

DNA-directed effects of electromagnetic field exposure (Short abstract)

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Electromagnetic fields (EMFs) were reported to affect DNA integrity, but the reproducibility of these findings was debated. We were able to reproduce effects in the Comet assay with human primary fibroblasts exposed to 50Hz EMF and concluded from further investigation that these are unlikely caused by a direct DNA damaging effect of the field. Consistently, follow-up studies showed that reactive oxygen species are not detectably increased in EMF-exposed cells nor does exposure of isolated DNA *in vitro* induce measurable DNA damage. We conclude that the Comet effect observed with EMF-exposed cells reflect cellular responses associated with DNA metabolism rather than the DNA itself.

## DNA-directed effects of electromagnetic field exposure (Long abstract)

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Evidence from genotoxicity studies indicated that exposure of human cells to electromagnetic fields (EMF) may give rise to DNA damage in their genomes. Given the potential implications for human health, we decided to replicate the key experiments underlying these observations and to investigate molecular causes and biological consequences.

We first focused on the validation of the DNA-directed effects in the Comet assay as observed previously with EMF exposed human fibroblasts. We were able to reproduce a slight increase in the Comet tailfactor/tailmoment for 50Hz EMF-exposed human primary fibroblasts, whereas effects in RF-EMF exposed cells were less consistent. Briefly, we found that intermittent (but not continuous) exposure of human primary fibroblasts to a 50 Hz EMF at 1 mT induces a slight but significant increase of DNA fragmentation in the Comet assay. Investigating this effect further, we found that it was detectable only in proliferating cells and correlated with a slight reduction of actively replicating cells (visualized by BrdU incorporation) and a concomitant increase of apoptotic cells (assessed by Annexin V staining) in exposed cultures. These observations led us to conclude that the causes of the Comet tailfactor/tailmoment increase are related to secondary cellular responses (DNA metabolism, apoptosis/necrosis) to the field rather than a direct generation DNA damage (Focke et al., 2010). Regarding RF-EMF, we were not successful in reproducing a genotoxic effect on ES-1 fibroblasts as published before. HR-1d human fibroblasts, however, showed a very small effect with borderline statistical significance in Comet assays following exposure to a talk modulated field at a SAR value of 1W/kg (1950 MHz). Owing to the weakness of these effects both, reproducibility by visual and automated Comet-analyses and dose-effect relationships were not entirely consistent.

We went on to develop tools allowing a direct assessment of potential impacts of ELF-EMFs on the DNA itself, on DNA-protein/enzyme interactions, or on DNA-relevant aspects of cell physiology. This involved the establishment of biochemical assays as well as of life-cell imaging procedures. So far, we used these methods to address whether ELF-EMFs have a potential to induce physical damage to DNA or to alter formation or levels of reactive oxygen species (ROS) in cells.

To discriminate between potential direct and indirect DNA effects, we investigated the impact of ELF-EMF on isolated DNA *in vitro*, i.e. in the absence of the complex cellular environment. To this end, we optimized a classical plasmid relaxation assay, which allows detection of a single DNA lesion in relatively large DNA molecules. Undamaged bacterial plasmids exist in a compact “super-coiled” conformational state, facilitating the re-establishment of proper base-pairing following rapid denaturation-renaturation. By contrast, plasmids with DNA-strand breaks assume a relaxed ring form that is fully unwound during denaturation and therefore more difficult to restore upon renaturation. The plasmid relaxation assay did not reveal an increase of damage following exposure of purified DNA to an intermitted 50Hz ELF-EMF signal, and the same held true for DNA exposed continuously.  $\gamma$ -irradiation with 15 and 30 cGy, however, a dose close to the detection limit of a standard alkaline Comet assay (5-10 cGy), gave rise to statistically significant increases of detectable DNA damage in this assay. Thus, these data from controlled *in vitro* experiments show that the Comet effects observed upon ELF-EMF exposure of cells cannot be explained by direct induction of DNA damage as in the case of ionizing radiation and most likely reflect the consequence of a complex biological response to the field.

Several reports indicated an increased production of free radicals and/or oxidative DNA damage as a possible cause for ELF-EMF-induced effects in the Comet assay. To evaluate this hypothesis in our setting, we combined classical Comet analyses with a pre-treatment with a purine DNA glycosylase (bacterial Fpg protein) that efficiently excises 8-oxo-G, a major oxidative lesion, from DNA. Fpg converts oxidative DNA base damage to DNA strand-breaks that then become detectable in the Comet assay. The Fpg-treatment did not enhance the tailfactor difference between sham- and ELF-EMF-exposed cells, whereas it did in control experiments  $H_2O_2$  exposed cells. Since 8-oxo-G can be regarded a highly sensitive marker of DNA oxidation, these data suggested that EMF induced DNA strand-breaks observed in Comet assays are not primarily a consequence of oxidative DNA damage. However, to address this critical issue from another angle, we aimed to measure ROS production/levels directly in cells while they are under ELF-EMF exposure. To this end, we established a fluorescence-based method for ROS measurement in single cells. As a dye, we chose the cell-permeable, non-fluorescent carboxy-H<sub>2</sub>DCFDA, which is activated by intracellular esterases and oxidized by ROS, thereby converted to a green-fluorescence emitting molecule. The resulting fluorescence intensity within a cell is considered proportional to the amount of ROS produced. To achieve the highest possible sensitivity for the assay, we evaluated three different methods for fluorescence detection and quantitation; 1) fluorescence activated cell sorting (FACS), 2) fluorescence quantitation in a plate reader, and 3) microscopic scanning and analysis of individual cells. Of these, the microscopic analysis proved to be the most sensitive. After validating the newly established life-cell method by control treatments with  $H_2O_2$  at a low to intermediate dose range, we measured ROS level in two cell-types exposed to ELF-EMF under four specific conditions; short continuous or intermittent (5min ON/10min OFF) exposure at high field strength (2mT, 30min), and long continuous or intermittent (5min ON/10min OFF) exposure at lower field strength (1mT, 15h). The short exposure was done with U2OS human cancer cells and HR-1d human primary fibroblasts, while the long exposure was done with HR-1d cells only, that previously showed Comet effects under similar conditions. None of the exposure conditions tested produced any detectable increase in ROS formation, corroborating our previous conclusion that changes in Comet tailfactors following ELF-EMF cannot be explained by increased free radical impact.

If not by direct formation of DNA damage, ELF-EMF exposure may show effects in the Comet assay because it affects DNA repair or other cellular DNA-transactions. To provide experimental tools to test this idea, we evaluated the feasibility and reliability of biochemical assays with recombinant proteins and synthetic DNA substrates to monitor EMF-effects on DNA-protein interactions and activities of DNA processing enzymes. As a start, we chose the endonuclease APE1, a critical enzyme in DNA base excision repair dealing with the bulk of DNA damage occurring cells. In kinetic experiments, we did not find any influence of a 50Hz EMF on the enzymatic activity or stability of APE1. This observation suggests that the EMF-dependent Comet effects unlikely originate from reduced APE1 activity and the accumulation of AP-sites in nuclear DNA. The assay nevertheless showed that such experiments are feasible and will allow research into possible mechanisms underlying the EMF induced Comet effects. To this end, the assay can be adapted to examine all enzymatic steps of DNA repair and other pathways of interest (e.g. DNA replication), which will be necessary to clarify whether or not EMFs have the potential to interfere with enzymatic DNA transactions.

In conclusion, the Comet tailfactor/tailmoment changes detectable after ELF-EMF exposure are not likely due to an elevated production of ROS and generally cannot be accounted for by direct induction of DNA damage. They may however be caused by cellular responses to the field that indirectly affect steady-state levels of genomic DNA strand-breaks.