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# ATHEM 2
# Athermal effects of electromagnetic field exposure associated with mobile communications

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**Final report to the project**

# ATHEM-2

# Athermal effects of electromagnetic field exposure associated with mobile communications

**A research project of the AUVA (Austrian Workers Compensation Board) in collaboration with**
**MUW: Medical University of Vienna**
**SL: Seibersdorf Laboratories GmbH**

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## Contributions - Project roles

**Initiator and Sponsor:** Austria Workers Compensation Board (AUVA), Vienna

**Project management:** Austria Workers Compensation Board (AUVA), Vienna

**Project coordination:** SCIgenia Research Support GmbH, 1090 Vienna

**Design and Realisation of a Human Exposure system**: Seibersdorf Labor GmbH, EMC & Optics, Seibersdorf

**Research on human cognitive brain function under exposure:** Medical University of Vienna, Center of Public Health, Institute of Environmental Hygiene, Vienna

**Research on *in vitro* Genotoxicology:** Medical University of Vienna, Internal Medicine I, Dept. Inst. for Cancer Research , Vienna

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## Executive Summary

The ATHEM-2 research project (Athermal effects of electromagnetic field exposure associated with mobile communications) continued earlier research of the ATHEM-1 project (2002-2008).

Mobile phones receive and emit radio frequency electromagnetic fields (RF-EMF). Everyone using such phones is exposed to these fields. The ATHEM-2 project investigated cognitive effects of so called athermal intensity RF-EMF exposure and, in particular, possible genotoxic effects of this exposure on human cells. One project focus was on *in vitro* (laboratory) experiments designed to reveal possible mechanisms of interaction with the genome.

The study protocol was reviewed and approved by external experts. All experiments were performed under double-blind conditions in order to generate data of the highest possible quality. Neither the tested volunteers, nor the scientific personal were aware of the exposure conditions used during experimentation and during sample analysis.

Experiments on human test subjects involved exposure of the cheek mucosa to RF-EMF for a two hour period on five consecutive days. The opposite cheek served as a source of control material. Cognition tests performed during exposure revealed a decreased reaction time and an increased error rate. For more complex tasks such as memory tests, both reaction time and error rate were observed to be increased.

Moderate genotoxic and cytotoxic effects were found in mucosal cells harvested two and three weeks after RF-EMF exposure. Data relating to pre-study, high-level exposure, were, furthermore, consistent with a possible accumulation of such effects.

The *in vitro* experiments on genotoxicity confirmed the existence of two types of cells – those that are sensitive to non-ionizing radiation, and those that are not. In addition, the present experiments confirmed the existence of a latency period (time between start of exposure and first incidence of effects).

DNA damage was shown to be caused in sensitive cells by RF-EMF-induced DNA oxidation, which is associated with an increased likelihood of DNA strand breaks. These effects were particularly pronounced in cells under additional metabolic stress. In sensitive cells we observed the triggering of very specific DNA repair pathways by RF-EMF exposure. This, on the one hand, confirms the presence of DNA damage, and at the same time indicates that the damage can be repaired to some extent. Induced DNA damage was, indeed, undetectable two hours after cessation of RF-EMF exposure.

Our observations do not indicate any acute adverse health effects of RF-EMF exposure. Nonetheless, a potential long term risk can not be excluded. Therefore, several preventative measures for reduction of exposure and associated risks are specified. The two most prominent recommendations are to transport a mobile phone in a bag rather than directly on the body, and to reduce the exposure of the head by using a headset or hands-free equipment whenever possible.

# 1 OCCUPATIONAL HEALTH AND SAFETY

by Hamid Molla-Djafari[1]

## 1.1 BACKGROUND

It requires a scientific approach to improve occupational health and safety in an active and objective way. The Austrian workers compensation board (AUVA) has launched studies on radiofrequent electromagnetic fields (RF-EMF) previously. For example, a scientific investigation on corpses verified the relation between the level of immission inside the head and the relevant exposure limits some years ago.

Since the advent of digital mobile communication in the mid 90's the exposure of the general public and workers to RF electromagnetic fields of mobile phones has significantly increased. According to the International Telecommunication Union - a specialized agency of the United Nations – there are more than 7 billion SIM-cards for mobile phone use worldwide (ITU, International Telecommunication Union, 2015).

Today we face a new type of exposure to RF-EMF of the general public. The investigation of possible health risks is a public responsibility. In view of some open issues on possible adverse health effects of the radiofrequency emitted by mobile phones more research is clearly needed.

With mobile communications the exposure to electromagnetic fields in the RF range (RF-EMF) has increased both among the occupationally exposed and the general public. Questions about application safety are raised. Private insurance companies have received the recommendation by reinsurance companies to exclude possible health risks in connection with EMF exposure from their insurance policies.

The possibility to exclude risks *a priori* does not exist. Alternatively, possible risks may be met by well-directed research. Based on these consideration the ATHEM-1 project was started by the AUVA already in 2002, aiming to objectively investigate possible effects of EMF exposure on biological systems. During the ATHEM-1 project it was observed - and meanwhile confirmed by international research – that exposure to RF-EMF causes effects in certain types of human cells. In order to exclude a possible risk of cancer it would be important to describe exposure conditions where no indications of risks for humans are observed.

## 1.2 EXPOSURE LIMITS

Exposure limits and preventive measures concerning RF-EMF immission are based on scientific data. It is well established that current exposure limits for legislative purposes protect the population from adverse effects related to tissue-heating. In communication, the EMF intensities are so low that critical heating of exposed tissue cannot be expected.

[1] redacted by Klaus Schiessl

However, considering this new type of exposure - never before microwave transmitters were widely used so close to the head - so called near-field exposure to RF-EMF has become very common.

For estimating the risk of exposure, there are different and contrary views, concerns and agendas giving raise to conflicts. For scientists this is a challenging situation. Established knowledge and avaiable research does not allow for final conclusions. With this background, the research project ATHEM-2 - like its predecessor ATHEM-1 - investigates possible interactions between electromagnetic fields and biological matter.

## 1.3 THERMAL AND ATHERMAL EXPOSURE EFFECTS

Scientists report on and discuss so-called athermal (i.e. heating-independent) effects of RF electromagnetic fields already for a few decades. If athermal effects with relevance for health and well-being exist, concepts for protection based on thermal effects require revison and modification.

While validity as well as compliance with thermal-based limiting values are commonly accepted, adverse biological effects are under investigation for almost two decades. In 2012 the Italian supreme court awarded a vocational disability pension to a manager with brain cancer, linking the disabling tumor to mobile phone use (occupational exposure). Thus, the court has recognized causality between very long and frequent cell phone use and the disease. There are also some epidemiological studies that have found a slightly enhanced risk of brain cancer for persons with extremely frequent, ongoing (more than 10 years) cell phone usage.

## 1.4 SOCIETAL ASPECTS OF EXPOSURE LIMITS

The public debate on possible adverse health effects from low frequency electromagnetic fields was intensified by the categorization of extremely low-frequent magnetic fields as category 2B – „ Possibly carcinogenic “ by the IARC (International Agency for Research on Cancer).[1] Since May 2013 also RF-EMF have been categorized as 2B-“Possibly carcinogenic” (2B).[2]

Exposure limits - as a societal issue - are based on scientific arguments and on political decisions. Scientific data provide the base for such decisions. Due to the possibility of different interpretation of the data it can be difficult to establish consense. To generate exposure limits that are widely accepted in every-day life a critical mass of consistent scientific data is required.

Some experimental results may not indicate harmfulness *per se* (e.g. influence on cognition) but may be relevant if exposure is combined with specific situations (e.g. operating a machine, driving a car).

In view of the popularity and acceptance of RF-EMF emitting devices, it is a pragmatic approach to recommend precautionary measures to reduce the exposure when using this technology. On one hand the public has right of health protection, on the other hand, exaggerated regulations and overstated restrictions of use would hamper an

innovative and popular technology. Therefore, to decide on the level of preventative measures high-quality, independent research in the field is required.

## 1.5 OBJECTIVES OF THE ATHEM RESEARCH

Research on athermal RF-EMF-induced biological effects always requires scientists with various backgrounds. Engineers need to collaborate with biologists, often it is necessary to find a common language before open issue can be worked on in a team. The complexity of biological systems renders research and the description of subtle exposure effects very difficult. The research during the previous ATHEM-1 project could resolve some inconsistencies within the field and contradictory results published in the international literature. Within the ATHEM-1 project different cell-types cultivated *in vitro* were exposed to RF-EMF under well-controlled conditions. We found both, insensitive cells and cells sensitive to RF-EMF exposure. The latter revealed exposure related effects only after a certain latency time (time between start of the exposure and first observation of e.g. DNA-lesions). The results of this first ATHEM project have led to the recommendation of preventive measures aiming on the prevention of possible risks. An educational video with examples of prudent use of the mobile phones and preventive measures was produced.

During the ATHEM-2 project we approached open issues and planned work packages as follows:

1. Design, construct, and apply objective and reproducible exposure conditions for double-blinded exposure experiments on humans.
2. Double-blindly investigate the effects of exposure to RF-EMF on cognitive performance and memory tasks.
3. Investigate possible cellular and gentoxicologic effects in humans with double-blinded *in vivo* experiments with volunteers.
4. Investigate exposure related effects on cellular DNA in double-blinded *in vitro* experiments, and
5. Investigate the cellular mechanisms of exposure related DNA-damage.

## 1.6 RESEARCH QUESTIONS AND OBJECTIVES

The specific topics are relevant for preventative measures, communicated to the general public and work force. The specific questions were:

1. **Latency Time**: determination of the exposure duration before exposure related effects are observable.
2. **Recovery Time**: determination of the time cells need to repair exposure related DNA lesions after the end of exposure.
3. **Dose threshold**: determination of the intensity (SAR value) which does not lead to exposure related effects (find a NOAEL - No Observed Adverse Effect Level); this includes the search for a dose-response relationship.
4. **Youth related risk**: investigation of whether or not the exposure sensitivity of metabolically active cells (typical for young persons) differs from inactive cells.
5. **Combined risks**: Investigation of possible enhancement of other environmental DNA damage by RF-EMF exposure.
6. **Relevance** of positive *in vitro* results for humans.
7. **Effects on humans** on cognition and cell biologically.

# 2 SCIENTIFIC OBJECTIVES

by Wilhelm Mosgoeller

## 2.1 RESEARCH GOALS

A principal goal of the ATHEM-2 project was to obtain reliable results within a complex research field. Exposure to radio frequency-electromagnetic fields (RF-EMF) and subsequent laboratory analyses were performed rigorously double-blinded throughout the project. Whilst (naturally) seeking to generate results as quickly as possible, in the ATHEM-2 project the priority was to avoid premature conclusions, and obtain reliable results.

Epidemiological studies are most important for the assessment of a cancer risk. However, epidemiological data alone, i.e. without basic research addressing possible underlying mechanisms, provides little insight into how to manage a possible risk. Investigations of human cells such as those performed in both ATHEM projects are, therefore, especially important in this regard.

## 2.2 RESEARCH QUESTIONS

Research focused on the effects of exposure to the high frequency-electromagnetic fields used by mobile phones (i.e. UMTS signals with frequencies ranging from 1.92 to 2.17 GHz).

Specific Absorption Rate (SAR) was rigorously controlled in each experiment in order to exclude a contribution of heating effects to the observed outcomes. The SAR value is a measure of the electromagnetic energy absorbed by tissue and, consequently, of a raise of its temperature. The intensity of exposure to radiofrequency electromagnetic fields is normally limited during mobile phone use to avoid effects related to tissue heating. In the present study, all experimental exposures were kept low enough to exclude any heating effects. The associated biological effects are therefore termed “athermal” or “non-thermal” effects.

### 2.2.1 INVESTIGATING BRAIN FUNCTIONS (COGNITIVE EFFECTS)

The ATHEM-1 project (completed in 2008) found RF-EMF exposure to reduce reaction time, but to increase the frequency of incorrect decisions. The present project provided an opportunity to replicate the experiments.

### 2.2.2 HUMAN (*IN VIVO*) EXPERIMENTS

This subproject investigated the impact of RF-EMF exposure on humans and the underlying mechanisms of any observed effects. Specifically, we investigated whether DNA damage associated with RF-EMF exposure *in vitro* (under laboratory conditions) is relevant under *in vivo* conditions.

The buccal mucosa of adult volunteers was exposed to a defined electromagnetic field and, two and three weeks later, gently scraped with a small tooth brush to obtain epithelial cells for evaluation of exposure-associated changes.

Cells harvested from the opposite buccal mucosa served as intraindividual controls for each study subject, (i) because exposure intensity rapidly diminishes with increasing distance from the source and, (ii) because of the absorbing characteristics of the intervening tongue tissue. Two different exposure intensities were used to allow evaluation of possible dose-response effects. All exposure parameters (intensity, exposed cheek side) were recorded under double-blinded conditions.

### 2.2.3 *IN VITRO* RESEARCH, INVESTIGATION OF DNA DAMAGE

Athermal RF-EMF exposure-related effects on cellular DNA were described by scientists, long before the start of the ATHEM-2 project, in laboratory animals.[3, 4] Discontinuous exposure of cultured cells (*in vitro*) lead to stronger effects than those observed following continuous exposure.[5, 6] Generally speaking, any exposure-related increase in DNA damage may reflect a long term risk, as already acknowledged in an Italian supreme court decision[2]

The ATHEM-1 project studied cultured human cells using two complimentary methods. DNA lesions and changes in protein synthesis were observed within the very same (sensitive) cells, whilst other cells exhibited no exposure-related alterations. The protein findings in the very same cells[7] implicitly confirmed the vulnerability of cells containing exposure-related DNA lesions. These findings have meanwhile been reproduced by international researchers.[8-10] RF-EMF exposure-associated DNA damage has also been observed in animal experiments.[4, 11, 12] The cellular mechanism underlying the formation of DNA lesions in sensitive cells was, however, unknown and/or a subject of scientific debate at the start of the project.

The EU REFLEX project found intermittent exposure to be biologically more effective than continuous exposure. Intermittent exposure conditions were, therefore, used for all cell experiments conducted in the course of the ATHEM-2 project. The exposure was in cycles of 15 minutes, the field was switched “on” for 5 minutes, and 10 minutes “off”, i.e. the field was effectively only “on” for one third of the exposure duration). This intermittent exposure results in a higher rate of DNA formation than continuous exposure. In other words: an association of increased damage with a decreased energy input provides strong evidence for a lack of correlation between the amount of electromagnetically induced heating and the extent of DNA damage. These effects are thus deemed “athermal”.

At first glance, research addressing the relationship between RF-EMF exposure and DNA damage has, to some extent, generated contradictory results. Many such “contradictions” can, however, be explained if exposure duration is considered. Negative results with short exposure duration (<2 hrs)[13-16] do not necessarily contradict positive effects following longer exposures.[8, 10] This issue was explored in

[2] In 2012, the Italian supreme court recognized a manager’s brain cancer as having being induced by heavy mobile phone use

greater depth in the ATHEM-2 project by investigating cells subjected to different exposure duration .

The following observations resolve other so-called “contradictions”, simply by taking into account some pertinent co-variables :

**Cell type:** Most investigations to date were done on lymphocytes.[15, 17-25] Exposure of these cells to RF-EMF typically does not cause DNA lesions, i.e. lymphocytes appear to be resistant to RF-EMF exposure. This finding does not, however, preclude the sensitivity of other cell types to RF-EMF exposure. A variety of studies describing sensitive cell types have been published since the start of the ATHEM-2 project, which include fibroblasts, neurons, trophoblasts, CHL cells and lymphoblastoid cell lines.[5, 6, 8, 10, 13, 26, 27] The ATHEM-2 project therefore investigated a range of cell lines in order to once more identify sensitive and insensitive cell types, and then focused on the cellular mechanisms underlying sensitivity in order to contribute to on-going debates.

**Latency time:** Exposure to athermal RF-EMF intensities, unlike exposure to ionizing radiation, does not yield immediately observable effects, particularly with short exposure duration. This indicates that different cellular mechanisms are linked to DNA damage caused by exposure to non-ionizing radiation. The latency time in sensitive cells (time interval between the start of exposure and the observation of lesions) varies from research team to research team (detection methodology), depends on the specific experimental setting and on the cell type. Published latency times range from 20 minutes,[9] through four hours,[7] to 16 hours.[8]

As described later in this report in more detail, oxidative stress and oxidation of DNA are key to understand RF-EMF exposure-related DNA damage. Oxidative status as a confounder of sensitivity could explain why some cells with a higher metabolic status, and therefore oxidative rate that is further increased by exposure to RF-EMF, are more sensitive to RF-EMF exposure than metabolically inactive cells.

**Recovery time:** DNA lesions in sensitive cells disappear after a certain time period following cessation of exposure. The ATHEM-1 project reported a recovery time of two hours for protein effects[7], in agreement with the findings of Franzellitti, et al. [8]. The ATHEM-2 project included a systematic investigation of the recovery time duration.

# 3 HUMAN EXPOSURE SYSTEM

Title of subproject:

## Exposure setup for provocation studies concerning possible effects of electromagnetic fields of UMTS mobile phones on the buccal mucosa

Acronym: Double blinded human-exposure system

A subproject of the research programme

## ATHEM 2

Prinicipal investigator and responsible author of report:

Gernot Schmid

Co-workers:

Stefan Cecil, Richard Überbacher

Seibersdorf Labor GmbH,

Section: EMC & Optics

## 3.1 BACKGROUND

In the last years, a multitude of *in vitro* studies have been published about the question of possible effects of radio- and microwave electromagnetic fields on the cellular level. Nevertheless, the data are not consistent.

For the comparison of *in vitro* results with results from human tests in the project ATHEM-2, we use the same type of electromagnetic fields in the exposure system for human experiments, as have been used for the exposure of cultured cells *in vitro*. For the *in vitro* experiments the facility sXc 1950 (IT'IS Foundation, Zurich, Switzerland) has been used for the cell expousre with UMTS signals. For human provocation experiments we developed an exposure system to deliver UMTS signals with characteristics as similar as possible to those used in the vitro experiments to targeted epithelial cells of the buccal mucosa. After exposure the buccal mucosa cells of the test subjects were harvested by standard smear procedures in certain time intervals, and were investigated for cytotoxic and gentoxic effects.

In the past, different concepts for head exposure setups in human provocation studies have been realized. Besides biological/medical aspects, the concept and the detailed and thorough dosimetric analysis of the exposure system are fundamental parts of a study design with high validity. Precisely defined exposure conditions and minimum inter- as well as intra-individual variations (in terms of radiation absorption in tissues) are key features of a high quality exposure setup. Particularly in the case of near field exposure, i.e., when the radiation source is close to the human body (head), very small variations of the relative position between head and antenna may lead to high fluctuations in the resulting absorption inside the tissue. An imprecise definition and control of the antenna position may therefore inevitably cause high intra- and inter-individual fluctuations of the exposure.

As the exposure system has to meet conditions governed by biological/medical considerations, a compromise between optimized exposure conditions and usability has to be found.

## 3.2 REQUIREMENTS FOR THE EXPOSURE SYSTEM

In this project, the buccal mucosa was the main target tissue because:

- the exposed cells can be easily (non-invasively) harvested
- the relatively short cell cycle of the buccal mucosa cells allows to analyse effects at different stages of cell maturation within 2-3 weeks

In the project ATHEM-2 the investigations focussed on possible effects of Radio Frequency ElectroMagnetic Fields (RF-EMF) of UMTS mobile phones on the cells of the buccal mucosa. In cooperation with the medical project partners, the following requirements for the exposure system were defined:

- minimal irritation and impairment of the volunteers
- randomized exposure, double blinded, on left or right side of the head
- two different exposure intensities (low/high)
- the exposure intensitiies close to the official exposure limits (SAR partial body limits for general population)
- minimize the exposure uncertainty (intra- and interindividual variability)
- high power efficiency (minimize amplifier costs)
- double blinded applications of the exposure by user-friendly control software
- monitoring and recording of the exposure-relevant system parameters during the experiments for quality assurance and safety of the volunteers in case of system failure

## 3.3 EXPOSURE SYSTEM CONCEPTION

### 3.3.1 OVERVIEW

Figure 3.1 shows the basic concept of the exposure system. During the exposure, the test subjects had a head set with the signal emitting antennas mounted on their head, and performed cognitive and memory tests on a computer while beeing exposed to RF-EMF. The whole setup was in a cabin (not shown) with RF-absorbers on all 4 side walls. The RF signal is generated and amplified according to the respective exposure setting, then transmitted to the right or left antenna of the headset by computer controlled switches (controlled by the exposure software, blinded for both volunteeer and experimenter). The actual exposure data were recorded in 10 second intervals and stored with a timestamp in encrypted form on the control computer.

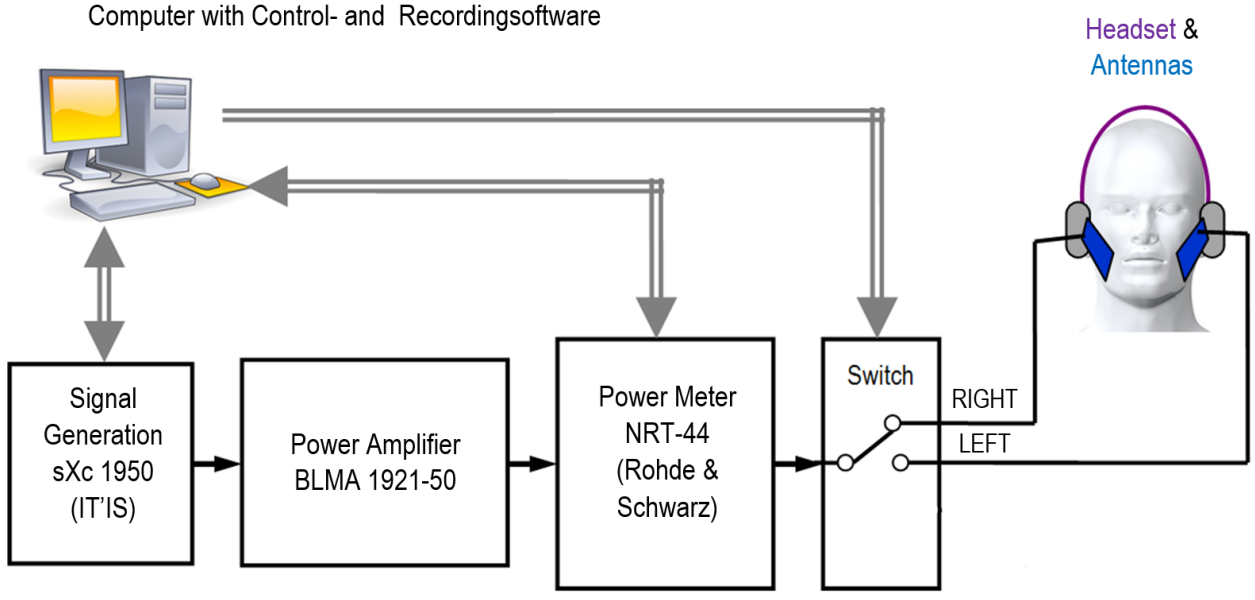


Figure 3.1: Schematic representation of the exposure system

### 3.3.2 SIGNAL GENERATION AND AMPLIFICATION

The generator for the RF signal creates an UMTS signal that has been developed by the IT'IS Foundation for exposure of cells *in vitro*. Basically the variability of possible signal characteristics of modern mobile communication technology is almost infinite, therfore only representative examples of signal characteristics can be chosen for provocation studies. For this project we chose signal characteristics leading to maximum power fluctuations of the RF signal.

In contrast to GSM phones UMTS-phones do not use a time division multiple access (TDMA) algorithm. UMTS phones do not transmit RF bursts but rather transmit a coded broadband signal. Nevertheless, due to the very efficient transmit power control implemented in UMTS-phones, UMTS mobile phones very unlikely transmit a continuous RF signal. In fact the transmission power is adjusted 1500 times per second in order to optimize the energy needed for ideal communication quality (receiving conditions). Because the fluctuations of the emitted power are determined by the receiving conditions, it is - in contrast to GSM, impossible to predict typical time domain signal characteristics for any UMTS transmission. Because it may be the low frequency spectral components to contribute to biological effects, a synthetic signal should contain a maximum of low frequency spectral components in the signal envelope.

Figure 3.2 shows the time course of the signal envelope of the used UMTS exposure signal (frequency 1950 MHz, bandwidth 5 MHz). The envelope of this signal contains many low frequency components up to 1500 Hz. This signal has been used in a variety of (international) *in vitro* studies. Therfore in the project ATHEM II, it was employed for the *in vitro* experiments during the project. Further details about the used signal can be found in.[28]

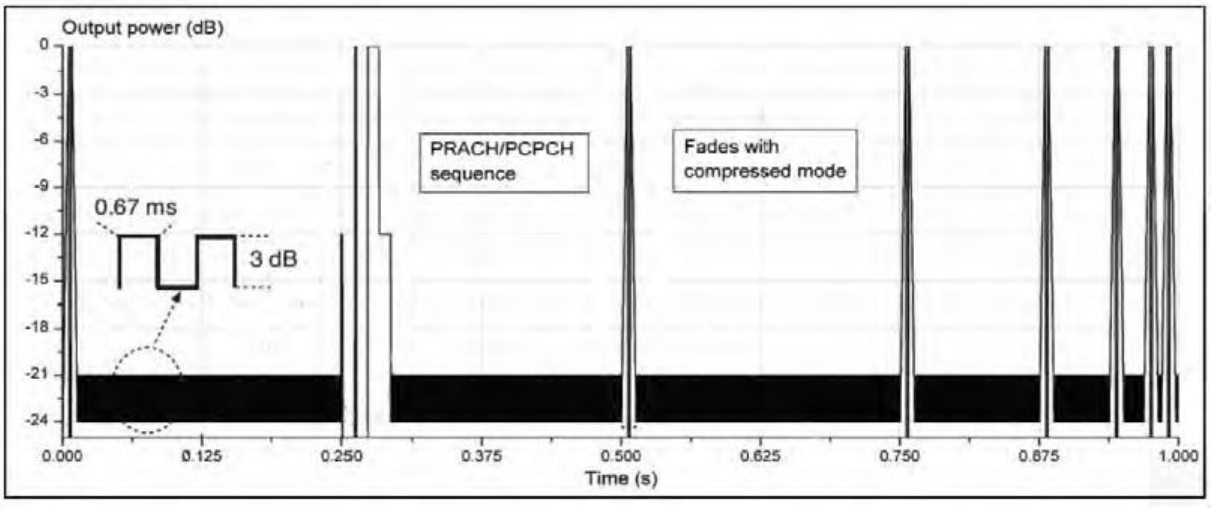


Figure 3.2: Schematic representation of the exposure signal

For the amplification we used a 50 W linear power amplifier model BLMA 1921-50 (Bonn Elektronik GmbH, Germany). All components for signal generation, signal amplification, and power measurements were built into a lockable 19“ rack (Figure 3.3). During the doubleblinded exposure experiments, the front panel of the 19“ rack was

covered by a intransparent foil to avoid possible unblinding due to a display-function of the locked in apparatus.

Figure 3.3: Signal generating hardware, amplifier and power meter, built in a 19“ rack (background) and laptop computer with control and recording software (foreground), installed at the Institut für Umwelthygiene, Kinderspitalgasse, 1090 Vienna

Basically there are 4 different exposure conditions (test conditions):

1. High exposure on the right side of the head (HR)
2. High exposure on the left side of the head (HL)
3. Low exposure on the right side of the head (LR)
4. Low exposure on the left side of the head (LL)

We used cells from the opposite (contralateral) side of the head as “non-exposed” or sham exposed control of the buccal mucosa.

One goal of the test system design was to achieve an average exposure level of 1.6 W/kg for the “High exposure” condition in the area of the buccal mucosa. The field intensity in the “Low exposure” condition should be sufficiently below the condition “high” to achieve a clear difference (without overlap) between high and low exposure.

### 3.3.3 HEADSET AND ANTENNAS

To minimize the interindividual variations of the exposure the relative position between antenna and head needs to be constant. Therefore the antennas were mounted on a headset, worn by the test subjects like a stereo headphone (Figure 3.4). The UMTS signal input to the antennas was delivered via a thin coaxial cables from above. The antennas were kept in constant position and distance to the cheek by spacers made of plastic, a material that does not interfere with the RF-EMFsignal.


Figure 3.4: Test subject wearing the headset with the antennas

The antennas were patch antennas (backplane 95 x 55 mm$^2$, patch 70 x 36,5 mm$^2$), operating at an frequency of 1950 MHz. They were arranged in a case of Rohacell® which is extremely light weight, and RF-transparent, with high mechanical stability. Figure 3.5 and Figure 3.6 show details of the antennas. On the facial side of the antenna case spacers are applied to ensure appropriate distance to the cheek. The correct adjustment for each individual, all 3 spacers had to gently touch the outer side of the cheek to realize a distance of 15 mm between patch antenna and cheek.

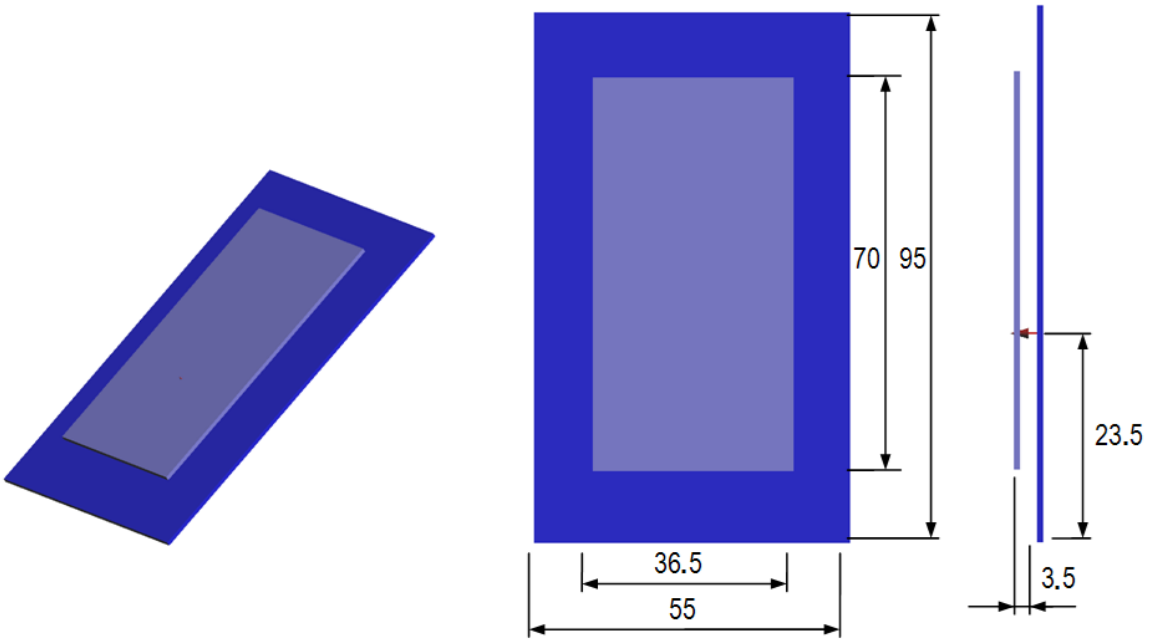


Figure 3.5: Scheme of the used patch antennas

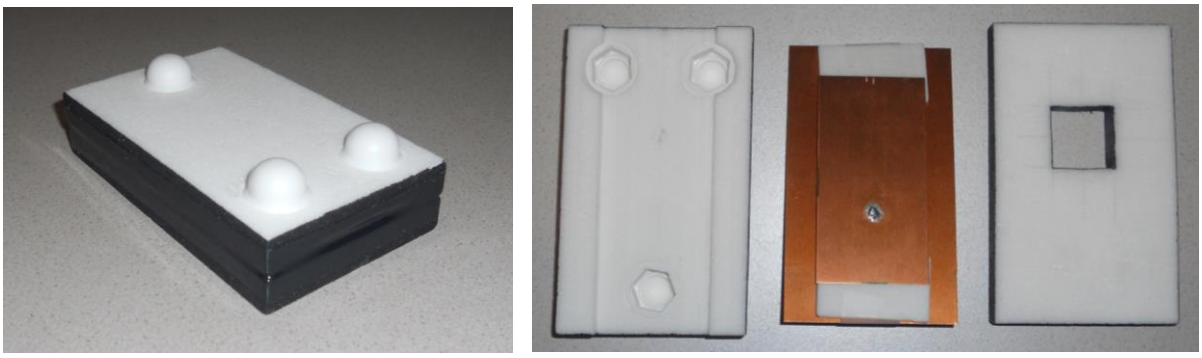

Figure 3.6: Pictures of the patch-antennas; left: antenna in a case of Rohacell®, right: opened antenna case (left: inside view of the case front, mid: antenna with flat spacers of Rohacell® for the patch, right: inside view of case back with the hole for the antenna connector)

We considered it important to avoid possible warming of the antenna, that could be perceived by the test subjects, which would pose a risk for premature unblinding of the test conditions. Measurements of the warming on the surface of the antenna at an input power of 1 W yielded a temperature elevation of less than 1.5 °C direct at the antenna surface (patch). This implies that considering the input power during the experiments (≤ 90 mW, see chapter 3.4.4) a noticeable warming can be excluded.

### 3.3.4 EXPOSURE CONTROL AND DATA RECORDING

The control and recording software (programmed in LabView, Version 8.0) offered an easy to understand and robust user interface. The application automatically controlled pseudo-randomly the exposure conditions in a double blinded way.

The experimenter chose the actual proband charactized by an unique ID. After headset positioning the experimental session was started on the computer. The sequence of presentations started automatically with the pseudo-randomly chosen exposure (LEFT/RIGHT, HIGH/LOW) for the pre-set exposure duration. During the whole session, the exposure parameters (including forward and backward power) were continuously measured and recorded. Together with possible interruptions, early abortions or discontinuities of any kind, the measured exposure data were encrypted, stored and secured on the control computer. One function of the software was to inform the experimenters in case of any system errors (defect of the antenna, cable break, etc.). The system would have given an alarm and the actual session gets interrupted. However, during the project all components worked properly. Figure 3.7 and Figure 3.8 show two exemplary screenshots of the control and saving software.

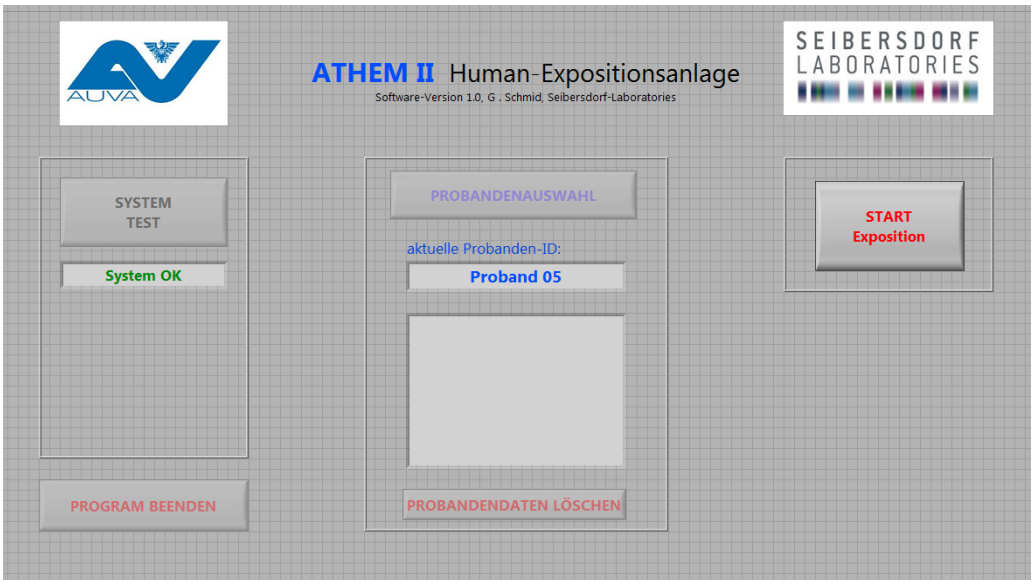


Figure 3.7: User interface of the control and data management software before exposure

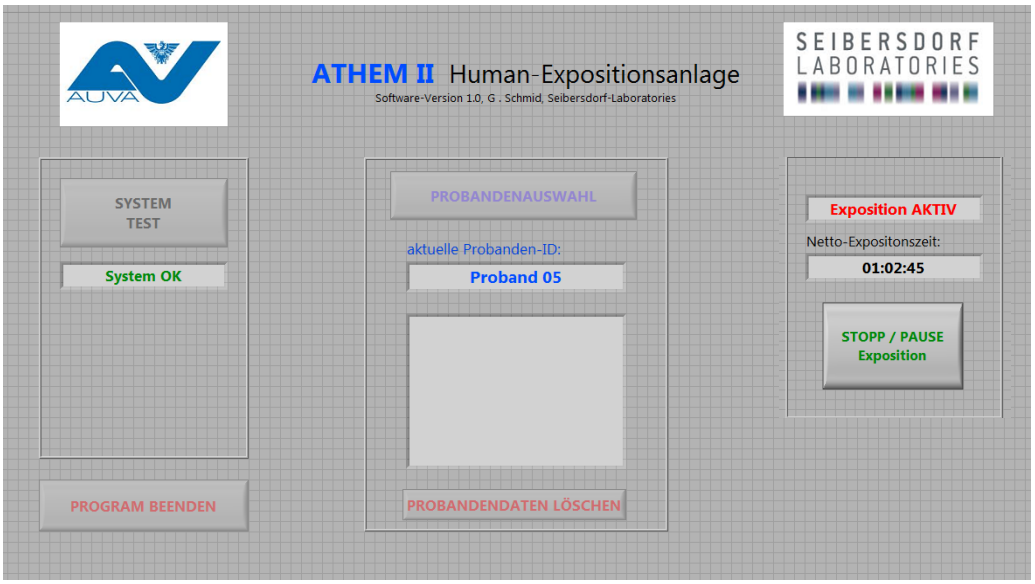


Figure 3.8: User interface of the control and data management software during exposure

## 3.4 DOSIMETRY AND DOSE VARIABILITY ESTIMATION

The dosimetric evaluation was an essential task to ensure a reliable implementation of the provocation studies. The most important steps were:

- **Selection of the antenna concept**: Already at this step, we employed simple numerical calculations or experimental measurements with antenna-prototypes to clarify whether or not the planned antenna concept can fulfill the requeirements (with regard to efficiency and radiation characteristic).
- **Development of a numerical antenna model:** Because the the RF absorption in various anatomical regions of the head and any part of the body cannot be determined experimentally, the dosimetric calculations were done on complex computer simulations and based on detailed numerical and anatomically correct head models. The development of such a numerical antenna model can be a challenging task. Finally the developed numerical antenna model was evaluated via computer simulations and experimental measurements to describe its accuracy.
- **Dose-finding:** After an antenna model which reflects the real antennas correctly was found, the next step was the dose-finding. For this task computer simulations with detailed anatomically correct head models were used to find out out how much RF antenna input power was required to obtain the desired SAR in the target tissues. For this specific task cable losses and antenna adjustment (and possible de-tuning of the antennas close to the head) were considered. We did not consider simplified head models (e.g. the standardized SAM-phantom for approval procedure for mobile phones) as useful approach for the dose finding. The SAM head model (and its dielectrical properties) is considered a conservative model, which overestimates the actual absorption in the human head, even by averaging over all different tissues.

- **Uncertainty estimation:** Finally the uncertainty range was implemented for the developed exposure system, considering possible intra- and interindividual variations of the exposure. We considered this step essential, because the technical design of the exposure system can have a significant impact on the variability. The most important input values for the uncertainty estimations are:

  - Variations (even small ones) of the relative position between antenna and head
  - Different sizes and forms of the head of the probands
  - Different dielectric properties of the tissues between the probands
  - Stability of the signal source and the RF amplifier (mostly neglectable compared to the other uncetrainty contributors)

After a literature search on the tissues in the area of the cheek, the optimization of the geometry of the antenna has been implemented on a numerical way based on numerical calculations with a simplified planar tissue model. The geometrically optimized antenna was built as prototype, and the corresponding numerical antenna model was validated. For dose-finding and estimation of the expectable uncertainties and the inter-individual range of variations of the relevant dosimetric quantities, numerical calculations with planar tissue models have been realized. The thicknesses of the tissues and the dielectric parameters have been varied between anatomical and physiological reasonable borders, independent from each other (dielectrical tissue parameter electrical conductivity $\sigma$ and relative permittivity $\varepsilon_r$, each one ±20% from the nominal value according to Gabriel, et al. [29] Finally, with numerical simulations using anatomical head models we tested how the dosimetric results from the calculations corresponded to the the planar tissue model calculations. All numerical calculations were done with the simulation platform SEMCAD X (Version 14.4, Schmid & Partner Engineering AG, Zurich, Swiss).

### 3.4.1 VALIDATION OF THE ANTENNA

For validation we performed SAR-measurements with the antenna in front of a homogenious flat phantom ($\sigma$=1,4 S/m $\varepsilon_r$=38) (Figure 3.9) and compared the the measurements with the numerical calculation. The tested distances to the flat phantom were 10 mm, 15 mm and 20 mm.

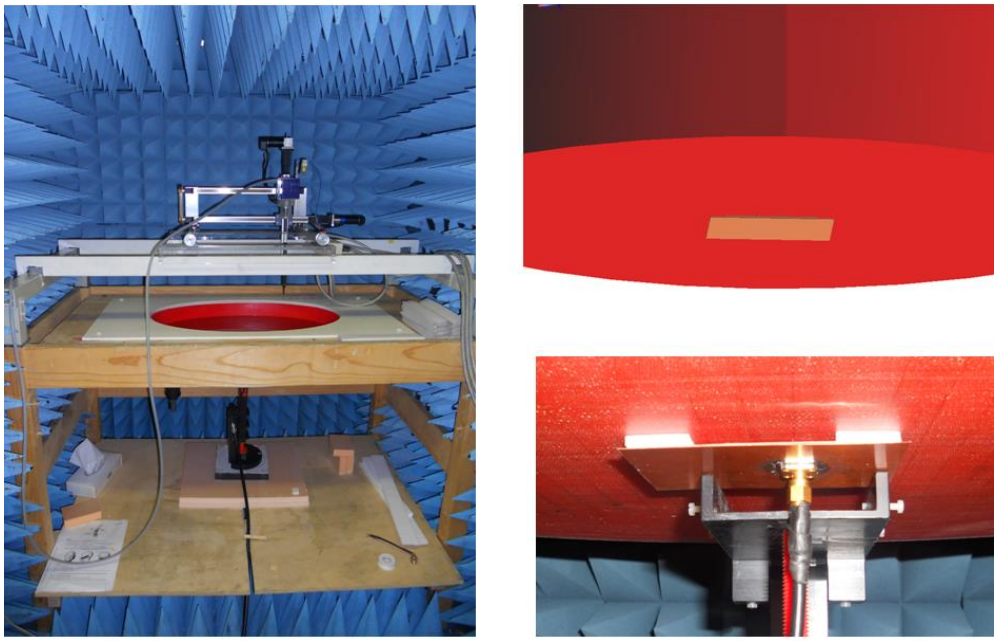

Figure 3.9: Arrangement for antenna validation, left and downright: measuring arrangement, upright: computed model

The match between measurements and calculations was satisfactory. The local SAR-distribution showed highest SAR deviation below ± 30%, the variabilityof the SAR averaged over 10 g (maxSAR10g) was less than ± 9%.

Furthermore, measurements and calculations with the homogenous flat phantom revealed a power efficiency of the maxSAR10g of about 22 W/kg per Watt antenna input.

### 3.4.2 NUMERICAL CALCULATIONS WITH PLANAR TISSUE MODELS

To calculate the SAR for the validated antenna model, we performed dosimetric calculations with a planar tissue model. Figure 3.10 shows the tissue model with skin, fat, muscles, buccal mucosa and attached another tissue in the oral cavity. The thicknesses of the layers and the dielectric tissue parameter were varied independently from each other in anatomically and physiologically reasonable boundaries (dielectric tissue parameter electrical conductivity $\sigma$ und relative permittivity $\varepsilon r$, each one ±20% from the nominal value after Gabriel, et al. [29]).

Table 3.1 shows the range of tested thicknesses of the considered tissue-layers. The oral cavity has been tested independently from each with the properties of 1) air, 2) tooth and 3) tongue, to estimate the possible dosimetric variation, i.e. varibility of the SAR in the buccal mucosa. The distance between the antenna and the model surface (skin) was 15±3 mm.

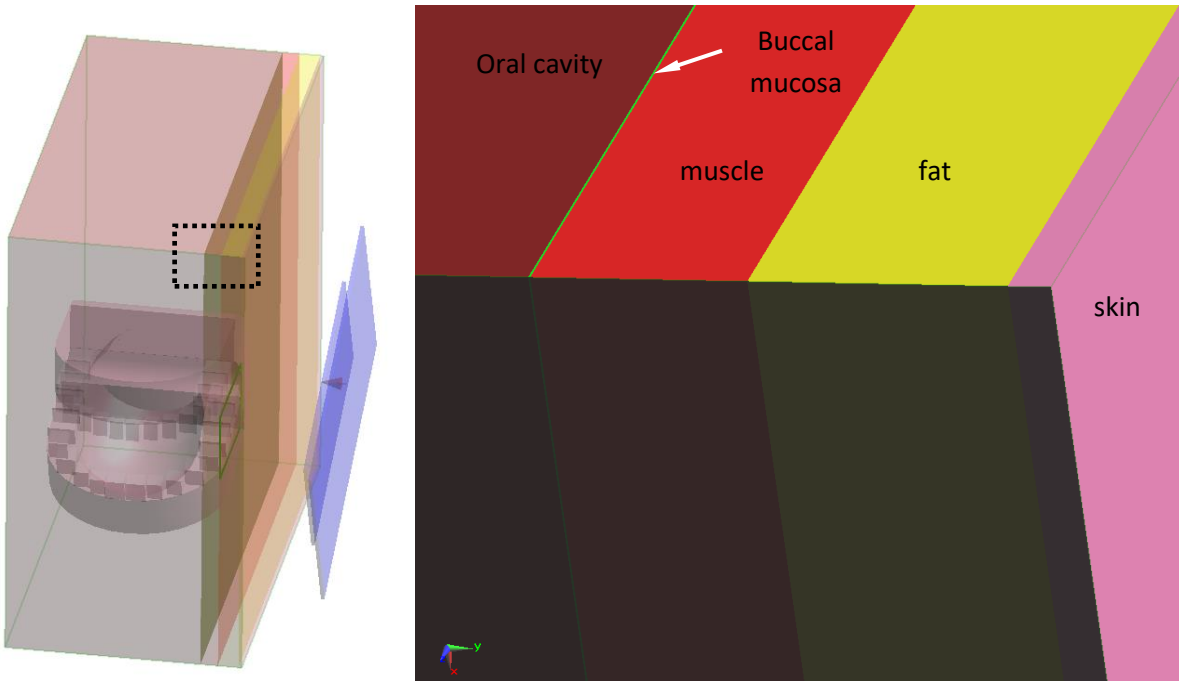


Figure 3.10: planar tissue model

Table 3.1: Tested thickness range of the tissues. The distance between antenna and skin was set to 15 ± 3 mm.

| Tissue | Average thickness | Thickness Range |
|---|---|---|
| Skin | 1.5 mm | 1 mm …. 2 mm |
| Fat | 6 mm | 3 mm …. 9 mm |
| Muscle | 5 mm | 2 mm …. 8 mm |
| Buccal mucosa | 50 µm | 20 µm …. 50 µm |

After 36 simulations the averaged SAR in the area of interest (green rectangle in Figure 3.11) of the buccal mucosa was found to be 18.5 W/kg (± 55%) per Watt antenna input.

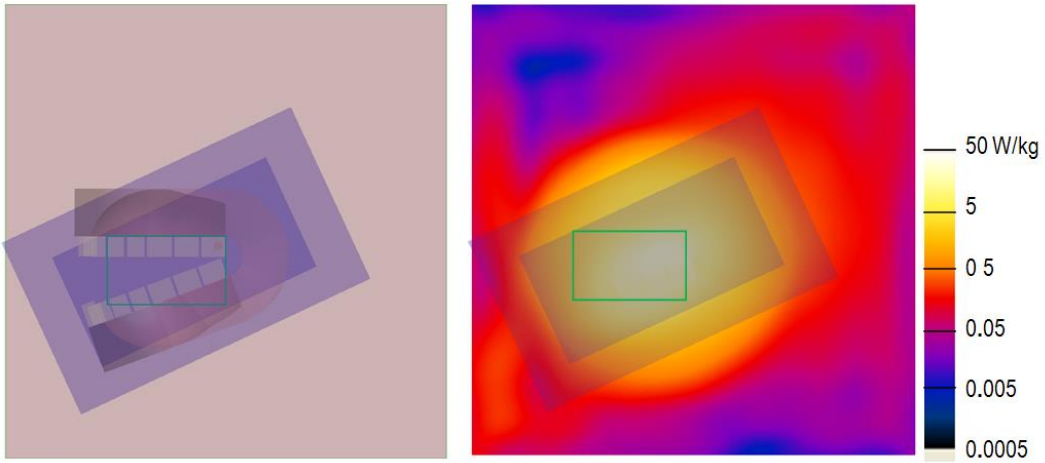


Figure 3.11: SAR-distribution in the buccal mucosa of the planar tissue model in nominal situation d = 15 mm at an antenna input power of 1 W

### 3.4.3 NUMERICAL CALCULATIONS WITH ANATOMICAL HEAD MODELS

For the estimation of the inter- and intra-individual expected SAR variability, different positions of the antenna relative to the head have been calculated based on the three head models. Additionally we varyied the dielectric tissue parameters (±20% from the nominal values according to Gabriel, et al. [29], the antennas were moved up, down, forward and backward by 10 mm from the nominal position. Furthermore we varied the distance between surface of the antenna and the skin by ± 3mm, and we studied the influence of metallic tooth fillings.

The calculations were performed with three different anatomical head models to cover a wide spectrum of the antecipated anatomical variabilty in the area of the cheeks (Figure 3.12). As the currently available anatomical head models offer only a limited spatial resolution, we added a representation of the buccal mucosa on each side of the head with about 35 µm thickness.

After 35 simulation runs, the averaged SAR over the area of interest in the buccal mucosa was 12.8 W/kg (± 49.7%) per Watt antenna input. The maxSAR10g was 19.0 W/kg (± 27.6%) per Watt antenna input.

The analysis of the homogeneity of the SAR in the target area showed, that at nominal antenna position over 76% of the mucous tissue were exposed to more than 50% of the SAR, and 93% of the Mucosa was exposed within over 32% of the maximum SAR value.

We found that metallic tooth fillings with direct contact to the buccal mucosa can lead to local SAR superelevations and modify the homogeneity of the exposure. Therefore, test persons with extensive tooth fillings were not recruited for the provocation tests.

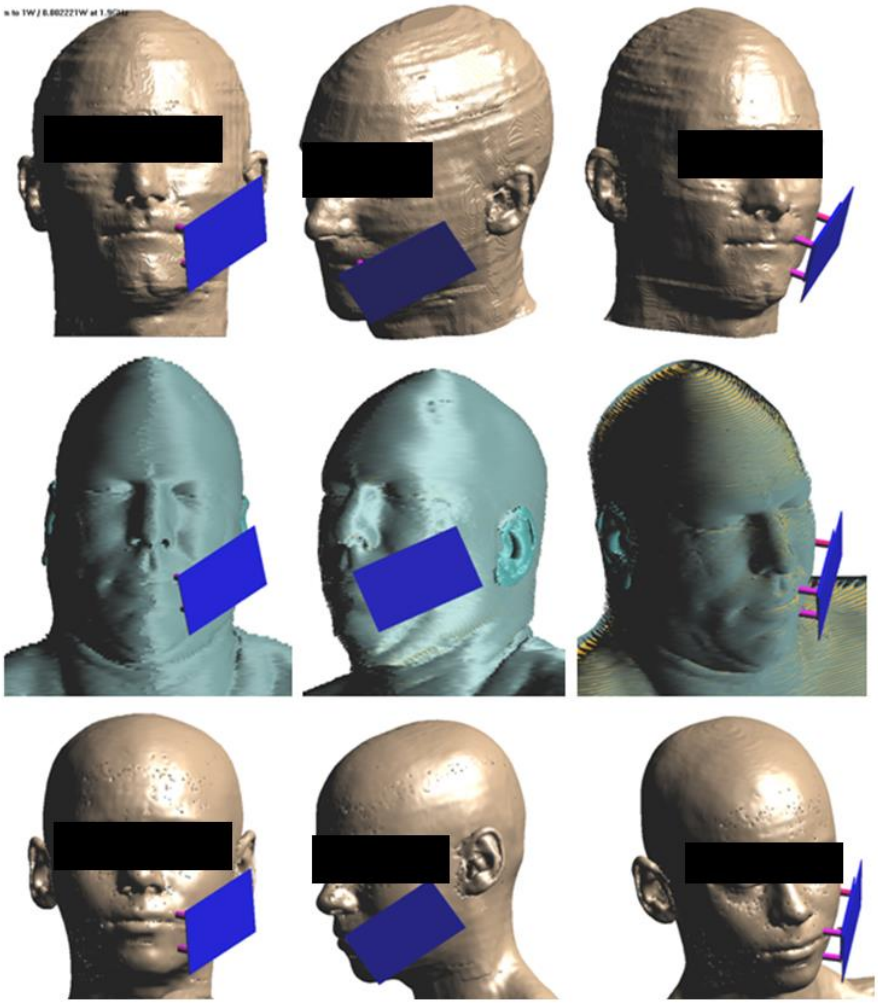

Figure 3.12: anatomical head models with antenna in normal position.

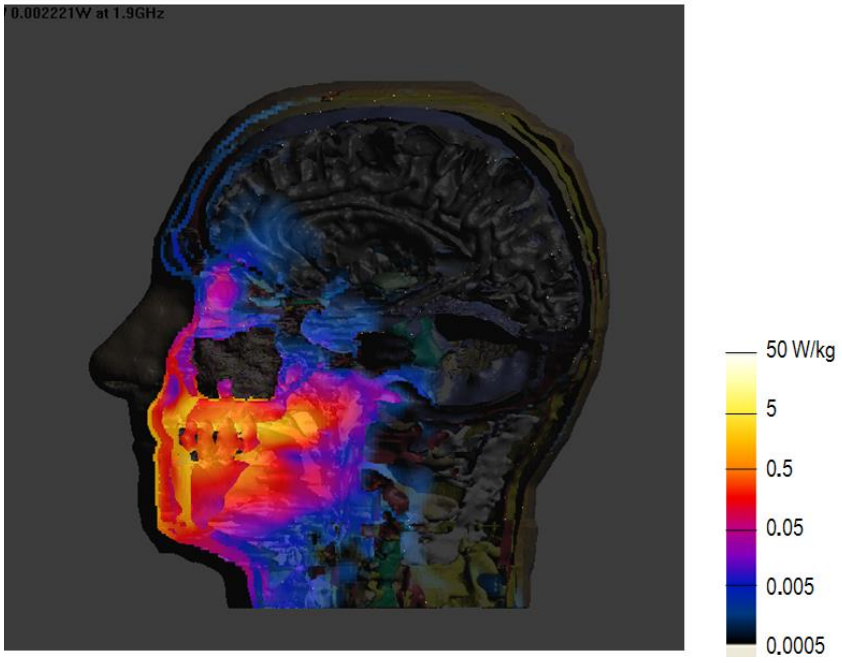


Figure 3.13: vertical SAR distribution through a part of the (arched) buccal mucosa of the anatomical head model “Duke” (34 year old adult) in standard situation d = 15 mm.The antenna input power was 1 W.

### 3.4.4 DEFINITION OF THE EXPOSURE LEVELS AND ANTENNA INPUT POWER

On the one hand, the exposure in the target area of the buccal mucosa should be as high as possible, on the other hand the maxSAR10g in the head of the probands must not go beyond the exposure limits determined by the International Commission for non ionizing radiation protection (ICNIRP; SAR of 2W/kg) for ethical reasons. The measurements and calculations at the homogeneous flat phantom which can be seen as conservative model, as well as from calculations at the anatomical head models, revealed a maxSAR10g of about 22 W/kg per Watt antenna input power. For the high exposure condition the effective antenna input power was 90 mW. At this antenna input power an averaged SAR in the area of interest (buccal mucosa) is 1.15 W/kg (± 49.7%, from calculations of anatomical head models) or 1.67 W/kg (± 55%, derived from calculations with the planar tissue model). Taken the results of both models together and estimate conservatively the average SAR in the buccal mucosa the SAR ranges from 0.58 – 2.59 W/kg.

Due to the inhomogenous SAR distribution (93% area, above 32% of the maximum SAR value), during “high exposure” in 93% of of the target area the exposure is above 0.19 W/kg. For the lower exposure intensity we chose a 15x lower SAR value as compared to the high exposure intensity. Thus, enough spacing and no overlap between the two exposure intensities for the human provocation tests can be guaranteed. The averaged antenna input power in the higher and lower exposure levels was:

$P_{ant,HIGH}$: 90 mW $\Rightarrow$ $SAR_{Mucosa\_avg,\ HIGH}$=1.59 W/kg (± 63%)

$P_{ant,LOW}$: 6 mW $\Rightarrow$ $SAR_{Mucosa\_avg,\ LOW}$=0.11 W/kg (± 63%)

### 3.4.5 EXPOSURE OF THE CONTROL SIDE (SHAM EXPOSURE)

For the statistical analysis, the buccal mucosa cells harvested from the contralateral (opposite) buccal mucosa served as non-exposed controls. We also determined the resulting (unintended) exposure on the opposite mucosa, based on numerical calculations by simulation of the cases shown in Table 3.2. We simulated various situations considering both, the EMF propagations in the mouth and coupling effects at the antenna at the opposite side (the inactive antenna was terminated with a 50 Ω load), and assuming a thin air gap (1-5 mm) between the palate and the tongue, or as worst case “no tongue”

In the worst case (no tongue) the contralateral exposure is increased 3 times but still clearly below the ipsilateral exposure level. It can be neglected even after the uncertainty of ± 63% is added.

| SAR-ratio ipsilateral / contralateral | | | |
|---|---|---|---|
| | | maximum SAR | average SAR |
| „normal tongue“,<br>small air gap between tongue and palate | | 24.6 dB<br>(factor 288) | 23.9 dB<br>(factor 245) |
| „without tongue“<br>(tongue modelled as air) | | 20.3 dB<br>(factor 107) | 19.2 dB<br>(factor 83) |
| SAR-variation ipsilateral / contralateral in extreme case „without tongue“ | | | |
| | | maximum SAR | average SAR |
| High exposure level | Ipsilateral | 6.90 W/kg | 1.73 W/kg |
| | Contralateral | 0.083 W/kg | 0.016 W/kg |
| Low exposure level | Ipsilateral | 0.45 W/kg | 0.12 W/kg |
| | Contralateral | 0.0054 W/kg | 0.0011 W/kg |

Table 3.2: Results of the calculation concerning (unwanted) exposure on the contralateral side.

## 3.5 EXPOSURE DATA, ANALYSIS AND UNBLINDING

The recorded exposure conditions during the tests were analysed in detail. All exposure parameters were lying within the expected ranges. Therefore the exposure conditions are within the predictions and valid. On December 9th, 2014 the exposure data were deblinded partly, on December 29th, 2014 the data were unblinded completely. Table 3.3 presents the exposure data allocation to the test people in anonymisied form (test people names in alphabetic order).

| No | Test person | Exposure condition | No | Test person | Exposure condition |
|---|---|---|---|---|---|
| 1 | Al. | HIGH RIGHT | 22 | Mü. | HIGH RIGHT |
| 2 | Am. | LOW LEFT | 23 | Op. | LOW RIGHT |
| 3 | Ba. A-K | HIGH LEFT | 24 | Pr. | LOW LEFT |
| 4 | Ba. | LOW LEFT | 25 | Ra.*) | HIGH LEFT |
| 5 | Be. | HIGH RIGHT | 26 | Rei. | HIGH LEFT |
| 6 | Br. | HIGH LEFT | 27 | Scha. | LOW LEFT |
| 7 | Fr. | LOW LEFT | 28 | Schi. | HIGH LEFT |
| 8 | Fu. | HIGH LEFT | 29 | Schn. | HIGH LEFT |
| 9 | Gr. | HIGH RIGHT | 30 | Schr. | LOW RIGHT |
| 10 | Gu. | LOW RIGHT | 31 | Schw. | LOW RIGHT |
| 11 | Hal. | HIGH LEFT | 32 | Se. | LOW RIGHT |
| 12 | Har. | HIGH RIGHT | 33 | Stei. | LOW RIGHT |
| 13 | He. | HIGH RIGHT | 34 | Step. | HIGH LEFT |
| 14 | Ho. | LOW RIGHT | 35 | Str. | LOW LEFT |
| 15 | Hö. | LOW LEFT | 36 | Stu. | HIGH RIGHT |
| 16 | Ir. | HIGH RIGHT | 37 | Sy. | HIGH RIGHT |
| 17 | Ka. | LOW RIGHT | 38 | Un. | LOW RIGHT |
| 18 | Ku. | HIGH RIGHT | 39 | Wa. | LOW RIGHT |
| 19 | Me. | LOW RIGHT | 40 | We. | LOW LEFT |
| 20 | Mos. | HIGH LEFT | 41 | Za. | LOW LEFT |
| 21 | Mosh. | HIGH LEFT | 42 | Zi. | HIGH RIGHT |

Table 3.3: exposure conditions of the test people (test people's names anonymized, in alphabetic order) - *) participated for only 1 session.

# 4 COGNITIVE EFFECTS

# Experimental Investigations on Cognitive Effects of Exposure to UMTS-Signals

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Co-workers:

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Assoz.Prof.Dipl.-Ing. Dr. Hans-Peter Hutter

Authors of report:

Ao.Univ. Prof. Dr. Michael Kundi

Ao.Univ. Prof. Dr. Wilhelm Mosgöller (Project-coordinator, Med. Univ. Wien)

## 4.1 RESEARCH QUESTIONS

The effects of radio frequency electromagnetic fields (RF-EMF) as used by mobile phones on cognitive functions have been frequently investigated. These investigations provide inconsistent results. Table 4.1 provides an overview of the publications available at 2015. 22 of the 43 published studies show and describe significant exposure related effects, the majority of these (N=14) describe an improved performance under exposure, e.g. a faster reaction or an increase of the number of tasks under exposure. Few investigations describe a shortened reaction time plus a trend for impaired performance under exposure (so called speed-accuracy trade-off).

In most investigations the expositions were short and the volunteers were tested only once. Whether or not the cognitive effects depend on the duration of the exposure is unknown. We were interested whether the effects become weaker or pronounced over time. In most reports published so far, there is no description whether exposure of the left or right side of the head is relevant. Most investigators exposed only one side. In view of the reported investigations we focused on 3 main research questions:

- Changes of cognitive effects during a longer exposure?
- Do the effects decrease or increase if retested for more days?
- Is the exposed side relevant (left or right side of the head)?

To answer these questions we designed a study protocol with double blinded randomized group allocation. Both, the investigated volunteers and also the investigators had no information about the actual exposure parameters.

Table 4.1. Overview of studies investigating cognitive effects of high frequency electromagnetic field exposure.
Legend to abbreviations: SRT…Simple Reaction Task; CRT…Choice Reaction Task; CW…Continuous Wave; MHz…Megahertz; PW…Pulsed Wave; RT…Reaction time; Exp…Exposure; sign…significant, EHS…Electro-hypersensitive, ELF-MF…extremely low frequent magnetic fields

| Study | Design | Participants | Exposure | Endpoint(s) | Results |
|---|---|---|---|---|---|
| **Preece et al. 1999** [30] | cross-over | 36 adults | 915 MHz CW and 217 Hz pulsed ~1 w 30 min during tests left side | cognitive test battery | significant shorter RT for CRT |
| **Koivisto et al. 2000** [31, 32] | cross-over without break double-blind | 48 adults right-handed (24m/24f) 18-34 y | GSM 902 MHz 0.65 W/kg 30 min during test left side | n-back test | significant shorter RT for 3-back |
| **Jech et al. 2001** [33] | cross-over consecutive days double-blind | 22 narcoleptic patients (9m/13f) 48±12 y | GSM900 0.06 W/kg 45 min test start after 5 min right side | attention test (odd.ball paradigm) | significant shorter RT |
| **Edelstyn & Oldershaw 2002** [34] | matched groups single-blind | 38 students, right-handed | regular GSM900 mobile 30 min left side (1.19 W/kg??) during tests | memory span forward/backward (digits, spatial allocation), serial subtraction, word fluency | 5 of 8 subtests significant effect of exposure improvement of memory and speed |
| **Lass et al. 2002** [35] | independent groups (exp/sham) | 100 adults (63m/37f) | 450 MHz, 7 Hz modulation 1.58 W/m² 10-20 min | 3 memory- and attention tests | significant higher variance in exp. group for 2 tests, significant less errors for 1 test |
| **Lee et al. 2003** [36] | matched groups (exp/sham) single-blind | 78 students (25m/53f) 20±1 y | GSM1800 60 min | tracking test vigilance test | significant stronger reduction of RT for exp. |
| **Smythe & Costall 2003** [37] | 3 independent groups (no/exp/sham) | 62 adults (33m/29f) 18-53 y | GSM1800. 0.79 W/kg. left side 15 min during learning phase | short and long-term memory | for males significant less errors for exposure, for females no difference |
| **Haarala et al. 2003, 2004** [38, 39] | cross-over 1 day break double-blind | 64 adults (32m/32f) 20-42 y | GSM 902 MHz 0.99 W/kg 65 min during test left side | SRT, 2CRT, 10CRT, vigilance test, subtraction test, verification test, stroop-test, n-back test | no effect on RT and errors |
| **Curcio et al. 2004** [40] | cross-over with baseline double-blind >2 days break | 20 adults right-handed (10m/10f) 22-31 y | GSM 902.4 MHz left side (helmet).0.5 W/kg 45 min vor/ 45 min during test | SRT, visuelle searching task, CRT, serial subtraction | significant shorter RT for SRT and CRT |

| Study | Design | Participants | Exposure | Endpoint(s) | Results |
|---|---|---|---|---|---|
| **Hinrichs & Heinze 2004** [41] | cross-over<br>break min 24 h ?<br>double-blind | 12 adults (2m/10f) 18-30 y | GSM 1.87 gHz<br>0.61 W/kg 30 min left side during learning (last 10 min) | recognition task | no effect on performance |
| **Hamblin et al. 2004** [42] | cross-over<br>1 week apart<br>single-blind | 12 adults (4m/8f) right-handed 19-44 y | GSM 894.6 MHz 0.87 W/kg right side 1 h during tests | reaction test | significant longer RT for exp. |
| **Krause et al. 2004** [43] | cross-over<br>without break<br>double-blind | 24 adults right-handed (12m/12f) 24,3±8,1 y | GSM900 mobile phone. 30 min left side(0.65 W/kg) during tests | modified sternberg memory test | significant more errors for exp. |
| **Maier et al. 2004** [44] | cross-over with baseline<br>double-blind | 11 adults<br>23-48 y | GSM900 left side (4 cm) between baseline and test (50 min) | auditive order threshold | significant less improvement for exposure |
| **Schmid et al. 2005** [45] | multiple cross-over<br>randomized<br>double-blind | 58 adults (29m/29f), 20-40y | UMTS 1.97 gHz<br>0.37/0.037 W/kg left side | fliquer fusion frequency tracking test, attention test, contrast sensitivity | no effect of exposure |
| **Besset et al. 2005** [46] | matched groups | 55 adults (27m/28f), 18-40y | GSM900 0.54 W/kg<br>2h (18-20:00). 5d/w.<br>6 weeks<br>3 d bl/28 d exp v.sham/14 d post | 22 neuropsych. tests<br>1 bl, 2 exp, 1 post | significant effects on RT change |
| **Preece et al. 2005** [47] | cross-over<br>1 day break<br>double-blind | 18 children (9m/9f)<br>10-12 y | GSM900 mobile phone left side 30-35 min (0.44/0.044 W/kg) during tests | 16 cognitive tests | significant effect for SRT<br>not significant after bonferroni correction |
| **Vecchio et al. 2012** [48] | cross-over<br>1 week break<br>double-blind | 11 adults right-handed (8m/3f) 24-63 y | GSM 902.4 MHz 0.25 w (0.5 W/kg)<br>45 min before test | CRT | significant improvement of RT for exp. not for sham |
| **Curcio et al. 2012** [49] | cross-over<br>1 week break<br>double-blind | 12 male adults right-handed 19-25 y | GSM 902.4 MHz 0.25 w (0.5 W/kg)<br>45 min before test | CRT | significant improvement of RT for exp., not for sham |
| **Haarala et al. 2005** [50] | cross-over<br>1 day break<br>double-blind | 32 children (16m/16f)<br>10-14 y | GSM 902 MHz mobile phone left side 50 min<br>(0.99 W/kg) during tests | SRT, 2CRT,10CRT, vigilance test, n-back | no effect on RT and errors |

| Study | Design | Participants | Exposure | Endpoint(s) | Results |
|---|---|---|---|---|---|
| **Keetley et al. 2006** [51] | cross-over with baseline<br>1 week break<br>double-blind | 120 adults (58m/62f) 18-70 y | GSM900 mobile phone 30 min left side before tests | audio-visual test, memory span, digit-symbol replacement, RT, CRT | signif. effects for 4 of 8 tests<br>improvement for one,<br>decline for other tests |
| **Russo et al. 2006** [52] | cross-over with<br>1 week break<br>double-blind | 168 adults (69m/99f) 17-41 y | 888 MHz<br>CW or GSM<br>left or right side 35-40 min during tests | SRT, 10CRT, vt, subtraction test | no effect of exp. |
| **Wilen et al. 2006** [53] | cross-over with baseline<br>min. 1 day break<br>(double)blind | 40 adults (32m/8f) 29-65 y<br>20 with sympthoms/ 20 controls | GSM900 mobile-signal<br>8.5 cm right side<br>0.8 W/kg 30 min between baseline and test | sternberg memory test<br>fliquer fusion frequency | no effect of exp.<br>no diff. between cases and controls |
| **Regel et al. 2006** [54] | cross-over<br>1 week break<br>double-blind | 33 ‚sensitive' (14m/19f)<br>84 ‚non-sensitive'<br>(41m/43f)<br>20-60 y | UMTS base-station signal<br>org.channel<br>left side (~2m. 25°) 45 min<br>SAR head: 45/4500 µW/kg during test | SRT, 2CRT, n-back, visual attention | 2CRT significant for sensitives<br>1-back precision significant for non-sensitives<br>not significant after adjustment for multiple endpoints |
| **Eliyahu et al. 2006** [55] | cross-over<br>latin square<br>single-blind<br>breaks? | 36 male adults right-handed 19-27 y | GSM 890.2 MHz 0.25 W. 2 h left/right side during tests (2 rounds) | spatial location test (FACE), letter location test, laterality test | in 3 of 4 tests reduced RT for exp. from left side and left hand reaction |
| **Hamblin et al. 2006** [56] | cross-over<br>1 week apart<br>double-blind | 120 adults (46m/74f) 108 right-handed 18-69 y | GSM 895 MHz 0.11 W/kg 30 min during test right or left | optical and acoustic reaction test | no effect of exp. |
| **Terao et al. 2006** [57] | cross-over<br>1 week break<br>double-blind | 16 adults (9m/7f) right-handed 23-52 y | 800 MHz, japanese system 0.054 W/kg. 30 min right side between pre- and post-test | CRT | RT after exp. shorter (not significant) |
| **Haarala et al. 2007** [58] | cross-over<br>1 week break<br>double-blind | 48 males, right-handed 24±2 y | 902 MHz CW or 217 Hz PW 0.25 W (0.738 W/kg) left or right side 90 min during test | SRT, 2CRT, 10CRT, vigilance test, subtraction test, verification test, n-back test | no effect of exp. |

| Study | Design | Participants | Exposure | Endpoint(s) | Results |
|---|---|---|---|---|---|
| **Cinel et al. 2007** [59] | cross-over + independent groups (CW/GSM) and (left/right) | 168 adults (54m/114f) 18-42 y | 888 MHz CW or GSM 1.4 W/kg 40 min left or right side | auditive order threshold | no effect of exp. |
| **Fritzer et al. 2007** [60] | independent groups single-blind | 20 male adults 22-37 y | GSM 900 MHz 1 W/kg 6 nights | digit connection attention test memory tests | no effect of exp. |
| **Regel et al. 2007** [61] | cross-over 1 week apart double-blind | 15 male adults right-handed 20-26 y | GSM 900 MHz 0.2 and 5 W/kg 30 min left side before sleep | SRT, CRT, n-back test | dose dependent increase of RT in all tests, significant for 1-back |
| **Curcio et al. 2008** [62] | cross-over 1 week apart double-blind | 24 adults (12m/12f) right-handed 19-36 y | GSM 902.4 MHz 0.25 W (0.5 W/kg) 3 x 15 min within. 85 min right side | SRT, sequence test | no effect of exp. |
| **Kleinlogel et al. 2008** [63] | cross-over 1 week apart double-blind | 15 male adults right-handed 20-35 y | GSM 900 MHz 1 W/kg UMTS 1950 MHz 0.1 and 1 W/kg 30 min left side | CRT | no effect of exp. |
| **Leung et al. 2011** [64] | cross-over min 4 days apart double-blind | 41 adolescents (13-15 y), 42 adults <40, 20 elderly (55-70 y) | GSM 849.6 MHz 0.7 W/kW-CDMA 1900 MHz 1.7 W/kg | odd-ball paradigm n-back test | lower precision for 3g exposure |
| **Riddervold et al. 2008** [65] | cross-over min 24 h break double-blind | 40 adolescents (17m/23f) und 40 adults (24m/16f) 31±4.5 y | 2140 MHz CW UMTS 2140 MHz UMTS-like 45 min. vertikal polarisation. 2.8 m distance 6-13 mW/m² | SRT, CRT, memory test, trail-making test | no effect of exp. |
| **Luria et al. 2009** [66] | independent groups (right/left/sham) single-blind | 48 male adults right-handed | GSM 890.2 MHz 0.54-1.09 W/kg. 1 h left/right side during tests | spatial location test | significant longer RT right hand for exp. from left side (hareuveny et al. indicated exp. not solely responsible) |
| **Wiholm et al. 2009** [67] | cross-over double-blind | 42 adults (21m/21f) 18-45 y | GSM 884 MHz 1.4 W/kg. 2.5 h left side during tests | labyrinth task | significant shorter length for exp. |
| **Riddervold et al. 2010** [68] | cross-over min 24 h break double-blind | 53 male emergency service employees, right-handed 25-49 y | TETRA 450 MHz 2 W/kg 45 min left side | SRT, CRT, memory test, trail-making test | small increase of RT |

| Study | Design | Participants | Exposure | Endpoint(s) | Results |
|---|---|---|---|---|---|
| **Sauter et al. 2011** [69] | cross-over<br>2 weeks apart<br>double-blind | 30 male adults right-handed 18-30 y | GSM 900 MHz<br>WCDMA 1966 MHz 2 W/kg.<br>7 h 15 min during tests | attention test<br>vigilance test<br>n-back test | significant change of RT for some tests, not significant after bonferroni correction |
| **Wallace et al. 2012** [70] | cross-over<br>min 1 week apart<br>double-blind and open | 48 EHS adults (19m/29f)<br>152 non-EHS adults (65m/67f) | TETRA 420 MHz 10 mW/m² (~0.3 mW/kg) 4.95 m distance 50 min during test | short-term memory<br>attention test | no effect of exp. |
| **Schmid et al. 2012** [71] | cross-over<br>1 week break<br>double-blind | 25 male adults right-handed 20-26 y | 900 MHz PW 2 u 8 Hz 2 W/kg<br>ELF-MF 0.176 µT 30 min left side | SRT, CRT, n-back test | significant shorter RT for mf exp. |
| **Loughran et al. 2013** [72] | cross-over<br>1 week break<br>double-blind | 22 adolescents (12m/10f) right-handed 11-13 y | GSM 900 MHz 0.35 and 1.4 W/kg left side. during tests | SRT, CRT, n-back test | no effect of exp. |
| **Sauter et al. 2015** [73] | cross-over<br>2 weeks apart<br>double-blind | 30 male adults right-handed 20-30 y | TETRA 385 MHz 1.5 W/kg and 6 W/kg 2 h 45 min left side during tests | attention tests memory tests | improved memory performance in 5 of 35 indicators |
| **Malek et al. 2015** [74] | cross-over<br>single-blind | 100 EHS persons<br>100 non-EHS persons | GSM 945 MHz 280 W/m².<br>GSM 1840 MHz 250 W/m².<br>UMTS 380 W/m² (2 m distance) | CRT, attention test<br>vigilance test | trend for longerr RT and better signal detection for vigilance test |

## 4.2 MATERIAL AND METHODS

### 4.2.1 EXPERIMENTAL DESIGN

The experimental protocol was based on a 2x2 group, double blinded investigation. Each volunteer was randomly allocated to one of four investigational conditions. The persons were RF-EMF exposed with the allocated exposure conditions, and tested for 5 consecutive days, per day for about 1 hour 50 minutes.

Table 4.2. Experimental design of four experimental groups differing by exposed side of the head and expousre intensity at the buccal cell layer.

| Exposed side / Intensity | Left | Right |
|---|---|---|
| SAR 0.1 W/kg | A | B |
| SAR 1.6 W/kg | C | D |

In the context with this chapter it is important to note that the indicated exposure values (W/kg) are labels only, they do not indicate the exact dose of the exposure of the brain. They describe the exposure of the buccal mucosa, which was the main target tissue for the human experiments (section 5). Under the chosen experimental conditions the highest exposure of the brain tissue was in the temporal lobe. The RF-EMF exposure was about 200 times below the indicated SAR. The SAR given in this chapter specifies merely the exposure group (high and low) and not the intensity of exposure for the brain.

Volunteers were seated in a cabin with RF-EMF absorbing walls and were exposed by antennas mounted on the head. The volunteers could move the head without limitation and almost without burden. There was an antenna on each side of the head, neither the subject nor the experimenter could detect which antenna was active during the experiment.

### 4.2.2 INCLUSION AND EXCLUSION CRITERIA

We recruited volunteers of both sexes, aged between 20 and 60 years, non-smokers, free of acute or chronic diseases, who do not use a mobile phone or use it for less than one hour per day. The recruited volunteers filed an agreement to the study protocol, to the electronic storage of their personal data and the use of the personal data for scientific purposes.

With respect to the research question in the subproject (buccal cell analysis, as described in section 5) the volunteers completed a diary from 2 weeks before, and 3 weeks after the exposure. In case the volunteers would use their mobile phone during the study participation, they were asked to use a headset. Volunteers were provided with a headset if needed.

### 4.2.3 STUDY PARTICIPANTS

From 42 enrolled subjects one dropped out after the first day for personal reasons. Due to time constraints and the tight schedule this person could not be replaced. Of the participants 21 were male and 20 female. Age was 29±10 years (22 to 56 years), 7 were left- and 34 right handed. The majority (30 subjects) had higher education, 9 had a university degree and 2 a college graduation.

20 subjects were randomly allocated to the exposure group “low” (SAR 0.1 W/kg), from these 9 were exposed at the left and 11 at the right side. High exposure (SAR 1.6 W/kg) was assigned to 21 subjects, 10 with left side and 11 with right side exposure.

### 4.2.4 EXPERIMENTAL PROCEDURES

For each participant exposure related cognitive effects were investigated every day for one week, following the same scheme (Table 4.3). The various tests were computer guided and presented automatically. The intermission between the specific tests was filled with an automated slide show.

After participants arrived at the laboratory a short interview about the current personal well-being was performed. This was followed by the mounting of the antennas. After an acclimation time of about 10 minutes the test persons filled in a questionnaire to report current mood and well-being. This was followed by a first series of cognitive tests and the measurement of the reaction time. The tests series was interrupted several times by a slide show. After participants completed the full program (about 2 hours) they again reported their actual mood and wellbeing.

After exposure and the demounting of the antennas, the volunteers received a short interview about particular sensations. The duration of each test run was about 2 and a half hour. The same test run was repeated every day from Monday to Friday, the slide shows varied and all tests were presented in a randomized fashion. On the last day after the test run we arranged dates for the study-exit visit and the sampling of the buccal cells (see chapter 5).

Table 4.3. Procedure for each experimental session. The durations given in minutes are average durations.

| Test/Presentation/Step | Duration (minutes) |
|---|---|
| **Application of antennas** | 2 |
| **Acclimatisation** | 10 |
| **Nitsch questionnaire** | 5 |
| **Simple reaction test (SRT)** | 3 |
| **Memory test initial presentation** | 2 |
| **Choice reaction test (CRT)** | 2 |
| **Memory test 1st examination** | 3 |
| **Slide show** | 10 |
| **Memory test 2nd examination** | 3 |
| **Perceptional speed** | 2 |
| **O2-test** | 5 |
| **Slide show** | 10 |
| **Memory test 3rd examination** | 3 |
| **Vigilance test** | 10 |
| **Slide show** | 10 |
| **Simple reaction test (SRT)** | 3 |
| **Choice reaction test (CRT)** | 3 |
| **Slide show** | 10 |
| **Vigilance test** | 10 |
| **Slide show** | 10 |
| **Nitsch questionnaire** | 5 |

### 4.2.5 COGNITIVE AND REACTION TESTS

In each of the 5 test runs a computer program presented a series of cognitive and reaction tests. The sequence is described in Table 4.3.

- **SRT – Simple Reaction Test**

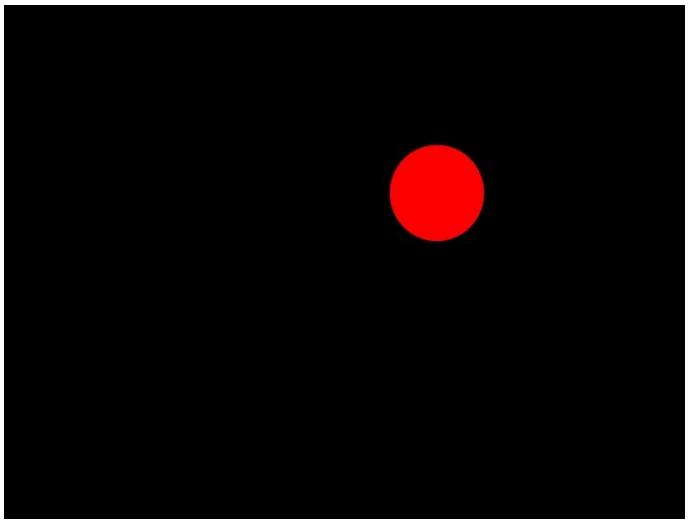

Figure 4.1. Example of a SRT (Simple Reaction Test) item

During the simple reaction test the volunteers respond as quickly as possible to the appearance of a colored shape (circle, sqare) irrespective of shape and color. The end point variable of this test was the average reaction time of 140 single reactions without the first 3 items.

- **CRT – Choice Reaction Test**

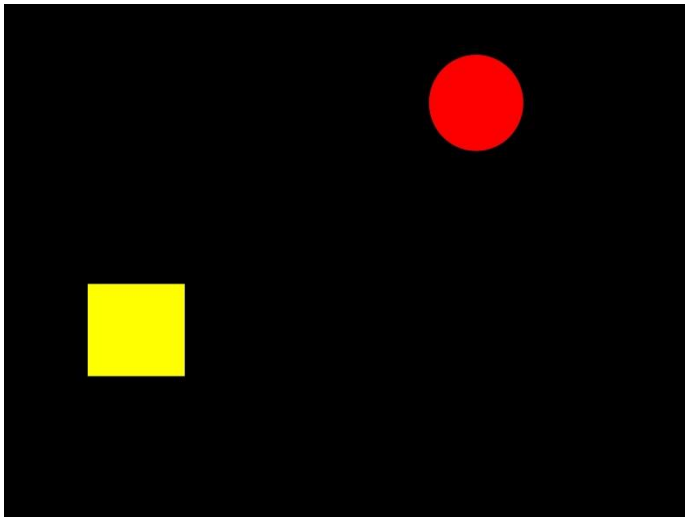

Figure 4.2. Example of a CRT (Choice Reaction Test) item

During the choice reaction test the volunteers responded with “Y” if two geometric figures matched in form or color, and responded with “N” if the pair did not match. This test provided 2 variables, i.e. the number of right or wrong answers and the mean reaction time over 140 items.

- **O2-test**

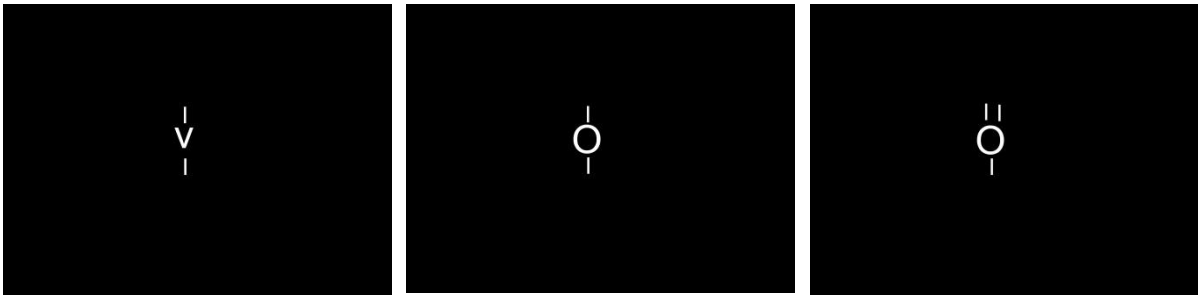


Figure 4.3. Three examples if O2-test items

During the so called O2 test the volunteers had to respond with key “Y” = “yes” if the letter ‚O‘ appears with 2 dashes (e.g. Figure 4.3 the middle symbol). In all other cases the correct answer was the key “N”. We analyzed the number of right and wrong answers and the mean reaction time of 120 items.

- **Perceptional speed test**

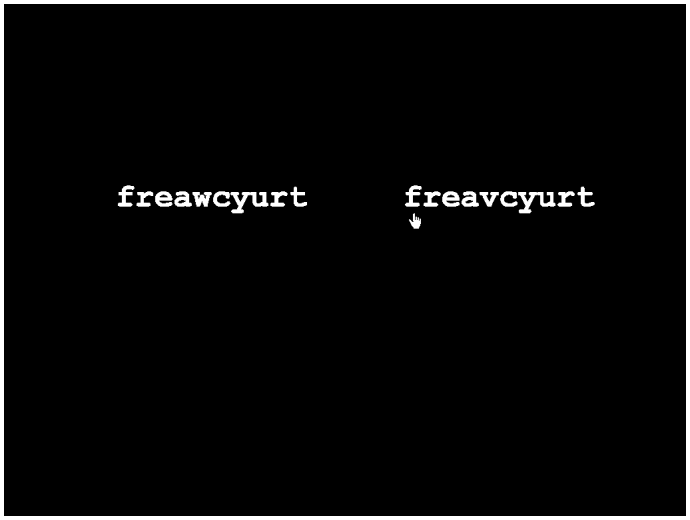


Figure 4.4. Example of a Perceptional Speed Test item.

For the perceptional speed test participants had to compare 2 series of letters and place the cursor as fast as possible under the only letter at the right series different from the left series. The analysis focused on the number of right or wrong reactions and the mean reaction time.

- **Vigilance test**

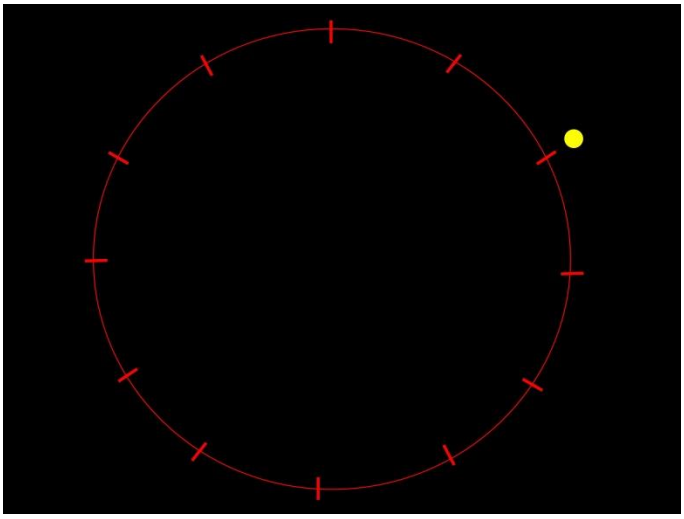

Figure 4.5. Vigilance test; the yellow point on the clock-face jumps from hour to hour.

The vigilance test estimates the degree the alertness and concentration of the test persons. The test consists of a clock-face and a marker (yellow point) that jumps every second from hour to hour. At random, the marker jumps over two hours instead. In this case, the test persons should react as quickly as possible and push a key. The number of right and wrong reactions (missed events, false alarms) as well as the time between event and reaction are analyzed.

- **Memory test**

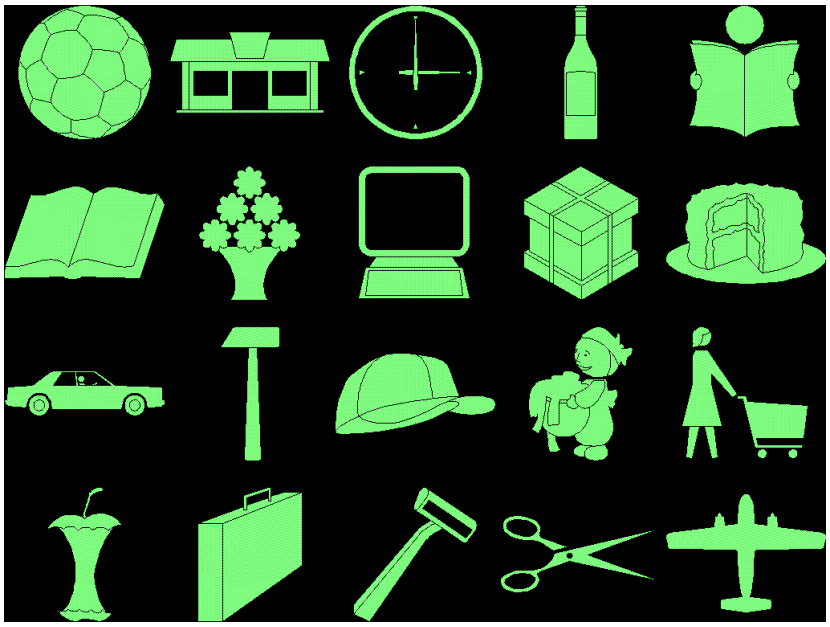

Figure 4.6. Example of the learning phase presentation of a memory test, 20 objects should be memorized for recall later in the session.

20 objects in similar appearance were presented on a screen for 40 seconds to memorize the items. After 3, 13 and 43 minutes participants were offered 10 memorized images and 10 distraction items that were not initially presented in random sequence. The task was to decide for each item if it was part of the first (memorized)

presentation or not. The number of correct decisions and the mean reaction time were analyzed.

### 4.2.6 STATE-OF-MOOD SCALE ACCORDING TO NITSCH

In addition to the cognitive reaction tests participants filled in the so called Nitsch questionnaire, a state-of-mood scale, both at the beginning and the end of each daily session. Participants were asked to indicate to which extent the current state was reflected by each of 20 attributes. The answers are then assigned to four subscales 1) effort disposition, 2) tension, 3) exhaustion, and 4) sleepiness. For statistical analysis these variables were transformed into so called stanine-values. To obtain these stanine-values a two-step procedure is applied, first the answers were area transformed based on tables of norms, second the sum of all items belonging to each subscale are stanine transformed according to standardization tables. Stanine-values reflect a specific area under the normal distribution. The mean of stanine-values in the norm population is 5 and the standard deviation is 2.

### 4.2.7 STATISTICAL EVALUATION OF COGNITIVE PERFORMANCE

The statistical evaluation of the cognitive performance and reaction times was done as follows:

**Reaction time**: The measured reaction times were logarithmically transformed because the distribution follows a log-normal distribution. Depending on whether the test was presented once or repeatedly during one session the statistical analysis was done by generalized estimation equations (GEE) with normally distributed variables similar to a 3- or 4-factor analysis of variance, including the factors SAR (0.1 vs. 1.6 W/kg), exposure side (left or right), test day (1 to 5) and, if applicable, first or second presentation during the test run.

**Counts of correct reactions**: The number of correct reactions was also analyzed by generalized estimation equations, assuming that the counts of correct reactions follow a binominal distribution. The factors were the same as described above for the reaction times. The number of wrong reactions or missed events was not statistically analyzed, because the number of items was always constant and therefore these counts are given by the difference between all tasks minus correct responses.

For the data analysis with GEE the differences in the measured values were attributed to the experimental factors. We analyzed main effects and interaction effects. The main effects were:

- SAR: differences independent of the side of the head, the section (first/second; early/late) during the test run and the test day, but depending on the SAR value during exposure (intensity of the RF-EMF)
- Side of the head: differences depending on the exposure to the left or right side of the head but independent of SAR, the section during the test run and the specific test day.

- Test day: differences between the test days irrespective of SAR, the exposed side of the head and of the section and day of the test run.
- Test section: differences between the first (early) and second (late) presentation during the daily test run but independent of SAR, the side of the head that has been exposed, or the test day.

To give an example: significant main effects for both SAR and exposed side of the head indicates that an effect occurred either very early or showed a trend depending on these factors that is not compensated by a difference at the beginning.

Significant interactions can be interpreted as follows:

- SAR*Side of the head: The difference of measurement between both intensities depends on the side of the head where exposure occurred
- SAR*Test day: The trend across test days differs depending on the intensity of exposure
- SAR* early or late presentation: the difference between the first or second presentation in the test run depends on the intensity of the exposure
- Side of the head*Test day: the results on the specific test days are different depending on the side of the head that has been exposed
- Side of the head*Test section: the difference between measurements from the first to the later exposure section depends on the side of the head that has been exposed
- Test day*Test section: The difference between the first and second section depends on the test day
- SAR*Side of the head*Test day: the trend over test days is different depending on the side of the head and the intensity of exposure
- SAR*Side of the head*Test section: the difference between the first section (hour) and second section (hour) depends on the exposed side of the head and the exposure intensity
- SAR*Test day*Test section: the difference between the early and late exposure section depends on the intensity of the exposure and on the day
- Side of the head*Test day* exposure section: the difference between the first and second exposure section depends on the side of the head that has been exposed and on the test day
- SAR*Side of the head*test day*Test section: the trend over the test days is different in the first or second exposure section and depends on the side of the head and intensity of the exposure

For all statistical analyses a p-value of $p<0.05$ was considered statistically significant. P-values above 0.05 and below 0.1 indicate a tendency.

## 4.3 RESULTS

### 4.3.1 SIMPLE REACTION TEST

This test was performed twice every day, once during the first and once during the second hour of exposure. Since we did not find a relevant difference between exposure on the left and right side we combined these results in Figure 4.7.

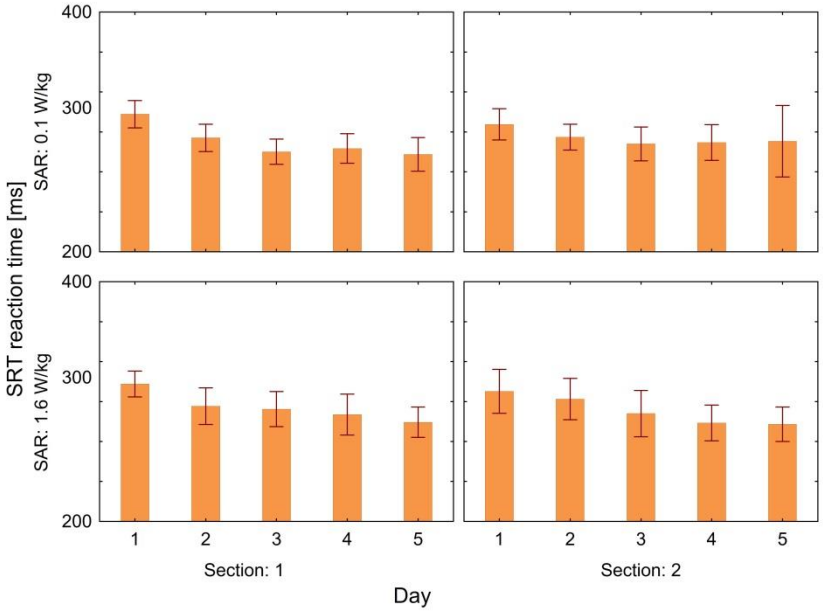


Figure 4.7. Simple reaction test, mean reaction times (and 95 % confidence intervals) on the 5 test days and at the first and second hour (section) during buccal exposure to 0.1 W/kg or 1.6 W/kg

The results concerning the reaction time show a highly significant training effect over the test days 1-5, the reactions became faster from day to day. Under high exposure this decrease of reaction time is more pronounced especially in the second hour (section) of exposure, however, the difference is not statistically significant (Table 4.4, section 1-2)

Table 4.4. Results of the analysis of reaction times by generalized estimation equations for the simple reaction test

| Source of variation | Statistic | Degrees of freedom | p-value | Significance |
|---|---|---|---|---|
| SAR | 0.00 | 1 | 0.969 | n.s. |
| Side of head | 0.26 | 1 | 0.608 | n.s. |
| Day (1-5) | 47.25 | 4 | <0.001 | highly significant |
| Section (1-2) | 0.02 | 1 | 0.888 | n.s. |
| SAR * Side of head | 0.36 | 1 | 0.547 | n.s. |
| SAR * Day | 2.83 | 4 | 0.587 | n.s. |
| SAR * Section | 1.71 | 1 | 0.191 | n.s. |
| Side of head * Day | 1.12 | 4 | 0.891 | n.s. |
| Side of head * Section | 0.05 | 1 | 0.829 | n.s. |

| Source of variation | Statistic | Degrees of freedom | p-value | Significance |
|---|---|---|---|---|
| Day * Section | 7.55 | 4 | 0.110 | n.s. |
| SAR * Side of head * Day | 0.23 | 4 | 0.994 | n.s. |
| SAR * Side of head * Section | 0.45 | 1 | 0.502 | n.s. |
| SAR * Day * Section | 6.66 | 4 | 0.155 | n.s. |
| Side of head * Day * Section | 6.26 | 4 | 0.180 | n.s. |
| SAR * Side of head * Day * Section | 9.18 | 4 | 0.057 | tendency |

### 4.3.2 CHOICE REACTION TEST

Also this test was performed twice (in the first and second section) every day. Figure 4.8 shows the results for reaction times and Figure 4.9 the fraction of correct reactions.

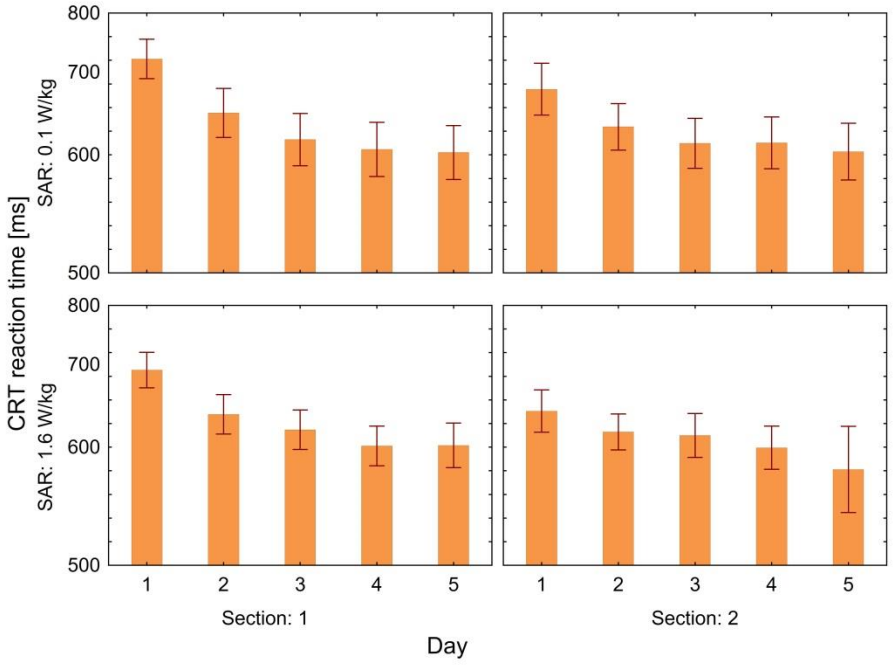


Figure 4.8. Choice reaction test, mean reaction times (and 95 % confidence intervals) on the 5 test days and at the first and second section during buccal exposure to 0.1 W/kg or 1.6 W/kg

Table 4.5. Results of the analysis of reaction times for the choice reaction test by generalized estimation equations

| Source of variation | Statistic | Degrees of freedom | p-value | Significance |
|---|---|---|---|---|
| **SAR** | 0.98 | 1 | 0.323 | n.s. |
| **Side of head** | 0.25 | 1 | 0.616 | n.s. |
| **Day (1-5)** | 48.49 | 4 | <0.001 | highly significant |
| **Section (1-2)** | 16.65 | 1 | <0.001 | highly significant |
| **SAR * Side of head** | 2.71 | 1 | 0.099 | tendency |
| **SAR * Day** | 8.34 | 4 | 0.080 | tendency |
| **SAR * Section** | 4.26 | 1 | 0.039 | significant |
| **Side of head * Day** | 5.18 | 4 | 0.270 | n.s. |
| **Side of head * Section** | 1.78 | 1 | 0.183 | n.s. |
| **Day * Section** | 29.93 | 4 | <0.001 | highly significant |
| **SAR * Side of head * Day** | 2.58 | 4 | 0.630 | n.s. |
| **SAR * Side of head * Section** | 0.84 | 1 | 0.358 | n.s. |
| **SAR * Day * Section** | 2.80 | 4 | 0.593 | n.s. |
| **Side of head * Day * Section** | 3.24 | 4 | 0.519 | n.s. |
| **SAR * Side of head * Day * Section** | 5.36 | 4 | 0.252 | n.s. |

The reaction time became significantly shorter from day to day. A similar trend is visible within a single day from first to second exposure section (especially during the first days). We observed a tendency for a stronger reduction over the days (p=0.08) for the higher exposure, and a statistically significant (p=0.039) stronger effect for the difference between the first and second section, with respect to the higher exposure intensity.

While the reaction times decreased from day to day and between the exposure sections on a single day, at the same time the fraction of correct reactions significantly decreased in the exposure group “high” (p=0.009), see Table 4.6.

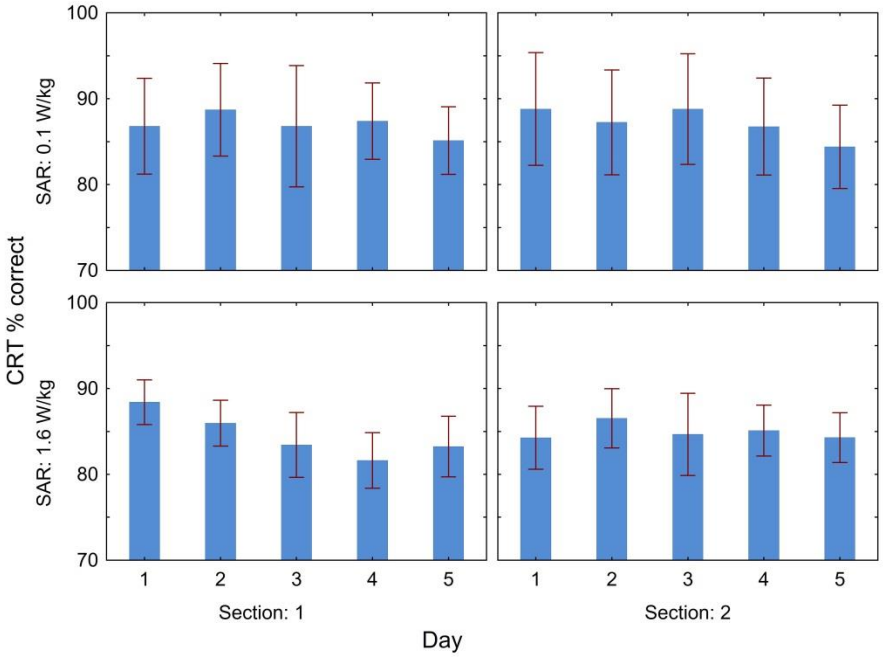


Figure 4.9. Choice reaction test, percentage of correct reactions (and 95 % confidence intervals) on the 5 test days and at the first and second hour during buccal exposure to 0.1 W/kg or 1.6 W/kg

Table 4.6. Results of the analysis of correct responses for the choice reaction test by generalized estimation equations

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 1.24 | 1 | 0.265 | n.s. |
| **Side of the head** | 1.31 | 1 | 0.253 | n.s. |
| **Day (1-5)** | 7.86 | 4 | 0.097 | tendency |
| **Section (1-2)** | 0.51 | 1 | 0.474 | n.s. |
| **SAR * Side of the head** | 0.05 | 1 | 0.818 | n.s. |
| **SAR * Day** | 3.19 | 4 | 0.527 | n.s. |
| **SAR * Section** | 0.00 | 1 | 0.971 | n.s. |
| **Side of the head * Day** | 3.24 | 4 | 0.518 | n.s. |
| **Side of the head * Section** | 2.94 | 1 | 0.086 | tendency |
| **Day * Section** | 4.15 | 4 | 0.387 | n.s. |
| **SAR * Side of the head * Day** | 2.55 | 4 | 0.637 | n.s. |
| **SAR * Side of the head * Section** | 1.07 | 1 | 0.301 | n.s. |
| **SAR * Day * Section** | 13.57 | 4 | 0.009 | highly significant |
| **Side of the head * Day * Section** | 3.02 | 4 | 0.555 | n.s. |
| **SAR * Side of the head * Day * Section** | 9.76 | 4 | 0.045 | Signifikant |

### 4.3.3 O2-TEST

This test was performed only once during a session. Figure 4.10 shows the mean reaction times and Figure 4.11 the percentages of correct reactions). Also for this test reaction times became shorter from day to day. In the exposure group ”high” there was a statistical tendency for shorter reaction times (p=0,054). Between the two exposure groups (high and low) the decrease of correct answers over the days was significantly different (p<0.001). There was a tendency for a difference depending on the side of the head that has been exposed: When exposing the right side of the head correct reactions increased from day to day. Exposure of the left side led to an increase only during the first 3 days (data not shown).

Table 4.7. Results of the analysis of reaction times for the O2 test by generalized estimation equations

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| SAR | 3.71 | 1 | 0.054 | Tendency |
| Side of the head | 0.01 | 1 | 0.917 | n.s. |
| Day (1-5) | 15.92 | 4 | 0.003 | highly significant |
| SAR * Side of the head | 0.00 | 1 | 0.947 | n.s. |
| SAR * Day | 1.91 | 4 | 0.752 | n.s. |
| Side of the head * Day | 4.68 | 4 | 0.322 | n.s. |
| SAR * Side of the head * Day | 1.95 | 4 | 0.745 | n.s. |

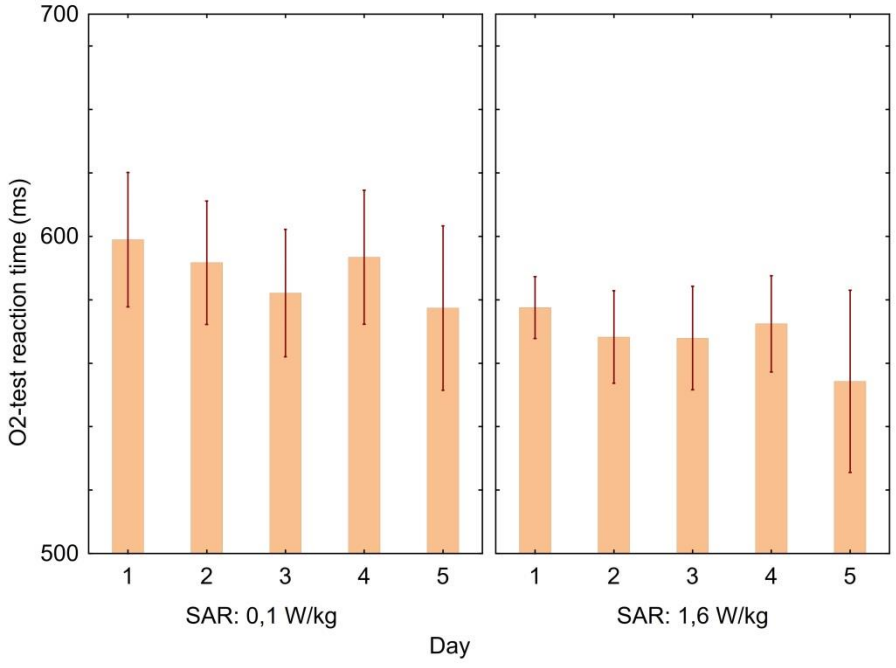


Figure 4.10. O2 test, mean reaction times (and 95 % confidence intervals) on the 5 test days during buccal exposure to 0.1 W/kg or 1.6 W/kg

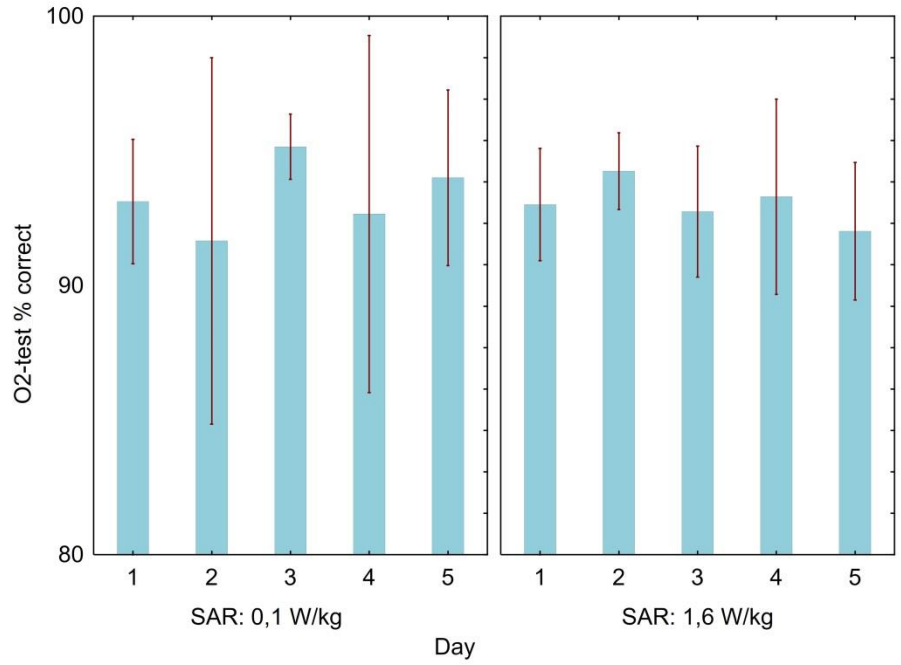


Figure 4.11. O2 test, mean percent of correct responses (and 95 confidence intervals) on the 5 test days during buccal exposure to 0.1 W/kg or 1.6 W/kg

Table 4.8. Results of the analysis of correct responses for the O2 test by generalized estimation equations

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 0.14 | 1 | 0.706 | n.s. |
| **Side of the head** | 0.00 | 1 | 0.973 | n.s. |
| **Day (1-5)** | 12.27 | 4 | 0.015 | significant |
| **SAR * Side of the head** | 1.12 | 1 | 0.289 | n.s. |
| **SAR * Day** | 26.56 | 4 | <0.001 | highly significant |
| **Side of the head * Day** | 12.76 | 4 | 0.013 | significant |
| **SAR * Side of the head * Day** | 6.57 | 4 | 0.161 | n.s. |

### 4.3.4 PERCEPTIONAL SPEED TEST

The perceptional speed test was presented once per the daily exposure (about in the middle of the test sequence).

Figure 4.12 shows the results for the reaction times and Table 4.9 the statistical analyses. Figure 4.13 shows the percent correct reactions, and Table 4.10 summarizes the statistical analysis. Reaction time decreased significantly over the days, this effect was more pronounced in the exposure group „high“. The fraction of correct reactions decreased over the days, the decrease was statistically significant lower in the exposure group “high”.

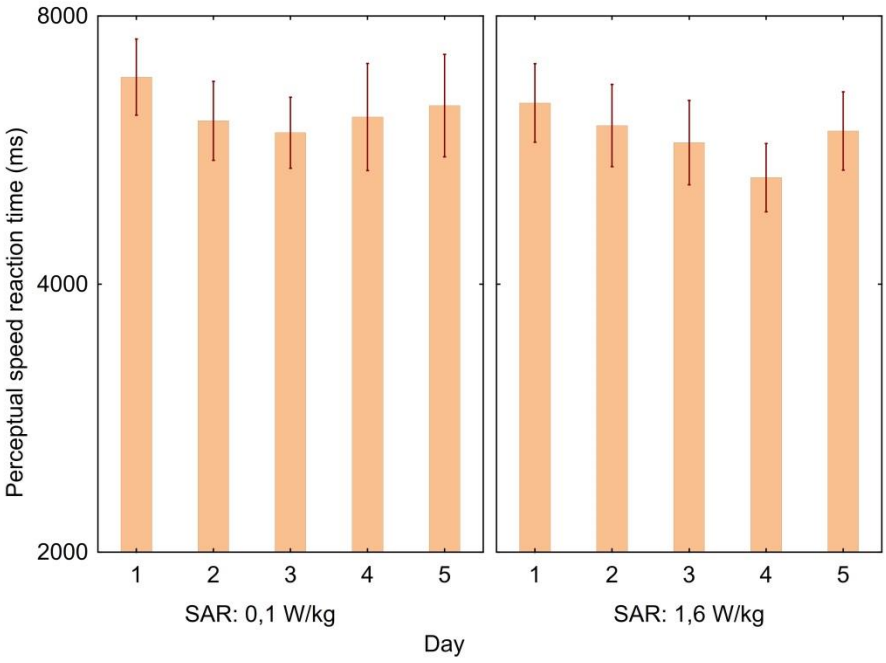


Figure 4.12. Perceptual speed test, mean reaction times (and 95 % confidence intervals) on the 5 test days during buccal exposure to 0.1 W/kg or 1.6 W/kg.

Table 4.9. Results of the analysis of reaction times for the perceptual speed test by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 1.01 | 1 | 0.315 | n.s. |
| **Side of the head** | 1.21 | 1 | 0.271 | n.s. |
| **Day (1-5)** | 37.01 | 4 | <0.001 | highly significant |
| **SAR * Side of the head** | 0.27 | 1 | 0.601 | n.s. |
| **SAR * Day** | 7.80 | 4 | 0.099 | tendency |
| **Side of the head * Day** | 3.15 | 4 | 0.533 | n.s. |
| **SAR * Side of the head * Day** | 10.40 | 4 | 0.034 | significant |

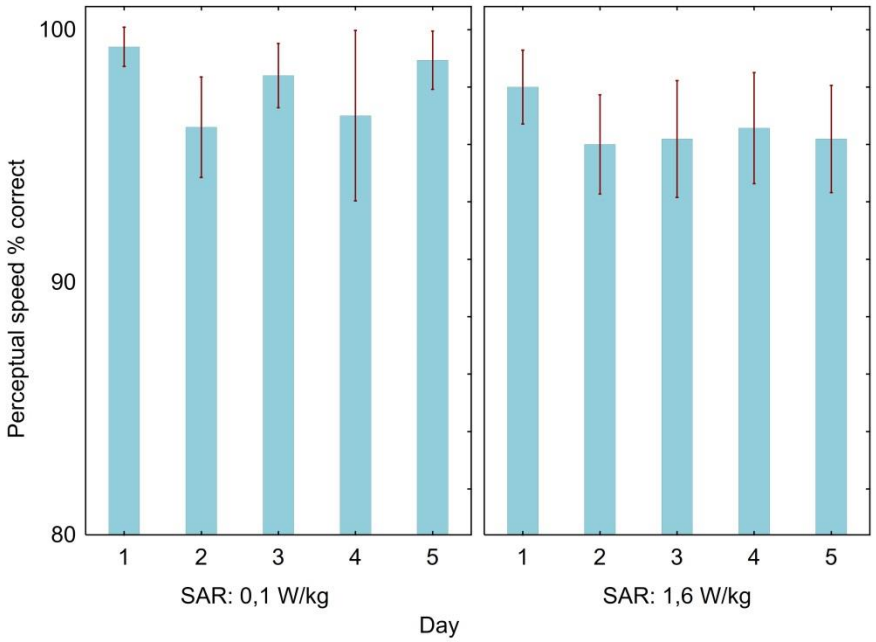


Figure 4.13. Perceptual speed test, mean percentage of correct responses (and 95 % confidence intervals) on the 5 test days during buccal exposure to 0.1 W/kg or 1.6 W/kg.

Table 4.10. Results of the analysis of correct responses for the perceptual speed test by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 5,71 | 1 | 0,017 | significant |
| **Side of the head** | 0,01 | 1 | 0,919 | n.s. |
| **Day (1-5)** | 19,69 | 4 | <0,001 | highly significant |
| **SAR * Side of the head** | 0,32 | 1 | 0,569 | n.s. |
| **SAR * Day** | 4,06 | 4 | 0,398 | n.s. |
| **Side of the head * Day** | 2,64 | 4 | 0,619 | n.s. |
| **SAR * Side of the head * Day** | 4,91 | 4 | 0,296 | n.s. |

### 4.3.5 VIGILANCE TEST

This test was performed twice on each test day. The results for reaction time are summarized in Figure 4.14 and the percentage correct reactions in Figure 4.15.

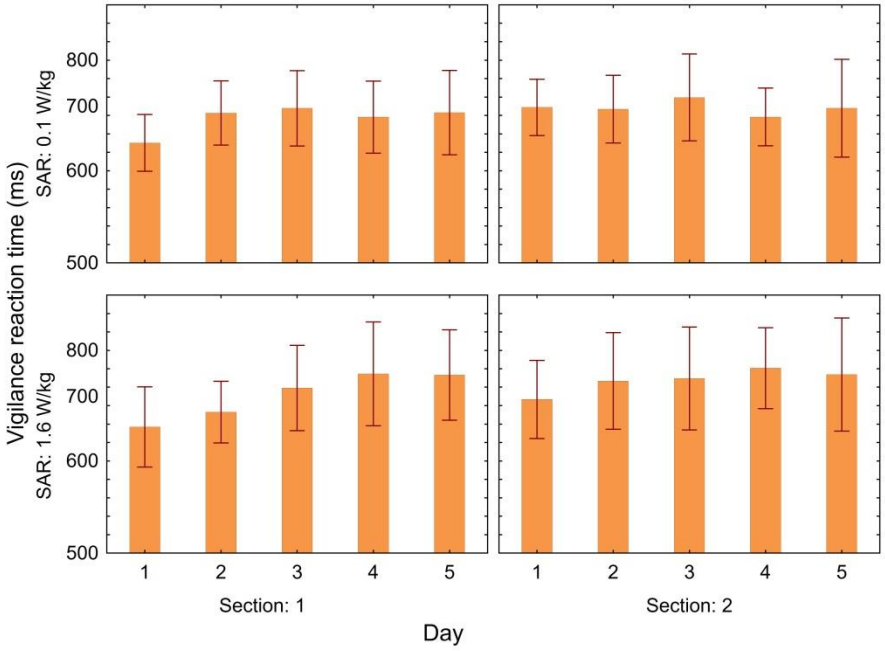


Figure 4.14. Vigilance test, mean reaction times (and 95 % confidence intervals) on the 5 test days and at the first and second presentation during buccal exposure to 0.1 W/kg or 1.6 W/kg.

The reaction time increased independently of the exposure intensity over the days and within the day (especially during the first days). Additionally, we observed a difference in the increase over test days depending on the side of the head, which has been exposed. The exposure dependent increase was more pronounced if the left side of the head was exposed.

The proportion of correct reactions decreased highly significantly in the exposure group „high“. Interestingly, this decrease was more pronounced in the first presentation on each day. A trend for the decrease being more pronounced if the left side of the head was exposed (Table 4.12).

Table 4.11. Results of the analysis of reaction times for the vigilance test by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 0.52 | 1 | 0.470 | n.s. |
| **Side of the head** | 2.26 | 1 | 0.133 | n.s. |
| **Day (1-5)** | 8.49 | 4 | 0.075 | tendency |
| **Section (1-2)** | 9.72 | 1 | 0.002 | highly significant |
| **SAR * Side of the head** | 0.04 | 1 | 0.847 | n.s. |
| **SAR * Day** | 3.30 | 4 | 0.509 | n.s. |
| **SAR * Section** | 0.14 | 1 | 0.710 | n.s. |
| **Side of the head * Day** | 4.98 | 4 | 0.289 | n.s. |
| **Side of the head * Section** | 5.09 | 1 | 0.024 | significant |
| **Day * Section** | 7.36 | 4 | 0.118 | n.s. |
| **SAR * Side of the head * Day** | 3.19 | 4 | 0.527 | n.s. |
| **SAR * Side of the head * Section** | 0.36 | 1 | 0.549 | n.s. |
| **SAR * Day * Section** | 3.10 | 4 | 0.541 | n.s. |
| **Side of the head * Day * Section** | 2.31 | 4 | 0.679 | n.s. |
| **SAR * Side of the head * Day * Section** | 7.32 | 4 | 0.120 | n.s. |

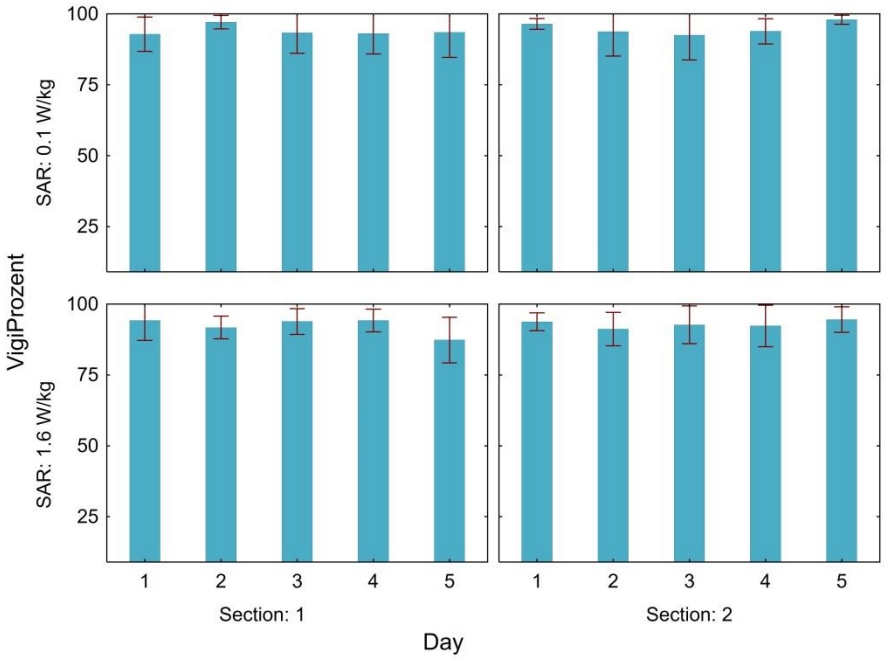


Figure 4.15. Vigilance test; mean percentage of correct responses (and 95 % confidence intervals) on the 5 test days and at the first and second presentation during buccal exposure to 0.1 W/kg or 1.6 W/kg.

Table 4.12. Results of the analysis of correct responses for the vigilance test by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 0.63 | 1 | 0.427 | n.s. |
| **Side of the head** | 0.27 | 1 | 0.605 | n.s. |
| **Day (1-5)** | 3.12 | 4 | 0.538 | n.s. |
| **Section (1-2)** | 0.59 | 1 | 0.442 | n.s. |
| **SAR * Side of the head** | 1.37 | 1 | 0.241 | n.s. |
| **SAR * Day** | 21.05 | 4 | <0.001 | highly significant |
| **SAR * Section** | 0.11 | 1 | 0.743 | n.s. |
| **Side of the head * Day** | 13.97 | 4 | 0.007 | highly significant |
| **Side of the head * Section** | 2.71 | 1 | 0.100 | tendency |
| **Day * Section** | 8.88 | 4 | 0.064 | tendency |
| **SAR * Side of the head * Day** | 8.11 | 4 | 0.088 | tendency |
| **SAR * Side of the head * Section** | 4.50 | 1 | 0.034 | significant |
| **SAR * Day * Section** | 14.41 | 4 | 0.006 | highly significant |
| **Side of the head * Day * Section** | 11.86 | 4 | 0.018 | significant |
| **SAR * Side of the head * Day * Section** | 6.09 | 4 | 0.193 | n.s. |

### 4.3.6 MEMORY TEST

Memory was tested 3 times during each test day. The first test 3 minutes after memorizing basically served as warming-up, and was not analyzed. The results of the other tests are summarized in Figure 4.16, the percentage of correct responses in Figure 4.17.

The reaction time was significantly shorter in the exposure group “high”. In both groups it increased from day 1 to day 2 significantly and remained high for the next days. The percentage of correct answers was significantly lower in the exposure group “high”. It was significantly reduced at the second day and remained at the low level. At every test day number of correct responses was lower at the last test.

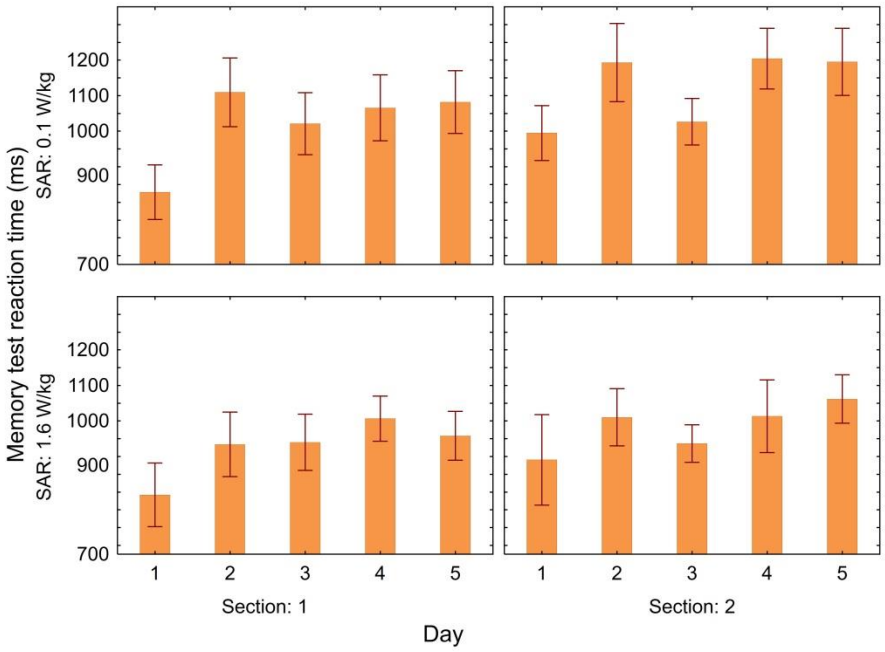


Figure 4.16, Memory test, mean reaction times (and 95 % confidence intervals) on the 5 test days and at the second (section 1) and third presentation (section 2) during buccal exposure to 0.1 W/kg or 1.6 W/kg.

Table 4.13. Results of the analysis of reaction times for the memory test by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 11.01 | 1 | <0.001 | highly significant |
| **Side of the head** | 1.40 | 1 | 0.237 | n.s. |
| **Day (1-5)** | 61.13 | 4 | <0.001 | highly significant |
| **Section (1-2)** | 34.58 | 1 | <0.001 | highly significant |
| **SAR * Side of the head** | 0.78 | 1 | 0.376 | n.s. |
| **SAR * Day** | 5.86 | 4 | 0.210 | n.s. |
| **SAR * Section** | 2.42 | 1 | 0.120 | n.s. |
| **Side of the head * Day** | 6.55 | 4 | 0.162 | n.s. |
| **Side of the head * Section** | 0.01 | 1 | 0.923 | n.s. |
| **Day * Section** | 27.23 | 4 | <0.001 | highly significant |
| **SAR * Side of the head * Day** | 10.49 | 4 | 0.033 | significant |
| **SAR * Side of the head * Section** | 1.19 | 1 | 0.275 | n.s. |
| **SAR * Day * Section** | 5.22 | 4 | 0.266 | n.s. |
| **Side of the head * Day * Section** | 2.04 | 4 | 0.729 | n.s. |
| **SAR * Side of the head * Day * Section** | 18.25 | 4 | 0.001 | highly significant |

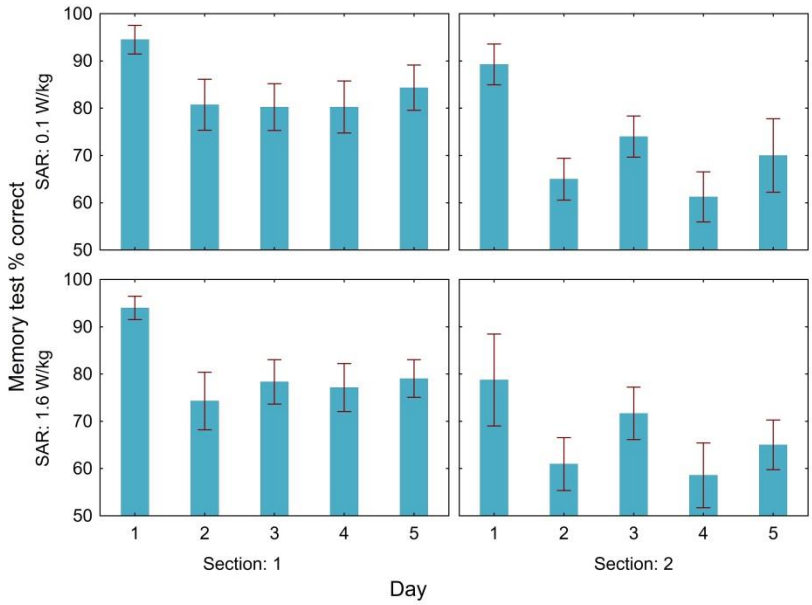


Figure 4.17. Memory test, mean percentage of correct responses (and 95 % confidence intervals) on the 5 test days and at the second and third presentation during buccal exposure to 0.1 W/kg or 1.6 W/kg.

Table 4.14. Results of the analysis of correct responses for the memory test by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 7.36 | 1 | 0.007 | highly significant |
| **Side of the head** | 0.53 | 1 | 0.465 | n.s. |
| **Day (1-5)** | 162.49 | 4 | <0.001 | highly significant |
| **Section (1-2)** | 157.18 | 1 | <0.001 | highly significant |
| **SAR * Side of the head** | 2.52 | 1 | 0.113 | n.s. |
| **SAR * Day** | 2.69 | 4 | 0.610 | n.s. |
| **SAR * Section** | 0.22 | 1 | 0.641 | n.s. |
| **Side of the head * Day** | 5.36 | 4 | 0.252 | n.s. |
| **Side of the head * Section** | 0.00 | 1 | 0.955 | n.s. |
| **Day * Section** | 14.73 | 4 | 0.005 | highly significant |
| **SAR * Side of the head * Day** | 14.37 | 4 | 0.006 | highly significant |
| **SAR * Side of the head * Section** | 6.67 | 1 | 0.010 | highly significant |
| **SAR * Day * Section** | 3.81 | 4 | 0.433 | n.s. |
| **Side of the head * Day * Section** | 1.12 | 4 | 0.892 | n.s. |
| **SAR * Side of the head * Day * Section** | 2.29 | 4 | 0.682 | n.s. |

### 4.3.7 STATE-OF-MOOD SCALE ACCORDING TO NITSCH

The rating of the state-of-mood was performed twice every day, before and after each session. Four sub-scales were analyzed: 1) effort disposition, 2) tension, 3) exhaustion, and 4) sleepiness. Because for tiredness and sleepiness scales high values indicate relaxed or alert, respectively, we converted these scales before statistical analysis.

Figure 4.18 shows means of ratings. Effort disposition remained about the same troughout all 5 test days, however, there was a tendency for a decrease from begin until the end of the test series within each day. Interestingly this decrease was less pronounced in the group “1.6 W/kg”. Tension increased from day to day, however, in the exposure group “high” less pronounced. Feelings of being relaxed and alert slightly increased during the test days, but within each day we observed a decrease, which was statistically significant. The exposure group „high“ (1.6 W/Kg) showed a less pronounced decrease of alertness in particular during the second half of the test week.

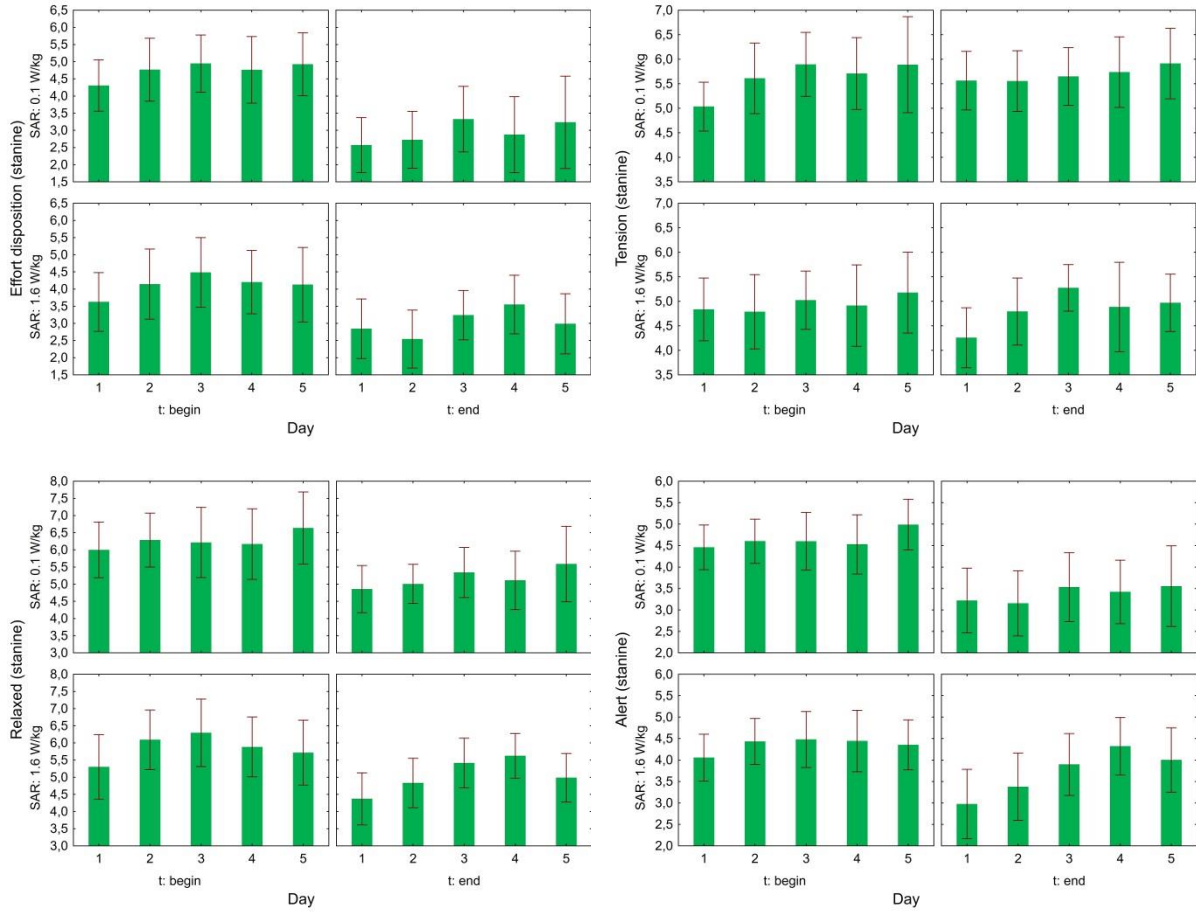


Figure 4.18. Mean stanine values (and 95% confidence intervals) of Nitsch subscales effort disposition, tension, relaxed, alert at the beginning and end of each of 5 experimental days for exposure groups 0.1 W/kg or 1.6 W/kg.

Table 4.15. Results of the analysis of the Nitsch subscale effort disposition by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 6.28 | 1 | 0.012 | significant |
| **Side of the head** | 0.37 | 1 | 0.541 | n.s. |
| **Day (1-5)** | 3.84 | 4 | 0.428 | n.s. |
| **SAR * Side of the head** | 0.33 | 1 | 0.564 | n.s. |
| **SAR * Day** | 3.94 | 4 | 0.414 | n.s. |
| **Side of the head * Day** | 6.04 | 4 | 0.196 | n.s. |
| **SAR * Side of the head * Day** | 16.53 | 4 | 0.002 | highly significant |

Table 4.16. Results of the analysis of the Nitsch subscale tension by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 0.72 | 1 | 0.395 | n.s. |
| **Side of the head** | 0.02 | 1 | 0.901 | n.s. |
| **Day (1-5)** | 0.25 | 4 | 0.993 | n.s. |
| **SAR * Side of the head** | 0.28 | 1 | 0.595 | n.s. |
| **SAR * Day** | 10.90 | 4 | 0.028 | significant |
| **Side of the head * Day** | 3.23 | 4 | 0.520 | n.s. |
| **SAR * Side of the head * Day** | 2.35 | 4 | 0.671 | n.s. |

Table 4.17. Results of the analysis of the Nitsch subscale exhaustion by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 0.60 | 1 | 0.438 | n.s. |
| **Side of the head** | 1.62 | 1 | 0.203 | n.s. |
| **Day (1-5)** | 4.19 | 4 | 0.381 | n.s. |
| **SAR * Side of the head** | 2.63 | 1 | 0.105 | n.s. |
| **SAR * Day** | 3.18 | 4 | 0.529 | n.s. |
| **Side of the head * Day** | 1.98 | 4 | 0.739 | n.s. |
| **SAR * Side of the head * Day** | 5.50 | 4 | 0.240 | n.s. |

Table 4.18. Results of the analysis of the Nitsch subscale sleepiness by generalized estimation equations.

| Source of Variance | Statistic | Degree of freedom | p-Value | Significance |
|---|---|---|---|---|
| **SAR** | 5.67 | 1 | 0.017 | significant |
| **Side of the head** | 0.19 | 1 | 0.666 | n.s. |
| **Day (1-5)** | 9.34 | 4 | 0.053 | tendency |
| **SAR * Side of the head** | 0.09 | 1 | 0.767 | n.s. |
| **SAR * Day** | 4.57 | 4 | 0.334 | n.s. |
| **Side of the head * Day** | 3.59 | 4 | 0.464 | n.s. |
| **SAR * Side of the head * Day** | 12.51 | 4 | 0.014 | significant |

## 4.4 SUMMARY OF RESULTS OF COGNITIVE PERFORMANCE

In this subproject 41 healthy adults (21 males and 20 females, aged 29±10 years) were tested. In an absorber cabin volunteers were double blinded exposed either on the right or left side of the head with a UMTS signal at two intensities. The specific absorption rate (SAR) was calculated for the oral mucosa and amounted to 0.1 W/kg or 1.6 W/kg for the two exposure groups. 20 persons were randomly allocated to the exposure group “low” (9 were exposed on the left and 11 were exposed on the right side of the head), 21 persons were allocated to the exposure group „high“ (10 were exposed on the left side and 11 on the right side of the head). Exposure took place on five consecutive days (Monday to Friday) at the same time of the day.

During the exposure, which lasted for roughly 2 hours per day, we performed cognitive tests and reaction tests. We observed significant learning effects of our volunteers, i.e. the reaction accelerated as the test time advanced. However, this accelerated reaction time was paralleled by a decrease of correct reactions. In contrast to the performance during reaction tests the reaction times increased significantly during the vigilance test and during the memory test. Again, at the same time the fraction of correct reactions decreased.

With respect to the exposure intensity we observed a stronger decrease of the reaction time during the choice reaction test and the perceptional speed test in the group with high exposure compared to the low exposure group. During the memory tests the reaction time was shorter in the high exposure group. The rate of errors was higher in the perceptional speed test and the memory tests in the high exposure group.

The ratings of the state-of-mood according to Nitsch showed trends depending on the exposure group (high, low). Tension decreased during the day in the exposure group „high“, and increased in the exposure group „low“, these effects were pronounced during the first days. The daily increase of sleepiness was stronger in the exposure group „high“ during the first days, and became weaker after some days. We observed no such changes in the exposure group „low“.

The results confirm key results of the previous ATHEM-1 project which showed a trend to faster reactions and a decrease of correct responses under RF-EMF exposure. This finding is in line with some previous publications (listed in section 4.1). Our results provide an explanation of controversially discussed results in the past. The main result of decreasing reaction times and decreasing accuracy during exposure was accompanied by a general trend in reaction time due to a certain learning effect. In addition, for some tests the difference was attributable to the intensity of the exposure.

The observed effects were depending on the complexity of the test conditions. Simple tests led to a reduction of reaction time and quality of the reaction. During complex tasks like memory tests we observed an increase of reaction time, and again a decrease of the reaction-quality.

# 5 HUMAN BUCCAL MUCOSA CELLS

# Experimental Investigation on Effects of UMTS Mobile-Phone Signals on Human Buccal Mucosa Cells

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## 5.1 BACKGROUND

In this subproject anomalies of cell nuclei in human buccal mucosa cells were investigated after exposure to radiofrequency electromagnetic fields (RF-EMF).

In the last couple of years investigations of micronuclei and other nuclear anomalies in human lymphocytes and other cells (e.g. buccal, nasal, urothelial cells) have gained importance concerning potential risk estimation from exposure to various environmental and occupational agents. On the one hand, there are comprehensive guidelines for performing these tests,[75-79] and on the other hand, these tests have been validated, the results indicate cancer risk as determined in epidemiological studies.[80] Hence, these tests may reveal increased risks in an exposed group at early an early stage (i.e. without having to wait for the rare event of a cancer case).

To date only few investigations of nuclear anomalies related to the use of mobile phones have been conducted. Smaller investigations with about 25 mobile phone users and controls from India[81, 82] resulted in increased frequencies of micronuclei in peripheral lymphocytes or buccal cells and of dicentric chromosomes.[83] A larger investigation in 85 mobile phone users and 24 controls also from India[84] also revealed increased frequencies of micronuclei in mobile phone users and higher rates of 'broken eggs' and binucleates. A field study from Germany[85] found no relationship between micronucleus frequency and duration of mobile phone use. However, only 13 from 131 participants did not use a mobile, and, on the other hand, only 33 subjects used a mobile for more than 3 hours per week. An investigation from Brazil[86] included 45 individuals that were subdivided into three groups based on the duration of use. There were no differences in rates of micronuclei, but a larger frequency of other nuclear anomalies such as broken eggs in subjects with more frequent mobile phone use was observed.

The evidence provided by those investigations on mobile phone usage rather speaks in favor of a gene- or gene-/cytotoxic effect, but overall results are not unambiguous and there are some methodological shortcomings. In the studies conducted so far exposure was assessed by the reported average intensity of mobile phone use. It has to be considered that the subjective assessment of mobile phone use may be biased. Furthermore, due to the life cycle of the investigated cell populations exposure during different time windows could have differential effects. Earlier exposure could affect basal cells from which differentiated cells originate, while more recent exposure could affect the development of emerging daughter cells.

When investigating exfoliated buccal mucosa cells, always a mixture of effects from chronic and acute exposure is observed, cumulative effects on some or all endpoints of nuclear anomalies cannot be excluded.

The greatest shortcoming, however, is dosimetry. Actual exposure of cells depends on the type and model of the mobile phone, on the position of the mobile during use, and the radiation conditions at the location of use. This introduces a strong variation of

exposure making it difficult to perform objective comparisons. Other shortcomings of available studies are the use of non validated protocols for the buccal mucosa assay.

### 5.1.1 NUCELAR ANOMALIES

Micronuclei are intracellular structures containing chromatin and surrounded by a nuclear membrane. They occur during cell division from loss of a chromosome (aneugenic effect) or of fragments of a chromosome (klastogenic effect) as a consequence of genotoxic damage. They can also be a consequence of an interference with the spindle apparatus during cell division. Other nuclear anomalies that indicate a genotoxic effect are nuclear buds (e.g. occurring from gene amplification) and broken eggs. Binucleated cells are produced from cytotoxic effects but also combinations of gene- and cytotoxic effects. Different stages of cell necrosis and apoptosis that could be caused by both genotoxic as well as cytotoxic effects are pyknosis, condensed chromatin, karyorrhexis and karyolysis.

In the following figure (Figure 5.1) a schematic overview of the different nuclear anomalies and their possible causes is shown.

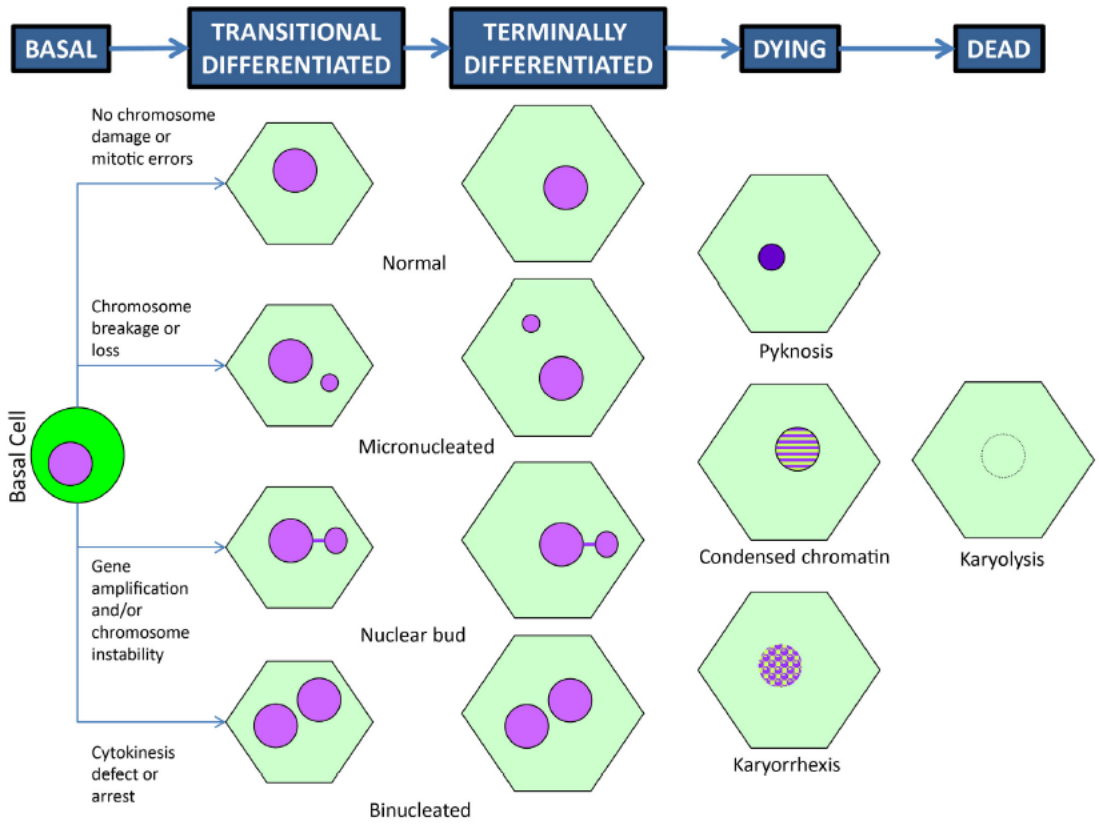


Figure 5.1. Overview of the devlopment of differentiated buccal mucosa cells with and without nuclear anomalies. It should be noted that cells with micronuclei, nuclear buds and bincleated cells can develop into dying or dead cells, while the opposite cannot occur. Graph from Bolognesi et al. 2013.[78]

Examples how these cells appear under the microscope with and without fluorescence filter are shown in Figure 5.2.

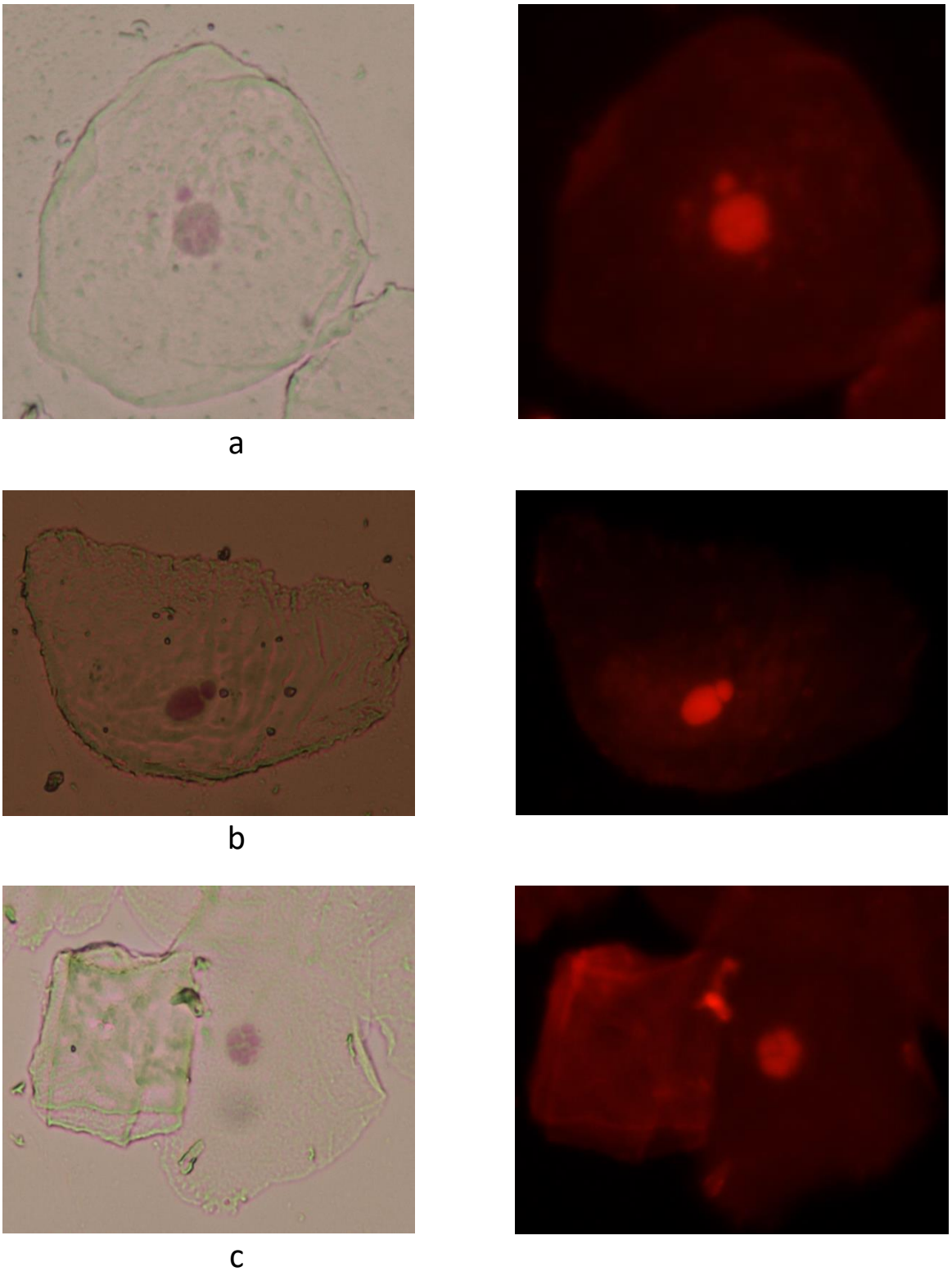


Figure 5.2. Examples of microphotographs of buccal mucosa cells. DNA has been stained with Feulgen (pink), cytoplasm with light green. Cells with 400-fold magnification under

transmission (left panel) and fluorescence filter (right panel); a) micronucleated cell; b) nuclear bud; c) kayrorrhexis (right cell) and karyolysis (left cell).

### 5.1.2 GOALS OF THE STUDY

The experimental procedures have addressed the methodological issues mentioned before, by the considering the following steps

1. Experimental intervention:
   a targeted exposure with predefined duration and intensity in the lab.
2. Defined observation period of 7 weeks,
   use of a headset, in case a mobile phone was used during this period
3. Double blind experimental procedure (Neither experimental subjects nor experimenter knew which side was actually exposed and at what intensity the exposure occurred.)
4. Direct comparison before to after exposure;
   collection of buccal cells before, 2 and 3 weeks after exposure
5. Intra-individual control by comparison of the exposed to the unexposed side od the head.
6. Performing the buccal cytome assay according to a validated protocol.[78]

## 5.2 MATERIAL AND METHODS

### 5.2.1 EXPERIMENTAL DESIGN

The experimental design is a 2x2-groups double-blind investigation. Subjects were allocated randomly to the experimental conditions. Each participant received the assigned exposure during 5 consecutive days with a daily exposure duration of about 1 h 50 min, at the same time of day.

Table 5.1, Experimental design of four experimental groups differing by exposed side of the head and exposure intensity

| Exposed side / Intensity | Left | Right |
|---|---|---|
| SAR 0.1 W/kg | A | B |
| SAR 1.6 W/kg | C | D |

Volunteers were seated in a cabin with RF-EMF-absorbing walls. The exposure was from antennas mounted on the head in such a way as to allow the volunteers to move the head without limitation and almost without burden. There was an antenna on each side of the head so that neither the subject nor the experimenter could detect which side is the exposed one. The procedure has been explained in greater detail in chapter 4.

### 5.2.2 INCLUSION AND EXCLUSION CRITERIA

Individuals aged 20 to 60 years of both sexes, non-smokers, and non-users of a mobile phone or with less than 1 h mobile phone use per day without acute or chronic diseases that could interfere with the experimental procedure of several hours each day were enrolled if they signed an informed consent.

Participants were informed that they have to fill in a diary over a period over two weeks before until three weeks after the experiment. Furthermore, in case they used a mobile phone, they had to use a headset (if they had no headset they were provided with such a device).

### 5.2.3 SAMPLE SIZE DETERMINATION

For the sample size determination the following issues were addressed:

1. Expected effect size
1. Variability of the primary endpoint
2. Statistical significance level
3. Statistical power

Ad 1. **Effect size:** In the investigation of Yadav and Sharma [84] 24 subjects that reported not using a mobile phone were compared to 85 regular users. The average duration of use was one hour and - according to the manufacturers - the mobile phones had a maximum SAR of between 0.34 and 0.95 W/kg. The difference in micronucleus frequency was highly significant associated with a standardized effect size (Cohen's d) of 1.64.

The investigation of Hintzsche and Stopper [85] in a sample of overall 112 subjects found 21 individuals using a mobile less than 5 years and 91 with more that 5 years mobile phone use. A weak effect of d=0.3 was obtained. The analyses of daily use revealed no relevant differences. Howevers, the groups were very small (some with only one person) and overall only 3 subjects had used the phone more than 1 h per day. Hence, it follows from the analysis of the literature that no definitive estimate of the effect size can be delineated but an effect size of d=1 should be detected under controlled experimental conditions.

Ad 2. **Variability:** With respect to the variability of the primary endpoint (in this case the rate of micronuclei) there are sufficient statistical data.[87, 88] In the expected range of low to medium frequencies the geometric variance across individuals (almost independent from the absolute magnitude of micronuclei) is about 0.8.

Ad 3. As **level of significance** of 5% error probability was chosen.

Ad 4. Statistical **power** was set to 80%.

Based on these assumptions, for the endpoint defined as the difference before to after exposure the standard deviation (for a correlation of 0.75 between time points) amounts to 0.89. The effect size that the study should detect is, therefore, 0.9.

The critical t-value is calculated as 2.021. This value is significant at a corrected significance level of 5% and a power of 80% if the sample size is 21 (see also Figure 5.3).

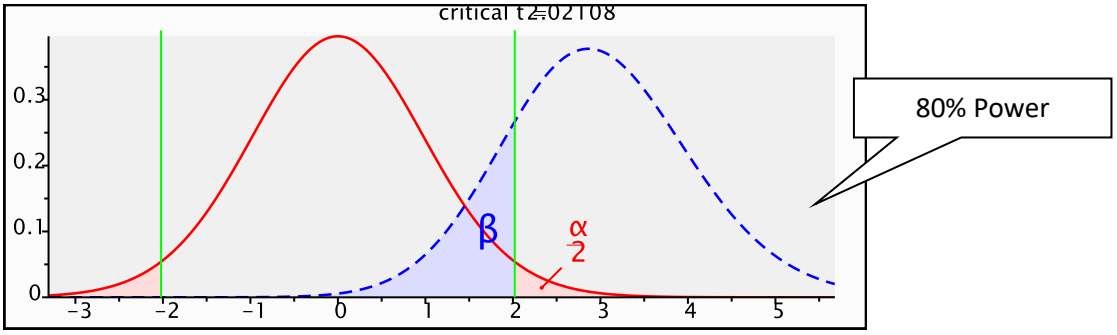


Figure 5.3. Plot of students t-distribution under the zero hypothesis (red ─) and the alternative hypothesis (blue ---) – Plot by G*Power 3.1.2. α: level of significance (0,05 = 5 %), β: 1-power = 0,2.

For two exposure levels 42 individuals were enrolled.

### 5.2.4 EXPERIMENTAL PROCEDURE

The procedure for the participants is schematically shown in Figure 5.4.

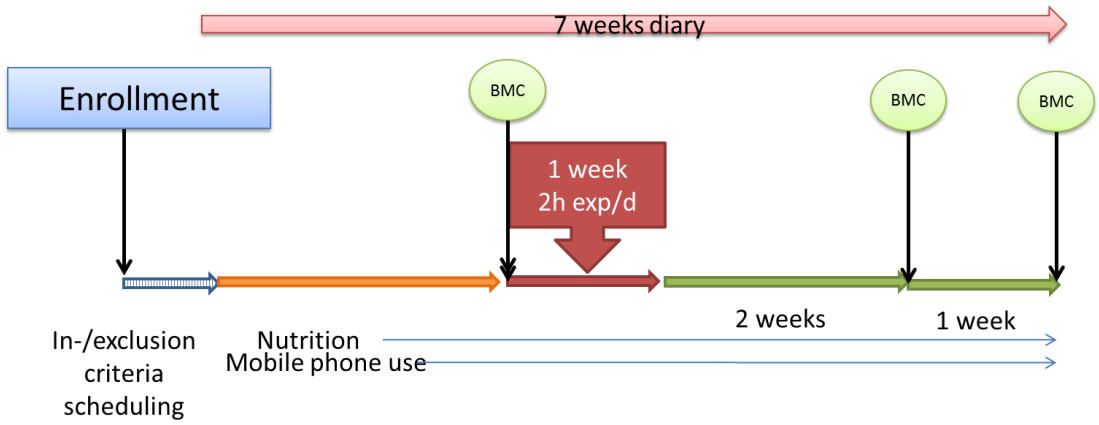


Figure 5.4. Procedure for the participants. The enrolled participants started entries into the diary two weeks before experimental exposure and continued until three weeks thereafter. Harvesting of buccal cells (BMC) was at the first day of the exposure week before exposure and two as well as three weeks after exposure.

On the first experimental day the assignment of the exposed side of the head and the exposure intensity (low or high) was randomly chosen. On the following days the identical experimental conditions were chosen by the system after identification of the volunteer by the full name. The exposure conditions were unblinded only after sample analysis was finished.

After the exposure and removal of the antenna a short interview about the subject's perceptions during the experiment was scheduled. Each day the total duration was approximately 2 ½ h. The same procedure was performed daily from Monday to Friday. At the last day the dates for collection of buccal cells were scheduled if not already fixed at enrollment.

### 5.2.5 EXPERIMENTAL SUBJECTS

From 42 enrolled subjects one had to quit participation after the first day for personal reasons. Due to time constraints and the tight schedule this person could not be replaced.

Of the participants 21 were male and 20 female. Age was 29±10 years (22 to 56 years), 7 were left- and 34 right handed. The majority (30 subjects) had higher education, 9 had a university degree and 2 a college graduation.

20 subjects were randomly allocated to the lower exposure group (SAR 0.1 W/kg) with 9 exposed at the left and 11 at the right side. High exposure (SAR 1.6 W/kg) was assigned to 21 subjects, 10 with left side and 11 with right side exposure.

### 5.2.6 DIARIES

Diaries were filled in for two to three weeks before the exposure, during and for three weeks after exposure week (overall 6-7 weeks). For each day the number of in- and outgoing call with the mobile phone was recorded, the total duration of calls as well as the total duration of calls without head set.

In addition, consumption of spicy meals, gingival bleeding and other events (e.g. dentist visit) that could have an influence on oral mucosa were recorded.

| 2. Woche | | | |
|---|---|---|---|
| | Handytelefonieren | | |
| Datum | Anzahl | Minuten | ohne Frspr. |
| Mo 21.01.2013 | | | |
| Di 22.01.2013 | | | |
| Mi 23.01.2013 | | | |
| Do 24.01.2013 | | | |
| Fr 25.01.2013 | | | |
| Sa 26.01.2013 | | | |
| So 27.01.2013 | | | |
| Anmerkungen: | | | |

| 3. Woche | | | |
|---|---|---|---|
| | Handytelefonieren | | |
| Datum | Anzahl | Minuten | ohne Frspr. |
| Mo 28.01.2013 | | | |
| Di 29.01.2013 | | | |
| Mi 30.01.2013 | | | |
| Do 31.01.2013 | | | |
| Fr 01.02.2013 | | | |
| Sa 02.02.2013 | | | |
| So 03.02.2013 | | | |
| Anmerkungen: | | | |

| 4. Woche | | | |
|---|---|---|---|
| | Handytelefonieren | | |
| Datum | Anzahl | Minuten | ohne Frspr. |
| Mo 04.02.2013 | | | |
| Di 05.02.2013 | | | |
| Mi 06.02.2013 | | | |
| Do 07.02.2013 | | | |
| Fr 08.02.2013 | | | |
| Sa 09.02.2013 | | | |
| So 10.02.2013 | | | |
| Anmerkungen: | | | |

| 2. Woche | | | |
|---|---|---|---|
| Datum | Scharfe Speise | Zahnfl. bluten | Sonst. |
| Mo 21.01.2013 | O | O | O |
| Di 22.01.2013 | O | O | O |
| Mi 23.01.2013 | O | O | O |
| Do 24.01.2013 | O | O | O |
| Fr 25.01.2013 | O | O | O |
| Sa 26.01.2013 | O | O | O |
| So 27.01.2013 | O | O | O |
| Anmerkungen: | | | |

| 3. Woche | | | |
|---|---|---|---|
| Datum | Scharfe Speise | Zahnfl. bluten | Sonst. |
| Mo 28.01.2013 | O | O | O |
| Di 29.01.2013 | O | O | O |
| Mi 30.01.2013 | O | O | O |
| Do 31.01.2013 | O | O | O |
| Fr 01.02.2013 | O | O | O |
| Sa 02.02.2013 | O | O | O |
| So 03.02.2013 | O | O | O |
| Anmerkungen: | | | |

| 4. Woche | | | |
|---|---|---|---|
| Datum | Scharfe Speise | Zahnfl. bluten | Sonst. |
| Mo 04.02.2013 | O | O | O |
| Di 05.02.2013 | O | O | O |
| Mi 06.02.2013 | O | O | O |
| Do 07.02.2013 | O | O | O |
| Fr 08.02.2013 | O | O | O |
| Sa 09.02.2013 | O | O | O |
| So 10.02.2013 | O | O | O |
| Anmerkungen: | | | |

Figure 5.5. Example for part of the diary for mobile phone use and other relevant incidents (in German language, as in the original experiment). The days with planned experimental exposure are marked pink.

### 5.2.7 BUCCAL MUCOSA CYTOM ASSAY

Immediately before the first exposure as well as 2 and 3 weeks after exposure buccal cells were harvested with a soft tooth brush from the participants separately from the left and the right cheek after they have rinsed their mouth twice with tap water.

Cells were then processed according to the protocol of Thomas et al. [76]. Samples were smeared with one drop of water via sterile pipettes gently to the end of glass slides. The slides were air dried for at least 10 minutes in the dark, and stained according to the standard Feulgen technique. Then they were rinsed with running tap water for 3 minutes, and were immediately stained with Schiff's reagent under dark condition at room temperature for 90 minutes and washed under running tap water for 5 minutes. The slides were counterstained with 0.2% (w/v) Light Green.

The slides were assigned a code number. The blinded assessment was done by a fluorescence microscope under 400 x magnification.

The first step consisted of counting 1000 differentiated cells and basal cells for all nuclear anomalies (micronuclei, nuclear buds, broken eggs, binucleates, condensed chromatin, karyorrhexis, karyolysis, and pyknosis), then further 1000 cells were inspected for micronuclei, nuclear buds and broken eggs.

## 5.3 STATISTICAL METHODS

Nuclear anomalies that are counted in the Cytom assay in 1000 cells (cytotoxic endpoints) or 2000 cells (genotoxic endpoints) usually follow a Poisson distribution. In the statistical analysis this is accounted for by application of so called generalized estimation equations (GEE). In this case a Poisson model with a log link was applied. Hence the parameter $\lambda$ of the Poisson distribution is modelled as a logarithmic function of the factors and covariables.

**Exposure to two intensities considering the contralateral side**

In the present case the analysis was conducted in two groups differing in exposure intensity (SAR 1.6 und 0.1 W/kg). Results obtained 2 and 3 weeks after exposure were compared to those immediately before first exposure. As an offset variable the contralateral side was used. As a consequence the results – expressed as odds ratios – indicate how much the base rate is increased or decreased after exposure corrected for results at the contralateral side. As covariates age and gender were included in the model. Pairwise comparisons of the rates 2 and 3 weeks after exposure against baseline were performed applying Bonferroni correction. Deviation from the Poisson model was checked and in case of overdispersion analysis was performed by the same procedure but using the negative binomial distribution.

**Exposure in correspondence with the side of mobile phone use**

In an additional analysis the side of the head that the test-person reported as predominantly used for the phone was considered:

1. cases where the side of the head of habitual use corresponded to the side of experimental exposure, and
2. those where there was no correspondence.

Persons that reported equal use on both sides were allocated to the corresponding group as well as those that reported no use of mobile phones.

## 5.4 RESULTS

### 5.4.1 STATISTICAL EVALUATION OF DIARIES

Table 5.2 shows average numbers of calls per week, duration of calls overall and those without headset. Results for the last week were omitted because this week was incomplete due to returning the diary on Thursday or Friday of the 7$^{th}$ week.

Table 5.2. Median and interquartile range of number of calls, duration of calls (minutes) and duration without hands-free set (minutes) during weeks 1 to 6.

| Group | Week | Number of calls | Duration of calls | Duration without hands-free set |
|---|---|---|---|---|
| 1.6 W/kg, right | 1 | 19.0 [16.0-27.0] | 66.0 [49.0-130.0] | 0.0 [0.0-3.0] |
| | 2 | 19.0 [13.0-36.0] | 116.0 [28.0-189.0] | 0.0 [0.0-1.0] |
| | 3 | 19.0 [13.0-37.0] | 86.0 [32.0-134.0] | 0.0 [0.0-0.6] |
| | 4 | 22.0 [13.0-30.0] | 116.0 [34.0-167.0] | 0.0 [0.0-4.0] |
| | 5 | 17.0 [11.0-29.0] | 66.0 [23.0-163.0] | 0.0 [0.0-1.0] |
| | 6 | 20.0 [16.0-39.0] | 98.0 [62.0-152.0] | 0.0 [0.0-5.0] |
| 1.6 W/kg, left | 1 | 30.5 [11.5-61.0] | 156.0 [41.5-248.3] | 0.0 [0.0-3.0] |
| | 2 | 23.0 [15.3-86.8] | 176.0 [16.8-248.3] | 0.0 [0.0-1.0] |
| | 3 | 27.0 [6.8-82.8] | 151.0 [11.3-221.8] | 0.5 [0.0-1.0] |
| | 4 | 35.0 [15.0-87.3] | 146.5 [64.5-293.3] | 0.0 [0.0-2.5] |
| | 5 | 30.0 [10.5-80.3] | 99.0 [37.5-332.0] | 0.0 [0.0-4.5] |
| | 6 | 34.0 [14.3-75.5] | 147.5 [36.0-268.3] | 0.0 [0.0-3.3] |
| 0.1 W/kg, right | 1 | 18.0 [14.0-49.0] | 38.0 [33.0-138.0] | 0.0 [0.0-1.0] |
| | 2 | 16.0 [14.0-33.0] | 59.0 [26.0-158.0] | 0.0 [0.0-3.0] |
| | 3 | 21.0 [13.0-37.0] | 64.0 [33.0-164.0] | 0.0 [0.0-1.2] |
| | 4 | 22.0 [12.0-56.0] | 84.0 [33.0-164.0] | 0.0 [0.0-4.0] |
| | 5 | 23.0 [16.0-54.0] | 48.0 [27.0-248.0] | 0.0 [0.0-2.0] |
| | 6 | 16.0 [12.0-57.0] | 43.0 [23.0-118.0] | 0.0 [0.0-0.0] |
| 0.1 W/kg, left | 1 | 16.0 [5.5-24.0] | 48.0 [10.0-149.0] | 0.0 [0.0-6.5] |
| | 2 | 10.0 [3.5-23.0] | 35.0 [9.5-105.5] | 0.0 [0.0-2.5] |
| | 3 | 16.0 [2.0-37.5] | 96.0 [5.0-141.0] | 0.0 [0.0-2.1] |
| | 4 | 8.0 [0.0-30.5] | 41.0 [0.0-110.0] | 0.0 [0.0-1.0] |
| | 5 | 9.0 [5.0-31.0] | 51.0 [18.5-120.5] | 0.0 [0.0-3.5] |
| | 6 | 10.0 [6.0-29.5] | 78.0 [23.0-101.5] | 0.0 [0.0-4.0] |

Days with gingival bleeding or spicy meals were too infrequent to compute and show averages. Only at 2 of 100 person-days spicy meals or gingival bleeding was reported.

Values were compared across groups for each week by Kruskal-Wallis tests. In no case a significant difference was obtained.

### 5.4.2 STATISTICAL EVALUATION OF MOBILE PHONE USE

The following table shows the side of the head the mobile phone was typically held by participants as well as the reported duration of mobile phone use outside the 7 weeks of filling in the diary stratified by exposure side during experiments.

Tabelle 5.1. Side of the head the participant reported to normally hold the mobile phone and average duration of phone calls, in relation to the experimental exposure during this investigation.

| | Exposed side of the head | | |
|---|---|---|---|
| | Left | Right | Total |
| Side of typical mobile phone use | n (%) | n (%) | n (%) |
| No use | 2 (10,5%) | 2 ( 9,1%) | 4 ( 9,8%) |
| Both sides | 5 (26,3%) | 3 (13,6%) | 8 (19,5%) |
| Left/predominantly left | 3 (15,8%) | 6 (27,3%) | 9 (22,0%) |
| Right/predominantly right | 9 (47,4%) | 11 (50,0%) | 20 (48,8%) |
| Minutes of calls/week: Median [quartile range] | 360 [5-900] | 300 [83-675] | 300 [75-900] |

It is obvious that the reported average duration of phone calls is usually substantially longer than during the experimental weeks. Habitual cell phone use the right side of the head was more frequent as compared to the left side.

### 5.4.3 STATISTICAL EVALUATION OF THE BUCCAL MUCOSA CYTOM ASSAY

#### Results for genotoxic endpoints

Typical endpoints to indicate genotoxic effects are micronuclei, nuclear buds and broken eggs.

As a first step it has been assessed whether mobile phone use before the experiment had an influence on the baseline rate of these nuclear anomalies at the side of the head of (predominant) mobile phone use.

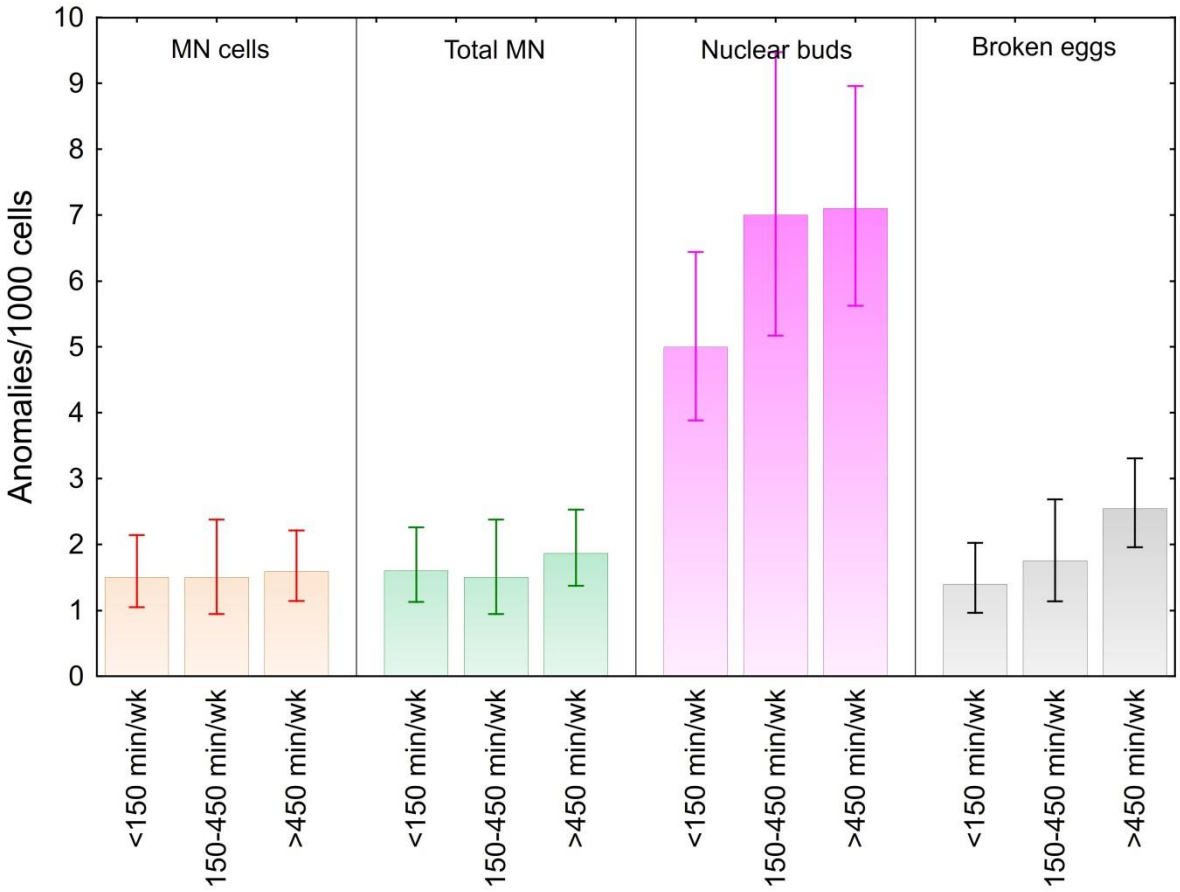


Figure 5.6. Rate of nuclear anomalies indicating genotoxic effects at baseline in relation to reported duration of mobile phone use (minutes/week) abbrevated min/wk at the side of the head of mobile phone use.

It turned out that nuclear buds and broken eggs increased significantly with increasing duration of calls (<150, 150-450 and >450 min/week).

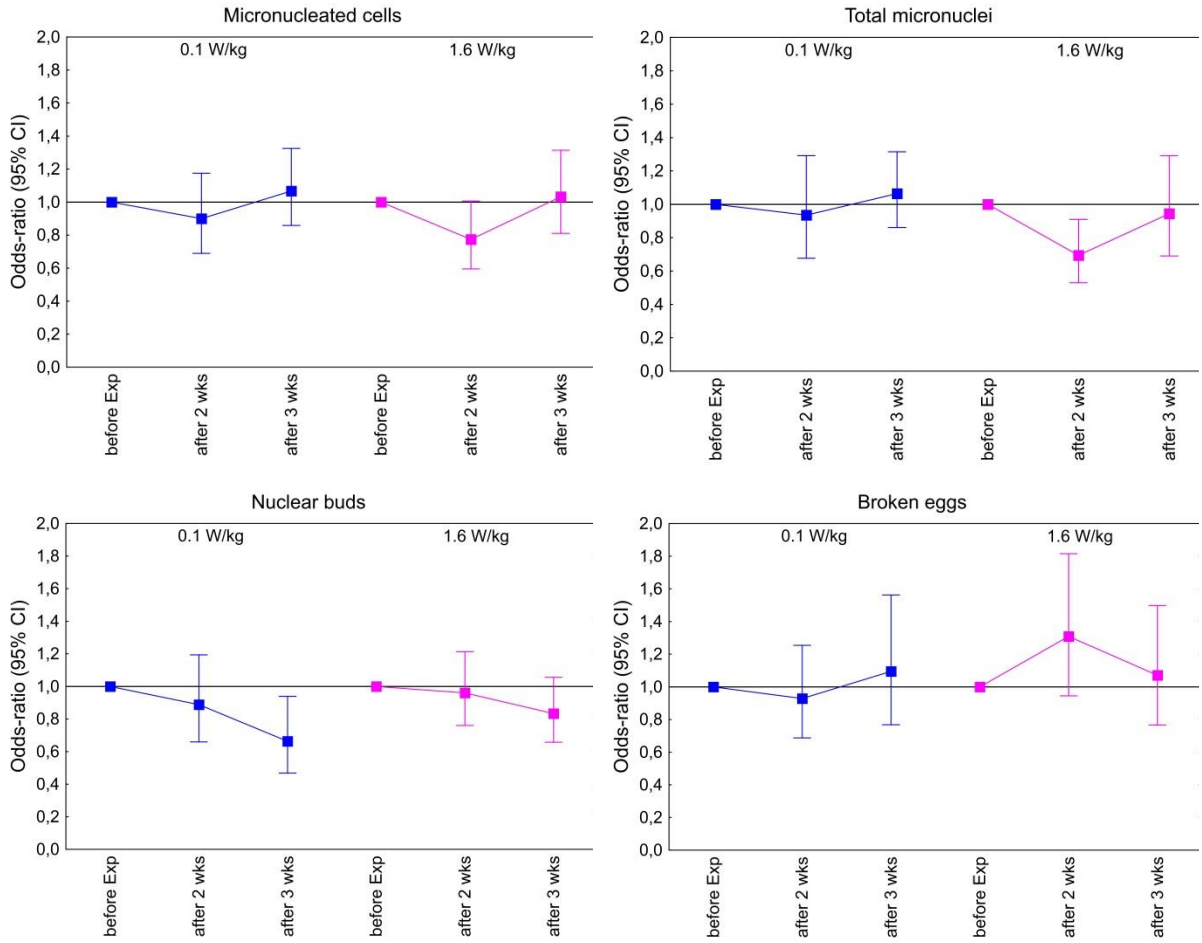


Figure 5.7. Odds ratio (and 95% confidence intervals) for nuclear anomalies indicating genotoxic effects (micronuclei, nuclear buds, and broken eggs) 2 and 3 weeks after exposure at SAR values of 0.1 or 1.6 W/kg relative to baseline values before exposure and relative to the contralateral unexposed side of the head.

For the total number of micronuclei two weeks after exposure a significant reduction occurred at 1.6 W/kg compared to baseline values and relative to the contralateral side. Three weeks after the exposure nuclear buds were reduced, after exposure at the lower exposure intensity significance was reached only. No systematic effects were observed for broken eggs.

Stratification into groups with exposure corresponding and not-corresponding to the habitual side of mobile phone use may give information about a possible modifying effect of exposure by mobile phone use outside the experimental exposure.

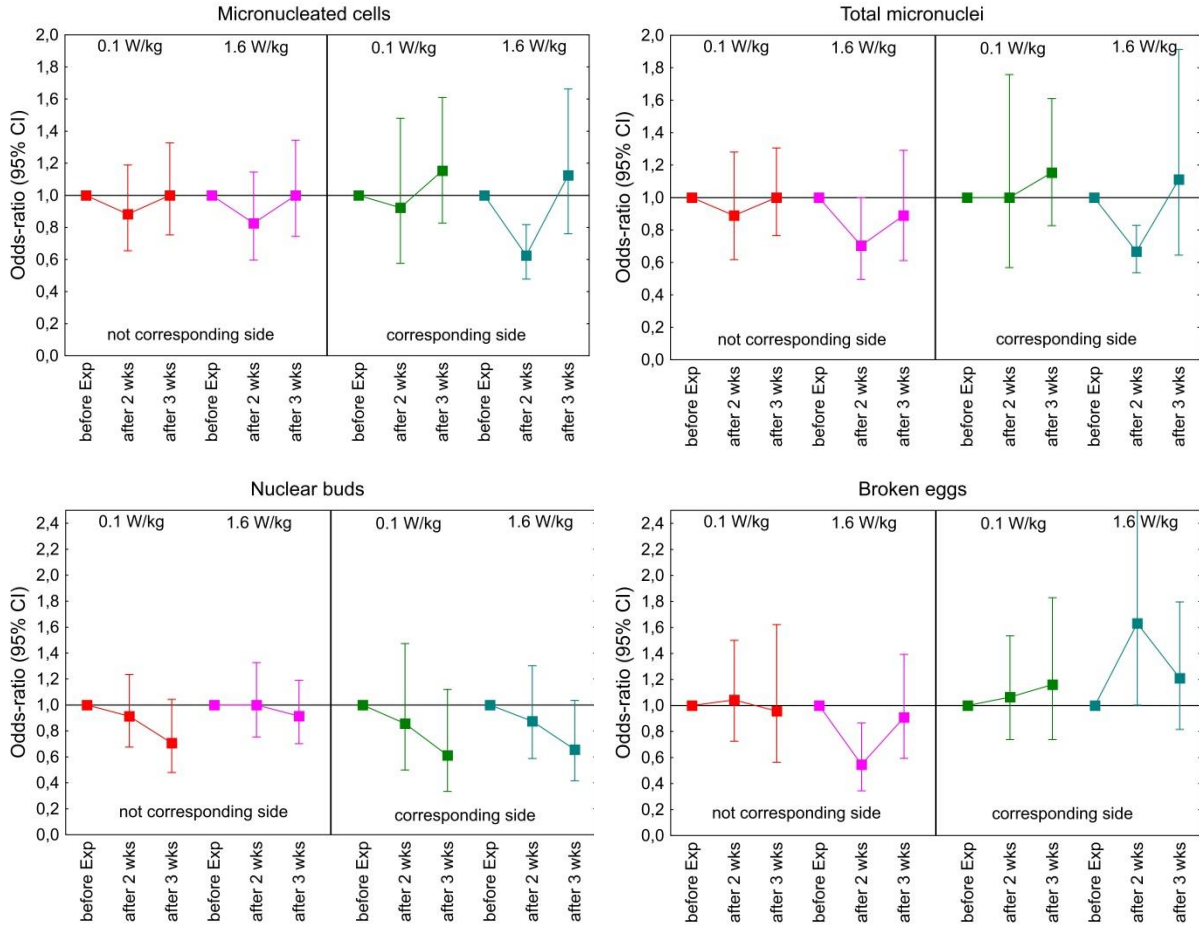


Figure 5.8. Odds ratio (and 95% confidence intervals) for nuclear anomalies indicating genotoxic effects (micronuclei, nuclear buds, and broken eggs) 2 and 3 weeks after exposure at SAR values of 0.1 or 1.6 W/kg relative to baseline values before exposure and relative to the contralateral unexposed side of the head stratified according to correspondence or not-correspondance of experimental exposure side with side of mobile phone use.

The exposure related effects on micronuclei and nuclear buds were stronger in participants with experimental exposure that corresponded to the habitual side of normal phone use. For broken eggs two weeks after exposure an increase was observed in those with corresponding exposure while those with not-corresponding exposure showed a significant decline.

Table 5.3 shows an overview of statistical evaluations for genotoxic endpoints.

Table 5.3. Tests of signficance for genotoxic endpoints for the factors week (week 2 and 3 against baseline), correspondance of the exposed side with the side of habitual mobile phone use and their interaction tested within a Poisson model. Shown are Wald chi² values and p values in brackets. Significant results are shown in bold.

| SAR | Nuclear anomaly | Week (W) | Correspondance (C) | WxC |
|---|---|---|---|---|
| 0.1 W/kg | Micronucleated cells | 1.95 (0.378) | 0.04 (0.844) | 0.46 (0.796) |
| 1.6 W/kg | Micronucleated cells | 29.59 **(<0.001)** | 3.80 (0.051) | 7.09 (0.029) |
| 0.1 W/kg | Total micronuclei | 0.93 (0.629) | 0.00 (0.946) | 0.44 (0.804) |
| 1.6 W/kg | Total micronuclei | 15.87 **(<0.001)** | 3.62 (0.057) | 0.82 (0.665) |
| 0.1 W/kg | Nuclear buds | 6.08 **(0.048)** | 0.07 (0.794) | 0.16 (0.924) |
| 1.6 W/kg | Nuclear buds | 4.22 (0.121) | 8.92 (0.003) | 1.60 (0.450) |
| 0.1 W/kg | Broken eggs | 3.29 (0.193) | 0.69 (0.407) | 2.09 (0.352) |
| 1.6 W/kg | Broken eggs | 6.24 **(0.044)** | 0.10 (0.756) | 7.65 (0.022) |

### Results for cytotoxic endpoints

Endpoints rather indicating cytotoxic effects are binucleates, condensed chromatin, karyorrhexis, karyolysis, and pyknosis. Results for these endpoints are presented in the following figures.

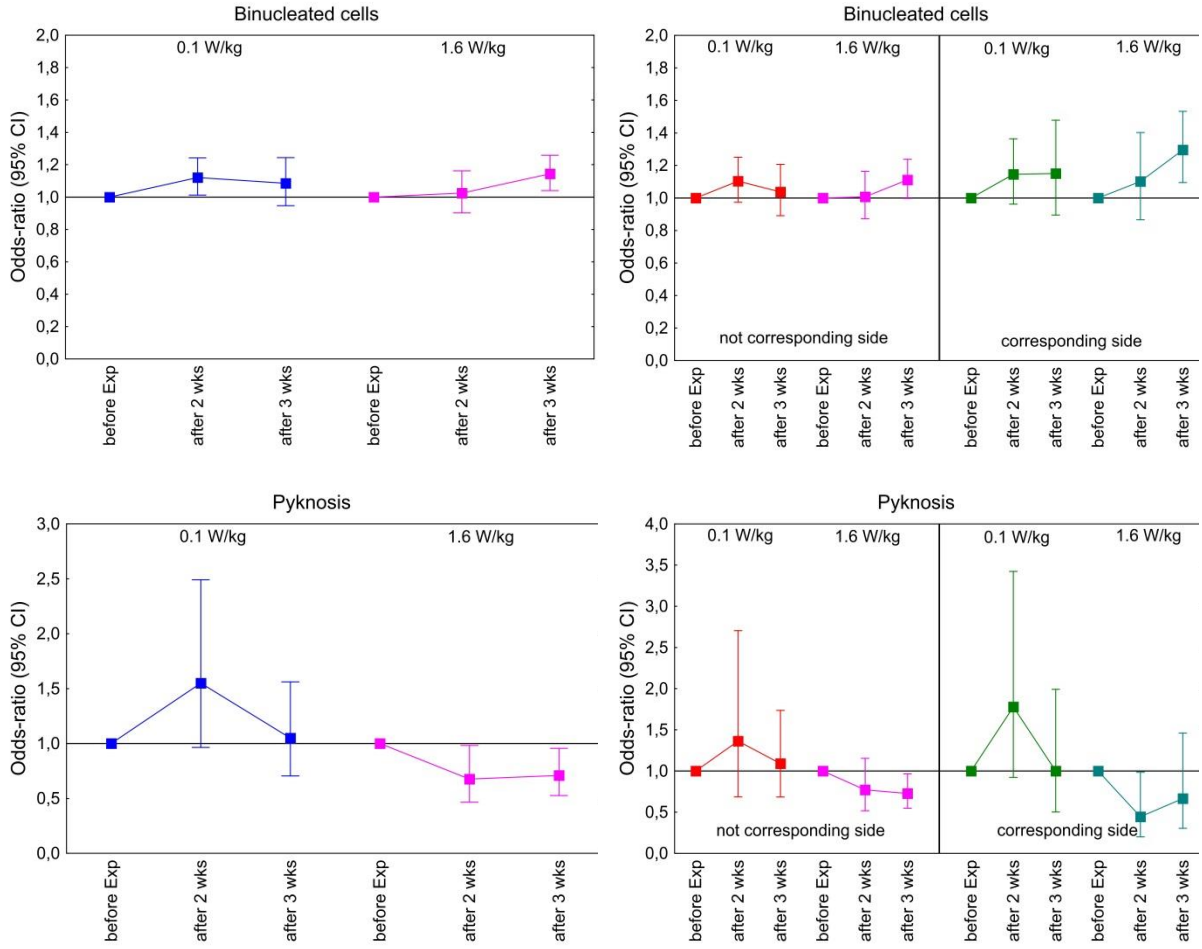


Figure 5.9. Odds ratio (and 95% confidence intervals) for nuclear anomalies indicating cytotoxic effects (binucleates and pyknosis) 2 and 3 weeks after exposure at SAR values of 0.1 or 1.6 W/kg relative to baseline values before exposure and relative to the contralateral unexposed side of the head overall (left panel) and stratified according to correspondence or not-correspondance of experimental exposure side with the reported side of mobile phone use (right panel).

Binucleates increased significantly at the higher exposure level (SAR 1.6 W/kg) after 3 weeks with a more pronounced effect for corresponding exposures. In contrast, pyknotic cells decreased significantly 2 and 3 weeks after exposure with a stronger effect in the group with corresponding exposure but, due to the larger variance, only significant after 2 weeks.

A significant increase was found for cells with condensed chromatin and karyorrhexis 2 and 3 weeks after exposure, with a stronger effect for karyorrhexis at the higher exposure level. Both anomalies showed a more pronounced effect in the group with side of exposure corresponding to habitual side of mobile phone use. This interaction

with correspondence was statistically significant for condensed chromatin and for karyorrhexis at the lower exposure level.

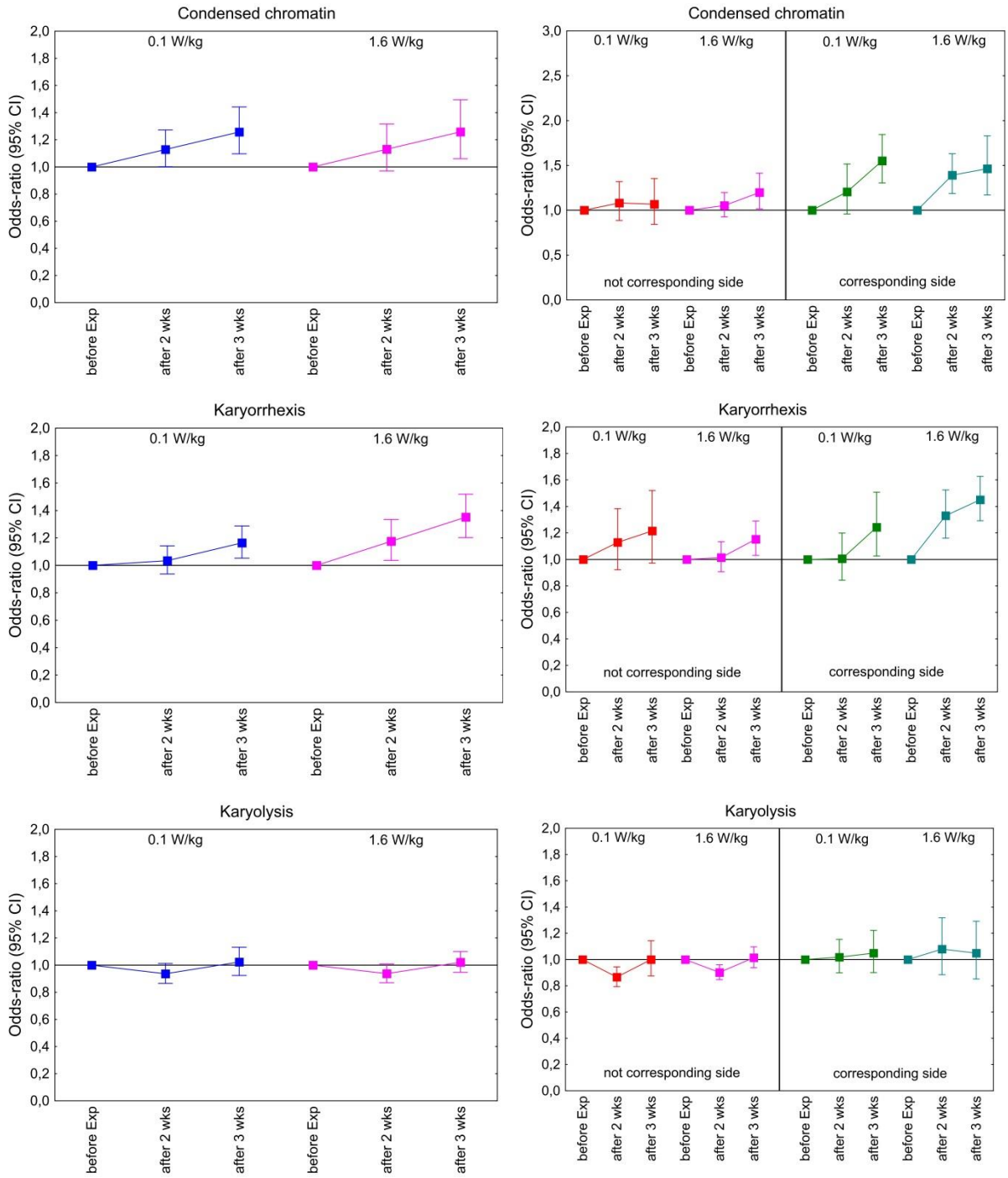


Figure 5.10. Odds ratio (and 95% confidence intervals) for nuclear anomalies indicating cytotoxic effects (condensed chromatin, karyorrhexis, and karyolysis) 2 and 3 weeks after exposure at SAR values of 0.1 or 1.6 W/kg relative to baseline values before exposure and relative to the contralateral unexposed side of the head overall (left panel) and stratified according to correspondence or not-correspondance of experimental exposure side with side of mobile phone use (right panel).

Karyolysis rather declined 2 weeks after exposure and after 3 weeks no difference to baseline occurred. Reduction after 2 weeks was statistically significant for the group with not-corresponding exposure.

Table 5.4 summarizes results of statistical evaluation of all cytotoxic endpoints.

Table 5.4. Tests of signficance for cytotoxic endpoints for the factors week (week 2 and 3 against baseline), correspondance of the exposed side with the side of habitual mobile phone use and their interaction tested within a Poisson model. Shown are Wald chi² values and p values in brackets. Significant results are shown in bold.

| SAR | Nuclear anomaly | Week (W) | Correspondance (C) | WxC |
|---|---|---|---|---|
| 0.1 W/kg | Binucleates | 5.07 (0.079) | 0.20 (0.651) | 0.57 (0.752) |
| 1.6 W/kg | Binucleates | 13.44 (**0.001**) | 6.12 (**0.013**) | 2.27 (0.322) |
| 0.1 W/kg | Karyorrhexis | 26.52 (**<0.001**) | 0.08 (0.782) | 6.11 (**0.047**) |
| 1.6 W/kg | Karyorrhexis | 8.31 (**0.016**) | 4.46 (**0.035**) | 0.97 (0.614) |
| 0.1 W/kg | Karyolysis | 6.24 (**0.044**) | 0.45 (0.504) | 5.59 (0.061) |
| 1.6 W/kg | Karyolysis | 0.98 (0.612) | 0.77 (0.381) | 4.12 (0.127) |
| 0.1 W/kg | Condensed chromatin | 11.56 (**0.003**) | 0.16 (0.685) | 7.40 (**0.025**) |
| 1.6 W/kg | Condensed chromatin | 16.93 (**<0.001**) | 0.04 (0.849) | 7.80 (**0.020**) |
| 0.1 W/kg | Pyknosis | 4.56 (0.102) | 0.04 (0.848) | 0.74 (0.691) |
| 1.6 W/kg | Pyknosis | 6.54 (**0.038**) | 0.03 (0.854) | 2.69 (0.261) |

## 5.5 SUMMARY AND DISCUSSION

In a double-blinded experiment healthy adults were exposed in an absorber cabin at the left or right cheek to simulated UMTS mobile-phone signals at a specific absorption rate (SAR) of 0.1 or 1.6 W/kg. 20 subjects were exposed at a SAR of 0.1 W/kg (9 at the left, 11 at the right side) and 21 subjects at 1.6 W/kg (10 at the left, 11 at the right side). Assignment of exposure conditions was random. Exposure took place daily from Monday to Friday at the same time of the day for about 2 hours.

Participants were asked to refrain from mobile phone use or to use it only with a hands-free set (head phones) two weeks before until three weeks after the experimental week. For the same time period participants filled in a diary logging daily mobile phone use, consumption of spicy meals, gingival bleeding and other events related to the oral cavity. Immediately before, as well as two and three weeks after exposure buccal cells were collected from the left and right cheeks using a soft brush and evaluated according to the protocol of Thomas et al. (2009)[76] for nuclear anomalies (‚Cytom assay').

Results for nuclear anomalies indicating genotoxic or cytotoxic effect were complex and not only depending on experimental conditions but in some cases also on habitual mobile phone use. The side of the head participants predominantly used for mobile phone calls has a modifying effect in one third of the investigated nuclear anomalies.

Following exposure, after two weeks the nuclear anomalies indicated rather a reduction of genotoxic effects (Figure 5.8). The so called “broken eggs” were the exception. These anomalies were reduced if the experimental exposure was on the other than habitual side of mobile phone use, but were significantly increased if the experimental exposure coincidently matched the habitual side of mobile phone use. This may indicate an accumulative effect of the experimental exposure and the telephone calls before the participation.

Except for pyknosis and karyolysis all endpoints indicating a cytotoxic effect show a significant increase occurred especially at the higher exposure level (SAR 1.6 W/kg). This was generally more pronounced in the group with exposure corresponding to habitual side of mobile phone use (Figure 5.10). Karyolysis occurs as the last step of necrosis and apoptosis and is the fate of all buccal mucosa cell developments until they are exfoliated. Pyknotic cells are considered as apoptotic cells with extreme condensation of chromatin. Cells with condensed chromatin may develop into pyknosis or karyorrhexis with pyknosis being much less frequent and it is unclear which factors are responsible for this switch.

Overall results of the Cytom assay exhibit disruption of cellular integrity in particular via cytotoxic pathways. However, cells in the final stage of decay (karyolitic cells) were less frequently found. This could indicate that processes expressing themselves in nuclear anomalies are consequences of attempts for repair and compensation. Cells with condensed chromatin and karyorrhexis have inactivated their genetic material. Within this context also results of a somewhat reduced number of nuclear anomalies indicating genotoxic damage may be interpreted, because there is an indirect

proportionality between frequency of genotoxic and cytotoxic anomalies. A cell trying to compensate cytotoxic interference (with pyknosis and karyolysis being endpoints of failure in this respect) cannot be subject to genotoxic interference. While cells with chromatin in condensed, inactive conformation may return into normal state of uncondensed DNA this is not the case once pyknosis or karyolysis have been reached.

In some cases stronger effects were observed in participants in which experimental exposure corresponded to the side of habitual mobile phone use (binucleates, condensed chromatin, karyorrhexis; Figure 5.9, Figure 5.10). This indicates that regular mobile phone use may induce cumulative effects to some degree. The effects of habitual exposure appear to be weak. Nevertheless they add to the effects associated with experimental exposure, a finding not considered in earlier studies and therefore may explain inconsistencies in previous reports.

# 6 GENETIC TOXICOLOGY

# Genotoxicity investigations concerning the identification of risks caused by long-term occupational exposure to mobile phone specific electromagnetic fields

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## 6.1 BACKGROUND

The scientific issues addressed in this subproject are based on the results of the project ATHEM-1 and on scientific publications published until 2011. During the project various modifications from the initial plan were realized; i. e. the integration of additional glioblastoma cell lines, as well as additional experiments concerning specific DNA-repair mechanisms.

All experiments focused on the potential genotoxic effects as a consequence of exposure of human cells to UMTS[3] radio frequency electromagnetic fields (RF-EMF).

### 6.1.1 WHAT IS DNA?

The DNA in the nucleus is the main storage molecule of the genetic information. Individual sections of the DNA encode for information in regard to the functions of individual cells and also for the structure and functions of the entire organism. Chemically, DNA is a double-stranded biopolymer. Each individual strand contains nucleotides which consist of one of 4 bases (adenine, guanine, cytosine and thymine) as well as a sugar moiety and a phosphate group. The DNA double strand is formed by the pairing of two complementary bases which are linked via hydrogen bonds. The pairs are formed between purine bases (adenine of guanine) which are linked to a respective pyrimidine base (cytosine or thymidine). Although the size of the bases differs, the distance between the individual bases in the strands is constant because one purine always pairs with a specific pyrimidine.[87]

The sequence of the bases in the DNA encodes for genes, i.e, information which is relevant for the cells. The genetic code can be compared with a sequence of letters which in correct sequence encode for a meaningful word. Similar as in human languages an exchange or lack of specific letters can change the meaning of a word. For example, bread→breed→bead. Single changes can result in another meaningful word (dissense gene) or in a nonsense word (gene).

In both cases the original information is lost. The detection or exchange of bases or fragments in the DNA may lead to a persisting change of the genetic code. When the alterations are not repaired before the replication of the cells, primary damage will lead to mutated genes. As a consequence, the protein for which a specific gene encodes cannot be produced in a proper way.[88]

### 6.1.2 DNA DAMAGE / LESION

Due to the importance to maintain the integrity of the genetic code, different intracellular DNA repair mechanisms have been developed during the evolution of the organisms. Certain proteins recognize damaged DNA bases and repair them. Normally, the repair process reestablishes the original sequence and genetic code. However, mistakes may occur during the repair processes. This will lead to loss of DNA bases and/or may cause other disturbances of the DNA molecule as well as

[3] UMTS, Universal Mobile Telecommunication System, the 3rd generation mobile telecommunication system prevalent in Europe

changes of the genetic information. Despite the efficiency of DNA repair mechanisms by various molecular mechanisms, a great number of DNA lesions necessitating frequent repair increases the risks for alterations of the genetic code.[89]

In this subproject, the potential effects of RF-EMF exposure on DNA damage, in particular on oxidation of DNA bases, were investigated. Furthermore, we analyzed also the impact of radiation on the induction of the most important repair processes.

## 6.2 METHODS

All experiments in this sub-project were conducted with cell cultures (*in vitro*).

### 6.2.1 ORIGIN OF THE CELL LINES

The cell lines were obtained from different institutions and stored deep frozen (

Table 6.1).

Before the use of the cells in the main experiments, the cells were checked for contaminations (bacteria and fungi), and tested for mycoplasma contaminations with a specific standard protocol.

### 6.2.2 CULTIVATION OF THE CELLS

The cells were cultivated under standard conditions in humidity saturated atmosphere with 5% $CO_2$ at 37°C in recommended media (

Table 6.1).

Table 6.1: Origin and cultivation conditions of the tested cell lines

| **Celll type** | **Cell line** | **Origin** | **Medium** |
|---|---|---|---|
| Oral mucosa epithelium | TR-146 | Dept. Dermatology, Harvard Medical School, USA | Dulbecco Medium with 10% fetal bovine serum. |
| Neuroblastoma | SH-SY5Y | Inst. of Neurophysiology and Neuropharmacology, Medical University of Vienna, Austria | Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1 mixture) supplemented with 2.0 mM L-glutamine and 12 % fetal bovine serum. |
| Liver carcinoma | HepG2 | Centre for Human Safety & Health, University of Leiden, Netherlands | Eagle's Minimal Essential Medium (EMEM), with 1.0 mM sodium pyruvate and 10 % fetal bovine serum |
| Fibroblast cells | ES-1 | Inst. of Arbeitsmedizin, Medical University of Vienna, Austria | Dulbecco Medium with 10% fetal bovine serum |
| Glioblastoma multiforme (GBM) "stem-like" cells | NCH421k | National Institute of Biology, Ljubljana, Slovenia | Minimum Essential Medium with 2.0 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 1.0 mM sodium pyruvate and 10% fetal bovine serum. |
| Glioblastoma cell lines | U-87 | | Dulbecco's Modified Eagles Medium (DMEM): Ham's F12 (1:1-mixture) with 2 mM L-glutamine, 12% fetal bovine serum, B-27 supplement, Heparin, bFGF und EGF |
| | U373 | | Dulbecco's Modified Eagles Medium (DMEM): Ham's F12 (1:1 mixture) with 2.0 mM L-glutamine and 10% fetal bovine serum |
| | U251 | | |

### 6.2.3 GENERAL DESIGN OF THE IN VITRO EXPERIMENTS

Typically, 5 x $10^5$ cells were seeded in each of three Petri dishes and cultured over night (24 h). The cells were exposed for 6, 16 or 24 h to different intensities (SAR between 0.25 W/kg and 1.0 W/kg) of the UMTS signal.

In each experiment cells were in parallel either exposed to the UMTS signal or sham exposed. Samples were blinded regarding the exposure condition during exposure and in subsequent evaluation steps. The unblinding (decoding which cells were UMTS exposed and which sham-exposed) was done after all laboratory investigations were completed.

The main method used for the investigation the of DNA lesions was the Comet assay. Depending on the specific research question the protocols varied. Comet assays were performed under:

- standard conditions
- with FPG (pretreatment with the lesion specific enzyme FPG)
- with Endo III (pretreatment with the lesion specific enzyme Endo III)
- and with $H_2O_2$ (pretreatment with hydrogen peroxide)

### 6.2.4 DETECTION OF SINGLE- AND DOUBLE-STRAND BREAKS (STANDARD COMET ASSAY)

The so-called “alkaline” version of the Comet assay was used in all experiments according to Tice et al.[90] to detect single- and double-strand breaks, apurinic and alkali-labile sites in DNA molecules. The cells were embedded in agarose gel and subsequently lysed (i.e. the cell membranes were destroyed).

#### Principle of the Comet assays

After RF-EMF exposure and pretreatment with FPG, Endo III or $H_2O_2$ lyzed cells are submerged in electrolyte buffer and then undergo electrophoresis under strictly controlled conditions. DNA, which has an overall negative charge, migrates towards the anode. The smaller the fragments the farther they migrate during a given period of time. Hence, the migration distance (length of the comet tail) reflects the size of the DNA fragments. The overall amount of DNA fragments in the tail is measured by the so-called tail intensity (the percentage of DNA in the tail). Intact DNA remains in the nucleus as it is too large to migrate. Staining of DNA with a DNA-specific fluorescent dye (propidium iodide) leads to images of damaged cells which are reminiscent of comets.

Intact cells and comets (formed as a consequence of DNA breaks) can be visualized under a fluorescence microscope. The light emission from the cell nuclei (intact DNA) and the comet tails (damaged DNA) can be recorded with a special (low light) camera and determined automatically by a image-analysis software[90] (see Figure 6.1).

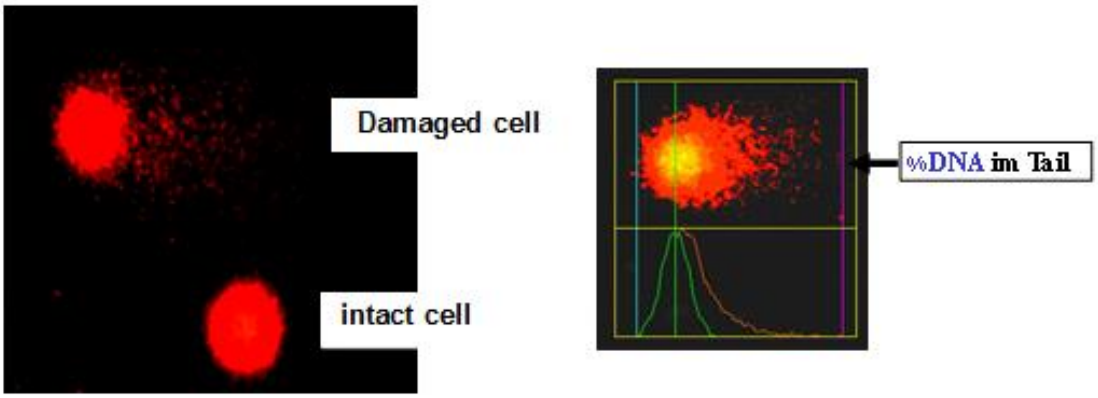



Figure 6.1: Schematic illustration of the comet analysis by use of a computer aided image analysis system (Comet Assay IV, Perceptive Instruments Ltd., UK).

In the past, different methods were developed to quantify DNA breaks based on images of cell nuclei and DNA-comets. Originally, the lengths of the comet tails were used as an indicator for the fragmentation of the DNA. However, the parameter “tail length” does not take into consideration the amount of DNA in the tail. It was shown that the size of the fragments does not necessarily correlate with the extent of DNA damage. A frequently used alternative endpoint is the so-called “tail moment”, which is defined as the product of the amount of DNA in the comet and its length. Also this parameter is not optimal since it puts too much weight on (perhaps only few) small fragments. The amount of fluorescence which can be quantified correlates not only with the DNA but also with the depth of the embedded cells in the gel.[91]

At present, the most frequently used parameter is the so-called “tail intensity” (% DNA in tail). This value is independent of the depth of the cell in the gel and is also independent of fluorescence-intensity variations. “Tail intensity” is defined as the fluorescence in the tail in relation to the total fluorescence. The value is 0% when a tail is not present and 100% when the entire DNA of a nucleus migrates into the gel. The latter result can be obtained in apoptotic (dead or dying) cells when the cell membrane was damaged and the DNA was fragmented before the cells were lyzed.[91]

**Number of analyzed nuclei per experiment**

It was found with computer aided measurements that the analysis of 50 cells per slide is a good compromise between efforts and precision.[90] This number of cells per slide is sufficient for a precise measurement of the tail intensities. Depending on the specific type of the experiment the Measurement error is between 1% and 7%. Of course it would be possible to evaluate a higher number of nuclei, but this would not lead to a substantial reduction of measurement errors. In the frame of the present project, three slides were prepared for each experimental condition (from each exposure experiment), 50 cells were counted per slide.

**Generation of data sets**

Following radiation exposure, pretreatment and electrophoresis, the results of the standard Comet assays were evaluated with a computer based image analysis system (Perceptive Instruments, UK). The transfer of the data from the files which were produced with this system was automatized (with Excel-Macros). All tail intensity data were documented and assigned to the specific exposure conditions in the individual experimental series (unblinding).

From each slide, the medians of tail intensities across 50 cells were calculated after the exclusion of apoptotic cells. As an endpoint “percentage of DNA in the tail” was calculated which is the ideal parameter for this type of experiments. As the rate of apoptosis was in all experiments very low and the median was used as the parameter for the distribution of tail intensities, the removal of apoptotoic cells had no effect on the results.

### 6.2.5 MEASUREMENT OF OXIDIZED DNA

To determine if oxidized bases are formed as a consequence of exposure, nuclei were treated with restriction enzymes. The formation of oxidatively damaged bases may lead to DNA strand breaks. The restriction enzymes recognize oxidized bases and cut the DNA strands at these sites. These experimentally produced DNA breaks at sites of oxidized lesions can be quantified with the standard comet assay procedure. In order to determine the amounts of oxidized DNA bases (purines and pyrimidines) the tail intensity is measured after the enzyme treatment and the value obtained with enzyme buffer (without enzyme) is subtracted from the value which is obtained after enzyme treatment. The results reflect the amount of oxidized bases and provide additional information concerning the extent and type of DNA oxidation (oxidized purines or pyrimidines).

For these experiments, the cells were transferred to slides after exposure or sham exposure and were lyzed overnight. Subsequently, the nuclei were treated with restriction enzymes which detect oxidized bases, followed by electrophoresis and determination of the % DNA in tail. Two different restriction enzymes were used, namely:

- Formamidopyrimidine DNA glycosylase (FPG) which detects oxidized purines and
- Endonuclease III (Endo III) which detects oxidized pyrimidines.

Furthermore, we investigated if RF-EMF exposure leads to increased sensitivity towards oxidative damage. Therefore, exposed and unexposed cells were incubated with hydrogen peroxide which leads to oxidative damage of DNA.

### 6.2.6 DETERMINATION OF DNA REPAIR PROCESSES

Different types of DNA repair processes eliminate specific forms of DNA damage. The activities of the so-called “base excision repair” (BER) and “nucleotide excision repair”

(NER) can be monitored by use of a modified protocol of the single cell gel electrophoresis (SCGE) comet assay. In the frame of the present study this approach was used to investigate the impact of RF-EMF exposure.

Base excision repair (BER) of DNA corrects a number of spontaneous and environmentally induced genotoxic or miscoding base lesions in a process initiated by DNA glycosylases. These enzymes eliminate damaged bases from the DNA, which leads to abasic sites. Such abasic DNA sites constitute a risk for mutations since it is possible that incorrect bases are inserted during the replication of the cells. A specific endonuclease recognizes abasic sites, and eliminates the damaged base. The gap is closed by DNA polymerase and finally fixed by a specific ligase.

Nucleotide excision repair (NER), repairs primarily UV-induced photoproducts (cyclopyrimidine dimers [CPDs], 6-4 photoproducts [6-4PPs]). Specific enzymes eliminate entire nucleotides and replace it. Finally, a new DNA strand is synthesized by DNA-polymerase and closed by a ligase (Figure 6.2).[92]

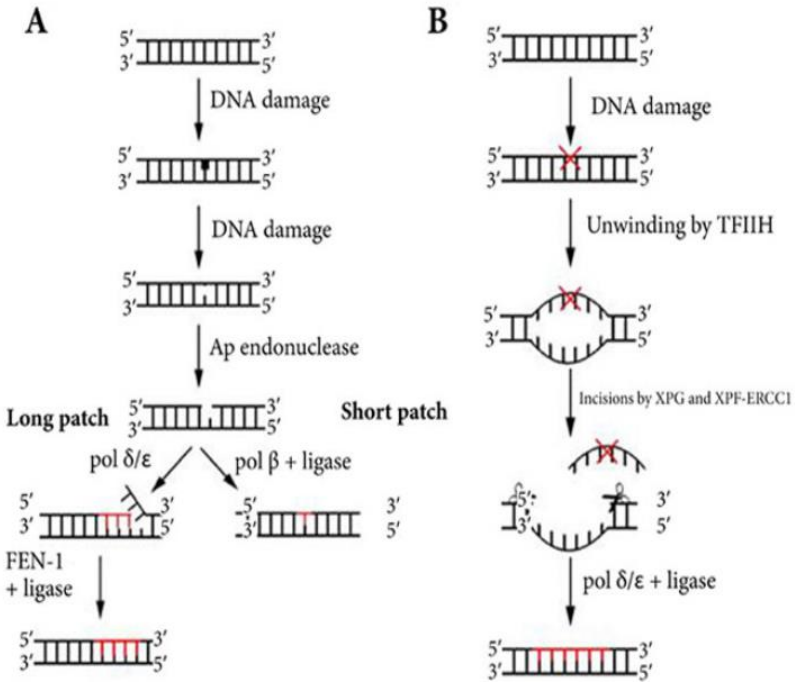



Figure 6.2: Simplified overviews of the base excision repair (BER) pathway and nucleotide excision repair (NER) pathway.[93]. (A) In BER the damaged base is first removed by DNA glycosylases after which AP endonucleases create a single strand break. Next either one or multiple new nucleotides are synthesized by DNA polymerases in short patch and long patch BER, respectively. FEN-1 removes the DNA flap in long patch BER and ligases seal the seams between original and newly synthesized DNA. (B) In NER, after the damage is recognized, the DNA helix is unwound by transcription factor II H (TFIIH). Next incisions are made both up- and downstream of the lesion by XPG and XPF-ERCC1, after which the DNA segment containing the lesion can be removed. Then the created gap is filled with newly synthesized DNA by DNA polymerases and sealed by DNA ligases.

In the present experiments we used modified protocols for the Comet assay to investigate the alterations of repair activities after RF-EMF exposure. These methods were developed by A. Collins[94] and are based on the induction of primary damage in so-called “reporter cells” by UV light or a chemical which is repaired by specific repair pathways. In other words, the induced damage is usually repaired either by BER or

NER. In order to determine whether a certain exposure induces these repair pathways we used an extract of the exposed cells that contained either increased amounts of repair enzymes or, if these enzymes have been used up, reduced amounts. By monitoring the extent of DNA damage in nuclei from reporter cells treated with cytosolic extracts of cells which had been exposed to EMF or sham exposed it is possible to determine differences in DNA strand breaks that indicate differences in the amount of repair activities of the test cells. This procedure provides information whether DNA repair processes are altered in exposed cells. Higher repair activates after RF-EMF exposure could be caused by induction of specific DNA damage. Reduced activities could indicate the increased use of repair enzymes without full replacement by new synthesis.

Figure 6.3 depicts schematically the experiments which were performed. Reporter cells (human liver tumor cell line HepG2) were exposed to UV-light in order to induce lesions which are specific for NER. For the investigation of the BER system, cells were treated with the photosensitizer Ro 19-8022. Subsequently the reporter cells were transferred to slides.

The test cells (glioblastoma cell lines U251 and U87) were exposed in parallel to different RF-EMF intensities or sham exposed, and the cytosolic extracts (containing DNA-repair enzymes) were isolated. Subsequently, the nuclei of the reporter cells were isolated and incubated with the extracts. The repair enzymes from the exposed test cells start to cut the damaged DNA in the first phase of the repair processes. Because the repair cannot be completed under these conditions, the DNA fragmentation rate in the reporter cells reflects the activity of repair enzymes in the test cells. The differences between exposed and sham exposed test cells reflects the induction of repair enzymes by RF-EMF exposure. In short: in this experimental series the determination of DNA fragments in the reporter cells is indicative for the activities of the BER and NER after RF-EMF exposure.

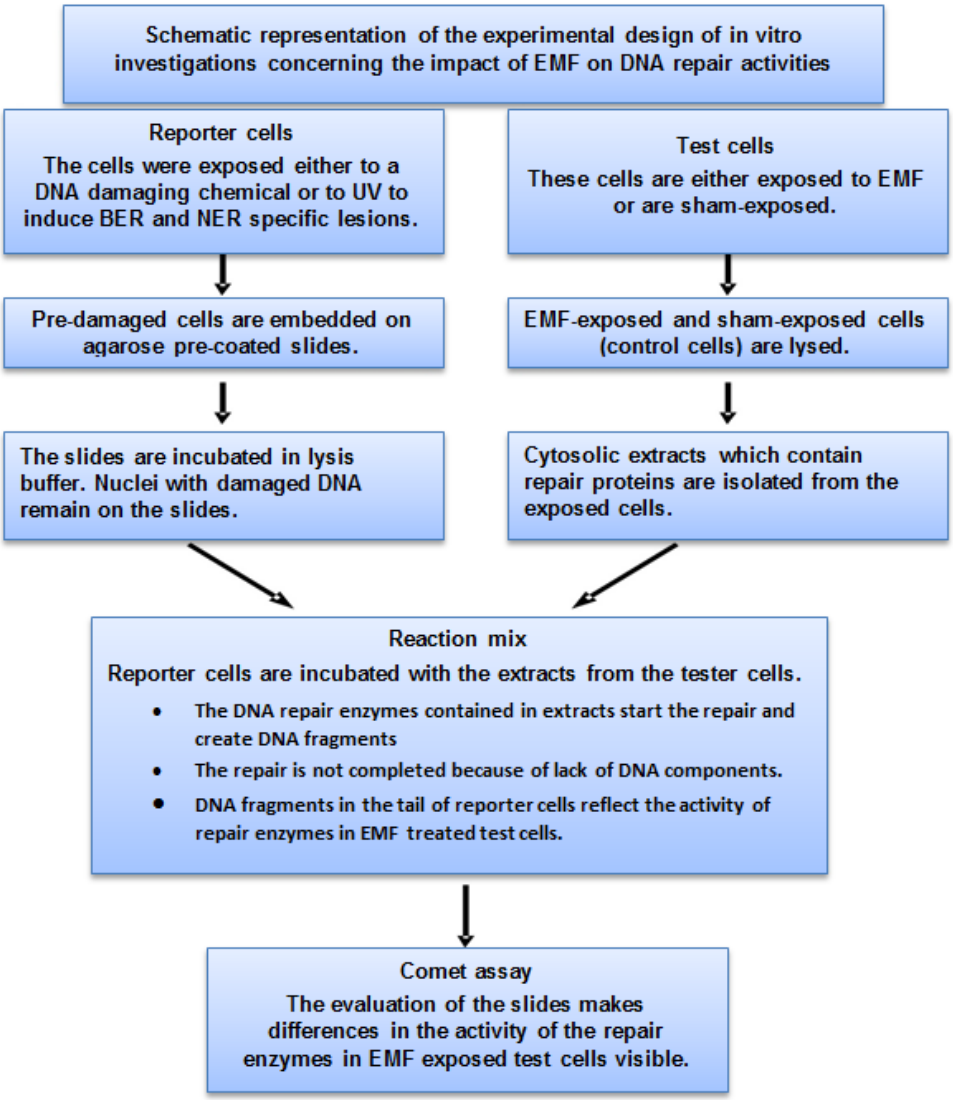


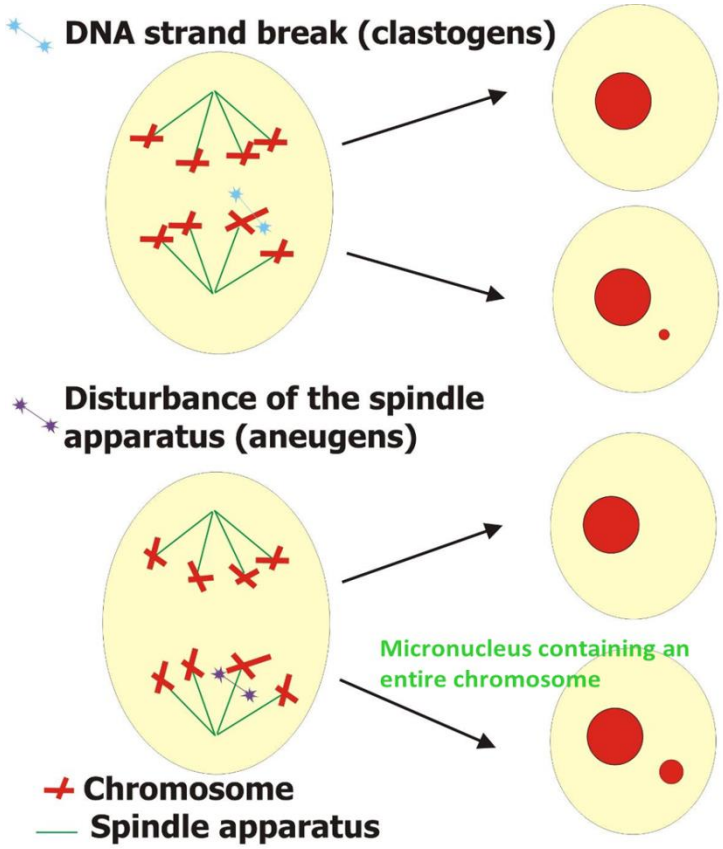

Figure 6.3: Schematic illustration of the DNA repair activity measurement induced by UMTS.

### 6.2.7 MICRONUCLEUS ANALYSIS EXPERIMENTS

Permanent chromosomal alterations (caused by primary DNA damage which was not efficiently repaired) may lead to morphologically detectable formation of DNA fragments which are not integrated in the nuclei of the cells after division. These structures are termed “micronuclei” (MN) and are DNA containing bodies in the cell cytoplasm and can be identified under a microscope.

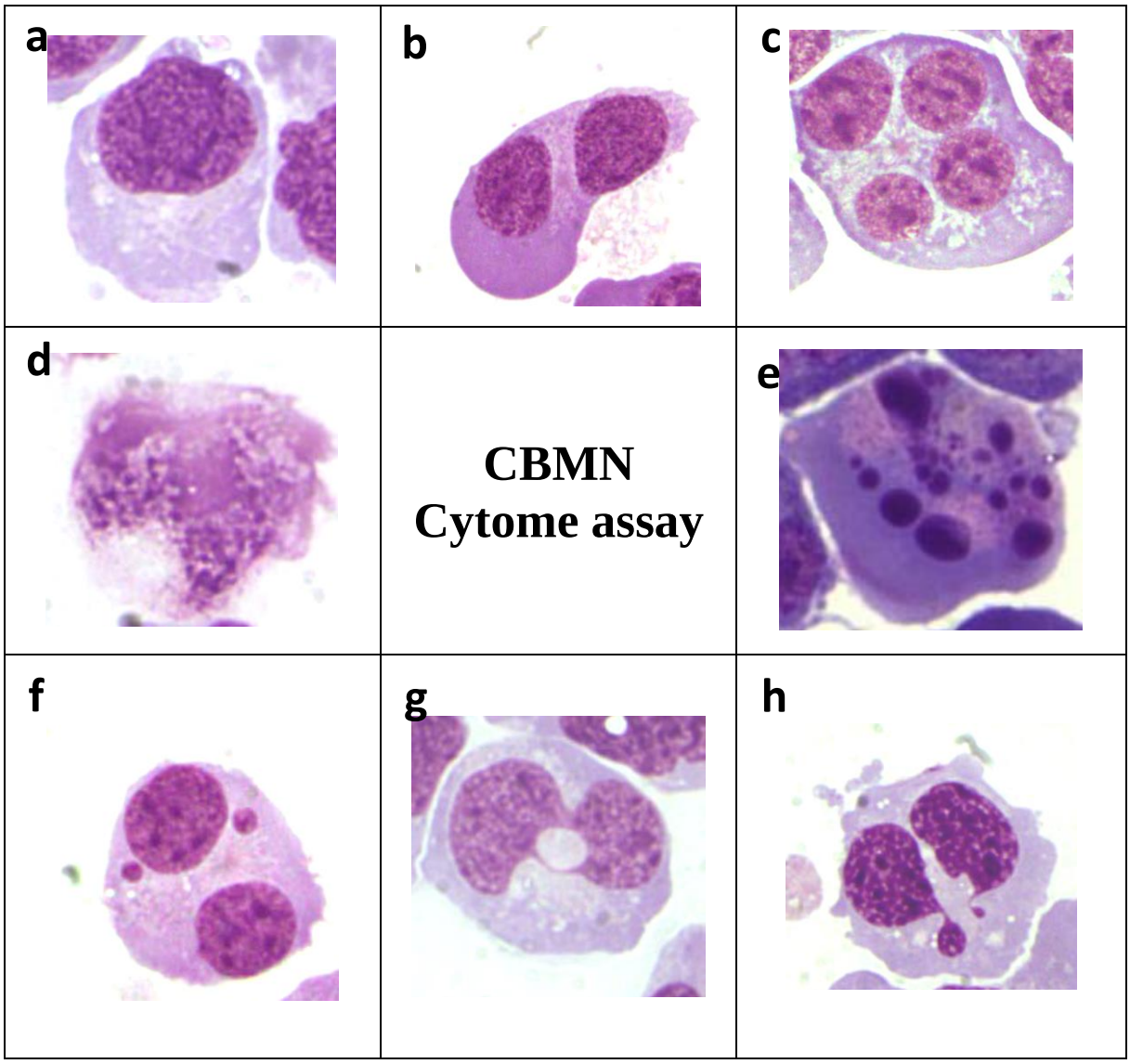
In order to investigate their formation after RF-EMF exposure we used the cytokinesis-block MN assay.[95] This method is based on the use of cytochalasin B which is added to the cells after the exposure and blocks cell division but does not inhibit the division of nuclei (Schema I). As a consequence, two nuclei are formed (so-called binucleated – BN cells). This approach enables to identify MN which are formed after one division of the nuclei. These structures can be identified in the cytoplasm microscopically and represent two forms of chromosomal aberrations, namely, aneuploidy (loss of individual chromosomes as a consequence of spindle disturbance) as well as chromosome breakage (clastogenicity). By use of the cytokinesis-block MN (CBMN)

assay it is possible to identify cells which underwent one mitosis after the RF-EMF exposure. This strategy avoids misleading results due to loss of MN or due to inhibition of the cell division.




Schema I: Mechanisms leading to the formation of micronuclei (MN).

After exposure to RF-EMF and cytochalasin B treatment for one generation the cells were fixed and stained, and the frequencies of mononucleated and BN cells as well as rates of multinucleated cells were determined. Furthermore, also the mitotic indices were calculated which provide information about the cell division. In addition, further nuclear anomalies were monitored, namely, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). These nuclear anomalies provide information about processes which are associated with genetic instability. NPB reflect dicentric chromosomes which are typically induced by radiation while NBUDs are formed as a consequence of gene amplification events. Also the rates of necrotic as well as apoptotic cells were determined. All experiments were conducted according to the standard protocol of Fenech et al.[96] (Schema II).




Schema II: Photomicrographs of the cells scored in the CBMN "cytome" assay. (a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) early necrotic cell; (e) late apoptotic cell; (f) BN cell containing one or more MNi; (g) BN containing an NPB (and a MN); (h) BN cell containing NBUDs. The ratios of mononucleated, BN, multinucleated, necrotic and apoptotic cells are used to determine mitotic division rate or NDI (a measure of cytostasis) and cell death (cytotoxicity). The frequency of BN cells with MNi, NPBs or NBUDs provides a measure of genome damage and/or chromosomal instability. For a wider selection of photomicrographs of different types of cells and biomarkers scored in the CBMN Cyt assay, refer to Fenech et al.[96]

### 6.2.8 STATISTICAL ANALYSES

#### Tail intensity data

For each experiment, three culture dishes were set up and exposed and three culture dishes sham exposed. From each of these cultures one slide was prepared and 50 cells were evaluated per slide.

Since the standard deviation of the tail intensity (%DNA) correlates with the means, an arcsine transformation was performed for the further statistical evaluation according to the following formula:

$$Tr(\%\text{DNA in tail}) = ArcSin(\sqrt{\%\text{DNA in tail}/100})$$

These transformations remove correlations between means and standard deviations of tail intensity values that would violate a precondition of the statistical model applied. After statistical calculations based on the transformed values, descriptive statistics were retransformed to tail intensity values in order to enable comparisons with data from earlier studies.

**Analysis of tail intensity data**

Statistical analyses were conducted with data obtained in all individual experiments by analysis of variance. The factors which were analyzed are:

- Exposure (difference between exposed and sham exposed cultures).
- SAR (specific absorption rate) in most experiments the doses 0.25, 0.50 and 1.0 W/kg were used.

In each experiment, unexposed controls were included for each dose (SAR). The analysis of the differences of the tail intensity values between exposed and non-exposed cells was performed on the basis of the individual experiments with linear contrasts (i.e. for each experiment direct comparisons between real and sham exposed cells were performed).

In the case of a significant difference (level of significance 5%) between exposed and sham exposed we marked this in all figures with asterisks unless indicated otherwise in the legends. We show in the graphs the means ± SDs of tail intensities in % DNA in tail. For each experiment relevant sources of variance are for example:

- **Exposure** (exposed, non-exposed),
  is the difference depending on the presence of EMF or not
- **SAR** (0.25, 0.5, 1.0 W/kg)
  is the difference related to the intensity of RF-EMF exposure
- **Interactions between exposure and SAR**:
  does the difference in exposed and non-exposed cells depend on SAR?

Table 6.2 shows an example of the way how results of statistical analyses are presented.

Table 6.2: Examples for the presentation of results as obtained from analysis of variance (ANOVA).

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 2,980 | 0,066 | Tendency |
| Exposure | 1 / 30 | 1,792 | 0,191 | n.s. |
| SAR * Exposure | 2 / 30 | 3,662 | 0,038 | sign. |

For each source of variance the so-called “statistical degrees of freedom” for numerator (FG-1) and denominator (FG-2) are indicated because the value F reflects a variance ratio that depends on these degrees of freedom.

If an effect of a specific source of variance exists, the variance in the numerator is bigger than the variance in the denominator, therefore hence F is bigger than 1. In the next step it is checked whether the effect is larger than random variations, i.e. the probability is calculated that such an effect or an even larger one occurs under the assumption of the zero hypothesis (which is the hypothesis of no effect). Is the probability smaller than 0.05 the zero hypothesis is rejected. Additionally, per convention, a value of p which is smaller than 0.01 is defined as highly significant and p values which are smaller than 0.1 are designated as a “tendency”. P values larger than 0.1 are regarded as not significant (abbreviation NS). The following results can be obtained in these analyses:

- None of the three sources of variance is significant (Figure 6.4A)
- Only exposure is significant (Figure 6.4B) i.e. exposed and non-exposed cells differ in regard to the tail intensities
- Only SAR is significant (Figure 6.4C)
- In this case the % DNA in tail in exposed and sham-exposed cells differs with different doses. Such an effect is mainly caused by factors other than the exposure
- Only the interaction is significant (Figure 6.4D);
  i.e. the difference between exposed and non-exposed cells depends on the SAR. This indicates that the exposure causes the effect
- Exposure as well as SAR are significant (Figure 6.4E)
  this means that a difference exists between exposed and non-exposed cells and additionally also a difference between the effects of different doses are seen
- Exposure and also interaction are significant (Figure 6.4F)
  A difference exists between exposed and non- exposed cells. This difference depends on SAR but it compensated by exposure effects of the doses
  Therefore, the factor SAR is not the main factor of the effect
- SAR and also the interactions are significant (Figure 6.4G)
  In this case a difference exits between different doses of radiation but the difference depends on whether exposed or non- exposed cells are concerned

The SAR effect compensates the exposure effects. Therefore, the factor “exposure” is not the main factor of the effect

- All three sources of variance are significant (Figure 6.4H)
  In this case a difference exists between exposed and non- exposed cells which depends on the dose of exposure. Therefore, the dose effect is not compensated and remains as a main factor

Figure 6.4 shows examples for the different results which can be obtained in the statistical analysis.

An interaction is only found if the difference is between exposed or non- exposed cells depends on SAR (Figure 6.4 examples: D, F, G and H).

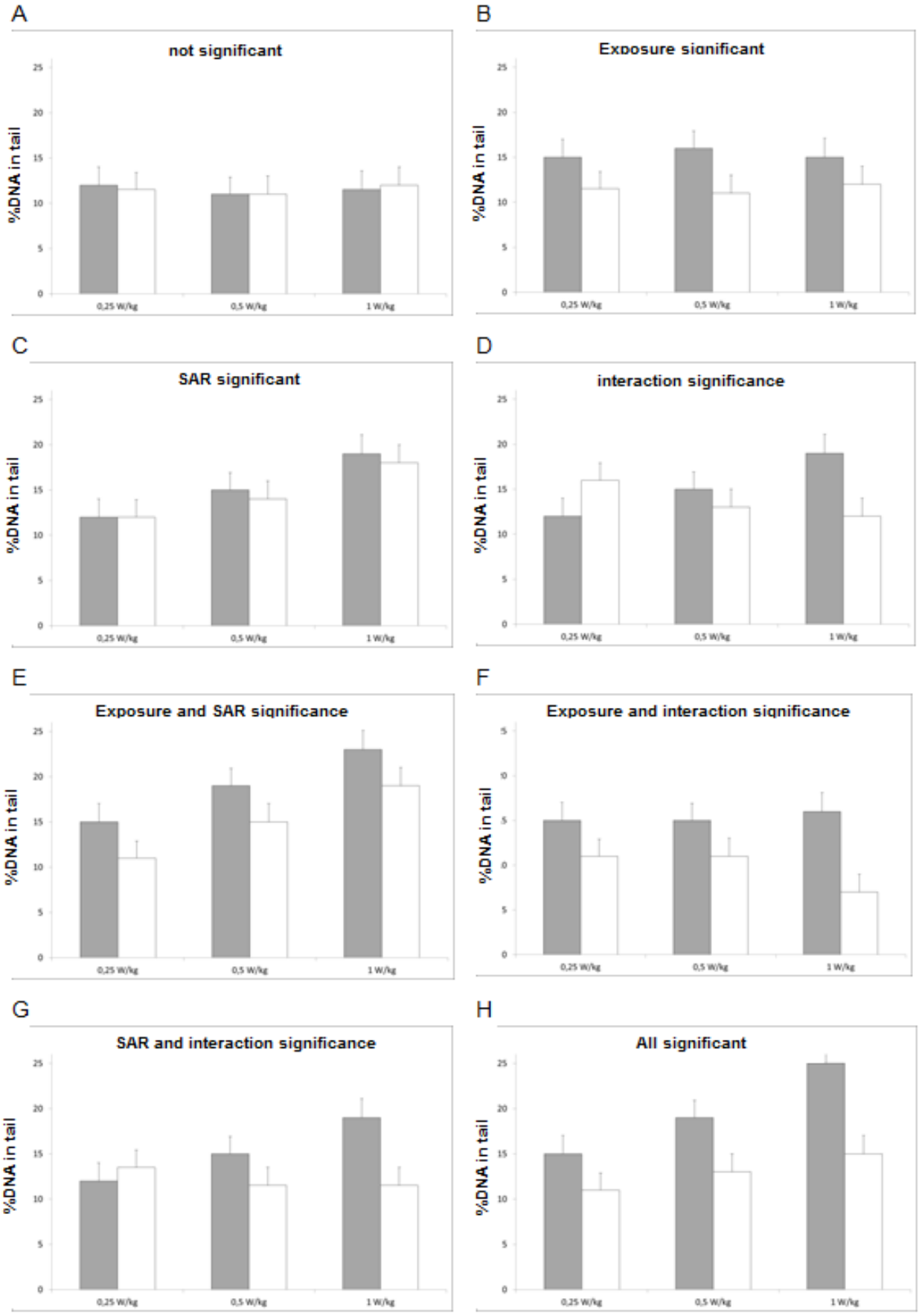



Figure 6.4: Fictional examples of possible results of the significance test. All combinations of possible outcomes are exemplified.

## 6.3 RESULTS

All in vitro experiments were conducted with the UMTS signal (1950 MHz) which is described in more detail in chapter 3 of this report.

### 6.3.1 SENSITIVE AND NON-SENSITIVE CELL LINES

The first series of experiments was conducted to find out which cell line is sensitive towards RF-EMF in regard to induction of DNA strand breaks.

In the first series of experiments 5 cell types were investigated. Four of them were used for the first time. One of them (ES-1) had been tested already in the former ATHEM-1 Project.

All 5 cell lines which were tested in the first trial:

- Human buccal epithelial line (TR-146)
- The neuroblastoma line SH-SY5Y
- The liver carcinoma line HepG2
- The fibroblast line ES-1
- The glioblastoma line U-87

In the first series of exposures the cells were cultivated under standard conditions.

The following exposure was used:

- UMTS signal
- SAR: 1.0 W/kg
- Duration of exposure was 16 h

After exposure, the cells were analyzed using the following conditions:

1. Without further treatment (Comet assay under standard conditions).
2. After pretreatment of cells with hydrogen peroxide ($H_2O_2$) in order to find out if the exposure leads to increased sensitivity to oxidative damage.
3. After pretreatment of isolated nuclei with the enzyme FPG to find out if RF-EMF exposure causes formation of oxidized purine bases.
4. After pretreatment of isolated nuclei with the enzyme Endo III in order to find out if RF-EMF exposure causes formation of oxidized pyrimidine bases.

For all statistical analyses we used the parameter “% DNA in tail”.

### Measurement of DNA damage (standard Comet assay)

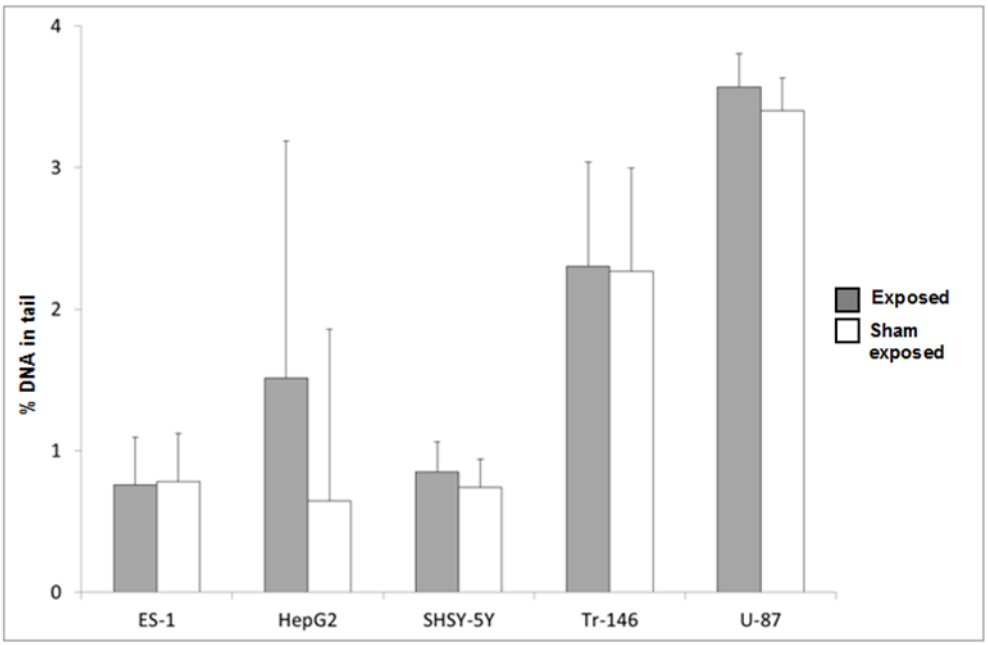



Figure 6.5: Results of the Comet assay in a RF-incubator under standard condtions after 16h exposure. All cell lines were exposed to a SAR of 1.0 W/kg. White bars represent sham exposed cells, grey bars exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

### Measurement of oxidized purines (FPG treatment)

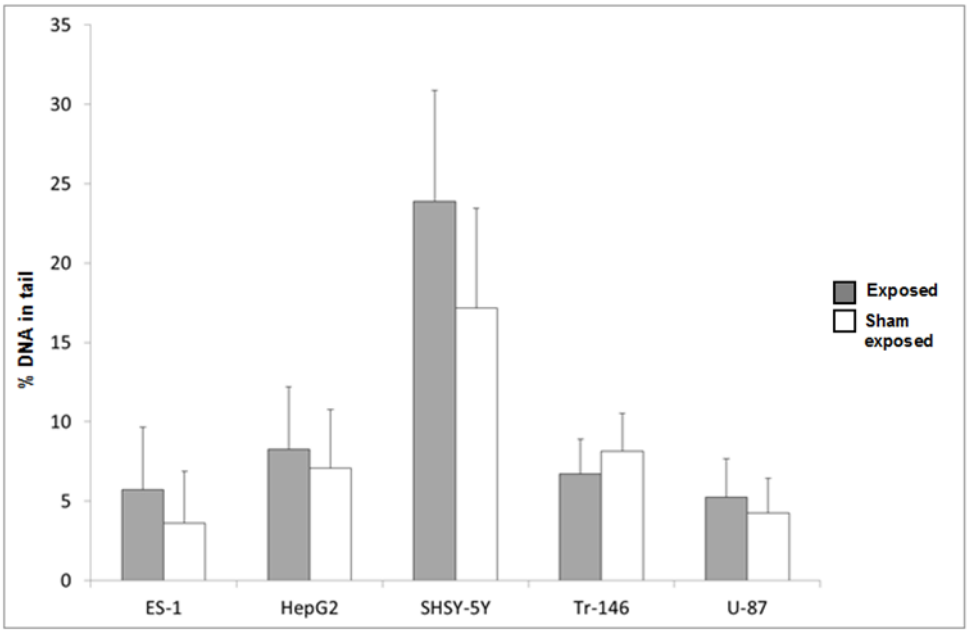



Figure 6.6: Results of the FPG Comet assays which enable the detection of oxidized purine bases. All cell lines were exposed to a SAR of 1.0 W/kg. White bars represent sham exposed cells, grey bars EMF-exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

### Measurement of oxidized pyrimidines (Endo III treatment)

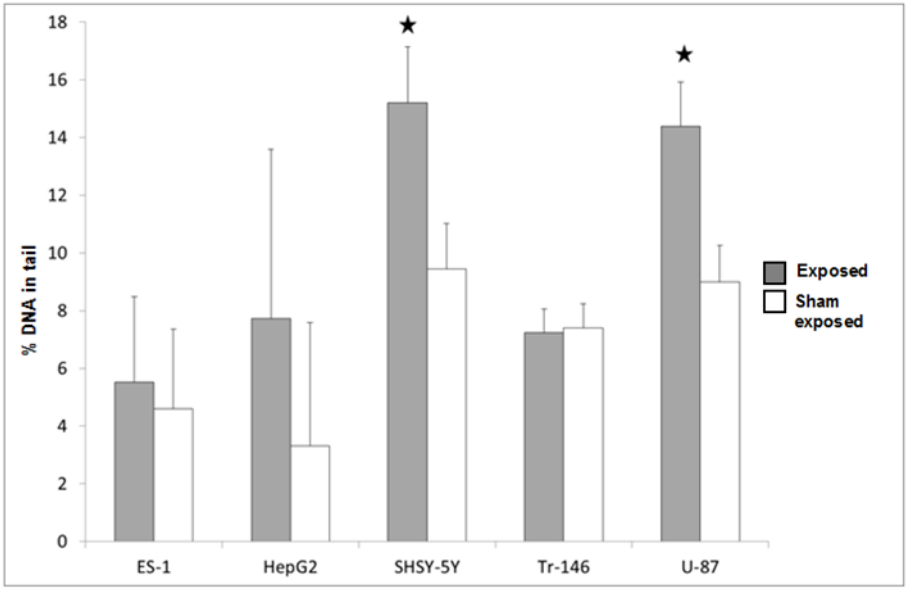



Figure 6.7: Results of the Endo III Comet assay after 16h exposure in a RF-incubator (SAR 1.0 W/kg). White bars represent sham exposed cells, grey bars EMF-exposed cells. Significant differences were seen between exposed and sham exposed SHSY-5Y cells and U87 glioblastoma cells. Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with★ ($p < 0.05$).

### Determination of reactive oxygen species (ROS) sensitivity (H2O2 treatment)

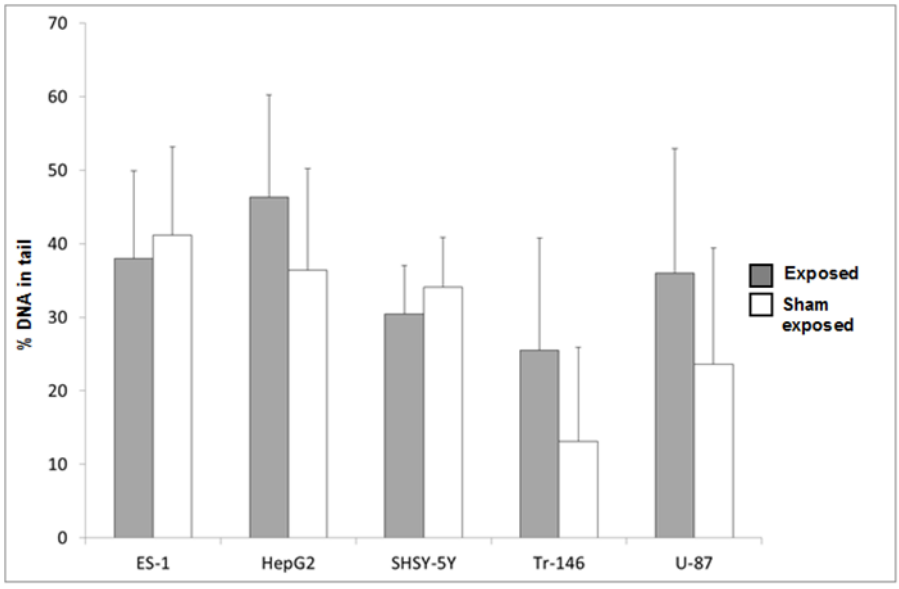



Figure 6.8: Result with H2O2-Comet Assay, investigation of the sensitivity to oxidation after 16 h exposure in a RF-incubator (SAR 1.0 W/kg). White bars represent sham exposed cells, grey bars EMF-exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

Increased values of % DNA in tail were seen under all experimental conditions in the liver cell line HepG2. The differences were pronounced when the experiments were carried out under standard conditions and after treatment of the cells with H2O2. Also in Endo III experiments an effect was seen. In order to find out if these experiments are reproducible, a further experimental series was conducted and the increased tail intensity could not be replicated. Therefore, we concluded that the results obtained with this cell line are not robust due to poor reproducibility.

## Summary of the first exposure series for the identification of the most sensitive cell line

Table 6.3: Summary of the results of the first experimental series (UMTS, SAR 1.0 W/kg, 16 hours).

| Cell line | Treatment (Endpoint) | Higher effect with exposed (B) / sham-exposed (U) | p- value | Significance |
|---|---|---|---|---|
| ES-1 Fibroblasts | Standard | U | 0.936 | n.s. |
| ES-1 Fibroblasts | Endo III | B | 0.660 | n.s. |
| ES-1 Fibroblasts | FPG | B | 0.407 | n.s. |
| ES-1 Fibroblasts | $H_2O_2$ | U | 0.642 | n.s. |
| TR-146, buccal derived cell line | Standard | B | 0.950 | n.s. |
| TR-146, buccal derived cell line | Endo III | U | 0.794 | n.s. |
| TR-146, buccal derived cell line | FPG | U | 0.409 | n.s. |
| TR-146, buccal derived cell line | $H_2O_2$ | B | 0.092 | tendency |
| U-87 Glioblastoma cell line | Standard | B | 0.424 | n.s. |
| U-87 Glioblastoma cell line | Endo III | B | <0.001 | highly s. |
| U-87 Glioblastoma cell line | FPG | B | 0.561 | n.s. |
| U-87 Glioblastoma cell line | $H_2O_2$ | B | 0.169 | n.s. |
| SHSY5Y, Neuroblastoma | Standard | B | 0.502 | n.s. |
| SHSY5Y, Neuroblastoma | Endo III | B | <0.001 | highly s. |
| SHSY5Y, Neuroblastoma | FPG | B | 0.192 | n.s. |
| SHSY5Y, Neuroblastoma | $H_2O_2$ | U | 0.335 | n.s. |
| HepG2 Liver hepatocellular carcinoma | Standard<br>repetition | B<br>U | 0.004<br>0.243 | highly s.<br>n.s. |
| HepG2 Liver hepatocellular carcinoma | Endo-III | B | 0.105 | n.s. |
| HepG2 Liver hepatocellular carcinoma | FPG | B | 0.572 | n.s. |
| HepG2 Liver hepatocellular carcinoma | $H_2O_2$<br>repetition | B<br>B | 0.068<br>0.211 | tendency<br>n.s. |

Interestingly, neither the standard method nor the comet assays with pretreatment yielded positive results when ES-1 fibroblasts were used. In the glioblastoma cell line U-87 differences between exposed and non-exposed cells were significant after Endo III treatment.

### 6.3.2 EXPERIMENTS WITH THREE FURTHER GLIOBLASTOMA CELL LINES

Since results found in the international scientific literature indicate that long-lasting exposure to RF-EMF may lead to higher incidence of brain tumors (glioblastoma) and since the first series of experiments initiated that glioblastoma cells are sensitive, three further glioblastoma derived cell lines were tested in subsequent experiments.

These cell lines were:

- NCH421k (“stem-like” cells)
- U373
- U251

The cells were cultivated under identical standard conditions as in the preceding experimental series.

The following exposure scheme was used:

- UMTS signal
- SAR: 3 doses
  - 0.25 W/kg
  - 0.5 W/kg and
  - 1.0 W/kg
- Duration of exposure: 16 h

The same experimental protocol was used as in the first experimental series.

After exposure, the cells were treated as follows:

1. No further treatment: detection of single- and double-strand breaks monitored in Comet assays under standard conditions.
2. Pretreatment of the nuclei with H2O2 to find out if RF-EMF exposure increases the sensitivity of cells towards oxidative DNA damage.
3. Pretreatment of the nuclei with the enzyme FPG in order to find out if RF-EMF exposure leads to formation of oxidized purine bases.
4. Pretreatment of the nuclei with the enzyme Endo III in order to find out if RF-EMF exposure leads to formation of oxidized pyrimidine bases.

For all statistical analyses we used the parameter “% DNA in tail”. Per experimental condition, three cultures were prepared and were either sham exposed or exposed to radiation. From each culture, one slide was prepared and 50 cells were evaluated per slide.

**NCH421k "stem-like" cells**

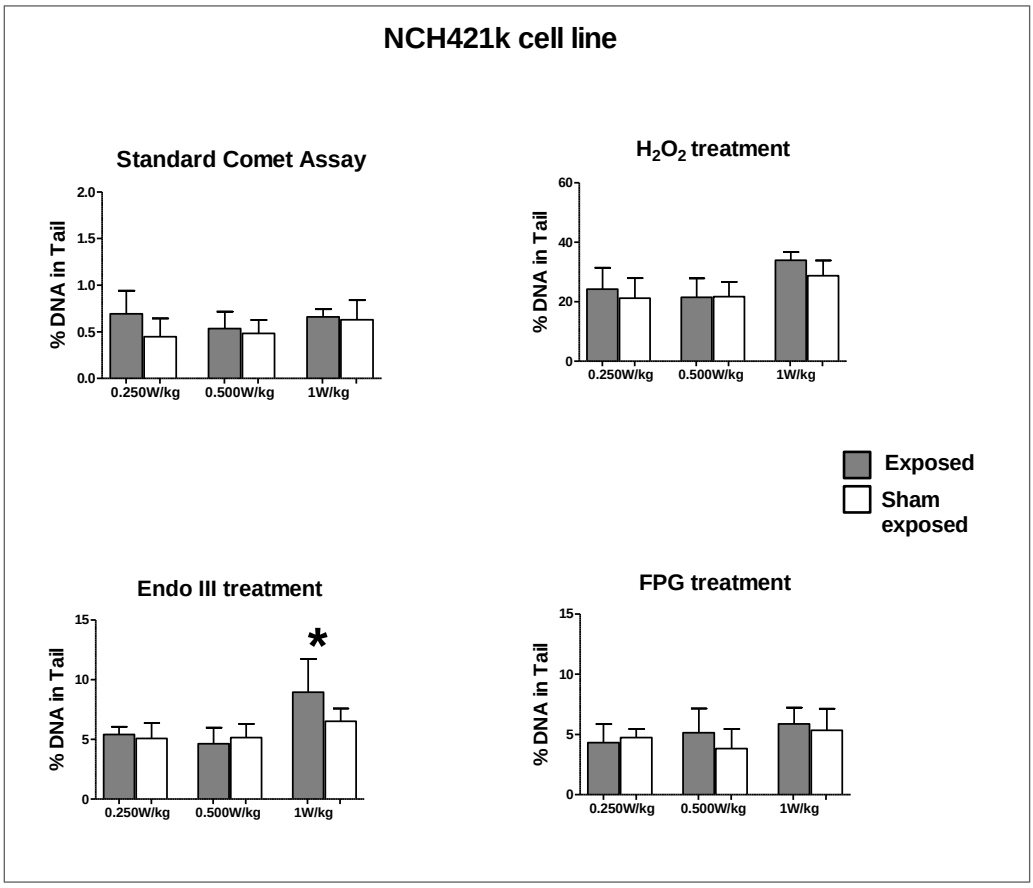



Figure 6.9: Results with glioblastoma multiforme (GBM) "stem-like" cells, NCH421k; 16 hours with different SAR. Standard Comet Assay: DNA breaks. H2O2 treatment: sensitivity to oxidation. Endo III: the detection of oxidized pyrimidine bases; FPG Comet assays: detection of oxidized purine bases. Bars represent means ±SD of results obtained with three cultures per experimental condition. Significant results are marked with★ ($p < 0.05$).

Under almost all experimental conditions higher values of the tail intensities were detected in exposed cells compared to non-exposed cells. However, only with a SAR of 1.0 W/kg of UMTS-irradiated cells a significant difference was seen after Endo III treatment. The results of these experiments are summarized in Table 6.4 to Table 6.7.

The significance which was seen after Endo III treatment for the factor SAR is based on increased levels after exposure to SAR of 1.0 W/kg (Table 6.4). With this dose a significant difference between exposed and non-exposed cells was found. This explains the statistical tendency which was observed after analysis of the interaction between SAR and exposure (Table 6.4).

For other experimental conditions no significant effect was detected. A statistical tendency was seen for the factor SAR with $H_2O_2$ treatment (Table 6.6). This effect is based on the increased values which were seen with a SAR of 1.0 W/kg (Figure 6.9). In this case we found also in sham-exposed cells slightly increased values. Therefore, it seems that this effect is only partly caused by the exposure.

Table 6.4: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidine bases. Glioblastoma multiforme (GBM) “stem-like” cells NCH421k; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 13.795 | <0.001 | highly sign. |
| Exposure | 1 / 30 | 1.683 | 0.204 | n.s. |
| SAR * Exposure | 2 / 30 | 2.821 | 0.075 | tendency |

Table 6.5: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma multiforme (GBM) “stem-like” cells NCH421k; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 27 | 2.119 | 0.140 | n.s. |
| Exposure | 1 / 27 | 0.917 | 0.347 | n.s. |
| SAR * Exposure | 2 / 27 | 1.393 | 0.265 | n.s. |

Table 6.6: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity towards oxidative damage. Glioblastoma multiforme (GBM) “stem-like” cells NCH421k; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 26 | 3.065 | 0.064 | tendency |
| Exposure | 1 / 26 | 0.140 | 0.711 | n.s. |
| SAR * Exposure | 2 / 26 | 0.168 | 0.846 | n.s. |

Table 6.7: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Glioblastoma multiforme (GBM) “stem-like” cells NCH421k; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 28 | 1.227 | 0.309 | n.s. |
| Exposure | 1 / 28 | 2.533 | 0.123 | n.s. |
| SAR * Exposure | 2 / 28 | 0.933 | 0.405 | n.s. |

**Results obtained with the glioblastoma cell line U251**

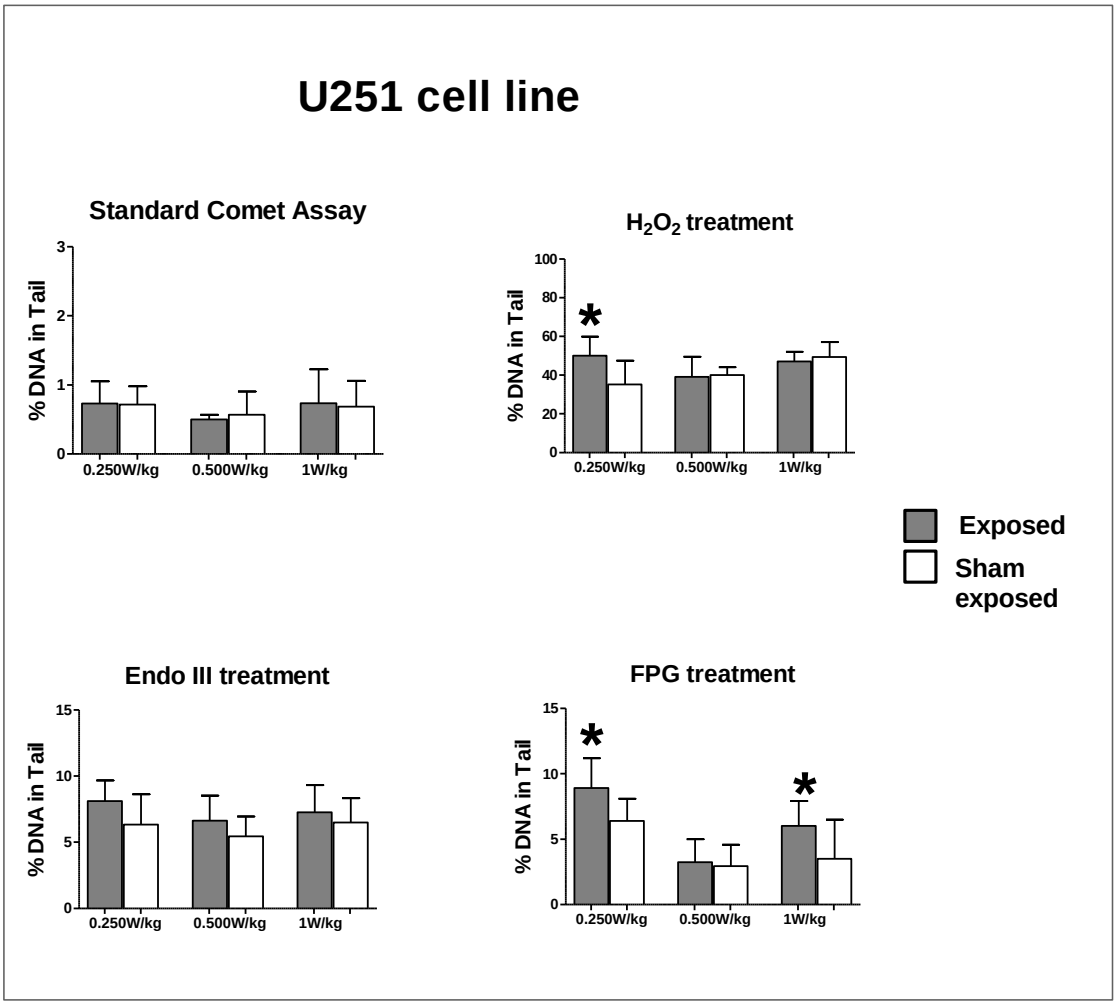



Figure 6.10: Results obtained with the glioblastoma cell line U251; exposure 16 hours with different SAR. Standard Comet Assay: DNA breaks. H2O2 treatment: sensitivity to oxygen radicals. Endo III: detection of oxidized pyrimidine bases; FPG Comet assays: the detection of oxidized purine bases. Bars represent means ± SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$).

Also in the glioblastoma line U251 higher values of the tail intensities (%DNA in tail) were found in exposed cells compared to sham-exposed controls under most conditions. Significant differences were found with the lowest exposure (SAR 0.25 W/kg) with H2O2 and with the highest exposure dose (SAR 1.0 W/kg) in FPG-treated cells (Figure 6.10).

Statistical analyses of all experimental factors showed for FPG-pretreated cell significant results for SAR and exposure. These results are based on the fact that the other exposures yielded higher tail intensities as the medium dose (SAR 0.5 W/kg). Also the difference between exposed and non-exposed cells was in this case significant. With Endo III treatment, a tendency for a difference between exposed and non-exposed cells was detected (Table 6.8).

Table 6.8: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidine bases. Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 1.056 | 0.361 | n.s. |
| Exposure | 1 / 30 | 4.008 | 0.054 | tendency |
| SAR * Exposure | 2 / 30 | 0.220 | 0.804 | n.s. |

Table 6.9: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 15.296 | <0.001 | highly sign. |
| Exposure | 1 / 30 | 5.120 | 0.031 | sign. |
| SAR * Exposure | 2 / 30 | 0.874 | 0.427 | n.s. |

After the evaluation of the results of Comet experiments with H2O2 treatment, significant interactions were found for “SAR” and “exposure”. This observation is based on the results obtained with SAR values of 0.5 W/kg and 1.0 W/kg (Table 6.10).

Table 6.10: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity to oxidative damage. Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 2.980 | 0.066 | tendency |
| Exposure | 1 / 30 | 1.792 | 0.191 | n.s. |
| SAR * Exposure | 2 / 30 | 3.662 | 0.038 | sign. |

Table 6.11: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 1.136 | 0.335 | n.s. |
| Exposure | 1 / 30 | 0.001 | 0.981 | n.s. |
| SAR * Exposure | 2 / 30 | 0.016 | 0.984 | n.s. |

**U373 Glioblastoma cell line**

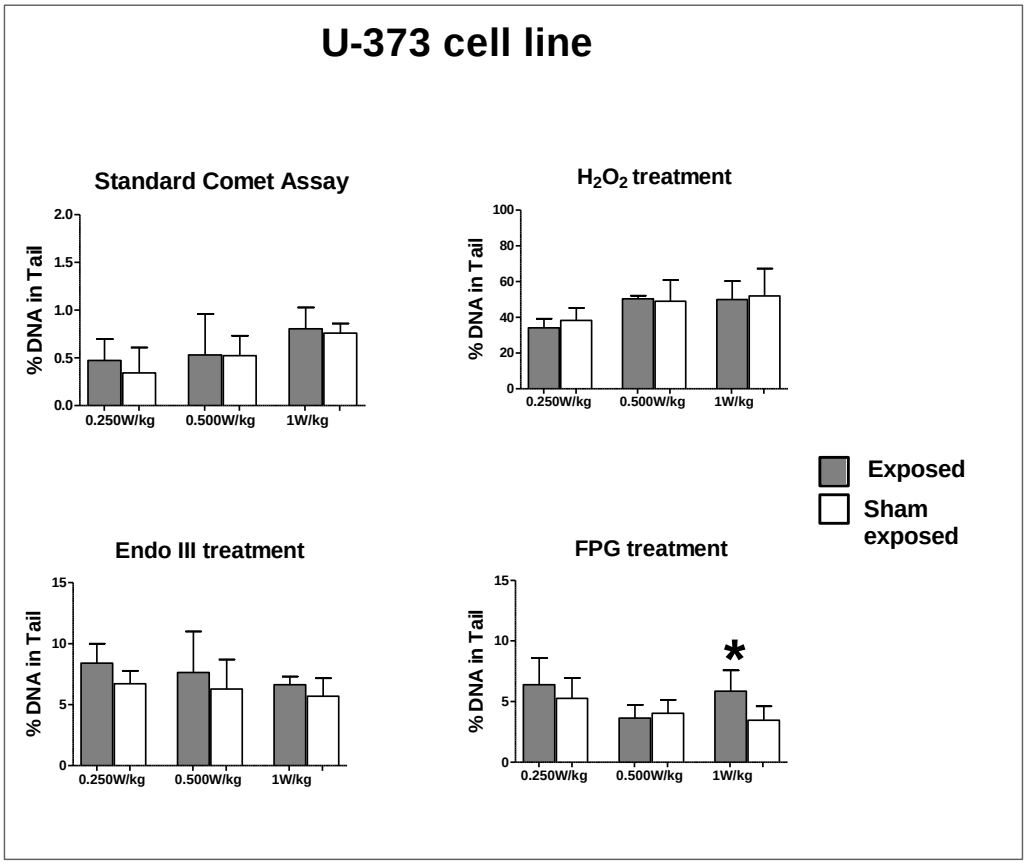



Figure 6.11: Results with the glioblastoma cell line U373; exposure 16 hours with different SAR. Standard Comet Assay: DNA breaks. H2O2 treatment: sensitivity to oxygen radicals. Endo III for the detection of oxidized pyrimidine bases; FPG Comet assays enable the detection of oxidized purine bases. Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$).

In general only slight effects were seen with this cell line. In FPG treated cells a significant difference between exposed cells and controls was detected with the highest dose (Fig. 6. 11).

The overall analyses of the results with ANOVA indicated that no significant differences exist with Endo III treated cells (Table 6.12). In the standard assays and in assays with H2O2 treatment only the factor SAR was significant (Table 6.14 und Table 6.15). The reason is that the exposed cells and sham-exposed cells shows higher tail intensities with increasing doses (Fig. 6.11), which means it is not caused by RF-EMF. Also in FPG Comet assays significant effects were detected (Table 6.13).

Table 6.12: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidine bases. Glioblastoma cell line U373; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 0.876 | 0.427 | n.s. |
| Exposure | 1 / 30 | 2.306 | 0.139 | n.s. |
| SAR * Exposure | 2 / 30 | 0.152 | 0.860 | n.s. |

Table 6.13: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma cell line U373; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 4.336 | 0.022 | sign. |
| Exposure | 1 / 30 | 3.558 | 0.069 | tendency |
| SAR * Exposure | 2 / 30 | 2.411 | 0.107 | n.s. |

Table 6.14: Results of analysis of variance (ANOVA) in Comet assays with H2O2 treatment. Glioblastoma cell line U373; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 30 | 8.663 | 0.001 | highly sign. |
| Exposure | 1 / 30 | 0.242 | 0.626 | n.s. |
| SAR * Exposure | 2 / 30 | 0.239 | 0.789 | n.s. |

Table 6.15: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U373; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 29 | 6.480 | 0.005 | highly sign. |
| Exposure | 1 / 29 | 0.388 | 0.538 | n.s. |
| SAR * Exposure | 2 / 29 | 0.504 | 0.609 | n.s. |

### 6.3.3 SAR DEPENDENT INDUCTION OF DNA STRAND BREAKS

SAR dependent induction of DNA strand breaks was monitored, since it was found in the literature that it is possible that the biological effects caused by high EMF do not

increase linearly with the dose. Therefore, two cell lines were evaluated after exposure to different SAR values.

The two cell lines were:

- The glioblastoma line U87
- The fibroblast line ES1

The cells were cultivated under standard conditions (37°C, 95% humidity, 5% CO2) as in previous experimental series.

The exposure was done as follows:

- UMTS signal
- SAR: 3 or 4 doses
  - 0.25 W/kg
  - 0.50 W/kg (only for U87)
  - 1.00 W/kg and
  - 1.50 W/kg (only for ES-1)
  - 2.00 W/kg (only for ES-1)
- Exposure duration: 16 h

Identical experimental conditions were used as in previous experiments.

The glioblastoma cell lines were tested as follows:

1. Analysis of the cells without further treatment: Comet assay under standard conditions for the detection of single and double strand breaks and apurinic sites.
2. Treatment with $H_2O_2$ to find out if the sensitivity towards ROS is increased.
3. Treatment of nuclei with FPG to find out if RF-EMF exposure causes an increase of the rate oxidised purine bases.
4. Treatment of nuclei with Endo III to find out if RF-EMF exposure causes an increase of the formation of the rate of oxidised pyrimidine bases.

The fibroblast cell line ES-1 was not treated with restriction enzymes since no significant differences were found in such experiments in the previous test series under identical experimental conditions (section 6.3.1). Therefore, the cells were only analyzed with the standard comet assay and after H2O2 treatment (Figure 6.12 and Figure 6.13).

### Fibroblast line ES-1

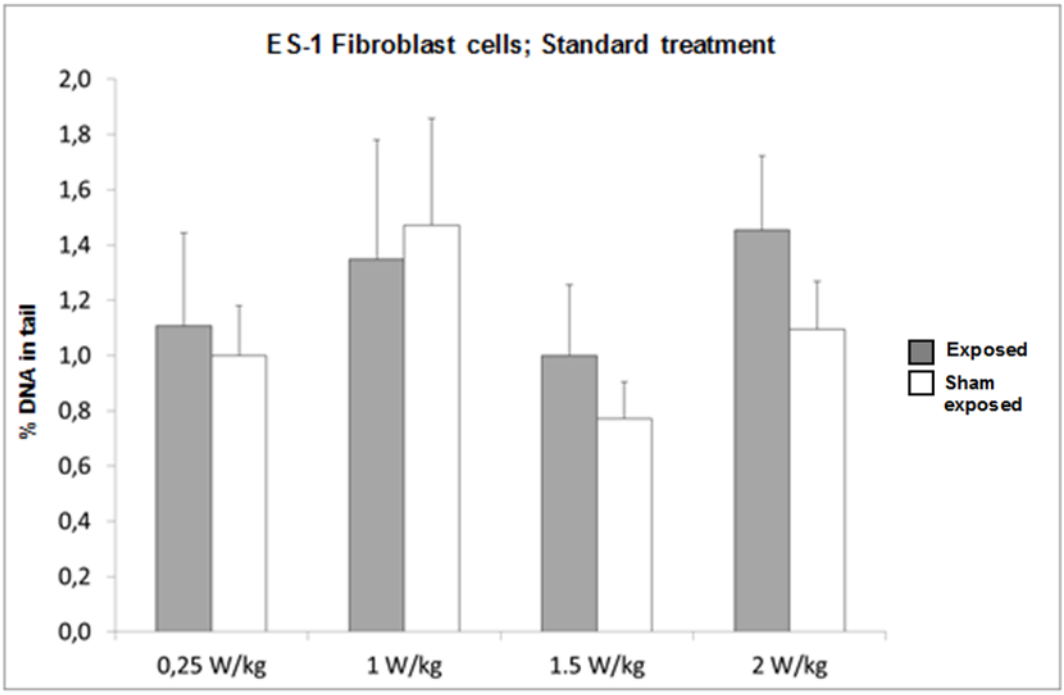



Figure 6.12: Fibroblasts ES-1; Comet assay under standard condition; UMTS exposure with 0.25, 1.0, 1.5 and 2.0 W/kg. %DNA in tail in exposed and sham exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

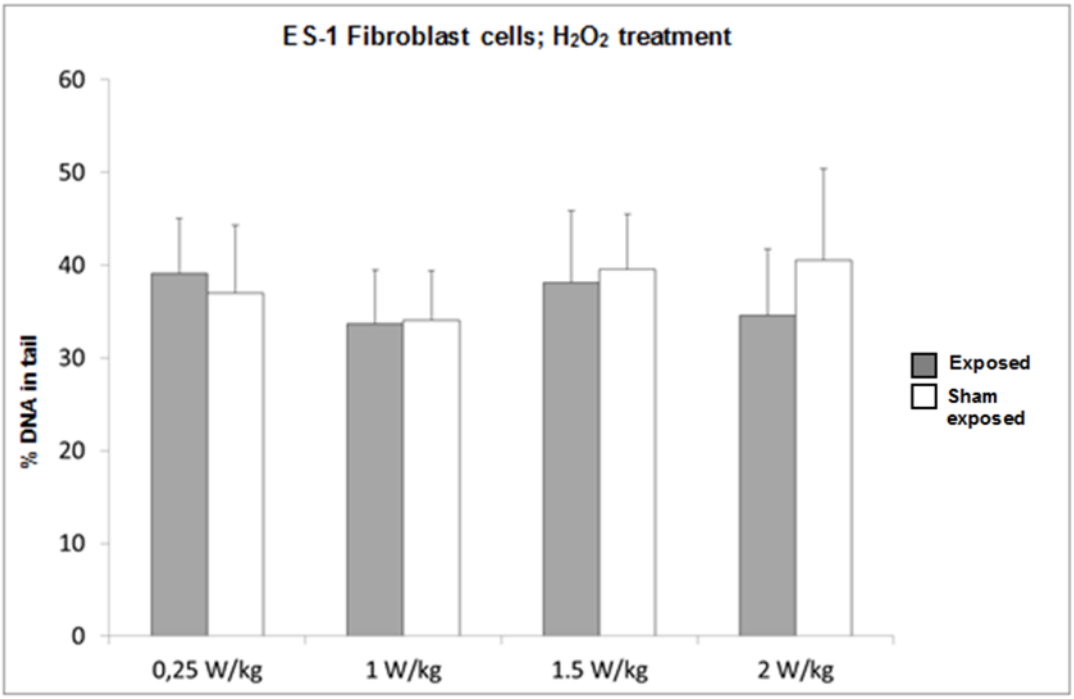



Figure 6.13: Fibroblasts cell line ES-1; treatment with $H_2O_2$ to investigate the sensitivity to oxidative damage; UMTS exposure to SAR values of 0.25, 1.0, 1.5 and 2.0 W/kg; *% DNA in tail;* Bars represent means ±SD of results obtained with three cultures per experimental condition.

In agreement with the results of the previous experiments, no significant differences were obtained in the fibroblasts. The overall analysis of variance indicates that neither the intensity of exposure nor the difference between exposed and non-exposed cells reveals statistically significant effects (Table 6.16 und Table 6.17).

Table 6.16: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Fibroblast line ES-1; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 1.0, 1.5 and 2.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 3/62 | 1.592 | 0.200 | n.s. |
| Exposure | 1/62 | 0.657 | 0.421 | n.s. |
| SAR * Exposure | 3/62 | 0.293 | 0.831 | n.s. |

Table 6.17: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity to oxidative damage. Fibroblasts cell line ES-1; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 1.0, 1.5 and 2.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 3/62 | 0.203 | 0.894 | n.s. |
| Exposure | 1/62 | 0.081 | 0.777 | n.s. |
| SAR * Exposure | 3/62 | 0.116 | 0.950 | n.s. |

### Glioblastoma cell line U-87

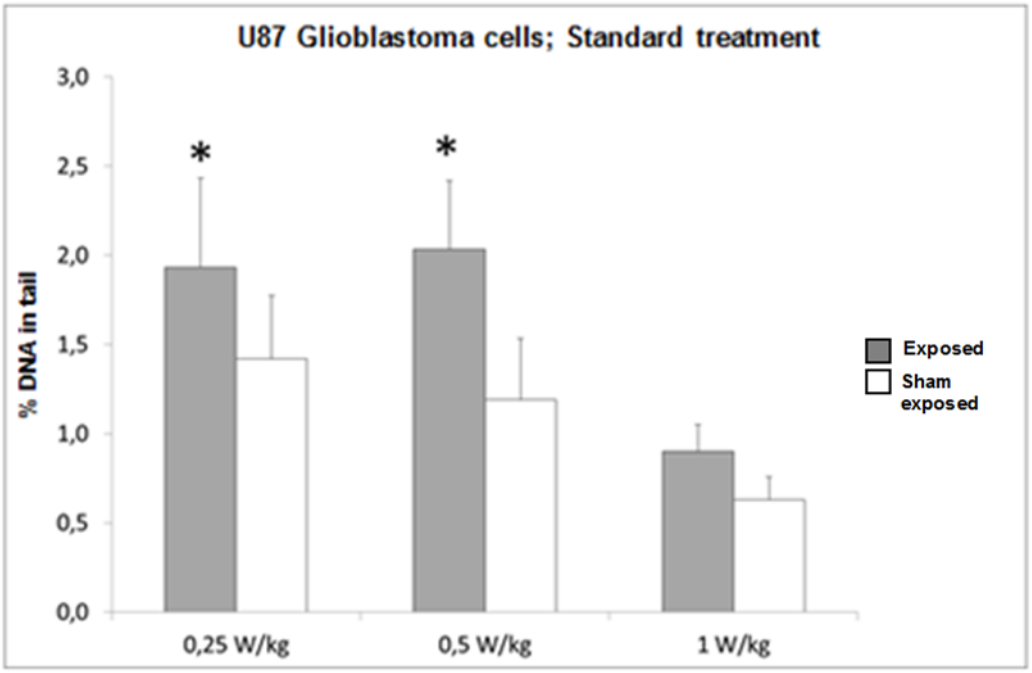



Figure 6.14: Results of Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$).

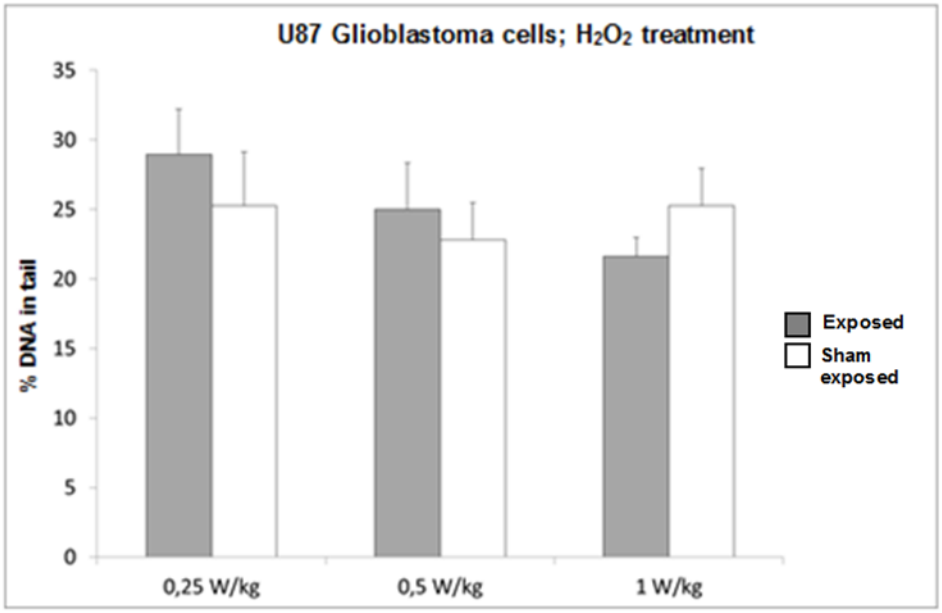



Figure 6.15: Results of Comet assays with $H_2O_2$ for the determination of the sensitivity to oxidative damage. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. *% DNA in tail* in exposed and sham exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

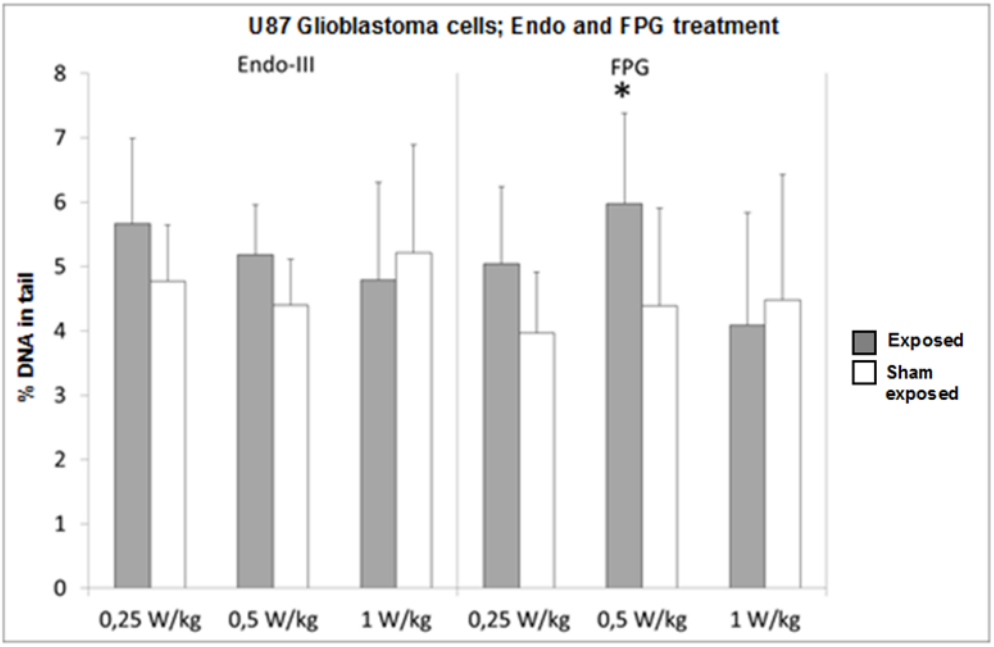



Figure 6.16: Glioblastoma cell line U87; 16 hours with different SAR doses. Endo III enables the detection of oxidized pyrimidine bases; FPG treatment enables the detection of oxidized purine bases. Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$).

A significant difference between exposed and non-exposed cells was found under standard conditions with a SAR of 0.25 and 0.5 W/kg and in FPG treated cells with 0.5 W/kg. Analysis of variance (Table 6.18) showed a significant effect caused by exposure, while with FPG treated cells (Table 6.21) the SAR effect of 0.5 W/kg was not sufficient to reach statistical significance.

Under standard conditions the factor SAR was significant but an inverse dose-response was detected i.e. higher values of the tail intensities were found with lower SAR doses. Results obtained with non-exposed cells show that these effects are partly caused by differences between the experiments with different SAR doses. Since differences between exposed and non-exposed cells become smaller with higher SAR values, it is possible that this difference masks a real dose effect.

It is known that such dose–response relations are possible when the exposure causes activation of counterregulation (e.g. induction of DNA repair processes).

Table 6.18: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/54 | 13.209 | 0.000 | highly sign. |
| Exposure | 1/54 | 6.741 | 0.012 | sign. |
| SAR * Exposure | 2/54 | 0.230 | 0.795 | n.s. |

Table 6.19: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity to oxidative damage. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/52 | 0.595 | 0.555 | n.s. |
| Exposure | 1/52 | 0.175 | 0.678 | n.s. |
| SAR * Exposure | 2/52 | 0.789 | 0.460 | n.s. |

Table 6.20: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidine bases. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/52 | 0.128 | 0.880 | n.s. |
| Exposure | 1/52 | 0.000 | 0.995 | n.s. |
| SAR * Exposure | 2/52 | 0.390 | 0.679 | n.s. |

Table 6.21: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/46 | 2.831 | 0.069 | tendency |
| Exposure | 1/46 | 1.749 | 0.193 | n.s. |
| SAR * Exposure | 2/46 | 0.885 | 0.419 | n.s. |

**Summary: SAR dependency**

In the cell line U87 but not in the line ES-1 an indication for an exposure related induction of DNA damage was observed. As mentioned above, we found under standard conditions and also after treatment of the nuclei with restriction enzymes significant induction of DNA migration.

It was not possible to detect clear dose-dependent effects but we found some indication for a U shaped effect. However, this observation was not consistent in all experiments.

### 6.3.4 EXPOSURE DURATION

Since it was described in the literature that DNA lesions are not observeable immediately after the start of exposure but may become visible after some time, three different exposure durations were monitored in the glioblastoma line U87, to test for a so called latency time.

**Methods**

To determine if the effect which was observed in the glioblastoma cell line U87 is influenced by the duration of exposure, experiments were conducted in which the cells were exposed for different periods of time.

After exposure, the cells were deep frozen in order to analyze them in one experiment. This strategy leads to reduction of the variability caused by individual working steps. After defrosting, Comet assays were conducted under standard conditions, with H2O2 treatment and after treatment of the nuclei with restriction enzymes (FPG/Endo III).

Different exposure durations were used:

- 6 h
- 16 h
- 24 h

As in previous experimental series, the cells were cultivated under standard condition (37°C, 5% CO2, 95% humidity).

The following exposures were used:

- UMTS signal
- SAR: three doses were tested
  - 0.25 W/kg.
  - 0.50 W/kg and
  - 1.00 W/kg

### Time dependency overview

Figures 6.17 - 6.20 depict the results of the experiments in which the time dependency of the exposure was investigated.

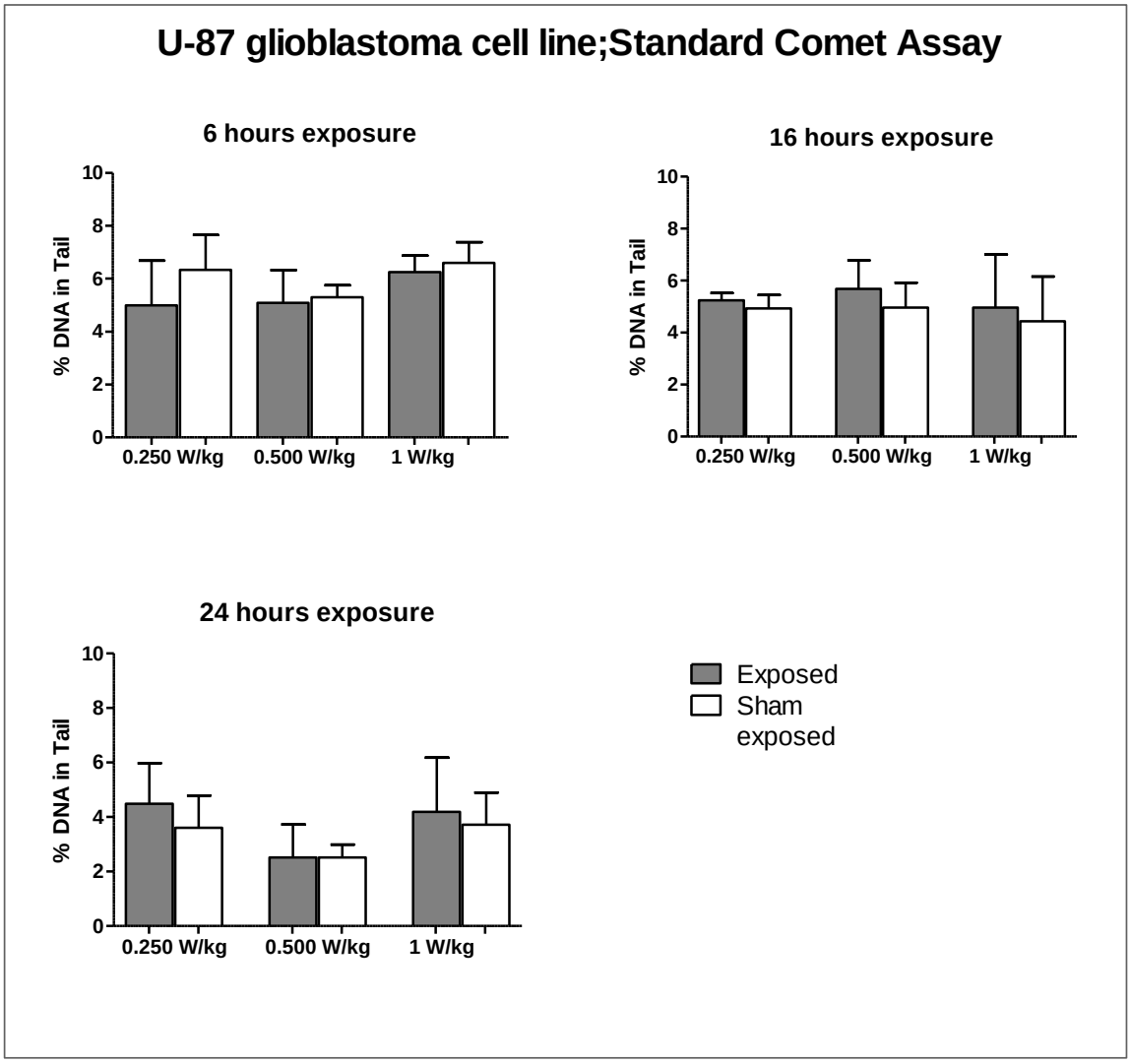



Figure 6.17: Results obtained with the glioblastoma cell line U87; Comet assays under standard condition; UMTS exposure with different SAR values 0.25 W/kg – 1.0 W/kg and different exposure durations 6, 16 and 24 hours. % DNA in tail in exposed and sham exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

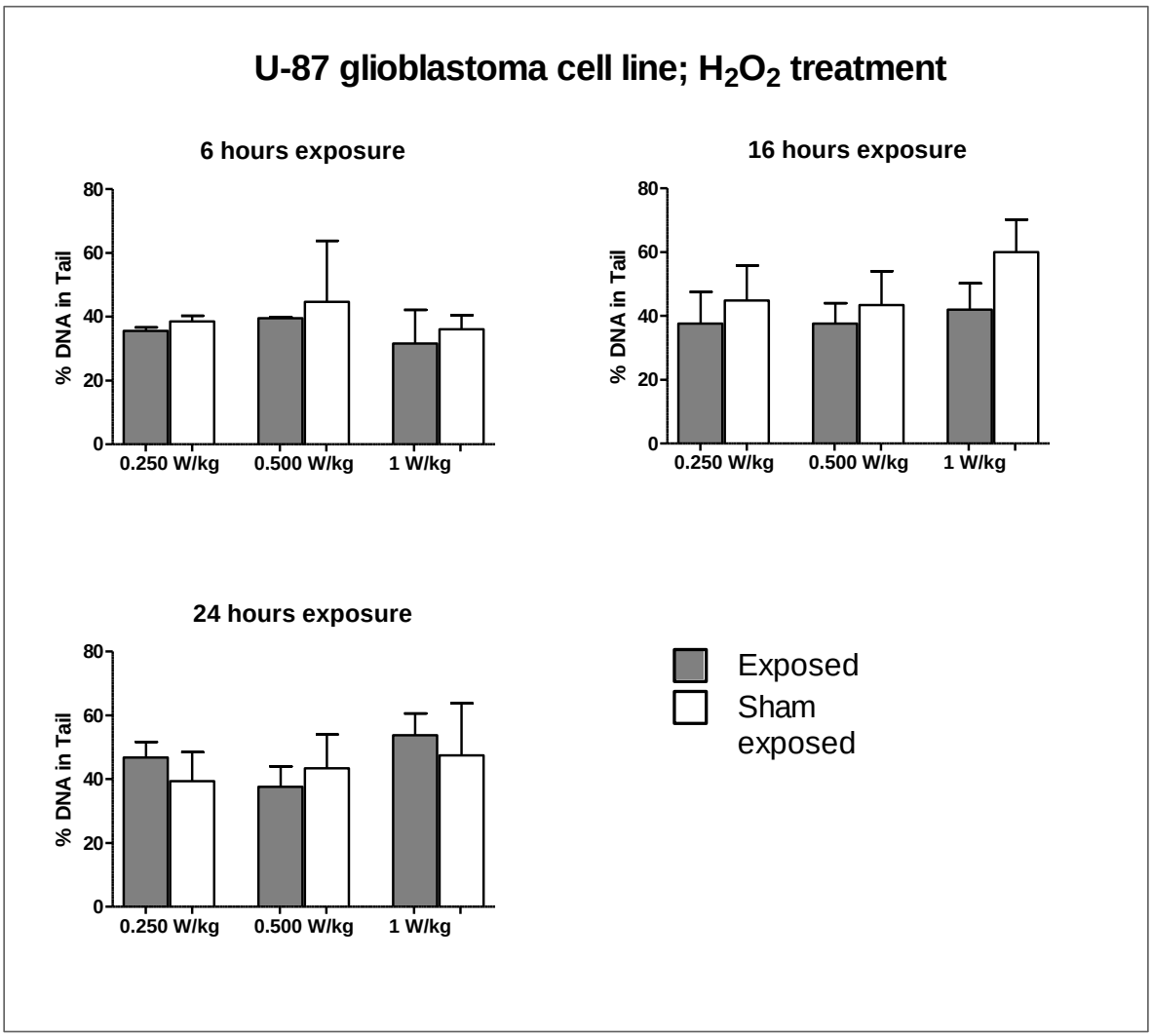



Figure 6.18: Results obtained with the glioblastoma cell line U87; H2O2 treatment; UMTS exposure with different SAR values 0.25 W/kg – 1.0 W/kg and different exposure durations 6, 16 and 24 hours; % DNA in tail in exposed and sham exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

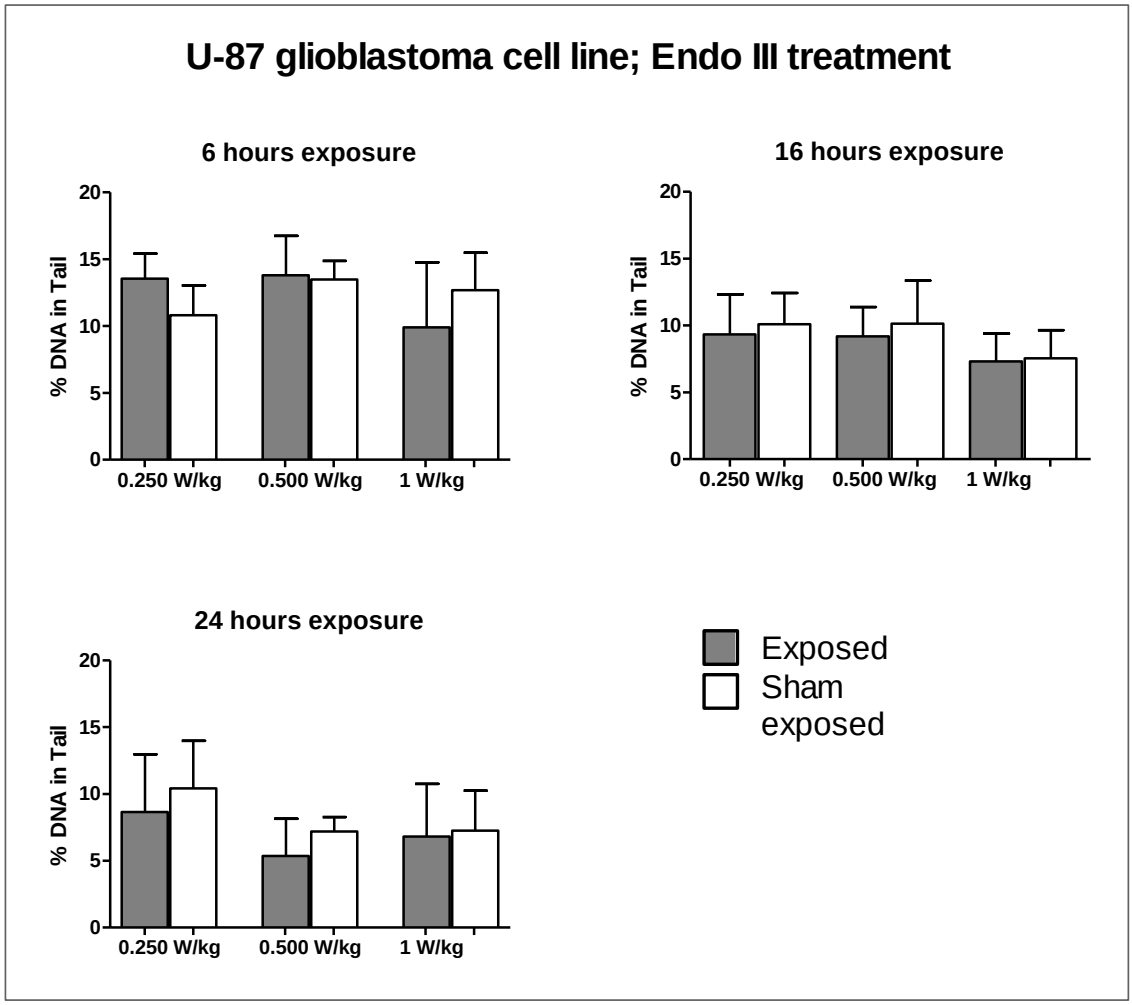



Figure 6.19: Results obtained with the glioblastoma cell line U87; Endo III treatment; UMTS exposure with different SAR values 0.25 W/kg – 1.0 W/kg and different exposure durations 6, 16 and 24 hours; % DNA in tail in exposed and sham exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition.

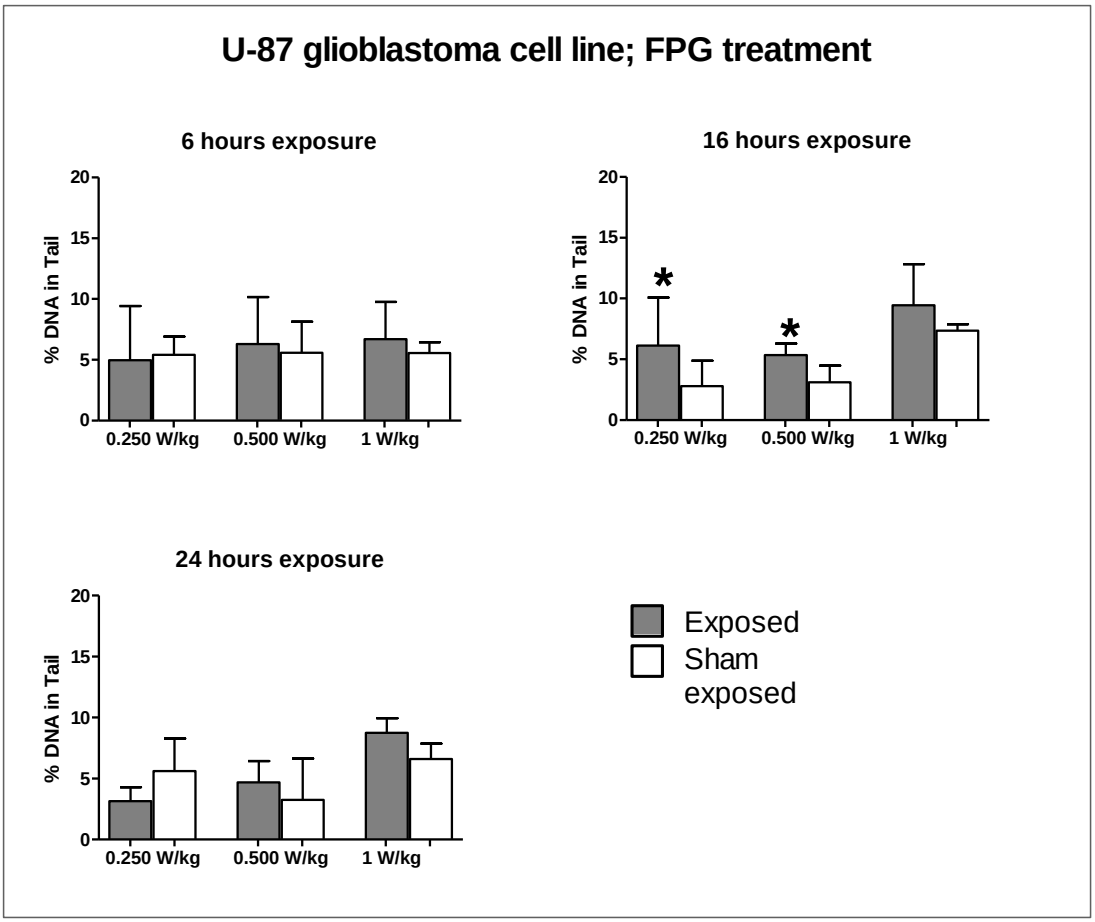



Figure 6.20: Results obtained with the glioblastoma cell line U87; FPG Comet Assay; UMTS exposure with different SAR values 0.25 W/kg – 1.0 W/kg and different exposure durations 6, 16 and 24 hours; % DNA in tail in exposed and sham exposed cells. Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★($p < 0.05$).

### Detailed evaluation of the results

#### Exposure duration 6h

No significant effects were seen under all experimental conditions after six hours exposure.

Table 6.22: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidin bases. Glioblastoma cell line U87; exposure duration 6 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 1.338 | 0.299 | n.s. |
| Exposure | 1 / 12 | 0.002 | 0.967 | n.s. |
| SAR * Exposure | 2 / 12 | 1.827 | 0.203 | n.s. |

Table 6.23: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma cell line U87; exposure duration 6 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 11 | 0.485 | 0.628 | n.s. |
| Exposure | 1 / 11 | 0.214 | 0.652 | n.s. |
| SAR * Exposure | 2 / 11 | 0.500 | 0.620 | n.s. |

Table 6.24: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity to oxidative damage. Glioblastoma cell line U87; exposure duration 6 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 1.179 | 0.341 | n.s. |
| Exposure | 1 / 12 | 0.885 | 0.365 | n.s. |
| SAR * Exposure | 2 / 12 | 0.019 | 0.981 | n.s. |

Table 6.25: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard conditions and enable the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U87; exposure duration 6 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 1.811 | 0.205 | n.s. |
| Exposure | 1 / 12 | 1.496 | 0.245 | n.s. |
| SAR * Exposure | 2 / 12 | 0.470 | 0.636 | n.s. |

**Exposure duration 16h**

A significant effect was seen in FPG Comet assays (Figure 6.20). The same observation was made also in earlier experiments. The analysis of variance indicated a significant effect of exposure and of the intensity (SAR). As in previous experiments, the lowest and highest exposure dose led to higher tail intensities. In the $H_2O_2$ experiments, a lower tail intensity was detected after an exposure for 16h in comparison to non-exposed cells (Fig. 6.18).

Table 6.26: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidine bases. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 1.644 | 0.234 | n.s. |
| Exposure | 1 / 12 | 0.272 | 0.612 | n.s. |
| SAR * Exposure | 2 / 12 | 0.038 | 0.962 | n.s. |

Table 6.27: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 16.006 | 0.004 | highly sign. |
| Exposure | 1 / 12 | 4.474 | 0.067 | tendency |
| SAR * Exposure | 2 / 12 | 0.236 | 0.640 | n.s. |

Table 6.28: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity to oxidative damage. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 2.253 | 0.148 | n.s. |
| Exposure | 1 / 12 | 5.263 | 0.041 | sign. |
| SAR * Exposure | 2 / 12 | 0.738 | 0.499 | n.s. |

Table 6.29: Results of analysis of variance in Comet assays (standard conditions) for the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 0.511 | 0.612 | n.s. |
| Exposure | 1 / 12 | 0.735 | 0.408 | n.s. |
| SAR * Exposure | 2 / 12 | 0.036 | 0.965 | n.s. |

### Exposure duration 24h

As after 16h exposure, significant exposure related effects were seen in FPG Comet assays after 24h (Table 6.31). We also observed a significant role of the exposure intensity, i.e. the size of the comets increased with the radiation dose (SAR). After H2O2 treatment the lowest value was detected with the median intensity (Figure 6.18).

Table 6.30: Results of analysis of variance (ANOVA) in Comet assays with Endo III for the detection of oxidized pyrimidin bases. Glioblastoma cell line U87; exposure duration 24 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 2.476 | 0.126 | n.s. |
| Exposure | 1 / 12 | 0.754 | 0.402 | n.s. |
| SAR * Exposure | 2 / 12 | 0.090 | 0.915 | n.s. |

Table 6.31: Results of analysis of variance (ANOVA) in Comet assays with FPG for the detection of oxidized purine bases. Glioblastoma cell line U87; exposure duration 24 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 19.381 | 0.002 | highly sign. |
| Exposure | 1 / 12 | 3.485 | 0.099 | tendency |
| SAR * Exposure | 2 / 12 | 0.005 | 0.945 | n.s. |

Table 6.32: Results of analysis of variance (ANOVA) in Comet assays with H2O2 for the determination of the sensitivity to oxidative damage. Glioblastoma cell line U87; exposure duration 24 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 4.778 | 0.030 | sign. |
| Exposure | 1 / 12 | 3.100 | 0.099 | tendency |
| SAR * Exposure | 2 / 12 | 0.046 | 0.955 | n.s. |

Table 6.33: Results of analysis of variance in Comet assays (standard conditions) for the detection of single and double strand breaks and apurinic sites. Glioblastoma cell line U87; exposure duration 24 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2 / 12 | 2.749 | 0.104 | n.s. |
| Exposure | 1 / 12 | 0.334 | 0.574 | n.s. |
| SAR * Exposure | 2 / 12 | 0.180 | 0.838 | n.s. |

### Summary of the experimental series concerning the effect of the exposure duration

In experiments concerning the formation of oxidized pyrimidine DNA bases (Endo III treatment) no significant difference in regard to “exposure” and “intensity” of the treatment (SAR) was found.

In experiments with oxidized purine bases (FPG treatment) exposure-dependent induction of DNA lesions was found after 16 and 24 h. The results suggest a U-shaped dose-response relation.

In experiments which concerned the sensitivity towards ROS (H2O2 treatment), a significant effect of radiation was detected after 24 h exposure. Additionally, there was also the tendency for a difference between exposed and non-exposed cells.

### 6.3.5 IMPACT OF SERUM DEPRIVATION

The goal of these experiments was to investigate if stress induced by serum deprivation leads to an increase of radiation-induced DNA strand breaks.

Earlier considerations as well as results of previous experiments led us to this assumption:

- It is known that ionizing radiation (X-rays, γ-rays, so-called low-LET-radiation, i.e. radiation with low linear energy transfer) causes only partly damage via direct interaction with the DNA molecules. The major part of the damage is induced indirectly via formation of reactive oxygen radicals.
- Serum deprivation leads to cellular stress. As a consequence, factors are synthesized not abundant under normal conditions. This additional biosynthesis (metabolic activation) may lead to increased sensitivity towards external factors.
- Serum deprivation induces a state that resembles “normal” conditions for glial cells i.e. in the brain, most cells are arrested.

#### Principle of test and overview of the methods

Serum deprivation causes a stress situation, which may have a negative impact on the “fitness” of the cells. Therefore, it is possible that cells which are cultivated in serum-free medium are more sensitive to exposure to DNA damaging factors. Therefore, an additional experiment was conducted in which the cells were cultivated in absence of serum and then exposed to RF-EMF.

In this experimental series, the cells were grown under standard conditions as in previous experiments (37° C, 5% CO2 atmosphere, 95% humidity). However, serum was not added to the cultivation medium. To ensure that the vitality of the cells was not affected, additional measurements were conducted in which the vitality of the cells was monitored by use of an adequate staining procedure.

##### Determination of the vitality of the cells

The cells were removed from the Petri dishes by trypsinization and transferred in 2.0 ml medium. From this suspension 20 µl were enumerated in a so-called Neubauer counting chamber to determine the number of cells. In each Petri dish 5 x 105 cells were grown in presence and absence of serum at standard conditions (37°C, 5% CO2 atmosphere, 95% humidity) for a period of seven days. Every 24 h, we counted the number of cells in 3 replica plates. The results were used to plot growth curves.

The numbers of living and dead cells were determined by use of the trypan blue exclusion technique. In all investigations we found that the vitality was higher than 80%.

After determination of the proliferation rate and of the vitality of the cells, the cells were exposed to radiation. Two glioblastoma cell lines were used in these experiments, namely:

- U87
- U251

The exposure duration was 16 h in all experiments .

The serum deprivation was performed over 5 days plus 16 h exposure to an RF-EMF field (UMTS signal) and different SAR values were used, namely:

- SAR 0.25 W/kg
- SAR 0.50 W/kg and
- SAR 1.00 W/kg

Subsequent investigations concerning the damage of the genetic material were conducted as described in the previous experimental series.

**Vitality of the cells before EMF exposure**

After cultivation and 2-3 passages the cells were exposed to EMF or sham-exposed in medium with and without serum for 16 h.

Figure 6.21 A and C depict the results of the survival curves of the cells after serum deprivation. The cells were cultivated in these experiments over a period of 7 days. Their numbers were significantly reduced after 5 days by 18-25%. This effect was observed with both cell lines. On the contrary, we found that the numbers of cells which were cultivated in presence of serum increased 6 fold respectively 3 fold. Also the vitality of cells was affected by serum deprivation (Figure 6.21 B and D). At the start of the incubation, the vitality was in the range of 97-99%; it decreased after 5 days to 80%, after 7 days the vitality was only 32% in cultures with U87 cells and 22% in cultures with U251 cells.

It is well known that a low vitality (below 80%) may lead to false-positive results in Comet assays. In order to avoid that it drops below this critical level of 80%, all subsequent experiments were performed after 5-days of serum deprivation. Only cells which had intact nuclei were evaluated for comet formation as it can be assumed that these cells are alive before the analysis.

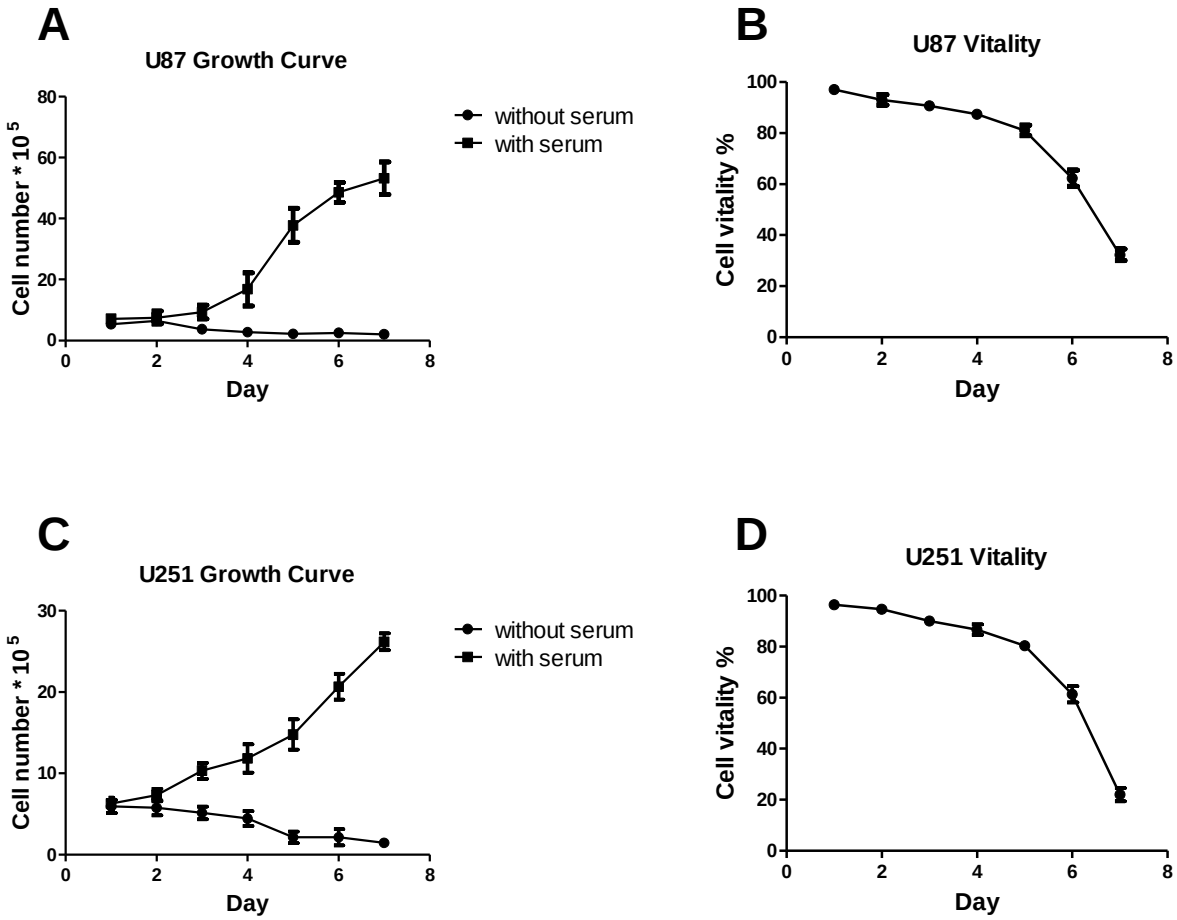



Figure 6.21: Growth kinetics of the glioblastoma lines U87 and U251 (A and C) and the impact of cultivation on the vitality of the cells in serum free conditions (B and D). 5x105 cells were seeded into Petri dishes and cultivated under standard conditions (5 % CO2. 37 C°), either in FCS supplemented medium or in serum free medium. After harvest by trypsinization, the cell numbers were determined in 24-hour intervals over a period of seven days. Symbols represent means ± SD of results obtained with three cultures per time point.

## Results of the exposure experiments

### Measurement of DNA damage (standard Comet assay)

The results of the two cell lines (U87 and U251) obtained under standard conditions are summarized in Figure 6.22.

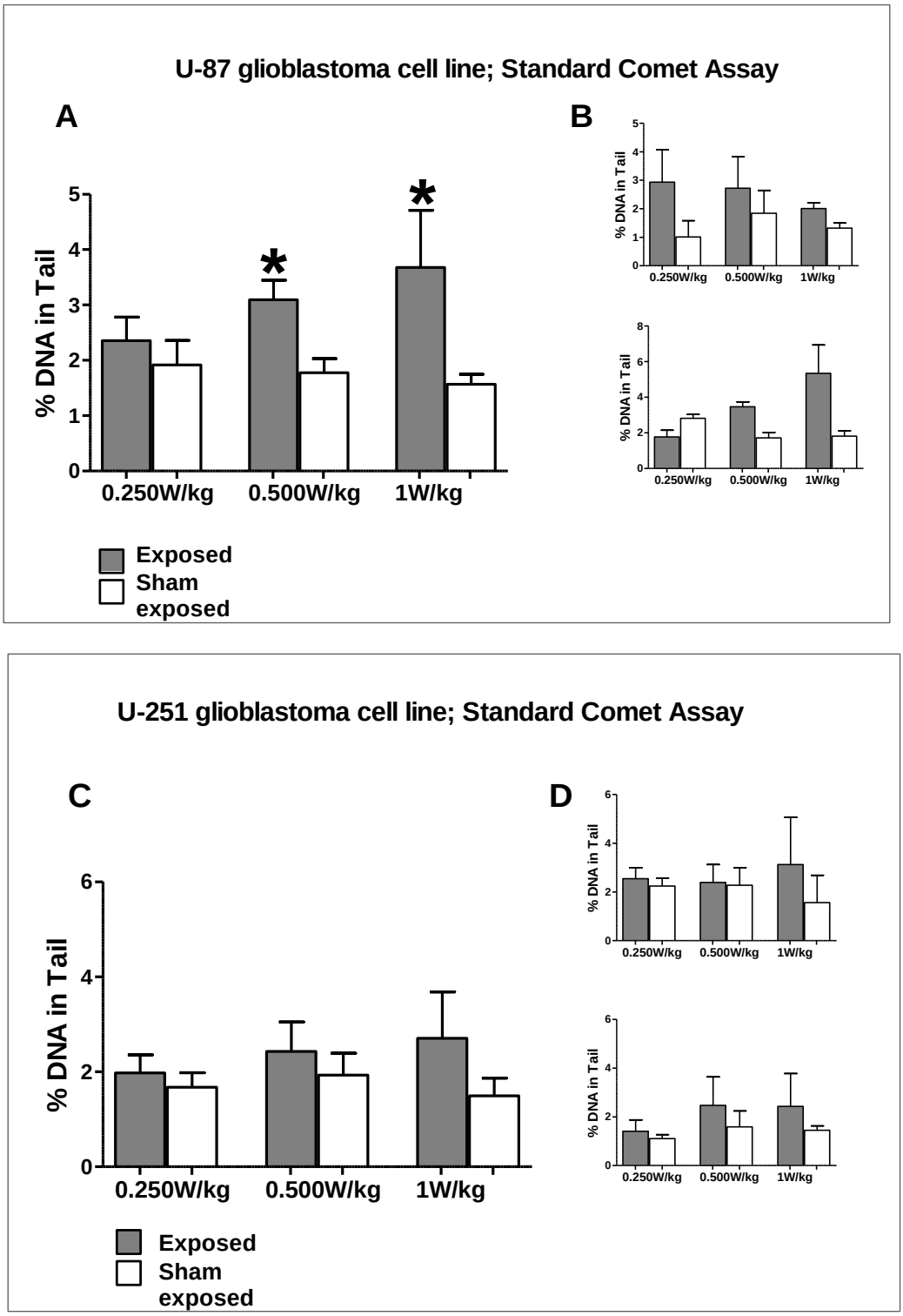



Figure 6.22: Results obtained with prestressed glioblastoma cell line U87 (overall result Fig.A and results of two independent experiments Fig.B); U251 (overall result Fig.C and results of two independent experiments Fig.D). Comet assays under standard conditions; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. % DNA in tail in exposed and sham exposed cells after cultivation under serum free conditions (5 days). Bars represent means ± SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$) in the overall results.

The glioblastoma line U87 showed a significant difference between exposed and sham-exposed cells (SAR 0.5 and 1.0 W/kg). The subsequent analysis of variance

showed an overall effect of exposure (Table 6.34). An increase of the tail intensity was found in all experiments in the glioblastoma cell line U251 (Figure 6.22) but did not reach the statistical significance (Table 6.34).

Table 6.34: U87 glioblastoma cell line under serum free conditions; Results of analysis of variance in standard Comet assays for the detection of DNA damage which were performed under serum free conditions (5 days). Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/30 | 0.39 | 0.681 | n.s. |
| Exposure | 1/30 | 10.23 | 0.003 | highly sign. |
| SAR * Exposure | 2/30 | 0.95 | 0.397 | n.s. |

Table 6.35: U251 glioblastoma cell line under serum free conditions; Results of analysis of variance in standard Comet assays for the detection of DNA damage which were performed under serum free conditions (5 days). Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/28 | 0.16 | 0.851 | n.s. |
| Exposure | 1/28 | 1.77 | 0.194 | n.s. |
| SAR * Exposure | 2/28 | 0.23 | 0.794 | n.s. |

**Measurement of oxidized pyrimidines (Endo III treatment)**

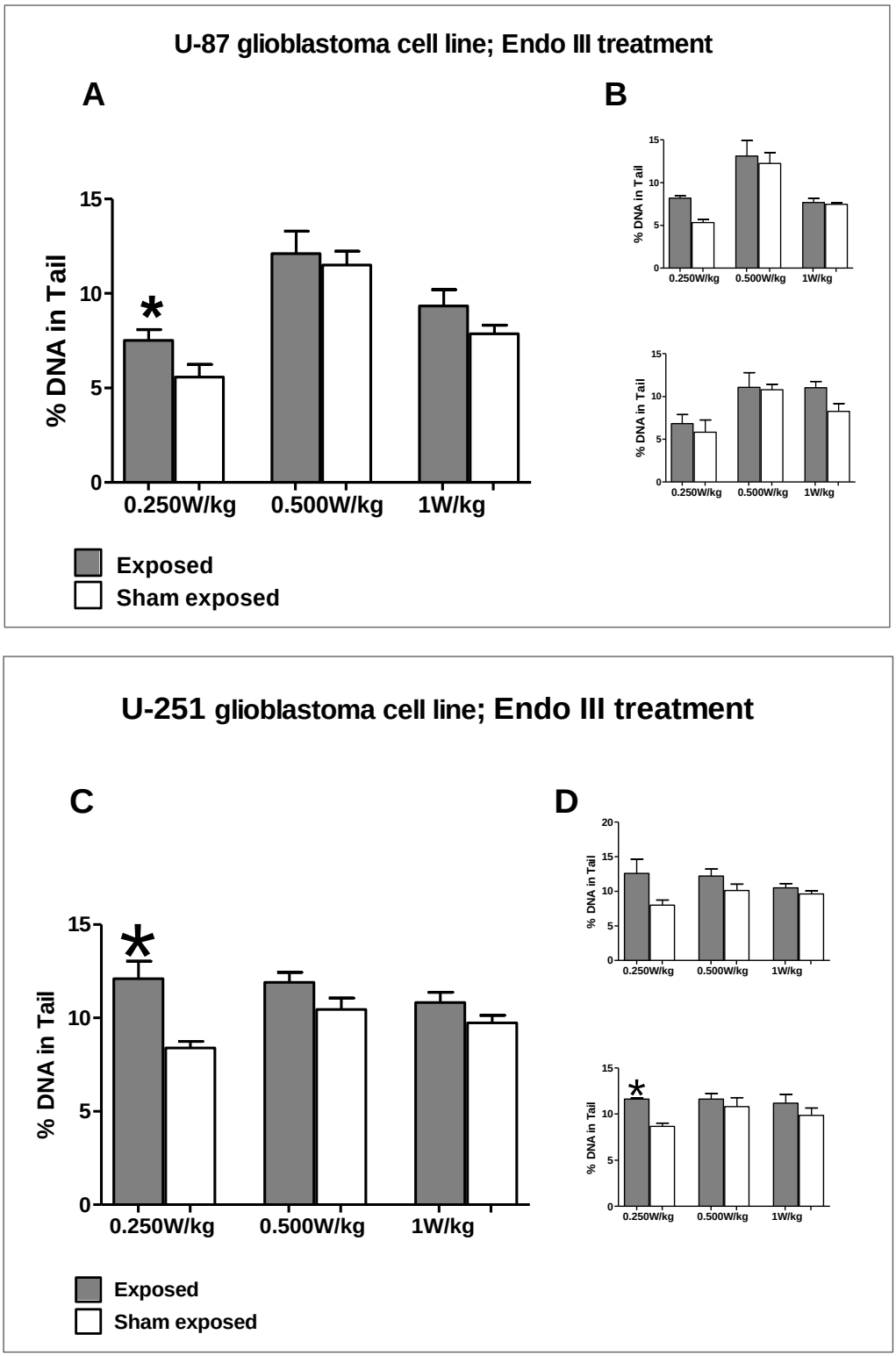



Figure 6.23: Results obtained with prestressed glioblastoma cell line U87 (overall result Fig.A and results of two independent experiments Fig.B); U251 (overall result Fig.C and results of two independent experiments Fig.D). Comet assays with Endo III treatment; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. % DNA in tail in exposed and sham exposed cells after cultivation under serum free conditions (5 days). Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$) in the overall results.

Significant differences between exposed and non-exposed cells were detected in line U87 and also in line U251. After the ANOVA - in both cell types we observed a significant exposure related effect (Table 6.36 und Table 6.37). In line U87 also the factor SAR was significant. These findings are based on the increase of the % DNA in tail value which was observed with a SAR of 0.5 W/kg compared to 0.25 W/kg (Table 6.36). In U251 cells, the SAR exposition was statistically significant. This can be explained by a strong difference between exposed and non-exposed cells with a SAR dose of 0.25 W/kg compared to other SAR values (Table 6.37).

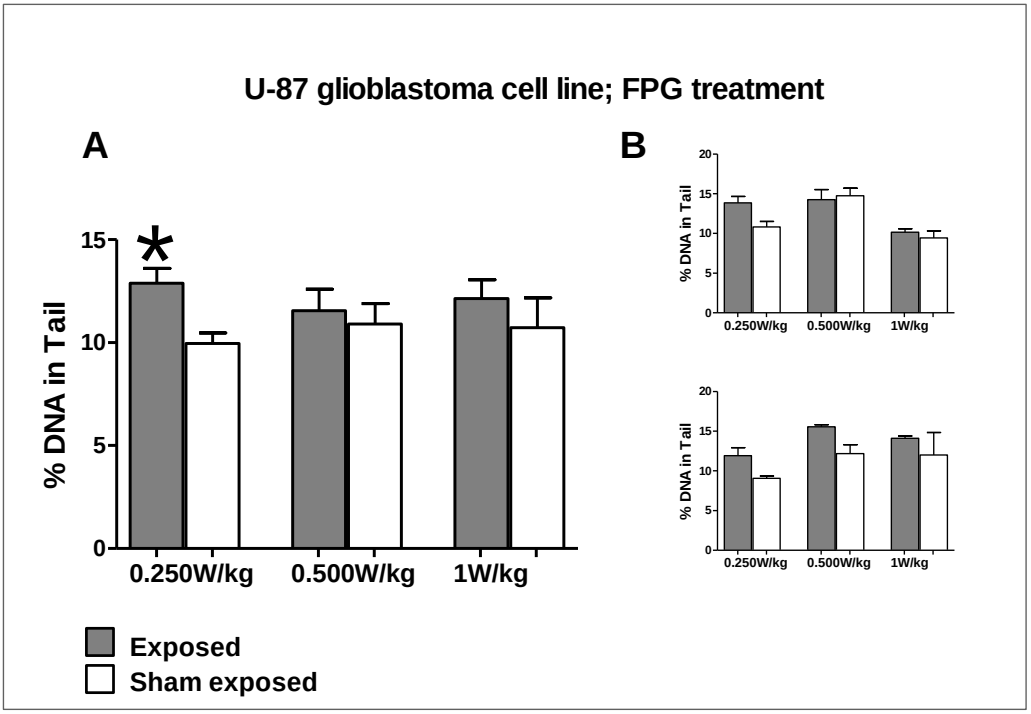
Table 6.36: U87 glioblastoma cell line under serum free conditions; Results of analysis of variance in Endo III Comet assays which were performed under serum free conditions (5 days). Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/30 | 25.39 | <0.001 | highly sign. |
| Exposure | 1/30 | 5.12 | 0.031 | sign. |
| SAR * Exposure | 2/30 | 0.77 | 0.473 | n.s. |

Table 6.37: U251 glioblastoma cell line under serum free conditions; Results of analysis of variance in Endo III Comet assays which were performed under serum free conditions (5 days). Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/29 | 2.11 | 0.139 | n.s. |
| Exposure | 1/29 | 6.34 | 0.018 | sign. |
| SAR * Exposure | 2/29 | 10.63 | <0.001 | highly sign. |

**Measurement of oxidized purines (FPG treatment)**



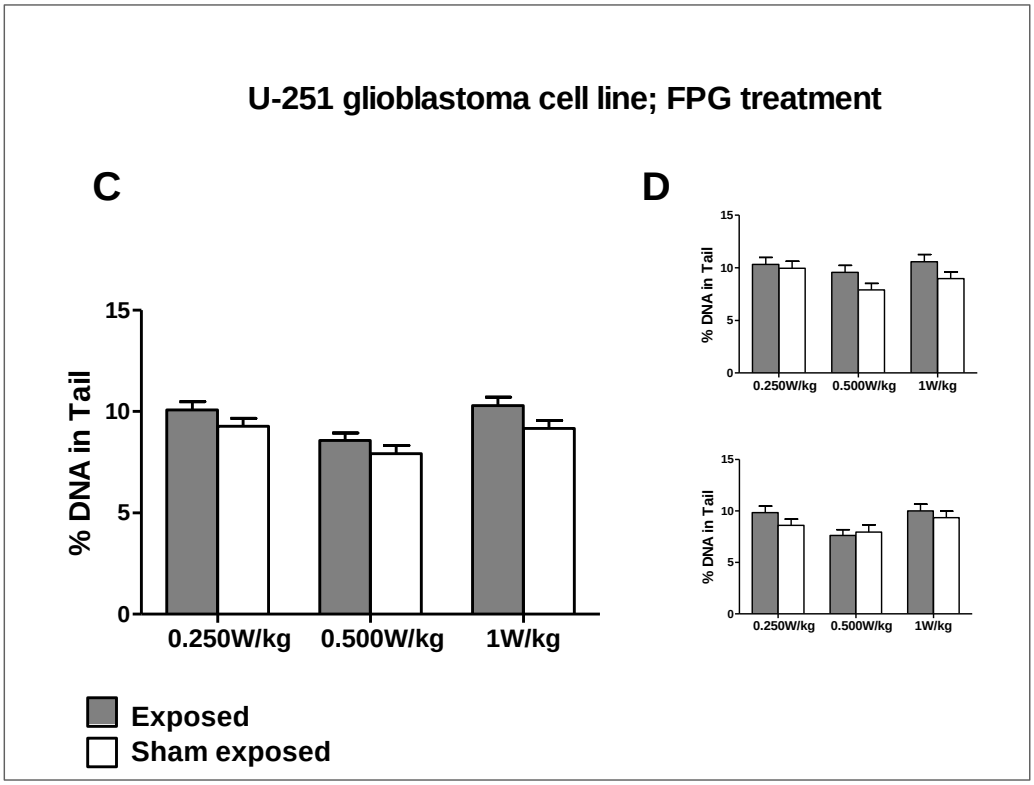




Figure 6.24: Results obtained with prestressed glioblastoma cell line U87 (overall result Fig.A and results of two independent experiments Fig. B); U251 (overall result Fig. C and results of two independent exposures and experiments Fig. D). Comet assays under FPG treatment; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. % DNA in tail in exposed and sham exposed cells after cultivation under serum free conditions (5 days). Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$) in the overall results.

Also in U87 cells exposure to 0.25 W/kg induced significant effects in comparison to sham-exposed cells (

Figure 6.24). Analyses of variance indicated for both cell lines (U87 and U251) a significant effect of exposure (Table 6.38 and

Table 6.39). In U251 line we found additionally a statistically significant difference between different SAR intensities. Exposure to 0.5 W/kg led to a significantly lower value as compared to other intensities (Table 6.39)

Table 6.38: U87 glioblastoma cell line under serum free conditions; Results of analysis of variance in FPG Comet assays which were performed under serum free conditions (5 days). Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50, and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/30 | 0.00 | 0.999 | n.s. |
| Exposure | 1/30 | 5.46 | 0.026 | sign. |
| SAR * Exposure | 2/30 | 1.14 | 0.333 | n.s. |

Table 6.39: U251 glioblastoma cell line under serum free conditions; Results of analysis of variance in FPG Comet assays which were performed un-der serum free conditions (5 days). Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure with SAR 0.25, 0.50 and 1.0 W/kg.

| Source of variance | Statist-degrees of freedom DF-1 / DF-2 | Statist. test value F | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/29 | 7.27 | 0.003 | highly sign. |
| Exposure | 1/29 | 5.71 | 0.024 | sign. |
| SAR * Exposure | 2/29 | 0.12 | 0.884 | n.s. |

**Summary of the results of experiments concerning the impact of cellular stress**

In contrast to the results obtained in experiments with serum, we found after serum deprivation also under standard conditions (i.e. without restriction enzymes and oxidative stress by H2O2) an effect with the UMTS signal. This effect was in the U87 line more pronounced as in line U251. In the latter cell type we observed with all SAR values increased DNA damage (% DNA in tail) as compared to sham-exposed cells; however, these differences were statistically not significant and not dose dependent.

Treatment of nuclei with the restriction enzymes (FPG and Endo III) caused in both lines significant effects of exposure but no clear dose dependency was seem, i.e. the effect did not increase with the SAR dose. The findings suggest that oxidized bases are formed more frequently when the cells are exposed to low doses.

### 6.3.6 RECOVERY OF CELLS AFTER SERUM DEPRIVATION AND UMTS EXPOSURE

In the ATHEM-1 project results were obtained in protein analyses which indicated that exposure induced effects disappear after 2 h. Therefore we investigated in the frame of the present study if a recovery phase after exposure leads to disappearance of comet formation.

#### Test principles and methodological overview

In order to address this problem, the cells were cultivated without serum (as described in chapter 6.3.5, and were exposed subsequently with RF-EMF).

The cells were cultivated in these experiments over a period of 5 days.

Two cell lines were used, namely:

- U87
- U251

The exposure duration was 16 h in all experiments

The cells were exposed to an UMTS signal (SAR of 1.0 W/kg).

After the exposure, serum free medium was replaced by serum supplemented medium and the cells were cultivated under these conditions for either:

- 0 h
- 1 h
- 2 h

DNA damage was monitored under standard conditions to find out if single- and double-strand breaks are detectable.

#### Results

The summary of results obtained with both cell lines are depicted in Fig. 6.25. After serum deprivation and UMTS exposure (SAR of 1.0 W/kg) increased DNA damage (%DNA in tail) was observed in both cell lines (U87 and U251). However, after a 1 h of recovery phase (in medium with serum) the extent of DNA damage was clearly reduced. After a two hour recovery phase (with serum) the exposure related effects disappeared completely in both cell lines.

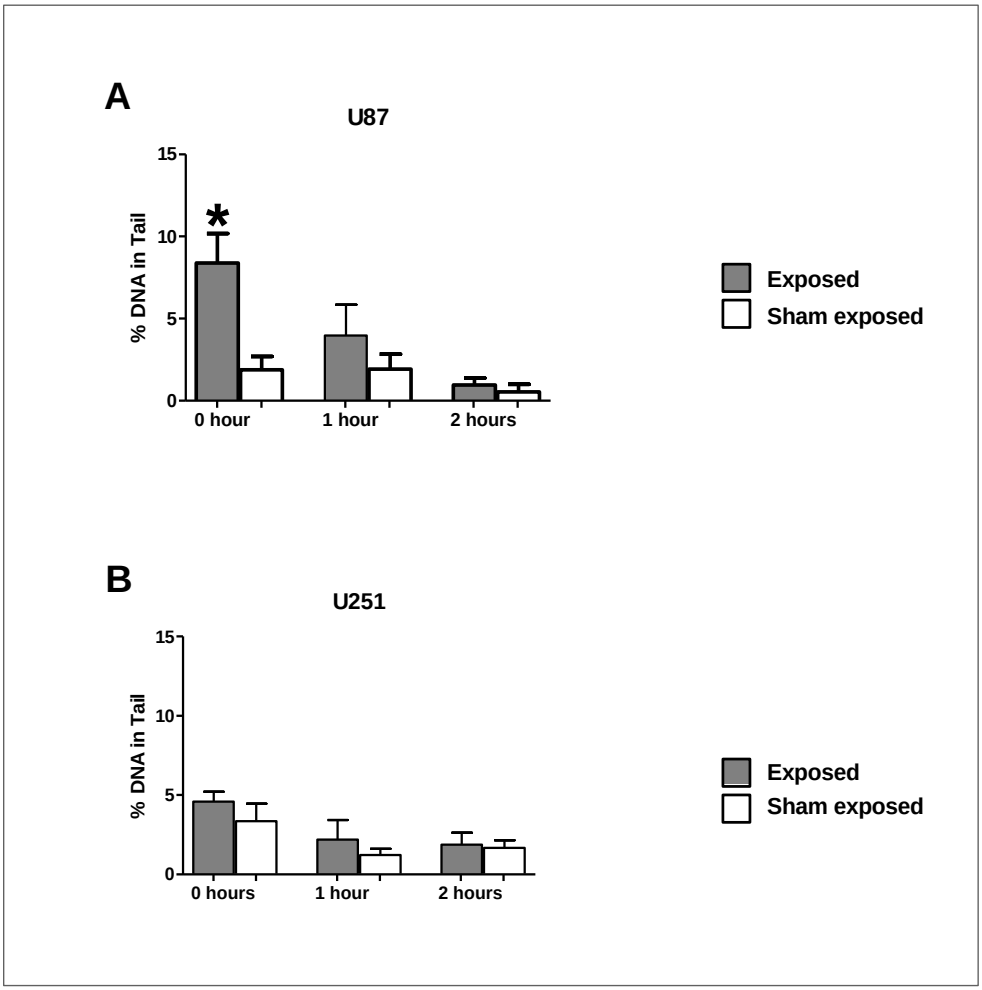



Figure 6.25: Results obtained with the glioblastoma cell lines U87 and U251. SAR 1.0 W/kg UMTS exposure for 16 hours; % DNA in tail in exposed and sham exposed cells after cultivation under serum free conditions (5 days). Bars represent means ±SD of results obtained with three cultures per experimental condition. Statistically significant results are marked with ★ ($p < 0.05$).

As in preceding experiments, also in these experiments a significant exposure effect was detected in line U87 (Table 6.40) while in U251 cells only a trend was observed (Table 6.41).

Table 6.40: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard condition. U87 glioblastoma cell line; after 5 days cultivation without serum; SAR 1.0 W/kg UMTS exposure for 16 hours with a recovery period of 0, 1, or 2 hours.

| Source of variance | DF-1 / DF-2 | F-value | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/48 | 21.852 | <0.001 | highly sign. |
| Exposure | 1/48 | 24.657 | <0.001 | highly sign. |
| SAR * Exposure | 2/48 | 5.637 | 0.019 | sign. |

Table 6.41: Results of analysis of variance in Comet assays for the detection of DNA damage which were performed under standard condition. U251 glioblastoma cell line; after 5 days cultivation without serum; SAR 1.0 W/kg UMTS exposure for 16 hours with a recovery period of 0, 1, or 2 hours.

| Source of variance | DF-1 / DF-2 | F-value | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/48 | 12.697 | 0.001 | highly sign. |
| Exposure | 1/48 | 3.593 | 0.082 | tendency |
| SAR * Exposure | 2/48 | 0.465 | 0.639 | n.s. |

### 6.3.7 UMTS EXPOSURE & BASE- AND NUCLEOTIDE-EXCISION REPAIR (BER AND NER)

Since damage of the genetic material causes mutations which may lead to adverse effects in the organism and also to cell death, a number of different repair mechanisms were developed during the evaluation, which eliminate different types of damage that are formed spontaneously or as a consequence of environmental factors, radiation and cell division. The most important repair systems in eukaryotic cells are base- and nucleotide-excision repair (BER and NER).

Aim of the experimental series was to find out if UMTS radiation affects these DNA-repair systems in glioblastoma cell lines.

#### Strategy

In order to monitor the extent of BER and NER two different lines were used. Specific types of damage were induced in a reporter cell line, which are specifically repaired by one of the two enzyme systems, i.e. either by NER or BER. In the case of NER experiments we used UV radiation (Stratalinker®, Stratagene, La Jolla, CA, USA). For BER experiments reporter cells were treated with the photosensitizing chemical Ro 19-8022 [(R)-1-[(10-chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl)carbonyl]-2-pyrrolidine-methanol] (Hoffman-La Roche, Basel, Switzerland).

The DNA damage was induced in the reporter cells (HepG2), a human derived hepatocellular carcinoma cell line.

Then the nuclei of the reporter cells were isolated and transferred to slides where they were incubated with cytoplasmic proteins of cells (U87 or U251) which had been exposed under defined conditions to UMTS. The subsequent electrophoresis step was conducted under standard conditions. The results of such experiments reflect the formation of comets which are formed as a consequence of migration of fragmented DNA in an electric field. The cytosolic extracts contain DNA repair proteins (enzymes); As mentioned above, they came from cells which were either sham-exposed or exposed with UMTS radiation.

The results which are described in subsequent chapters were obtained in three independent experimental series. For each of them three cultures were made in parallel. Increased comet formation in this experimental system reflects increased BER and NER activities. As a negative control, reaction buffer alone was used and as

positive controls, a specific restriction enzyme (Endo IV, causes DNA fragmentation) was used.

### Impact of radiation on the activity of NER

The results of experiments which were obtained in the Comet assay in which the activity of NER was monitored are summarized in Figure 6.26 and Figure 6.27.

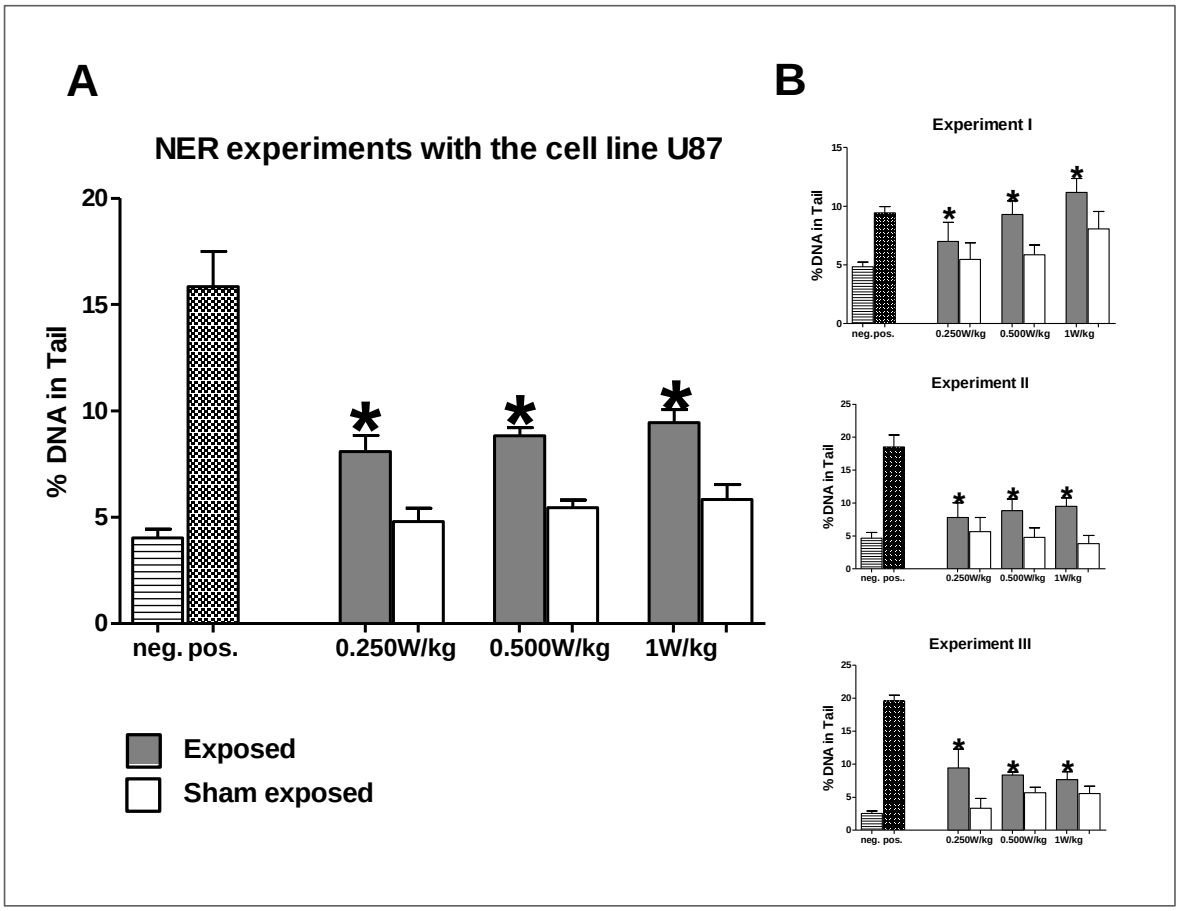



Figure 6.26: Results of the NER measurement with the cell line U87. UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg; % DNA in tail of the reporter cell line (HepG2 cells) after incubation with the exposed or sham exposed glioblastoma cell line U87 (overall result Fig.A and results of three independent experiments Fig.B). Also shown are the results of the negative control (buffer) and positive control (Endo IV treatment). Bars represent means ±SD of results obtained with three cultures per experimental condition (Fig.B). Statistically significant results are marked with ★ ($p < 0.05$).

Figure 6.27 shows the effect of EMF exposure on the activity of NER in U251cells.

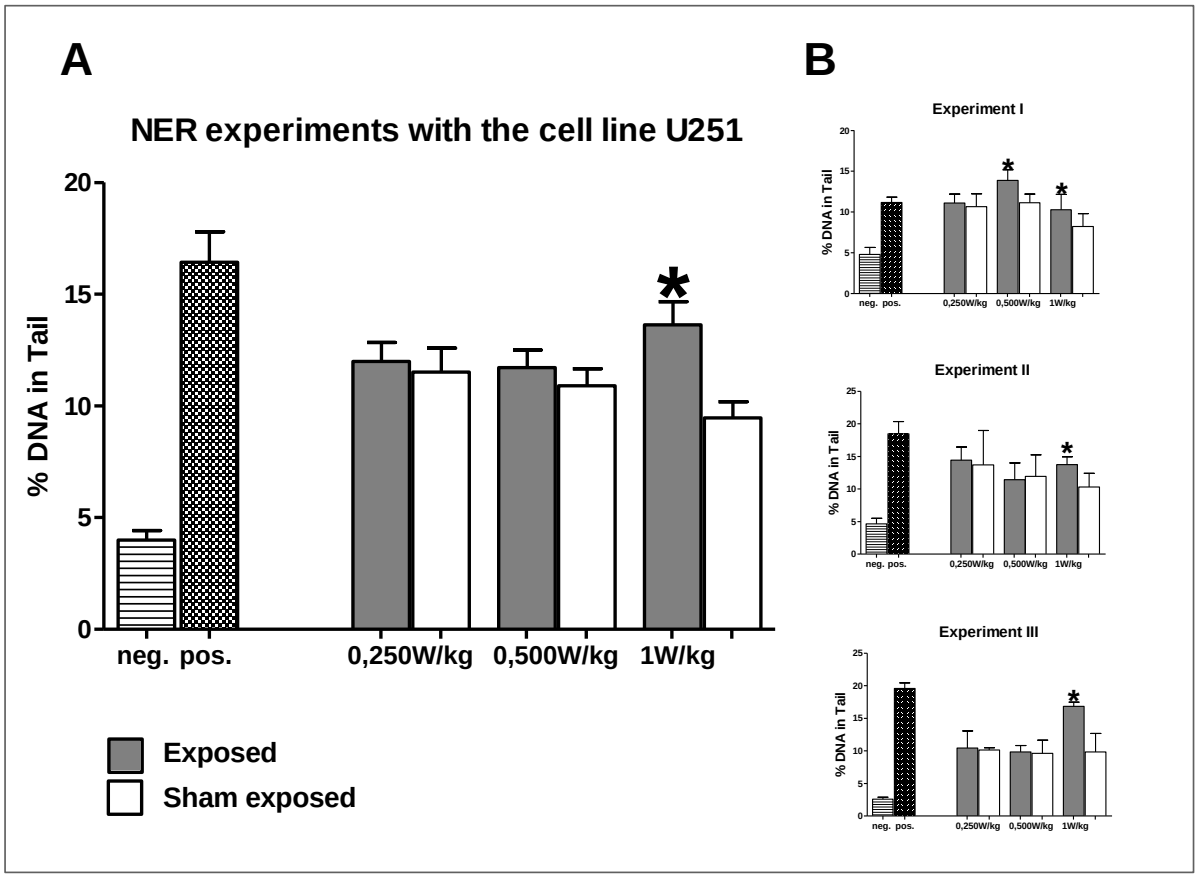



Figure 6.27: Results of the NER measurement with the cell line U251. UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg; % DNA in tail of the reporter cell line (HepG2 cells) after incubation with the exposed or sham exposed glioblastoma cell line U251 (A, overall result; B, results of three independent experiments). Also shown are the results of the negative control (buffer) and positive control (Endo IV treatment). Bars represent means ±SD of results obtained with three cultures per experimental condition (B). Statistically significant results are marked with ★ ($p < 0.05$).

In the experiments with the glioblastoma line U87 a significant increase of the tail intensity was detected with all SAR doses (from 0.25 till 1.0 W/kg, Table 6.42). Also in line U251 an increase of DNA damage was seen in nuclei of the reporter cells after incubation with cytosolic proteins of exposed cells. However, the effect was only with the highest intensity (SAR 1.0 W/kg) statistically significant. Analyses of variance of both cell lines indicated a significant exposure effect (Table 4.43).

Table 6.42: Results of analysis of variance for the NER activity Measurements. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. Subsequently, the reporter cell line HepG2 was treated with the extract of either exposed or alternatively sham exposed glioblastoma cell line U87.

| Source of variance | DF-1 / DF-2 | F-value | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/48 | 2.873 | 0.066 | tendency |
| Exposure | 1/48 | 48.378 | <0.001 | highly sign. |
| SAR * Exposure | 2/48 | 0.088 | 0.916 | n.s. |

Table 6.43: Results of analysis of variance for the NER activity Measurements. Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. Subsequently, the reporter cell line HepG2 was treated with the extract of either exposed or alternatively sham exposed glioblastoma cell line U251.

| Source of variance | DF-1 / DF-2 | F-value | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/48 | 0.092 | 0.913 | n.s. |
| Exposure | 1/48 | 6.479 | 0.014 | sign. |
| SAR * Exposure | 2/48 | 2.416 | 0.100 | n.s. |

### Impact of UMTS EMF-exposure on the activity of BER

The results which were obtained with both cell lines are summarized in Figure 6.28 and Figure 6.29.

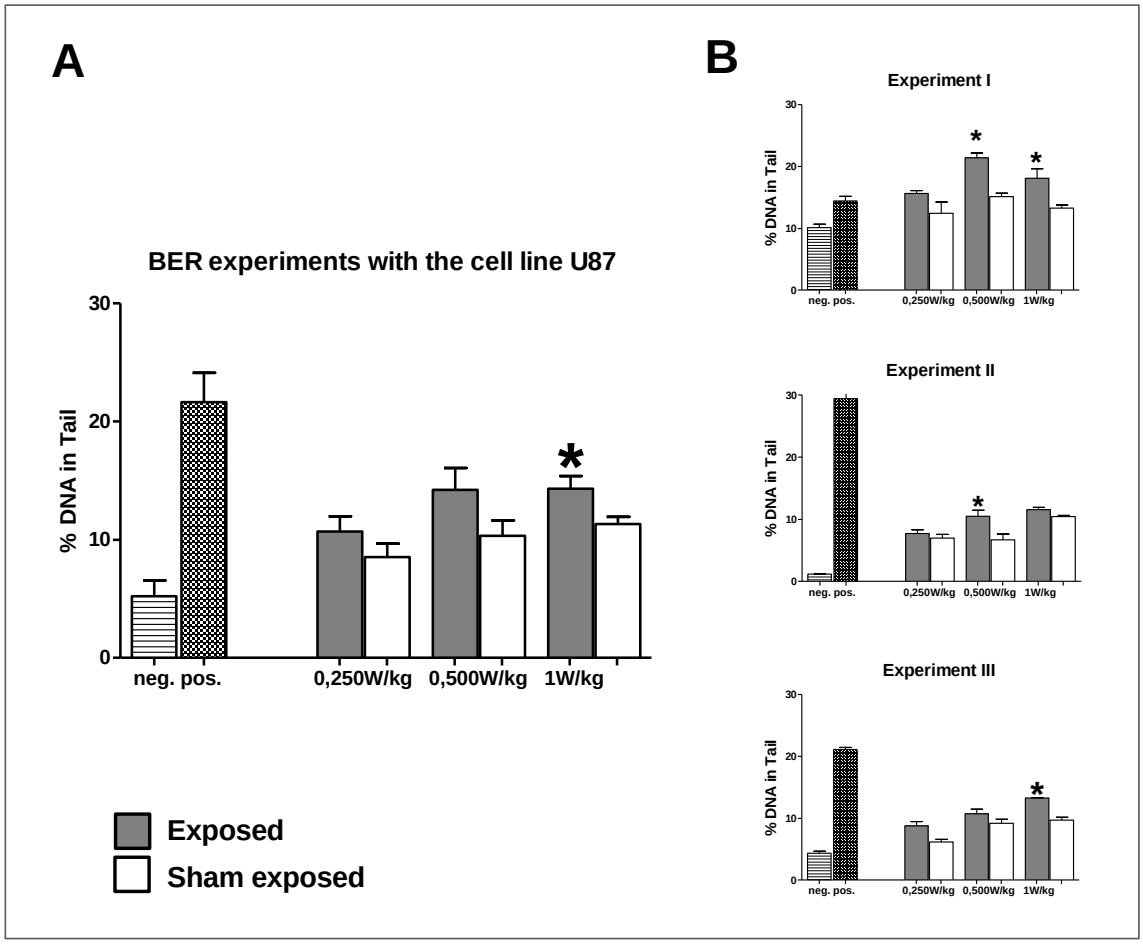



Figure 6.28: Results of the BER measurement with the cell line U87. UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg; % DNA in tail of the reporter cell line (HepG2 cells) after incubation with the exposed or sham exposed glioblastoma cell line U87 (overall result Fig.A and results of three independent experiments Fig.B). Also shown are the results of the negative control (buffer) and positive control (FPG treatment). Bars represent means ±SD of results obtained with three cultures per experimental condition (Fig.B). Statistically significant results are marked with ★ ($p < 0.05$).

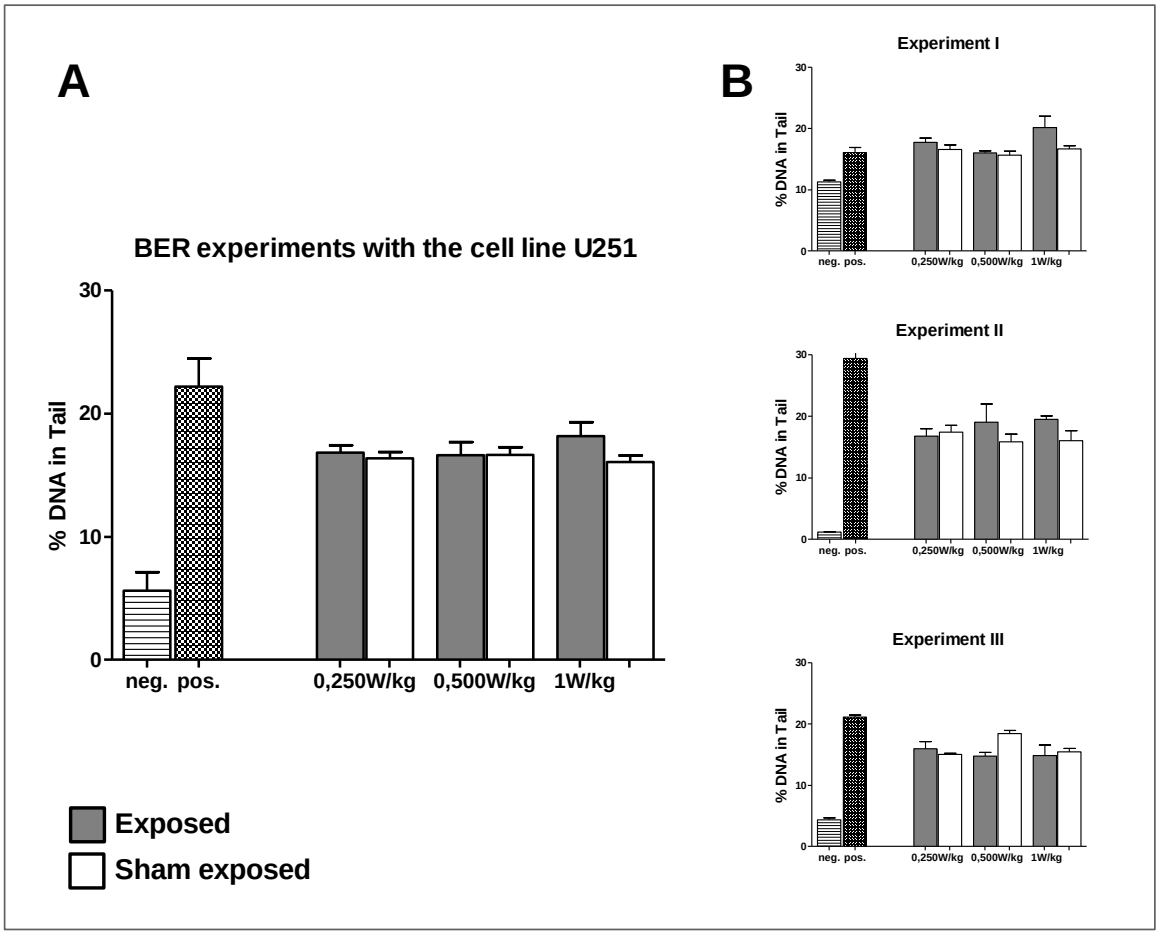



Figure 6.29: Results of the BER measurement with the cell line U251. UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg; % DNA in tail of the reporter cell line (HepG2 cells) after incubation with the exposed or sham exposed glioblastoma cell line U251 (overall result Fig.A and results of three independent experiments Fig.B). Also shown are the results of the negative control (buffer) and positive control (FPG treatment). Bars represent means ±SD of results obtained with three cultures per experimental condition (Fig.B). Statistically significant results are marked with ★ ($p < 0.05$).

The investigation of the influence of radiation on the acitivty of BER indicated a significant exposure effect with a SAR value of 1.0 W/kg in U87 cells (Fig. 6.28). Also analysis of variance indicates that exposure has a significant impact (Table 6.44). We also found a significant dose effect with cellular extracts of U87 glioblastoma cells (Table 6.44).

After exposure of U251 cells at a SAR of 1.0 W/kg a non-significant increase of the tail intensities was found (Figure 6.29).

Table 6.44: Results of analysis of variance for the BER activity. Glioblastoma cell line U87; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. Subsequently, the reporter cell line HepG2 was treated with the extract of the exposed or alternatively sham exposed glioblastoma cell line U87.

| Source of variance | DF-1 / DF-2 | F-value | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/48 | 22.260 | <0.001 | highly sign. |
| Exposure | 1/48 | 25.721 | <0.001 | highly sign. |
| SAR * Exposure | 2/48 | 0.268 | 0.766 | n.s. |

Table 6.45: Results of analysis of variance in for the BER activity. Glioblastoma cell line U251; exposure duration: 16 hours; UMTS exposure to SAR 0.25, 0.50 and 1.0 W/kg. Subsequently, the reporter cell line HepG2 was treated with the extract of the exposed glioblastoma cell line U251.

| Source of variance | DF-1 / DF-2 | F-value | p-value | Significance |
|---|---|---|---|---|
| SAR | 2/48 | 0.388 | 0.680 | n.s. |
| Exposure | 1/48 | 1.257 | 0.267 | n.s. |
| SAR * Exposure | 2/48 | 1.069 | 0.351 | n.s. |

### Summary: UMTS exposure and DNA repair

The experimental series concerning the impact of UMTS exposure (SAR 0.25-1.0 W/kg) yielded clear results which showed that the signal causes induction of repair enzymes in particular of nucleotide excision repair (NER). In U87 glioblastoma cells distinctively stronger effects were observed compared to the cell line U251. In regard to base excision repair (BER), our results indicate exposure dependent induction of this pathway as well. However, the effect was significant in the U-87 cell line and with the highest SAR value only.

### 6.3.8 UMTS EXPOSURE AND THE FORMATION OF MICRONUCLEI

The results which were obtained in the preceding experiments indicate that EMF exposure in particular in serum-free cultivated cells (stressed cells) can lead to DNA damage. However, as described in section 6.3.7, we showed also that the exposure can activate DNA repair. Therefore, it was relevant to investigate if the induction of DNA breaks which was found in the Comet experiments leads to persisting chromosomal damage or if the damage which leads to comet formation is repaired.

For the investigation of chromosomal damage we performed micronucleus (MN) assays with two glioblastoma cell lines:

- U87
- U251

As a positive control we used the cytostatic drug mitomycin C (0.01 µg/ml, Sigma-Aldrich Chemicals Co., USA).[95, 97] The cells were incubated with the drug for 16 h and grown under standard conditions as in the previous experiments (37° C, 5% CO2 atmosphere, 95% humidity).

The exposure was as follows:

- UMTS signal
- SAR three doses:
  - SAR 0.25 W/kg
  - SAR 0.50 W/kg
  - SAR 1.00 W/kg

Two independent exposure experiments and analyses were performed with 16 h exposure to UMTS

**MN assay with the cell lines U87.**

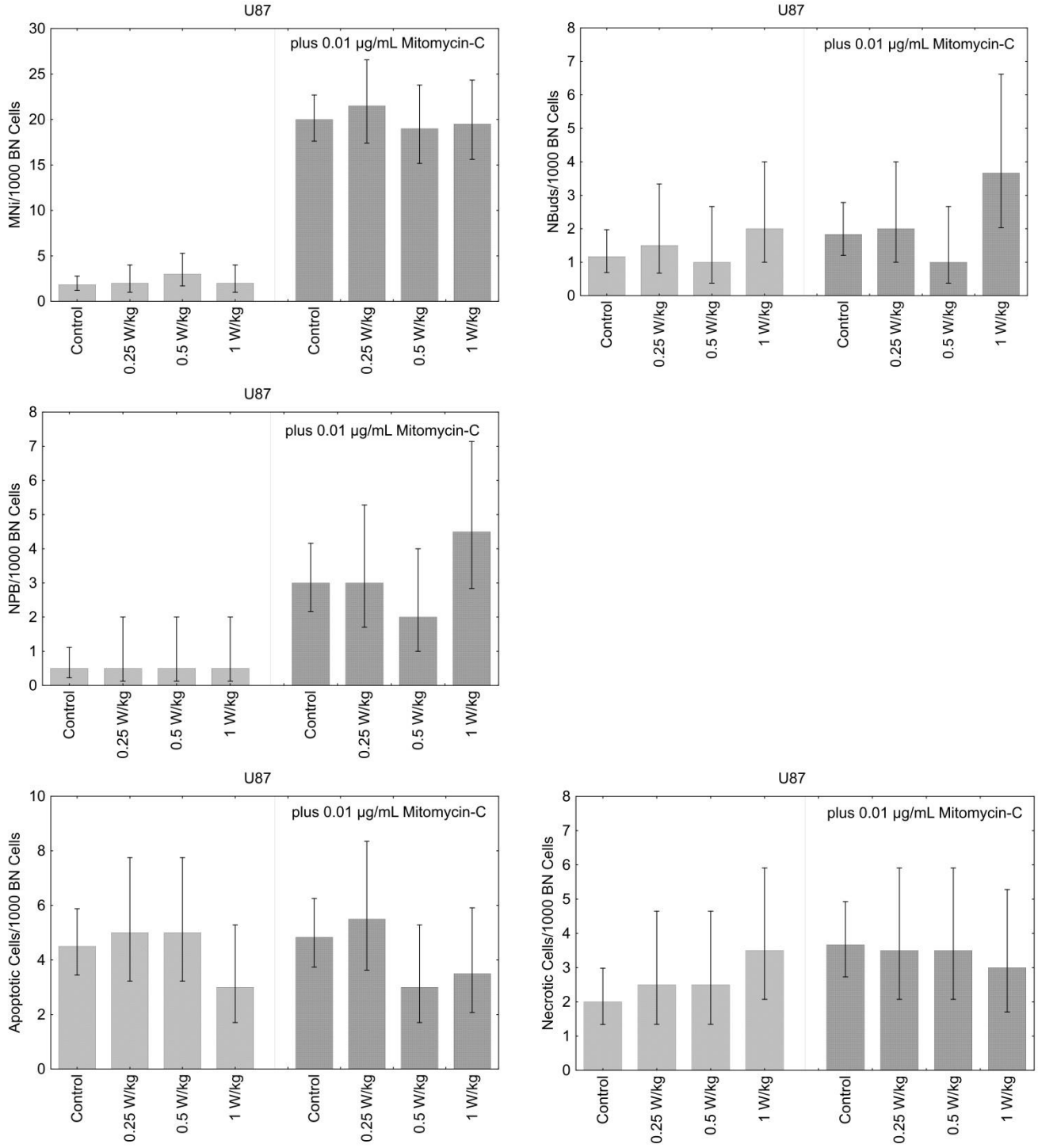



Figure 6.30: Results obtained in MN cytome experiments with the cell line U87. The Y-axis shows the number of cellular abnormalities which were counted per 1.000 cells. Sham exposed cells (control) and exposed cells with a SAR from 0.25, 0.50 to 1.0 W/kg for 16 hours in presence and absence of 0.01 µg/mL mitomycin C (positive control). We analyzed micronuclei in binuclear cells (BiN MN), nuclear buds (NBUDs), nuclear bridges (NPB), apoptotic and necrotic cells.

**MN assay with the cell line U251.**

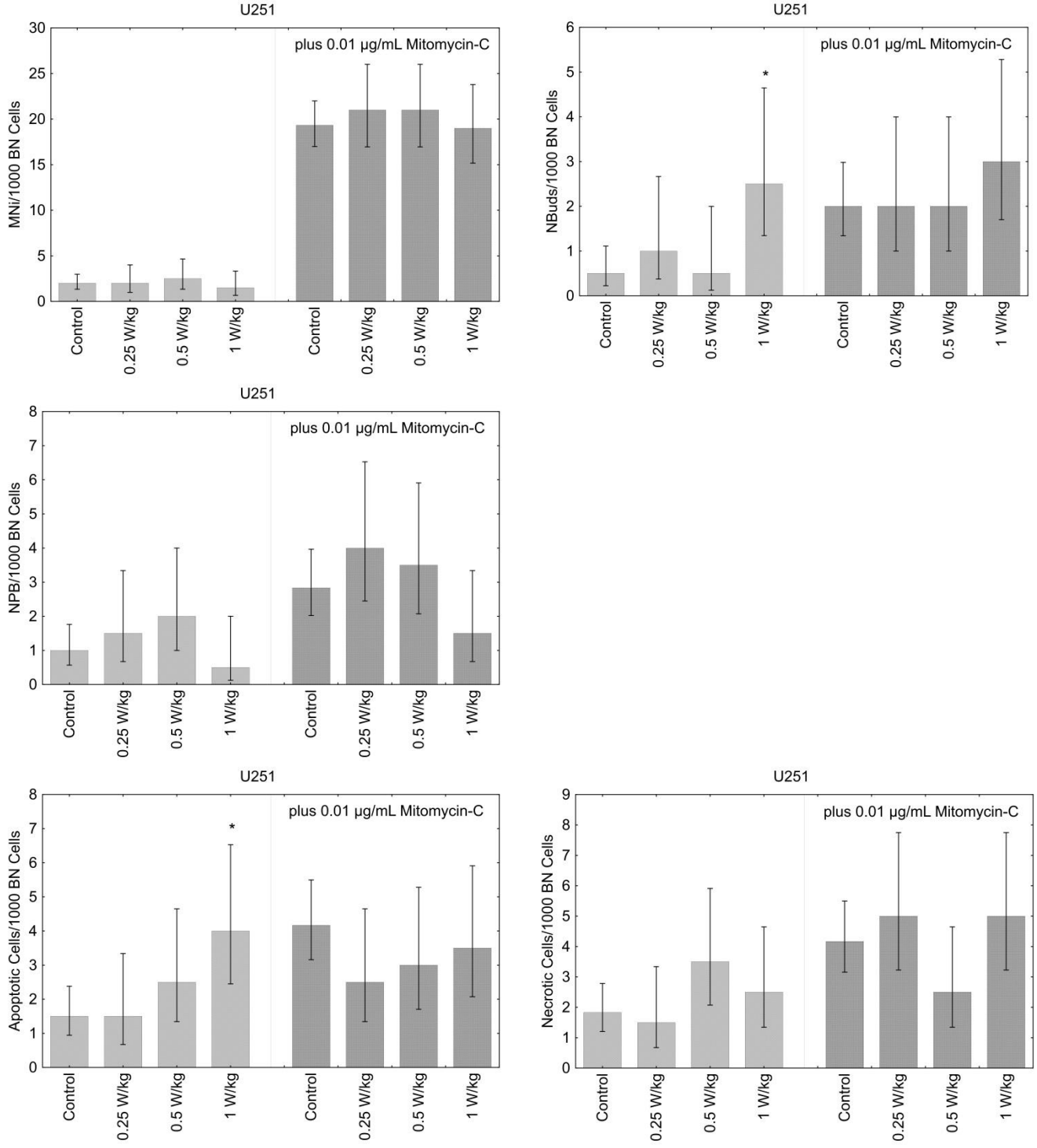



Figure 6.31: Results obtained in MN cytome experiments with the line U251. The Y-axis shows the number of cellular abnormalities which were counted per 1.000 cells. Sham exposed cells (control) and exposed cells with a SAR from 0.25, 0.50 to 1.0 W/kg exposure for 16 hours in presence and absence of 0.01 µg/mL mitomycin C (positive control). We analyzed micronuclei in binuclear cells (BiN MN), nuclear buds (NBUDs), nuclear bridges (NPB), apoptotic and necrotic cells. Statistically significant results are marked ★ ($p < 0.05$).

For the statistical evaluation of these experiments we used, in contrast to the Comet assay experiments, a general linear model (GLM) for Poisson-distributed variables and a log-link (i.e. we assumed that the logarithm of the parameter of the Poisson distribution is a linear function of independent variables).

The analysis indicates that no differences were found between UMTS exposed and non-exposed cells with two exceptions (Table 6.46). The number of apoptotic cells was significantly increased in the cell line U251 at the highest dose (Figure 6.31). Furthermore we saw also an increase of cells with Nbuds.

Table 6.46: Results obtained in MN cytome experiments with two glioblastoma cell lines U87 and U251; 16 hours UMTS exposure (SAR 0.25. 0.5 and 1.0 W/kg); in presence and absence of 0.01 µg / mL mitomycin C (as a positive control). We analyzed micronuclei in binuclear cells (BiN MN), nuclear buds (NBUDs), nuclear bridges (NPB), apoptotic and necrotic cells. For the statistical evaluation of these experiments a general linear model (GLM) for Poisson-distributed variables and a log-link was used.

| Cell line | Mitomycin-C | Endpoint | Wald Chi² (FG=3) | p-value | Significance |
|---|---|---|---|---|---|
| U87 | without | Necrotic cells | 2.77 | 0.428 | n.s. |
| U87 | with | Necrotic cells | 0.38 | 0.944 | n.s. |
| U251 | without | Necrotic cells | 4.75 | 0.191 | n.s. |
| U251 | with | Necrotic cells | 3.89 | 0.273 | n.s. |
| U87 | without | Apoptotic cells | 2.41 | 0.491 | n.s. |
| U87 | with | Apoptotic cells | 4.04 | 0.257 | n.s. |
| U251 | without | Apoptotic cells | 9.35 | 0.025 | sign. |
| U251 | with | Apoptotic cells | 2.79 | 0.424 | n.s. |
| U87 | without | BIN MN | 1.97 | 0.580 | n.s. |
| U87 | with | BIN MN | 0.70 | 0.873 | n.s. |
| U251 | without | BIN MN | 0.99 | 0.804 | n.s. |
| U251 | with | BIN MN | 0.84 | 0.840 | n.s. |
| U87 | without | NPB | 0.00 | 1.000 | n.s. |
| U87 | with | NPB | 4.05 | 0.256 | n.s. |
| U251 | without | NPB | 4.24 | 0.237 | n.s. |
| U251 | with | NPB | 4.64 | 0.200 | n.s. |
| U87 | without | NBUDs | 1.95 | 0.582 | n.s. |
| U87 | with | NBUDs | 6.04 | 0.110 | n.s. |
| U251 | without | NBUDs | 11.64 | 0.009 | highly sign. |
| U251 | with | NBUDs | 1.52 | 0.678 | n.s. |

## 6.4 SUMMARY OF THE RESULTS OF THE IN VITRO GENOTOXICITY TESTS

This project-part focussed on in vitro experiments in order to verify or refute findings which were obtained in the ATHEM-1 project. We found in the first experimental series that sensitive and less sensitive cells lines exist and investigated the molecular mechanisms which lead to damage of the genetic material in sensitive cell types.

The central questions were: “How can weak electromagnetic fields lead to DNA breaks?” and “Which role can be attributed to cellular DNA repair mechanisms?”

The method which was used in most experiments is the so-called “Comet assay”. According to different questions we modified the protocol of this procedure. To clarify if oxidized DNA bases are formed, we used lesion specific enzymes (FGP and Endo III); to find out if the radiation increases sensitivity to reactive oxygen species (ROS), we treated the cells with hydrogen peroxide (H2O2) after UMTS exposure. For the BER and NER experiments nuclei of a reporter cell line were damaged with NER/BER specific factors. All Comet assay experiments were analyzed with a computer-aided image analysis system.

On the basis of the results of the first experimental series which indicated that the glioblastome cell line U87 is possibly sensitive to UMTS exposure, we performed further studies with three additional brain derived cell lines and found that two of them had no increased sensitivity towards RF-EMF exposure.

To answer the question whether the sensitivity of the cells depends on the duration of exposure, we conducted additional series of experiments which addressed this question. In our experiments 6 h exposure did not cause significant DNA damage. After serum deprivation, which leads to cell cycle arrest, we found increased sensitivity of the cells towards UMTS exposure. However, after a recovery period of 1 h the extent of DNA strand breaks declined and after a 2 h recovery the effect disappeared completely.

In order to find out if exposure leads to micronuclei (MN) formations (which are a consequence of structural and numerical chromosomal aberrations), we conducted additional MN cytome experiments and found that the exposure of cells for 16 h does not lead to persistent DNA damage.

In conclusion, we found in this project, that some cell lines are insensitive to RF-EMF. However, we detected also a number of sensitive cell lines in which DNA damage could be detected after certain RF-EMF exposures. Further experiments with the most sensitive cell types indicated that oxidation of DNA bases may play a role. It is also notable that in UMTS-exposed cells clear evidence of induction of DNA repair processes was found.

# 7 COORDINATOR'S PROJECT SUMMARY

by Wilhelm Mosgoeller

The ATHEM-2 project systematically investigated cellular mechanisms that are typical of cells that are sensitive to RF-EMF exposure. As in the previous project (ATHEM-1) we found both sensitive and insensitive cells. The sensitive cells were investigated in more detail in order to find cellular mechanisms underlying the formation of exposure-associated DNA lesions.

Two mechanisms appear to be important: 1) oxidative DNA damage, and 2) stiumulation of production of proteins involved in the cellular DNA repair.

## 7.1 EXPOSURE SYSTEM FOR TESTING HUMANS

In order to be able to relate the results of *in vitro* experiments to observations with human subjects, the same RF-EMF signal (UMTS) was utilized for both *in vitro* and *in vivo* investigations. The system for double blinded human exposure was developed and built in the Seibersdorf Laboratories (chapter 3). This system enabled objective research in accordance with the highest possible scientific standards, including prospective, randomized, controlled, and double blind exposure and data analysis. Exposure conditions were withheld from both volunteers and investigators during exposure, data processing and analysis.

The exposure system for volunteer testing was designed to allow a precise exposure of buccal epithelial cells inside the mouth. During these experiments the exposure intensity for the buccal cells was comparable to the everyday situation, i.e. head exposure during a mobile phone call with the mobile phone positioned next to the ear. The comparison of cells harvested from the left and right buccal mucosa, before and after exposure, allowed an analysis of the effect of controlled electromagnetic field exposure upon this tissue.

The exposure system exhibited a high degree of dosimetric reliability. The antennae (signal source) were validated and anatomical models were used to calculate and define signal strength.

## 7.2 COGNITIVE EFFECTS

Exposure of the volunteers' buccal cells to defined UMTS fields was inevitably associated with an exposure of the basal areas of the brain, though at lower intensities. Each session, therefore, included an evaluation of neural functions and cognitive effects of RF-EMF exposure using standard tests. The volunteers performed simple and complex reaction tests in order to assess awareness, vigilance, and memory functions. Each volunteer, moreover, provided a subjective self-rating of his/her actual well-being at the end of the session. Data analysis focussed on reaction time and decision accuracy during exposure.

Our findings confirm effects already described in the ATHEM-1 project report. Whilst reaction times tend to shorten during RF-EMF exposure, this was, however, accompanied by an increased error rate.

Reduced reaction time during exposure to electromagnetic fields has been described many times in various peer-reviewed publications, and could even be deemed a beneficial exposure-related effect. That said, the increased incidence of mistakes during exposure corroborates the recommendation not to use mobile phones whilst driving, or performing other tasks requiring full attention.

### 7.3 GENO- AND CYTOTOXIC EFFECTS

Buccal mucosal samples for laboratory analysis were collected from volunteers prior to, and following RF-EMF exposure. The level of buccal mucosa exposure during normal mobile phone use is unknown. For the project in question, exposure was designed to be comparable to that around the ear during a phone call. Exposure levels were well below limiting values for RF-EMF that are designed to prevent thermal effects.

The cytogenetic and cytotoxic effects of RF-EMF exposure were investigated using cell samples taken before and after exposure. During laboratory analysis, neither the test person nor the investigators knew whether the left or right side of the mouth (buccal mucosa) was exposed or sham-exposed. Cell sample differences before and after exposure on the sham-exposed side reflect the natural variability of the analysed endpoints. Systematic differences between left and right, pre- and post-exposure samples on the exposed side indicate RF-EMF exposure-related effects.

Field studies, by their nature, suffer from various methodological problems that can be overcome by conducting investigations under more controlled conditions. One methodological shortcoming of field studies is the difficulty of determining exposure intensities under everyday life conditions. In field studies, the exposure intensity can always be only a rough estimate, based as it is on asking the study participants about the type of mobile phone used by them and how they actually use (hold) it.

The present subproject was, in contrast, designed as a prospective, randomized and controlled cohort study, using data from published field studies to calculate the study power and cohort size.

Volunteer diaries provided a further analysis endpoint. Information on daily phone usage (i.e. frequency, side of the head predominantely used during calls) allowed for the analysis of cumulative effects of RF-EMF exposure. The coincidental experimental exposure on the same side the subject (ipsilateral side) normally uses the phone, coulde be associated with more frequent cellular changes than on the contralateral side, particularly for the heaviest users of mobile phones.

Cytogenetic and cytotoxic investigations both revealed *in vivo* exposure-related effects. The observed correlation between exposure-related effects and side and frequency of normal phone usage, is consistent with a time-dependent accumulation

of exposure-related cellular effects. However, at present this is a preliminary finding warranting further testing in a study tailored to address it more specifically.

## 7.4 DNA-LESIONS AND CELLULAR MECHANISMS

The largest subproject focused on cytogenetic changes and genotoxicologic effects in cells caused by *in vitro* RF-EMF exposure. Induction of DNA lesions by exposure to a high frequency electromagnetic field at athermal intensities was widely reported prior to the start of the present project.[9, 10]

### 7.4.1 RF-EMF-SENSITIVE AND INSENSITIVE CELLS

Exposure-related DNA lesions have been observed both in cultured cells *(in vitro)* and in laboratory animals (*in vivo)*.[98, 99] Based on ATHEM-1 project findings, we reported the existence of sensitive and insensitive cells with respect to vulnerability to RF-EMF-induced DNA damage.[6]

In an attempt to confirm this key ATHEM-1 project result, five cell lines from different tissues were investigated *in vitro*. While we found a lower level of exposure-associated DNA lesions than in previous investigations,[6] we nonetheless confirmed the central finding that cells vary in their sensitivity to RF-EMF (section 6.3.1). The sensitive cells included a glioblastoma cell line. Interestingly this result was reproduced with a further glioblastoma cell line.

The observation of both sensitive and insensitive cell types in the very same project can end a long debate. Positive and negative findings were frequently considered as inconsistent result while the interpretation that different cell lines can react differently to RF-EMF exposure received too little attention so far.

### 7.4.2 LATENCY TIME

The *in vitro* experiments revealed that formation of DNA lesions is subject to a latency time. RF-EMF exposure-related effects were not observed after the shortest applied exposure (6 hours) but longer exposures can be associated with significant levels of DNA lesions.

This latency time (time interval between start of exposure and occurrence of DNA lesions) indicates that DNA lesions induced by electromagnetic field exposure can be attributed to a cellular mechanism other than that responsible for the effects of e.g. radioactive exposure. The observation of a latency time can, thus, explain so-called “contradictory findings” of earlier studies which only investigated short exposure duration. Published latency time following RF-EMF exposure vary according to the experimental system used (20 minutes,[9] 2 or 4 hours,[7] and 16 hours[8]). Our current results lie within the reported range.

### 7.4.3 OXIDATIVE DNA LESIONS

Experiments designed to elucidate underlying cellular mechanisms clearly revealed an increased level of oxidative DNA lesions caused by high frequency electromagnetic fields in only a subset of the cell lines studied. Oxidized DNA is a well-known precursor

to DNA strand breaks. Together with the results of a plethora of published investigations, this finding provides strong support for an indirect mechanism of RF-EMF exposure-related DNA breaks. Oxidation renders DNA strands “brittle”. The occurrence of oxidative changes in cells following EMF exposure has been extensively reviewed by Yakymenko, et al. [100] 93 of one hundred investigations of oxidative effects related to electromagnetic field exposure in the athermal (low dose) range, reported an effect. These observed oxidative changes can explain how fields that are too weak to break chemical bonds in DNA can nevertheless cause an increased level of DNA strand breaks in sensitive cells.

### 7.4.4 DOSE-RESPONSE RELATIONSHIP

We did not succeed in finding an intensity threshold for the biological activity of electromagnetic fields, i.e. a so-called NOEL (No Observed Effect Level), and were, thus, unable to define an EMF exposure intensity not leading to DNA lesion formation in sensitive cells. In some cases a low dose was, in fact, observed to cause stronger effects than a higher dose. This pattern was, however, not consistently observed so that the present results can not contribute to the establishment of a clear dose-response relationship.

### 7.4.5 ROLE OF CELLULAR ACTIVITY

In order to investigate the role of metabolic cellular activity, as well as the effects of combined exposure, cultured cells were maintained in serum-free medium, as is commonly performed to model cell stress *in vitro*. A serum-free growth medium "forces" cells to synthesize factors which, under normal culture conditions, are provided by the medium, and, thereby, increases metabolic activity. Cells were pre-stressed in this manner and then exposed to RF-EMF.

Induction of cell stress prior to exposure to RF-EMF at different SAR intensities, was consistently observed to increase sensitivity to RF-EMF exposure. Of 36 individual experiments performed, 34 revealed a higher level of DNA lesions in the exposed group. We previously showed a correlation between cell activation and sensitivity to RF-EMF exposure in the course of the ATHEM-1 project. Whilst inactive lymphocytes failed to exhibit any EMF exposure-related effects, a slight increase in the rate of protein synthesis was observed in EMF-exposed activated lymphocytes.[7].

Given that RF-EMF exposure-related effects may accumulate with time, the finding that metabolically active cells are more sensitive than less active cells to RF-EMF exposure, is most relevant for children and teenagers, simply because their bodies contain a higher proportion of metabolically active cells and because they can naturally be expected to live longer than older persons. Young persons, thus, stand to benefit most from the preventative measures described in chapter 8.

### 7.4.6 OTHER SYNERGISTIC EFFECTS

The first experiments to reveal DNA lesions following RF-EMF exposure in 1995 and 1996,[3, 4] and which considered specific replication studies,[101] in retrospect, provide

an early hint that certain narcotics can synergise with RF-EMF-exposure to increase the level of DNA lesions in laboratory animals.

Further such synergisms are, moreover, evident. The work of Tillmann, et al. [102] for example, revealed increased effects of the carcinogen N-ethyl-N-nitrosourea (ENU) in laboratory animals exposed to 4.8 W/m² RF-EMF (whole body UMTS-signal), a finding that has, moreover, been independently reproduced.[103]

This mechanistic principle is further corroborated by the aforementioned *in vitro* stress model. Cell stress synergises with RF-EMF to increase the rate of DNA lesion formation which may, in turn, be indicative of increased risk.

### 7.4.7 RECOVERY TIME AFTER END OF EXPOSURE

A report from Franzellitti, et al. [8] describing repair of exposure-related DNA lesions within 2 hours accords well with an ATHEM-1 project finding, namely that exposure-associated protein changes were no longer detectable two hours after EMF exposure.[7] The investigation, within the framework of ATHEM-2, of the persistence of DNA lesions following cessation of exposure, revealed a disappearance of these lesions within a comparable time frame.

### 7.4.8 ROLE OF DNA REPAIR

To investigate the influence of RF-EMF (UMTS signals) on DNA repair mechanisms in glioblastoma cell lines, we conducted experiments involving two cell lines. DNA damage was elicited in the first cell line (reporter cells) using controlled conditions, whilst protein extracts were prepared from the cells of interest following exposure to defined UMTS signals. The DNA repair protein-containing protein extract from RF-EMF-exposed cells was then applied to the reporter cells (containing pre-damaged DNA). Fragmented DNA determined by standard comet assay directly reflected the level of repair enzymes in sham-exposed and RF-EMF-exposed cells.

The reproducibly elevated DNA repair enzyme levels in RF-EMF exposed cells indicated the activation of DNA repair protein synthesis by RF-EMF exposure. Protein synthesis is generally activated in cells which sense an increased level of DNA lesions. The exposure-related increase reported here therefore provides further evidence that RF-EMF exposure caused DNA lesion formation. The activation of the repair system described in Chapter 6 would not have occurred in the absence of DNA lesions.

We subsequently investigated if exposure-related DNA damage can be repaired or becomes permanent instead. Can the activated repair system repair exposure-induced DNA lesions before they give rise to so-called epigenetic changes such as chromosome damage as an indicator of permanent DNA damage?

The so-called micronucleus assay was used to investigate chromosomal fragments as surrogates of irreparable DNA lesions. Two glioblastoma cell lines were exposed (and sham-exposed) to three different signal EMF intensities for 16 hours. With few exceptions, no significant differences were found between exposed and sham-exposed cells, implying that most exposure-related DNA-lesions were repairable, and that a single acute exposure may not produce measurable permanent DNA damage.

In the context with the observed evidence for accumulation of permanent DNA damage even minor effects on the DNA are relevant, they underpin recommendations calling for a minimization of personal exposure, especially for younger persons with a higher life expectancy.

## 7.5 SUMMARY AND OUTLOOK

The present project revealed various significant effects of RF-EMF (UMTS) exposure on cognitive endpoints and in experiments on human cells. We, furthermore, identified cellular mechanisms which plausibly confirm and account for exposure-related DNA lesions. The most important observations were:

1. Exposure to high frequency electromagnetic fields can increase the rate of formation of oxidative DNA lesions
2. RF-EMF exposure can trigger highly specific cellular repair mechanisms

Experiments with human volunteers revealed that RF-EMF exposure impacts the integrity of buccal epithelial cells. These changes were, moreover, more pronounced in persons who reported the highest levels of cellular phone use, which is consistent with an accumulation of exposure-related cellular changes over time.

Measures to reduce the possible risks of RF-EMF exposure are discussed in the following chapter.

# 8 CONCLUDING REMARKS AND PREVENTIVE MEASURES

by Hamid Molla-Djafari[4]

## 8.1 PROTECTIVE AND PREVENTATIVE MEASURES IN ELECTROMAGNETIC FIELDS

Protective and preventative measures in electromagnetic fields (EMF) in the RF frequency range can be divided into two categories of effects upon biological tissues, namely "thermal", i.e. heating-related effects and "athermal" effects. These measures are valid for RF exposure in general, and are not only applicable to mobile communications. These measures comprise 1.) generic protective measures to avoid any harm induced by heating, and 2.) preventative measures taking into account possible adverse health effects caused by EMF-exposure related athermal phenomena.

In both cases, the three most important aspects of protection are: Distance, Duration of Exposure, and Shielding.

## 8.2 PROTECTION IN ELECTROMAGNETIC FIELDS

### 8.2.1 MEASURES FOR EXPOSED PERSONS (GENERAL PUBLIC AND OCCUPATIONAL EXPOSURE)

The preferred protective measure is avoidance of any exposure whatsoever. Since, this is, however, rarely possible, consideration must be given to the three factors, *Distance, Duration of Exposure, and Shielding*.

The distance from the EM radiation source should be as great as possible because field strength decreases quickly with increasing distance (according to the squared or cubed inverse of the distance, depending on source type and geometry).

The duration of exposure should be minimized by reducing the time spent in the exposed area, or by switching off the source. If such measures are insufficient, or cannot be realized, shielding can reduce exposure. It may be applied directly to the source (preferable), or around the exposed persons. Personal protective equipment that reduces EMF intensity as in a Faraday cage is available for use in certain situations. For room or source shielding the choice of shielding material is critical and to be selected according to the prevailing EMF frequency. Highly conductive metals such as copper or aluminium provide good field damping in the RF frequency range, but are largely ineffective at low frequencies. Materials with a high magnetic permeability must be used instead in such cases.

Persons carrying active implanted medical devices (AIMD, for example a cardiac pacemaker) require special attention, and exposure evaluation must take into account the information provided by the manufacturer. Pacemaker wearers should also avoid contact with charged objects.

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### 8.2.2 MEASURES FOR MANUFACTURERS

The emitted power of electromagnetic fields can be reduced by careful engineering, for example through optimization of electrodes and antennae, shielding of power supply lines, etc. Organisational measures, in particular restricted source access, can also reduce the emission level.

### 8.2.3 MEASURES FOR FACILITY OPERATORS

RF facility and device operators can contribute to a significant emission reduction by optimization the RF-EMF source (transmitter), by reduction of emitted power, or by tuning the angle of emission. Such technical measures take priority over organizational measures.

Organizational measures for operators of RF transmitters comprise:

- increase distance between transmitter and exposed persons
- post signs and pictograms to warn persons with AIMD (active implanted medical devices) like cardiac pacemakers, readily visible directly at the EMF-source, or at the entrance to the area
- avoid exposure of workers with AIMD (e.g. pacemakers) to intense EMF sources
- restrict access to areas of intense EMF exposure
- instruct employees about EMF-related health risks

Technical measures (for RF transmitters only) comprise:

- limitation of emitted power to the lowest necessary level
- adjustment/optimization of the direction of emission
- adjustment of antenna gain
- adjustment of the angle of emission
- adjustment of transmitter elevation

If the above measures fail to provide an adequate reduction of emission appropriate shielding is required.

## 8.3 TIPS FOR MOBILE PHONE USERS

1. Keep mobile phone away from your head: use the speaker, a head-set, or Bluetooth® hands-free set.
2. Choose a phone with a low SAR value and radiation connect factor (more info at www.handywerte.de, or www.bfs.de/bfs).
3. Keep your phone in a bag. Do not wear it directly on the body, especially if you are on the move (e.g. travelling by public transport, etc.).
4. In the car: activate the speaker, use a Bluetooth headset, or, better still, use the mobile phone with an external antenna.
5. Use the phone primarily in areas with good signal (e.g. not in the basement or in an elevator).
6. Refrain from extremely long conversations. Athermal effects only appear after a long exposure.
7. For long conversations, use a landline.
8. While waiting for the connection, do not hold your phone to the head.
9. Write text messages instead of calling.
10. Take a break after a long mobile phone conversation. Athermal effects decrease after the end of the exposure.

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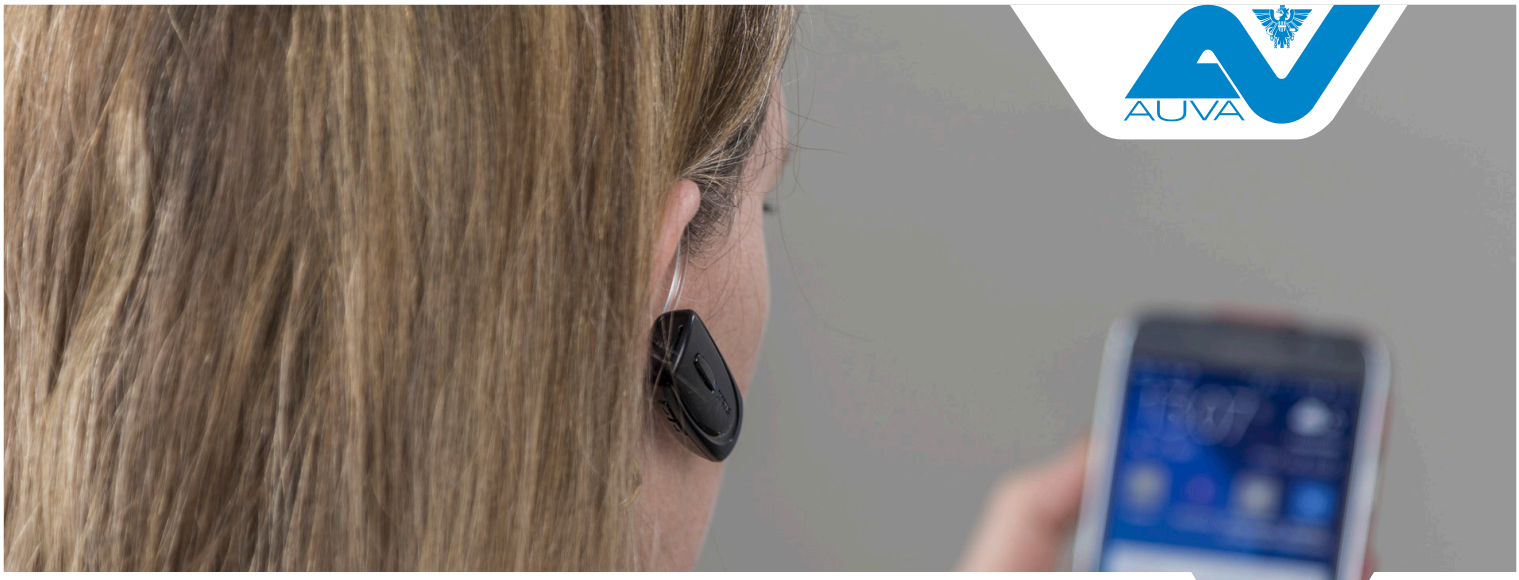

# ATHEM 2
# Athermal effects of electromagnetic field exposure associated with mobile communications

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