



Pulsed or continuous electromagnetic field induce p53/p21-mediated apoptotic signaling pathway in mouse spermatogenic cells *in vitro* and thus may affect male fertility



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ABSTRACT

The impact of electromagnetic field (EMF) on the human health and surrounding environment is a common topic investigated over the years. A significant increase in the electromagnetic field concentration arouses public concern about the long-term effects of EMF on living organisms associated with many aspects. In the present study, we investigated the effects of pulsed and continuous electromagnetic field (PEMF/CEMF) on mouse spermatogenic cell lines (GC-1 spg and GC-2 spd) in terms of cellular and biochemical features *in vitro*. We evaluated the effect of EMF on mitochondrial metabolism, morphology, proliferation rate, viability, cell cycle progression, oxidative stress balance and regulatory proteins. Our results strongly suggest that EMF induces oxidative and nitrosative stress-mediated DNA damage, resulting in p53/p21-dependent cell cycle arrest and apoptosis. Therefore, spermatogenic cells due to the lack of antioxidant enzymes undergo oxidative and nitrosative stress-mediated cytotoxic and genotoxic events, which contribute to infertility by reduction in healthy sperm cells pool. In conclusion, electromagnetic field present in surrounding environment impairs male fertility by inducing p53/p21-mediated cell cycle arrest and apoptosis.

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1. Introduction

Humans are constantly exposed to extremely low frequency electromagnetic field (ELF-EMF) due to the urban development and technological progress (Bekhite et al., 2016; Gye and Park 2012). A significant increase in the electromagnetic field concentration arouses public concern about the long-term effects of EMF with higher than natural frequency on living organisms (Dini and Abbro, 2005; Gye and Park, 2012). The danger of the EMF action depends mainly on the exposure time, frequency, intensity of electric current and the distance from the electrical conductor (Gryz et al., 2014; Gye and Park 2012; Lee et al., 2014; Zhao et al., 2015).

The male reproductive system cells such as spermatids and spermatocytes are particularly sensitive to potential DNA damaging factors such as EMF. Most recent reports suggest that even a

short exposure may negatively impact on reproduction and sexual functions disturbing spermatogenesis and contributing to male infertility. Numerous evidence indicate that exposure to EMF may reduce sperm quality in humans or animals, including a decrease in motility and sperm viability, morphometric abnormalities, as well as increased levels of oxidative stress (Lee et al., 2014; Liu et al., 2015a, 2015b; Markkanen et al., 2008). Despite a number of studies, biological effects and mechanisms of apoptosis pathways in spermatogenic cells exposed to EMF are further not clear. It is suggested that genetically abnormal cells are eliminated by spontaneous apoptosis as a defense mechanism during spermatogenesis. However, in order to understand this mechanism fully, signaling pathways involved in this process should be explained (Gye and Park, 2012). Moreover, based on epidemiological studies, it was suggested that exposure to EMF may be carcinogenic to humans, but theoretical considerations suggest that direct DNA damage is unlikely (Markkanen et al., 2008).

The electromagnetic field exposure at home and also at work becomes an important issue in the field of public health in the context of reduced male individual fertility. Despite number of experiments performed, the question of whether biological effects

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and mechanisms of EMF actions are harmful to human health is still unanswered. Due to this fact and the lack of literature data focused on the male fertility reduction as a cause of EMF action, we chose the mouse germ cells for our research: 1) GC-1 spg cell line – cells between spermatogonia type B and primary spermatocytes and 2) GC-2 spd cell line – spermatocytes. Furthermore, GC-1 spg and GC-2 spd cell monocultures represent a perfect spermatogenesis system for understanding a complex cellular processes involving the proliferation and differentiation of male reproductive system producing germ cells. The experimental setup was designed to explore the complex mechanisms involved in multi-pathway response to 2, 50 and 120 Hz frequencies of EMF, which humans are continuously exposed in natural environment. The main aim of the present study was to investigate whether extremely low frequency electromagnetic field may induce stress-related effects on cellular and biochemical features, such as mitochondrial metabolism, morphology, proliferation rate, viability, cell cycle progression, oxidative stress balance and proteins involved in mentioned processes.

2. Materials and methods

2.1. Cell lines and cell culture

The mouse spermatogonia germ cell line, GC-1 spg (ATCC: CRL-2035) and mouse spermatocyte cell line, GC-2 spg (ATCC: CRL-2196) were grown and maintained at 37 °C in DMEM with 4.5 g/l glucose and 1 mM sodium pyruvate (without L-glutamine), supplemented with 10% FBS and antibiotic mix solution (100 U/ml penicillin, 0.1 mg/ml streptomycin, 29.2 mg/ml L-glutamine). Cell lines were kept in humidified atmosphere, at 37 °C in the presence of 5% CO₂. Adherent cells were passaged every 3 days by trypsinization. For all experimental procedures, cells were seeded at constant density of $1.5 \times 10^3/\text{cm}^2$, 24 h before electromagnetic field exposure.

2.2. Electromagnetic field (EMF) exposure system and exposure conditions of cell cultures

The EMF was generated using Magneris unit (Astar) for a low-frequency magnetic field therapy. The device was applied with flat CP-type applicator, consisting of two element (CP-ID1, CP-ID2) equipped with cooling system. The applicator generates the magnetic flux density in the range of 0–10 mT (0–100 Gs) and the maximum nominal induction in center of the CP applicator is 2.5 mT (25 Gs). The EMF system was designed and constructed in Poland (Bielsko-Biala). The absolute electromagnetic radiation was determined using GM04 magnetic field gaussmeter (Hirst Magnetic Instruments Ltd, UK) supplied with a transverse Hall probe as standard. Gaussmeter and probe were calibrated to standard traceable to the National Physical Laboratory (NPL). The irradiation setup parameters were previously described by [Koziorowska et al. \(2017\)](#) ([Koziorowska et al., 2017](#)).

In short, in this study cell culture dishes were placed on geometrical center of magnetic field area at a reference distance of 10 cm from EMF source. The peak value of magnetic induction range from 2.5 mT at the center to 8 mT at the outer edge of the culture flasks or plates. Cells were exposed to different types of electrical waveforms over a wide range of frequencies (in this study 2, 50, 120 Hz). Sinusoidal shape of magnetic field as well as continuous and pulsed (1s/1s) mode of emission were applied. Unless otherwise stated, cells were exposed to EMF for 2 h, left 48 h for post-exposure recovery and then analyzed. The natural environmental static magnetic field from Earth was neglected.

2.3. One-step growth curve

As usually, cells were seeded at a density of $1.5 \times 10^3/\text{cm}^2$ and after 24 h exposed to EMF for 2 h. Then, live cell number was counted every 24 h for up to 72 h with the simple trypan blue exclusion assay.

2.4. Cell metabolic activity assay – MTT

24, 48 and 72 h after EMF exposure, MTT solution was added to cell culture medium at a final concentration of 1 mg/ml. After 4 h incubation at 37 °C, crystals were dissolved in DMSO and absorbance was read at 595 nm and 620 (measurement and reference wavelengths, respectively). The results are presented as %, while readings for non-treated control are considered as 100%.

2.5. Cell viability

Fluo Cell Double Staining Kit was used for simultaneous fluorescent staining of viable and dead cells according to the manufacturer's instructions. Fluorescent microphotographs were taken using InCell Analyzer and results are presented as % of total cell number. A minimum of 1000 cells was counted in each sample.

2.6. Cell cycle analysis

The cell cycle profile was evaluated using DNA Cell cycle plug-in from ImageJ software on photographs taken after nuclei visualization during cellular viability assessment. Results are presented as % of cells in each of G0/G1, S and G2/M phases. A minimum of 1000 cells was counted in each sample.

2.7. Cellular oxidative stress parameters

Detection of superoxide and nitric oxide production was evaluated using DHE and DAF-FM probes, respectively (both final concentrations 5 μM) according to [Mytych et al. \(2015\)](#) ([Mytych et al., 2015](#)) and minimum of 1000 cells was analyzed.

2.8. Western blot analysis

Whole cell protein lysates and Western Blot analyses were done according to [Mytych et al. \(2014\)](#) ([Mytych et al., 2014](#)). Used primary antibodies were: anti-β-actin (#PA1-16889), anti-p21 (#PA5-701151), anti-p53 (#700439), anti-NF-κB (#PA5-37658) (Thermo Scientific), anti-Bcl-2 (#sc-7382) (Santa Cruz), anti-active caspase 3 (#NB100-56113) (Novus Biologicals) and secondary: anti-mouse (#A9044) or anti-rabbit (#A0545) (Sigma).

2.9. Statistical analysis

All experiments were conducted at least in triplicate. The statistical analysis of the results was performed using GraphPad Prism ver. 6.0. All results represent the mean ± SD of at least 3 independent experiments. Differences between control and test samples were assessed with one-way analysis of variance with Dunnett post hoc test. A p-value of <0.05 was considered as statistically significant between groups.

3. Results

3.1. Effect of EMF on cellular morphology and proliferation

At the beginning, the GC-1 spg and GC-2 spd cell lines were exposed to different frequencies of EMF and one-step growth curves were performed to assess the growth kinetics

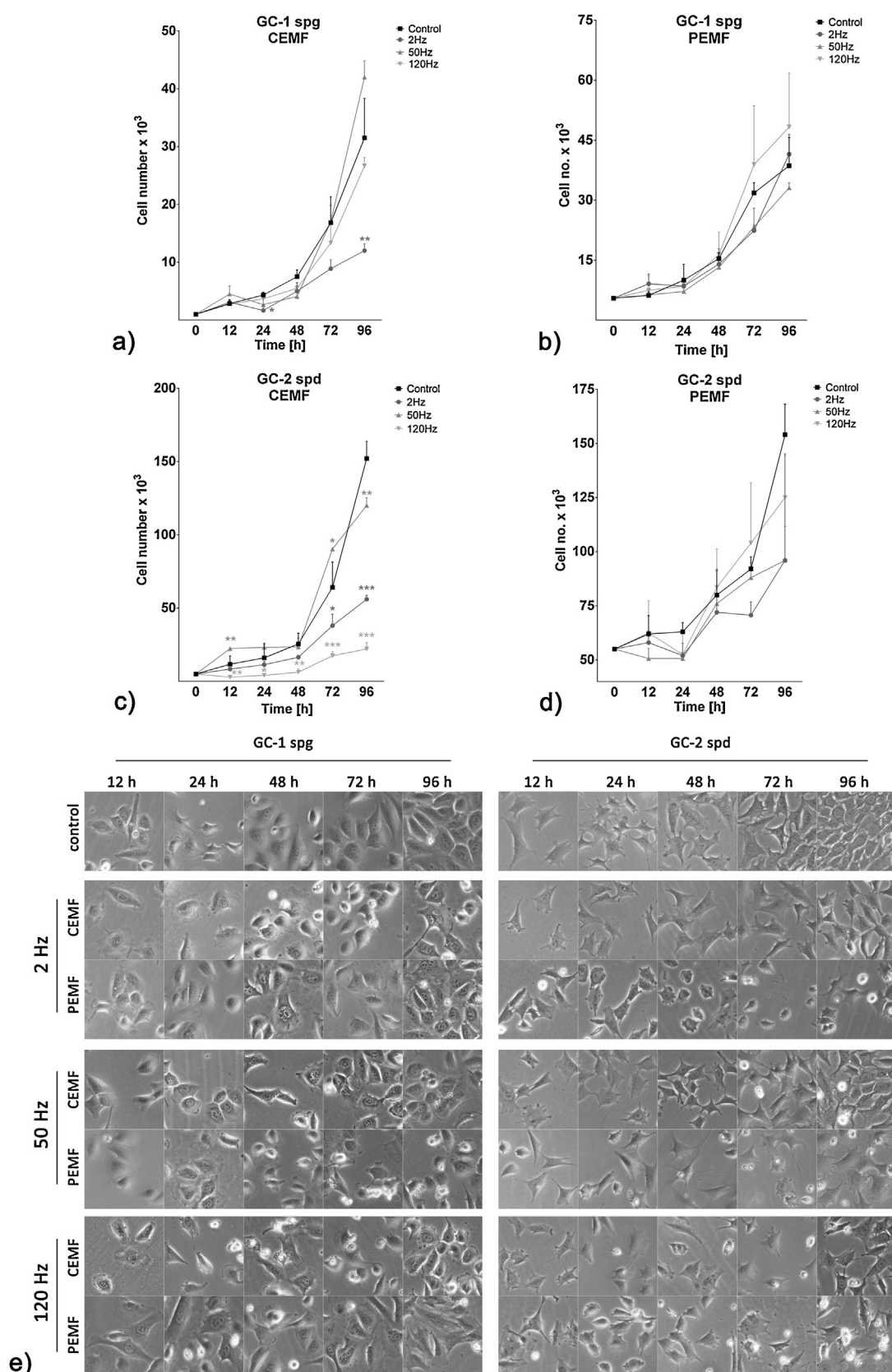


Fig. 1. Visualization of growth kinetics corresponding to each of EMF parameters (a–d) and cellular morphology (e). Cells were exposed for 2 h, left 96 h for post-exposure recovery and then analyzed. The reference distance between cells and EMF source was 10 cm. Bars indicate SD, $n = 3$, ** $p < 0.01$, * $p < 0.05$, no indication – no statistical significance (one-way ANOVA and Dunnett's a post hoc test).

corresponding to each of EMF parameters (Fig. 1a–d). Specifically, GC-1 spg and GC-2 spd count was evaluated at 12, 24, 48, 72 and 96 h following EMF treatment. The obtained results show that the CEMF at 2 Hz frequency decreased cell growth and proliferation of GC-1 spg (reduction to almost 40%) after 96 h. Simultaneously, in the case of GC-2 spd cells treated with CEMF we observed a statistically significant cell growth reduction to approximately 40%, 80%, 15% after 96 h (2 Hz, 50 Hz, 120 Hz, respectively) (Fig. 1a–b). In opposite, no significant differences in growth kinetics were observed after exposure to PEMF in both GC-1 spg and GC-2 spd cell lines (Fig. 1c–d).

Next we evaluated the effect of EMF on GC-1 spg and GC-2 spd in terms of morphological changes. PEMF resulted in elongated and fibroblast-like shapes in GC-1 spg cells, while GC-2 spd cells kept polygonal shape with more regular dimensions, except 2 Hz frequency where cells were characterized by more spherical-like shape, but still grown attached to the substrate. Furthermore, GC-1 spg and GC-2 spd cells exposed to different intensities of CEMF did not show any changes in cellular or nuclear morphology (Fig. 1e). Based on this results and on the fact that morphological changes may result from ROS-mediated modulation of proteins playing a key role in regulation of actin dynamics we were interested in analysis of intracellular redox balance and that we tested in the next part of study.

3.2. EMF-induced oxidative and nitrosative stress imbalance

As expected, 48 h after EMF exposure, we observed statistically significant upregulation in redox balance directly correlated with electromagnetic field parameters in wide range of frequencies in all tested conditions (Fig. 2a–h). Simultaneously, an increased activation of NF- κ B transcription factor in rapid response to cellular oxidative stress was noted (Fig. 2i–l). Continuing, intensified reactivity of superoxide and NO may lead to DNA extensive damage and concomitant cell cycle arrest and DDR pathways activation.

3.3. Cell cycle distribution analysis

We found reduction in the G0/G1 cell cycle phase population in both analyzed cell lines. Overall, the cause may be cell cycle arrest and accumulation in G2/M phase, while in some cases an increase in S phase. Based on these findings, we also focused our attention on expression patterns of proteins especially involved in cell cycle phase regulation and DDR machinery.

Measurement of DNA content indicated that, a number of GC-1 spg cells exposed to CEMF fell in the G2/M phase, what correlated with increasing EMF frequency (Fig. 3a–b). Furthermore, quantitative immunoblots analysis showed upregulation of p21 protein synthesis with no differences in p53 profile in comparison to control cells. Noteworthy, GC-1 spg cells exposed to PEMF showed an increase in S phase cell population at 2 and 50 Hz frequency with cell cycle arrest in G2/M phase at 120 Hz. Protein analysis in this condition showed p53 downregulation with no difference in p21 protein profile (Fig. 3c–f).

Exposure of GC-2 spd cells to CEMF resulted in increase of cell number in S phase after treatment with 50 and 120 Hz, whereas p21 and p53 proteins expression were downregulated for all electromagnetic field parameters tested. Consistently, GC-2 spd cells exposed to PEMF indicated increase in G2/M phase in all tested systems (Fig. 3. g–h). Additionally, detailed protein expression analysis showed a p21 constant level while p53 synthesis was upregulated (Fig. 3i–l).

3.4. Metabolic activity and cell viability

Oxidative stress may also directly affect mitochondria functions. Interestingly, results did not show the reduction in the metabolic activity of GC-1 spg cells exposed to EMF (Fig. 4a).

In the case of GC-2 spd cells, we noted frequency-dependent decreasing tendency in metabolic activity. The most pronounced differences were observed for 120 Hz for both modes of emission (to 75% and 90% in comparison to control, respectively) (Fig. 4b).

If DNA damage response machinery fails, apoptotic death program can be activated which was certainly confirmed in the case of GC-2 spd cell line treated with CEMF, while GC-1 spg cells remained unchanged (Fig. 4c–e).

Moreover, the apoptotic cell death could be related to caspase pathway activation. Exposure of GC-1 spg cell line to the CEMF resulted in active caspase 3 downregulation concomitant with Bcl-2 level decrease. Although, in the case of 2 Hz of PEMF we observed downregulation of active caspase 3 and Bcl-2 (Fig. 4f–h).

On the other hand, CEMF resulted in simultaneous upregulation of active caspase 3 in GC-2 spd, while PEMF caused active caspase 3-independent Bcl-2 increase (Fig. 4i–k).

4. Discussion

Literature data suggest that high risk of male infertility may be correlated with EMF exposure (Gye and Park 2012; Lee et al., 2014), however the exact molecular mechanisms underlying these disorders were not proposed. Therefore, in this study we decided to provide more detailed data on this topic.

To date, the literature provides many, sometimes contradictory information on the effects of EMF on the rate of proliferation and cell viability in *in vitro* studies. It is assumed that CEMF activates adaptive mechanisms in cells protecting their genome from harmful effects (Liu et al., 2015b), while PEMF exposure results in more severe damages and formation of DNA breaks. However, some other studies show completely diverse effects (Cho et al., 2012; Kim et al., 2010; Yan et al., 2010; Zhou et al., 2011). The explanation of these differences may be due to the different metabolic, differentiation or ROS and RNS status of tested cells (Ayrapetyan and Markov, 2006). Also, the differences in cellular proliferation rate and viability are directly correlated with the EMF frequency and time of exposure (Bedir et al., 2015; Kiray et al., 2013; Lee et al., 2014). Here, we noted PEMF-induced decrease in cell number what is in agreement with others (Ceccarelli et al., 2013; Vigano et al., 2014). Moreover, morphological analysis revealed more changes in cells exposed to PEMF, which is also supported by observations made by others (Esposito et al., 2013; Luo et al., 2012). Continuing, one of the reasons of reduction in the cell count is induction of apoptotic cell death and to date, potential biophysical mechanism of this kind of cell death initiation in spermatogenic cells due to the EMF exposure was not fully understood (Artacho-Cordon et al., 2013). In this study, we observed upregulation in caspase 3 level correlated with the increased frequency value of EMF in both cell line tested. The obtained results are confirmed by other researchers, who noted simultaneous increase in apoptotic cell number, oxidative stress and morphological changes (Tayefi et al., 2010). Further, it was hypothesized that EMF exposure results in increased ROS production and changes in the expression of genes involved in the activation of apoptosis by Bcl-2, Bax, caspase-3 and cytochrome C pathways and thus in apoptotic cell death (Liu et al., 2015a). In the present study, a decrease in the Bcl-2 expression level due to the EMF treatment was observed in GC-1 spg cells suggesting induction of apoptosis. Also, there are numerous scientific evidence about consequences of decreased level of Bcl-2 protein, such as extension of G0/G1 cell cycle phase.

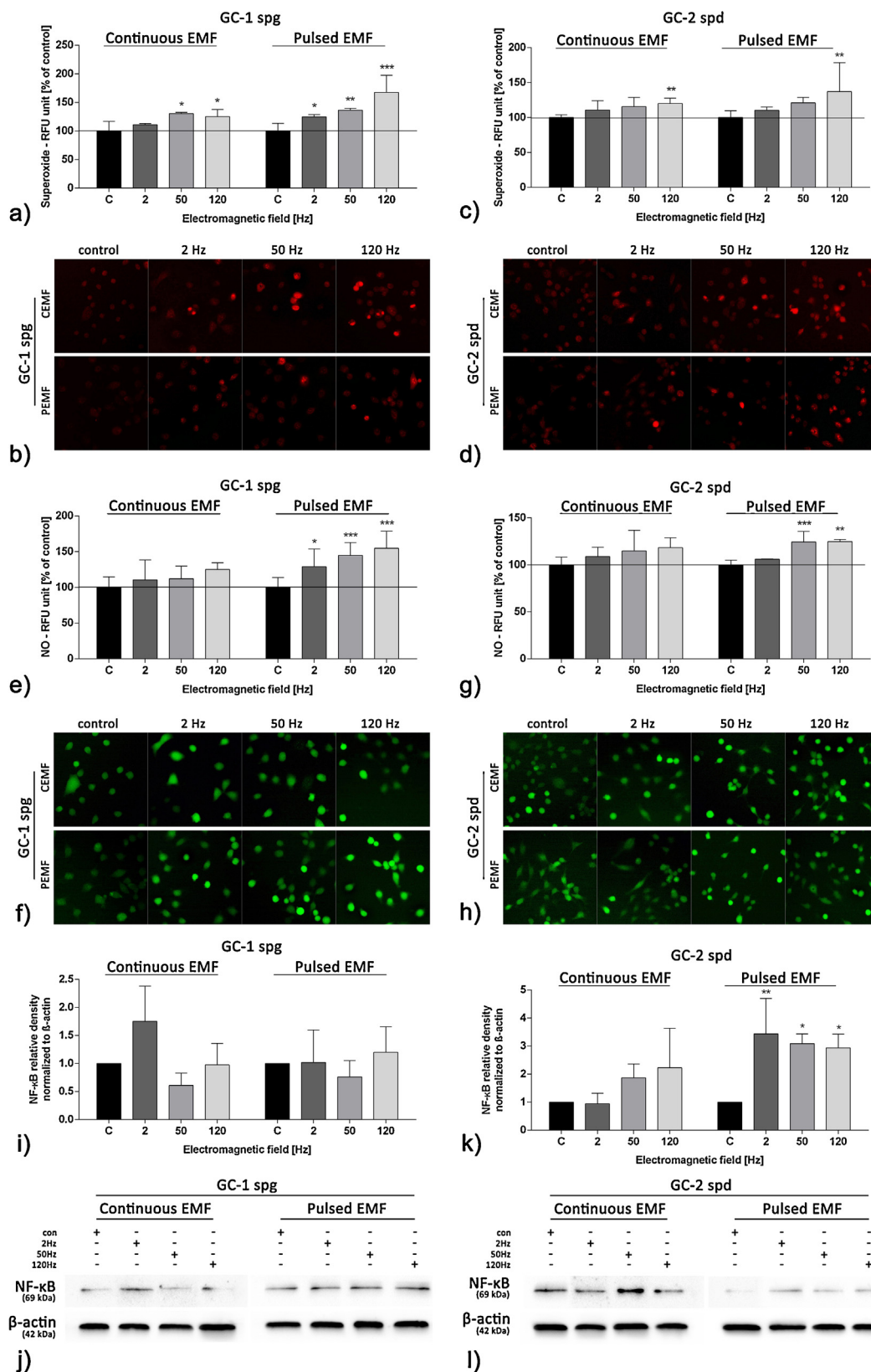


Fig. 2. EMF-induced changes in superoxide (a–d) and nitric oxide (e–h) production as relative fluorescence units (RFU) and protein involved in redox balance control (i–l). Representative Western Blots are shown. Cells were exposed for 2 h, left 48 h for post-exposure recovery and then analyzed. The reference distance between cells and EMF source was 10 cm. Bars indicate SD, n = 3, ***p < 0.001, **p < 0.01, *p < 0.05, no indication – no statistical significance (one-way ANOVA and Dunnett's a post hoc test). Red fluorescence – Texas Red (superoxide), green – FITC (nitric oxide).

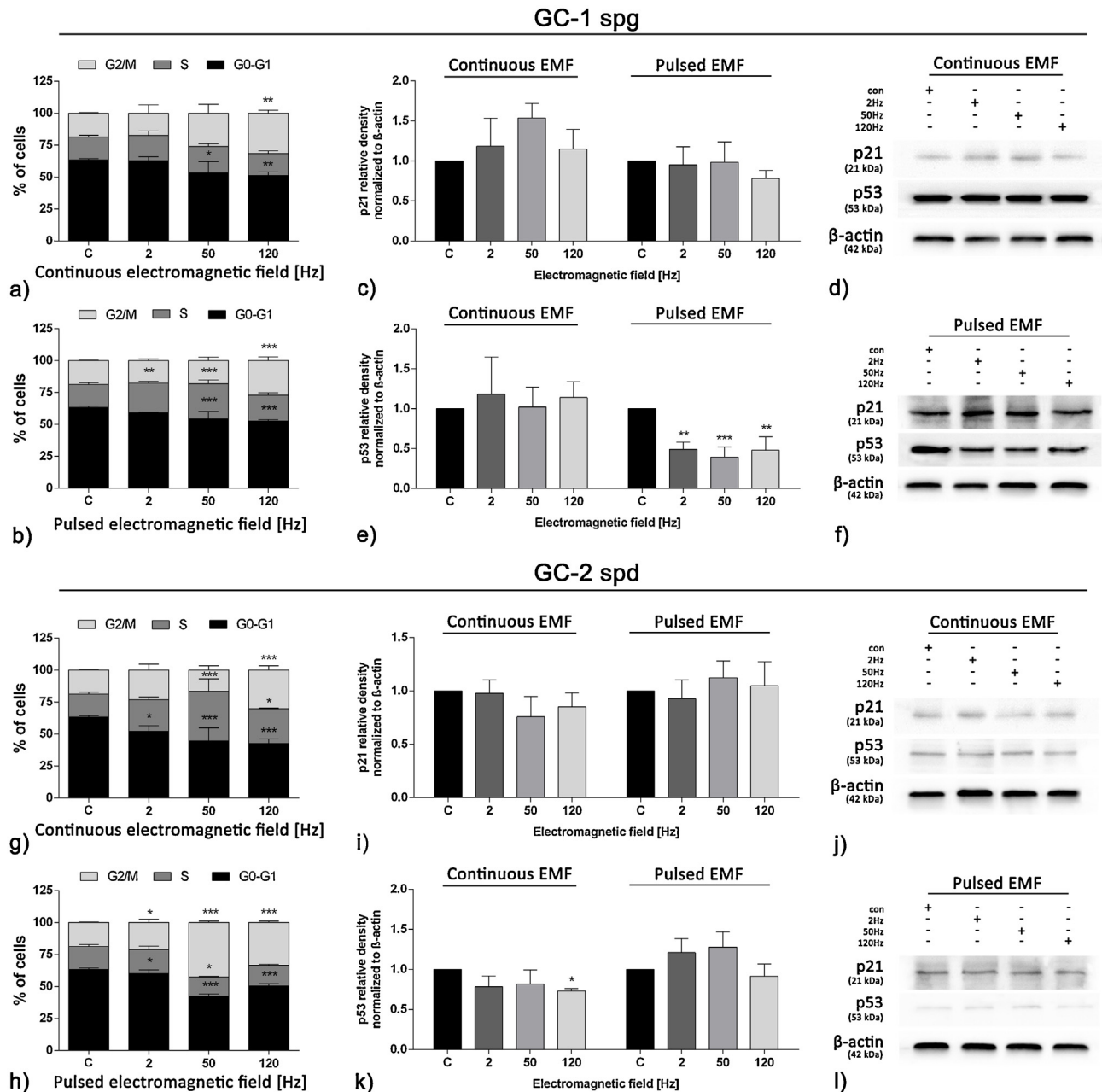


Fig. 3. Effects of EMF on spermatogenic cells in terms of cell cycle progression (a–b, g–h) and activation of DNA damage response pathway (c–f, i–l). Representative Western Blots are shown. Cells were exposed for 2 h, left 48 h for post-exposure recovery and then analyzed. The reference distance between cells and EMF source was 10 cm. Bars indicate SD, n = 3, ***p < 0.001, **p < 0.01, *p < 0.05, no indication – no statistical significance (one-way ANOVA and Dunnett's a post hoc test).

Furthermore, Bcl-2 protein level may be downregulated by p53, which contributes to the initiation of apoptosis (Cory et al., 2003; Simpson et al., 1999).

The male reproductive system cells, such as spermatids, spermatocytes and sperm cells are particularly sensitive to DNA damaging factors due to their function and structure i.e. small amount of cytoplasm and low level of antioxidant enzymes (Duan et al., 2015). Furthermore, male germ cells are also characterized by sensitivity to oxidative stress because their cell membranes are rich in unsaturated fatty acids and do not have the ability to repair DNA (Walczak-Jedrzejowska et al., 2013). EMF was already shown to induce ROS and RNS production causing disturbances in cells (Zeni et al., 2007). Here we report oxidative and nitrosative stress due to the CEMF and PEMF exposure in spermatogenic cells as well

as activation of antioxidant defense controlled by NF-κB transcription factor responsible for maintaining redox balance. NF-κB protein upregulation or translocation to nucleus may occur in response to DNA damage caused by oxidative stress after all (Kato et al., 2003). Similarly, to Natarajan et al. (2006) (Natarajan et al., 2006), we observed EMF-induced increased expression of NF-κB protein. Further, increased expression of NF-κB followed by caspase 3 activation confirms apoptosis as a result of permanent DNA damage due to the EMF treatment. Although, when DNA damage is not severe, cell cycle arrest program is activated thus allowing to repair DNA damage. In this study, analysis of the cell cycle showed EMF-induced decrease in the number of cells in the G0/G1 phase and simultaneous increase in the number of cells in G2/M. The number of cells in G0/G1 was never lower as the number

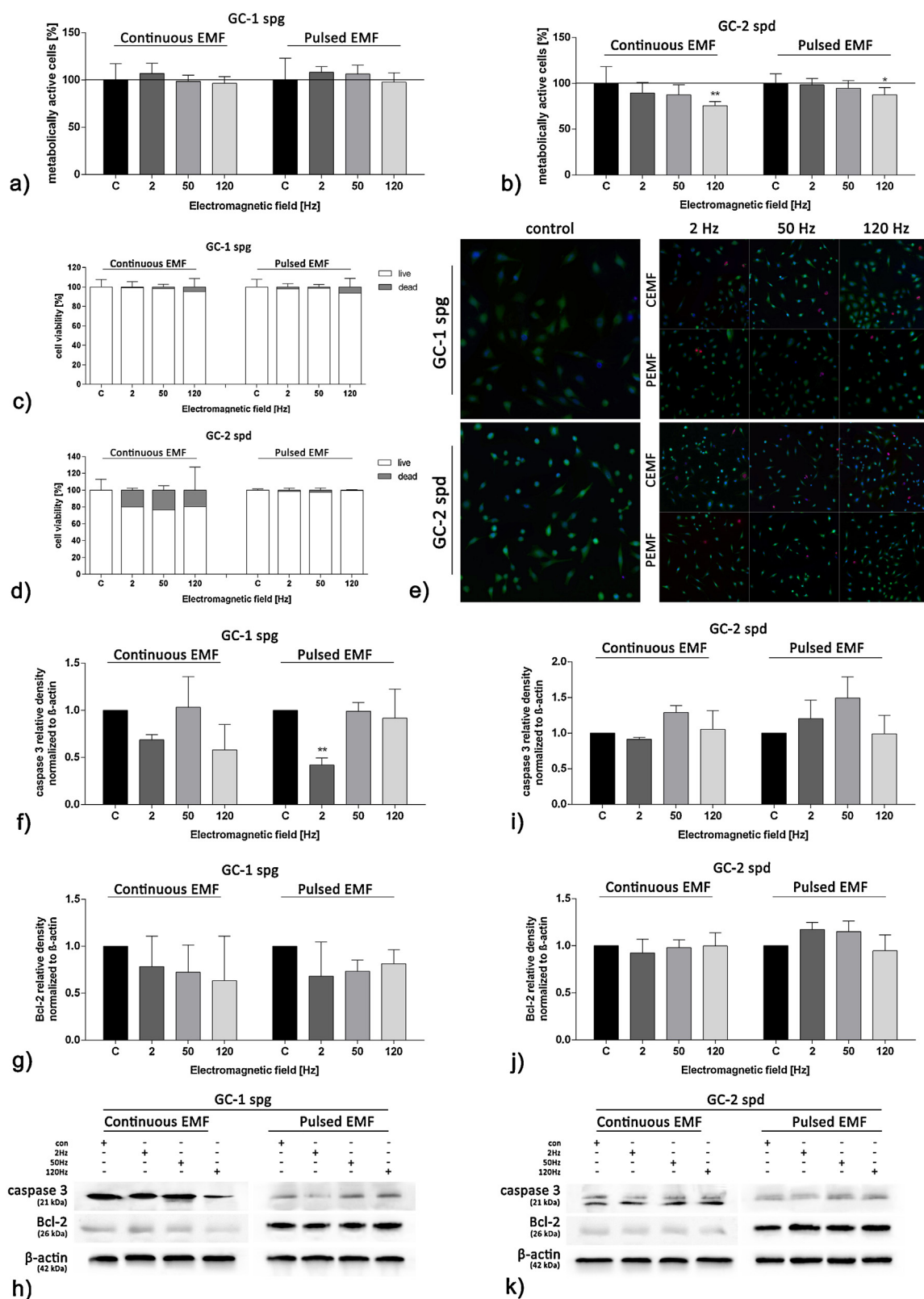


Fig. 4. EMF-induced changes in mitochondrial activity (a–b), cell viability (c–e) and proteins involved in apoptotic pathway synthesis (f–k). Representative Western Blots are shown. Cells were exposed for 2 h, left 48 h for post-exposure recovery and then analyzed. The reference distance between cells and EMF source was 10 cm. Bars indicate SD, $n = 3$, ** $p < 0.01$, * $p < 0.05$, no indication – no statistical significance (one-way ANOVA and Dunnett's a post hoc test). Green fluorescence – FITC (live cells), red – Texas Red (dead cells), blue – DAPI (nuclei).

of cells in G2/M phase. These results confirm cell cycle arrest as a consequence of external factors action (Sun et al., 2009). In contrast, Anbarasan et al. (2016) showed that PEMF at different frequencies promotes the passage of cells from G0/G1 to S and G2/M cell cycle phase (Anbarasan et al., 2016). Moreover, recent reports indicate that EMF causes increase in proliferating cells at a constant level of cells in G0/G1 and G2/M phases (Stavroulakis, 2003). The same conclusion was reached by (Liu et al., 2015a) and colleagues but quite different effects were observed by (An et al., 2015) and (Huang et al., 2014). However, these difference may be due to different types of cells and their origin. DNA damage pathways are controlled by p21 and p53 proteins (Abbas and Dutta 2009; Mihara et al., 2003). In this study, we observed increase in p21 protein expression in GC-1 spg cells exposed to CEMF. This may be due to the formation of numerous DNA breaks under EMF treatment. Further, the p53 protein mediates not only cell cycle arrest, but also DNA repair, cellular metabolism and Bcl2-dependent or independent apoptosis (Wu et al., 2015). In our study, we observed PEMF-induced increase in total p53 protein level in GC-2 spd cells, what supports theory of cell cycle arrest activation intended to repair DNA damages caused by external factors.

In conclusion, our results strongly suggest negative impact of EMF on spermatogenic cells. EMF-induces oxidative and nitrosative stress and leads to DNA damage. As response cells activate cell cycle arrest program and when damage is too severe, caspase-dependent apoptosis is initiated to remove abnormal cells. In consequence, this protects germ cells to be transferred to another generation but also directly reduces the pool of functional sperm cells and directly contributes to infertility.

5. Conclusions

Electromagnetic field present in surrounding environment may impair male fertility by inducing p53/p21-mediated cell cycle arrest and apoptosis based on mouse spermatogenesis *in vitro* model.

Conflict of interest

None declared.

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