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RESEARCH ARTICLE

Non-Contact Electric Field May Induced Higher CD4, CD8, Caspase-8, and Caspase-9 Protein Expression in Breast Tumor Tissue of Rats (*Rattus norvegicus* Berkenhout, 1769)

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Abstract Non-contact electric field therapy has been studied as one of less invasive and safe alternative methods in cancer therapy. Some studies reported that non-contact electric field therapy with low intensity (50 - 60 V/m) and medium frequency (150 kHz) could slower the rat's breast tumor growth and did not cause significant damage on the rat's kidney and liver. However, further study needed to evaluate the potency of non-contact electric field to be developed into effective cancer therapy. Thus, we investigated the immune response of non-contact electric field therapy through the detection of CD4 and CD8 protein expression and the mechanism of apoptosis through the detection of caspase-8 and caspase-9 proteins on breast tumor tissue of rats. Twenty four rats were divided into 4 groups: 1. non-induction - non-therapy (NINT), 2. non-induction - therapy (NIT), 3. induction - non-therapy (INT), and 4. induction - therapy (IT). DMBA (7,12-dimethylbenz(α)anthracene) 20 mg/kg body weight was used to induce breast tumor formation. Rats with breast nodules that had reached 1 cm in size were exposed to an ECCT device with 150 kHz and 50 - 60 V/m electric fields. Further, the breast tissues were collected for routine histological and immunohistochemistry preparation for CD4, CD8, caspase 8, as well as caspase 9 detection. The data were statistically analyzed using the Mann-Whitney U-Test method, with a significance level of $p < 0.05$ using the SPSS 16.0 version. The scoring results were compared between the INT and IT groups. Our results showed that non-contact electric field therapy could suppress breast tumor growth and improve its histological structure. Interestingly, the breast tissue of IT group qualitatively had slightly more necrotic and apoptotic cells than that of INT group. Moreover, the IT group showed higher CD4 and CD8 as well as higher caspase 8 and caspase 9 expression. Collecting all the data together, we concluded that non-contact electric field therapy potent to improve histological structure of breast tumor.

Keywords: Non-contact electric field, immunity, apoptosis, breast tumor, *Rattus norvegicus.*

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Introduction

Breast cancer incidences reach up to 11.7% of the total cases of malignancies in women globally on 2020, which is also the highest cancer case in Indonesia [57]. There were 16.6% or around 5,858 new cases of breast cancer with 9.6% or 22,430 fatalities [60]. Unfortunately, more than 80% of breast cancer cases in Indonesia are already found at an advanced stage, thus the treatment is more complex [28].

Various cancer therapies have been developed, such as chemotherapy, radiotherapy, immunotherapy, and surgery [51]. However, these therapies may cause side effects, including hair loss, diarrhea, constipation, abdominal pain, infection, appendicitis, arthritis, skin erythema, fatigue, aplastic anemia, nausea, hematoma, lymphedema, skin color alteration, cancer sores, bleeding, vomiting, itching, body

weakening, as well as organs failure [46,52,51,42,35,6]. Therefore, an alternative less-invasive cancer-therapy needs to be developed to avoid the many side effects caused by the therapy.

Electric field therapy is one of less invasive and safe methods as an alternative in cancer therapy [22,1,13]. There are two types of electric field therapies namely, dynamic electric field and non-contact electric field therapies. Dynamic electric field therapy is applied by direct contact between the electricity-generating plate/electrode with the skin surface area around the tumor with side effects in the form of irritation in the area that is in contact with the electrodes [20]. Tumor Treating Fields (TTFields) therapy is known to shorten the treatment time and increase survival rates in human glioblastoma cancer [21,54,19]. Non-contact electric field therapy could be an alternative in reducing the irritation because it does not require direct skin contact [9].

Giladi *et al*. [13] showed that electric field therapy with medium frequency (100-300 kHz) and low intensity can reduce microtubule polymerization, multinucleate formation, decreases cell viability when the duration of therapy is extended, and induces caspase-mediated apoptosis. Uniquely in this study, the therapy effect was more seen on cells that were actively dividing, such as cancer cells, and less on normal cells. In vivo study in mice induced by breast tumors and treated with 100 kHz electric field showed inhibition of tumor nodule growth of more than 67% without any abnormality in normal mice breast tissue [1]. The immune response is a natural mechanism for limiting the growth of cancer cells, primarily through an adaptive immune response that involves T lymphocytes as the main effector and can be used as an indicator of the immune response of the tumor-host [24].

The presence of Tc and Th cells can be detected by the presence of specific proteins, CD8 and CD4 proteins, which are transmembrane glycoproteins on the surface of T cytotoxic and T helper lymphocytes. Methods using these protein markers have been widely used as a way to analyze and assess the prognosis of tumour tissue. For example, Rathore *et al*. [47] reported that TILs (tumor-infiltrating lymphocytes) were associated with a good prognosis in the immune response against cancer. Alamsyah *et al*. [1] reported that the expression of CD8 protein in breast tumor tissue treated with Electro-Capacitive Cancer Therapy (ECCT) with 100 kHz and 18 Vpp (50-60 V/m) electric field was significantly higher than in untreated breast tumor tissue. The high infiltration of lymphocytes into tumour tissue indicates the higher level of patient safety. Research on detecting the presence of CD4+ and CD8+ lymphocytes in breast cancer can be a good indicator in representing the host immune response mechanism compared to other markers [61].

The optimal frequency of the electric field in treating breast cancer cell cultures (in vitro) MCF-7 and MDA-MB-231 types is 150 kHz [13]. Nurhidayat *et al*. [36] reported that the total leukocyte count in rats treated with a medium-frequency (150 kHz) and low-intensity (18 Vpp or 50-60 V/m) non-contact electric field was higher than in the group without therapy. This result suggested that the non-contact electric field therapy was safe for leukocyte and platelet profiles. Furthermore, Pratiwi *et al*. [43] reported that a non-contact electric field therapy with the same frequency and intensity significantly increased caspase-3 protein expression. Activation of this executor caspase is initiated by the presence of caspase-8 and caspase-9 as apoptosis initiator.

Caspase-8 and 9 have a role as initiators of extrinsic and intrinsic pathway apoptosis in tumor development. Caspase-8 is crucial for the tumor microenvironment as well as the primary tumor cells because it controls the immune response, the activation of B and T lymphocytes, also the differentiation and polarization of macrophages. If energy availability is low and caspase-8 inhibitors are present to inhibit the apoptotic process and the phagocytic capacity of macrophages is decreased, the apoptotic process can transform into necroptosis and secondary necrosis [5, 55].

Breast tumor therapy in white rats exposed to 150 kHz and 18 Vpp (50-60 V/m) electric field showed a tendency for a slower growth rate of tumor nodules compared to the untreated group of rats, although not significant [43]. The same frequency and intensity also did not cause significant damage on the rat's kidney and liver [36]. These studies suggested medium-frequency and low-intensity non-contact electric field have potency to be developed into effective cancer therapy. Thus, it needs to be studied further, especially regarding the immune response of non-contact electric field therapy through the detection of CD4 and CD8 protein expression and the mechanism of apoptosis through the detection of caspase-8 and caspase-9 proteins.

Materials and Methods

Experimental Animals and Samples Collection

This research was conducted under Ethical Clearance Number: 00029/04/LPPT/2018 from the Ethics Commission of the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, Yogyakarta and a series of the previous research conducted by Pratiwi *et al.* [43]. The materials, tools, and methods for experimental animals and samples collection in this study were the same as those used in Pratiwi *et al.* [43] research. Federer Formula was used to determine the rat number for the experimental design, which included 4 treatment groups for minimal biological replication of rats. In this study, 24-five-weeks-old female rats (Sprague Dawley strain) were employed. Rats were randomly divided into 4 groups: 1. non-induction - non-therapy (NINT) group which was not induced by DMBA or 7,12-dimethylbenz(α)anthracene (given corn oil as DMBA solvent 10 times orally, each 0.5 mL, twice a week within 5 weeks) and did not treat with non-contact electric field therapy, 2. non-induction - therapy (NIT) group which was not induced by DMBA, but treated with non-contact electric field therapy, 3. induction - non-therapy (INT) group is induced by DMBA 20 mg/kg body weight but did not treated with non-contact electric field therapy, 4. induction - therapy (IT) group, was induced by DMBA 20 mg/kg body weight and treated with non-contact electric field therapy. Rats from the NIT and IT groups were placed in a rat cage with an external alternating current-electric field (AC-EF) of 150 kHz and low intensity (18 Vpp; 50–60 V/m) between two capacitive electrodes. As a result, we only observed the bare minimum of tissue samples needed for biological replication, which was three tissue samples per treatment group.

Histological Examination and Immunohistochemistry

The samples were prepared using the paraffin method referring to the Bancroft & Cook [4] method with some modifications. Nodules were fixed using 10% NBF, washed using 70% alcohol, dehydrated using graded alcohol, cleared using toluol, and embedded in paraffin 57-60°C. The samples were sectioned for 5 µm of thickness and stained with Ehrlich hematoxylin-eosin.

The IHC method was performed in accordance with the protocol of Starr Trek Universal HRP Detection System (Biocare Medical; cat.no BRR 700 AH, AL10). The material for the IHC staining consisted of CD4 antibody (Abcam, cat.no ab203034, Cambridge, UK, 1:750), CD8α antibody (Abcam, cat.no ab33786, Cambridge, UK, 1:750), anti-caspase-8-antibody (Abcam, rabbit monoclonal antibody [cat.no ab108333]), anti-caspase-9-antibody (Abcam, rabbit polyclonal antibody [cat.no ab52298]). Samples were deparaffinized with xylol and rehydrated using graded alcohol. Samples were heated in citrate buffer at pH 6 using a microwave for antigen retrieval. Samples were dripped with 0.3% H_2O_2 solution in PBS and 0.3% H_2O_2 in methanol-PBS for endogenous peroxidase blocker, then background snipper solution was added as a protein blocker. Samples were incubated with dilution of primary antibodies anti-CD4, anti-CD8, anti-caspase 8, or anti-caspase 9, and 1% BSA-PBST as secondary antibody in 1:500 ratio then stored at 4°C overnight. After that, samples were dripped with Trekkie Universal Link for 30 minutes, then dripped with TrekAvidin-HRP (label). Samples were removed to a dark place to be dripped with DAB (chromogen) and then counterstained using hematoxylin. Next, samples were dehydrated using graded alcohol. Samples were immersed in xylol and then covered with entellan. The samples were then observed with the light microscope Leica ICC50 E.

Histological Scoring

Histopathological characterization of the rat's breast tissue was carried out according to pathological diagnostic criteria in human breast tissue. Pathological diagnostic criteria were observed in stages, starting from normal breast tissue samples, usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). Usual ductal hyperplasia is characterized by widening of the terminal duct, budding and extension of the duct into the surrounding fatty tissue area, irregular curvature of the lumen, presence of phagocytic cells, proliferative luminal epithelial cells, formation of a second lumen, morphological diversity of cells in size, as well as no atypia. Atypical ductal hyperplasia is characterized by enlargement of normal epithelial cells, as well as the increasing ratio of nuclei to cytoplasm and increased basophilicity. Ductal carcinoma *in situ* is characterized by the presence of atypical epithelial cells in the ducts, dense morphology, and central comedo necrosis. Invasive ductal carcinoma is characterized by the infiltration of cancer cells into the interstitial tissue and pushes through the basement membrane [12].

The samples of IHC preparations for tumor nodules in the INT and IT groups were observed using a Leica light microscope with 60-75 fields of view at 400 x magnification for each treatment group. Furthermore, manual cell counting was carried out using ImageJ software to calculate the percentage of positive expression of CD4, CD8, caspase-8, and caspase-9 proteins in rat's breast tumor tissue. The percentage was calculated by counting the number of positive DAB staining cells (brown) divided by the

total cells in each field of view, then multiplied by 100% [58]. The quantification results then compared between the INT and IT groups. In addition, the area of necrosis was scored on tumor tissue samples in the INT and IT groups using ImageJ software. The percentage area of necrosis was calculated as the ratio between the area of necrosis and the total area of the tumor, then multiplied by 100% [7].

Data Analysis

The data were statistically analyzed using the Mann-Whitney U-Test method, with a significance level of $p < 0.05$ using the SPSS 16.0 version. The scoring results were compared between the INT and IT groups.

Results and Discussion

Non-Contact Electric Field Therapy Improved the Histological Structure of Rat Breast Tumor

Non-contact electric field therapy is reported to inhibit the growth of cancer tissue both *in vitro* and *in vivo* [1,2]. Mujib *et al.* [33] stated that non-contact electric fields increased cancer cell death in oral cancer tissues. Furthermore, non-contact electric field therapy does not cause abnormalities in normal tissue which making this therapy as a better alternative for cancer treatment [1]. So far, there are few studies that reporting the effect of non-contact electric field therapy with low intensity (50 - 60 V/m) and medium frequency (150 kHz) on the healing process of cancer tissue. Thus, we studied the effect of non-contact electric field therapy performed using ECCT in breast tumors induced by DMBA.

Margot and Bonnie [30] reported that the induction of DMBA at 15–20 mg/kg body weight caused papillary carcinoma in the breasts of rats. Our results showed the appearance of tumor nodules characterized by massive cell proliferation (Figure 1C and 1D). Thus, adipose tissue could not be observed as in normal breasts. Tumor tissue is characterized by multi-layered glandular epithelial cells, the actively divide nuclei of connective tissue, and a lot of lymphocytes infiltration [49].

The breast tissue of control group, either non-induced without therapy (NINT) or with electric field therapy (NIT) groups showed normal conditions consisting of parenchymal tissue and interstitial tissue (Figure 1A and 1B). The induction of DMBA in both IT and INT groups caused uncontrolled proliferation of epithelial cells lining the lumen and showed the structure of adenocarcinoma tissue in the breast (Figure 1C and 1D). In the INT group (Figure 1C), the connective tissue structure was less clear, the lumen was relatively narrower, and the epithelial cells varied in shape and size (nuclear pleomorphism). The IT (Figure 1D) group showed a relatively good connective tissue structure with clearly visible myoepithelial cells, wider lumens, and uniform shape and size of epithelial cells.

The IT group had a better histological structure, while the INT group ndicated more malignant tumor growth and proliferated tumor cells, which suppress the surrounding connective tissue, Pratiwi *et al.* [43] in their study showed that the rate of cell proliferation and malignancy in rat breast tumor tissue treated with a static 150 kHz and 18 Vpp (50-60 V/m) electric field was significantly lower, confirmed by the lower PCNA (proliferating cell nuclear antigen) and ErbB2 (growth factor receptor) protein expression levels in the IT group. Hence, the observations of rat breast tumor tissue in the present study showed that non-contact electric field therapy could suppress tumor growth (Figure 1D).

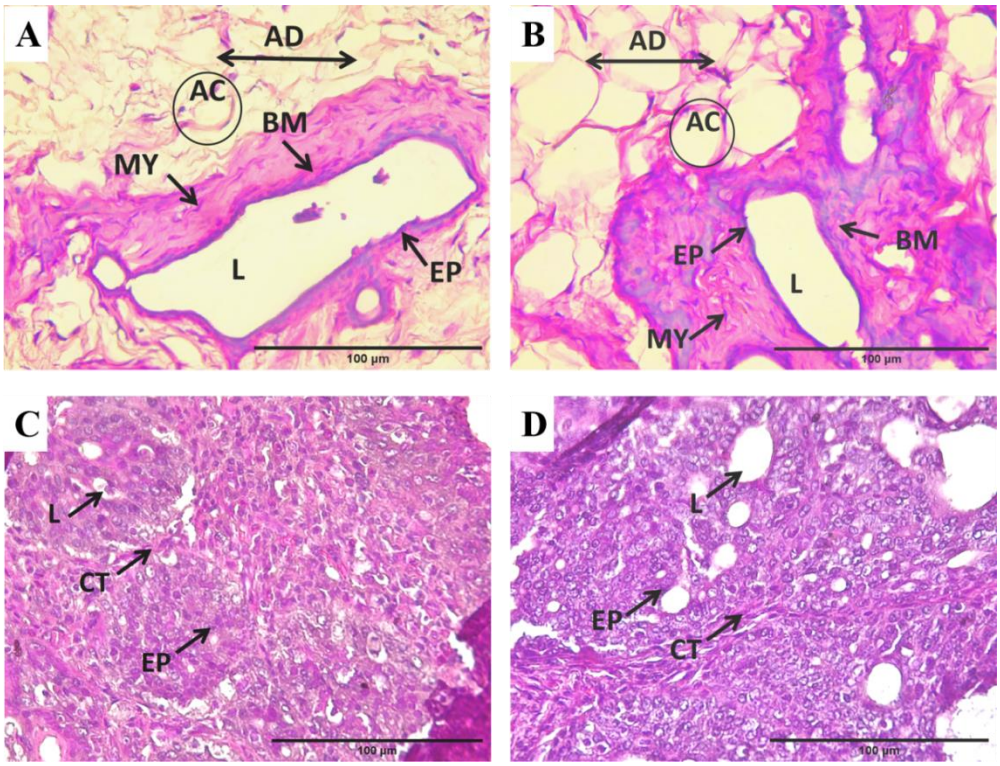

Figure 1. Histological structure of rat breast on non-induction - non-therapy (NINT) group (A), non-induction – therapy (NIT) group (B), induction - non-therapy (INT) group (C), induction - therapy (IT) group (D). AD: Adipose; L: Lumen; AC: Acinus; MY: Myoepithel; EP: Epithelial cell; BM: Basal membrane; CT: Connective tissue. Hematoxylin-eosin staining. Scale bar: 100 µm

Necrosis and apoptosis have an important role in the tumor cells suppression. However, the precise mechanism and how these functions are compromised during cancer development are yet to be clarified. This study showed that there were necrotic and apoptotic cells in the breast tissue of INT group and IT group, which the breast tissue of IT group qualitatively had slightly more necrotic and apoptotic cells than that of INT group (Figure 2). The necrosis area in the IT group (4.28 ± 5.58%) was higher than the INT group (3.19 ± 4.50%), however it showed insignificant difference based on the Mann Whitney-test ($p < 0.05$). Pratiwi *et al*. [43] also reported that the rate of apoptosis in the IT group was 4.75% higher than in the INT group INT group and was characterized by the number of cells expressing caspase-3 protein in the IT group.

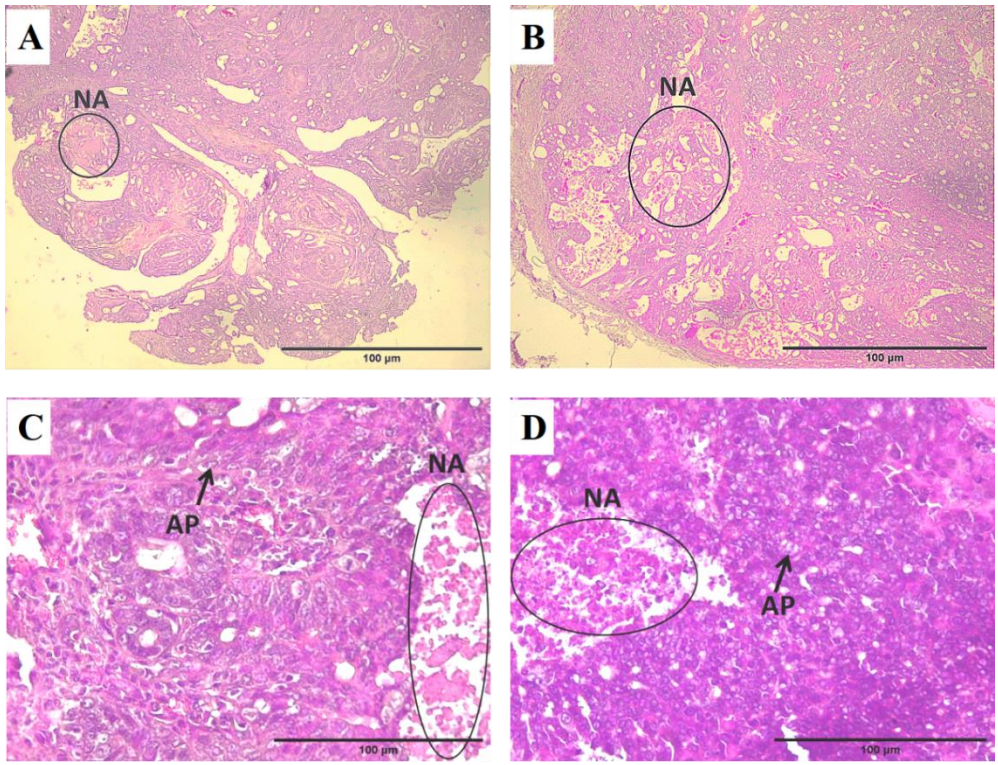

Figure 2. Necrosis area and apoptosis in the rat's breast tumor tissue of induction – non-therapy (INT) group (A and C) and induction - therapy (IT) group (B and D). The IT group has a broader necrosis area and slightly more apoptotic cells compared to that of the INT group. NA: Necrosis area; AP: Apoptosis. Hematoxylin-eosin staining. Scale bar: 100 µm

Besides of apoptosis, there is also necroptosis (programmed necrosis) which has a dual role in inhibiting and metastasizing of tumor cells. Necroptosis can increase tumor metastasis when inflammatory process following necrosis induces angiogenesis, cell proliferation, and gene mutations mediated by cytokines and ROS. On the other hand, necroptosis can inhibit tumor growth if the inflammatory process triggers immune cell infiltration and cytotoxic T cell activation [14]. Tomes *et al.* [59] and Lee *et al.* [25] stated that necrosis can occur in solid tumors due to hypoxia and/or high aerobic glycolysis caused by a lack of blood supply to actively dividing cells. Here we connected the tumor nodules growth and texture with the inflammatory process during necrosis. Pratiwi *et al*. [43] reported that the solid tumor texture became soft and fluid after ECCT exposure, despite the fact that the significant tumor size did not decrease. The appearance of CD-68 proteins (macrophages) was also significantly higher. In this case, our study also showed higher expression of CD4+ and CD8+ in the IT group. Fluid present in the tumor nodules may arise due to the infiltration of pus, blood, and immune cells in the treated tumor tissue. We suspect that necroptosis after non-contact electric field therapy in this study tends to inhibit the tumor growth because the inflammatory process triggers higher immune response in the tumor nodules. Cellular mechanisms regarding the process of necroptosis in tumor cells need to be studied further to determine which role of necroptosis is more dominant in tumor tissue treated with non-contact electric fields (tumor inhibition or tumor metastasis). Furthermore, to understand better how immune system and apoptosis play a role in this therapy, we evaluate protein expressions of CD4, CD8, caspase-8, and caspase-9 of INT and IT groups.

Non-Contact Electric Field Therapy Induced Higher CD4+ Lymphocyte Expression in the Rat's Breast Tumor Tissue

The IHC results of rat breast tumors treated with INT and IT showed that distribution of CD4+ lymphocytes tend to be found in blood vessels and at some points areas of necrosis. According to Hunter *et al.* [16] and Murphy & Weaver [34] CD4+ lymphocytes were found in many areas of blood vessels apart from lymph vessels. CD4+ lymphocyte infiltration at several points in the necrosis areas is caused by the recruitment of immune cells to eliminate necrotic cells [48].

The results in Figure 3 and Figure 4 showed that the mean score of CD4+ lymphocyte infiltration in the IT group exposed to ECCT's electric field was higher than that in the INT group (0.081 ± 0.019% and 0.248 ± 0.054%, respectively). Increased CD4+ lymphocytes infiltration may indicate higher cancer tissue elimination rate, because CD4+ lymphocytes play a significant role in the priming process of CD8+ lymphocytes as well as in the secondary expansion and activation of CD8+ memory T cells [17].

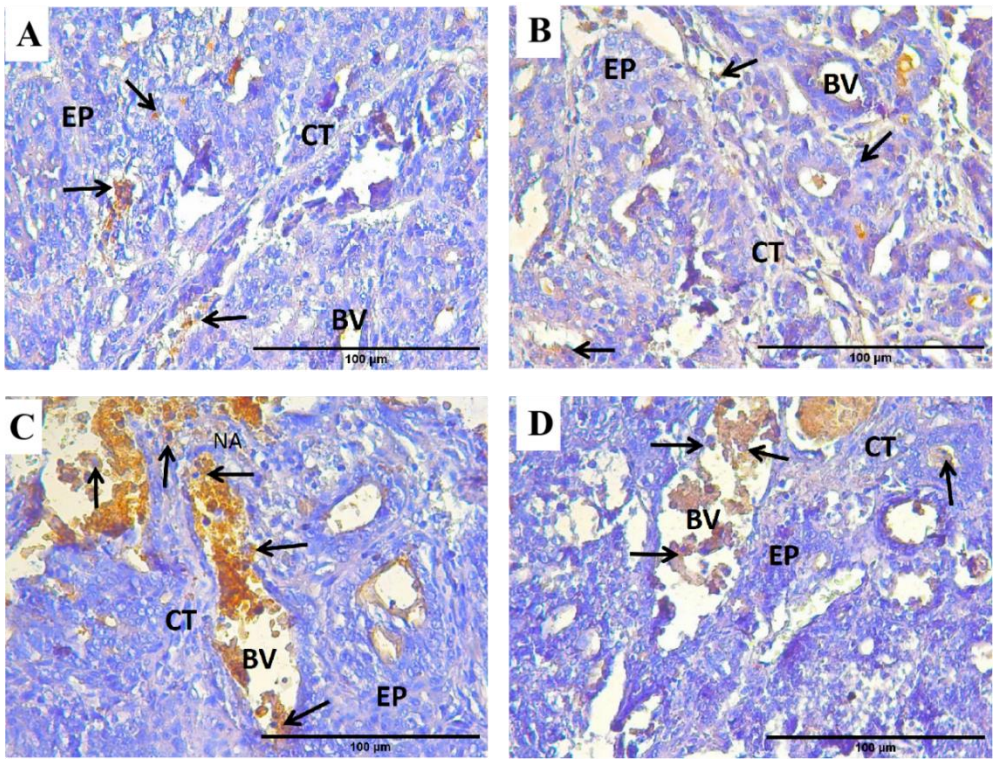

Figure 3. Immunohistostaining of the rat's breast tumor using CD4 antibody (n=3) on INT group (A and B) and IT group (C and D). Non-contact electric field therapy increases the expression of CD4 in IT group rats. Arrow (↑): Positive expression of CD4; EP: Epithelial cell; CT: Connective tissue; BV: Blood vessel. Scale bar: 100 µm

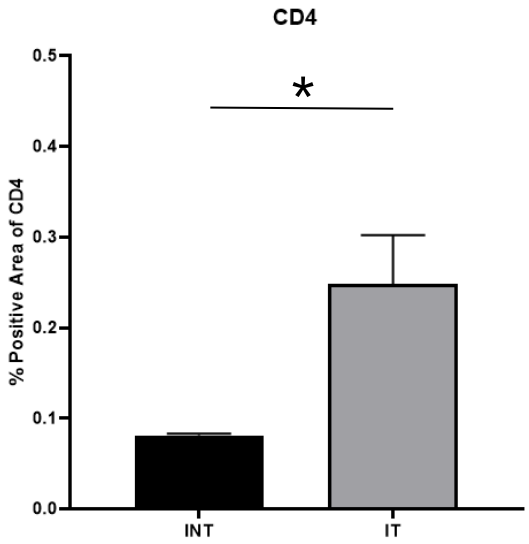

Figure 4. Area percentage of CD4+ lymphocytes expression (n=3) of induction - non-therapy (INT) group and induction - therapy (IT) group in the rat's breast tumor tissue. Non-contact electric field therapy significantly increases the expression of CD4 in IT group rats. Analysis with the Mann-Whitney U-Test method. *: $p < 0.05$. Immunohistochemistry staining

The anti-tumor immune response relies on CD4+ (Th1) lymphocytes by producing IFNγ, which mediates the expansion, differentiation, and activation of tumor-specific CD8+ cytotoxic T cells that induce cell lysis. The higher CD4 expression in IT treatment indicated a higher immune response. CD4+ lymphocytes and macrophages are crucial in mediating senescence surveillance (monitoring of cell aging by immune cells) of pre-malignant tumors and preventing cells from entering the malignant tumor development phase, which will lead to cancer surveillance. In this phase, CD4+ and CD8+ T cells responded to play a significant role in assisting malignant tumor cells elimination [37].

Huang *et al.* [15] reported CD4+ Th1 lymphocytes and CD8+ lymphocytes are dominant in carrying out the immunosurveillance function in the early stages of cancer development. However, the dominant subset of CD4+ switches to Treg and Th17 cells in the late stages of cancer development, which contribute to the promotion of tumor development.

Our results interestingly showed the opposite results from the study of Alamsyah *et al*. [2]. They used an ECCT device with an electric field frequency of 100 kHz which showed CD4+ lymphocytes expressions were higher in the INT group with distribution locations around blood vessels and areas of necrosis than IT group. The presence of CD4+ T cells in the tumor areas also may not always indicate tumor elimination and exposure of electric field can decrease CD4+ T cells polarization. According to the study of Su *et al.* [56] in breast cancer, there is also a naive CD4+ T cell type that is positively correlated with infiltration of Treg cells, which inhibits the immune response to cancer elimination. Macchetti *et al.* [27] reported CD4+ lymphocytes that infiltrate tumor sites play a role in assisting metastasis through lymph nodes in early-stage breast cancer. In other words, determining the results of a more specific analysis regarding the prognosis of therapy with non-contact electric fields requires further research on the dominance of the T-helper type (especially the Th1 type) found in tumor tissue in therapeutic and non-therapy treatments.

Non-Contact Electric Field Therapy Induced Higher CD8+ Lymphocyte Expression in the Rat's Breast Tumor Tissue

The results showed that the distribution of CD8+ lymphocytes tended to congregate in the necrotic area of the IT treatment and spread during the INT treatment (Figure 5). Necrosis can trigger inflammation indicated by the presence of CD8+ lymphocytes. It it due to lymphocytes participation in inflammation response, through their cytotoxic granules or granzyme S inducing pro-inflammatory cytokines [31, 40].

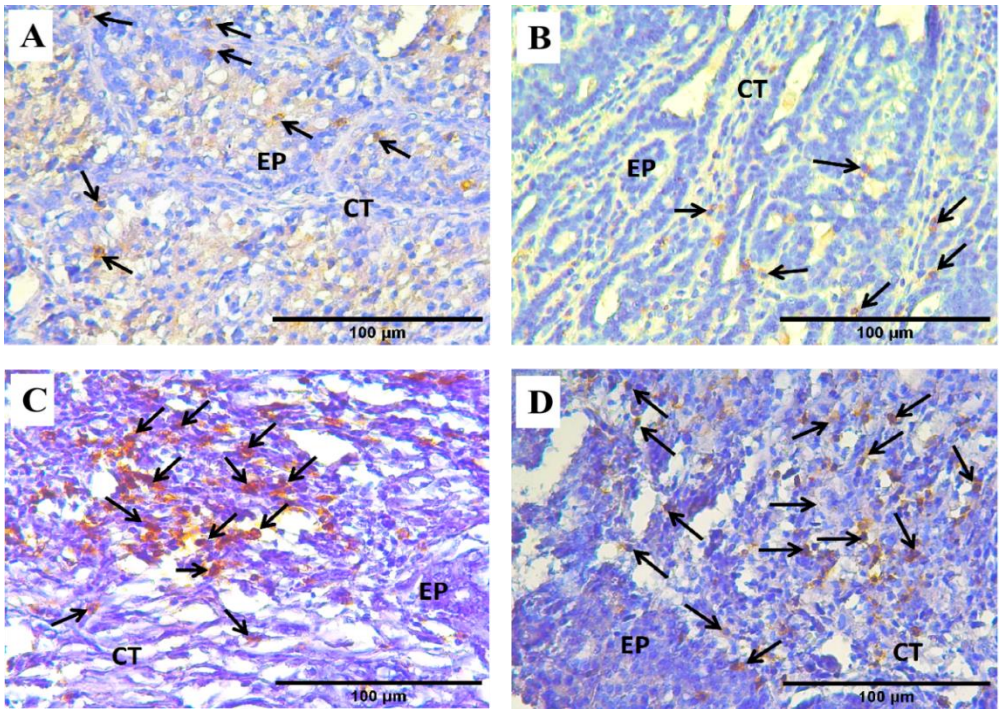

Figure 5. Immunohistostaining of the rat's breast tumor using CD8 antibody (n=3) on INT group (a,b) and IT group (c,d). Non-contact electric field therapy increases the expression of CD8 in IT group rats. Arrow (↑): Positive expression of CD8; EP: Epithelial cell; CT: Connective tissue; BV: Blood vessel. Scale bar: 100 µm

Statistical analysis results of the percentage of CD8+ lymphocytes area as shown in Figure 6 showed a significant difference between the INT and IT groups, whereas the area of CD8+ cell in the INT group (0.2896 ± 0.0461%) was lower than that in the IT group (0.3695 ± 0.0438%). This showed tumor tissue that received electric field therapy from ECCT with a frequency of 150 kHz and an intensity of 50 - 60 V/m had a higher CD8+ cell infiltration rate than the group without therapy. A higher infiltration of CD8+ lymphocytes indicates an increased immune response for the cancer elimination process, since it plays a significant role in the initiation of cancer cell apoptosis [34].

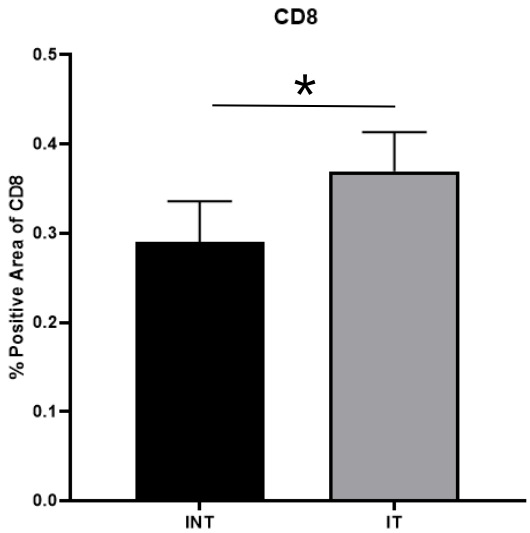

Figure 6. Area percentage of CD8+ lymphocytes expression (n=3) of induction - non-therapy (INT) group and induction - therapy (IT) group in the rat's breast tumor tissue. Non-contact electric field therapy significantly increases the expression of CD8 in IT group rats. Analysis with the Mann-Whitney U-Test method. *: $p < 0.05$. Immunohistochemistry staining

The infiltration value of CD8+ lymphocytes in the IT group indicated a higher cancer cell elimination process. The elimination process involves two general pathways, a pathway facilitated by the expression of FasL and TNF-α and a pathway facilitated by cytotoxic granules from CD8+ lymphocytes [34]. The cytotoxic granule pathway, also known as the granzyme pathway, has many enzyme types to initiate intrinsic and extrinsic apoptosis at the end of the pathway, especially granzyme B which activates caspases 3, 7, and 9 [8].

The combined data of lymphocytes expressing CD4 and CD8 proteins give rise to the possibility of the electric field of ECCT exposure with a frequency of 150 kHz and an intensity of 50 - 60 V/m to delay the development of the rat's breast tumors in a malignant stage. Pratiwi *et al.* [43] which also used the same ECCT and electric field frequency (150 kHz) showed decreased expression of metastases-promoting genes (*CCl2* and *IL18*) in the IT treatment and increased in the INT group, indicating therapy with this non-contact electric field may be possible to slow the progression of tumors to become more malignant.

This study was in line with the results of Alamsyah *et al.* [2], which showed CD8 expression in the IT group was significantly higher ($p < 0.05$) compared to the INT group after exposed to 100 kHz non-contact electric field. The CD8+ lymphocytes were distributed around blood vessels, tumor tissue areas, and areas of necrosis. However, this study shows opposite progression of CD4/CD8 ratio. The Study of Alamsyah *et al.* [2] shows decreasing value of of CD4/CD8 Ratio after therapy that suggested a good prognosis in rat breast cancer. Meanwhile this study showed that the CD4/CD8 ratio of INT group (0.28) is lower than the IT group (0.67). The difference between the result may indicated the different effect of electric field frequency (100 and 150 KHz) to CD4 and CD8 expression in breast cancer.

Lin *et al.* [26] reported that electric fields can increase the motility of lymphocytes by triggering directional migration towards the cathode (in direct currents), and can activate intracellular signaling kinase mechanisms such as phosphorylation of Erk1/2 (extracellular signal-regulated kinases 1 or 2) and Akt (serine threonine kinase) which are involved in cell motility. This mechanism could be the reason of the increase of CD8+ lymphocytes infiltration. The molecule involved in cell motility, namely actin, can react at alternating current (AC) by affecting its polarity, making actin molecules migrate towards the maximum electric field intensity, and line up to bridge the space between the electrodes [3].

The electric field might change cell membrane potential, which can trigger cell hyperpolarization [53]. Kadir *et al.* [18] reported that the membrane potential of cancer cells is more depolarized than normal cells. Cells that are more depolarized also have a higher potential for metastasis, and conditioned hyperpolarization can reduce cancer cells invasion and migration [41]. The proportion of average percentage positive areas in this study ranged from 0.02-0.3% of the total scoring area. This is concordant with the research by Sawe *et al.* [50] which reported that CD8+ and CD4+ lymphocytes in breast cancer tissue and normal breast tissue usually have a mean area percentage of less than 1%, with an increase in cancer tissue about 2-fold for CD8+ lymphocytes and 5-fold for CD4+ lymphocytes. The results of scoring the mean percentage of positive areas for CD4+ lymphocytes showed a lower value than the results for CD8+ lymphocytes, indicating that the possibility of induction of cytotoxic T cells by Thelper at the time of observation had not occurred massively.

Non-Contact Electric Field Therapy Induced Higher Caspase 8 Expression in the Rat's Breast Tumor Tissue

The breast tumor tissue in the IT group showed more positive expression of caspase-8 compared to that in the INT group (Figure 7). The results shown in (Figure 8) revealed that caspase-8 expression in the IT group was significantly higher (1.43 ± 0.67%) than that in the INT group (0.83 ± 0.47%). Caspase-8 and caspase-9 protein in the INT group was more abundant in the myoepithelial cell area, while in the IT group it was more in the epithelial cell area. Myoepithelium can suppress the growth of tumor cells towards the connective tissue. The induction of myoepithelial death by caspase-8 in the INT group has implications for the more malignant tumor because the myoepithelial function as a tumor suppressor is reduced or lost. Damage to the myoepithelium can trigger growth factors, angiogenic factors, and ROS in microenvironment of cells, thereby triggering the proliferation of more malignant tumor cells [38]. Whereas in the IT group, caspase 8 was selectively more abundant in the epithelial cell area. In DMBA-induced rats, carcinogenesis occurs in mammary epithelial cells. DMBA undergoes metabolic activation to form its active metabolite, dihydrodiolepoxides, which can damage DNA and form DMBA-DNA adducts [45].

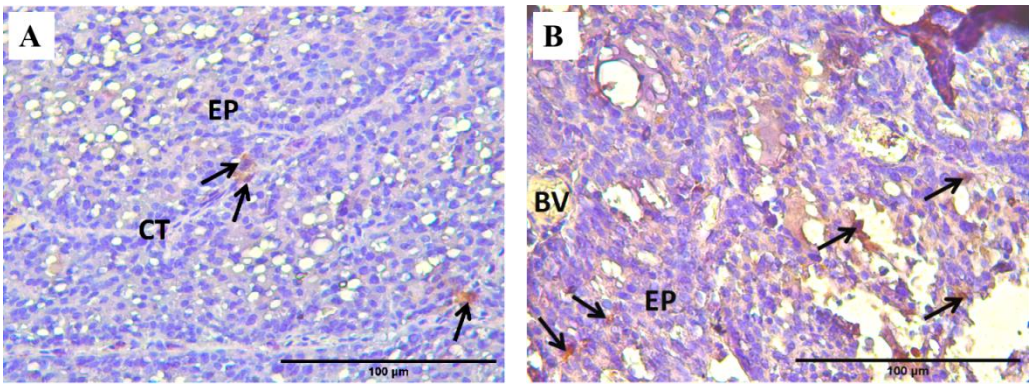

Figure 7. Immunohistostaining of the rat's breast tumor using caspase-8 antibody (n=3) on INT group (a) and IT group (b). Non-contact electric field therapy increased the expression of caspase-8 in IT group rats. Arrow (↑): Positive expression of caspase-8; EP: Epithelial cell; CT: Connective tissue; BV: Blood vessel. Scale bar: 100 μm

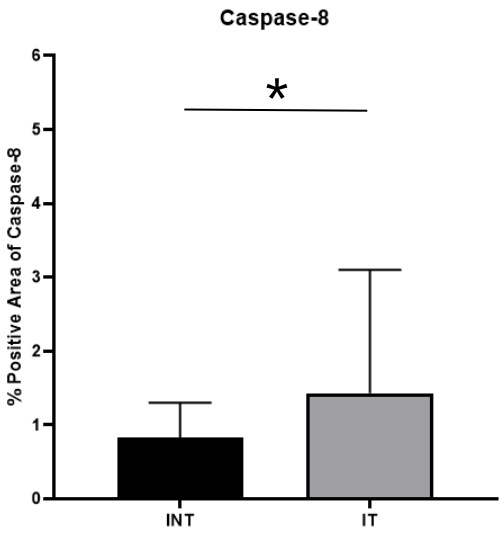

Figure 8. Area percentage of caspase-8 expression (n=3) of induction - non-therapy (INT) group and induction - therapy (IT) group in rat breast tumor tissue. Non-contact electric field therapy significantly increases the expression of caspase-8 in IT group rats. Analysis with the Mann-Whitney U-Test method. *: $p < 0.05$. Immunohistochemistry staining

The higher caspase-8 expression in the IT group is comparable to the expression of caspase-3 protein as the executor of apoptosis, which was also significantly higher in the therapy group [43]. The extrinsic apoptosis pathway is activated in response to ligands that bind to members of the death receptor superfamily, leading to activation of caspase-8 as the initiator, followed by caspase-3 as the executor. One of the common ligands and death receptors in the extrinsic pathway are FasL and FasR, which are associated with cytotoxic T cells [11]. Non-contact electric field therapy could induce apoptosis in the extrinsic pathway by influencing the migration of immune cells play a role in activating the caspase 8 protein, one of which is cytotoxic T cells through the Fas pathway. These data are in line with the higher infiltration value of CD8+ cells (cytotoxic T cells) in the IT group.

Non-Contact Electric Field Therapy Induced Higher Caspase 9 Expression in the Rat's Breast Tumor Tissue

The breast tumor tissue in the IT group also showed more positive expression of caspase-9 compared to that in the INT group (Figure 9). The results shown in (Figure 10) revealed that the caspase-9 expression in the IT group (4.05 ± 1.26%) was significantly higher compared to that of the INT group (1.52 ± 0.87%).

Intrinsic apoptosis pathway can be triggered through mitochondrial release of cytochrome c, leading to the formation of Apaf-1 and cytochrome c complex, thereby activating caspase-9 as the initiator followed by caspase-3 as the executor [44]. Non-contact electric field therapy has been shown to potentially induce the intrinsic pathway of apoptosis in the epithelial cell area of the rat's breast tumor tissue. On the other hand, degradation of myoepithelial cell in INT group promotes breast tumor progression and metastasis, although the exact mechanisms of this phenomenon are still unclear [29]. Activation of caspase-9 is closely related to the activity of cytotoxic T cells that trigger intracellular pro-apoptotic signals in the perforin and granzyme pathways [39].

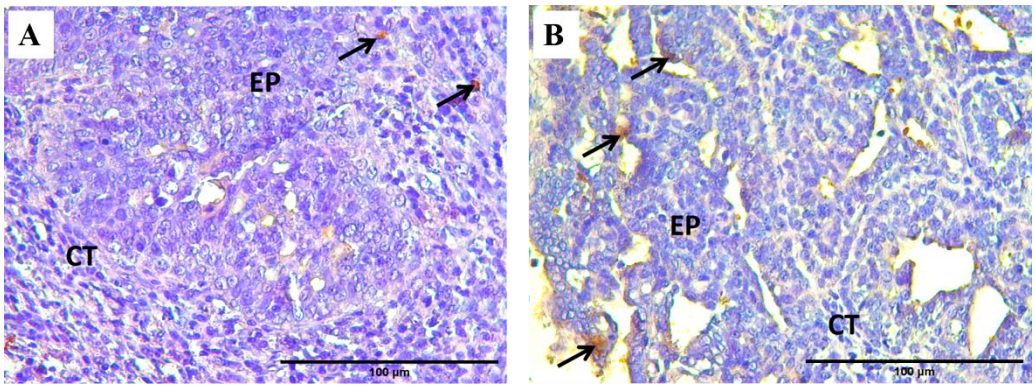

Figure 9. Immunohistostaining of the rat's breast tumor using caspase-9 antibody (n=3) on INT group (a) and IT group (b). Non-contact electric field therapy increased the expression of caspase-9 in IT group rats. Arrow (↑): Positive expression of caspase-9; EP: Epithelial cell; CT: Connective tissue. Scale bar: 100 µm.

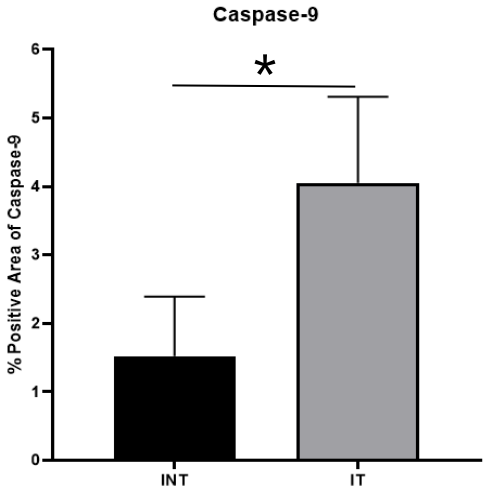

Figure 10. Area percentage of caspase-9 expression (n=3) of induction - non-therapy (INT) group and induction - therapy (IT) group in rat breast tumor tissue. Non-contact electric field therapy significantly increases the expression of caspase-9 in IT group rats. Analysis with the Mann-Whitney U-Test method. *: $p < 0.05$. Immunohistochemistry staining

Overall results showed the expression of caspase-9 in rat breast tumor tissue was higher than caspase-8, because in vertebrates, the majority of apoptosis occurs through the intrinsic pathway [23]. Intrinsic apoptotic pathway also has a close relationship with the extrinsic pathway. Ligation of the type 2 cell death receptor (Bcl-2) in the extrinsic apoptotic pathway can trigger a complex signaling cascade that activates the pro-apoptotic proteins Bid and Bax. Both of these proteins will activate caspase-3 (executor) through MMP (mithocondrial membrane permeabilization) in the intrinsic pathway, so that caspase-9 expression will also increases [23].

The regulation of cell death that occurs in tumor cells is very complex. Apoptosis and necrosis have a close relationship in suppressing tumor cell growth. The therapy increased apoptosis in the rat's breast tumor tissue, which was characterized by higher expression of caspase-8 and caspase-9 in the IT group. However, the results also showed a higher necrosis area in the treatment group, although not significant. This can occur due to the process of necroptosis associated with apoptosis. The apoptotic process can turn into necroptosis (programmed necrosis) if energy availability is low and caspase-8 inhibitors are present to inhibit the apoptotic process [55]. In addition, the decrease in the phagocytic capacity of macrophages can turn cells from undergoing apoptosis into cells that undergoing secondary necrosis [5].

The higher necrosis area in the IT group may be the effect of higher apoptosis in the IT group compared to the INT group. Apoptosis that occurs can turn into necroptosis or secondary necrosis depending on

the condition of the tumor being treated. When the rate of apoptosis is high, it is possible that the amount of energy and phagocytic capacity of macrophages are decreased and resulting a secondary necrosis. The results of necrosis area also showed insignificant numbers, so it could be concluded that non-contact electric field therapy with a frequency of 150 kHz and an intensity of 50 - 60 V/m does not have a significant effect on the necrosis of rat breast tumor tissue.

Conclusions

In conclusions, exposure of non-contact electric field with a frequency of 150 kHz and an intensity of 50 - 60 V/m in the rat's breast tumor tissue (*Rattus norvegicus* Berkenhout, 1769) can significantly increase the percentage of lymphocytes that express CD4 and CD8 proteins compared to the group without therapy. The percentage of lymphocytes that express CD8 was found to be higher than those that express CD4. The CD4/CD8 ratio of the INT group (0.28) is lower than the IT group (0.67). The level of necrosis tends to be higher in the treated breast tumor tissue compared to untreated rats, but the result was not significant. In addition, this therapy shows significantly higher protein expression of caspase-8 and caspase-9 in the treatment group compared to the untreated group. Thus, non-contact electric field therapy has a potential to improve the effectively of breast tumor treatment.

Conflicts of Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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