

A pre-exposure to RF-EMF can enhance the immune responses of mice following *Salmonella Typhimurium* and *Klebsiella pneumoniae* infections

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ABSTRACT

► Original article

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Background: The question of whether low levels of non-ionizing radiations such as the radiofrequency electromagnetic fields (RF-EMF) can induce the same positive immune responses remains unanswered. This study aimed to investigate the effects of non-ionizing RF-EMF on some parameters of the immune system in an animal model following infection with *Salmonella Typhimurium* and *Klebsiella pneumoniae*. **Materials and Methods:** Male BALB/c mice were exposed to RF-EMFs generated by a common GSM mobile phone for 3 days. Animals were infected with *K. pneumoniae* or *S. Typhimurium* on the 4th day. On the 7th day after injection, blood samples were collected by cardiac puncture. The specific antibodies against bacteria were determined by agglutination method and serum levels of the cytokines were measured using the ELISA method. Moreover, the leukocytes count was measured using a cell counter. **Results:** The levels of specific antibodies against bacteria were higher in non-irradiated mice compared to irradiated mice. There were no significant differences between the irradiated and non-irradiated mice regarding the total blood leukocyte count. The mean serum levels of IFN- γ and IL-17 after infection with *K. pneumoniae* were significantly higher in the irradiated mice ($p < 0.001$). **Conclusions:** Low levels of RF-EMF can stimulate the immune responses in the mice pre-exposed to RF-EMF. This study provides further evidence supporting that exposure to certain levels of RF-EMF can stimulate the immune system. These adaptive responses may be applied to cope with the increased susceptibility of the astronauts to infections during a deep space mission.

Keywords: *Salmonella typhimurium*, *Klebsiella pneumoniae*, microwave radiation, IL-17.

INTRODUCTION

The immune system plays a key role in preventing infectious diseases. The immune system is a complex network of highly specialized cells and organs that work together to protect the body against foreign agents⁽¹⁾. Electromagnetic radiations are exposures from natural and manmade sources. The source of electromagnetic radiation is accelerating changes and electromagnetic radiation manifests itself as an oscillating electric and magnetic field. Until now, the natural electromagnetic background was relatively constant, but the situation changed markedly and precipitously with the development of modern communications and electrical power systems. The environment is now heavily laden with manmade electromagnetic fields from radio, TV, microwaves, mobile phones and many other sources⁽²⁾.

Salmonella is a significant member of the Enterobacteriaceae family. These organisms cause severe diseases in humans and animals^(3, 4). *S. typhimurium* causes enterocolitis with vomiting and diarrhea⁽⁴⁾. *K. pneumoniae*, is another important bacterium of this family, causes pneumonia and can produce a severe hemorrhagic necrotizing consolidation of the lung, urinary tract infection and bacteremia^(3, 4). Electromagnetic radiation has several effects on the bacteria and could change the antibacterial sensitivity^(5, 6), growth rate⁽⁷⁾ and other physicochemical properties⁽⁸⁾.

Some studies show that low levels of radiation cause stimulatory effects on immune responses⁽⁹⁻¹³⁾. The aim of this study was to investigate the effects of radiofrequency electromagnetic fields (RF-EMFs) on some parameters of the immune system including humoral immunity, peripheral leukocytes count and serum levels of TH1 (IFN- γ), TH17 (IL-17) and Treg (TGF- β) cells in the animal model BALB/c mice following infection with *S. typhimurium* and *K. pneumoniae*.

MATERIALS AND METHODS

Ethical considerations

This study was approved by the Ethical Committee of Bushehr University of Medical Sciences with reference number B-92-15-10. The experiments were conducted at INIRPRC, Shiraz University of Medical Sciences in accordance with the ethical recommendations of the *Animal Research Ethical Committees of the Bushehr University of Medical Sciences and Shiraz University of Medical Sciences*.

Animals

The male BALB/c mice (6-8 weeks old) were used in this study. The mice were kept in a temperature-controlled condition with a 12-hours light/12-hours dark cycle and received standard laboratory food and water. All mice were housed in a distinct room where the testing procedure was performed to minimize any potential stress.

Lethal dose (LD50) determination

For this purpose, 100 male BALB/c mice were taken. Animals (weight of 20-25 g) were divided into 10 individual groups including 10 animals in each group. They were given standard food and water, kept at room temperature 21 ± 1 °C, under 12 h light/12 h dark condition. *S. typhimurium* (PTCC number 1709) and *K. pneumoniae* (PTCC number 1290) were cultured at 37 °C in the nutrient broth (Merck, Germany) for 24 hours distinctly. Different concentration of fresh bacterial culture was measured using a spectrophotometer at optical density 625 nm (OD₆₂₅). Subsequently, the animals were infected with serial dilutions of each bacterium via intraperitoneal injection to determine lethal dose 50 (LD50). Animals were monitored for 30 days and their survival was evaluated daily.

Experimental design (in vivo study)

In this part, 88 male BALB/c mice were

randomly divided into equal 11 groups (eight animals in each group). Groups 1 and 6 were kept under the same radiation 1 hour per day for 3 days, groups 2 and 7 were kept under the same radiation 2 hours per day for 3 days, groups 3 and 8 were kept under the same radiation 4 hours per day for 3 days, groups 4 and 9 were kept under same radiation 6 hours per day for 3 days, groups 1-5 were infected with *S. typhimurium* and groups 6-10 were infected with *K. pneumoniae*. Groups 5 and 10 were infected with bacteria without any radiation exposure. Group 11 as a control group was kept under the same experimental condition (no exposure, no bacteria). The male BALB/c mice were exposed to radiation using a GSM mobile phone for 3 days and then infected with *K. pneumoniae* or *S. typhimurium* on the 4th day (table 1). On the 7th day after injection, the blood samples were collected by cardiac puncture. The samples were centrifuged at 3000 rpm for 10 minutes. Then serum samples were obtained and stored at -20 °C.

Measurement of specific antibodies against bacteria

The specific antibodies against bacteria were determined by the agglutination method. Two-fold dilutions of the sera were made in phosphate buffered saline (PBS) with a total volume of 0.2 ml. Then, 0.2 ml volumes of the

bacterial suspension were added to 96 well micro-plates. The plates were then incubated at 37 °C for one hour and observed for agglutination using an inverted microscope. The results of anti-bacterial antibody were measured.

Measurement of serum cytokine levels

Serum cytokine levels of IFN- γ , IL-17, and TGF- β were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits produced by eBioscience (USA). According to the manual's instructions, sensitivity levels of the IFN- γ , IL-17, and TGF- β kits were 15.0, 16.0, and 8.0 pg/ml, respectively.

Determination of the blood leukocyte count

Total and differential leukocyte counts were carried out on the peripheral blood samples. Total cell counts were made using a hemocytometer (Sysmex). Giemsa-stained blood films were used for differential counts.

Statistical analysis

The results were recorded as mean \pm SD. Differences in variables were analyzed using Student's t-test, ANOVA, Mann-Whitney U, and Kruskal-Wallis as appropriate and P values <0.05 were considered as significant. All data were analyzed by SPSS 16 software (Chicago, IL).

Table 1. Experimental design.

Animal Groups	Number of Animals	Daily Exposure Time (hr)			IP Injection
		Day 1	Day 2	Day 3	Day 4
Group 1 Group 6	8 8	1	1	1	<i>Salmonella</i> <i>Klebsiella</i>
Group 2 Group 7	8 8	2	2	2	<i>Salmonella</i> <i>Klebsiella</i>
Group 3 Group 8	8 8	4	4	4	<i>Salmonella</i> <i>Klebsiella</i>
Group 4 Group 9	8 8	6	6	6	<i>Salmonella</i> <i>Klebsiella</i>
Group 5 Group 10	8 8	-	-	-	<i>Salmonella</i> <i>Klebsiella</i>
Group 11	8	6	6	6	No Bacteria

RESULTS

In order to determine the optimum bacterial concentration, the LD50 test was carried out. LD50 results are shown in figure 4-5 (Kaplan-Meier Diagrams). LD50 for *S. typhimurium* was 10^6 CFU/ml and for *K. pneumoniae* 10^7 CFU/ml. The specific antibodies against bacteria in non-irradiated mice were higher than the irradiated mice. The antibody titer of *S. typhimurium* in the non-exposed group was 1/256, while these levels after 1, 2, 4 and 8 hours of exposure were 1/128, 1/8, 1/16 and 1/8, respectively. On the other hand, the antibody titer of *K. pneumoniae* in the non-exposed group was 1/256, while these levels after 1, 2, 4 and 8 hours of exposure were 1/32, 1/16, 1/64 and 1/8, respectively.

There are no significant differences in the

total leukocyte count were observed between irradiated and non-irradiated mice (figures 1, 2a and 2b). No significant differences were observed between irradiated and non-irradiated mice in the mean serum level of TGF- β (figure 3c and 3d).

For mice groups which infected by *K. pneumoniae*, the mean serum levels of IFN- γ was significantly higher in irradiated mice compared to non-irradiated mice ($p < 0.001$) (figure 2d). On the other hand, the mean serum levels of IL-17 in aforementioned irradiated mice were significantly higher than non-irradiated mice ($p < 0.001$) (figure 3b).

For mice groups which infected by *S. typhimurium*, no significant differences were observed between irradiated and non-irradiated mice in their mean serum levels of IFN- γ and IL-17 cytokines (figures 2c and 3a).

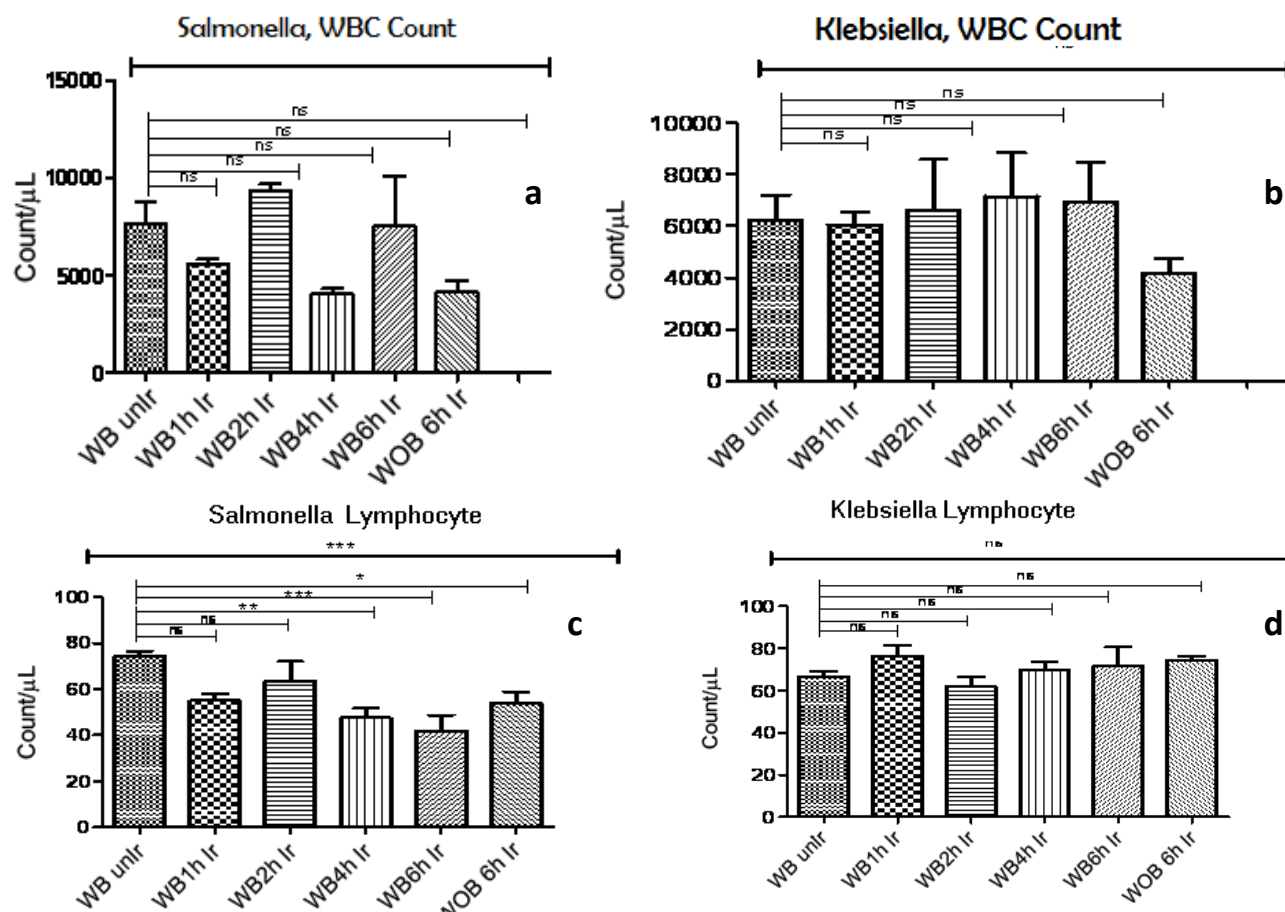


Figure 1. Comparison of WBC count in exposed and non-exposed group for different bacteria a. *S. typhimurium* b. *K. pneumoniae* c. Comparison of lymphocyte count in exposed and non-exposed group for *S. typhimurium* d. Comparison of lymphocyte count in exposed and non-exposed group for *K. pneumoniae*. WB: with bacteria, WOB: without bacteria, lr: irradiated, Unlr: unirradiated.

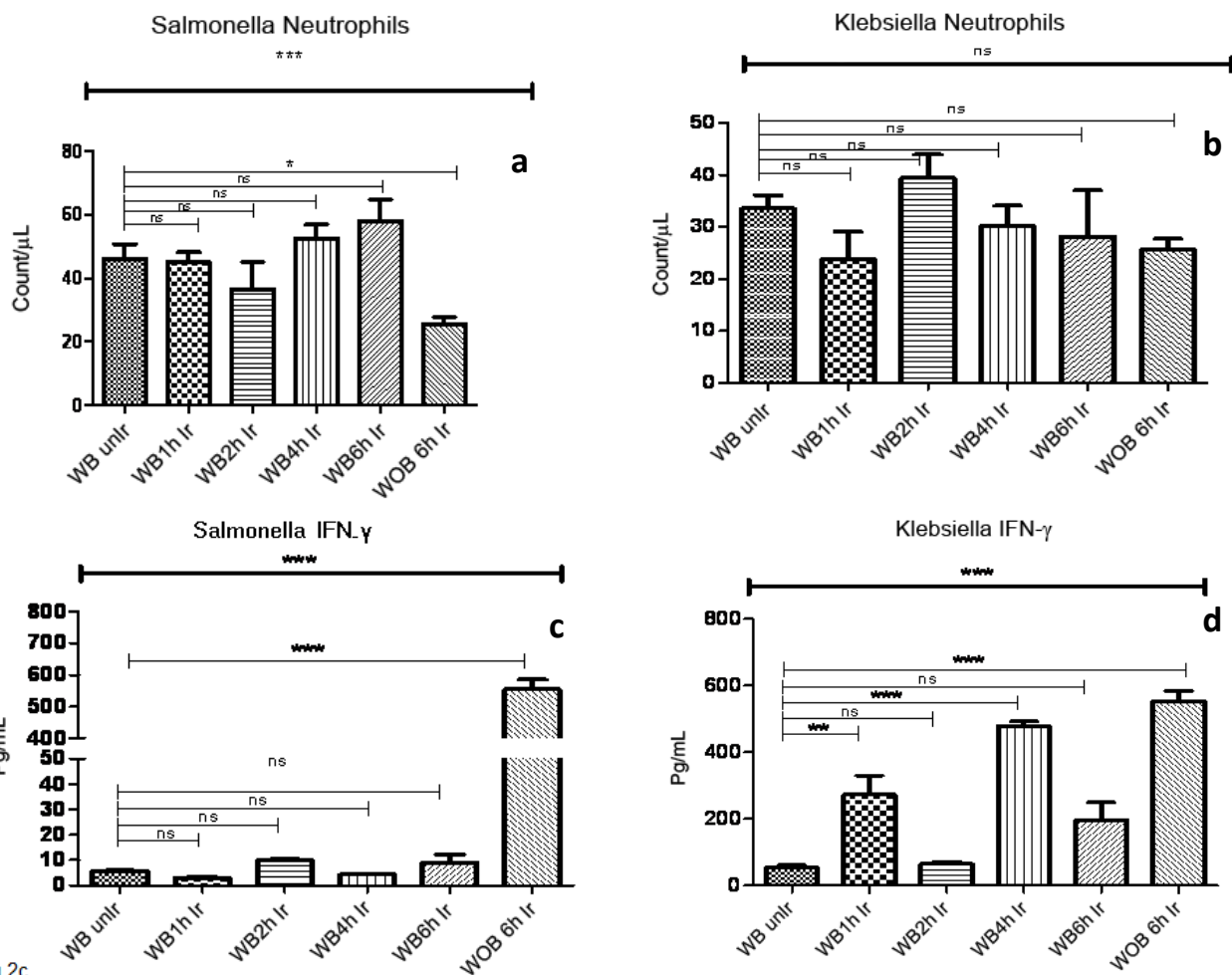


Figure 2.a. Comparison of the Neutrophil count in the exposed and non-exposed groups for *S. Typhimurium* b. and *K. pneumoniae* c. Comparison of serum level of IFN- γ in the exposed and non-exposed groups for *S. Typhimurium* d. and *K. pneumoniae*. WB: with bacteria, WOB: without bacteria, Ir: irradiated, Unir: unirradiated.

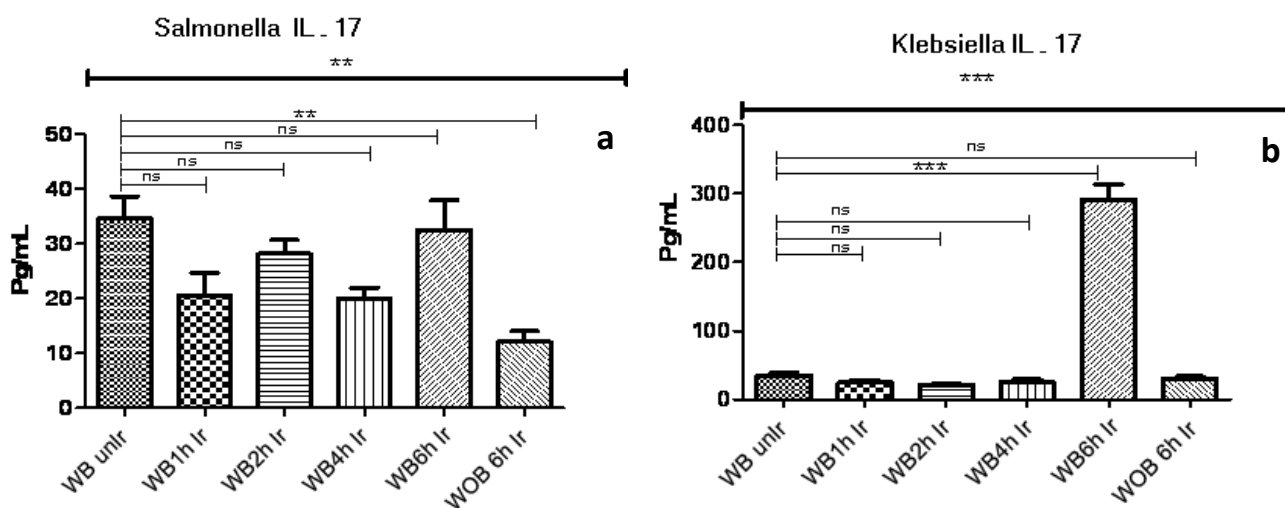


Figure 3.a. Comparison of serum level of IL-17 in the exposed and non-exposed groups for *S. Typhimurium* b. and *K. pneumoniae*.

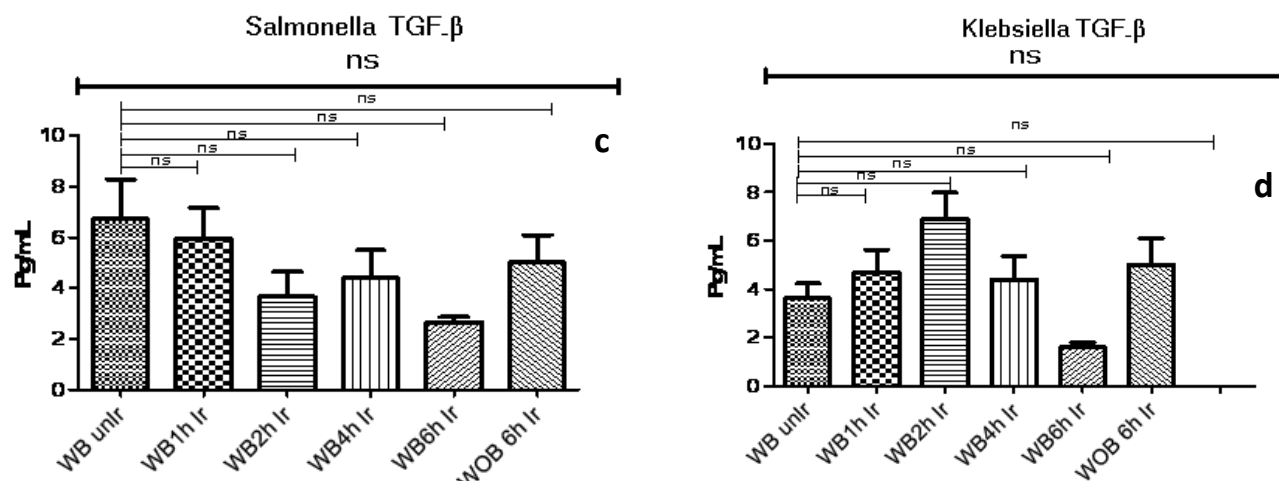


Figure 3.c. Comparison of serum level of TGF-β in the exposed and non-exposed groups for *S.Typhimurium*. d. and *Klebsiella pneumoniae*. WB: with bacteria, WOB: without bacteria, Ir: irradiated, Unir: unirradiated.

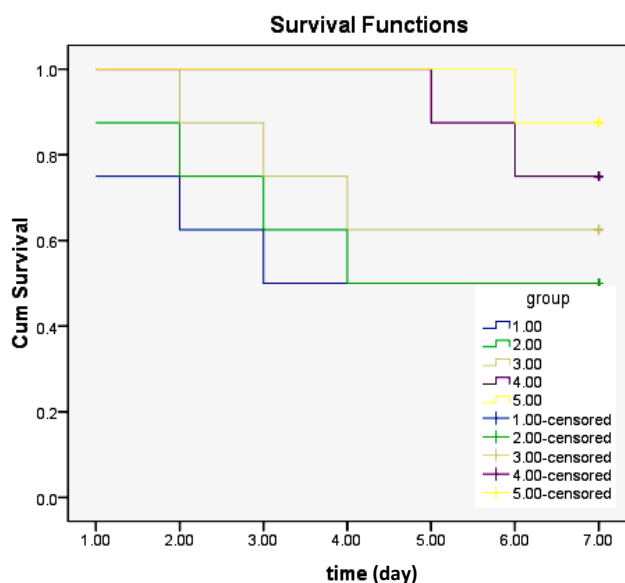


Figure 4. Kaplan-Meier Analysis after Infection with *S. Typhimurium*

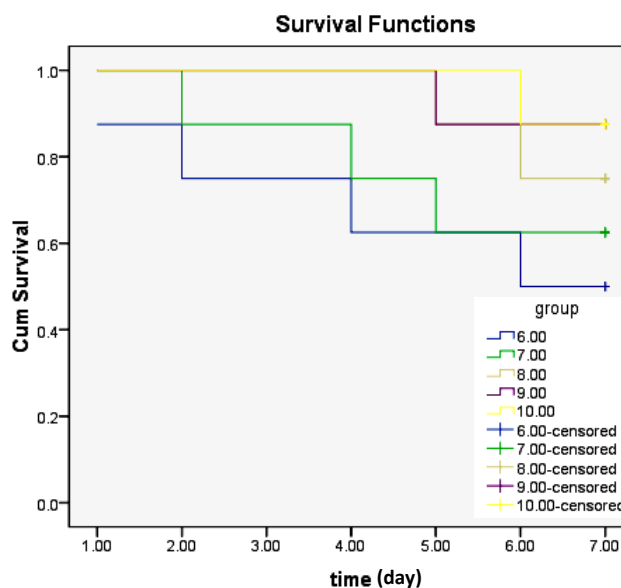


Figure 5. Kaplan-Meier analysis after infection with *K. pneumoniae*

DISCUSSION

DeBruyn and DeJager showed that exposure to radiation induces an inflammatory response (14,15). The inflammatory response is useful in short time due to in long time it can damage tissues (16). Selmaoui *et al.* has shown an elevated IL-8 level in the young men that exposed to radiation (17). In this study, we found increased IL-17 and IFN-γ level in mice that exposed to radiation. Our findings are supported by the report of Aldinucci and Pessina who

found an increase of IL-8 and IFN-γ level after exposure and neutrophils were decreased in peripheral blood counts because they migrated to the tissues after exposure (18). Goraca *et al.* showed that the response to exposure by the immune system was dose-dependent and is also observed in this study (19).

Other studies have shown that the adaptation effect of radiation depends on the experimental design. The studies which performed based on the non-ionization radiation as the energy of each photon is not too high it should be better to

use power and SAR which can do some changes in the tissues and cells. Some evidence indicates that different stress condition makes a similar effect on the immune cells ⁽²⁰⁾.

IFN- γ cytokine is produced by the NK cells and TH₁ cells. Therefore, it seems that microwave radiation could increase the activity of IFN- γ producing cells. One of the main functions of IFN- γ is converting TH₀ to TH₁ cells which are very important for cell-mediated immunity. The cell-mediated immune response is very important for eradication of bacteria, viruses and also tumor cells. Interestingly our results show that microwave radiation can boost the immune responses. It is worth noting that this stimulatory phenomenon is in line with the numerous reports which showed that exposure to both ionizing and non-ionizing radiations may lead to enhanced activity of the immune system ⁽²¹⁻²³⁾.

The results of the current study show that after injection of *K. pneumoniae* in the group which was not exposed to microwave radiation, the IFN- γ level is significantly low in comparison to the groups which exposed with radiation for 1 and 4 hours (figure 2d). In our investigation, in the infected group with *K. pneumoniae* in 2-hour exposure to radiation in compared to the groups which were more exposed to the radiation, the number of WBC were significantly raised. While WBC count in the exposed and non-exposed group didn't show differences (figure 1b). We did not observe any alteration in the group infected with *K. pneumoniae*. In this study, the neutrophil count in the group which was infected by *K. pneumoniae* was higher compared to the group which was not infected by the bacteria (figure 2b). This phenomenon may happen as a result of stress.

Furthermore, in this study, we showed that after injection of *K. pneumoniae* in the control group which was not exposed to radiation, the IL-17 levels were significantly lower than the groups which were exposed to radiation (figure 3b). As mentioned earlier, IL-17 produced by some cells such as TH₁₇ and it seems that microwave photon could increase the activity of TH₁₇ cells. One of the main functions of IL-17 is the induction of chemokine's and recruitment of

neutrophils to the infection site. So, our results suggest that microwave photon maybe protects us against bacterial infections.

The decrease in neutrophil number in the groups which exposed to radiation needs to explain more in details. Based on the Elmusharaf theories in 2007, the number of neutrophils reduce the result of phagocytes colonization and also alter in the location of these cells. If the production of reactive oxygen species (ROS) in the previous study well documents, then the increase of neutrophils in this study is in the line of Elmusharaf and Wiese theories ^(24, 25).

One of the reasons for increasing the levels of IFN- γ and IL-17 may be because of ROS and recruitment of the phagocytes, exposed with the radiation lead the immune response to the inflammation ^(18, 19). In our study amount of IFN- γ in the group which only exposed with the radiation compared to the others were dramatically high.

The level of IL-17 in the group exposed and was not with the radiation and bacteria changes were not significant but in an inter-group comparison which 6 hours exposure, the levels of IL-17 compared to the other time of exposure could show the stress is the reason for this increasing (figure 2b). While the levels of IL-17 changes in the group exposed to both radiation and bacteria was significantly higher than the other groups (figure 3b). As radiations could release the IL-17 and the role of IL-17 against bacteria we can conclude that presence of radiation and bacteria for releasing the IL-17 have a synergistic effect ^(14, 15, 26, 27).

Antibody titer against bacteria in the exposed group was lower than non-exposed. Probably radiation could provoke the immune system and decrease the bacterial proliferation, then, an antibody against bacteria in exposed mice was reduced. The inter-group comparison showed that there is a different pattern in lymphocyte, neutrophil, IFN and IL-17 level and it indicated that time of exposure is related to the number of cells and cytokine release (figures 1-3) ^(24, 28, 29). If we want to have an appropriate response against the intracellular bacteria like *Salmonella*, the innate and adaptive immune responses must be in a good condition in

exposure with infectious agent; macrophage and neutrophil will be activated and some cytokine such as IL-1, 6, 12, 18, TNF- α , and IFN- γ will rise (30-32). Results of previous studies showed that RF is suitable to increase the immune system of the animal against the damage but in the lethal dose of radiation (33). For clear interpretation of the effect of radiation, we should pay attention to the shape, direction of the body, body volume, frequency, time of exposure, the power of radiation, environment, the mass of exposure and SAR (29). It is clear that local and systemic radiation have a different effect on the immunologic and hematologic index. These changes always are transitional and reverse the effects (29).

Based on the SCENIHR there is obvious evidence that shows the radiation has a different effect (34). There are some report showed the advantages of radiation but in the low frequency (19, 35, 36). There is a study showed that the radiation has a good effect on the immune responses (26). Cuppen *et al.* in 2006 showed that the radiation could increase the cytokine production (27). Some investigators showed that radiation could stimulate the immune responses both in the cells and cytokine production (25, 35-39). Some investigators believe that exposure to radiation for a short time could provoke the immune responses. These effects start from the molecules and then will be observed in the levels of cells (19, 25, 35, 36, 39, 40).

In the study done by Aldinuchi and Pessina, they showed an increased level of IFN and IL-6 after 24 hours of exposure to radiation (18). Exposure to radiation for a short time also has a proliferative effect on the lymphocytes (37). In some studies, free radicals were responsible for the induction of phagocytosis effect of phagocytes (19, 37, 41-43). In addition, when the body was exposed for a long time with the radiation, an opposite effect was observed (44). Several studies showed the beneficial effect of radiation if exposure is not for a long time. Some studies exhibit the relation between electromagnetic and immune cells but the more experimental test is needed (45).

Exposure to radiation these days is inevitable, hence, it seems dose of radiation

must be adjusted to minimize damage. While there is some consideration of exposure to radiation, on the other hand, some studies showed the therapeutic effect of radiation, the limitation of these studies is related to the lack of experimental study (38, 45). Exposure to radiation induces the oxidative stress and accelerates the inflammation in the cells (45). Cuppen in 2007 reported that exposure to electromagnetic radiation induces the stress on the cells and alerts the immune system (39). In a recent study, in the mice, some leukocyte parameters were different. The differences were not in the number of WBC for the groups were exposed to radiation but the differences were observed in CD3 and CD4 (24). Radiation stimulates the inflammation (14, 15) and inflammation in a short time is beneficial but if it takes place for a long time maybe damages the body (16). In conclusion, radiation has affected the regulation of inflammation (45).

CONCLUSION

These results are in line with the reports indicating that non-ionizing radiation can stimulate the immune system. Findings of this study support this hypothesis that exposure to radiofrequency radiation can stimulate the immune system activities against bacterial infection in mice.

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Int. J. Radiat. Res., Vol. 18 No. 2, April 2020

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