry results in L929 cells treated with ZrO_2 nanoparticle (20 nm).

Treatment		Examined cell number	early apoptotic cell (%)	late apoptotic cell (%)	live cell (%)	necrotic cell(%)
50 µg/ml	1th repeat	3624	14.6	3.3	75.6	6.5
	2th repeat	3216	11.8	4.6	76.9	6.7
	3th repeat	3420	13.2	3.9	76.2	6.6
100 µg/ml	1th repeat	2407	12.3	4.3	76.7	6.7
	2th repeat	2492	14.3	3.3	77.5	4.8
	3th repeat	2445	13.3	3.8	77.1	5.8
150 µg/ml	1th repeat	2831	10.4	2.8	80.2	6.6
	2th repeat	2420	13.1	3.4	79.8	3.8
	3th repeat	2625	11.7	3.1	80.0	5.2
NC	1th repeat	4800	0	0	100	0
	2th repeat	4765	0	0	100	0
	3th repeat	4782	0	0	100	0
PC	1th repeat	3692	12.5	3.9	77.7	5.9
	2th repeat	3723	13.0	3.0	76.0	8.0
	3th repeat	3707	12.7	3.5	76.9	6.9

2.3. Cell culture design

L929 mouse fibroblast cells were grown in Roswell Park Memorial Institute media (RPMI 1640) media with %10 fetal bovine serum, %1 antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) and sodium pyruvate. Cells were incubated in 37 °C % 5 CO_2 incubator. Cell media was refreshed once in every 4 days until they reach confluency to be used in the experiments.

2.4. ZrO and H_2O_2 treatment of L929 cells

L929 cells were put in 10^6 cells/well concentration in 1 ml fresh

Table 4
Statistical results in L929 cell treated with ZrO_2 nanoparticle (20 nm) in flow cytometry.

Treatment	Examined cell number mean ± SE	early apoptotic cell (%) mean ± SE	late apoptotic cell (%) mean ± SE	Live cell (%) mean ± SE	Necrotic cell (%) mean ± SE
50 µg/ml	3420 ± 117,7***	13,2 ± 1,40***	3,93 ± 0,65***	76,23 ± 0,65***	6,60 ± 0,10***
100 µg/ml	2448 ± 24,5***	13,3 ± 1,00***	3,80 ± 0,50***	77,1 ± 0,40***	5,76 ± 0,95***
150 µg/ml	2625 ± 205,5***	11,7 ± 1,35***	3,10 ± 0,30***	80,0 ± 0,20***	5,20 ± 0,20***
Negative Control	4782 ± 10,1	0,00 ± 0,00	0,00 ± 0,00	100 ± 0,00	0,00 ± 0,00
Positive control	3396 ± 225,8***	12,7 ± 0,25***	3,46 ± 0,45***	76,8 ± 0,85***	6,93 ± 1,05***

***$p < 0.001$ compared with negative control.

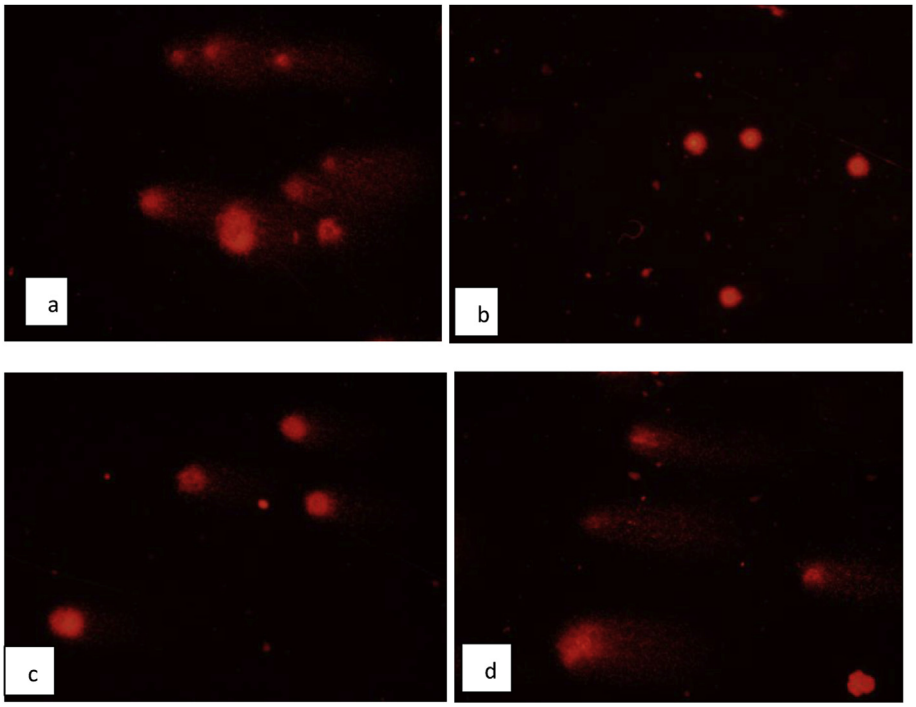


Fig. 1. Comet views in ethidium bromide-stained L929 Mouse Fibroblast Cells.(a.Type2-3 b. Type 0 c.Type 3 d. Type 4).

complete RPMI as described above into 24-well plates, then they were rested overnight in 37 °C 5% CO_2 incubator. We tested 50, 100 and 150 µg/ml of 10 µm filter sterilized ZrO_2's effect on L929 cells. 10 mM of sterile H_2O_2 was put into 1 mL media of overnight rested cells in the COMET positive control wells. Cells were treated with the ZrO_2 and H_2O_2 for 72 h in 37 °C at incubator with 5% CO_2. Afterwards cells were collected into eppendorf tubes and centrifuged at 2000 rpm to get rid of supernatants, then cells were resuspended in PBS and this washing step was repeated 3 times. After getting rid of the last supernatant phase the cells were counted and 100.000 cells were used for COMET assays and 100.000 cells of the each condition were stained with Annexin V and PI dyes to analyze the apoptotic levels via flow cytometry. All experimental conditions were tested as a triplicate.

2.5. Comet assay method/oxidative damage/genotoxicity assay

Comet assay was performed under alkaline conditions according to the method of Singh et al. [24] with slight modifications. Microscopic slides were covered by a thin layer of 0.5% normal melting agarose (NMA) at about 52 °C (dissolved in $Ca^{2+}$and Mg^{2+} free phosphate buffer saline (PBS). Eppendorf tubes were placed in water bath at 35 °C. 100 µl of L929 cell suspension were diluted with 1 ml of PBS in eppendorf tube. Then 110 µl of mixtures were mixed with 110 µl of LMA (0.5%). 110 µl of this mixture was spreaded on NMA-coated slides using micropipette and immediately was covered with coverslip. Slides were conserved in fridge at +4 °C for 20 min. The coverslips were slowly removed from top of slides. Then slides were placed in chalets including lysis solution and conserved for 1 h in fridge in dark. Slides were placed on a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (300 mM NaOH + 1 mM EDTA) to allow DNA unwinding before electrophoresis. Electrophoresis was conducted at 20 °C using 25 V and 300 mA for 25 min. The above steps should be carried out in dark to avoid DNA damage. After electrophoresis, slides were placed in neutralizing buffer (pH = 7.5) for 15 min. Then slides were placed in chilled ethanol for 10 min. DNA was stained with ethidium bromide (0.1 mg/ml, 1:4) and the slides were examined with a fluorescent microscope (BX51, Olympus, Japan). 100 cells was considered for microscopic evaluation.

Two parameters were evaluated in comet assay analyses;
1. Genetic damage index 2. Damaged cell percent, in following formula;
Genetic damage index: 0xType 0 + 1xType I + 2xType II + 3xType III + 4xType IV
Damaged cell percentage: TypeII + TypeIII + TypeIV

Comet assay scores show levels of UD (undamaged, 0), Type 1 (low damaged), Type II(moderate damaged), Type III (high damaged), and Type IV (ultra high damaged).

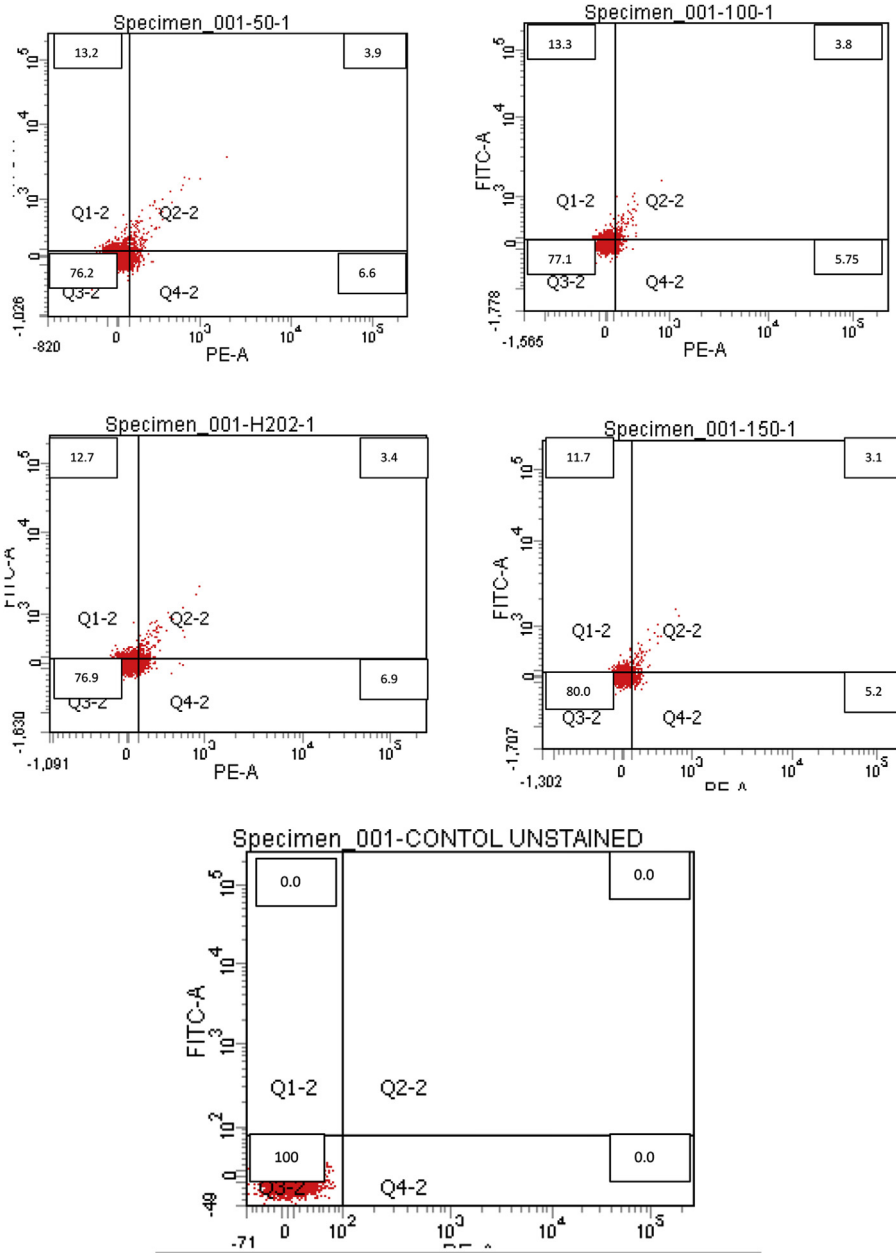


Fig. 2. The cells were exposed to different ZrO_2 nanoparticle concentration for 72 h. Cell apoptosis rate after Annexin V-FITC/PI staining was detected by flow cytometry.

2.6. Flow cytometry method/apoptosis assay

To detect the apoptosis, Annexin V-FITC apoptosis detection kit with PI was used. The kit is based on observation of the translocation of the membrane phosphatidylserine from the inner side of plasma membrane to cell surface. In flow cytometry analyses, late apoptotic cell, early apoptotic cell, live cell and necrotic cell were counted. Cells were washed 2 times with cold PBS and resuspended with 1 x Binding Buffer. 100 µl of this prepared mixture was transferred to 5 ml culture tubes. 5 µl FITC Annexin V and 5 µl PI were added to each tube and mixed and tubes was left in the dark for 15 min 400 µl 1 x Binding Buffer was added to all tubes and read on a flow cytometry device.

2.7. Statistical analysis

Data were compared by one-way variance analysis. All statistical analysis was performed using the Statistica 13.0 package program. Multiple comparisons among groups (negative control and ZrO_2 gropus) were performed by Tukey test. Pearson Correlation test was used to determined the relationship among parameters. $P < 0.05$ was considered as level of significance.

3. Results

Tables 1 and 2 represent the comet assay values (Type 0–4) in L929 cells treated with ZrO_2 nanoparticle (20 nm) and Tables 3 and 4 indicates the flow cytomery results of L929 cells. Fig. 1 presents the comet assay views (a-d). Fig. 2 shows cell viability in L929 cells line exposed to different concentrations of 20 nm ZrO_2 nanoparticle for 72 h. Parallel to the increase in the concentration of ZrO_2, an increase in GDI and DCP parameters was observed (Fig. 3). In increasing concentrations of the nanoparticle, there was an increase in early apoptotic, late apoptotic, and necrotic cell counts (Fig. 4). Table 5 represents results of correlation between comet analysis and flow cytometry data.

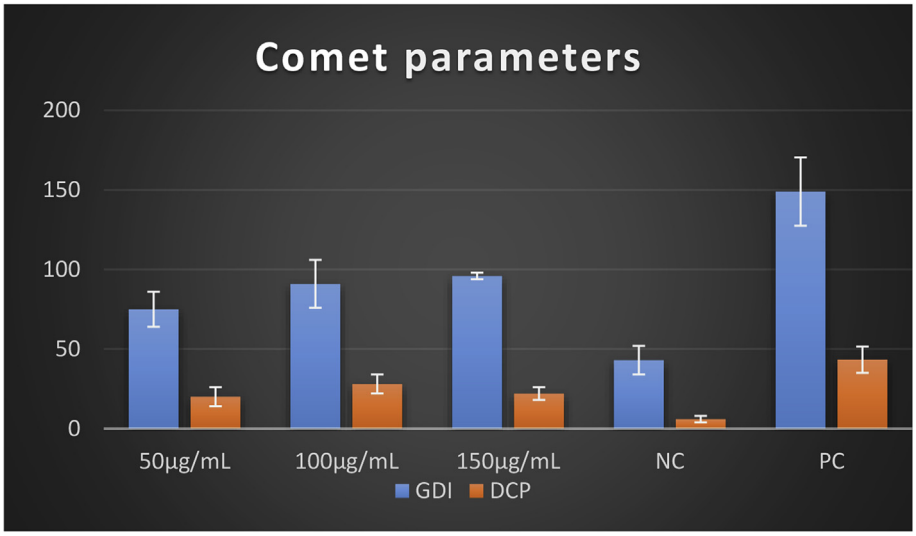
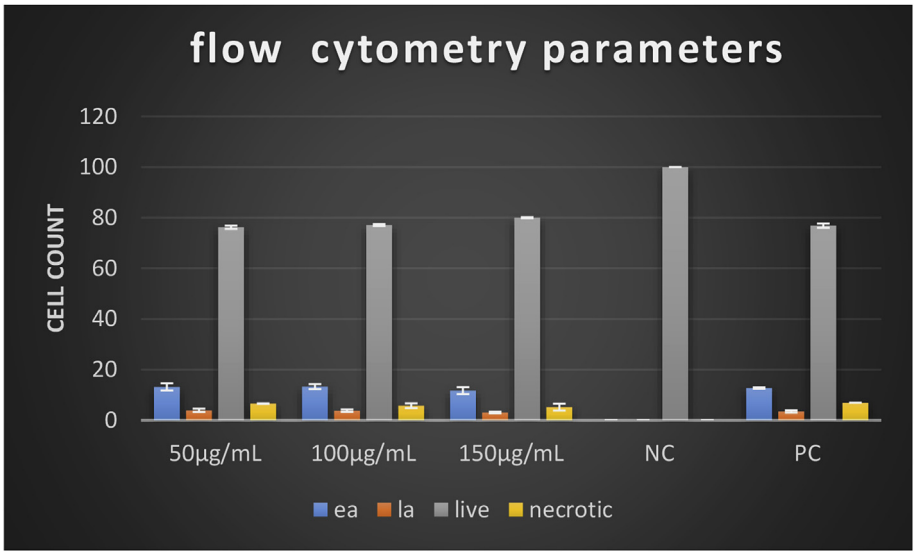


Fig. 3. Frequency of GDI and DCP in relation to ZrO_2 nanoparticle concentrations in L929 cells.



Ea: early apoptotic, la: Late apoptotic

Fig. 4. Frequency of early apoptotic, late apoptotic, live and necrotic cells in relation to ZrO_2 nanoparticle concentrations in L929 cells.
Ea: early apoptotic, la: Late apoptotic.

Table 5
Pearson correlation analysis results between comet assay parameters and flow cytometry data.

	Early apoptotic cells	late apoptotic cells	Live cells	Necrotic cells	GDI
late apoptotic cells	,935***				
Live cells	-,988***	-,960***			
Necrotic cells	,903***	,898**	-,954***		
GDI	,629*	,570*	-,664**	,728**	
DCP	,678**	,641*	-,713**	,758**	,971***

***very high correlation, ** high correlation, * low correlation.
GDI: genetic damage index, DCP: damaged cell percentage.

4. Discussion

In this study, the genotoxic and apoptotic effects of 20 nm ZrO_2 nanoparticles were investigated on L929 cells treated with 50, 100, and 150 µg/ml concentrations. Comet and flow cytometry analysis were used to determine the genetic damage and cell viability in L929 cells. The relationship between genetic damage and apoptotic effect was determined.

20 nm ZrO_2 nanoparticle showed statistically significant genotoxic and apoptotic effects on L929 cells, and this effect was dose dependent. GDI and DCP values obtained from the comet analysis increased at higher concentrations.

Based on flow cytometry analysis, there were toxic effects on fibroblasts by ZrO_2 nanoparticle and this effect was statistically significant. Moreover, this effect was more robust at higher concentrations of the nanoparticle. Flow cytometry results showed an increase in early apoptotic, late apoptotic and necrotic cell counts at all concentrations. There is a correlation between flow cytomery data and comet anaysis

parameters.

Previous studies gave controversial results in terms of genetic damage and cell viability both *in vivo* and *in vitro* with different cell types. In these studies; the effect of nanoparticles with different properties, sizes and concentrations were examined. Due to the discrepancies between the studies, the genotoxic and apoptotic effect of ZrO_2 nanoparticle on the mammalian cells is still now known. Our group aimed to decipher the effect by this study.

Song et al. [25] determined the cytotoxicity of silver and zinc nanoparticles (90 nm) in concentrations of 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml in human epithelial colorectal adenoma cells and they found that these nanoparticles caused cellular damage. Zinc nanoparticles were shown to have more cytotoxic effect at those same concentrations than those of silver nanoparticles. It was reported that zinc nanoparticles have dose-dependent effect on the cell viability and toxicity. Suppression of SOD levels at which the zinc nanoparticles caused cellular oxidative stress was determined by changes in the release of reactive oxygen species and changes in GSH levels. Song et al. [25] found that zinc and silver nanoparticles had negative impact on cell-viability. This was determined by CCK-8 analysis and that there was a significant decrease in the cell viability of nanoparticle treated cells compared to the negative controls. Silver and zinc nanoparticles have been shown to have an adverse effect on the cell viability. The effect of zinc and silver nanoparticles on the cell viability coincides with the effect of the ZrO_2 nanoparticle on L929 fibroblast cells in our study. Karunakaran et al. [26] examined the cytotoxic effects of nano and micro particles of the ZrO_2 and The effect of TiO_2 on 3T3 mouse embryonic fibroblast cells was also tested by previous studies. The cytotoxic effects of different doses of ZrO2 and TiO2 (12, 5, 25, 50, 100 and 200 μg/ml) in nanoscale and microsize were tested by MTT assay. They found that the percentage of live cells had a dose dependent decrease. ZrO_2 at micro size reduced the cell viability from 96.7% to 62.3%, and at the nanoscale ZrO_2 decreased the cell viability from 97.0% to 73.3%. A statistically significant decrease in cell viability is consistent with the current cytometry data of our study. In another study, Aude-Garcia et al. [27] examined the toxicity mechanism of ZnO and ZrO_2 nanoparticles on the macrophages; they reported that ZnO nanoparticle dispersed in the culture medium and formed nanoscale shrinkage, ZnO nanoparticle had toxic effect but ZrO_2 nanoparticle had no toxicity on macrophages. Dhanalekshmi and Meena [28] reported that the $AgZrO_2$ application has antimicrobial activity and ZrO_2 nanoparticle intercalates between the strands of DNA and this might be its mechanism of action as anti-microbial agent. This effect on the bacteria is the result of inhibition in DNA replication. Di Virgilio et al. [29]. reported that the crystalline and amorphous SiO_2ZrO_2 nanoparticles induced genotoxicity and cytotoxicity of (782 ± 19 and 891 ± 34 nm) on the human osteosarcoma cell line (MG-63). They have determined that both the crystal and amorphous nanoparticles can penetrate into the cell. They reported that both of these nanoparticules cause an increase in micronucleus frequency and DNA damage. In our study, ZrO_2 was reported to cause DNA damage. Flow cytometry data supports the apoptotic effect. Increased Type IV cell death levels in the comet analysis overlaps with the apoptotic effect that we observed by the flow cytometry analysis. Demir et al. [30] evaluated the genotoxic effects of titanium, zirconium and aluminum nano and microparticles in Drosophila. They reported that nanoparticles both in micro- and nanoscale dimensions did not have genotoxic effects in their analysis. Otero-González et al. [31] have investigated the effects of various nanoparticles (Mn_2O_3, Fe, ZrO_2, TiO_2, Fe_2O_3) on oxygen uptake and membrane integrity in yeast cells. They reported that only Mn_2O_3 nanoparticles suppressed the oxygen uptake and impaired membrane integrity, but other nanoparticles had no significant effect on the yeast cells. Lindberg et al. [32] tested whether pigmented and unadulterated titanium dioxide had any genotoxic effects in lung cells of C57BL/6J mice or not. It has been reported that additive titanium dioxide causes both an increase in the micronucleus frequency and DNA damage by the comet analysis of C57BL/6J mice in lung cells. Genetic damage is reported to be as a results of inflammation.

5. Conclusion

According to the results obtained from the Comet analysis, it was observed that the ZrO_2 nanoparticles caused a statistically significant genotoxic damage on the mammalian fibroblast cells in a dose dependent manner. High correlation rates between GHI and HHY parameters, were obtained by Comet analysis and it suggests that ZrO_2 nanoparticles are the reason of the observed genotoxic damage. It was determined that ZrO_2 nanoparticles caused statistically significant early, late apoptotic effects and necrotic effects in a dose dependent fashion by the flow cytometry analysis. Among the parameters studied in the flow cytometry analysis, there was a positive strong correlation suggesting that ZrO_2 nanoparticles had an apoptotic effect on the fibroblasts. In the comet analysis, Type 4 comet images indicating the GHI value especially support apoptotic activity of our nanoparticles. The presence of a high correlation rate between the comet analysis and the flow cytometry parameters indicates that the two methods complement each other. In this study, it is supported that ZrO_2 nanoparticles have apoptotic and genotoxic effects on L929 mammalian fibroblast cells. We believe that studies in different cell types under different conditions will allow us more efficient and safe use of nanoparticles in versatile areas of modern human life.

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Appendix A. Supplementary data

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.cbi.2018.09.017

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