Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: L. Verschaeve, R.Antonissen, A. Baeyens, A. Vral, A. Maes (2019) Fluorescence In Situ Hybridisation Study of Micronuclei in C3A Cells Following Exposure to ELF-Magnetic Fields. *Caryologia* 72(2): 45-51. doi: 10.13128/cayologia-254

Published: December 5, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Fluorescence In Situ Hybridisation Study of Micronuclei in C3A Cells Following Exposure to ELF-Magnetic Fields

Luc Verschaeve[1,2,*], Roel Antonissen[1], Ans Baeyens[3], Anne Vral[3], Annemarie Maes[1]

[1] *Sciensano, Risk and Health Impact Assessment Service, Brussels, Belgium*
[2] *Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium*
[3] *Department of Basic Medical Sciences, Ghent University, B-9000 Ghent, Belgium*
*Corresponding author: Luc.verschaeve@sciensano.be

Abstract. Human C3A cells were exposed to extremely low frequency (50 Hz) magnetic fields (ELF-MF's) up to 500 μT. They were subjected to the micronucleus assay using a Fluorescence In Situ Hybridization (FISH) technique with an in-house pan-centromere probe. We found no increased frequency in micronucleated cells and no change in the proportion of centromere positive over centromere negative micronuclei compared to the unexposed control cells. These results are in accordance with some, but in contradiction with other previously published investigations underlining that effects of environmental ELF-EMF's on cellular DNA may be very subtle and that small changes or environmental influences may determine the outcome of a (geno)toxicity study. Interestingly, a low-level (5μT) exposure resulted in less than the background micronucleus frequency.

Keywords. 50 Hz magnetic fields, FISH staining, micronuclei, centromere staining, genotoxicity.

INTRODUCTION

Overall, there is little experimental or theoretical evidence that extremely low frequency magnetic fields (ELF-MF's) from power lines or other man made sources in the environment can be genotoxic. Given the level of energy involved, it is difficult to accept that they are able to directly interact with genomic structures. The results of most *in vitro* and *in vivo* genetic toxicology studies involving ELF-MF's have been negative and therefore there is a general consensus that they, especially at normal (moderate) exposure levels, are not directly mutagenic (Bergqvist et al. 2003; Vijayalaxmi and Prihoda 2009). Yet, some papers did report effects suggesting that ELF magnetic fields may interact with DNA or, most often, with DNA-damaging agents, hence being co-genotoxic (e.g., Tofani et. al. 1995; Lai and Singh 1997; Singh and Lai 1998; Bergqvist et al. 2003; Cho and Chung 2003; Ding et al. 2003;

Moretti et al. 2005; Vijayalaxmi and Obe 2005; Juutilainen et al. 2006; EHC 2007; Ruiz-Gómez and Martinez-Morillo 2009; Markkanen 2009; Udroiu et al. 2006). According to some of these studies ELF-magnetic fields are able to enhance, but not to start a mutagenic (DNA damaging) effect. Some of the above mentioned papers also indicate that EMF-MF's exposure may, alone or in conjunction with another agent, be able to promote the occurrence of aneuploidy caused by an aneugen via a mechanism involving the neuroendocrine system (Maes et al. 2016a). Jin et al. (2015) however, provided evidence that ELF-MF's alone do not induce either G2/M arrest or aneuploidy, even when administered in combination with different stressors. Only a few papers reported so far on possible aneugenic or co-aneugenic effects of electromagnetic fields (Udroiu et al. 2006; Maes and Verschaeve 2012; Maes et al. 2016a). On previous investigations (Maes et al. 2016a,b) we reported increased levels of especially nuclear buds and large micronuclei in cells that were exposed to 50 Hz ELF-MF's. This indicated that the magnetic fields may, at least in particular cells, situations and exposure levels induce gene amplification (buds) and aneuploidy. In the present paper we further explore this possibility by using fluorescence in situ hybridisation (FISH) with a pan-centromeric probe. The main objective was to verify our previous results and to investigate whether potential ELF-MF's induced micronuclei (MN) were predominantly centromere-positive or centromere-negative, respectively suggesting an aneugenic or clastogenic effect.

MATERIALS AND METHODS

ELF-MF exposure unit

The exposure unit was a cylindrical coil (380 turn coil, 42 cm long, 20 cm inner diameter) which allowed the exposure of cell cultures to a nearly constant magnetic field (with a tolerance of a few percent). With this device, cell cultures could be exposed to different 50 Hz magnetic field amplitudes ranging from 0 up to about 2500 μT. More details about the exposure unit can be found elsewhere (Maes et al. 2000; Verheyen et al. 2003; Mineur 2009). We exposed cell cultures to magnetic fields of 5, 10, 50, 100 and 500 μT. The ambient magnetic field was 0.02±0.01 mT.

Cell cultures and ELF-MF exposure

Human hepatic C3A cells (Brunschwig Chemie B.V, Amsterdam, the Netherlands) were grown in 24 well plates in Dulbecco's modified Eagle's culture medium supplemented with 10% foetal calf serum. The cell density was 200.000 cells/well. Plates were incubated at 37°C and 5% CO_2. Humidity was maintained using a water bath containing milli-Q water inside the incubator. After 24 h of incubation, a magnetic field producing a determined magnetic flux density (5, 10, 50, 100, or 500 μT) was applied for another 24 h. Following exposure to the magnetic field, cells were blocked in their binucleated (BN) telophase stage with cytochalasin B (4.5 μg/ml, Merck). Another 24h later cells were fixed with methanol/acetic acid (3/1) and spread onto well-cleaned microscope slides. Magnetic flux densities were chosen based on our previous experiments (Maes et al. 2000, 2016a,b; Verheyen et al. 2003). Each exposure was accompanied by its own unexposed (negative) control culture (0 μT). Methyl methane sulfonate (MMS, 15μg/ml) was used as a positive control. It was found to induce micronuclei as expected (results not shown). Both control cultures were incubated away from the coil at a distance where no ELF-MF, other than the ambient field could be measured.

Two independent investigations were conducted. In the first experiment magnetic flux densities of 5, 10, 50, 100 and 500 μT were investigated. The second study was conducted on new exposed cell cultures and fresh slides using magnetic flux densities of 5, 50 and 500 μT.

Fluorescence In Situ Hybridization (FISH)

In the first set of experiments, FISH was performed on the slides using an in-house pan-centromeric probe, labelled with spectrum orange. For more details about this probe and the FISH protocol we refer to Baeyens et al. (2011) and Vral et al. (2016). In the repeat study, FISH was performed using a FITC-labeled PNA (peptide nucleic acid) probe, specific for centromeric sequences, from Panagene (centFAM 5nmol, PN-CN001-005 Eurogentec, Belgium). The protocol, described in detail by M'Kacher et al. (2014), for centromere staining of dicentric chromosomes was followed. At the end of both FISH procedures, the slides were counterstained and mounted with DAPI-vectashield (H-1200, Labconsult, Belgium).

FISH-DAPI stained slides were analysed with the Metafer 4 platform (MetaSystems GmbH, Altlussheim, Germany) connected to a motorized Zeiss AxioImager M1 microscope (Zeiss, Oberkochen, Germany). Detailed information regarding the MSearch slide scanning procedure, stage movement, focusing and image acquisition are detailed in Willems et al. (2010). For analyses of micronuclei the MNScore module for Metafer MSearch was used. This software allows automated MN scoring in binucleated (BN) cells using a 10x objective. The auto-

matically selected BN cells were then checked manually (false BN cells and false positive or negative micronuclei were removed) and only confirmed BN cells with micronuclei were scanned via the Autocapt image acquisition software using a 40x objective. The autocapt images were then manually viewed for the presence of centromeres. Bad/non-interpretable FISH images were rejected. For more details about the MN-centromere analysis we refer to Vral et al. (2016). The number of investigated cells was set to 2.500 but the actual number of analysed cells was sometimes less (see Table 1). Two slides (approximately 1.250 cells) were analysed per exposure.

RESULTS AND DISCUSSION

The results of the two independent investigations are summarized in Table 1. The table data indicate that micronucleus frequencies were not increased following ELF-MF's exposures up to 500 μT. According to this analysis we observe no clear differences in number of MN between the tested situations. No clear difference or trend in the percentage of centromere-negative or centromere-positive MN can be observed as well. According to these data 50 Hz ELF-MF's do not change the proportion of centromere-positive over centromere-negative MN, neither do they induce elevated micronucleus frequencies.

This does not coincide with our previous results on the same cells that were exposed in the same way to magnetic fields of the same magnetic flux densities. In our previous investigation (Maes et al. 2016b) we also performed two independent experiments but slides were stained with Giemsa. Currently we used a fluorescence in situ hybridisation procedure but there is no reason why the different staining methods should influence the results. Both investigations were also performed by the same persons ruling out the possible variation in micronucleus frequencies obtained by individual laboratories and scorers (Fenech et al. 2003).

As mentioned before, the literature reveals the presence of positive as well as negative results following exposure of cells or organisms to (weak) ELF-MF's. We, for example, did not find increased micronucleus frequencies in peripheral human white blood cells after exposure to ELF-MF's up to 800 μT using the same experimental set up (Verheyen et al. 2003). Moreover, Loberg et al. (2000) also did not find evidence for the hypothesis that magnetic fields interact with genotoxic agents to induce adverse biological effects in either normal or genetically susceptible human cells. The same holds true for the investigation of Ding et al. (2003)

Table 1. Summary of two independent experiments on ELF-MF's exposed C3A cells following FISH-staining of micronuclei.

	Number of Binucleated cells	MN/1000 cells	MN+	MN-	% CM+	% CM-	MN+/ MN-
Experiment 1							
5 μT	2500	10	5.2	4.8	52	48	1.1
control	2500	19.2	14	5.2	72.17	27.08	2.7
10 μT	2500	12	7.2	4.8	60	40	1.5
control	2500	14	8.8	5.2	62.86	37.14	1.7
50 μT	2500	18	14.4	3.6	80	20	4
control	2500	13.6	11.2	2.4	82.35	17.65	4.7
100 μT	2500	16.8	12.8	4	76.19	23.81	3.2
control	2500	18.8	13.6	5.2	72.34	27.66	2.6
500 μT	2500	12	8	4	74.29	25.71	2
control	2500	14	10.4	3.6	66.67	33.33	2.9
Experiment 2							
5 μT	3432	20.69	15.15	5.54	73.24	26.76	2.7
Control	3423	21.62	16.36	5.26	75.68	24.32	3.1
50 μT	1629	22.71	14.12	2.46	89.19	10.81	5.7
Control	1093	20.13	16.47	3.66	81.82	18.18	4.5
500 μT	2193	22.8	15.04	7.75	66	34	1.9
control	3021	28.8	16.88	5.3	81.61	18.39	3.2

where Chinese hamster ovary cells were exposed to a 60 Hz ELF-MF at 5 mT field strength. In this investigation, MN were evaluated by immunofluorescence staining using anti-kinetochore antibodies from the serum of Scleroderma (CREST syndrome) patients. No statistically significant difference in the frequency of MN was observed between sham exposed and 24 h ELF-MF's exposed cells. The number of spontaneous kinetochore-positive and kinetochore-negative MN was, as in our present investigation, not affected by exposure to an ELF magnetic field alone. However, Kesari et al. (2016) reported increased micronucleus frequencies at 10 and 30 μT in SH-SY5Y neuroblastoma cells using a flow cytometry method. Other examples of positive and negative results on ELF-MF's exposed cells and organisms were presented by Heredia-Rojas et al. (2017).

Actually, the absence of independent replication has been a consistent feature of experimental studies searching for biological effects of weak ELF-(electro) MF's (ELF-EMF's). As pointed out by Foster and Skufca (2016) many scientific results can't be replicated, leading to serious questions about what's true and false in the world of research. Many reasons, especially involving statistical inadequacies or different experimental factors

or errors of unknown nature, can be evoked. Effects of environmental ELF-EMF's on cellular DNA are believe to be very subtle (Heredia-Rojas et al. 2018) and therefore small experimental changes or environmental influences may determine the outcome of a (geno)toxicity study. Also, different cell types (e.g., healthy lymphocytes versus a cancer cell line), but especially the different physiological state of the cells may account for different susceptibilities and consequently different results (Fenech 1998). It was for example shown that cells from aged donors and leukemic patients respond to ELF-EMF's exposure differently than 'other' (normal) cells (Cadossi et al. 1992), and that DNA damage was found in cells from Turner syndrome patients but not in cells from healthy individuals (Scarfi et al. 1997a). The same authors found that Turner syndrome subjects showed a lower spontaneous and mitomycin C-induced micronucleus frequency, in comparison with healthy subjects (Scarfi et al. 1996). On the other hand, in another publication they did not report a different response between normal cells and cells from Turner syndrome patients (Scarfi et al. 1997b). The viability of goldfish that were infected by a parasite increased substantially when the fish were exposed to very low levels of ELF-MF's (Cuppen et al. 2007) giving another example of possible different effects according to the health status of a cell or organism. Some more cases are also reported in the literature.

We used HepG2/C3A cells mainly because they have nitrogen metabolizing activity comparable to perfused rat livers, which was an important asset in some of our other studies. They are a clonal derivative of Hep G2 hepatocellular carcinoma cells, with an unstable chromosome number of 45-60. Perhaps another batch and cell passage may also be responsible for small physiological changes that ultimately may influence the MN frequency. Although cellular passaging was not found to influence significantly hASCs's secretome properties (Serra et al., 2018), increased cell passage number was found to alter P-glycoprotein expression in Caco2 cell (Senarathna and Crowe, 2015). Previously, Gloy et al. (1994) already reported that the response of membrane voltage to ATP and angiotensin II in rat mesangial cells was influenced by cell culture conditions and passage number. Furthermore, Peiser et al. (1993) reported that more micronuclei were always detected in cells of higher passages than of lower passages showing that metabolic and genetic characteristics of permanently growing cells differ remarkably depending on the culture passage. Unfortunately, we do not recall whether the cells used in our independent investigations were from a different batch and/or different cell passages. The different background levels of MN found in our different investigations may yet show that there are some differences in cell behaviour from one experiment to the other. In our previous investigation (Maes et al., 2016b) we obtained background MN yields of 5-9MN/1000 BN cells (Giemsa stain; experiment done in 2014-2015), whereas we now had background micronucleus frequencies of approximately 13-19MN/1000 BN cells in the first experiment (2016) and 20-29MN/1000 BN cells (2018) in the second experiment (FISH staining). Examples of micronucleated C3A cells are given in Figure 1.

An important finding of our previous investigation (Maes et al. 2016b) was also that low-level ELF-MF exposures resulted in micronucleus frequencies that were lower than in the unexposed control cells. Actually, this was also observed here. However, this was only substantial (and statistically significant according to the binomial test described by Kastenbaum and Bowman 1970) in our first experiment. Here the micronucleus frequency in cells exposed to 5 μT was 10MN/1000 BN cells compared to almost the double (19.2MN/1000 BN cells) in the controls. This may possibly indicate that low-level exposures to ELF-MF's, as environmental stimuli, can activate DNA repair mechanisms which then result in the repair of 'spontaneous' DNA damage which is not repaired in unexposed cells. This may be more or less comparable to the adaptive response which was already described in earlier investigations. Adaptive response is a phenomenon in which cells that were pre-exposed to extremely low and non-toxic doses of a toxic agent build-up a resistance to the damage induced by subsequent exposure to a

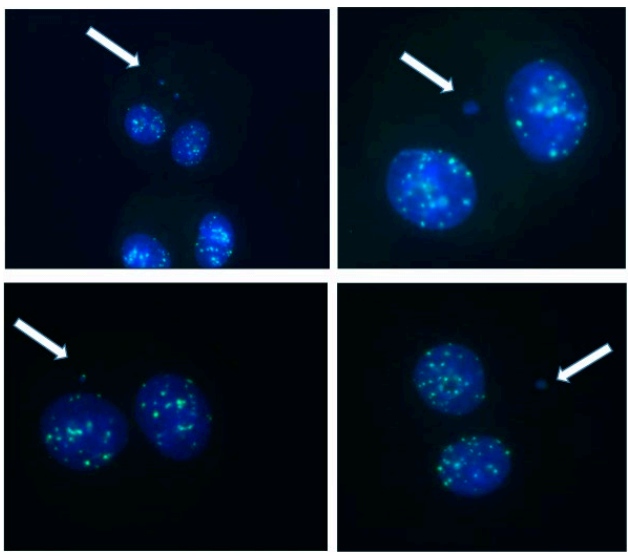

Fig. 1. Examples of binucleated C3A cells with micronuclei following FISH staining. Centromere positive cells are at the left.

higher and toxic dose of the same, similar (in action) or another toxic agent (Vijayalaxmi et al. 2014). There are indications that ELF-EMF's may also elicit such response and therefore may even have beneficial instead of detrimental properties, at least after short exposure times. Some previous research also proposed that low-frequency magnetic fields might play a positive role in cardiac tissue against ischemia reperfusion injury via regulating ROS production and NO/ONOO– balance (Ma et al. 2013).

Although the present study was not able to associate ELF-MF's with genotoxicity it is evident that there is no consensus reached yet on the alleged association between ELF-electromagnetic fields (and ELF-MF's in particular) and adverse health effects in humans. Yet, this remains an important issue, especially in view of the transition from nuclear and fossil to renewable energy sources for power production which leads to the necessity to optimize and expand the existing power grid and to construct several high voltage AC and DC power lines across the concerned countries (for the time being this is for example the case in Germany where nuclear power plants are all about to be dismantled).

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Johanna Aernoudt and Ms. Toke Thiron of the Department of Basic Medical Sciences of Ghent University for their excellent practical assistance.

This paper was part of the research performed within the BBEMG (Belgian BioElectroMagnetics Group); cf. http://www.bbemg.be/.

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