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An Investigation of the Potential of Apoptotic and Genetic Damage of Bismuth Oxide Nanoparticle on MDBK Cell Line

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ABSTRACT

Background: Today, nanoparticles have been used in almost all areas of life thanks to advances in nano-scale technology.Among the available nanoparticles, Bismuth oxide nanoparticle is widely used in many products in terms of its properties such as antibacterial, antitumor and cytotoxic activity and antifungal.

Objective: In this study, we aimed to determine the in vitro genetic damage potential and apoptotic effects of Bismuth oxide nanoparticle having 90 - 210 nm size range in MDBK cell line cultures.

Material and Methods: In this study; single cell gel electrophoresis (COMET) method and flow cytometry method were used to determine genotoxic and apoptotic effect.The MDBK (Madin Darby Bovine Kidney) cells were treated with Bismuthoxide at three concentrations of 30µg / ml, 60 µg / ml and 90µg / ml.

Results: No significant difference was found between the negative control group and Bismuthoxide groups for GDI and DCP parameter. When all the concentrations were considered, there was a significant difference between negative control and other groups for early and/or late apoptotic, necrotic cell and number of live cells ($p < 0.05$).

Keywords: MDBK (Madin Darby Bovine Kidney) cell line;Genetic Damage;Bismuth Oxide Nanoparticle;flow cytometry.

INTRODUCTION

Nanotechnology is a reciprocal discipline that explores and manipulates physical substances on the nanometers scale, representing a unique combination of natural, fine, computer and material lores [1]. With the development of nanotechnology nano- sized accoutrements similar as nanocrystals, nanoparticles and nanotubes can be produced. The NPs, which have gained great significance in recent times, constitute the main base of nanotechnology. Nanomaterials are characterized by a crucial point of the combination with the widely small size and face area, irrespective of natural and physicochemical parcels, similar as size, structure, composition and shape [2].

Currently, the use of nanoparticles in numerous areas of nanotechnology needs to be used. thus, it's necessary to examine the implicit goods of nanoparticles to cover mortal and environmental health [3]. Despite the advantages and advances of nanoparticles to the world, there has been serious debate about the dispersion of nanotechnology, and therefore the implicit pitfalls associated with its disadvantages in the world lately are anticipated [4].

Nanoparticles can be classified in different ways according to theirproperties.Metal nanoparticles are prepared from essence precursors [5]. Bismuth, the chemical symbol" B" asemi-metal. Bi2O3 is one of the important essence oxides and provides a suitable terrain for biomoleculeadsorption.Bi2O3 nanoparticles have better adsorption parcels than regular sized patches due to their lesser advantages and new parcels(advanced specific face, further face free energy and good electrochemical stability,etc.) [6]. In a study, Abudayyak et al 2017

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[7]. have delved the poisonous goods in order to estimate the toxin of Bi2O3 nanoparticles in mammalian cells(HepG2 hepatocarcinoma cell), order(NRK- 52E renal epithelial cell), intestine(Caco- 2 colorectal adenocarcinoma cell) and lung(A549 lung melanoma cell). Recent studies have shown that essence nanoparticles have cytotoxic and genotoxic goods on humans [8]. Bismuth oxide is seen as the least poisonous heavy essence for humans and is extensively used in medical operations for good antibacterial parcels. Bismuth oxide nanoparticles have been used in different sizes in vivo and in vitro trials [7,6].

One of the branches of toxicology, inheritable toxicology, is a discipline that examines the changes in DNA motes of cells during the normal natural functioning of the organism and plays an important part in the evaluation of inheritable damage caused by differentagents. Mutagenic agents or mutagens on the DNA patch show their goods directly or laterally by binding to proteins synthesized according to genomic information. The crucial motes and pathways involved in DNA damage are towel damage, aging, cancer, gravidity and some inheritable and multifactorial conditions [9].

Genotoxic effect can be measured by numerous different ways and methods .The single cell gel electrophoresis, Comet assay, system grounded on the principle of determination of fractures on the DNA patch at the gene position has set up wide use in the determination of numerous DNA damage and form, and in the bio monitoring and exposure studies. Comet system is a simple, sensitive and rapid-fire system because it has come a useful and favored system in inheritable toxicology studies [10, 11, 12].

Each cell has a certain life span. There's a controlled balance between cell death and cell proliferation. Apoptosis, which is one of the cell death types, refers to the petal that's separated from the fallen splint or flower in Greek.Cell death due to DNA damage is performed by apoptosis, necrosis and autophagy. Apoptosis, which is one of these different forms of cell inactivation, is the main pathway after DNA damage [13]. Flow cytometry is the dimension of the characteristics of cells or patches in a flowing fluid. Cells or patches suspended in inflow cytometry are passed through a cube illuminated by ray light; when the cells pass through the light, the signals they give are anatomized. The source of the signals formed may be the physical parcels of the cell, similar as size and granularity; There may also be colorful fluorochromes which bind to the cell. therefore information can be collected about colorful features similar as immunophenotype of the cell or flyspeck, DNA content, enzyme conditioning, cell membrane eventuality, viability [14, 15].

Some in vivo studies have shown that different NPs, including ZnO, can be retained in the order [16]. Order cells wereshown to be dependable for detecting a NP cure- response. In our study, we used the Madin- Darby doggy order(MDCK) as an in vitro model of renal epithelial cells and aimed to assess the possible cellular toxin/ apoptotic and genotoxicity medium of Bi2O3nanoparticles and the correlation between inflow cytometry parameters and comet assayparameters. The study was designed to probe their cytotoxicity, genoto- oxidative damage and apoptotic eventuality in MDBK cells.

MATERIALS AND METHODS

Chemicals

Bismuth oxide(Bi2O3)(High chastity,99.95, CAS Number1314-23-4) was attained from US Research Nanomaterials, Inc. According to the manufacturer, Bismuth oxide Nanopowder, Crystal Phases monoclinic, APS 90- 210 nm, Color unheroic, Morphology shape near globular True viscosity5.89 g/ cm3. A stock result of Bi2O3(500 µl) using steriled distilled water was prepared and stored at 4 °C in darkness. DMEM and fetal Shin serum were bought from Sigma. Other chemicals or detergents used in this study were of cell culture, HPLC, or logical grade. The size of nanoparticle was measured using nanosizer at Advanced Technology, Education, Research and Application Center in Mersin University. The size of nanoparticle was,2 nm(**Figure 1**).

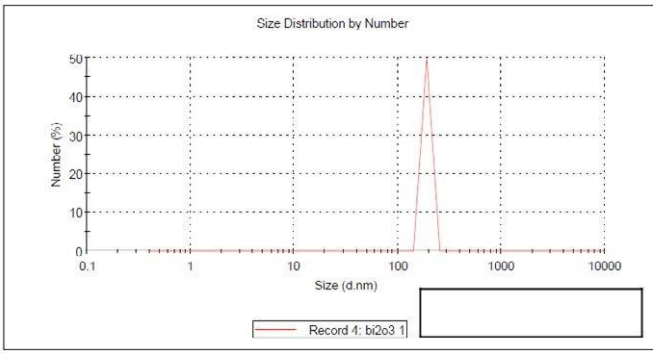

Figure 1: Results of Size Measurement of Bi2O3 Nanoparticle

The selectionof cure and the size of nanoparticle

The studies performed with Bi2O3 reported that the attention of 30 µg/ ml showed the genotoxic and cytotoxix effect in numerous cell lines [17,18]. Thus; in this study, flyspeck size and attention were named asnm and 30 µg/ ml, 60 µg/ ml and 90 µg/ ml, independently.

Cell culture design

MDBK cells were grown in DMEM media with 10 fetal bovine serum, 1 antibiotics(100 µg/ ml penicillin and 100 µg/ ml streptomycin) and sodium pyruvate. Cells were incubated in 5 CO-2incubator at 37 °C. Cell media was refreshed formerly in every 7 days until they reach confluency (80) to be used in the trials.

Bi2O3 and H2O2 treatment of MDBK cells

MDBK cells were put in 4x105cells well attention in 1 ml fresh complete DMEM as described over into 12- well plates, also they were rested overnight in 37 °C 5 CO2 incubator. We tested 30, 60 and 90 µg/ ml of 20 µm sludge castrated Bi2O3's effect on MDBK cells. 10mM of sterile H2O2 was put into 1 mL media of overnight rested cells in positive control wells for COMET assay. Cells were treated with the Bi2O3and H2O2 for 72 h in 37 °C at incubator with 5 CO2. latterly cells were collected into eppendorf tubes and centrifuged at 2000 rpm to get relieve of supernatants, also cells were resuspended in PBS and this washing step was repeated 3 times. After getting relieve of the last supernatant phase the cells were counted and100.000 cells

were used for COMET assays and100.000 cells of the each condition were stained with Annexin V and PI colorings to dissect the apoptotic situations via inflow cytometry. All experimental conditions were tested as a quadruple.

Comet assay/ genoto- oxidative damage

Comet assay was performed under alkaline conditions according tomethod of [19]. with slight variations. Slides were carpeted by a thin subcaste of 0.5 normal melting agarose(NMA) dissolved in Ca2 and Mg2 free phosphate buffer saline(PBS) at about 50 °C. Eppendorf tubes were placed in water bath at 35 °C and also 100 µl of MDBK cell suspense was adulterated with 1 ml of PBS in eppendorf tube also 100 µl of fusions were mixed with 100 µl of LMA(0.5). 100 µl of this admixture was spreaded on NMA- carpeted slides using micropipette and incontinently was covered with coverslip. Slides were conserved in fridge at 4 °C for 20 min. The coverslips were sluggishly removed from top of slides. also slides were placed in baches including lysis result and conserved for 1 h in refrigerator in dark. Slides were placed on a vertical gel electrophoresis unit filled with fresh electrophoretic buffer(300 mM NaOH 1mM EDTA) to allow DNA unwinding before electrophoresis. Electrophoresis was conducted at 20 °C using 25 V and 300 mama for 25 min. The below way should be carried out in dark to help DNA damage. After electrophoresis, slides were placed in negativing buffer(pH = 7.5) for 15 min. also slides were placed in stupefied ethanol for 10 min. After staining procedure with ethidium platitude(0.1 mg/ ml, 14) and the slides were examined with a fluorescent microscope(BX51, Olympus, Japan). 100 cells were considered for bitsy evaluation.100 cells were counted from each attention and the counted cells were classified in five groups according to their damage position Type 0, Type 1, Type 2, Type 3 and Type 4.

Two parameters were estimated in comet assay analyses;

1. inheritable damage indicator 2. Damaged cell chance, in following formula;

inheritable damage indicator 0xType 0 1xType I 2xType II 3xType III 4xType IV

2. Damaged cell chance TypeII TypeIII TypeIV

Comet assay scores show situations of UD(undamaged, 0), Type 1(lowdamaged), Type II(moderate damaged), Type III(high damaged), andType IV(ultra-high damaged).

Flow cytometry system/ apoptosis Assay

To determine cellular apoptosis or necrosis, Annexin V- FITCapoptosis discovery tackle with PI was used. The tackle is grounded on observation of the translocation of the membrane phosphatidylserine from the inner side of tube membrane to cell external- face, which can be fluently detected by staining with a fluorescentdye Annexin V, a protein that has a high affinity for phosphatidylserine, conjugated toFITC.In inflow cytometry analyses, late and early apoptoticcell, live cell and necrotic cell werecounted.The cells are grouped according to their staining parcels in the following **Table 1**. roughly 4x105 cells were passed through inflow cytometry to determine the quantum of drift of the MDBK cells exposed to bismuth oxide nanoparticles to the apoptotic process.

Table 1. Cell staining properties

	PI	Annexin V
Earlyapoptotic cells	-	+
Lateapoptotic cells	+	+
Live cells	-	-
Necrotic cells	+	-

Statistical analysis

The normalcy control for all parameters was performed with the Shapiro Wilk's test. STATISTICA13.0 analysis program was used to estimate the data attained as a result of the experimental protocols. The attention applied to determine whether there's a statistically significant difference between the results were compared between both the positive control values and the negative control values. The mean of the data attained from the experimental protocols was used for all analyzes. The difference between the groups was anatomized by Kruskal Wallis program. When assessing, p value(confidence interval) was taken as0.05. Pearson correlation analysis was applied to determine the relationship between Comet and flow cytometry parameters. Research Instruments

RESULTS

In the present study, genotoxicity measures of the medications attained from the MDBK cells exposed to three attention of Bi2O3 nanoparticle(30 µg/ ml, 60 µg/ ml and 90 µg/ ml) were determined by Comet test system. Cell viability was determined by inflow cytometry analysis.

Comet Analysis Results

Results of comet analysis attained from MDBK cell line are shown in **Table 2**. The images attained under the luminescence microscope are shown in **Figure 2**. An increase in the attention of GDI and DCP was observed in resemblant with the increase in attention(**Figure 3**). No significant difference was set up between the negative control group and the 30µg/ ml and 60µg/ ml and 90µg/ ml attention groups in terms of GDI parameter. No significant difference was set up between the negative control group and the attention group of 30 µg/ ml and 60 µg/ ml

Table 2. Resultsof CometAssayAnalysis in MDBK celltreatedwith Bi2O3nanoparticle (90-210 nm ~191,2)nm)

Treatment Bi2O3	Repeatnumber	Tip 0	Tip I	Tip II	Tip III	Tip IV	GDI	DCP
30 µg/ml	1threpeat	69	22	8	1	0	41	9
	2threpeat	80	15	2	0	0	30	5
	3threpeat	77	17	5	1	0	30	6
	4threpeat	62	32	5	0	0	45	6

60 µg/ml	1threpeat	82	15	3	1	0	21	3
	2threpeat	85	11	3	1	2	20	4
	3threpeat	96	3	1	1	0	5	1
	4threpeat	86	8	4	1	0	22	6
90 µg/ml	1threpeat	88	8	4	0	0	16	4
	2threpeat	97	1	1	1	0	7	2
	3threpeat	92	4	2	0	0	15	4
	4threpeat	84	11	2	2	0	25	5
NC	1threpeat	86	11	2	1	0	18	3
	2threpeat	84	13	3	0	0	19	3
	3threpeat	77	17	5	1	0	30	6
	4threpeat	86	12	2	0	0	16	2
PC (H2O2-10 mM)	1threpeat	34	42	14	7	3	103	24
	2threpeat	26	32	21	14	7	144	24
	3threpeat	30	37	17	11	5	124	33
	4threpeat	24	38	20	12	6	138	38

and 90µg/ ml for the DCP parameter(**Table3**).

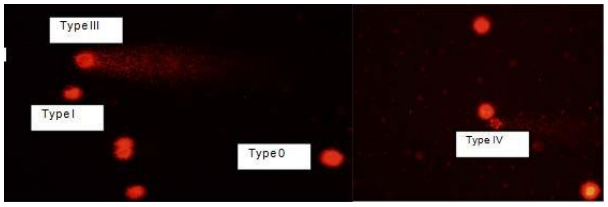

Figure 2. Comet views in ethidium bromide-stained MDBK Cells

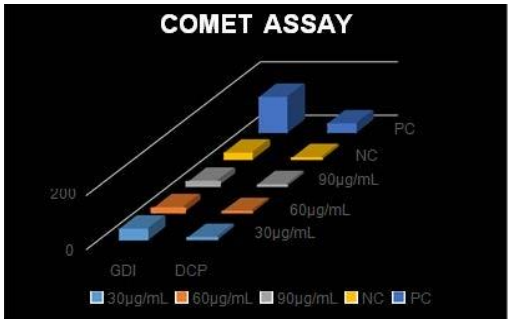

Figure 3. Frequency of GDI and DCP in relation to Bi2O3 nanoparticle concentrations in MDBK cells.

Table3. Statistical results in MDBK celltreatedwith Bi2O3nanoparticle(90-210 nm ~191.2nm) in Genetic damage Index and Damaged cell percent in Comet assay

Treatment	GDI±SE	DCP±SE
30 µg/mlBi2O3	36.5±7.68	6.50±1.73
60 µg/ml Bi2O3	17.0±8.04	3.50±2.08
90 µg/ml Bi2O3	15.7±7.36	3.75±1.25
Negative Control	20.75±6.29	3.50±1.73
Positivecontrol H2O2	127.25±18.2*	29.75±6.94*

Flow Cytometry Analysis Results

When 30 µg/ ml attention was considered, there was a significant difference between negative controland other groups for early and late apoptotic, necrotic cell and number of live cells(p<0.05). Considering the 60 µg/ ml and 90 µg/ ml attention, there was a statistically significant difference between the other groups in terms of early apoptotic, necrotic and live cell counts(p<0.05). There was a significant difference between positive control and negative control in terms of early and late apoptotic, necrotic cell and number of live cells(p<0.05).

DISCUSSION

In the present study, genotoxicity measures of the medications attained from the MDBK cells exposed to three attention of Bi2O3 nanoparticle(30 µg/ ml, 60 µg/ ml and 90 µg/ ml) was determined by Comet test system. Two parameters, GDI and DCP, were estimated in comet assay system. Cell viability was determined by inflow cytometry. In the inflow cytometry system, the cells were estimated according to their early and late apoptotic, feasible and necrotic parcels. Bi2O3(1912 nm) nanoparticle showed an increase in GDI and DCP values on MDBK cells, but this increase is n't significant statistically .Based on inflow cytometry analysis, there were poisonous goods on MDBK cells by Bi2O3 nanoparticle and this effect was statistically significant. also, this effect was more robust at advanced attention of the nanoparticle. Flow cytometry results showed an increase in early apoptotic, late apoptotic and necrotic cell counts at all attention. A high correlation was set up between the Comet analysis parameters(r = 0.962). There was a medium/ low correlation between Comet and flow cytometry analysis parameters(r = 0.446,0.440,0.361-0.2820.343-0.266)

Controversial results have been reported in data on inheritable damage and cell viability in in vivo and in vitro studies with different cell types in which the goods of different types, sizes and attention of set nanoparticles are examined.

It has been reported that some bismuth oxide compounds exhibit antibacterial activity and have antimicrobial, antifungal activity. during this area,in the study performed by [20] the bactericidal activity of Bismuth colloidal nanoparticles has been tested with Streptococcus mutans and it's shown to inhibit the growth of Streptococcus mutans. The fungicidal activity of bismuth oxide nanoparticles against Candida albicans was investigated and their antibiotic abilities were analyzed. Bismuth oxide nanoparticles of 77nm size are reported to increase C. albicans growth (85% reduction in colony size) and to inhibit biofilm formation and to point out antimicrobial activity. These results are reported to be more effective than those obtained

with the most effective oral antiseptic and commercial antifungal agents, chlorhexidine, nystatin and terbinafine.These results suggest that bismuth oxide colloidal nanoparticles could also be a very interesting candidate as a fungicidal agent for incorporation into an oral antiseptic. it's compatible with our study in terms of toxic effect when compared on a cellular basis. [7] evaluated the toxic effects of Bi2O3 nanoparticles in four different cell types; liver (HepG2 hepatocarcinoma cell), kidney (NRK-52E renal epithelial cell), intestine (Caco-2 colorectal adenocarcinoma cell) and lung (A549 lung carcinoma cell). it's determined that Bi2O3 nanoparticles (~149.1 nm) were easily obtained by all cells and showed cytotoxic / genotoxic effects.Host necrobiosis pathways were apoptosis in HepG2 and NRK-52E cells and Bi2O3 nanoparticles were observed to have necrotic effect in A549 and Caco-2 cells.In also our study, Bi2O3 nanoparticle has necrotic effect on MDBK cells. When two studies are evaluated, they support one another .In addition, the rise in 8-hydroxy deoxyguanine (8-OHdG) levels was reported by [21] as a marker of DNA damage in response to oxidative stress. Increased levels of 8-hydroxy deoxyguanine (8-OHdG) are important to demonstrate damage to DNA. The reactive oxygen species change by influencing the bases in DNA and therefore the products formed by the changing bases include more than 20 samples, like thymine peroxyl radicals, hydroxy hydroperoxide, thymine glycol, 5-hydroxymethyluthuracil, 5-formyluracil and 5-hydroxy-5-methylhydantoin. mentioned. 8-OHdG (8-hydroxy deoxyguanosine) is that the most well-known of these damaged bases. Therefore, 8-OHdG form oxidative modified DNA is employed to determine the amount of DNA damage. Hydroxyl radicals (OH) interact within the 8th position in the guanine molecule to cause oxidation. The oxidative damage of the modified DNA leads to 8-OHdG (8-hydroxy deoxyguanosine). additionally , Cu+2 ions bind with high affinity especially to guanine bases of DNA and interact with H2O2 and are reported to contribute to DNA damage.

Ahamedet al.(2019)investigated the dose-dependent cytotoxicity and apoptosis response of the Bi2O3 nanoparticles on the human carcinoma cell line (MCF-7) and reported that the potential cytotoxicity mechanisms of the Bi2O3 nanoparticles were formed by oxidative stress [22]. They showed that Bi2O3 nanoparticles reduce cell viability and induce membrane damage during a dose range of 50-300µg / ml depending on the dose. In a physicochemical study, they showed that the Bi2O3nanoparticles had a crystalline structure and a spherical form with a mean structure of 97nm, and showed that Bi-2O3nanoparticles reduced cell viability in toxicity studies and induced membrane damage during a concentration range of 50-300µg / ml.In addition, exposure of the MCF-7 cells to the Bi2O3 nanoparticles revealed that the expression level of Bcl-2, Bax and caspase-3 genes resulted in apoptotic response. In our study, the presence of medium and low correlation between the flow cytomere and comet analysis parameters showed that the 2 analyzes were supported by each other. additionally , within the previous studies [23,24] it had been reported that the increase in the Tip4 comet parameter can be taken as an indicator of apoptotic response, although the rise in comet analysis is not statistically significant compared to the negative control in DNA damage parameters. the rise in apoptotic gene expression levels in MCF7 cells coincides with the Comet data of our study.

In another study, Akbarzadeh et al [25].Investigated the consequences of bismuth oxide folate and 5-aminolevulinic acid (5-ALA) on oral epidermoid carcinoma cells (CB) and lung cancer (A549) cell lines. during this study, the cytotoxic effect of bismuth oxide nanoparticle on cells was evaluated both alone and together with folate (5-ALA). KB and A549 cells were incubated with Bi2O3 nanoparticles and folate-5-ALA-conjugated Bi2O3 nanoparticles at 10, 20, 50 and 100 µg/ ml concentrations. 0020Cytotoxic effect was tested using MTT method. additionally , nanoparticle-induced apoptosis within the treated cells was obtained using Caspases-3 activity assay and flow cytometry analysis. Bi203 nanoparticles with a mean of 19.2 ± 6.5 nm successfully synthesized were then conjugated with 5-ALA and folate. Bi2O3 or folate conjugated nanoparticles were easily get into by the cells during a concentration-dependent manner and determined to exhibit cytotoxic effects. Significant necrobiosis was recorded at concentrations of more than 50 µg / ml for both compounds. The prepared nanoparticles showed low cytotoxicity at low incubation times. However, increased concentrations of nanoparticles and cytotoxicity were reported to be increased. In our study, within the flow cytometry analysis, a rise in the parameters of early and necrotic cell counts showed that the cause of cell death is supported by the data. Because the particle size of the nanoparticle used is different, it's not appropriate to compare two studies in terms of particle size, but it's similar in terms of the response of KB and A549 cells to nanoparticles. Liu et al. (2017) investigated human toxicity of the bismuth nanoparticle in human embryonic kidney cells (HEK293) during a study in which they were taken into the cell by renal cells, induced autophagy and increased the quantity of LC3II protein, and therefore the bismuth nanoparticle had toxic effects on embryonic kidney cells[16]. it's a similarity with the toxicity effect of our study. Shakibaie et al.(2018) reported that the bismuth nanoparticles of bacterial origin between 20 and 120 nm have cytotoxic potential in cancer cells like A549, MCF-7 and 3T3 normal fibroblast cells [26, 27]. Chemical structures of bismuth nanoparticles and organic and inorganic compounds on their surfaces are shown to play an effective role in their cytotoxicity. The toxic effect of bismuth nanoparticle was according to flow cytometry results in our study. Reus et al.(2018) investigated the consequences of 250 nm bismuth nanoparticles on BALB / c3T3 cells synthesized by LASIS method [18]. They reported that the morphological structure of the cell was disrupted by the introduction of nanoparticles into the cells and necrobiosis was related to the apoptotic process using the TUNEL method. Because the tactic we use in our study is a method based on the determination of apoptotic cells, the rise in both early and late apoptotic cells in flow cytometry analysis supports the results of the study conducted by Reus et al. In another study, Bogusz et al. (2018) evaluated the toxic effects of Bi(OH)3 and α-Bi2O3 nanoparticleson malignant gliosarcoma 9L and MCF-7human carcinoma cells.clonogenicassay displaysa deathrate of over 90% in 9L andMCF-7 cells for a concentration of 50 µg/mL after incubation for 24 h.for Bi(OH)3 and α-Bi2O3 nanoparticles [17]. In contrast to, at thesame concentration, the nanomaterials exhibit no remarkable mortality in normal Madin-Darby caninekidney cells.

CONCLUSION

According to the results obtained from the Comet analysis, it

wasobserved that the Bi2O3nanoparticles has not a big genotoxic effect on the MDBK cells in a concentration- dependent manner. High correlation rateswere obtained between GDI and DCP parametersby Comet analysis.It was determined that Bi2O3 nanoparticles caused statistically significant early, apoptotic and necrotic effects during a dose dependent fashion by the flow cytometry analysis. There was a positive strong correlation among the parameters studied within the flow cytometry analysissuggesting that Bi2O3 nanoparticles had an apoptotic effect on the MDBK cells. The presence of a high correlation rate between the comet analysis and therefore the flow cytometry parameters indicates that the two methods complement each other. during this study, it's supported that Bi2O3nanoparticles have apoptotic effects on MDBK cells. We believe that studies in several cell types under different conditions will allow us more efficient and safe use of nanoparticles in versatile areas of modern life.

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