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Pulsed electromagnetic field potentiates etoposide-induced MCF-7 cell death

Sung-Hun Woo[1,#], *Bohee Kim*[1,#], *Sung Hoon Kim*[1,2], *Byung Chul Jung*[1,3], *Yongheum Lee*[4,*] *& Yoon Suk Kim*[1,*]

[1]Department of Biomedical Laboratory Science, College of Software and Digital Healthcare Convergence, Yonsei University, Wonju 26493, [2]Department of Biomedical Laboratory Science, Korea Nazarene University, Cheonan 31172, Korea, [3]Department of Nutritional Sciences and Toxicology, University of California, Berkeley, CA 94720, USA, [4]Department of Biomedical Engineering, College of Software and Digital Healthcare Convergence, Yonsei University, Wonju 26493, Korea

Etoposide is a chemotherapeutic medication used to treat various types of cancer, including breast cancer. It is established that pulsed electromagnetic field (PEMF) therapy can enhance the effects of anti-cancer chemotherapeutic agents. In this study, we investigated whether PEMFs influence the anti-cancer effects of etoposide in MCF-7 cells and determined the signal pathways affected by PEMFs. We observed that co-treatment with etoposide and PEMFs led to a decrease in viable cells compared with cells solely treated with etoposide. PEMFs elevated the etoposide-induced PARP cleavage and caspase-7/9 activation and enhanced the etoposide-induced down-regulation of survivin and up-regulation of Bax. PEMF also increased the etoposide-induced activation of DNA damage-related molecules. In addition, the reactive oxygen species (ROS) level was slightly elevated during etoposide treatment and significantly increased during co-treatment with etoposide and PEMF. Moreover, treatment with ROS scavenger restored the PEMF-induced decrease in cell viability in etoposide-treated MCF-7 cells. These results combined indicate that PEMFs enhance etoposide-induced cell death by increasing ROS induction–DNA damage–caspase-dependent apoptosis. [BMB Reports 2022; 55(3): 148-153]

INTRODUCTION

Breast cancer, the most commonly diagnosed cancer, is the leading cause of cancer death in women worldwide (1). Various treatments, including surgery, radiation therapy, and hormone therapy have been developed for breast cancer management (2), in addition to several chemotherapeutic drugs (3). However, using chemotherapeutic drugs is accompanied by a major obstacle: extended exposure leads to the cancer cells adapting to chemotherapy, resulting in a reduction in drug efficacies and an induction of resistance (4, 5). Therefore, new strategies to use low doses with improved drug efficacies in cancer chemotherapy have consistently been suggested (6). Recently, it has been widely reported that various complementary and alternative medicines (CAMs) can be used as an adjuvant treatment during chemotherapy to minimize chemoresistance (7).

Pulsed electromagnetic fields (PEMFs), one such CAMs, is a well-known non-invasive method that produces pulsed microcurrents into the whole body or a specific region (8). Several studies suggested that PEMFs affect the potency of anti-cancer drugs. A study examined that PEMF exposure increased doxorubicin-induced cytotoxicity in both MDA-MB-231 and HBL-100 cells (7). Moreover, Qi Han *et al*. reported that pre-exposure to a 50 Hz EMF for 12 h significantly increases the cytotoxic effect of 5-fluorouracil (5-FU) on MCF-7 cells (9). In addition, PEMFs have been reported to affect intracellular signaling molecules in various cancer types, such as chondrosarcoma, neuroblastoma, and breast cancers (10).

Etoposide is used as an anti-cancer drug for various types of cancer, including lung cancer, lymphoma, leukemia, neuroblastoma, ovarian cancer, and breast cancer (11-13). It has been reported that oral treatment with etoposide is a valid and safe option for pretreated metastatic breast cancer patients. It can also be considered for patients that are failing other approaches but are suitable for chemotherapy (13). However, etoposide is not mainly or solely used in chemotherapy owing to it being highly cytotoxic and poorly water-soluble. Administering high doses of etoposide, therefore, requires significant fluid volumes because of this poor water-solubility. It can also cause heart failure, hypotension, or hypersensitivity in some patients. Another type of etoposide, called etoposide phosphate, can be more conveniently administered; however, it is not more effective than using etoposide alone yet more expensive (12). Therefore, developing complementary methods for using low doses of etoposide is required. Etoposide is known to induce apoptosis

*Corresponding authors. Yoon Suk Kim, Tel: +82-33-760-2860; Fax: +82-33-760-2195; E-mail: yoonsukkim@yonsei.ac.kr; Yongheum Lee, Tel: +82-33-760-2863; Fax: +82-33-760-2920; E-mail: koaim@yonsei.ac.kr
#These authors contributed equally to this work.

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by forming a ternary complex with topoisomerase II and DNA in various breast cancer cell lines, including MCF-7, MDA-MB-231, HCC1937, and HCC3153 (11, 14). This ternary complex prevents re-ligation of DNA strands, leading to their breakage (12). It has been reported that etoposide also induces apoptosis through G2/M cell cycle arrest, cleavage of caspase-9 and PARP, and activation of ERK1/2 and Akt in MCF-7 breast cancer cells (15).

There are no reports thus far that discuss the effects of co-treatment with PEMFs and etoposide on breast cancer cells. Therefore, in this study, we examined whether PEMFs potentiate the anti-cancer effect of etoposide in MCF-7 cells. We observed that PEMF stimulations resulted in a further reduction in cell viability in etoposide-treated MCF-7 cells. Based on our results, we have concluded that PEMF stimulations can act as a potential adjuvant therapy for etoposide-based treatment of breast cancer.

RESULTS AND DISCUSSION

PEMF stimulation enhances etoposide-induced MCF-7 cell death

PEMFs are known to be used as an adjuvant therapy for chemotherapy against a variety of cancers (6); therefore, we initially examined whether PEMF stimulations affect the anti-cancer effect of drugs, including amentoflavone, cyclophosphamide, methotrexate, and etoposide. MCF-7 cells were treated with each anti-cancer drug and incubated for 2 days. During this period, the cells were stimulated with a 1 h long PEMF session (2.5 mT at 70 Hz) thrice a day. PEMF stimulations alone (PEMF group) did not affect the cell viabilities of MCF-7 cells or those treated with amentoflavone, cyclophosphamide, or methotrexate (Fig. 1A-C). However, treatment with etoposide and PEMF (indicated as the ETO + P group) significantly reduced the cell viability of MCF-7 cells compared with treatment with etoposide alone (ETO group) (Fig. 1D). These results suggest that PEMF influences etoposide-induced cell death and does not have cytotoxic effects on MCF-7 cells when used alone.

To examine whether PEMF exposure times could influence the viability of cells treated with etoposide, MCF-7 cells were treated with 1 μM etoposide and a 1 h long PEMF session (2.5 mT at 70 Hz) thrice a day for indicated times (12, 24, or 48 h). Viable cells were then enumerated using trypan blue dye exclusion assay (Fig. 1E). Cell viability showed no variations among all groups 12 h after the etoposide treatment. However, 24 h after the etoposide treatment, the cell viability of the ETO + P group was reduced by approximately 30% compared with that of the ETO group. Furthermore, 48 h after the etoposide treatment, the cell viability of the ETO + P group was reduced by nearly 50% compared with that of the ETO group (Fig. 1E). These results indicate that PEMFs potentiate etoposide-induced cell death in a time-dependent manner.

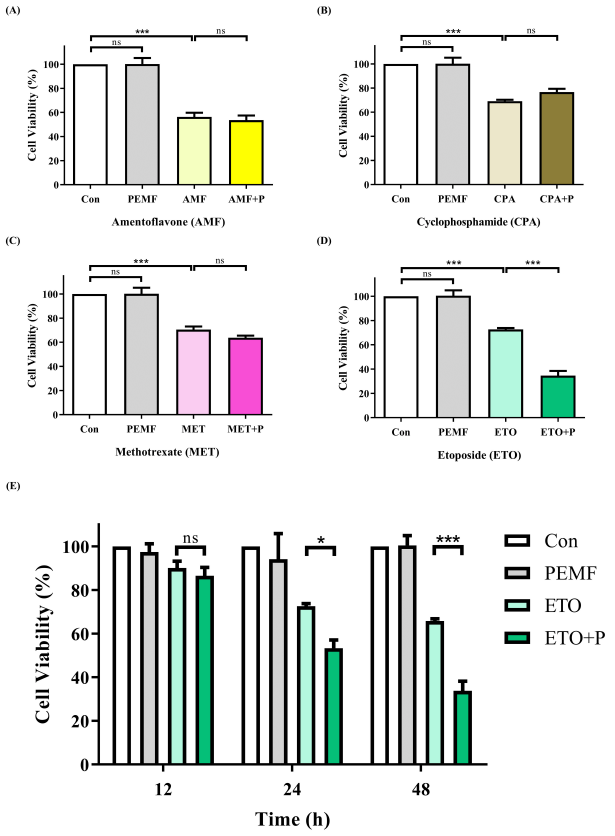

Fig. 1. Increase of etoposide-induced MCF-7 cell death in response to PEMF stimulation. MCF-7 cells were treated with (A) 100 μM amentoflavone (AMF), (B) 0.5 mM cyclophosphamide (CPA), (C) 10 nM methotrexate (MET), and (D) 1 μM etoposide (ETO) followed by incubation for 2 days. The treated cells were stimulated with or without a 1 h PEMF session (2.5 mT at 70 Hz) thrice a day for 2 days. (E) MCF-7 cells were treated with 1 μM etoposide and maintained for the indicated periods (12, 24, or 48 h). Etoposide-treated cells were stimulated with or without PEMF for the indicated periods. Subsequently, cell viability was analyzed. The Con group indicates non-treated cells. The PEMF group (PEMF) indicates cells stimulated with PEMF only. The Drug group (e.g. ETO) indicates cells stimulated with an anti-cancer drug alone, and the Drug + P group (e.g. ETO + P) indicates cells treated with anti-cancer drugs and PEMF. The number of viable cells in the control group was set as 100%. Data were the mean ± SEM of three independent experiments. P values were determined using Student's *t*-test (ns, not significant, $^{**}P < 0.05$ and $^{***}P < 0.001$).

Etoposide-induced MCF-7 cell death is enhanced via increased activation of caspase-dependent pathways by PEMF stimulation

It has been reported that etoposide induces cell death through an up-regulation of apoptosis (16). Thus, we investigated whether PEMFs enhance etoposide-induced cell death via an up-regulating apoptotic pathway. MCF-7 cells were treated with 1 μM etoposide and stimulated with a 1 h long PEMF session

(2.5 mT at 70 Hz) thrice a day for an indicated time (0, 12, 24, or 48 h). Thereafter, cells were harvested to analyze apoptosis-related proteins. PARP cleavage is a well-known applicable marker for apoptosis (17) and, thus, it was analyzed to identify whether PEMF stimulations affect etoposide-induced apoptotic cell death in MCF-7 cells. PARP cleavage was further increased in the ETO + P group than in the ETO one (Fig. 2A). However, the expression of the cleaved PARP remained the same in cells treated with PEMF stimulations alone. These results suggest that PEMF alone does not induce apoptotic cell death and it potentiates etoposide-induced apoptotic cell death in MCF-7 cells.

Caspase-3 and -7 are known to be upstream molecules of PARP in the apoptotic pathway (18); therefore, their activations were analyzed. Caspase-3 activation was not detected; however, caspase-7 activation was increased in the ETO group and further elevated in the ETO + P group (Fig. 2B). Cleavage of caspase-8 and -9 is known to be upstream events for caspase-3 and -7 activation (17); therefore, cleaved caspase-8 and -9 were assessed. Caspase-8 cleavage was increased in the ETO group compared with the control. However, cleavage of caspase-8 in the ETO and ETO + P groups was the same. Meanwhile, caspase-9 cleavage was up-regulated in the ETO group and further increased in the ETO + P group (Fig. 2C). These results corroborate the fact that PEMF enhances etoposide-induced apoptotic cell death via caspase-9, caspase-7, and PARP-dependent pathways.

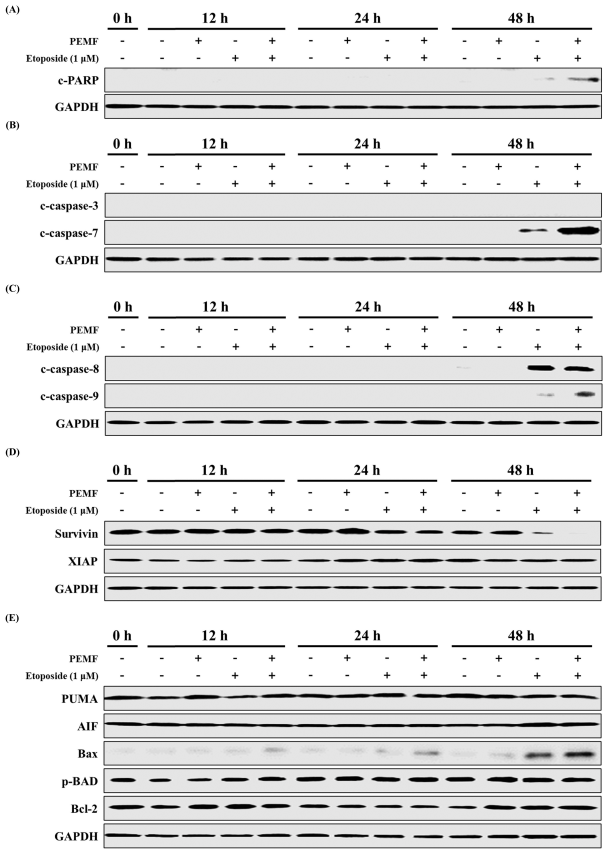

Fig. 2. PEMF-induced increased activation of apoptotic molecules in etoposide-treated MCF-7 cells. MCF-7 cells were treated with the 1 μM of etoposide and exposed to PEMFs for 2 days. During this period, cells were harvested for protein preparation at the times indicated (0 h, 12 h, 24 h, and 48 h after treatment with ETO). (A) PARP cleavage, (B) activation of caspase-3 and -7, (C) activation of caspase-8 and -9, (D) survivin and XIAP protein levels, (E) amounts of PUMA, AIF, Bax, phosphorylated BAD and Bcl-2 were analyzed using western blot assay. GAPDH was used as an internal control.

Survivin and X-linked inhibitor of apoptosis protein (XIAP) are known to inhibit the activity of caspases (18-20). Therefore, we also examined the expression levels of survivin and XIAP. The XIAP expression was the same for the ETO and ETO + P groups. However, the expression of survivin was reduced in the ETO group and further weakened (almost undetected) in the ETO + P group (Fig. 2D). These results imply that PEMFs induce an increased down-regulation of survivin in etoposide-treated MCF-7 cells, leading to enhanced activation of apoptotic caspases. We then examined whether PEMFs affect the expressions of apoptosis-related proteins in etoposide-treated MCF-7 cells. The expressions of p53 upregulated modulator of apoptosis (PUMA), apoptosis-inducing factor (AIF), phosphorylated BCL2-associated agonist of cell death (p-BAD), and B-cell lymphoma 2 (Bcl-2) remained the same. However, the expression of Bax was increased in the ETO group and further elevated in the ETO + P group. PEMFs alone did not affect the Bax expression (Fig. 2E). These results show that PEMFs can increase cell death by up-regulating the Bax expression in etoposide-induced MCF-7 cells. Bax is a well-known upstream molecule for caspase-9 activation; therefore, it is probable that PEMFs increase the Bax expression. This in turn causes further activation of caspase-9, leading to increased cell death in etoposide-treated MCF-7 cells. These results combined imply that PEMF increases etoposide-stimulated cell death by enhancing the Bax expression, caspase-9/7 activation, and PARP cleavage.

PEMF stimulation increases activation of DNA damage-related molecules induced by etoposide in MCF-7 cells

Several studies reported that etoposide induces DNA damage in cancer cells, leading to cell death (21). Thus, we examined whether PEMF stimulations affect etoposide-induced DNA damage in MCF-7 cells. The cells were treated with 1 μM etoposide, exposed to a 1 h long PEMF session (2.5 mT at 70 Hz) thrice a day for the indicated times and harvested. Proteins related to the DNA damage were then analyzed using western blotting. H2AX phosphorylation, known to be associated with the DNA damage response, can be used as a marker for DNA damage (22); it was, thus, first examined. The ETO group exhibited up-regulated H2AX phosphorylation compared with the control and increased H2AX phosphorylation was observed in the ETO + P group (Fig. 3A). However, the PEMF group showed no changes in the H2AX phosphorylation compared with the control. These results corroborate that PEMF enhances etoposide-

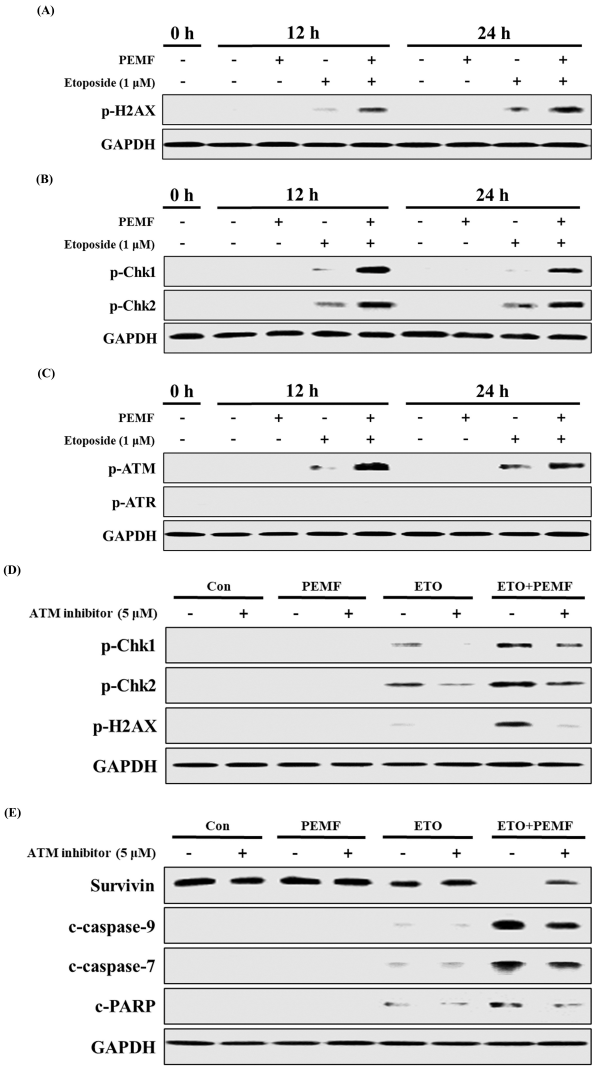

Fig. 3. PEMF-induced enhanced phosphorylation of DNA damage-related molecules in etoposide-treated MCF-7 cells. 1 μM etoposide was used to treat MCF-7 cells. Subsequently, the cells were exposed to PEMFs for the indicated times (0 h, 12 h, 24 h after treatment with ETO). Phosphorylation of (A) H2AX, (B) Chk-1 and Chk-2, and (C) ATM and ATR, were analyzed using western blot assay. 1 μM etoposide with or without 5 μM ATM inhibitor were used to treat MCF-7 cells. Cells were then exposed to PEMFs for 24 h. Phosphorylation levels of (D) Chk1, Chk2, and H2AX, (E) expression level of survivin and cleavage of caspase-9, caspase-7, and PARP were analyzed by western blot assay.

induced DNA damage in MCF-7 cells.

The DNA damage-associated signaling pathways consist of a variety of proteins, such as ataxia-telangiectasia, mutated (ATM), ATM and Rad3-related (ATR), Checkpoint kinase 1 (Chk1), and Chk2 (23, 24). Therefore, we examined whether PEMF affects DNA damage-associated signaling molecules in etoposide-treated MCF-7 cells. Chk1 and/or Chk2 are well-known upstream molecules of H2AX; thus, phosphorylated Chk1 (p-Chk1) and Chk2 (p-Chk2) were assessed. The ETO group exhibited increased p-Chk1 and p-Chk2, and the phosphorylation of Chk1 and Chk2 was more enhanced in the ETO + P group (Fig. 3B). ATM and/or ATR are well-known upstream molecules of Chk1/Chk2; therefore, phosphorylated ATM (p-ATM) and ATR (p-ATR) were assayed. The phosphorylation of ATM was increased to a larger degree in the ETO + P group than in the ETO group. However, the phosphorylation of ATR was undetected (Fig. 3C). These results imply that PEMF stimulations increase etoposide-induced phosphorylation of ATM, Chk1/Chk2, and H2AX, indicating that PEMF potentiates etoposide-induced DNA damage. This in turn leads to increased cell death in etoposide-treated MCF-7 cells.

We then examined whether the etoposide-induced DNA damage enhanced by PEMFs leads to an increase in caspase-dependent apoptotic cell deaths in etoposide-treated MCF-7 cells. The cells were pretreated with a 5 μM ATM inhibitor for 1 h. The cells were subsequently treated with 1 μM etoposide and stimulated with a 1 h long PEMF session (2.5 mT at 70 Hz) thrice a day for the indicated times. Phosphorylation of Chk1, Chk2, and H2AX was weakened by ATM inhibition, confirming ATM to be an upstream molecule of Chk1, Chk2, and H2AX (Fig. 3D). In addition, the cleavage of caspase-9, caspase-7, and PARP was reduced and the ETO/PEMF-induced down-regulation of survivin was recovered by inhibiting ATM (Fig. 3E). These data combined suggest that PEMF enhances etoposide-induced caspase-dependent pathways by increasing DNA damage.

MCF-7 cell death induced by etoposide is increased via PEMF-stimulated up-regulation of intracellular ROS

It has been demonstrated that etoposide induces intracellular ROS generation, leading to cell death (25). Thus, we examined whether PEMFs affect ROS generation in etoposide-treated MCF-7 cells. The cells were treated with 1 μM etoposide and stimulated with PEMF for 1 h (2.5 mT at 70 Hz) thrice a day for 48 h (Fig. 4A). The PEMF group showed no difference in the ROS level compared to the control (Con) group. However, the intracellular ROS level of the ETO group was slightly increased, and a significant increase of the same was observed in the ETO + P group (Fig. 4A, B). These results show that PEMF stimulations potentiate intracellular ROS induction in etoposide-treated MCF-7 cells.

We then examined whether the PEMF-induced increase in ROS generation leads to increased DNA damage and caspase-dependent apoptotic cell death in etoposide-treated MCF-7 cells. The cells were pretreated with 10 μM N-acetyl-l-cysteine (NAC, ROS scavenger) and treated with 1 μM etoposide. After which, the cells were stimulated with a 1 h long PEMF session (2.5 mT at 70 Hz) thrice a day. Treatment with a ROS scavenger diminished the ETO/PEMF-induced enhanced phosphorylation of DNA-damage-related molecules, such as ATM, Chk1, Chk2, and H2AX (Fig. 4C). On the other hand, when MCF-7

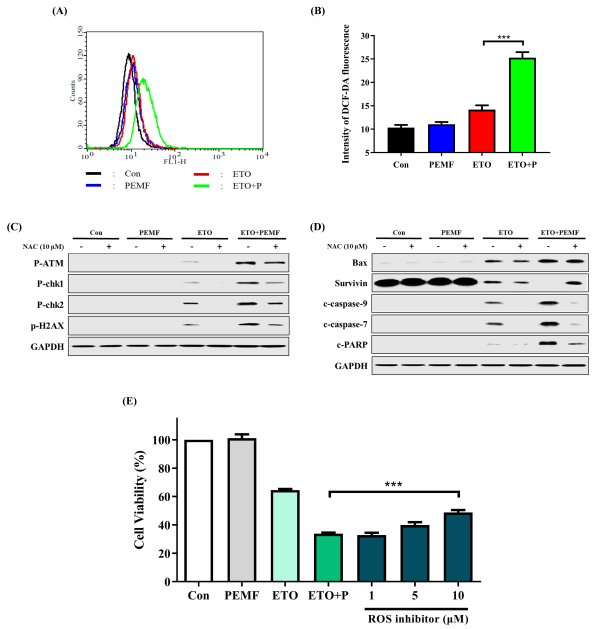

Fig. 4. Increase of etoposide-induced MCF-7 cell death by PEMF-stimulated up-regulation of intracellular ROS. MCF-7 cells were treated with 1 μM etoposide and maintained for 2 days. During this incubation period, cells were stimulated with or without PEMFs. The cells were stained with DCF-DA and the fluorescence intensity, displayed as (A) histogram and (B) bar graph, was determined by flow cytometry. The fluorescence intensity of the Con group was set to 101. Data were the mean ± SEM of three independent experiments. P values were determined using Student's *t*-test (ns, not significant, and ***P < 0.001). MCF-7 cells were treated with 1 μM etoposide in the presence or absence of 10 μM ROS scavenger (NAC) and exposed to PEMFs for 12 h. (C) The phosphorylation of Chk1, Chk2, and H2AX; (D) expression of Bax and survivin; and cleavage of caspase-7, caspase-9, and PARP were analyzed by western blotting. (E) MCF-7 cells were treated with the 1 μM etoposide in the presence or absence of 1, 5, or 10 μM ROS scavenger (NAC) and exposed to PEMFs for 48 h. Cell viability analysis was performed. The cell viability of Con group was set to 100%. Data were the mean ± SEM of three independent experiments. P values were determined using Student's *t*-test (ns, not significant and ***P < 0.001).

cells were pretreated with ATM inhibitor and then treated with etoposide and PEMF, inhibition of ATM did not alter intracellular ROS level under all conditions tested implying that PEMF-induced increase of DNA damage does not affect PEMF-induced enhancement of intracellular ROS induction in etoposide-treated MCF-7 cells (Supplementary Fig. 1). Moreover, the ROS scavenger reduced ETO/PEMF-induced cleavage of PARP, caspase-9, and caspase7 (Fig. 4D). The ETO/PEMF-induced down-regulation of survivin was recovered by treatment with a ROS scavenger (Fig. 4D). Meanwhile, the expression of Bax showed no variations regardless of the ROS scavenger treatment showing that PEMF-induced up-regulation of Bax is caused by other pathway(s) excluding ROS generation in etoposide-treated MCF-7 cells. These results suggest that PEMF-induced up-regulation of intracellular ROS increases DNA damage and caspase-dependent apoptotic pathways in etoposide-treated MCF-7 cells. In addition, we determined whether the PEMF-induced increase in ROS generation affects etoposide-induced MCF-7 cell death. Reduced cell viability resulting from treatment with etoposide and PEMF was recovered by treatment with a ROS scavenger in a dose-dependent manner (Fig. 4E). These results show that the PEMF-stimulated ROS potentiates etoposide-induced MCF-7 cell death.

In conclusion, this study demonstrated that PEMF enhances etoposide-induced MCF-7 cell death by up-regulating the ROS induction–DNA damage–caspase-dependent pathway. These results allude to the possibility of PEMF being used as adjuvant therapy to increase the efficacy of etoposide in chemotherapy against breast cancer.

MATERIALS AND METHODS

See supplementary information for Materials and Methods.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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