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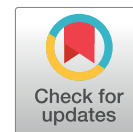
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# Relative expression of *IL-10* and *TNF-α* mRNA of kidney and spleen tissues of rat with and without mammary tumor after exposed to alternating current electric field

Nafisa Khoirun Widiastri<sup>1</sup> , Anyсах Nur Fauziyah<sup>1</sup> , Firman Alamsyah<sup>2</sup> , Rarastoeti Pratiwi<sup>1\*</sup>

<sup>1</sup>Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia

<sup>2</sup>Center for Medical Physics and Cancer Research, Ctech Labs Edwar Technology, Tangerang, Banten, 15143, Indonesia

\*Corresponding author: Teknika Selatan St., Sendowo, Sinduadi, Mlati, Sleman, Yogyakarta 55281. Email: [rarastp@ugm.ac.id](mailto:rarastp@ugm.ac.id)

## ABSTRACT

**Background:** Cancer therapy based on alternating current electric field exposure, Electro-Capacitive Cancer Therapy (ECCT), has been pre-clinically tested for its effectiveness in breast tumor inhibition. However, concerns regarding the safety of electric field (EF) exposure to vital organs have been raised. In the kidney and spleen, *IL-10* and *TNF-α* play roles in the response to inflammation.

**Objective:** This research aimed to evaluate the safety of electric field exposure to vital organs, by assessing the expression of *IL-10* and *TNF-α* mRNA in the kidney and spleen of rats, both with and without breast tumors.

**Methods:** Female rats were divided into four groups; non-induction-non-therapy (NINT), non-induction-therapy (NIT), induction-non-therapy (INT), and induction-therapy (IT). Rats were induced by 7,12-dimethylbenz[a]anthracene (DMBA) at a dosage 20 mg/Kg body weight and exposed to electric fields for 10 hours, followed by a 2-hour rest period. Total RNA from the kidney and spleen was isolated, cDNA was synthesized by reverse transcription PCR, and analysis of *IL-10* and *TNF-α* mRNA expression was performed by RT-qPCR method. Data were analyzed using the Livak formula, GraphPad Prism 9.0 software, and significance test ANOVA ( $\alpha = 0.05$ ).

**Results:** Alternating current electric field exposure had no effects on the expression of *TNF-α* and *IL-10* mRNA in the kidney and spleen of rats. Specifically, no effect was observed on *TNF-α* mRNA expression in all groups, and there was no effect on *IL-10* mRNA expression in the spleen. Only the INT group showed a significant increase in *IL-10* mRNA expression in the kidney ( $p < 0.05$ ).

**Conclusion:** Exposure of alternating current electric field did not affect the relative mRNA expression of *TNF-α* and *IL-10* in the kidneys and spleen of rats.

**Keywords:** Electric field, *IL-10*, kidney, mRNA, spleen, *TNF-α*, vital organs

## Introduction

Breast cancer is the most common cancer in women in the world [1]. The high number of breast cancer's survivor that continues to increase shows the importance of effective cancer therapy management. However, common cancer therapy management such as radiotherapy and chemotherapy has various side effects including prolonged pain, infertility, and hair loss. Side effects are also seen psychologically in patients who are more susceptible to depression, sleep and cognitive problems [2]. Therefore, an alternative

therapy is needed to minimize the negative impact of cancer therapy.

Electro Capacitive Cancer Therapy (ECCT), which employs exposure to alternating current (AC) electric fields, is an emerging alternative approach to cancer therapy. Preliminary study shows that treatment involving low-voltage (18Vpp) medium-frequency (100 kHz) electric field (EF) exposure *in vitro* can inhibit MCF-7 growth by 28-39% and cause carcinoma cell shrinkage in mice after 2 weeks of EF exposure [3]. ECCT can also have an anti-proliferative effect on breast tumor, in

terms of the down-regulation of *IL-18* and *CCL-2* whose roles in the development of breast tumor [4]. EF exposure has been studied for its effects on kidney's histological structure and function, with findings indicating no induced histological damage [5]. In addition, creatinine levels remained within the normal range, indicating EF exposure did not impair kidney function [6]. Similarly, spleen health indicators, such as erythrocytes and leukocyte counts, hemoglobin levels, hematocrit values, and erythrocyte index in Swiss strain mice, remained stable after exposure to EF fields and DMBA induction [7]. From the previous study, there was no effect of EF exposure on kidney and spleen, in terms of its function for both tissue and histological structure for kidney.

Electric field exposure's effect on the activity of the immune system such as macrophages could be studied through gene expression related to macrophages with pro-inflammatory and anti-inflammatory characteristics. EF may cause the stress oxidative that leads to tissue damage causing inflammation [8]. Some of the cytokines related to inflammatory response are tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin 10 (IL-10). TNF- $\alpha$  acts as an early mediator of inflammation and is important to the activation of various pro-inflammatory cytokines [9]. IL-10 is an anti-inflammatory cytokine that plays a role in limiting the host's immune response to pathogens thereby preventing further tissue damage and maintaining homeostasis [10]. However, the molecular response involving both TNF- $\alpha$  and IL-10 mRNA expression has not been studied. This research aims to address this gap by studying the molecular response of both inflammatory-related genes after EF exposure.

The kidney, an essential player in the body's excretory system and immune system homeostasis [11,12], often serves as a crucial parameter in disease treatment research. The spleen, being the biggest lymphoid organ in the body, plays important role in adaptive immunity, housing various immune cells subset such as B cells, T cells, macrophage, and dendritic cells distributed across its red pulp, white pulp, and marginal zone [13]. The effect of EF exposure on the inflammation response in the

spleens of both normal and breast tumor-bearing rats has not been studied further. Therefore, it is necessary to study the gene expression related to the inflammatory response in the spleen. In this study, both normal and breast tumor-bearing rats were exposed to EF to examine the effect of EF towards non-target organs, such as the kidney and spleen, which have important roles.

This research was conducted in order to study the expression of mRNA gene related to inflammation response, especially TNF- $\alpha$  and IL-10 mRNA expression in kidney and spleen of normal and breast-tumor-bearing Sprague-Dawley (SD) rats induced with DMBA and exposed to medium-frequency AC electric field.

## Methods

### Animals and induction of mammary tumors

This research utilized kidney and spleen samples from Sprague-Dawley rats (*Rattus norvegicus*, Berkenhout, 1769), which were preserved in RNALater solution (Invitrogen; cat. no. AM7024) and stored at -20°C. The samples were collected under Ethical Clearance no. 00029/04/LPPT/IV/2018. The tissue samples included kidneys and spleens from rats, both induced and not-induced by 7,12-dimethylbenz[a]anthracene (DMBA). The samples were grouped into four groups, with six biological replicates in each: non-induced and non-treated (NINT) as the control, non-induced but treated (NIT) as the placebo, induced but non-treated (INT), and induced and treated (IT). The induction was carried out using DMBA (20 mg/kg body weight), administered orally twice a week for a total of 10 times over 5 weeks. For the NIT and IT groups, ECCT was carried out through exposure to an AC electric field with a frequency of 150 kHz and a voltage of 18 Vpp. The ECCT procedure was conducted for a duration of 2 x 5 hours daily over 21 days, with a 2-hour break each day [4].

### Total RNA isolation

Total RNA isolation was performed according to the Direct-zol™ RNA miniprep Plus (ZymoScience; cat. no. R2072) protocol, with a few modifications.

**Table 1.** Primer sequence of *IL-10*, *TNF- $\alpha$* , and *GAPDH*.

Gene		Sequence (5' → 3')	Product length
<i>IL-10</i> [14]	Forward	GCAGGACTTTAAGGGTTACT	264 bp
	Reverse	TTCATGGCCTTGTAGACACC	
<i>TNF-<math>\alpha</math></i> [15]	Forward	AGCATGATCCGAGATGTGGAA	101 bp
	Reverse	AATGAGAAGAGGCTGAGGCACA	
<i>GAPDH</i> [16]	Forward	AGTCCCCATCCCAACTCAGC	138 bp
	Reverse	TTTTTGAGGGTGCAGCGAAC	

28 31 The heart, kidney and spleen samples were removed from the RNALater solution, weighed, and crushed in Trisure solution. Following this, an equal volume of chloroform was added and the mixture was homogenized. RNA extraction was carried out by using a refrigerated centrifuge at a speed of 12,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a 1.5 mL tube and an equal volume of ethanol was added, followed by homogenization. Half of the mixture was then moved to a column on the collection tube and centrifuged for 1 minute. The supernatant was discarded. The process was repeated with the remaining solution.

5 Next, 400  $\mu$ L RNA wash buffer was added to the column, centrifuged for 1 minute, and the supernatant was discarded. A DNase solution was prepared by mixing DNase and DNA digestion buffer in a 1:15 ratio, totaling 80  $\mu$ L in volume. The homogenized mixture was added to the column and left to incubate for 15 minutes. The column was then washed with 400  $\mu$ L of Directzol RNA, followed by 1-minute centrifugation. The supernatant was discarded, and this step was repeated. Afterward, 700  $\mu$ L RNA wash buffer was added to the column, centrifuged for 3 minutes, and the supernatant was discarded. Finally, the RNA was eluted with 50  $\mu$ L nuclease-free water (NFW) solution. The RNA concentration was measured using Nanodrop spectrophotometer, with absorbance measured at  $\lambda = A_{260}/A_{280}$  (Spectrophotometry Nanodrop®).

### cDNA synthesis

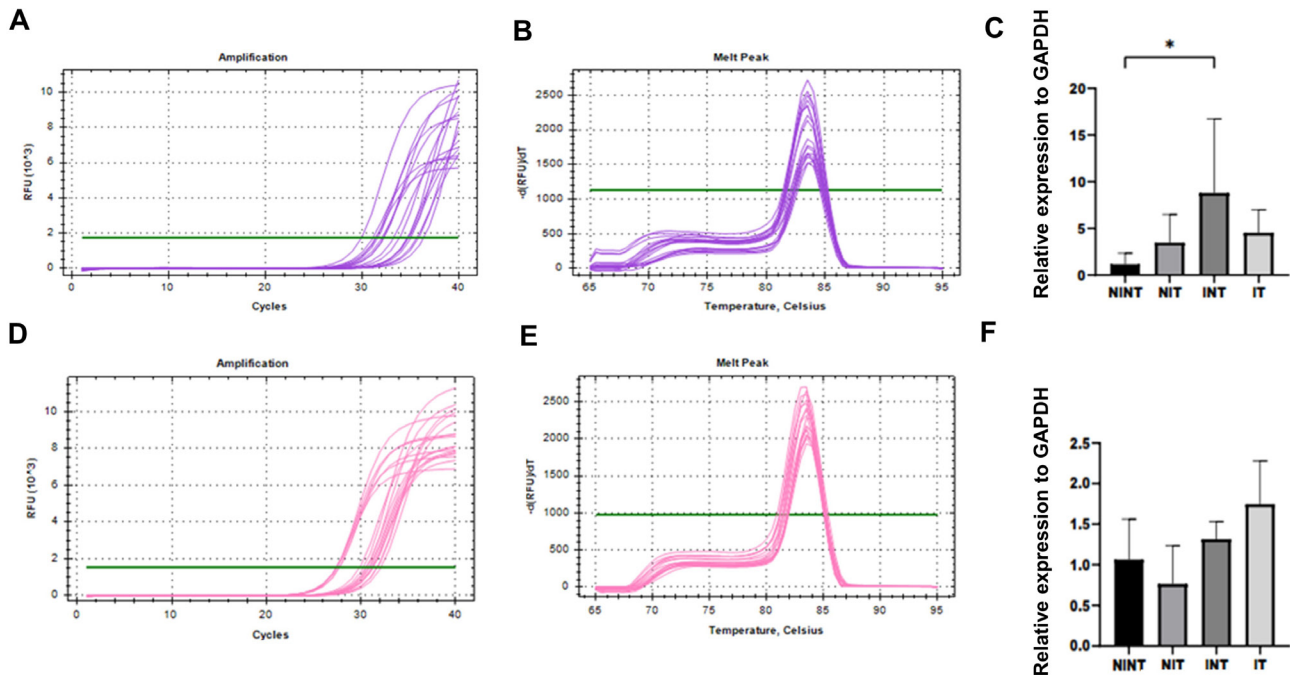
15 3 The synthesis of cDNA was performed referring to ReverTra Ace qPCR RT Master Mix with gDNA

remover by Toyobo protocol. A solution mixture was prepared containing 5  $\mu$ L of 4x DN Master Mix, 5  $\mu$ L of 5x RT Master Mix II, and 10  $\mu$ L of a mixture of RNA and NFW solution. The Master Mix solution contains a random and OligodT primer. All RNA concentrations were equalized into 500 ng/ $\mu$ L. Once the mixture was ready, the PCR process was performed with the following steps: genomic DNA removal (37°C, 5 minutes), amplification (37°C for 15 minutes, 50°C for 5 minutes, and 98°C for 5 minutes), and followed by a hold (4°C, 5 minutes).

### Gene expression analysis using RT-qPCR

The cDNA obtained from the previous step was analyzed using RT-qPCR method referring to SensiFAST SYBR® No-ROX (BIO-98005) protocol. This study focused on three genes: two genes of interest (*TNF- $\alpha$*  and *IL-10*), and one reference gene (*GAPDH*). The temperature of each gene was optimized using this method as well. For the quantification of gene expression, a mixture was prepared that consisted of 5  $\mu$ L of SYBR reagent, 0.4  $\mu$ L of both reverse primer and forward (primer sequences, Table 1), 3.2  $\mu$ L of NFW solution, and 1  $\mu$ L of cDNA template. For the negative control, the same mixture components were used, excluding the cDNA template. Afterward, the RT-qPCR method was performed with the following steps: activation (95°C, 2 minutes), denaturation (48°C, 5 sec), annealing (*TNF- $\alpha$* , *IL-10*, and *GAPDH*, 60 °C for 10 sec), and extension (72°C, 20 sec) using RT-qPCR Machine (Biorad CFX 96). The primers used in this study were optimized for the optimum annealing





**Figure 1.** Relative mRNA expression of *IL-10* in kidney and spleen after ECCT treatment. Amplification and melt peak curve of *IL-10* in (A and B) kidney and (D and E) spleen. Relative expression of *IL-10* mRNA (C) in the kidney and (F) in the spleen. Error bars show standard deviations of six replications; \*p < 0.05

temperature. Agarose gel electrophoresis of the qPCR amplicon was performed to verify the specificity of the primers. As the result, a single band of each gene was formed, explaining that the primers were specific.

## Data analysis

The expressions of the gene were shown as Cq value and analyzed using Livak Method formula [17]. Normalization of Cq value of the gene target compared to the reference gene was performed with the following equation.

$$\Delta Cq_{(test)} = Cq_{(target, test)} - Cq_{(GAPDH, test)}$$

$$\Delta Cq_{(calibrator)} = Cq_{(target, calibrator)} - Cq_{(GAPDH, calibrator)}$$

$$\Delta\Delta Cq = \Delta Cq_{(test)} - \Delta Cq_{(calibrator)}$$

Afterward, the ratio between relative gene expression or fold change from each group was measured and compared to the control as the representation of normal condition with the following formula;  $2^{-\Delta\Delta Cq}$ . The significance of fold change for each group was analyzed with ANOVA test using GraphPad Prism 9.0 software.

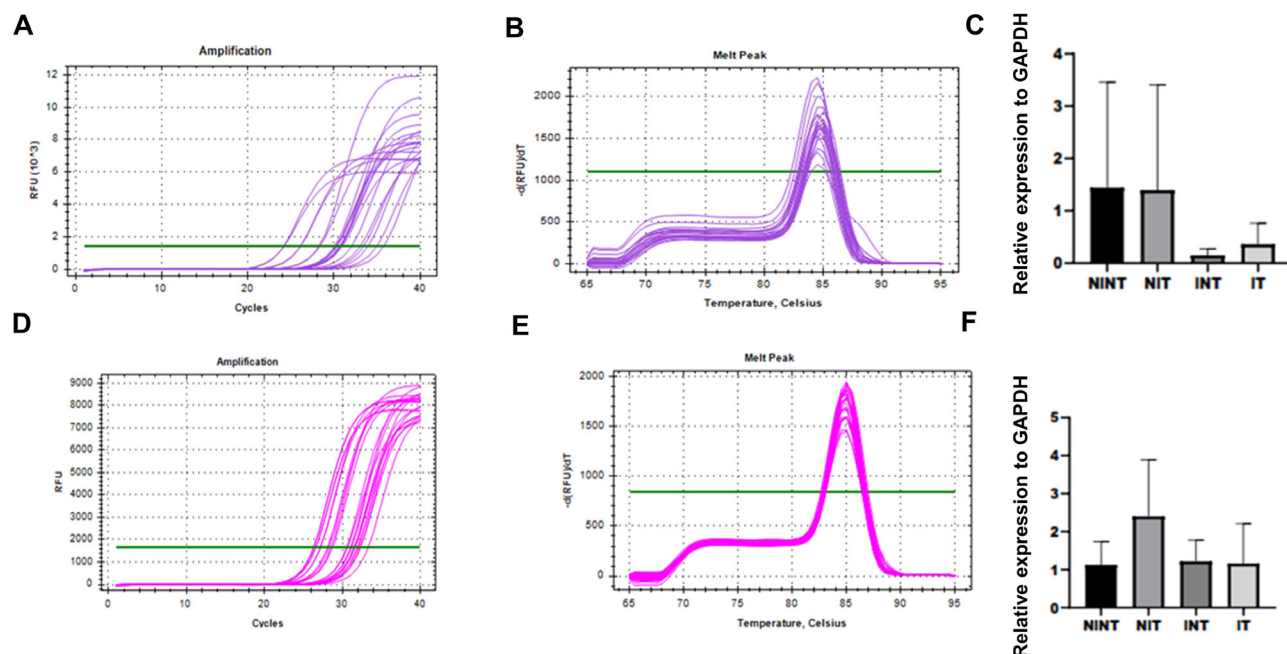
## Results

### Gene expression analysis result

The mRNA relative expression of *IL-10* (Figure 1) and *TNF- $\alpha$*  (Figure 2) were obtained as the result in this study. The change of expression level for each gene was shown as fold change or the change of Cq value.

Quantification results of *IL-10* mRNA relative expression in the kidney (Figure 1E.) showed that there was no effect of EF exposure for NIT and IT groups compared to the control, even though there were increased tendencies. Meanwhile, for INT group, there was an increase (upregulated) ( $p < 0.05$ ) after EF exposure. In the spleen (Figure 1F.), there was no effect of EF exposure for all groups compared to the control, even though there were increased tendencies as well.

Quantification results of *TNF- $\alpha$*  mRNA relative expression in kidney (Figure 2E) and in spleen (Figure 2F) showed that there was no effect of EF exposure for all groups compared to the control (NINT), though there were down tendencies.



**Figure 2.** Relative mRNA expression of *TNF- $\alpha$*  in kidney and spleen after ECCT treatment. Amplification and melt peak curve of *TNF- $\alpha$*  in (A and B) kidney and (D and E) spleen. Relative expression of *TNF- $\alpha$*  mRNA (C) in kidney and (F) in spleen. Error bars show standard deviations of six replications; ns:  $p > 0.05$

## Discussion

In this research, *IL-10* and *TNF- $\alpha$*  mRNA expression in kidney and spleen of normal and breast-tumor rats after exposure to AC electric fields with medium frequency (150 kHz) and low voltage (18 Vpp) were quantified. The gene of GAPDH is always expressed in every cell in any condition, during normal or pathophysiological conditions, hence it was used as a reference gene [18]. This study reveals that there was no effect of EF exposure towards the expression of *IL-10* and *TNF- $\alpha$*  mRNA expression in the kidney and spleen. In addition, the induction of DMBA towards rats can cause the increase of *IL-10* mRNA expression.

*IL-10* mRNA expression level in kidney was not affected by EF exposure in NIT and IT groups, though there were increasing tendencies. *IL-10* secretes anti-inflammatory cytokines that play a role in cell regeneration [19]. The cell cycle and cytokinesis process will be disrupted when the cell receives electric field exposure [3]. Disrupted cell cycle leads to cell damage that induces inflammatory response. The body has mechanisms to deal with cell damage and maintain

the homeostasis state, one of the mechanisms is by secreting *IL-10* as an anti-inflammatory agent. In addition, the expression level of *IL-10* mRNA in renal tissue was significantly increased in INT group. *IL-10* may respond to inflammatory and carcinogenic agents such as DMBA. Oral induction of DMBA allows the infiltration of renal tissue. DMBA may also induce oxidative stress. According to Haddad and Fahlman [20], *IL-10* plays a role in inflammatory and oxidative stress response as an antioxidant agent. The significant increase of this gene may be associated with its response in dealing with oxidative stress caused by DMBA. There are two pathways that may change the *IL-10* secretion, namely Redox pathway and ROS pathway [20]. In this study, DMBA may induce oxidative stress that alters ROS equilibrium in the cell. When the equilibrium of ROS is altered, there will be a change in the equilibrium and affect the phosphorylation process which has an impact on gene expression so that the expression level of *IL-10* is changed [20]. In this study, the main source of increasing *IL-10* mRNA expression was not clearly observed. It was because in this study, total RNA from every cell of the tissue were

used, making it not available to examine whether it is from the nephron cells or macrophages cells that are related to inflammatory response. Therefore, future research to differentiate the source of increased *IL-10* mRNA expression is needed.

*IL-10* mRNA expression in the spleen was not changed in NIT group, though there were decreasing tendencies. The result showed there was no effect of electric field exposure to the spleen organ without DMBA induction. Meanwhile, in INT and IT groups, there was no change with increased tendency in relative expression of *IL-10* gene. Induction of DMBA as a carcinogenic could potentially increase reactive oxygen species (ROS). Increased ROS at high levels may cause oxidative stress on target cells, damage to macromolecule level, inflammation, tumor growth, metastasis and at the highest level may cause cell death [21]. *IL-10* as an anti-inflammatory cytokine has roles in inhibiting ROS through inhibition of NF- $\kappa$ B activation and proteolytic degradation of I $\kappa$ B- $\alpha$  inhibitor subunits. NF- $\kappa$ B is also known to be involved in the activation of pro-inflammatory cytokines and regulates the transcription of various pro-inflammatory cytokines that cause inflammatory responses in tissues [22].

There was no effect of EF exposure towards *TNF- $\alpha$*  mRNA relative expression in the kidney for NIT, INT, and IT groups although there were decreasing tendencies. In NIT and IT groups, there were decreasing tendencies for both groups. *TNF- $\alpha$*  cytokine is a pro-inflammatory cytokine that the overexpression of *TNF- $\alpha$*  itself might initiate inflammation responses in tissue. By suppressing the expression of this gene, tissue damage may be reduced.

For the INT group, there was no effect of EF exposure towards *TNF- $\alpha$*  expression level, though there were decreased tendencies for all of the groups. Compared to NIT and IT groups, INT group had the biggest decrease tendency. It may be caused by DMBA induction disturbing the homeostasis and equilibrium of the cell. In this study, there was an increase of *IL-10* in the

same group. *IL-10* can suppress the expression of pro-inflammatory cytokines such *TNF- $\alpha$*  [23].

Overall, the decreasing tendency of *TNF- $\alpha$*  may be correlated to the increase and the increasing tendency of *IL-10* relative mRNA expression in the kidney in this study. *IL-10* might suppress the expression of *TNF- $\alpha$*  through post-transcriptional mechanisms, inhibition of NF $\kappa$ B and p38 MAPK activation, and the inhibition of Janus Kinase 1 Pathway (JNK1) [24]. Related to the increase and increasing tendency of *IL-10* gene expression in this study, the expression of *TNF- $\alpha$*  had a decreasing tendency. However, the gene expression change after the electric field exposure is not significant, so it can be considered that there was no effect on the relative expression level of *TNF- $\alpha$*  in the kidney.

There was no change with the increasing tendency in *TNF- $\alpha$*  mRNA relative expression in spleen tissue for NIT, INT, and IT groups compared to the control. The result shows that the AC electric field had no effect on *TNF- $\alpha$*  mRNA relative expression in the spleen. Referring to previous study [25], there was stress potential on experimental animals and decreased immunity after an electric field (heat shock). These stresses might cause a decrease in lymphocyte recovery, the quantity increase of the splenocyte that is indicated by tissue swelling.

*TNF- $\alpha$*  belongs to pro-inflammatory cytokines that appear at the beginning of infection, therefore it may be easily expressed when there is an inflammatory response as surveillance [26]. In the spleen, *TNF- $\alpha$*  plays a role in the formation of Fibroblastic Reticular Cells (FRCs), hence the absence of *TNF- $\alpha$*  indicates the abnormality of FRCs in the spleen. *TNF- $\alpha$*  is activated by two kinds of receptors, TNFR1 and TNFR2, that initiate the transduction pathway. TNFR1 is related to the cytotoxic induction responses as immune surveillance. Meanwhile, TNFR2 plays a role in the activation, migration, and proliferation of the cell. Both of the receptors may cause inflammation response, tissue degeneration, and cell proliferation [27].

Exposure to AC electric fields may affect the dielectric characteristic of tissue, especially on its relative permittivity. Permittivity shows the effect of organ polarization after electric field exposure. The polarization will appear following the change of internal cell charge as the result of exposure to external electric fields [28].

Normal cells have lower permittivity than tumor or cancer cells, estimated to be around 1:6.4 and 1:3.4. This difference is due to histological and physiological conditions on both kinds of cells in which cancer cells have higher water quantity and blood vessels [29]. The membrane potential ( $V_m$ ) in tumor cells is higher and depolarized compared to normal cells where the range of  $V_m$  in normal cells tends to be more negative. The  $V_m$  value indicates a high rate of cell proliferation in tumor cells [30]. This is in accordance with the previous explanation where the permittivity of tumor cells is higher and it is easier to change polarization so that exposure to an electric field has a more significant effect on tumor cells than normal cells.

Permittivity is related to the content of the tissue. Kidney has high dielectric properties, such as permittivity and conductivity, due to its water content [31]. Spleen tissue has fairly high permittivity compared to the kidney due to the number of blood vessels and blood cells related to its function as a lymphatic organ and blood circulation. Nonetheless, Kuwahara [32] stated tumor cells have much higher permittivity because they have a lot of blood vessels to support metastasis. In addition, tumor cells have higher water content than normal cells. It may cause there was no effect of EF exposure towards normal cells, such as kidney and spleen in this study.

Previous study revealed that EF exposure doesn't significantly induce kidney histological damage [5]. In addition, after exposure to EF, kidney had no change in its function based on the level of creatinine that remained in the normal range [6]. Related to spleen function as the lymphatic organ that involves a lot of blood

cells, exposure of EF had no effect on the number of erythrocytes, leukocyte count, hemoglobin level, hematocrit value, and erythrocyte index of Swiss strain mice induced by DMBA and exposed to AC electric field [7]. Septiani [33] also stated that low-voltage medium-frequency AC electric field therapy was relatively safe for the hematological profile of Sprague Dawley rats induced by DMBA. Therefore, exposure to an AC electric field does not cause a significant polarity change in the normal spleen.

## Conclusion

The exposure of electric field therapy with medium frequency (150 kHz) and low voltage (18 Vpp) had no effects on the relative mRNA expression of TNF- $\alpha$  and IL-10 genes in the kidney and spleen, making it a potential alternative treatment for breast cancer. To understand the safety of ECCT exposure to the kidney and spleen, the study of inflammation-related genes such as TGF- $\beta$  and IL-6 is needed.

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## Author contributions

Conceptualization, F.A.; Methodology, R.P.; Investigation, N.K.W. and A.N.F.; Writing – Original Draft, N.K.W. and A.N.F.; Writing – Review & Editing, N.K.W., A.N.F., and R.P.; Funding Acquisition, N.K.W., A.N.F., and R.P.; Resources, N.K.W., A.N.F., and R.P.; Supervision, F.A., and R.P.



## Declaration of interest

The authors declare that they have no conflicts regarding the interest of this study.

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