

Non-contact electro capacitive cancer therapy (ECCT) modulate the mRNA expression of p53, Apaf-1, survivin, NF- κ B, TSP-1 and bFGF in DMBA-induced breast cancer rat

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ABSTRACT

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Breast cancer is the most common cancer that causes death in women in the world. Cancer development is facilitated by the inhibition of apoptosis and induction of angiogenesis. Current cancer therapy still encounters problems in the form of recurrence, resistance, and side effects of drugs. Non-contact static electric field therapy, electro capacity cancer therapy (ECCT) with medium frequency, is a therapy developed to inhibit the proliferation of tumor cells. This study aimed to determine the mRNA expression of p53, Apaf-1, survivin related to apoptosis and NF- κ B, bFGF and TSP-1 related to angiogenesis in rat breast tumor tissue after ECCT frequency of 150 kHz. Breast tissue samples and rat breast tumor nodules stored in RNA later at -20°C were used. The tissue was obtained from the non-induction non-therapy (NINT) group, induction non-therapy (INT), non-induction therapy (NIT), and induction therapy (IT). mRNA expression of p53, Apaf-1, NF- κ B, bFGF and TSP-1 were analyzed using qRT-PCR and calculated with the Livak formula. Data were analyzed using one-way Anova and post-hoc LSD. The results showed that, mRNA expression of p53, Apaf-1 and TSP-1 in the IT group increased significantly, and mRNA expression of survivin and bFGF decreased significantly compared to the INT group. However, the expression of NF- κ B mRNA in the IT group remained the same as in the INT group. In conclusion, ECCT with a frequency of 150 kHz upregulates p53, Apaf-1 and TSP-1 mRNA expression and downregulates survivin and bFGF mRNA expression but have no effect on NF- κ B mRNA expression in rat breast tumor tissue.

ABSTRAK

Kanker payudara merupakan kanker paling umum dan menjadi penyebab kematian tertinggi pada wanita di seluruh dunia. Perkembangan kanker diperantarai oleh penghambatan apoptosis dan induksi angiogenesis. Terapi kanker saat ini masih menghadapi berbagai masalah berupa kekambuhan, resistensi, dan efek samping obat. Terapi medan listrik statis non-kontak, *electro capacity cancer therapy* (ECCT) dengan frekuensi menengah, merupakan terapi yang dikembangkan untuk menghambat proliferasi sel tumor. Penelitian ini bertujuan untuk mengetahui ekspresi mRNA p53, Apaf-1, survivin yang berhubungan dengan apoptosis serta NF- κ B, bFGF, dan TSP-1 yang berhubungan dengan angiogenesis pada jaringan tumor payudara tikus setelah diberi perlakuan ECCT dengan frekuensi 150 kHz. Sampel jaringan payudara dan nodul tumor payudara tikus yang disimpan dalam RNA later pada -20°C digunakan dalam penelitian ini. Jaringan tersebut berasal dari kelompok non-terapi non-induksi (NINT), non-terapi induksi (INT), terapi non-induksi (NIT), serta terapi induksi (IT). Ekspresi mRNA p53, Apaf-1, NF- κ B, bFGF, dan TSP-1 dianalisis menggunakan qRT-PCR dan dihitung dengan rumus Livak. Data dianalisis menggunakan one-way Anova dan post-hoc LSD. Hasil penelitian menunjukkan bahwa ekspresi mRNA p53, Apaf-1, dan TSP-1 pada kelompok IT meningkat secara signifikan, dan ekspresi mRNA survivin serta bFGF menurun secara signifikan dibandingkan kelompok INT. Namun, ekspresi mRNA NF- κ B pada kelompok IT tetap sama seperti pada kelompok INT. Simpulan, ECCT dengan frekuensi 150 kHz meningkatkan ekspresi mRNA p53, Apaf-1, dan TSP-1 serta menurunkan ekspresi mRNA survivin dan bFGF, tetapi tidak memberikan efek terhadap ekspresi mRNA NF- κ B pada jaringan tumor payudara tikus.

Keywords:

breast cancer;
ECCT;
apoptosis;
angiogenesis;
gene expression

INTRODUCTION

For the last decades, breast cancer, categorized as in situ and invasive (infiltrating) carcinoma, is the most common malignancy cancer type influencing women and acts as the death-leading cause for women in the world after cervical cancer.¹⁻⁴ Globally, with an estimated 2.26 million cases expected to be recorded in 2020, breast cancer is the most widely diagnosed cancer, the most common cause of cancer death among women, and a serious global health concern.^{3,5,6} Currently, the damaging side effects of breast cancer therapies associated with psycho-neurological and postoperative symptoms in these patients designate as a critical public health concern.⁷⁻⁹ Furthermore, anti-cancer therapy of breast tumors is challenging due to the progression of disease that is highly metastatic to the brain, bone, lung, and liver.^{1,7,10} Thus, novel technologies in the management of breast cancer, and optimal approaches to target breast cancer are challenging to develop. Electric-based cancer therapy has reportedly been utilized as an alternative non-drug co-therapy for a variety of symptoms associated with cancer, including cancer-related pain, vomiting, postoperative intestinal obstruction, stiffness, lethargy, hot flashes, anxiety, sleeplessness, and others.^{11,12} Previous studies have been confirmed that electro capacitive cancer therapy (ECCT) inhibit the growth of breast cancer cells and tumor tissue in rat and induces body immune system to destroy tumor cells.¹³⁻¹⁷ Importantly, molecular biomarkers associated with apoptosis and angiogenesis are some of the critical prognostic patterns used in evaluating of hallmarks of cancer.¹⁸⁻²⁰ Therefore, the electro-capacity therapeutic effect especially related to biomarkers of apoptosis and angiogenesis in enhancing the efficacy of breast cancer treatment holds promising to be evaluated.

Electro capacitive cancer therapy

(ECCT) is a constructed-sub-systems as a non-contact alternating current (AC) electric field (EF)-based cancer therapy that uses the low-intermediate intensity electro-static wave sources (<30Vpp) and low-intermediate frequencies (<100-200 kHz) to generate a relatively larger electrical field intensity near the tissue interfaces of peripheral and intra-tissue malignancy.^{13,15,23} Previous pre-clinical and clinical studies have been demonstrated that ECCT low-frequency (100 kHz) and low intensity (18 Vpp) effectively arresting tumor proliferation, inhibiting tumor growth, inducing tumor cell's apoptosis without adversely affecting the leukocyte profile.^{13-17,22-26} Furthermore, ECCT can inhibit the proliferation of breast tumors by decreasing the expression of PCNA, and ErbB2, increasing caspase 3, and macrophage CD68 on histological observations, as well as down-regulated CCL-2 and IL8 genes.^{26,27} Furthermore, it was reported that exposure to low-intensity(18Vpp)andmedium-frequency (100 kHz) non-contact electric fields inhibited mammary tumor growth in mice by inducing CD8+ T cells activation, inhibiting M1 to M2 macrophage polarization, and decreasing CD4/CD8 ratio leading to tumor cell death.¹⁴

Impaired regulations of apoptosis and angiogenesis process are two of the hallmarks of cancer.^{28,29} Moreover, gene expression analysis can offer prognostic and predictive characteristics that aid in defining tumor biomarkers and creating more targeted treatments.³⁰ Since cancer cells have lower levels of apoptotic protease activating factor-1 (Apaf-1) than normal cells, they are well recognized to be resistant to apoptosis.³¹ In the apoptosis intrinsic pathway, as cytochrome c is released from mitochondria, Apaf-1 determines whether it will remain stable or break down.³² Interestingly, the apoptosis process can be blocked by inhibitors of apoptosis proteins (IAPs), mainly survivin/BIRC5.³³ In breast cancer previous studies, survivin expression has been found to be associated

with chemotherapy resistance, a bad prognosis, and increasing expression.^{34,35}

In another line, tumor cell proliferation can trigger the process of tumor angiogenesis, the mechanism of which is regulated by several pathways, namely protein kinases, cell cycle, and transcription factors.^{36,37} In many different cancers, including breast cancer, tumor cell angiogenesis can encourage tumor growth, facilitate tumor progression, and increase tumor aggressiveness.^{38–40} Some of the mediators and signaling pathways associated with tumor angiogenesis include hypoxia inducible factor-1a (HIF-1a), nuclear factor kappa-B (NF- κ B) and vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF).^{38,41,42} The NF- κ B transcription factor will regulate target genes that influence the process of breast tumor invasion, metastasis and angiogenesis such as bFGF and VEGF.⁴³ In addition, thrombospondins (TSPs) -1 and -2 were one of the first inhibitors of angiogenesis proteins identified, a property later attributed to their interactions with endothelial cell surface receptors.⁴⁴ The expression levels of the tumor suppressor gene p53, oncogenes like myc and ras, and TGF- activity are all tightly correlated with the involvement of TSP-1 in tumor growth.⁴⁵

(DMBA) is a well-established polycyclic aromatic hydrocarbon used to induce mammary carcinogenesis in rodents by causing DNA adduct formation and mutagenic transformation in mammary epithelial cells. The DMBA-solvent (corn oil) group serves as the vehicle control to differentiate the biological effect of the carcinogen itself from potential nonspecific effects of the solvent. The DMBA-induced group represents the tumor-bearing model to evaluate ECCT's therapeutic modulation of molecular markers associated with apoptosis and angiogenesis. In the present of study, we elucidate the mRNA expression of p53,

Apaf-1, survivin, NF- κ B, bFGF and TSP-1 related to angiogenesis in rat breast tumor tissue after ECCT frequency of 150 kHz. This gene panel was selected to represent complementary molecular pathways through which ECCT may exert antitumor effects: p53 and Apaf-1 as pro-apoptotic markers, survivin and NF- κ B as anti-apoptotic regulators, and TSP-1 and bFGF as opposing angiogenic modulators. The 150 kHz frequency was selected as it represents the optimal therapeutic window shown to modulate cellular polarization without causing dielectric heating or membrane damage in previous ECCT optimization studies.

MATERIAL AND METHODS

Research design

Female *Sprague-Dawley* rats aged 6–8 wk (180–200 g) were used. Animals were housed under controlled conditions (temperature 22 ± 2 °C, humidity $55 \pm 10\%$, 12:12 h light–dark cycle) with ad libitum access to food and water. Each experimental group consisted of six rats (n = 6). The design of this study was an experimental-based study with a post-test-only control group design that used four treatment groups i.e. 1) rats administered corn oil (vehicle) without DMBA induction or ECCT exposure (NINT/normal control); 2) DMBA solvent-induced animal group and treated with ECCT (NIT); 3) rats induced with DMBA (20 mg/kg BW, orally once a week for 5 consecutive wk) to establish mammary tumors without ECCT treatment (NIT/DMBA-induced tumor); 4) rats induced with DMBA and subsequently treated with ECCT once tumors reached palpable size (DMBA-induced + ECCT/IT).

Sample and ethical clearance

The sample used in this study was nodule breast tumor tissue which was a collection from the Laboratory of Biochemistry, Faculty of Biology, Universitas Gadjah Mada. The samples

were obtained from rats that had been induced with DMBA at a dose of 20 mg/kgBW, which were administered orally twice a week for 5 wk. Animal trials in the NIT and IT groups were treated by the ECCT for 2x5 hr per day for 21 d. On the 22nd day, the animals were sacrificed for tissue isolation.²⁶

Non-contact electro capacitive cancer therapy (ECCT) was applied using a parallel-plate capacitive system operating at 150 kHz and 18 Vpp (equivalent to ~1.5 V/cm field strength). The electrode plates were positioned 3 cm apart, enclosing the tumor-bearing region without direct contact with the skin. Each session lasted 4 hr per day for 14 consecutive d. All treatments were performed in a temperature-controlled environment ($22 \pm 2^\circ\text{C}$)

This research was carried out at the Genetic Engineering Laboratory, Biotechnology Study Program, Graduate School, Universitas Gadjah Mada. This study used an ethical clearance (EC) certificate with number: 00029/04/LPPT/2018 obtained from the Institutional Animal Care and Use Committee of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta.

Quantitative RT-PCR

Total RNA was isolated from tissue samples using Direct-zol RNA Microprep Kits (Cat.No. R2072, Zymo Research, CA, USA) according to the manufacturer's recommendations. Total RNA was quantified by NanoDrop spectrophotometer (MaestroGen, MaestroNano, Hsinchu, Taiwan) respectively.

Samples of cDNA were synthesized from total RNA using the cDNA synthesis kit (Cat.No.BIO-65053, SensiFAST, Bioline, London, UK). According to the manufacturer's recommendations, 500 ng/ μL of template cDNA was put to the final volume of 20 μL of the reaction mixture. qPCR was performed with the SYBR Green method (Cat. No. BIO-98005, SensiFAST, Bioline, London, UK) by a real-time PCR system (Biorad CFX96, Biorad, California, USA). Quantitative Reverse Transcriptase PCR cycle parameters included 2 min at 95°C , then 40 cycles of denaturation (5 sec at 95°C), annealing (10 sec at $58\text{--}60^\circ\text{C}$), and elongation process (5-20 sec at 72°C). GAPDH was used as the reference gene. The sequences of the primers are presented in TABLE 1.

TABLE 1. The sequences of the primers

Gene	Sequence	Annealing temperature
p53	Forward: 5'-CCAAGAAGGACCAGTCTAC-3' Reverse: 5'-GAGGCAGTCAGTCTGAGTC-3'	60°C
Apaf-1	Forward: 5'-ACCTGAGGTGTCAGGACC-3' Reverse: 5'-CCGTCGAGCATGAGCCAA-3'	58°C
Survivin	Forward: 5'-CCTTTGCTGGGCAACCTGGA-3' Reverse: 5'-GCCTTGCACAACCCTCCCTT-3'	60°C
NF- κ B	Forward: 5'-TTCCCTGAAGTGGAGCTAGGA-3' Reverse: 5'-CATGTCGAGGAAGACACTGGA-3'	60°C
TSP-1	Forward: 5'-TCGGGGCAGGAAGACTATGA-3' Reverse: 5'-ACTGGGCAGGGTTGTAATGG-3'	59°C
bFGF	Forward: 5'-TGCCAGAGCCTGC TCTTAAC-3' Reverse: 5'-GATGCCACGGAGATAAGCGA-3'	60°C
GADPH	Forward: 5'-TGACAACCTTGGCATCGTGG-3' Reverse: 5'-GGGCCATCCACAGTCTTCTG-3'	60°C

Statistical analysis

Data analysis was performed using Biorad CFX managerTM software to obtain quantification cycle (Cq), quantification curve and melting curve values from the qRT-PCR results. Changes in gene expression related to apoptosis and angiogenesis in the therapy and non-therapy groups can be identified by comparing the ΔCq value of the related gene with the ΔCq value of the control or calibrator. The ΔCq value of each therapy and non-therapy group was normalized using the housekeeping gene by the Livak formula⁴⁶ and the foldchange values were obtained. The foldchange values obtained were processed and analyzed using SPSS 20.0 to test data normality with Shapiro Wilk to determine the distribution of data and homogeneity test with the Lave test. The data was then subjected to a one-way Anova parametric different test to compare differences between groups and then continued with the LSD post-hoc test.

RESULTS

ECCT regulate the relative mRNA expression of p53, Apaf-1, and survivin related to apoptosis

Analysis of gene expression related to apoptosis aims to determine the molecular mechanism of ECCT in inducing apoptosis of cancer cells through the genes involved in the process, especially p53, Apaf-1, and survivin. The results are demonstrated in FIGURE 1. Relative mRNA expression of p53 and Apaf-1 in breast tumor nodules (INT) showed a significant decrease compared to normal breast tissue (NINT). Based on the results of the study, ECCT therapy with a frequency of 150 kHz significantly induced p53 and Apaf-1 mRNA expression in breast tumor (IT) nodules from untreated (INT) tumor nodules. Whereas p53 and Apaf-1 mRNA expression in breast tumor nodules (IT)

did not differ significantly from normal breast tissue (NINT) ($p > 0.05$). In addition, ECCT therapy with a frequency of 150 kHz was able to significantly inhibit survivin expression in breast tumor nodules (IT) compared to untreated tumor nodules (INT) ($p = 0.031$). Meanwhile, survivin expression in breast tumor nodules (IT) was not significantly different from normal breast tissue (NINT) ($p = 0.335$). Interestingly, ECCT exposure in normal breast tissue (NIT) did not make a significant difference of p53, Apaf-1 and survivin mRNA expression compared to normal breast tissue that was not exposed to an electric field (NINT).

ECCT regulate the relative mRNA expression of NF- κ B, TSP-1, and bFGF related to angiogenesis

Angiogenesis is very important in metastasis and cancer progression. Therefore, knowing the molecular mechanisms through the genes involved particularly NF- κ B, TSP-1 and bFGF is one of the ECCT therapeutic approaches to prevent angiogenesis in breast cancer. The results show that ECCT therapy with a frequency of 150 kHz was able to significantly increase TSP-1 mRNA expression and decrease bFGF mRNA expression significantly in breast tumor nodules (IT) compared to untreated tumor nodules (INT). Whereas the expression of TSP-1 in breast tumor nodules (IT) was not significantly different from normal breast tissue (NINT) ($p = 0.039$). On the other hand, NF- κ B expression in the ECCT-treated tumor group (IT) was significantly decreased compared to normal breast tissue (NINT) ($p = 0.041$). However, compared to the untreated tumor group (INT), ECCT did not induce a further significant inhibition of NF- κ B expression ($p > 0.05$). Interestingly, ECCT exposure to normal breast tissue (NIT) did not significantly differ in the expression of TSP-1, bFGF and NF- κ B compared to untreated normal breast tissue (NINT).

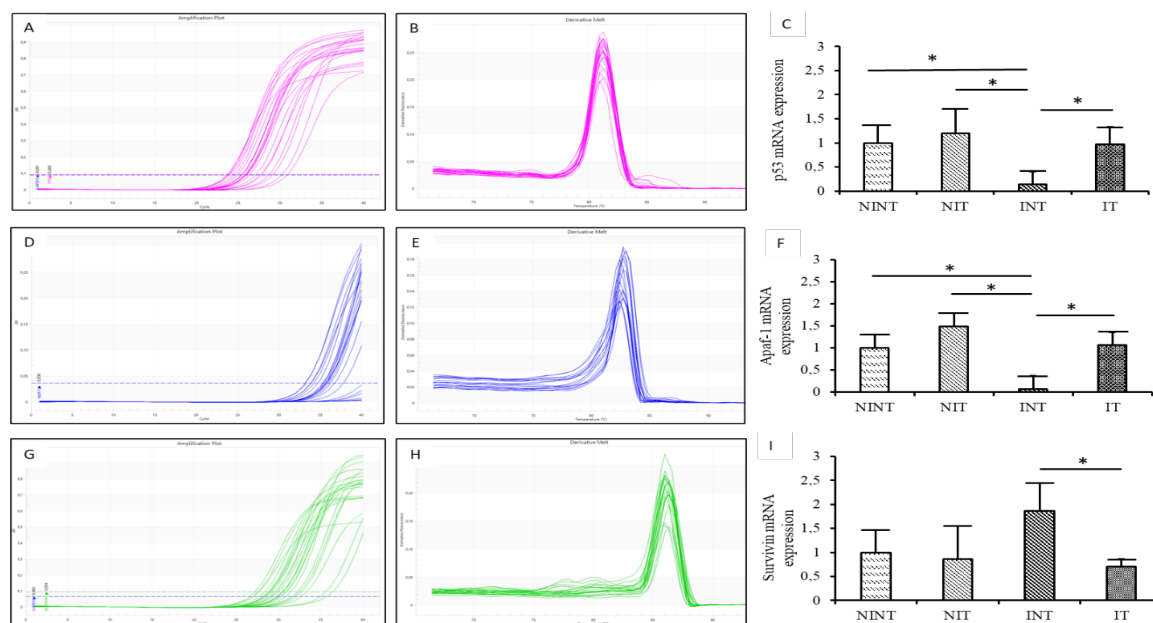


FIGURE 1. Relative mRNA expression of p53, Apaf-1, and survivin related to apoptosis in rat breast tumor tissue after ECCT. Amplification, melt peak chart and mRNA relative expression of p53 (A, B, C), Apaf-1 (D, E, F) and survivin (G, H, I). NINT: DMBA solvent-induced animal without ECCT group; NIT: DMBA solvent-induced animal group and treated with ECCT; INT: DMBA-induced animal group without ECCT; and IT: DMBA-induced and ECCT-treated animal groups. (n=6±SD), *p<0.05.

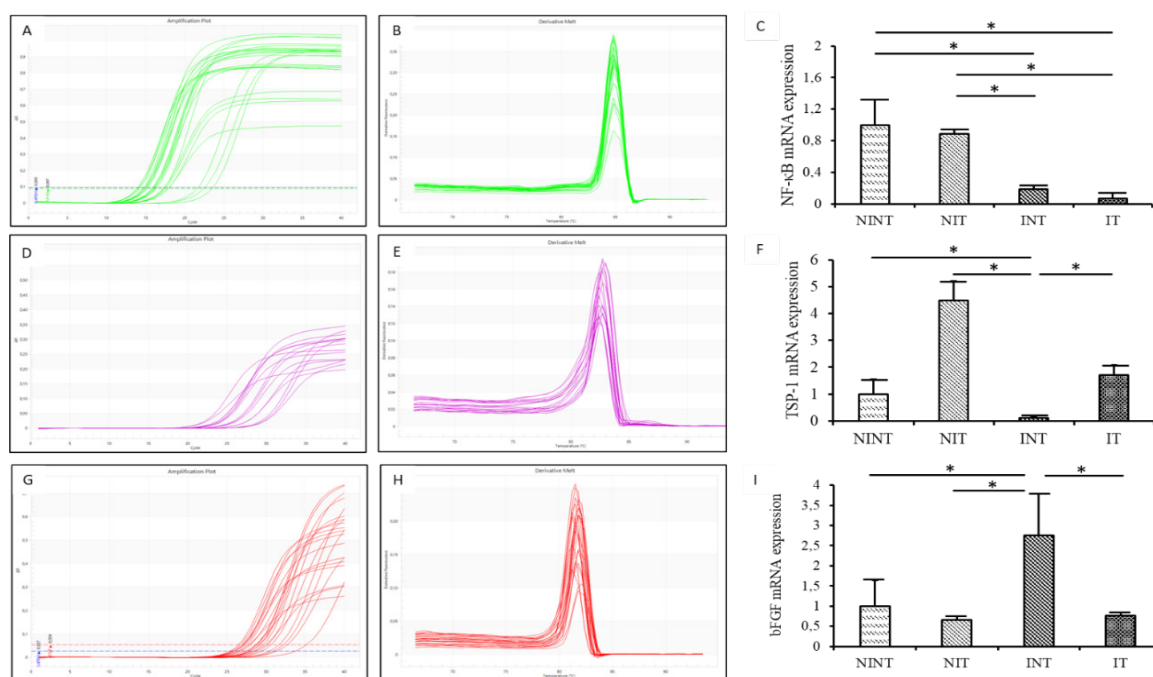


FIGURE 2. Relative mRNA expression of NF-κB, TSP-1 and bFGF related to angiogenesis in rat breast tumor tissue after ECCT. Amplification, melt peak chart and mRNA relative expression of NF-κB (A, B, C), TSP-1 (D, E, F) and bFGF (G, H, I). NINT: DMBA solvent-induced animal without ECCT group; NIT: DMBA solvent-induced animal group and treated with ECCT; INT: DMBA-induced animal group without ECCT; and IT: DMBA-induced and ECCT-treated animal groups. (n=6±SD), *p<0.05.

DISCUSSION

Upregulation of p53, Apaf-1 mRNA expression and downregulation of survivin mRNA expression are indicated as a result of apoptotic signaling induced by the ECCT electric field mainly through the intrinsic mitochondria-mediated pathway in tumor cells but does not affect normal cells. The differences between cancer cells and normal cells are found in their metabolism, division, differentiation, migration, and apoptotic pathways. Previous studies which reported that ECCT was able to increase apoptosis of squamous cancer cells and HeLa cells and decrease their proliferation compared to normal cells, indicated that cancer and normal cells showed different biological responses to external disturbances such as electric field irradiation.^{16,27} This is presumably because the electrical properties of the two cells are different. A previous study explained that the fluorescence time of nicotinamide adenine dinucleotide (NADH), which plays an important role in electron transport, is different between cancer cells and normal cells that have the same origin.⁴⁷

Exposure to electric fields from ECCT is thought to trigger DNA damage which causes p53 to be activated. P53-mediated upregulation of pro-apoptotic genes can mediate both extrinsic and intrinsic apoptotic pathways. The extrinsic pathway includes several TNFR (tumor necrosis factor receptor) family death receptors and, through the creation of DISC (death-inducing-signaling-complex), results in cascades of activation of caspases, including caspase-8 and caspase-3, which are main key of the apoptotic pathway.⁴⁸ Fas, DR5 (death receptor-5), and PERP (p53 apoptosis effector related to PMP-22) are the death receptors most frequently implicated in extrinsic apoptosis. In the intrinsic case, p53 regulates the expression of the Bcl-

2 family, including Bax (Bcl-2-associated X), Noxa, PUMA (P53-upregulated modulator of apoptosis), and BID (BH3 interacting domain death agonist).⁴⁹ In response to stress, Bax forms a homodimer and releases cytochrome C from mitochondria. Cytochrome C, Apaf-1 and pro-caspase-9 then form a complex called the apoptosome, where caspase-9 is activated and drives caspase-3, caspase-6, and caspase-7, which induce cell death.⁵⁰ A previous study showed that the expression of caspase-3 in immunohistochemical observations of breast tumor tissue increased after being exposed to ECCT 150 kHz.⁵¹ This is in line with the results of the current study showing that ECCT can trigger breast tumor cell apoptosis through the apoptotic pathway mediated by p53 and caspase-3.

The two main processes involved in tissue expansion are vasculogenesis and angiogenesis, which in cancer is called metastasis.¹² The pathogenesis of cancer metastases consists of a series of successive and covert steps, which include growth, angiogenesis, detachment, invasion, intravasation, restoration of life in the circulation, endothelial cell adhesion, and growth in distant organs.⁵² Angiogenesis is a major factor that plays a role in cancer progression and expansion, especially in solid tumors.⁵³ Angiogenesis, the formation of new blood vessels with capillary branching, plays an important role in the pathology of tumor growth, where the response is influenced by pro-angiogenic and anti-angiogenic growth factors.⁵⁴

The results of this study showed that the ECCT frequency of 150 kHz increased the expression of TSP-1 indicating that exposure to an electric field in cancer cells was able to inhibit angiogenesis via the TSP-1 pathway, which is thought to correlate with p53. DNA damage in response to exposure to an electric field result in an increase

in ATM and ATR, which causes CHK1 and CHK2 phosphorylation, then leads to p53 activation.⁴⁹ Activated P53 will then translocate into the nucleus and increase TSP-1 gene transcription, which is a potential inhibitor for cancer cell angiogenesis.⁵⁵

The same result also occurred in the bFGF mRNA expression, where its expression in tumor cells was also suppressed after being exposed to ECCT with a frequency of 150 kHz. FGF-2/bFGF, also known as basic FGF, is a major pleiotropic regulatory protein in the processes of differentiation, proliferation, migration, and maintenance of vascular endothelial cells.⁵⁶ The angiogenic activity of bFGF occurs first by binding to FGFR, integrin, and heparin sulfate proteoglycans, then activation of the Ras/MAP-kinase pathway which then phosphorylates the transcription factor C-Jun/C-Fos/C-myc to increase transcription of the VEGF gene.^{53,54} In addition, the expression of FGF2 and FGFR in normal cells is upregulated and downregulated by receptor internalization signals, but FGF2/FGFR signaling in cancer cells is dysregulated.⁵⁷ The healthy cells are in cooperative interactions and mostly run on a mitochondria-regulated metabolism, generating 36 ATP via the Krebs cycle.^{58,59} In contrast to cancer cells, which have a high proliferation rate, the required flux energy is greater. More intensive use of energy can activate glycolytic energy production especially under hypoxic conditions. Mesenchymal cancer cells are mostly hypoxic, thus showing a high level of glycolysis.⁶⁰ So that the response of angiogenesis in cancer cells and normal cells is different.

On the other hand, ECCT did not affect NF- κ B expression in breast tumor tissue. NF- κ B is one of the upstream of bFGF and VEGF gene expression, where if NF- κ B binds to IKK and is activated, it will then translocate into the nucleus and increase the expression of bFGF

and VEGF, pro-angiogenesis genes.⁶¹ It is suspected that downregulation of bFGF is not only initiated by NF- κ B, but also by other mediators such as AP-1 and HIF-1 α . A possible mechanism of interaction between the electric field and the cell is that the electric field changes the plasma membrane potential by increasing the activation of the voltage-gate calcium channel (VGCC), a Ca²⁺ ion channel, which then triggers an intracellular signal transduction cascade response.⁶² A previous study reported that electric field therapy in glioblastoma cells was able to inhibit angiogenesis by reducing the expression of HIF1 α , VEGF, and MMP2.¹² Another study also reported that after 10 d after 120 Hz electric field therapy with magnetic induction intensities of 10, 15, or 20 mT, the tumor size of mice was significantly smaller than the control group.⁶³ Furthermore, exposure to an electric field with 15 Hz 1.2 mT was able to increase the expression of Ang-2 and bFGF but had no significant effect on the expression of Tie-2, Ang-1, and VEGF.⁶⁴

This study contributes to the growing body of evidence that non-contact electro-capacitive fields can modulate key apoptotic and angiogenic pathways in mammary carcinogenesis. By simultaneously profiling six molecular targets, this research provides an integrated view of ECCT's multifaceted biological impact. However, as the findings are based on mRNA expression levels, further validation at the protein and functional level is warranted to confirm mechanistic implications.

CONCLUSION

In conclusion, the observed modulation of apoptotic and angiogenic gene expression suggests that ECCT with a frequency of 150 kHz might represent a promising non-invasive adjunct therapy for breast cancer management. Future studies should explore dose-response

relationships and combine ECCT with standard chemotherapeutics or targeted agents to assess synergistic potential.

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