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Cytotoxic and genotoxic effects of 50nm Gold Nanorods on mouse splenocytes and human cell lines

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Abstract

The study was designed to: 1) to evaluate the cytotoxic potential effects of different concentrations of 50nm AuNRs on mouse splenocytes chromosomal aberrations; 2) to examine the effect of different concentrations of 50nm AuNRs on human lung cancer (A549), Hepatic cancer (HepG2), colorectal cancer (Caco-2) cell lines, and normal lung (CCD-19Lu) cell line as a control. Cytotoxicity was evaluated using MTT (3-(4,5-dimethizzol-zyl)-2,5-diphenyl tetrazolium bromide) assay; 3) cell cycle assay was conducted using flow cytometry. Results indicated that 50nm AuNRs induced chromosomal aberrations in cultured mouse splenocytes in a dose dependent manner. All the examined doses of 50 nm AuNRs were cytotoxic to mice splenocytes, and it induced most the aberration types, structurally (including chromatid gaps, chromatid breaks, deletions, and fragments, and numerically which represented as diploidy when compared with negative control. While 6.25 µg/ml 50nm AuNRs was safe when applied to cultured splenocytes. Also, results showed that 50nm AuNRs induced profound cytotoxicity in cancer cells of human colon cancer (Caco-2) (IC50 = 73.36), human liver cancer (HepG2) (IC50 = 67.72), human lung cancer cell line (A549) (IC50 = 33.97), respectively. Moreover, AuNRs has a cytotoxic activity on normal lung (CCD-19Lu) (IC50 = 545.5). Flow cytometric analysis demonstrated that 50nm AuNRs has a cytotoxic effect on human carcinoma cells (HepG2, CaCo2, A549, and CDD-19Lu) cells through the increased G2/M phase cell cycle arrest. Conclusion, these data indicate that 50nmAuNRs has a cytotoxic and genotoxic effects on mouse splenocytes and human normal and cancer cell lines at a concentration dependent manner.

Keywords: Gold Nanorods, cell lines, cell viability assay, flow cytometry, chromosomal aberrations

1. Introduction

Nanomaterials can exhibit various properties at the nanoscale due to their greatly expanded surface area. Nanoparticles (NPs) may have a variety of biological side effects, making them potentially more dangerous to living things (**Barar 2015; Yah 2013**). The normal operation of biological systems and human health could both be endangered by NPs' high reactivity. It is crucial to assess the genotoxicity of various nanoparticles, and significant scientific effort has been focused in this direction (**Gupta and Xie 2018**). Currently, gold nanoparticles (AuNPS) are employed in a wide range of applications, including biolabeling, catalysis, electrochemistry, antimicrobial (**Plotnikov *et al.* 2016**), gene/drug delivery, cancer diagnostics, and treatment (**Perevezentseva *et al.* 2014**). (**Abdoon *et al.* 2016**). However, there is a dearth of shared standard procedures for nanotoxicology as well as reputable scientific sources on the possibly detrimental consequences of AuNPs. Only recently has this issue begun to be slowly addressed. Despite widespread efforts, the outcomes are frequently inconsistent, particularly when it comes to the in vitro and in vivo toxicity of nanoparticles (**Savage *et al.* 2019)**. Although gold nanoparticles are known to be cytotoxic and genotoxic in vitro, cell culture results did not support this (**Vales 2020; Sani *et al.* 2021**). Genetic alterations such as DNA damage and mutations, particularly structural or chromosomal changes, are used to study genotoxicity (**Rodriguez-Rocha, et al. 2011; Zaahkouk et al. 2015**). The chromosomal aberration assay determines the substances that can alter chromosomal structure and produce genotoxicity in cultured mammalian cells, and are associated

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with a number of malignancies and genetic disorders in humans (**Pfeiffer et al. 2000**).

Two in vitro investigations that looked into the impact of Au-NPs on chromosomal aberrations in mammalian cells came up empty in this context. Non-significant abnormalities were discovered in Chinese Hamster ovarian cells after 20 hours of exposure to 14-nm Au-NPs in comparison to the negative control (**George et al. 2017**). On in vitro human cell growth, cytotoxicity and programmed cell death (apoptosis) were investigated. Genes associated with apoptosis and cell cycle arrest were also examined in the CoCa-3 cell line (**Rashad *et al*. 2018; Rashad *et al.* 2019).** In addition to proving the metal's anticancer properties, cadmium chloride decreased therapeutic efficacy in cancerous cells at relatively low doses when compared to non-cancerous cells (**Mousa *et al*., 2022**).

Also, Xia *et al*. (2006) revealed no discernible change in the frequency of chromosomal abnormalities between untreated control cells and Chinese hamster lung fibroblasts treated with Au-NPs (5, 20 or 50 nm). Additionally, mice spermatogonia cells after receiving AuNPs injections intraperitoneally show no chromosomal abnormalities (**Zakhidov *et al*., 2012**). The flow cytometry study of ZnSo4-damaged HepG2 cells showed a significant increase in apoptosis and an arrest of the cell cycle in the G2/M phase. Additionally, after being exposed to ZnSo4 in high concentrations, HepG2 cell lines showed (**Mousa *et al*., 2022**).

Despite their significance in many scientific fields, nanoparticles are now understood to exhibit unanticipated toxicity to mammalian cells and to cause cell cycle arrest. Additionally, the usage of AuNPs with diverse physicochemical qualities caused the outcomes of several investigations to vary (**Li *et al*. 2018**). **Choudhury *et al*. (2013)** showed that microtubule damage caused by gold nanoparticles (GNPs) caused cell cycle arrest at the G0/G1 phase and was localised to the tubulin/microtubule system. Therefore, more research is required to determine the methods via which AuNPs may cause cell cycle arrest. This study is the first to assess the biosafety of 50nm AuNRs using mouse splenocyte chromosomal aberrations, comet assay, and flow cytometry on cancer cell lines as mutagenic, and/or carcinogenic materials.

2. Materials and Methods

Ethical approval

The National Research Centre of Egypt's Committee for Animal Care authorized all the procedures carried out in the current study, which complied with the US National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). (Proj ID: LGA 03 15 0032).

Synthesis and characterization of 50 nm AuNRs

Utilizing the seed-growth technique, 50 nm AuNRs were created using the method **Murphy *et al*. (2011)**. A V-630 UV-VIS Spectrophotometer was used to determine the absorption spectra of AuNRs solutions (Jasco, Japan). A strong absorption band with a maximum at 808 nm, caused by the electronic oscillation of the nanorod's electrons across its long axis, and a weak range at 530 nm, polarised along the nanorod's short axis, caused by the oscillation of the nanorod electrons along the short axis. Images from a JEOL JEM 2010 TEM running at 200 kV accelerating voltage were taken using transmission electron microscopy (TEM) techniques.

Experimental design

Experiment 1: Effect of 50nm AuNRs on chromosomal aberrations in mice splenocytes.

In Milli-Q water, 50 nm AuNRs were utilised at doses corresponding to those given to humans (6.25, 12.5, 25, 50, 75, and 100 g/kg body weight). It depends on the formula for the mortality of the 50 nm AuNRS that was tested. Mortality (M) equals m/MW times 1/V Where V is the volume of the media and m is the mass of the gm in vivo and in vitro. The corresponding concentrations used were (0.01, 0.0075, 0.005, 0.0025, 0.00125, and 0.0006)/MW.

Cell culture

Swiss mice from the Animal House of the National Research Centre in Giza, Egypt, were employed in the current experiment. The spleen was taken out, cleaned with RPMI 1640 media, diced into small pieces, and the cells centrifuged at 1000 rpm for 10 min. Splenocytes cells were cultivated in culture dishes at a density of 2 x 105/dish.

Chromosomal aberration assay

Splenocytes cells were cultured in culture plates (Falcon) containing RPMI 1640 medium plus 15% fetal calf serums, antibiotics (100 units/ml of penicillin, and 100 mg/ml streptomycin) and 2 mg /ml concanavalin A, then maintained in 5%CO2 incubator at 37oC. The culture was exposed to the tested concentrations of the golden rod nanoparticles (AuNRs). Colchicine was added to the culture cells 2 h prior to harvesting to metaphase arresting. Cell pellet was suspended in a 0.075 M KCl at 37oC for 15 min. The cells were fixed in methanol: acetic acid (3:1 v/v), dropped onto clean and dry slides, air dried and stained with Giemsa in phosphate buffer (pH 6.8). Hundred metaphase spreads were scored per treatment, metaphases were examined under light microscope at magnification of 1500x. Structural and numerical aberrations were recorded.

Experiment 2: Cytotoxic effect of different concentrations of 50nm AuNRS on human cancer cell lines HepG2, CaCO-2, A549 and normal cell line CCD-19Lu.

Cell lines and cell culture

The American Type Culture Collecting (ATCC), Manassas, Virginia, USA provided the human lung cancer cell line A549, the human hepatocytes carcinoma cell line HepG2, the human colorectal cancer cell line CaCo-2, and the normal human lung cell line CDD-19Lu for the current work. In addition to 10% heat-inactivated foetal bovine serum (Gibco, Grand Island, New York, USA; Cat.no.10099133) and 1% penicillin/streptomycin, cells were grown in DMEM medium (Gibco, New York, USA; Cat.no.11995073) (Thermo Fisher Scientific; Waltham, MA, USA; Cat.no. SV30082). DMEM medium was used to grow the cells. (Gibco, New York, USA; Cat.no.11995073) They were planted at a density of 1 104 cells/well in a 96-well flat-bottomed microtiter plate and augmented with 10% heat-inactivated foetal bovine serum (Gibco, Grand Island, New York, USA; Cat.no.10099133) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA; Cat.no.SV30082). Cells were

cultivated in 100 l of media and incubated at 37 °C and 5% CO2 for 24 hours to produce cultures that were 70% to 80% confluent. Different doses of 50nm AuNRs were added, and the medium was then aspirated and replaced with new DMEM. The cells were then grown for a further 24 hours at 37°C and 5% CO2, as previously described **(Rashad *et al.* 2019)**.

Cell viability determination by MTT assay (determination of IC50)

10 l of the 12 mM MTT stock solution (5 mg/ml MTT in sterile PBS) were added to each well at the conclusion of the incubation period. After that, the plate was incubated at 37 °C for 4 hours. The purple formazan crystal that had developed at the bottom of the wells was removed, and it was then dissolved in 100 l of DMSO (Sigma Aldrich, St. Louis, MO, USA; Cat. no. 673439) for 20 minutes. There was also a negative control that involved adding 10 mL of the MTT stock solution to 100 mL of medium on its own. On an ELISA reader, the absorbance at 570 nm was read (StatFax-2100, Awareness Technology, Inc., USA). Estimated as (OD of X-treated sample - OD of blank) / (OD of control - OD of blank) 100%, the percentage of surviving cells. Plotting the experiment's outcomes required the construction of sigmoidal and dose-dependent curves. Three separate experiments were used for the assays, which were run in triplicate. Using the sigmoidal curve, the concentration of 50 nm AuNRs inhibited 50% of cells (IC50) was determined.

Experiment 3: Cell cycle assay using flow cytometry

One tenth of a million HepG2, CaCo2, A548, and CDD-19Lu cells were sown in a 10-cm culture dish with complete media and allowed to grow there overnight. After 6 hours of replacing the media with serum-free DMEM, the cells were given a 48-hour treatment with the reagents at the specified doses. By trypsinization, treated cells were collected. Cells were fixed in 70% ethanol at 20 °C for 3 hours after being thrice washed with ice-cold PBS. The cells were placed in a suspension of 100 ml PBS, stained with 100 ml of propidium iodide (PI) solution and 50 ml of RNase A solution (100 g/ml), and then let to sit in the dark for 30 to 60 minutes. In the previously disclosed Attune flow cytometer (Applied Bio-system, US), the labelled cells were read **(Rashad *et al.* 2018).**

Statistical analysis

The expression of all data was means standard error (SE). With the use of SPSS 18.0 software, one-way ANOVA was used to assess the statistical significance. When P 0.05, values were deemed statistically significant. The Tukey's Honestly Deviation (HSD) test was used to compare the means.

3. Results and Discussion

Experiment 1. Effect of 50nm AuNRs on chromosomal aberrations in mice splenocytes.

Table 1 showed that the effect of different concentrations of 50nm AuNRs on cultured mouse splenocytes. Results indicated that 50nm AuNRs induced chromosomal aberrations in cultured mouse splenocytes in a dose dependent manner. All the examined doses of 50nm AuNRs were cytotoxic to mice splenocytes, and it induced, most the aberrations types, structurally (including chromatid gaps, chromatid breaks, deletions, and fragments, Figure 1) and numerically which represented as diploidy when compared with negative control, Whereas 6.25 µg/ml 50nm AuNRs was safe when applied to cultured splenocytes according to the genotoxicity assay.

Table (1): Percentage of chromosomal aberrations induced by using 50 nm AuNRs on cultured mice splenocytes.

Conc. 50 nm AuNRs µg/ml (Equivalent to human doses)	Chromosomal aberrations No / metaphase	Types of chromosomal. Aberrations.		Mean of Ch. Aberr. + SE
		Structural aberrations	Numerical aberrations	
0	3 /65	3	-	4.62 ± 0.34
6.25	4/76	4	-	5.3 ± 1.67
12.5	5/80	5	-	6.25 ± 0.67 *
25	6/94	6	-	6.4 ±0.89 *
50	7/91	7	-	7.7 ± 0.23 **
75	8/101	7	1	7.9 ± 0.12 **
100	9/94	8	1	9.6 ± 0.34 **

* Significant at (P< 0.05), ** Significant at (P< 0.01)

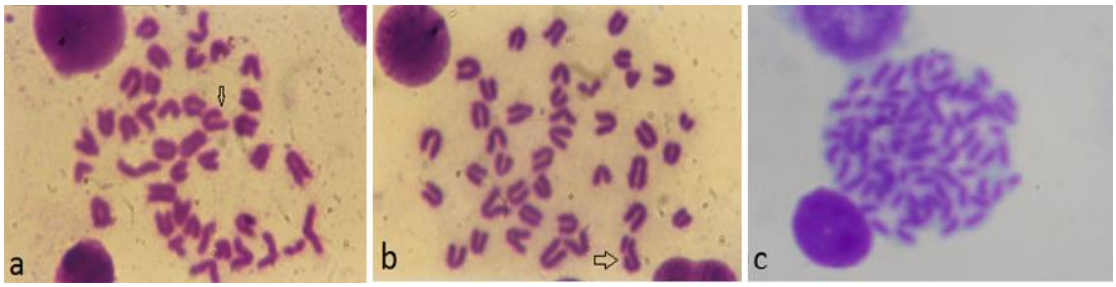

Fig. 1. Photomicrograph showing deletion, chromatid break and diploid metaphases in mice splenocytes metaphases treated with 50 nm AuNRs, a) deletion, b) chromatid break as structural aberration type, where in c) is a diploid metaphase as numerical aberration type.

2. Cytotoxic effect of 50nm AuNRs on human cancer and normal cell lines (MTT assay)

Table 2 compares the vitality of HepG2, Caco-2, and A549 cancer cells to the positive control CCD-19Lu cells to show the cytotoxic effect of various doses of 50nm AuNRs. As shown in Fig. 2, 50nm AuNRs had cytotoxic effect on both cancerous and healthy lung cell types. These results supported the existence of a cytotoxic impact and showed that 50nm AuNRs lowered cell viability in both malignant and non-malignant cells.

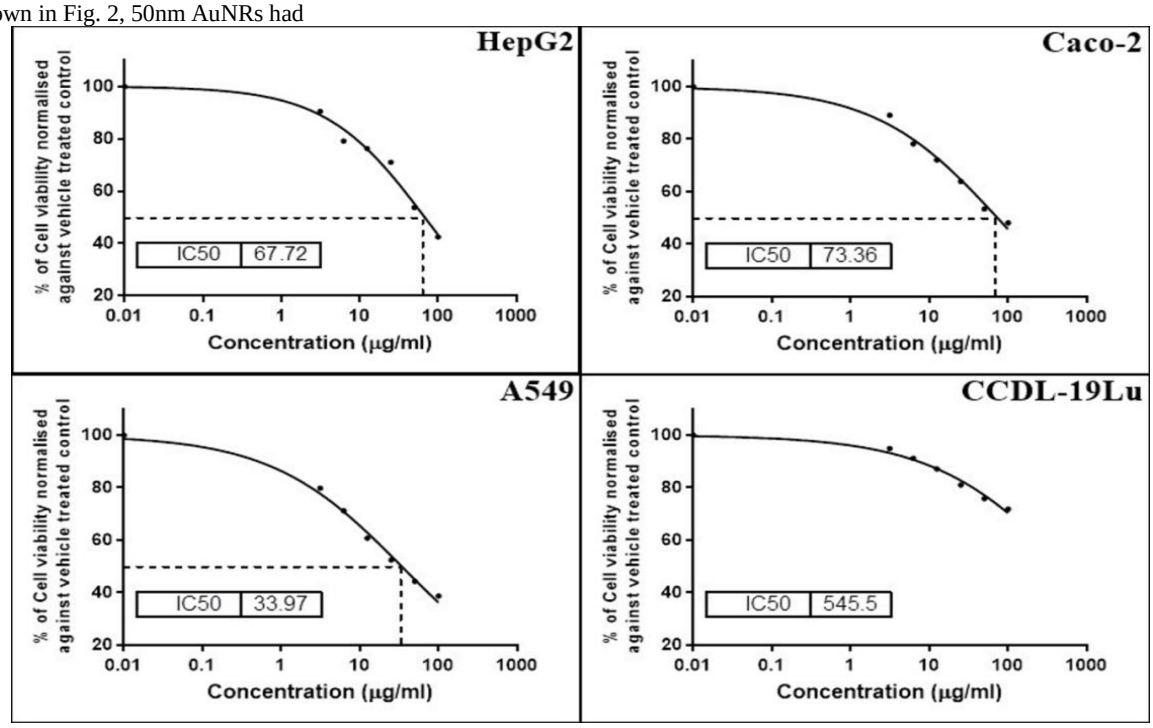

Fig. 2. Dose-dependent growth inhibition as a positive control against. 50nm AuNRs Non-malignant human lung cells (CCD-19Lu cells) and malignant human cells (HepG2, Caco-2, A549). Viability was quantified by MTT assay. Results are mean ± (n = 3). *p < 0.05 to α compared with the control.

Table (2): The effect of Gold on the viability percentages of the four cell lines.

Cell line	Conc. ug/ml	Mean O.D	Viability %	Death %	IC50
HepG2	100	1.091	42.48	57.52	67.72
	50	1.379	53.70	46.30	
	25	1.825	71.05	28.95	
	12.5	1.958	76.25	23.75	
	6.25	2.032	79.11	20.89	
	3.125	2.324	90.48	9.52	
Caco-2	100	0.637	48.11	51.89	73.36
	50	0.708	53.44	46.56	
	25	0.846	63.90	36.10	
	12.5	0.954	72.05	27.95	
	6.25	1.036	78.25	21.75	
	3.125	1.180	89.09	10.91	
A549	100	0.635	38.70	61.30	33.97
	50	0.727	44.27	55.73	
	25	0.861	52.47	47.53	
	12.5	0.998	60.79	39.21	
	6.25	1.168	71.15	28.85	
	3.125	1.310	79.83	20.17	
CCD-19Lu	100	1.747	71.87	28.13	54.55

	50	1.844	75.86	24.14	
	25	1.969	81.01	18.99	
	12.5	2.119	87.18	12.82	
	6.25	2.216	91.19	8.81	
	3.125	2.307	94.94	5.06	

3. Effect of 50nm AuNRs on cell cycle of HepG2, CaCo-2, A549, and CDD-19Lu cells

The cell cycle distribution of HepG2, Caco-2, A549, and CCD-19Lu cells was influenced by gold nanorod concentrations of 73.36, 67.72, 33.97, and 545.5 g/ml. In the control and Gold nanorods, the G0/G1 phase decreased from (62.5% to 27.3%; 62.3% to 43.4%; 62.6% to 32.4%; 66.4% to 67.4%) and the S phase percentage decreased from (16.3% to 18.2%; 15.2% to 18.9%; 15.1% to 17.1%; 15.8% to 13.2%), respectively (Figure, 1). When cells were incubated with either the control (19.4%) or gold nanorods (21.2% to 54.5%; 22.5% to 37.7%; 22.3% to 50.5%; 19.8% to 19.4%), the proportion of cells in the G2/M phase significantly increased. These findings demonstrated a considerable buildup of HepG2, Caco-2, A549, and CCD-19Lu cells in the G2/M phase and supported the hypothesis that Gold nanorods cause a cell cycle to arrest in the G2/M phase, which is a lethal effect (Table 4). After significant HepG2, Caco-2, A549, and CCD-19Lu viability inhibition occurred, an effort was made to determine the cytotoxic and genotoxic impact of the Gold nanorods on cell cycle arrest using flow cytometry based on cell cycle distribution. Figure (4) demonstrated that HepG2, Caco-2, A549, and CCD-19Lu cells treated with Gold nanorods performed better than the control group. These findings demonstrated a large buildup of HepG2, Caco-2, A549, and CCD-19Lu cells in the G2/M phase and demonstrated that 50nm AuNRs significantly increases cytotoxicity and genotoxicity by inducing G2/M phase cell cycle arrest (Table 3).

Table (3): The percentages of cancer cells in different phases of cell cycle in HepG2, CaCo2, A549, and CDD-19Lu cells.

	HepG2		CaCo2		A459		CDD-19Lu	
Phases	G1	G2	G1	G2	G1	G2	G1	G2
G0/G1	62.5±2.5[a]	27.3±1.5[b]	62.3±3.5[a]	43.4±2.3[b]	62.6±3.3[a]	32.4±1.8[b]	66.4±3.7	67.4±3.5
S	16.3±1.9	18.2±1.8	15.2±3.4	18.9±4.5	15.1±2.9	17.1±2.8	15.8±2.6	13.2±1.8
G2/M	21.2±1.2[b]	54.5±3.6[a]	22.5±1.8 [b]	37.7±2.8[a]	22.3±3.8 [b]	50.5±4.5[a]	19.8±3.8	19.4±4.5

Values are expressed as mean ± SEM, n = 7. Means within the same row for each cell type carrying different superscript letters are significantly different ($P \leq 0.05$).

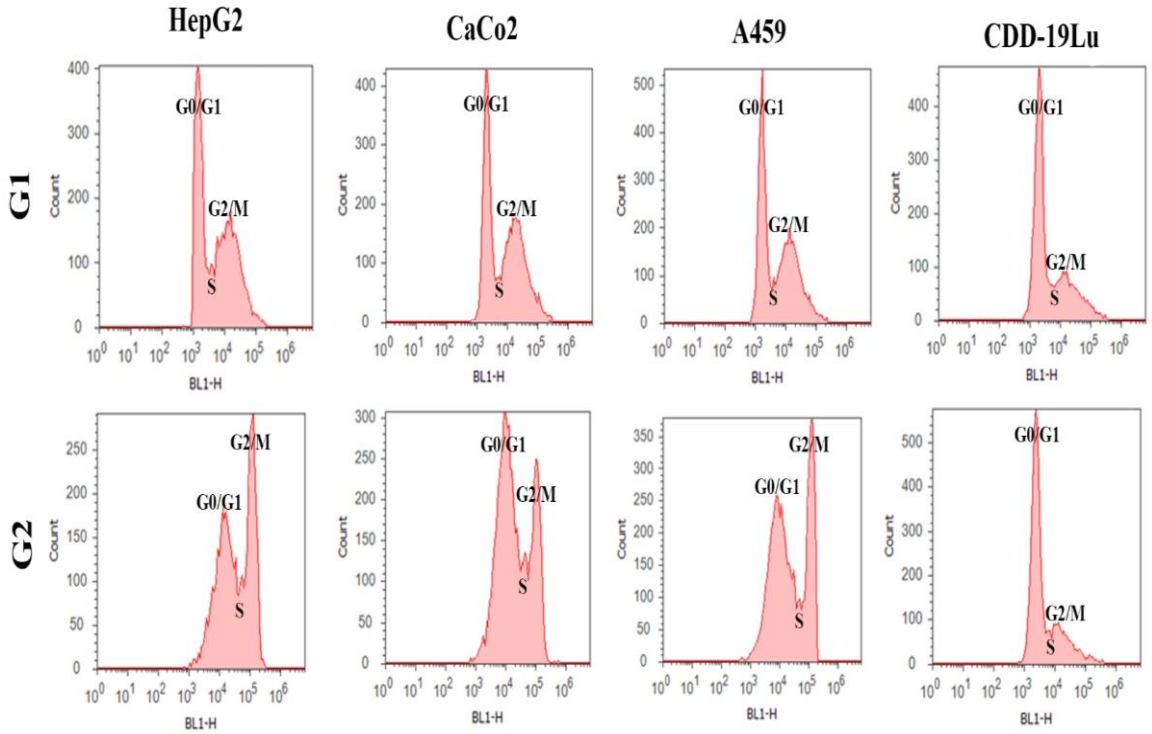

Fig. 3. Cell cycle histograms as measured by flow cytometry show effect of X on cell cycle of HepG2, CaCo2, A549, and CCD-19Lu cells. The X-axis represents the PI fluorescence based on the DNA content and the Y-axis represents the number of cells in each phase.

Conclusion

Given that any changes to DNA have the potential to cause cell death, tissue dysfunction, and the emergence of cancer, the genotoxicity of gold nanoparticles is a crucial element of toxicology. The genotoxic effects of 50nm AuNRs on both healthy and cancerous cells were examined in this work. For the study, four genotoxicity tests—chromosomal aberrations, the Cell Viability (MTT) Assay, and the Cell Cycle Assay Using Flow Cytometry were carried out.

Our findings showed that the two types of chromosomal abnormalities are strongly induced by gold nanorods (GNPs) at the investigated levels.structurally and numerically, on cultured mice splenocytes.

Chromosomal aberrations induced by (GNPs) on cultured mice splenocytes were dose dependent, as in case of many chemicals (**Amer *et al*. 2000 and Aboul-ela 2002**).

Gold nanorods (GNPs) 50 nm has a significant genotoxicity with tested concentrations, 100, 75, 50, 25 and 12.5 equivalent to the human doses. But using the least concentration 6.25, GNPs was save and did not induce any significance effect. So, the concentration 6.25 can be used with human treatment as save and harmless.

In this study, incubation of mouse splenocytes in vitro with different concentrations of 50nm AuNRs produced both types of aberrations, structurally (Chromatid gaps, chromatid breaks, deletions, and fragments) and numerically aberrations which represented as diploidy. Similarly, 5 nm and 15 nm Au NPs were able to inhibit cell proliferation by apoptosis and to induce chromosomal damage (**Di Bucchianico *et al*. 2014**). This could be due to the oxidative stress generated after exposure to these nanoparticles exposure that leads to cell proliferation, reduction in ATP production and mitochondrial damage which in turn impairs energy-dependent DNA repair mechanisms resulting in DNA damage (**El Mahdy *et al*. 2014**). In contrast, the AuNPs were found to be non-genotoxic using chromosomal aberration assays (**George *et al.* 2017**). This discrepancy could be attributed to the shape or size of AuNPs used.

In the current work, the results showed significant accumulation of 50nm AuNRs in HepG2, Caco-2, A549 and CCD-19Lu cells at the G2M phase and confirmed that Gold nanorods has cytotoxic effect via induction of G2M phase arrest of the cell cycle. Starting from 12.5 µg to 100 µg AuNRs concentrations was added to media showed DNA chromosomal abnormalities in cultured mouse splenocytes. Additionally, in human liver cell lines (HepG2), Lung cancer (A459), colon cancer (Caco2) and normal lung (CCD-19Lu). Gold nanorods was the most effective on the viability A549 cell reduction among the two cells where the all concentrations had significant effect while Caco-2 was the lowest without significant change. Flow cytometric analysis demonstrated that treatment of human HepG2, CaCo2, A549, and CCD-19Lu cells with Gold nanorods increased G2/M phase cell cycle arrest. Gold nanorods (GNPs) 50 nm has a significant genotoxicity with tested concentrations, 100, 75, 50, 25 and 12.5 equivalent to the human doses. But using the least concentration 6.25, GNPs was safe and did not induce any cytotoxic effect. So, the concentration 6.25 can be used with human treatment as save and harmless. In concomitant, BSA-GNPs induced G2/M arrest of RAW264.7 cells through microtubule stabilization (**Li *et al.* 2018**). The G2/M phase of both the cell cycle influences the intracellular movement of nanoparticles, which are internalised by cells but just not ejected from them (**Kim *et al.* 2011**). Kinesin 5A level was raised by the buildup of BSA-GNPs in lysosomes, which led to the stability of microtubules (including the promotion of tubulin polymerization and inhibition of tubulin depolymerization) (**Cardoso *et al*., 2009; Chen *et al*. 2015**), blocking chromosomal partition and causing G2/M cell cycle halt by increasing CDH1 (**Touati *et al.* 2015**). **Choudhury *et al.* (2013)** reported that bare GNPs induce G0/G1 arrest by causing microtubule damage.

Conclusion, these data indicate that 50nmAuNRs has a cytotoxic and genotoxic effects on mouse splenocytes and human normal and cancer cell lines at a concentration dependent manner.

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Author's contribution

ASSA, SER design the work, EAA and SER conduct the experimental work and the paper were prepared by ASSA and SER. Each author contributed to the serious debate, data analysis, and text revision.

Availability of data

The dataset obtained and analysed from this work were presented at the text and the original data will be available upon request.

Competing Interests

The authors declare that they have no competing interests.

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