Radiation-induced DNA Double Strand Breaks and Their Modulations by Treatments with *Moringa oleifera* Lam. Leaf Extracts: A Cancer Cell Culture Model

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ABSTRACT

Gamma radiation brings deleterious effects upon human cells by inducing oxidative stress and DNA damages. Antioxidants have been shown to confer protective effects on irradiated normal cells. *Moringa oleifera* Lam. is a widely used nutritional supplement with antioxidant activities. This report showed that antioxidant-containing supplements, in addition to protecting normal cells, could protect cancer cells against genotoxic effects of gamma radiation. γ-H2AX immunofluorescent foci were utilized as an indicator of radiation-induced DNA double strand breaks. MCF-7 human breast adenocarcinoma cells were irradiated with 2-8 Gy gamma radiation. A linear relationship between the formation of γ-H2AX foci and radiation dose was observed with an average of 10 foci per cell per Gy. A 30-minute pretreatment of the cells with either the aqueous or the ethanolic extract of *M. oleifera* leaves could partially protect the cells from radiation-induced DNA double strand breaks. A pretreatment with 500 µg/mL aqueous extract reduced the number of foci formed by 15% when assayed at 30 minutes post-irradiation. The ethanolic extract was more effective; 500 µg/mL of its concentration reduced the number of foci among irradiated cells by 30%. The results indicated that irradiated cancer cells responded similarly to nutritional supplements containing antioxidants as irradiated normal cells. These natural antioxidants could confer protective effects upon cancer cells against gamma radiation

INTRODUCTION

Radiation therapy has long been accepted as a conventional treatment of malignant tumors. Gamma radiation is a low linear-energy-transfer radiation often used in radiation therapy. It principally acts via ionization of cellular water molecules, generating DNA-damaging free radicals, especially superoxide and hydroxyl radicals, resulting in oxidative DNA damage and double strand breaks, eventually leading to apoptosis [1-3]. Typical therapeutic doses may range from 20 to 80 Gy, which are given in fractions of 1.8 – 3 Gy each, depending on the type of cancer.

An early event in ionizing radiation induced DNA damage response is the rapid phosphorylation of histone H2AX to become γ-H2AX, which often serves as an indicator of the presence of DNA double strand breaks [4]. Discernable foci of DNA damage response components can be observed, which colocalize with phosphorylated H2AX [5]. γ-H2AX foci are induced vary rapidly within a few minutes upon irradiation; the number of foci reaches its maximum by 30 minutes to an hour. Then, the level of γ-H2AX declines gradually over time together with the reduction in the number of its foci. This decline has been shown to correlate with the activity of DNA damage repair components which are recruited to the double strand break sites. By 24 hours, γ-H2AX level will be reduced to almost its background level.

The observed damages brought about by gamma and other ionizing radiation are mainly caused by radiation-induced reactive oxygen species and radicals within and outside the cells. Many antioxidants have been shown to counter the effects of ionizing radiation including gamma rays. Antioxidant thiols confer protective effects on normal human lymphocytes against gamma radiation [6]. *N*-acetylcysteine, ascorbic acid, sodium ascorbate, co-enzyme Q10, α-lipoic acid, L-selenomethionine, and vitamin E succinate and their combinations have been shown to protect

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MCF-10A normal human breast epithelial cells against many types of ionizing radiation including gamma rays [7].

Due to this protective effect on normal cells, some patients with solid cancers requiring radiation treatments opted to take nutritional supplements with antioxidant activities for protection purposes. However, within cancer cells, radiation-induced oxidative stress is required to induce DNA strand breaks and eventual death. Therefore, consumptions of nutritional supplements or herbal products with antioxidant activity might hamper the action of ionizing radiation against cancer cells, especially if these cells respond similarly to the treatments of antioxidants as normal cells do. To address this question, an experimental system was set up using MCF-7 human breast adenocarcinoma cells as a cancer cell model and extracts of *Moringa oleifera* Lam. as antioxidants-containing nutritional supplements. Leaf extracts of *M. oleifera* had been shown to contain antioxidant phenolics including gallic acid, chlorogenic acid, ferulic acid, caffeic acid, and egallic acid [8]. The question was asked whether the extracts of *M. oleifera* leaves could protect breast cancer cells against radiation-induced DNA double strand breaks. γ-H2AX foci were utilized to monitor the extent of radiation-induced DNA damage.

EXPERIMENTAL METHODS

Cell culture

MCF-7 human breast adenocarcinoma cells (American Type Culture Collection, USA) were cultured in MEM medium (Invitrogen, USA) supplemented with 110 mg/L sodium pyruvate, non-essential amino acids, 10% fetal bovine serum, 100 µg/L streptomycin and 100 U/L penicillin in a 5% CO_2 incubator at 37^{o}C (Binder, Bohemia, NY, USA). The culture medium was replaced every 3 days. Cells were removed by a treatment with 0.25% trypsin and 0.1% EDTA.

Preparation of *Moringa oleifera* Lam. leaf extracts

Powdered dried leaves of *Moringa oleifera* Lam. were obtained from a commercial source. The aqueous extract was prepared 1:10 (w/v) in boiling distilled water for 10 min and left to cool down at room temperature for 1 h, filtered and lyophilized in a ScanVac freeze dryer (Labogene, Denmark). The ethanolic extract was prepared 1:10 (w/v) in 95% ethanol by shaking at room temperature for 24 h, filtered and evaporated in a Hei-Vac rotary evaporator (Heidolph Instruments, Germany). The extracts were both stored at -20°C until use.

Treatments with *Moringa oleifera* Lam. leaf extract

MCF-7 cells were seeded in 60 mm culture dishes. After 1 - 2 days of growth, the culture was replenished with fresh medium. To test the effects of *M. oleifera* leaf extracts on MCF-7 cells, the aqueous and the ethanolic extracts of *M. oleifera* were dissolved in phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO), respectively. They were filter-sterile and added directly to the culture medium at the concentration of 500 µg/mL for 30 minutes before subsequent irradiation. The concentration of DMSO was kept at 1% or less in the treated medium.

Radiation treatments

Cells were seeded and grown for two days to sub-confluence. On the day of radiation treatment, the growth medium was replaced with a fresh batch prior to and after irradiation. Cells were irradiated with gamma radiation from a ^{60}Co source in the culture medium at 0.50 – 0.70 Gy/min (Gems Irradiation Center, Thailand Institute of Nuclear Technology). Radiation absorbed doses were monitored using optically-stimulated luminescence dosimeters provided by Nuclear Technology Service Center, Thailand Institute of Nuclear Technology.

Immunofluorescence staining

Treated cells were fixed 30 min post-irradiation with cold 3.7% formaldehyde in phosphate-buffered saline for 10 min, blocked with 3% bovine serum albumin and 0.1% Triton X-100, immunostained with 2µg/mL mouse anti-phospho-histone H2AX (Ser139) (clone JBW301) (EMD Millipore, Billerica, MA, USA) for 1 h at room temperature, blocked and incubated with Alexa 488 goat anti-mouse $IgG_1(\gamma 1)$ secondary antibodies (Life Technologies Corporation, Carlsbad, CA, USA) at 1:500 dilution, and counterstained with 60 µg/mL bisbenzimide. The samples were washed at least three times with Tris-buffered saline between each step. For microscopic observation, they were mounted in ProLong® Gold (Life Technologies Corporation, USA). Gamma H2AX foci were observed with fluorescence imaging using an Olympus IX71 inverted

microscope (Olympus, USA). Images were captured with Olympus DP70 camera (Olympus, USA).

RESULTS AND DISCUSSION

Effects of gamma radiation

Cultured MCF-7 cells responded to gamma radiation similarly to other cell types. Upon irradiation with 2 – 8 Gy gamma rays, certain morphological changes could be observed at 30 minutes post-irradiation. Enlargement of cell and nuclear volume was specifically noted, which became more pronounced at higher radiation dose (Fig. 1a – d). Cells also appeared more rounded with increasing radiation dose. These observations were similar to what had been previously reported on irradiated MCF-7 and normal breast epithelial cells [9,10]. Alteration in plasma membrane morphology and reorganization of actin filament close to the plasma membrane had also been reported.

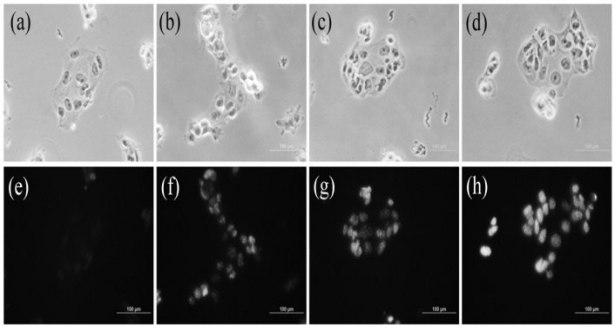

Fig. 1. MCF-7 breast adenocarcinoma cells 30 min post-irradiation, showing cell morphology (a – d) and γ-H2AX foci (e – h), which represent sites of DNA double strand breaks. Cells were irradiated with gamma radiation at 0 (a, e), 2 (b, f), 4 (c, g) and 8 (d, h) Gy. Images on the top and bottom panels are of the same sets of cells.

Irradiated MCF-7 cells were assayed for DNA double strand breaks by immunofluorescence staining with anti-γ-H2AX antibodies at 30 minutes post-irradiation. Without radiation treatment, no γ-H2AX foci were observed indicating that the background level of DNA double strand breaks was almost undiscernible. Upon gamma irradiation, foci of DNA double strand breaks were detected within the cell nuclei, appearing as bright spots when observed under the microscope (Fig. 1e – h). The number of foci increased linearly with increasing radiation dose from 2 to 8 Gy, yielding 10 γ-H2AX foci per cell per Gy on average. This observation confirmed the linear correlation between the number of DNA double strand breaks and radiation dose, which had been reported for other cell types [11]. The average number of foci was also similar to what was observed for irradiated normal human lymphocytes [12].

Effects of *Moringa oleifera* Lam. extracts

Moringa oleifera Lam. is a widely-used nutritional supplement, which has been shown to possess both antioxidant and anticancer activities including activities against breast cancer [8,13,14]. Aqueous and ethanolic extracts of *M. oleifera* were prepared and supplemented to MCF-7 cells for 24 hours. At 500 μg/mL, the aqueous extract appeared relatively non-toxic. No significant morphological changes were observed, and the cells remained attached to the substratum. On the other hand, the ethanolic extracts appeared toxic at 500 μg/mL. Treated cells were rounded and most of the cells became detached (data not shown). Therefore, for our purposes, cells were treated with *M. oleifera* extracts for only 30 minutes. With this treatment duration, no major changes in cellular morphology could be observed (Fig. 2a – c). When assayed for γ-H2AX foci, no significant changes in the foci number were detected (Fig. 2d – f), indicating that the aqueous and the ethanolic extracts of *M. oleifera* did not induce significant numbers of DNA double strand breaks in MCF-7 breast cancer cells.

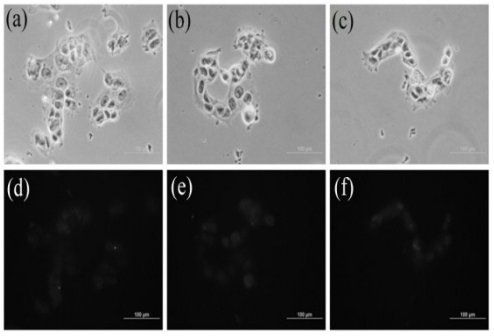

Fig. 2. MCF-7 breast adenocarcinoma cells after 30 min treatments with extracts of *Moringa oleifera* Lam.: 0 μg/mL (a and d), 500 μg/mL aqueous extract (b and e) and 500 μg/mL ethanolic extract (c and f). The top panels (a – c) show cell morphology and the bottom panels (d – f) show γ-H2AX foci of the same sets of cells.

Effects of treatment combinations

Aqueous and ethanolic extracts of *M. oleifera* were supplemented to MCF-7 breast cancer cells 30 min prior to and during gamma irradiation of 0, 2, 4, and 8 Gy. Cells were assayed for DNA double strand breaks at 30 minutes post-irradiation by immunofluorescence staining for γ-H2AX foci. As previously discussed, minimal DNA breaks were observed in untreated cells, and the number of

γ-H2AX foci per cell increased with increasing radiation dose. Not much differences in cellular and nuclear morphology were seen when cells were treated with *M. oleifera* extracts. However, the number of γ-H2AX foci per cell decreased when cells were treated with either the aqueous or the ethanolic extracts prior to and during irradiation, comparing to irradiated cells without treatments with either of the extracts. This observation held true for all irradiation doses from 2 to 8 Gy (Fig. 3). The ethanolic extract appeared more effective as the number of foci in pre-treated cells (Fig. 3f, l, r) was more reduced than cells pre-treated with the aqueous extract (Fig. 3e, k, q). Treatments with the ethanolic extract caused 30% reduction in the number of foci or events of DNA double strand breaks among irradiated cells, while treatments with the aqueous extract only brought about 15% reduction in DNA double strand break events.

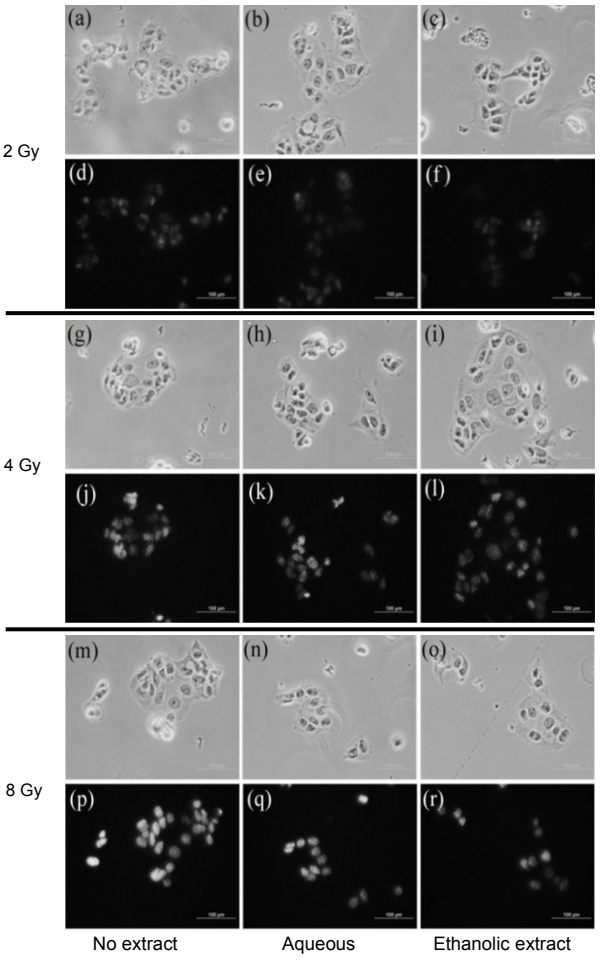

Fig. 3. Treatment combinations of gamma radiation and leaf extracts of *Moringa olerfera* Lam. MCF-7 cells were pre-treated for 30 minutes without the extracts (a, d, g, j, m, p) or with 500 µg/mL of either the aqueous (b, e, h, k, n, q) or the ethanolic extract (c, f, i, l, o, r). Radiation doses were indicated on the left of the panels. Phase-contrast images (a – c, g – i, m – o) showed cell morphology. Fluorescence images (d – f, j – l, p – r) showed γ-H2AX foci, which indicated the sites of DNA double strand breaks.

Moringa oleifera Lam. leaf extracts reduced radiation-induced DNA breaks

DNA double strand breaks are a type of gamma radiation-induced damage which is especially important. First, it is the major type of damage found resulting from gamma radiation. Experiments by Roots *et al*. [15] showed that the ratio of DNA double strand breaks to single strand breaks induced by 1 Gy gamma radiation could be as high as 110 in mammalian cells. Second, DNA double strand breaks are potentially lethal if left unrepaired, due to destabilization of the genome. It was estimated that natural causes resulted in 10 DNA double strand breaks per cell per day in normal human cells. γ-H2AX foci have been widely used to track DNA double strand break events in various cell types due to their close correlation with the number of DNA double strand breaks as assayed by the decay of incorporated ^{125}I [16]. In this report, it was shown that gamma radiation additionally induced approximately 10 DNA double strand breaks per cell per Gy in MCF-7 human breast adenocarcinoma cells, visualized as γ-H2AX foci. This number of breaks was comparable to the numbers reported for gamma-irradiated human lymphocytes [12], but very low comparing to the numbers reported for irradiated human lung fibroblasts where as high as 75 breaks were reported per cell after 2 Gy X-irradiation [17]. The observed differences indicated that the extent of radiation-induced damage to the DNA is cell-type dependent. Rothkamm and Löbrich (2003) have also shown that significantly different numbers of DNA breaks were induced in different fibroblast cell lines irradiated with the same radiation dose [17].

The ability of radiation to produce adverse effects on cancer cells constitutes the basis for the use of radiotherapeutic approach to cure cancer. Ionizing radiation induced oxidative stress among irradiated cells leading to an increased level of intracellular reactive oxygen species, lipid peroxidation and oxidative damage to the DNA [18-21] as we have observed in irradiated MCF-7 cancer cells. *Moringa oleifera* Lam. is one of many plants which have been shown to possess antioxidant activities and suspected to have ameliorative effects against ionizing radiation. The leaf extracts exhibited 1,1-diphenyl-2-picrylhydrazyl, superoxide and hydroxyl radical scavenging activities, inhibitory effects on lipid peroxidation, and protective effects against oxidative damage to naked DNA *in vitro* [8,22-26]. This report assayed the ability of the aqueous and the ethanolic extracts of *M. oleifera* leaves to

protect MCF-7 cells from the genotoxic effect of acute gamma radiation from 2 to 8 Gy. Interestingly, both types of extracts were able to protect the cells from radiation-induced DNA double strand breaks as evidenced by the reduction of γ-H2AX foci upon pre-treatments with either of the extracts.

Many herbal extracts with antioxidant activities have been shown to exhibit protective effects upon irradiated normal cells. These extracts must be administered prior to radiation treatment. For example, extracted zingerone, a phenolic alkanone from ginger, was shown to exert radioprotective effects on Chinese hamster fibroblast cells (V79) when given to the cells 1 h prior to radiation exposure [27]. Studies have been conducted to investigate *M. oleifera*'s protective effects against radiation-induced oxidative stress. Swiss albino mice were injected with a total dose of 150 mg/kg body weight of 50% methanolic extract of *M. oleifera* leaves as a single or five daily doses prior to a whole-body gamma irradiation at 4 Gy. Supplementation of the extract was able to reduce percent aberrant erythrocytes as seen by metaphase chromosome aberration and micronucleus induction [28], indicating the radioprotection efficacy of *M. oleifera*. Although not a scientific study, in Thailand, there has been a report that the aqueous extract of *M. oleifera* could alleviate radiation toxicity in cancer patients receiving radiation therapy [13], another support for its radioprotective ability.

CONCLUSION

The above studies showed the radio-protective effects of antioxidant herbal extracts upon normal cells. The study reported here is one of a few that investigated these effects on irradiated cancer cells. The results of this investigation indicated that a 30-minute pre-treatment with *M. oleifera* leaf extracts yielded 15 – 30% reduction in radiation-induced DNA double strand breaks in MCF-7 human breast cancer cells. This observation suggested that irradiated cancer cells respond similarly to natural antioxidants as irradiated normal cells. Radiation protection that was to be conferred upon normal cells would also be perceived by cancer cells. Additional investigation into the effects of antioxidant pretreatments on the eventual survival or death of irradiated cancer cells would be an important next step in understanding the implications of the radio-protective effects of antioxidant supplements on cancer patients.

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