



Immunomodulatory role of non-ionizing electromagnetic radiation in human leukemia monocytic cell line[☆]

Himanshi Yadav^a, Rajeev Singh^{a,b,*}

^a Department of Environmental Studies, Satyawati College, University of Delhi, Delhi, 110052, India

^b Department of Environmental Science, Jamia Millia Islamia (A Central University), New Delhi, 110025, India

ARTICLE INFO

Keywords:

Non-ionizing electromagnetic radiation
Radiofrequency radiation
Innate immune system
Phagocytosis
Superoxide
Nitrite
Free radical
Monocytes
Pro-inflammatory cytokine

ABSTRACT

In daily life, people are usually exposed to radiofrequency radiations (RFR). The effects of RFR on human physiology have been a major source of controversy since the WHO declared that these radiations are a type of environmental energy that interacts with the physiological functioning of the human body. The immune system provides internal protection and promotes long-term health and survival. However, the relevant research on the innate immune system and radiofrequency radiation is scant. In this connection, we hypothesized that innate immune responses would be influenced by exposure to non-ionizing electromagnetic radiation from mobile phones in a cell-specific and time-dependent manner. To test this hypothesis, human leukemia monocytic cell lines were exposed to 2318 MHz (MHz) RFR emitted by mobile phones at a power density of 0.224 W/m² in a controlled manner for various time durations (15, 30, 45, 60, 90, and 120 min). Systematic studies on cell viability, nitric oxide (NO), superoxide (SO), pro-inflammatory cytokine production, and phagocytic assays were performed after the irradiation. The duration of exposure seems to have a substantial influence on the RFR-induced effects. It was noticed that after 30 min of exposure, the RFR dramatically enhanced the pro-inflammatory cytokine IL-1 α level as well as reactive species such as NO and SO generation as compared to the control. In contrast, the RFR dramatically reduced the phagocytic activity of monocytes during 60 min of treatment when compared to the control. Interestingly, the irradiated cells restored their normal functioning until the final 120-min of exposure. Furthermore, mobile phone exposure had no influence on cell viability or TNF- α level. The results showed that RFR exhibits a time-dependent immune-modulatory role in the human leukemia monocytic cell line. Nevertheless, more research is needed to further determine the long-term effects and precise mechanism of action of RFR.

1. Introduction

The widespread use of wireless communication systems and the rapid growth of mobile phone users have exposed the public to a high level of risk from non-ionizing electromagnetic fields (Yadav et al., 2022). As a result, there is increasing awareness about the potential detrimental impacts of electromagnetic radiation (EMR) exposure on human health (Saliev et al., 2019). The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) are involved in investigating the link between cancer and mobile phone

usage. As a result, radiofrequency electromagnetic radiation has been categorized by the IARC as possible carcinogenic to humans (Group 2B). In addition, during the last few decades, the global mobile telephony business has grown at an exponential rate. It has been noticed that mobile data usage has steadily climbed year after year, and was expected to reach 53.4 trillion Megabytes (MBs) by 2021. Furthermore, data use has increased by 11.2 trillion MB since last year. Herein, wireless data traffic is expected to nearly quadruple to 240 trillion MB per year by 2027, according to Ericsson (CTIA, 2022).

Therefore, exposure to RFR by humans appears to be a major

Abbreviations: RFR, (Radiofrequency radiation); EMR, (Electromagnetic radiation); SAR, (Specific absorption rate); Min, (minutes); ELF, (electromagnetic low frequency); NO, (Nitric oxide); SO, (Superoxide); FACS, (Fluorescence-activated cell sorting); PMA, (Phorbol 12-myristate 13-acetate); NBT, (Nitro Blue Tetrazolium); MTT, [3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide]; MHz, (Megahertz); MBs, (Megabytes); LPS, (Lipopolysaccharide); Pg, (Picogram).

[☆] This paper has been recommended for acceptance by Wen Chen.

* Corresponding author. Department of Environmental Science, Jamia Millia Islamia (A Central University), New Delhi-110025, India.

E-mail address: 10rsingh@gmail.com (R. Singh).

<https://doi.org/10.1016/j.envpol.2023.121843>

Received 1 March 2023; Received in revised form 15 May 2023; Accepted 16 May 2023

Available online 17 May 2023

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epidemiological issue on a global scale, and it is critical to thoroughly understand how such types of radiation work and cause the potential health impacts. In this series, several research studies on the impact of varying radio-frequency ranges on human health, including fertility and cancer, have been reported in recent years. Nevertheless, the majority of studies focused on male reproductive health with RFR exposures of 850–2400 MHz and a specific absorption rate (SAR) range of 0.15 W per kilogram (W/kg) to 5 W/kg have shown significant variations in sperm parameters. While several studies with similar endpoints reported negative findings, turning it into a subject of intense debate. Several genotoxic studies on RFR on various cell lines are also controversial but carcinogenicity studies mostly showed negative results with respect to RFR (Kocaman et al., 2018; Yadav et al., 2021; Gupta et al., 2020; Jangid et al., 2022; Negi and Singh, 2021). However, the impact of mobile phone radiation on immunological health has received the least attention. In a study, Pei et al. (2019) reported that mobile phone radiation at 1800 MHz and 1900 MHz reduces the phagocytosis activity and increases apoptosis in neutrophils after 6 weeks of exposure, while no effect on neutrophil chemotaxis could be observed. A study found that EMR from mobile phones weakens rats' immune system. The exposure of 1 h/day for 30 days with a 900 MHz RFR and SAR of 0.9 W/kg, significantly reduced the immunoglobulin levels and blood cell count, except neutrophil and monocytes, which were found to increase. Further, the above alterations were found to be more pronounced in the group exposed to 2 h/day for 30 days (Ola and Mona., 2017). Another study reported altered iNOS levels when nitrite was added to stimulated RAW 264.7 macrophages following exposure to 1ns ultra-wideband (UWB) pulses repeated at 600 Hz with a SAR of 0.106 W/kg (Seaman et al., 2002). El-Gohary & Said (2017), reported that RFR of 900 MHz emitted from a mobile phone at a SAR of 0.9 W/kg could modulate serum parameters of albino wistar rat after 30 days of exposure for a duration of 1 h/day. The exposure decreased the immunoglobulin levels (IgM, IgG, IgA, and IgE); and the total count of lymphocyte, leukocyte, basophil and eosinophil, at a significant level as compared to the control. These alterations were recorded to be more pronounced in the group that was exposed for 2 h/day. These authors concluded that the exposure to mobile phone radiation compromises the immune system of rats (El-Gohary and Said, 2017). In a study, after stimulation with PMA and LPS, monocytes differentiated into macrophage, and showed an exponential increase in NFκB amount, but NFκB expression was decreased after microwave exposure of 2450 MHz at 900 W. Also, cytokine expression was found to be altered by microwave radiation. Thus, inhibitory effects of these radiations were found on monocytes stimulated with PMA and LPS (Li et al., 2012). In spite of such investigations, no consensus has been established with respect to RFR impact on immune health because the available literature is limited and the experiments undertaken either revealed inconsistencies in the results or were conducted in different cell types or with extra-low electromagnetic frequency (ELF) (Gulati et al., 2020; Yadav et al., 2022).

Because immune cells regulate an individual's health on a systemic level a proper understanding of how mobile phone radiation interacts with these cells is crucial. Furthermore, the immune system is an extremely sensitive model for studying such interactions since it responds mostly to every internal and external stimuli that is foreign to our system. The primary role of the immune system is to protect the host body by developing a defence mechanism against foreign invaders and producing antibodies for the elimination of pathogens and aberrant cells within the body (Møller et al., 2020). The first line of defence is provided by innate immune cells like monocytes or macrophages, which react by producing inflammatory mediators (Rosado et al., 2018). Hence, innate immune functions can be closely investigated in monocytes. In this context, THP-1 cells have been employed as an *in vitro* cell model of human monocytes and macrophages in research studies reporting on inflammatory disorders (Chanput et al., 2010, 2014; Sharif et al., 2007; Hjort et al., 2003; Ueki et al., 2002).

In view of the above mentioned issues, in this study we used THP-1

cells to investigate the influence of mobile phone radiation on innate immune responses for various durations of time. As of now, to the authors' knowledge, limited research has investigated the time-dependent effect of mobile phone radiation on specific immune cells, namely monocytes. Therefore, we conducted experiments using mobile phone exposure in controlled conditions in monocytes to evaluate the impact of cell-phone usage for different time durations (15, 30, 45, 60, 90, and 120 min) that are typically utilized by people. The minimum and maximum exposure times that we used were 15 min and 120 min, respectively. Hence, in the present study, the role of mobile phone radiation on THP-1 cell functions has been examined by quantification of superoxide production, nitrite production, phagocytic activity, cell viability and pro-inflammatory cytokine levels.

2. Materials and methods

2.1. Cells culture

The experiments were conducted with the THP-1 monocytic cell line, acquired from the American Type Culture Collection (ATCC®, USA), which is routinely cultured in RPMI-1640 medium (Himedia), supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Gibco™) and 1% Penicillin and Streptomycin (Sigma). The cells were maintained at 37 °C in humidified air containing 5% CO₂. Routinely, THP-1 cells were cultured in T-75 flasks in a 20 mL complete cell culture medium. They were sub-cultured every two to three days (twice a week) at a concentration of 8×10^5 to 1×10^6 cells/mL when cells were around 80% confluent. This concentration was achieved by seeding approximately 1×10^6 cells per flask. Pre-warm complete cell culture medium was added at 5% CO₂ and 37 °C, and cells were counted with the help of haemocytometer. Next, 8×10^5 cells were taken directly from the suspension, added to 20 mL complete cell culture medium, and transferred to one T75 flask.

2.2. Reagents and culture media

Reagent-grade chemicals were mostly obtained from Sigma and Himedia. Heat-inactivated fetal bovine serum (FBS, Himedia), Rpmi-1640 (Himedia), FITC-labelled Zymosan/Saccharomyces cerevisiae Bio-Particles (Invitrogen). Penicillin, streptomycin, Phorbol 12-myristate 13-acetate (PMA), nitro blue tetrazolium (NBT), MTT [3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide] were procured from Sigma-Aldrich Chemicals (St. Louis, MO). Dimethyl sulphoxide (DMSO), 2-phenoxyethanol, methanol, triton X-100, N-(1-naphthyl) ethylene diamine dihydrochloride (NEDD), sulphanilamide and other routine chemicals were purchased from Sigma/Merck.

2.3. Cell exposure

We used the RF exposure unit based on a 4G mobile phone (a Redmi Note 7). RF exposure and Sham exposure were performed simultaneously at a power density of 0.224 W/m² for varying time durations in different incubators at 37 °C with 5% CO₂. Cells were exposed in 15 mL round-bottom tubes at a concentration of 1×10^6 cells/mL in each tube. As per the manufacturer's claim, the mobile phone has a SAR of 0.962 W/kg (head) and 0.838 W/kg (body) (distance 15 mm). Calibration was accomplished by measuring the physical parameters of the cell-phone with the Narda system 520. The SAR was calculated using the theoretical computation of Allen et al. (1975) or by using the formula $SAR = \sigma[E]^2/\rho$ (where σ is the conductivity, ρ is the density and E is the electric field). As a result, the maximum and minimum SAR values were calculated to be 0.5 W/kg and 0.06 W/kg, respectively. Herein, during the exposure, no temperature change was observed. Further, taking all probable uncertainties into consideration, the SAR value at all locations within the irradiated samples was always far below thermal effects. Since the background conditions or fields in our experiment were

identical for both the sham exposure and the real exposure, the differences that were observed cannot be attributed to these conditions.

2.4. Experimental design

The exponentially growing human leukemia monocytes (THP-1) were treated concurrently as follows:

- Control or Sham exposure: Cell cultures are irradiated through the mobile phone's switch off mode.
- RFR exposure: Cell cultures are irradiated through a mobile phone in talk mode.

All the experiments were carried out at 15, 30, 45, 60, 90, and 120 min of exposure durations. After irradiation, the cell suspension was processed for the different assays.

2.5. In vitro experiments

2.5.1. Nitrite assay

After the irradiation, the cells were incubated with 10 µg/mL LPS for 24 h. After that, cell-free supernatant was collected and processed for the nitrite assay. The nitrite assay was done by the method of Rai & Singh. (2010a; 2011). Briefly, the cell supernatant was mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% NEDD, and 2.5% H₃PO₄). After 15 min of incubation at room temperature, the absorbance was measured at 540 nm using an ELISA plate reader (Bio-Rad). The concentration of nitrite is calculated by plotting a standard curve and has been expressed as micromolar (µM).

2.5.2. Superoxide assay

The nitro blue tetrazolium assay, which measures the generation of superoxide radicals in monocytes, was used to assess the production of superoxide anion by the method of Rai & Singh., (2009, 2010b). Following the radiation exposure, the THP-1 cells were incubated in a medium containing 1 mg/mL NBT and 1 µg/mL PMA for 2 h. The cells were then collected by centrifugation (200 g, 5 min) and washed with 1X PBS, and fixed with methanol. The reduced intracellular product, formazan, was dissolved by adding 20 µl of 0.1% triton X-100, 120 µl of 2 M KOH, and 140 µl DMSO. The absorbance was measured at 620 nm by an ELISA plate reader (Bio-Rad).

2.5.3. Phagocytosis assay

2.5.3.1. Microscopic analysis. To measure the phagocytic activity, heat-killed yeast cells (*Saccharomyces cerevisiae*) were used as target cells. The heat-killed yeast was made by heating the commercial baker's yeast (3 mg/mL PBS, pH 7.8) at 70–80 °C for 15 min. Cells were incubated with heat-killed yeast for 90 min, washed three times with PBS to avoid the excess yeast, fixed in 100% methanol and incubated for 5 min to dry, and stained with Giemsa for 15 min. Without any predetermined sequence or scheme, approximately 200 phagocytes per slide were examined and counted for each radiation time point with an Olympus microscope at 40x resolution. All the technical details on each slide were covered before counting. Phagocytes that engulf one or more yeast cells are counted as positive cells. The formulae for the percentage phagocytosis and the phagocytic index are as follows: (a) percentage phagocytosis: number of phagocytic cells showing phagocytosis per 100 phagocytic cells observed, (b) phagocytic index - the average number of engulfed yeast per positive cell x percentage phagocytosis.

2.5.3.2. Flow cytometry analysis. THP-1 cells were assessed by flow cytometry, as described earlier by Carneiro et al. (2014). The cells (1 × 10⁶ cells/well/mL) were irradiated with mobile phone radiation at different time points. Phagocytosis analysis in the monocytic cells was

assessed using BD FACSCalibur (BD Biosciences, USA) by FITC-labelled Zymosan *Saccharomyces cerevisiae* BioParticles staining. Monocytes were incubated with FITC-labelled yeast suspensions at a multiplicity of infection (MOI) of 1M:5Y (Monocytes: yeast ratio) for 30 min, at 37 °C and 5% CO₂. After incubation, plates were kept on ice to stop phagocytosis, and cells were rinsed thrice with 1 × PBS to remove unbound yeasts. After washing with 1 × PBS, the THP-1 was finally resuspended in a small volume of ice-cold 1 × PBS (containing 1% FCS and 0.01% sodium azide). Ten thousand events for once were scored for phagocytes using BD FACSCalibur (BD Biosciences, USA) and subsequently analyzed using the Cellquest pro 6.1 software. The results are expressed as a percentage of phagocytes.

An acquisition protocol was defined to measure forward scatter (FSC), side scatter (SSC), green fluorescence, and red fluorescence on a logarithmic scale. Each sample was collected for 30 s at the slowest flow rate to minimize the coincidental appearance of free yeasts and monocytes in the laser beam. An FSC threshold was set to gate out debris. Cells and free yeast cells were discriminated by combined measurements of FSC and SSC. Monocytes were gated in the R1 area. The control with monocytes alone was used to define the R1 gates.

2.5.4. Trypan blue exclusion assay

The viability of monocytes was determined through the trypan blue dye exclusion technique. Next, 10 µl of THP-1 cell suspension (~1 × 10⁵) was kept for 3 min at 37 °C with a 1:1 dilution of 0.4% trypan blue dye (Sigma). Thereafter, 10 µl of dye-stained cell suspension were further added to the hemocytometer and viewed under a microscope. The number of unstained and stained cells showed the number of viable and damaged cells, respectively. After counting the total number of cells, the percentage of viable cells was computed.

2.5.5. MTT assay

The viability effect of the treatments was assessed using the MTT-based assay according to the protocol reported by Mosmann (1983) and Singh & Rai (2008) with a slight modification. In this approach, the cells were exposed at various time points. After radiation exposure at different time durations, cells were washed and incubated in 100 µl of medium containing 0.5 mg/mL MTT for 2 h in a 96-well culture plate. The cells were washed and solubilized by adding 150 µl of DMSO. After 15 min of incubation at room temperature, the absorbance was measured at 570 nm by an ELISA plate reader (Bio-Rad).

2.5.6. Enzyme-linked immunosorbent assay of TNF-α and IL-1α

The cells were irradiated with mobile phone radiation at different time points. For IL-1α, the cells were then stimulated with 10 µg/mL lipopolysaccharide (LPS) for 24 h, and for TNF-α, 50 ng/mL LPS was given for 1 h. The above-mentioned effective dose and duration of LPS treatment were determined by performing pilot experiments and using information from the available literature (Wataha et al., 1996; Długosz et al., 2019; Liu et al., 2018). Monocyte supernatant that was non-irradiated and radiation treated for different time duration (15, 30, 45, 60, 90, and 120 min) was used for IL-1α and TNF-α cytokine evaluation using commercial ELISA kits (Cayman, USA). Herein, the basic principle for the ELISA assay was employed. For this, the quantitative solid phase sandwich enzyme immunoassay technique was used, where a monoclonal antibody specific for either IL-1α or TNF-α was coated onto the microtiter plate provided in the kit. Duplicate readings for the standard and triplicate readings for the control and treated samples were taken and averaged. The average absorbance of standards, controls, and samples was used for the calculation of the cytokine concentration using a standard curve. Results were expressed as a picogram (pg) of IL-1α, TNF-α per mL of supernatant fluid.

2.5.7. Quantification and statistical analysis

The data were obtained from at least three repeated experiments performed in triplicate for each of the parameters studied. The data from

all three repeated experiments were pooled for statistical analysis. Prism 8 (GraphPad, USA) and Microsoft Excel (v15.0) were used for statistical analysis. A one-way ANOVA test was used, and the Dunnett's multiple comparisons test was subsequently applied to evaluate the statistical significance. The value represents the mean $\pm$ SEM of three independent experiments ($n = 9$ for each assay); differences were considered statistically significant at a value of $p < 0.05$.

3. Results

3.1. Nitric oxide production

Nitric oxide levels in THP-1 cells were assessed after exposure to RFR for various time durations using the Griess reagent, and results are shown in Fig. 1. The statistical analysis through ANOVA showed that NO levels are dependent on the duration of exposure. Further, the groups that differed significantly from the rest were separated using a multiple-comparison approach. After exposure, according to the combined data of all replicates, the NO level in THP-1 cells begins to rise at 15 min ($p > 0.05$), continues to rise at 30 min ($p < 0.0001$), then decreases at 45 min ($p > 0.05$), rises again at 60 and 90 min ($p < 0.01$), eventually reaches control levels at 120 min ($p > 0.05$). It was noticed that among various exposure durations, 30 min of exposure appeared to be more effective than 15, 45, 60, 90, and 120 min of exposure. The NO levels in mobile phone exposure groups were found to be highly significant at 30, 60, and 90 min ($p < 0.0001$, $p < 0.01$, $p < 0.01$ respectively) compared to the control. However, there was no significant difference in the groups that were exposed to the RFR for 15, 45, and 120 min compared to the control.

3.2. Superoxide production

After being treated with RFR for varied intervals of time, superoxide levels in THP-1 cells were measured using the NBT test (Fig. 2). Statistical analysis through ANOVA showed that SO level is dependent on irradiation time. The groups that differed significantly from the rest were separated using a multiple-comparison approach. After exposure, SO levels in THP-1 cells begin to rise at 15 min ($p < 0.001$), continue to rise at 30 min ($p < 0.0001$) and 45 min ($p < 0.01$), then decline at 60 and 90 min ($p > 0.05$), eventually reaching control levels at 120 min ($p > 0.05$). Among various exposure durations, 30 min of exposure appeared to be more effective than 15, 45, 60, 90, and 120 min of exposure. The SO levels in mobile phone exposure groups were found to be highly significant in 15, 30, and 45 min ($p < 0.001$, $p < 0.0001$, $p < 0.01$, respectively) compared to the control. However, there was no significant difference in the groups that were exposed to the RFR for 60, 90, and 120 min.

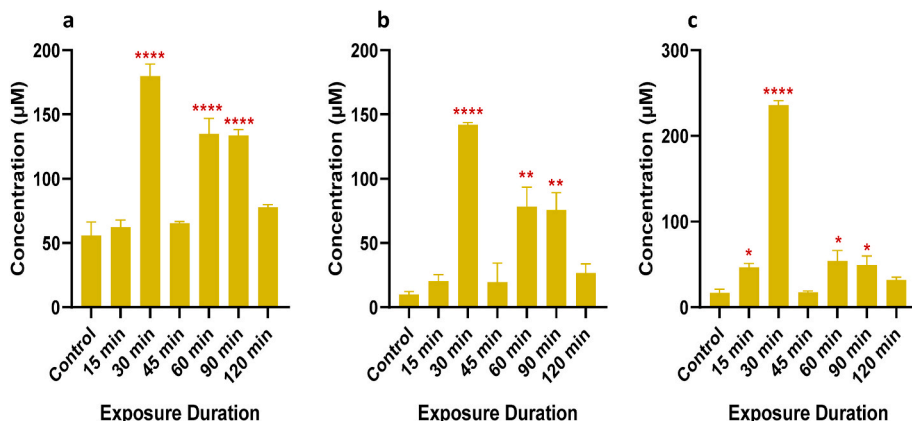


Fig. 1. Effect of Non-ionizing electromagnetic radiation emitted from mobile phone on nitrite release by THP-1 cells. Value represents the mean of one independent experiment ($n = 3$ for each group) and the respective results of three independent experiments were shown as a, b and c. Error bars indicate the standard error of mean (SEM) within each group. Significant differences in relation to the non-treated (control) group are also shown: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

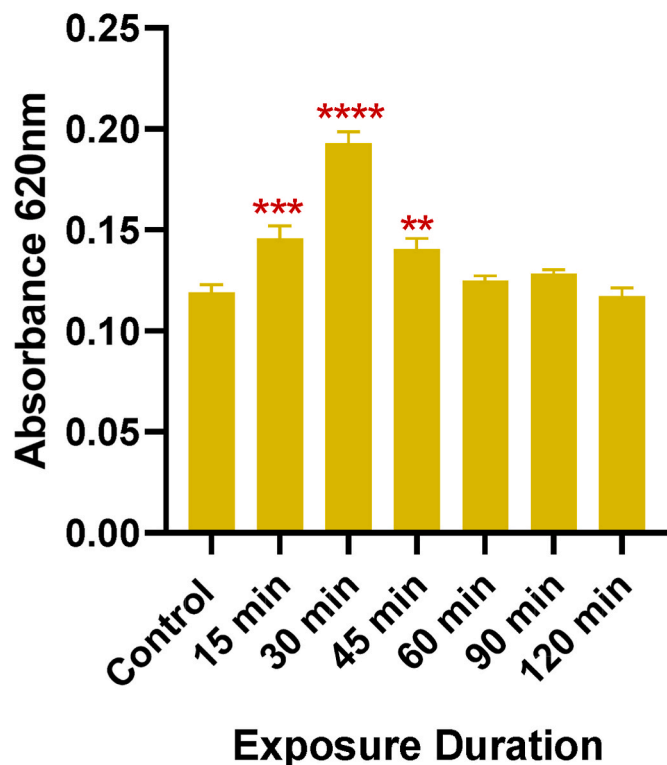


Fig. 2. Reduction of NBT by Non-ionizing electromagnetic radiation-induced superoxide ion radicals in THP-1 cells, monitored by measuring absorbance at 620 nm. Value represents the mean of three independent experiments ($n = 9$ for each group). Error bars indicate the standard error of mean (SEM) within each group. Significant differences in relation to the non-treated group (control) are also shown: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

3.3. Phagocytic activity: microscopic analysis

The phagocytic activity of THP-1 cells stimulated with heat-killed yeast cells after RFR exposure was assessed by microscopy. The percentage phagocytosis and phagocytic index were calculated, and the results are represented in Fig. 3. The groups that differed significantly from the rest were separated using an ANOVA multiple comparison approach. It was noticed that mobile phone radiation inhibited phagocytosis in a time-dependent manner. When subjected to different exposure durations, THP-1 cells' phagocytic index and percentage phagocytosis both revealed a similar trend of decreased phagocytosis, while their levels of significance varied. In comparison to other exposure times, 60 min seems to be more effective than 15, 30, 45, 90, and 120

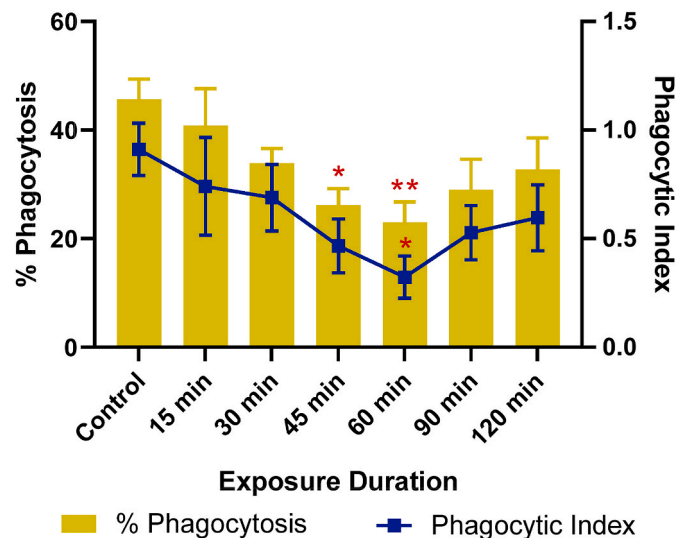


Fig. 3. Effect of Non-ionizing electromagnetic radiation emitted from mobile phone on percentage phagocytosis and phagocytic index by phagocytes of THP-1 cells. Value represents the mean of three independent experiments ($n = 9$ for each group). Error bars indicate the standard error of mean (SEM) within each group. Significant differences in relation to the non-treated group (control) are also shown: * ($p < 0.05$); ** ($p < 0.01$).

min. At 60 min, there was a maximum inhibition of the phagocytic index and % phagocytosis ($p < 0.05$ and $p < 0.01$, respectively) in comparison to the control. The percentage phagocytosis, however, was also substantially altered at 45 min ($p < 0.05$), in contrast to the phagocytic index, which was found to be insignificantly affected at 45 min ($p > 0.05$). At all other time points (15, 30, 90, and 120 min), the phagocytic index and percentage phagocytosis were slightly reduced, but no significant differences compared to the control could be evaluated ($p >$

0.05). Thus, the observation suggests a transient effect of RFR on phagocytosis.

3.4. Phagocytic activity: FACS analysis

To confirm the microscopic observations regarding phagocytosis, the flow cytometry assay was performed using FITC-labelled *Saccharomyces cerevisiae* (Zymosan Bioparticles). The percentage of phagocytes with internalized zymosan bioparticles after different exposure periods and without exposure (control) was analyzed. Monocyte auto-fluorescence was calibrated using a suspension of unstained monocytes before treatment with yeast cells. Following that, FITC-stained monocytes were examined. Fig. 4a shows the dot plot of unstained monocytes, and Fig. 4b shows the average percentage of cells that phagocytosed bioparticles.

A noticeable decrease in percentage phagocytosis after RFR exposure was observed to be 58% at 60 min ($p < 0.05$) and 56% at 90 min ($p < 0.05$) in comparison to non-irradiated cells (64%). Percentage phagocytosis was also found to be slightly decreased, starting at 64.46% (control) to 64% (15 min of exposure), 60% (30 min of exposure), and 62% (45 min of exposure), and decreasing again to 59% (120 min of exposure), but no significant differences could be evaluated ($p > 0.05$).

3.5. Proinflammatory cytokine (IL-1 α , TNF- α) secretion

The secretions of pro-inflammatory cytokines, as immunologic functions, were evaluated by ELISA test in THP-1 cells following RFR exposure to determine if RFR exposure may affect cellular functions. It was interesting to see that the RFR exposure for varying periods dramatically altered the IL-1 α levels (Fig. 5), but it did not alter TNF- α levels in THP-1 cells (Fig. 6). The TNF- α levels were slightly induced but did not show any considerable difference at any of the time points (15, 30, 45, 60, 90, and 120 min) ($p > 0.05$) compared to the control. According to the combined data of all replicates, the IL-1 α levels in THP-1 cells begin to be affected at 15 min of RFR exposure, where the secretion

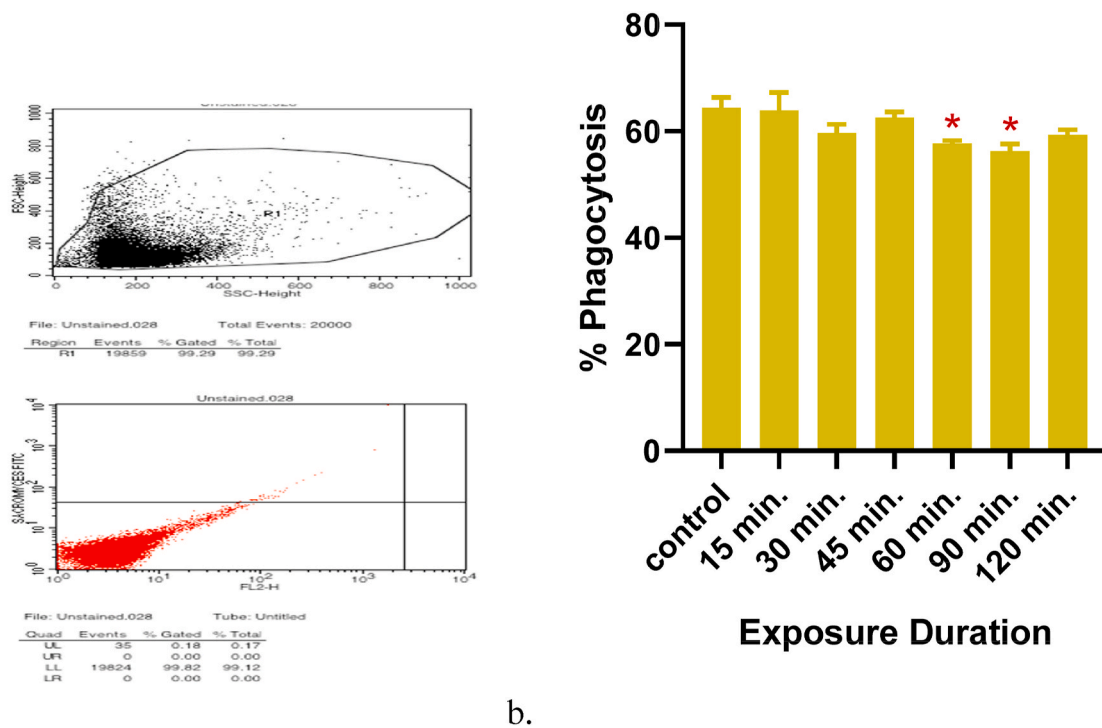


Fig. 4. a: Phagocytosis assay by flow cytometry. Dot plot of unstained monocytes alone that were used to gate live cells. b: Effect of mobile phone radiation on phagocytic activity by flow cytometry. Value represents the mean of three independent experiments ($n = 9$ for each group). Error bars indicate the standard error of mean (SEM) within each group. Significant differences in relation to the non-treated group (control) are also shown: * ($p < 0.05$).

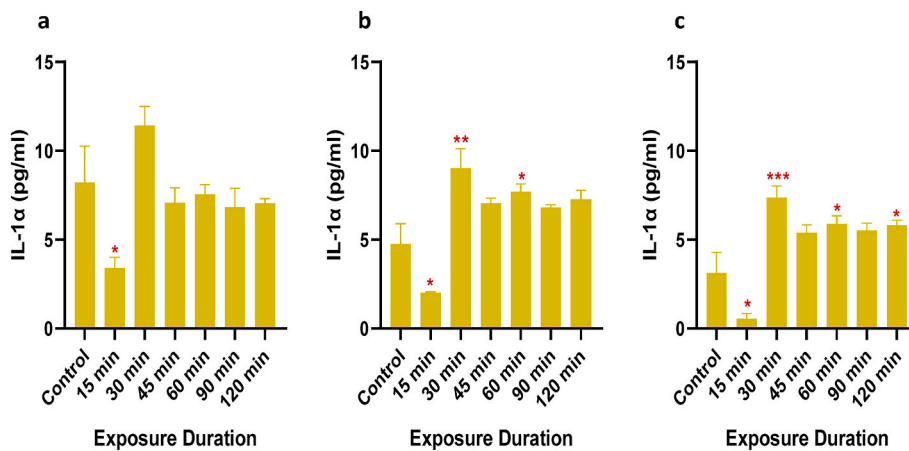


Fig. 5. Effect of Non-ionizing electromagnetic radiation emitted from mobile phone on IL-1 α production of THP-1 cells. Value represents the mean of one independent experiment ($n = 3$ for each group) and the respective results of three independent experiments were shown as a, b and c. Error bars indicate the standard error of mean (SEM) within each group. Significant differences in relation to the non-treated group (control) are also shown: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

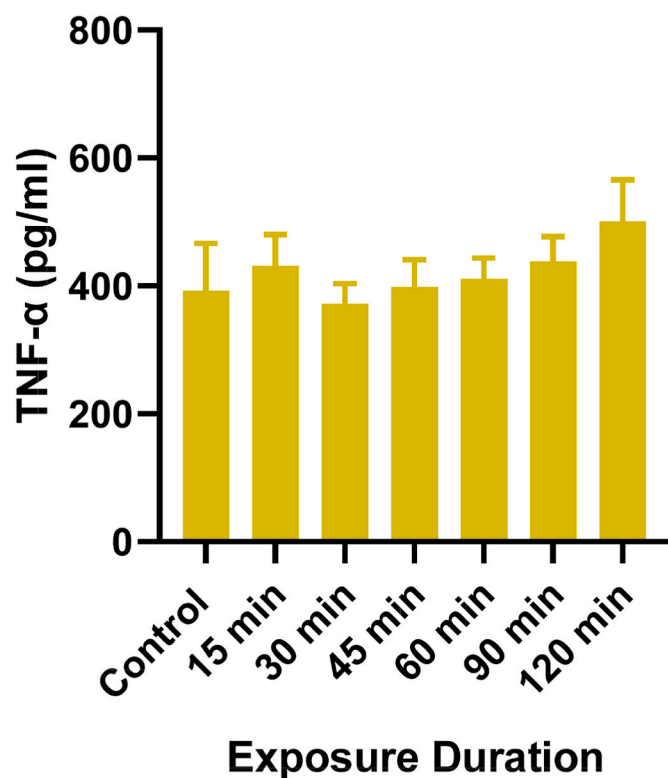


Fig. 6. Effect of Non-ionizing electromagnetic radiation emitted from mobile phone on TNF- α production of THP-1 cells. Value represents the mean of three independent experiments ($n = 9$ for each group). Error bars indicate the standard error of mean (SEM) within each group.

is substantially inhibited ($p < 0.01$) compared to the control, then the cells show a surge in IL-1 α levels at 30 min of exposure ($p < 0.001$), and then return to normal by 120 min of exposure ($p > 0.05$). It was observed that, in comparison to other exposure times, 15 and 30 min appeared to be more efficient than 45, 60, 90, and 120 min. The maximum secretion was noted at 30 min of exposure ($p < 0.001$), and the minimum was recorded at 15 min of exposure ($p < 0.01$). There was no discernible difference in IL-1 α cytokine level compared to the control at any of the other time points (45, 60, 90, and 120 min) ($p > 0.05$).

3.6. Cell viability study

To evaluate the cytotoxic effect of RFR, we initially used the trypan

blue exclusion assay and then followed the MTT assay. The THP-1 cells were processed immediately after exposure, and the results are represented in Fig. 7. According to the trypan blue assay, the viability of cells is recorded to be approximately 97% in all cases. The MTT data showed no significant difference between the irradiated cells' viability at different time durations and non-irradiated (control) cells ($p > 0.05$). Hence, it is concluded that the mobile phone radiation at different time duration did not affect the cell viability.

4. Discussion

Until now, research study devoted to *in vitro* experimental evidence related to the time-dependent effect of RFR exposure on monocytes has been limited (Yadav et al., 2022). Hence, research in this area is

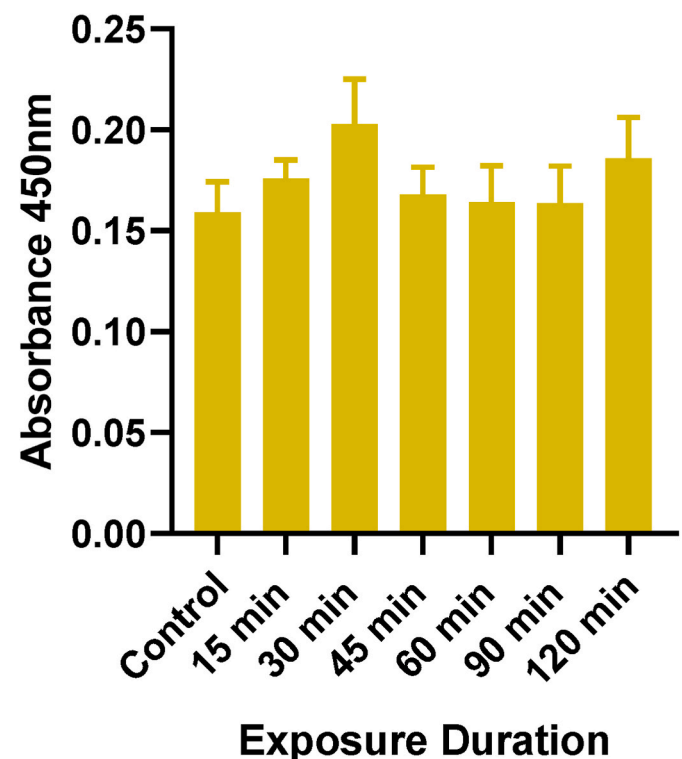


Fig. 7. Cell viability evaluation by MTT assay. Formazan absorbance expressed as a measure of cell viability in THP-1 cells after exposure to Non-ionizing electromagnetic radiation emitted from mobile phone. Value represents the mean of three independent experiments ($n = 9$ for each group). Error bars indicate the standard error of mean (SEM) within each group.

becoming more and more essential in light of our rising exposure to cell-phone radiation. The objective of the present study was to examine the non-thermal effects of 2318 MHz RFR from a 4G mobile phone at a power density of 0.224 W/m^2 on monocytes over a range of exposure durations. The duration of exposure to cellular phones is an important factor, but the exact duration differs from individual to individual. Hence, setting the duration for experimental conditions is a complicated matter. In this study, we chose the varying periods of exposure, i.e., 15, 30, 45, 60, 90, and 120 min which are common cell-phone usage durations among individuals. We found that RFR exposure in THP-1 cells affects the immune system in a time-dependent manner. When comparing different exposure times, 30 min seemed to be the most effective because most of the parameters began to change at this time. Also, the mean exposure period for cell-phone use by most individuals is considered to be around 30 min (Khan, 2008). After 30 min of mobile phone exposure, there is a considerable increase in nitrite and superoxide levels, however, after 60 min, there is a decrease in phagocytic activity. Further, with no change in cell viability, the IL-1 α responses of THP-1 cells were also observed to be impacted at 15 and 30 min of exposure.

In the immune response, such as the eradication of pathogens or the modulation of inflammatory pathways, reactive small molecules such as superoxide, and nitric oxide, are necessary to play significant roles as immunotoxicants as well as immunomodulators (Yadav et al., 2022). In our study, the NO levels in mobile phone exposure groups were found to increase at 30, 60, and 90 min. However, the secretion dropped to approximately normal in groups exposed to RFR for 15, 45, and 120 min. Several investigations are in agreement with our data, where nitric oxide (NO) levels were found to increase with short exposure times of 30 min in various irradiated tissues (Yariktas M et al., 2005; Esmekaya et al., 2016; Ozguner et al., 2005, 2006). The *in vivo* studies with similar exposure parameters i.e., exposure to 900 MHz RFR with a power density of 1.04 mW/cm^2 for 30 min/day for 10 days, found that NO levels were significantly higher in the exposed group compared to the control (Yariktas M et al., 2005; Ozguner et al., 2005, 2006). The other study with an exposure frequency of 900 MHz with electric (E) and magnetic field (H) strengths of $E = 14.310 \pm 3.3897 \text{ V/m}$, and $H = 0.0386 \pm 0.012 \text{ A/m}$, respectively, also showed increased NO levels after exposure (Esmekaya et al., 2016). In other studies, NO levels in rat's brain tissue were elevated after exposure to 900 MHz, 915 MHz, and 2450 MHz frequencies for 1 h each day for seven days and one month (Ilhan et al., 2004; Asl et al., 2020). In addition, Dasdag et al. (2008) showed that 900 MHz exposure at a power density of 0.0782 mW/cm^2 for 2 h/day for 7 days for 10 months had no significant effect on serum NO levels in the exposed group as compared to the sham group (Dasdag et al., 2008). From the perspective of the RFR effect at a particular duration, the articles mentioned above support our results.

This indicates that the effect of RFR on NO level is dependent on exposure duration, and the observed variation in effects over different exposure durations may be caused by the cells' inbuilt ability to adapt to the adverse environment (RFR). The RFR therefore raises NO levels at 30 min, and cells attempt to adapt by reducing NO to a level that is close to normal at 45 min. However, the RFR continuously attempts to induce NO rise, which causes its secretion to increase to a significant level at 60 min and 90 min and to reach a similar level to control at 120 min (Fig. 1). In addition, the oscillating nature of calcium signalling might also be the reason for such results (Camello-Almaraz et al., 2006; Bertero; Maack, 2018; Davidson and & Duchon, 2006; Putney, 1998). Thus, the observations suggest a transient effect of RFR on NO production. The ROS increase was also found to be transient in UCB cells after UMTS exposure (Durdik et al., 2019). Similarly, the effect of RFR on SO level in our study was found to be transient. In this way, the RFR elevates SO levels constantly for 15 and 30 min of exposure. Thereafter, as cells seek to adapt, the SO level begins to drop after 45 min, reaching a level that is approximately normal at 60, 90, and 120 min (Fig. 2). Only a few data have been reported regarding the NO and SO levels in immune cells

following exposure to RFR. These investigations show that exposure to RFR increases NO levels (Esmekaya et al., 2011). In contrast, some researchers found no effect on NO and SO levels in immune cells (Hook et al., 2004; Lantow et al., 2006). This might be due to the difference in experimental protocols (source of radiation, duration of exposure, or SAR value). Thus, RFR here could transiently modulate the inflammatory response by altering SO and NO levels.

The pro-inflammatory cytokines are markers of the innate immune response and are released by activated immune cells immediately after any environmental stress (Yadav et al., 2022). In line with this facts our current findings demonstrate a significant increase in IL-1 α secretion in THP-1 cells upon RFR exposure for 30 min. But this secretion was temporary, as the secretion was at a negligible difference with control after 45 min of exposure. The maximum secretion was noted at 30 min of exposure and the minimum at 15 min. There was no discernible difference in IL-1 α cytokine level compared to control at any of the other time points (45, 60, 90, and 120 min) (Fig. 5). Consistent with this, the IL-1 β decreased significantly at 15 min of short ELF exposure in irradiated fibroblast cells (Gómez-Ochoa et al., 2011), and 2 h of RFR exposure does not affect IL-1 secretion (Hirose et al., 2010). RFR initially (at 15 min) appears to prevent THP-1 cells from secreting IL-1 α , but as a defence mechanism against external stimuli, the cells strive to overcome the block, causing the secretion to surge around 30 min before returning to normal levels. This could be a possible explanation for the observed trend in IL-1 α levels. Furthermore, TNF- α levels were found to be unaltered as no remarkable difference was noted in exposed groups at any time points as compared to control (Fig. 6). In line with this finding, other researchers reported no effect on TNF- α and IL-1 α after exposure for 8 h in monocytes by GSM1950 MHz (Tuschl et al., 2006). Several studies on microglial cells also report no effect on TNF- α production after RFR exposure (Thorlin et al., 2006; Hirose et al., 2010; Su et al., 2018). There are studies that have reported increased release of TNF- α after RFR exposure (Lu et al., 2014; Singh et al., 2020). Such contrasting data could be the result of *in vivo* experimentation or a difference in cell types and exposure parameters.

Phagocytic activity is one of the key roles played by monocytes, and altering this characteristic in monocytes would compromise the integrity of the immune response (Chiu and Bharat, 2016). Microscopy and flow cytometry were used to examine the phagocytic activity in the THP-1 cell line, and we have found that exposure to mobile phones decreases it in both instances. The phagocytic activity was reduced maximally at 60 min and increased gradually afterwards reaching to a level similar to control at 120 min of exposure to mobile phone radiation (Figs. 3 and 4).

Studies by other researchers show consistency with our results, indicating RF exposure at 2450 MHz for 30 min reduces phagocytosis in murine macrophages. The effect is temporary because it disappears after the irradiation stops (Mayers and Habeshaw, 1973). Due to the interaction of one or two RF, macrophages showed a decrease in phagocytic activity after 24 or 72 h (Su et al., 2018; López-Furelos et al., 2018). Another study reported that 20 min of exposure to pulsed waves at 2450 MHz and 6 W/kg SAR decreased microglial phagocytosis and increased the secretion of pro-inflammatory cytokines, as well as the generation of NO (He et al., 2014). On the other hand, phagocytic activity was found to increase when macrophage cell lines were exposed to non-ionizing radiation (Sueiro-Benavides et al., 2021; Zafra et al., 1988). However, exposure to 2450 MHz and 2 or 10 W/kg SAR for 4 or 24 h on the HL-60 cell line had minimal to no influence on phagocytosis at 2 W/kg SAR or at 10 W/kg SAR (Koyama S et al., 2015). Such contrasting results could be explained on the basis of varying exposure settings, cell types, and cell's pre-stimulation (Dini, 2010). Thus, the data suggested that mobile phone radiation could decrease cellular phagocytic activity, at least for a shorter duration.

Although we did not look into the signal transduction pathways that are involved in the up-regulation of NO, other studies have shown that reactive oxygen compounds such as superoxide could be produced due

to stress from radiation exposure which may serve as a defence mechanism and can increase NO production. It has been suggested that NO and the stress response are related (Ozguner et al., 2005; Stefano et al., 2001; Xiao et al., 1998). The mechanism by which the RFR interferes with the cell's generation of oxygen has been shown by enhancing NADH oxidase activity (Friedman et al., 2007). In some of the studies, it has been reported that RFR exposure could stimulate voltage-gated calcium channels, causing calcium to influx and triggering the generation of nitric oxide (NO) (Pall, 2018). In addition, this voltage-dependent increase in extracellular calcium channels (Cho et al., 1999), the transmission of the charge to the DNA (Blank and Goodman, 2008), or intracellular calcium deposits (Ross et al., 2016), might be the reason for increased expression of proinflammatory cytokines in macrophages (Brown et al., 2004), which also affects phagocytic activity (Eichwald and Kaiser, 1995). In this way, RFR induces SO, NO, and IL-1 secretion in THP-1 cells, which might subsequently decrease the phagocytic activity of THP-1 cells following RFR exposure. The decrease in phagocytic activity of THP-1 cells may be attributed to the rise in IL-1 levels. Additional information that supports our hypothesis experimentally reveals that IL-1 rise can decrease the phagocytosis function in microglial cells (van der Zee et al., 1995; Koenigsknecht-Talboo and Landreth, 2005; Pan et al., 2011). Therefore, in the present investigation, it is estimated that the elevated levels of IL-1 α at 30 min after RFR exposure may have suppressed the phagocytic responses 60 min after exposure. Nevertheless, to investigate the exact mechanism underlying this process, additional studies are needed. Altogether, the results show that RFR has the potential to interfere with the innate immune system, but that the immune system will adapt to RFR, just as it usually does with other stressors.

5. Conclusion

The results of the present study suggest that exposure to RFR may modify innate immune responses in a time-dependent manner. The 30-min exposure seems to be the most effective among the different exposure durations studied, which is noteworthy as it corresponds to the time duration for which people tend to use their cell-phones the most. After 30 min of mobile phone use, it was noticed that there was a noticeable rise in reactive species like superoxide, nitric oxide, and the proinflammatory cytokine IL-1 α . Additionally, following exposure for 60 min, THP-1 cells' phagocytic response was found to be dramatically reduced. The elevated level of IL-1 α around 30 min might be the reason for the decreased phagocytic response around 60 min of exposure in THP-1 cells.

Furthermore, the adverse effects detected in THP-1 cells at certain durations of mobile exposure were found to be transient and restored to approximately normal levels around 120 min of exposure. Additionally, mobile phone radiation had no impact on TNF- α levels or the viability of THP-1 cells during the given period. Overall, the results obtained in the present study showed that RFR can affect the functions of the human innate immune system, but it also showed that the immune system may be able to adjust to radiofrequency radiation, just as it usually does in response to other stresses. Since very limited literature in this field is accessible for comparison and reference, further research should be done on the other factors involved therein, like signal transduction pathways, to better understand how cell-phone radiation affects monocytes. Further studies are also needed to analyze the long-term effects of mobile phone use and the relationship among NO, SO production, phagocytosis, and pro-inflammatory responses.

Author contribution

H Y performed the experiments and wrote the manuscript. R S wrote, designed, analyzed and supervised the study.

Grant support

This work was financially supported by the Indian Council of Medical Research, (Grant No.-5/10/FR/9/2018-RBMCH), New Delhi, India-110029.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

Authors gratefully acknowledge the financial support from Indian Council of Medical Research. The first author is also thankful to Council of Scientific and Industrial Research (CSIR) for providing fellowship.

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