

The 1800 MHz Radiofrequency Electromagnetic Fields Can Lead to a Reduction in the Number of CD4+T Cells, IL-2, IL-10, and IL-17a on PBMC Cultures

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Abstract- Although there have been many studies on the effects of electromagnetic fields on humans, scientists still have no agreement on the effects found because several studies showed no statistically significant effects. The effects of radiofrequency electromagnetic fields exposure on the immune system are varied, ranging from no effects to genotoxic effects on lymphocytes. Our study aimed to investigate whether exposure to 1800 MHz radiofrequency electromagnetic fields (RF-EMF) in variable durations and distances could lead to the dysregulation of T helper 1, 2, and 17. The peripheral blood mononuclear cells (PBMCs) cultures from healthy human subjects were exposed to 1800 MHz RF-EMF, with durations of 15, 30, 45, and 60 minutes and distances of 5 and 25 cm. We evaluated the effects of RF-EMF exposure on the number of CD4+ T cells, IL-2, IL-10, and IL-17a after 48 hours of culture with the flow cytometer. The closer the distance, the lower the number of CD4+ T cells. The longer the exposure, the lower the number of CD4+ T cells and the number of IL-2, IL-10, and IL-17a decreases significantly. CD4+ T cells expressing IL-2 increased significantly with the increase of the duration of 1800 MHz RF-EMF exposure (15, 30, and 45 min), but decreased at 60 minutes of exposure when compared to PBMCs without exposure. Sixty minutes of PBMC exposure to RF-EMF with a distance of 5 cm causes a significant reduction in the number of CD4+ T cells, the expression of IL-2, IL-10, and IL-17a.

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Introduction

The rapid development of mobile technology in modern life raises public concerns and fears about the possible effects of EMF exposure on health for users and the general population exposed for 24 hours from base transceiver stations (1,2). However, there is no evidence of the adverse effects of long-term EMF exposure. Some countries accept guidelines from the International Commission on Non-Ionizing Radiation Protection (ICNIRP) but apply precautionary approaches or other to implement more conservative boundaries than those proposed by the World Health Organization (WHO), ICNIRP, or other international organizations (2).

Radiofrequency ranges from 3 kHz to 300 GHz (3-5).

Exposure to RF-EMF is associated with non-wired communication devices, a technology widely used with several cellular phones registered currently estimated at 5 billion. Environmental exposures are also associated with radiofrequency emitted by cellular phone base transceiver stations, televisions, and radio towers. While operating, cellular telephone antennas, emit RF-EMF, which can penetrate 4-6 cm into the human brain (6).

Various studies have shown that EMF disrupts the body's energy system, thus causing various health problems (7,8). Those studies mentioned that EMF might disrupt immune function through stimulating various allergic and inflammatory responses, autoantibodies, as well as affecting the tissue repair process. These disorders increase the risk of various diseases, including

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malignancy (for example, leukemia in children) and can trigger or cause flares in some autoimmune diseases (2,7).

In recent years, there has been evidence of the role of immunology and oxidative stress on long-standing EMF exposure. The investigated markers, including lymphocytes, activated macrophages, and secretion of several inflammatory factors such as interleukin-1 (IL-1), tumor necrosis factor (TNF), prostaglandin (PG), reactive oxygen species (ROS), lipid peroxides (LP). Reactions occur due to these metabolites play a role in the occurrence of malignancies after prolonged EMF exposure (8).

In addition to the unclear mechanism, several studies on the adverse effects of radiofrequency EMF on the immune system showed different results. Therefore, we conducted an RF-EMF exposure experiment (1800 MHz frequency) on CD4+ Th1, Th2, and Th17 cells in PBMC cultures with different distances and durations of exposure. The objective of the study was to investigate the effect of exposure to RF-EMF fields that can increase or decrease Th1, Th2, and Th17 CD4 + cells.

Materials and Methods

Study design

Our study was experimental research conducted in a laboratory with a randomized post-test-only controlled group. Peripheral blood mononuclear cells (PBMCs) cultures were mixed in vitro with RPMI 1:10 culture media and divided into 13 treatment groups. PBMCs were exposed to 1800 MHz EMF-RF with various durations and distances. PBMCs were cultured for 48 hours 37° C. Next, PBMCs were examined using a flow cytometer for CD4+ T cells expressing IL-2, IL-10, and IL-17a.

Subjects and samples

Subjects

Venous blood was collected from 4 healthy volunteers (age 20 to 35 years). The samples (12 mL) were taken from the antecubital vein, using a 21G needle into vacuum tubes containing heparin (Becton Dickinson Biosciences) as the anticoagulant. All subjects had previously given their informed consent. Ethics approval was obtained from the Medical Faculty of Universitas Brawijaya Research Ethics Committee (letter number: No. 146/EC/KEPK/05/2018).

Samples

The sample was obtained consecutively on the healthy control of healthy volunteers of students and employees in the Medicine Faculty of Universitas Brawijaya.

PBMCs sample size was calculated using the following formula: $p(n-1) \geq 15$; $9(n-1) \geq 15$, $n \geq 2.667$, n: Number of samples for each treatment, and p: Number of treatments. From the calculation, 2.667 was the minimum number of samples for each group. In this study, the number of samples in each group as 4. The total sample was 36.

Variables

The independent variable in this study is an electromagnetic field with a frequency of 1800 MHz. While the dependent variables are CD4+ T cells that express IL-2, IL-10, and IL-17a.

PBMCs isolation

PBMCs were purified from heparinized peripheral venous blood samples using Ficoll-Hypaque gradients, $d=1.077$ g/mL (Sigma-Aldrich Co. LLC.). PBMCs were washed with phosphate-buffered saline (PBS) and resuspended in 300 μ L RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% glutamine/penicillin/streptomycin at a concentration of 1×10^6 cells/ml in multiwell plates and a tissue-culture incubator at 37° C, 5% CO₂. Before cultured, PBMCs were exposed to 1800 MHz EMF, with four different durations of exposure (15, 30, 45, and 60 minutes) and two different distances (5 cm and 25 cm) at 37° C. After 48 hours of culture, PBMCs were harvested, in which ten μ g/ml Brefeldin A (Golgiplug) (BD Pharmingen, San Diego, CA, USA) was given 5-6 hours prior. Golgiplug was given so that cytokines are not overly secreted into the supernatant .

Cells were taken with a micropipette and put into a 1.5 ml Eppendorf tube and centrifuged 2500 rpm for 3 minutes. The formed pellet is washed 2-3 times with 1 ml PBS. PBMCs are ready for staining for flow cytometry examination.

Sample preparation and flow cytometric analysis of CD4, Th17, IL-2, and IL-10

Cell surface staining was performed using mouse anti-human monoclonal antibodies (mAbs) anti-CD4 fluorescein isothiocyanate (FITC) (BD Pharmingen). The staining was performed by adding 20 μ L of each mAb to 100 μ L of separated PBMCs in the same tube, followed by 30 minutes incubation in the dark at room temperature. The tubes were washed twice with FACs buffer .

Next, fixative and permeabilizing solutions were added, followed by intracellular staining using 20 μ L of Fastimmune Anti-Human IL-2 phycoerythrin conjugate (PE) (BD Pharmingen), Mouse Anti-Human IL-10 PE

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(BD Pharmingen), Mouse Anti-Human IL-17a PE (BD Pharmingen). The cells were incubated for 30 minutes in the dark at room temperature and washed twice with FACs buffer.

Finally, 0.5 mL of phosphate-buffered saline was added to the washed cells prior to the measurement of CD4+ cells, IL-17a, IL-2, and IL-10. Sample analysis was performed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, California, USA). FACs-acquisition and analysis were performed with FACs Cell Quest Pro software (BD Biosciences). Samples were initially examined for the frequency of CD4+ T cells.

EMF RF signal generator

VSG25A Vector Signal Generator is a 100 MHz to 2.5 GHz vector signal generator, which displays a wave generator that can be set at several frequencies from 54 kHz to 180 MHz. The RF-EMF generators are obtained from The Signal Hound® company. In the study, 1800 MHz frequency and specific absorption rate (2 w/kg) (SAR) were used to recreate mobile phone radiofrequency radiation at four different duration (15, 30, 45, and 60 minutes) and two different distances (5 cm and 25 cm). The Generator was placed in a cube box made of aluminum layered with lead (32 cm×32 cm) at a temperature of 37° C, with procedures similar to the study by Sulalah *et al.*, 2019, and Resvina *et al.*, 2019 (9,10).



Figure 1. VSG25A Vector Signal Generator, The Signal Hound® company

Treatment group

PBMCs in this study were divided into several treatment groups: 1. PBMCs without exposure, 2. PBMCs with exposure to 1800 MHz RF-EMF, a distance of 5 cm for 15 minutes of exposure, 3. PBMCs with exposure to 1800 MHz RF-EMF, a distance of 5 cm for 30 minutes of exposure, 4. PBMCs with 1800 MHz RF-EMF exposure, a distance of 5 cm for 45 minutes of exposure, 5. PBMCs with 1800 MHz RF-EMF exposure, a distance of 5 cm for 60 minutes of exposure, 6. PBMCs with 1800 MHz RF-EMF exposure, a distance of 25 cm for 15 minutes of exposure, 7. PBMCs with 1800 MHz RF-EMF exposure, a distance of 25 cm for 30 minutes of

exposure, 8. PBMCs with 1800 MHz RF-EMF exposure, a distance of 25 cm for 45 minutes of exposure, and 9. PBMCs with 1800 MHz RF-EMF exposure, a distance of 25 cm for 60 minutes of exposure.

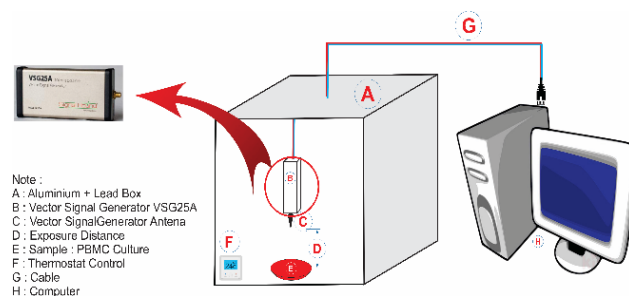


Figure 2. Schematic of the instrument for 1800 MHz EMF RF exposure

Statistical analyses

Statistical differences between experimental groups were determined by paired student t-test. Quantitative data were expressed as the mean and standard deviation. Comparisons between two groups were tested for statistical significance using One Way Anova and Tukey test or the nonparametric Kruskal Wallis and Mann Whitney U-test as appropriate. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS statistical software (version 20, SPSS Inc., Chicago, IL, USA).

Results

Table 1 showed the effect of EMF RF on the number of CD4+ T cells. The data showed the distance of the source of exposure to the PBMCs has a direct relationship to the number of CD4+ T cells. However, the duration of exposure is inversely correlated with the number of CD4+ T cells. On 25 cm exposure, there was an increase in the number of CD4+ up to 45 minutes, but at 60 minutes of exposure, there was a significant decrease.

Table 2 showed that the number of IL-2-expressing CD4+ T cells has a positive correlation with the duration of 1800 MHz RF-EMF exposure (15, 30, and 45 min). But the number is decreasing at 60 minutes of exposure when compared to PBMCs without exposure.

Table 3 showed that the longer the exposure, the number of CD4+ T cells expressing IL-10 significantly decreases. On 5 cm exposure, there was an increase until 45 minutes, but at 60 minutes, there was a significant decrease when compared to the control. On 25 cm exposure, there was a significant increase, except for the 45 minutes of exposure.

Table 4 showed that the longer the exposure, the

number of IL-17a-expressing CD4+ T cells significantly decreases, and increases only at 15 minutes of exposure with a distance of 5 cm.

Figures 3, 4, and 5 showed flow cytometric analysis

of CD4+ T cells, CD4+ T cells expressing IL-2, IL-10, and IL-17a, with and without 1800 MHz RF-EMF exposure.

Table 1. The comparison of the number of CD4+ T cells between the two groups based on variable exposure durations

1800 MHz	Duration (in minutes)	CD4+ (mean±SD)	P	Statistical Test
5 cm	60 ^{ac}	21.34±1.95	0.000	Kruskal-Wallis → Mann Whitney
	45 ^b	16.57±0.42		
	30 ^c	21.20±0.53		
	15 ^d	26.74±0.61		
	Control ^{de}	26.86±3.14		
25 cm	60 ^a	26.99±2.11	0.000	Anova → Tukey
	45 ^b	34.03±3.01		
	30 ^b	35.58±1.62		
	15 ^b	33.72±1.44		
	Control ^c	26.86±3.14		

Groups with the different superscripts differ significantly ($P<0.05$).

Table 2. The comparison of the number of IL-2-expressing CD4+ T cells between the two groups based on variable exposure durations

1800 MHz	Duration (in minutes)	IL-2 (mean±SD)	P	Statistical Test
5 cm	60 ^a	15.26±0.38	0.001	Kruskal-Wallis → Mann Whitney
	45 ^b	21.26±0.94		
	30 ^c	28.43±0.54		
	15 ^d	37.14±0.67		
	Control ^e	17.73±8.87		
25 cm	60 ^a	20.93±0.21	0.000	Anova → Tukey
	45 ^{bd}	26.72±1.04		
	30 ^c	19.13±0.60		
	15 ^d	25.90±0.57		
	Control ^e	17.73±8.87		

Groups with different superscripts differ significantly ($P<0.05$).

Table 3. The comparison of the number of IL-10-expressing CD4+ T cells between the two groups based on variable exposure durations

1800 MHz	Duration (in minutes)	IL-10 (mean±SD)	P	Statistical Test
5 cm	60 ^a	9.95±0.81	0.002	Kruskal-Wallis → Mann Whitney
	45 ^b	22.71±0.79		
	30 ^c	28.19±1.30		
	15 ^d	37.51±0.91		
	Control ^e	19.64±12.59		
25 cm	60 ^a	26.43±0.19	0.000	Anova → Tukey
	45 ^b	13.56±0.17		
	30 ^c	21.33±0.68		
	15 ^d	29.36±0.80		
	Control ^e	19.64±12.59		

Groups with different superscripts differ significantly ($P<0.05$).

Table 4. The comparison of the number of IL-17 expressing CD4+ T cells between the two groups based on variable exposure durations

1800 MHz	Duration (in minutes)	IL-17a (mean±SD)	P	Statistical Test
5 cm	60 ^a	22.57±0.98	0.000	Anova → Tukey
	45 ^b	28.28±0.60		
	30 ^c	28.19±1.30		
	15 ^d	41.95±1.40		
	Control ^e	31.58±12.48		
25 cm	60 ^a	25.44±0.84	0.000	Anova → Tukey
	45 ^b	24.02±0.34		
	30 ^c	29.86±0.56		
	15 ^d	15.67±0.50		
	Control ^e	31.58±12.48		

Groups with different superscripts differ significantly ($P < 0.05$)

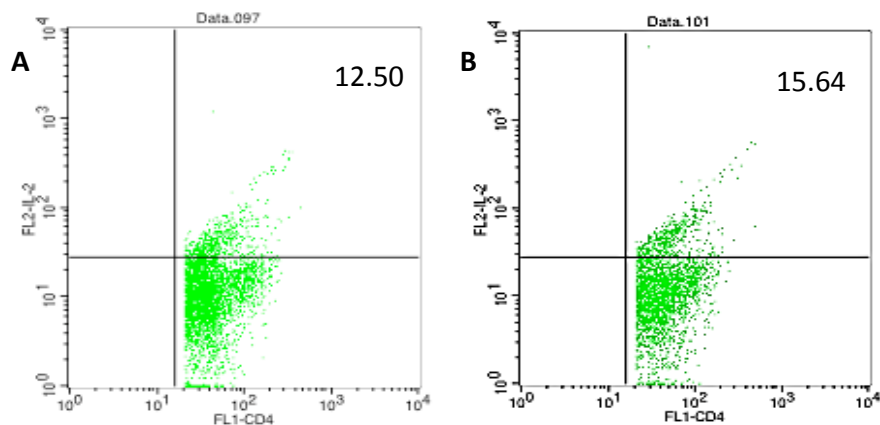


Figure 3. Flow cytometric analysis showing the percentage of IL-2-expressing CD4+ T cells: A. Control, without exposure, B. Increased with 1800 MHz RF-EMF exposure, 5 cm, 45 minutes

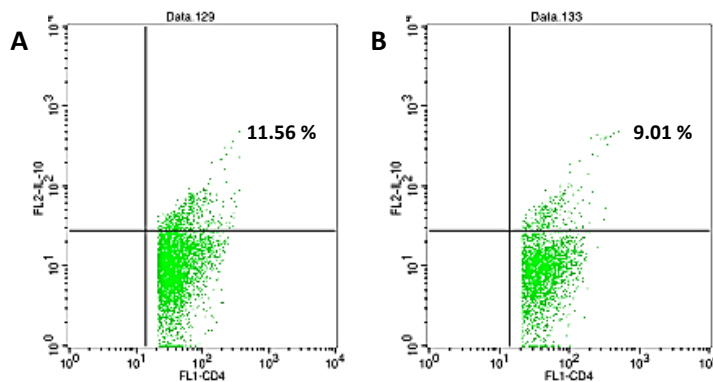


Figure 4. Flow cytometric analysis showing the percentage of IL-10-expressing CD4+ T cells: A. control, without exposure, B. Decreased with 1800 MHz RF-EMF exposure, 5 cm, 60 minutes

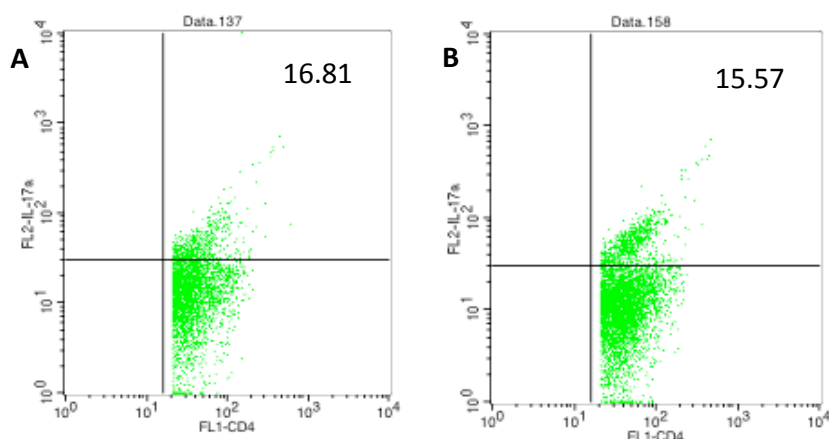


Figure 5. Flow cytometric analysis showed the percentage of IL-17a-expressing CD4+ T cells: A. control, without exposure, B. Decreased with 1800 MHz RF-EMF exposure, 5 cm, 60 minutes

Discussion

Studies on the biological effects of EMF, such as power-frequency, radiofrequency, and microwaves at the cellular level, are increasing in number. Many types of EMF are widely used in various places daily (11,12). Exposure to EMF as non-infection or external stress factors on the immune system is not less important than infection factors and internal stress (12,13). It has been shown that low-frequency EMF can act at the cellular level affecting various cell functions, including cell proliferation and differentiation, apoptosis, DNA synthesis, RNA transcription, protein expression, protein phosphorylation, and oxidation-reduction (re-dox) (14).

The immune cells respond in a very sensitive and effective way to infection factors or internal stress factors, including bacteria, intracellular parasites, and viruses, and to non-infection factors or external stress factors including nutrition, toxins, alcohol, foreign bodies, and also EMF exposure (12). The result of studies on the effects of electromagnetic fields on the immune system is important for evaluating their adverse effects. Laboratory studies on cells aim to explain the fundamental mechanism of the relationship between RF-EMF exposure and its effects on the cells of the cellular immune system.

In the study, PBMCs exposed to RF-EMF for 30, 45, and 60 minutes could significantly decrease the number of CD4+ T cells when compared with PBMCs control without exposure. The mechanism of the decrease might be through apoptosis. On exposures at a distance of 25 cm for 15, 30, and 45 minutes there was an increase in CD4+ cell number compared to the control group without

exposure. But, there was a tendency to decrease at a longer duration of exposure. This temporary increase was likely due to the normal cellular immune reaction to the external stimuli. RF-EMF more often causes a decrease in cell number rather than stimulates cell proliferation. The reduction in the number of CD4+ T cells was greater at 5 cm exposure compared to 25 cm, which showed that distance was affecting the magnitude of the exposure effect.

The main criteria for evaluating cellular reactions to external factors are cell growth and survival, which depends on the extent of the effect. Severe damage inhibits or suppresses cell growth and causes cell death. Very rarely, cell growth is accelerated by external factors, except by specific growth factors. Tian *et al.*, reported that at Specific Absorption Rate (SAR) >20 W/kg, cell survival rates decreased (15). Takashima *et al.*, compared the effect of 2,450 MHz EMF on high SAR on growth, survival, and cell cycle (16). The results showed that they were not significantly affected (17).

Several studies have shown an increase in apoptosis in human myelogenous leukemic cell lines, HL-60 and ML-1, in thymocytes and macrophages of mice exposed to long-term extremely low frequency (ELF)-EMF. A study has shown a decrease in apoptosis in lymphocytes exposed to 100 μ T (50 Hz) for 16 hours without mitogen (18). But a study by Hirose *et al.*, showed no significant difference in the percentage of cell apoptosis (Annexin V affinity assay) observed in groups exposed to RF signals (2,142.5 MHz, SAR up to 800 mW/kg) and negative controls (16,19,20).

In our study, a decrease in CD4+ T cells after exposure to 1800 MHz RF-EMF was followed by a

decrease in IL-2 production significantly on the distance of 5 cm and a duration of 60 minutes, when compared with cells without exposure. Conversely, a decrease in IL-2 production can result in a decrease in CD4+ T cell proliferation. IL-2 is a prototypical autocrine T-cell growth factor and is required for *in vitro* T-cell proliferation. IL-2 is an important factor in determining the magnitude of T-cell and NK-cell responses *in vivo*. IL-2 is also important in programming CD8 memory T cells, which undergo secondary expansion in viral infections. IL-2 also plays a role in promoting tolerance and preventing autoimmunity (21). Decreased IL-2 production due to exposure to RF-EMF may lead to a susceptibility to viral infections, a tendency to suffer from inflammatory bowel disease (IBD), low immune tolerance, and autoimmune diseases.

The increase in IL-2 production on 15, 30, and 45 minutes of exposure appears to be temporary, as what happened with CD4+ T cells. But, there was a tendency for IL-2 to decrease with the increasing duration of exposure. On RF-EMF exposure with a distance of 25 cm, the results obtained were inconsistent. The decrease in IL-2 production at a distance of 5 cm RF-EMF exposure was greater than the 25 cm (15.26 ± 0.38 versus 20.93 ± 0.21). The data showed that the distance from the EMF source to cells influences the magnitude of the effect.

The results of our study differ from a study by Tuschl *et al.*, regarding the adverse effects of Global System for Mobile communications (GSM) modulated RF fields on the functional competence of human immune cells. But, the exposure was done with 1950 MHz basic GSM, SAR of 1 mW/g with intermittent mode (5 min "on," 10 min "off") for 8 hours. No significant effects were found, with the conclusion that emissions from cellular telephones do not adversely affect the human immune system (IL-1, -2, and -4; INF-g; and INF-a) (22).

In our study, the longer the exposure, the number of CD4+ T cells expressing IL-10 decreased significantly. At an exposure distance of 5 cm, IL-10 increased significantly compared to controls, but there was a tendency to decrease. At a distance of 5 cm with an exposure duration of 60 minutes, IL-10 decreased significantly compared to control, whereas, at a distance of 25 cm, the results were inconsistent.

The major function of IL-10 is as an anti-inflammatory and immunosuppressive cytokine. IL-10 strongly inhibits the production of pro-inflammatory cytokines. It inhibits macrophage antigen presentation and decreases expression of MHC class II, adhesion molecules, and the costimulatory molecules CD80 (B7.1) and CD86 (B7.2). The importance of IL-10 as an

endogenous inhibitor of cell-mediated immunity is emphasized by the finding that IL-10-deficient mice develop the autoimmune disease and exaggerated inflammatory responses (21). From our study on IL-10 CD4+, it could be implied that a decrease in IL-10 due to long exposure to RF-EMF would be potentially harmful to the body, which may increase the risk of autoimmune disease and exaggerate inflammatory responses.

IL-17a is a pro-inflammatory interleukin produced mainly by Th17 cells, associated with many inflammatory diseases of contact delayed-type hypersensitivity and airway (23). Like IL-2 and IL-10, a decrease in CD4+ T cells was followed by a decrease in cytoplasmic IL-17a production. In our study, the expression of IL17a as a representation of Th17 cells showed a significant decrease in PBMCs exposed to 1800 MHz RF-EMF, except for 15 minutes exposure at the closest distance (5 cm). The longer the exposure, the number of CD4+ T cells expressing IL-17a decreases significantly. It could be implied that CD4+ T cells exposed to 1800 MHz RF-EMF might suffer a decrease in IL-17a production, which could result in an increased risk of extracellular bacterial and fungal infections. Interestingly, decreasing IL-17a is actually beneficial for autoimmune diseases, but how about the effects of chronic/long-term exposure still needs further study.

The level of exposure decreases with increasing distance from the transmitter (5). The highest local exposure is when using cellular or cordless telephones. In addition to these environmental exposures, RF-EMF exposure from radars and microwaves can occur, and safety rules must be applied (24-26). The intensity of radiation received will be inversely proportional to the square of the distance between the object receiving radiation and the source of radiation. The farther the distance from the source, the radiation intensity will decrease. But, the closer to the radiation source, the greater the radiation intensity will be (24). The proposition was consistent with the result of our study. The decrease in CD4+ cell number and the cytokines produced was greater at exposure from 5 cm than the 25 cm. However, at an exposure distance of 25 cm, the results were inconsistent.

Interaction can occur through the thermal or non-thermal mechanism. The thermal mechanism occurs as a result of temperature changes in the tissue caused by the RF field (4,27,28). The non-thermal mechanism is not directly related to changes in temperature but is more related to other changes in the tissue caused by electric or magnetic fields (4,28).

A number of interaction mechanisms are based on the

excitation of molecular vibrations such as sound waves, by the RF field. In excitation, both energy and momentum are conserved. For energy conservation, the photon energy from the RF field must match the photon energy from the vibration. Conservation of momentum can be much more needed. RF wavelengths must match ultrasonic wavelengths (28).

Federal Communications Commission (FCC) and other regulatory agencies claimed that only factors with thermal mechanisms affecting health. Therefore, exposure limits are set based only on thermal effects. Nevertheless, many studies, *in vivo* and *in vitro*, proved that significant harmful biological effects occur due to the effects of non-thermal RF exposure (28,29). In our study, the temperature was maintained within the physiological range, 36.5-37° C, to eliminate thermal effects disrupting the cellular immune system. Thus, changes that occurred in our study must be due to non-thermal mechanisms.

The following are various mechanisms of the effects of EMF exposure to the immune system proposed by various studies: 1. Oxidative stress induced by EMF radiation due to the production of reactive oxygen species (ROS) and lipid peroxidase, 2. Increased influx of Ca²⁺, 3. Redistribution of plasma membrane receptors and activation of protein kinase C. 4. Genotoxic effects on lymphocytes, 5. Apoptosis and proliferation, 6. Heat/thermal related effects (RF thermogenic level), 7. Autoantibody production.

We conclude that 60 minutes of PBMC exposure to RF-EMF with a distance of 5 cm causes a significant reduction in the number of CD4⁺ T cells and the expression of IL-2, IL-10, and IL-17a. Immune cells recognize electromagnetic fields at low exposure levels and produce biochemical stress responses. Exposure to electromagnetic fields at low levels (non-thermal) can impair immune function if it occurs continuously for a certain period of time. Scientific evidence shows that the current safety standards for low-frequency EMF are inadequate because most of the standards were made by only considering thermal effects. Therefore, the authorities need to re-evaluate safety standards.

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