



OPEN Cobalt oxide nanoparticles disrupt cellular homeostasis through oxidative genomic instability and mitochondrial stress, driving necrosis in non-small lung cancer cells

Hanan R. H. Mohamed^{1✉}, Shahd H. Elnawasani², Basma A. Mohamed², George M. Hakeem², Maria Nagy², Rawan Essam², Ayman Diab² & Gehan Safwat²

Non-small cell lung cancer (NSCLC) remains one of the most aggressive and treatment-resistant malignancies worldwide, with current therapies often limited by poor tumor specificity, resistance, and systemic toxicity. Nanoparticle-based therapeutics offer a promising strategy to overcome these challenges. Cobalt oxide nanoparticles (Co_3O_4 NPs), owing to their distinctive redox properties and capacity to generate reactive oxygen species (ROS), have emerged as potential anticancer agents; however, their intrinsic effects against NSCLC cells and the underlying mechanisms remain poorly understood. This study therefore investigates the novel cytotoxic and pro-apoptotic effects of pristine Co_3O_4 NPs in non-small A549 lung cancer cells using an integrated mechanistic approach. Cell viability was measured with the sulforhodamine B (SRB) assay, while genomic DNA damage, ROS generation, mitochondrial membrane potential, and apoptosis induction were evaluated using alkaline comet assay, 2',7'-dichlorofluorescein diacetate, rhodamine-123 dye, and flow cytometry, respectively. Mechanistic insights were further supported by quantitative real-time PCR analysis of apoptosis- and mitochondria-related genes (*p53*, *ND3*, and *Bcl-2*). A 72-hour exposure to Co_3O_4 NPs various concentrations significantly reduced A549 cell viability in a concentration-dependent manner with a markedly low IC_{50} value of 34.17 $\mu\text{g/ml}$. Moreover, treating A549 cells with the IC_{50} concentration (34.17 $\mu\text{g/ml}$) of Co_3O_4 NPs caused excessive ROS production, severe genomic DNA damage, and dramatic mitochondrial depolarization. Gene expression analysis revealed remarkable upregulation of pro-apoptotic *p53* and mitochondrial *ND3* gene expression, alongside significant downregulation of anti-apoptotic *Bcl-2* gene expression. Despite *p53* activation the pronounced mitochondrial dysfunction and bioenergetic collapse favored necrotic cell death over classical apoptosis. Flow cytometric analysis confirmed this shift, showing a substantial increase in necrotic A549 cells alongside a marked reduction in apoptotic A549 populations following Co_3O_4 NPs exposure. These findings collectively provide integrated evidence that pristine Co_3O_4 NPs induce oxidative stress, genomic DNA damage, mitochondrial dysfunction, and cell death in A549 NSCLC cells. The predominance of necrotic cell death under the conditions examined suggests that severe oxidative and mitochondrial stress contributes to the observed cytotoxic response. Further studies using NSCLC models with different *p53* statuses, appropriate normal-control cells, and in vivo systems are required to establish the selectivity, reproducibility, and broader biological relevance of these effects.

Keywords Co_3O_4 NPs, ROS generation, Genomic DNA damage, Mitochondrial damage and *p53*-associated stress and apoptotic signaling

¹Department of Zoology, Faculty of Science, Cairo University, Giza, Egypt. ²Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), 6th of October City, Egypt. ✉email: hananeeyra@cu.edu.eg

Lung cancer remains one of the leading causes of cancer-related mortality worldwide, accounting for approximately 18% of all cancer deaths¹. Among its subtypes, non-small cell lung cancer (NSCLC) constitutes around 85% of all lung cancer cases, making it the most prevalent form^{2,3}. The major risk factors for NSCLC include tobacco smoking, exposure to environmental carcinogens, genetic predisposition, and lifestyle factors. While smoking is the predominant risk factor, a significant proportion of cases arise in non-smokers due to exposure to air pollution, occupational hazards, and genetic susceptibility^{4,5}.

Despite advances in surgery, radiotherapy, targeted therapy, immunotherapy, and standard platinum-based chemotherapies like cisplatin and carboplatin, the treatment of advanced NSCLC remains a major clinical challenge^{6–10}. Conventional chemotherapy remains a cornerstone for advanced and metastatic cases, functioning primarily by inducing severe DNA damage on rapidly dividing malignant cells^{6–8}. However, the long-term efficacy of these regimens is severely restricted by therapeutic resistance, pronounced tumor heterogeneity, and profound off-target toxicities, including nephrotoxicity, neurotoxicity, myelosuppression, and severe gastrointestinal distress^{6–10}. These severe side effects severely compromise patient quality of life, underscoring an urgent need for novel therapeutic approaches capable of damaging cancer cells through distinct cellular mechanisms while minimizing systemic adverse toxic effects.

Nanotechnology has attracted considerable interest in cancer research because nanoparticles can interact with cancer cells through multiple physicochemical and biological mechanisms¹¹. Among metal oxide nanoparticles, cobalt oxide nanoparticles (Co_3O_4 NPs) are of particular interest because of their distinctive physico-chemical properties: high surface area, biocompatibility and unique redox and catalytic properties. Co_3O_4 NPs possess a mixed-valence $\text{Co}^{2+}/\text{Co}^{3+}$ spinel structure that enables efficient redox cycling and surface catalytic activity, promoting the generation of reactive oxygen species (ROS) under biological conditions^{12–14}. This feature is particularly relevant in cancer therapy, as tumor cells, including NSCLC, often exhibit elevated basal oxidative stress and weakened antioxidant defenses, rendering them more susceptible to further ROS insults^{15–17}. Moreover, cobalt-based nanomaterials have demonstrated pro-apoptotic, DNA-damaging, and mitochondria-targeting effects in several cancer models, supporting their potential as selective anticancer agents. Transition metal oxide nanoparticles are also increasingly explored as nano-therapeutics because of their physicochemical stability, tunable surface characteristics, and compatibility with functionalization strategies for biomedical applications^{15–17}. Recent studies have demonstrated the strong and selective cytotoxicity of Co_3O_4 NPs against human aggressive melanoma A375 and colorectal CT26 cancer cells^{14,17}. Collectively, these findings supporting further investigation of Co_3O_4 NPs cellular effects as redox-active nanoplatforms for treatment of other cancer types.

However, the biological and therapeutic responses of lung cancer cells particularly NSCLC to Co_3O_4 NPs remain fully uncharacterized, particularly for pristine, unmodified Co_3O_4 NPs. Despite the anticancer activity of cobalt oxide-based nanomaterials have been reported in lung cancer models, available studies, including that of Jayapalan and colleagues¹⁸, have investigated formulated, doped, surface-modified, or hybrid nanostructures, making it difficult to distinguish the effects attributable specifically to pristine Co_3O_4 NPs from those associated with additional components or synergistic interactions. Moreover, important mechanistic gaps remain, as most prior work has focused mainly on general cytotoxicity or a limited set of apoptotic markers without integrating genotoxic, oxidative, mitochondrial, and gene-regulatory responses within a unified experimental framework.

Accordingly, the present study was designed to estimate the biological and therapeutic effects of pristine, unmodified Co_3O_4 NPs to provide a systematic, multi-level evaluation of Co_3O_4 NPs-induced cellular stress responses in non-small A549 lung cancer cells. By linking oxidative stress generation to genomic DNA damage, mitochondrial dysfunction, and p53-dependent apoptotic signaling through combined functional and molecular analyses, this study clarifies the sequence and interrelationship of events underlying Co_3O_4 NPs-mediated cytotoxicity, thereby moving beyond descriptive cytotoxicity reports toward a more mechanistic and nanoparticle-specific understanding. To address this research gap, we explored the therapeutic potential of Co_3O_4 NPs against NSCLC by assessing A549 cancer cell viability using the sulforhodamine B (SRB) assay, evaluating genomic DNA integrity and mitochondrial membrane potential using the alkaline comet assay and rhodamine-123 dye, respectively, and measuring intracellular ROS generation using 2',7'-dichlorofluorescein diacetate. Furthermore, changes in apoptosis-related gene expression and apoptotic induction were analyzed using quantitative real-time PCR (qRT-PCR) and Flow Cytometry, respectively. This integrated assessment provides a more comprehensive characterization of the cellular responses of A549 NSCLC cells to pristine Co_3O_4 NPs and helps define the relationship among oxidative stress, genomic damage, mitochondrial dysfunction, gene-expression changes, and cell death.

Materials and methods

Source and characterization of Co_3O_4 NPs

The Co_3O_4 NPs used in this study were commercially obtained from Sigma Chemical Company (St. Louis, MO, USA) as a black powder, with a manufacturer-reported primary particle size of ≤ 50 nm (Product No. 637025; CAS No. 1308-06-1). The nanoparticles were stored in a dry, dark environment at room temperature and freshly dispersed in dimethyl sulfoxide (DMSO) immediately before each experiment using a standardized ultrasonication procedure. The identity and physicochemical characteristics of the Co_3O_4 NPs were determined using complementary techniques, as previously described¹⁴. X-ray diffraction (XRD; XPERT-PRO, PANalytical, Almelo, Netherlands) confirmed the crystalline structure and purity of Co_3O_4 , with characteristic diffraction peaks at 18.99° , 31.31° , 36.86° , 38.66° , 44.75° , 59.35° , and 65.21° (Fig. 1). Transmission electron microscopy (TEM) showed cubic-spherical particles with a mean primary particle size of 20.08 nm (Fig. 1). Dynamic light scattering (DLS; Malvern Zetasizer Nano Series, Malvern Instruments, Westborough, MA, USA) showed an average hydrodynamic diameter of 691.7 nm and a zeta potential of -2.11 mV (Fig. 1). The substantially larger hydrodynamic diameter compared with the TEM-derived primary particle size indicates that Co_3O_4 NPs

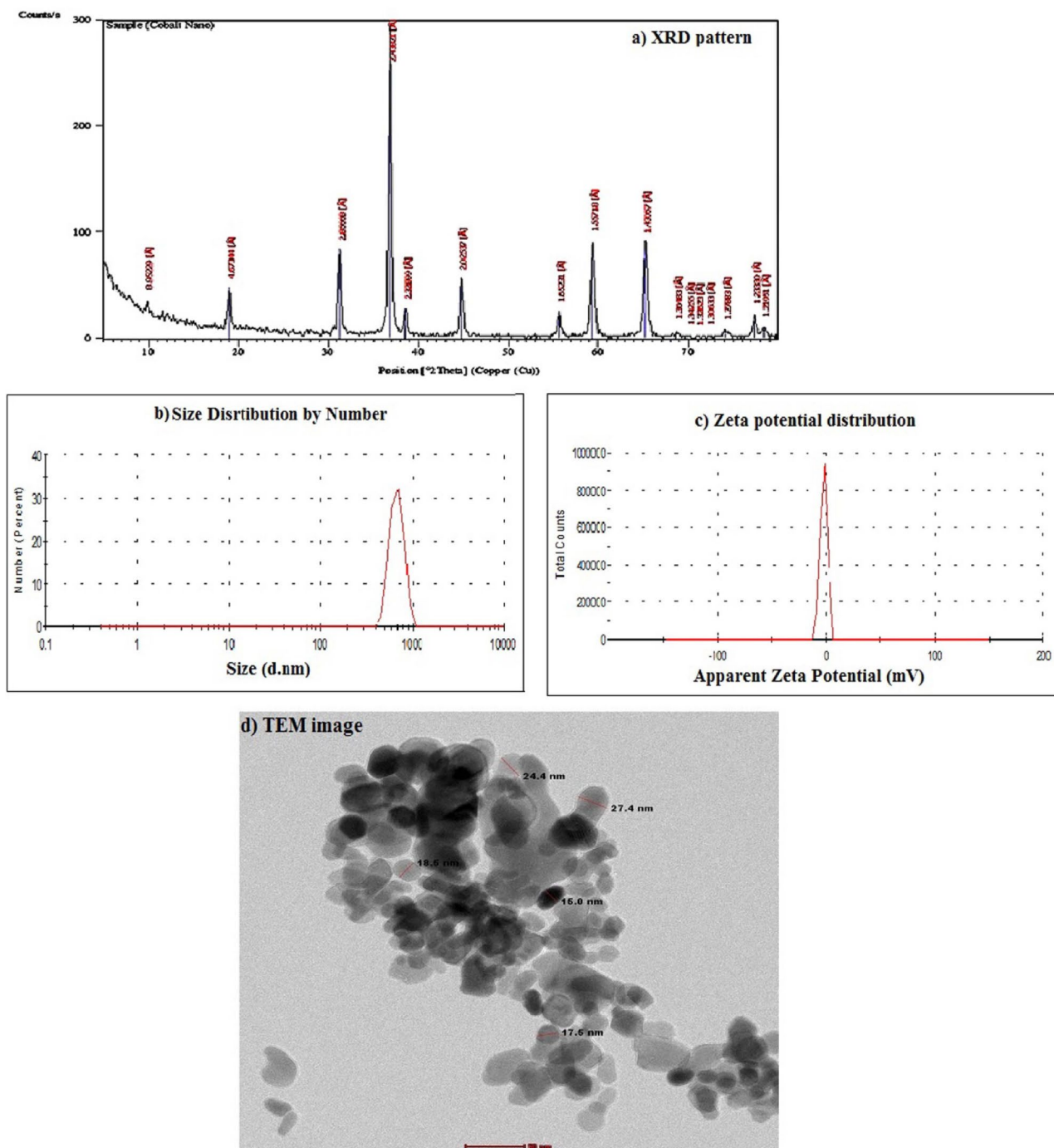


Fig. 1. Physicochemical characterization of Co_3O_4 NPs as reported by Mohamed et al.¹⁴, **(a)** X-ray diffraction (XRD) pattern confirming crystalline structure and purity; **(b)** particle size distribution by dynamic light scattering (DLS); **(c)** zeta potential distribution indicating surface charge and colloidal stability; **(d)** transmission electron microscopy (TEM) image showing cubic-spherical morphology and uniform dispersion.

underwent aggregation/agglomeration in the aqueous dispersion. Accordingly, the DLS value was interpreted as the hydrodynamic size of the dispersed particle aggregates rather than as the primary nanoparticle diameter. Ultrasonication was applied immediately before treatment to obtain a reproducible nanoparticle suspension and minimize variability in dispersion among experiments.

Preparation of Co₃O₄ NPs suspension for cell treatment

Co₃O₄ NPs suspensions were freshly prepared before each experiment using the same DMSO dispersion and ultrasonication procedure. The required concentrations were subsequently prepared by dilution with the appropriate cell-culture medium. The same commercial Co₃O₄ NPs preparation and standardized dispersion procedure were used throughout the experiments to maintain consistency between independent experimental replicates. The final concentration of DMSO in the cell cultures was maintained at a level that did not affect cell viability, and corresponding vehicle controls were included. To minimize microbial and endotoxin contamination, Co₃O₄ NPs suspensions were prepared and handled under aseptic conditions using sterilized glassware and plasticware and sterile, cell-culture-grade reagents. Fresh nanoparticle suspensions were prepared before each experiment using the same standardized dispersion and ultrasonication procedure. Appropriate vehicle controls were prepared and treated under identical conditions to distinguish nanoparticle-associated effects from those related to the vehicle or experimental handling. These procedures were consistently applied throughout the experiments to improve the reliability and reproducibility of the *in vitro* assessments.

Cell culture

Human non-small lung cancer A549 cells were procured from Nawah Scientific Inc. (Mokatam, Cairo, Egypt) and were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/mL streptomycin. The A549 cancer cells were maintained under optimal conditions in an incubator at 37 °C with 5% CO₂ to support proper growth. To enhance methodological rigor, routine quality control measures were implemented for all cell cultures. Cells were periodically screened for mycoplasma contamination using a PCR-based mycoplasma detection assay according to the manufacturer's instructions, and only mycoplasma-free cultures were used for experiments. In addition, passage number was carefully controlled to reduce phenotypic drift; cells were used within a defined low-to-moderate passage range (passages 5–20 after thawing). Cell morphology and growth characteristics were also regularly monitored to confirm culture health and consistency. These practices were applied to ensure the validity and reproducibility of the experimental outcomes.

Assessment of Co₃O₄ NPs cytotoxicity on A549 Cells

The influence of Co₃O₄ NPs on the viability of A549 lung cancer cells was assessed using the Sulforhodamine B (SRB) cytotoxicity assay^{19,20}. Lung cancer A549 cells were seeded in 96-well plates at a volume of 100 µl per well and incubated for 24 h in complete DMEM medium. Subsequently, the cells were exposed to five different concentrations of Co₃O₄ NPs (0.2, 2, 20, 200, and 2000 µg/ml) for 72 h. This concentration range was selected based on preliminary dose-finding experiments to capture minimally cytotoxic to highly cytotoxic levels, enabling accurate IC₅₀ determination while avoiding nonspecific toxicity, in line with established *in vitro* cytotoxicity and nanotoxicology guidelines^{21–23}.

Following the treatment, cells were fixed with cold trichloroacetic acid, washed, and stained with 0.4% SRB for 10 min at room temperature in the dark. Excess dye was removed with 1% acetic acid, plates were dried overnight, and protein-bound SRB was solubilized in Tris base solution. Absorbance at 540 nm was measured using a BMG LABTECH[®] FLUOstar Omega microplate reader, and cell viability was expressed relative to untreated control cells. The half-maximal inhibitory concentration (IC₅₀) of Co₃O₄ NPs against A549 cancer cells were calculated from dose–response curves generated from three independent replicates, using GraphPad Prism software.

Treatment schedule

A549 lung cancer cells were cultured in T25 flasks under optimized conditions, including complete DMEM medium, pH 7.4, 37 °C, and 5% CO₂. Cells were divided into untreated (control) and Co₃O₄ NPs-treated groups. Control cells were exposed to DMSO (<0.1%), whereas treated cells were incubated with Co₃O₄ NPs for 72 h at the determined IC₅₀ concentration in this study using SRB assay. Following treatment, cells were harvested by trypsinization, centrifuged, and washed twice with ice-cold PBS. The harvested cells were immediately stored at –80 °C in PBS to preserve RNA integrity for subsequent molecular analyses. RNA quality and integrity were subsequently verified by Nanodrop (A₂₆₀/A₂₈₀ ratios) and agarose gel electrophoresis prior to downstream assays. All treatments were performed in triplicate to ensure accuracy, reproducibility, and statistical reliability of the experimental results.

Screening ROS generation within A549 cells

The impact of Co₃O₄ NPs on ROS generation in A549 lung cancer cells was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye^{24,25}. A549 cell suspensions were incubated with an equal volume of 20 mM DCFH-DA for 30 min at room temperature in the dark. The dye readily entered the cells and was converted into the fluorescent compound dichlorofluorescein (DCF) in the presence of intracellular oxidants, with fluorescence intensity used as an indicator of ROS generation. The cell–dye suspensions were then transferred onto clean glass slides and the spread thin film was examined using an epifluorescence microscope at 200× magnification. Images were acquired from the control and Co₃O₄ NP-treated groups using identical microscope and acquisition settings, including the same objective, illumination conditions, exposure time, and detector gain. The same image-processing and fluorescence-quantification procedures were applied to all groups. Fluorescence intensity was quantified using Fiji/ImageJ software to compare ROS-generated fluorescence between untreated and Co₃O₄ NP-treated cells.

Measurement of genomic DNA damage

Genomic DNA damage in untreated (control) and Co₃O₄ NPs-treated A549 lung cancer cells was assessed using the alkaline comet assay (single-cell gel electrophoresis) following established protocols^{26,27}, with modifications to ensure statistical robustness. Briefly, a 15 µl suspension of A549 cells was mixed with 60 µl of 0.5% low-melting-point agarose and spread onto slides pre-coated with 1% normal-melting agarose. Slides were allowed to solidify at room temperature and then immersed in cold lysis buffer containing freshly added DMSO and Triton X-100, followed by incubation in the dark at 4 °C for 24 h. After lysis, slides were equilibrated in freshly prepared alkaline electrophoresis buffer (pH 12) for 15 min to allow DNA denaturation, and electrophoresis was performed at 25 V and 300 mA for 30 min in the same buffer. The slides were subsequently neutralized, fixed, air-dried, and stained with ethidium bromide for visualization. To ensure statistical reliability, 50 cells were analyzed per slide, and three independent slides were prepared for each treatment condition. Comet images were captured and analyzed using COMETSCORE™ software, and DNA damage parameters; including tail length, % DNA in tail, and tail moment, were reported as mean ± standard deviation (SD) from these replicates.

Estimation of mitochondrial membrane potential integrity

The impact of Co₃O₄ NPs on mitochondrial membrane potential in lung A549 cancer cells was assessed using the fluorescent Rhodamine 123 dye^{28,29}. A suspension of A549 cancer cells was mixed with an equal volume of Rhodamine 123 fluorescent dye (10 mg/ml) and incubated in the dark for 1 h at 37 °C. The cells were subsequently washed twice with PBS, spread as a thin film on clean sterile slides, and visualized using an epifluorescence microscope at 200× magnification. Rhodamine 123 fluorescence images were acquired under identical microscope and acquisition settings for untreated control and Co₃O₄ NP-treated A549 cells, including the same objective, illumination conditions, exposure time, and detector gain. Images were processed and analyzed using the same procedure, and fluorescence intensity was quantified using Fiji/ImageJ software. The fluorescence signal was presented in the green channel corresponding to Rhodamine 123 fluorescence. The relative decrease in fluorescence intensity in Co₃O₄ NP-treated A549 cancer cells was considered indicative of mitochondrial membrane-potential loss.

Flow cytometric analysis of apoptosis

Apoptosis and necrosis induction in A549 lung cancer cells were analyzed using dual-channel Flow cytometry with Annexin V-FITC and propidium iodide (PI) staining. Briefly, both adherent and non-adherent cells were collected 72 h after treatment with Co₃O₄ NPs at the IC₅₀ concentration (34.17 µg/ml). Cells were washed twice with cold PBS, resuspended in binding buffer, and incubated with Annexin V-FITC and PI according to the manufacturer’s instructions. Data acquisition was performed using a BD FACSCalibur flow cytometer. The gating strategy included forward scatter (FSC) versus side scatter (SSC) to exclude debris, and FSC-H versus FSC-A to remove doublets. Cells were then analyzed for Annexin V and PI fluorescence to identify viable cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), late apoptotic cells (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁻/PI⁺). Quantitative data were obtained from at least 10,000 events per sample and expressed as mean ± SD. Statistical significance between treated and control groups was determined using an unpaired Student’s t-test (*p* < 0.05). This approach ensured accurate, reproducible measurement of apoptotic and necrotic subpopulations in response to Co₃O₄ NPs exposure.

Quantification of gene expression

The mRNA expression levels of apoptotic *p53*, anti-apoptotic *Bcl2*, and mitochondrial NADH dehydrogenase 3 (*ND3*) genes were measured in A549 lung cancer cells using quantitative real-time PCR (qRT-PCR). Total RNA was extracted from untreated and Co₃O₄ NPs-treated lung cancer cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. RNA quality and purity were assessed using Nanodrop, with A₂₆₀/A₂₈₀ ratios between 1.8 and 2.0 considered acceptable, and RNA integrity was confirmed by agarose gel electrophoresis.

A total of 1 µg of high-quality RNA was reverse-transcribed into complementary DNA (cDNA) using the cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed for each gene using the StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix and pre-designed primers listed in Table 1^{30–32}. Gene expression levels were normalized to the housekeeping gene

Gene	Strand	Primer’s sequences
GAPDH	Forward	5'-GAAGGTGAAGGTCGGAGTCA-3'
	Reverse	5'-GAAGATGGTGATGGGATTTC-3'
ND3	Forward	5'-CGCCGCCTGATACTGGCAT-3'
	Reverse	5'-CTAGTATTCCTAGAAGTGAG-3'
BCL-2	Forward	5'-TCCGATCAGGAAGGCTAGAGT-3'
	Reverse	5'-TCGGTCTCCTAAAGCAGGC-3'
P53	Forward	5'-CAGCCAAGTCTGTGACTTGCACGTAC-3'
	Reverse	5'-CTATGTCGAAAAGTGTTTCTGTGCATC-3'

Table 1. Sequences of primers used in qRT-PCR.

GAPDH, and relative fold changes were calculated using the comparative Ct ($\Delta\Delta C_t$) method. All measurements were performed in triplicate, and results are presented as mean $\pm$ SD.

Statistical analysis

All experimental data obtained in this study were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) and are presented as mean $\pm$ standard deviation (SD) from independent experiments. Prior to analysis, data were checked for normality and homogeneity of variance where applicable. Statistical comparisons between untreated control and Co_3O_4 NPs-treated A549 lung cancer cells were performed using an unpaired Student's t-test. A p -value < 0.05 was considered statistically significant, and significance levels are consistently indicated in the tables and figures.

Results

Co_3O_4 NPs exhibit strong cytotoxicity against A549 cells

The SRB cytotoxicity assay results quantitatively demonstrated that Co_3O_4 NPs exert a significant cytotoxic effect on A549 lung cancer cells in a concentration-dependent manner (Fig. 2). Treatment with increasing concentrations of Co_3O_4 NPs (0.2, 2, 20, 200, and 2000 $\mu\text{g}/\text{ml}$) for 72 h led to a progressive and statistically significant reduction in cell viability compared with untreated control A549 cells. Cell survival data were derived from independent experiments and analyzed statistically, with significance indicated in Fig. 2. The IC₅₀, representing the concentration required to reduce cell viability by 50%, was calculated from the dose–response curve and determined to be 34.17 $\mu\text{g}/\text{mL}$. These quantitative and statistically validated findings confirm the dose-responsive cytotoxic activity of Co_3O_4 NPs against A549 lung cancer cells.

Co_3O_4 NPs cause excessive ROS in A549 cells

Staining of lung cancer A549 cells with DCFH-DA dye demonstrated that exposure of A549 lung cancer cells to Co_3O_4 NPs at the IC₅₀ concentration (34.17 $\mu\text{g}/\text{ml}$) for 72 h resulted in a significant elevation ($p < 0.001$) of intracellular ROS generation level (Fig. 3). This was evidenced by a significantly higher fluorescence intensity in treated A549 cancer cells relative to untreated control cells. Quantitative analysis confirmed that the increase in ROS production was statistically significant ($p < 0.001$), indicating pronounced oxidative stress in response to Co_3O_4 NPs exposure.

Co_3O_4 NPs cause dramatic genomic DNA damage

Results of the alkaline Comet assay revealed a dramatic induction of genomic DNA damage in A549 lung cancer cells following exposure to Co_3O_4 NPs (Table 2; Fig. 4). Quantitative DNA damage parameters; including tail length, %DNA in the tail, and tail moment, were all significantly increased ($p < 0.01$) after 72 h treatment with the IC₅₀ concentration (34.17 $\mu\text{g}/\text{ml}$) compared to untreated control A549 lung cancer cells. The quantitative results are summarized in Table 2, while representative comet images illustrating intact versus damaged nuclei are presented in Fig. 4, together supporting the genotoxic impact of Co_3O_4 NPs exposure under the tested conditions.

Co_3O_4 NPs cause dramatic loss in mitochondrial membrane integrity

Fluorescence analysis of Rhodamine-123-stained A549 lung cancer cells demonstrated a marked reduction in mitochondrial membrane integrity in A549 cells following 72 h exposure to Co_3O_4 NPs at the IC₅₀ concentration (Fig. 5). Co_3O_4 NPs-treated A549 lung cancer cells showed a pronounced decrease in fluorescence intensity relative to untreated control cells, and quantitative analysis confirmed that this reduction was statistically significant ($p < 0.001$). Figure 5 presents both quantitative measurements and qualitative representative

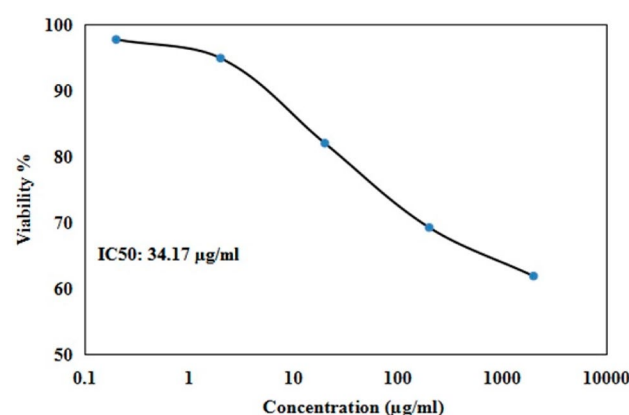


Fig. 2. Concentration-dependent effect of Co_3O_4 NPs on A549 NSCLC cell viability. A549 cells were exposed to Co_3O_4 NPs at five concentrations (0.2, 2, 20, 200, and 2000 $\mu\text{g}/\text{mL}$) for 72 h, and cell viability was determined using the sulforhodamine B (SRB) assay. The IC₅₀ value was calculated from the fitted dose–response curve using GraphPad Prism. Cell viability decreased with increasing Co_3O_4 NP concentration, with a significant reduction observed at the highest concentrations compared with untreated cells ($p < 0.001$). Data are presented as mean $\pm$ SD from three independent experiments.

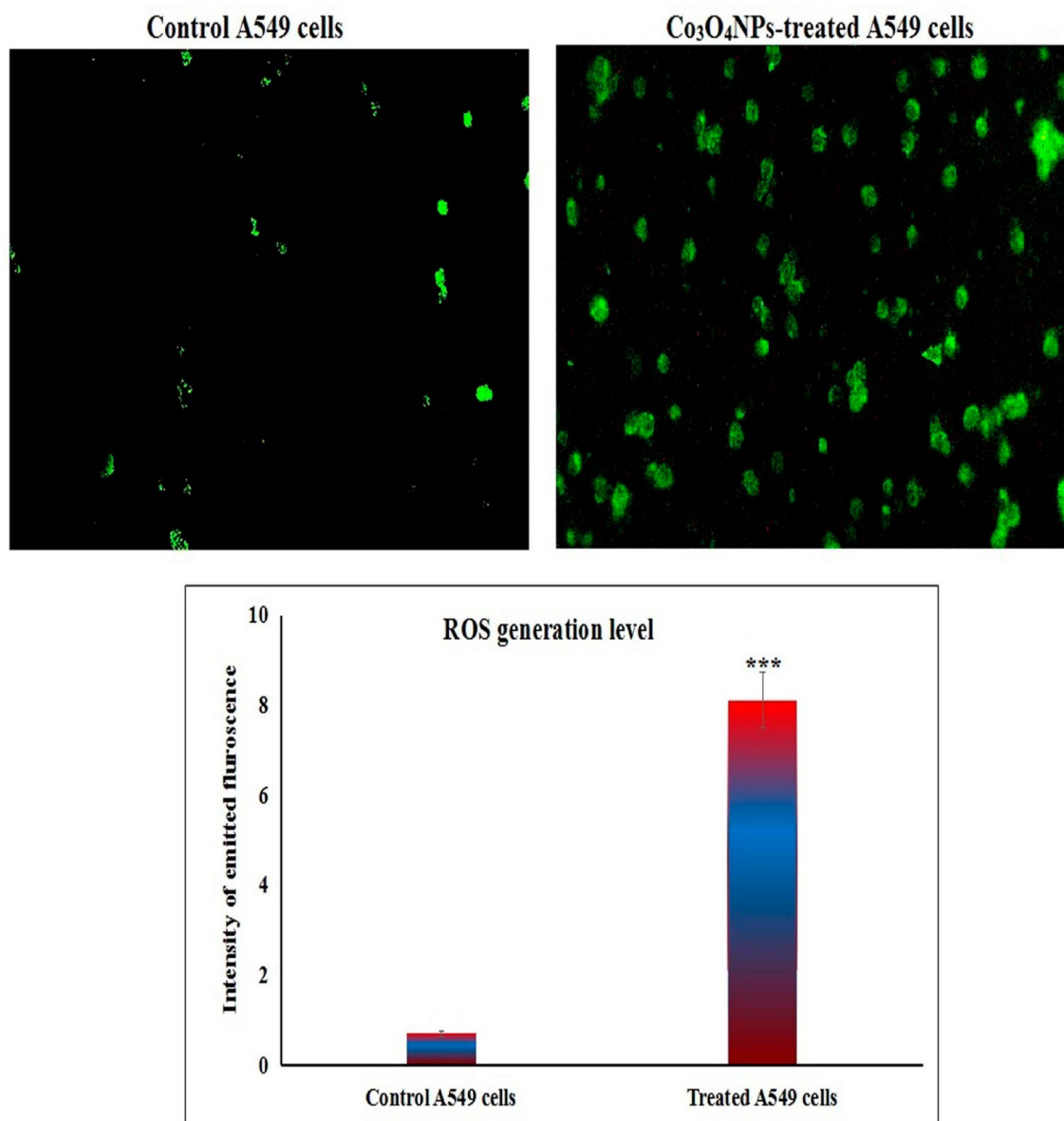


Fig. 3. Intracellular reactive oxygen species (ROS) generation in A549 cells following 72 h treatment with Co_3O_4 NPs at the IC50 concentration (34.17 $\mu\text{g}/\text{ml}$). Representative fluorescence images of untreated control cells (left) and Co_3O_4 NPs-treated cells (right) are shown. Three replicates were used and fluorescence intensity reflects ROS levels. Quantitative analysis of ROS generation is included in the figure and indicated statistical significant difference at $p < 0.001$.

Cells	Treatment ($\mu\text{g}/\text{ml}$)	DNA damage indicating parameters		
		Tail length (px)	%DNA in tail	Tail moment
A549 cells	Untreated (0.00)	5.77 ± 1.08	16.09 ± 0.94	1.05 ± 0.08
	Co_3O_4 NPs IC50 (34.17)	$12.42 \pm 1.07^{**}$	$27.28 \pm 1.36^{**}$	$3.65 \pm 0.53^{**}$

Table 2. Induction of genomic DNA damage in the untreated (control) and treated A549 cells with the IC50 concentration of Co_3O_4 NPs for 72 hour. Results are expressed as mean $\pm$ SD. **: Indicates statistical significant difference from the compared untreated control A549 cells at $p < 0.01$ using *independent student t-test*.

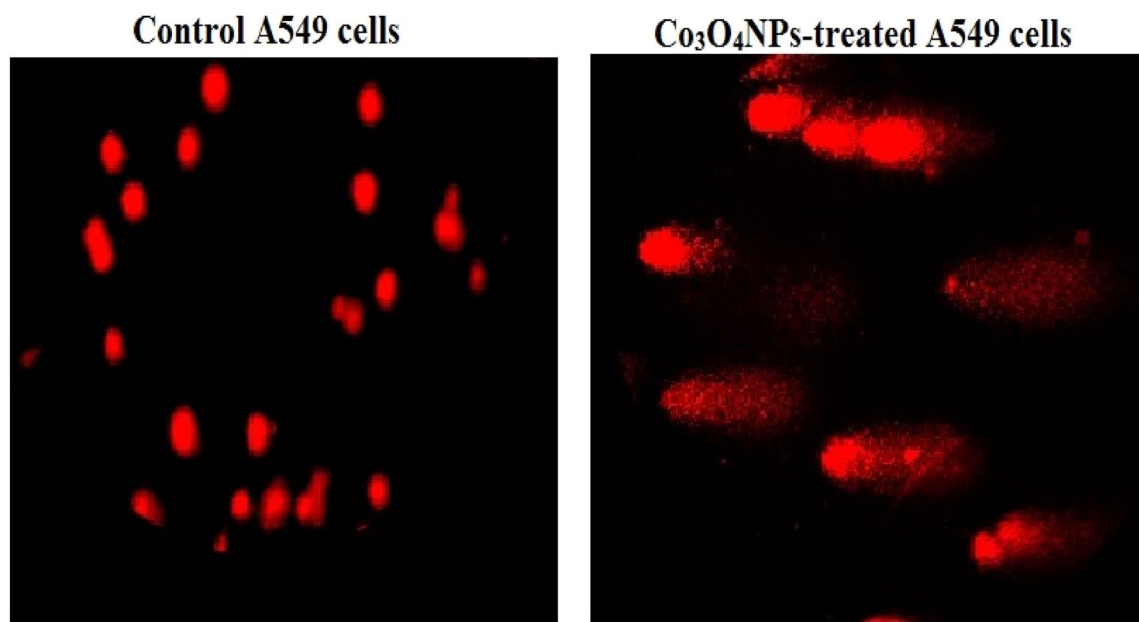


Fig. 4. Representative alkaline comet assay images of A549 cancer cells following $\text{Co}_3\text{O}_4\text{NP}$ exposure. Representative images of scored comet nuclei from untreated control and $\text{Co}_3\text{O}_4\text{NP}$ -treated A549 cancer cells exposed to the IC_{50} concentration ($34.17 \mu\text{g/ml}$) for 72 h. The corresponding quantitative comet assay parameters, including tail length, %DNA in tail, and tail moment, are presented in Table 2.

fluorescence images, providing complementary numerical and visual evidence of mitochondrial depolarization consistent with activation of the intrinsic apoptotic pathway.

$\text{Co}_3\text{O}_4\text{NPs}$ stimulate cell death in A549 lung cancer cells

As illustrated in Fig. 6, exposure of A549 lung cancer cells to the IC_{50} concentration of $\text{Co}_3\text{O}_4\text{NPs}$ ($34.17 \mu\text{g/ml}$) for 72 h led to a substantial increase in necrotic cell population, which significantly rose by 2.46-fold compared with untreated control cells ($p < 0.001$). This pronounced necrotic response indicates that $\text{Co}_3\text{O}_4\text{NPs}$ compromise plasma membrane integrity and induce cell death through a non-apoptotic pathway. In contrast, early apoptotic cells significantly decreased to 0.39-fold of the control ($p < 0.05$), and late apoptotic cells were markedly reduced to 0.55-fold relative to untreated cells ($p < 0.01$), suggesting that classical programmed apoptosis was not the predominant mode of cell death under these experimental conditions. These quantitative shifts were consistently observed across all replicates and are further supported by the representative Flow Cytometry plots in Fig. 6, which visually depict the distribution of necrotic and apoptotic populations. Together, these findings indicate that $\text{Co}_3\text{O}_4\text{NPs}$ preferentially induce necrotic cell death in A549 cells, with a relative suppression of apoptosis, highlighting a distinct cytotoxic profile of $\text{Co}_3\text{O}_4\text{NPs}$ in this lung cancer model.

$\text{Co}_3\text{O}_4\text{NPs}$ significantly dysregulate *p53*, *Bcl2* and *ND3* gene expression

The findings of qRT-PCR analysis demonstrated a significant dysregulation of key apoptosis- and Mitochondrial function-related genes in A549 lung cancer cells following 72 h exposure to the IC_{50} concentration ($34.17 \mu\text{g/ml}$) of $\text{Co}_3\text{O}_4\text{NPs}$. As presented in Table 3, treated A549 cancer cells exhibited statistically significant upregulation of the pro-apoptotic gene *p53* and mitochondrial *ND3* ($p < 0.01$), accompanied by a marked downregulation of the anti-apoptotic *Bcl-2* gene ($p < 0.001$) compared with untreated control cells. These results provide quantitative evidence that $\text{Co}_3\text{O}_4\text{NPs}$ exposure promotes apoptotic signaling at the transcriptional level, consistent with the observed induction of mitochondrial-mediated apoptosis.

Discussion

The rising global incidence of lung cancer, particularly non-small cell lung cancer (NSCLC), highlights the urgent need for innovative and effective therapeutic strategies. Conventional treatments, especially chemotherapy, often suffer from significant drawbacks such as systemic toxicity, the development of drug resistance, and low selectivity for cancer cells^{10,33}. These limitations emphasize the necessity of exploring alternative approaches. One promising avenue is the use of nanomaterials, particularly metal oxide nanoparticles, owing to their distinctive physicochemical and biological properties. Among these, $\text{Co}_3\text{O}_4\text{NPs}$ have gained attention for their redox activity, catalytic behavior, and capacity to generate ROS^{14,33}. Despite their potential, the therapeutic effects of $\text{Co}_3\text{O}_4\text{NPs}$ on A549 lung cancer cells remain largely unexplored. Therefore, this study aims to investigate the potential anticancer activity of $\text{Co}_3\text{O}_4\text{NPs}$ by assessing their impact on A549 cell viability, ROS generation, genomic DNA damage, mitochondrial disruption, and apoptosis induction.

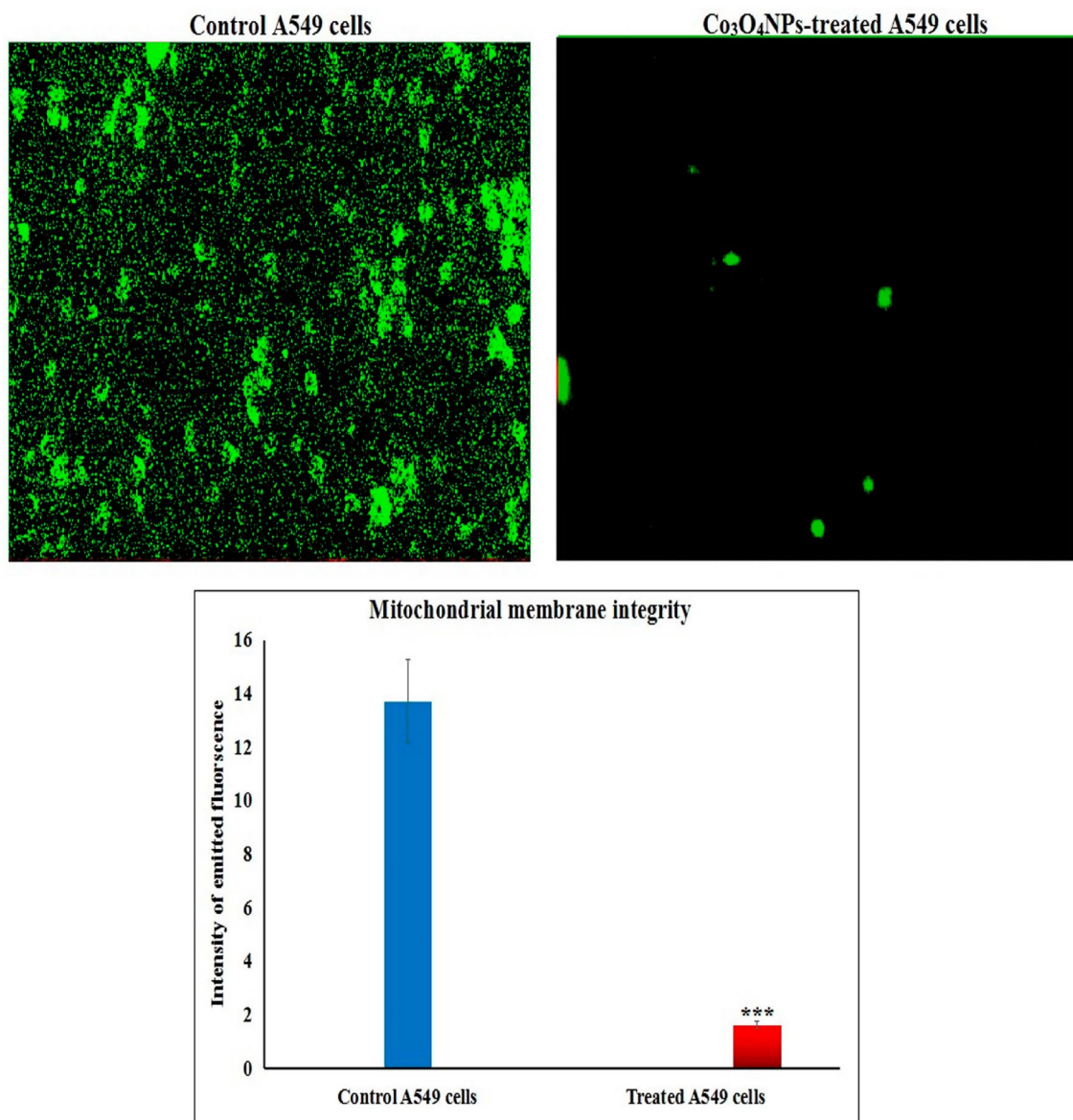


Fig. 5. Mitochondrial membrane potential in A549 cells following 72 h exposure to $\text{Co}_3\text{O}_4\text{NPs}$ at the IC50 concentration ($34.17 \mu\text{g/ml}$) measured using rhodamine-123 staining. The figure presents representative fluorescence images and quantitative analysis of fluorescence intensity, showing a significant loss of mitochondrial membrane potential in treated cells at $p < 0.001$ compared with control A549 cells.

The substantial discrepancy between the primary particle size determined by TEM (20.08 nm) and the hydrodynamic diameter measured by DLS (691.7 nm) in the physicochemical characterization conducted by Mohamed et al.¹⁴ reflects the different principles of these two techniques and the tendency of $\text{Co}_3\text{O}_4\text{NPs}$ to form aggregates in aqueous dispersion. TEM measures the primary particle dimensions in the dry state, whereas DLS measures the hydrodynamic size of particles and dispersed aggregates suspended in suspension. The larger DLS diameter therefore indicates the presence of aggregated particles under the aqueous conditions used for characterization. In the present study, $\text{Co}_3\text{O}_4\text{NPs}$ suspensions were subjected to standardized ultrasonication immediately before dilution and cell exposure to disperse particle clusters and achieve a more homogeneous suspension. This procedure was applied consistently to all experimental preparations to minimize aggregation and ensure comparable nanoparticle exposure across treatment groups.

Our SRB cytotoxicity assay revealed that $\text{Co}_3\text{O}_4\text{NPs}$ exhibit strong cytotoxic effects against A549 lung cancer cells (Fig. 7), as evidenced by a pronounced, concentration-dependent decrease in cell viability following exposure to five different concentrations (0.2, 2, 20, 200, and 2000 $\mu\text{g/ml}$) over 72 h. Notably, the calculated IC50 value was as low as $34.17 \mu\text{g/ml}$, indicating high potency. These findings are consistent with recent reports demonstrating the significant cytotoxicity of $\text{Co}_3\text{O}_4\text{NPs}$ against various human cancer cell lines, including

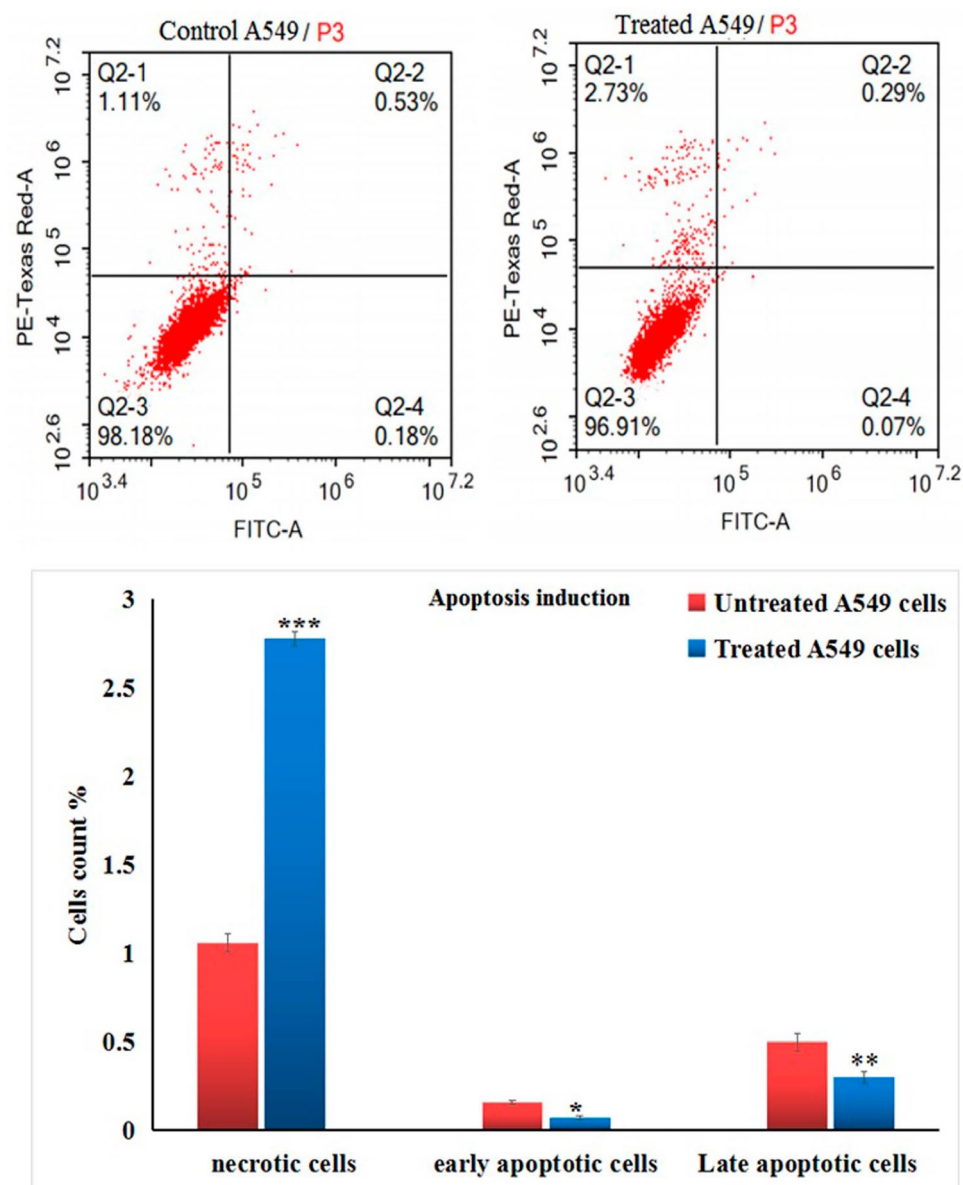


Fig. 6. Flow cytometric analysis of apoptosis in A549 cells after 72 h treatment with Co_3O_4 NPs at the IC50 concentration (34.17 $\mu\text{g}/\text{ml}$). Quadrant distribution: Q2-1, necrotic cells; Q2-2, late apoptotic cells; Q2-3, viable cells; Q2-4, early apoptotic cells. The figure includes representative dot plots and quantitative data, showing a marked increase in early and late apoptotic cells in treated groups relative to untreated controls.

Cells	Treatment ($\mu\text{g}/\text{ml}$)	fold change in the expression level of:		
		p53	ND3	Bcl2
A549 cells	Untreated(0.00)	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
	Co_3O_4 NPs IC50 (34.17)	2.57 $\pm$ 0.41**	2.33 $\pm$ 0.23**	0.71 $\pm$ 0.03***

Table 3. The expression level of apoptotic p53, mitochondrial ND3 and anti-apoptotic Bcl2 genes in the untreated (control) and treated A549 cells with the IC50 concentration of Co_3O_4 NPs for 72 hour. Results are expressed as mean $\pm$ SD. **, ***: Indicates statistical significant difference from the compared untreated control cells at $p < 0.01$ and 0.001 , respectively using independent student t-test.

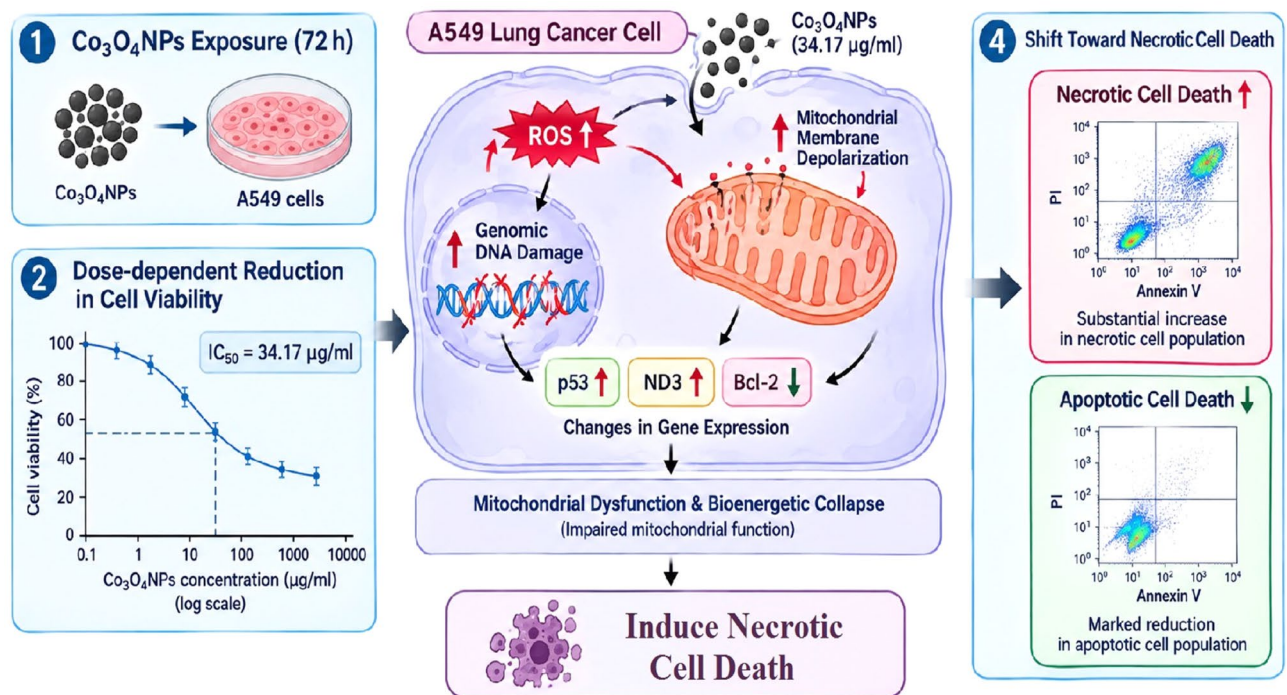


Fig. 7. Integrated mechanistic evidence for Co₃O₄NPs-induced necrotic cell death in non-small A549 small lung cancer cells.

A-375 melanoma cells, Hep-G2 hepatocellular carcinoma cells, CT26 colorectal cancer cells, and mouse colon adenocarcinoma cells^{15–17,34}.

The mechanisms by which Co₃O₄ NPs induce cancer cell death remain a subject of intense investigation, thus to further investigate the underlying mechanisms of Co₃O₄ NPs induced strong cytotoxicity, ROS generation level, integrity of genomic DNA and mitochondrial membrane potential alongside with apoptosis induction 72-hour exposure of A549 cells to the IC₅₀ concentration (34.17 µg/ml) of Co₃O₄ NPs were assessed. A marked increase in fluorescence intensity emitted from Co₃O₄ NPs-treated A549 cells confirmed intensive ROS generation by Co₃O₄ NPs in A549 cancer cells, consistent with recent findings. These results suggest that Co₃O₄ NPs-induced cytotoxicity in A549 cancer cells is predominantly mediated by excessive ROS production, which disrupts cellular redox balance and causes oxidative damage to vital biomolecules, including DNA, lipids, and proteins. This ROS-induced stress ultimately compromises cellular integrity and triggers apoptotic cell death^{15–17,35–37}.

In this study, the elevated ROS level induced by Co₃O₄ NPs was closely associated with severe genomic DNA damage in A549 lung cancer cells, as evidenced by significant increases in key DNA damage parameters: tail length, %DNA in tail, and tail moment measured using the alkaline Comet assay. ROS-mediated genomic DNA damage occurs through various mechanisms, such as the formation of single-strand and double-strand breaks, base modifications, and DNA crosslinking. These lesions, detected by the Comet assay in Co₃O₄ NPs-treated A549 cells, compromise the genomic integrity, resulting in mutations, chromosomal fragmentation, and, ultimately, cell death, especially since a single double-strand break can be sufficient to trigger apoptosis. DNA damage activates critical repair pathways, including the DNA damage response; however, excessive ROS level can overwhelm these repair mechanisms, leading to failure and the initiation of apoptotic processes^{14,38–40}.

In addition to ROS-induced genomic damage, mitochondrial dysfunction plays a pivotal role in the cytotoxic effects of Co₃O₄ NPs. Mitochondria are central regulators of cellular energy metabolism and apoptosis. In healthy cells, they maintain the integrity of the mitochondrial membrane potential, which is crucial for efficacy ATP production. However, under conditions of oxidative stress, such as those induced by excessive ROS generation in Co₃O₄ NPs-treated A549 cancer cells, the mitochondrial membrane potential collapses, resulting in mitochondrial membrane depolarization and mitochondrial dysfunction. A dramatic loss of mitochondrial membrane potential was demonstrated in A549 cells treated with the IC₅₀ concentration of Co₃O₄ NPs, as evidenced by a notable decrease in fluorescence intensity, indicating that oxidative stress was sufficient to impair mitochondrial membrane integrity^{14,41,42}. Consequently, ROS-associated mitochondrial stress and loss of membrane potential together with severe genomic damage represent a critical event in the intrinsic apoptosis pathway, driving A549 cells toward apoptotic cell death.

These data collectively indicate that Co₃O₄ NPs induce cell death in A549 lung cancer cells through two closely interconnected mechanisms: ROS-mediated genomic DNA damage and mitochondrial dysfunction. The simultaneous induction of oxidative DNA damage and mitochondrial dysfunction provides a dual cytotoxic pathway that effectively compromises cancer cell survival. Such convergent stress signals are known to activate both the intrinsic apoptotic pathway and necrotic cell death, critical mechanisms for eliminating damaged or dysfunctional cells, depending on the severity of cellular damage^{14,43}. In this context, exposure to Co₃O₄ NPs for

72 h resulted in a marked upregulation of pro-apoptotic *p53* and mitochondrial *ND3* gene expression, together with a significant downregulation of the anti-apoptotic *Bcl-2* gene.

These transcriptional changes indicate activation of cellular stress responses and mitochondrial-associated signaling pathways linked to intrinsic apoptotic regulation. However, the pronounced oxidative stress and severe mitochondrial disruption observed in Co_3O_4 NPs-treated cells appear to exceed the threshold for apoptotic execution in a substantial cell population, thereby favoring a shift toward necrotic cell death rather than apoptosis alone. This indicates that, although a subset of cells may initiate mitochondria-mediated apoptotic signaling, excessive ROS generation and extensive mitochondrial collapse likely drive a predominant necrotic outcome. Accordingly, Co_3O_4 NPs exert a dual cytotoxic effect, involving activation of apoptotic signaling alongside necrotic cell death, where the final balance between these two processes is determined by the magnitude of oxidative stress and the extent of mitochondrial integrity loss.

Flow cytometric analysis verified that Co_3O_4 NPs treatment induces both apoptosis and necrosis in A549 lung cancer cells, as evidenced by clear shifts in necrotic and apoptotic cell populations. Notably, exposure to Co_3O_4 NPs at the IC₅₀ concentration (34.17 $\mu\text{g}/\text{ml}$) caused a 2.46-fold increase in necrotic cells, alongside marked reductions compared to untreated control cells. These findings demonstrate that Co_3O_4 NPs-induced A549 cell death under these conditions occurs through a necrotic pathway rather than an apoptotic mechanism. This pronounced necrotic response is likely driven by excessive ROS generation that overwhelms cellular antioxidant defenses, resulting in irreversible plasma membrane injury and mitochondrial dysfunction^{15–44}. Furthermore, Co_3O_4 NPs-induced oxidative stress can provoke direct severe genomic DNA damage and causes a critical drop in intracellular energy levels, conditions that drive cells toward necrosis rather than energy-dependent programmed apoptosis. This explanation is supported by the dramatic genomic DNA damage observed in the Co_3O_4 NPs-treated A549 lung cancer cells and is consistent with previous studies demonstrating necrosis as the primary mode of cell death following exposure to metal oxide nanoparticles in cancer cells^{14,45}.

Interestingly, the pronounced reduction in both early and late apoptotic A549 cell fractions noticed following Co_3O_4 NPs exposure indicates that, at the tested concentration, these nanoparticles do not predominantly activate a classical, caspase-dependent apoptotic program. Instead, the data demonstrate a partial bypass of canonical apoptosis, likely due to excessive and rapid ROS accumulation that inflicts acute mitochondrial injury. Severe oxidative stress is well recognized to induce lipid peroxidation, protein oxidation, and mitochondrial DNA damage, ultimately leading to severe mitochondrial membrane permeabilization and collapse of the mitochondrial membrane potential^{146,47}. Once mitochondrial dysfunction surpasses a critical threshold, ATP production level declines markedly, depriving cells of the energy required to execute the tightly regulated steps of apoptosis. Under such bioenergetically compromised conditions, necrotic or necroptotic-like cell death becomes more likely than orderly apoptosis^{46,47}.

The significant overexpression of *p53* observed in Co_3O_4 NPs-treated A549 cancer cells confirms activation of a strong cellular stress response triggered by oxidative DNA lesions and mitochondrial perturbations. *p53* is a central stress sensor that transcriptionally regulates genes involved in cell-cycle arrest, DNA repair, senescence, and apoptosis⁴⁸. Under moderate stress, *p53* can effectively promote mitochondrial apoptosis through induction of pro-apoptotic *Bcl-2* family members and facilitation of mitochondrial outer membrane permeabilization. However, under conditions of overwhelming oxidative and metabolic stress, *p53* activation may coincide with severe mitochondrial failure and loss of ionic homeostasis, conditions that favor necrosis rather than apoptosis⁴⁸. Therefore, in the present context, *p53* upregulation likely reflects the severity of cellular stress rather than the successful execution of a complete apoptotic cascade. Importantly, A549 cells harbor wild-type *p53*, which enables a functional *p53*-dependent stress response. This characteristic should be considered when interpreting the present results. NSCLC cell lines with mutant or dysfunctional *p53* frequently exhibit impaired apoptotic signaling, altered redox regulation, and different thresholds for mitochondrial injury. Consequently, their response to Co_3O_4 NPs-induced stress may differ quantitatively or qualitatively. While the current findings clearly demonstrate *p53* activation in a *p53*-competent model, they do not imply that *p53* is the sole mediator of cytotoxicity. Instead, *p53* appears to act in concert with broader oxidative and mitochondrial damage pathways. Future comparative studies using *p53*-mutant NSCLC models would therefore be valuable to determine how *p53* status modulates sensitivity and death modality following Co_3O_4 NPs exposure.

Moreover, the concurrent significant overexpression of the mitochondrial *ND3* gene provides additional evidence for a mitochondrial-centered mechanism of toxicity. *ND3* gene encodes a core subunit of complex I of the electron transport chain, and its dysregulation is associated with impaired electron transfer and increased electron leakage, which in turn enhances superoxide generation and amplifies mitochondrial ROS generation⁴⁹. Elevated *ND3* gene expression in stressed cells may therefore represent either a compensatory response or a marker of respiratory chain disturbance, both of which can intensify oxidative damage and accelerate mitochondrial collapse⁴⁹. Notably, such mitochondrial disturbances can occur independently of *p53* status, supporting the idea that Co_3O_4 NPs cytotoxicity is not solely dependent on *p53*-mediated pathways. Collectively, these findings suggest that Co_3O_4 NPs compromise mitochondrial integrity through multiple, converging mechanisms; however, confirmation in *p53*-mutant models is necessary to determine whether comparable mitochondrial susceptibilities exist across a wider NSCLC spectrum.

Simultaneously, marked downregulation of the anti-apoptotic gene *Bcl-2* gene expression reduces mitochondrial membrane stabilization and lowers the threshold for membrane permeabilization. Although *Bcl-2* suppression typically sensitizes cells to apoptosis, its protective influence becomes secondary when mitochondrial injury is extensive. Under severe ROS overload and respiratory chain disruption, mitochondrial swelling, permeability transition pore opening, and ATP depletion can progress so rapidly that caspase activation becomes inefficient or incomplete, thereby shifting the balance from apoptosis toward necrotic death^{47–49}. Collectively, the simultaneous significant overexpression of *p53* and *ND3* alongside remarkable suppression of *Bcl-2* reflects a cellular state characterized by extreme oxidative stress, respiratory dysfunction, and bioenergetic crisis. While

these molecular signatures are often associated with apoptosis, their combined severity in Co₃O₄NPs-treated A549 lung cancer cells appears to overwhelm apoptotic machinery and favor necrosis as the predominant death modality. This interpretation is consistent with the observed reduction in apoptotic populations and underscores that the severity and kinetics of mitochondrial injury, strongly influenced by nanoparticle dose and reactivity, are critical determinants of death modality. At the same time, acknowledging the wild-type *p53* background of A549 cells highlights the need for validation in *p53*-mutant NSCLC models to fully establish the generalizability of these mechanisms^{46–49}.

Despite the precise mechanism by which Co₃O₄NPs induce mitochondrial dysfunction remains to be fully elucidated. The pronounced mitochondrial depolarization, *ND3* upregulation, and *Bcl-2* downregulation observed in A549 lung cancer cells following Co₃O₄NPs exposure may result either from direct nanoparticle–mitochondria interactions or as a secondary consequence of ROS-mediated oxidative stress. Metal oxide nanoparticles, including Co₃O₄NPs, have been reported to accumulate within mitochondria, where they can directly disrupt electron transport chain complexes, promote electron leakage, and compromise membrane integrity^{14,50–52}. Concurrently, excessive ROS generation can damage mitochondrial lipids, proteins, and DNA, triggering mitochondrial permeability transition and bioenergetic collapse^{14,50–52}. In our study, the rapid accumulation of ROS and extensive oxidative DNA damage suggest that mitochondrial impairment is likely amplified by oxidative stress, though direct interactions cannot be excluded. Future investigations employing high-resolution imaging, mitochondrial-targeted fluorescent probes, and ROS scavengers will be critical to distinguish between direct nanoparticle effects and ROS-mediated secondary damage, thereby clarifying the mechanistic basis of Co₃O₄NPs-induced mitochondrial dysfunction in NSCLC cells.

Based on the integrated findings discussed above shown in Fig. 7, the present study provides novel insights into the intrinsic anticancer potential of pristine Co₃O₄NPs in non-small A549 lung cancer cells, without the use of additives, surface modifications, or hybrid formulations. Unlike previous studies that primarily reported general cytotoxicity or employed hybrid nanostructures, this work offers a systematic, multi-level mechanistic evaluation that links excessive ROS generation, genomic DNA damage, mitochondrial respiratory disruption, and stress-responsive gene modulation within a unified experimental framework. This integrative approach allows a more precise delineation of intracellular events underlying Co₃O₄NPs-mediated cytotoxicity and advances current understanding beyond descriptive toxicity toward nanoparticle-specific mechanisms of action. Notably, the data indicate that although *p53* signaling is activated in A549 cells, which harbor wild-type *p53*, Co₃O₄NPs-induced cell death is not exclusively governed by classical *p53*-dependent apoptosis. Instead, the combined evidence of pronounced mitochondrial dysfunction, *ND3* dysregulation, *Bcl-2* suppression, and a marked reduction in apoptotic cell fractions suggests that severe oxidative and metabolic stress overwhelms apoptotic execution, favoring necrotic cell death. This distinction is critical, as it highlights mitochondrial collapse and bioenergetic failure as central determinants of cell death modality. Accordingly, the *p53* response in this model appears to reflect the magnitude of cellular stress rather than the successful completion of a canonical apoptotic cascade. Given that *p53* status varies widely among NSCLC subtypes, responses to Co₃O₄NPs exposure may differ in *p53*-mutant or *p53*-deficient contexts, underscoring the need for caution when generalizing these findings.

Several limitations of the present study should be acknowledged. First, all experiments were conducted in vitro using a single *p53*-proficient lung cancer cell line, which does not fully represent the molecular heterogeneity of NSCLC or the complexity of the in vivo tumor microenvironment. In addition, mechanistic interpretation is primarily based on transcriptional analysis without complementary protein-level validation (e.g., Western blot analysis of *p53* and related apoptotic proteins or caspase activity assays), which limits the ability to confirm pathway activation at the functional protein level. These aspects should be addressed in future studies to strengthen mechanistic validation. Moreover, the study focused on acute and intermediate cellular responses, while long-term effects, nanoparticle biodistribution, and potential off-target toxicity in normal cells were not evaluated. Future investigations should therefore include comparative analyses across NSCLC models with distinct *p53* statuses, incorporation of three-dimensional tumor systems, and in vivo studies to assess pharmacokinetics, biodistribution, and systemic safety. Collectively, these future directions will be essential for defining the broader applicability and translational relevance of Co₃O₄NPs as anticancer agents, while also informing their rational and safe development for biomedical use.

Conclusion

In conclusion, this study demonstrates that pure Co₃O₄NPs exhibit potent anticancer activity against non-small A549 lung cancer cells through a multi-faceted mechanism involving excessive ROS generation, severe genomic DNA damage, profound mitochondrial depolarization, and activation of stress-responsive pathways. The observed upregulation of pro-apoptotic *p53* and mitochondrial *ND3*, together with marked downregulation of the anti-apoptotic *Bcl-2* gene, indicates activation of cellular stress and intrinsic mitochondrial death pathways. However, the Flow-Cytometric findings showed a predominance of necrotic cell death, suggesting that severe oxidative and mitochondrial stress may favor necrotic cell death under the conditions examined. These findings provide an integrated characterization of the cellular responses of A549 cells to pristine Co₃O₄NPs and highlight the interplay among oxidative stress, genomic damage, mitochondrial dysfunction, and cell death. Nevertheless, the present study is limited to a single *p53*-proficient cell line, and responses may differ in NSCLC cells harboring mutant or deficient *p53*, limiting the generalizability of the results. Additional factors, including nanoparticle size, shape, surface charge, and concentration, may also influence cellular responses. Future studies should therefore evaluate Co₃O₄NPs effects across multiple cancer and normal cell lines, consider *p53* status as a variable, and incorporate three-dimensional tumor models and in vivo assessments to clarify pharmacokinetics, biodistribution, systemic toxicity, and therapeutic selectivity. Collectively, these investigations will be essential to determine the translational potential of Co₃O₄NPs as safe and effective targeted anticancer agents.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Hanan RH Mohamed designed the study, performed molecular experiments, wrote the manuscript, and conducted statistical analysis. Shahd H. Elnawasani, George Mossad, Maria Nagy, Basma A. Mohamed, Rawan Essam: Performed experimentations and contributed to writing the manuscript. Gehan Safwat, Ayman Diab, and all authors: Reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to H.R.H.M.

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