ISSN 2449-8955 European Journal of Biological Research *Research Article*

DOI: http://dx.doi.org/10.5281/zenodo.4447339

Investigation of the effects of 50 Hz electromagnetic field on the lifespan of the red blood cells in vitro

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Received: 17 September 2020; **Revised submission:** 13 January 2021; **Accepted:** 18 January 2021

http://www.jbrodka.com/index.php/ejbr

ABSTRACT: In recent years, studies have indicated that electromagnetic fields (EMFs) may have harmful effects on human health. The effects on human health of the 50 Hz extremely low frequency EMF (ELF-EMF), which is often used in daily life, are still controversial. In our study, we investigated the in vitro effects of 50 Hz ELF-EMF on the lifespan of erythrocytes, which have no nucleus and organelles, and are therefore relatively more sensitive compared to other cells in the body to any harmful effect that may come from outside. Whole blood obtained from healthy volunteers was exposed to 50 Hz, 0.3 mT ELF-EMF over 35 days. After this time, erythrocytes (red blood cell, RBC) counts in blood, hematocrit (HCT) value, main corpuscular volume (MCV), and erythrocyte osmotic fragility (EOF), an indicator of aging, were examined. At the end of 35 days, RBC and HCT were decreased while MCV and EOF were increased in the blood samples of both the EMF-exposed group and the non-exposed group. However, while there were no statistically significant changes in terms of RBC counts, and HCT between the two groups, it was observed that MCV and EOF increased significantly less in the EMF-exposed group compared to the non-exposed group. These results suggest that 50 Hz ELF-EMF exposure does not affect the lifespan of erythrocytes in vitro, but it may extend erythrocytes' lifecycles due to a reduction in osmotic fragility of the erythrocytes in in vivo conditions.

Keywords: Red blood cell; Erythrocyte; Osmotic fragility; Electromagnetic field; ELF-EMF.

1. INTRODUCTION

Since the use of electrical devices is increasing day by day, living beings that are close to them are affected by the electromagnetic field (EMF) while these electromagnetic devices run. Since the powerline frequency is 50 Hz (60 Hz in some countries), these beings are mostly exposed to EMFs in this frequency band. In many previous studies, it was mentioned that the devices that run on powerline frequency may harm living beings with the EMFs they create. Short-term EMF exposure studies on nerve and heart tissues, which can be stimulated by EMFs, it was stated that 50 Hz extremely low exposure EMF (ELF-EMF) affects the electrical activity of the brain and decreases the speed of neural transmission, as well as the heart rate [1-6]. In contrast, the structural functions of the tissues have mainly examined in long-term exposure studies; such studies have shown that ELF-EMF may cause childhood cancers, developmental disorders, cancers in adults,

reproductive dysfunction, cardiovascular disorders, depression, suicide, neurobehavioral effects, immunological modifications, and neurodegenerative diseases like Alzheimer's disease and amyotrophic lateral sclerosis [7, 8]. However, the results obtained from both short-term and long-term exposure studies have not been confirmed in subsequent studies [9-13]. The reason for the differences between the results of the studies may have occurred because the changes caused by the exposure are compensated by the beings' internal systems. This idea suggests that the 50 Hz ELF-EMF exposure may have a greater effect on cells with lower compensation ability.

Erythrocytes are blood tissue cells that have the main function of carrying oxygen to tissues. They contain a protein called hemoglobin that has iron in its structure and has a lifespan of 100-120 days in the circulation [14]. In 1 ml of blood, although the amounts are different in men and women, there are 4.5-5.5 million erythrocytes on average. In contrast to the other cells, erythrocytes do not engage in DNA replication and protein synthesis, since they do not contain a nucleus [15]. Erythrocytes should contain with their own intracellular enzyme proteins after they develop until they die. For this reason, when erythrocytes are compared to other cells, the ability to compensate for the probable response that may occur because of EMF exposure is quite low when compared to that of the other cells in the organism.

Because erythrocytes cannot defend themselves against harmful exposures sufficiently, it is thought that exposure studies carried out in vitro on erythrocytes will represent a good model to research the cellular effects of ELF-EMF. There is no exposure study based on this idea in the literature. For this reason, it was planned in this study to examine the effect of 50 Hz ELF-EMF on the lifespan of erythrocyte cells in in vitro conditions.

2. MATERIALS AND METHODS

2.1. Ethical approval

Approval was received from the Muğla Sıtkı Koçman University Faculty of Medicine Clinical Studies Ethics Committee.

2.2. Setup

An Ndustrio (Ekofan, İzmir, Turkey) brand blood storage cabinet was used. Two bobbins (Pasco EM-6724, Roseville, CA, US) with 500 coils with inner diameters of 18.6 cm and external diameters of 22.1 cm were placed parallel to each other and spaced 21 cm apart, as shown in Fig. 1. Only a glass shelf with 4 mm thickness was placed between two bobbins. These two bobbins were fed in series with a waveform generator with an amplifier (Pasco PI-8127, Roseville, CA, USA); in this way, a Helmholtz coil setup was built. In the Helmholtz coil system, it is assumed that the magnetic flux between two bobbins is parallel and homogeneous [16]. The cables that feed the bobbins are fed through air-tight holes in the blood storage cabinet and the device that generates the waveform is placed outside of the incubator. A container in which the blood samples are kept is placed in the middle of the bobbins, as illustrated in Fig. 1.

2.3. Electromagnetic field

The frequency (50 Hz), waveform (sinus), and voltage (10 V; maximum output voltage of the device) of the electricity to be generated was is set on the device. The frequency and the waveform of the EMF generated between the bobbins was tested with a Powerlab 16/35 (Adinstruments, New South Wales, Australia) data-recording device, and the magnetic flux density of the EMF was tested with a Chauvin Arnoux

CA42 (Paris, France) 0-400 kHz Gaussmeter. The current passing through the bobbins at 10 V was 0.09 A. The magnetic flux density generated in the middle of the bobbins in this current was measured as 0.3 mT. The magnetic flux density between the bobbins when the power supply was off was not higher than 1 μTesla and the electric field intensity was not higher than 1 V/m.

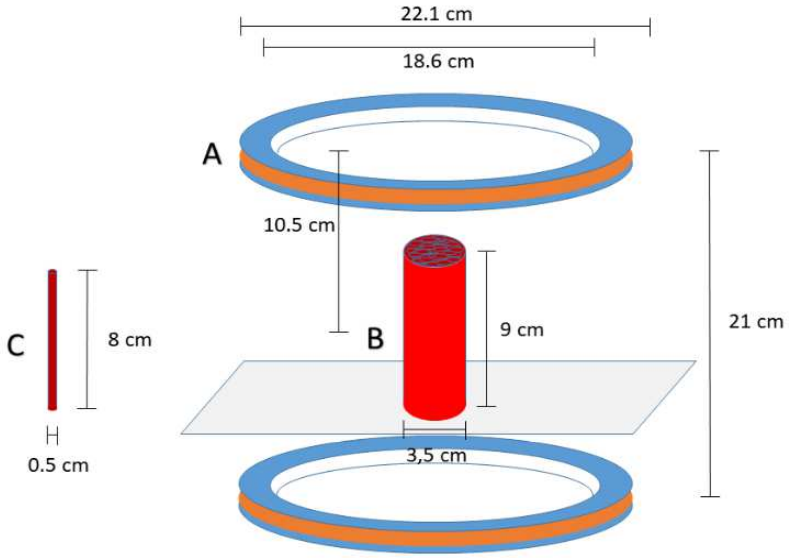

Figure 1. Helmholtz coil setup. Two bobbins (A) are placed in parallel and connected in series electrically. A container (B) containing blood samples (C) is placed on a plastic glass in the middle of two bobbins.

2.4. Temperature control of the blood cabinet

The temperature of the cabinet was set as +4°C. This temperature was monitored during the experiment with a temperature probe (MLT12, Adinstruments, New South Wales, Australia) and a Powerlab 16/35 (Adinstruments, New South Wales, Australia) data-recording device.

2.5. Source of the erythrocytes

All blood products were obtained from 60 male volunteers who applied to the "Ministry of Health Muğla Sıtkı Koçman University Training and Research Hospital Transfusion Center" (Muğla, Turkey) as donors for "Complete Blood Donation" and whose blood types were "O Rh (+)". The criteria under the topic title of "Blood Donor Selection" defined in the "National Blood and Blood Products Guide" were used in the selection of donors (Table 1) [17].

2.6. Receiving the blood from the donor and transferring it to the laboratory

Complete blood was obtained by entering the collecting vein in the forearm through a cannula. 450 ml of blood was collected in a PVC blood collection bag (Kansuk, İstanbul, Turkey) with a leukocyte filter. There was 63 ml of standard citrate-phosphate-dextrose-adenine (CPDA-1) solution in the blood collection bag. Two blood samples with 1 ml of blood in average were taken from each of the blood collection bags and the sample numbers on them were recorded. Afterwards, these blood samples were placed in blood storage cabinet at +4°C. The duration between the time blood was taken from the donor and the time the experiment began was not more than 24 hours and strict attention was paid to keeping the blood at +4°C during blood transfer.

Table 1. Criteria under the topic title of "Blood Donor Selection" defined in the "National Blood and Blood Products Guide" (Ministry of Health, 2011).

1.	Age range	Must be 19–66 years
2.	Donation frequency	Should be longer than 90 days for men and 120 days for women
3.	Pulse	Should be regular and between 50 and 100 in a minute
4.	Body temperature	Should not be above 37.5°C
5.	Body weight	Must be at least 50 kg
6.	Blood pressure	Systolic pressure should be 90–180 mm Hg and diastolic pressure should be 60–100 mm Hg
7.	Hemoglobin level	Should be 13.5–18.0 g/dl for men and 12.5–16.5 g/dl for women
8.	Medical history	Should not have a known disease
9.	Alcohol	Should not consume alcohol 12 hours in advance
10.	Drug use	Should not use drugs before donation (rejection is applied according to the appropriate time period according to the pharmacokinetics qualities of the drug. For example, the period is 5 days for aspirin and piroxicam)
11.	Blood volume to take	Volume of the blood donation should be 450 mL ± 10% except for the anticoagulant solution

2.7. Formation of the groups

Two experimental groups are formed, namely the EMF group (n=30) and the control group (n=30). As the experiment groups were carried out, sample selections for each group were realized at random.

2.8. Procedure

For the EMF group, laboratory tests were studied from one of the pair of blood samples belonging to the donors. The second blood sample was placed in the EMF setup, as shown in Fig. 1. According to the "National Blood and Blood Products Guide," the lifetime of complete blood that contains CPDA-1 for transfusion is accepted as 35 days [17]. For this reason, blood samples were left under EMF exposure for 35 days in total. At the end of the exposure, laboratory tests from all blood samples were studied again. The same procedure was applied using the same setup for the control group blood. However, the waveform generator was closed during the incubation of control blood and no EMF was generated in the setup.

2.9. Laboratory analyses

Laboratory analyses were carried out on the bloods both before the experiment started and after they were left in the exposure setup for 35 days.

2.9.1. Cell count

An erythrocyte dilution pipette (Marienfeld-Superior, Königshofen, Germany) was filled with blood to the 0.5 line. Then, it was filled with Hayem solution up to the 101 line (a mixture of 0.5 g of mercury chloride, 5 g of sodium sulfate, 1 g of sodium chloride, and 200 ml of distilled water). In this way, the blood was diluted 200 times. Afterwards, a drop of diluted blood was dripped onto a counting chamber (Thoma, Marienfeld-Superior, Königshofen, Germany) covered with a lamella. The used counting chamber consisted of a chamber with sides of 1 mm and a length of 0.1 mm. The total volume of this chamber was 0.1 mm^3 (1 mm x 1 mm x 0.1 mm). The large square with sides of 1 mm was divided into 16 squares with three longitudinal and transverse lines. When three lines were excluded, the side of each of these squares was 0.2 mm and the volume was 0.004 mm^3 (0.2 x 0.2 x 0.1 mm). The cells in counting chamber are counted with the

help of an optical microscope (Eclipse Ci-L, Nikon Instruments, Tokyo, Japan) at 400x magnification. For the determination of erythrocyte number, four squares at the corners of the 16 squares and one of the squares in the middle of the chamber were counted. The total volume through which the cell count was realized was 5 (the number of counted squares) x 0.02 = 0.1 mm^3. Consequently, a total cell number was found with the formula of total cell number = counted cell number x 200 (dilution coefficient) / 0.02 mm^3 (total volume of the five counted squares). In the counting process, leukocytes and thrombocytes, which were not filtered and infrequently observed, were not taken into consideration.

2.9.2. Determination of hematocrit (HCT)

HCT provides the rate that the total erythrocyte volume covers in the total blood volume, and it is calculated as follows:

$$\text{HCT} = \frac{\text{Total Erythrocyte Volume}}{\text{Total Blood Volume}}\ x\ 100$$

A heparin-containing capillary tube (Nris, Vitrex, Herlev, Denmark) was filled to the 3/4 of the tube with blood to be analyzed. Then, one tip of the tube was closed with glass putty. The capillary tubes were placed in an HCT centrifuge (MicroCL 17, Thermo Fisher Scientific, Osterode, Germany). Then, these tubes were centrifuged for 5 minutes at 12,500 rpm. Afterwards, the rate of erythrocyte volume / total blood volume in the tubes was found using an HCT scale.

2.9.3. Main corpuscular volume (MCV)

MCV shows the average volume of erythrocytes in a sample. This value is calculated as follows:

$$\text{MCV (fL)} = \frac{\text{HCT (\%)}}{\text{RBC count (mill/uL)}}\ \text{x}\ 10$$

2.9.4. Osmotic fragility test

For each osmotic fragility test, a total of six 5 ml tubes were used with the following contents prepared before the study: 1) 0.9% NaCl, 2) 0.6% NaCl, 3) 0.5% NaCl, 4) 0.4% NaCl, 5) 0.3% NaCl, and 6) distilled water. Then, 0.05 ml of blood was pipetted in these tubes. There was a waiting period for 20 minutes and then the tubes were placed in a centrifuge (Nüve NF800, Istanbul, Turkey) and centrifuged for 5 minutes at 3,000 rpm. Following this, liquid at a volume of 0.1 ml was obtained from the remaining supernatant on the tubes and absorbance was read at 540 nm with a spectrophotometer (Spectramax i3, Molecular Devices, Sunnyvale, CA, USA). In the absorbance reading, solutions that did not include pipetted blood were used as a blind sample. Then, Tube 1 (0.9% NaCl solution) and Tube 6 (distilled water) were considered as the negative (0% hemolysis) and positive (100% hemolysis) controls, respectively. The percentages of the hemolysis values in Tubes 2, 3, 4, and 5 were found by comparing the absorbance of the Tubes 1 and 6. In addition, CH20, CH50, and CH80 (sodium solution in which 20%, 50%, and 80% of total erythrocytes were hemolyzed) values are found for each blood sample by the acquiring regression formula from the hemolysis values.

2.10. Statistical analysis

Before the study, the sample size was calculated with GraphPad StatMate 2.0 (GraphPad Software, San Diego, CA, USA; Windows 8.1) software to prevent Type II error (false negative). The following setup was used in the calculation of the sample size: alpha 0.05, beta 0.2, and power 0.8. Post hoc power analysis was carried out with the parameters acquired after the study using the same software and setup. Whether all values

acquired from the study were compatible with normal distribution was evaluated using the D'Agostino–Pearson omnibus normality test and the Shapiro–Wilk normality test. In the study, the percentage change at the end of the 35th day was determined using the following formula:

$$\text{change value } \% = \frac{(\text{last value} - \text{first value})}{\text{first value}} \text{ x } 100$$

Whether there was a statistical difference between % change value parameters belonging to the control and EMA groups was evaluated using the Mann-Whitney U test. In all cases, the alpha value was selected as 0.05, and a p-value below 0.05 was accepted as significant; and p-value below 0.0001 was accepted as very significant.

3. RESULTS

The erythrocyte counts, the HCT and the MCV values, and statistical results are shown in Table 2.

Table 2. Hemogram values and statistical comparison between groups.

	Control Group (n=30)			EMF Group (n=30)			P value of the % changes
	1st day	35th day	% changes	1st day	35th day	% changes	
RBC count ($x10^6/mm^3$)	4.69 ± 0.62	4.57 ± 0.59	-1.99 ± 0.18	4.70 ± 0.51	4.61 ± 0.56	-1.72 ± 0.24	0.40
Hematocrit (%)	43.6 ± 5.80	42.5 ± 5.46	-2.82 ± 0.40	44.5 ± 4.93	43.6 ± 5.01	-2.67 ± 0.38	0.52
Main corpuscular volume (fL)	92.6 ± 7.13	93.4 ± 7.01	1.06 ± 0.14	92.6 ± 6.48	92.8 ± 6.64	0.95 ± 0.12	0.0068**

3.1. Erythrocyte count analysis

The erythrocyte count in cubic millimeters decreased at a rate of 1.99±0.18% in the control group and 1.72±0.24% in the EMF group at the end of the 35-day period. There was no statistically significant difference between the % changes in the two groups in terms of erythrocyte counts (p=0.40).

3.2. HCT analysis

The HCT value decreased at a rate of 2.82±0.40% in the control group and 2.67±0.38% in the EMF group at the end of the 35-day period. There was no statistically significant difference between the % changes in the two groups in terms of HCT (p=0.52).

3.3. MCV analysis

The MCV value decreased at a rate of 1.06±0.14% in the control group and 0.95±0.12% in the EMF group at the end of the 35-day period. MCV increased less in the EMF group compared to the control group; a highly statistically significant difference was found between % changes in the two groups (p=0.0068).

3.4. Osmotic fragility analysis

From the osmotic fragility tests, the % hemolysis values of erythrocytes in 0.9%, 0.6%, 0.5%, 0.4%, 0.3%, and 0.0% NaCl solutions are shown in Table 3 and the osmotic fragility curves drawn by means of these values is given in Fig. 2a and 2b. At the end of 35-day period, a shift to the right (i.e., an increase in

osmotic fragilities) was observed in the osmotic fragility curves of erythrocytes belonging to both the control group and the EMF group.

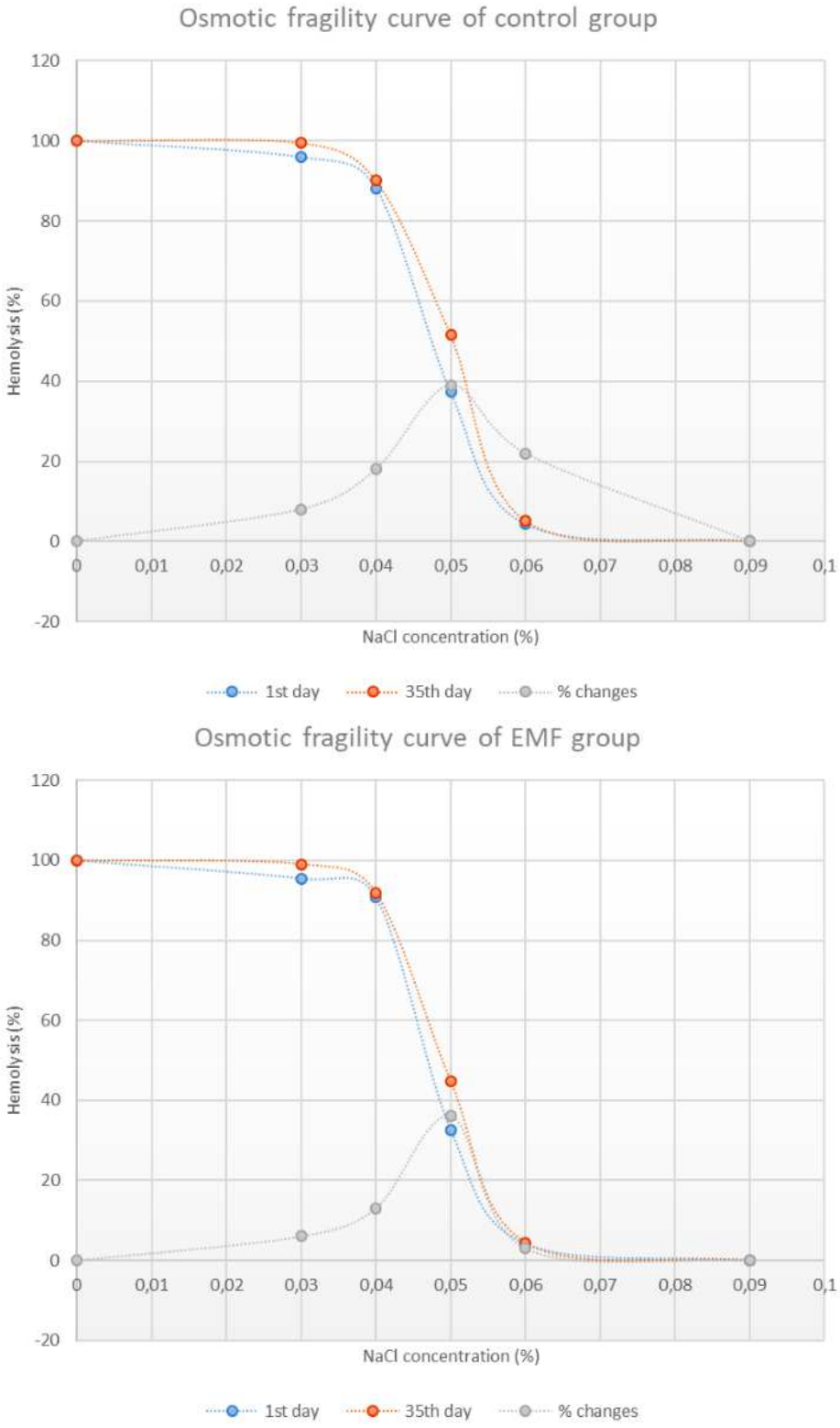

Figure 2. a) Osmotic fragility curve of control group, b) Osmotic fragility curve of EMA group.

Table 3. Osmotic fragility tests result and statistical comparison between groups. CH20, CH50 and CH80 show the sodium solution in which 20%, 50%, and 80% of total erythrocytes are hemolyzed.

		Control Group (n=30)			EMF Group (n=30)			P value of the % changes
		1st day	35th day	% changes	1st day	35th day	% changes	
Osmotic Fragility (% hemolysis)	0.9% NaCl	0	0	0	0	0	0	>99
	0.6% NaCl	4.28 ± 0.87	5.11 ± 1.22	0.22 ± 0.15	4.15 ± 0.81	4.39 ± 1.01	0.03 ± 0.10	< 0.0001***
	0.5% NaCl	37.3 ± 5.15	51.5 ± 8.95	0.39 ± 0.07	32.6 ± 5.59	44.8 ± 6.68	0.36 ± 0.05	0.033*
	0.4% NaCl	88.2 ± 15.1	90.1 ± 16.4	0.18 ± 0.07	90.7 ± 16.3	91.8 ± 17.2	0.13 ± 0.08	0.38
	0.3% NaCl	95.9 ± 9.16	99.5 ± 8.45	0.08 ± 0.07	95.5 ± 9.72	99.0 ± 8.54	0.06 ± 0.44	0.88
	0.0% NaCl	100	100	0	100	100	0	>99
Osmotic Fragility (% NaCl concentration)	CH20	0.54 ± 0.03	0.57 ± 0.04	5.62 ± 1.56	0.53 ± 0.03	0.55 ± 0.03	3.74 ± 1.05	0.0019**
	CH50	0.47 ± 0.03	0.50 ± 0.03	6.44 ± 1.84	0.47 ± 0.03	0.48 ± 0.04	2.74 ± 1.76	0.0004**
	CH80	0.41 ± 0.03	0.43 ± 0.05	4.97 ± 1.19	0.41 ± 0.04	0.42 ± 0.05	3.23 ± 1.16	0.036*

According to the data acquired, it was seen that erythrocyte fragility in 0.6% NaCl solution increased at a rate of 0.22±0.15% in the control group and 0.03±0.10% in the EMF group at the end of the 35-day period. Erythrocyte fragility in 0.6% NaCl solution increased less in the EMF group; a highly statistically significant difference was found between the % changes in the two groups ($p<0.0001$). It was observed that erythrocyte fragility in the 0.5% NaCl solution increased at a rate of 0.39±0.07% in the control group and 0.36±0.05 % in the EMF group. As in the 0.6% NaCl solution, erythrocyte fragility in 0.5% NaCl solution increased less in the EMF group, % changes in the two groups were statistically significant ($p=0.033$). At the end of the 35-day period, the erythrocyte osmotic fragility increased in both the control group and EMF group in 0.4% and 0.3% NaCl solutions. However, there was no statistical significance between the two groups in terms of changes in this increase ($p=0.38$, $p=0.88$, respectively).

CH20, CH50, and CH80 (NaCl solutions in which 20%, 50%, and 80% of total erythrocytes were hemolyzed) values calculated with regression formula acquired by using % hemolysis values are shown in Table 3. At the end of the 35th day, it was found that CH20 increased at rate of 5.62±1.56% in the control group and 3.74±1.05% in the EMF group, CH50 increased at a rate of 6.44±1.84% in the control group and 2.74±1.76% in the EMF group, and CH80 value increases at a rate of 4.97±1.19% in the control group and 3.23±1.16% in the EMF group. The CH20, CH50, and CH80 values increased less in the EMF group compared to the control group and a statistically significant difference was observed between the % changes in the two groups ($p=0.0019$, $p=0.0004$, and $p=0.036$, respectively).

4. DISCUSSION

Our aim in the study was to examine possible effect of 50 Hz ELF-EMF exposure on the erythrocyte lifespan under in vitro conditions. A limited number of studies have examined the effects of EMF exposure at different frequencies and intensities on blood parameters in the literature; moreover, there has been no consensus on the acquired results. Dasdag et al. stated that 50 Hz ELF-EMF increases hematocrit values but does not affect other parameters [18]. Çakır et al. did not encounter any kind of change in erythrocyte number and HCT value when they applied ELF-EMF exposure to rats [19]. Bonhomme-Faivre et al. observed a decrease in the erythrocyte number and HCT value on the 20th day of ELF-EMF exposure, but they did not reach the same conclusion for longer exposures [20]. All of these studies were in vivo studies. The research carried out by Bonhomme-Faivre et al. suggests to us that the results caused by an exposure to EMF that can

effect erythrocytes might be compensated for in time by an increase or decrease in the erythrocyte production speed by living organisms. For this reason, if there is an influence caused by EMF in the erythrocyte lifespan, we think that it can only be shown through an in vitro study.

According to the findings acquired from this study, over 35 days of 50 Hz ELF-EMF exposure, there were no statistically significant changes in terms of the change rates of RBC counts, and HCT between the two groups. It was observed that MCV and EOF in the EMF-exposed group showed a significantly lower reduction compared to the non-exposed group. Since there were no differences in terms of erythrocyte number, and in relation to this, in the HCT value at the end of 35 days, we saw that the EMF exposure in the dosage we applied did not affect the erythrocyte lifespan under in vitro conditions. In addition, MCV and osmotic fragilities of erythrocytes under EMF exposure increased less compared to the control group; therefore, we think that EMF in the dosage we applied decreased the forces that affect the erythrocyte membrane and cause fragility.

Since erythrocytes do not contain organelles such as DNA and mitochondria, they do not die by apoptotic mechanisms seen in other body cells. Their cause of death is completely related with physical forces: As erythrocytes get old, the intracellular antioxidation/oxidation balance deteriorates; as a result of this deterioration, oxidation of membrane lipids occurs. As a consequence of oxidation, cell membranes become more and more stiff and become more fragile. Thus, the osmotic fragility of the aging cells increases. Normally, the erythrocyte diameter is 8 microns in its thickest place, but the diameter of a systematic capillary is 5 microns. For this reason, erythrocytes become stuck when they pass through the capillaries and their shapes change. Cell membranes of old erythrocytes, especially in places where the vessel diameter is less as 3 microns, cannot endure the pressure and undergo lysis. In this way, after an erythrocyte is produced in the bone marrow, it remains in circulation for around 120 days. According to the study results, it is possible to say that since 50 Hz EMF decreases osmotic fragility, it may also slow down aging. If EMF slows down the aging of erythrocytes, it would be reasonable to expect erythrocyte number and HCT values in cubic millimeters to be higher compared to the non-exposed group. However, we did not observe a significant change in either parameter in our study. We think that this was the case because we did not use an in vivo environment in our study. Therefore, even if erythrocytes get old and cell membranes become more fragile, since they are not exposed to a physical stress, such as passing through capillary vessels, erythrocytes may not die under in vitro conditions; this may be why there was no difference between the two groups in terms of the results for cell number and HCT. If the blood used in the experiment were to be transfused to another patient later on, it would probably have a longer lifespan compared to the blood of the control group, since the blood of the EMF group would be less fragile.

According to the data we acquired from the study, 50 Hz ELF-EMF exposure had the effect of retarding erythrocyte aging. We did not encounter any kind of information that presented a probable mechanism. It may be that 50 Hz ELF-EMF effects oxidation/antioxidation systems have a direct effect on the cell membrane. It has been claimed that ELF-EMF exposure increases oxidative stress in studies carried out using serum, lung, nerve, myocardial, and squamous cancer cells[21–27]. Again, in some studies realized using brain tissues and muscle tissues, it was claimed that ELF-EMF exposure decreases oxidative stress [28-30]. In addition, there was no change encountered in terms of oxidative stress in serum and pheochromocytoma cells in recent exposure studies [31, 32]. As this makes the clear, the effect of ELF-EMF on oxidation/antioxidation systems in the literature is contradictory. We think that the answer to the question

regarding how 50 Hz ELF-EMF exposure affects erythrocytes over oxidation/antioxidation systems can be elucidated through studies focusing on intracellular biochemical and molecular analysis.

5. CONCLUSION

In this study, we observed that 50 Hz ELF-EMF exposure does not affect the lifespan of erythrocytes in vitro, but it may extend erythrocytes' lifecycles due to the reduction of the osmotic fragility of the erythrocytes in in vivo conditions. These findings can potentially be used in the future to extend the preservation period in environments like blood banks where blood products are stored. In addition, we hope that the study results will guide scientists and policymakers who are interested in EMF pollution.

Authors' Contributions: SE and OE performed conception and design, development of methodology, experiments and statistical analysis, interpreted the results and writing of the manuscript. AZ supervised the study. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This study was approved by the Mugla Sitki Kocman University Clinical Research Ethics Committee with the number 2014/16.

Acknowledgments: This manuscript was developed from a master thesis titled 'Investigation of Possible Effects of Extremely Low Frequency Electromagnetic Fields into Erythrocyte Lives'. The project was supported by Mugla Sitki Kocman University Scientific Research Projects Unit, project number 2014/071.

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