

Original Article

An Investigation of the Effects of Raw Garlic on Radiation-induced Bystander Effects in MCF7 Cells

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Abstract

Introduction

Radiation-induced bystander effect (RIBE) is a phenomenon in which radiation signals are transmitted from irradiated cells to non-irradiated ones, inducing radiation effects in these cells. RIBE plays an effective role in radiation response at environmentally relevant low doses and in radiotherapy, given its impact on adjacent normal tissues or those far from the irradiated tumor. Reactive oxygen species contribute to RIBE induction. Therefore, the present study was conducted to investigate the possible inhibitory effects of garlic, as an antioxidant-containing plant, on RIBE.

Materials and Methods

MCF7 cells, treated with raw garlic extracts, were irradiated by ⁶⁰Co gamma rays, and their culture medium was transferred to non-irradiated autologous bystander cells. Percentage cell viability and micronucleus formation in both irradiated and bystander cells were examined and compared with corresponding cell groups, not treated with garlic.

Results

Treatment with garlic extract reduced the number of micronucleus-containing cells in both irradiated and bystander cells. However, it only increased the percentage cell viability in bystander cells, not the irradiated ones.

Conclusion

RIBE was effectively suppressed by raw garlic extracts. Inhibitory effects of raw garlic may be of particular importance for exposure to environmentally relevant low doses, where RIBE dominates direct radiation effects. They are also partially important for addressing the limited therapeutic gain of radiotherapy, as they may only increase the percentage cell viability of bystander cells, not the directly irradiated tumor cells. However, more comprehensive in-vivo research regarding garlic treatment duration is required to support the obtained results.

Keywords: Allium Sativum; Antioxidant; MCF7 Cells, Radiation-Induced Bystander Effect; Raw Garlic

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1. Introduction

Ionizing radiations interact with organic compounds and water in cells. The interaction between radiation and water molecules results in the generation of free radicals, which attack DNA and act as genotoxins. DNA lesions lead to gene mutation and subsequent cancerous transformations. Based on the above description, DNA is the main target of ionizing radiation (target theory) and the magnitude of DNA lesions determines the probability of cancer development.

However, radiation-induced bystander effect (RIBE), which is defined as radiation responses observed in non-irradiated cells, may contribute to cancer induction, as well. As a result, RIBE complicates the radiation dose-response relationship, especially at low doses in which indirect (non-targeted) radiation effects predominate.

It is accepted that reactive oxygen species (ROS), including free radicals, play an important role in the induction of RIBE. In some studies, the inhibitory effects of ROS scavengers on lesions, induced by RIBE, have been demonstrated [1-7]. In addition, external antioxidants such as vitamins E and C have been reported to decrease the number of micronuclei, formed in bystander cells. [8]

Garlic (*Allium sativum L.*) is a plant, which has been used in herbal medicine as early as 3700 BC, given its therapeutic efficacy [9]. This plant contains flavonoids and sulfur-containing compounds with antioxidant properties. In some studies, reviewed by Borek^[9], the ability of garlic to enhance endogenous antioxidants such as glutathione peroxidase, superoxide dismutase, and catalase has been demonstrated.

Moreover, it has been revealed that garlic ingredients are able to directly scavenge ROS [9, 10]. Morihara et al. showed that aged garlic extract scavenges superoxide radicals in a dose-dependent manner [11]. They also showed that garlic significantly inhibits the production of superoxide in human neutrophils. Chang et al. studied the radioprotective effects of alkyl thiosulfate,

derived from *Allium* vegetables. DNA-induced damages in irradiated cells were significantly decreased when they were incubated with garlic-derived 2-propenyl thiosulfate.

[12].

Radioprotective effects of garlic have been also illustrated in animal models [13, 14]. Although garlic has antioxidant properties, it is clastogenic in high amounts and acts as a pro-oxidant with some side-effects [10]. Anemia, weight loss, and growth inhibition have been observed in rats, which were fed raw garlic for a long period of time [10]. In 32D and 32Dp210 cell lines, garlic induced apoptosis. Avci et al. concluded that the oxidant potential of garlic extract may play a part in its possible anti-cancer potential. [15]

The present study investigated the possible protective effects of raw garlic extract on RIBE. Garlic is used in various preparations such as raw, aged, heat-treated, and powder, each with its own specific properties. Raw garlic homogenate has the above-mentioned pro-oxidant and antioxidant potentials. However, as garlic is usually consumed raw, the effects of raw garlic on the magnitude of damages, induced in bystander cells, were investigated.

2. Materials and Methods

2.1. Cell Culture

A breast cancer cell line (MCF7) was provided by Pasteur institute in Tehran, Iran. The cells were grown in RPMI1640 culture medium (Gibco, Germany) and supplemented with 10% fetal bovine serum (Biosera, England), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Sigma, USA); they were maintained at 37°C in a humidified atmosphere with 5% CO₂. Subconfluent cells were subcultured in 10 cm² flasks and 96-well plates to prepare the experimental samples.

2.2. Garlic Extract Preparation

Fresh garlic was purchased at the peak of maturity from a local market in Mashhad, Iran. Garlic bulbs were separated, peeled, and

washed with distilled water. After drying, a total of 100 g of washed garlic bulbs and 50 mL of distilled water were added to a grinder and crushed. The mixture was filtered using a filter paper. The solution was centrifuged at 4000 rpm for 20 min and the supernatant was separated. Final concentration of the garlic extract in solution was found to be 500 mg/mL (50% W/V). The extract was frozen and kept at -20°C , as long as necessary.

2.3. Determination of Non-Toxic Concentration of Garlic Extract

The cells were incubated with 50-500 $\mu\text{g/mL}$ of garlic extract. Two and six hours later, the cells were washed and fed with a fresh medium, respectively. Finally, percentage cell viability was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cellular proliferation assay.

2.4. MTT assay

In MTT assay, the yellow tetrazolium MTT is reduced by metabolically active cells. The resulting intracellular purple formazan can be solubilized and measured by spectrophotometry. To perform the test, culture media of wells, containing the examined cells, were removed. Ten μL of MTT (Sigma, St. Louis, MO) and 100 μL of fresh medium were added to individual wells and incubated for 4 hours.

Then, the culture media in the wells were replaced with 200 μL of dimethyl sulfoxide (Sigma, USA) and mixed for 10 minutes to dissolve the converted dye. Finally, light absorbance was measured at 545 nm, using a multi-well scanning spectrophotometer (Stat Fax 2100, USA). The cell survival percentage was measured in comparison with the control group.

2.5. Evaluation of Bystander Effect and Direct Radiation Response

The cells were cultured in 96-well plates (2×10^3 cells per well) and 10 cm^2 flasks (2×10^5). After 24 hours, they were irradiated with 0.5, 2, and 4 Gy gamma rays, emitted from a ^{60}Co teletherapy unit (Theratron,

Phoenix model, average dose rate of 0.71Gy/min) at room temperature. To locate the adherent cells in the depth of maximum dose, the height of the culture media of the irradiated dishes was increased to 5mm.

The plates were used to perform the MTT assay and determine the percentage cell viability as a direct radiation response. The cells in flasks were used as target cells to induce bystander effect in the non-irradiated cells, cultured in non-irradiated 96-well plates. For this purpose, culture media of the irradiated flasks were extracted one hour after irradiation, were passed through 0.2 μm filters (Orange Scientific, Belgium), and were added to non-irradiated bystander cells. The MTT assay was performed for bystander cells, as well as the irradiated ones.

2.6. Study of the effect of garlic extract on direct radiation response

The cells were cultured in 96-well plates, as mentioned above, and were left to grow and adhere to the bottom of the wells. Five groups of cells were prepared, which are as follows:

- Control 1: sham-irradiated cells;
- Control 2: sham-irradiated cells, treated with 100 $\mu\text{g/mL}$ of garlic extract for two hours;
- Group T1: irradiated cells;
- Group T2: irradiated cells, treated with 100 $\mu\text{g/mL}$ of garlic extract for two hours before irradiation; and
- Group T3: irradiated cells, treated with 100 $\mu\text{g/mL}$ of garlic extract during the irradiation.

Irradiation was performed by 2 Gy ^{60}Co gamma rays, and MTT assay was performed after 7 days to measure the percentage cell viability. A parallel experiment was performed, in which the cells were cultured in 10 cm^2 flasks, and the micronucleus assay was performed to evaluate their response to radiation.

2.7. Study of The Effects of Garlic Extract on RIBE

Target cells were cultured in 10 cm^2 flasks. They were divided into five groups similar to

the categorization of directly irradiated cells, mentioned above. One hour after irradiation, the culture media extracted from the target flasks were filtered and transferred to bystander cells, previously cultured in 96-well plates. Bystander cells were divided into five corresponding groups (control 1, control 2, and groups B1, B2, and B3; prefix ‘T’ was replaced by ‘B’ in bystander groups), based on the culture medium they received.

A parallel experiment was performed in which bystander cells were cultured in 10 cm² flasks and received medium from the corresponding target flasks. Bystander cells in the plates were used for MTT assay, and bystander cells, cultured in 10 cm² flasks, were applied for the micronucleus assay.

2.8. Micronucleus assay

The cytokinesis-block micronucleus assay was performed, based on studies by Albertini et al. and Fench [16,17]. In brief, at the time of medium transfer, cytochalasin B 1 µg/mL was added to both bystander and target flasks. Cells in the flasks were incubated for 21 hours and washed with phosphate-buffered saline; finally, they were fixed with a 3:1 methanol-to-acetic acid solution (Merck, Germany) three times. After drying, the cells were stained with 5% Giemsa (Merck, Germany) for 7 minutes.

Walls of the flasks were removed and the cells were scored at 400× magnification to determine the number of micronucleated cells per 1000 bi-nucleated cells (MC).

2.9. Statistical analysis

SPSS version 21 was used to perform statistical analysis. Data distribution was normal, thus one-way ANOVA and Tukey's multiple comparison tests were performed to compare groups at P<0.05. Percentage cell viability in directly irradiated and bystander groups was presented as the mean of at least three independent experiments, replicated in 8 wells. The yields of MC were presented as the mean of 6 independent experiments.

3. Results

3.1. Raw garlic toxicity

Figure 1 represents percentage cell viability of different cultures, incubated with different concentrations of garlic for two and six hours, respectively. Raw garlic was non-toxic up to 100 µg/mL, and there was no significant difference between the two groups exposed to garlic for two and six hours, respectively (P>0.05); therefore, in the following experiments, the cells were incubated with 100 µg/mL of the garlic extract for two hours.

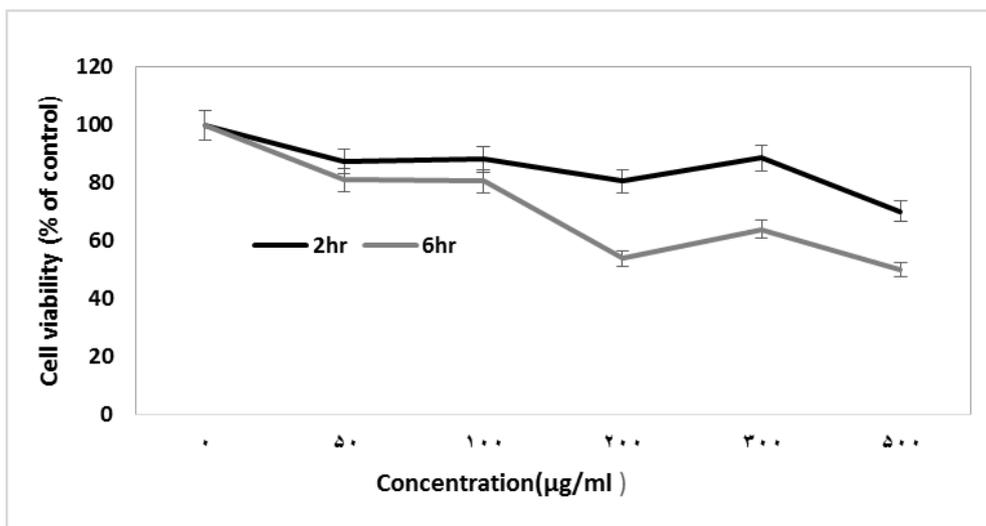


Figure 1. Percentage viability of cells, exposed to different concentrations of raw garlic for 2 and 6 hours, respectively. Each point represents mean±SD of at least three independent experiments replicated in 8 wells.

3.2. Percentage cell viability of directly irradiated and bystander cells at different radiation doses

Figure 2 shows the radiation response of target and bystander cells. Percentage cell viability

of all groups, compared to the controls was statistically different ($P < 0.001$). However, in the bystander cell groups, unlike the directly irradiated cells, radiation response was independent of dose ($P > 0.05$).

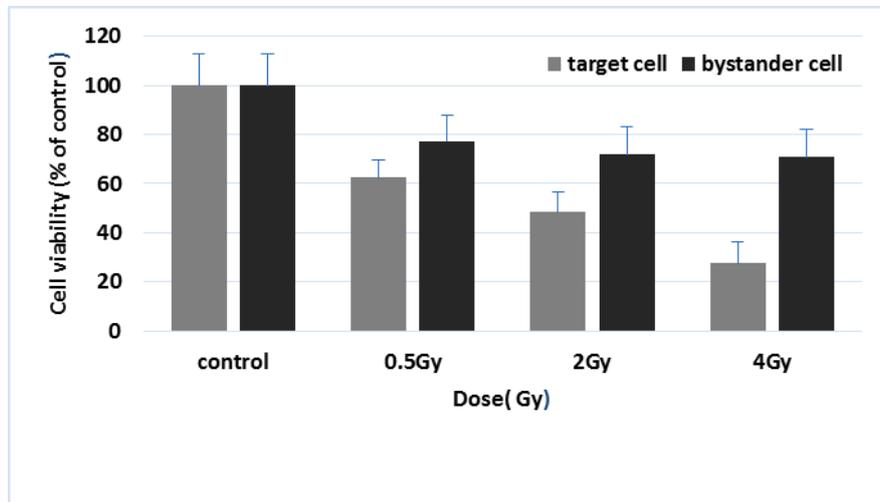


Figure 2. Percentage cell viability of target and bystander cells, exposed to different doses of gamma rays

Table 1. Percentage cell viability and MC frequency of target groups, irradiated with 2 Gy

Groups	Cell viability \pm SD*	MC value \pm SD
Control 1 (without garlic)	98.41% \pm 8.70	62.00 \pm 5.00
Control 2 (with garlic)	89.06% \pm 13.00	58.50 \pm 2.00
T1 (without garlic)	48.38% \pm 8.00	231.80 \pm 4.40
T2 (treated with 100 μ g/mL of garlic for two hours before irradiation)	44.48% \pm 7.00	144.83 \pm 6.70
T3 (treated with 100 μ g/mL of garlic during irradiation)	58.66% \pm 12.50	147.40 \pm 10.00

* Standard deviation

Table 2. Percentage cell viability and MC frequency of bystander groups, receiving culture medium from 2Gy irradiated cells

Groups	Cell viability \pm SD*	MC value \pm SD
Control 1 (without garlic)	100% \pm 13.00	65.00 \pm 8.50
Control 2 (with garlic)	85.18% \pm 7.50	66.50 \pm 0.70
B1 (received medium from T1)	69.08% \pm 10.70	119.60 \pm 5.30
B2 (received medium from T2)	80.07% \pm 13.50	87.20 \pm 8.10
B3 (received medium from T3)	88.68% \pm 9.70	77.40 \pm 12.80

* Standard deviation

3.3. The Effects of Garlic Extract on Direct Radiation Response

Based on the results obtained from the MTT assay, the garlic extract had no effect on direct radiation response (Table 1). Percentage cell viability of groups T2 and T3, despite being treated with garlic extract, was still different from the control groups ($P < 0.001$), and similar to T1 group ($P > 0.05$); however, the

micronucleus assay revealed that they were protected by the garlic extract. Although MC values of T2 and T3 groups were still statistically different from those of control groups ($P < 0.001$), they decreased from 231.8 to 144.83 in T1 and to 147.4 in T2 and T3 groups, respectively ($P < 0.001$).

3.4. The Effects of Garlic Extract on Radiation Bystander Response

MTT assay showed that non-toxic concentration of garlic extract could protect bystander cells. Percentage cell viability of B2 and B3 groups increased, which was not different from the control groups ($P=0.16$ for B2 and 0.641 for B3 group). MC values of B2 and B3 bystander cells also decreased from 119.6 to 87.2 and 77.4 , respectively (Table 2). Even though MC frequency of B2 group was still higher than the controls ($P = 0.016$), it was significantly lower than B1 group ($P < 0.001$).

4. Discussion

Detrimental effects of ionizing radiation have provoked investigators worldwide to search for natural and synthetic materials with radioprotective properties. Medicinal herbs are of high importance in this regard given their lower toxicity, compared to chemical medications. The present study was designed to determine whether raw garlic, as a natural diet, can prevent or decrease RIBE in MCF7 cells. The results clearly indicated that garlic extract decreased the nuclear damage to MCF7 cells, which occurred as a result of either direct irradiation or RIBE.

The mitigation of direct radiation injuries by garlic has been reported by other investigators [12, 14, 18], and has been attributed to antioxidant properties of garlic [19]. Since free radicals also play an important role in the induction of bystander effect [1-7], the underlying RIBE radioprotective mechanism of garlic may be linked either directly or indirectly to its antioxidant capabilities through free radical scavenging. Suppression or reduction of RIBE by other antioxidants such as vitamins supports this suggestion. [8, 20]

The results showed that the extent of radioprotection by garlic is not a function of administration timing (i.e., MN frequencies of B2 and B3, as well as T2 and T3, were not statistically different). In addition, as we only added the garlic extract to the target cells, it can be concluded that the inhibitory effect of

raw garlic has been exerted through the inhibition of bystander signal production or bystander signal transition from target cells to bystander ones. In comparison with our method, Law et al. treated both target and bystander cells with green tea catechin, as a radioprotector, and suppressed RIBE in Chinese hamster ovary cells [21]. As catechin existed in both target and bystander cell cultures, it is not clear whether catechin has affected the target or bystander cells to prevent RIBE.

To the best of our knowledge, no investigation has been conducted on the effect of garlic on RIBE. However, RIBE inhibitory effects of other natural nutrients such as catechin [21], ascorbic acid [20], and vitamins C and E [8] have been demonstrated. In the study by Law et al., catechin only affected RIBE and had no radioprotective effect on directly irradiated cells, i.e., the number of micronucleated cells did not change as a result of catechin administration in target cells. [21] This observation is not similar to our result with respect to the direct radioprotective effect of garlic. Raw garlic decreased micronucleus formation in directly irradiated cells, as well as bystander cell groups. Besides the differences in radioprotectors used in these two studies, the variation in the results may be related to different radiations applied in these two studies. We irradiated cells with low linear energy transfer (LET) gamma rays, while they used high LET alpha particles to irradiate target cells. Low LET radiations predominantly attack DNA through free radicals that can be scavenged by garlic ingredients. However, direct DNA damages induced by alpha particles in directly irradiated cells obviously could not be eliminated by catechin in green tea.

Garlic could not increase the percentage cell viability of target cells, examined via MTT assay, although it decreased the frequency of micronucleated cells, measured by the micronucleus assay in target cell groups. Similarly, in other investigations, different effects of radioprotectors on various end points

have been observed. Kinashi *et al.* investigated the radioprotective effects of ascorbic acid 2-glucoside *in vivo* [20]. They examined apoptosis and micronucleus formation in spleen cells of mice under local radiation of head. They fed mice with ascorbic acid 2-glucoside. It significantly decreased the number of micronucleated cells, while the decrease of apoptotic splenocytes was not statistically significant. In another study carried out by Knopacka *et al.*, vitamin C or E did not influence the level of apoptotic bystander cells, whereas they decreased the number of micronucleated cells. [8] These authors suggested that factors causing micronucleation in bystander cells are regulated by apoptosis-independent pathways. Ineffectiveness of raw garlic extract in the percentage cell viability of target cells may be a noticeable observation. It can be concluded that garlic extract, as a radioprotector to prevent RIBE in normal tissues of patients on radiotherapy, would not increase the percentage cell viability of tumor cells, which are directly irradiated.

In this study, we reported some evidence for radiation-induced bystander response in MCF7 cells. This observation supports the results by Shao and colleagues [22]. They irradiated a fraction of MCF7 cells within a population with a precise number of helium-3, and found micronuclei in non-irradiated cells. However, despite the constant RIBE response observed in this study, they found that the number of micronucleated cells, induced in the whole population, was proportional to the number of helium particles attacking target cells. Such a difference may be attributed to different experimental conditions in the two studies (low LET radiation and medium transfer technique in the current study versus high LET helium particles for the irradiation of a fraction

of cells within the population in the mentioned study).

In addition to radioprotective effects, garlic has a pro-oxidant potential and acts as an anti-cancer medication [10, 15]. If the same components are responsible for both radioprotection and toxicity, it may be used in form of targeted molecules transferred by nanoparticles in tumor cells, to enhance therapeutic ratio in radiotherapy. It is not unwise to suppose that molecules accumulate selectively in tumors via targeted nanoparticles and partially diffuse in normal tissues (with low concentration) simultaneously act as a radiosensitizer and a radioprotector and eventually enhance the therapeutic ratio.

5. Conclusion

We demonstrated that raw garlic has the potential to reduce the detrimental effects of radiation, induced either directly or as a result of RIBE in MCF7 cells. Hence, garlic is recommended to be included in human diets, as its ability to mitigate RIBE may be of particular importance for exposure at environmentally relevant low doses, where RIBE dominates direct radiation effects. However, further comprehensive *in vivo* research regarding garlic treatment duration is required to support the obtained findings.

Acknowledgment

The authors would like to thank the vice-president for research of Mashhad University of Medical Sciences (MUMS) for funding this study. The authors are also grateful to the pharmaceutical research center of MUMS for providing the raw garlic extracts.

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