Evaluation of the protective effects of folic acid on the lung exposed to 900-MHZ electromagnetic field: A stereological and histopathological study

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Abstract
There is strong scientific evidence that radio frequency (RF) radiation is harmful to life. Exposure to radiation may cause lung toxicity and respiratory disorders. Folic acid (FA) is one of the powerful antioxidants that minimize oxidative stress in the biological system. In this study, we evaluated the effectiveness of the FA against the EMF-induced potential negative effects on the lung. Twenty-four male Wistar albino rats were divided into the four groups; control group (Cont), electromagnetic field group (EMF), FA treated group (FA), and electromagnetic field exposure + FA treated group (EFA). After the routine histological procedures, volumes of the alveoli, bronchioles and blood vessels have been estimated by the Cavalieri principle. It was found that a significant decrease in the mean volume of alveoli, bronchioles and blood vessels in EMF group in comparison of the Cont group (p<0.01). Besides this, histopathological analysis demonstrated that there was impaired lung structure, shrunken alveoli, and increased thickness of the alveolar wall in the EMF group sections. In the EFA group, significant protective effects were observed in the structures volumes and histopathology (p<0.01). These findings corresponded with the antioxidant effect of FA treatment. Our results suggest that FA protected alveoli, bronchioles, and blood vessels against EMF-induced lung injury. Thus FA has the potential to be a therapeutic agent.

Keywords: electromagnetic fields, folic acid, lung injury, Cavalieri Principle, stereology, antioxidant

1. Introduction
The exposure to electromagnetic fields (EMF) is increasing as a parallel of the developing technology. Many of electrical equipment especially mobile phones, emits the EMF, which ultimately negatively affects the human health. Several studies have reported that EMF caused cognitive impairment (1), depressive disorder (2), anxiety-like behaviour (3) and obsessive-compulsive disorder-like behaviour in brain (4). Moreover, exposure to EMF has been associated with reproductive function failure such as decreasing of sperm motility, inhibin B, prolactin, activin B, FSH and LH hormone levels in male Sprague dawley rats (5). Saygün et al. (6) have found a decrease the serum testosterone levels in the rats exposed to 2.45 GHz EMF.

EMF causes an increasing the free radicals by the Fenton reaction in the tissues; this may change the cellular balance and leads to the cell damage (7,8). The oxidation products are harmful for the cellular components such as lipids, proteins, and DNA. Furthermore, the oxidative stress is associated with the apoptotic and necrotic cell death (9). It is known that, H2O2 plays an important role in carcinogenesis due to its diffusion capability throughout the mitochondria and across the cell membranes (10). Apart from this, EMF may have cytotoxic, genotoxic and carcinogenic effects on different organ (11-13). It was shown that the increased levels of reactive oxygen cause breast cancer as well as cell loss and dysfunction (14). Soffritti et al. (15) detected that increased genotoxicity in several tissues such as; heart, liver, pancreas, brain, skin, kidneys. In the tissue, generation of large amounts of free radicals can also disrupt the intracellular antioxidant defence mechanism. In this regard, it is very important that an antioxidant treatment can be applied to minimize possible cell damage. FA is a water-
soluble vitamin with a high antioxidant capacity. It is involved in the synthesis, repair and methylation of DNA or RNA thus functions in the prevention of the cancer formation (16). It has been reported that FA inhibits EMF-induced oxidative brain damage (17). In a stereological study, it was found that folic acid has protective effects on the kidney against the side effects of exposure to electromagnetic radiation (18). Therefore, in this study we aimed to investigate the potential protective role of FA against the 900-MHZ EMF exposure in the lung.

2. Material and methods

2.1. Animals

Twenty-four male Wistar albino rats (12 weeks old, weighing 250 ± 50 g) were obtained from Experimental Animal Center of the Ondokuz Mayis University (Samsun, Turkey). All the experimental procedure was approved by the Ethical Committee at the Ondokuz Mayis University (HADYEK/2013-23, 27.08.2014). Animals were randomly divided into four experimental groups with six rats each group (n=6). The animals were maintained ad libitum feeding schedule, housed individually in a 12-h light: 12-h dark cycle (lights on at 08.00 am–08:00 pm) at a temperature of 22 ± 2°C and 45–55% humidity. All experiments were performed in accordance with the guidelines of the European Community Council for experimental animal care.

2.2. Groups

Control (Cont): The rats were not received any treatment and EMF exposure. Electromagnetic field exposure (EMF): The rats were placed in a special apparatus and exposed to 900 MHz EMF for one hour per day (1:30-14:30) during the 21 days. No treatment was given to this group (17). Folic acid (FA): FA was given by gavage during the 21 days (50 mg/kg/day of FA, dissolving in 2 ml distilled water). This group was not received EMF exposure (17). EMF+FA: During the 21 days, FA was given by gavage (50 mg/kg/day of FA, dissolving in 2 ml distilled water) and subsequently animals were placed EMF apparatus and they were exposed to 900 MHz EMF one hour (13:30-14:30) per day (17).

2.3. Electromagnetic field exposure system

The system we use for EMF exposure; it is a signal generator (Microwave Test Transmitter, Set Electronic Ltd, Turkey) with 1-2 watt output (PW-Pulse Wave) that can operate at 900-1800 MHz frequency. This system works by connecting a 16-section round cage made of polycarbonate with a monopole antenna that can emit electromagnetic waves. In the setup, the antenna is placed in the center, equidistant and perpendicular to all the partitions in the cage, in order to provide an equal distribution of EMF from the monopole antenna to the compartments. In addition, the power density in the area close to the monopole antenna was precisely measured with the electric field probe (EXTECH RF EMF strength meter) during the experiment. For exposure, rats are positioned equidistant from each other with their heads facing the monopole antenna. The rats belonging to the EMF groups were exposed to 900 MHz EMF for 1 hour a day at the same point for 21 days (17).

2.4. Histological and stereological procedure

End of the experimental processes, the tissues of animals were fixed by cardiac perfusion (10% formalin). The lung tissues, after the processed of using routine histological techniques, were embedded in paraffin and were cut the section (10 μm) by using the microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany) then stained with hematoxylin-eosin (H&E).

2.5. Stereology

The calculation of the reference volume by the Cavalieri method is based on multiplying the surface area of the object by the average section thickness. Surface areas of the sections can be estimated using the point-counting grid that is consists of systematic points (+), which is separated at an equal distance from each other. Each of the points in this grid represents a unit grid area between four points. After superimposing of the grid randomly on the sections, total point numbers that are hit to related area or region would give an estimation of surface area. When the area of a point multiplies with the total number of points intersecting with the structures in the section, the total area is calculated (19, 20).

\[ V_{ref} = \Sigma P_i \cdot \hat{t} \]
\[ P_i = P(a) \]

\( V_{ref} \) is the reference volume, \( \Sigma P_i \) is the total number of point superimposed to related area in the sections, \( \hat{t} \) is the average section thickness and \( P(a) \) represents the area represented by a point (20).

For this method, sampling interval determined by the pilot study and cross-sectional images were taken in tissue sections with a 4X objective (Olympus BX43, Center Valley, PA). By using the Image J program (Image Processing and Analysis in Java, NIH, USA), the point counting grid was placed on the images and calculated interested structures' (alveoli, bronchioles and vessel) area.

2.6. Statistical analysis

After the normality test, data were analyzed with the One-way ANOVA (Tukey-Post Hoc Test). Results were expressed as the means ± SEM. Statistical analyses were performed on SPSS 20.0 for Mac IBM Corporation (SPSS Inc., Chicago, IL, USA). All statistical values under 0.05 were considered significant.

3. Results

3.1. The mean volume of alveoli

The mean volume of alveoli in EMF group was significantly decreased compared to the Cont group (p<0.01). It was observed that significant increase in the alveoli volume of EFA group compared to the EMF (p<0.01). Moreover, there were no significant differences between the Cont and EFA group’s alveoli volume (p>0.05), Table 1, Fig.1.

3.2. The mean volume of bronchioles

The mean volume of bronchioles in the EMF group was significantly decreased compared to the Cont group (p<0.01).
Furthermore, the volume of bronchiole in the EFA group showed significant increase compared to the EMF groups \((p<0.01)\). There were significant differences between the Cont with the EFA groups \((p<0.01)\), Table 1, Fig. 2.

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Alveoli</th>
<th>Bronchioles</th>
<th>Blood vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>CE 0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>CV 0.06</td>
<td>0.07</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>EMF</td>
<td>CE 0.01</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>CV 0.04</td>
<td>0.02</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>CE 0.05</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>CV 0.07</td>
<td>0.03</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>EFA</td>
<td>CE 0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>CV 0.04</td>
<td>0.04</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

3.3. **The mean volume of blood vessels**

The mean volume of blood vessels in the lung was decreased in the EMF groups compared to the Cont group \((p<0.01)\). In contrary to this, a significant increase was found in the EFA groups compared to the EMF group \((p<0.01)\). There were differences between the Cont and EFA \((p<0.05)\).

### 3.4. Histopathological results

Histopathological observations were done in the HE stained sections. In the Cont group, general structure of lung were look in normal, the alveoli components were clearly observed; its walls thickness was normal. The bronchioles and vessels have well-structured layers and their lumens were seen open. The alveolar wall in the EMF group was thicker than Cont and EFA groups. Most of the area was seen with edematous. Also, alveolar haemorrhage was seen in the alveolar walls in the EMF exposed group. In the EMF group alveoli there is pronounced blood cells infiltration, which are neutrophil, lymphocytes and monocyte were seen. Also, it was observed that the wall of the bronchioles was split and the epithelial cells were damaged in the EMF exposed group. On the other hand, in the EFA group, the protective effect of folic acid was seen in the alveoli, bronchioles and vessels structures since they look very healthy and all structures could be seen clearly. It is observed that there is a clear delineation of the alveoli walls as well as the bronchial walls well organized in the EFA group. Similarly, In the EFA group, the vessels were well preserved and their walls were visible (Figs. 4-6).

4. **Discussion**

Today, individuals are exposed to electromagnetic waves throughout their lives, starting from the womb. The effects of electromagnetic waves, which are also defined as environmental pollution factors, on the human body are undoubtedly an issue that needs to be investigated. For this aim, in the present study, we evaluated that the protective effects of FA on EMF exposed lung tissues. Studies have reported that the radiation emitted by mobile phones can cause oxidative stress by increasing free oxygen radicals (ROS) in various tissues (21, 22). It has been shown that EMF exposure causes abnormal changes in intracellular antioxidant defence systems such as MDA and GSH due to excessive production of ROS in the body (17, 21). Odacı et al. (23) found that prenatal exposure to 900-MHz EMF causes a significant decrease in the total number of granule neurons in the dentate gyrus.

3.3. **The mean volume of blood vessels**

The mean volume of blood vessels in the lung tissue (mean± SEM). (*) and (**) show statistical differences under \(p<0.05\) and \(p<0.01\) level, respectively.

**Blood vessels**

![Graph showing the mean volume of blood vessels between groups in the lung tissue. (*) and (**) indicate statistical differences under \(p<0.05\) and \(p<0.01\) level, respectively.]

Fig. 1. The graph shows the mean alveolar volume between the groups in the lung tissue (mean± SEM). (*) and (**) show statistical differences under \(p<0.05\) and \(p<0.01\) level, respectively.

Fig. 2. The graph shows the mean volume of bronchioles between the groups in the lung tissue (mean± SEM). (**) Show statistical differences under \(p<0.01\) level.

Fig. 3. The graph shows the mean volume of blood vessels between the groups in the lung tissue (mean± SEM). (*) and (**) show statistical differences under \(p<0.05\) and \(p<0.01\) level, respectively.
Similarly, it was found that pyramidal cell number significantly reduced in the EMF exposed group (24). The dentate gyrus and hippocampus cell loss may be mediated by the induction of oxidative stress in the nerve tissues by the EMF.

Fig. 4. Light microscopic images were taken from the sections of the alveolar region of the lung. There was no clear delineation of the alveolar structures in the EMF group. Moderate pulmonary oedema was detected in the EMF exposed group. Based on histological structure a significant protective effect of the FA treatment was observed in the EFA group. It was observed that the general morphology of the cells in these group alveoli was seen healthy. Not only all structures were preserved but also the alveolar cell borders were clear in the EFA group. The FA ameliorates the side effect of EMF on the lung tissue. *; Healthy alveoli, Hematoxylin and eosin staining

Fig. 5. Light microscopic images shows bronchioles in the lung. The normal structured bronchioles (star) were seen in the Cont, FA and EFA groups. It is noticed that there was heavily impaired wall (arrow) of bronchioles in the EMF group. Hematoxylin and eosin staining

Fig. 6. Light microscopic images shows that blood vessels. The stars indicate the normal structured vessels in the Cont and EFA groups. In the EMF group sections, it was observed that the vascular wall was thinned and the endothelial integrity was impaired (arrow). It is seen that FA administration has protective effects on the vessels in the EFA group. Hematoxylin and eosin staining

Yahyazadeh et al. (25) investigated the effect of 900-MHZ EMF on the lung. They showed that mean volumes of alveoli, bronchioles and blood vessels were significantly decreased in EMF group compared to the Cont group. They also detect histopathological alterations such as irregularity of the epithelial border and dispersion of connective tissue in bronchiole in the EMF group sections. Baltacı et al. (26) found that 50-Hz EMF leads to increases of MDA level in the lung tissue. Although GSH levels were also increased in response to increased MDA levels in this study, they were insufficient to restore MDA levels to control values. In the light of these findings, the researchers concluded that despite the increased antioxidant system activity, the increased tissue damage in the lung tissue as a result of exposure to EMF could not be prevented. Another study reported that exposure to a 50-Hz EMF increased the levels of SOD and TBARS in lung tissue (10). Similarly, Seyhan and Canseven (27), reported that exposure to 50-Hz EMF negatively affects the antioxidant defence systems in many organs such as the spleen, skin, lung, kidney and brain. According to all these studies results, it is clear that EMF causes to tissue damage by negatively affecting the antioxidant defence system. In several in vivo studies with the mice and rats, the EMF is thought to be cause cell death (apoptosis) (8, 28, 29). It has been reported that folic acid has free radical scavenging properties and antioxidant activity (30). The antioxidant activity of folic acid is mediated by many mechanisms that can increase total antioxidant capacity (TAC) and reduce ROS formation (30). In one study, it was reported that folate intake supports lung functions and is beneficial (31). In a meta-analysis study, it was reported that folic acid supplementation could significantly improve markers in the antioxidant defence system by increasing serum GSH and TAC.
concentrations and decreasing serum MDA concentrations (32). Kivrak et al. (17) reported that folic acid showed neuroprotective effects in the brain cells due to the its antioxidant activity. In another study, folic acid administration in diabetic rats has been shown to reduce oxidative damage (33).

In conclusion, we observed that EMF causes a reduced volume of blood vessels, bronchioles volume, and alveolar volume in the lung. This might cause serious side effects on the oxygen-carrying capacity of the organ. According to these results, it would be suggested that EMF might lead to impairment in the lung structure, so it causes loss of function. FA, that antioxidant properties are known, ameliorated these effects.

Based on the stereological results, although FA reduced the negative effects of EMF, it could not normalize bronchiole and blood vessel volumes. The inability of FA to show sufficient antioxidant effect may be due to insufficient dose or duration of use. There is a need for new studies on the duration and dose of FA.

On the other hand, although we attribute the protective effects of FA to its antioxidant properties, the lack of biochemical methods limits our study. We believe that this subject needs to be investigated using different techniques such as biochemical analysis of lung tissues, electron microscopic observation, and immunohistochemical staining processes for a better understanding of the side effect of EMF exposure on the lung.

Conflict of interest statement
The authors have declared that there is no conflict of interest.

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