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Radiosensitizing Effect of Bromelain Using Tumor Mice Model via Ki-67 and PARP-1 Inhibition

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Abstract

Recent reports have shown that bromelain (BL), a pineapple extract, acts as an adjuvant therapy in cancer treatment and prevention of carcinogenesis. The present study was designed to investigate the possible mechanisms by which BL could radiosensitize tumor cells *in vitro* and in a mouse tumor model. BL has shown a significant reduction in the viability of the radioresistant human breast carcinoma (MCF-7) cell line using cell proliferation assay. The in vivo study was designed using the Ehrlich model in female albino mice, treated with BL (6 mg/kg b. wt., intraperitoneal, once daily for 10 days) 1 hour before exposure to a fractionated dose of gamma radiation (5 Gy, 1 Gy for 5 subsequent days). The radiosensitizing effect of BL was evident in terms of a significant reduction in tumor volume, poly ADP ribose polymerase-1 (PARP-1), the proliferation marker Ki-67 and nuclear factor kappa activated B cells (NF-κB) with a significant elevation in the reactive oxygen species (ROS) content and lipid peroxidation (LPO) in tumor cells. The present findings offer a novel insight into the radiosensitizing effect of BL and its potential application in the radiotherapy course.

Keywords

bromelain, MCF-7, Ehrlich, gamma radiation, radiosensitizing effect

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Introduction

Radioresistance is the adjustment of tumor cells toward the radiation therapy and developing resistance to the radiation beams by using multiple genes, factors, and mechanisms. Also, it obstructs the improvement of cancer patients' treatments with radiation leading to radiation therapy failure and poor prognosis.[1,2] This is most likely to occur in cancer cells of some breast tumors which contain lower ROS levels and enhanced ROS defenses compared to equivalent non-tumorigenic cells, which may contribute to tumor radioresistance.[3] Moreover, one of the major limitations of radiotherapy (RT) is that the cells of solid tumor become deficient in oxygen. The more hypoxic the tumors are, the more resistant they are to the effects of radiation; because oxygen causes the radiation damage to DNA.[4,5] So, fractionated irradiation gives chance for reoxygenation between fractions, leading to an increase in the efficiency of RT and, to a decrease of normal tissue complications.[6] Besides, the usage of radiosensitizers could overcome these limitations and improve the treatment. Radiosensitizers are substances that render cells more susceptible to RT.[7] Targeting for DNA repair-deficient mechanisms, inhibiting tumor cell proliferation, and increasing lipid peroxidation and ROS contents are considered major hallmarks of sensitization to resistance.[8-10]

Proliferative activity of cancer cells is an important factor for assessing the biological behavior of carcinoma, predicting clinicopathological and prognostic significance. Ki-67 is widely used to reflect proliferation in breast, lung and gastric carcinomas to distinguish between tumors with low and high proliferative activity.[11-13] PARP inhibitors are becoming important actors in many resistant cancer cells;

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they are also promising drugs for combined treatments particularly with radiotherapy.[14,15]

The synthetic therapeutics have shown limited improvement in treatment in addition to their side effects. Given that, searching for natural radiosensitizers is needed to establish a potential future drug in cancer therapy as they are believed to be safer than synthetic ones. Using natural radiosensitizers, such as genistein, curcumin, and quercetin in combination with RT was found to be more beneficial than synthetic compounds.[1]

Bromelain (BL) belongs to proteolytic enzymes. It contains an SH- group which is essential for its catalysis.[16] It has a wide range of therapeutic benefits such as anti-platelet aggregation, anti-obesity, cardioprotective, anti-inflammatory and analgesic.[17-20] Very recently, it has been found that BL treatment has significantly weakened the SARS-COVID-2 infection in VeroE6 cells and COVID-19[21,22]; moreover, it has been also studied during acute asthma and it displayed a decrease in markers of *lung* inflammation.[23] BL also has anti-metastatic and anti-proliferative effects, besides increasing autophagy and apoptosis of cancer cells.[24-26] Polyenzymatic proteolytic drugs were found to improve the results of RT and prevent post-radiation fibrous changes and side effects.[27] Thus, utility of systemic enzyme therapy is capable of improving the quality of life so it could be beneficial for cancer patients.[28] The strategies used to improve radiotherapy aim to increase the effect on the tumor or to decrease the effects on normal tissues. The radioprotection effect of BL on normal cells has been evaluated recently,[29] hence the current work is designed to study the radiosensitizing mechanism of BL in vitro as well as in vivo through inhibiting proliferation and DNA repair mechanisms of tumor cells, aiming to find a potential adjuvant therapy for cancer patients.

Materials and Methods

In Vitro MTT Cell Proliferation Assay

Cell viability of human breast carcinoma cells (MCF-7) was evaluated in vitro by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[30,31] to confirm the antitumor and radiosensitizing activity of BL. Cell culture MCF-7 cell line (Tissue Culture Unit, The Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt) was cultured in growth medium containing 10% FBS, 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Four groups were used in this study: First: MCF-7 cells (served as a control). Second: MCF-7 with double-fold dilutions of BL in PBS (0.009-5 mg/ml). Third: MCF-7 cells exposed to gamma radiation (6 Gy).[32] Fourth: MCF-7 incubated with double-fold dilutions of BL for 1 hour before irradiation (IRR). Cells were checked under microscope for any physical signs of toxicity, for example partial or complete loss of the monolayer, shrinkage and rounding or cell granulation. Finally, 20 ul of MTT reagent (Bio Basic Inc., Canada) was added. The plate was incubated for 1 to 4 hours to allow the MTT to be metabolized and optical density was measured on a microplate reader at a wavelength of 590 nm. Each sample made in triplicate.

$$\text{Viable cell } \% = (\text{Absorbance of samples} - \text{Absorbance of blank}) / (\text{Absorbance of control} - \text{Absorbance of blank}) \times 100$$

The inhibitory concentration 50% (IC_{50}) is the dose of a drug that reduces the viability to 50%, and it was calculated using non-linear regression analysis.

Animals

Adult female Swiss albino mice weighing 25 to 30 g were obtained from the breeding unit of the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt. This study was approved to be performed by the Committee of Scientific Ethics at Faculty of Pharmacy, Al-Azhar University, Egypt, following the guidelines for animal use. The animals were housed in plastic cages (10 mice/cage) under proper environmental conditions that is 12 hours dark/light cycle, good ventilation condition and temperature, and 40% to 55% humidity at the NCRRT animal house, fed with nutritionally adequate standard diet pellets, provided with water ad libitum. Mice were left 1 week for acclimatization on the lab environment before starting the experiment. They were monitored during the study according to a standardized method by means of monitoring sheets and scoring chart.[33] Mice were treated gently; toughness, pressure, and squeezing were avoided. Injection was done by a professional staff member.

Tumor Transplantation

Ehrlich ascites carcinoma (EAC) cell line was supplied by serial sub-culturing at the National Cancer Institute, Cairo University, Egypt. It was implanted in each donor (female Swiss albino mice) by intraperitoneal (i.p) injection of 2.5×10^6 cells/22 g b. wt, and allowed to multiply.[34] To count the EAC cells before i.p injection, the bright line hemocytometer was used and trypan blue was added to count the viable cell under microscope.[35] The solid tumor was obtained by the intramuscular inoculation of 0.2 ml of 1×10^6 viable EAC cells in the right lower limb of each mouse.[36] Mice with a palpable solid tumor diameter (10 mm^3) that was maintained within 7 to 10 days after inoculation were used for the study.

Pilot Studies

Pilot experiments were carried out in order to evaluate the following:

(a) Any toxic effects of BL and the most proper dose. Five groups of female mice were used to choose the most effective dose of BL. The first group included normal mice injected with PBS, while the other 4 groups examined the daily injection of BL (6 and 12 mg/kg) for 10 days, each dose examined alone and with irradiation.

The mortality rate, body weight change and complete blood picture were examined. It was found that BL in 6 mg/kg dose was safer than 12 mg/kg, with low to no mortality occurrence. So this dose was selected for the further studies.

(b) The radiosensitizing effect of BL on tumor cells (ESC) was studied using different irradiation models. Five groups of female mice were implanted with EAC to form solid tumors. The first group served as untreated ESC-bearing mice injected with PBS, while the second and third groups examined the effect of the irradiation doses, 1 Gy × 5 per 5 days and 2 Gy × 3 per 3 days respectively. The fourth and fifth groups examined the effect of chosen dose of BL (from the pilot study (a)) with gamma irradiation doses 1 Gy × 5 and 2 Gy × 3 respectively.

Tumor volume and weight as well as the levels of tumor LPO were estimated, in addition to the histopathological examination of the solid tumors from which the tumor area percentage was determined. It was found that 1 Gy × 5 irradiation dose was more effective.

Animal Grouping

Forty animals were randomly divided into 4 groups (10 mice each). Group 1: mice bearing Ehrlich solid tumor (EST) injected i.p with phosphate buffered saline (PBS) for 10 days. Group 2: mice bearing EST were exposed to a fractionated dose of γ-radiation (1 Gy × 5) daily (the last 5 days of the injection) and injected i.p with PBS for 10 days. Group 3: mice bearing EST received freshly prepared BL dissolved in PBS (6 mg/kg, i.p according to a pilot study), daily for 10 days, BL purchased from Merck KGaA Co. (Darmstadt, Germany). Group 4: mice bearing tumor were subjected to combined treatment, BL daily injection for 10 days then irradiation (1 Gy) 5 times, where BL treatment was 1hr before irradiation process. Mice were scarified 3 days after last irradiation dose.

Radiation Processing

Whole body γ- irradiation of mice was carried out using Cesium 137 Gamma cell-40 biological irradiator (Nordion-Canada), installed in the NCRRT, Cairo, Egypt. The dose rate was 0.675 Gy/minute during the experimental period. Daily correction for humidity, barometric pressure, and temperature were made.

Estimation of Tumor Volume

Two-dimensional measurements of solid tumors were estimated using Vernier caliper on daily basis and documented data used for applying the following equation: $\textbf{\textit{Tumor volume}} = \frac{1}{2}(length \times width^2)$ [37] and the change in tumor volume was calculated as $Vf - Vi / Vi \times 100$, where V_f is the final volume and V_i is the initial volume.

Parameters Assessed in Ehrlich Solid Tumor (EST)

Ki-67 immunohistochemical (IHC) analysis. Formalin-fixed (10%), paraffin-embedded, 4 μm-thick sections of EST tissue of the right thigh muscles mounted on positive charged slides were used for immunohistochemical staining. Immunohistochemical staining of Ki-67 (a tumor cell proliferation marker) was carried out according to the manufacturer's instructions (Ab: 275R-16 Cell Marque—USA). After addition of antigen retrieval with 10 mM sodium citrate buffer (pH = 6) for 10 minute, endogenous peroxidases were blocked by 3% H_2O_2 in PBS for 10 minute, the slides were then incubated overnight with primary antibodies against Ki-67 (1:200 dilution) at 4°C and then slides were washed and incubated with power stain poly HRP (horseradish peroxidase). DAB (3,3'-diaminobenzidine) kit for mouse and rabbit detection system from Genemed—USA (Cat# 52-0017) was used to visualize any antigen-antibody reaction in the tissues. The sections were counterstained with hematoxylin dye and digitally imaged (X400) using light microscope (CX21, Olympus, Japan).

For IHC quantification, each sample scores for at least 5 high power fields (3 animals per group). The % of Ki-67 immunostained cells was defined as the percentage of the number of positively nuclear stained cells with respect to the total number of cells in the entire field of the specimens.[38]

Molecular analysis. The mRNA levels of PARP-1 and NF-κB genes; and of the housekeeping gene β-actin were measured by real time polymerase chain reaction (RT-PCR). Total RNA was isolated from tumor tissues using Qiagen tissue extraction kit (Qiagen, USA) in accordance with the manufacturer's instructions. The extracted RNA (0.5-2 μg) was used for cDNA conversion using high capacity cDNA reverse transcription kit (Fermentas, USA) and 12.5 μl reaction volume SYBR chemistry in Applied Biosystems 2720 Thermal Cycler, USA to amplify PCR under the following conditions: 50°C for 2 minute, 95°C for 10 minute, and 40 cycles at 60°C for 1 minute and 72°C for 1 minute; using primers mentioned in Table 1.

Table 1. Sequences of Primers for Real-Time Quantitative PCR.

Gene	Forward primer	Reverse primer
PARP-1 NM007415.2	5′- CCATCGACGTCAACTACGA -3′	5′-GTGCGTGGTAGCATGAGTGT -3′
NF-κB DQ020180.1	5′- CAATGGCTACACAGGACCA -3′	5′-CACTGTCACCTGGAACCAGA -3′
β-Actin NC000071.6	5′-GCGTGGGGACAGCCGCATCTT-3′	5′- ATCGGCAGAAGGGGCGGAGA -3′

Table 2. Cytotoxic Activity of Bromelain and/or γ-Irradiation (6 Gy) Against Human Breast Carcinoma Cell Line (MCF-7).

	Viability %	
Bromelain concentration (mg/ml)	Non irradiated MCF-7	Irradiated MCF-7
5	34.2 ± 0.005*	21.3 ± 0.006*,#
2.5	49.1 ± 0.014*	35.1 ± 0.007*,#
1.25	80.3 ± 0.001*	57.2 ± 0.006*,#
0.625	31.2 ± 0.002*	74 ± 0.007*,#
0.312	95.8 ± 0.01	79.5 ± 0.009*,#
0.156	99.8 ± 0.012	83.4 ± 0.002#
0.078	99.7 ± 0.018	89.7 ± 0.007
0.039	99.7 ± 0.001	92.4 ± 0.016
0.019	100 ± 0.011	100 ± 0.022
0.009	100 ± 0.014	100 ± 0.007
MCF-7 cells (BL = 0 mg/ml)	100 ± 0.014	100 ± 0.014
IC_{50} (mg/ml)	0.801 ± 0.02625	0.735 ± 0.04631

Each value indicates the mean of 3 records ± S.E. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison tests.
*Significant versus control MCF-7, where #:significant versus irradiated MCF-7 at $P < .05$. IC_{50} ± S.E values were calculated using non-linear regression analysis.

For expression of results, the comparative ΔΔCT method was used for relative mRNA quantification of target genes, normalized to an endogenous reference β-actin and a relevant control, equal to $2^{-\Delta\Delta CT}$. ΔΔCT is the difference between the mean $\Delta CT_{(sample)}$ and mean $\Delta CT_{(control)}$, where $\Delta CT_{(sample)}$ is the difference between the mean CT (sample) and the mean CT (β-actin) and $\Delta CT_{(control)}$ is the difference between the mean CT (control) and the mean CT (β-actin).

Poly ADP ribose polymerase-1 (PARP-1) protein analysis. PARP-1 level was determined by ELISA technique using a readymade kit (MyBiosource, USA) according to manufacturer's instructions in the tumor homogenate. PARP-1 was calculated after plotting the standard curve and expressed as ng/ml.

Oxidative stress analysis. LPO was estimated by measurement of malondialdehyde (MDA) formation using the thiobarbituric acid method [39]. A modified technique of Vrablic et al[40] was used to measure the generation of ROS by the intracellular conversion of nitro blue tetrazolium (NBT) to formazan by the action of superoxide anion.

Statistical Analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA). The groups were compared by Tukey-Kramer test, tumor volumes monitoring along days measured through the experiment were analyzed by 2-way ANOVA followed by Bonferroni's posttest and the graphs were sketched using GraphPad Prism (ISI® Software, USA) version (5) software. Data were presented as mean ± standard error (S.E) and P values $<.05$ were considered significant.

Results

Effect of BL/irradiation on MCF-7 Cells Viability

We examined the radiosensitizing effect of BL on MCF-7 cells by performing MTT assay. From Table 2, exposure of MCF-7 cells to 6 Gy γ-radiation showed a non-significant decrease in cell viability compared to control untreated cells reflecting the radioresistance of MCF-7 cell line. BL pretreatment showed significant cytotoxic activity, causing a decrease in the absorbance readings indicating the presence of less viable cells (IC50 value of 0.801 mg/ml); however the most prominent cytotoxic effect appeared when the MCF-7 cells were subjected to BL and then to γ-radiation compared to control or irradiated cells. The IC50 value was decreased to 0.735 mg/ml. In addition, BL treatment produces partial or complete loss of the monolayer and shrinkage of the cells, as well dead cells could be observed

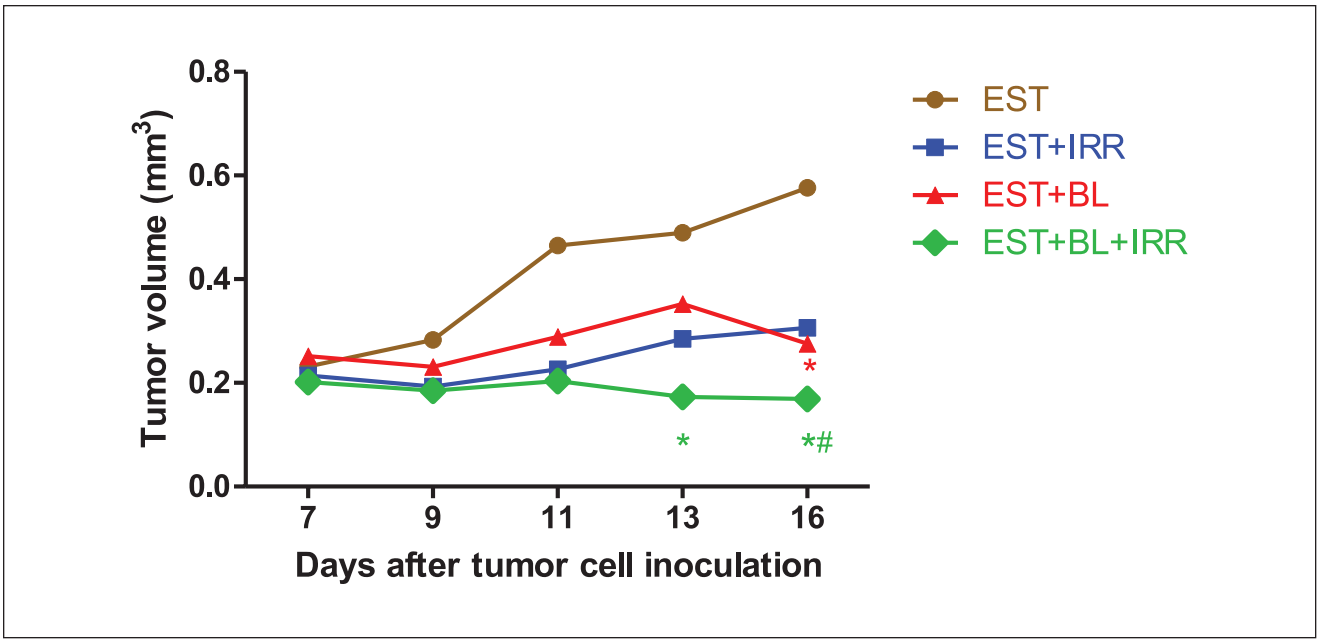

Figure 1. The progression in tumor volume starting from day 7 after cancer cells inoculation, values shown as mean ± S.E. Statistical analysis was carried out by two-way ANOVA followed by Bonferroni's posttest.
*Significant versus EST group, where #: significant versus irradiated-EST group at $P < .05$.

aggregated together and floating in the medium according to BL concentration.

Effect of BL/irradiation on Tumor Volume

Regarding the documented observations of tumor volume growth in Figure 1, beginning from the appearance of tumor, after 7 days from challenge with viable Ehrlich tumor cells, the treatment of EST bearing mice with BL alone showed significant decrease in tumor volume at day16 compared to the untreated EST group. Treatment of EST bearing mice with BL, and then exposure to radiation showed a significant decrease in tumor volume at day 13 compared to the untreated EST group at day 16 compared to untreated and irradiated EST groups. Table 3 shows that the tumor volume was increased by 50.13% in the control EST group, while in the γ- irradiated EST group it was increased by 29.06%, but BL treatment resulted in 20.49% increase, however the combined treated group showed almost no increase in tumor volume (0.09%).

Effect of BL/irradiation on Tumor Cell Proliferation Index (Ki-67) in EST Bearing Mice

To find out how tumor cell proliferation is affected by BL/irradiation treatment, immunohistochemistry assay was employed on EST to compare the nuclear expression of Ki-67 protein in different groups. As seen in Figure 2a to d, treatment with BL reduced Ki-67 expression, with the lowest expression observed in the combination therapy group.

Table 3. Tumor Volume Change Percent of EST-Bearing Mice Treated With IRR (1 Gy × 5) and/or BL (6 mg/kg).

Groups	Tumor volume change (%)
EST	50.13 + 2.8
EST + IRR	29.06 + 2.8*
EST + BL	20.49 + 1.4*
EST + BL + IRR	0.09 + 0.009*,#

The values shown are mean ± S.E of data, *: Significant versus EST group, where #: significant versus irradiated-EST group at $P < .05$. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison tests.

Ki-67 index was calculated for each group as the percentage of the positively stained cells relative to the total number of cells. Mean Ki-67 index was 10.7% for the BL + EST-irradiated group, 16.32% for the BL + EST group and 27.31% for EST-irradiated group, compared with 50% in the untreated EST group (Figure 2e); indicating that combination therapy significantly more effective than single agent therapy reflecting the beneficial effect of BL in sensitizing tumor cells toward radiotherapy.

Effect of BL/irradiation on PARP-1 Level and Relative Gene Expression of PARP-1 and NF-κB in EST Bearing Mice

To test other mechanisms by which BL could sensitize tumor cells toward radiation therapy, PARP-1 (a DNA repairing protein) level as well gene expression were estimated. It was

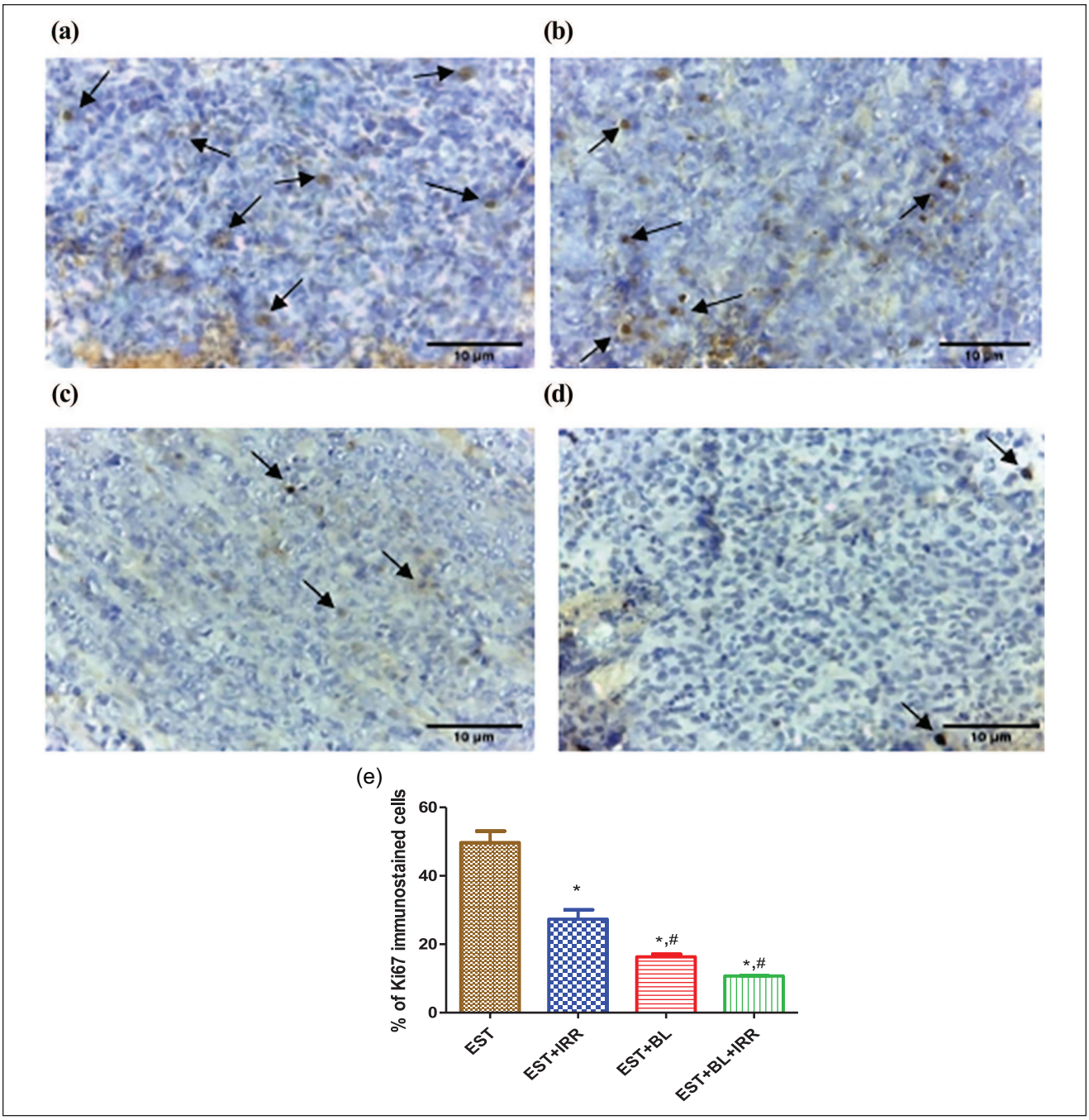

Figure 2. Immunohistochemical analysis of the expression of Ki-67 in the EST xenografts of the right thigh muscles counterstained with hematoxylin. (a) High nuclear expression (+++) for untreated-EST group. (b) Moderate nuclear expression (++) for irradiated-EST group. (c) Low (+) nuclear expression for bromelain-EST treated group. (d) Low (+) to non-nuclear expression for BL + irradiated-EST group, (400×), arrows represent the Ki-67 positivity. (d) Ki-67 index values expressed as mean of 15 records from 3 animals ± S.E. (e) Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison tests.

*Significant versus EST group, were #: significant versus irradiated-EST group at $P < .05$.

found that, both level and mRNA expression of PARP-1 were inhibited in the tumor tissues upon treatment with BL or radiation compared to the Ehrlich untreated group. However the combined therapy showed the maximum inhibitory effect compared with irradiated or untreated Ehrlich bearing groups, reflecting a potential radiosensitizing effect of BL (Figure 3).

NF-κB, a proliferation marker, was measured in the current study to confirm the potential radiosensitizing effect of

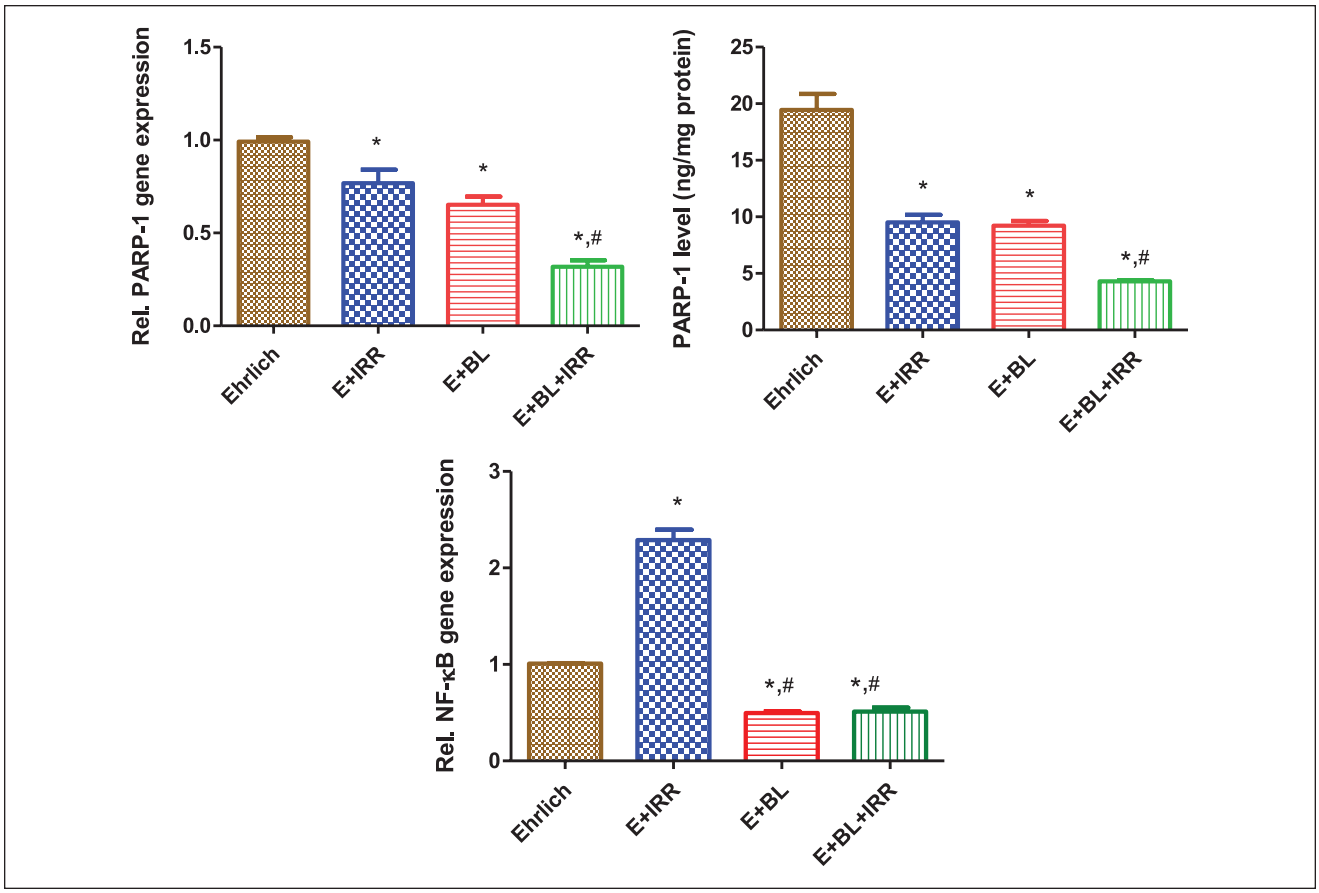

Figure 3. PARP-1 levels, relative gene expression of PARP-1 and NF-κB in tumor tissues of EST bearing mice treated with IRR (1 Gy × 5) and/or BL (6 mg/kg). Each value represents the mean of 5 records ± S.E. Statistical analysis was carried out by 1-way ANOVA followed by Tukey-Kramer multiple comparison tests.
*Significant versus EST group, where #: significant versus irradiated-EST group at $P < .05$.

BL. It was found that the NF-κB mRNA gene was highly expressed upon radiation treatment showing apparent resistance, while BL treatment reflected a sensitizing effect through the significant inhibition of NF-κB compared with the control untreated-EST and irradiated-EST (Figure 3).

Effect of BL/irradiation on MDA and ROS levels in EST bearing mice. Biomarkers of oxidative stress, MDA and ROS, are important tools in assessment of cancer therapy. Their high level indicates the degree of apoptosis and proliferation.[41] In this study bromelain treatment alone or prior to radiation exposure showing significant increase in ROS and MDA levels in tumor tissues compared to control EST or irradiated-EST groups (Figure 4).

Discussion

Many factors may affect the radioresistance of cancer cells such as reoxygenation, DNA repair, apoptosis, and proliferation. Hypoxia of the solid tumor tissue is considered a major cause radioresistance. The current study overcomes this problem by using a fractionated dose of radiation to permit tumor reoxygenation between fractions and makes the tumor more sensitive to radiotherapy.[42] Treatment of MCF-7 cell line with BL alone or prior to irradiation showed a significant decrease in cell viability compared to control and irradiated cells, indicating that BL possesses in vitro radiosensitizing activity against the radioresistant MCF-7 cell line. The radioresistance of MCF-7 breast carcinoma cells was mention before in the study of Jänicke et al.[43] Previous reports showed that the in vitro treatment of tumor cell lines with BL results in inhibition of cell growth and invasion capacities.[26,44,45] It has been reported that BL has a tumoricidal activity in vitro via induction of ROS and subsequent mitochondrial membrane depolarization, later on leading to oxidative stress as well as through its autophagy action.[46,47] The anticancer effect of BL has been credited mainly to its protease component through digestion and diffusion in tumor cells.[25,26] As another possible mechanism, BL has been shown to selectively induce apoptosis in tumor cells by upregulating p53 and Bax expression. This facilitates the release of cytochrome c into the cytosol, and the

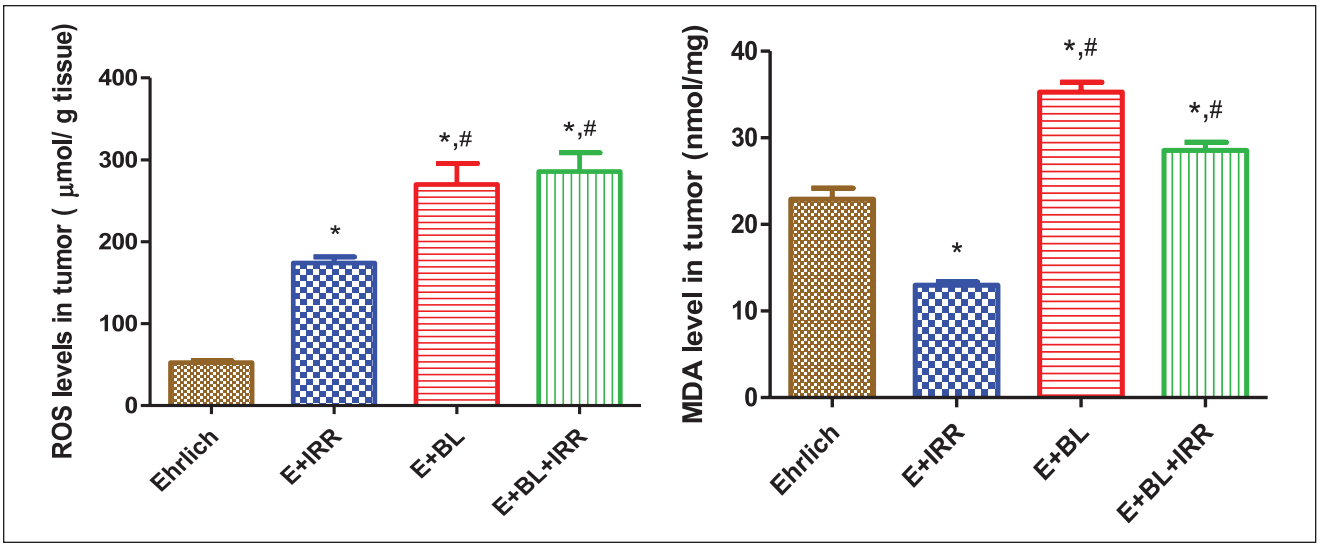

Figure 4. Levels of MDA and ROS in tumor tissues of ESC bearing mice treated with IRR (1 Gy × 5) and/or BL (6 mg/kg). Each value represents the mean of 6 records ± S.E. Statistical analysis was carried out by 1-way ANOVA followed by Tukey-Kramer multiple comparison tests.
*Significant versus Ehrlich group, where #: significant versus irradiated group at $P < .05$.

heme iron of cytochrome c would act as a catalytic center to enhance ROS production which in turn would provide a background for the development of apoptosis.[23,48]

Since the growth of most cancers is partly dependent on proliferation of the tumor cells, Ki-67 and NF-κB (a proliferative markers) have been assessed in the current study. Here, the regression of Ki-67 and NF-κB in BL treated groups indicates impairments of EST proliferation. However, when comparing the BL+IRR group with the IRR group, a low percentage of Ki-67 positive cells and inhibition of NF-κB expression occurred. The NF-κB expression signal was triggered in the IRR samples due to the increase in ROS levels and protein kinase which in turn activates NF-κB expression.[49,50] Previous study of El-Naa et al[51] has shown that the activity of Ehrlich solid tumors excised from mice could be inhibited by down-regulation of NF-κB and Ki-67 expression, since radiosensitizing drugs should inhibit proliferation.[52] Hence, we could postulate BL as a radiosensitizer. An assessment of malignancy in some tumor entities has shown that Ki-67 is highly susceptible to protease treatment.[53] A study by Amini et al[54] reported that combined treatment of BL and N-acetyl cysteine inhibited cell proliferation and tumor growth in in vitro and in vivo gastrointestinal tumor model through the reduction of Ki-67 expression. Several studies have explored the impact of NF-kB inhibition on radiosensitivity in various models.[55,56] For instance, cisplatin, one of the main chemotherapeutic and radiosensitizing agents, through inflicting DNA damage has been shown to inhibit NF-κB activity and expression of antiapoptotic genes.[57] Moreover, it was shown that BL-induced blockage of NF-κB/Cox2/PGE2 pathway may inhibit the inflammatory response in tumor resulting in suppression of tumor progression.[58,59] Further, it was considered that drugs which inhibit NF-κB, COX-2, and PGE2 activity may have a potential cancer treatment potency.[60]

PARP-1 is a multitasking enzyme that controls many intracellular processes, including DNA repair, signaling and transcription. The proliferating cancerous and non-cancerous cells such as macrophages possess high PARP-1 expression.[61] To gain more insight into the radiosensitizing action of BL, PARP-1 activity and relative gene expression were assessed in the current study. EST bearing mice treated with BL and exposed to radiotherapy revealed significant PARP-1 inhibition in comparison with either irradiated or untreated EST bearing mice. These data again support our conclusion about BL treatment, that it could overcome the radioresistance of tumor cells. Other reports indicated that PARP-1 inhibition effectively sensitizes cancer cells to IRR in both in vitro and in vivo models.[8,62,63] Additionally, in previous studies BL showed an inhibition in PARP-1 cellular proteins, indicating that BL induces cell death mainly through apoptosis and regression in DNA repair mechanisms.[64,65] Since the essential role of PARP is double and single strand break (DSB and SSB) recognition and repair, the underlying mechanism of radiosensitization by PARP inhibitors has been suggested to be caused by a delay in DSB and SSB repair processing, resulting in DNA break accumulation and cell death,[66,67] especially for tumors with DNA repair defects, such as BRCA mutations.[14] In addition, vasoactivity has been reported for some PARP inhibitors which may modulate the levels of hypoxia within tumors. Indeed, as hypoxic cells are more radioresistant than oxic

cells, the reoxygenation of hypoxic tumors could be of therapeutic benefit.[42] Inhibition of PARP activity can sensitize hypoxic cancer cells and the combination of ionizing radiation and PARP inhibition has the potential to improve radiotherapy.[8,68]

ROS synthesized during mitochondrial respiration exacerbate the DNA damage caused by radiotherapy.[69] ROS levels may influence the extent to which cancer cells are resistant to therapies like radiotherapy.[3,70] LPO has been used as an indicator for ROS-caused damage to cell membranes. MDA is one of the paramount end-products of peroxidation of polyunsaturated fatty acids and is commonly used to estimate oxidative stress.[41,71] In the present study, the MDA level in irradiated groups was significantly lower than the non-irradiated EST group. while the levels of MDA and ROS were found to be increased in the tumor tissues of EST bearing mice upon treatment with either BL alone or combined with IRR, in comparison with both untreated and irradiated EST bearing group. The low MDA levels in irradiated samples may be due to the radioresistant nature of the solid tumor. Therefore, there is no damage to the cell membrane to produce MDA. While the increased level of MDA in the cancer non-irradiated group may be due to the pattern of MDA-metabolizing enzymes in the tumor cells, the lipid composition of the cell membranes with a different level of peroxidizable substrates, such as polyunsaturated fatty acids, and the presence of inflammatory cells, which can increase the level of diffusible MDA from the tumor surrounding tissues.[72,73] Previous reports suggested that BL induced ROS production in monocytes of patients suffering from X-linked agammaglobulinemia and in carcinoma A431 and melanoma A375 cells causing subsequent mitochondrial membrane depolarization later on leading to oxidative stress and also through its autophagy action.[46,58,74] Also, treatment with BL has been demonstrated to induce ROS and reactive nitrogen species in macrophages as well as to decrease levels of free glutathione (GSH).[65] Depletion of GSH content may increase the radiation sensitivity of tumor cells due to a reduction in the radioprotective reaction.[75] This action of BL has been attributed to its cysteine protease activity as cysteine is an amino acid that limits GSH biosynthesis (ROS quencher).[76] This may account for the high level of ROS in tumor tissues, which in turn react with the polyunsaturated fatty acids of lipid membranes and induces lipid peroxidation.[73,77] These outcomes reflect the apoptotic and killing effect of BL together with inhibiting Ki-67 and PARP-1, which give BL the opportunity to act as a significant radiosensitizer.

Conclusion

These findings may prove bromelain to be a novel addition to radiotherapy. BL possessed a radiosensitizing action through increasing LPO and ROS production in tumor tissue, inhibition of repair of DNA strand breaks and inhibition of proliferation. Ki-67, PARP-1, and NF-κB are useful prognostic biomarkers in the evaluation of BL as a radiosensitizer.

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Declaration of Conflicting Interests

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