

„Risk Evaluation of Potential Environmental Hazards From Low Energy (REFLEX) Using Sensitive In Vitro Methods“

Editors' remarks:

A final report on the REFLEX program on risk evaluation of hazards from electromagnetic fields that has been widely discussed in the public is now available. An experienced geneticist, Mrs. Vijayalaxmi from the Department of Radiation Oncology at the University of Texas (USA) has reviewed this report for critical evaluation. An author of many publications herself, she intimately knows the methods used by scientists in the field. In the last few years, she not only spoke to scientists involved in this program during meetings and in personal conversations, but used the opportunity to observe test performance at various labs herself.

The report is written in concise style, as is the usual approach in writing reviews for peer reviewed journals. It may therefore be difficult to understand to outsiders in some parts. In spite of the evaluative terms normally applied in such reviews, the author abstains from polemics. Our goal was to present the text in its original form, including the author's evaluations and conclusions. The publication of this review is meant as a contribution to the debate, supplementing the many reports in the media dealing with this project.

Critical on the results of final report

Vijayalaxmi

The following analysis exclusively deals with the results presented in the final report of REFLEX on DNA single-strand breaks (SSB), DNA double-strand breaks (DSB), chromosome aberrations (CA) and micronuclei (MN) in mammalian cells after in vitro exposure to extremely low-frequency electromagnetic fields (ELF-EMF) and radio-frequency fields (RF-EMF).





remarks

DNA damages in the 2004 on the REFLEX program

Summary

At the outset, it is important to recognize that the known basic mechanism of interaction between biological material and ELF-EMF, and between biological material and RF-EMF are different: induction of electric currents in the case of former and generation of heat or increase in temperature in the case of latter. The knowledge of such mechanism(s) is crucial to establish a causal relationship between ELF-EMF and RF-EMF exposure and human disease. The in vitro investigations are relevant and offer several advantages. They: (a) can be conducted in controlled environments, (b) are useful for exploring 'potential' mechanisms, (c) can include large sample size, and (d) can generate data within a relatively short time. However, in vitro exposure of, especially, continuously growing cultured cells will never mimic 'real life' situation and thus, the results can not be extrapolated directly to human exposure in living environment and at work place.

The following 4 investigators conducted the experiments, in the REFLEX project, to determine whether or not exposure of freshly collected and/or continuously growing, cultured mammalian cells to ELF-EMF and/or RF-EMF, in vitro, has potential adverse effects on SSB, DSB, CA and/or MN. (1) Rudolf Tauber, Berlin, Germany, (2) Hugo Rüdiger, Vienna, Austria, (3) Anna Wobus, Gatersleben, Germany, and (4) Angeles Trillo, Madrid, Spain.

Rudolf Tauber (Participant # 2), Berlin, Germany

(1) HL-60 cells were used to conduct the experiments. They are a continuously growing cell-line derived from a human patient suffering from leukemia (cancer). Therefore, HL-60 cells should NOT be considered as 'normal' cells. However, the use of HL-60 cells in RF-EMF investigations is important. Inclusion of a 'normal' cell type in the experiments, concurrent with HL-60 cells, would have helped in understanding whether or not the response to RF-EMF exposure was different between normal cells and cancer cells.

(2) Detailed information on the precise number of cells in S-phase (cycling cells), cells in apoptosis (instead of dot-plots in Figures 80, 81 and 82) would have been helpful to indicate the absence of significant differences between RF-EMF-exposed and sham-exposed cells. This information is important for the validity of data and the conclusions from the comet assay.

(3) Since a great majority of HL-60 cells are known to be aneuploid, the reported incidence of MN in sham-exposed control cells is very low, ~4/1000 binucleate cells, i.e., <1 aneuploid cell, break or fragment in 100 first division metaphases.

Hugo Rüdiger (Participant # 3), Vienna, Austria

(1) Cell specificity is very common in biological response to chemical, biological and/or physical agents.

Based on the epidemiological evidence linking ELF-EMF exposure with leukemia (section 1.0, Introduction), one would expect that human blood lymphocytes are the target cells for ELF-EMF-induced genotoxicity. The absence of increased primary DNA damage (SSB/DSB) in human blood lymphocytes exposed to ELF-EMF is not only intriguing but also puzzling.

(2) Cultured human diploid fibroblasts and SV40 transformed rat granulosa cells were used for investigations. It is known that the altered characteristics which are acquired during the continuous growth period of cultured fibroblasts and SV40 transformed cells are not usually observed in freshly collected cells. Thus, the response of these cultured cells exposed to ELF-EMF or RF-EMF may not be the same as that observed in freshly collected cells.

(3) Examination and conclusions drawn from peripheral blood lymphocytes and monocytes obtained from a single donor will not allow the determination of inter-individual variability.

(4) Results from concurrent positive control cells (all Tables and Figures) and sham-exposed cells (some Tables and Figures) are not included. Data from such cells are not only essential but also required under GLP (Good Laboratory Practice) regulation for valid comparison with ELF-EMF- and RF-EMF-exposed cells.

(5) Comet Assay: (a) Very simple version of neutral comet assay was used to determine the DSB. The procedure did not involve the treatment of the cells with proteinase K and ribonuclease A (Lai and Singh, *Int J Radiat Biol.*, 69, 513-521, 1996; *Bioelectromagnetics*, 18, 156-165, 1997) to digest the proteins and to degrade the nuclear matrix so that the DNA is no longer super-coiled to drive out the DSB into the comet tail. Hence, the results from the neutral comet assay as well as the interpretation of the data may not be accurate. (b) Continuously growing cells with cell cycle duration of 24-30 hours were used for all experiments (except human blood lymphocytes, Figures 20 and 21). During prolonged ELF-EMF-, RF-EMF-

and sham-exposures (especially 24 hours) a number of 'normal' cells will enter into the process of semi-conservative DNA synthesis with several 'replication forks' (cycling cells). In the comet assay, the DNA from such replication forks will easily dissociate and emerge as strand breaks into the comet tail, resulting in 'normal cycling cells' displaying 'increased tail length' and thus mimic 'damaged cells'. The number of cycling cells in each exposure condition is not determined. Alteration in cell cycle, if any, in ELF-EMF- and RF-EMF-exposed cells (relative to sham-exposed cells) will result in 'cycling cells' being classified as 'damaged cells' which, in turn, will have an impact on the multiplication method (cells in type Ax2.5, cells in type Bx12.5, cells in type Cx30.0, cells in type Dx67.5 and cells in type Ex97.5) used to 'derive' the tail factor. (c) Mitochondrial membrane potential (MMP) was investigated as a measure of cells undergoing apoptosis (i.e., cells with severely fragmented DNA). In the comet assay, cell in apoptosis exhibit numerous DNA strand breaks and such cells will undoubtedly be classified in category E, which, in turn, will have a tremendous impact on the multiplication method (Ex97.5) used to 'derive' the tail factor. (i) Detailed data on MMP in ELF-EMF-exposed cells are not presented in section 3.1.1.1. The variances observed in the expression of mitochondrial and ribosomal genes in human fibroblasts were high (section 3.1.4.6). (ii) The results on MMP in cells exposed to RF-EMF were inconsistent (section 3.2.1.2). It is summarized that an indirect effect of RF-EMF exposure on apoptosis through modulating the expression of various genes and proteins can not be excluded (section 3.2.3.6). Thus, the questions related to the effect of ELF-EMF and RF-EMF exposure on MMP/apoptosis are unresolved: a definitive answer is critical for the overall assessment of comet assay results. (d) The multiplication method, x2.5, x12.5, x30.0, x67.5 and x97.5, used to derive the 'tail factor' for the cells in categories of A, B, C, D



and E, respectively, is extremely arbitrary. A 'potential' increase in the numbers of 'cycling cells' (with replication fork-induced strand breaks) and 'apoptotic cells' (with severally fragmented DNA) in ELF-EMF- and RF-EMF-exposed samples (relative to sham-exposed samples) will certainly contribute to their misclassification as 'damaged cells'. For every 1% increase in apoptotic cells (classified as 'E') in ELF-EMF- and RF-EMF-exposed cells, the tail factor will increase by a value of 1. (e) The 'tail factor' has not been validated as a measure of SSB and DSB by any other research group (who conducts the comet assay) anywhere in the world. The multiplication method and the 'derived' tail factor add considerable doubt to the overall comet data and conclusions. (f) Considering that there was an increase in SSB/DSB in cell exposed to ELF-EMF, at field strength as low as 35 mđT, it is difficult to extrapolate the over all results in terms of adverse human health effects since the ELF-EMF exposure for people living in an average home will be at much smaller average field strength, <1 mđT (Lancet, 354, 1925-1931, 1999).

(6) Chromosomal aberrations (CA): (a) There is no mention of the addition of bromodeoxyuridine to the culture medium to distinguish the cells in their first, second or later mitotic metaphase. Since it is customary to examine the cells in their first mitotic metaphase only to evaluate CA, it is not clear how the cells in first mitotic metaphase are differentiated from the second or later division metaphase. (b) Gaps are usually the 'unstained/achromatic' regions of the chromosome(s), and are not generally considered as 'serious aberrations' such as breaks and acentric fragments. Gaps are typically seen in chromosomes that are not fixed well in methanol+acetic acid mixture. Metaphases exhibiting 24-58% gaps (Tables 6 and 21) are not acceptable for analysis and to draw conclusions. (c) The incidence of chromatid/chromosome breaks in 'normal cells', reported in published literature, can be up to 2%. Hence, the 2.2% chromo-

some breaks (Table 6) reported in ELF-EMF-exposed cells can be considered as 'normal'.

(7) Micronuclei (MN): (i) Some of the fragmented DNA in apoptotic cells may have been erroneously classified as MN by untrained and unsupervised individual(s). (ii) It is surprising to find the incidence of MN in sham-exposed cells (0.5/100 cells), positive control cells (16.8/100 cells) and negative control cells (0.4/100 cells) are the same in Figure 14 (ELF-EMF experiment) and Figure 95 (RF-EMF experiment). The same data appear to have been used for both ELF-EMF and RF-EMF exposures. If this is true, it is extremely disguised utilization of the same data.

(8) Fluorescence in situ hybridization: A total of 24,000 metaphases were examined from the slides used for fluorescence in situ hybridization (probes for 22 autosomes + X and Y chromosomes to analyze 1000 metaphases for each chromosome). This involved enormous extremely time-consuming microscopic work. The reason(s) for not reporting the detailed data is not clear.

(9) Re-evaluation in independent laboratories: The results reported in APPENDIX-I are dramatically different between different independent laboratories. Variations in staining techniques and the number of cells examined will not contribute to such significant differences in the data (there could be an absence or inadequate mutual agreement in the criteria/procedure used for evaluation of the slides). More importantly, the positive control cells could not be either evaluated due to a low number of assessable cells or did not exhibit the expected increase in MN. Hence, the overall data are not acceptable for comparison with the observations reported earlier by Hugo Rüdiger (Participant # 3) and to draw any type of conclusion(s). The re-evaluation is ineffective and unrewarding.



[Anna Wobus, \(Participant # 4\), Gatersleben, Germany](#)

(1) The same comments, mentioned above, with respect to the comet data apply here. It is not clear why the detailed data are not presented either in Table(s) or in Figure(s) in the report.

[Albert Kolb\(Participant # 7\), Hannover, Germany](#)

(1) The same comments, mentioned above, with respect to the comet data apply here. It is not clear why the detailed data from CHO cells and HeLa cells are not presented in the report.

[Overall assessment](#)

(1) The conclusions in the REFLEX report are debatable since several data are questionable.
(2) The absence of critical and detailed information on 'cycling cells' and 'apoptotic cells' exacerbate the

use of 'derived' tail factor as a measure of DNA single and double strand breaks (SSB/DSB) in the comet assay.

(3) Several questions can be raised from the data presented on chromosomal aberrations (CA).

(4)Some of the fragmented DNA in apoptotic cells may have been erroneously classified as micronuclei (MN) by untrained and/or unsupervised individual(s).

(5)It is discomfoting that the re-evaluation data reported in APPENDIX-I are dramatically different between different independent laboratories. Perhaps, this could be an indication that the original observations are at best equivocal, and at worst flawed.

(6)The intriguing results certainly require independent replication investigations.

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