

Indirect effects of interference of two emerging environmental contaminants on cell health: Radiofrequency radiation and gold nanoparticles

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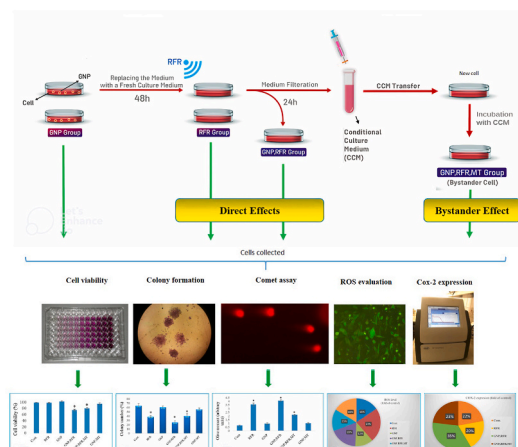
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HIGHLIGHTS

- Radiofrequency radiation (RFR) has non-targeted effects on health.
- Interference of RFR and Gold nanoparticle (GNP) increased cell death indirectly.
- COX-2 expression did not change in RFR exposed, GNPs contaminated and bystander cells.
- RFR elevated ROS levels but did not change double strand break in the cells.

GRAPHICAL ABSTRACT



Abbreviations: RFR, radiofrequency radiation; BE, bystander effect; GSM, Global System for Mobile Communication; SAR, specific absorption rate; ROS, reactive oxygen species; NMs, Nanomaterials; GNPs, gold nanoparticles; SPR, surface plasmon resonance; MN, micronuclei; CCM, conditioned culture medium; CHO, Chinese Hamster Ovary; RPMI, Roswell Park Memorial Institute; EDTA, Ethylene diamine tetra acetic acid; PBS, phosphate buffered saline; GMSK, Gaussian filtered Minimum Shift Keying; CST, Computer Simulation Technology; HFSS, High frequency simulating system; MTT, tetrazolium salt; DMSO, dimethyl sulfoxide; CBMN, Cytokinesis-block micronucleus; DCFH-DA, 2',7', Dichlorofluorescein diacetate; MT, medium transfer; SEM, standard error of mean; ANOVA, analysis of variance; H₂O₂, Hydrogen peroxide; NO, nitric oxide; DSB, double strand breaks; SSB, single strand breaks; TGF, transforming growth factor; COX-2, cyclooxygenase-2; HMGB1, high-mobility group box 1; SOD, superoxide dismutase; GPx, glutathione peroxidase; NOX, NADPH oxidase; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; Ptg2, prostaglandin-endoperoxide synthase 2; Rplp0, ribosomal protein lateral stalk subunit P0; PTT, Photo Thermal Therapy; RFA, Radiofrequency Ablation.

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ABSTRACT

Background: The global need for wireless technologies is growing rapidly. So, we have been exposed to a new type of environmental pollution: radiofrequency radiation (RFR). Recent studies have shown that RFR can cause not only direct effects but also indirect or non-targeted effects such as the bystander effect (BE). In this study, we investigated the BE induced by RFR in the presence of gold nanoparticles (GNP). Moreover, we studied the expression of cyclooxygenase-2 (COX-2).

Methods: Non-toxic dose of 15-nm GNP was used to treat the Chinese Hamster Ovary (CHO) cells. After 48 h of incubation, cells were exposed to 900 MHz GSM RFR for 24 h. Then we collected the cell culture medium of these cells (conditioned culture medium, CCM) and transferred it to new cells (bystander cells). Cell deaths, DNA breaks, oxidative stress and COX-2 expression were analyzed in all groups.

Results: The results showed that RFR increased metabolic death in cells treated with GNP. Inversely, the colony formation ability was reduced in bystander cells and RFR exposed cells either in the presence or absence of GNP. Also, the level of reactive oxygen species (ROS) in GNP treated cells showed a significant reduction compared to those of untreated cells. However, RFR-induced DNA breaks and the frequencies of micronuclei (MN) were not significantly affected by GNP. The expression of COX-2 mRNA increased in RFR GNP treated cells, but the difference was not significant.

Conclusion: Our results for the first time indicated that RFR induce indirect effects in the presence of GNP. However, the molecular mediators of these effects differ from those in the absence of GNP. Also, to our knowledge, this is the first study to show that COX-2 is not involved in the bystander effect induced by 900 MHz RFR.

1. Introduction

With the development of wireless communication technology and smart devices, we are increasingly exposed to a new growing type of environmental pollution: Electromagnetic pollution (Bektas et al., 2020; Wang et al., 2022). The prevalence of Radiofrequency radiation (RFR¹) has notably risen over the past three years, primarily due to the surge in teleworking and tele-education facilitated by the internet and mobile phones during the COVID-19 pandemic. Despite the apparent benefits of these exposures, such as economic instability, unemployment, job retention, etc., concerns persist regarding their impact on health, particularly for future generations (Cantürk Tan et al., 2022; Tan et al., 2022). RFR has been classified as 'Group 2B (Possibly carcinogenic)' due to the scientific evidence linking to brain tumors (Humans, 2013; Harrell, 2017). There are a large number of studies that have investigated the effects of radiofrequency radiation on cancer, reproductive system, bones and muscles, diabetes and etc. (Bodewein et al., 2022; Jooyan and Mortazavi, 2022; Kenny et al., 2022; Nyberg et al., 2022; Bektas et al., 2023).

In humans and other complex multicellular organisms, organs and cells are connected through blood, lymph, intercellular fluids, the endocrine and nervous systems (Sherwood, 2015). Thus the activity and characteristics of the cells and organs at different locations can be influenced by other cells, known as the bystander effect (BE). The BE refers to the phenomenon where cells not affected by chemical or physical factors exhibit the effects observed in cells that were directly affected (not-targeted cells), a kind of indirect effect. The BE is well known and documented for ionizing radiation (Smolarz et al., 2022; Moloudi et al., 2023). Recently, genome instability has been reported in mesenchymal stem cells not directly exposed to radiation (Nogueira-Pedro et al., 2022; Zhang et al., 2023). Also, Zhang and coworkers induced BE utilizing microbeam in the level of a cell (Zhang et al., 2022), but has recently been reported regarding to non-ionizing radiation (NIR) (Mortazavi et al., 2015; Jooyan et al., 2019; Zeni et al., 2021). It further complicates our understanding of RFR effects on the body.

BE occurs through intercellular communication between targeted and bystander cells in two primary processes: gap junction for neighboring cells, secretion of soluble substances for distant cells. In this case, different factors such as molecules, cytokines, vesicles, etc., are involved (Chen et al., 2020; Du et al., 2020). We previously showed that ROS mediates intercellular communication signaling in the bystander effect (BE) induced by radiofrequency radiation (RFR) (Jooyan et al., 2019), as reported for ionizing radiation. For instance, The role of oxidative stress

and its signaling path way in bystander cells has been investigated in Mukherjee and colleague s study (Mukherjee et al., 2022). Actually, BE is comparable to inflammatory reactions due to the activation of cytokines, ROS, RNS, and cyclooxygenase 2 (COX-2). High COX-2 expression has been linked to BE signaling induced by X and Gamma radiations (Zhou et al., 2005; Lyng et al., 2006; Kobayashi and Konishi, 2018; Tang et al., 2023).

The health impact of RFR covered the entire living space will be further complicated by the presence of other factors such as nanomaterials (NMs). NMs are materials with dimensions ranging between 1 and 100 nm (Burri and Bellucci, 2008). They are progressively prevalent due to advancements in nanotechnology, so increasing exposure to nanoparticles has become inevitable (Nowack and Bucheli, 2007). The unique properties of gold nanoparticles (GNPs) including low cellular toxicity and ease of biological bonding, leading to their use in various fields, from clinical cases and pharmacy to electronics and aerospace engineering. So, GNPs are introduced into our bodies and environment through multiple pathways and applications (Agtuca, 2014; Aqeel et al., 2022). Metal nanoparticles respond to the magnetic fields of RFR and potentially amplifying the energy transfer to these particles resulting in heat generation (Rahme and Holmes, 2015). Several studies have reported the use of nanoparticles, including gold nanoparticles, in combination with radio waves in non-invasive therapies (Gannon et al., 2008; Ashikbayeva et al., 2019; Sametova et al., 2022).

RFR emitted from mobile phones in cells encountered with gold nanoparticles can elicit undesirable direct and indirect effects on living systems. Our previous study identified the indirect effects of RFR on CHO cell lines, known as the BE (Jooyan et al., 2019). So far, no studies have explored the indirect effects of RFR combined with other factors. This project, for the first time, aimed to unveil this phenomenon by examining the bystander effect of RFR in the presence of GNP using an in vitro cell model. We also investigated the role of the key COX-2 gene in the BE induced by non-ionizing radiation, given its involvement in the mechanism of ionizing radiation-induced BE. In this regard after RFR exposure of GNP-treated cells, their mediums were transferred to new cells and then DNA damages, cell deaths, oxidative stress and gene expression were studied. In addition, for the first time, we evaluated whether bystander effects induced by non-ionizing and ionizing radiation have different or similar mechanisms.

2. Material and method

2.1. Cell line and culture condition

This study was conducted using Chinese Hamster Ovary (CHO) cell line provided from the National Cell Bank of Iran (Pasteur Institute, Iran). The cells were grown in 60 mm² petri dishes containing 5 cc RPMI-1640 medium (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco), penicillin (Sigma, USA), and streptomycin (Jaberebn-Hayan, Tehran, Iran) at 37 °C with 5% CO₂ and 95% humidity. 0.25% trypsin and 0.03% EDTA (Ethylene diamine tetra acetic acid, Sigma) in phosphate buffered saline (PBS) solution were applied to subculture confluent cells.

2.2. Gold nanoparticle synthesis

The Turkic method was utilized to synthesize gold nanoparticles (Kimling et al., 2006; Shcherbakov et al., 2020). Briefly, 6 µl of 0.38 M K [AuCl₄] was added to 9 ml of deionized water and stirred at 600 rpm at

the temperature of 70 °C. After 2 min, 9 mg of sodium citrate solution was added. By forming the colloidal solution containing the gold nanoparticles, the solution color gradually changes to dark red. First, the heater was turned off, and after 20 min, when the solution reached to room temperature, the stirrer was stopped. Hydrodynamic particle size distributions were subsequently measured using light scattering (DLS; Nanobrook 90Plus; Brookhaven; USA) at 25 °C.

2.3. Gold nanoparticle cytotoxicity

To assess the concentration of gold nanoparticles for treatment, GNP cytotoxicity was evaluated. Cells were cultured at a density of 10,000 cells per well in 96- well plates. The following day, these cells were treated with different concentrations of nanoparticles (1, 10, 50, 250 and 1000 nM) for 24 and 48 h. Subsequently, the culture medium containing nanoparticles was replaced with fresh culture medium, and the cell viability was determined using MTT test and the trypan blue dye exclusion method. The Non-cytotoxic concentration of nanoparticles was selected to treat cells for subsequent experiments.

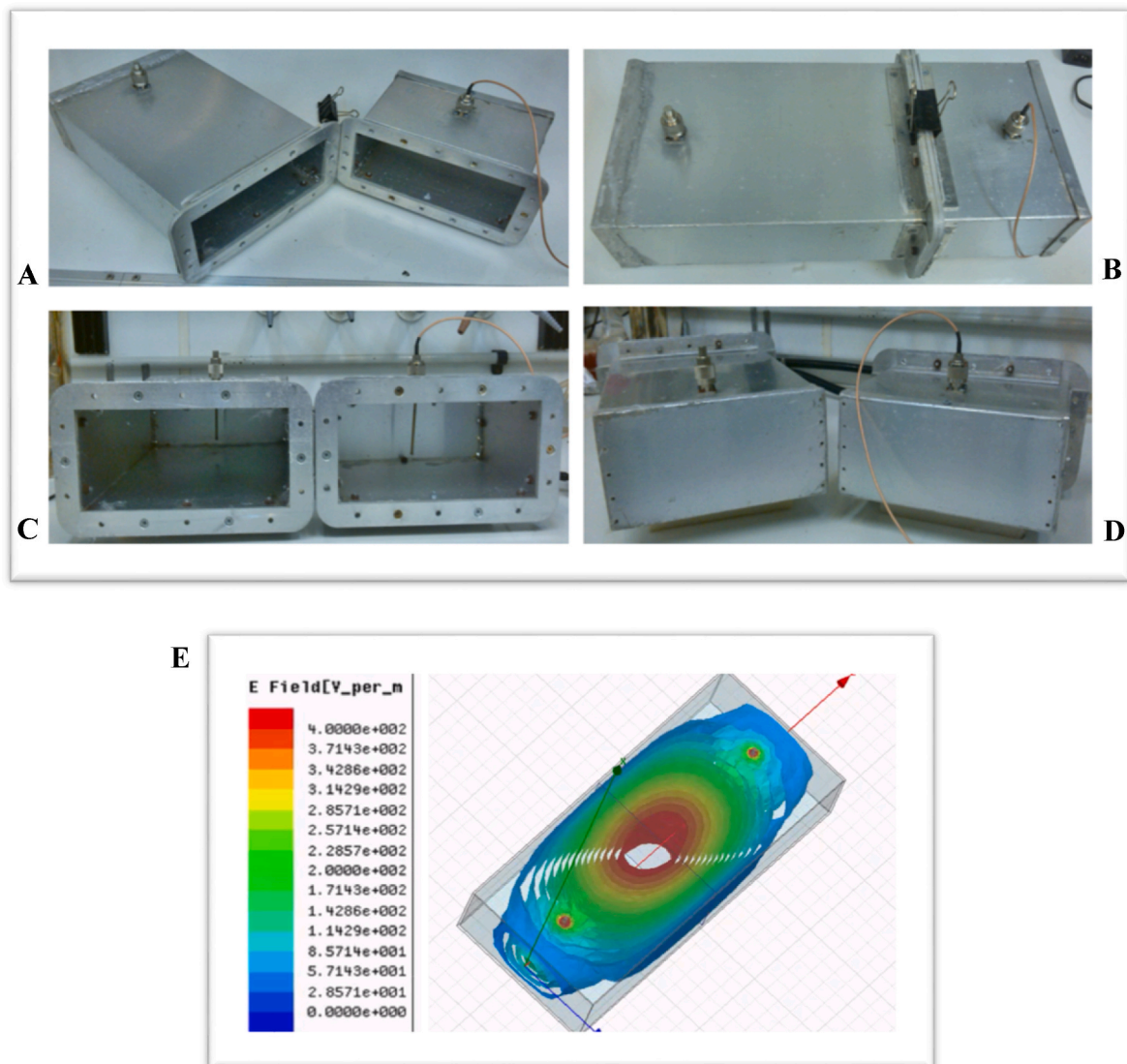


Fig. 1. The Configuration of the cavity used to generate a standing wave on cell samples. A) the cavity divided into two unequal parts. B) The two parts securely assembled using clips for precise positioning. C) Antennas positioned near the cavity's ends. D) Small pores created on both sides of the cavity to facilitate air and moisture exchange. E) HFSS simulation showcasing the electromagnetic field distribution within the cavity. The illustration demonstrates a uniformly progressive field within the cavity, with intensity increasing from the antennas toward the center (blue to red zones). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.4. RFR exposure set up

The exposure set up consisted of a signal generator and a cavity. Both of which were previously detailed in our prior publication. Briefly, the signal generator amplifies a Gaussian Minimum Shift Keying (GMSK) modulation randomly generated digital signal in one of the standard GSM frequency channels. For this study, the output power was set at 2 Watts in “Talk mode” and a specific software was used to select the GSM 900 MHz frequency channel. The signal bandwidth, modulation and duty cycle were 200 kHz, 217 Hz and 0.125 respectively.

A TE101 cavity at the frequency of 900 MHz was made to generate a standing wave. This cavity was divided into two unequal parts to facilitate easy insertion of the sample (Fig. 1A and B). The appropriate length of antennas to store electromagnetic energy inside the cavity was determined using the full-wave electromagnetic simulation via HFSS (Fig. 1C). Additionally, the software illustrated the total electric field distribution with vertical polarization excitation within the cavity showcasing the lowest field intensity near the antennas and the highest intensity at the cavity's center, where the samples were placed (Fig. 1E).

Simulation using CST (Computer Simulation Technology) and HFSS (High frequency simulating system) software revealed specific absorption rate (SAR) values ranging between 0.2 and 0.4 w/kg in the biological samples. To insure uniformity in temperature, the sterilized cavity was placed in the incubator 12 h prior to each experiment. furthermore, to maintain similar CO₂ and moisture levels to that of the incubator during exposure, the holes were created at both ends of the cavity (Fig. 1D).

2.5. RFR exposure of GNP treated cells

Cells were cultured at a density of 5000 cells per well in 96 well plates a day before the experiment. The exponentially growing cell were incubated with gold nanoparticles at a final concentration of 10 nm for duration of 24 and 48 h. Following this, the culture medium of the cells of the GNP/RFR group was replaced with fresh medium and then exposed to RFR for 24 h. After a 4 h interval, MTT assay, cell cloning ability, DNA instability and intracellular ROS levels were investigated in the cells according to described methodologies below.

2.6. Investigation of BE of RFR in the presence of GNP

The culture medium transfer (MT) method was applied to explore the indirect effects (bystander effect) of RFR. Logarithmically growing cells were treated with gold nanoparticles in a 35 mm plate at a cell density of 60% for 48 h, followed by exposure to RFR for an additional 24 h. After a 12-h interval post-exposure, the cell culture medium was collected and filtered with a 0.22 µm pore size filter to remove the cells from the medium. This culture medium was called “conditioned culture medium (CCM)”. This CCM was transferred to new cells whose medium had been spirited at that moment, termed “bystander cells” and subsequently kept in the incubator for 12 h. For this experiment, six groups were established:

- 1) RFR group: Cells exposed to RFR for 24 h.
- 2) Cont. group: Cells neither exposed to RFR nor treated with GNP, with no medium transfer.
- 3) GNP group: Cells treated with GNP for 48 h.
- 4) “GNP,RFR” group: Cells treated with GNP for 48 h, followed by exposure to RFR for 24 h after replacing their culture medium with fresh medium.
- 5) “GNP,RFR,MT” group: Cells receiving CCM (bystander cells).
- 6) “GNP,MT” group: Cells receiving the medium from cells treated with GNP for 48 h.

2.7. Metabolic cell death

The MTT assay was employed to assess metabolic cell death or cell viability. Metabolically active cells reduce 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide salt (MTT) to generate water-insoluble purple formazan crystals. for the test, 10 µL MTT solution (Sigma, USA) (5 mg/mL PBS) was added to cells in the exponential growth phase. After the formation of formazan crystals, the media were aspirate, and 100 µL dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. Subsequently, absorbance was measured at wavelengths of 570 and 630 nm using a BioTek plate reader. The absorbance directly correlates with the number of live cells, indicating the total amount of formazan produced (Al-Ziaydi et al., 2020). Additionally a group of cells were not exposed to RFR, along with medium without cells and DMSO, were examined as negative controls. The experiment was repeated three times.

2.8. Reproductive cell death

The clonogenic assay was conducted to assess cell reproductive death, aiming to distinguish cells that are metabolically active but unable to form colonies. The assay was performed as described by Puck and Marcus (1956). Briefly, 100 cells were cultured in 35 mm plates and following incubation at 37 °C, 5% CO₂, and 95% humidity for 7 days, the sets of cells containing more than 50 cells (a colony) were scored using a light Olympus microscope with a 10X objective lens. Non-treated cells were considered the control to calculate plating efficiency (PE) and survival fraction (SF). Experiments were done thrice, and at least three plates were seeded for each group. All details and relating formula are available in our previous study (Bigdeli et al., 2016).

2.9. DNA damage (alkaline single-cell gel electrophoresis)

The alkaline single-cell gel electrophoresis, also known as the comet assay, is regarded as a sensitive method to detect DNA damages, encompassing single strand breaks (SSBs) and double strand breaks (DSBs). Its methodology was adopted as per Lee et al.' study: (Lee et al., 1996). Briefly, Approximately 30,000 cells combined with 1% low-melting-point agarose (Sigma-Aldrich) was pipetted onto a microscope slides coated with a thin layer of 1% agarose. To lyse the cells and then DNA unwinding, slides were gently immersed in the lysis buffer and denaturation buffers respectively, followed by electrophoresis. Subsequently, the slides were immersed in the neutralization buffer and were stained with ethidium bromide. Following these steps, A fluorescence microscope (Axioskop 2 plus; Zeiss, Jena, Germany), equipped with an excitation filter of 510–560 nm and a 590 nm filter, was utilized to capture images at a 200x magnification. At least 3000 cells were visually analyzed for each group and analyzed by Comet Score 1.5 software (TriTek Corp., Sumerduck, USA). The extent of DNA damages visualized as a comet with a head and a tail was evaluated. The Olive tail moment a commonly used parameter, was calculated according to formula (3). This value is expressed in arbitrary units (a.u.):

$$\text{Olive Moment (OM)} = (\text{Tail Mean} - \text{Head Mean}) \times \text{DNA amount in the Tail (\%)}$$

Cells irradiated with 4 Gy X-ray and cells without any treatment were regarded as positive and negative controls, respectively. The experiment was repeated three times. To know, the buffer compositions and the details of the steps, see reference (Jooyan et al., 2019).

2.10. DNA double strand breaks (micronucleus assay)

The Micronucleus assay, also called Cytokinesis-block Micronucleus (CBMN) assay, is a standard method used to determined DSB (Fenech and Morley, 1986). It operates by impeding cytokinesis through

inhibiting actin of filament polymerization and the formation of contractile microfilaments via cytochalasin B. This drug has no impact on nucleus division resulting in the formation of cells with two nuclei (binucleated cells) was formed. In instances of chromosome aberration, these binucleated cells can contain one or more micronuclei (MN) (Sommer et al., 2020), small nuclei formed during the anaphase of mitosis or meiosis.

The adherent method was employed to conduct this assay as described with details before (Jooyan et al., 2019). Briefly, following the end of exposure, the cell medium was replaced with fresh medium and 6 µg/ml of cytochalasin B was added. The cells were then incubated for 24 h, were fixed and stained with Giemsa 10% (Sigma-Aldrich). Roughly 1000 binucleated cells were analyzed at a magnification of $\times 400$ for each group and the number of MNs was recorded.

2.11. Intracellular and intercellular level of ROS

Fluorescence microscopy and flow Cytometry were utilized to evaluate ROS levels within cells by using 2',7'-Dichloro fluorescein diacetate (DCFH-DA). For both techniques, the culture media of treated cells were replaced with fresh media and DCFH-DA was added at a final concentration of 20 µg/ml, allowing them to incubate at 37 °C for 45 min.

In the case of fluorescence microscopy, cells were washed several times with PBS and the fluorescence intensity of intracellular ROS was observed using a 20 $\times$ lense. , More than 50 field, each containing about 50 cells, were analyzed for each group.

For the flow cytometry, after 45 min incubation, cells were detached, washed and resuspended in BPS. The fluorescence emission of 12,000 cells was recorded using the CyFlow Space flow Cytometer (Partec, Germany) using FloMax soft-ware. This experiment was carried out on cells treated with 100 µM H₂O₂ for 4 h as positive control. These measurements were repeated three times.

To evaluate intercellular oxidative stress, the level of ROS in the culture media was determined using chemiluminescence spectroscopy. The procedure was carried out according to Jooyan et al. and Kadhim et al.'s studies (Jooyan et al., 2019; Kadhim et al., 2023).

2.12. COX- 2 expression

a) RNA extraction and quantification

To extract RNA, The High pure RNA Isolation kit (Roche, Germany) was employed according to the protocol. The obtained RNA precipitate was suspended in DEPC water (Water Free Nuclease) and stored at -70 °C until cDNA synthesis.

The RNA concentration and absorptions was determined through optical density measurements using 1000Nano Drop (DE, Wilmington Technologies Nanodrop). The sample's absorbances were measured at three wavelengths: 260 nm, 280 nm, and 230 nm.

b) cDNA synthesis

The cDNA synthesis was conducted using Reagent RT TM Prime Script kit (Bio Takara, Japan) the described instructions. Hexamer random primers were utilized for the cDNA synthesis process. To ensure uniform cDNA concentrations across samples, an aliquot was withdrawn, containing 1 µg of RNA, and the final volume was adjusted to 20 µl. The original stock was stored in the freezer and working samples were refrigerated until the experiments were performed.

c) Real-time PCR

Real time PCR was performed on the cDNA samples using SYBR Green MasterMix-Low Rox (GENAXXON, Korea) in the Rotor Gene system (Corbett Research, Mortlake, NSW, Australia). Specific primers

were selected targeting a segment of the COX-2 gene sequence using NCBI blast-primer software. The ribosomal protein lateral stalk subunit P0 (Rplp0) gene sequence was chosen as the housekeeping gene to normalize the mRNA expression levels and control error between samples. The primers sequences are shown in Table 1.

PCRs reactions were performed in a final volume of 20 µL containing 18 µL of the mix and 2 µL of the cDNA template. The mix contained SYBR Green MasterMix-Low Rox and pairs of primers for the two genes at a final concentration of 10 µM. The temperature profile and quantification were managed by the time-real 6plex Q Gene-Rotor PCR. Data analysis and curve melt analysis were performed using software installed on the Rotor-Gene-Q 2019 device. The PCR conditions were as follows: one cycle at 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. After each annealing and extension step, the SYBR Green fluorescence was measured at 60 °C. Post- PCR, a melting curve analysis was conducted by gradually increasing the temperature from 55 to 95 °C.

All samples were duplicated and C_t (cycle threshold) was averaged from the two measurements for data analysis. To assess gene expression, the following formulas was employed: $\Delta\Delta C_t$ was calculated according to as formula (4) and $\Delta\Delta C_t$ (delta-delta Ct) was determined by formula (5) (Al-Shammari et al., 2020):

$$\Delta C_t = C_t \text{ of the target gene} - C_t \text{ of the reference gene} \quad (4)$$

$$\Delta\Delta C_t = \Delta C_t \text{ of the experiment sample} - \Delta C_t \text{ of the control sample} \quad (5)$$

2.13. Statistical analysis

The results were analyzed using SPSS 22 software. Data were presented as means $\pm$ standard error of the mean (SEM). For continuous variables, one-way analysis of variance (ANOVA) and Tukey's post hoc testing was applied to compare the means. A p-value of 0.05 was considered as the threshold for statistical significance.

3. Results

3.1. Characterization of gold nanoparticles

The Turkevich method was applied to synthesis GNPs. A red solution was formed at the end of the synthesis process. The nanoparticle size was determined using light scattering (DLS; Nanobrook 90Plus; Brookhaven; USA), which revealed an average nanoparticle size of 15 nm. These Nanoparticles were stabilized by citrate within an aqueous solution (Haddada et al., 2020). No redshift was observed during examinations, indicating the absence of aggregation.

3.2. GNP cytotoxicity

The results presented in Fig. 2 illustrate a reduction in cell viability

Table 1
Sequence of RT-PCR primers. Sequence of cox-2 and Rplp0 gene primers and results of their confirmation using Primer Blast software.

Cox-2: <i>Cricetulus griseus</i> prostaglandin-endoperoxide synthase 2 (Ptgs2) mRNA				
	Sequence (5'>3')	Length	Tm	GC%
Forward primer	CTCAGCAAGGAAGTCACTCACT	21	60.00	52.38
Reverse primer	TAGAATCCAGTCCGGGTACAGT	22	60.02	50.00
Control (<i>Cricetulus griseus</i> ribosomal protein lateral stalk subunit P0 (Rplp0))				
	Sequence (5'>3')	Length	Tm	GC%
Forward primer	GCAGACAACGTGGGCTCCAAGCAGAT	26	69.29	57.69
Reverse primer	GGTCTCTCTTGTGAACACGAAGCCC	26	69.05	61.54

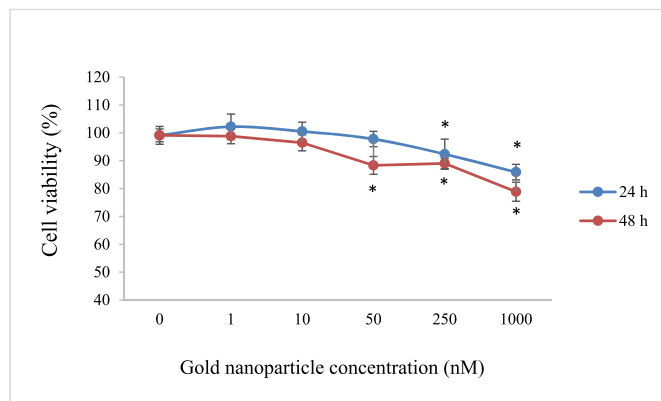


Fig. 2. Cell viability at various concentrations of gold nanoparticles. * indicates a significant difference ($P < 0.05$) from other groups, as determined by ANOVA test; the data are presented as mean $\pm$ SEM derived from at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with increasing concentrations of GNP. However, significant reductions in cell viability were observed only at concentrations of 250 and 1000 nM compared to the control samples. This trend was similarly observed in the cells treated with GNP for 48 h where there was a lower viability compared to those treated for 24 h, although this reduction was not significant except for concentrations of 50 nM. Based on these findings, we selected the concentration of 10 nM (not-toxic dose) and 48 h of treatment to perform experiments.

3.3. Metabolic death

The MTT assay utilized to demonstrate metabolic activity or cell viability revealed a significant increase in cell death in samples treated with both gold nanoparticles and radiofrequency radiation (GNP, RFR group) compared to the control samples (24.88% vs. 0.9%). A similar increase in cell death was observed in the GNP, RFR, MT group, affirming the presence of the bystander effect (BE) induced by RFR in the presence of GNPs. The cell viability of other groups did not change and was the same as to that of the Cont. group. (See Fig. 3A).

3.4. Reproductive death

The cell cloning assay was used to investigate reproductive death. Results showed that the number of colonies remained unchanged in cells

treated with GNP and GNP, MT group in comparison to Cont. group (60.8% and 55.6% vs 60.5%). However, the colony formation ability of cells exposed to RFR after pre-treatment with GNP significantly decreased to 25.08% ($P < 0.05$). This reduction was even notably lower than that observed in the cell exposed to RFR (38.4%). Additionally, the number of colonies decreased in GNP, RFR, MT group compared to both GNP and Cont. groups, yet significantly more than observed in the GNP, RFR group (Fig. 3B). This observation confirms the occurrence of the BE.

3.5. DNA damages

As it was illustrated in Fig. 4A, treatment of cells GNP with did not significantly alter the OM compared to its levels in untreated cells (0.98 vs. 0.73). However, RFR exposure of cells pre-treated with GNP led to a notable increase in OM compared to control group (4.1 vs. 0.73); Although, the value in GNP, RFR group did not significantly differ from the RFR group. Exposure to RFR for 24 h also resulted in a significant increase in OM compared to the control sample ($P < 0.05$). Fig. 4B displays comet images as observed through fluorescence microscopy.

3.6. DNA double strand breaks (micronucleus assay)

The presence of MN in binucleated cells is a sign of DSB. The number of MN in cells treated with gold nanoparticles was 11.7%, which did not significantly differ from the number in untreated cells (10.36%) ($P > 0.05$). The RFR exposure of cells pre-treated with GNP showed an increase in the number of MN (15.5%), yet this increase was not statistically significant. Likewise, RFR exposure for 24 h did not significantly alter the number of micronuclei in cells compared to non-exposed cells (13.4% vs 10.36%). A 6 Gy X-ray exposure was applied as a positive control.

Binucleated cells and MN were shown in Fig. 5, where in addition to MNs, anaphase bridges were observed. Anaphase bridges signify DNA double strand breaks and the formation of dicentric chromosomes. They emerge in anaphase when sister chromosomes are pulled toward two cell poles (Acilan et al., 2007). As there were no significant changes in the number of MN in the GNP, RFR group compared to the control group, the assessment of medium transfer and BE was not conducted via this method.

3.7. Production of ROS

The experimental results showed that cells treated with gold nanoparticles and exposed to RFR for 24 h demonstrated a significant reduction in the level of cellular ROS to 0.77 fold of the control ($p <$

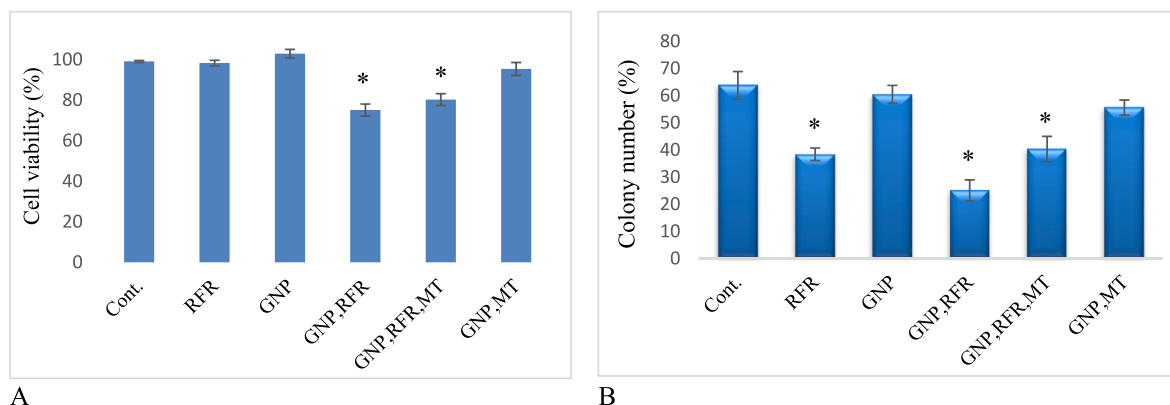


Fig. 3. Percentage of cells demonstrating no metabolic or reproductive death. A) Cell viability indicates cells that did not experience metabolic death. GNP treated cells exposed to RFR exhibited significantly reduced cell viability, as did cells receiving conditioned cell medium (CCM). B) Cells that have not succumbed to reproductive death can produce colonies. RFR, GNP treated cells exposed to RFR and bystander cells have shown reduced potential to generate colonies. * indicates a significant difference ($P < 0.05$) in comparison to other groups, Statistical analysis between the groups was determined by ANOVA; The data are presented as SEM averages from at least three independent experiments. The abbreviations of the groups have been described in 2–6 section.

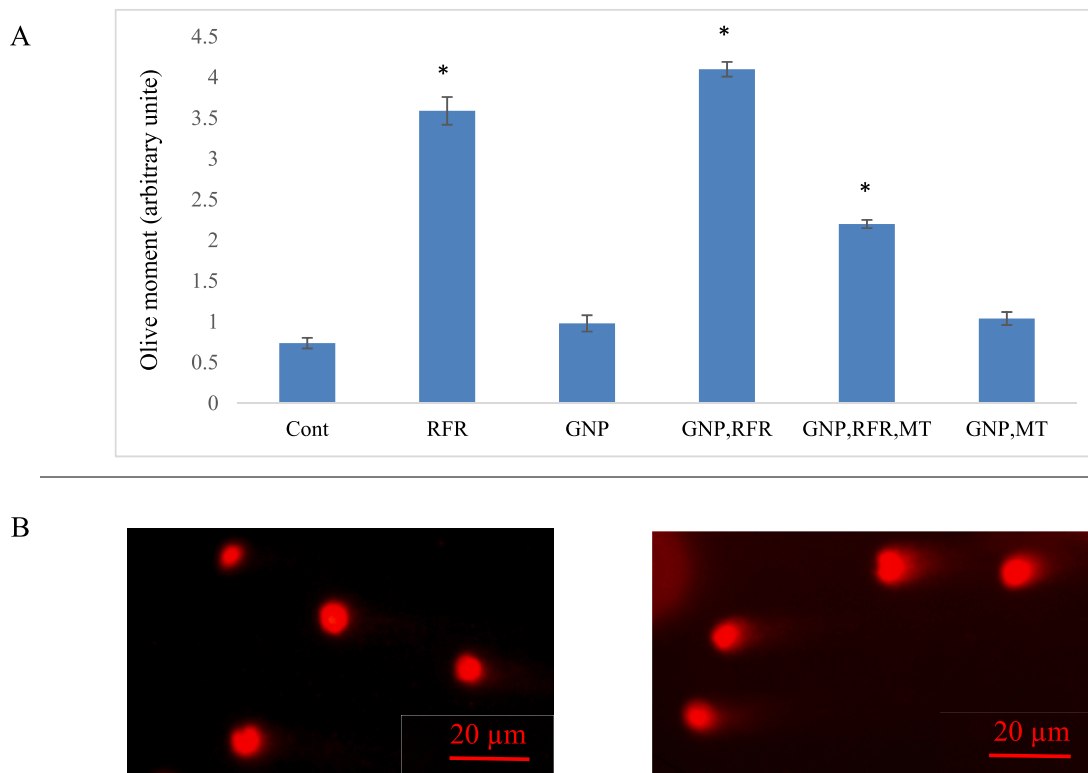


Fig. 4. DNA damage assessment based on comet assay results. A) The amount of olive moment reflects DNA damages (DSB and SSB). B) Comet images demonstrate cells in single gel electrophoresis assay in the control samples (left) and samples exposed to RFR (right). * indicates a significant difference ($P < 0.05$) in comparison to the Cont. group. Statistical analysis among the groups was determined via ANOVA; The data were presented as SEM averages from a minimum of three independent experiments. The abbreviations of the groups have been described in 2–6 section.

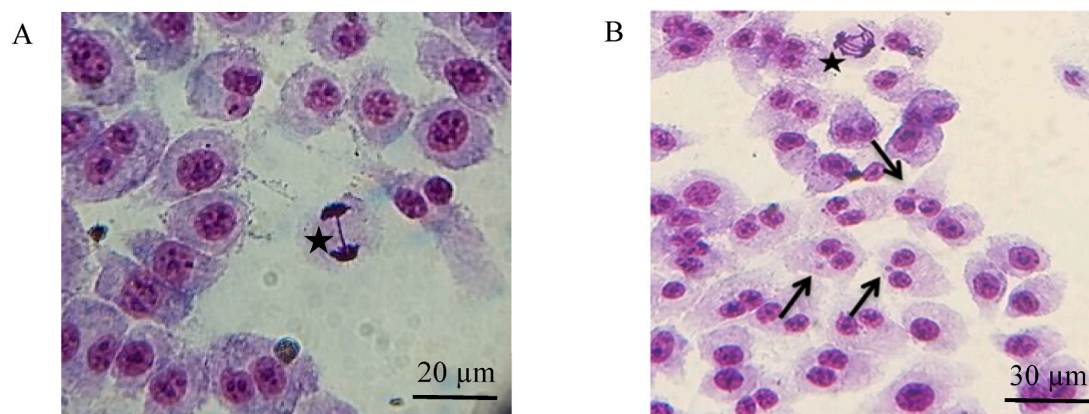


Fig. 5. Results of MN assay. A) binucleated cell and an anaphase bridge (black stare) in control samples. B) binucleated cells and MNs (arrows) in positive control samples. As it can be seen there is a cell with several anaphase bridge. The frequency of MN did not significantly change in GNP treated cell in comparison to that in control sample.

0.05). However, when cells were subjected to RFR alone, there was a notable increase in ROS production compared to the control samples, but in the presence of gold nanoparticles mitigated this increase, demonstrating no significant difference compared to non-RFR-exposed

or GNP treated cells (1.1 fold of control). The level of ROS in the GNP, RFR, MT and GNP, MT groups remained the same as the control. Furthermore no significant changes were observed in the level of intercellular ROS levels in all groups compared to the control samples

(data not shown).

3.8. COX-2 gene expression

The analysis of the extracted RNA indicated its purity, as the ratio of absorption at 260 nm–280 nm was 1.88 and at 230 nm–260 nm was 2.1, confirming the purity of the RNA and the absence of protein or other contaminants (Gallagher, 1998).

The gene expression of COX-2 was evaluated semi-quantitatively via Real-time PCR using Green SYBR P16 dye. The rate of changes in gene expression ($\Delta\Delta Ct$) compared to the control group was calculated. As it was presented in Table 2, no significant difference was found between the cells exposed to RFR for 24 h and the control group ($p > 0.05$). The COX2 mRNA expression in bystander cells was 1.05 fold that of the control which was not significantly different from the control or RFR groups. GNP led to an increase in COX2 expression compared to the other groups, but this increase was statistically not significant.

Cont.: Cells not exposed to RFR or treated with GNP; no culture medium transfer was performed. RFR: Cells exposed to RFR for 24 h. GNP, RFR: Cells treated with GNP for 48 h, then exposed to RFR after replacing the culture medium. GNP, RFR, MT: Cells received CCM (bystander cells).

4. Discussion

Communication technologies have exposed humans and the biota to a new type of environmental pollution: Radiofrequency radiation. Despite extensive scientific research, its biological effects remain an ongoing subject of investigation (Gulati et al., 2020). Health concerns regarding RFR effects increased as it became evident that these effects could be exacerbated through indirect ways or interactions of RFR with other factors. Nanoparticles such as GNP, are one of the factors that we encounter more than before due to conscious use or their release into the environment and its unconscious contamination (Jabir et al., 2022).

Our study discovered that RFR exposure in the presence of GNP caused cell death. Reduced cell viability was also observed in bystander cells not directly exposed to GNP and RFR. There are several studies showed that adding citrate colloids coated gold nanoparticles to the cell culture medium before RFR exposure reduces cell viability (Gannon et al., 2008; Glazer and Curley, 2010; Kruse et al., 2011; Anaraki and Dadashi, 2023). The cytotoxicity of nanoparticles is depending on nanoparticle size and RF frequency. Thikra Mustafa and coworkers proved that lower frequency RFR was most effective in killing the cancer cells in the present of larger particles (25 nm), while at a higher frequency (13.56 MHz) of RF, smaller sized (15 nm) caused more cell death (Mustafa et al., 2013). The efficacy of GNPs in killing cancer cells upon RFR exposure has been considered a non-invasive method for cancer treatment, utilizing Photo Thermal Therapy (PTT) or Radiofrequency Ablation (RFA). These methods act based on increasing temperature (Cardinal et al., 2008; Rejinold et al., 2015; Ashikbayeva et al., 2019; Narasimh an et al., 2020; Xie et al., 2023). Conduction-band electrons on the surface of gold nanoparticle, exhibiting Surface Plasmon Resonance (SPR), oscillate or excite upon interaction with electromagnetic radiation, resulting in heat generation (Rahme and Holmes, 2015). However, our experiments did not reveal any temperature rise in the culture

medium of GNP pre-treated cells exposed to RFR. Temperature toxicity induced by RFR is depending on NP concentration and exposure duration to RFR; lower doses of gold nanoparticles and shorter RFR exposure cause lower toxicity (Kruse et al., 2011). Although our cells were treated with GNP for 48 h, the concentration was lower compared to other studies. Additionally, the power used was essential; other studies employed powers above 100 W, whereas the power we used was 2 W (Glazer and Curley, 2010; Shenoi et al., 2011; Corr et al., 2012; Narasimh an et al., 2020).

Some of the paradigms for NP-mediated toxicity include ROS generation and subsequent oxidative stress (Manke et al., 2013). However, our data revealed a reduction in ROS levels in cells treated with GNP, with no changes in ROS levels in cells treated with RFR after GNP pre-treatment, while they indicated ROS increment after RFR exposure. Surprisingly, GNP not only had no toxic effect but also showed a protective effect. The antioxidant activity of AuNPs has been reported by Duy and coworkers who showed that it increased with the decrease of AuNPs size. For instance, 10 nm gold NPs had the strongest effect in reducing ROS levels compared to 20, 38, and 53 nm particles (Duy et al., 2013). The antioxidant activity of ascorbate peroxidase and superoxide dismutase enzymes also increased due to the impact of Gold nanoparticles in a concentration dependent manner (Jadczak et al., 2020). The low concentrations of GNP used in our study may fall within the window of antioxidant concentrations. This is a beneficial effect, particularly since these cells are normal and non-cancerous. Previous studies have shown that nanoparticles can kill cancer cells but have no effect on normal cells, consistent with this observation (Mahmood et al., 2022). Despite some studies reporting the cytotoxicity of GNP and similar metals (Ibrahim et al., 2021).

Our data further revealed that RFR exposure of cells treated with GNP significantly reduce the cell cloning capacity compared to RFR-exposed groups. However, no significant changes were observed in DNA damages investigated by comet and MN assays. The frequency SSB or DSB was similar to those found in cells exposed to RFR without any pretreatment. Therefore, gold nanoparticles and RFR only exhibited a synergistic effect only in cells deaths, either metabolically or reproductively. This was not due to increased genetic damage or ROS production as observed for FRF group. The reduced colony formation ability of cells exposed to RFR compared to the control sample was accompanied by an increase in the level of ROS and DNA SSB damages; therefore, the mechanism of cell death induced by GNP upon RFR seems to be different from that by RFR. Although no change was observed in the temperature of the cell medium during or after RFR exposure of GNP pre-treated cells, disruption in cell life may result from heat! Once the GNPs are internalized, they serve as target molecules for increased intracellular heat when exposed to an external electromagnetic field. In the case of RFA, which aim to destroy cancer cells, high power RFR is applied, releasing so much heat that it raises the ambient temperature (Gannon et al., 2008). This temperature increment above 40 °C (104 °F) can be life-threatening. However, the excited plasmons of GNP via low power RFR (less than 2 W) used in our experiment did not release enough thermal energy to detect in cell culture. Still it may have been sufficient to disrupt cell life: a kind of an increment in local temperature in the vicinity of nanoparticles and consequently activating some cell death signaling pathways (Collins, 2018).

Bystander cells (from the GNP,RFR, MT group) also showed reproductive death and DNA damages. This was evidently not due to increased local temperatures, as these cells were neither treated with GNP nor exposed to RFR. The bystander effect can occur through transfer factors to bystander cells in two ways: 1) through gap junction to adjacent cells that are in contact with the directly exposed cells. 2) via the extracellular environment, such as blood or lymph *in vivo* or medium in *in vitro* conditions, in the case of cells distant from the exposed cells (Chen et al., 2020; Du et al., 2020). As we applied the medium transfer method, the second way was the main mechanism for inducing the BE. In our previous study, ROS were introduced as a mediator molecule

Table 2
COX-2 gene expression in the mRNA level.

	Cont.	RFR	GNP, RFR	GNP,RFR, MT (Bystander Cells)	P- Value
COX-2 expression (fold of control)	1	0.09 ± 0.03	1.6 ± 0.04	1.05 ± 0.05	p > 0.05

Data is presented as mean ± standard deviation. Statistical analysis was conducted using analysis of covariance (ANCOVA) with a significance level set at less than 0.05.

responsible for triggering molecular signals in the BE induced by RFR (Jooyan et al., 2019). The extracellular ROS of the cell measured by analyzing medium from GNP-RFR exposed cells, did not show a significant increase compared to control. Taking a closer look at the present results and the previous study, we found that the DNA damages in the bystander cells received CCM from RFR, GNP groups were not as severe as those observed in the bystander cells received CCM. This finding confirmed that there are other signaling molecules that stimulate the BE, especially when CCM obtain from cells treated with two factors (in this case, GNP and RFR). In addition to ROS, another intercellular molecular signaling such as Ca^{++} , nitric oxide, 5-hydroxytryptamine, L-DOPA, glycine, nicotine, cytokines (interleukin 8, TGF- β etc.), reactive nitrogen species, and extracellular vesicles are considered candidates for further investigation (Marín et al., 2015; Hargitai et al., 2021).

To better comprehend the mechanism of BE induced by RFR alone or in the presence of GNP, the expression of COX-2 was evaluated. COX-2 can be induced by various growth factors, cytokines, and several stress factors (Deeb et al., 2008). The activation of COX-2 signaling in inflammatory reactions has been well established. BE also is recognized as an inflammatory response, and high COX-2 expression has been observed in bystander effect induced by ionizing radiation (Zhou et al., 2005; Hei et al., 2008; Tang et al., 2023). This gene is also a downstream target for the mitogen-activated protein kinase (MAPK) pathway, which is involved in the radiation response. The MAPK pathway is required for COX-2 and subsequent ROS activation is strongly indicated in the bystander signaling. Therefore, the association of COX-2 expression with non-ionizing radiation effects and the bystander effect induced by it is worth considering. In our study, no significant changes were observed in COX-2m RNA levels in bystander cells. Attempts to understand the mechanisms of the BE have shown that they are complex and involve multiple pathways (Sztamári et al., 2019). Thus, other inflammation factors such as high-mobility group box 1 (HMGB1), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS) may be involved in the BE induced by RFR (Chen et al., 2018). Also, we found no alteration in COX-2 expression in cells exposed to RFR. In accordance with our results, the COX2 protein expressions were not affected by RFR exposure in mouse microglial cells in the study done by Lu Y and et al. The authors suggested that expression of inflammatory factors stimulated by RFR exposure is in cell-dependent (Lu et al., 2014). In contrast to our findings, overexpression of cyclooxygenase enzyme under the influence of RFR has been reported, but in chronic exposure condition (2 h/day for three months) (Hussein et al., 2016) and in *in vivo* study in mouse astrocyte type I cells at the frequency of 1800 MHz (Lu et al., 2014). However, RFR exposed cells pre-treated by GNP showed a slight increase in COX-2 expression. As COX-2 has been introduced as a non-classical heat-responsive gene (Rossi et al., 2012). Although there is no strong evidence, it is suggested that heat produced in cells (as discussed before) trigger cell death and SSB signaling in cells of GNP, RFR groups. Overexpression of COX-2 has also been reported in thermal ablation by RFR (Kumar et al., 2017).

The limitation of our present study lies in not studying other factors apart from COX-2 and in using only one type of cells. These factors will be considered for further examination in future studies.

5. Conclusion

Nanoparticles have a wide range of applications and benefits in various fields, but their release into the environment and accumulation in biological materials can have adverse effects on the biosphere and humans. They can also interact with other factors, exacerbating their harmful effects. When cells are contaminated with small amounts of gold nanoparticles and exposed to prolonged RFR, it is cytotoxic. However, these conditions do not lead to significant genetic damages or oxidative stress compared to those induced by RFR alone. Increased cell death also extends to bystander cells, indicating the nonlinear effects of RFR in the

presence or absence of nanoparticles. The mechanism of this process is more complex and requires further studies compared to the mechanisms of BE induced by ionizing radiation. This study also suggests a potential therapeutic benefit: using gold nanoparticles and radiofrequency radiation to kill cancer cells.

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CRediT authorship contribution statement

Najmeh Jooyan: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Seyed Mohammad Javad Mortazavi:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Bahram Goliaei:** Supervision, Validation, Writing - review & editing. **Reza Faraji-Dana:** Conceptualization, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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