

Genotoxic and Cytotoxic Research Results and their Relevance for the Estimation of possible Adverse Health Effects caused by Low Level Electromagnetic Fields

**A NIRMED Statement
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Commenting the REFLEX Project: Risk Evaluation of Potential Environmental Hazards From Low Energy Electromagnetic Field Exposure Using Sensitive *in vitro* Methods

1 Preface

The REFLEX project is a contribution to the actual discussion about possible adverse health effects resulting from exposure to either ELF (Extreme Low Frequency Electromagnetic Fields – 50/60 Hz Power Lines) or RF (Radio Frequency, 30 MHz – 3 GHz) in doses below the guideline levels.

There is well accepted scientific evidence that ELF-EMF causes induced electric fields in the organism, while RF leads to temperature rise. Guidelines and limits protect the population against any adverse health effects caused by these mechanisms. Since science cannot totally exclude any possible unknown adverse health effects of ELF or RF in doses below the guideline levels much research has been done in the past decades and is still going on.

This research is focussed in four quite different areas (with decreasing relevance for health effect estimation): (i) human *in vivo* studies; (ii) human epidemiological studies; (iii) animal *in vivo* studies and (iv) *in vitro* studies with tissues and cells.

As physicians we know that the human organism is an extremely complex and dynamical system permanently reacting to a large number of different stimuli and regulating itself due to internal as well as to external environmental interacting factors. In our daily life we are exposed to thousands of chemical and physical agents which are capable of causing serious damage in cells and tissue. A lot of these agents are classified as definitively carcinogenic but are still produced and applied in agriculture and even in food production.

2 The idea behind REFLEX

The main goal of the REFLEX project was to investigate the effects of ELF and RF on single cells *in vitro* at the molecular level below the energy density defined by the

present safety levels. Most, if not all chronic diseases, including cancer and neurodegenerative disorders, are of diverse and heterogeneous origins. This variability is generated to a great extent by a relatively small number of critical events, such as gene mutations, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis). Gene mutations, cell proliferation and apoptosis are caused by or result in altered gene and protein expression profiles. The convergence of these critical events is required for the development of chronic diseases. Therefore, the REFLEX project was designed to answer the question whether any of these disease-causing critical events could occur in living cells after EMF exposure. Failure to observe the key critical events in living cells *in vitro* after EMF exposure would suggest that further research efforts in this field could be suspended and financial resources should be reallocated for the investigation of more important issues.

Therefore, the idea behind the REFLEX project was to investigate a necessary condition when studying carcinogenesis: If ELF-and RF-exposure appears unlikely in providing any evidence for occurrence of genotoxic effects *in vitro*, no further efforts should be done for future studies of increased incidence of malignancies associated with EMF exposure. From a mathematical (statistical) point of view REFLEX hypothesized no effects of EMF on the basic mechanisms of carcinogenesis or neurodegenerative disorders.

3 The findings of REFLEX

In fact the outcome of the REFLEX project will be a contribution under the condition of publication to the effects of low frequency and RF fields on the *in vitro* responses of cells. A large number of responses were examined in a wide variety of cell types, and included effects on cell proliferation, chromosomal damage and programmed cell death (apoptosis) as well as gene and protein expression profiles. RF field exposures were performed at levels roughly equivalent to the maximum advised for humans in existing exposure guidelines.

The strength of REFLEX was the adoption of similar exposure conditions and toxicological and molecular biology methodologies for evaluation of possible ELF and RF effects (on genes, differentiation, gene expression and protein targeting, immune responses, apoptosis and transformation in cells). Secondly, the adoption of the post-genomic technologies (DNA micro-arrays and proteomics) enables very large numbers of potential cellular effects to be examined simultaneously without prejudice.

The **ELF** data obtained in the course of the REFLEX project when all published may show that ELF has effects on primary cell cultures of human fibroblasts and on other cell lines. However, only the ELF genotoxicity results of the comet assay in fibroblasts is currently published (Ivanicsits et al., '02,'03a '03b). The genotoxicity experiments were obtained in two laboratories (Rüdiger & Tauber) and further replication by Bersani & Scarfi (in press) appears to have failed. ELF generated DNA

strand breaks in fibroblasts at a significant level at a flux density as low as 35 μ T. There was a strong positive correlation between both the intensity and duration of exposure to ELF and the increase in single and double strand DNA breaks and micronuclei frequencies (unpublished). Surprisingly this genotoxic effect on fibroblasts was only observed when cells were exposed to intermittent ELF, but not to continuous exposure. Responsiveness of fibroblast to ELF increased with the age of the donor and in the presence of specific genetic repair defects. The effect also differed among the other types of cells examined. In particular, lymphocytes from adult donors were not responsive (unpublished). Chromosomal aberrations were also observed after ELF exposure of human fibroblasts (unpublished).

In relation to the effects of ELF on differentiation, and function of embryonic and neuronal stem cells: the following observations were made in different REFLEX laboratories:

- 1) ELF at a flux density of about 2 mT upregulated the expression of early genes, such as p21, c-jun and egr-1, in p53-deficient mouse embryonic stem cells, but not in healthy wildtype cells (Czyz et al., 2004 a & b);
- 2) ELF (0,1 mT) increased the proliferation rate of neuroblastoma cells (unpublished); and
- 3) ELF (0,8 mT) enhanced the differentiation of mouse stem cells into cardiomyocytes (unpublished). However, no clear-cut and unequivocal effects of ELF on DNA synthesis, cell cycle, cell differentiation, cell proliferation and apoptosis were found (unpublished).

With respect to radiofrequency electromagnetic fields (**RF**), data showed that RF produced genotoxic effects in fibroblasts, granulosa cells and HL60 cells (unpublished). Cells responded to RF exposure between SAR level 0,3 and 2 W/kg with a significant increase in single and double strand DNA breaks and in micronuclei frequency (unpublished). Chromosomal aberrations in fibroblasts were observed after RF exposure (unpublished).

RF at a SAR of 1.5 W/kg upregulated the expression of neuronal genes in neuronal precursor cells and of early genes in p53-deficient embryonic stem cells, but not in wild-type cells (Czyz et al,' 04).

Proteomic analyses on human endothelial cell lines showed that exposure to RF-EMF changed the expression and phosphorylation of numerous, largely unidentified proteins (Leszczynski et al '02). Among these proteins is the heat shock protein hsp27, a marker for cellular stress responses. There was no evidence that RF-EMF affected processes such as cell proliferation, apoptosis or immune cell functionality (unpublished).

For both ELF and RF, the results of the whole genome cDNA micro-array and proteomic analyses indicated that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (unpublished).

The results presented in the REFLEX project need to be considered in the context of existing published studies. It is well accepted from the totality of the available evidence that RF fields do not possess sufficient energy to cause direct damage to DNA. Many research groups worldwide have investigated the molecular and cellular effects of RF fields using *in vitro* cell systems. After reviewing the available evidence, the independent Advisory Group on Non-Ionising Radiation (AGNIR, 2003) concluded that 'although there has been a wide range of diverse exposures and biological models investigated, no consistent pattern has emerged from the cellular studies of RF exposure'.

It was noted that positive findings have not been confirmed by other independent studies; similar experiments fail to confirm each other or may even show contradictory results. Overall is concluded that:

'In aggregate the research does not give cause for concern. The weight of evidence now available does not suggest that there are adverse health effects from exposures to RF fields below guideline levels, but the published research on RF exposures and health has limitations, and mobile phones have only been in widespread use for a relatively short time. The possibility therefore remains open that there could be health effects from exposure to RF fields below guideline levels; hence continued research is needed.'

Some results from the REFLEX project appear to challenge the AGNIR conclusions. For example, genotoxic effects were observed in fibroblasts, granulosa cells and HL60 cells. In various assays, RF exposed cells demonstrated an increase in single and double strand DNA breaks, and in micronucleus frequency; chromosomal aberrations were also seen in fibroblasts. Changes in the expression of specific genes, and alterations to proteins in human cell lines were also seen. There was no evidence that RF fields affected cell proliferation, apoptosis or immune function.

The reported effects appear to show very high levels of specificity with regard to cell type, exposure condition and the biological endpoint under consideration. Taken together, the results may suggest that certain types of exposure can cause genetic damage in certain cell types. However, if RF fields do cause genotoxic or carcinogenic effect, a consistent pattern of responses would be expected. Similarly these responses would be expected to be consistent in different cell types exposed to the same fields. Evidence of a consistent dose-response relationship would also strengthen the plausibility of any response.

While some responses do seem to have been repeated by different laboratories, others do not appear to have been seen consistently across the project. Also some only occurred in one cell type and not in others, and some changes were observed at one field intensity but not at higher or lower intensities.

Overall this inconsistency does not suggest that robust responses have been observed and the extent to which these effects may be due to experimental artefacts is unclear. The physiological significance of some of the reported changes, for

example in the changes in gene and protein expression, were commented on by the authors themselves and their biological relevance questioned.

The cytotoxic and genotoxic effects can only be evaluated with full experimental controls and replications. The normal variability might be rather wide; therefore small differences might be within these normal ranges.

The experimental paradigm has to be the same in the replications, i.e. the investigations on each cytological parameter. The origins of cells, lymphocytes, fibroblasts have to be specified as from healthy donors, and the age as well as the absence of repair deficiencies has to be mentioned.

In summary, the REFLEX project provides some novel observations regarding the potential of RF fields to affect cellular and molecular processes *in vitro*. The results will no doubt stimulate further highly focused research to confirm or refute the findings. In due course, it is expected that AGNIR and other review bodies will examine the REFLEX project report, as well as any associated articles that may appear in the peer-reviewed literature.

Since unpublished results can not be evaluated and compared one has to wait until all the REFLEX projects are published in peer reviewed journals.

4 The relevance of the REFLEX findings

When commenting on the relevance of the preliminary REFLEX findings, one has to address two different aspects:

1. Do we have convincing results and what do the results mean?
2. Assumed the reported findings would be confirmed, what is the consequence for daily life?

When considering the first topic, one has to take into account that at the beginning of the REFLEX project **no** hypotheses have been formulated. Therefore, the reported results are far away from any confirmatory testing. A large number of parameters were investigated (even by each research group) and under these circumstances findings by chance must be expected.

For the calculation of any statistical significance, consideration of multiple testing is mandatory. Therefore, all the results published up to now only have descriptive value. No statistically significant effect confirming a hypothesis has been reported. Therefore, the outcome of the study is considered to be hypothesis generating only.

All reported findings must be subject to confirmatory testing of these hypotheses, which in a next step can be formulated, being fully aware of the REFLEX outcome. Only if the hypotheses based on the preliminary descriptive findings of the REFLEX project were confirmed in follow-up investigations, then a clear and statistically

significant effect of EMF on the in vitro experiments described in the REFLEX project could be considered as proven.

When focusing on the second point the question arises: what have in vitro studies to do with in vivo studies? As described before the REFLEX project created some novel findings many of them still require anonymous peer review and publication. Future studies should include extensive independent replications of the key observations reported, initially using the same methodologies.

If all the reported findings would be confirmed then biological effects of EMF on in vitro cell growth and DNA damage must be taken into consideration. The consequences of these effects would depend particularly also on the DNA repair capacity and the apoptosis in vivo. On the other hand the accumulation of potentially carcinogenic mutations, or the coupling of DNA damage depend on environmental or nutritional factors modulating the impact of EMF on the cells.

That means one cannot imagine any conclusive evidence when evaluating in vitro findings in relation to human health. The REFLEX data have made a contribution (as yet mostly unpublished) to the data base relating to genotoxic and phenotypic effects of both ELF and RF on in vitro cellular systems. The genotoxic and phenotypic effects, which have been reported within REFLEX, do not show a causal link between RF or ELF exposure and any adverse health effects. The data neither preclude nor confirm a health risk due to EMF exposure nor was the project designed for this purpose.

5 NIRMED Physician's Summary

In general there is a large gap between several controversial in vitro research results and established science as evidence for adverse human health effects. Therefore, scientific work established a catalogue of criteria (e.g. the Bradford-Hill criteria) to establish scientific evidence for setting guidelines to protect human health.

Bradford-Hill Criteria

- **Temporal Relationship:** Exposure always precedes the outcome.
- **Strength:** defined by the size of the association as measured by appropriate statistical tests.
- **Dose-Response Relationship:** An increasing amount of exposure increases the risk.
- **Consistency:** The association is consistent when results are replicated in studies in different settings using different methods.
- **Plausibility:** The association agrees with currently accepted understanding of pathological processes.
- **Consideration of Alternate Explanations:** In judging whether a reported association is causal, it is necessary to determine the extent to which

researchers have taken other possible explanations into account and have effectively ruled out such alternate explanations.

- **Experiment:** The condition can be altered (prevented or ameliorated) by an appropriate experimental regimen.
- **Specificity:** This is established when a single putative cause produces a specific effect.
- **Coherence:** The association should be compatible with existing theory and knowledge.

Exposure Criteria: In addition to the basic Bradford Hill criteria in all EMF studies the exposure criteria should be well-defined and harmonized in order to be comparable.

Taken together all research results have to be interpreted in the context of all past and present studies and should be weighted based on their methodological efforts. Dealing with the REFLEX project there are three basic limitations:

- (i) In vitro studies cannot claim for any causal linkage; they can only give hints whether and what kind of possible interactions in isolated cells or tissue can be observed. According to the biological relevance of various study designs (see above) in vitro studies do not sufficiently contribute. Any result – even if replicated in confirmatory studies – must be considered as a necessary but not sufficient condition for possible health effects.
- (ii) The results inside the REFLEX project are surprisingly inconsistent. Therefore, **no** unambiguous and clear-cut findings have been reported yet and the extent to which confounders or experimental artefacts may be responsible remains unclear. The physiological significance of some of the reported changes, for example in the changes in gene and protein expression, were commented on by the authors themselves and their biological relevance has been questioned.
- (iii) Compared to the wide variety of findings in this research area most of the results cannot show any convincing evidence for biological effects of either ELF or RF. However, the REFLEX report is hypotheses generating and should be subject to confirmatory.

Therefore, for the time being national and international expert bodies as well as governmental agencies have not drawn any conclusions about possible causal linkages between EMF exposure (below the guidelines) and serious human adverse health effects. Based on epidemiological findings only high voltage power lines (50/60 Hz) have been associated with a possible risk for childhood leukemia.

What is the NIRMED message?

From the scientific and physician's point of view the REFLEX results are interesting contributions to the actual discussion concerning some assumed interacting mechanisms of ELF and RF with cells and tissues in vitro. However, these results are not confirmed.

Therefore, the REFLEX results do not appear to give reason to reassess the health risk of EMF or, least of all, to discuss lowering of the guideline limits. Any precautionary consequence from the in vitro results presented up to now should be carefully kept in mind until more scientific evidence will be available.

On the other hand, if future and well-designed confirmatory studies fail to replicate these in vitro effects, in line with the weight of evidence this would be a strong argument against any physiological relevance for adverse health effects caused by low level electromagnetic fields.

6 References

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