Research Article

Bromelain Enhances the Anti-tumor Effects of Cisplatin on 4T1 Breast Tumor Model In Vivo

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Abstract

Background: This study aimed to evaluate the antitumor enhancing effect of bromelain consumption on 4T1-challenged mice treated with cisplatin. **Methods:** Mice challenged with 4T1 triple-negative breast cancer cells received water, bromelain, cisplatin, or bromelain + cisplatin treatment for 28 days. Tumor size was measured, and lung metastasis was evaluated by clonogenic assay. Expression of tumor inflammatory genes of the harvested tumor was quantified by polymerase chain reaction array and ELISA (enzyme-linked immunosorbent assay). **Results**: All treatments significantly reduced the size of tumor and lung metastasis, with combination treatment showing the best effect. Also, bromelain alone and combination treatment showed downregulation of the expression of tumor inflammatory genes (Gremlin [GREM1], interleukin 1β [IL-1β], interleukin-4 [IL-4], nuclear factor κB subunit 1 [NFκB1], and prostaglandin-endoperoxide synthase 2 [PTGS2]), tumor nitric oxide level, and serum IL-1β, and IL-4 levels. On the other hand, cisplatin treatment increased the expression of selected inflammatory markers. **Conclusion:** This study suggests that bromelain treatment could potentiate the antitumor effect of cisplatin on triple-negative breast cancer 4T1 cells through modulating the tumor environmental inflammation.

Keywords

antitumor, bromelain, cisplatin, inflammation, in vivo

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Introduction

Breast cancer remains one of the major health care issues in women worldwide today. Globally, it contributes to the highest new cases and deaths among all types of cancer in women.[1] Among different classes of breast cancer, triple-negative breast cancer (TNBC) is one of the most aggressive breast cancer subtypes associated with poor prognosis.[2] Various advancements in chemotherapy for treating breast cancer have been made. Most chemotherapy treatments administer well-known drugs such as docetaxel, tamoxifen, and cisplatin to treat breast cancer.[3] Efficacy of TNBC chemotherapy treatment is limited due to the lack of specific therapeutic molecular targets in TNBC.[2] The use of platinum DNA cross-linking agents such as cisplatin as neoadjuvant in treating TNBC patients had achieved encouraging outcome in a few phases II and III clinical trials.[4] However, cisplatin treatment also has been reported to induce inflammation.[5] The inflammation occurring in TBNC[2] or caused by chemotherapeutic agents may reduce the sensitivity of breast cancer against the treatment and promotes tumor progression, including metastasis.[6] Therefore, control of TNBC inflammation is required to improve the clinical outcome.

Many of the breast cancer chemotherapies are administered in combination with other drugs, adjuvants, or monoclonal antibodies.[3] Combination treatment of 2 or more

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different drugs or herb-drug cocktails has been of interest in combating various diseases, especially cancer.[7,8] Dietary supplements or herbs have long been used to modulate the immune system as well as to increase the efficacy of drugs consumed.[9] Several fruits, including pineapple, were reported with immunomodulatory[10] and anti-inflammatory effects.[11] Bromelain is a collection of proteases derived from pineapple. It is well absorbed when consumed orally with minimal side effects and has been reported to have immunostimulatory and anti-inflammatory effects.[10-12] In addition, bromelain isolated from stem pineapple has been reported with in vivo antitumor effect on P-388 leukemia, sarcoma (S-37), Ehrlich ascitic tumor, Lewis lung carcinoma, MB-F10 melanoma, and ADC-755 mammary adenocarcinoma models, which may be attributed to the immunomodulatory and anti-inflammation effects of bromelain.[13]

Our previous preliminary study has demonstrated a significant cytotoxic effect of bromelain in combination with cisplatin in killing and inhibiting cancer cells in vitro; nevertheless, further in vivo study is required to confirm our findings.[14] This study was conducted to evaluate the antitumor and anti-inflammatory effects of bromelain and bromelain plus cisplatin on TNBC 4T1-challenged mice model. The result from this study will provide additional information to the current findings as research on the antitumor effect of bromelain is still limited, particularly on the effect of bromelain on aggressive inflammatory TNBC.

Materials and Methods

Chemicals and Reagents

Bromelain, derived from pineapple stem, was purchased from Sigma, Ronkonkoma, NY (Catalog Number: B5144). Cisplatin cis-diammineplatinum(II) dichloride was purchased from Nacalai-Tesque (Nacalai, Japan; Catalog Number: D3371). Cell culture media, RPMI-1640, was purchased from Sigma (Sigma, Ronkonkoma, NY) while other cell culture reagents, trypleE, fetal bovine serum, and penicillin-streptomycin, were purchased from Gibco (Life Technologies, Carlsbad, CA). Collagenase IV was purchased from Fisher Bioreagents Brands (Fisher, Pittsburgh, PA).

Cell Maintenance

4T1 cells were purchased from the Animal Tissue Culture Collection (ATCC, Manassas, VA). The cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA) and 1% penicillin-streptomycin (Gibco). The cells were cultured and incubated in a 37°C incubator with 5% CO_2.

Experimental Animals, Tumor Inoculation, and Treatment

All studies involving animals were conducted in compliance with the Universiti Putra Malaysia's ethical guidelines as approved by the Animal Ethics Committee (UPM, Selangor, Malaysia). The approval number obtained was UPM/IACUC/AUP-R098/2014. For the pilot in vivo cancer study, 8-week-old, female BALB/C mice (n = 28), weighing from 22 to 25 g, were obtained from the animal house of Monash University Malaysia (Subang Jaya, Malaysia). The mice were acclimatized to the laboratory environment for 1 week before commencing the experiment. Around 1×10^5 of 4T1 cells in 100 µL media was inoculated in the mice mammary fat pad. Subsequently, the mice were randomly divided into 4 groups: untreated (control), bromelain only, cisplatin only, and combination of bromelain and cisplatin groups, with each group bearing 7 mice. The mice were fed with standard food pellet and water. For the treatment, the bromelain and combination of bromelain and cisplatin groups were fed with 25 mg/kg of bromelain by oral gavage at 100 µL every day for 28 days of treatment, and the dose was selected based on previous researches.[13,15] The cisplatin treatment was administered at 1 mg/kg intraperitoneally in the cisplatin only group, and the combination group received 25 mg/kg of bromelain orally, and 1 mg/kg of cisplatin intraperitoneally every other day for 28 days of treatment. The dose was selected based on a previously published report.[16] After the designated treatment time, the mice were then sacrificed via cervical dislocation, and the tumors and lungs were harvested for further analyses.

Clonogenic Assay

Lungs were harvested from the untreated, bromelain-treated, cisplatin-treated, and bromelain/cisplatin-treated mice after 28 days of treatment. The lungs were chopped and mechanically digested using sterile scissors and scalpels into small pieces. Then, the pieces of lungs were incubated in collagenase D for 30 minutes at 37°C. Afterward the digested parts of lungs were passed through a 70 µm cell strainer. The single suspension cells were then centrifuged at 2000 rpm for 5 minutes and washed with phosphate-buffered saline twice. Subsequently, the cells were resuspended in 10 mL of RPMI 1640 supplemented with 60 µM of 6-thioguanine. A 100× dilution was performed for all of the single-cell suspension harvested from lungs and was plated in 6 well plates. The plates were incubated in a 37°C humidified CO_2 incubator for 7 days. After the incubation period, the supernatant from the wells was removed, and the attached cells, which are the metastatic 4T1 cells that are resistant to 6-thioguanine treatment, were fixed with methanol for 30 minutes and, later, stained with 0.5% crystal violet for 1 hour. The number of colonies per each well was counted based on the number of

dots and size (average size ~0.1 mm^2). Plating efficiency (%) was calculated by dividing the number of colonies counted to the number of cells plated.

RNA Extraction From the Harvested Tumors and RT^2 Polymerase Chain Reaction Array Analysis

Tumors harvested from the untreated, bromelain, cisplatin, and bromelain + cisplatin treated mice were later stored in RNA for 24 hours. Next, the tumors were mechanically disrupted by freezing the samples in liquid nitrogen and meshing using mortar and pestle. RNA extraction from the tumors was performed using the RNeasy Plus Mini Kit (Qiagen, Germantown, MD). Then, the tumors were homogenized in 600 µL of lysis buffer using Qiashredder (Qiagen). The homogenization process was conducted by centrifuging the samples at 14 000*g* for 5 minutes. This process was repeated twice until a homogenous lysate was obtained. Then, the lysate was passed through individual DNA removal spin columns before retaining the RNA in RNeasy spin mini columns (Qiagen). The columns were then washed several times with the provided wash buffers. Total RNA was eluted in 50 µL of RNase-free water, and the concentration and purity were immediately measured on a nano spectrophotometer (Beckman Coulter, Atlanta, GA). Then, the RNA from each of the samples was kept at −80°C until further analysis. cDNA conversion was performed using the RT^2 First Strand Kit (Qiagen). Briefly, 1 µg of the RNA from each of the samples were diluted in water and gDNA elimination buffer to a total volume of 10 µL. The mixture was incubated at 42°C for 5 minutes, before preparing the cDNA synthesis cocktail. Then, after adding the cocktail, the cDNA synthesis was performed at 42°C for 15 minutes, followed by 95°C for 5 minutes. Next, the RT^2 qPCR (quantitative polymerase chain reaction) Master Mix was set up using the cDNA and master mix provided, and the mixture was loaded into the Inflammatory Response and Autoimmunity Arrays PCR array. The PCR reaction was run on the CFX96 Biorad system according to the user manual. The gene name, refseq number, and RT^2 catalog number for each of the genes tested are appended as a Supplementary file (available online).

Cytokine Analysis of Interleukin-1β and Interleukin-4

Cytokine analysis of interlukin-1β (IL-1β) and IL-4 in the serum of the untreated, bromelain, cisplatin, and bromelain + cisplatin treated mice was performed using the ELISA MAX Standard kit (BioLegend, San Diego, CA). Briefly, antibodies for IL-1β and IL-4 were fixed in the wells of 96-well plates overnight. The following day, after a series of washing and blocking, the samples were incubated in the plates for 2 hours. Subsequently, the wells were then stained and measured colorimetrically using a microplate reader (Biotek Instruments, Winooski, VT). The value of absorbance of each sample was calculated against the respective control.

Detection of Nitric Oxide Levels

To detect the level of nitric oxide (NO), the Griess reagent Kit (Sigma) was used according to the user's manual. The harvested tumors were mixed with the Griess reagent and deionized water before being incubated for 30 minutes at room temperature. Next, the absorbance of the mixture was measured at 548 nm using the µquant microplate reader (Beckman Coulter).

Statistical Analysis

All assays were repeated in 3 independent experiments. Means ± standard deviations were compared for each group by 1-way analysis of variance (ANOVA) and Duncan's multiple range test using SPSS 16.0 statistical software (IBM, Armonk, NY). P values $< .05$ were considered statistically significant comparing with the untreated control.

Results

The Combination of Bromelain and Cisplatin Enhanced the Reduction of Tumor Size Versus Bromelain and Cisplatin Alone

Based on Figure 1A and B, the tumor weight and size of all the treated groups were smaller than the untreated group. Bromelain showed similar effects in controlling the tumor weight and size as the cisplatin (Figure 1A and B), but cisplatin was more effective in suppressing the lung metastasis of 4T1 cells (Figure 1C and D). Comparatively, the combination of bromelain and cisplatin had the lowest weight and size than the bromelain alone and cisplatin alone treatments. The combination treatment had an approximately 47% reduction of tumor weight compared with the untreated. Additionally, the combination treatment also managed to decrease the weight of the tumor by 40% comparing with bromelain alone and 32% to cisplatin alone treatments. Moreover, the same pattern can be observed based on the lung clonogenic assay, and the combination treatment had the lowest number of colonies of all the other groups, as illustrated in Figure 1C and D.

The Combination of Bromelain and Cispatin Greatly Affected the Expression of Inflammation-Related Genes Versus Bromelain and Cisplatin Alone

RT^2 PCR array was performed to detect the difference in the level of expression of cancer-related genes in the harvested

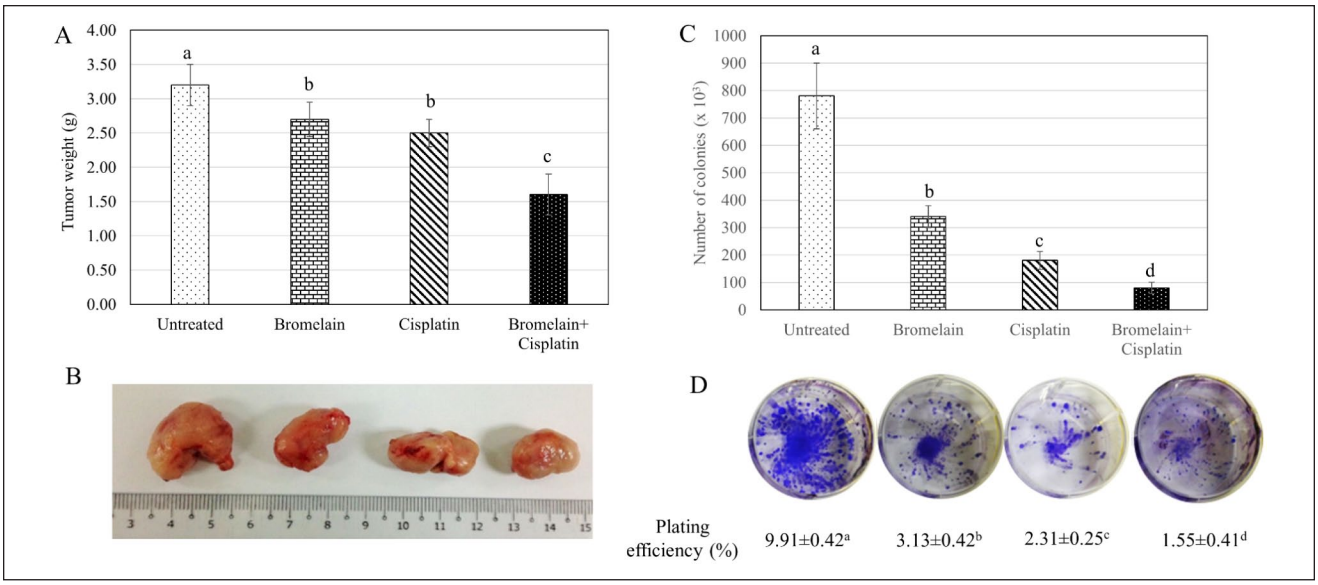

Figure 1. (A) Average weight and (B) representative size of the tumors harvested from the untreated, bromelain, cisplatin, and bromelain + cisplatin treated mice. (C) and (D) are lung metastasis analysis via clonogenic assay. Colonies of metastasized 4T1 cells to the lungs were stained purple with 0.5% crystal violet. Values represent the mean ± SEM (n = 7). Different letters indicate significant differences between treatment groups ($P < .05$).

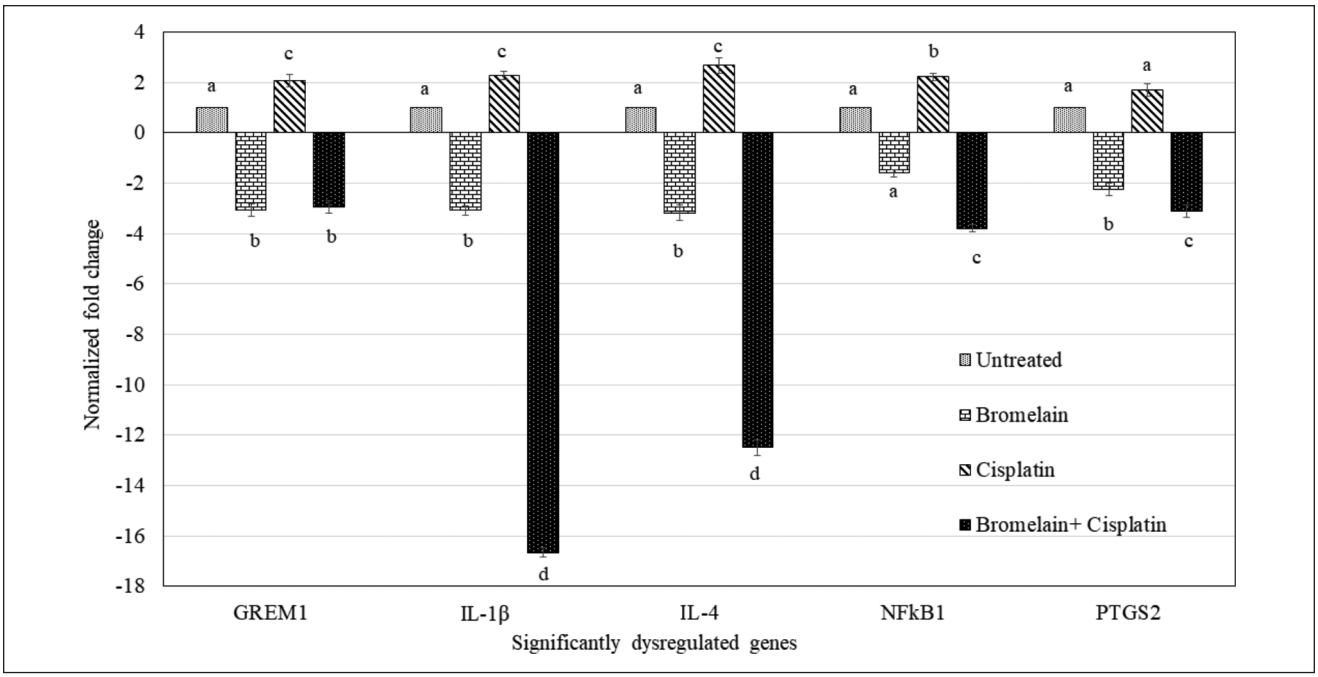

Figure 2. Relative normalized expression of inflammatory genes (GREM1, IL-1β, IL-4, NFκB1, and PTGS2) in the tumors harvested from the untreated, bromelain, cisplatin, and bromelain + cisplatin treated mice. Values represent the mean ± SEM (n = 7). Different letters indicate significant differences between treatment groups ($P < .05$).

tumors after 28 days of treatment. As illustrated in Figure 2, the overall expression of the combination treatment was substantially different from the bromelain and cisplatin alone treatments. For cisplatin-treated tumor, inflammatory-related genes (gremlin-1 [GREM1], IL-1β, IL-4, nuclear factor κB subunit 1 [NFκB1], and prostaglandin-endoperoxide synthase 2 [PTGS2]) showed significant upregulation comparing with the untreated group. On the other hand, the bromelain

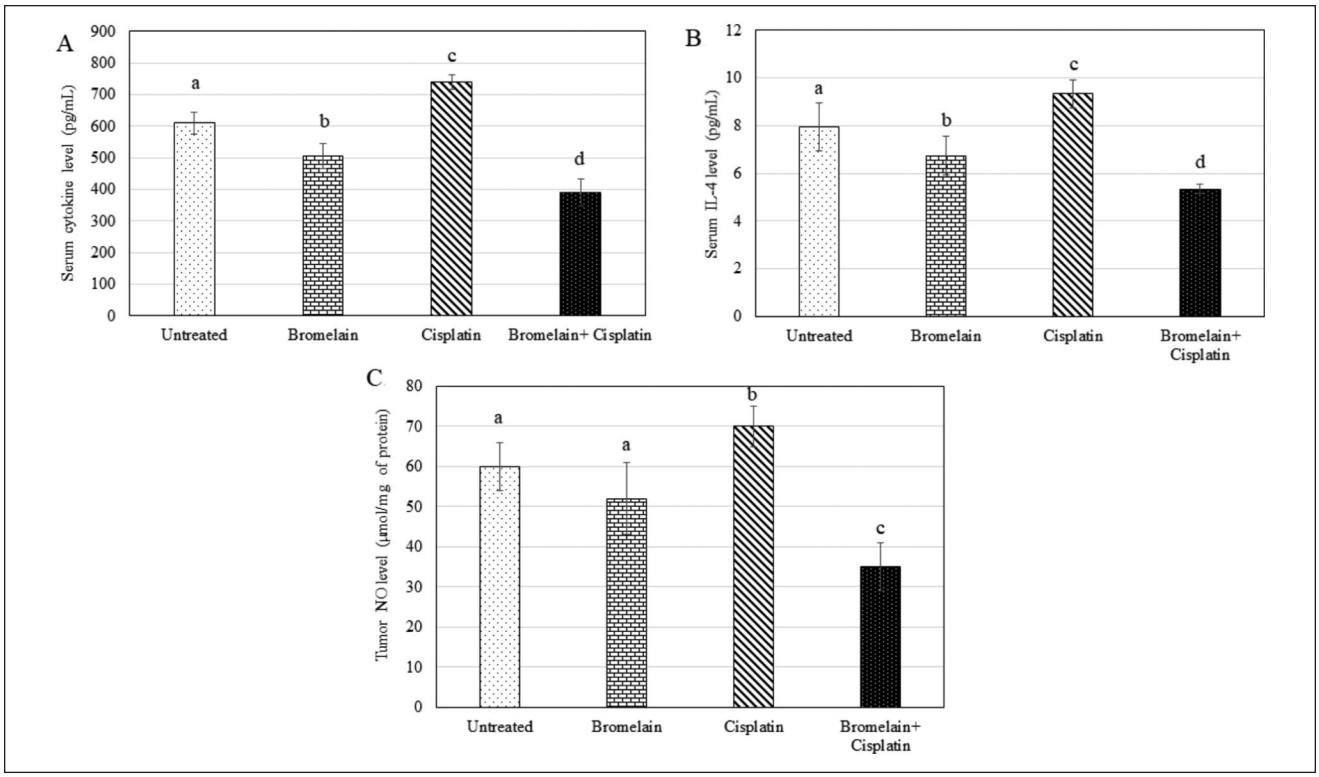

Figure 3. Average levels of serum (A) IL-1β, (B) IL-4, and (C) tumor NO level of the untreated, bromelain, cisplatin, and bromelain + cisplatin treated mice. Values represent the mean ± SEM (n = 7). Different letters indicate significant differences between treatment groups ($P < .05$).

and combination treatments had downregulation in the inflammatory-related genes. Although the combination treatment caused changes similar to that of bromelain alone, it downregulated the inflammatory-related genes with higher fold changes comparing with the bromelain treatment.

The Combination of Bromelain and Cisplatin Regulated the Levels of IL-1β and IL-4 Cytokines

As shown in Figure 3A and B, the serum levels of proinflammatory IL-1β and immunosuppression IL-4 cytokines were recorded as being highest in the cisplatin-treated group. On the other hand, bromelain and combination treatments were observed with significantly lower levels of serum IL-1β and IL-4 cytokines. The trend of the serum IL-1β and IL-4 cytokines levels (Figure 3A and B) were the same as the gene expression analysis (Figure 2). Overall, combination treatment showed the lowest level of serum IL-1β and IL-4 versus the other groups.

The Combination of Bromelain and Cisplatin Decreased the Levels of Pro-Inflammatory Mediator Nitric Oxide Levels

The level of NO was detected in the tumors of the untreated and treated mice. As shown in Figure 3C, the level of NO in the combination treatment group had the lowest value. On the other hand, cisplatin alone recorded an increased level of NO comparing with the untreated group. Bromelain alone treatment also reduced the NO level but was not significant compared with the untreated group.

Discussion

The usage of pineapple as therapeutic medicine has long been applied in several native cultures in tropical and subtropical countries. Bromelain is a cocktail of various proteases that can be extracted from the stem of pineapple and many reports proposed that these phytomedical compounds contribute to the bioactivities of pineapple.[12] For instance, bromelain is commonly used as anti-inflammatory medication, especially after injuries or surgeries.[17] Bromelain has also been commercialized as an herbal supplement, especially in inflammation relief therapy.[18] Besides, bromelain was also proclaimed to have cytotoxic effects on gastrointestinal carcinoma cell lines in vitro and could increase the survival rate of mice challenged with slow-growing mammary and Lewis lung cancer models.[13] However, the effect of bromelain on aggressive cancer and its combination with other herbs or drugs have not been extensively studied yet. In this study, cisplatin alone and bromelain alone managed to reduce the size of the tumor (Figure 1A and B). Besides, bromelain

significantly reduced the metastasis of 4T1 cells to lung (Figure 1C and D), which is similar to the anti-metastasis effect that was reported in the Lewis lung cancer model.[13] These results strengthened the previous findings by Baez et al,[13] where the antitumor effects of bromelain were active on both slow-growing and aggressive mammary tumors in vivo. In terms of combination treatments, they should not be antagonistic toward one another. An ideal combination should have additive effects or even better enhancement with minimal side effects.[19] In this study, the combination treatment of bromelain and cisplatin managed to enhance the reduction of both the weight and size of the tumor compared with the single treatments (Figure 1A and B).

TNBC microenvironment inflammation[2] and chemotherapeutic drugs-induced inflammation[5] have been associated with breast cancer metastasis and progression that may lead to poor prognosis. Previous study[13]and reviews[12,20] have postulated that the antitumor and anti-metastasis effects of bromelain may be contributed by its anti-inflammatory effect. However, the regulation of the inflammatory genes expression by bromelain in the aggressive TNBC tumor model remains unclear. Thus, this study evaluated the regulation of inflammatory gene expression in the tumor of 4T1 TNBC mice model treated with bromelain, cisplatin, and the combination of the 2 treatments. Based on the gene expression study, suppression of inflammatory-related genes (GREM1, IL-1β, IL-4, NFκB1, and PTGS2) was observed in the bromelain-treated tumor while overexpression of these genes was observed in the cisplatin-treated tumor (Figure 2). GREM1 is the bone morphogenic protein antagonists involved in inflammation via activation of NFκB.[21] Overexpression of GREM1 in TNBC is associated with poor prognosis while suppression of it was related to delayed tumor progression.[22] Activation of the NFκB gene promoted the expression of pro-inflammatory elements, including expression of IL-1β, PTGS2, and inducible nitric oxide synthase genes, which are commonly upregulated in inflammatory breast cancer.[23] In addition, some of the chemotherapeutic agents such as cisplatin also enhanced inflammation in the tumor that contributes to subsequent chemo-resistance and metastasis.[5] Suppression of TNBC inflammation may delay the tumor progression and favor the treatment outcome.[24] In this study, although cisplatin treatment was able to control the tumor size and lung metastasis, it was also associated with the overexpression of the pro-inflammatory genes and a higher level of NO in the tumor comparing with the untreated mice.

On the other hand, bromelain treatment had also downregulated the expression of these pro-inflammatory genes and the level of NO in the tumor. Interestingly, concurrent treatment of bromelain and cisplatin even lowered the level of the pro-inflammatory genes and NO in the tumor, which may contribute to the best effect in controlling the tumor size and the lung metastasis among all groups of 4T1 challenged mice. Previous study has reported that some nonsteroidal anti-inflammatory drugs such as the cyclooxygenase-2 inhibitor JTE-522 potentiated the antitumor effect of cisplatin in gastric cancer cells through inhibition of NFκB,[25] which is similar to the enhancing effect of bromelain with cisplatin on 4T1 TNBC cells in vivo.

Conclusion

Bromelain and cisplatin both positively reduced the tumor size and lung metastasis of 4T1-challenged mice. However, regulation of tumor inflammation by bromelain and cisplatin are different, whereas bromelain showed an anti-inflammation effect while cisplatin was observed with a pro-inflammation effect. Co-treatment of bromelain and cisplatin showed enhancement of the inhibitory effect in tumor size, metastasis, and tumor inflammation. Thus, this study demonstrates promising results for bromelain-based cancer therapy with cisplatin for breast cancer that can be further explored.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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