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P. PISZCZEK[1], K. WOJCIK-PIOTROWICZ[2], B. NOWAK[3], P. GUZDEK[4], P. NOVAK[5], J. PYTKO-POLONCZYK[5], K. GIL[1], J. KASZUBA-ZWOIŃSKA[1]

PHAGOCYTOSIS OF LATEX BEADS BY A HUMAN MONOCYTIC MONO MAC 6 CELL LINE AND EFFECTS OF LOW-FREQUENCY ELECTROMAGNETIC FIELD INTERACTION

[1]Department of Pathophysiology, Jagiellonian University Medical College, Cracow, Poland; [2]Department of Biophysics, Jagiellonian University Medical College, Cracow, Poland; [3]Department of Immunology, Jagiellonian University Medical College, Cracow, Poland; [4]Lukasiewicz Research Network - Institute of Microelectronics and Photonics, Warsaw, Poland; [5]Institute of Dentistry, Department of Integrated Dentistry, Jagiellonian University Medical College, Cracow, Poland

Some studies have shown that electromagnetic fields (EMFs) may impact immune response cells and their functions. The first stage of the defense from pathogens is innate immunity encompassing phagocytosis and phagocytosis-related intracellular effects. Our work aimed to determine the influence of a low-frequency electromagnetic field (7 Hz, 30 mT_{rms}) on the phagocytosis process of latex beads (LBs), the production of reactive oxygen species (ROS), and viability changes in a human monocytic Mono Mac 6 (MM6) cell line as an experimental model of the phagocytosing cells in *in vitro* cell culture conditions. For these purposes, cells were firstly activated with infectious agents such as lipopolysaccharide (LPS), Staphylococcal enterotoxin B (SEB), or the proliferatory agent phytohaemagglutinin (PHA), and then a phagocytosis test was performed. Cell viability and range of phagocytosis of latex beads by MM6 cells were measured by flow cytometry, and the level of ROS was evaluated with the use of a cytochrome C reduction test. The obtained results revealed that applied EMF exposure mainly increased the necrosis parameter of cell death when they were pre-stimulated with SEB as an infectious factor and subsequently phagocytosed LBs (P=0.001). Prestimulation with other agents like LPS or PHA preceding phagocytosis resulted in no statistically significant changes in cell death parameters. The level of ROS depended on the used stimulatory agent, phagocytosis, and/or EMF exposure. The obtained effects for EMF exposure indicated only a slight decrease in the ROS level for cells phagocytosing latex beads and being treated with SEB or PHA, while the opposite effect was observed for LPS pre-stimulated cells (data not statistically significant). The results concerning the viability of phagocytosing cells, the effectiveness of the phagocytosis process, and the level of radical forms might result from applied EMF parameters like signal waveform, frequency, flux density, and especially single EMF exposure.

Key words: *immunomodulation, electromagnetic fields, phagocytosis, cell viability, reactive oxygen species, cytochrome C, lipopolysaccharide, phytohaemagglutinin, Staphylococcal enterotoxin B*

INTRODUCTION

Many studies are showing that electromagnetic fields may influence living organisms, their systems, organs, and tissues/cells, including the immune system (1). A properly functioning immune system plays an important role in defense from infectious agents and/or other harmful environmental factors (2). The first line of defense from pathogens is innate immunity, encompassing phagocytosis. The primary evolutionary function of phagocytosis was a nutritional role, and even now this function has been preserved in bacteria and protozoa. Phagocytosis is also engaged in tissue remodeling, removal of apoptotic cells, cellular organelles, and especially in immune defense mechanisms in humans and animals (3). Neutrophils and monocytes in peripheral blood and tissue macrophages are effector cells crucial for non-specific cellular immune response at its initial stages, thus changes in their function or quantity may lead to weakening or enhancing of the immune response. Additionally, other cells play an important role in the organism's defense *e.g.* eosinophils in the case of parasitic infections, dendritic cells, astrocytes, as well as endothelial cells of small blood vessels. Phagocytosis is an example of an innate immune response, but when carried out by monocytes/macrophages, it triggers an acquired immune response mechanism through antigen-presenting cell (APC) function. Because phagocytosis is a mechanism acting in the first line of defense against pathogens (4), it fulfills a very important role in maintaining the proper homeostasis of the organism. Since neutrophils and monocytes/macrophages can perform phagocytosis very effectively, they are called

professional phagocytes (5). A crucial role in the performance of efficient phagocytosis of nanoparticles is please remove played activation, which may be induced by the receptor-dependent pathway with the usage of infectious agents like lipopolysaccharide (LPS), Staphylococcal enterotoxin B (SEB), or the proliferatory agent phytohaemagglutinin (PHA). A consequence of phagocytosis triggered by infectious or proliferatory agents is a release of reactive oxygen species (ROSs) (6-9) synthesized upon NADPH oxidase activation during the first phase of the process, followed by an oxygen-independent killing mechanism occurring in the phagolysosome. Some studies performed on macrophages have shown the impact of electromagnetic fields on enhanced ROSs production (10). Moreover, the EMF may act in combination with other factors like black carbon, a very damaging air pollutant present in the environment, and in this way modulate macrophage immune response and apoptosis (11). Furthermore, the role of EMF in the modulation of cell viability, including immune system cells, has been presented (1, 12-16). Following these studies, exposure to various EMFs might change the immune response activity of immune cells through changes in cell viability parameters. The present study was focused on determining the effect of a low-frequency electromagnetic field (7 Hz, 30 mT_{rms}) on MM6 cell line viability (apoptosis, late apoptosis, and necrosis), as well as on the efficacy of phagocytosis and production of ROS during phagocytosis assay with latex beads. Our study aimed to address the question of whether EMF exposure affect innate cellular response mechanisms like phagocytosis, as it involves many intracellular signaling routes, which might be potential 'receivers' of the EMF's dose.

MATERIAL AND METHODS

Cell culture

The human monocytic Mono Mac 6 (MM6) cell line originated from the Ziegler-Heitbrock German Collection of Microorganisms and Cell Cultures. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA; Life Technologies, Carlsbad, CA, USA), containing 10% (v/v) fetal calf serum (Gibco, Grand Island, NY, USA; Life Technologies, Carlsbad, CA, USA) heat-inactivated, and L-glutamine 0.2 M and gentamicin 50 mg/ml (Sigma-Aldrich, St. Louis, MO, USA). MM6 cells were cultured in an incubator at maintained physical parameters such as temperature (37ºC), carbon dioxide concentration (5%), and humidity (90%). The viability of cells was assayed by the trypan blue exclusion method and counted with a hemocytometer (Fuchs-Rosenthal chamber). The experiments were performed on cells at the logarithmic phase of growth, under conditions of >98% viability. For experiments, Mono Mac 6 cells were seeded into 96-well (Nest Biotechnology, Wuxi, China) culture plates and grown at an initial density 2×10^5 cells/well for experiments. The MM6 cell culture was chosen for the following reasons: i) it functions as an experimental model of phagocytosing cells in *in vitro* conditions; ii) it possesses stable properties in a wide range of external conditions, which ensures the repeatability of experimental results.

Electromagnetic fields exposure system

A low-frequency sinusoidal electromagnetic field generator (7 Hz, 30 mT_{rms}) was designed and provided by the Institute of

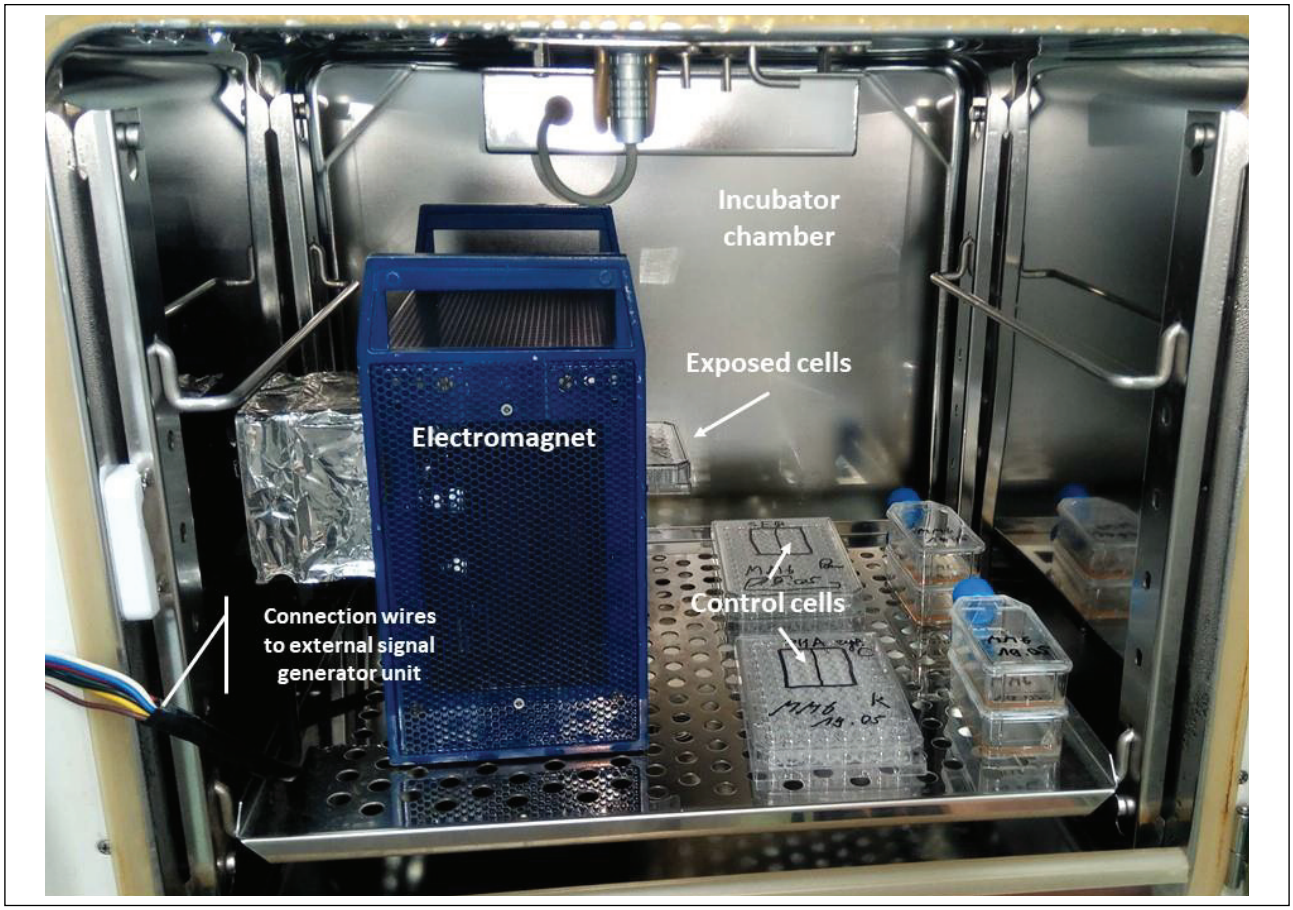

Fig. 1. View of the exposure system in the incubator chamber.

Electron Technology (Cracow, Poland). Details of the experimental setup were previously described (13), *Fig. 1*. In brief, the parameters of the EMF stimulation were set at the externally placed signal generator unit which feeds the electromagnet (element indicated inside the incubator). The electric signal from the signal generator unit (alternating current) was calibrated to obtain an alternating magnetic field with chosen magnetic flux densities. The magnetic field homogeneity in the sample volume was tested and ensured with an accuracy of a few % by using a Gaussmeter (F.W. Bell 6010, Bell Technologies, Houston, TX, USA). The background magnetic field for static- or alternating components was as follows <(0.05±0.01) mT or <(0.03±0.01) rms mT, respectively. The MM6 cells seeded on 96-well plates were placed in the exposure device and maintained in the incubator at 5% CO_2 and 37.0 ± 0.1ºC. Control cells were kept in the same conditions, except for the EMF application. A single EMF stimulation was performed for 3 h and each experiment was repeated at least four times, with threefold repetition per each experimental point. The choice of parameters for the applied electromagnetic field was mainly related to the following reasons: i) bio-activity (16) according to one of the practicable theoretical models (17); ii) proximity to the frequency range of signal transduction in cells; iii) exclusion of thermal effects; iv) continuation of previous studies with LF-EMFs on cell viability parameters, cytokine production in peripheral blood mononuclear cells and leukocytic cell lines (13, 18-21) and similar ones.

Phagocytosis assay with latex beads

The experimental methodology was conducted strictly according to the diagram presented in *Fig. 2*. After 24 hours from the cell culture seeding, cells were stimulated with one of the three stimulation agents - lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA), Staphylococcal enterotoxin B (SEB, Sigma-Aldrich, St. Louis, MO, USA) or phytohaemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO, USA), all at the same concentration of 1 µg/ml (19, 22). Cells without a stimulatory agent constituted the control group. After a subsequent 24 hours, 3.64×10^{11} latex beads (100 nm LBs) suspended in 20 µl PBS (Sigma-Aldrich, St. Louis, MO, USA) were added to each sample with a phagocytosis assay, and then cells were placed in the EMF generator and exposed to the electromagnetic field for 3 hours. The latex beads concentration was adjusted according to the assumed artificial experimental model of phagocytosis because LBs mimic extracellular pathogens. The number of used latex beads per sample was similar to some other studies with alive pathogens (23). Simultaneously, an EMF-unexposed phagocytosis assay was performed. For phagocytosis estimation by the flow cytometry method, the latex beads used were labeled with an orange fluorophore (emission wavelength: λ_{em}=540 nm) and added to each phagocytosis assay, respectively. Phagocytosis was stopped by placing the cell culture plates on ice (4ºC).

Flow cytometry evaluation of phagocytosis assay

Phagocytosis of orange fluorophore-labeled latex was estimated by flow cytometry analysis. After stopping the phagocytosis test with orange labeled latex beads, the cells were transferred into 5 ml tubes and washed three times with 5 ml ice-cold PBS (Sigma-Aldrich, St. Louis, MO, USA). Then, the cells were resuspended in 200 µl ice-cold PBS set on ice, and measured in the appropriate channel with a BD FACS Calibur flow cytometer. For each sample, no less than 10^4 events were acquired.

Cytochrome C reduction test

A cytochrome c reduction test was used for the detection of reactive oxygen species level in MM6 cell cultures, based on another study (24). The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA; Life Technologies,

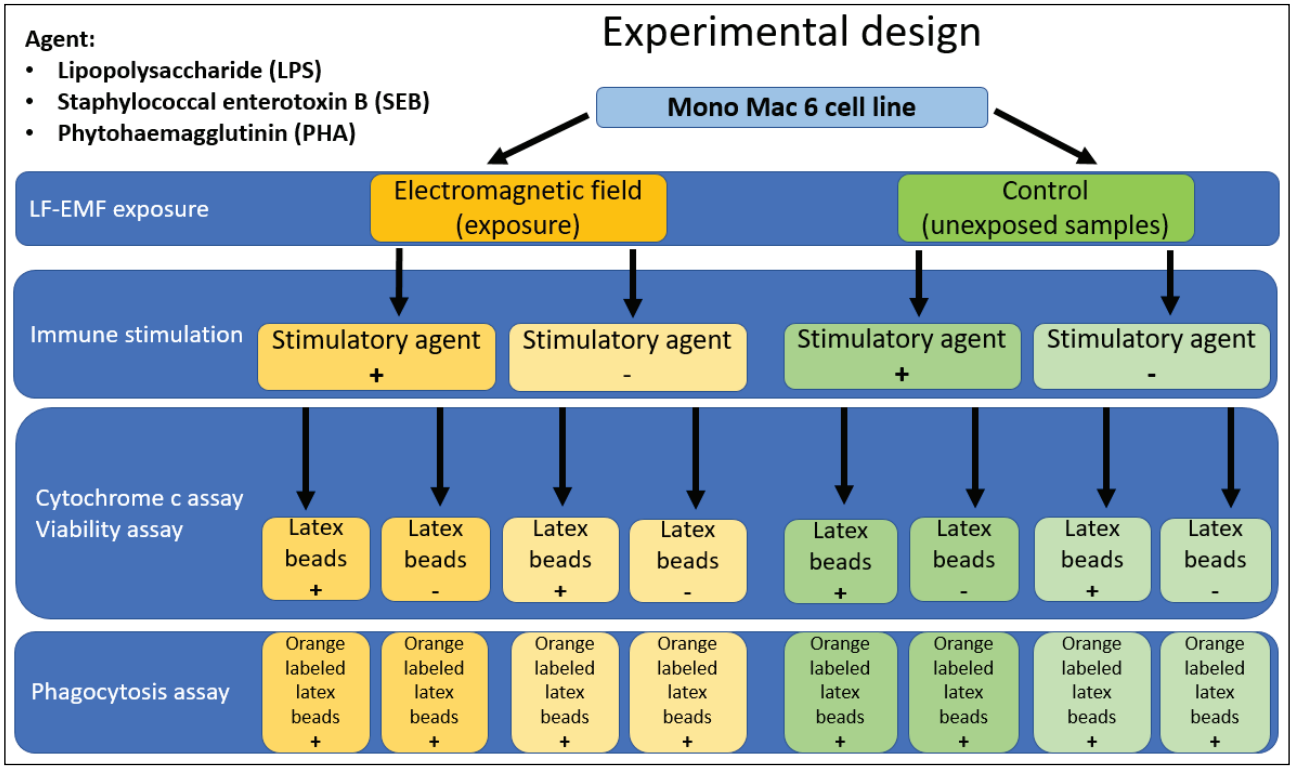

Fig. 2. Scheme (flow chart) of experimental procedure.

Carlsbad, CA, USA) phenol red-free medium and exposed to EMF or not exposed (control). All samples were supplemented with 100 µM cytochrome C (Sigma-Aldrich, St. Louis, MO, USA) in PBS/EDTA, for 1 h. In the next step, samples were centrifuged for 5 min. at 195×g, and 100 µl/well of supernatant was transferred onto a 96-well plate for measurement of absorbance (at 550 nm) with the use of an ELISA-reader (Ultra Microplate Reader, ELx800, Bio-Tech Instruments Inc., Vinooski, VT, USA), in five-fold repetition per each sample.

Flow cytometry cell viability analysis

The MM6 cell cultures were harvested 24 h after phagocytosis assay, washed three times with cold PBS (Sigma-Aldrich, St. Louis, MO, USA), then resuspended in 1× binding buffer (1.0 mmol/L HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH=7.4, 140 mmol/L NaOH, 2.5 mmol/L $CaCl_2$) (BD Biosciences, Franklin Lakes, NJ, USA) and stained with annexin V-APC (AnV-APC) labeling and propidium iodide (PI), strictly according to the manufacturer's recommendations. Before flow cytometric analysis, 400 µl of 1× binding buffer was added, and cells were analyzed on a BD FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the use of BD CellQuest Pro software. Annexin V-APC - propidium iodide staining was applied to estimate cell viability parameters as early apoptosis (AnV-positive cells), late apoptosis and necrosis (AnV and PI-positive cells), and necrosis (PI-positive cells). Controls to set up compensation and quadrants encompassed unstained cells, cells stained with Annexin-V-APC alone or with only PI. A minimum of 10^4 events were collected on each sample.

Statistical analysis

The normal distribution of the variables was tested with the Shapiro-Wilk test; not all data presented normality. Statistical evaluation of the experimental data was performed with the U-Mann Whitney and Kruskal-Wallis tests considering $P<0.05$ as the minimum level of significance. Results are presented as mean values ±standard deviation (±SD) of at least four independent experiments with threefold repetition per experimental point. The insignificant differences between compared groups were not marked in the figures. All statistical analyses were done with the use of SPSS software (SPSS 26.0, SPSS Inc.) for Windows.

RESULTS

It is commonly known that phagocytes play a crucial role in innate immune response and initiate an acquired response when phagocytosing cells are APCs (8, 9, 24). We have investigated whether exposure to a low-frequency electromagnetic field may affect a crucial immune response mechanism such as phagocytosis, resulting in oxygen free radical release. In the experimental schedule, we assessed the viability of phagocytosing cells upon phagocytosis assay and the effectiveness of the process. Both activities were evaluated by flow cytometry analysis.

Reactive oxygen radical production - cytochrome C assay

Reactive radical species are produced after the engulfment of phagocytosed material in phagocytosing cells. In this study, MM6 cells internalized latex nanoparticles, following 24-hour-long activation of cells by infectious (LPS, SEB) or proliferatory (PHA) agent and after undergoing electromagnetic field stimulation for the phagocytosis assay. The specificity of our experimental procedure requires the comparison between respective exposed/control groups arranged in pairs, according to *Table 1*. This means, that each EMF-exposed sample had its unexposed control equivalent.

Table 1. Exposed groups versus control groups in the performed experiments.

Exposed group	Control group
EMF	C
EMF-LPS/SEB/PHA	C-LPS/SEB/PHA
EMF-LB	C-LB
EMF-LPS/SEB/PHA-LB	C-LPS/SEB/PHA-LB

LPS, lipopolysaccharide; SEB, Staphylococcal enterotoxin B; PHA, phytohaemagglutinin; EMF low-frequency electromagnetic field; C, Control; LB latex beads.

The level of oxide radical anions measured in the cytochrome C assays varied, in accordance with the stimulatory agent used (LPS, SEB, or PHA), latex beads phagocytosis, and/or EMF exposure. In general, ROS synthesis was significantly lowered in the samples without latex beads in comparison to the respective ones, where latex beads (LBs) were phagocytosed by MM6 cells (*Fig. 3A*, *3B*) (except for results obtained for the application of PHA; *Fig. 3C*). Nevertheless, it is seen that the applied electromagnetic stimulation did not induce any statistically significant changes in ROS production when comparing EMF-exposed samples to the respective controls considered in the experimental procedure (*Table 1*, *Fig. 3A-3C*). The obtained effects for EMF exposure indicated only a slight decrease in the ROS level for samples phagocytosing latex beads and being treated with SEB or PHA, while the opposite effect was observed for LPS pre-stimulated cells.

Phagocytosis assay by flow cytometry analysis

The effectiveness of the MM6 cell phagocytosis assay was estimated by cytometric measurement of the uptake of latex nanoparticles labeled with an orange fluorophore. In each experiment, pre-activation of the MM6 cells was performed according to the experimental scheme (LPS, SEB, PHA), followed by LBs phagocytosis combined with EMF exposure (*Fig. 2*). The obtained results indicate insignificant changes in the percentage of fluorescence measured for LPS, PHA, or SEB pretreated cells and/or exposed to EMF. Specifically, the phagocytic activity of MM6 cells pre-stimulated with LPS or PHA and exposed to EMF was only slightly diminished compared to the unexposed samples (*Fig. 4A-4C*). In contrast, for the simultaneous stimulation with the SEB agent and EMF, a weak increase in fluorescence was noticed compared to the respective control sample, (*Fig. 4B*).

Cell viability parameters evaluation upon phagocytosis test

1. Early apoptosis

The results presented in *Figs. 5A-5C* show the changes in the population of early apoptotic Mono Mac 6 cells (AnV-positive) exposed to EMF alone or combined with an infectious/proliferatory agent, in the presence/absence of latex nanoparticles. A statistically insignificant increase in the number of early apoptotic cells was noticed in EMF-exposed samples compared to the control. Also, nearly no difference was observed for cells simultaneously stimulated with EMF or LPS as an infectious agent, upon performed phagocytosis of LBs (*Fig. 5A*).

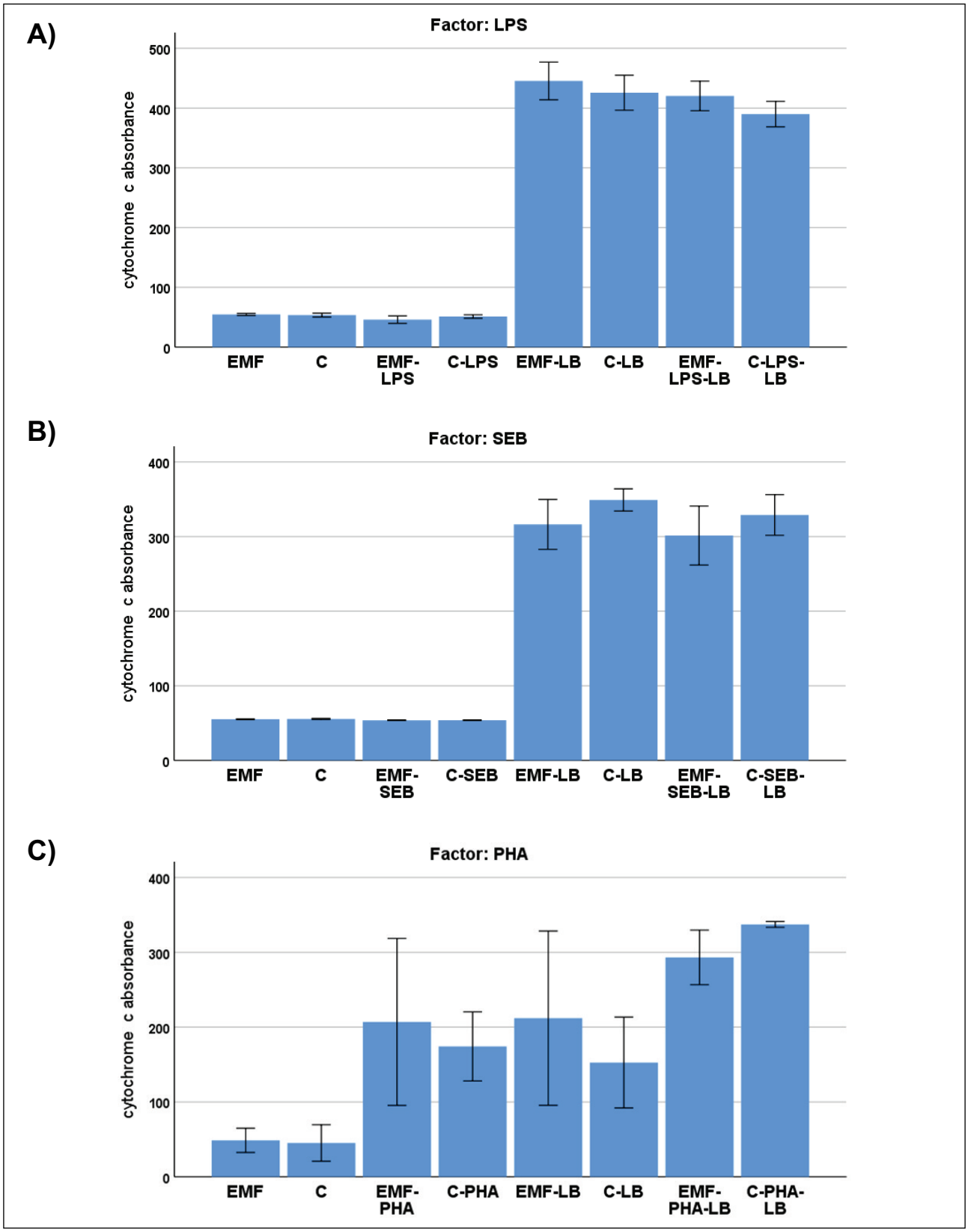

Fig. 3. Superoxide radical anion production in Mono Mac 6 cells after exposure to EMF with or without infectious or proliferatory factors, and/or latex beads. A): LPS, lipopolysaccharide; B): SEB, Staphylococcal enterotoxin B; C): PHA, phytohaemagglutinin. Results are presented as the mean ±SD. There were no statistically significant differences between compared groups (*Table 1*); P>0.05 (not indicated in the graph).

Abbreviations: C, Control; C-LB, cells phagocytosing latex beads; C-LPS/SEB/PHA-LB, LPS/SEB/PHA pretreated cells phagocytosing latex beads; EMF, low-frequency electromagnetic field; EMF-LB, EMF exposed cells phagocytosing latex beads; EMF-LPS/SEB/PHA, EMF and LPS/SEB/PHA treated cells; EMF-LPS/SEB/PHA-LB, EMF and LPS/SEB/PHA treated cells phagocytosing latex beads; LPS/SEB/PHA, LPS/SEB/PHA pretreated cells;

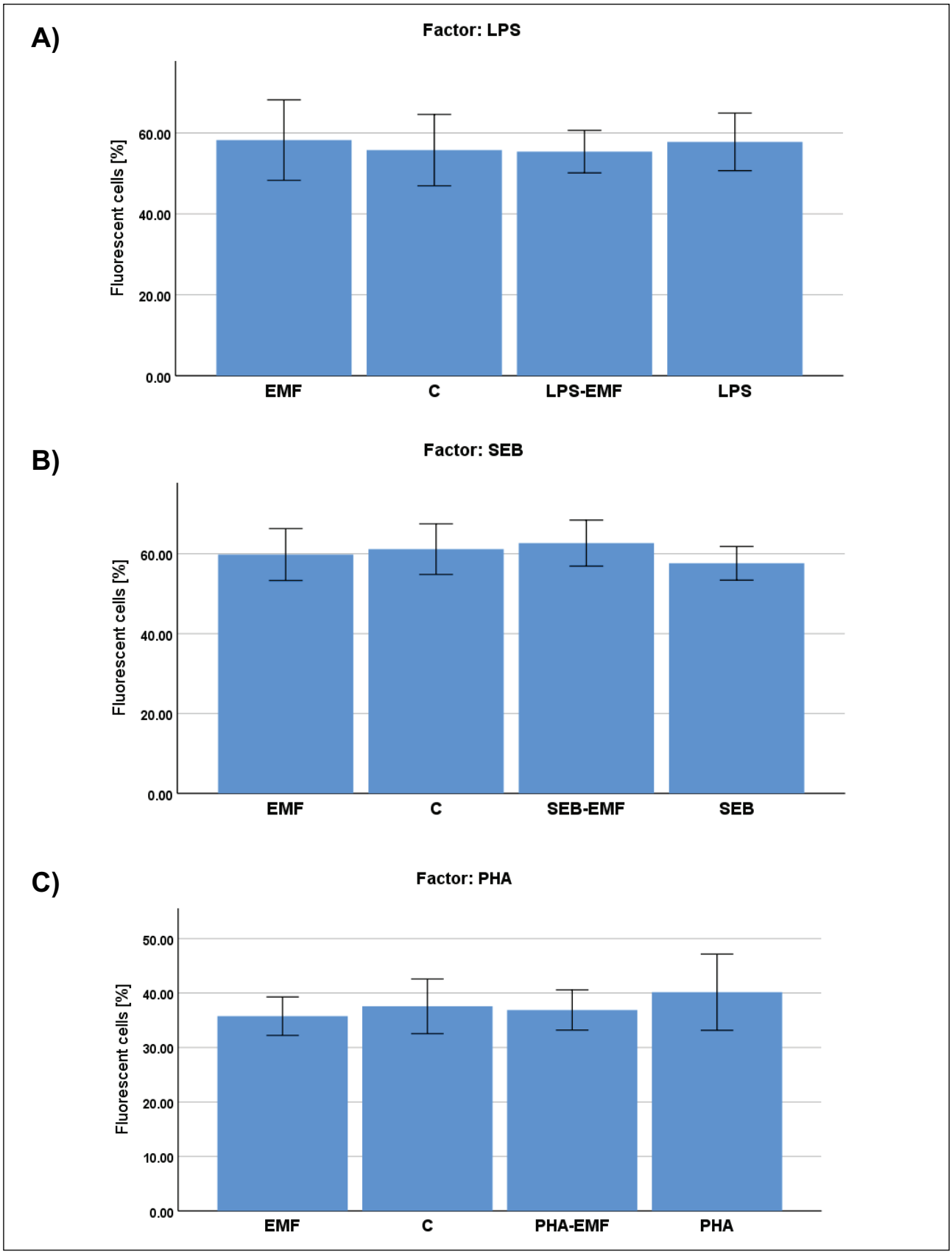

Fig. 4. The percentage of cells phagocytosing latex nanoparticles labeled with an orange fluorophore exposed to a low-frequency electromagnetic field with or without infectious/proliferatory agents: A): LPS, lipopolysaccharide; B): SEB, Staphylococcal enterotoxin B; C): PHA, phytohaemagglutinin. Results are presented as the mean ±SD. There were no statistically significant differences between compared groups (*Table 1*); P>0.05 (not indicated in the graph).
Abbreviations: C, Control; C-LPS/SEB/PHA, LPS/SEB/PHA pretreated cells; EMF, low-frequency electromagnetic field; EMF-LPS/SEB/PHA, EMF and LPS/SEB/PHA treated cells;

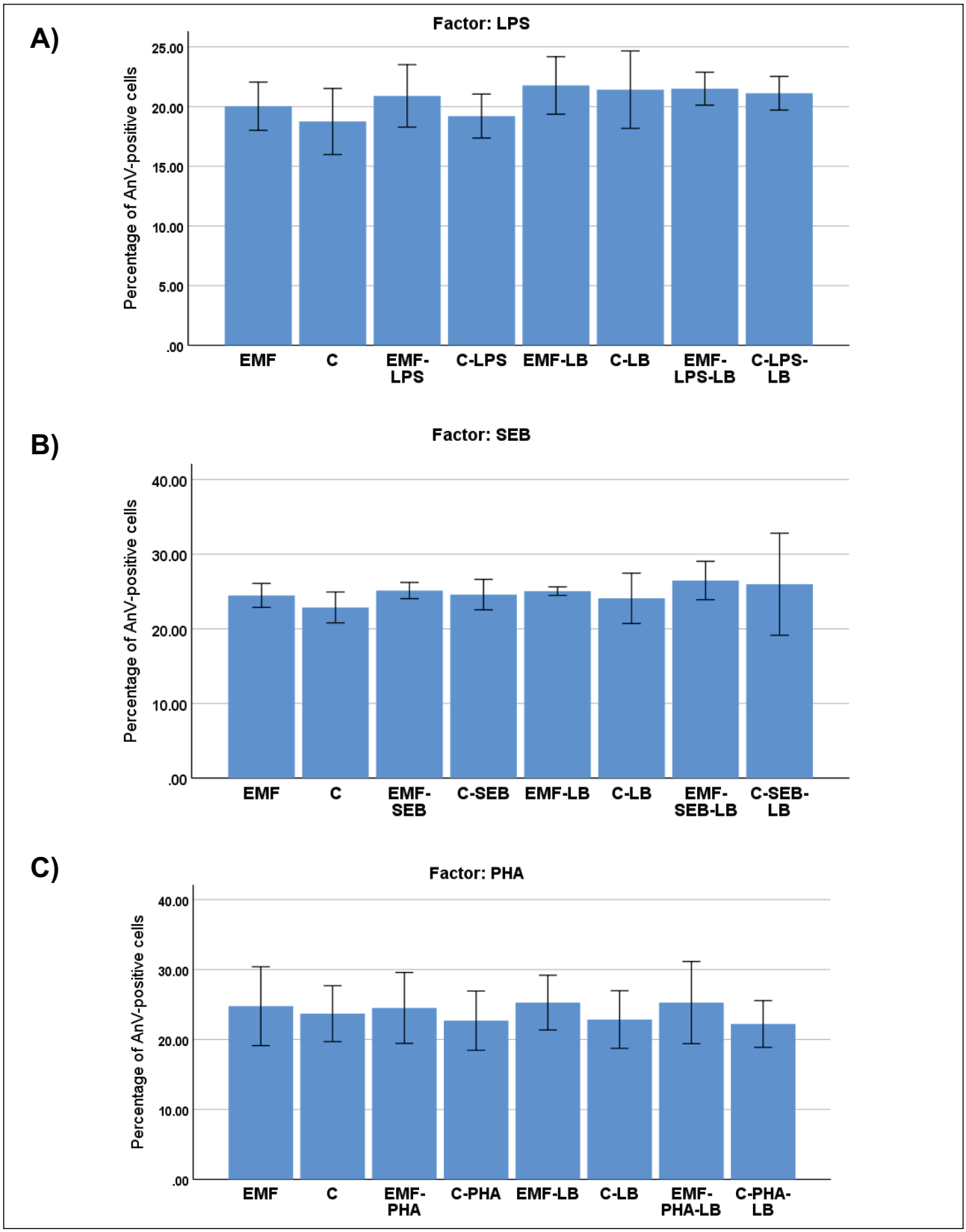

Fig. 5. The percentage of MM6 early apoptotic cells (AnV-positive) exposed to EMF with or without infectious/proliferatory agents, and/or latex beads: A): LPS, lipopolysaccharide; B): SEB, Staphylococcal enterotoxin B; C): PHA, phytohaemagglutinin.
Results are presented as the mean ±SD. There were no statistically significant differences between compared groups (*Table 1*); P>0.05 (not indicated in the graph).
Abbreviations: C, Control; C-LB, cells with the latex beads; C-LPS/SEB/PHA, LPS/SEB/PHA pretreated cells; C-LPS/SEB/PHA-LB, LPS/SEB/PHA pretreated cells phagocytosing latex beads; EMF, low-frequency electromagnetic field; EMF-LB, EMF exposed cells phagocytosing latex beads; EMF-LPS/SEB/PHA, EMF and LPS/SEB/PHA treated cells ; EMF-LPS/SEB/PHA-LB, EMF and LPS/SEB/PHA treated cells phagocytosing latex beads.

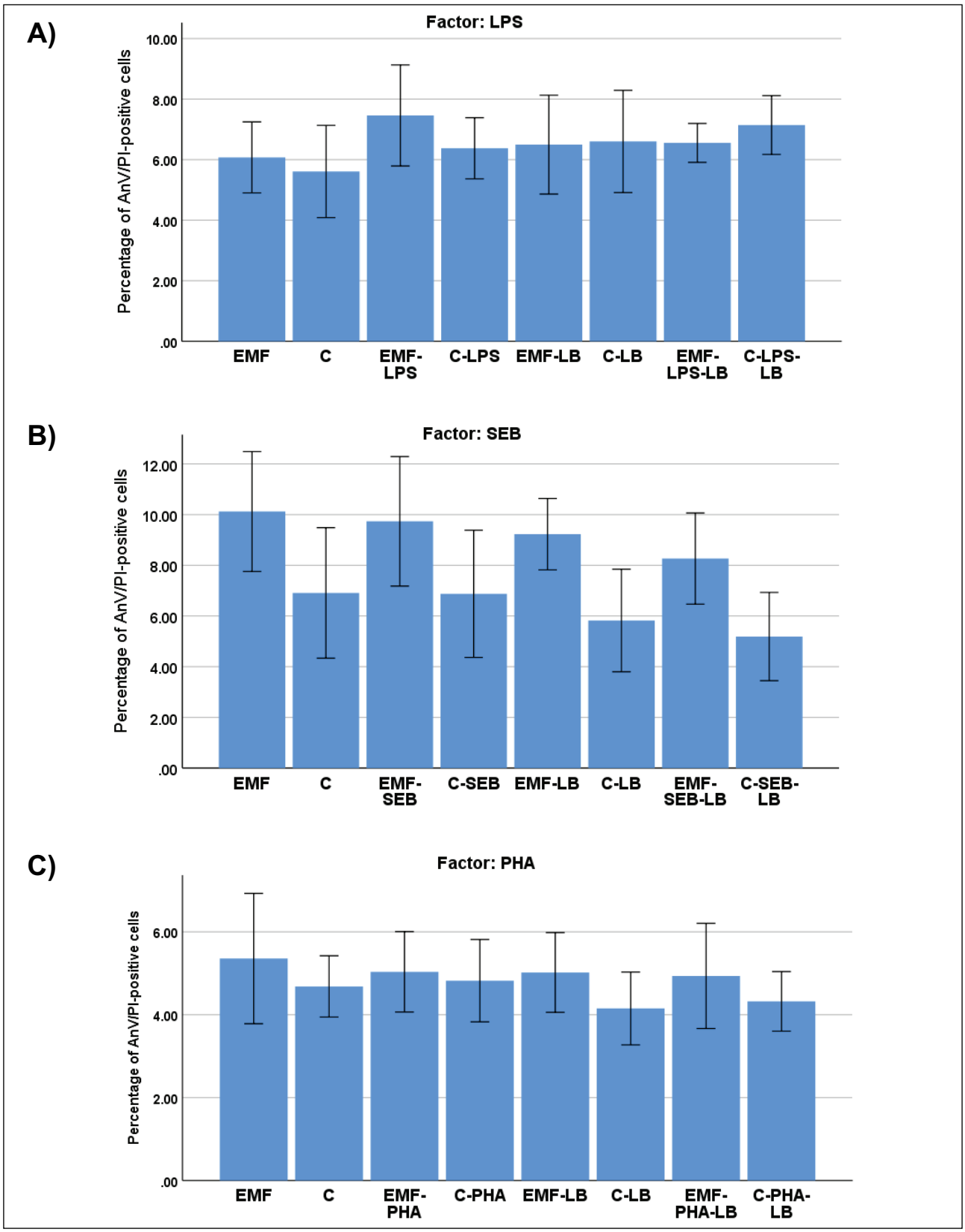

Fig. 6. The percentage of MM6 late apoptotic and necrotic cells exposed to a low-frequency electromagnetic field with or without infectious/proliferatory agents, and/or latex beads: A): LPS, lipopolysaccharide; B): SEB, Staphylococcal enterotoxin B; C): PHA, phytohaemagglutinin. Results are presented as the mean ±SD. There were no statistically significant differences between compared groups (*Table 1*); P>0.05 (not indicated in the graph).

Abbreviations: C, Control; C-LB, cells phagocytosing latex beads; C-LPS/SEB/PHA, LPS/SEB/PHA pretreated cells; C-LPS/SEB/PHA-LB, LPS/SEB/PHA pretreated cells phagocytosing latex beads; EMF, low-frequency electromagnetic field; EMF-LB, EMF exposed cells phagocytosing latex beads; EMF-LPS/SEB/PHA, EMF and LPS/SEB/PHA treated cells; EMF-LPS/SEB/PHA-LB, EMF and LPS/SEB/PHA treated cells phagocytosing latex beads.

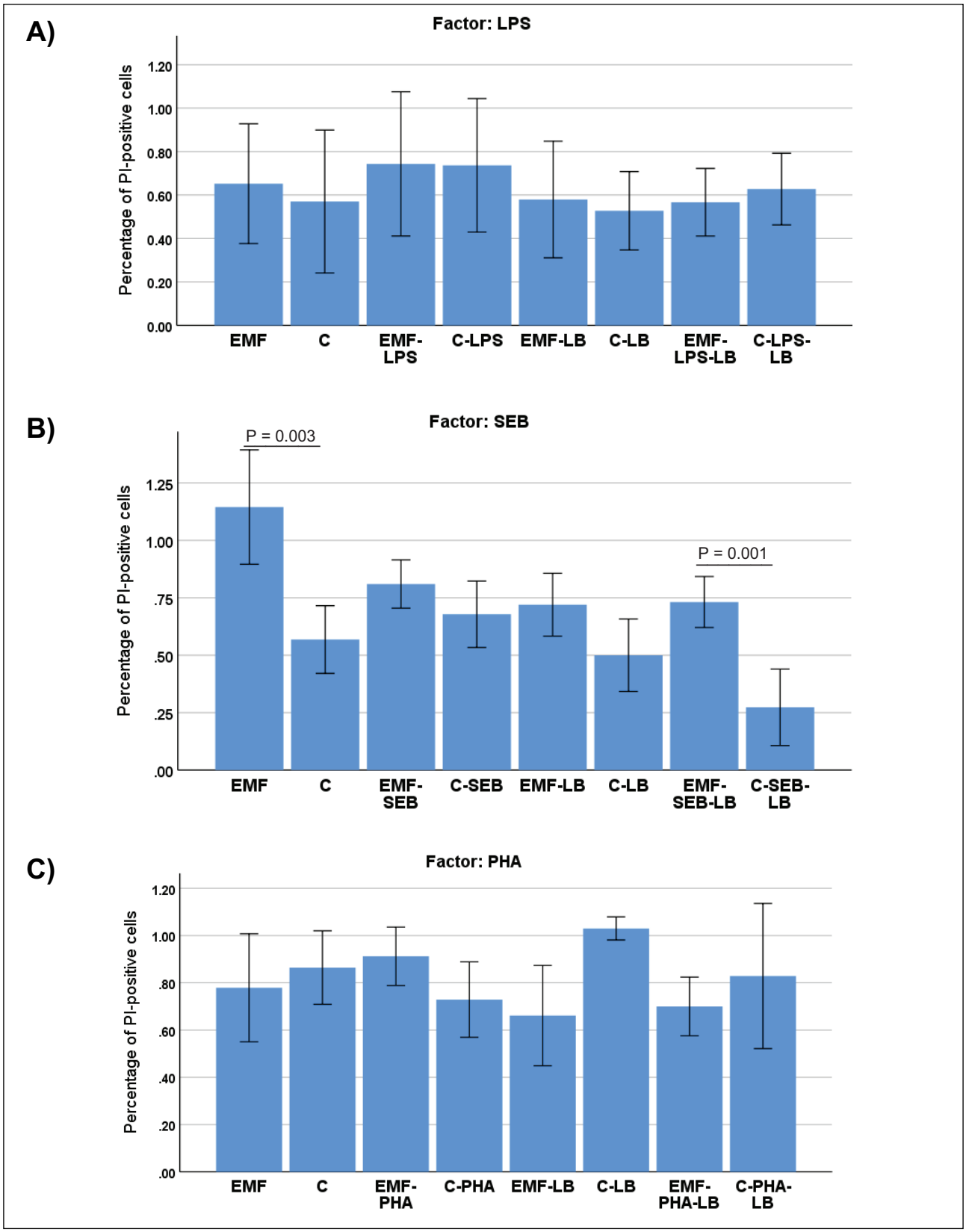

Fig. 7. The percentage of MM6 necrotic cells (PI-positive) exposed to the low-frequency electromagnetic field with or without infectious/proliferatory agents, and/or latex beads: A): LPS, lipopolysaccharide; B): SEB, Staphylococcal enterotoxin B; C): PHA, phytohaemagglutinin. Results are presented as the mean ±SD. Statistically significant differences between compared groups (*Table 1*) were presented in the graph ($P<0.001$ and $P<0.003$), other data $P>0.05$.

Abbreviations: C, Control; C-LB, cells phagocytosing latex beads; C-LPS/SEB/PHA, LPS/SEB/PHA pretreated cells; C-LPS/SEB/PHA-LB, LPS/SEB/PHA pretreated cells phagocytosing latex beads. EMF, low-frequency electromagnetic field; EMF-LB, EMF exposed cells phagocytosing latex beads; EMF-LPS/SEB/PHA, EMF and LPS/SEB/PHA treated cells; EMF-LPS/SEB/PHA-LB, EMF and LPS/SEB/PHA treated cells phagocytosing latex beads.

Similarly, when cells were pre-stimulated with SEB, virtually no changes in early apoptosis were observed in all experimental groups (*Fig. 5B*). In the presence of the proliferatory agent (PHA) and EMF action, a slight increase in AnV-positive cells was noticed, but this was not statistically significant (*Fig. 5C*).

2. Late apoptosis and necrosis

Analysis of late apoptosis in MM6 cells (AnV-positive and PI-positive cells) revealed nearly no changes in the case of LPS pretreatment and LBs phagocytosis, regarding the related control sample (*Fig. 6A*). Additionally, a slight increase in the percentage of late apoptotic cells was observed when cells were exposed to EMF and LPS-treated (without latex beads) compared to the LPS control. In the case of SEB application, all cells exposed to EMF showed a slightly increased population of late apoptotic cells in comparison to the respective unexposed ones (*Fig. 6B*). Finally, for PHA pre-stimulated cells exposed to EMF, a small enlargement of the late apoptosis range was observed upon the latex nanoparticles phagocytosis process (*Fig. 6C*). Nevertheless, none of the indicated differences were statistically significant.

3. Necrosis

The third assessed cell viability parameter was necrosis, evaluated as a percentage of PI-positive cells. The obtained results revealed that EMF exposure did not influence necrosis in LPS pretreated cells and phagocytosing latex beads, no effect was seen also in cells without phagocytosis of LBs (*Fig. 7A*). In the experiment with SEB pre-stimulation (*Fig. 7B*), statistically significant differences were noticed between cells phagocytosing LBs and exposed to EMF concerning the control (not exposed to EMF), as well as for respective samples, not phagocytosing LBs. Finally, PHA pre-stimulated MM6 cells upon EMF exposure revealed a slight increase in necrosis, while the phagocytosing LBs cells also exhibited a diminished percentage of necrotic cells in comparison to the respective control; nevertheless, both changes were statistically insignificant (*Fig. 7C*).

DISCUSSION

Our work aimed to determine if exposure to an electromagnetic field during phagocytosis of latex beads by pre-activated MM6 cells influences the efficacy of phagocytosis, ROS production in the process, and viability of the phagocytosing cells. The obtained results show that the applied electromagnetic field does not result in statistically significant differences between exposed and unexposed cells for any of the viability parameters, except for SEB used as a pre-activation agent. Many studies show both, similar or opposite effects. For instance, in the study (25), the authors did not observe significant differences in the viability of human dermal fibroblast cells between samples exposed to EMF (5 Hz, 0.25, 0.5, and 0.8 mT; 50 Hz, 0.8 mT) and control ones, evaluated with the trypan blue method. Similarly, in another study (26), no significant changes in mouse spermatocyte-derived GC-2 cell viability, measured by a cell counting kit-8 (CCK-8), between cells stimulated with EMF (50 Hz, 3 mT) and sham ones were detected. No changes in human SH-SY5Y neuroblastoma cell viability were noticed either for an applied 50 Hz EMF with a flux density of 1 mT (27). The lack of effect concerning cell viability, evaluated with the use of the MTT, was observed for a low-frequency EMF (50 Hz, 200 G) acting on human A2780 ovarian cancer cells as well (28). Some other authors have evaluated the influence of EMF (50 Hz, 0.1 mT–1.0 mT) on C2C12 myoblast culture cell viability by the trypan blue exclusion test, and found no significant bio-effect resulting from EMF action (29). Similar findings were discussed for human adipose tissue-derived mesenchymal stem cells (30). However, another research group did not observe changes in the cell viability of cultured neurons in a single exposure to EMF (50 Hz, 2 mT), while repeated electromagnetic stimulation significantly decreased these cell viability (31). Also, no changes in the level of viable cells were observed for mouse embryonic fibroblasts exposed to EMF (50 Hz, 2.0 mT) (32). Nevertheless, in the study (33) it was shown that EMF (2–20 Hz; 0.1–20 mT) alone did not cause any remarkable changes in MCF7 cancer cell viability measured with the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method despite the difference in the chosen frequency and intensity of the applied electromagnetic field, but in the presence of magnetic nanoparticles (MNPs) significant descending trends in MCF7 cell viability were observed in an EMF frequency- and intensity-dependent manner. On the other hand, some studies have concluded that EMFs might affect intracellular homeostasis and signal transduction pathways. For instance, it was found that a low-frequency electromagnetic field (7 Hz, 30 mT_{rms}) applied three times for 4 hours with 24-hour intervals between stimulations on the human bladder microvascular endothelial cell line (HMVEC-Bd) and LPS-activated MM6 coculture evoked a decrease of all viability parameters (early apoptosis, late apoptosis, and necrosis) (19). An analogous effect was observed for hippocampal neurons exposed to EMF (50 Hz, 8 mT) with the use of an MTT reduction test (34), as well as for B16F10 cancer cells stimulated with 7.83 Hz EMF (natural Schumann resonance frequency) (35). There are also studies presenting opposite effects triggered by EMF. For instance, the increased viability of dental pulp stem cells (DPSCs) in an MTT assay under EMF (50 Hz, 1 mT) exposure was detected (36). In another study where cancer cells (U87_MG, 143B) and noncancerous cells (BJ, HEK) were exposed to various EMFs with a frequency of 2, 20, 30, 40, 50, and 60 Hz and magnetic induction in the range of (2–6) mT, significant differences in cell viability dependent on cell types, applied frequency, and magnetic flux density were observed (37). More precisely, in the case of 143B cell exposure to EMF (2 Hz, 2–4 mT), an increase of viable cells was noticed, whilst for 20 Hz no changes were observed except for stimulation performed in EMF with 6 mT magnetic flux density. Moreover, for the stimulation parameters (30–60 Hz and 2–6 mT), a reduction in 143B cell viability was evoked. Beyond the frequency and intensity of EMF, the time of exposure is also critical for various biological parameters, e.g. cell viability, expression of proteins, gene transcription, cytotoxicity, production of ROS, etc. For example, it has been stated that exposure of leiomyosarcoma (LMS) cells to 10 Hz–1 MHz EMF for 45 min over 2 days did not exert a cytotoxic effect; however, extended to 120 min or 300 min time of exposure, this resulted in a significant inhibition of LMS viability (38). All of these data indicate that electromagnetic stimulation might modify/influence many intracellular processes in various cell types depending on the applied parameters of the EMF itself. In the present study, exposure to the used low-frequency EMF did not induce significant changes in the production of free radicals in any of the experimental arrangements. This is in line with the results obtained by other researchers (39). Additionally, in the study (40), exposure to 50 Hz, 1 mT EMF did not change ROS in SH-SY5Y human neuroblastoma cells. However, it was also observed that EMF (50 Hz, 30 μT) may increase ROS in rat C6 glioma cells (41) and that 16-minutes of stimulation with EMF (50 Hz, 1 mT) combined with cisplatin results in increased ROS level and antioxidant enzyme activity in AT478 murine squamous carcinoma cells loaded with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) (42). A similar effect was obtained in human lymphocytes exposed to EMF (60 Hz, 0.8 mT) using 2',7'-dichlorofluorescein diacetate (DCF-

DA) (43), and also in a study where changes of ROS level due to 1-hour exposure to (50 Hz, 0.25 G–2 G) EMF of human keratinocytes was measured applying an oxidation-sensitive dihydrorhodamine (DHR) probe (44). In contrast, antioxidant capacity and intracellular ROS level were reduced due to extremely low-frequency electromagnetic field (75 Hz, 1 mT) action on RAW264.7 cells (45). In turn, in another study, the 2',7'-dichlorofluorescein diacetate (DCFH-DA) and flow cytometry were used to determine the effect of pulsed EMF (180–195 Hz; 10, 40, and 60 μT) on ROS production in human neutrophils in peripheral blood in an *in vitro* study. It was shown that the applied EMF slightly inhibited the activity of unstimulated cells, as opposed to neutrophils treated with PMA and stimulated with EMF (46). Some other data focused on evaluation of ROS with a DCFDA cellular ROS detection assay has revealed that pulsed EMF (pulse duration of 1.3 ms and frequency of 75 Hz, yielding a 0.1 duty cycle) reduces hypoxia-stimulated ROS level in two neuronal cell models, human neuroblastoma-derived SH-SY5Y cells and rat pheochromocytoma PC12 cells and in microglial N9 cells. It was demonstrated that the applied EMF significantly lowered hypoxia-induced ROS production in all the model cell types, after 24 or 48 hours of exposure (47). In the study of Kozlowska *et al.* (48), the applied low-frequency electromagnetic field (50 Hz, 2 h) has modified the transcriptomic profile of the endometrium isolated from pigs during peri-implantation period. The authors have presented the EMF-related changes in the expression of 1561 transcriptionally active regions (TARs) of genes encoding proteins, which play an important role in crucial biological processes like proliferation and metabolism in endometrial tissue (48). In the group of 461 evaluated (differentially expressed genes) DEGs, 156 were up-regulated (34%), 305 were down-regulated (66%) and 341 (74%) had known biological functions. They also indicated that NOS3 and SERPINE1, which were up-regulated as a result of EMF treatment, are located in the apelin signaling pathway (KEGG). The apelin/APJ system might be also involved in the cardiovascular system, adipose tissue and obesity related disorders or physiological functions by influencing blood vessels and can be a marker of oxidative stress during pregnancy (48). The consequences of the oxidative stress might be more extensive for various stress factors and biological models. For instance, in the study of Li *et al.* (49) it was shown, that higher level of lipid peroxidation products may enhance the myocardial cardiac injury in rats treated with isoproterenol (ISO) through the decline in the activities of the most important endogenous antioxidant enzyme systems and increased expression of pro-apoptotic signaling proteins (*e.g.* Bax, cytochrome C and caspases 3 and 9). Nevertheless, the pretreatment with 6-Shogaol protected against the intrinistic pathway of programmed cell death evoked by ISO, which indicates on its free radical scavenging properties (49). The 6-Shogaol as a potent pharmacotherapeutic applied in rats with ISO-induced myocardial damage, caused reduction of oxidative stress, inflammation, and programmed cell death (49). It is also known that in the activated macrophages, superoxide radical production is associated with phagocytosis (6). Our results did not show any influence of the used EMF on phagocytosis and ROS production, which is in agreement with another study where the percentage of microparticles phagocytosed by cells exposed to EMF was not significantly different compared to the control group (50). Nevertheless, some results have indicated an increase of phagocytosis activity in the RAW264.7 macrophage cell line when a high-frequency electromagnetic field (2450 GHz, SAR 0.4060 W/kg) was applied (11). These diverse effects might be caused by the different frequency ranges of the applied electromagnetic fields. In the case of our study, the lack of significant changes in particular parameters might be related to the single not repeated EMF exposure (only during phagocytosis test). However, in general, also other stimulation parameters (*e.g.* signal waveform, frequency, flux density) may play a role. Nevertheless, the observed bio-effects may also be cell type-dependent, as it is known that the same physical factor can trigger diverse signal pathways in different cell types. To summarize, investigations of phagocytosis as an innate immune response effector mechanism combined with EMF action would appear to be an entirely new issue raised in the studies. Currently, there are no other published experimental data showing the one of these three agents (LPS, SEB, PHA) and/or low-frequency EMF on phagocytosis process and phagocytosis-related intracellular effects. In this context, even a negative result also indicates whether a particular stress factor may influence/modulate intracellular processes at all. For future research prospects testing the cell response in frequency- and/or amplitude-dependent manner of EMF might complete the description of studied phenomenon. Nevertheless, the presented experiments are the first steps in studying the phagocytosis process and related signal transduction pathways under some stress external conditions like exposure to electromagnetic field. Another important issue needed extensive studies is related to EMF-exerted changes in genes and proteins expression which play a crucial role in phagocytosis as an innate cellular immune response mechanism (51).

The obtained results revealed, that the used electromagnetic field did not influence phagocytosis and ROS production. Nevertheless, EMF exposure in combination with SEB infectious factor in LBs phagocytosing MM6 cells caused a slight increase in the level of necrosis, which may enhance the proinflammatory effect. The lack of significant changes in the effectiveness of the phagocytosis process and level of free radicals might result from the applied EMF parameters, in particular the time of stimulation and signal repetition (single exposure). The observed bio-effects may also be cell type-dependent, as it is known that the same physical factor can trigger diverse signal pathways in different cell types. Therefore, further studies concerning various parameters of EMF itself are needed to understand the potential relation between phagocytosis as an innate immune response effector mechanism and EMFs - as an external stress factor. Additionally, the investigation of proteins and genes expression engaged in signal transduction in the phagocytosis process under EMF exposure is still an open issue. These kinds of studies are embedded in the mainstream interests of current immunology, biophysics, and environmental sciences.

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Author's address: Piotr Piszczek, Department of Pathophysiology, Jagiellonian University Medical College, Czysta street 18, 31-121 Cracow, Poland.
E-mail: piotr.piszczek@doctoral.uj.edu.pl