Open Access

OBM Genetics

Original Research

Role of DNA Damage and MMP Loss in Radiosensitization-Induced Apoptosis by Ellagic Acid in HeLa Cells

Vidhula R Ahire[1, 2, *], Amit Kumar [3], Sushma Bhosle [4], Kaushala Prasad Mishra[5, 6]

1. Anticancer Bioscience, Ltd.,Chengdu 640000, China; E-Mail: vidhula.ahire@gmail.com
2. J. Michael Bishop Institute of Cancer Research, Chengdu 640000, China
3. RB&HSD, Bhabha Atomic Research Center, Trombay, Mumbai, India; E-Mail: amithere4@rediffmail.com
4. Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332, USA; E-Mail: sushma.bhosle@bme.gatech.edu
5. Ex Bhabha Atomic Research Center; E-Mail: mishra_kaushala@rediffmail.com
6. Foundation for Education and Research, Ghatkopar (E), Mumbai, India

* **Correspondence:** Vidhula R Ahire; E-Mail: vidhula.ahire@gmail.com

Academic Editor: Tapan K Bera

Special Issue: Molecular Cancer Therapeutics

OBM Genetics
2021, volume 5, issue 2
doi:10.21926/obm.genet.2102129

Received: September 22, 2020
Accepted: March 30, 2021
Published: May 14, 2021

Abstract

Ellagic acid (EA) is a polyphenol found in grapes, pomegranates, walnuts, etc. exhibits anti-cancer properties. The current study was conducted to understand the radiosensitizing role of EA on HeLa cells. Monotherapy of EA and radiation was initially studied on HeLa cells. The addition of EA before the radiation treatment subsequently made DNA more susceptible to damage thereby developing DNA beaks, which are known to be lethal for cell survival. This was evaluated by performing comet and γ-foci formation assay. Other assays which included cell-cycle distribution, clonogenic cell survival assay, mitochondrial membrane drop, and apoptosis were performed to evaluate the effect of EA with radiation. Our results demonstrate that, when cells were exposed to the combinatorial treatment of EA (10µM) and 2Gy of γ-radiation there was augmented cell death, lesser cell-proliferation, reduction in

the colony-forming ability, increased DNA tail length, more number of γ-foci persisting even after 24h, enhanced apoptosis, augmented drop in the mitochondrial membrane potential and a G1 cell-cycle arrest. These results suggest that EA exhibits not only anti-cancer properties in terms of cell-death but also exhibits a radiosensitizing effect when given in combination with γ-radiation. Thus, it can be concluded that EA not only exhibits anticancer effects but also has potential in radiosensitizing HeLa cells.

Keywords
Ellagic acid; radiotherapy; HeLa; G1 arrest; γ-foci formation

1. Introduction

Although, cervical cancer exhibits a declining trend it remains a leading health issue for Indian women [1]. Because of its great benefits radiotherapy remains the default choice of treatment for advanced-stage cervical cancer patients. Nevertheless, clinicians face a major challenge of local recurrence and radio-resistance in the course of treatment [2]. Acquiring tumor radio-resistance can be attributed to the hypoxia in the microenvironment of the tumor [3, 4] and repair of the damaged DNA in cancer cells [5]. Higher radiation dose can lead to several severe side effects whereas a low dose may not be able to exert sufficient toxic effects on the tumor cells. Reducing the radiation dose and increasing the radio-sensitivity are logical strategies in gaining improved patient survival. In recent decades, a great deal of knowledge has been acquired on radiation-induced apoptosis paving the way in manipulating and controlling the cancer radio-response which will subsequently promote in achieving better clinical outcomes [6]. Since the efficacy of radiotherapy primarily depends on radiosensitivity, we strategized to use a herbal compound that would not only act as an anticancer-radiosensitizing agent at low concentrations but also reduce the severe side effects caused during the patient treatment [6]. Such compounds are known to enhance the radiation-associated tumor cell killing [7-13]. Recent studies have shown differential mechanisms of various polyphenols that prevent the incidence and development of cancer [14, 15]. Ellagic acid is one such polyphenol known for its anticancer activity in various cell lines which includes prostate, breast, colorectal, skin, osteogenic sarcoma, cervical, etc. [4-6]. It naturally occurs as phenol in grapes, pomegranates, nuts, black currents, strawberries, pecans, green tea raspberries, and walnuts [8, 9, 16-18]. Our previous lab studies on EA have shown it to be a potent anti-cancer and an antioxidant agent [8, 19, 20]. Seeram NP et al. reported the detection of EA in human plasma at the highest concentration of 31.9 ng/ml after 1h post- ingestion, however, it was speedily eliminated within 4h [21, 22]. Hence to overcome these limitations, diverse schemes for oral administration have been suggested which include, self-emulsifying systems, inclusion complexes, solid dispersions, polymorphs, and micro and nanoparticles [23]. In the current study, our focus was to understand the effect of EA and 2Gy irradiation on DNA damage and its repair kinetics, which was investigated by comet and gamma-foci formation respectively, cell cycle distribution and apoptosis by propidium iodide incorporation, reproductive cell death by clonogenic assay and drop in the mitocohondrial membrane potential by Mitotracker.

2. Experimental

2.1 HeLa Cell Culture, EA Treatment, and Cell Irradiation.

The human cervical cell line HeLa was acquired from National Center for Cell Sciences, Pune, India. The cells were grown in DMEM (Gibco Co. USA) supplemented with fœtal bovine serum (FBS) 10%, 100 units/ml penicillin, and 200µg/ml streptomycin (Gibco Co. USA). EA (E-2250) was procured from Sigma. To prepare the stock solution, EA (1, 10, 100µM) was dissolved in DMSO and stored at 4°C. Freshly prepared dilutions in media with FBS were used for all experiments. HeLa cells were incubated in a humidified atmosphere at 37°C in a 5% CO2 environment. Radiation treatment on HeLa cells was performed at RT using 60^{Co}γ-rays at a dose rate of 1Gy/ min (Bhabhatron-II, Panacea Technologies, and Bangalore, India). For combination treatments, EA was added to the growing HeLa cultures 2-3h before irradiation.

2.2 Cell Viability by Trypan Blue

To determine the number of viable cells after the treatment of EA and radiation alone or in combination the trypan blue assay was performed. The intact membrane of live cells excludes this dye exhibiting a clear cytoplasm whereas the dead cells take up the dye exhibiting blue color. HeLa cells were initially treated with 1, 10, 100 µM of EA. Cells were also given a monotherapy of 0, 2, 4, 6 Gy of radiation dose. The cell death was studied for EA and radiation doses as a single treatment. Later a combination treatment of 10µM EA and 2Gy was imparted to the cells. 2-3h before the radiation treatment HeLa cells were treated with 10 µM of EA. All the treatments were given for 24, 48, or 72h at normal culture conditions. Cell death was characterized by 0.04 % w/v trypan blue. To understand the cell morphology and apoptotic cells phase-contrast images were taken at 48h.

2.3 Immunohistochemistry Studies for DSB

To evaluate the effects of combined treatment of EA and 2Gy on the number of DSBs, γ-foci formations were studied. Cells were seeded on a coverslip with a cell density of $3x10^5$. The coverslips were placed in a culture plate 2-3h later when the HeLa cells were attached to the coverslip. Media supplemented with FBS was added to the culture plates. The following day, cells were treated with 10µM EA and or 2Gy irradiation. Cells were then fixed at various time points (5mins, 15mins, 3h, and 24h) with 2% paraformaldehyde (PFA) to study the DNA repair kinetics. Cells were permeabilized with Igepal and washed with PBS. A primary mouse monoclonal anti-γ-H2AX antibody was used to stain the foci. After the PBS wash, cells were stained with a secondary antibody which was a goat- antimouse-Cy3 antibody. Antifade mounting media containing DAPI was used to counter-stain the nuclei. Coverslips were placed upside down to score the foci under the confocal microscope (510META, Carl Zeiss, Germany) with a 63X objective. 200 cells per dose/time point were scored to calculate the average number of foci per cell.

2.4 Clonogenic Assay for Reproductive Potential

Cell survival was studied on HeLa cells after the treatment of the combination of EA and 2Gy irradiation for 48h and 72h. Clonogenic assay was performed as described by Franken et al. [24].

Initially cells were seeded in 6 well plates and placed in an incubator with 5% CO_2 and 37°C. The cells were then treated with 10µM EA and or radiation (0-6Gy). Culture media was replaced after 48 and 72h. HeLa cells were allowed to grow until adequate-sized colonies grew up to 12-14 days. Cells were then stained with 0.5% crystal violet. Number of colonies formed per plate was noted down to assess the surviving fraction.

2.5 Comet Assay for DNA Damage

Single-cell DNA damage can be studied by the comet assay. Different treatments can lead to DNA damage. After cell permeabilization, thenegatively charged damaged and fragmented DNA migrates to the anode when electrophoresed for 30 mins. The longer tail formation is associated with a longer comet formed i.e. more is the damage of DNA. Intact DNA does not migrate. The comet assay was performed as described by Olive and colleagues [25]. Slides were initially coated with 1% low-grade melting agarose. Later cells exposed to various treatments were pelleted and homogenized with 0.5% low melting agarose and pipetted on top of the pre-coated agarose slides. The slides were exposed to chilled alkaline lysis buffer for 1h. The slides were electrophoresed at 30V for 20 mins. Finally, the slides were stained with propidium iodide and scored under the fluorescence microscope (Leica).

2.6 Cell Cycle Analysis and Apoptosis by PI Flow Cytometric Assay

Cancer treatments are usually designed to push cancer cells to undergo apoptosis. In some cases, the cells are also arrested in a particular phase of the cell cycle before undergoing apoptosis. Apoptotic cells are characterized by a change in the shape of the nuclei and DNA fragmentation that consequently leads to loss of DNA from the nucleus. PI is a DNA binding fluorochrome that allows a precise evaluation of cellular DNA content by flow cytometric analysis [26]. In our experiments, 1 x 10^6cells were treated with radiation, EA alone, or in combination for 48h. HeLa cells were washed with PBS, centrifuged, and re-suspended in 300µl of PBS. Fixation was done with chilled 70% ethanol. Cells were then incubated with 1 mg/ml RNase for 1h at 37°C after the PBS wash. Propidium iodide (PI) (50 µg/ml) was used to stain the DNA content. FACS was performed using the Partec Cyflow cytometer.

2.7 Mitochondrial Membrane Potential ($\Delta\psi_m$) Drop by Mitotracker

As mitochondria are associated with the cell's capability to produce ATP for cellular processes by oxidative phosphorylation; it is one of the major indicators of cell health. Certain dyes are lipophilic cationic and have the potential to accumulate in the matrix space of the mitochondria in an inverse proportion to the $\Delta\psi m$ in a Nernstian fashion [27-29]. More the negative $\Delta\psi m$ (more polarized) more will be the accumulation of the dye. To understand the effect on the mitochondrial potential of HeLa cells, 3x10^5cells were grown on a sterile coverslip and then treated with EA and or 2Gy radiation. 48h later cells were fixed for 20 mins with 4% paraformaldehyde. They were then treated for 20 min with media containing 40 nM of MitoFlour. Normal culture conditions were maintained during this incubation process. After a wash with a warm medium, the cells were mounted with an anti-fade mounting medium with DAPI for counterstaining. Images were procured by Carl Zeiss Confocal Microscope with a 63Xobjective.

2.8 Statistics

All represented *in vitro* data have been performed at least three times, represented with ± standard error of the mean. Prism Graphpad version 7 was used to analyze cell survival and apoptosis, using statistical software using a non-parametric student's t-test. Data is represented as a mean + SD from 4 independent experiments and is statistically significant at $p<0.05$ as compared to control (* $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$, and **** $p\leq0.0001$).

3. Results

3.1 EA Sensitizes HeLa Cells to γ-Radiation

To explore the drug sensitivity of HeLa cells were treated with different doses of EA (1, 10 and 100 µM) and the (%) cell death as observed in Figure 1A, left panel as observed using the trypan blue assay. It was observed that 10µM of EA could kill cells effectively. Also, the best results were obtained when HeLa cells were treated with EA for 48h. Also when HeLa cells were treated with radiation doses (0-6Gy) and a dose-dependent increase in the cell death was observed (Figure 1A, right panel). For all further experiments, 2Gy was selected as a dose for its clinical relevance. To understand if the HeLa cells get radiosensitized by EA, they were exposed to combinatorial therapy of EA and 2Gyradiation.

Figure 1B exhibits the cell death (%) for treatment of EA for 3-time points (24, 48, and 72h). Significant cell death was observed in HeLa cells at 48h when treated with EA 10µM and 100µM. Also, 2Gy γ-radiation treatment exhibits a significant amount of cell death. Although 4Gy and 6Gy exhibits more cell-killing than 2Gy, in consideration of clinical relevance, 2Gy was selected for further experimentations. Besides, the percentage of cell death in HeLa cells that underwent the dual treatment of EA and 2Gy significantly enhanced when compared to the treatments in standalone cases. Figure 1C depicts the colony-forming ability of HeLa cells after treatments of IR alone or in combination if IR and EA (10µM). This can be deduced in the survival curve in the graph of Figure 1C. The combined treatment of EA and 2Gy radiation exhibited steeper curves than the curve of the radiation treatment alone. EA has significantly ($p<0.05$) been able to sensitize cells to irradiation.

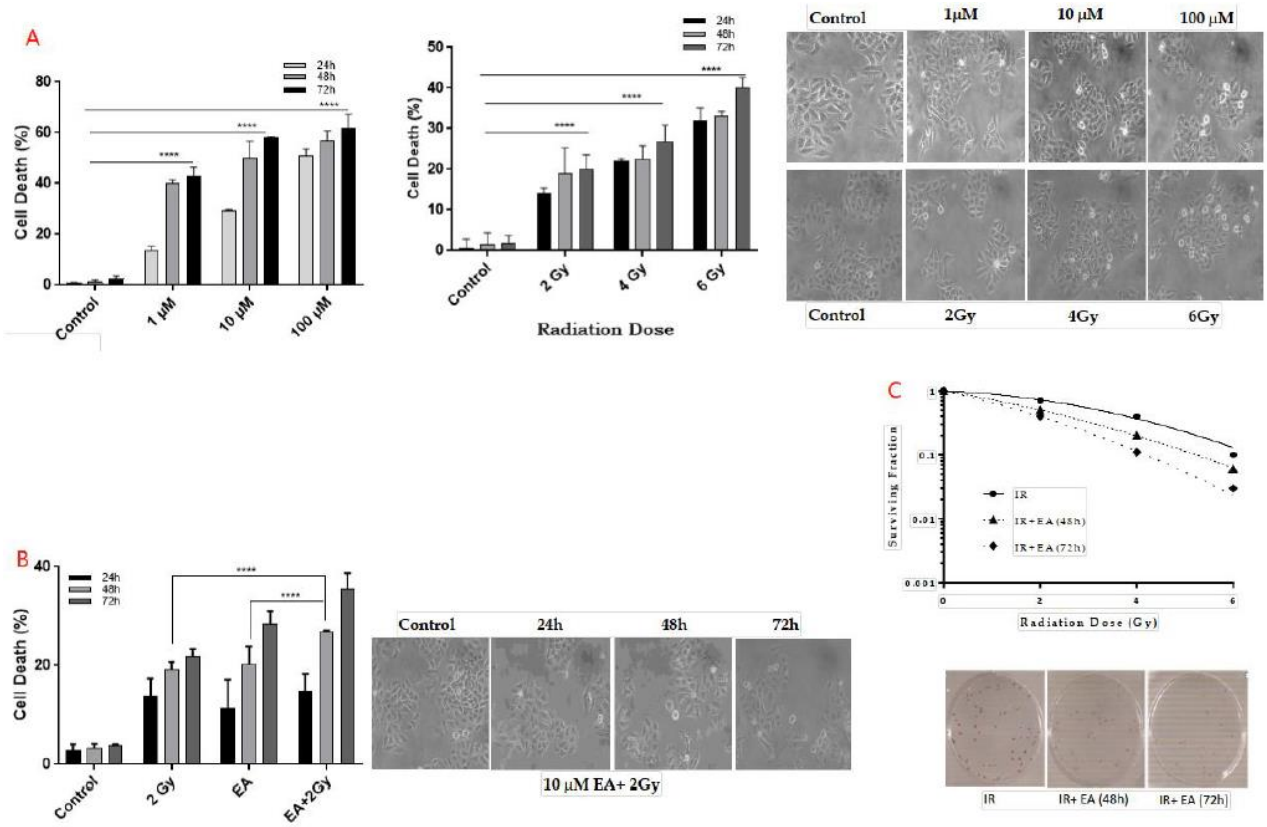

Figure1 Enhanced radiation induced cytotoxicity in HeLa cells by ellagic acid.(A) Cell death (%) of HeLa cells after individual treatment of EA and ionizing radiation at various EA concentrations and radiation doses. Phase contract images included. (B) Cell death of HeLa cells after irradiation and EA treatment alone or in combination at 24, 48 and 72h time-point as deduced by trypan blue assay. Phase contract images included. (C) Histogram and pictorial representation of cell surviving fraction of HeLa cells after EA (10 µM) treatment in combination with 2 Gy radiation as deduced by cell survival assay. Data is represented as a mean + SD from 4 independent experiments and is statistically significant at p≤0.05 as compared to control (**** p≤0.0001).

3.2 EA Enhances Radiation-Induced DNA Damage

Comet assay was performed to evaluate whether HeLa cells have increased DNA damage after the combined treatment of EA and γ-radiation, to test if EA directly induced DNA damage (Figure 2). Figure 2A represents comets of treated HeLa cells. Irradiation with 2Gy increased the tail length, and a significantly longer tail length ($p<0.05$) was observed in the EA + 2Gy treatment. Interestingly, EA treatment alone did not exhibit a significant tail length (scale bar 10 µm). The radiation dose of 2Gy induced more significant damage in HeLa cells than that of EA treatment when compared to control. However, a prominent and statistically significant ($p = 0.0001$) DNA damage was seen in the group which was treated with EA + 2Gy.

More than 100 cells were quantified per treatment and are represented in the histogram. A study was performed to understand DNA repair kinetics. In Figure 2B nuclei were stained to study the γ-H2AX foci formation which is a marker of DSB DNA damage. The nuclei are stained blue and the γ-foci are stained red. Each red dot denotes a DSB. HeLa cells exhibited a clear increase in γ-

foci after the combined treatment of EA and 2Gy than the treatment individually. After EA only a minor increase in foci was found. The histogram in Figure 2B clearly shows the highest number of foci been seen at the 15 min time point which reduced in the 3h and 24h.

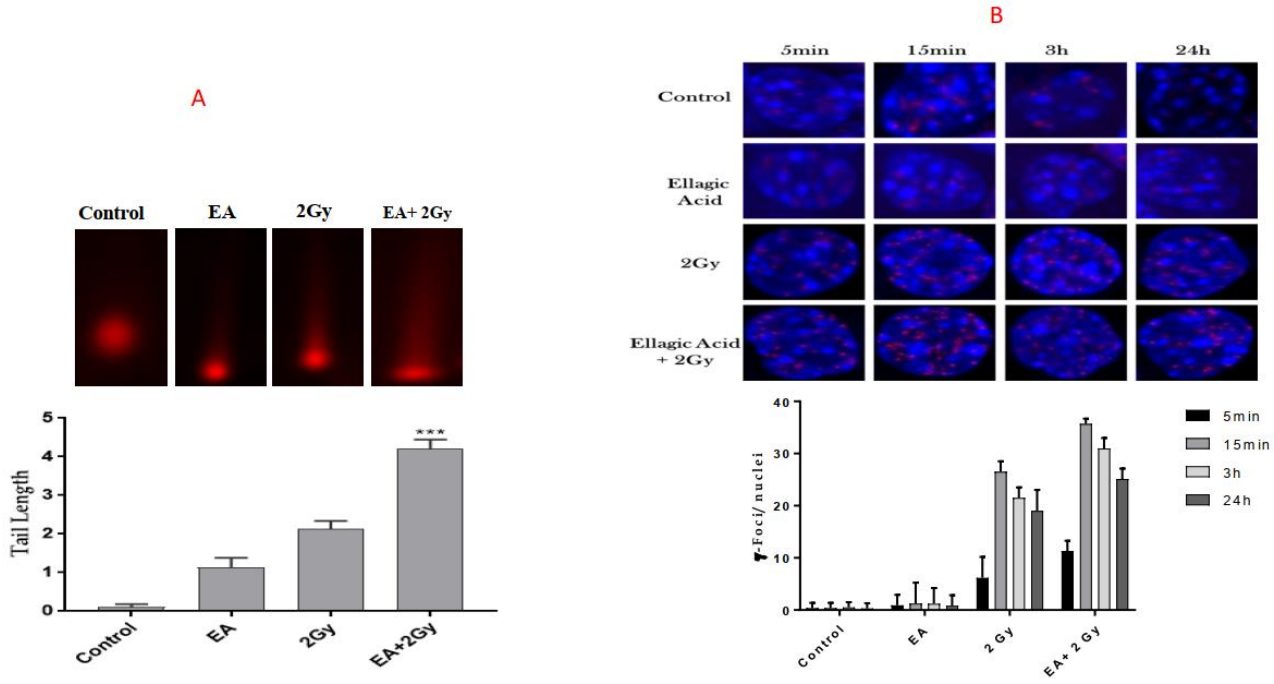

Figure 2 EA mediated augmented radiation induced cytotoxicity exhibiting higher DNA damage in form of comet tails with scale bar 10 µm. EA + 2Gy showed significantly ($p<0.001$) more DNA damage than the other treatment groups (A) and gamma foci (B) formation after the EA and radiation treatments alone or in combination. Data is represented as a mean + SD from 4 independent experiments and is statistically significant at $p\leq0.05$.

3.3 EA Influences the Cell Cycle Distribution and Apoptosis in γ-Irradiated HeLa Cells

Post 48h, cell cycle distribution was studied (Figure 3A) in HeLa cells treated with EA, 2Gy, and EA + 2Gy. In control cells, a high percentage of cells were observed in the G2 phase of the cell cycle than the G1 phase. Amongst the single treatments of EA and 2Gy radiation, high G1 arrest was seen in the cells treated with 2Gy. But enhanced effects were seen in cells that received the dual treatment of EA and 2Gy. HeLa cells exhibited a significant ($p<0.0001$) arrest in the G1 phase of the cellcycle.

Apoptosis was also measured 48h after the treatments of EA and 2Gy alone or in combination by flow cytometry. In Figure 3A HeLa cells exhibited augmented apoptosis in the combinatorial treatment as compared to control and individual treatments of EA and 2Gy. In the monotherapy with EA, only a minor increase in apoptosis was seen which was a bit higher in 2Gy. The same can be seen in Figure 3B, the right panel where the cells undergoing apoptosis are marked with red arrows.

Figure 3C, left panel exhibits the drop in the mitochondrial potential of HeLa cells after EA and 2Gy irradiation treatment alone or in combination. A heightened drop in the mitochondrial potential was observed in HeLa cells that underwent the dual treatment of EA and 2Gy radiation

than the mono treatment of EA or 2Gy. It can be correlated with the diffusion of mitotracker dye as seem to be heightened in EA + 2Gy as compared to the mono- treatment of EA or 2Gy.

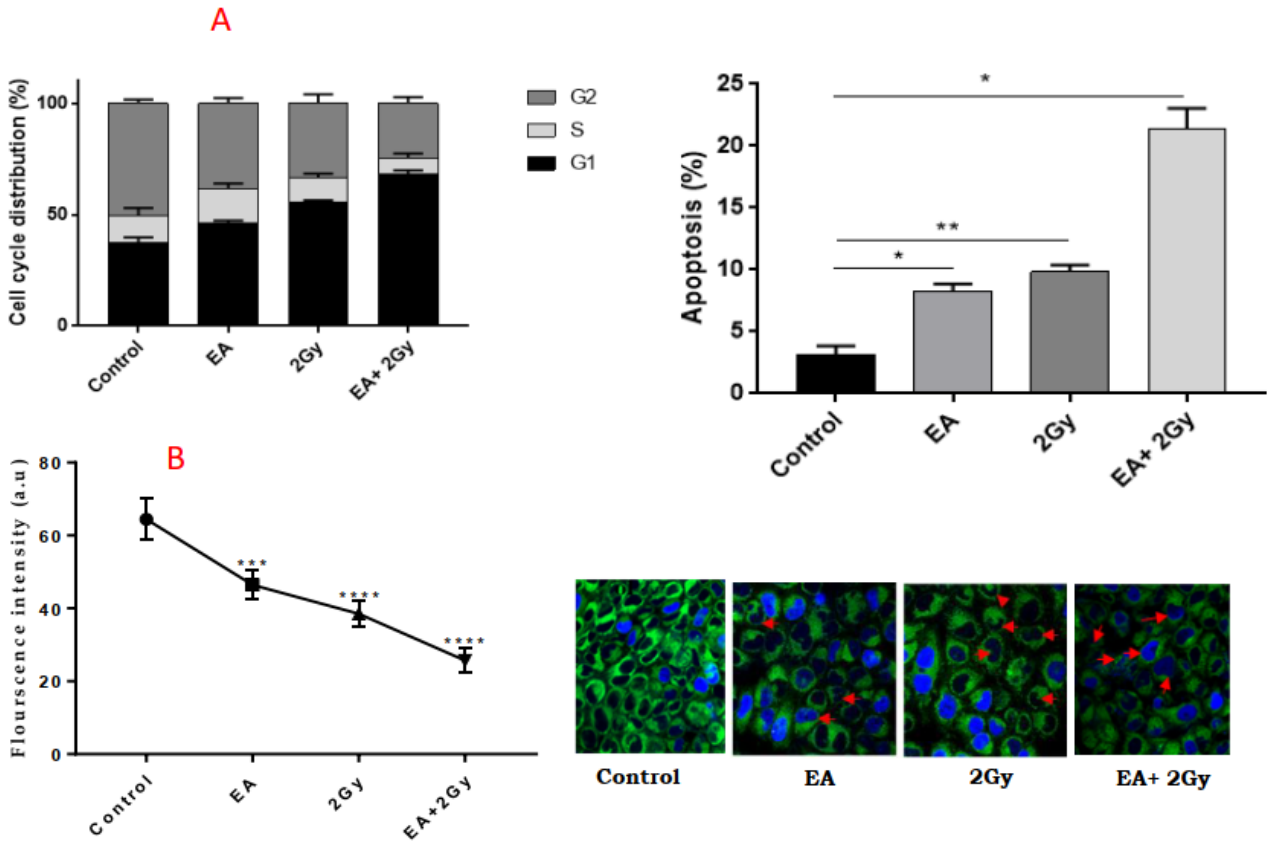

Figure 3 Radiation induced cytotoxicity enhanced by EA through the mitochondria pathway of apoptosis. (A) exhibits the cell cycle distribution and apoptosis after the cells were treated with EA and radiation alone or in combination. The G1 population in the treatment groups showed a significant ($p<0.0001$) arrest in the G1 phase as compared to the control group. Also, apoptosis is significantly higher in EA, 2Gy and EA + 2 Gy than that compared to control (B) Drop in mitochondrial membrane potential after HeLa cells were treated with EA and 2Gy alone or in combination. (C) Morphological view of cells undergoing apoptosis (red arrow) and diffusion of mitotracker from the treated cells leading to a drop in the membrane potential of mitochondria. Data is represented as a mean + SD from 4 independent experiments and is statistically significant at $p\leq0.05$ as compared to control (* $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$ and **** $p\leq0.0001$).

4. Discussion

Although conventional therapies like radio- and chemotherapy are used in cancer treatment for decades, the limitation of abating the recurrence and radio-resistance remains a big challenge. Researchers are therefore studying the adjuvants, which can, not only increase the toxicity to tumor cells but also simultaneously impart radiosensitivity. Imparting radiotherapy often makes these tumor cells radio-resistant. A radiosensitive tumor is easier to cure than a radio-resistant

tumor, which subsequently leads to metastasis and local recurrence. It is, therefore, necessary to understand that radio- sensitivity of tumors plays a critical role in determining the therapeutic efficacy of radiotherapy. EA is one such adjuvant [8, 9, 11, 30] which has been showing potential in anticancer studies (Figure 1). EA when used in combination with radiation, DNA integrity was salvaged at a higher rate than the treatment of EA and or radiation alone. EA alone did not show much damage to DNA. 10 μM of EA assisted in reducing the cell survival with radiation in combination and induced HeLa cells radiosensitization. This proves that EA has the potential of inducing radiosensitization in HeLa cells. To support this we performed various assays.

It is a known fact that γ-irradiation causes DNA double-strand breaks where DNA repair proteins tend to accumulate. DNA damage was studied by the comet assay and the γ-H2AX foci formation. It was seen the EA when combined with ionizing radiation showed maximum DNA damage in form of the highest tail length as indicated in Figure 2A. This interestingly co-related with the maximum γ-H2AX formation in the cells, which underwent the dual treatment. The peak of H2AX phosphorylation was observed at 15 minutes after radiation exposure. Although the number of foci is reduced (Figure 2B) after 24h they were still significantly high for the cells to survive. This suggests that cancer cells attempted to repair the DNA damage but not all the damage could be repaired. Although some of the foci or the DNA damage was repaired, a large number of foci were still persistent indicating the difficulty of cell survival due to genomic instability, which will subsequently, makes cells undergo apoptosis. Our results suggest the HeLa cells when exposed to γ-irradiation lose their reproductive potential or colony-forming ability and exhibit the incompetency to undergo proliferation when combined with EA. This subsequently enabled the HeLa cell sensitization and prompted cell reproductive death (Figure 1C). These cells could not regain their reproductive potential even after the supplementation of fresh media. This indicates that even at 2Gy treatment, a low dose as 10μM EA can radiosensitize HeLa cells.

Consequently, this stimulates the checkpoint proteins arresting the advancement of the cell cycle. Due to enhanced DNA damage in HeLa cells and compromised DNA damage repair and a prolonged checkpoint arrest induced by EA, the cells were eventually arrested in the G1 phase of the cell cycle. The combinatorial effects of EA and ionizing radiation were more pronounced. A heightened G1 arrest was seen compared to monotherapies of EA and 2Gy ionizing radiation and lead to apoptosis (Figure3A).

In cancer, one of the evident organelle dysfunction is observed in the mitochondria. The mitochondrial membrane potential is an indicator of the health of the cell. Drop in the mitochondrial potential is always associated with cells that would be pushed to apoptosis. There was a significant decrease in the MMP of HeLa cells that were sensitized to EA before being exposed to γ-irradiation. The commencement of apoptosis, alterations in MMP that lead to cytochrome c release, and therefore a diffused green fluorescence was observed indicating the loss of MMP. More the diffusion more is the loss of the mitochondrial membrane potential as can be seen in. Also, the changes in the nuclei shape are associated with cells being pushed to apoptosis. Some of the associated apoptotic alterations include the convolution of nuclei (Figure 3B), chromatin condensation, externalization of phosphatidylserine, DNA laddering, cell shrinkage, etc. It can be concluded that a clinically relevant 2Gy radiation dose, when combined with a concentration as low as 10μM of EA, has the potential to enhance the radiation effect in HeLa cells by radiosensitizing the cells. This is indicated by inhibiting cell proliferation, arresting the cells in the G1 phase, and inducing apoptosis through the mitochondrial-dependent apoptosis pathway.

Acknowledgement

The authors would like to thank University Grant Commission, Government of India for the RGNF-Senior Research Fellowship (201112-RGNFSC-MAH-114) granted to Vidhula Ahire for her research. We would also like to thank the authorities of Bhabha Atomic Research Centre for permitting the usage of facilities to conduct the above-mentioned work.

Author Contribution

V.A, A.K., and K.P.M., devised the project, the main conceptual ideas and proof outline. V.A. carried out the experiments. V.A., and S.B., contributed in manuscript writing whereas A.K., and K.P.M., took the lead in providing critical feedback and helped shape the research, analysis and manuscript.

Competing Interests

The authors have declared that no competing interests exist.

References

1. Sreedevi A, Javed R, Dinesh A. Epidemiology of cervical cancer with special focus on India. Int J Womens Health. 2015; 7: 405-414.
2. Huang EY, Wang CJ, Chen HC, Fang FM, Huang YJ, Wang CY, et al. Multivariate analysis of para-aortic lymph node recurrence after definitive radiotherapy for stage IB-IVA squamous cell carcinoma of uterine cervix. Int J Radiat Oncol Biol Phys. 2008; 72: 834-842.
3. Rofstad EK, Sundfør K, Lyng H, Trope CG. Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis. Br J Cancer. 2000; 83: 354-359.
4. Höckel M, Schlenger K, Aral B, Mitze M, Schäffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res. 1996; 56: 4509-4515.
5. Parliament MB, Murray D. Single nucleotide polymorphisms of DNA repair genes as predictors of radioresponse. Semin Radiat Oncol. 2010; 20: 232-240.
6. Qin C, Chen X, Bai Q, Davis MR, Fang Y. Factors associated with radiosensitivity of cervical cancer. Anticancer Res. 2014; 34: 4649-4656.
7. Ahire VR, Mishra KP, Kulkarni GR. Apoptotic radiosensitivity of cervical tumor cells enhanced by ellagic acid. Eur J Biotechnol Biosci. 2015; 3: 56-58.
8. Madhukar Bhosle S, Ahire VR, Henry MS, Thakur VS, Huilgol NG, et al. Augmentation of radiation-induced apoptosis by ellagic acid. Cancer Invest. 2010; 28: 323-330.
9. Ahire VR, Mishra KP. Ellagic acid radiosensitizes tumor cells by evoking apoptotic pathway. J Radiat Cancer Res. 2016; 7: 71-78.
10. Girdhani S, Bhosle SM, Thulsidas SA, Kumar A, Mishra KP. Potential of radiosensitizing agents in cancer chemo-radiotherapy. J Cancer Res Ther. 2005; 1: 129-131.
11. Bhosle SM, Huilgol NG, Mishra KP. Enhancement of radiation-induced oxidative stress and cytotoxicity in tumor cells by ellagic acid. Clin Chim Acta. 2005; 359: 89-100.

12. Kumar B, Joshi J, Kumar A, Pandey BN, Hazra B, Mishra KP. Radiosensitization by diospyrin diethylether in MCF-7 breast carcinoma cell line. Mol Cell Biochem. 2007; 304: 287-296.
13. Sandhya T, Lathika KM, Pandey BN, Mishra KP. Potential of traditional ayurvedic formulation, Triphala, as a novel anticancer drug. Cancer Lett. 2006; 231: 206-214.
14. Rigacci S, Miceli C, Nediani C, Berti A, Cascella R, Pantano D, et al. Oleuropein aglycone induces autophagy via the AMPK/mTOR signalling pathway: A mechanistic insight. Oncotarget. 2015; 6: 35344-35357.
15. Fetoni AR, Paciello F, Mezzogori D, Rolesi R, Eramo SL, Paludetti G, et al. Molecular targets for anticancer redox chemotherapy and cisplatin-induced ototoxicity: The role of curcumin on pSTAT3 and Nrf-2 signalling. Br J Cancer. 2015; 113: 1434-1444.
16. Girish C, Pradhan SC. Drug development for liver diseases: Focus on picroliv, ellagic acid and curcumin. Fundam Clin Pharmacol. 2008; 22: 623-632.
17. Jurenka J. Therapeutic applications of pomegranate (Punica granatum L.): A review. Altern Med Rev. 2008; 13: 128-144.
18. Ahire V, Das D, Mishra KP, Kulkarni GR, Ackland L. Inhibition of the p53 Y220C mutant by 1-hydroxy-2-methylanthraquinone derivatives: A novel strategy for cancer therapy. J Environ Pathol Toxicol Oncol. 2016; 35: 355-364.
19. Ahire V, Kumar A, Mishra KP, Kulkarni G. Ellagic acid enhances apoptotic sensitivity of breast cancer cells to γ-radiation. Nutr Cancer. 2017; 69: 904-910.
20. Bhosle SM, Huilgol NG, Mishra KP. Apoptotic index as predictive marker for radiosensitivity of cervical carcinoma: Evaluation of membrane fluidity, biochemical parameters and apoptosis after the first dose of fractionated radiotherapy to patients. Cancer detect prev. 2005; 29: 369-375.
21. Seeram NP, Lee R, Heber D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (Punica granatum L.) juice. Clin Chim Acta. 2004; 348: 63-68.
22. González-Sarrías A, García-Villalba R, Núñez-Sánchez MÁ, Tomé-Carneiro J, Zafrilla P, Mulero J, et al. Identifying the limits for ellagic acid bioavailability: A crossover pharmacokinetic study in healthy volunteers after consumption of pomegranate extracts. J Funct Foods. 2015; 19: 225-235.
23. Zuccari G, Baldassari S, Ailuno G, Turrini F, Alfei S, Caviglioli G. Formulation strategies to improve oral bioavailability of ellagic acid. Appl Sci. 2020; 10: 3353.
24. Franken NA, Rodermond HM, Stap J, Haveman J, Van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006; 1: 2315-2319.
25. Olive PL, Banáth JP. The comet assay: A method to measure DNA damage in individual cells. Nat Protoc. 2006; 1: 23-29.
26. Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc. 2006; 1: 1458-1461.
27. Ehrenberg B, Montana VA, Wei MD, Wuskell JP, Loew LM. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. Biophys J. 1988; 53: 785-794.
28. Johnson LV, Walsh ML, Bockus BJ, Chen LB. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J Cell Biol. 1981; 88: 526-535.
29. Lemasters JJ, Ramshesh VK. Imaging of mitochondrial polarization and depolarization with

cationic fluorophores. Methods Cell Biol. 2007; 80: 283-295.
30. Ahire V, Mishra KP, Kulkarni GR. On the mechanism of cellular toxicity in breast cancer by ionizing radiation and chemotherapeutic drugs. J Environ Pathol Toxicol Oncol. 2014; 33: 69-82.