ORIGINAL ARTICLE

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Ozone combined with doxorubicin exerts cytotoxic and anticancer effects on Luminal-A subtype human breast cancer cell line

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SUMMARY

OBJECTIVE: We aimed to examine the potential anticancer effects of ozone applied after chemotherapeutic treatment with different concentrations of doxorubicin in Luminal-A subtype of human breast cancer cell line (MCF-7) and compare the results with effects on L929 fibroblast cell line.

METHODS: Both cell lines were incubated with increasing doses of doxorubicin (1–50 μM) for 24 h at 37°C. Then, half of groups were incubated with 30 μg/mL ozone for 25 min as combination groups. Cell viability was analyzed by MTT assay, apoptosis by flow cytometry, and levels of tumor necrosis factor alpha, transforming growth factor beta, and matrix metalloproteinase-2 and MMP-9 by immunocytochemistry.

RESULTS: Doxorubicin + ozone treatment enhanced viability of L929 ($p<0.01$) but reduced viability of MCF-7 compared to only doxorubicin-applied cells without ozone treatment ($p<0.001$). This combined treatment also enhanced apoptotic effect of doxorubicin on MCF-cells ($p<0.001$), but not on L929. It significantly increased all protein levels of L929 compared with those of other groups ($p<0.05$ for tumor necrosis factor alpha and MMP-2; $p<0.01$ for transforming growth factor beta and MMP-9). This treatment reversed the effect of doxorubicin on tumor necrosis factor alpha levels and considerably reduced MMP-2 and MMP-9 levels of MCF-7 compared with those of control group ($p<0.01$ and $p<0.001$, respectively).

CONCLUSION: Ozone treatment potentiated the apoptotic and anticancer activities of doxorubicin in MCF-7 cells and showed repairing and healing effect on healthy fibroblast cells, which were damaged from cytotoxic effects of chemotherapeutic agent. MCF-7 cells may acquire sensitivity against the doxorubicin combined with ozone treatment through activating tumor necrosis factor alpha, MMP-2, and MMP-9 expressions.

KEYWORDS: Breast cancer. Doxorubicin. MCF-7. Ozone. Fibroblast.

INTRODUCTION

Ozone therapy is an alternative treatment of remarkable clinical interest in the field of oncology[1], showing different effects at different concentrations (changing between 1 and 50 μg/mL) on different organs[1,2]. The ozone treatment was demonstrated to enhance anticancer effects of conventional anticancer drugs like 5-fluorouracile and to exert anti-inflammatory effects on colon cancer[3] and melanoma cells[4]. Moreover, ozone can alter the tumor microenvironment by reducing the production of cytokines involved in the survival and chemoresistance of cancer cell.[5]

Breast cancer is the most common type of cancer in women and second most common reasons of cancer-related mortalities. According to the reports of World Health Organization, 1.2 million new cases are diagnosed each year, with more than 500,000 deaths in all over the world[6]. Breast cancer could be classified into at least five subtypes: Luminal-A, Luminal-B, HER2, basal, and normal subtypes according to the immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Luminal-A cancers are low grade, tend to grow slowly, and have the best prognosis. The immune profile of Luminal-A type breast cancer is ER+, PR+/–, and HER2–, and these cancers are responsive to the endocrine therapy[7]. The addition of chemotherapy to endocrine therapy for breast cancer generally provides little benefit. However, the value of chemotherapy in all patients with Luminal-A breast cancer is controversial[8].

Of the cell lines commonly incorporated into the in vitro models of cancers, ER-positive luminal A cell line MCF-7 has been intensively used to investigate the effectiveness of anticancer therapies[9]. However, L929 is a noncarcinogenic murine cell line that has been used as a control group, especially in cancer studies[10].

From a medical point of view, the investigation of ozone treatment could be of great interests to interpret the ozone-related

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multiple effects in the cancer patients. The aim of this study was to examine the potential in vitro anticancer effects of ozone applied after a chemotherapeutic treatment with different concentrations of doxorubicin in MCF-7 human breast cancer cell line and compare the results with the effects on L929 fibroblast cell line.

METHODS

Cell culture and experimental groups

L929 fibroblast cell line and MCF-7 human breast cancer cell line purchased from ATCC (USA) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. When cells grown as adherent monolayer reached 70–80% confluency, they were passaged using trypsin EDTA (Sigma, USA).

L929 was used as a control group, while MCF-7 was used as an experimental group. Both cell lines were grown for 24 hours to reach to a number of 1×10^4 cells/well, then incubated with increasing doses of doxorubicin (ranged from 1 to 50 µM) for 24 h at 37°C. At the end of incubation, half of groups were incubated with 30 µg/mL ozone for 25 min, produced with Medical Ozone Generator (Turkozone® Blue S) in a specific setup, following the same method described in literature[5].

MTT assay

The cell viability (%) was determined with MTT assay kit (Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich, Missouri, ABD, Cas: 298-93-1). After incubation, both cell lines were washed slowly in PBS at pH 7.4, mixed with 10 µl MTT, and incubated for 3 h at 37°C. Cell viability was measured at 540 nm using a spectrophotometer (SPECTROstar® Nano [ABS]) and then calculated according to following formula: Viability = (Sample–Blank) / (Control–Blank). All experiments were repeated for three times in 3 different weeks.

Flow cytometry

Apoptosis and necrosis at 24 h after treatment with increasing doses of doxorubicin and ozone were detected using flow cytometric assay by staining with Annexin V 7-Aminoactinomycin D (7-AAD) kit (Invitrogen/Biolegend, San Diego, CA, USA). As per the manufacturer's protocol, MCF-7 and L929 cells (8×10^5 cells/well) were seeded in 6-well plates containing 2 mL of medium and incubated at 37°C for 24 h. After the cells were harvested individually into Eppendorf tubes, they were washed twice with cold PBS. Then, all cells were centrifuged at 1500 rpm (2819×*g*) for 5 min and the supernatant was removed. Annexin V binding buffer was added, and cells were counted as 10^6 cells/mL. Then, 5 µl Annexin V+5 µl 7-AAD were added, cells were incubated for 15 min at room temperature in dark. Later, 400 µl binding buffer was added on ice, and the percentages of apoptosis and necrosis were analyzed by flow cytometry (BD Accuri™ C6 Plus).

Immunocytochemistry

The immunocytochemical analysis of tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), and matrix metalloproteinase-2 (MMP-2) and MMP-9 was performed using a horseradish peroxidase/AEC Detection IHC Kit (ScyTek Laboratories, USA, cat no: ACD002) according to the literature[11]. Primary antibodies of TNF-α (Santa Cruz Biotechnology Cas: 52B83, Lot: G2018), TGF-β (ThermoFisher Scientific, MA, USA, product no: MA5-16949), MMP-2 antibody (Invitrogen, USA, MA5-13590), and MMP-9 antibody (Invitrogen, USA, MA5-15886) were diluted at 1/100, and 150 µl of diluted antibodies were added onto 1×10^4 cells. Stained cells were scored as follows: 0: no staining, 1: mild staining, 2: moderate staining, and 3: strong staining, and a total score was given between 0 and 300.

RESULTS

Ozone treatment enhanced antiproliferative effects of doxorubicin on MCF-7

The morphological analysis divulged that cell proliferation of both L929 and MCF-7 was greatly inhibited by 5 µM doxorubicin compared to the control groups, while addition of ozone to the treatment reversed the cytotoxic effect of doxorubicin in L929 but conversely enhanced this effect in MCF-7 cells. MTT assay revealed that the increasing doses of doxorubicin significantly decreased the cell viabilities of both cells, and the effective dose was 5 µM, especially for MCF-7 ($p<0.001$; Figure 1). Addition of ozone after doxorubicin treatment showed more cytotoxicity on the viability of MCF-7 cells than those of L929 cells. Interestingly, the ozone treatment after application of increasing doses of doxorubicin significantly enhanced the viability of L929 cells ($p<0.01$) but reduced the viability of MCF-7 compared to the only doxorubicin-applied cells without ozone treatment

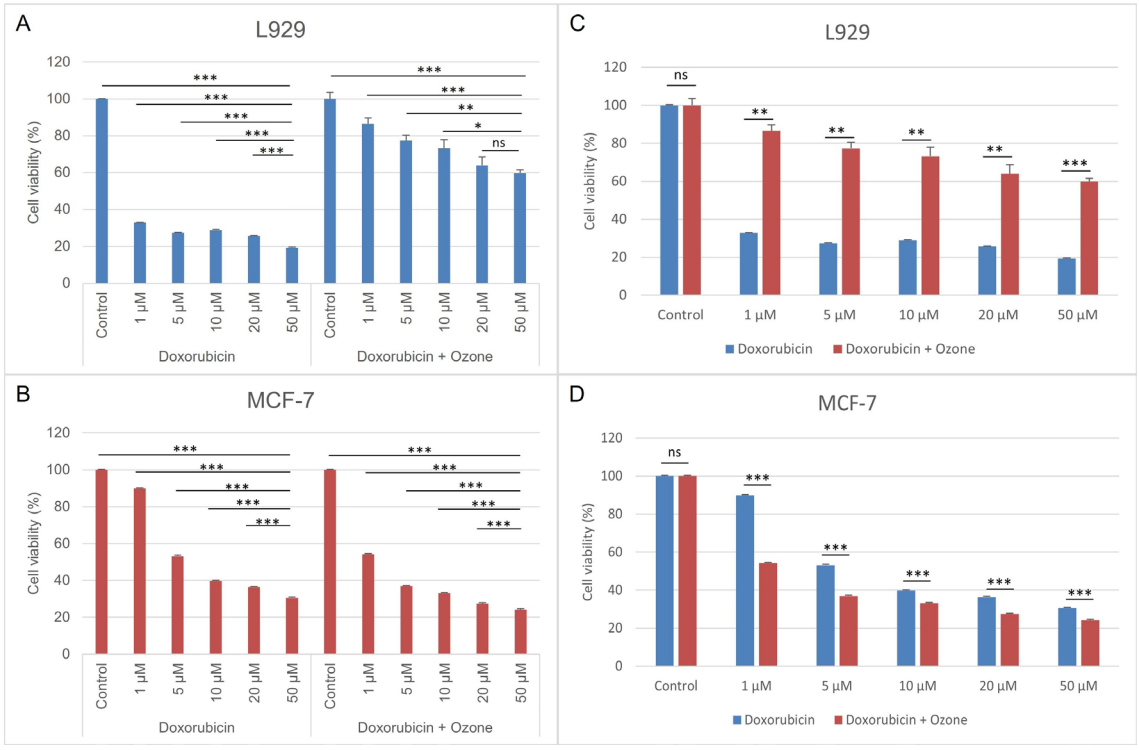

Figure 1. The cell viabilities (%) of L929 and MCF-7 cells calculated according to the results of MTT assay. The intragroup comparisons of cell viabilities of L929 (A) and MCF-7 (B) cells treated with increasing doses of doxorubicin (ranged from 1 to 50 µM). The intergroup comparisons of cell viabilities of L929 (C) and MCF-7 (D) cells treated with or without ozone. All data shown are given as mean±standard deviation of three different experiments performed independently. *p<0.05, **p<0.01, and ***p<0.001 are the statistically significance levels. ns: not significant.

(p<0.001; Figure 1). These results suggest that the doxorubicin treatment alone may inhibit the cell viability of both L929 and MCF-7 cells in a dose-dependent manner, and addition of the ozone application mainly enhanced the antiproliferative effect of doxorubicin on the MCF-7, but not on L929 cells.

Ozone treatment enhanced apoptotic effect of doxorubicin on MCF-7 cells

Flow cytometric analysis showed that MCF-7 cells were relatively more resistant to 5 µM doxorubicin alone as compared to L929 cell line (p<0.001; Figure 2). L929 cells that were treated with doxorubicin combined with ozone showed a significantly higher ratio of cell death compared to untreated cells (p<0.001) and significantly lower ratio compared to doxorubicin-alone-treated cells (p<0.001). MCF-7 cells that were treated with 5 µM doxorubicin combined with ozone showed a considerably higher ratio of cell death compared to both untreated and doxorubicin-alone-treated cells (p<0.001). These results strongly suggest that the combination of ozone with doxorubicin enhanced the apoptotic effect of doxorubicin on MCF-7 cells, but not on L929 cells.

Ozone treatment combined with doxorubicin altered the expressions of TNF-α, MMP-2, and MMP-9 in MCF-7 cells

Immunocytochemical analysis showed no significant difference between the scores of control group and 5 µM doxorubicin-alone group of L929 cells for TNF-α, TGF-β, MMP-2, and MMP-9 proteins (Table 1). However, the combination treatment significantly increased the scores of L929 cells for all proteins compared with those of the other groups (p<0.05 for TNF-α and MMP-2; p<0.01 for TGF-β and MMP-9). For MCF-7 cells, doxorubicin alone did not change the scores of TGF-β, MMP-2, and MMP-9 immunostainings but strongly increased the score of TNF-α immunostaining compared with the control

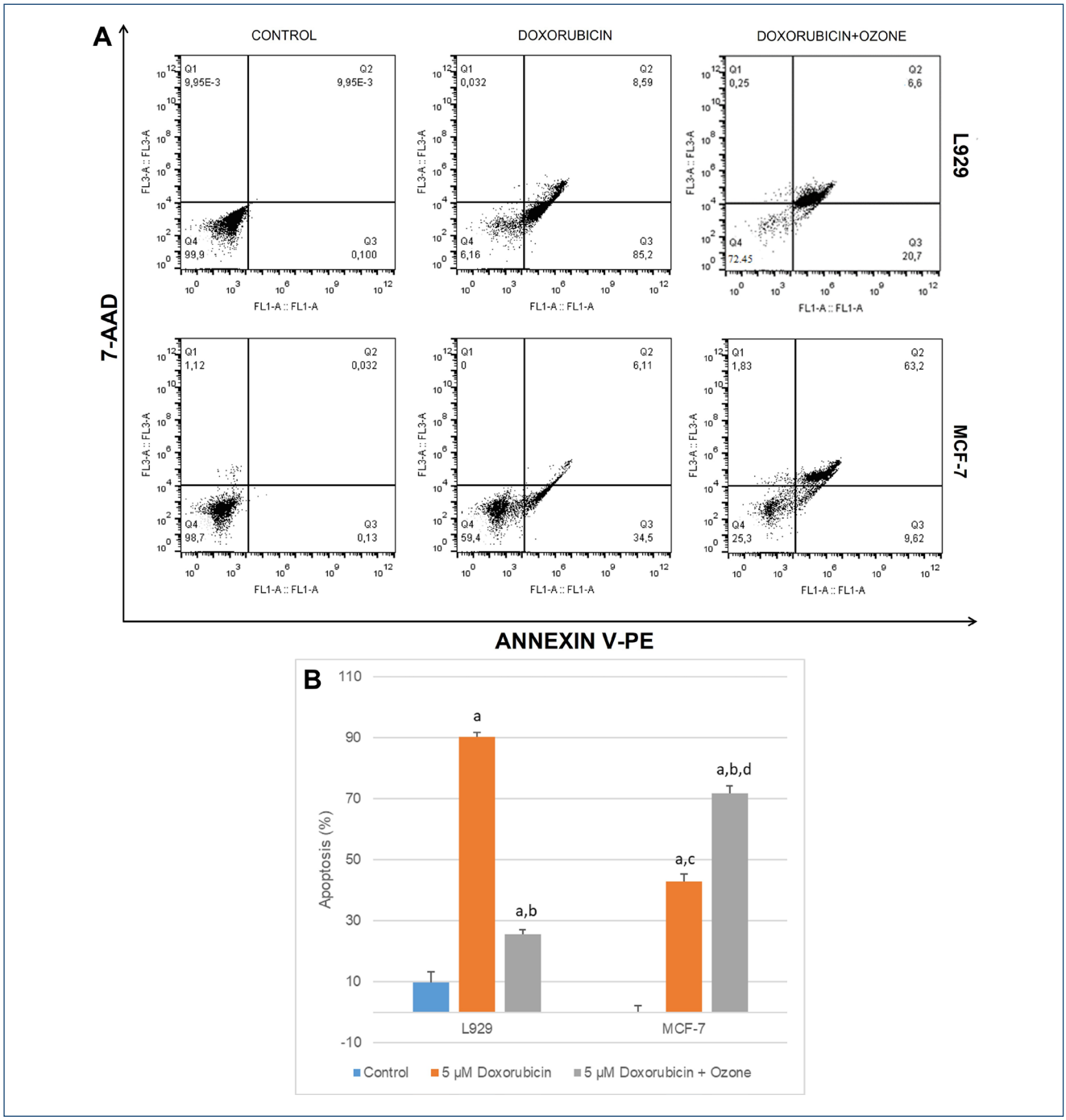

Figure 2. Flow cytometric analysis of Annexin V-PE/7-AAD-stained L929 and MCF-7 cells. A: Flow cytometry results are exhibited as dot plots; B: Bar graph representation of quantitative results of flow cytometry. Quantitative data are shown as mean±standard deviation of three different experiments performed independently. [a] p<0.0001 vs. control groups; [b] p<0.0001 vs. 5 µM Dox groups; [c] p=0.0003 vs. 5 µM doxorubicin-treated L929 cells; [d] p=0.0001 vs. 5 µM doxorubicin + ozone-treated L929 cells.

group (p<0.01). In contrast, the combination treatment with ozone reversed the effect of doxorubicin on TNF-α levels and considerably reduced MMP-2 and MMP-9 immunostaining of MCF-7 cells compared with the control group (p<0.01 and p<0.001, respectively). In addition, the combination treatment significantly reduced MMP-9 immunostaining of MCF-7 cells compared with the doxorubicin-alone group (p<0.001) (Table 1). These results suggest that the ozone treatment combined with doxorubicin altered the expressions of TNF-α, MMP-2, and MMP-9 in MCF-7 cells, but not that of TGF-β.

Table 1. Immunocytochemical findings for L929 and MCF-7 cell lines.

Cell	Protein	Control	5 μM doxorubicin	5 μM doxorubicin + ozone	p-value
L929	TNF-α	140.0±22.4	120.0±27.4	190.0±22.4[a]	0.0242
	TGF-β	180.0±27.4	220.0±27.4	300.0±0.0[b]	0.0096
	MMP-2	130.0±27.4	110.0±22.4	200.0±0.0[a]	0.0156
	MMP-9	190.0±22.4	200.0±0.0	266.0±8.9[b]	0.0087
MCF-7	TNFα	180.0±27.4	280.0±27.4[b]	230.0±27.4	0.0094
	TGF-β	300.0±0.0	300.0±0.0	294.0±13.4	0.3679
	MMP-2	300.0±0.0	250.0±0.0	220.0±27.4[b]	0.0022
	MMP-9	296.0±8.9	298.0±4.5	272.0±4.5[c,d]	<0.0001

[a]p<0.05 and [d]p<0.001 vs. 5 μM doxorubicin group. [b]p<0.01 and [c]p<0.001 vs. control group.

DISCUSSION

Several screening and diagnostic methods have been developed for the patients with breast cancer; therefore, most get their diagnoses and treatment at earlier stages. The modified radical mastectomy (MRM) and breast-conserving surgery (BCS) are standard treatment modalities in breast cancer surgery, while the sentinel lymph node biopsy (SLNB) or axillary lymph node dissection (ALND) could also be applied depending on the involvement of axillary lymph nodes. Although the incidence of breast cancer has been increasing, the mortality rates have decreased by developing anticancer therapies due to these developments in diagnostic and therapeutic facilities[12]. Increased efforts are being made to find a novel, effective, and safe anticancer treatment for human breast cancer[13,14]. One of these efforts uses the ozone as an innovative method for decreasing the inflammation status in preclinical and clinical experiences. Ozone therapy showed its harmless effects and increased efficiency of complex treatment of patients with radiation reactions and skin lesions on the areas of irradiation[15]. Therefore, we examined the potential success of combination of ozone with doxorubicin in treatment for Luminal-A subtype human breast cancer cell line MCF-7 and compared the results with L929 fibroblast cell line and observed the cytotoxic and anticancer effects of combination therapy on cellular models. We also analyzed the possible biological mechanisms involved in these effects and found that MCF-7 cells acquire sensitivity against the doxorubicin combined with ozone treatment through activating numerous pathways, which include TNF-α, MMP-2, and MMP-9 expressions.

Ozone was shown to be able to increase the cytotoxicity of some chemotherapeutics including 5-fluorouracile and cisplatin and to decrease interleukin secretion in human colon cancer cells[3]. A 24-h incubation with ozone was proved to decrease the cytotoxicity of doxorubicin in skin fibroblasts and cardiomyocytes by mediating its anti-inflammatory effects, and the best cytoprotective effect of ozone was reached to 30 μg/mL[5]. In the present study, same effective dose of ozone was used for combination treatment with doxorubicin and enhanced cytotoxicity of doxorubicin in breast cancer cells but reversed this effect in fibroblast cells. These results suggest that the synergistic effect of ozone treatment with chemotherapeutics may change according to the type of cells and tissues and the malignancy.

During the development of medical treatments, ER positivity in the tumor has gained importance. In the literature, ER positivity has been reported in 60–65% of breast cancers[16]. ER-positive Luminal-A-subtype breast cancers compose at least half of all new breast cancer diagnoses due to the high proliferative mitotic activity. This subtype shows a well prognosis and its metastasis is mostly limited to the bone. Several in vitro studies have reported some anticancer effects of chemotherapeutic agents used in ER-positive breast cancers, and a very good response is obtained especially in luminal tumors[17]. Mostly used Lumina-A-subtype MCF-7 cells acquire doxorubicin resistance through activating or inhibiting numerous pathways, which include apoptosis, inflammation, or metastasis. It was reported that doxorubicin resistance was induced by an increased drug efflux through upregulating expression of transporters such as P-glycoprotein and multidrug resistance protein-1[18] or phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and glycogen synthase kinase-3β (GSK-3β) in doxorubicin-resistant MCF-7 cells[19]. Increased expression levels of the antiapoptotic protein Bcl-2 along with activation of MAPK pathways have been demonstrated in doxorubicin-resistant MCF-7 cells[20]. Any treatment targeting apoptotic pathways can be promising to enhance the effectiveness of standard chemotherapeutic regimens while protecting the safety of cancer treatment. In the present study, ozone treatment after doxorubicin incubation was able to induce apoptotic cell death with

its intrinsic ability to suppress the viability of MCF-7, but not of L929. Therefore, the ozone treatment potentiated the apoptotic and anticancer activities of doxorubicin in MCF-7 cells and showed a repairing and healing effect on healthy fibroblast cells, which were damaged from cytotoxic effects of the chemotherapeutic agent.

TNF-α can be produced by tumor cells, infiltrating immune cells, and stromal cells in tumor microenvironment. Patients with different advanced cancers have elevated TNF-α expression in biopsies and in the plasma[21]. Soluble TNF-α is well known to be involved in all steps of tumor development, including tumorigenesis, proliferation, angiogenesis, metastasis, and subverting the immune responses[22]. Moreover, soluble TNF-α induces resistance to BRAF inhibitors in melanoma cells[23] and to cisplatin chemotherapy in malignant pleural mesothelioma[24]. The expression of transmembrane TNF-α was found to be correlated with the disease severity and doxorubicin resistance. Both in vitro and in vivo studies reported that suppressing transmembrane TNF-α expression increased the sensitivity of breast cancer cells toward doxorubicin[25]. In consistent with the literature, the present study demonstrated that doxorubicin alone significantly increased the levels of TNF-α but the combination treatment with ozone reduced these levels in MCF-7 cells, suggesting that ozone treatment may enhance the sensitivity of breast cancer cells against chemotherapy.

Multiple pathways contribute to cancer aggressiveness, and one of these pathways implied in the invasion by cancer cells involves the MMPs, which breakdown and remodel the extracellular matrix proteins[26]. Increased expression of MMP-2 and MMP-9 have been proposed as the prognostic marker in breast cancer[27]. Mohammed et al. investigated the effects of sublethal doxorubicin treatment in noninvasive MCF-7 cells and found that doses of 0.6 μM or less of doxorubicin led to increased migration and invasion by increasing the induction and secretion of MMP isoforms, including MMP-2 and MMP-9[28]. The present study showed that 5 μM doxorubicin alone did not change the levels of MMP-2 and MMP-9 in MCF-7 cells, but addition of ozone treatment successfully reduced these levels. These results confirm the activated invasive program in MCF-7 cells can be inhibited by a combination treatment with doxorubicin and ozone.

CONCLUSION

Overall, our results suggest that an ozone application may aid MCF-7 cells to overcome the resistance against chemotherapies. Considering the success of exposure to ozone after treatment with doxorubicin, this in vitro study is a pilot study for preclinical studies for the effective and safe treatment of human breast cancer. These effects could be of great interest in future oncologic studies for the management of the chemoresistance phenomena of malignancies against many drugs like the doxorubicin.

AUTHORS' CONTRIBUTIONS

OOK, AGY: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, and Writing – review & editing.

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