Electric Field-Based Cancer Therapy Induces the Expression of *HMGB1* and *PD-L1* mRNA Genes on Breast Tumor of Female Rats

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Abstract

Electro Capacitive Cancer Therapy (ECCT) is an electric field-based cancer therapy method using intermediate frequency (150 kHz) and low intensity (18 Vpp). High Mobility Group Box 1 (*HMGB1*) is a cytokine related to damage-associated molecular patterns (DAMPs) secreted by dead cells. The expression of Programmed Death Ligand 1 (*PD-L1*) is ligand present on the surface of tumor cells and its expression is associated with the increase in the number CD8+ T lymphocytes. This study aims to examine the effect of ECCT exposure on the expression of *HMGB1* and *PD-L1* genes on the breast tumor, brain, and liver tissues of Rattus norvegicus (Berkenhout, 1769). The tissues were obtained from the previous studies stored in RNAlater (-20˚C). Female rat tissues of the previous study from four treatment groups, namely the control group (NINT), non-DMBA-induction with therapy (NIT), DMBA-induction with non-therapy (INT), and DMBA-induction with therapy (IT). Gene expression was analyzed using the RT-qPCR. Statistical t-test with a $p<0.05$ significance level was performed using GraphPad Prism 9.4.0 software. The result shows *HMGB1* and *PD-L1* mRNA genes were both significantly expressed in breast tumor samples. The liver and brain samples of normal rats did not show any significant changes in the activity of these genes after exposure to the electric field. This study indicates that exposure to electric fields may trigger the expression of *HMGB1* and *PD-L* on the rat's breast tumor samples. This study also provides information related to the safety of ECCT in healthy organs of female rats, especially the brain and liver.

Keywords: *ECCT, breast tumor, HMGB1, PD-L1, IFN- γ.*

INTRODUCTION

Electro-capacitive Cancer Therapy (ECCT) is a cancer therapy device based on an intermediate-frequency (100 kHz) low-intensity (18 Vpp) electric field. Cancer therapy based on exposure to an intermediate frequency (100-300 kHz) low-intensity (1-3 V/cm) electric field has an inhibitory effect on proliferating cells (Kirson, *et al*., 2004).

Alamsyah, *et al*. (2015) showed that exposure to the ECCT electric field resulted in a decrease in the size of tumor tissue by more than 67% and a

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decrease in the number of proliferating cells. Pratiwi, *et al.* (2020) reported that the antiproliferative effect on cancer cells after exposure to the ECCT electric field decreased CCL2 and IL18 gene expression. Exposure to electric fields can activate tumor suppressor factor p53 and cause tumor growth to be inhibited (Mujib, *et al*., 2017).

High Mobility Group Box 1 or *HMGB1* is a cytokine acting as a damage-associate molecular pattern (DAMP). *HMGB1* is expressed by the cells during stress or by the dead cells. *HMGB1* plays a role in dendritic cell maturation and antigen presentation to cytotoxic T cells (Dong, *et al*., 2022). *HMGB1* blockade in breast tumors causes a drastic decrease in T lymphocytes (Hubert, *et al*., 2021).

Programmed death ligand 1 (*PD-L1*) is a ligand of the programmed death 1 (PD-1) receptor on the surface of T cells. The *PD-1*/*PD-L1* interaction causes immune tolerance by blocking the activity of T lymphocytes so that the immune system does not carry out excessive activities, resulting in damage to healthy cells. In certain conditions such as tumors, *PD-L1* binds to *PD-1* causing blockade of the immune response by inhibiting T cell activity (Kythreotou, *et al*., 2018).

Interferon γ (*IFN-γ*) is an inflammatory cytokine known for its antiviral and immunomodulatory characteristics (Horras, *et al*., 2011), as well as its role in the apoptosis induction of various cells (Dai and Krantz, 1999), immunosurveillance, and tumor growth suppression (Castro, *et al*., 2018). Kaplan, *et al*. (1998) reported that non- *IFN-γ* sensitive rats with *IFN-γ* receptor-1 deficiency experience faster tumor metastasis. Its expression and pro-inflammatory activity is associated with the M1 polarized macrophages, especially in the liver (Wang, *et al*., 2021).

ECCT only targets proliferating cells, so this therapy is considered safe for healthy tissue. *In vivo* research conducted by Alamsyah, *et al*. (2015) showed no significant changes in the complete blood profile of control mice post-therapy. Alamsyah also reported no signs of abnormalities on the skin and breast tissue in placebo mice.

Our investigation uses samples from female rats that have previously been exposed to an electric field using an ECCT cage. Although ECCT only targets proliferating cells, in the previous study (Pratiwi, *et al*., 2020), the whole-body parts of the rats were exposed to the electric field. Hence studies related to the safety of the device, especially in healthy organs, must be carried out.

The purpose of this study is to provide evidence that exposure to a low-intensity intermediate-frequency electric field is capable to activate the production of the cytokine *HMGB1* and the expression of ligand *PD-L1*. We also provide information for the first time regarding the safety of the device to healthy tissue; liver, and brain of female rats after being exposed to ECCT by analyzing the activity of *HMGB1* and *PD-L1*.

METHODS

This study was conducted at the Laboratory of Biotechnology of the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada (UGM). This research uses breast, brain, and liver samples of female rats (Rattus norvegicus Berkenhout, 1769; SD strain) obtained from a previous study by Pratiwi, *et al*. (2018) with ethical clearance No. 00029/04/LPPT/IV/2018. In the aforementioned study, female rats are subjected to 4 types of treatment focusing on DMBA induction for tumor growth and electric field therapy, each treatment consist of 6 rats as individual replication; Non-Induced Non-Therapy rats as the negative control (NINT), rats treated with only the electric field therapy and no DMBA induction (NIT), rats that are only treated with DMBA induction and no electric field therapy (INT), and rats treated with DMBA induction and electric field therapy (IT). After breast tumors were developed, the rats were sacrificed, and the tissue samples of tumors and various organs were obtained.

The sample of breast, brain, and liver samples are subjected to total RNA isolation using RNA ZymoResearch–Direct-zol RNA Miniprep Plus R2072 (California, US), followed by RT-PCR cDNA synthesis using Bioline SensiFAST cDNA Synthesis Kit BIO-65053 (Ohio, US), and finally gene quantification by qPCR method using Bioline SensiFAST SYBR No-ROX Kit BIO-98005 (Ohio, US). The qPCR protocol for each cycle includes polymerase activation at 95˚C (2 mins), denaturation at 95˚C (5 secs), annealing at 60˚C for *HMGB1* and *PD-L1* and 62.6˚C for interferon γ (10 secs), and extension at 72˚C (2 mins). The primers used for gene target amplification are HMGB1 F: TTCTGTTCTGAGTACCGCCCA R: TTGTCATCCGCAGCAGTGTT (Liu, *et al.*, 2019), PD-L1 F: TGAAAGTCAACGCTCCATACC R: CTCAGCCTGGCACATTAGTT (Takaki, *et al.*, 2020), and Interferon γ F: GCAAAAGGACGGTAACACGA R: TGCTGATGGCCTGGTTGTC (Lin, *et al.*, 2016). Target genes are normalized using GAPDH F: TGACAACTTTGGCATCGTGG R: GGGCCATCCACAGTCTTCTG (Taki, *et al.*, 2014). The relative expression of the target gene is calculated using Livak's formula and statistically analyzed using the GraphPad Prism 9.4.0 software. Result significance is determined by t-test with $p<0.05$ value considered as significant.

RESULTS

Relative mRNA Expression of *HMGB1* and *PD-L1* of Rat's Breast Tumor Tissue

The observation result of the *HMGB1* gene is shown in Figure 1. (A, B, E). The amplification chart of the *HMGB1* gene shows Cq values in the range of 20 to 30 (A). A single peak was obtained and shown on the melt peak curve at the tempera-

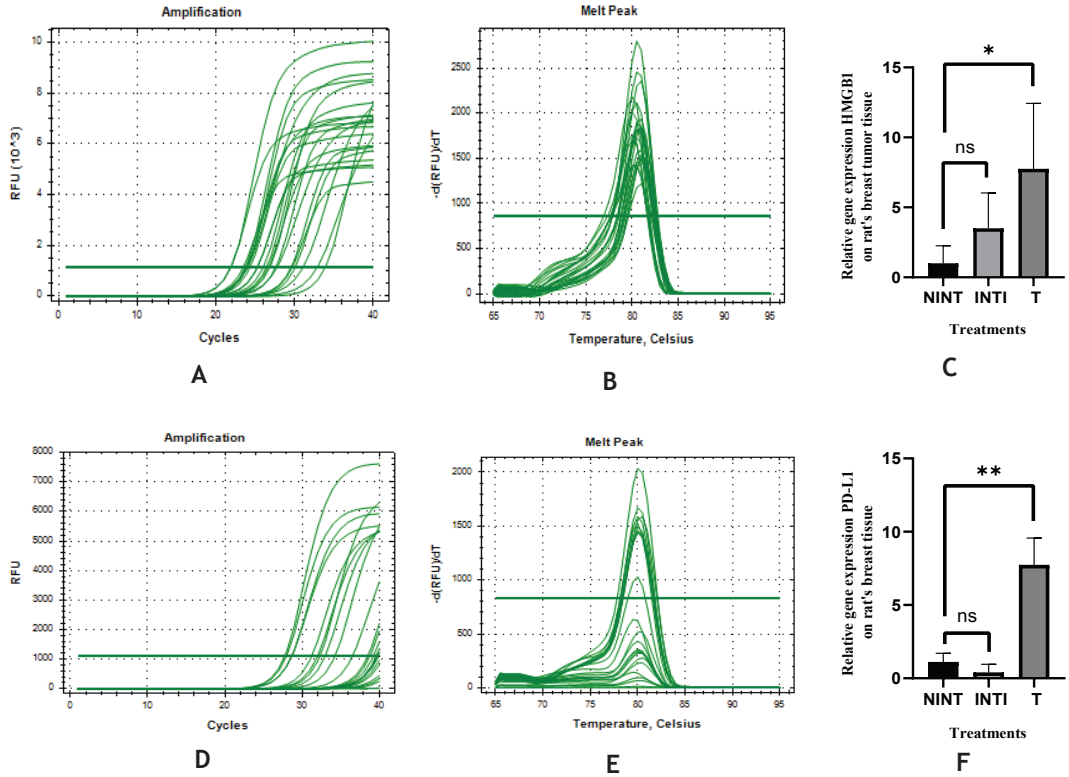

Figure 1. Relative mRNA expression of *HMGB1* and *PD-L1* genes on female rat's breast tumor tissue before and after ECCT treatment. Amplification and melt peak charts of (A and B) *HMGB1* and (C and D) *PD-L1*. Relative expression of mRNA (E) *HMGB1* and (F) *PD-L1* genes. The standard deviation of the mean data experiment shows **, $p<0.01$, n=6.

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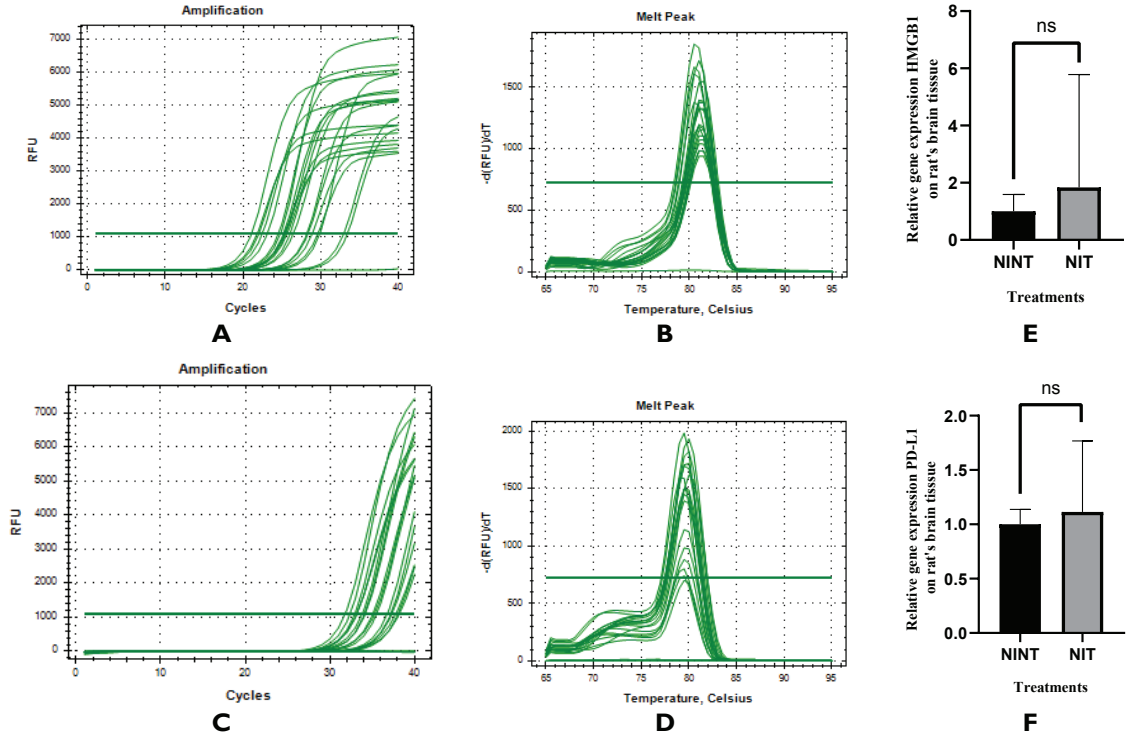

Figure 2. Relative mRNA expression of *HMGB1* and *PD-L1* genes on female rat's brain tissue before and after ECCT treatment. Amplification and melt peak charts of (A and B) *HMGB1* and (C and D) *PD-L1*. Relative expression of mRNA genes (E) *HMGB1* and (F) *PD-L1*, ns=$p \geq 0.05$, n=6.

ture of 81°C (B). This single peak in the melt curve indicates that the gene primers used in the amplification process are specific.

Statistical analysis shows a generally up -regulated expression of mRNA *HMGB1* with using GAPDH as reference gene (E). The up-regulation occurred on the tumor samples of both treatments with and without ECCT exposure. The elevated level of *HMGB1* on the INT samples occurs due to the molecular response of the tissues caused by tumor induction, even though a significant level was not achieved. The significant expression of *HMGB1* was observed one the IT treatment in which the tumor samples were exposed to ECCT treatment ($p<0.05$).

The amplification chart of *PD-L1* genes (C) shows a Cq value range between 20 to 40 for the target gene. The single peak is shown at the temperature of 80˚C (D), indicating the specificity of the primer.

Statistical analysis shows breast tumor tissue of rats that were not given electric field therapy, the INT treatment group, showed a decrease in the *PD-L1* gene expression with GAPDH as reference gene; this decrease, however, has not reached a significant level. Instead, this study revealed that the elevated level of the *PD-L1* gene expression was highly significant ($p<0.01$) in breast tumor tissue after ECCT therapy was given.

Relative mRNA Expression of *HMGB1* and *PD-L1* on Rat Brain Tissues

Observation shows a single peak on the melt peak curve at the temperature of 81°C for both genes (Figure 2A-D). The single peak in the melt curve indicates that the gene primers used in the amplification process are quite specific.

The relative mRNA expression is depicted in the form of a histogram (Figure 2 E-F). The charts

show the expression of both *HMGB1* and *PD-L1* using GAPDH as reference gene on the brain tissue of healthy rats after being exposed to ECCT treatment. Although the electric field therapy causes a slight increase in the expression of target genes, the values were microscopic and does not reach statistical significance (p>0.05). Hence, we may conclude based on the experiment and statistical analysis that exposure to ECCT does not cause any significant changes in the level of *HMGB1* and *PD-L1* genes in the brain tissue of healthy rats.

Relative mRNA Expression of *HMGB1*, *PD-L1*, and Interferon γ on the Liver Sample of Healthy Female Rats Before and After Exposure to ECCT

The amplification chart depicted in Figure 3. shows a single peak on the melt peak curve in the temperature of 82˚ (B), 80.5˚ (D), and 79˚C (F) for *HMGB1*, *PD-L1*, and Interferon γ genes respectively. The single peak in the melt curve indicates that the gene primers used in the amplification process are specific.

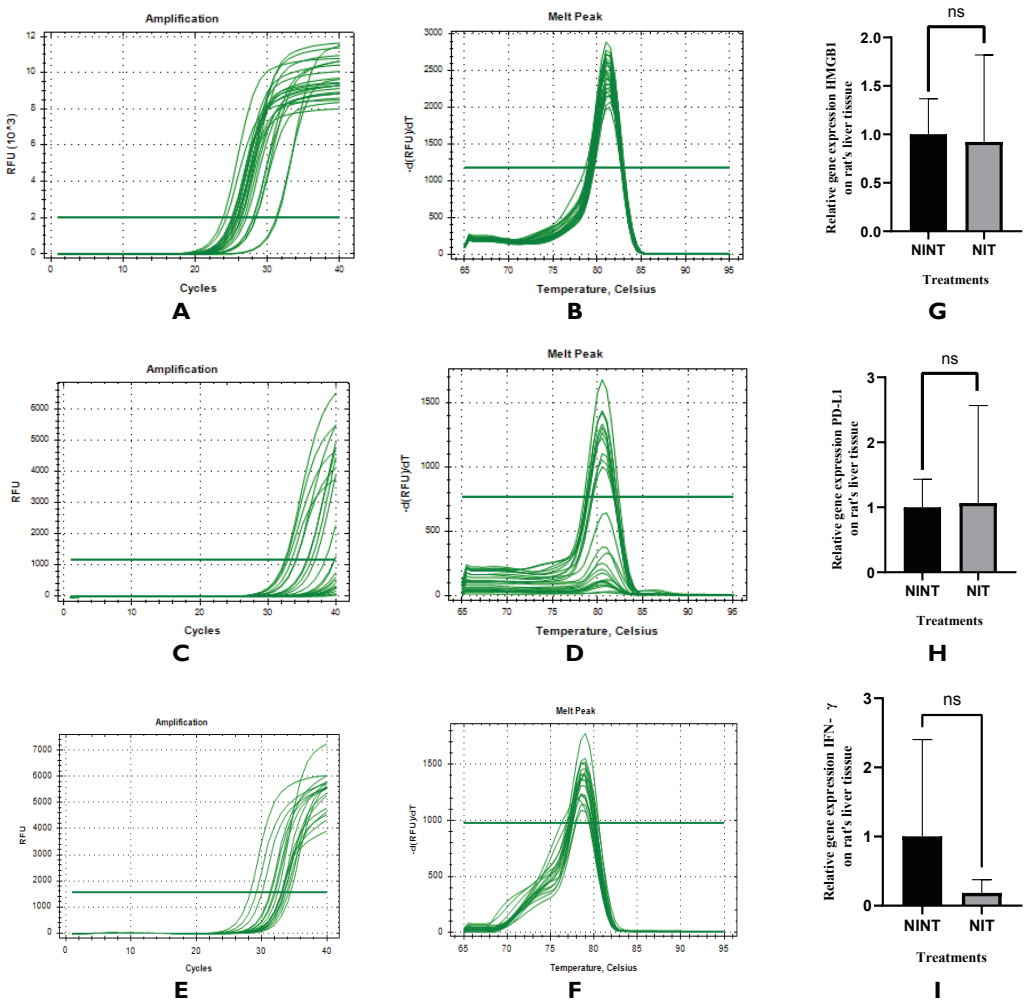

Figure 3. Relative mRNA expression of HMGB1, PD-L1, and interferon γ genes on liver tissue of healthy female rats before and after ECCT treatment. Amplification and melt peak charts of (A and B) *HMGB1*, (C and D) *PD-L1*, and (E and F) Interferon γ. Relative expression of mRNA genes (G) *HMGB1*, (H) *PD-L1*, and (I) Interferon γ, ns=p≥0.05, n=6.

The quantification results of the relative mRNA expression of the target gene; *HMGB1* and *PD-L1* showed no significant changes in the liver tissue of healthy rats after exposure to ECCT. The activity of the interferon *γ* gene after the exposure was down-regulated ($p>0.05$) using GAPDH as reference gene. However, the down-regulation of interferon γ has not yet reached a significant level. This indicates that exposure to an electric field has no significant effect on the activity of the *HMGB1*, *PD-L1*, and interferon γ genes in the liver tissue of healthy female rats.

DISCUSSION

Electric fields with low intensity (2 V/cm) and intermediate frequency (100–300 kHz) are shown to have inhibitory effects on proliferating cells (Kirson, *et al*., 2004). During exposure to an external electric field, cells undergo proliferation arrest and destruction. Mitosis began normally in treated cells exposed to an external electric field; however, it was delayed for some time (average within 2 h) before cytokinesis (Kirson, *et al*., 2004). The electric field exposed to tumor tissue is able to interfere with the activity of microtubules and septin filaments which are the main components in the cell division process, causing abnormal chromosome segregation to occur and resulting in cell death (Giladi, 2016).

The release of *HMGB1* to the cell surface as DAMP is one of several characteristics of Immunogenic Cell Death (ICD) (Krysko, *et al.*, 2012). ICD refers to the activation of the immune response during cell death through the transformation of non-immunogenic tumor cells to immunogenic ones. *HMGB1* is one of the main markers of ICD that plays a role in dendritic cell maturation and antigen presentation to cytotoxic T cells (Dong, *et al*., 2022). *HMGB1* released by dead tumor cells will bind to the TLR4 receptor on dendritic cells and cause antigen cross-presentation on tumor cells (Apetoh, *et al*., 2007). This indicates that HMGB1 expression affects the maturation process of dendritic cells so that they can present tumor cell antigens. CD8+ receptors on T cells will then recognize these tumor cell antigens (Dong, *et al*., 2022). Exposure to an electric field was able to induce the release of the cytokine *HMGB1* in rat breast tumors as a marker of ICD, suggesting that cell death due to exposure to electric fields is immunogenic.

In most tumors, *PD-L1* expression is associated with a poor prognosis. Schalper, *et al.* (2014) stated that a high relative expression of *PD-L1* mRNA in breast tumors was closely related to an increase in lymphocyte count and longer recurrence-free survival (RFS). A meta-analysis conducted by Ibrahim, *et al.* (2014) and Lotfinejad, *et al.* (2020) showed that *PD-L1* expression causes an increase in TIL levels which is closely associated with a good prognosis in triple-negative breast tumors ($p<0.001$). *PD-L1* expression in breast tumors is due to an increase in CD8+ T cells which indicates a good prognosis (Garcia-Diaz, *et al*., 2017). This statement is proven by Alamsyah, *et al*., (2021) that exposure to the ECCT electric field in rat breast tumors results in a decreased CD4/CD8 ratio, in other words, an increase in CD8+ which indicates a better prognosis. During tumor formation, CD8+ T cells release several cytokines such as interferon γ onto the cell surface. Interferon γ is a potent inducer of *PD-L1*. We also demonstrated that exposure to ECCT electric fields in rats' breast tumors led to up-regulation of interferon γ. The result of the activity of interferon γ on breast tumors after exposure to ECCT will be published separately. Hence, the expression of *PD-L1* on breast tumors is not an immune response blockade, but rather an anti-tumor response by CD8+ T cells via secretion of interferon γ which is a *PD-L1* inducer.

The production of HMGB1 as a DAMP is able to activate the production of *IFN-γ* (Gao, *et al*., 2019). Western blot analysis conducted by Garcia-Diaz, *et al*. (2017) showed that the increase in *PD-L1* was strongest through the interferon Th2 *INF-γ* pathway. In this study, exposure to an electric

field in the rat liver caused an insignificant decrease in INF-γ expression, meaning it does not sufficiently influence *PD-L1* activity. Since the production of INF-γ is relatively uninterrupted, it does not affect *PD-L1* activity. Lin, *et al*. (2018) also reported that exposure to static electricity with an intensity above 56.3 kV/m resulted in temporary oxidative stress in the liver, however, this does not result in oxidative damage to the liver. In addition, Lin's research used a high-intensity electric field 50 times stronger than ECCT. Hence, the exposure to low-intensity intermediate-frequency electric field does not affect the activity of *HMGB1*, *PD-L1*, and *IFN-γ* in the brain and liver samples of female rats.

CONCLUSION

Exposure to a intermediate-frequency (150 kHz) and low-intensity (18 Vpp) ECCT device led to a significant expression of *HMGB1* and *PD-L1* mRNA genes in breast tumors samples of tumor-induced female rats.

The expression of *HMGB1* and *PD-L1* does not show any significant changes in the brain tissue of healthy rats. Significant changes are not seen in the expression of *HMGB1*, *PD-L1*, and interferon γ on liver samples of female rats. This experiment gives us information regarding the safety of exposure to electric fields on healthy tissues although further research still needs to be carried out.

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