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to my wonderful mother, for her measureless love and support.

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TABLE OF CONTENTS

LIST OF FIGURES	V
LIST OF TABLES	VI
LIST OF ABBREVIATIONS	VIII
1. Introduction	1
1.1. Does exposure to mobile phone pose long term health hazard in humans: the current state of knowledge	2
1.2. Cell phone specific EMF and SAR values	2
1.3. Biological effects	5
1.4. Result of genotoxicity studies	6
1.4.1. Endpoints in genotoxic experiments.....	6
1.4.2. Results of experiments with primary cell lines.....	7
1.4.3. Genotoxicity experiments with laboratory rodents.....	12
1.5. Do mobile phone specific EMF cause genetic damage in humans?	13
1.6. Results of carcinogenicity studies	14
1.6.1. Results of carcinogenicity studies with rodents	14
1.6.2. Results of carcinogenicity studies with humans	16
1.7. Classification of the carcinogenic risk caused by mobile phone specific electromagnetic fields by the IARC	17

1.8. Design of the present study.....	18
1.9. The comet assay	19
1.9.1. Calibration of the SCGE assay.....	20
1.9.2. Determination of the cell vitality.....	20
1.9.3. Use of comet assay in genetic toxicology	20
1.9.4. Quantification of the comet formation.....	21
1.10. The exposure apparatus	21
2. Material and Methods	25
2.1. Chemicals.....	25
2.2. Reagents for SCGE assays.....	26
2.3. Preparation of slides for the single cell gel electrophoresis assays (SCGE)	27
2.4. Cell culture and cultivation.....	27
2.5. Determination of the mitotic activity	31
2.6. The comet assay (general scheme)	31
2.6.1. SCGE experiments under standard conditions	31
2.6.2. SCGE experiments with lesion specific enzyme	32
2.6.3. SCGE with hydrogen peroxide treatment.....	32
2.6.4. Electrophoresis and analyzing	33
2.7. Experiments with RF-exposure	36
2.7.1. Determination of the acute toxicity	36

2.7.2. UMTS exposure	36
2.7.3 The cryopreservation process for the most sensitive cell lines	37
3. Results	38
3.1. Determination of the mitotic activities	38
3.2. Calibration experiments.....	40
3.2.1. Experiments with hydrogen peroxide	40
3.2.2. Impact of treatment of isolated nuclei with different dilution of restriction enzymes.....	42
3.2.3. Impact of deep freezing (cryopreservation) on the sensitivity of the indicator cells.....	42
3.3. Investigation of the genotoxic effects caused by RF exposure.....	45
3.3.1. Sensitivity of the different cell lines to 1.0 W/kg	45
3.3.2. Dose response effects of mobile phone specific RF exposure.....	48
4. Discussion.....	51
4.1. Background values of the untreated controls	51
4.2. Results of SCGE assays after RF-exposure	53
4.2.1. Overview of earlier in vitro experiments with mammalian cell lines.....	53
4.2.2. Results of earlier SCGE studies.....	55
4.2.3. Induction of DNA damage in the glioblastoma line U-87	56
4.3. Conclusions and outlook	58

5. Abstract	X
6. Zusammenfasssung	XII
7. References.....	XIV
8. Appendix.....	XX

LIST OF FIGURES

Figure 1.	Electromagnetic spectrum.....	3
Figure 2.	Use of different indicator cell lines in vitro experiments with cell phone specific EMF.	10
Figure 3.	Endpoints which were used in vitro experiments with cell phone specific EMF.....	10
Figure 4.	The sXc1950 installed system..	23
Figure 5.	Mechanical and electronic design of the UMTS exposure system	24
Figure 6.	Morphology of the different indicator cell lines	30
Figure 7.	Schematic illustration of the comet assay.	35
Figure 8.	Double blind protocol of the exposure apparatus.....	37
Figure 9.	The Growth kinetics of the different cell lines.....	39
Figure 10.	The DNA migration after exposure to different concentrations of H ₂ O ₂	41
Figure 11.	Results of comet assay with three dilutions of the restriction enzymes ..	43
Figure 12.	Results of the comet assay with freezing the cells after 16 h exposure in a RF-incubator.....	44
Figure 13.	Results of the comet assay after 16h exposure in a RF-incubator.....	47
Figure 14.	Results of the comet assay with U-87 after 16h exposure in a RF-incubator.....	49
Figure 15.	Results of the comet assay with ES-1 after 16 h exposure in a RF-incubator.....	50
Figure 16.	Overview on endpoints which were used in RF-studies	55

LIST OF TABLES

Table 1. Communications Technologies (Environment & Human Health, Inc.[3]).	4
Table 2. Examples for the positive results of in vitro experiments with cell phone specific EMF.	8
Table 3. The sources, abbreviation, CAS number and the application of the used chemicals and materials	25
Table 4. The background level of DNA migration in the different cell lines	52
Table 5. Results of the comet assay with the ES-1 cell line after 16h exposure in a RF-incubator.	XX
Table 6. Results of the comet assay with the U-87 cell line after 16h exposure in a RF-incubator.	XXI
Table 7. Results of the comet assay with the SH-SY5Y cell line after 16h exposure in a RF-incubator.	XXII
Table 8. Results of the comet assay with the Tr-146 cell line after 16h exposure in a RF-incubator.	XXIII
Table 9. Results of the comet assay with the HeoG2 cell line after 16h exposure in a RF-incubator.	XXIV
Table 10. Percentage of DNA in tail, after exposure of U-87 to various SAR levels under standard conditions	XXVI
Table 11. Results of the ANOVA analysis with the U-87 cell line under standard conditions.	XXVII
Table 12. Percentage of DNA in tail, after exposure of U-87 to various SAR levels and H ₂ O ₂ treatment	XXVIII

Table 13. Results of the ANOVA analysis with the U-87 cell line with H ₂ O ₂ treatment.....	XXIX
Table 14. Percentage of DNA in tail, after exposure of U-87 to various SAR levels after Endo III treatment.....	XXX
Table 15. Results of the ANOVA analysis with the U-87 cell line after Endo III treatment.....	XXXI
Table 16. Percentage of DNA in tail, after exposure of U-87 to various SAR levels after FPG treatment.....	XXXII
Table 17. Results of the ANOVA analysis with the U-87 cell line after FPG treatment.....	XXXIII
Table 18. Percentage of DNA in tail, after exposure of ES-1 to various SAR levels (0.250 W/kg, 1.0 W/kg, 1.5 W/kg and 2.0 W/kg) under standard conditions.....	XXXIII
Table 19. Results of the ANOVA analysis with the ES-1 cell line under standard conditions.....	XXXIV
Table 20. Percentage of DNA in tail, after exposure of ES-1 to various SAR levels with H ₂ O ₂ treatment.....	XXXV
Table 21. Results of the ANOVA analysis with the ES-1 cell line with H ₂ O ₂ treatment.....	XXXVI

LIST OF ABBREVIATIONS

CA	Chromosomal aberration
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DSB	Double strand breaks
EB	Ethidium bromide
EMF	Electromagnetic fields
Endo III	Endonuclease III
ES-1	Human diploid fibroblast line
FPG	Formamidopyrimidine glycosylase
GHz	Gigahertz
GSM	Global System for Mobile Communications
h	Hour
HepG2	Human hepatocellular carcinoma line
H ₂ O ₂	Hydrogenperoxide
HPRT	Hypoxanthine phosphorybosyl transferase
IARC	International Agency for Research on Cancer
kHz	Kiloherz
LTE	Long Term Evolution
MHz	Megahertz
MN	Micronuclei
MTD	Maximal tolerated dose
OH	Hydroxyl radical
OR	Odds ratio
PD	Petri dishes
RF	Radio-frequency
RF-EMF	Radiofrequency electromagnetic fields
ROS	Reactive oxygen species
SAR	Specific absorption rate

SCGE	Single cell gel electrophoresis assay
SD	Standard deviations
SH-SY5Y	Human neuroblastoma line
SSB	Single strand breaks
TR-146	Human buccal line
U-87	Human glioma line
UMTS	Universal Mobile Telecommunications Service
WHO	World Health Organization

1. Introduction

The present thesis describes the results of experiments, which were conducted with cell lines of human origin, to elucidate if exposure of humans to electromagnetic fields (EMF) emitted by cell phones may causes DNA-instability.

It has been estimated that 6.8 billion of individuals are currently exposed to this type of nonionizing radiation (ICT statistic from ITU International) and a number of investigations have raised concerns in regard to possible health effect of mobile phones.

The introduction section of this thesis defines the types of EMF to which phone users are exposed (chapter 1.2). Furthermore, the current evidence concerns biological effects investigated in in vitro studies with cells (chapter 1.4.2), followed by an overview on investigations with laboratory rodents (chapter 1.4.3) and human epidemiological studies (chapter 1.5).

In the subsequent section (1.9), the principle of the comet assay, which was used in the current study, is outlined and a justification of the selection of the different cell lines is given at the end of this section.

1.1. Does exposure to mobile phone pose long term health hazard in humans: the current state of knowledge

The debate on the possible adverse effects of EMF emitted by mobile phones started already in the middle of the 1990's, but the observation time was too short to indicate if RF-EMF exposure may have a negative impact on humans. In this context, earlier investigations are notable which were conducted to investigate effects of exposure to radio frequency (RF) fields from other sources such as radio-transmitters, TV towers and radars. These studies were reviewed in an article by Kundi et al. [1] and despite methodological problems, there are some indications of an increased risk, especially in respect to leukemia. An Italian group suggested increased levels of leukemia in children living in the vicinity of high-power radio transmitters [2].

In 2011, the International Agency for Research on Cancer (IARC) classified electromagnetic fields as “possibly carcinogenic to humans” (Group 2B), this classification is based on evidence for increased risks for gliomas and acoustic neuroma which were found to be associated with wireless phone use in epidemiological studies. Also other types of radiation were assessed in regard to cancer risks, e.g. those emitted by radio transmitters.

1.2. Cell phone specific EMF and SAR values

The part of the electromagnetic spectrum which is defined as radio-frequency fields ranges between 3.0 (or in some definitions 30) kilohertz (kHz) and 300 gigahertz (GHz) (fig. 1). Only specific frequency bands are used for communication services including mobile phones and wireless local area network (wifi) [3].

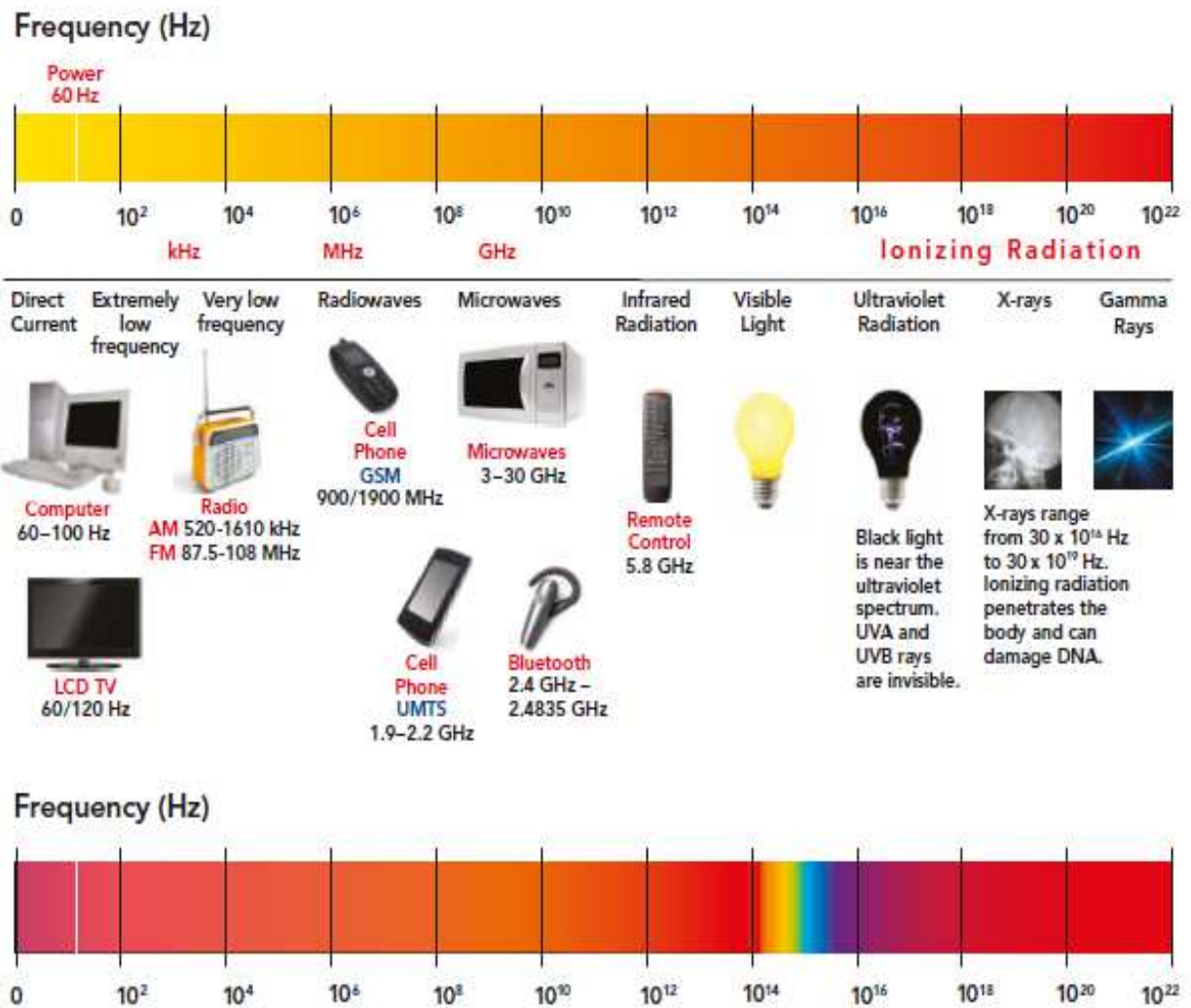


Figure 1. Electromagnetic spectrum (Environment & Human Health, Inc., [3])

Table 1 show the frequencies which are used for communication technologies. At the turn of the millennium, UMTS phones (Universal Mobile Telecommunications Service) entered the market and replaced partly the previous GSM (Global System for Mobile Communications) type devices although GSM is still widely used for phone transmissions. At present, a new generation with higher bandwidth is developed (LTE: Long Term Evolution).

Table 1. Communications Technologies (Environment & Human Health, Inc.[3]).

Communication Technologies			
YEAR INTRODUCED	MOBILE PHONE	TYPE	FREQUENCY
1980s	1G	Analog phones	450 and 900 MHz
1990s	2G	Digital (GSM)	900 and 1900 MHz
2000	3G	UMTS	1900– 2200 MHz
2011	4G	LTS	2000– 8000 MHz (frequencies not yet allocated)

Most cell phones operate at frequencies between 450 and 2100 MHz, with peak powers in the range between 0.1 and 2.0 watt [4]. The exposure of users decreases as a function of the distance from the phone.

An important parameter describing the exposure of biological tissues or part or the whole body of an organism is the specific absorption rate (SAR), which is a measure of the rate at which energy is absorbed by the body. It is defined as the energy absorbed per unit of time and per unit of mass of a tissue, specified in watts per kilogram (W/kg).

In the US, SAR limits were established by the Federal Communications Commission

(FCC) in 1996 and remained unchanged since then. In Europe, the SAR limits were defined by the European Committee for Electrotechnical Standardization (CENELEC), they are based on recommendations of the International Commission on Non-Ionizing Radiation Protection (ICNIRP). For the use of mobile phones the current value is 2.0 W/kg, averaged over a volume of 10 grams of contiguous tissue [3].

1.3. Biological effects

In principle, two types of effects of RF fields are known namely (i) thermal and (ii) non-thermal effects [5]. The first are characterized by an increase of the temperature of exposed tissues, cells or organisms and biological effects may occur due to this phenomenon. The human body has a number of homeostatic mechanisms by which increase of the temperature (due to external high temperatures and/ or physical activities) can be reduced; these mechanisms are also operative in regard to prevention of adverse effects caused by EMF. The definition of the present SAR limits is based on the temperature increase due to physical activity.

Numerous experimental studies were published with exposures below the doses which cause thermal effects that reported biological effects. However, due to dosimetric issues as well as failures to reproduce the findings, no consensus was reached concerning the biological consequences of low dose exposures. In 2011, a study was published by the US National Institutes of Health (NIH) which showed in 47 healthy people using a cell phone for a 50-minute call, that metabolism in the region of the brain closest to the cell phone antenna was significantly affected and correlated with the estimated intensity of the electromagnetic field. The cell phone model which was used had a specific absorption rate of 0.901 W/kg, which is less than the SAR limit of 2.0 W/kg for cell phones defined by FCC. Although the health impact of these findings is unknown, the findings provides evidence that RF-EMF exposure by cell phone use affects brain functions in humans at levels below the SAR [6].

The molecular mechanisms which account for non-thermal effects are currently not established, but several hypotheses were developed, involving interactions with cellular membranes or membrane structures such as voltage gated calcium channels. Biophysical mechanisms may also involve resonance phenomena, these interactions may cause oscillation and destabilization of biologically important molecules [5].

Since acute and short term effects were detected in a variety of endpoints such as EEG (Electroencephalography) changes, effects on brain metabolism or sleeping patterns there is an ongoing discussion whether these biological effects are relevant for human health. The most important question concerns potential long term adverse effects, such as cancer and/or malformation of the offspring, reduced fertility or accelerated aging.

It is known, that instability of the genetic material plays a causal role in the etiology of these diseases [7]; therefore, this thesis focuses on the question whether EMF have an impact on the integrity of the genetic material. The current status of research regarding the carcinogenicity and DNA damaging properties of mobile phone specific EMFs are described in the following chapters.

1.4. Result of genotoxicity studies

1.4.1. Endpoints in genotoxic experiments

Genotoxicity test procedures can be categorized in three major groups [8]: (i) tests in which primary lesions (such as bulky DNA adduct) are measured, (ii) “indicator” assays which concern the detection of phenomena which are associated with DNA damage (e.g. induction of repair processes) and (iii) classical mutation assays which are procedures in which gene mutations and changes of chromosomal integrity (breakage or clastogenicity and aneuploidy) are measured.

The comet or single cell gel electrophoresis (SCGE) assay detects single (SSB) and double strand breaks (DSB) which reflect the DNA damage (for details see chapter 1.9)

classical chromosomal aberration (CA) analysis with human cell lines have nowadays been replaced by micronucleus assays (MN) and reflect both, clastogenic as well as aneugenic effects. The most widely used gene mutation assay is the Salmonella/microsome test which was developed by B. Ames [9] and HPRT experiments with Chinese hamster cells [10].

1.4.2. Results of experiments with primary cell lines

Results of studies which were conducted with primary cells and stable cell lines have been reviewed by Verschaeve et al. in 2010 [11]. In the meantime, four additional studies have been published [12-15]. In total, 51 articles appeared between 1990 and 2013, which covered this topic, selected positive findings are listed in the table 2.

The diagrams in figure 2 and figure 3 shows the type of cell lines and the endpoints which were used in the different EF-studies. It can be seen that most of the investigations were conducted with peripheral blood cells (47%) and that the most used method was the SCGE assay (46%).

Table 2. Examples for the positive results of in vitro experiments with cell phone specific EMF.

Indicator cells	Endpoints	Exposure conditions	Exposure time	References
Cytogenetic investigations				
Human-hamster hybrid cells	Spindle disturbances	835 MHz, Max SAR 0.06 W/kg	2 h	[16]
Human lymphocytes	Sister chromatid exchanges and cell proliferation	954 MHz (GSM), SAR 1.5 W/kg	2 h	[11]
Human white blood cells	Aneuploidy detection	830 MHz, SAR 1.6-8.8 W/kg	72 h	[17]
Human white blood cells	Chromosomal aberration test	954 MHz (GSM), SAR 1.5 W/kg	30 min and 120 min	[18]
Human white blood cells	Micronucleus test	1909.8 MHz (GSM), SAR 5.0 and 10.0 W/kg	24 h	[19]
Primary cultured neurons	HPLC analysis of 8-OHdG	1800 MHz, SAR 2 W/kg	24 h	[20]
SCGE assay				
Embryonic stem cell-derived neural progenitor cells	SSB and DSB	1.71 GHz (GSM), SAR 1.5 W/kg	6 h	[21]
Humans lens epithelial cells	SSB	1.8 GHz, SAR 1.0-3.0 W/kg	2 h	[22]
Humans lens epithelial cells	SSB and DSB	1.8 GHz, SAR 1.0-4.0 W/kg	2 h	[23]

Table 2. Examples for the positive results of in vitro experiments with cell phone specific EMF, continuous.

Indicator cells	Endpoints	Exposure conditions	Exposure time	References
SCGE assay				
Humans lens epithelial cells	SSB and DSB	1.8 GHz, SAR 1.0-4.0 W/kg	2 h	[27]
Human diploid fibroblasts	SSB and DSB	1800 MHz, SAR 2.0 W/kg	16 h	[28]
Human fibroblast ES-1	SSB	1950 UMTS, SAR 0.1 W/kg	8 h	[29]
Human lymphoblastoid cells	SSB	813.5 MHz, SAR 0.0024 and 0.026 W/kg	2 h and 21 h	[24]
SHSY-5Y neuroblastoma cells	SSB and DSB with ROS	872 MHz (GSM), SAR 5.0 W/kg	1 h	[25]
Human trophoblast cells (HTR-8/SV neo)	SSB and DSB	217 Hz (GSM), SAR 2.0 W/kg	16 h and 24 h	[26]

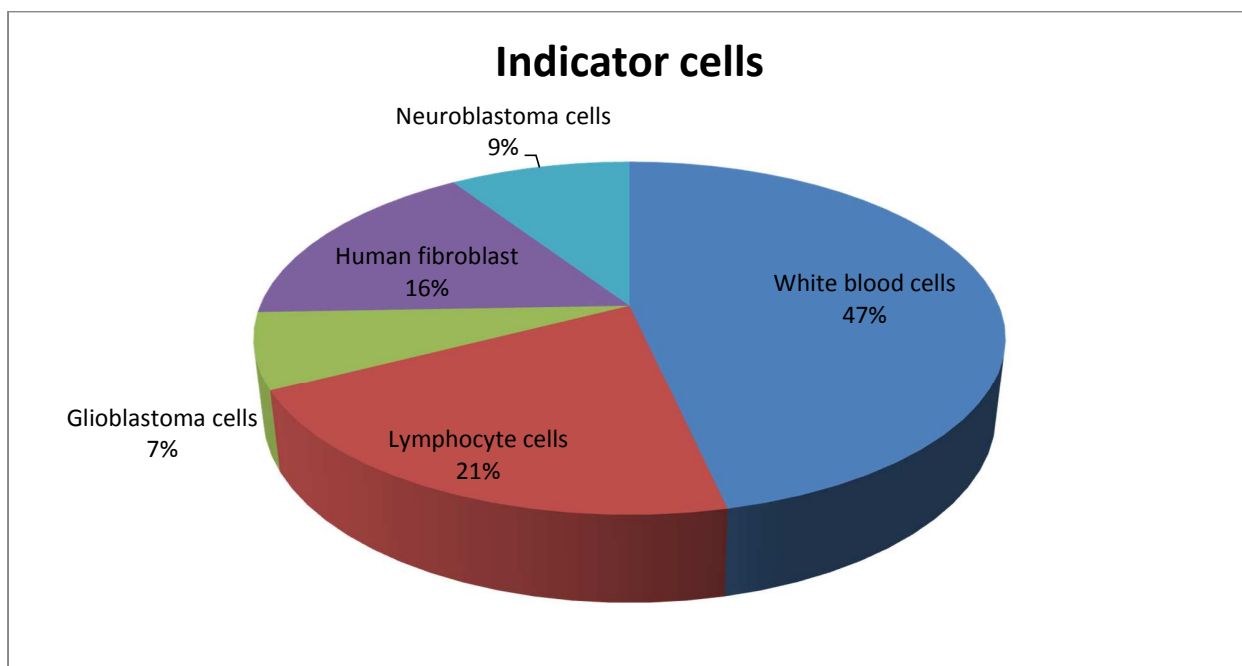


Figure 2. Use of different indicator cell lines in vitro experiments with cell phone specific EMF.

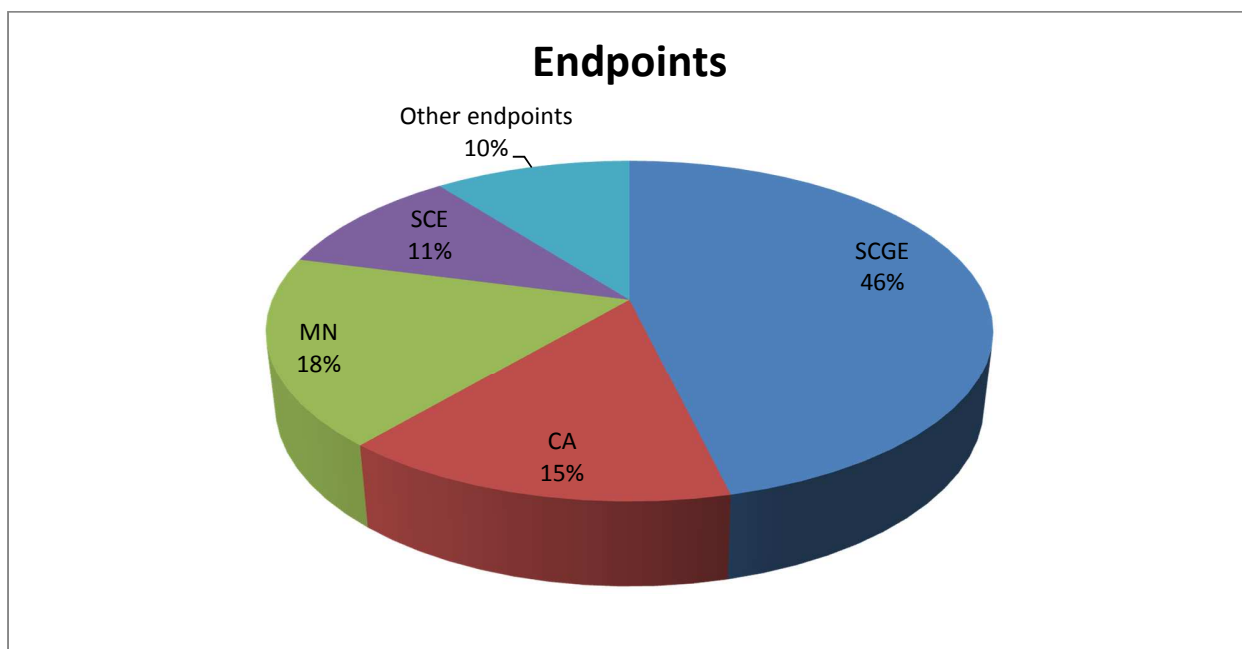


Figure 3. Endpoints which were used in vitro experiments with cell phone specific EMF.

Nicolova et al. [27] found a moderate induction of DNA DSB and up-regulation of the DNA damage inducible GADD45 gene by GSM-signals but no increase of the frequencies of CA and sister chromatid exchanges (SCE). This observation suggests that RF-induced DNA breaks may be repaired, and that no conversion to chromosomal damage and homologous recombination occurs. The authors concluded that the responses were not associated with detectable changes in cell physiology.

Lixia et al. [28] investigated the effects of a 1.8 GHz GSM signal in human lens epithelial cells and found no significant DNA damage after 2 h exposure at SARs of 1.0 and 2.0 W/kg following a 0 up to 140 min incubation time after exposure. However, significant DNA damage was found when exposure was at a SAR of 3.0 W/kg with 30 min exposure period, with longer incubation times no effects were found.

The same research group found also an induction of DNA SSB in human lens epithelial cells after 2 h exposure to 1.8 GHz fields at SARs of 3.0 and 4.0 W/kg [11]. These results indicate that 1.8 GHz RF repairable DNA damage is induced in the cells at an exposure level below 3.0 W/kg, while damage caused by 4.0 W/kg is irreversible.

Franzellitti et al. [29] provided data, which indicate that high frequency electromagnetic fields with a carrier frequency and modulation scheme typical for the GSM signal may affect DNA integrity, although they previously reported no increased levels of DNA damage in human trophoblast cells following a different exposure regimes [30].

Taken together, the results of the investigations are controversial and mainly negative findings were obtained. Therefore, it is not possible to reach any conclusion regarding the outcome of these studies at present.

1.4.3. Genotoxicity experiments with laboratory rodents

In total 29 investigations have been conducted with mice or rats. The most frequently used endpoints are MN formation and induction of comets which were determined in most trials in peripheral blood cells [11].

The majority of studies that was published so far did not find convincing evidence for DNA damage after acute or chronic exposure to RF-fields [31, 32]. In a study by Malyapa et al. [33] which investigated DNA damage in rat brain cells after in vivo exposure to 2450 MHz radiation no significant DNA damage was reported. In contrary, Sakar et al. [34], found alterations of the lengths of a DNA microsatellite sequence in cells from the brain and testes of mice which were exposed to 2.45 GHz fields in 1985.

Additionally, Lai and Singh [35] demonstrated that acute exposure to low-intensity RF radiation increased DNA strand breaks in brain cells of rats. Also Behari et al. [36] found a significant increase in comet heads, tail lengths and tail moments in exposed brain cells. The authors concluded that chronic exposure may cause significant damage in the brain, which may be lead to tumor promotion. Also in an in vitro study, induction of MN was found in a brain cell culture model [37]. This later experiments were conducted since human studies indicate that EMF cause neural tumors (see below).

Finally, an interesting study from Kesari et al. [38], which focused on the impact of EMF on the integrity of the genetic material in sperm cells. These findings indicate that mobile phone specific EMF causes a decrease in the sperm number, which leads to infertility.

Overall, the results of these investigations are controversial and mainly negative findings were obtained. It can be concluded that the available data do not provide convincing evidence of EMF induced genetic effects in laboratory rodents.

1.5. Do mobile phone specific EMF cause genetic damage in humans?

Only few studies addressed the question if occupational RF-exposure causes genetic damage. Maybe this lack of information is due to the difficulties in obtaining the consent of a sufficient number of RF-exposed subjects. Studies with volunteers exposed to mobile phone radiation may suffer from selection bias and may be flawed by other environmental exposures to pollutants and difficulties in finding appropriate control populations. In total, 8 investigations were found which were published during 2001-2013 [11].

In all these studies, effects were compared between mobile phone users and non-users. Most investigations concerned DNA migration and MN formation in peripheral blood cells. Furthermore, few studies were conducted with exfoliated buccal mucosa cells and one with hair follicle cells. It has been criticized that the experimental design of these studies are problematic because the assessment of exposure of the individuals was often based on the payment for the mobile use and differences in the exposure between short telephone calls and long telephone calls were not taken in consideration.

Three Indian research groups investigated the cytogenetic effects in mobile phone users. The first studied CA in peripheral blood lymphocytes, MN in buccal mucosa cells, and DNA damage (comet assay) in peripheral blood cells [11]. Increased levels of DNA damage, CA and MN were found in the phone users. Also a second group found increased levels of CA and SCE in the white blood cells of (mainly smoking and alcohol consuming) users. Furthermore, they found indication of a comutagenic/synergistic effect as lymphocyte cultures of mobile phone users that were exposed to 10 ng/mL of mitomycin C had higher levels of SCE and CA compared to MMC exposed cells from subjects who did not use phones [11]. Finally, a third group reported on increased frequencies of micronucleated exfoliated buccal cells [39], while no indication for increased MN was seen in buccal cells in a recent German study [40]. However one of

the main short-comings of this study is relatively small number of controls subjects (n=13).

A very interesting finding from a Turkish group concerns the increase in DNA breaks in human which were monitored in SCGE experiments hair root cells which were collected around the ears. This study is difficult to interpret since the method is not standardized and the viability of the indicator cells was not monitored [41].

1.6. Results of carcinogenicity studies

1.6.1. Results of carcinogenicity studies with rodents

According to the guidelines which were established by the US National Toxicology Program (NTP-Guidelines) for the testing of chemical compounds, the concentrations of substances should be as high as possible. Therefore, the “maximal tolerated dose” (MTD) is usually determined in preliminary experiments and used in the main studies which are conducted with 50 animals of each sex per experimental group. The MTD is defined as the dose which causes marginal sign of toxicity. This strategy is based on the assumption that it is possible to extrapolate from animal experiments with high dose and a relatively low number of animals per group to the exposure situation of humans which are usually exposed to chemicals at much lower doses over a long time period.

In the case of EMF, it is problematic to use this concept since radiation with doses which are only 1-2 folds over the allowed SAR levels cause thermal effects. Since an increase of the temperature will cause misleading results, only doses can be tested which are close to the allowed human exposure level. These problems could be solved by use of a high number of animals per exposure group, which would enable the detection of weak effects; alternatively it is feasible to use tumor-prone animals which are predisposed to cancer.

The results of animal studies are summarized in a review of Juutilainen et al. [42]. In total, 16 trials were conducted with mice or rats which were exposed to EMF (15 studies with GSM and 1 study with UMTS) and no evidence for induction of tumors was observed in any of these experiments. However, the group sizes were in these studies quite low (i.e. in the range between 35 and 120 animals/treatment groups). The animals were exposed to SAR levels between 0.13-1.4 W/kg and the limit for mobile phone use is 2.0 W/kg for the human head (European Committee for Electrical Standardization (CENELEC)). Therefore, no firm conclusions can be drawn in regard to the safety of humans on the basis of the outcomes of these studies.

In 1997 Repacholi, the head of the WHO expert group on the health effects of non-ionizing radiation published a study which raised international concern [43]. He used a mouse strain which is predisposed to the developed lymphomas (E μ -Pim1-mice). Due to their increased sensitivity these animals are an interesting model. In this investigation, the EMF-field exposed animals had significant increase of lymphoma.

Since the publishing of this study, two follow-up trials with the same mouse strain were published which were conducted under similar conditions and no evidence for induction of cancer was seen in these experiments [42] .

Another example for the use of animals is experiments with AKR/J mice which are characterized by a lifelong expression of an N-tropic, ecotropic murine leukemia virus. This strain is widely used in experimental cancer research as the animals have high leukemia rate (60-90 %). The animals were exposed to 0.4 W/kg and no effects on lymphoma were observed [42]. In some trials C3H/HeJ mice were used which have increased rates of mammary tumors. Most of the studies concluded that RF field exposure did not affect the development of tumors of the animals [42]. Only in one investigation a statistically significance were observed [44]. Furthermore, a study with Patched1 knock-out mice was performed. This is an animal model for multiorgan

tumorigenesis in which exposure of newborn animals to GSM radiation was shown to cause development of brain tumors [45].

1.6.2. Results of carcinogenicity studies with humans

In total, results of one cohort study and of six case-control studies are available.

A Danish cohort study included 420.095 participants and 257 cases of glioma were recorded between 1982 and 1995 [46]. This study found no increased risk of tumors in the central nervous system.

Three early case-control studies [47-49] were performed in a time when the extent of mobile phone use was low, i.e. the users had low cumulative exposures, the time since first use of a mobile phone was short and effect estimates were imprecise. Time-trend analyses did not show an increased rate of brain tumors after the increase in mobile phone use. However, these studies have substantial limitations because most of the analyses examined trends until the early 2000s only. Such analyses are uninformative if excess risk only manifests more than a decade after phone use begins, or if phone use only affects a small proportion of cases e.g., the most heavily exposed, individuals.

The INTERPHONE study was a multicenter case-control study which involved 13 nations. It was to assess whether radiofrequency radiation exposure from cell phones is associated with tumor risks in particular with the incidence of glioma, meningioma, acoustic neuroma and parotid gland tumors [50]. This non-blinded, pooled analysis included 2708 glioma cases and 2972 controls. The interview-based case-control study was designed to have a sufficiently high statistical power to detect a 1.5-fold increase in risk 5 to 10 years from the commencement of cell phone use. Overall, no s risk of glioma or meningioma was observed with use of mobile phones.

A Swedish research group conducted a pooled analysis of two studies which concern the association between mobile and cordless phone users in regard to the incidents of

glioma, acoustic neuroma and meningioma [51]. 1148 glioma cases (ascertained 1997-2003) and 2438 controls were included. Mailed questionnaires and telephone interviews were performed to obtain information on the exposures and covariates of interest, including use of mobile and cordless phones (response rates 85 % and 84 %, respectively). Participants who had used a mobile phone for ≤ 1 year had an odds ratio (OR) for glioma of 1.3. The effect increased with increasing time since first use and with total call time. Maximal OR values were 3.2 (for more than 2000 h of use). Ipsilateral use of the mobile phone was associated with higher risk. Similar findings were reported for use of cordless phones.

An additional study from Japan with 787 participants [52], found an increased risk for acoustic neuroma was associated with mobile phone use (maximal OR 3.08).

1.7. Classification of the carcinogenic risk caused by mobile phone specific electromagnetic fields by the IARC

In 2011, the International Agency for Research on Cancer (IARC) in Lyon evaluated the possible carcinogenicity risks caused by mobile phones. The expert group classified RF-EMF as “possibly carcinogenic to humans” (Group 2B). This conclusion was justified on the bases of the available data and the main facts are summarized in an article which published in Lancet Oncology [53]. For meningioma, parotid-gland tumors, leukemia, lymphoma, and other tumor types, the working group found the available evidence insufficient to reach a conclusion on the potential association with mobile phone use. However, they noted that there is “limited evidence in humans” for the carcinogenicity of RF-EMF, based on positive associations between glioma and acoustic neuroma and exposure to RF-EMF from wireless phones.

The IARC experts evaluated more than 40 studies that assessed the carcinogenicity of RF-EMF in rodents. An increased total number of malignant tumors were found in RF-EMF-exposed animals in one of the seven chronic bioassays. Increased cancer

incidences in exposed animals were noted in two of twelve studies with tumor-prone animals and in one of 18 studies using initiation-promotion protocols. Four of six cocarcinogenicity studies showed an increased cancer incidence after exposure to RF-EMF in combination with known carcinogens; however, it was noted that the predictive value of this type of study for human cancer is unknown. Overall, the Working Group concluded that there is “limited evidence” from experimental animals in regard to the carcinogenicity of RF-EMF.

1.8. Design of the present study

The present study was designed on the basis of the existing data. As mentioned above, a new type of mobile phone entered the market in 2000 which is using UMTS and only two investigations have been published which concern the impact of this form of EMF on the genetic stability of mammalian cells [54, 55]. One of them was published by Schwarz et al. in 2008 [54] and was strongly criticized [56]. The authors used in this investigation human fibroblasts which were exposed to different doses and time periods and found evidence for significant effects but not time or dose depended. In a follow up study, which was conducted by another group (Speit et al., [55]), the results could be not reproduced.

Five different cell lines were used in the present investigations which were selected on the basis of the results of earlier investigations. The human derived fibroblast ES-1 cell line was chosen since it has been used in an earlier study. The authors found after 24 h exposure at 0.05 W/kg a significant induction of comets [54]. Lerchl stated in a comment that: *“The critical analysis of the data given in the figures and the tables furthermore reveal peculiar miscalculations and statistical oddities which give rise to concern about the origin of the reported data.”* [56]. Therefore it was of interest to find out if the findings are reproducible or not.

The human glioblastoma cell line was included due to the fact that epidemiological studies indicate that EMF exposure causes glioblastoma (see chapter 1.6.2). For the same reason also neuroblastoma SH-SY5Y was employed. Further cell lines were HepG2 and the buccal line TR-146. The human liver derived line has inducible phase I and phase II enzymes and is able to activate and detoxify xenobiotics and reflects the metabolism of xenobiotics in the human body better than other metabolically incompetent cells [57]. The cell line TR-146, which is derived from a human neck metastasis of buccal epithelial origin [58], expresses ultrastructural characteristics of normal human buccal epithelial cells, e.g. intermediate filaments, microvilli-like processes, and lack of complete keratinization [59]. This cell line was employed since exfoliated buccal cells of cell phone exposed individual will be tested as a part from the ongoing ATHEM project in a human intervention trial and due to the fact that oral cells are exposed to high EMF doses.

Prior to the main experiments, the growth kinetics of the different cell lines were conducted under defined experimental conditions, furthermore calibration experiments with hydrogen peroxide (H_2O_2) and with lesion specific enzymes were performed.

The impact of deep-freezing on the sensitivity and viability of different cell lines was studied in a further calibration experiments.

In the first series of the main experiments the cell lines were exposed to 1.0 W/kg; this exposure was chosen because it is the limit of commonly used mobile phones [60]. Subsequently, two cell lines were treated with multiple doses in order to investigate possible dose-response relationships.

1.9. The comet assay

The SCGE assay is based on the determination of DNA migration in an electric field. Already in 1984 Ostling and Johanson [61] published a protocol for experiments which

were carried out under neutral conditions with isolated nuclei to detect DSB. Subsequently, Singh et al. [62] developed protocols by SCGE assays in which the nuclei are incubated in alkaline lysis buffer. This procedure has the advantage that not only DSB but also SSB and apurinic site can be detected.

1.9.1. Calibration of the SCGE assay

We used in the present trial H_2O_2 to establish calibration curves in SCGE experiments. This chemical was also used as a positive control in the main experiments, and in combination with RF-exposure to draw conclusions if exposure leads to alterations of the sensitivity of the cells towards ROS induced DNA damage.

H_2O_2 is produced endogenously by several physiological processes, such as the inflammatory respiratory burst and during oxidative phosphorylation. It is a natural source of oxidative damage in cells, causing a variety of DNA lesions, including SSB and DSB, DNA damage due to H_2O_2 results from production of the hydroxyl radical ($\bullet\text{OH}$) in the presence of transition metal ions such as iron via the Fenton reaction, in which H_2O_2 is reduced in the presence of metal ions [63].

1.9.2. Determination of the cell vitality

It has been shown that acute toxic effects may cause misleading results in SCGE experiments [64], therefore the “vitality” of the cells was determined by trypan blue (0.4 %) exclusion method which indicates whether cell membranes are intact. The method is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not [65].

1.9.3. Use of comet assay in genetic toxicology

Over the past decade, the comet assay become a standard method for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, as well as, fundamental research in DNA damage and repair [66]. It can

be not only used in experiments with cell lines in vitro but also in animal experiments and human studies [66]. In contrast to other genotoxicity tests, such as MN assays, SCE and gene mutation assays no cell divisions are required to observe effects.

In order to standardize the SCGE tests, international guidelines have been developed and published by different expert groups [64, 67]. In the present study, the experiments were conducted according to these guidelines. It is notable that also an OECD guideline for in vitro experiments which is at present in preparation.

1.9.4. Quantification of the comet formation

The extent of DNA damage can be quantified by visual (manual) scoring, with computer added automated software scoring system with an image capture device attached to the microscope. The most commonly used parameters are the tail length, the tail moment and tail intensity (% DNA in tail). But at present it is recommended by Collins [66] to use the tail intensity.

1.10. The exposure apparatus

The waveguide-based and computer-controlled exposure system (figure 4) which was used in the present study was developed by the Foundation for Research on Information Technologies in Society (IT'IS, Zurich, Switzerland). The apparatus has power efficiency for SAR of up to 17.0 W/kg, a nonuniformity of the SAR distribution of less than 26 % and provides a temperature load of less than 0.03 °C per W/kg average SAR. It is based on two short-circuited waveguides operating at the frequency of 1950 MHz. The setup has the dimensions $h \times w \times d = 450 \times 250 \times 580 \text{ mm}^3$. The waveguide is placed inside an incubator (HeraCell 240 CO₂ incubator, Kendro Laboratory, Germany), which provides the necessary environmental conditions for the cell cultures (temperature, humidity, CO₂). A commercial broadband coax-to-waveguide coupler was used to excite the waveguides. Reflected power is terminated in a 50 load. Six 35 mm ø Petri dishes

are exposed per waveguide chamber. They are arranged in three pairs and positioned in the waveguide chamber (figure 5). A dish holder assures the correct positional arrangement. The distances of the dish centers to the short are 95 mm, 188 mm and 280 mm. Tight exposure and environmental control is realized by field sensors, temperature sensors for the air environment, and by an optimized air-flow system based on ventilators, with a common adit for the two waveguides. The voltage output of the sensor was calibrated to the square of the H-field inside the area. Since no field probes for WCDMA (Wideband Code Division Multiple Access) were available, calibration of the monopole sensor was performed via true RMS power measurements: First, the H-field as a function of input power was determined for an unmodulated exposure (CW) using an H3DV3 field probe (SPEAG). Then the sensor voltage as a function of WCDMA input power was recorded and finally identified with the CW H-field at the same average power level.

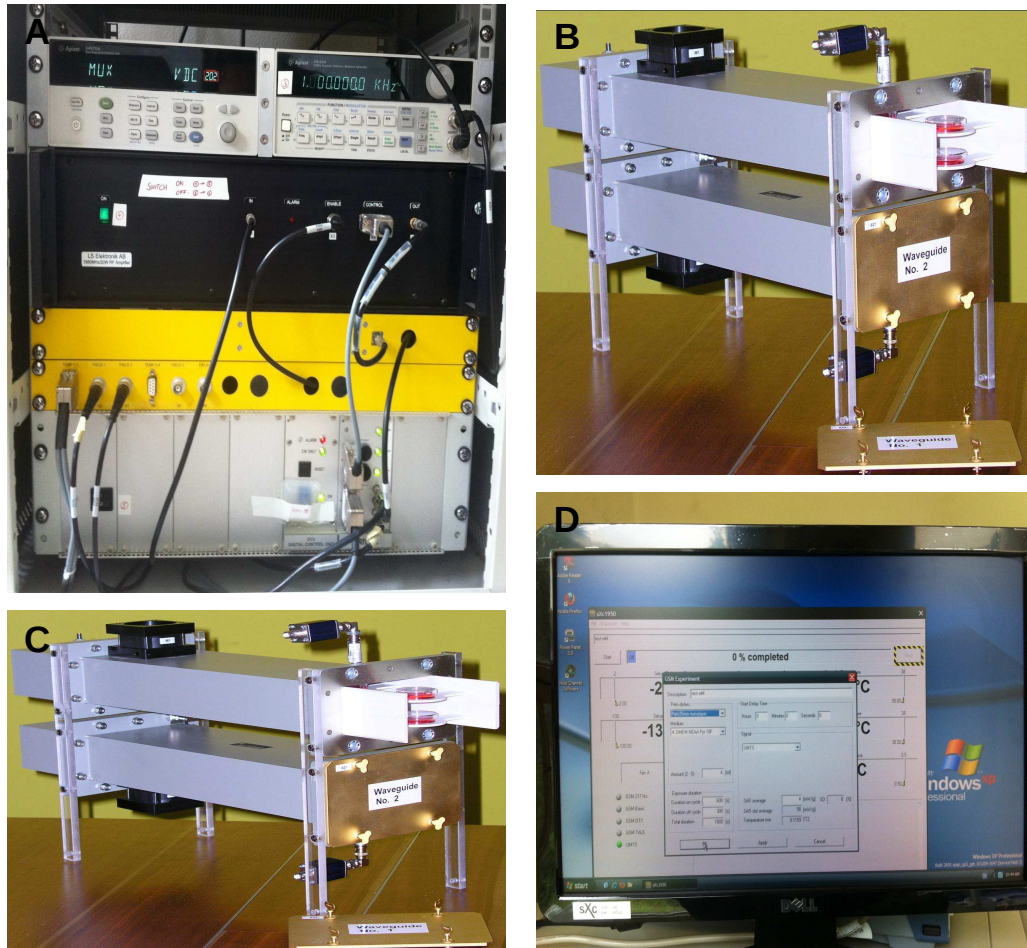


Figure 4. The sXc1950 installed system. The apparatus was designed to test EMF exposures of the third generation (3G) of mobile communication. The setup is based on two waveguide chambers that are blindly excited by a fully computer-controlled signal unit: A the two waveguides placed inside an incubator, B the dimensions of the setup, C and D the computer-controlled exposure system.

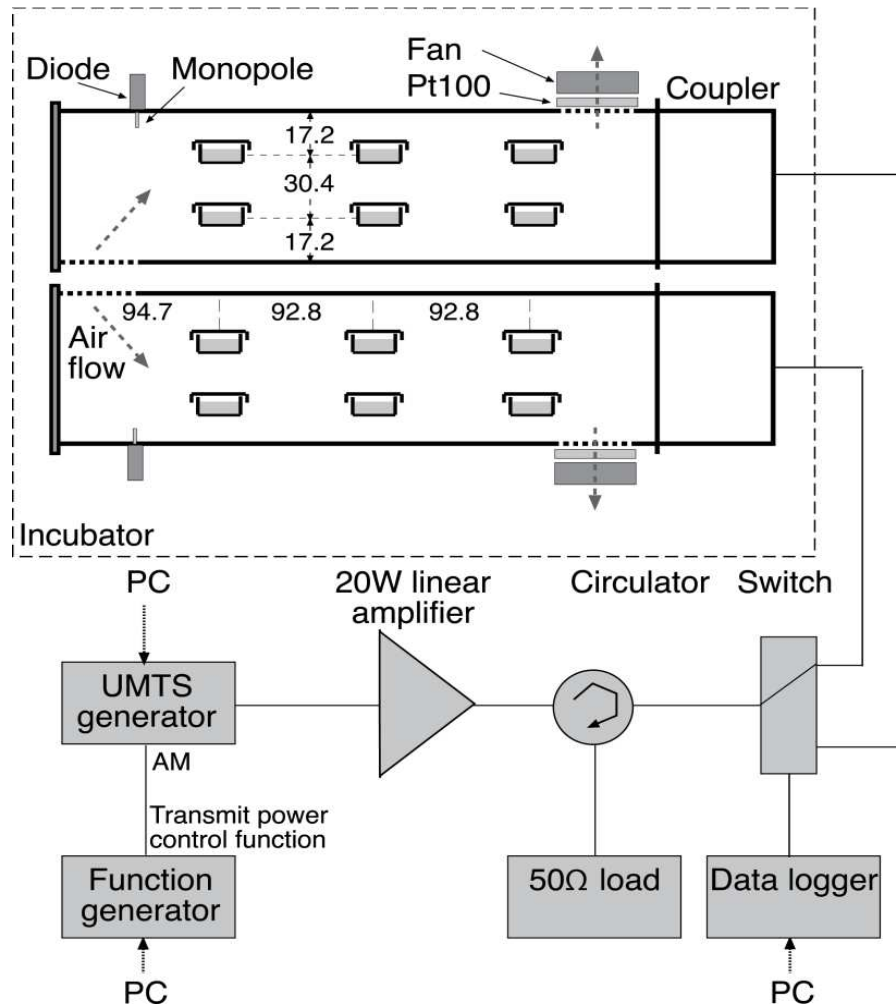


Figure 5. Mechanical and electronic design of the UMTS exposure system. Inner dimensions of the R18 waveguides: 64.8 x 129.6 x 425 mm³ (height x width x length), (IT'IS Foundation, Zurich, Switzerland).

2. Material and Methods

2.1. Chemicals

The table 3 summarized the sources of the chemicals which were used in the experiments.

Table 3. The sources, abbreviation, CAS number and the application of the used chemicals and materials

Chemicals	Abbreviation	Company	CAS Registry Numbers	Application
Comet Assay				
2-Amino-2-(hydroxy-methyl)-1.3-propandiol	Trizma base	Sigma-Aldrich ¹⁾	77-86-1	Lysis solution
Bovine serum albumin	BSA	Sigma-Aldrich ¹⁾	9048-46-8	Enzyme buffer
Dimethyl sulfoxide	DMSO	Roth ²⁾	67-68-5	Lysis solution, freezing of cells
Disodium-Ethylendiamin-tetraacetic acid	Na ₂ -EDTA	Sigma-Aldrich ¹⁾	6381-92-6	Lysis solution, enzyme buffer, electrophoresis buffer
Dulbecco's Phosphate buffered saline (10X), Ca und Mg free	DPBS	PAA Laboratories GmbH ³⁾		Washing and buffer dilutions
Ethidium bromide	EtBr	Sigma-Aldrich ¹⁾	1239-45-8	Staining solution
Trypan blue		Sigma-Aldrich ¹⁾	72-57-1	Determination of the viability
Hydrogen chloride	HCl	Sigma-Aldrich ¹⁾	7647-01-0	pH adjustment
Normal melting point agarose	NMP Agarose	Invitrogen Life Technologies ⁴⁾		Slide preparation
Low Melting Point Agarose	LMP Agarose	Invitrogen Life Technologie ⁴⁾		Slides for SCGE experiments
Sodium chloride	NaCl	Lactan ⁵⁾	7647-14-5	Lysis solution, enzyme buffer
Sodium hydroxide	NaOH	Sigma-Aldrich ¹⁾	1310-73-2	Lysis solution, enzyme buffer, electrophoresis buffer
t-Octylphenoxy-polyethoxyethanol	Triton X-100	Sigma-Aldrich ¹⁾	9002-93-1	Lysis solution

Table 3. The sources, abbreviation, CAS number and the application of the used chemicals and materials, continuous.

Chemicals	Abbreviation	Company	CAS Registry Numbers	Application
Enzyme (Comet Assay)				
Endonuclease III (10UG)	Endo III	Sigma-Aldrich ¹⁾		Detection of oxidative pyrimidine bases
Formamidopyrimidine glycosylase (10 UG)	FPG	Sigma-Aldrich ¹⁾	78783-53-6	Detection of oxidative purine bases
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Hepes	Sigma-Aldrich ¹⁾	7365-45-9	Enzyme buffer
Test substance				
Hydrogenperoxide	H ₂ O ₂	Sigma-Aldrich ¹⁾	7722-84-1	Induction of DNA oxidative damage

1) Sigma-Aldrich, Steinheim, Germany; 2) Roth, Karlsruhe, Germany; 3) PAA Laboratories GmbH, Pasching, Austria; 4) Invitrogen Life Technologies, Paisley, Scotland; 5) Lactan, Graz, Austria.

2.2. Reagents for SCGE assays

Modified Dulbecco's PBS (phosphate-buffered saline): 100 mL of 10 x DPBS were dissolved in 900 mL distilled water and stored at room temperature.

Lysis solution: 146.1 g NaCl, 37.2 g Na₂EDTA and 1.2 g Trizma base dissolve in 1.0 liter of distilled water. The pH was adjusted to 10.0 with NaOH pellets (approximately 7.0 g of NaOH pellets). The solution was stored in a refrigerator at 4 °C.

Immediately before use, Triton X-100 (1.0 %) and DMSO (10.0 %) were added and afterwards the solution was stored in a refrigerator at 4 °C for several hours.

Electrophoresis buffer: 84.0 mL of NaOH (10 M) and 14.0 mL of Na₂EDTA (200 mM) were dissolved in 2800 mL cold distilled water. The pH range was between 12.5 and 13.0.

Neutralization Buffer per liter: 48.5 g Trizma base added to approximately 800 mL dH₂O and adjusts the pH to 7.5 with HCl, brings the volume up to 1000 mL with dH₂O and the solution was stored and refrigerated at 4 °C.

Enzyme reaction buffer: 19.04 g HEPES, 14.9 NaCl, 0.372g Na₂EDTA and 0.4 g BSA were dissolved in one liter of distilled water and adjusted to pH 8.0 with potassium hydroxide (KOH).

2.3. Preparation of slides for the single cell gel electrophoresis assays (SCGE)

The slides were dipped into 1.0 % normal melting agarose (NMA) which had been prepared in 1x phosphate-buffered saline (DPBS). Subsequently, they were dried overnight and stored at room temperature until use. Slides for experiments with lesion specific enzymes were coated with 2 x 80 µl 1.0 % NMA in PBS and covered with a 20 x 20 mm coverslip for a least 5 min to allow the agarose to solidify. The coverslips were removed and dried overnight at room temperature.

When the slides were completely dry, the cells were suspended in a 0.5 % low melting agarose solution prepared in 1 x DPBS at 37 °C and immediately pipetted on to the coated slides. Subsequently, they were covered with coverslips, and then placed on ice to allow the agarose to solidify. After 10 min the coverslips were removed and the slides stored at room temperature.

2.4. Cell culture and cultivation

The cell lines were collected from different sources and stored deep frozen. Prior to the experiments they were analyzed for contaminations with mycoplasmas, bacteria and fungi.

The human diploid fibroblast line (ES-1 cells) was isolated from a healthy donor (male, 6 years old) and cultured in Dulbecco medium supplemented with 10 % foetal bovine serum. The cells were obtained from Alexander Pilger (Institute of Occupational Medicine, AKH, Austria).

Human glioma U-87 cells (glioblastom-astrocytoma, grade IV) are a highly malignant anaplastic glioma clone derived from a 44-year-old Caucasian woman. The cell line was cultured in monolayer cultures in minimum essential medium with 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 1.0 mM sodium pyruvate and 10.0 % foetal bovine serum. The cells were obtained from Metka Filipič, (National Institute of Biology, Slovenia).

The human buccal line (Tr-146) was derived from a human neck metastasis originating from a buccal carcinoma of a 67 year-old female [58] and was cultured in Dulbecco Medium supplemented with 10.0 % foetal bovine serum. The cells were obtained from James G. Rheinwald (Dermatology Institute of Boston, USA).

The human neuroblastoma line (SH-SY5Y) is one of three serially isolated neuroblast clones of the human neuroblastoma cell line SK-N-SH which was established in 1970 from a metastatic bone tumor [68]. The cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1 mixture) supplemented with 2 mM L-glutamine and 10 % foetal bovine serum. The cells were obtained from Stefan Böhm (Medical University of Vienna, Austria).

Human hepatocellular carcinoma line (HepG2) was isolated in 1979 by Aden et al. [69] from a primary hepatoblastoma of an 11-years old Argentine male. The cells were cultured in Eagle's Minimal Essential Medium supplemented with 1 mM sodium pyruvate and 10 % foetal bovine serum. The cells were provided by Firouz Darroudi (University of Leiden, Netherlands).

All cells were grown in 75 cm² flasks in a standard tissue culture incubator at 37 °C in humidified atmosphere in presence of 5 % CO₂. The media were changed every second day. The cells were subcultivated till a 70 % confluence was reached. Subsequently, they were washed with 1 x DPBS (pH 7.4) and then trypsinised for 2 to 5 min at 37 °C. Reseeding was done at 1000 cells/cm² density for standard cultivation and with higher densities for the comet assay.

The morphology of the different types of indicator cells is shown in figure 6 (A-E).

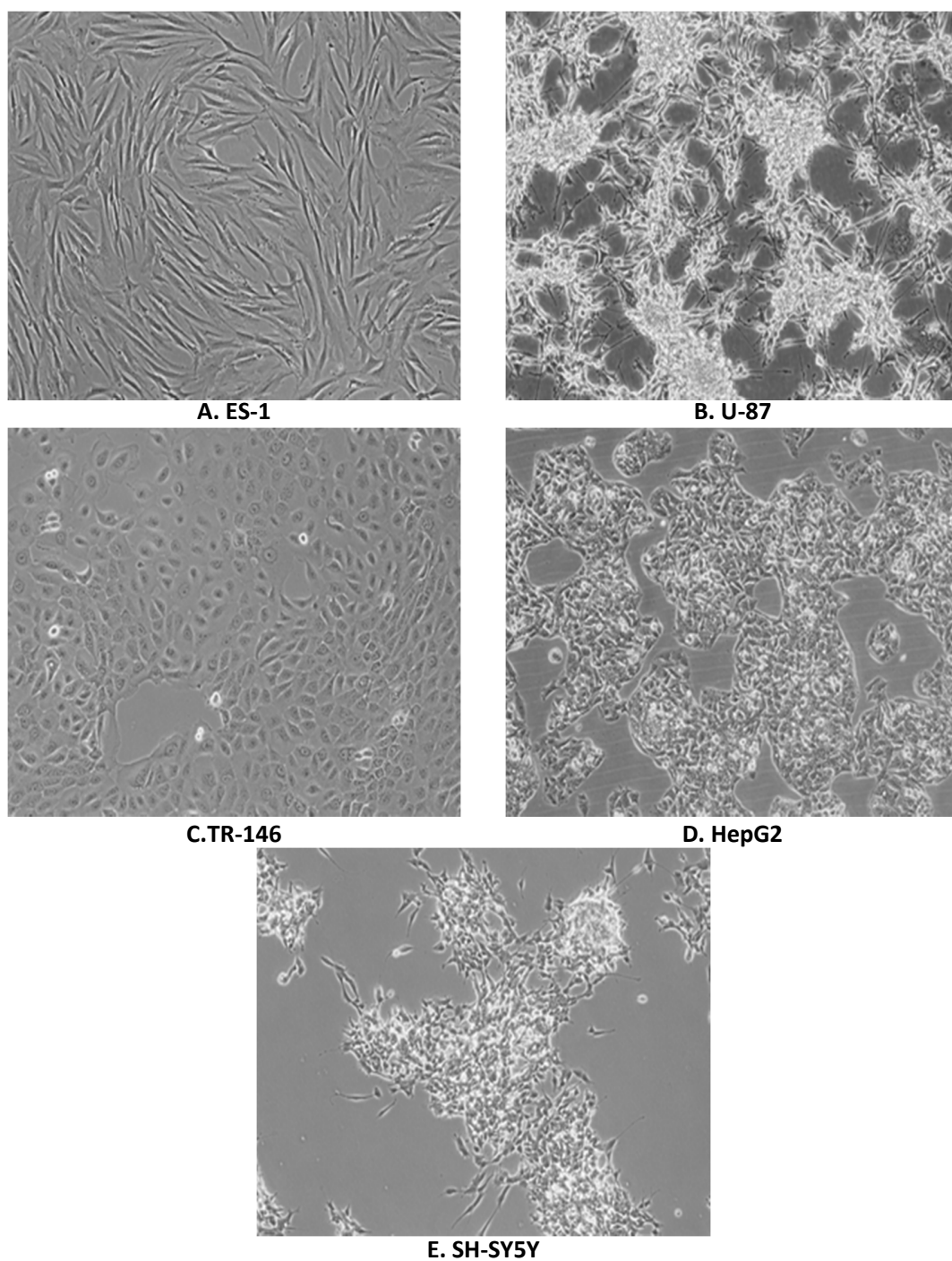


Figure 6. Morphology of the different indicator cell lines. A. ES-1: diploid fibroblasts, B. U-87: glioma cell line, TR-146: buccal cell line, HepG2: hepatocellular carcinoma cells and E. SH-SY5Y: neuroblastoma cells.

2.5. Determination of the mitotic activity

Prior to the main experiments the growth kinetics of the different cell lines was determined. The cells were cultivated as described above in Petri dishes (ø 60 mm). After harvesting by trypsinization, the cell numbers were determined with a light microscope in a Neubauer chamber (LO - Laboroptik Company, Germany), in 24 h intervals over a period of seven days (for each time point, three wells were evaluated).

2.6. The comet assay (general scheme)

The experiments were conducted according to international guidelines [64]. Three different protocols were used: (A) the standard protocol which enables the detection of SSB and DSB under alkaline conditions [64], (B) a modified version to determine formation of oxidatively damaged purines and pyrimidines by use of lesion specific enzyme (restriction enzyme) [66], (C) treatment of the cells with H₂O₂ in order to assess alterations of the sensitivity of the cells towards ROS [70].

Figure 7 shows the different procedures which were used. For each experimental point, three slides were prepared in parallel and the tail intensities measured in 150 cells with a computer added analyses system. Before the main experiments with EMF exposure, calibration experiments were conducted in order to optimize the experimental conditions.

2.6.1. SCGE experiments under standard conditions

The cells were collected by trypsinization, afterwards 5×10^5 cells were spread onto precoated agarose slides (1.5% NMPA). In order to isolate the nuclei of the cells, the slides were treated at least 1 h at 4 °C in lysis solution. Subsequently, they were placed for 30 min in cold electrophoresis buffer for 30 (unwinding), before the electrophoresis was started (30 min). The gels were neutralized by washing (two times with the

neutralization buffer and subsequently one time with dH₂O) and after drying, stained with ethidium bromide (20 µg/mL).

2.6.2. SCGE experiments with lesion specific enzyme

The measurements were performed as described by Collins [66]. For the SCGE assay with lesion specific enzymes 50.000 cells were transferred on each precoated slide. After the lysis step, the slides were washed twice in enzyme reaction buffers (pH 8.0) for 8 min and 50 µl of the enzyme solutions or the respective amounts of buffer were added to the gel, then the slides were covered with cover slips and were incubated at 37 °C in humid atmosphere with Endo III for 45 min and FPG for 30 min. After the treatment, the glass cover slips were removed and the slides placed in the electrophoresis chamber (C.B.S. Scientific Company, USA).

To determine the optimal enzyme concentrations, calibration experiments were performed as suggest by Collins [71]. The enzymes were diluted with enzyme reaction buffer in three different concentrations 1:3000, 1:5000 and 1:7000 to find the optimal concentration for the main experiments.

2.6.3. SCGE with hydrogen peroxide treatment

In the main experiments, the cells were exposed to 30 µM H₂O₂ for 10 min on ice. This protocol [70] was used on the basis of the results of calibration experiments in which the cells were treated with H₂O₂ under different conditions.

A. Treatment of the cells on slides placed on ice: The cells were detached by trypsinization from flasks and resuspended in low-melting-point agarose (1 x 10⁵ cells/mL) and embedded on sides. The slides were immersed in H₂O₂ solution (on ice) for 10 min. After the treatment, the slides were washed with 1 x DPBS to remove excess H₂O₂ (2 x 8 min).

B. Treatment of the cells in 24-well plates at 37°C: Cells were plated in a 24 multi well plate (1 mL at 5×10^6 cells per mL) and were allowed to attach for 24 hours at 37 °C. Subsequently, they were treated with H₂O₂ at 37 °C. After the treatment, the wells were washed with 1 x DPBS to remove H₂O₂ (2 x 8 min). Subsequently, the cells were detached with trypsin from the wells and embedded in normal melting agarose slides.

C. Treatment of the cells on slides in an incubator: After trypsinization, the cell suspension was spread on slides, treated with H₂O₂ and kept at 37 °C for 10 minutes.

2.6.4. Electrophoresis and analyzing

The slides were placed in an ice cold horizontal electrophoresis chamber (26 cm x 40 cm, C.B.S. Scientific Co., California, USA) containing cold electrophoresis buffer (pH $\geq$ 13) for 30 min to allow unwinding of the DNA. Subsequently, electrophoresis was conducted for 30 min (25 V; at 4 °C), the gels were washed two times with neutralization buffer (8 min) and rehydrated with distilled water. After drying at room temperature, the slides were stained with ethidium bromide (20 µg/mL) and the tail intensities measured by use of a computer added image analysis system (Comet Assay IV, Perceptive Instruments Ltd., UK, [72]). For each experimental point, three cultures were made in parallel and 50 cells were analyzed per culture (in total 150 cells per experimental point).

All results were analyzed by use of GraphPad Prism, version 4.0 (GraphPad Software, Inc., San Diego, CA; USA). Results are reported as means $\pm$ standard deviations (SD).

All statistical analyzes were conducted with the transformed values, because the percentage values have a variation, which correlates with the average value. To eliminate this correlation, an arcsine transformation must be performed for further statistical analysis. This is obtained according to the following formula:

$$Tr \% DNA \text{ in tail} = \text{Arcsin } \% DNA \text{ in tail}/100$$

For representing the data in the tables 5-21, the statistical results were transformed back. The results of the SCGE assays were analyzed by student t-test and one-way ANOVA; p values ≤ 0.05 were considered as statistically significant.

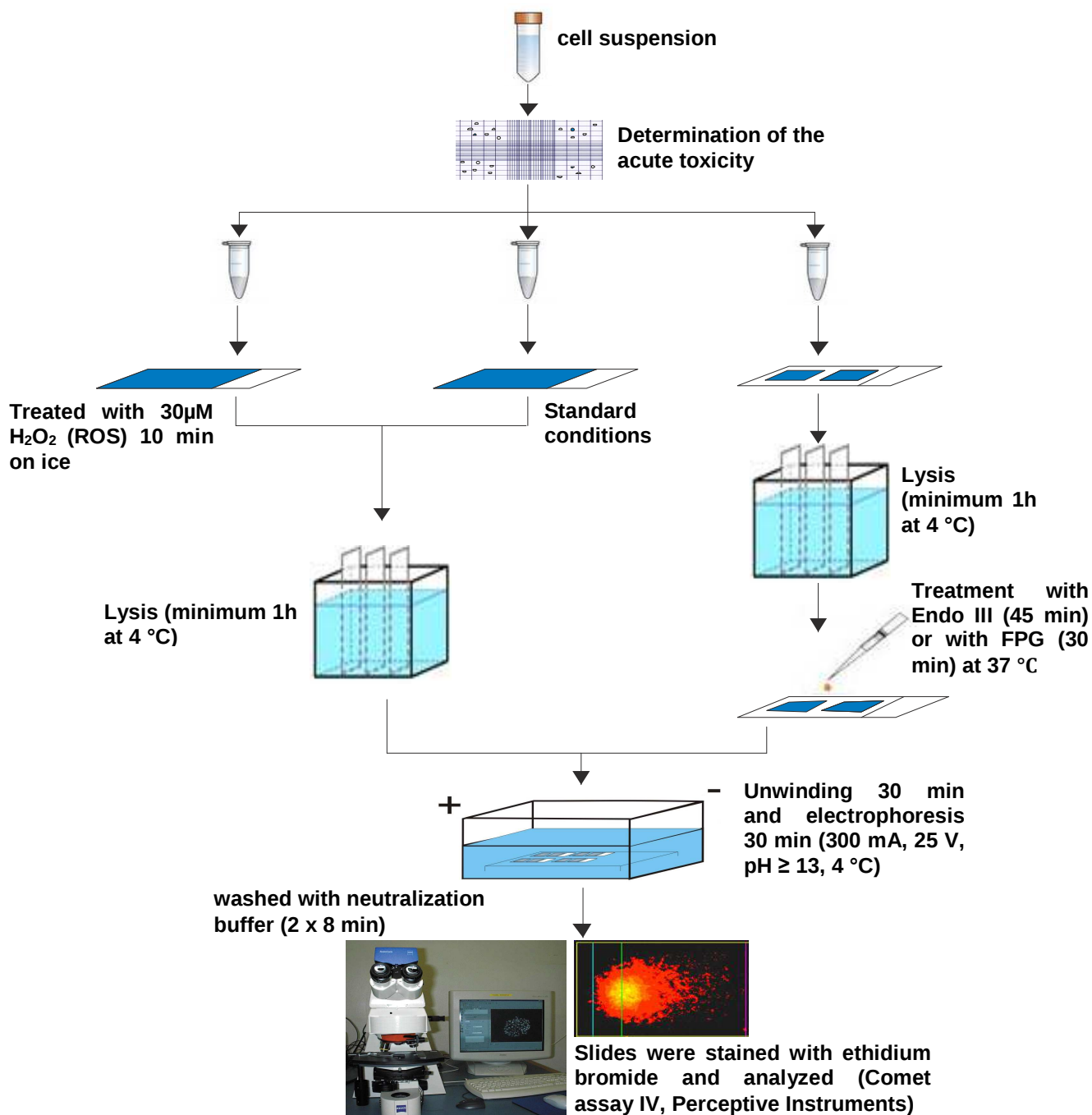


Figure 7. Schematic illustration of the comet assay. After determination of the vitality, cell suspension was transferred on the slides. In the standard experiments the slides were treated with the lysis solution at 4 °C (minimum 1 h) and subsequently placed in the electrophoresis chamber. In experiments with H₂O₂, the cells were treated with 30 µM H₂O₂ on ice for 10 min. When the modified protocol with the restriction enzymes was used, the slides were treated after lysis with 1.3000 Endo III for 45 min and 1:5000 FPG dilution for 30 min. After the treatment, the slides were exposed to alkaline buffer for 30 min before electrophoresis at 300 mA (25 V, pH 13) for 30 min at 4 °C. After drying, the slides were stained with ethidium bromide and analyzed.

2.7. Experiments with RF-exposure

The experiments with mobile specific RF-exposure were realized after several calibration steps. The different cell lines were exposed in a first series to 1.0 W/kg for 16 hours to UMTS (1.950 MHz). Subsequently, the cells were treated in presence and absence of hydrogen peroxide (30 μ M, 10 min on ice); in addition the nuclei were isolated and treated with the two lesion specific enzymes. In a second series of experiments, the most sensitive cell lines were exposed to different doses.

2.7.1. Determination of the acute toxicity

The acute toxicity of the cells was determined in each experiment with trypan blue (0.4 %) dye exclusion technique [65] with an improved Neubauer hemocytometer (Paul Marienfeld GmbH, Lauda-Königshofen, Germany). The cell suspensions were mixed 1:1 with the staining solution. DNA damage was only analyzed in cells from cultures in which the viability was ≥ 80 %, as acute toxic effects may cause false positive results [64].

2.7.2. UMTS exposure

The exposure apparatus which was used in all experiments is described in chapter 1.10. To study the effects of RF-exposure 5×10^5 cells of the different cell lines were seeded into 35 mm Petri dishes ($\varnothing$ 35mm, Nunc) and allowed to attach to the bottom of the flasks for 24 hours before to UMTS exposure. Six 35 mm dishes were exposed simultaneously in one waveguide chamber, in the other chamber the cells were sham exposed (see figure 8). The cells were treated intermittently (cycles of 5 min on, 10 min off) because it is assumed that modulated HF-EMF is more effective than continuous exposure [73].

The experimental setting was stored in coded files and uncovered by the IT IS foundation in Zurich per e-mail. Each cell line was exposed independently since each medium requires a different program. For the determination of the SAR dependency, the

cells were exposed for 16 hours of intermittent exposure (5 min on and 10 min off) to a SAR level 1.0 W/kg. In the second series of experiment the most sensitive cell lines were exposed to different exposure levels; each cell line was tested twice.

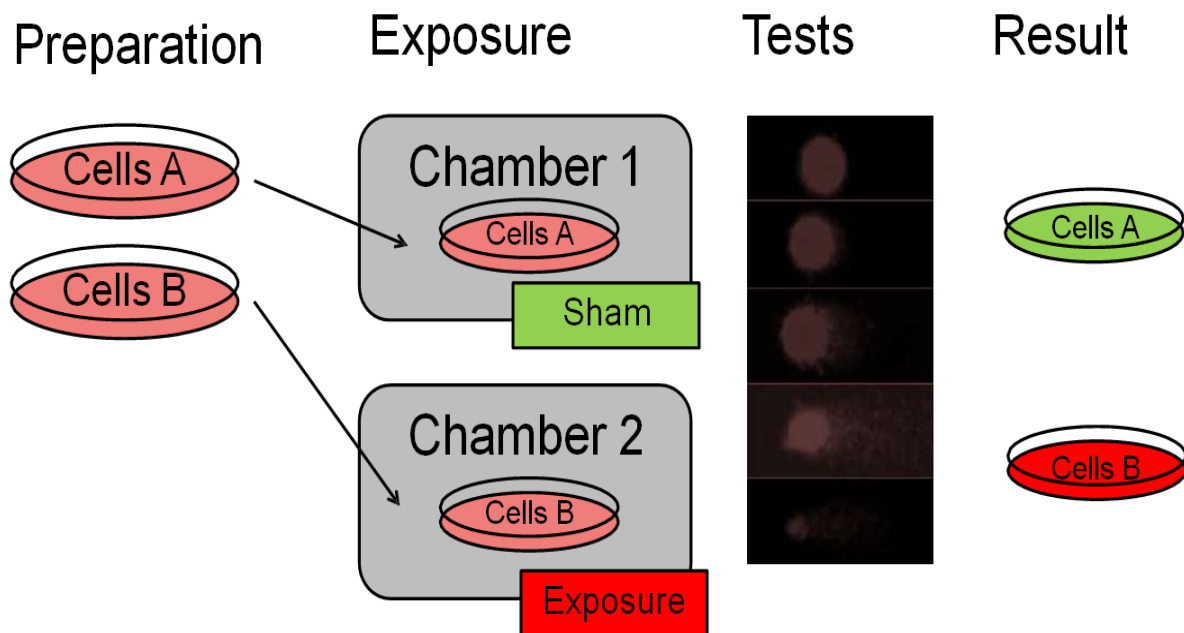


Figure 8. Double blind protocol of the exposure apparatus. After seeding the cells 12 nunc Petri dishes were used, 6 were placed in each wave guide. One of the waveguides (chamber) is exposed, the other is only sham exposed. After the exposure, cells were analyzed with different SCGE protocols.

2.7.3 The cryopreservation process for the most sensitive cell lines

In the second part of the study, the most sensitive cell lines (U-87 and ES-1) were exposed to different UMTS dose, detached by trypsinization from the flasks, resuspended with 5 % DMSO and frozen overnight in -80 °C and then transferred to liquid nitrogen. The cells were stored deep frozen and defrozen after 2 weeks and the results were compared to those which were obtained with freshly cultivated cells. After thawing the cells, the comet assay was performed according to the protocol of Tice et al. [64].

3. Results

This chapter consists of three sections. The first part describes the results of investigations concerning the growth kinetics of the different cell lines. The second paragraph contains results of calibration experiments. The last section presents the results of the main experiments which were conducted to investigate the genotoxic effects of EMF. In the first experimental series, all cell lines were analyzed after treatment with 1.0 W/kg. In subsequent trials, the glioblastoma derived cell line and the fibroblasts which were considered to be the most interesting ones, were exposed to different doses.

3.1. Determination of the mitotic activities

Fig.9 A-E depicts the growth curves which were obtained with the different cell lines. The cells were cultivated in culture dishes ($\varnothing$ 60 mm) and the cell numbers were determined under a light microscope in Neubauer chamber, in 24 h intervals over a period of seven days.

It can be seen that the fastest growth rate was observed with the HepG2 cell line, followed by the neuroblastoma cell line SH-SY5Y. The buccal line Tr-146 and glioblastoma line U-87 had an identical doubling time, which was longer than that of the liver cells and neuroblastoma cells; the mitotic activity of the fibroblasts was substantially lower as that of the other cell types.

The doubling times were determined by use of a specific software program (<http://www.doubling-time.com/compute.php>). The values increased in the order HepG2 (31)> SH-SY5Y (33)> Tr-146 (43) $\geq$ U-87 (43) > ES-1 (48). Numbers in parentheses indicate the doubling times in hours.

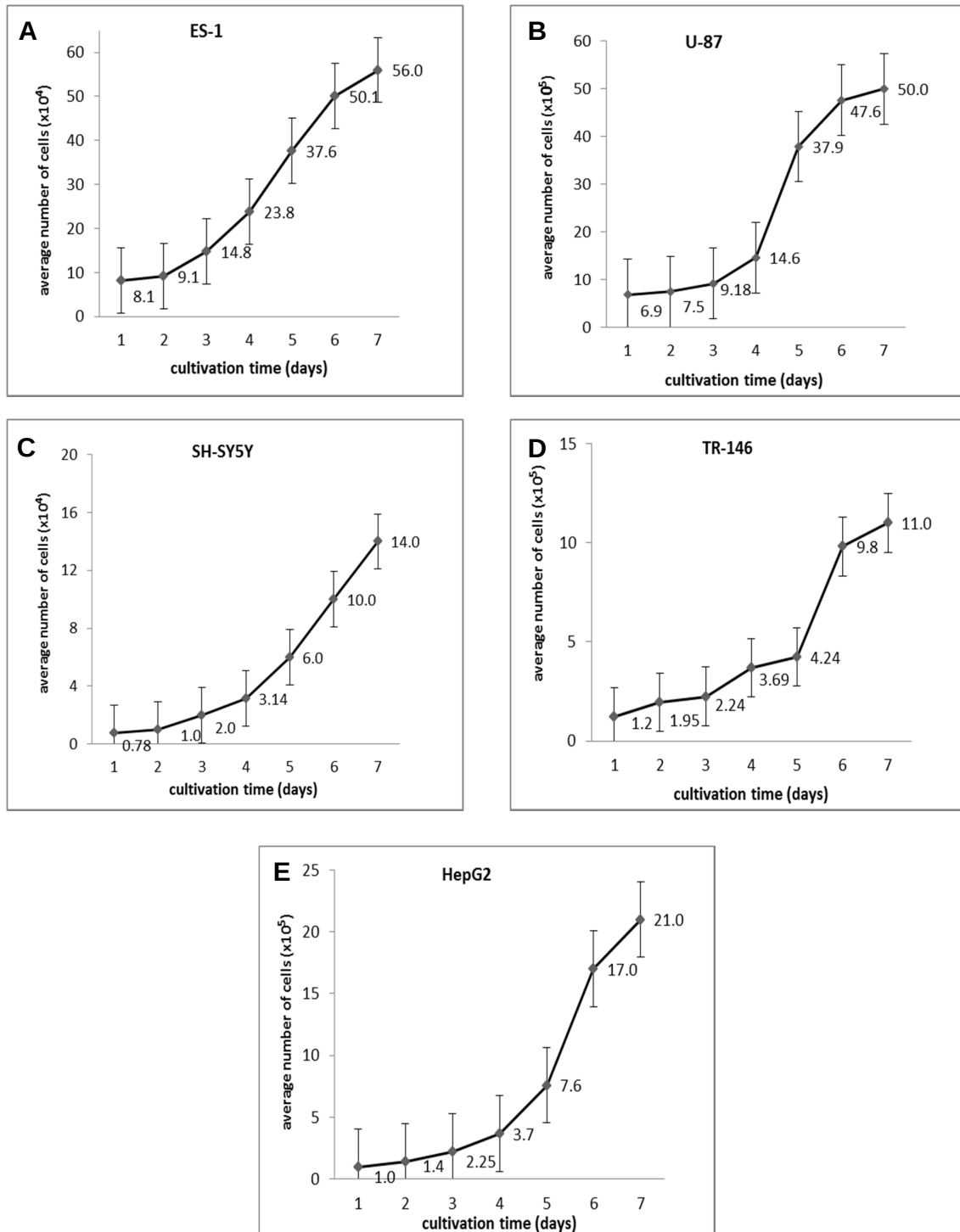


Figure 9. The Growth kinetics of the different cell lines. The cells were seeded into small size Petri dished ($\varnothing$ 60 mm) under standard conditions (5% CO_2 , 37 $^\circ\text{C}$). After harvest of the cells by trypsinization, the cell numbers were determined in a Neubauer chamber. The points represent means $\pm$ SD of results obtained with three cultures (3 wells per time point).

3.2. Calibration experiments

Prior to the main experiments, calibration trials were conducted with all five cell lines in which the formation of comets was analyzed in untreated cells and after hydrogen peroxide exposure under identical experimental conditions.

HepG2 cells and TR-146 cells have been used in earlier SCGE trials with ROS challenge [74, 75], while the fibroblast ES-1 line was only investigated in an EMF study by Diem et al. and Schwarz et al. [54, 76], and no data from H₂O₂ treatment were available.

In order to optimize amounts of the lesion specific enzymes, different dilutions of FPG and Endo III were tested in SCGE assays [66].

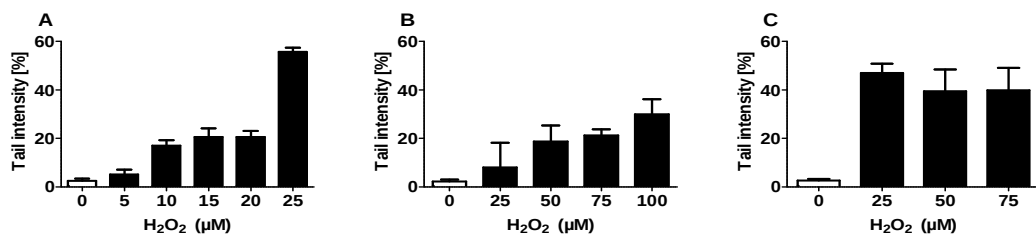
Furthermore, the impact of deep-freezing on the sensitivity of the cells was studied in a separate experiment.

3.2.1. Experiments with hydrogen peroxide

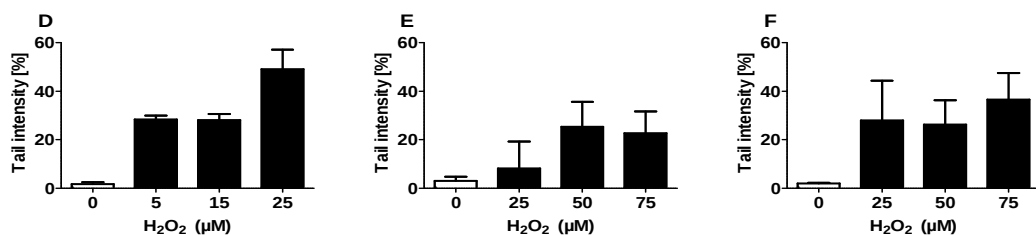
As described in chapter 2.6.3, the cells were treated with different concentrations of H₂O₂ under different experimental conditions. The cells were placed on slides and treated on ice (A), treated in 24-wells at 37 °C (B), on slides in an incubator at 37 °C (C), the treatment time was in all experimental series 10 min (fig. 10). For all cell lines, the optimal concentration of H₂O₂ which was used in the subsequent main experiments as a positive control was 30 µM.

We found that the standard deviations are smaller when the cells were treated on slides placed on ice as compared to results obtained after exposure in 24-well plates at 37 °C. Therefore, this protocol was used in the RF-experiments.

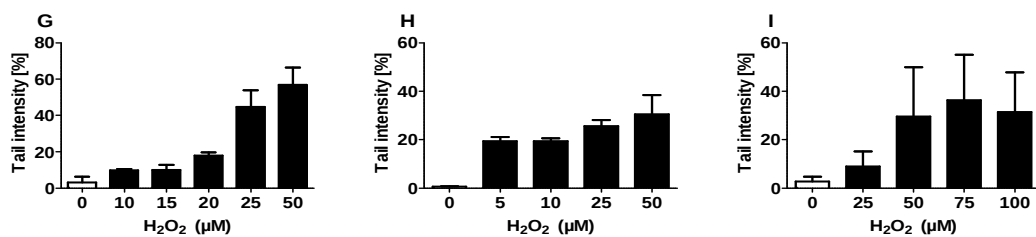
ES-1



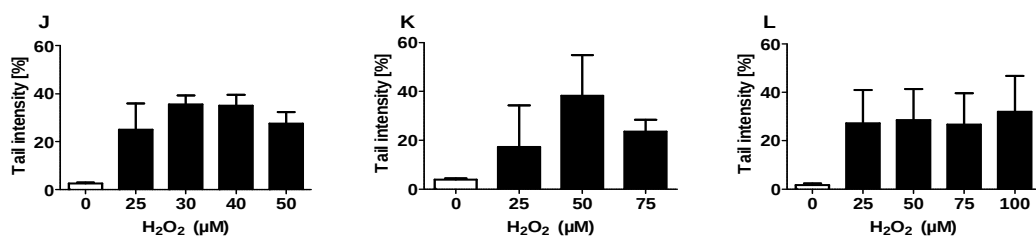
U-87



SHSY-5Y



TR-146



HepG2

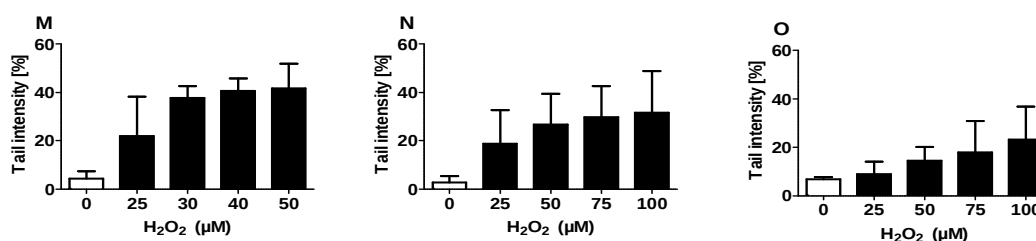


Figure 10. The DNA migration after exposure to different concentrations of H₂O₂.The exposure time was in all cases 10 min. (A, D, G, J, M) treatment of cells on slides which were placed on ice, (B, E, H, K, N) treatment in 24-wells at 37°C (C, F, I, L, O) treatment of cells on slides in the incubator at 37°C. PBS was used as a negative control. Bars represent means $\pm$ SD of results obtained with three plates per experimental point.

3.2.2. Impact of treatment of isolated nuclei with different dilution of restriction enzymes

Since the activity of the restriction enzymes declined as a function of the storage time and it can be not excluded that the extent of oxidatively damaged DNA bases differs in the individual cell-lines.

Calibration experiments were conducted to establish the optimal amount of the enzymes as suggested by Collins [71]. The nuclei were isolated from the untreated cells as described in material and methods (chapter 2.6.2) and were exposed to different dilutions of the restriction enzymes Endo III for 45 min and FPG for 30 min. Subsequently, electrophoresis was conducted under standard conditions and the nuclei were stained with ethidiumbromide.

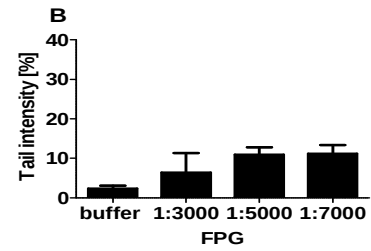
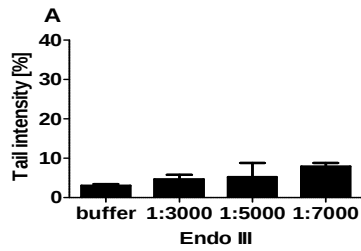
It can be seen in figure 11 that maximal DNA migration in all cell lines was observed with Endo III with the 1:3000 dilution of the stock solution, in the case of FPG, the optimal concentration was the 1:5000 dilution.

3.2.3. Impact of deep freezing (cryopreservation) on the sensitivity of the indicator cells

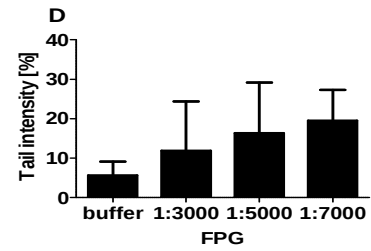
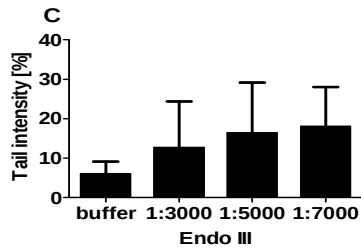
The experiment was performed with two cell lines U-87 (fig 12.A) and ES-1 (fig 12.B). Figure 12 (A and B) depicts the impact of deep freezing on the sensitivity of the indicator cells. The comet formation was analyzed in untreated culture and after treatment with H₂O₂ (30 µM, 10 min on ice) with and without freezing.

Furthermore, no significant differences in the extent of spontaneous DNA damage were seen between unfrozen and frozen cells under the different experimental conditions. It can be seen in figure 12 (C and D), that the cell vitality was still high after deep freezing (≥ 90).

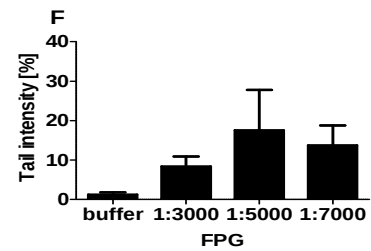
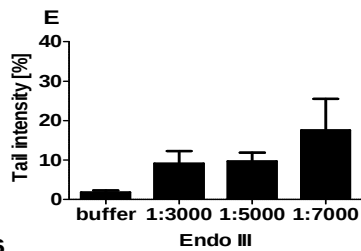
ES-1



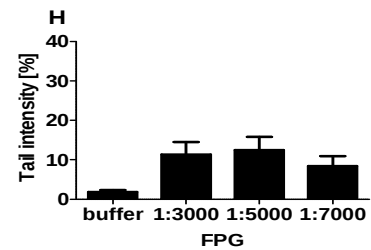
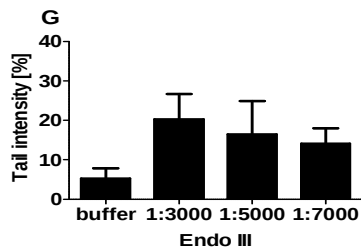
U-87



SHSY-5Y



TR-146



HepG2

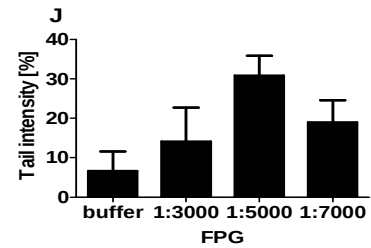
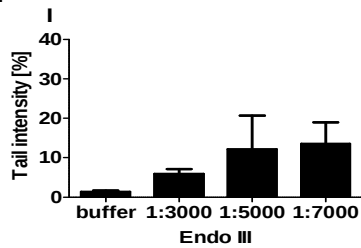


Figure 11. Results of comet assay with three dilutions of the restriction enzymes. Nuclei were isolated from the different cell lines and treated with the different concentration of the restriction enzymes. The buffer was used as a negative control. Bars represent means $\pm$ SD of results obtained with three plates per experimental point.

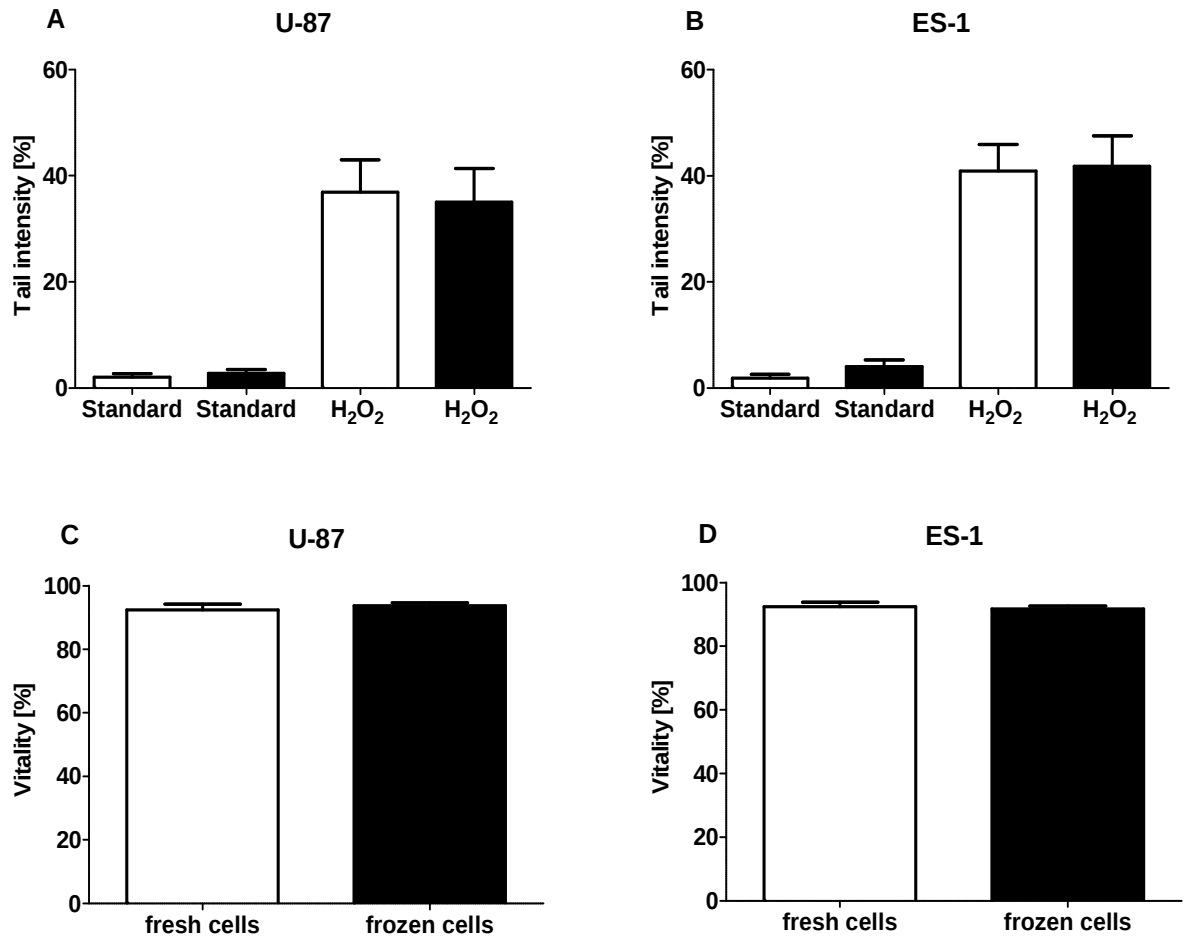


Figure 12. Results of the comet assay with freezing the cells after 16 h exposure in a RF-incubator. White bars represent the fresh collected culture and the black bars the cells after the cryopreservation procedure. Bars represent means $\pm$ SD of results obtained with three cultures per experimental point.

3.3. Investigation of the genotoxic effects caused by RF exposure

This chapter describes the main results and consists of two paragraphs. The first concerns the comparative analysis of the sensitivity of the five different cell lines after exposure to 1.0 W/kg. The induction of comets was analyzed in all experiments under standard conditions, after treatment of the nuclei with lesion specific enzymes and after treatment of intact cells with ROS (hydrogen peroxide exposure). The second paragraph contains results from experiments which were conducted with different doses with two cell lines, glioblastoma U-87 and fibroblast ES-1. The former one was chosen on the basis of the outcome of the single dose measurements, the second because positive results have been published in an earlier study [54].

3.3.1. Sensitivity of the different cell lines to 1.0 W/kg

The findings which were obtained with all cell lines under identical experimental conditions are summarized graphically in fig. 13. All lines were exposed to 1.0 W/kg (exposure period 16 h), in parallel also non exposed cultures were included.

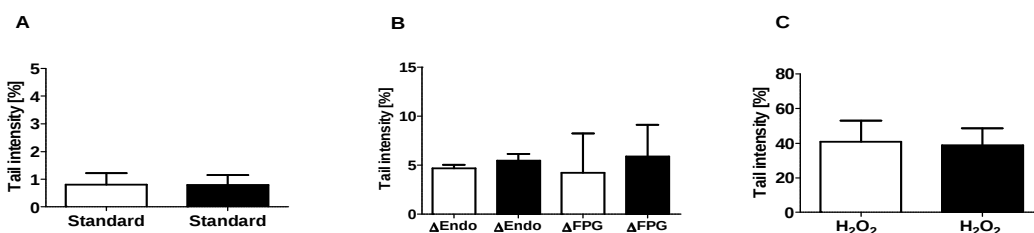
It can be seen that no induction of DNA migration was detectable when the experiments were conducted under standard conditions (fig 13). The results which were obtained with the lesion specific enzymes are shown in the middle of the graph (the left bars depict the findings with Endo III and the right bars concerns the results with FPG).

The black bars which reflect the DNA migration after treatment of the nuclei with restriction enzymes, shows the extent of comet formation after subtraction of the values which were obtained with the respective enzyme buffers. In other words the tail intensity values are indicative for the formation of DNA migration, which is attributing to oxidatively damaged purines and pyrimidines.

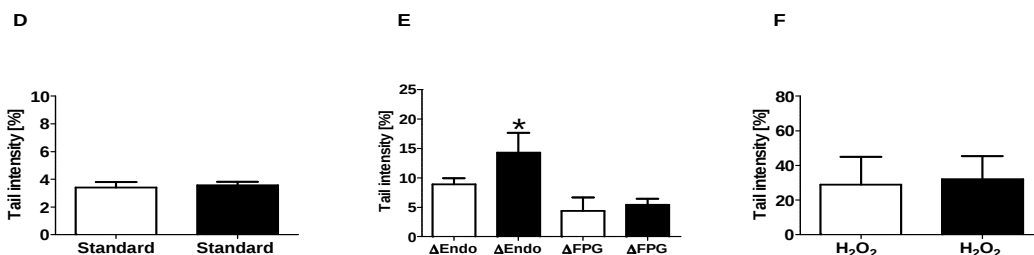
It can be seen in figure 13.E that the extent of DNA migration due to formation of Endo III sensitive sites was in the glioblastoma cell line (U-87) increased in comparison to the

background value in the unexposed control cultures. Also in the line SH-SY5Y a significant Endo III effect was seen. With all other cell lines, no or marginal differences were detected. Also after treatment of the different cell lines with ROS (hydrogen peroxide) after exposure to the EMF field, did not lead increase the extent of DNA migration.

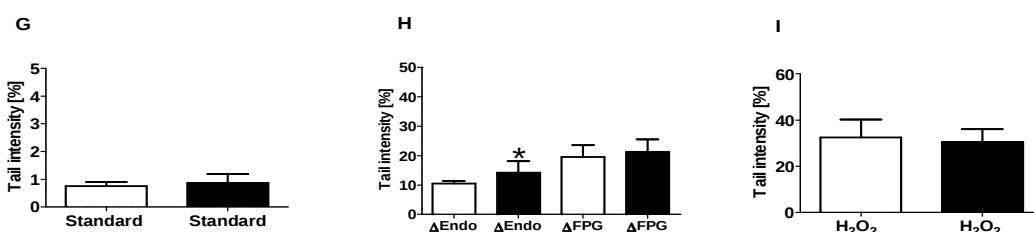
ES-1



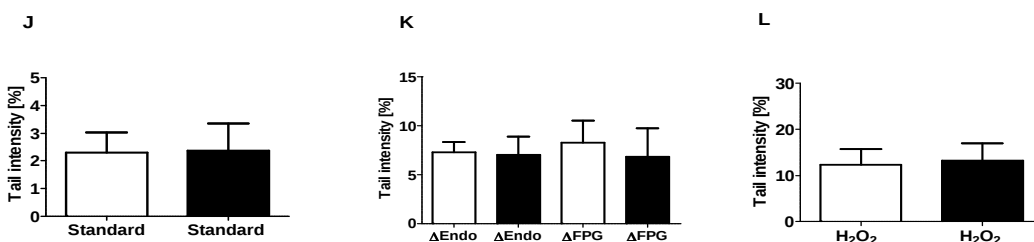
U-87



SHSY-5Y



TR-146



HepG2

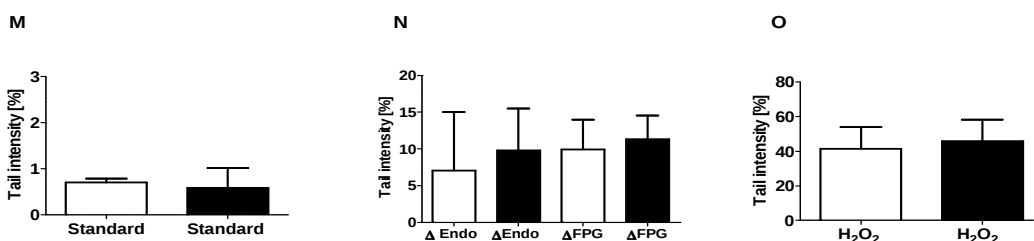


Figure 13. Results of the comet assay after 16h exposure in a RF-incubator. All cell lines were exposed to 1 W/kg. White bars represent sham exposed cells and black bars “wave on” exposed cells. Bars represent means $\pm$ SD of results obtained with three cultures per experimental point. Stars indicate the statistical significant ($p < 0.05$).

3.3.2. Dose response effects of mobile phone specific RF exposure

The findings which were obtained in the U-87 line with both lesion specific enzymes indicate that RF-exposure may cause an increase of oxidative DNA damage. Therefore, the cells were treated in subsequent experiments with different exposure doses. The results are summarized in figure 14.

It can be seen that a significant effect was detected in these SCGE assays under standard conditions (fig 14.A), when the cells were exposed to 0.250 W/kg and 0.500 W/kg. Also with the highest dose an increase was found, however this effect did not reach significance.

It is also notable, that the extent of DNA-migration was increased when the nuclei were treated with lesion specific enzymes (fig 14.C and 14.D). In the case of ENDO III, a higher percentage of DNA in the tail was seen with all three dose levels. However, all p-values were ≥ 0.05 and the effect did not reach statistical significance. Also with FPG, the extent of comet formation was increased at all three doses, again the effect not reach significance. However, in the combined evaluation effects were seen in all three doses.

No clear EMF-effects were seen in the ROS-exposure assays (H_2O_2), but a clear induction of DNA migration was detected after treatment of the cells with the peroxide itself (fig 14.B).

Further dose response experiments were conducted with the fibroblast line ES-1 (figure 15). It can be seen that no induction of DNA migration was found under the present conditions. Also in experiments with ROS (H_2O_2) treatment no impact of RF-exposure on the extent of comet formation was seen.

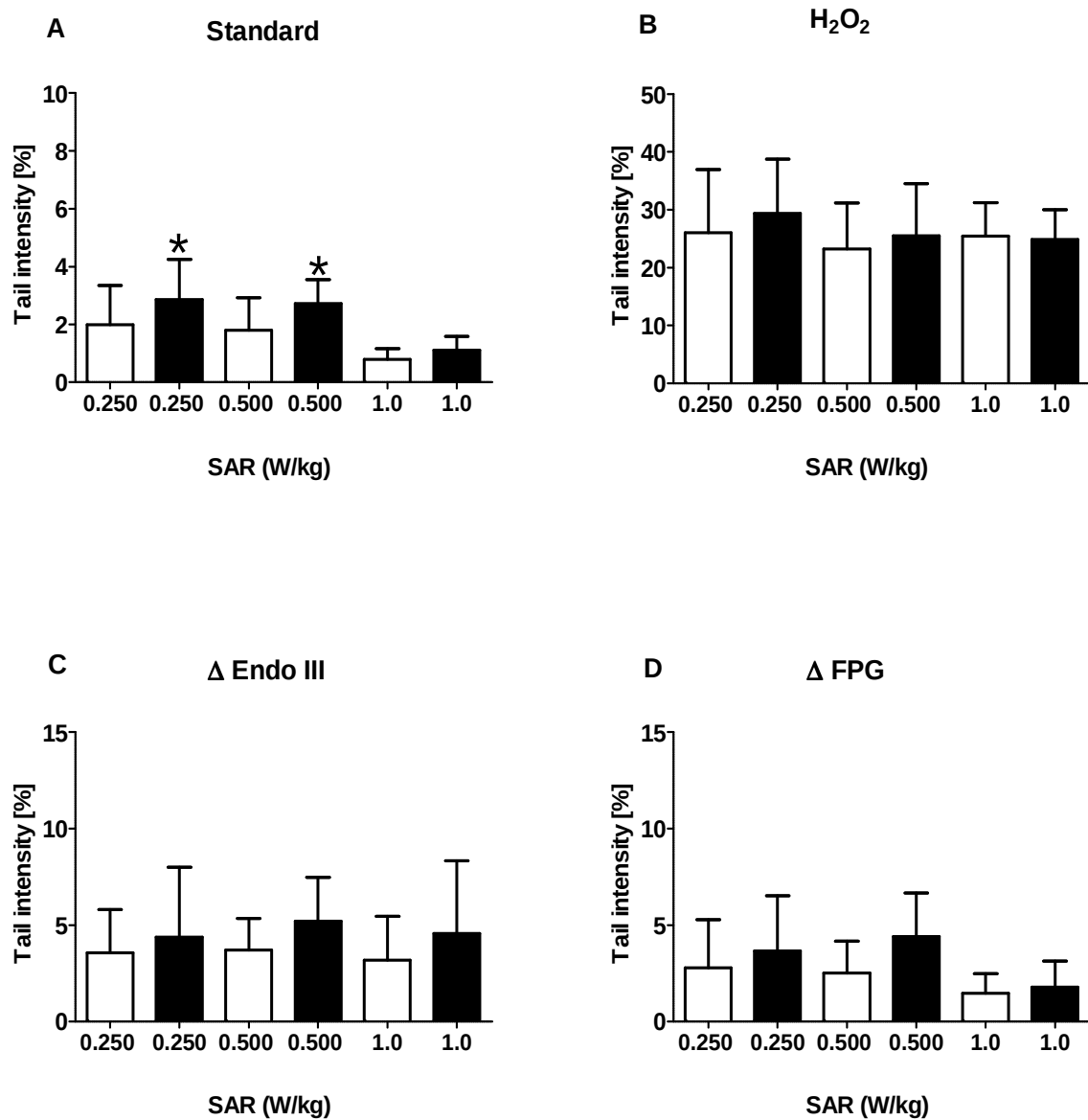


Figure 14. Results of the comet assay with U-87 after 16h exposure in a RF-incubator (0.250 W/kg, 0.500 W/kg and 1.0 W/kg). White bars represent sham exposed cells, black bars EMF-exposed cells. Bars represent means $\pm$ SD of results obtained with three cultures per experimental point. Stars indicate the statistical significant ($p < 0.05$).

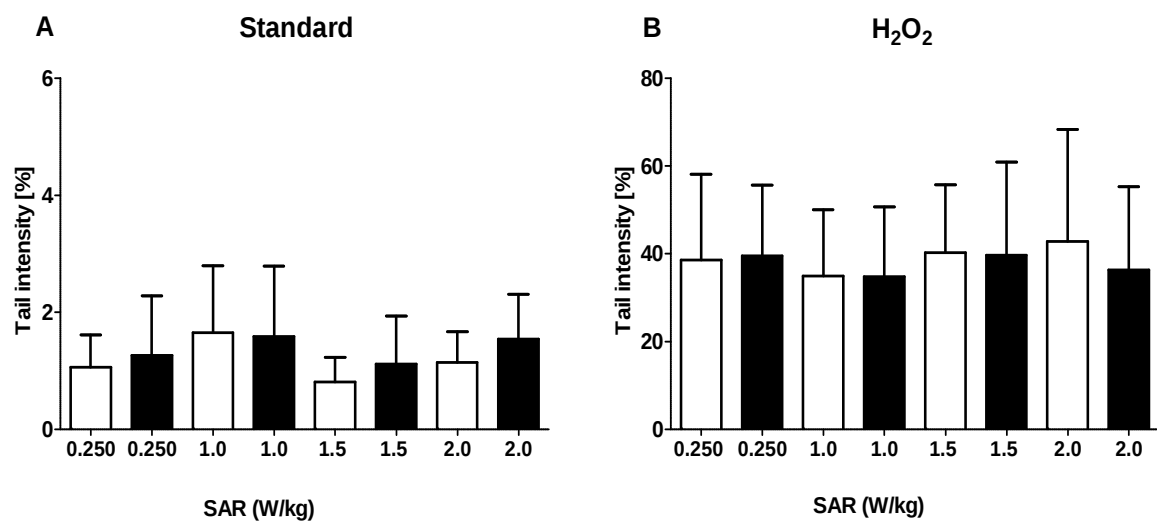


Figure 15. Results of the comet assay with ES-1 after 16 h exposure in a RF-incubator (0.250 W/kg, 1 W/kg, 1.5 W/kg and 2 W/kg). White bars represent sham exposed cells and black bars EMF-exposed cells. Bars represent means $\pm$ SD of results obtained with three cultures per experimental point.

4. Discussion

The discussion section consists three chapters. The first concerns the background levels which were detected in untreated control cultures with the different protocols. The second paragraph describes the results which were obtained after RF exposure of the cells. The findings of these experiments are discussed in comparison with the results of earlier in vitro experiments. The last part of the discussion section contains concluding remarks and suggestions for future research.

4.1. Background values of the untreated controls

The extent of comet formation which was found in the five cell lines with the different protocols is summarized in table 4. It can be seen, that the levels of spontaneous DNA damage vary quite strongly, i.e. in the glioma cells (U-87) the % DNA in tail was almost five-fold higher as in SH-SY5Y, ES-1 and HepG2 (in which values between 0.7 and 0.78 were found).

The different cell lines have been used in earlier SCGE studies, and the extent of comet formation which was found in these trials under standard conditions is in general in the same order of magnitude. For ES-1, a value of ~4.0 was reported by Diem et al. [76]; He et al. [77] published a value of ~1.0 from an experiment with SH-SY5Y cells. The corresponding values for HepG2 and Tr-146 cells are 3.35 and 11.21 [78].

Also in regard to the sensitivity towards ROS, substantial differences were seen. The ranking order of H₂O₂ sensitivity declined in the order HepG2> ES-1> SH-SY5Y> U-87> TR-146. It is possible that the differences in the responses reflect the efficiency of the antioxidant defense systems of the individual cell lines. HepG2 cells were used in earlier experiments with H₂O₂ challenge by An et al. [79], and an average induction of 37.56 % DNA in tail was reported, while Koller et al. [75] published a lower value (11.6).

However, the results can be not directly compared due to differences in the exposure time cultivation conditions (treatment of the cells on slides versus treatment in 24-wells).

Table 4. The background level of DNA migration in the different cell lines. It represents the mean of % of DNA in tail $\pm$ SD.

Protocol	HepG2	TR-146	ES-1	SH-SY5Y	U-87
Standard	0.70 $\pm$ 0.11	2.27 $\pm$ 1.83	0.78 $\pm$ 1.10	0.74 $\pm$ 0.38	3.40 $\pm$ 3.14
H₂O₂	41.90 $\pm$ 16.95	13.15 $\pm$ 14.82	41.17 $\pm$ 19.50	34.11 $\pm$ 11.52	23.59 $\pm$ 24.36
Endo III	3.32 $\pm$ 6.73	7.40 $\pm$ 1.93	4.61 $\pm$ 11.81	9.43 $\pm$ 3.77	8.99 $\pm$ 3.65
FPG	7.06 $\pm$ 7.44	8.17 $\pm$ 6.69	3.62 $\pm$ 15.34	17.17 $\pm$ 11.69	4.42 $\pm$ 7.14

As shown in the graphs (page 43), a clear increase of DNA migration was seen when isolated nuclei were treated with the lesion specific enzymes. These findings indicate that the enzymes which were used had a sufficiently high activity. It is interesting that the extent of endogenously oxidized purines (FPG treatment) was in the SH-SY5Y cell line approximately two-fold higher as in TR-146 and HepG2 and five-fold higher than in ES-1 and U-87.

Also after Endo III treatment strong differences in comet formation were observed. In these experiments the most pronounced effect was detected in the neuron derived cells, followed by the glioblastoma line (U-87).

As in the case of the studies with ROS treatment, the differences of the findings which were obtained with the individual cell lines may reflect differences in the antioxidant defense mechanisms.

4.2. Results of SCGE assays after RF-exposure

As described above, clear negative results were obtained in the first experimental series with 1.0 W/kg in three out of five cell lines. In the neuroblastoma cell line SH-SY5Y moderate, but significant increase of comet formation was detected in Endo III treatment, also in the glioblastoma cells a positive result was found with this enzyme.

The further experiments were conducted with two cell lines. Fibroblasts were tested since it was reported in earlier publications that this cells are sensitive to RF-exposure [54, 76]. Additionally, dose response experiments were conducted with the glioblastoma line to find out if the positive result can be reproduced. This line is of particular interest in the fact, that induction of glioblastomas were found in epidemiological studies in mobile phone users [51].

As described in the results (chapter 3.3.2), we observed again increased comet formation with FPG after RF- exposure of the cells. This effect was seen with all doses but failed to reach significance. Furthermore, also increases were observed with the second lesion specific enzyme Endo III and the cumulative evaluation of both experimental series indicates that this effect is significant (see appendix, table 14).

4.2.1. Overview of earlier in vitro experiments with mammalian cell lines

In total 75 in vitro studies with eukaryotic cells are mentioned in a comprehensive overview of Vijayalaxmi and Prihoda [80] which appeared in 2012. In the meantime computer-aided literature search in a biomedical database (PubMed) showed that 10 additional studies appeared in the last two years.

The majority of experiments were conducted with white blood cells from human (62 investigations) [14, 80, 81], other human cell lines were derived from glioblastomas (2 investigations) [80], fibroblasts were used in 2 trials [54, 76].

In addition, some studies were published in which human lens epithelial cells were used (3 investigations) [15, 80]. Experiments with rodent cells were conducted with cell lines which are widely used in routine genotoxicity testing such as Chinese hamster V79 [80].

A variety of endpoints was used in these experiments. Figure 16 shows that in most studies DNA migration was monitored in SCGE assays followed by MN tests and CA assays. In few trials other endpoints such as induction of SCE and HPRT-mutations were monitored.

The SCGE experiments were conducted in most investigations under standard conditions; only in few experimental series the cells were treated with radical generating chemicals. According to our knowledge, no data have been published so far which concern the formation of oxidized DNA bases with restriction enzymes.

It is notable that a variety of different exposure conditions was studied: 57 investigations concerned the effects of mobile phone specific fields, additionally also the impact of other exposures (e.g. microwaves) was analyzed. In regard to the present study it is relevant that only two earlier investigations were conducted with UMTS [54, 82].

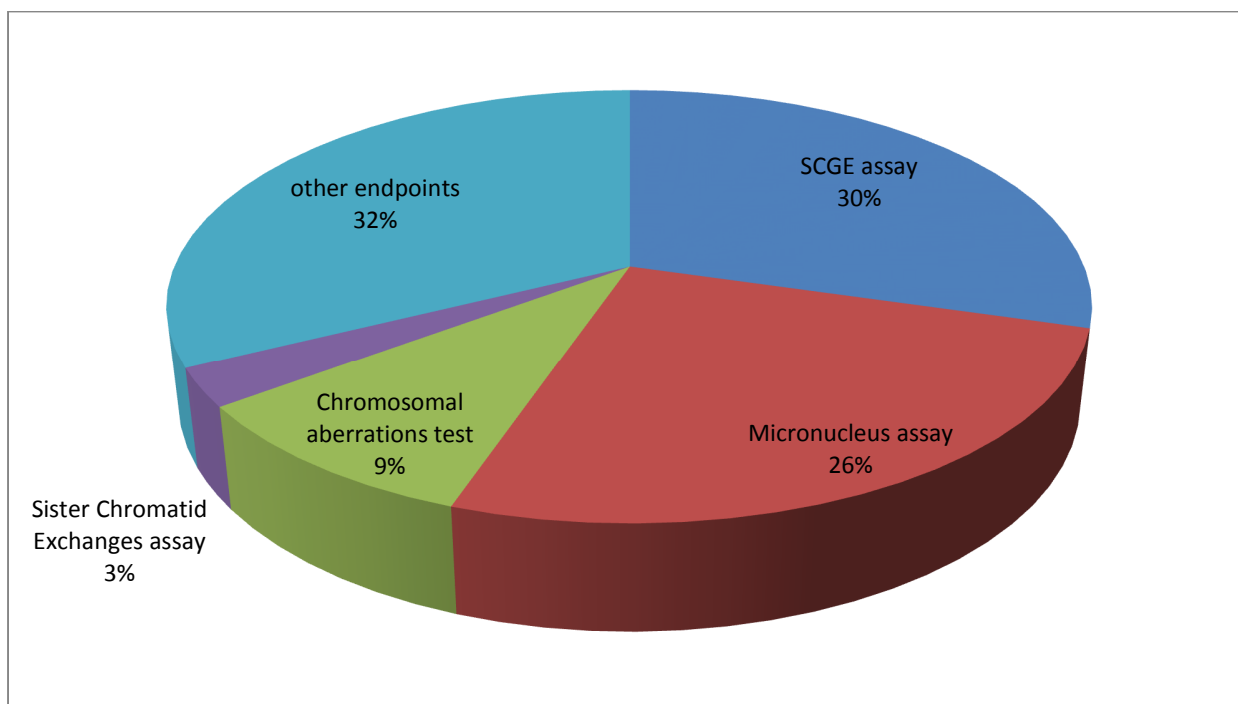


Figure 16. Overview on endpoints which were used in RF-studies (include microwaves, mobile phones, etc.). The diagram is based on studies described in the review of Verschaeve et al. [11] and on computer aided search in biomedical database.

In most RF-studies, negative results were found, regardless of the experimental model and radiation type.

It is mentioned in a statement of Rüdiger [83], that genotoxic effects of RF-EMF fields are described in 30 publications. However, a closer look of the individual studies shows that many endpoints do not reflect DNA damage in a strict sense.

4.2.2. Results of earlier SCGE studies

Apart from the articles which are described in the meta-analysis of Vijayalaxmi and Prihoda [80] four new papers were published in which comet formation was used as an endpoint [14, 15, 41, 84]. Only in 12 publications positive findings were reported. Three of them come from Sun et al. (Zhejiang University, China) who works with human lens epithelial cells [22, 23, 85], two came from the Medical University of Vienna from Diem et

al. and Schwarz et al. [54, 76]. Furthermore, an Italian group detected a positive effect in a human trophoblast HTR-8/SVneo cell line [29]. It is also notable, that positive findings were repeatedly reported in vivo experiments with rats by Lai et al. [86] who analyzed comet formation in brain cells after exposure of the animals.

The only investigation which is directly comparable to the present study in regard to the experimental model has been published by Schwarz et al. [54]. The authors used ES-1 cells and in addition also human lymphocytes. The cells were exposed in the same type field generator, to doses between 0.5-2.0 W/kg. Subsequently, the authors analyzed the extent of comet formation by visual scoring and found significant induction of comets in the fibroblasts.

This study was criticized by Lerchel [56], who stated that the standard deviation revealed a comprehensively low coefficient of variation which was in contrast to earlier studies by the same group. Additionally, he also stated the shortcomings of the statistical analysis. A response to the comments of Lerchel was published in 2009 by Rüdiger [83].

In regard to the results of the present investigation, it is notable that the standard deviations which we obtained in the same cells were substantially higher and that no indication for induction of comets was seen in any of the experiments. In this context it is notable that also Speit et al. [55] repeated the experiment with ES-1 cells with the same equipment under identical exposure conditions and found no indication for a positive result in his experiments.

4.2.3. Induction of DNA damage in the glioblastoma line U-87

As described in detail in the results section, we found an indication for formation of oxidatively damaged purine in experiments with FPG (fig. 11 E, page 43). Furthermore, we detected also a significant effect in the first series with Endo III (fig. 13 E, page 47). Also in the follow-up study the percentage DNA in tail was higher after RF-exposure, but this effect was not significant.

It was postulated in earlier investigations that ROS formation plays a role in the genotoxic properties of RF. For example, Xu et al. [15] found evidence for slightly increased concentration of ROS in human skin fibroblasts after 24h exposure to 3.0 W/kg with 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, which enables the detection of intercellular radicals.

In two investigations it was hypothesized that ROS formation due to the Haber-Weiss reaction occurs as a consequence of RF-exposure [87, 88]. Zmyslony et al. [87] found DNA damage in comet assay with lymphocytes under standard conditions when the cells were exposed to 50 Hz in presence of FeCl₂. However they did not confirm this assumption by additional biochemical investigations. Further evidence comes from a study with cultured plants (tobacco cells), in which a decrease of the activities of antioxidant enzymes (CAT and ascorbate peroxidase) and an increase of lipid peroxidation was detected after treatment with 10 and 30 mT (millitesla) [88]. Also Lai et al. [86] placed the involvement of Fe ions on they reported on comet formation in brain neurons of rats after treatment of the animals with 0.01 mT for 24 h. These effects were attenuated when the rats there pretreated with an iron chelator.

The impact of ROS on induction of comet formation in rat brains by RF was confirmed to a certain extent by the finding of Kesari et al. [89] who detected a decrease of SOD and an increase of catalase after treatment of the animals with 50 GHz.

Table 4 (page 52) shows that the SH-SY5Y and U-87 have substantial higher levels of endogenous formation of oxidative purines and pyrimidine. It is an indication that these cell lines may be more sustainable to oxidative damage as the other cell types which were used in the present study.

It is interesting that evidence for induction of oxidative DNA damage was restricted in the present study to cells from the nervous system (SH-SY5Y) and to glioblastoma derived cells (U-87). As described in the introduction, several epidemiological findings indicate

that RF induces glioblastomas in human. Therefore a number of investigations have been conducted with cultured neuronal cells [80]. Furthermore, also in vivo investigations were carried out with laboratory rodents. The results of these investigations are reviewed in a paper by Kesari et al. [90]. The review concludes that the regular and long term use of RF can have a negative effect upon biological system especially on brain (e.g. reduction of the memory function, increased ROS levels, and changes in gene and protein expression).

4.3. Conclusions and outlook

Taken together, the results of the present study shows that a dose of 1.0 W/kg does not cause measurable effects in buccal, liver and fibroblast cell lines.

However, some indication for induction of oxidative damage was found in cell lines from the nervous system. This observations support the results of earlier findings which indicate that RF-induced oxidative damage causes comet formation in brain tissue of rodents.

On the base of these findings, it can be tentatively assumed that the molecular basis of induction of glioblastomas, which was found to be associated in humans as a consequence of frequent use of cell phones, may be due to induction of oxidative DNA damage in cells of the central nervous system. However, more experimental evidence is required to harden this assumption.

5. Abstract

The potential health effects of mobile phone specific electromagnetic fields (EMF) are currently controversial discussed. In 2012, the IARC classified EMF (Electromagnetic fields) radiation into group 2B (possibly carcinogenic to humans) and it was postulated in several studies that the induction of tumors may be due to different molecular mechanisms, including DNA damage and oxidative stress. Aim of the present study was the investigation of the induction of oxidative and non-oxidative DNA-damage and of alterations of the sensitivity in human cell lines to reactive oxygen species (ROS) as a consequence of exposure to Universal Mobile Telecommunications Service (UMTS) radiation. According to our knowledge, only one in vitro study has been published so far with this type of EMF in which the indication of DNA damage was investigated. In the present study inductions of single and double strand breaks and formation of oxidatively damaged DNA base and changes of the sensitivity to H₂O₂ were monitored. Induction of DNA damage was determined with single cell gel electrophoresis assay (SCGE) in five human derived cell lines namely in the human diploid fibroblast cell line (ES-1), in human glioblastoma (U-87) cells, in the human buccal derived cell line (Tr-146), in a human neuroblastoma line (SH-SY5Y) and in a human hepatocellular carcinoma line (HepG2). The SCGE assay, which was conducted according to international guidelines, is based on the determination of DNA migration in an electromagnetic field. With ES-1 fibroblasts clear negative results were obtained under all experimental conditions. These findings are of interest since positive results have been detected with this cell line in a previous study with UMTS. However, these results could not be reproduced in following up experiments. Also with HepG2 cells and buccal cells Tr-146 clear negative results were obtained, while induction of formation of oxidized purines were detected in the neuronal cell line SH-SY5Y after exposure to 1.0 W/kg. Furthermore, the U-87 cell-line indicates an increased formation of oxidized DNA bases. The effect was not dose dependent, but significant in the experiments with the restriction enzyme ENDO III in the first experimental series. Also under standard conditions and in the case of FPG, negative effects were seen in the second experimental series. The significance was

seen, when the data were evaluated in a pooled analysis. Taken together, our findings suggest that mobile phone specific EMF, which are currently used may induce oxidative DNA damage in glioblastoma and possibly also in the neuroblastoma cell line (SH-SY5Y). These observations are of interest in regard to epidemiological studies in which increased rates of glioblastoma were observed in mobile phone users. However, further verification is required in order to substantiate the present findings.

6. Zusammenfassung

Die gesundheitlichen Effekte von Handy spezifischen elektromagnetischen Feldern werden derzeit kontroversiell diskutiert. 2012 qualifizierte die Internationale Agentur für Krebsforschung (IARC) EMF-Strahlung als Gruppe 2B "möglicherweise krebserregend für Menschen" und es wurde in verschiedenen Untersuchungen postuliert, dass die Induktion von Tumoren durch verschiedene, molekulare Mechanismen erfolgt, unter anderem durch DNA-Schäden und oxidativem Stress. Ziel der vorliegenden Studie war die Untersuchung von oxidativen und nicht-oxidativen DNA-Schäden sowie von Veränderungen der Empfindlichkeit humaner Zelllinien gegenüber Schädigung reaktive Sauerstoffspezies (ROS) als Folge von der Exposition mit UMTS-Strahlung. Nach unserer Kenntnis wurde bisher nur eine in vitro-Studie publiziert, in der diese Art von EMF-Strahlung in Bezug auf die Auslösung von DNA Schäden untersucht wurde. In der vorliegenden Studie wurde die Induktion von Einzel-und Doppelstrangbrüchen sowie die Bildung oxidativ geschädigter DNA Basen sowie Veränderungen der Empfindlichkeit gegenüber Wasserstoffperoxid untersucht. Die DNA Schädigung wurde in Einzelzellgelelektrophorese Assay in fünf verschiedenen humanen Zelllinien untersucht, nämlich in der humanen diploiden Fibroblasten Linie ES-1, die Glioblastoma Linie U-87, Neuroblastoma SH-SY5Y Zelllinie, Leberkarzinom HepG2 und in der epithelialen Zelllinie Tr-146. Die SCGE Experimente wurden entsprechend internationalen Richtlinien durchgeführt, und basiert auf die Messung der DNA-Migration in einem elektromagnetischen Feld. Eindeutig negative Ergebnisse wurden mit der ES-1 Fibroblasten Zelllinie unter allen experimentellen Bedingungen erhalten. Diese Erkenntnisse sind von Interesse, da positive Resultate mit dieser Zelllinie in einer früheren Studie mit UMTS erhalten wurden. Allerdings konnten diese Resultate in dieser Studie nicht reproduziert werden. Auch mit HepG2 Zellen und mit der bukkalen Zelllinie Tr-146 wurden eindeutig negative Resultate erhalten. Die Induktion der Bildung von oxidiertem Pyrimidin wurde in der neuronalen Zelllinie SH-SY5Y und in der U-87 Zelllinie nach einer Bestrahlung von 1.0 W/kg ermittelt. In der zweiten Versuchsserie wurde ein Effekt unter Standardbedingungen sowie FPG beobachtet. Dieser Effekt war nicht dosisabhängig jedoch wurde die Signifikanz nach einer gepoolten Analyse der Daten,

bestätigt. Insgesamt deuten die Ergebnisse, dass Handy-spezifischen EMF, die derzeit Verwendung sind oxidative DNA-Schäden in Glioblastom und möglicherweise auch in der Neuroblastom-Zelllinie SH-SY5Y verursachen. Diese Beobachtungen sind von Interesse in Hinblick auf epidemiologische Studien, in denen Hinweise auf erhöhte raten von Glioblastomen bei Handy-Nutzer beobachtet wurden. Allerdings sind weitere experimentelle Untersuchungen erforderlich, um diese vorliegenden Ergebnisse zu bestätigen.

7. References

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8. Appendix

Table 5. Results of the comet assay with the ES-1 cell line after 16h exposure in a RF-incubator. The cell line was exposed to 1.0 W/kg and treated according to the different protocols¹. Numbers indicate means and 95 % CI.

ES-1			
Experimental conditions	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
Endo III	1	3.32	1.33
	2	6.37	7.93
	3	7.29	6.02
	Mean value (95% CI)	5.53 (1.41 – 12.11)	4.61 (0.00 – 17.98)
	P=0.369		
FPG	1	3.87	0.60
	2	4.68	3.61
	3	9.25	9.06
	Mean value (95% CI)	5.73 (0.90 – 14.36)	3.62 (0.84 – 20.89)
	P=0.268		
H ₂ O ₂	1	30.62	58.51
	2	28.86	36.55
	3	32.58	47.75
	4	43.45	22.04
	5	57.85	54.35
	6	35.78	29.97
	Mean value (95% CI)	38.01 (27.07 – 49.61)	41.17 (26.43 – 56.78)
	P=0.340		
Standard	1	0.43	1.26
	2	1.17	0.48
	3	0.77	0.69
	Mean value (95% CI)	0.76 (0.12 – 1.95)	0.78 (0.11 – 2.03)
	P=0.475		

¹ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 6. Results of the comet assay with the U-87 cell line after 16h exposure in a RF-incubator. The cell line was exposed to 1.0 W/kg and treated according to the different protocols.². Numbers indicate means and 95 % CI.

U-87			
Experimental conditions	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
Endo III	1	13.72	7.46
	2	13.04	9.15
	3	16.51	10.48
	Mean value (95% CI)	14.39 (10.19 – 19.18)	8.99 (5.57 – 13.13)
	P=0.008		
FPG	1	7.03	5.72
	2	2.58	1.87
	3	6.75	5.82
	Mean value (95% CI)	5.24 (0.66 – 13.79)	4.24 (0.31 – 12.33)
	P=0.330		
H ₂ O ₂	1	17.68	19.29
	2	37.10	57.25
	3	47.20	26.47
	4	59.79	21.01
	5	18.67	5.89
	6	39.79	20.38
	Mean value (95% CI)	35.98 (19.59 – 54.26)	23.59 (8.64 – 43.09)
	P=0.119		
Standard	1	3.32	3.68
	2	3.80	3.13
	3	3.60	
	Mean value (95% CI)	3.57 (3.00 – 4.19)	3.40 (0.78 – 7.76)
	P=0.290		

² As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 7. Results of the comet assay with the SH-SY5Y cell line after 16h exposure in a RF-incubator. The cell line was exposed to 1.0 W/kg and treated according to the different protocols.³. Numbers indicate means and 95 % CI.

SH-SY5Y			
Experimental conditions	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
Endo III	1	16.78	11.27
	2	16.73	8.20
	3	12.32	8.95
	Mean value (95% CI)	15.22 (9.31 – 22.26)	9.43 (5.90 – 13.70)
	P=0.014		
FPG	1	32.46	14.69
	2	27.36	14.34
	3	13.45	22.98
	Mean value (95% CI)	23.89 (4.42 – 52.42)	17.17 (7.15 – 30.38)
	P=0.173		
H ₂ O ₂	1	27.63	36.43
	2	31.44	21.24
	3	25.21	40.25
	4	40.41	36.79
	5	26.82	43.68
	6	32.03	27.68
	Mean value (95% CI)	30.49 (24.96 – 36.30)	34.11 (25.45 – 43.33)
	P=0.200		
Standard	1	0.68	0.70
	2	0.68	0.92
	3	1.24	0.62
	Mean value (95% CI)	0.85 (0.26 – 1.78)	0.74 (0.41 – 1.18)
	P=0.306		

³ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 8. Results of the comet assay with the Tr-146 cell line after 16h exposure in a RF-incubator. The cell line was exposed to 1.0 W/kg and treated according to the different protocols⁴. Numbers indicate means and 95 % CI.

Tr-146			
Experimental conditions	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
Endo III	1	8.50	7.33
	2	6.27	8.29
	3	7.01	6.63
	Mean value (95% CI)	7.23 (4.70 – 10.26)	7.40 (5.48 – 9.59)
	P=0.421		
FPG	1	5.06	7.65
	2	5.54	6.02
	3	10.04	11.22
	Mean value (95% CI)	6.72 (1.74 – 14.63)	8.17 (2.92 – 15.75)
	P=0.268		
H ₂ O ₂	1	13.57	14.39
	2	16.77	10.79
	3	40.85	33.70
	4	56.70	15.73
	5	24.81	3.37
	6	9.42	8.23
	Mean value (95% CI)	25.53 (9.48 - 46.12)	13.15 (4.69 - 25.01)
	P=0.078		
Standard	1	3.48	1.72
	2	1.60	2.07
	3	2.01	3.12
	Mean value (95% CI)	2.30 (0.54 - 5.24)	2.27 (0.85 - 4.35)
	P=0.481		

⁴ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 9. Results of the comet assay with the HeoG2 cell line after 16h exposure in a RF-incubator. The cell line was exposed to 1.0 W/kg and treated according to the different protocols⁵. Numbers indicate means and 95 % CI.

HepG2			
Experimental conditions	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
Endo III	1	2.39	0.50
	2	6.98	6.22
	3	3.67	1.38
	4	13.42	9.81
	5	16.43	2.45
	Mean value (95% CI)	7.72 (1.98 - 16.78)	3.32 (0.33 - 9.22)
	P=0.092		
FPG	1	10.66	8.64
	2	7.15	4.94
	3	4.02	3.04
	4	12.29	5.79
	5	6.75	15.47
	6	10.02	
	Mean value (95% CI)	8.25 (5.26 - 11.85)	7.06 (2.55 - 13.59)
	P=0.311		
H ₂ O ₂	1	52.73	43.30
	2	52.16	53.17
	3	47.18	23.23
	4	52.55	37.60
	5	49.29	56.90
	6	21.11	34.09
	Mean value (95% CI)	45.53 (32.19 - 59.19)	41.11 (28.21 - 54.68)
	P=0.283		

⁵As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 9. Results of the comet assay with the HeoG2 cell line after 16h exposure in a RF-incubator. The cell line was exposed to 1.0 W/kg and treated according to the different protocols⁶, continuous.

HepG2			
Experimental conditions	Experimental conditions	Experimental conditions	Experimental conditions
Standard	1	0.93	0.71
	2	0.42	0.64
	3	1.29	0.81
	4	0.18	0.71
	5	0.45	0.59
	6	0.25	0.77
	Mean value (95% CI)	0.52 (0.19 - 1.02)	0.70 (0.62 - 0.79)
	P=0.168		

⁶As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 10. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) under standard conditions. The cell line was exposed to 1.0 W/kg and treated according to the different protocols⁷. Numbers indicate means and 95 % CI.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg	1	2.42	1.88
	2	2.43	0.51
	3	1.75	1.04
	4	0.92	1.38
	5	2.06	1.16
	6	2.81	1.01
	Mean value (95% CI)	2.01 (1.32 - 2.84)	1.12 (0.69 - 1.66)
		P=0.013	
0.500 W/kg	1	1.65	1.67
	2	2.83	1.42
	3	1.77	0.52
	4	2.81	2.29
	5	2.46	2.03
	6	2.06	0.46
	Mean value (95% CI)	2.24 (1.73 - 2.82)	1.29 (0.58 - 2.28)
		P=0.025	
1.0 W/kg	1	1.07	0.95
	2	0.65	0.64
	3	0.74	1.04
	4	0.98	0.20
	5	1.97	1.26
	6	1.25	0.63
	Mean value (95% CI)	1.07 (0.66 - 1.59)	0.74 (0.37 - 1.23)
		P=0.106	

⁷ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 10. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) under standard conditions. The cell line was exposed to 1.0 W/kg and treated according to the different protocols⁸, continuous.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg Repeat experiment	1	5.39	2.62
	2	4.55	4.32
	3	3.37	3.95
	Mean value (95% CI)	4.40 (2.21 - 7.29)	3.59 (1.65 - 6.24)
		P=0.184	
0.500 W/kg Repeat experiment	1	3.55	2.79
	2	3.60	3.94
	3	3.82	1.06
	Mean value (95% CI)	3.66 (3.31 - 4.02)	2.44 (0.12 - 7.61)
		P=0.141	

Table 11. Results of the ANOVA analysis with the U-87 cell line under standard conditions.

Source of variance	df1/df2	F	p-value
Exposition	1/3	90.394	0.002
SAR	2/4	5.012	0.081
Experiment	3/2	20.360	0.063

⁸ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 12. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) and H₂O₂ treatment⁹. Numbers indicate means and 95 % CI.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg	1	34.79	10.42
	2	23.38	34.90
	3	28.37	30.94
	4	26.16	33.30
	5	41.06	42.56
	6	44.17	29.63
	Mean value (95% CI)	32.76 (24.29 - 41.85)	29.61 (18.08 - 42.64)
	P=0.304		
0.500 W/kg	1	13.55	19.87
	2	27.81	32.80
	3	40.25	19.40
	4	27.27	21.10
	5	31.34	35.21
	6		31.57
	Mean value (95% CI)	27.56 (16.10 - 40.76)	26.39 (19.13 - 34.36)
	P=0.414		
1.0 W/kg	1	18.00	25.56
	2	24.03	32.04
	3	19.76	29.13
	4	26.82	16.97
	5	19.02	23.58
	6	22.50	
	Mean value (95% CI)	21.61 (18.20 - 25.22)	25.27 (18.34 - 32.89)
	P=0.111		

⁹ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 12. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) and H₂O₂ treatment¹⁰, continuous.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg Repeat experiment	1	26.90	21.58
	2	26.36	12.04
	3	13.31	19.13
	Mean value (95% CI)	21.81 (5.83 - 44.28)	17.38 (6.75 - 31.62)
	P=0.230		
0.500 W/kg Repeat experiment	1	22.34	18.37
	2	13.41	18.51
	3	28.10	12.34
	Mean value (95% CI)	20.94 (5.84 - 42.14)	16.30 (8.39 - 26.19)
	P=0.190		

Table 13. Results of the ANOVA analysis with the U-87 cell line with H₂O₂ treatment.

Source of variance	df1/df2	F	p-value
Exposition	1/4	3.263	0.144
SAR	2/4	2.886	0.165
Experiment	3/2	9.509	0.119

¹⁰ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 14. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) after Endo III treatment¹¹. Numbers indicate means and 95 % CI.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg	1	9.29	3.55
	2	6.54	4.50
	3	0.55	0.77
	4	3.89	7.02
	5	7.67	7.38
	Mean value (95% CI)	4.95 (1.18 - 11.13)	4.22 (1.26 - 8.79)
	P=0.376		
0.500 W/kg	1	3.58	2.33
	2	1.85	1.39
	3	7.85	6.55
	4	9.07	1.77
	5	2.97	4.27
	6	4.17	5.03
	Mean value (95% CI)	4.59 (2.14 - 7.90)	3.31 (1.53 - 5.74)
	P=0.187		
1.0 W/kg	1	4.64	3.80
	2	0.00	6.29
	3	1.87	3.67
	4	4.86	0.47
	5	5.82	0.79
	6	5.11	15.77
	Mean value (95% CI)	3.04 (0.62 - 7.20)	4.01 (0.52 - 10.55)
	P=0.343		

¹¹ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 14. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) after Endo III treatment¹², continuous.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg Repeat experiment	1	6.52	5.56
	2	0.61	1.68
	3	5.14	3.84
	Mean value (95% CI)	3.51 (0.27 - 17.25)	3.51 (0.28 - 10.15)
	P=0.500		
0.500 W/kg Repeat experiment	1	3.26	3.41
	2	3.73	4.25
	3	5.23	3.98
	Mean value (95% CI)	4.04 (1.91 - 6.90)	3.87 (2.86 - 5.03)
	P=0.403		

Table 15. Results of the ANOVA analysis with the U-87 cell line after Endo III treatment.

Source of variance	df1/df2	F	p-value
Exposition	1/3	0.008	0.935
SAR	2/4	0.746	0.533
Experiment	3/4	0.669	0.647

¹² As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 16. Percentage of DNA in tail, after exposure of U-87 to various SAR levels (0.250 W/kg, 0.500 W/kg and 1.0 W/kg) after FPG treatment¹³. Numbers indicate means and 95 % CI.

U-87			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg	1	1.79	4.56
	2	1.71	1.50
	3	5.62	3.56
	4	0.84	0.65
	5	2.98	1.11
	6	4.41	
	Mean value (95% CI)	2.65 (1.11 - 4.83)	2.03 (0.52 - 4.51)
	P=0.279		
0.500 W/kg	1	2.50	0.44
	2	8.19	3.49
	3	5.11	0.76
	4	6.76	2.96
	5	0.32	
	Mean value (95% CI)	3.92 (0.69 - 9.64)	1.65 (0.10 - 4.99)
	P=0.118		
1.0 W/kg	1	4.84	3.23
	2	1.74	0.16
	3	0.73	2.70
	4	0.68	
	5	1.37	
	Mean value (95% CI)	1.63 (0.33 - 3.88)	1.65 (0.39 - 9.89)
	P=0.495		
0.25 W/kg Repeat experiment	1	8.65	3.29
	2	3.48	6.92
	3	4.49	3.13
	Mean value (95% CI)	5.34 (0.79 - 13.58)	4.30 (0.73 - 10.64)
	P=0.304		
0.5 W/kg Repeat experiment	1	2.06	3.98
	2	6.60	2.09
	3	8.25	3.20
	Mean value (95% CI)	5.28 (0.15 - 16.97)	3.04 (1.10 - 5.90)
	P=0.151		

¹³ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 17. Results of the ANOVA analysis with the U-87 cell line after FPG treatment.

Source of variance	df1/df2	F	p-value
Exposition	1/4	7.767	0.048
SAR	2/6	4.857	0.053
Experiment	3/4	0.154	0.949

Table 18. Percentage of DNA in tail, after exposure of ES-1 to various SAR levels (0.250 W/kg, 1.0 W/kg, 1.5 W/kg and 2.0 W/kg) under standard conditions¹. Numbers indicate means and 95 % CI.

ES-1			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg	1	2.69	2.05
	2	3.04	1.72
	3	0.38	1.14
	4	0.90	0.72
	5	0.41	0.58
	6	0.99	0.62
	7	0.74	1.44
	8	0.92	0.71
	9		0.57
	Mean value (95% CI)	1.11 (0.49 - 1.97)	1.00 (0.64 - 1.44)
	P=0.380		
1.0 W/kg	1	2.27	1.92
	2	2.84	2.02
	3	3.56	0.96
	4	0.21	0.42
	5	0.26	3.30
	6	2.28	0.72
	7	0.88	1.42
	8	0.61	0.55
	9	1.35	3.53
	Mean value (95% CI)	1.35 (0.58 - 2.43)	1.47 (0.75 - 2.43)
	P=0.412		

Table 18. Percentage of DNA in tail, after exposure of ES-1 to various SAR levels (0.250 W/kg, 1.0 W/kg, 1.5 W/kg and 2.0 W/kg) under standard conditions, continuous¹⁴

ES-1			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
1.5 W/kg	1	1.50	0.63
	2	2.96	0.89
	3	0.48	0.64
	4	1.50	0.58
	5	0.93	0.49
	6	0.55	0.51
	7	1.20	0.96
	8	0.61	1.76
	9	0.30	
	Mean value (95% CI)	1.00 (0.52 - 1.63)	0.77 (0.50 - 1.10)
	P=0.208		
2.0 W/kg	1	1.69	0.79
	2	2.08	1.64
	3	2.63	2.22
	4	1.26	1.13
	5	1.30	1.18
	6	0.47	1.01
	7	0.80	0.82
	8	1.07	1.09
	9	2.60	0.43
	Mean value (95% CI)	1.46 (0.92 - 2.10)	1.10 (0.75 - 1.51)
	P=0.123		

Table 19. Results of the ANOVA analysis with the ES-1 cell line under standard conditions.

Source of variance	df1/df2	F	p-value
Exposition	1/2	0.278	0.650
SAR	3/6	3.853	0.074
Experiment	2/1	5.490	0.284

¹⁴ As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Table 20. Percentage of DNA in tail, after exposure of ES-1 to various SAR levels (0.250 W/kg, 1.0 W/kg, 1.5 W/kg and 2.0 W/kg) with H₂O₂ treatment¹. Numbers indicate means and 95 % CI.

ES-1			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
0.250 W/kg	1	17.75	7.55
	2	24.66	10.47
	3	29.74	23.36
	4	44.72	61.12
	5	70.36	52.07
	6	42.25	45.62
	7	45.70	46.25
	8	41.06	48.02
	9		52.55
	Mean value (95% CI)	39.10 (25.83 - 53.26)	36.97 (21.38 - 54.10)
	P=0.413		
1.0 W/kg	1	19.53	17.80
	2	8.61	16.64
	3	16.49	14.66
	4	47.09	32.28
	5	47.23	39.88
	6	36.85	50.09
	7	52.79	49.12
	8	42.85	50.87
	9	41.91	43.14
	Mean value (95% CI)	33.67 (21.30 - 47.29)	34.07 (22.60 - 46.59)
	P=0.479		

Table 20. Percentage of DNA in tail, after exposure of ES-1 to various SAR levels (0.250 W/kg, 1.0 W/kg, 1.5 W/kg and 2.0 W/kg) with H₂O₂ treatment, continuous¹⁵.

ES-1			
SAR	Experimental points	Exposed % DNA in tail	Sham-exposed % DNA in tail
1.5 W/kg	1	9.10	20.11
	2	16.23	13.25
	3	13.35	52.78
	4	44.99	38.14
	5	50.13	50.23
	6	43.36	46.53
	7	66.37	51.25
	8	57.78	50.34
	9	55.21	
	Mean value (95% CI)	38.12 (21.58 - 56.22)	39.56 (26.30 - 53.64)
P=0.443			
2.0 W/kg	1	16.09	3.77
	2	10.68	19.20
	3	7.68	9.12
	4	49.73	47.14
	5	47.03	56.79
	6	53.90	56.25
	7	50.33	54.71
	8	49.68	61.90
	9	41.50	76.02
	Mean value (95% CI)	34.61 (19.69 - 51.27)	40.53 (19.85 - 63.16)
P=0.313			

Table 21. Results of the ANOVA analysis with the ES-1 cell line with H₂O₂ treatment.

Source of variance	df1/df2	F	p-value
Exposition	1/2	1.864	0.304
SAR	3/6	0.568	0.656
Experiment	2/6	1.029	0.486

¹As described in material and methods (chapter 2.6), for the statistical evaluation transformed data were used.

Curriculum Vitae

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