



Micronucleus frequency in buccal mucosa cells of mobile phone users

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ABSTRACT

Mobile phones are being used extensively throughout the world, with more than four billion accounts existing in 2009. This technology applies electromagnetic radiation in the microwave range. Health effects of this radiation have been subject of debate for a long time, both within the scientific community and within the general public. This study investigated the effect of mobile phone use on genomic instability of the human oral cavity's mucosa cells. 131 Individuals donated buccal mucosa cells extracted by slightly scraping the oral cavity with a cotton swab. Every participant filled out a questionnaire about mobile phone use including duration of weekly use, overall period of exposure and headset usage. 13 Individuals did not use mobile phones at all, 85 reported using the mobile phone for three hours per week or less, and 33 reported use of more than three hours per week. Additionally, information on age, gender, body weight, smoking status, medication and nutrition was retrieved. For staining of the cells a procedure using α -tubulin-antibody and chromomycin A₃ was applied. Micronuclei and other markers were evaluated in 1000 cells per individual at the microscope. A second scorer counted another 1000 cells, resulting in 2000 analyzed cells per individual. Mobile phone use did not lead to a significantly increased frequency of micronuclei.

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1. Introduction

Mobile phones are being used extensively throughout the world. According to the European Information Technology Observatory, there were more than four billion accounts in 2009. Especially in newly industrialized countries like India and China prevalence of mobile phones is still increasing at enormous rates. But also in Western Europe and North America the markets for telecommunication applications are still growing even though penetration rates (subscriptions per inhabitants) have exceeded 100% already. Especially young people employ mobile phones not only for calls but also for messages, data transfer, games and other applications (source: <http://www.eito.com/>).

This technology is based upon electromagnetic radiation of the microwave frequency range. Frequency and modulation standard of the radiation vary depending on the region of the world. In Germany standards of the so called second and third generation are applied. The most important second generation standard is "Global System for Mobile Communication" (GSM) which uses frequencies of around 900 MHz and 1800 MHz. Throughout the world this standard is used in more than 670 networks in over 200 countries. Higher frequencies of around 2100 MHz are used by the most common third generation standard "Universal Mobile Telecommu-

nication System" (UMTS). This system is used in more than 100 countries. For all those frequencies (up to 300 GHz) the International Commission on Non-Ionizing Radiation Protection (ICNIRP) has established guidelines for exposure limitations (ICNIRP, 2009) that have been adopted by national regulations in many countries.

Effects of radiofrequency (RF) radiation on biological systems on all levels from the organism to single molecules have been subject of debate for a long time not only within the scientific community but also within the general public. Epidemiologic investigations mostly focused on a putative connection between mobile phones and cancer. Whereas some authors do see a link, e.g. Khurana et al. (2009), most of the investigations cannot confirm an elevated risk (for review see (Moulder et al., 2005; Schuz et al., 2009)).

There have been investigations showing alterations of enzyme activity (Ammari et al., 2008; Friedman et al., 2007), others did not see any changes (Huang et al., 2008). Contradicting results are also found for cell proliferation (Capri et al., 2004; Lee et al., 2008), production of reactive oxygen species (Guney et al., 2007; Hoyto et al., 2008) and many other biological endpoints. Some effects have been consistently observed in replicate experiments, e.g. spindle disturbances (Schmid and Schrader, 2007; Schrader et al., 2008).

More than 100 studies investigated the effect of RF radiation on DNA. With regard to gene expression, some studies have found effects while other studies were not able to detect any alterations (McNamee and Chauhan, 2009). Some investigations found genotoxic effects, e.g. Luukkonen et al. (2009), whereas the majority of studies could not confirm these findings (Huang et al., 2008; Speit et

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Table 1
Characteristics of the study population (Mean, [Min–Max]).

		Total	Male	Female
Gender		100.0%	41.2%	58.8%
Age [years]		26.1 [19–84]	27.4 [19–84]	25.2 [20–64]
	19–24	67.2%	63.0%	70.1%
	25–39	26.7%	29.6%	24.7%
	40–84	6.1%	7.4%	5.2%
Body-mass-index [kg/m ²]		22.0 [17.0–31.2]	23.0 [17.6–31.2]	21.3 [17.0–30.5]
	<20	22.1%	5.6%	33.8%
	20–25	64.9%	77.8%	55.8%
	>25	13.0%	16.6%	10.4%
Smoking status	Non-smoker	84.8%	81.5%	87.0%
	<10 cigarettes/day	7.6%	11.1%	5.2%
	≥10 cigarettes/day	7.6%	7.4%	7.8%
Headset usage	Non-users	78.6%	78.3%	78.9%
	Users	21.4%	21.7%	21.1%
Overall period of exposure	<5 years	18.8%	20.5%	17.6%
	5–10 years	64.3%	63.6%	64.8%
	≥10 years	16.3%	15.9%	17.6%
Duration of phone use [h/week]	0	9.9%	14.8%	6.5%
	0–3	64.9%	66.7%	63.6%
	3–6	13.7%	11.1%	15.6%
	6–9	9.2%	4.0%	10.4%
	9–12	1.5%	0.0%	2.6%
	12–15	0.8%	0.0%	1.3%

al., 2007) (for review see (Verschaeve and Maes, 1998; Vijayalaxmi and Prihoda, 2008)).

The micronucleus test is one of the most established procedures to measure DNA damage (OECD, 2007; Stopper and Müller, 1997). It was originally developed by Boller and Schmid (1970) and later improved by Fenech and Morley (1985) by introducing the application of cytochalasin B. The test quantifies the occurrence of small nuclear bodies termed micronuclei. They contain broken chromosome fragments in case of a clastogenic event and/or whole chromosome in case of an aneugenic event. Generally micronuclei emerge during mitosis when chromosomes or chromosomal fragments are not distributed to the daughter nuclei but lag behind and are surrounded by their own nuclear membrane. Laboratory protocols have been validated and standardized comprehensively (Bonassi et al., 2001; Fenech et al., 2003a,b; OECD, 2007; Reifferscheid et al., 2008; von der Hude et al., 2000) for lymphocytes and cell lines. The micronucleus frequency in peripheral blood lymphocytes correlates with the risk for cancer and cardiovascular disease (Bonassi et al., 2007; Murgia et al., 2007) making it a reliable biomarker. According to recent suggestions other anomalies like nucleoplasmic bridges or nuclear buds may be scored in parallel with micronuclei, making the micronucleus test a “cytome” assay (Fenech, 2006).

Micronuclei can also be counted in exfoliated cells (Majer et al., 2001), especially in buccal mucosa cells (Thomas et al., 2009; Titenko-Holland et al., 1994). This technique was originally developed by Stich et al. (1982a,b) and its validation in interlaboratory comparison studies is currently underway (Bonassi et al., 2009; Holland et al., 2008). It has been used to investigate the effects of malignancies (Benner et al., 1994), pesticides (Bortoli et al., 2009), smoking (El-Setouhy et al., 2008) and other influences.

Besides the advantage of a very fast and easy cell extraction, the buccal mucosa micronucleus test is especially suitable to analyze effects of RF radiation because the oral cavity is located within the area of exposure. So far only one investigation applied this assay to evaluate whether mobile phone use is associated with genomic instability in buccal cells (Yadav and Sharma, 2008). The authors found more than twice as many micronuclei in mobile phone users compared to non-users. They also described an increase of the

micronucleus frequency in relation to the overall period of exposure.

The present study was executed to verify whether those results could be confirmed in a different population using a larger cohort and counting double the amount of cells to increase statistical power and using a different, DNA-specific staining procedure.

2. Materials and methods

2.1. Chemicals and solutions

All chemicals were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Extraction solution contained 0.9% sodium chloride in demineralized water. Washing buffer contained 0.01 M Tris–HCl, 0.1 M EDTA and 0.02 M sodium chloride in demineralized water. For the staining procedure anti- α -tubulin-antibody solution was diluted 1:50 and chromomycin A₃ was dissolved in phosphate buffered saline (PBS) containing 150 mM magnesium chloride, giving a final concentration of 100 mM of chromomycin A₃.

2.2. Participants

Before initiation this study was approved by the Ethical Advisory Board of the hospital of the University of Würzburg (203/08). By statistical power analysis, a minimum group size of $n=9$ was calculated to detect the same increase in micronucleus frequencies seen by Yadav and Sharma (Yadav and Sharma, 2008). Participants were recruited (a) from students by announcement in lectures, (b) from university employees by internal communication, and (c) from the general public by newspaper announcement. All individuals received written information about the purpose of this study and the approval of the Ethical Advisory Board. Written consent to participation was requested and additionally a questionnaire about duration of daily mobile phone use, overall period of exposure and usage of headsets was filled out. Supplementary information including gender, age, body height and weight, smoking status, medication and nutrition habits was retrieved. Special attention was paid to including age-matched non-users. Proband characteristics are shown in Table 1.

To ensure that the test procedure actually allows detecting chromosomal damage, positive controls were added. These consisted of four patients receiving radiotherapy (25–29 single doses of 2.0 Gy or 2.3 Gy of γ -radiation during four weeks before sampling) compared to four healthy control individuals. Patient characteristics are included in Table 2.

2.3. Sample preparation

Cells were extracted by rubbing a cotton swab connected to a small wooden stick in both inner cheeks, applying light pressure while moving the swab up and down and turning it. Then, cells were gathered in a tube containing 5 ml of extraction

Table 2

Micronucleus frequencies per 1000 cells of patients receiving radiotherapy and control individuals.

	Age	Gender	Radiation Doses	MN-containing cells	Total number of micronuclei
Controls					
# 1	29	Male	–	1.0	1.0
# 2	21	Male	–	1.5	1.5
# 3	20	Female	–	0.4	0.4
# 4	23	Female	–	0.5	0.5
Mean $\pm$ SEM				0.8 $\pm$ 0.3	0.8 $\pm$ 0.3
Patients					
# 1	64	Male	28 $\times$ 2.0 Gy	166.0	226.0
# 2	61	Male	25 $\times$ 2.3 Gy	190.7	279.8
# 3	68	Male	27 $\times$ 2.3 Gy	107.0	130.0
# 4	70	Male	29 $\times$ 2.2 Gy	61.5	78.5
Mean $\pm$ SEM				131.3 $\pm$ 29.1*	178.6 $\pm$ 45.5*

* $p < 0.05$ vs. controls.

solution. This procedure was repeated with a new swab collecting the cells in the same solution. Cells were centrifuged (1000 rpm, 5 min) and resuspended in washing buffer. This washing step was repeated twice. Subsequently, around 6000 cells were brought onto pre-cleaned microscope slides by cyto-centrifugation (1000 rpm, 5 min). Slides were stored in methanol (-20°C) for at least one hour to fix cells. For staining, slides were air-dried and incubated with anti- α -tubulin-antibody solution (1:50) for four hours at 37°C . Thereafter slides were rinsed with PBS and cells were incubated with chromomycin A_3 solution for five minutes at room temperature. Finally, this preparation was mounted with Dabco mounting medium. Before analysis slides were coded to guarantee blinded evaluation.

2.4. Scoring

Evaluation was performed using a Nikon Eclipse 55i fluorescence microscope with $400\times$ magnification. Each of the two scorers evaluated 1000 cells of each study participant, giving a total number of cells analyzed per individual of 2000. Only results presented in Table 2 were evaluated by a separate scorer. Main outcome of this study was the frequency of micronuclei. The following criteria had to be fulfilled to regard a nuclear anomaly as a micronucleus: (a) lying inside the cytoplasm but outside the main nucleus, (b) staining pattern similar to the main nucleus, (c) size approximately 1/16 to 1/3 of the main nucleus and (d) roundish shape. Furthermore the frequency of binucleated cells and fragmented nuclei were counted. Condensed chromatin could not be distinguished without doubts from fragmented nuclei. Neither pyknosis nor karyolysis was observed. In case a cell contained more than one micronucleus, this was registered in order to determine not only the number of micronuclei but also the number of micronucleated cells. Since cells with more than one micronucleus appeared very rarely, results are presented as total micronucleus frequency. Exemplary pictures are shown in Fig. 1.

2.5. Statistics

Statistical analysis was performed using the t -test and the Mann-Whitney- U test with the help of the software Statistica 8.0.

3. Results

3.1. Study cohort

In total, samples of 131 individuals were evaluated. Table 1 shows the most important characteristics of this cohort. The mean age was 26.1 years ranging from 19 to 84. Mean age of low ($0 \leq t < 1$ h/week), medium ($1 \leq t \leq 2$ h/week) and high ($t > 2$ h/week) mobile phone users was 30.2, 22.9 and 24.4 years respectively. The study population consisted of 41.2% males and 58.8% females. Body-mass indices ranged from 17.0 to 31.2 kg/m^2 (mean 22.0 kg/m^2). 22.1% of the individuals lay below, 64.9% within, and 13.0% above the normal span from 20.0 to 25.0 kg/m^2 . 7.6% of all the individuals were heavy smokers (10 or more cigarettes per day), 7.6% were light smokers (less than ten cigarettes per day) and 84.8% were non-smokers. The most commonly reported medication was oral contraception (26.7%). Some individuals reported allergies or occasional use of analgesics (6.9%). Notably, no chemotherapeutic drugs or X-ray-treatments were reported. The majority of the participants

did not have special nutrition habits; 7.6% reported being vegetarians. 21.4% employed a headset for telephoning, 78.6% did not. 9.9% had never used a mobile phone at all. The users consisted of 18.8% short-term users (less than five years), 64.3% mid-term users (five to ten years) and 16.3% long-term users (ten years or more). The primary parameter was the time of mobile phone use per week. As mentioned before, 9.9% used no mobile phone at all. 64.9% used the phone up to three hours, 13.7% used it more than three and up to six hours and 11.5% used their mobile phone for more than six hours per week.

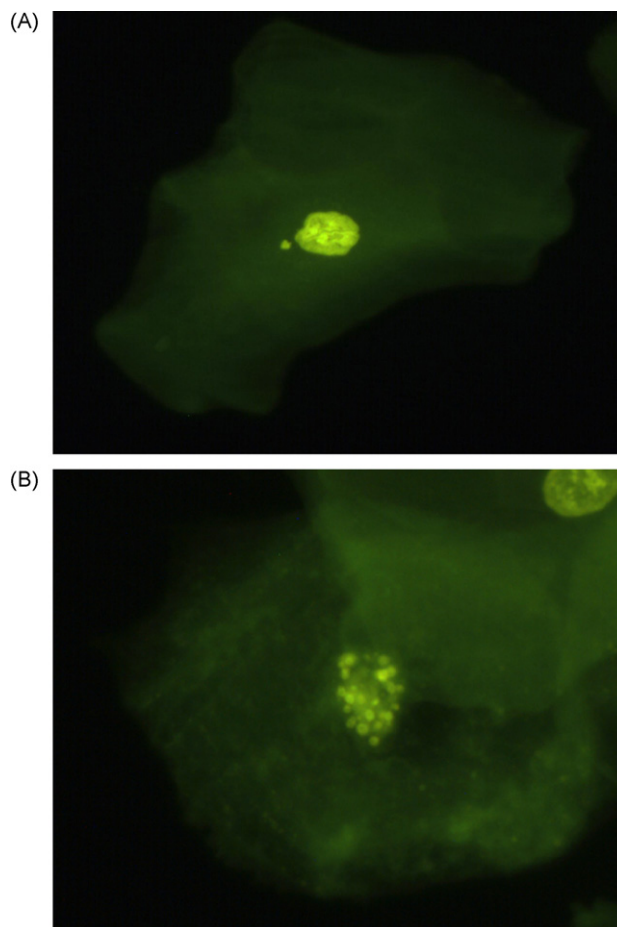


Fig. 1. Pictures of buccal mucosa cells: (A) cell containing one micronucleus and (B) cell with a fragmented nucleus.

Table 3Frequencies of micronuclei and fragmented nuclei per 1000 cells depending on various life style factors (Mean $\pm$ SEM).

		Micronuclei	Fragmented nuclei
Gender	Male ($n = 54$)	1.27 (± 0.15)	0.44 (± 0.12) [*]
	Female ($n = 77$)	1.44 (± 0.15)	0.12 (± 0.03) [*]
Age [years]	19–24 ($n = 88$)	1.38 (± 0.13)	0.29 (± 0.07)
	25–39 ($n = 35$)	1.34 (± 0.19)	0.16 (± 0.06)
	40–84 ($n = 8$)	1.38 (± 0.59)	0.22 (± 0.13)
Body-mass-index [kg/m ²]	<20 ($n = 29$)	1.47 (± 0.30)	0.29 (± 0.17)
	20–25 ($n = 85$)	1.26 (± 0.11)	0.22 (± 0.05)
	>25 ($n = 17$)	1.76 (± 0.36)	0.34 (± 0.14)
Smoking status	Non-smoker ($n = 111$)	1.45 (± 0.12)	0.26 (± 0.06)
	<10 cigarettes/day ($n = 10$)	1.05 (± 0.26)	0.25 (± 0.17)
	≥ 10 cigarettes/day ($n = 10$)	0.83 (± 0.25)	0.15 (± 0.11)
Headset usage	Non-users ($n = 92$)	1.38 (± 0.13)	0.30 (± 0.07)
	Users ($n = 25$)	1.26 (± 0.25)	0.08 (± 0.04)
Overall period of exposure	<5 years ($n = 21$)	1.13 (± 0.25)	0.25 (± 0.08)
	5–10 years ($n = 72$)	1.42 (± 0.15)	0.22 (± 0.08)
	≥ 10 years ($n = 19$)	1.18 (± 0.28)	0.26 (± 0.12)

^{*} $p < 0.05$ male vs. female (fragmented nuclei).

3.2. Cytogenetic results

The positive controls (Table 2) showed a very high response to ionizing radiation in terms of an increase in micronucleus frequencies. The buccal mucosa cells of these patients frequently contained more than one micronucleus; therefore results are presented as micronucleus-containing cells and total number of micronuclei.

Table 3 shows micronucleus frequencies depending on the various parameters inquired in the questionnaire. No significant difference was observed between males and females, depending on age, between low, normal and high body-mass-index, and depending on smoking status. The number of fragmented nuclei also was

not changed dependent on these variables except for gender, where males had a statistically significant ($p < 0.05$) higher number of fragmented nuclei. Micronucleus frequency was not changed between headset users and non-users. A decrease in fragmented nuclei was observed in headset users as a statistically non-significant trend. There was no difference between people who have used the phone for less than five, five–nine, or ten and more years.

Fig. 2 shows micronucleus frequencies depending on the duration of weekly mobile phone use. Both graphs (A) and (B) of this figure originate from the whole dataset, but the individuals were grouped differently. In (A) individuals were grouped according to consistent increase in time interval per column, leading to big differences in sample sizes; in (B) grouping was done according to

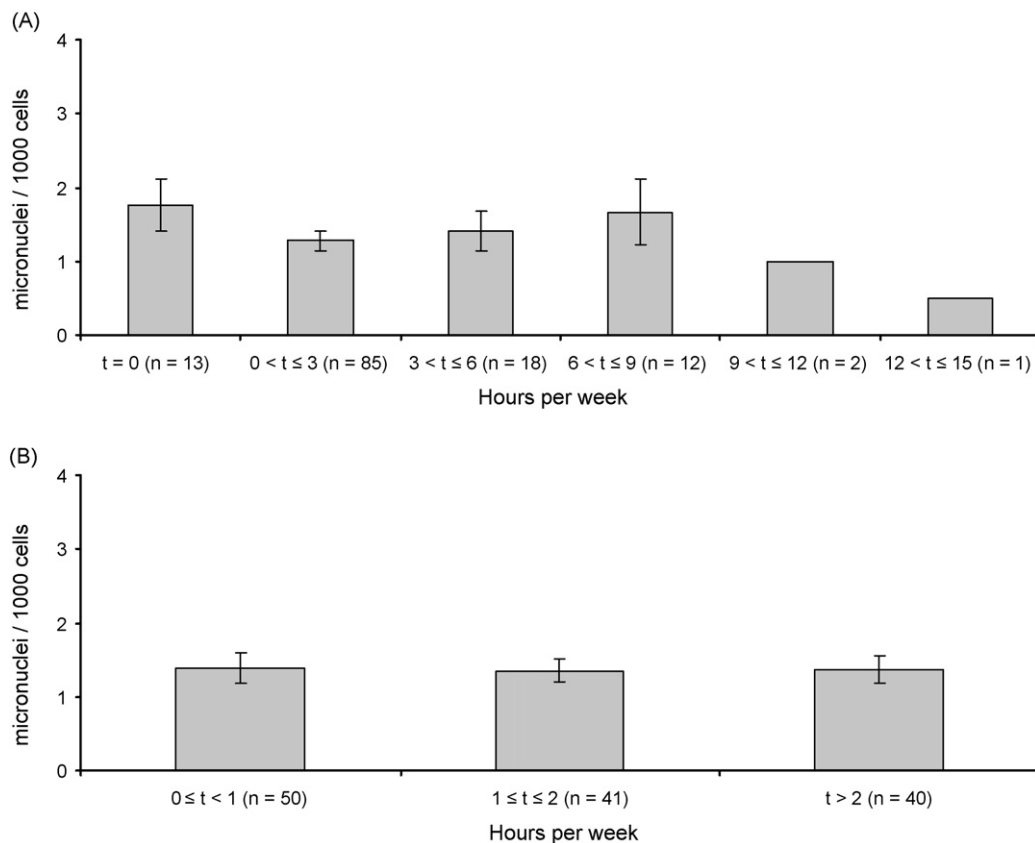


Fig. 2. Micronucleus frequency depending on weekly phone use. Individuals were grouped to achieve equal time intervals (A) or equal sample sizes (B) between columns [Mean $\pm$ SEM].

Table 4

Frequencies of fragmented nuclei per 1000 cells depending on mobile phone use (Mean $\pm$ SEM).

Mobile phone use [h/week]		Fragmented nuclei
$t=0$	($n=13$)	0.38 (± 0.19)
$0 < t \leq 3$	($n=85$)	0.31 (± 0.07)
$3 < t \leq 6$	($n=18$)	0.08 (± 0.06)
$6 < t \leq 9$	($n=12$)	0.04 (± 0.04)
$9 < t \leq 12$	($n=2$)	0.00 (± 0.00)
$12 < t \leq 15$	($n=1$)	0.00
$0 \leq t < 1$	($n=50$)	0.24 (± 0.06)
$1 \leq t \leq 2$	($n=41$)	0.43 (± 0.14)*
$t > 2$	($n=40$)	0.09 (± 0.04)*

* $p < 0.05$: $1 \leq t \leq 2$ vs. $t > 2$.

equal sample size per column, leading to different time intervals. This was done because in (A) sample sizes were partly very small and differed a lot, making direct comparison difficult. No significant differences were observed. The statistical analysis was repeated (a) excluding the smokers and (b) analyzing the data of the two scorers separately (not shown). These analyses equally showed no influence of mobile phone use on the micronucleus frequencies. The number of fragmented nuclei showed a decreasing trend with duration of phone use (Table 4). Different grouping revealed that individuals using the mobile phone for 1–2 h exhibited more fragmented nuclei than people using it less or more.

4. Discussion

The controversy about whether RF radiation causes effects on biological systems besides the well-documented and widely accepted heating at higher radiation powers has been going on for a long time and is almost as old as the wide-spread application of mobile phone technology. Even though a large number of studies ranging from tests on cellular systems over animal investigations to large epidemiological surveys have been conducted, this question has not been solved conclusively. Today the majority of studies do not show effects, but the scientific community is far from reaching a consensus on this topic (Khurana et al., 2009; Moulder et al., 2005). The hypothesis that non-ionizing radiation does not provoke effects at low power intensities is supported by the biophysical basics of the interaction between radiation and biological systems, which do not readily provide mechanisms explaining those effects. So called non-thermal effects have repeatedly been put up for discussion. This term can describe two different phenomena that have to be treated separately. The first one comprises effects which have been demonstrated only at very high field intensities and thus always coincide with heating effects but are thought to be themselves not due to the heating process. The second comprises effects at very low intensities which do not lead to a detectable increase in temperature. These effects have been claimed very often but could never be confirmed consistently (Foster, 2000).

One of the biggest concerns of the general public is the connection between mobile phone use and cancer. This link can only be confirmed or rejected by epidemiological surveys and with some limitations by animal studies. However, in vitro investigations and human biomonitoring studies using genotoxicity endpoints are very useful tools to supplement the body of evidence. The buccal micronucleus test is a very reliable, widely used assay to measure not only DNA damage but also chromosomal instability and cell death (Thomas et al., 2009). The cell extraction is minimally invasive making sampling fast and easy. This test is especially suitable for investigating the influence of mobile phone use because mobile phones are located near the cheek during calls, making the oral mucosa one of the most exposed tissues of the human body. It is theoretically possible to differentiate between left and right cheek

to determine side differences, which might be considered informative, because some epidemiological studies suggested an ipsilateral increase in brain cancers but not on the contralateral side (Khurana et al., 2009). However, in this study this discrimination was not done, because mobile phone use is very often not limited to one side. Even if some people might have a preferential side, many individuals will change the side between and also during calls. Furthermore, exposure intensity differences between both cheeks (inside) have not been quantified according to our knowledge.

For extraction of the cells, use of wooden spatulas or different brushes is very popular. Here, a cotton swab was used, because this method assures good cell recovery and is convenient for the participants. Furthermore, only differentiated cells were observed during scoring the slides, making evaluation easier because no basal cells had to be considered while counting. It is not recommended to score micronuclei in basal cells, and the ratio between basal and differentiated cells is mostly only dependent on the force with which cells were extracted and the number of samplings (Thomas et al., 2009). It can be assumed that no basal cells were observed because the extraction method was not as invasive into the cheek tissue as others, indicating that this procedure can be recommended for future investigations. Participants were asked in detail about their habits of using the mobile phone because the main aim of this study was to determine putative influences of mobile phone radiation. However, exposure to other sources of electromagnetic radiation of similar frequencies (e.g. using cordless phones at home, living very near to base stations, using wireless internet systems) has not been inquired and an influence cannot be excluded for sure.

The staining method for analyzing buccal mucosa cells is one of the critical steps during this assay (Casartelli et al., 1997). For nucleus staining it is essential to use DNA-specific stains. Nersesyan et al. (2006) showed that there can be an apparently more than four-fold increase in micronucleus frequency between smokers and non-smokers using non-DNA-specific stains. Using DNA-specific stains on slides from the same subjects, no statistical significant difference could be observed. This indicates that micronucleus formation is overestimated strongly when non-DNA-specific stains are used. In this study anti- α -tubulin-antibody was used to stain the cytoplasm (Weber et al., 1975) and chromomycin A₃ was used to stain the nucleus (Crissman and Tobey, 1990). This method can be recommended for future studies because it has several advantages: (a) it gives stable, long-lasting preparations that can be mounted with normal mounting media; (b) the staining procedure is simple and robust; and (c) nuclei in yellow appear in a clearly different and thereby easily and quickly distinguishable color in contrast to the cytoplasm in green.

This study did not show any statistically significant changes in micronucleus frequency depending on age, gender, body-mass-index and smoking status. This is in agreement with most of the studies using the buccal mucosa micronucleus test (Holland et al., 2008). These findings do not correlate with results in the micronucleus test in lymphocytes where older individuals and females usually tend to have more micronuclei. Samples from males exhibited more fragmented nuclei than those from females in our dataset. However, all frequencies of fragmented nuclei were still in a very low range (less than 0.5 fragmented nuclei/1000 cells). Future studies are needed to support and explain this finding.

Patients receiving high dose radiation therapy showed a clear increase in micronucleus frequency demonstrating that the micronucleus test in buccal mucosa cells is capable of detecting genotoxic exposures.

So far there has been one study investigating the effect of mobile phone use on micronucleus frequency in buccal mucosa cells (Yadav and Sharma, 2008). That study found a statistically significant increase in micronuclei in mobile phone users com-

pared to non-users. It was not distinguished between the different durations of weekly mobile phone use but the study individuals were grouped according to the overall period of exposure. The authors declared a positive correlation between the overall period of exposure and micronuclei. In contrast, our study did not detect any influence of mobile phone use, neither with respect to the duration of weekly use nor to the overall period of exposure. The somewhat fluctuant results of fragmented nuclei frequencies are difficult to explain and suggest a high variability of this endpoint. Several points might explain the different outcomes for micronucleus frequencies between our study and that by Yadav et al. (Yadav and Sharma, 2008). First, 2000 cells from 131 individuals in contrast to 1000 cells from 109 individuals in the study by Yadav et al. were analyzed here, resulting in more than double the amount of evaluated cells. This lends the study an increased statistical power but does not necessarily explain the difference. But in addition, staining techniques differed. We used chromomycin A₃, which is a DNA-specific stain (Crissman and Tobey, 1990). Yadav et al. (Yadav and Sharma, 2008) used orcein which is not a DNA-specific stain and thus might not only stain DNA-containing micronuclei but also other artifacts that are not associated to genomic instability (Nersesyan et al., 2006). It also is known that the use of betel quid and other similar preparations is widely popular in India and has been found to result in increased buccal mucosa micronucleus frequencies (Kausar et al., 2009). Yadav et al. might not have had the chance to exclude this confounder reliably from their study. The different baseline level (micronucleus frequencies in controls) could be attributed to the different staining procedures. Alternatively it could reflect a population variation caused by different distributions of genotypes of the participants in the study populations. Furthermore, differences in micronucleus frequencies within a population might reflect exposure to a certain agent, but could also represent an underlying genomic instability.

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Conflict of interest

None.

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References

- Ammari, M., Lecomte, A., Sakly, M., Abdelmelek, H., de-Seze, R., 2008. Exposure to GSM 900 MHz electromagnetic fields affects cerebral cytochrome c oxidase activity. *Toxicology* 250, 70–74.
- Benner, S.E., Lippman, S.M., Wargovich, M.J., Lee, J.J., Velasco, M., Martin, J.W., Toth, B.B., Hong, W.K., 1994. Micronuclei, a biomarker for chemoprevention trials: results of a randomized study in oral pre-malignancy. *Int. J. Cancer* 59, 457–459.
- Boller, K., Schmid, W., 1970. Chemical mutagenesis in mammals. The Chinese hamster bone marrow as an in vivo test system. *Hematological findings after treatment with trenimon*. *Humangenetik* 11, 35–54.
- Bonassi, S., Biasotti, B., Kirsch-Volders, M., Knasmueller, S., Zeiger, E., Burgaz, S., Bolognesi, C., Holland, N., Thomas, P., Fenech, M., 2009. State of the art survey of the buccal micronucleus assay—a first stage in the HUMN(XL) project initiative. *Mutagenesis* 24, 295–302.
- Bonassi, S., Fenech, M., Lando, C., Lin, Y.P., Ceppi, M., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Jia, C., Di Giorgio, M., Ferguson, L.R., Fucic, A., Lima, O.G., Hrelia, P., Krishnaja, A.P., Lee, T.K., Migliore, L., Mikhalevich, L., Mirkova, E., Mosesso, P., Muller, W.U., Odagiri, Y., Scarff, M.R., Szabova, E., Vorobtsova, I., Vral, A., Zijno, A., 2001. Human MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ. Mol. Mutagen.* 37, 31–45.
- Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulka-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis* 28, 625–631.
- Bortoli, G.M., Azevedo, M.B., Silva, L.B., 2009. Cytogenetic biomonitoring of Brazilian workers exposed to pesticides: micronucleus analysis in buccal epithelial cells of soybean growers. *Mutat. Res.* 675, 1–4.
- Capri, M., Scarcella, E., Fumelli, C., Bianchi, E., Salvioli, S., Mesirca, P., Agostini, C., Antolini, A., Schiavoni, A., Castellani, G., Bersani, F., Franceschi, C., 2004. In vitro exposure of human lymphocytes to 900 MHz CW and GSM modulated radiofrequency: studies of proliferation, apoptosis and mitochondrial membrane potential. *Radiat. Res.* 162, 211–218.
- Casartelli, G., Monteghirfo, S., De Ferrari, M., Bonatti, S., Scala, M., Toma, S., Margarino, G., Abbondandolo, A., 1997. Staining of micronuclei in squamous epithelial cells of human oral mucosa. *Anal. Quant. Cytol. Histol.* 19, 475–481.
- Crissman, H.A., Tobey, R.A., 1990. Specific staining of DNA with the fluorescent antibiotics, mithramycin, chromomycin, and olivomycin. *Methods Cell Biol.* 33, 97–103.
- El-Setouhy, M., Loffredo, C.A., Radwan, G., Rahman, R.A., Mahfouz, E., Israel, E., Mohamed, M.K., Ayyad, S.B., 2008. Genotoxic effects of waterpipe smoking on the buccal mucosa cells. *Mutat. Res.* 655, 36–40.
- Fenech, M., 2006. Cytokinesis-block micronucleus assay evolves into a “cytome” assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat. Res.* 600, 58–66.
- Fenech, M., Bonassi, S., Turner, J., Lando, C., Ceppi, M., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Bigatti, M.P., Bolognesi, C., Cao, J., De Luca, G., Di Giorgio, M., Ferguson, L.R., Fucic, A., Lima, O.G., Hadjidekova, V.V., Hrelia, P., Jaworska, A., Joksic, G., Krishnaja, A.P., Lee, T.K., Martelli, A., McKay, M.J., Migliore, L., Mirkova, E., Muller, W.U., Odagiri, Y., Orsiere, T., Scarfi, M.R., Silva, M.J., Sofuni, T., Surrallés, J., Trenta, G., Vorobtsova, I., Vral, A., Zijno, A., 2003a. Intra- and inter-laboratory variation in the scoring of micronuclei and nucleoplasmic bridges in binucleated human lymphocytes, results of an international slide-scoring exercise by the HUMN project. *Mutat. Res.* 534, 45–64.
- Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003b. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat. Res.* 534, 65–75.
- Fenech, M., Morley, A.A., 1985. Measurement of micronuclei in lymphocytes. *Mutat. Res.* 147, 29–36.
- Foster, K.R., 2000. Thermal and nonthermal mechanisms of interaction of radio-frequency energy with biological systems. *IEEE Trans. Plasma Sci.* 28, 15–23.
- Friedman, J., Kraus, S., Hauptman, Y., Schiff, Y., Seger, R., 2007. Mechanism of short-term ERK activation by electromagnetic fields at mobile phone frequencies. *Biochem. J.* 405, 559–568.
- Guney, M., Ozguner, F., Oral, B., Karahan, N., Mungan, T., 2007. 900 MHz radiofrequency-induced histopathologic changes and oxidative stress in rat endometrium: protection by vitamins E and C. *Toxicol. Ind. Health* 23, 411–420.
- Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech, M., 2008. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. *Mutat. Res.* 659, 93–108.
- Hoyto, A., Luukkainen, J., Juutilainen, J., Naarala, J., 2008. Proliferation, oxidative stress and cell death in cells exposed to 872 MHz radiofrequency radiation and oxidants. *Radiat. Res.* 170, 235–243.
- Huang, T.Q., Lee, M.S., Oh, E.H., Kalinec, F., Zhang, B.T., Seo, J.S., Park, W.Y., 2008. Characterization of biological effect of 1763 MHz radiofrequency exposure on auditory hair cells. *Int. J. Radiat. Biol.* 84, 909–915.
- ICNIRP, 2009. ICNIRP statement on the “Guidelines for limiting exposure to time-varying electric, magnetic, and electromagnetic fields (up to 300 GHz)”. *Health Phys* 97, 257–258.
- Kausar, A., Giri, S., Mazumdar, M., Giri, A., Roy, P., Dhar, P., 2009. Micronucleus and other nuclear abnormalities among betel quid chewers with or without sadagura, a unique smokeless tobacco preparation, in a population from North-East India. *Mutat. Res.* 677, 72–75.
- Khurana, V.G., Teo, C., Kundi, M., Hardell, L., Carlberg, M., 2009. Cell phones and brain tumors: a review including the long-term epidemiologic data. *Surg. Neurol.* 72, 205–214, discussion 214–205.
- Lee, J.J., Kwak, H.J., Lee, Y.M., Lee, J.W., Park, M.J., Ko, Y.G., Choi, H.D., Kim, N., Pack, J.K., Hong, S.I., Lee, J.S., 2008. Acute radio frequency irradiation does not affect cell cycle, cellular migration, and invasion. *Bioelectromagnetics* 29, 615–625.
- Luukkainen, J., Hakulinen, P., Maki-Paakkanen, J., Juutilainen, J., Naarala, J., 2009. Enhancement of chemically induced reactive oxygen species production and DNA damage in human SH-SY5Y neuroblastoma cells by 872 MHz radiofrequency radiation. *Mutat. Res.* 662, 54–58.
- Majer, B.J., Laky, B., Knasmueller, S., Kassie, F., 2001. Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at ele-

- vated risk of genetic damage and in chemoprevention trials. *Mutat. Res.* 489, 147–172.
- McNamee, J.P., Chauhan, V., 2009. Radiofrequency radiation and gene/protein expression: a review. *Radiat. Res.* 172, 265–287.
- Moulder, J.E., Foster, K.R., Erdreich, L.S., McNamee, J.P., 2005. Mobile phones, mobile phone base stations and cancer: a review. *Int. J. Radiat. Biol.* 81, 189–203.
- Murgia, E., Maggini, V., Barale, R., Rossi, A.M., 2007. Micronuclei, genetic polymorphisms and cardiovascular disease mortality in a nested case-control study in Italy. *Mutat. Res.* 621, 113–118.
- Nersesyan, A., Kundi, M., Atefie, K., Schulte-Hermann, R., Knasmüller, S., 2006. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. *Cancer Epidemiol. Biomarkers Prev.* 15, 1835–1840.
- OECD, 2007. Guideline for the testing of chemicals draft proposal for a new guideline 487: in vitro micronucleus test, 3rd Draft. In: OECD (Ed.) 487.
- Reifferscheid, G., Ziemann, C., Fieblinger, D., Dill, F., Gminski, R., Grummt, H.J., Hafner, C., Hollert, H., Kunz, S., Rodrigo, G., Stopper, H., Selke, D., 2008. Measurement of genotoxicity in wastewater samples with the in vitro micronucleus test: results of a round-robin study in the context of standardisation according to ISO. *Mutat. Res.* 649, 15–27.
- Schmid, E., Schrader, T., 2007. Different biological effectiveness of ionising and non-ionising radiations in mammalian cells. *Adv. Radio Sci.* 5, 1–4.
- Schrader, T., Munter, K., Kleine-Ostmann, T., Schmid, E., 2008. Spindle disturbances in human-hamster hybrid (A(L)) cells induced by mobile communication frequency range signals. *Bioelectromagnetics* 29, 626–639.
- Schuz, J., Lagorio, S., Bersani, F., 2009. Electromagnetic fields and epidemiology: an overview inspired by the fourth course at the International School of Bioelectromagnetics. *Bioelectromagnetics* 30, 511–524.
- Speit, G., Schutz, P., Hoffmann, H., 2007. Genotoxic effects of exposure to radiofrequency electromagnetic fields (RF-EMF) in cultured mammalian cells are not independently reproducible. *Mutat. Res.* 626, 42–47.
- Stich, H.F., Curtis, J.R., Parida, B.B., 1982a. Application of the micronucleus test to exfoliated cells of high cancer risk groups: tobacco chewers. *Int. J. Cancer* 30, 553–559.
- Stich, H.F., Stich, W., Parida, B.B., 1982b. Elevated frequency of micronucleated cells in the buccal mucosa of individuals at high risk for oral cancer: betel quid chewers. *Cancer Lett.* 17, 125–134.
- Stopper, H., Müller, S.O., 1997. Micronuclei as a biological endpoint for genotoxicity: a minireview. *Toxicol. in Vitro* 11, 8.
- Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmüller, S., Fenech, M., 2009. Buccal micronucleus cytome assay. *Nat. Protoc.* 4, 825–837.
- Titenko-Holland, N., Moore, L.E., Smith, M.T., 1994. Measurement and characterization of micronuclei in exfoliated human cells by fluorescence in situ hybridization with a centromeric probe. *Mutat. Res.* 312, 39–50.
- Verschaeve, L., Maes, A., 1998. Genetic, carcinogenic and teratogenic effects of radiofrequency fields. *Mutat. Res.-Rev. Mutat. Res.* 410, 141–165.
- Vijayalaxmi, Prihoda, T.J., 2008. Genetic damage in mammalian somatic cells exposed to radiofrequency radiation: a meta-analysis of data from 63 publications (1990–2005). *Radiat. Res.* 169, 561–574.
- von der Hude, W., Kalweit, S., Engelhardt, G., McKiernan, S., Kasper, P., Slacik-Erben, R., Miltenburger, H.G., Honarvar, N., Fahrig, R., Gorlitz, B., Albertini, S., Kirchner, S., Utesch, D., Potter-Locher, F., Stopper, H., Madle, S., 2000. In vitro micronucleus assay with Chinese hamster V79 cells—results of a collaborative study with in situ exposure to 26 chemical substances. *Mutat. Res.* 468, 137–163.
- Weber, K., Pollack, R., Bibring, T., 1975. Antibody against tubulin: the specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.* 72, 459–463.
- Yadav, A.S., Sharma, M.K., 2008. Increased frequency of micronucleated exfoliated cells among humans exposed in vivo to mobile telephone radiations. *Mutat. Res.* 650, 175–180.