

## Perspectives Revisited - The Buccal Cytome Assay in Mobile Phone Users

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**ABSTRACT** Buccal cell preparations previously scored for micronuclei were re-investigated for genomic instability and other biomarkers to assess DNA damage, cell-proliferation and cell-death in healthy mobile phone users (n=25; 30.96±2.09y) using mobile phones for 3-5y and the non-mobile phones users (n=25; 32.28±2.01y) according to the buccal micronucleus cytome (BMCyt) assay which was then not available. The frequency of micronuclei (13.66x), nuclear buds (2.57x), basal (1.34x), karyorrhectic (1.26x), karyolytic (2.44x), pyknotic (1.77x) and condensed chromatin (2.08x) cells were highly significantly (p=0.000) increased in mobile phone users whereas the binucleated cells (4.03x) and repair index (8.36x) showed significant decrease (p=0.000). DNA damage and nuclear anomalies scored in BMCyt assay are indicative of genetic damage that has not been repaired and this may predispose the mobile phone users to malignancy and cytotoxicity ramifications. Therefore, despite the benefits of communication technology, measures need to be taken so that better connectivity is not at expense of health.

### INTRODUCTION

The Indian mobile-phone subscriber base has crossed 960.58 million (TRAI 2015) and continues to increase without abatement. Rather, mobile telephony has become ubiquitous and an integral way of life across all strata. However, the use of low-frequency radiofrequency electromagnetic radiations in mobile phones for communication is of concern for human health in the wake of WHO stating that cell phone use is “possibly carcinogenic” (IARC 2011). This has again kindled interest in determining the genetic damaging effects from cell (mobile) phone usage (Daroti et al. 2015; Shah et al. 2015). However documentations on exposures from radiofrequency radiations (RFR) continue to be equivocal in recent literature as genetic damage effects (Ozgun et al. 2014; Gulati et al. 2015; Shah et al. 2015; Zalata et al. 2015) and no effects (Vijayalaxmi et al. 2013, 2015; Kumar et al. 2015; Zhu et al. 2015) have been documented.

In view of this controversy, and the earlier commentary of Vijayalaxmi et al. (2007) on Gandhi and Singh (2005) on cytogenetic investigations in mobile phone users compared to those

on healthy non-users, the same buccal smear permanent preparations (earlier scored only for the presence of micronuclei) were re-scored in accordance with the buccal micronucleus cytome assay (Thomas et al. 2009) which was then not available. The buccal epithelial cells are optimal for human biomonitoring as the sample collection is minimally-invasive and the assessment of micronuclei (MN) is a useful genetic damage biomarker of endogenous and exogenous exposures (Thomas et al. 2011). Furthermore, the varied biomarkers scorable by the buccal micronucleus cytome (BMCyt) assay are very informative because of their association with increased risk for accelerated ageing, cancer and neurodegenerative diseases (Thomas and Fenech 2011).

The assay (Thomas et al. 2009) entails scoring of micronucleated cells (chromosomal damage) as well as other biomarkers viz. of DNA damage (nuclear buds), cell-proliferation (basal and binucleated cells) and of cell-death (karyorrhectic, karyolytic, condensed chromatin and pyknotic cells) in the oral epithelium, which is composed of four strata of structural, progenitor, and maturing cell populations. These include the basal cell layer (stratum basale), prickle cell layer (stratum spinosum), and the keratinized layer at the surface. The oral epithelium maintains itself by continuous cell renewal whereby new cells produced in the basal layer by mitosis migrate to the surface replacing those that are shed. The basal layer contains the cells that may

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express genetic damage (chromosome breakage or loss). The daughter cells, containing/not containing MN, differentiate eventually into the prickle cell layer and the keratinized superficial layer, and then exfoliate into the buccal cavity with a turnover of 7-21 days. Some cells may degenerate and manifest as cells with condensed chromatin, fragmented nuclei (karyorrhectic cells), pyknotic nuclei, or the nuclear material may be completely lost (karyolytic or "ghost" cells). In some cases, the cells may be arrested at the binucleated stage (cytokinesis defect) or may exhibit nuclear buds (also known as "broken eggs" in buccal cells) which are biomarkers of gene amplification (Holland et al. 2008; Thomas et al. 2009).

For the present communication, buccal epithelial cell populations comprising various cell types and their ratios were quantified and, along with the previously scored micronuclei in healthy mobile phone users, were compared to those in age- and gender-matched controls. The rationale of the appropriateness of this assay is that continuous exposure to low-intensity electromagnetic microwaves during mobile phone usage may lead to genotoxic effects in the buccal mucosal cells. In the absence of any other exposures, the close proximity of the buccal tissue to the positioning of the cell phones during telephonic conversations increases chances of RFR exposure with more to buccal mucosa than to any other tissue. This may manifest after cell division as unreparable genetic damage and/or as sustained genetic alterations, probably the consequence of genetic defect (s) in cell-cycle checkpoints (Fenech et al. 2011).

### Objectives

The aim of the present study was to assess the buccal cytome of mobile phone users and non-users for DNA damage, cell proliferation and cell death biomarkers.

### MATERIAL AND METHODS

The details of the study (following ethical approval) and the method for buccal cell-preparations (after informed consent) have been extensively described previously (Gandhi and Singh 2005). The present paper describes the application of the scoring criteria on coded buccal epithelial cell-preparations for a comprehen-

sive evaluation of the buccal cytome, additionally scoring for DNA damage, cell proliferation and cell-death markers as recommended and validated by Thomas et al. (2009).

Buccal epithelial cells (2000 cells per participant; 1000 per slide) were scored for micronuclei and nuclear buds among which a total of 1000 cells (500/slide) were scored for the various cell types viz. basal, binucleated, condensed chromatin, karyorrhectic, pyknotic and karyolytic cells. The record of the frequency of micronucleated cells was re-examined and on finding no differences, the frequency of micronucleated cells was retained. The results on other scored markers are also reported in per cent frequency for markers of DNA damage, cell-proliferation and cell-death cells  $\pm$  standard error of mean. Repair index (RI), which is the degree of genotoxicity, was calculated from the summation of karyorrhectic cells and karyolytic cells divided by the sum of micronucleated cells and cells with nuclear buds (Celik et al. 2010).

### Statistical Analysis

The students' t-test was used to compare the frequencies of biomarkers between mobile phone users and non-mobile phone users. The Pearson correlation, analysis of variance (ANOVA) regression and step-wise regression analyses were carried out to find any association (if any) between genetic damage and the study variables. The level of significance was set at  $\leq 0.05$ .

### RESULTS

The results of the study reveal a highly significant ( $p=0.000$ ) increase in chromosomal damage as evidenced by the frequency of micronucleated cells and cells with nuclear buds in mobile phone users. Cell-death events were also highly significantly ( $p=0.000$ ) increased (condensed chromatin cells, karyolytic cells, pyknotic cells, karyorrhectic cells ( $p=0.006$ ) as was cell proliferation (basal cells;  $p=0.051$ ). There was significant decrease in cell-proliferation (binucleated cells;  $p=0.000$ ) and of repair index ( $p=0.000$ ) in the users in comparison to non-users (Table 1). The results reveal that aneugenic/ clastogenic events (Surrallés et al. 1997; Jyoti et al. 2015) showed a marked statistical increase ( $p=0.000$ ) evidenced as an almost 14x elevation of frequen-

**Table 1: The Buccal Micronucleus Cytome (BM cyt) assay in mobile phone users and controls**

<i>The BM cyt biomarkers</i>		<i>Mobile phone users (n=25) (percent frequency ±S.E.M.)</i>	<i>Significant- fold increase/decrease</i>	<i>Control group (n=25) (percent frequency ±S.E.M.)</i>	<i>p-value</i>
<i>DNA Damage Markers</i>	Micronucleated cells	0.820***±0.940	13.66	0.060 ± 0.03	0.000
	Nuclear buds	0.108***±0.009	2.57	0.042 ± 0.007	0.000
<i>Cell- Proliferation Markers</i>	Basal cells	0.268* ±0.025	1.34	0.200 ± 0.023	0.051
	Binucleated cells	0.124 ±0.041	4.03†	0.500***± 0.042	0.000
<i>Cell- Death Markers</i>	Karyorrhectic cells	1.316** ±0.063	1.26	1.044 ± 0.070	0.006
	Condensed chromatin cells	0.516***±0.046	2.08	0.248 ± 0.023	0.000
	Karyolytic cells	1.328***±0.085	2.44	0.544 ± 0.043	0.000
	Pyknotic cells	0.796***±0.058	1.77	0.448 ± 0.038	0.000
Repair Index (RI)		1.340 ±0.06	8.36†	11.21 ± 1.34***	0.000

A p value  $d \leq 0.05$  indicates significant differences, †significant decrease

cy of micronucleated basal cells. Frequency of nuclear buds (~2.6x) also exhibited significant ( $p=0.000$ ) elevation in mobile phone-users thereby implying that there is marked DNA amplification as nuclear buds are extruded by recombinational mechanisms to form minute chromosomes which can be replicated and/or eliminated by nuclear budding, transiently becoming MN, before being extruded from the cell to form microcells (Shimizu et al. 2000). Amplification events arise because of elimination of amplified DNA or because of DNA repair defects complexed possibly with excess chromosomes from aneuploid cells and may also result because of defective separation of sister chromatids at anaphase due to failure of decatenation (Fenech et al. 2011).

The number of basal cells and binucleated cells are indicators of cell-proliferation; in the present study, the mobile phone users had significantly increased fully differentiated basal cells (1.34 fold;  $p=0.051$ ) than in non-users. Considering that the buccal epithelium has a the basal cell layer, a prickle cell layer, and the keratinized layer at the surface (Thomas et al. 2009), the newly arising basal cells in the basal layer migrate to the surface replacing those that are shed (Holland et al. 2008). The observed increase in basal cells implies altered regenerative potential of the buccal mucosal layer which could be an effect of RFR exposure from continuing mobile phone use. An increase in basal cells was reported with increased duration of mobile phone usage in a study by Ros-Llor et al. (2012). There was a highly significant ( $p=0.000$ ) decrease (4.03x) in binucleated cells in mobile phone users. However, such cytokinetic event has been

reported in buccal cells of mobile phone users and computer users (Rajkokila et al. 2011).

Among the cell-death markers, karyorrhectic cells were (1.26 fold) significantly increased ( $p=0.006$ ), pyknotic, condensed chromatin and karyolytic cells almost doubled and karyolytic cells increased by 2.5x times. These cells represent transient stages of cell-apoptosis and/or cell-necrosis (Thomas et al. 2009). Karyorrhectic cells exhibit fragmented nuclei and are formed in later stage of apoptosis (Yadav and Jaggi 2015). Pyknotic cells appear as cells with shrunken nuclei and result from the process of cell-death representing the mechanism of nuclear disintegration (Thomas et al. 2009); condensed chromatin cells are manifestations of early stages of apoptosis resulting from rapid proteolysis of nuclear matrix proteins; and on reaching the advanced stages of necrosis and apoptosis, karyolysis results (Yadav and Jaggi 2015).

Repair Index (RI) was almost eight times decreased ( $p=0.000$ ) in mobile phone users implying cytotoxic effects. Repair index, which represents the degree of genotoxicity (Celik et al. 2010) showed an 8x decrease ( $p=0.000$ ) emphasizing that the frequencies of karyolytic and karyorrhectic cells are increased which could be because of radiofrequency radiation exposure causing nuclear disintegration. In literature, karyorrhectic cells were also reported to be slightly increased (1.29x) in mobile phone users (Yadav and Sharma 2008).

## DISCUSSION

The significantly increased frequency of buccal cells with micronuclei, binucleates, karyorrhexis, condensed chromatin cells, py-

knosis and karyolysis observed in the study participants is probably from the use of mobile phones which use microwaves in the RFR range given that the participants were healthy with no other incidental/accidental/workplace exposure(s). The observations find similarity with the work of Daroit et al. (2015) using buccal mucosa to screen for micronucleated cells, nuclear buds and binucleates as well as of Rekhadevi et al. (2009) micronuclei in buccal epithelial and DNA damage in peripheral blood lymphocytes of mobile phone users, of Gulati et al. (2015) documenting increased frequency of micronuclei in cells of buccal cavity of mobile phone users. However some contrary reports exist: Ros-Llor et al. (2012) on buccal cells of mobile phone users, of Hook et al. (2004) on DNA damage in Molt-4 cells and of Vijayalaxmi et al. (2013) on peripheral blood lymphocytes. The study design, cell types, techniques and genetic endpoints screened may account for these differences. The assessment of DNA damage in epithelial cells collected from the oral cavity is optimal for assaying use of mobile phones as these cells are in direct line of contact when phone is in use; also the collection of buccal epithelia is a minimally-invasive method useful for monitoring populations exposed to genotoxic agents (Bonassi et al. 2009). The choice of buccal epithelium for assessment of genetic damage is further optimal as on one hand it has a unique proliferative response which allows cellular population to maintain a constant rate of cell divisions, while the cell-proliferative response in epithelial cells induces proneness of cells to DNA damage on the other (Torres-Bugarín et al. 2014). Scoring epithelial cells for DNA damage further gains significance as nearly 90 percent of all cancers are derived from epithelial cells (Holland et al. 2008). The additional end-points scored in the BMCyt assay are also very informative biomarkers as these include measures of chromosomal instability and gene amplification (via nuclear buds), cytokinesis arrest due to aneuploidy (via binucleated cells), and cell-death (karyorrhectic and pyknotic cells) events. Therefore, the micronucleogenic cells present in epithelial tissue provide a monitor for individuals or populations exposed to mutagenic, genotoxic, or teratogenic events (Torres-Bugarin et al. 2014).

The cytome assay has revealed significant genetic damaging effects, cytokinetic defects as

well as cytotoxicity in buccal epithelial cells of mobile phone users. The almost 14 fold increase in aneugenicity/ clastogenicity manifested as micronuclei is chromosomal damage retained after DNA repair resulting from the consequence of aneugenic/ clastogenic events which occurred 1-3 weeks before in the basal epithelium layers (Thomas et al. 2009). The mobile phone users had been using phones for 3-5y and therefore have had continuous exposure from the RFR.

Nuclear buds are indicators of gene amplification and therefore are a direct measure of DNA damage because nuclear buds arise as elimination of amplified DNA and/or causes of defects in DNA repair (Nersesyan 2005); these also may be the precursors of micronuclei (Shimizu et al. 1998).

Non-programmed cell-death, as observed in the significantly elevated karyorrhectic cells, karyolytic, condensed chromatin and pyknotic cells, occurs because of the inability of the cell nucleus to maintain its integrity and hence causes cells to die. The stages in order of deteriorating nuclear integrity are condensed chromatin cells, karyorrhectic cells, pyknotic and the karyolytic cells (Thomas et al. 2009). In the present study at the time of sampling, the mobile phone users had maximum karyolytic followed by karyorrhectic, pyknotic and condensed chromatin cells similarly indicating significantly increased loss-of-nuclear integrity. Such effects have also been observed in literature. The frequency of karyolytic cells was 7.09 fold increased than the karyorrhectic cells in mobile phone users (Yadav and Sharma 2008) and in study by Rajkokila et al. (2011) 1.84x in mobile-phone and computer users, probably from the RFR exposure.

Cell-proliferation is a measure of healthy buccal epithelium with its constant rate of cell-division to maintain a balance between exfoliating epithelial cells and their replacement (Torres-Bugarin et al. 2014). Mobile phone users of the present study had a marginal increase of basal cells while cytokinetic defects exhibited a decrease compared to the occurrence of these events in the non-users. In documented studies also, cell-proliferation was reported to be altered in human epithelial amnion cells (Velizarov et al. 1999) when exposed to 960 MHz frequency and in human skin fibroblasts (Pacini et al. 2002) exposed to Global System for Mobile Communication (GSM) cellular phone radiofrequency.

The observed decrease in RI is a manifestation of increased karyorrhectic and karyolytic cells and decreased frequency of micronucleated cells and nuclear buds in mobile phone users indicating an alteration of homeostasis of the buccal epithelium. Such an alteration can occur during the degenerative processes of nuclear buds to micronuclei and karyorrhectic to karyolytic cells causing clastogenetic changes. These may cause mutagenetic effects which ultimately can lead to carcinogenesis, progressively increasing with concurrent cell-death events (Ramirez and Saldanha 2002; Celik et al. 2010). The consequences of decreased RI can eventually lead to more genetically-damaged cells. In fact the micronucleated cells arise from dysfunction of mitotic apparatus in preceding mitosis or because of clastogenicity causing chromosome breakage (Falck et al. 2002). The events of karyolysis and karyorrhexis and of MN and DNA damage have also been reported in mobile phone users and computer users (Yadav and Sharma, 2008; Rajkokila et al. 2011). Also an elevated frequency of nuclear buds in exfoliated cells of oral epithelium has been reported in mobile users (Souza et al. 2014).

The ramifications of increased genomic damage as observed in the present study in terms of statistically increased micronucleated cells and nuclear buds, and of cell-death and cell-proliferation biomarkers underlie long-term consequences. Since micronuclei result from clastogenicity (chromosomal breakage) and/ or aneugenicity (chromosomal loss), these events imply chromosomal instability (Luzhna et al. 2013), and furthermore the association between increased MN frequencies and risk of cancer is also well validated (Bonassi et al. 2011). In fact, chromosomal changes and genetic instability (as also observed in the participants of the present study) are underlying factors in carcinogenesis and therefore, the identification of individuals at high risk for cancer has public health implications (Bonassi et al. 2005).

Despite the fact that the mechanism by which RFR used in mobile telephony can induce genetic damage is not understood, various hypotheses have been put forward on the bases of biochemical and physiological alterations observed on RFR exposure (Singh and Kapoor 2014). A study by Blank and Goodman (2009) reported that electromagnetic radiations, as used for cell telephony, can penetrate unattenuated into cells

and directly interact with the DNA and other cell constituents. An interaction of the electromagnetic field with biological systems can also initiate oxidative stress which may induce DNA damage via free radical generation (Phillips et al. 2009; Consales et al. 2012). Oxidative stress has in fact also been documented after cell-phone radiation exposure on male reproductive system (Desai et al. 2009) and in hippocampus in rats (Kerman and Senol 2012), which with antioxidant supplementation prevented apoptosis in male albino rats (Ibrahim and Gharib 2010). Cell-proliferation rate in hepatocarcinoma cells (Hep G2) was also decreased by RFR exposure (Ozgur et al. 2014). Over-expression of ornithine decarboxylase from microwave radiations is also linked to progression of cancer as studied in rats (Paulraj and Behari 2012). These studies therefore emphasize that oxidative stress is one of the mechanisms associated with increased genotoxic effects of the RFR radiations.

However, effects of RFR exposure causing genetic damage continue to be contradictory (Daroit et al. 2015; Zhu et al. 2015). Some current reports show concordance with the results of the present study. Shah et al. (2015) reported significant increase in chromosomal damage in blood samples exposed to *in vitro* cell phone radiation. A significant increase in sperm DNA fragmentation percent, *CLU* gene expression and *CLU* levels alongwith significant decrease in sperm physiology and activity in semen samples of individuals exposed to cell phone radiation for one hour in comparison to the non-exposed individuals have been reported (Zalata et al. 2015). Gulati et al. (2015) reported a significant increase in micronuclei assessed in buccal cells and tail moment in peripheral blood lymphocytes in individuals residing near mobile phone base station in comparison to those not exposed to such radiations. Others have also reported cytogenetic alterations in animal models and human cells exposed to RF of 915MHz (Garaj-Vrhovac et al. 2009) and 1250-1350 MHz (Garaj-Vrhovac and Orescanin 2009). Earlier studies on humans include those of Zotti-Martelli et al. (2005) who observed micronucleus frequencies *in vitro* in peripheral blood lymphocytes exposed to microwaves while Yadav and Sharma (2008) reported increased frequency of micronucleated buccal cells in human exposed *in vivo* to mobile phone radiations. Significant increases in mean DNA damage, micronuclei and

chromosomal aberrations were also reported in mobile phone users in comparison to non-users (Rekhadevi et al. 2009). Elevated DNA damage in peripheral blood leukocytes and chromosomal damage in buccal cells of mobile phone users have already been earlier reported (Gandhi and Anita 2005; 2011; Gandhi and Singh 2005, 2011; Yadav and Sharma 2008; Rekhadevi et al. 2009).

However, Hintzsche and Stopper (2010) and Ros-Llor et al. (2012) reported no significant increase in frequency of micronuclei in buccal mucosal cells of mobile phone users in comparison to the controls. Other contrary findings without significant effect of radiofrequency radiations include the work of Hook et al. (2004) on exposure of Molt-4 cells to 847.74 MHz code-division multiple-access (CDMA), 835.62 MHz frequency-division multiple-access (FDMA), 813.56 MHz iDEN(R) (iDEN), and 836.55 MHz time-division multiple-access (TDMA) which neither induced DNA damage nor apoptosis. Kim et al. (2008) also reported no clastogenic effect of 835 MHz radiofrequency electromagnetic fields on mammalian cells. A study by Vijayalaxmi et al. (2013) reported no significant differences in human peripheral blood lymphocytes exposed to modulated wideband code division multiple access (WCDMA) and continuous wave (CW) radiofrequency exposures (900 MHz) in comparison to the controls. Scarfi et al. (2006) reported that exposure to radiofrequency radiation (900 MHz, GSM signal) failed to affect micronucleus frequency and cell proliferation in human peripheral blood lymphocytes in culture. Dominant lethal mutations also did not show an increase when the unexposed female mice were mated to RF-exposed (900 MHz RFR) male mice in comparison to those mated to sham-exposed mice (Zhu et al. 2015).

The commentary of Vijaylaxmi et al. (2007) on Gandhi and Singh (2005) and Gandhi and Anita (2005) is also mainly on similar lines. These incidentally are two separate studies with none of the study participants repeated and hence comprise general information and experimental data from different individuals. The other comments are responded to here. The information gathered, sample collection (buccal and blood) and laboratory work were all conducted at the same moment in time with subsequent publication of the results (Gandhi and Singh 2005; Gandhi and Anita 2005). In no way were good laboratory practices and research ethics compro-

mised and so there was no involvement of any changes affecting the documented data.

For clarification purposes it is reiterated that the publications of Gandhi and Singh (2005) and Gandhi and Anita (2005) are separate investigations on distinct study participants with their associated data. A structured validated questionnaire was administered using a face-to-face interview method and included specific queries on phone models and sets with duration of phone use. It was observed that phone replacements were not common at the time nor was use of a hands' free-device. The SAR value, as a plausible exposure index, was used for comparison as also documented widely in literature at that time. Details on placement of phone sets when not in use are also specifically mentioned in the two studies.

The comment regarding non-documentation of reports on lack of genetic damage effects from RFR exposure, it is pointed out that the last paragraph of the discussion in Gandhi and Singh (2005: 263) and in the last portion of the discussion in Gandhi and Anita (2005: 100), the studies contrary to our findings have been clearly mentioned. In response to the statement about processing of samples, the methodology in both the manuscripts impeccably states that the blood samples and buccal smear preparation were processed within 2-3h of collection. It also needs to be clarified that "+/-" implies standard errors of mean (S.E.M.) as depicted clearly in the tabulated results (Gandhi and Singh 2005; Gandhi and Anita 2005).

Pertaining to comments specifically for Gandhi and Singh (2005), statistical analysis as given in Table I had revealed that the control group matched the mobile users with respect to age, sex, diet ( $\chi^2 = 0.987$ ,  $p=0.32$ ,  $df=1$ ) and socioeconomic status ( $\chi^2=1.86$ ,  $p=0.17$ ,  $df=1$ ). Also as the groups matched for diet preferences, therefore diet could not directly be a probable cause of the elevated genetic damage. Socio-economic status (middle and high) by both groups was self-reported and also exhibited a match. Regarding frequency and amount of consumption of non-vegetarian diet, this is a personal preference irrespective of affordability. In view of socioeconomic status (SES), it needs to be pointed out that Punjab has a very robust *gross domestic product (GDP)* index and the state is an economically developed state (Government of Punjab 2013). Therefore, persons with middle

and high SES (as are the participants of the present study) can well afford a daily preferred non-vegetarian diet.

Regarding the comments about the control group, it is clearly mentioned that the control group comprised healthy individuals who had not and did not use mobile phones; they had no incidental and occupational exposure(s). Also neither of the groups resided in the vicinity of any cell phone base station. Against this information, the conditions of a 'real' control group are clearly met. Also the statement at the end of discussion, is a general statement and is not meant to be extrapolated to the study participants and does not pertain to the control group under study.

Inconsistencies 'a-c' (Gandhi and Singh 2005) are errors which have crept in due to a shift of the columns on removal of the table grids. The column with heading 'daily frequency of calls' has two sub-parts IN and OUT. The data under OUT have been shifted under the heading IN of duration of calls (min) while "daily exposure" does not have IN and OUT categorizations. So accordingly, the daily exposure for MM1 is nearly 13h (12.70h) only viz. the duration of incoming calls is 22min with daily 20 calls ( $22 \times 20 = 440\text{min}$ ), on this basis the total exposure for incoming calls comes out to be ( $440/60 = 7.33\text{h}$ ) 7.33h. Similarly with daily 23 outgoing calls, each of about 14min ( $23 \times 14 = 322\text{min}$ ), the exposure from outgoing calls comes out to be ( $322/60 = 5.366 = 5.37\text{h}$ ) 5.37h. Hence total daily exposure from both incoming and outgoing calls is ( $7.33 + 5.37 = 12.70\text{h}$ ) 12.70h. The authors are apologetic deeply and upset about the misinterpretation created. As for comment 'd' (MM1 had been using mobile phone for 4.5 years being a student with a daily exposure of 12.70h), the data are self-reported and under signatures while the comment 'e' (regarding diet of middle socio-economic class in controls), this has been dealt with above.

Regarding the methodology for cell culturing, short-term culturing of peripheral blood lymphocytes (Moorhead et al. 1960) was carried out using PHA (phytohemagglutinin) as a mitogen, and as per the methodology adopted, BrdU (bromodeoxyuridine) was not used. The rationale for scoring 72h cultured lymphocytes without using BrdU was that repaired genetic damage was being scored in mobile phone users with similar cultures from controls (non-users). The

observed anomalies were documented and included centromere separation, acrocentric associations and triploidy; these were significantly elevated in mobile phone users. Though these may not be considered 'serious aberrations', yet their significant increase from those in controls imply cytotoxicity with cytokinesis and cell-proliferation defects. Incidentally, such defects have now been also documented in the buccal epithelial while scoring the buccal cytochrome assay as increase in micronucleated cells, nuclear buds, basal cells, karyorrhectic cells, condensed chromatin cells, karyolytic cells and pyknotic cells.

The observed per cent aberrant metaphases in controls were low (10.66 %) because the controls were healthy participants with no recent past illnesses or exposures. Also the low incidence of MN (0.06%) observed in controls implies that the controls were healthy, and their diets probably rich in antioxidants. In studies on healthy controls from this part of the region, similarly low MN frequencies have been reported viz. 0.0013 in buccal mucosal cells (Sambyal et al. 2004), 0.042 in uterine smears of cervix cancer patients (Gandhi and Kaur 2003) and are in accordance with earlier reports in exfoliated buccal cells of engine repair workers, taxi drivers and traffic police (Karahalil et al. 1999). Also low per cent aberrant metaphases in normal study participants have been documented at 13.02 percent (Kamboj and Sambyal 2006).

The present observations (data) on Buccal micronucleus cytochrome assay (Thomas et al. 2009), an assay which has been well-validated from the same laboratory as the Cytokinesis block micronucleus (CBMN) assay (Fenech 2007), are responses to comments 'a' (regarding consideration of cell proliferation/cell cycle) and 'b' (regarding other nuclear anomalies and low frequency of MN in controls). Nuclear anomalies such as condensed chromatin, pyknosis, karyorrhexis, etc. are cell-death parameters with distinctive nuclear features and in no manner at all can be confused with MN.

In the paper by Gandhi and Anita (2005), the study was on DNA damage in peripheral blood leukocytes (PBL) of mobile phone users with evidence of chromosomal damage simultaneously observed by scoring MN on unstimulated blood lymphocytes (Xue et al. 1992) even though the latter has not found wide-spread use. Nonetheless, scoring of MN in unstimulated blood lymphocytes provides good measure of unre-

pairable (chromosomal) damage, though the assay needs validation. In the Gandhi and Anita (2005) study, DNA damage has been observed as repairable genetic damage while the concurrently observed MN are manifestations of retained genetic damage after DNA repair. Regarding the MN frequency in controls, it was 0.05% (and not 0.0006) which is a gross error the authors deeply regret.

Also as mentioned in the methodology in Gandhi and Anita (2005), the comet assay on PBL of mobile phone users and controls was performed concurrently. Standard comet assay methodology as given by Singh et al. (1988) and as modified by Ahuja and Saran (1999) was followed as documented clearly in the manuscript and therefore the details of the protocols were not given. DNA migration length measured by visual scoring using an occulo-micrometer is an acceptable method used for scoring DNA damage in the comet assay (Collins et al. 2003, 2008). In fact, there is good synchronization between visual scoring and image analysis for silver-stained comets (Garcia et al. 2007).

Correlation analysis of the data of Gandhi and Singh (2005) has revealed a significant association of MN with duration of mobile phone usage ( $p=0.022$ ) and duration of daily incoming call ( $p=0.037$ ) (Gandhi and Singh 2011). In the latter, the analysis of variance showed a significant association of the frequency of micronuclei with SAR ( $p=0.040$ ) value of mobile phones and exposure per day ( $p=0.050$ ) whereas the per cent aberrant metaphases were associated significantly to SAR ( $p=0.034$ ). Regression analysis showed significant association respectively of both, MN and per cent aberrant metaphases, with SAR ( $p=0.030$ ;  $0.050$ ), duration of mobile phone usage ( $p=0.020$ ;  $0.005$ ) and with exposure per day ( $p=0.016$ ); this was however lost on step-wise regression analysis. Vijayalaxmi et al. (2007) have mentioned regarding the step-wise regression only but make no clear mention of the results of correlation/ regression analyses they performed.

The above clarifications on the commentary of Vijayalaxmi et al. (2007) in no way undermine the significant findings reported in Gandhi and Singh (2005) and Gandhi and Anita (2005).

### CONCLUSION

The present study on the buccal cytome assay in mobile phone users reiterates earlier findings and provides adjunct data on highly sig-

nificant ( $p=0.000$ ) increase in Chromosomal (micronucleated cells) and DNA (nuclear buds) damage and cell-death (karyorrhectic, pyknotic, karyolytic and condensed chromatin cells) and cell-proliferation (basal cells) markers have been observed. These results emphasize the presence of chromosomal and genomic instability as well as cytotoxicity and cytostatic effects in buccal epithelial cells of mobile phone users in the absence of any incidental/occupational exposure.

### RECOMMENDATIONS

Caution in the use of mobile phone users is recommended in the light of the present findings.

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