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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.

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% CO_2. Adherent cells were passaged every 3 days by trypsinization. For all experimental procedures, cells were seeded at constant density of $1.5 \times 10^3/cm^2$, 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 $1.5 \times 10^3/cm^2$ 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