

Table 4. Publications which reported no significant oxidative effects after RFR exposure.

Reference	Biological system exposed	RFR exposure	Effects reported
(Hook et al., 2004)	Mammalian cells <i>in vitro</i>	835.62 MHz (frequency-modulated continuous-wave, FMCW) and 847.74 MHz (code division multiple access, CDMA), SAR = 0.8 W/kg, for 20–22 h	FMCW- and CDMA-modulated RFR did not alter parameters indicative of oxidative stress.
(Ferreira et al., 2006a)	Rat whole body	800–1800 MHz, from cell phone	No changes in lipid and protein damage, and in non-enzymatic anti-oxidant defense in frontal cortex or hippocampus.
(Ferreira et al., 2006b)	Pregnant rat whole body	RFR from cell phone	No differences in oxidative parameter of offspring blood and liver, but increase in erythrocytes micronuclei incidence in offspring.
(Dasdag et al., 2003)	Rat whole body	Cell phone RFR, SAR = 0.52 W/kg, 20 min/day for 1 month	No alteration in MDA concentration.
(Demirel et al., 2012)	Rat whole body	3G cell phone RFR, “standardized daily dose” for 20 days	No difference in GSH-Px and CAT activity in eye tissues, in MDA and GSH levels in blood.
(Khalil et al., 2014)	Human head/whole body	Cell phone RFR (talking mode) for 15 or 30 min	No relationship between exposure and changes in the salivary oxidant/anti-oxidant profile.
(de Souza et al., 2014)	Human head/whole body	Cell phone RFR	No difference in the saliva from the parotid gland exposed to cell phone RFR to the saliva from the opposite gland of each individual.

low-intensity RFR, which could be evoked both by the direct oxidative damage and by disturbed cellular signaling.

Oxidative effects and non-cancer health effects of RFR

A new medical condition, so-called electrohypersensitivity (EHS), in which people suffer due to RFR exposure, has been described (Johansson, 2006). Typically, these persons suffer from skin- and mucosa-related symptoms (itching, smarting, pain, heat sensation), or heart and nervous system disorders after exposure to computer monitors, cell phones and other electromagnetic devices. This disorder is growing continuously: starting from 0.06% of the total population in 1985, this category now includes as much as 9–11% of the European population (Hallberg and Oberfeld, 2006). In Sweden, for example, EHS has become an officially recognized health impairment.

To that, a high percentage, up to 18–43% of young people, has recently been described to be suffering from headache/earache during or after cell phone conversations (Chu et al., 2011; Yakymenko et al., 2011). Likewise, a number of psychophysical and preclinical disorders including fatigue, irritation, headache, sleep disorders, hormonal imbalances were detected in high percent of people living nearby cell phone base transceiver stations (Buchner and Eger, 2011; Santini et al., 2002).

An allergy reaction to RFR in humans has been confirmed by a significant increase in the level of mast cells in skin of persons under exposure to electromagnetic devices (Johansson et al., 2001). Likewise, higher level of degranulated mast cells in dermis of EHS persons has been detected (Johansson, 2006). In turn, the activated mast cells can release histamine and other mediators of such reactions which include allergic hypersensitivity, itching, dermatoses, etc.

Importantly, an implication of ROS in allergic reactions is rather clear nowadays. For example, in case of airway allergic inflammation, the lung cells generate superoxide in nanomolar concentrations following antigen challenges (Nagata, 2005). Then, mast cells generate ROS following aggregation of FcεRI, a high-affinity IgE receptor (Okayama, 2005). In addition, pollen NADPH oxidases rapidly increase the level of ROS in lung epithelium (Boldogh et al., 2005); and removal of pollen NADPH oxidases from the challenge material reduced antigen-induced allergic airway inflammation. Thus, it seems plausible that EHS-like conditions can be attributed at least partially to ROS overproduction in cells due to RFR exposures.

Oxidative effects and potential carcinogenicity of RFR

During recent years, a number of epidemiological studies indicated a significant increase in incidence of various types of tumors among long-term or “heavy” users of cellular phones (Yakymenko et al., 2011). Briefly, reports pointed to the increased risk in brain tumors (Cardis et al., 2010; Hardell and Carlberg, 2009; Hardell et al., 2007), acoustic neuroma (Hardell et al., 2005; Sato et al., 2011), tumors of parotid glands (Sadetzki et al., 2008), seminomas (Hardell et al., 2007), melanomas (Hardell et al., 2011) and lymphomas (Hardell et al., 2005) in these cohorts of people. To that, a significant increase in tumor incidence among people living nearby cellular base transceiver stations was also reported (Eger et al., 2004; Wolf and Wolf, 2007). Similarly, experimental evidences of cancer expansion in rodents caused by long-term low-intensity RFR exposure were published (Chou et al., 1992; Repacholi et al., 1997; Szmigielski et al., 1982; Toler et al., 1997). To that, activation of ODC was detected in RFR-exposed cells (Hoyto et al., 2007). ODC is involved in

processes of cell growth and differentiation, and its activity is increased in tumor cells. Although overexpression of ODC is not sufficient for tumorigenic transformation, an increased activity of this enzyme was shown to promote the development of tumors from pre-tumor cells (Clifford et al., 1995).

Significant overproduction of ROS leads to oxidative stress in living cells, induces oxidative damage of DNA and can cause malignant transformation (Halliwell and Whiteman, 2004; Valko et al., 2007). It is known that in addition to mutagenic effects, ROS play a role as a second messenger for intracellular signaling cascades which can also induce oncogenic transformation (Valko et al., 2006). Earlier we hypothesized (Burlaka et al., 2013) that low-intensity RFR exposure leads to dysfunctions of mitochondria, which result in overproduction of superoxide and NO, and subsequently to ROS-mediated mutagenesis. To that, it is well established that oxidative stress is associated with carcinogenesis; for instance, the oxidative stress elicited by Membrane-Type 1 Matrix Metalloproteinase is implicated in both the pathogenesis and progression of prostate cancer (Nguyen et al., 2011). Similarly, a progressive elevation in mitochondrial ROS production (chronic ROS) under both hypoxia and/or low glucose, which leads to stabilization of cells via increased HIF-2 α expression, can eventually result in malignant transformation (Ralph et al., 2010). These data, together with the strong experimental evidences on activation of NADH oxidase under RFR exposure (Friedman et al., 2007) suggest that low-intensity RFR is a multifactorial stress factor for living cell, significant feature of which is oxidative effects and potential carcinogenicity as a result.

Conclusions

The analysis of modern data on biological effects of low-intensity RFR leads to a firm conclusion that this physical agent is a powerful oxidative stressor for living cell. The oxidative efficiency of RFR can be mediated via changes in activities of key ROS-generating systems, including mitochondria and non-phagocytic NADH oxidases, via direct effects on water molecules, and via induction of conformation changes in biologically important macromolecules. In turn, a broad biological potential of ROS and other free radicals, including both their mutagenic effects and their signaling regulatory potential, makes RFR a potentially hazardous factor for human health. We suggest minimizing the intensity and time of RFR exposures, and taking a precautionary approach towards wireless technologies in everyday human life.

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Declaration of interest

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References

- Abdel-Rassoul, G., El-Fateh, O. A., Salem, M. A., et al. (2007). Neurobehavioral effects among inhabitants around mobile phone base stations. *Neurotoxicology* 28:434–440.
- Abu Khadra, K. M., Khalil, A. M., Abu Samak, M., et al. (2014). Evaluation of selected biochemical parameters in the saliva of young males using mobile phones. *Electromagn. Biol. Med.* 32:72–76.
- Agarwal, A., Desai, N. R., Makker, K., et al. (2009). Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: An in vitro pilot study. *Fertil. Steril.* 92:1318–1325.
- Akbari, A., Jelodar, G., Nazifi, S. (2014). Vitamin C protects rat cerebellum and encephalon from oxidative stress following exposure to radiofrequency wave generated by BTS antenna mobile. *Toxicol. Mechanisms Methods* 24:347–352.
- Al-Damegh, M. A. (2012). Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. *Clinics* 67:785–792.
- Avci, B., Akar, A., Bilgici, B., et al. (2012). Oxidative stress induced by 1.8 GHz radio frequency electromagnetic radiation and effects of garlic extract in rats. *Int. J. Radiat. Biol.* 88:799–805.
- Ayata, A., Mollaoglu, H., Yilmaz, H. R., et al. (2004). Oxidative stress-mediated skin damage in an experimental mobile phone model can be prevented by melatonin. *J. Dermatol.* 31:878–883.
- Aynali, G., Naziroglu, M., Celik, O., et al. (2013). Modulation of wireless (2.45 GHz)-induced oxidative toxicity in laryngotracheal mucosa of rat by melatonin. *Eur. Arch. Oto-Rhino-Laryngol.* 270: 1695–1700.
- Balci, M., Devrim, E., Durak, I. (2007). Effects of mobile phones on oxidant/antioxidant balance in cornea and lens of rats. *Curr. Eye Res.* 32:21–25.
- Baohong, W., Jiliang, H., Lifan, J., et al. (2005). Studying the synergistic damage effects induced by 1.8 GHz radiofrequency field radiation (RFR) with four chemical mutagens on human lymphocyte DNA using comet assay in vitro. *Mutat. Res.* 578:149–157.
- Belyaev, I. (2010). Dependence of non-thermal biological effects of microwaves on physical and biological variables: Implications for reproducibility and safety standards. *Eur. J. Oncol. Library* 5: 187–217.
- Belyaev, I. Y., Koch, C. B., Terenius, O., et al. (2006). Exposure of rat brain to 915 MHz GSM microwaves induces changes in gene expression but not double stranded DNA breaks or effects on chromatin conformation. *Bioelectromagnetics* 27:295–306.
- Bilgici, B., Akar, A., Avci, B., et al. (2013). Effect of 900 MHz radiofrequency radiation on oxidative stress in rat brain and serum. *Electromagn. Biol. Med.* 32:20–29.
- Blank, M., Soo, L. (2001). Electromagnetic acceleration of electron transfer reactions. *J. Cell Biochem.* 81:278–283.
- Blank, M., Soo, L. (2003). Electromagnetic acceleration of the Belousov-Zhabotinski reaction. *Bioelectrochemistry* 61:93–97.
- Bodera, P., Stankiewicz, W., Zawada, K., et al. (2013). Changes in antioxidant capacity of blood due to mutual action of electromagnetic field (1800 MHz) and opioid drug (tramadol) in animal model of persistent inflammatory state. *Pharmacol. Rep.* 65: 421–428.
- Bohr, H., Bohr, J. (2000a). Microwave-enhanced folding and denaturation of globular proteins. *Phys. Rev. E* 61:4310–4314.
- Bohr, H., Bohr, J. (2000b). Microwave enhanced kinetics observed in ORD studies of a protein. *Bioelectromagnetics* 21:68–72.
- Boldogh, I., Bacsai, A., Choudhury, B. K., et al. (2005). ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J. Clin. Investig.* 115: 2169–2179.
- Buchner, K., Eger, H. (2011). [Changes of clinically important neurotransmitters under the influence of modulated RF fields—A long-term study under real-life conditions]. *Umwelt -Medizin-Gesellschaft* 24: 44–57.
- Budi, A., Legge, F. S., Treutlein, H., et al. (2007). Effect of frequency on insulin response to electric field stress. *J. Phys. Chem. B.* 111: 5748–5756.
- Burlaka, A., Selyuk, M., Gafurov, M., et al. (2014). Changes in mitochondrial functioning with electromagnetic radiation of ultra high

- frequency as revealed by electron paramagnetic resonance methods. *Int. J. Radiat. Biol.* 90:357–362.
- Burlaka, A., Tsybulin, O., Sidorik, E., et al. (2013). Overproduction of free radical species in embryonal cells exposed to low intensity radiofrequency radiation. *Exp. Oncol.* 35:219–225.
- Byus, C. V., Kartun, K., Pieper, S., et al. (1988). Increased ornithine decarboxylase activity in cultured cells exposed to low energy modulated microwave fields and phorbol ester tumor promoters. *Cancer Res.* 48:4222–4226.
- Calabrese, E. J. (2008). Hormesis: Why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem.* 27:1451–1474.
- Campisi, A., Gulino, M., Acquaviva, R., et al. (2010). Reactive oxygen species levels and DNA fragmentation on astrocytes in primary culture after acute exposure to low intensity microwave electromagnetic field. *Neurosci. Lett.* 473:52–55.
- Caraglia, M., Marra, M., Mancinelli, F., et al. (2005). Electromagnetic fields at mobile phone frequency induce apoptosis and inactivation of the multi-chaperone complex in human epidermoid cancer cells. *J. Cell. Physiol.* 204:539–548.
- Cardis, E., Deltour, I., Vrijheid, M., et al. (2010). Brain tumour risk in relation to mobile telephone use: Results of the INTERPHONE international case-control study. *Int. J. Epidemiol.* 39:675–694.
- Cenesis, M., Atakisi, O., Akar, A., et al. (2011). Effects of 900 and 1800 MHz electromagnetic field application on electrocardiogram, nitric oxide, total antioxidant capacity, total oxidant capacity, total protein, albumin and globulin levels in guinea pigs. *Kafkas Üniv. Vet. Fakültesi Dergisi* 17:357–362.
- Céspedes, O., Ueno, S. (2009). Effects of radio frequency magnetic fields on iron release from cage proteins. *Bioelectromagnetics* 30: 336–342.
- Cetin, H., Naziroglu, M., Celik, Ö., et al. (2014). Liver antioxidant stores protect the brain from electromagnetic radiation (900 and 1800 MHz)-induced oxidative stress in rats during pregnancy and the development of offspring. *J. Matern.-Fetal Neonat. Med.* 72:1915–1921.
- Chou, C. K., Guy, A. W., Kunz, L. L., et al. (1992). Long-term, low-level microwave irradiation of rats. *Bioelectromagnetics* 13:469–496.
- Chu, M. K., Song, H. G., Kim, C., et al. (2011). Clinical features of headache associated with mobile phone use: A cross-sectional study in university students. *BMC Neurol.* 11:115.
- Clifford, A., Morgan, D., Yuspa, S. H., et al. (1995). Role of ornithine decarboxylase in epidermal tumorigenesis. *Cancer Res.* 55: 1680–1686.
- Consales, C., Merla, C., Marino, C., et al. (2012). Electromagnetic fields, oxidative stress, and neurodegeneration. *Int. J. Cell Biol.* 2012: 683897.
- Dasdag, S., Akdag, M. Z., Kizil, G., et al. (2012). Effect of 900 MHz radio frequency radiation on beta amyloid protein, protein carbonyl, and malondialdehyde in the brain. *Electromagn. Biol. Med.* 31:67–74.
- Dasdag, S., Akdag, M. Z., Ulukaya, E., et al. (2009). Effect of mobile phone exposure on apoptotic glial cells and status of oxidative stress in rat brain. *Electromagn. Biol. Med.* 28:342–354.
- Dasdag, S., Bilgin, H., Akdag, M. Z., et al. (2008). Effect of long term mobile phone exposure on oxidative-antioxidative processes and nitric oxide in rats. *Biotechnol. Biotechnol. Equip.* 22:992–997.
- Dasdag, S., Zulkuf Akdag, M., Aksen, F., et al. (2003). Whole body exposure of rats to microwaves emitted from a cell phone does not affect the testes. *Bioelectromagnetics* 24:182–188.
- De Iuliis, G. N., Newey, R. J., King, B. V., et al. (2009). Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. *PLoS One* 4: e6446.
- de Souza, F. T., Silva, J. F., Ferreira, E. F., et al. (2014). Cell phone use and parotid salivary gland alterations: No molecular evidence. *Cancer Epidemiol. Biomarkers Prevent.* 23:1428–1431.
- Demirel, S., Doganay, S., Turkoz, Y., et al. (2012). Effects of third generation mobile phone-emitted electromagnetic radiation on oxidative stress parameters in eye tissue and blood of rats. *Cutan. Ocul. Toxicol.* 31:89–94.
- Desai, N. R., Kesari, K. K., Agarwal, A. (2009). Pathophysiology of cell phone radiation: Oxidative stress and carcinogenesis with focus on male reproductive system. *Reprod. Biol. Endocrinol.* 7:114.
- Deshmukh, P. S., Banerjee, B. D., Abegaonkar, M. P., et al. (2013). Effect of low level microwave radiation exposure on cognitive function and oxidative stress in rats. *Indian J. Biochem. Biophys.* 50: 114–119.
- Diem, E., Schwarz, C., Adlkofer, F., et al. (2005). Non-thermal DNA breakage by mobile-phone radiation (1800 MHz) in human fibroblasts and in transformed GFSH-R17 rat granulosa cells in vitro. *Mutat. Res.* 583:178–183.
- Dutta, S. K., Ghosh, B., Blackman, C. F. (1989). Radiofrequency radiation-induced calcium ion efflux enhancement from human and other neuroblastoma cells in culture. *Bioelectromagnetics* 10: 197–202.
- Eger, H., Hagen, K., Lucas, B., et al. (2004). [Influence of the proximity of mobile phone base stations on the incidence of cancer]. *Environ. Med. Soc.* 17:273–356.
- Enyedi, B., Niethammer, P. (2013). H₂O₂: A chemoattractant? *Methods Enzymol.* 528:237–255.
- Esmekaya, M. A., Ozer, C., Seyhan, N. (2011). 900 MHz pulse-modulated radiofrequency radiation induces oxidative stress on heart, lung, testis and liver tissues. *Gen. Physiol. Biophys.* 30:84–89.
- Ferreira, A. R., Bonatto, F., de Bittencourt Pasquali, M. A., et al. (2006a). Oxidative stress effects on the central nervous system of rats after acute exposure to ultra high frequency electromagnetic fields. *Bioelectromagnetics* 27:487–493.
- Ferreira, A. R., Knakievicz, T., Pasquali, M. A., et al. (2006b). Ultra high frequency-electromagnetic field irradiation during pregnancy leads to an increase in erythrocytes micronuclei incidence in rat offspring. *Life Sci.* 80:43–50.
- Forman, H. J., Ursini, F., Maiorino, M. (2014). An overview of mechanisms of redox signaling. *J. Mol. Cell Cardiol.* 73:2–9.
- Friedman, J., Kraus, S., Hauptman, Y., et al. (2007). Mechanism of short-term ERK activation by electromagnetic fields at mobile phone frequencies. *Biochem. J.* 405:559–568.
- Furtado-Filho, O. V., Borba, J. B., Dallegrave, A., et al. (2014). Effect of 950 MHz UHF electromagnetic radiation on biomarkers of oxidative damage, metabolism of UFA and antioxidants in the livers of young rats of different ages. *Int. J. Radiat. Biol.* 90:159–168.
- Gandhi, O. P., Morgan, L. L., de Salles, A. A., et al. (2012). Exposure limits: The underestimation of absorbed cell phone radiation, especially in children. *Electromagn. Biol. Med.* 31:34–51.
- Garaj-Vrhovac, V., Fucic, A., Horvat, D. (1992). The correlation between the frequency of micronuclei and specific chromosome aberrations in human lymphocytes exposed to microwave radiation in vitro. *Mutat. Res.* 281:181–186.
- Garaj-Vrhovac, V., Gajski, G., Pažanin, S., et al. (2011). Assessment of cytogenetic damage and oxidative stress in personnel occupationally exposed to the pulsed microwave radiation of marine radar equipment. *Int. J. Hyg. Environ. Health.* 214:59–65.
- Garson, O. M., McRobert, T. L., Campbell, L. J., et al. (1991). A chromosomal study of workers with long-term exposure to radio-frequency radiation. *Med. J. Austral.* 155:289–292.
- Georgiou, C. D. (2010). Oxidative stress-induced biological damage by low-level EMFs: Mechanism of free radical pair electron spin-polarization and biochemical amplification. *Eur. J. Oncol.* 5:63–113.
- Goodman, R., Blank, M. (2002). Insights into electromagnetic interaction mechanisms. *J. Cell Physiol.* 192:16–22.
- Griendling, K. K., Sorescu, D., Ushio-Fukai, M. (2000). NAD(P)H oxidase: Role in cardiovascular biology and disease. *Circ. Res.* 86: 494–501.
- Guler, G., Tomruk, A., Ozgur, E., et al. (2012). The effect of radiofrequency radiation on DNA and lipid damage in female and male infant rabbits. *Int. J. Radiat. Biol.* 88:367–373.
- Guney, M., Ozguner, F., Oral, B., et al. (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.
- Gürler, H. Ş., Bilgici, B., Akar, A. K., et al. (2014). Increased DNA oxidation (8-OHdG) and protein oxidation (AOPP) by low level electromagnetic field (2.45 GHz) in rat brain and protective effect of garlic. *Int. J. Radiat. Biol.* 90:892–896.
- Guzy, R. D., Schumacker, P. T. (2006). Oxygen sensing by mitochondria at complex III: The paradox of increased reactive oxygen species during hypoxia. *Exp. Physiol.* 91:807–819.
- Hallberg, O., Oberfeld, G. (2006). Letter to the editor: Will we all become electrosensitive? *Electromagn. Biol. Med.* 25:189–191.
- Halliwell, B. (1991). Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. *Am. J. Med.* 91: 14S–22S.

- Halliwell, B. (2007). Biochemistry of oxidative stress. *Biochem. Soc. Trans.* 35:1147–1150.
- Halliwell, B., Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? *Br. J. Pharmacol.* 142:231–255.
- Hamzany, Y., Feinmesser, R., Shpitser, T., et al. (2013). Is human saliva an indicator of the adverse health effects of using mobile phones? *Antioxid. Redox. Signal.* 18:622–627.
- Hardell, L., Carlberg, M. (2009). Mobile phones, cordless phones and the risk for brain tumours. *Int. J. Oncol.* 35:5–17.
- Hardell, L., Carlberg, M., Hansson Mild, K. (2005). Case-control study on cellular and cordless telephones and the risk for acoustic neuroma or meningioma in patients diagnosed 2000–2003. *Neuroepidemiology* 25:120–128.
- Hardell, L., Carlberg, M., Hansson Mild, K., et al. (2011). Case-control study on the use of mobile and cordless phones and the risk for malignant melanoma in the head and neck region. *Pathophysiology* 18:325–333.
- Hardell, L., Carlberg, M., Ohlson, C. G., et al. (2007). Use of cellular and cordless telephones and risk of testicular cancer. *Int. J. Androl.* 30: 115–122.
- Hardell, L., Carlberg, M., Soderqvist, F., et al. (2007). Long-term use of cellular phones and brain tumours: Increased risk associated with use for > or = 0 years. *Occup. Environ. Med.* 64:626–632.
- Hardell, L., Eriksson, M., Carlberg, M., et al. (2005). Use of cellular or cordless telephones and the risk for non-Hodgkin's lymphoma. *Int. Arch. Occup. Environ. Health* 78:625–632.
- Hayden, M. S., Ghosh, S. (2011). NF-kappa B in immunobiology. *Cell Res.* 21:223–244.
- Hong, M. N., Kim, B. C., Ko, Y. G., et al. (2012). Effects of 837 and 1950 MHz radiofrequency radiation exposure alone or combined on oxidative stress in MCF10A cells. *Bioelectromagnetics* 33:604–611.
- Hook, G. J., Spitz, D. R., Sim, J. E., et al. (2004). Evaluation of parameters of oxidative stress after in vitro exposure to FMCW- and CDMA-modulated radiofrequency radiation fields. *Radiat. Res.* 162: 497–504.
- Hou, Q., Wang, M., Wu, S., et al. (2014). Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells. *Electromagn. Biol. Med.* 34:85–92.
- Hoyto, A., Juutilainen, J., Naarala, J. (2007). Ornithine decarboxylase activity is affected in primary astrocytes but not in secondary cell lines exposed to 872 MHz RF radiation. *Int. J. Radiat. Biol.* 83:367–374.
- Hyland, G. J. (2000). Physics and biology of mobile telephony. *Lancet* 356:1833–1836.
- ICNIRP. (1998). Guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields (up to 300 GHz). *Health Phys.* 74:494–522.
- Ilhan, A., Gurel, A., Armutcu, F., et al. (2004). Ginkgo biloba prevents mobile phone-induced oxidative stress in rat brain. *Clin. Chim. Acta.* 340:153–162.
- Inoue, M., Sato, E. F., Nishikawa, M., et al. (2003). Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Curr. Med. Chem.* 10:2495–2505.
- Jelodar, G., Akbari, A., Nazifi, S. (2013). The prophylactic effect of vitamin C on oxidative stress indexes in rat eyes following exposure to radiofrequency wave generated by a BTS antenna model. *Int. J. Radiat. Biol.* 89:128–131.
- Jelodar, G., Nazifi, S., Akbari, A. (2013). The prophylactic effect of vitamin C on induced oxidative stress in rat testis following exposure to 900 MHz radio frequency wave generated by a BTS antenna model. *Electromagn. Biol. Med.* 32:409–416.
- Jing, J., Yuhua, Z., Xiao-qian, Y., et al. (2012). The influence of microwave radiation from cellular phone on fetal rat brain. *Electromagn. Biol. Med.* 31:57–66.
- Johansson, O. (2006). Electrohypersensitivity: State-of-the-art of a functional impairment. *Electromagn. Biol. Med.* 25:245–258.
- Johansson, O., Gangi, S., Liang, Y., et al. (2001). Cutaneous mast cells are altered in normal healthy volunteers sitting in front of ordinary TVs/PCs – results from open-field provocation experiments. *J. Cutan. Pathol.* 28:513–519.
- Kahya, M. C., Naziroğlu, M., Çiğ, B. (2014). Selenium reduces mobile phone (900 MHz)-induced oxidative stress, mitochondrial function, and apoptosis in breast cancer cells. *Biol. Trace Elem. Res.* 160: 285–293.
- Kang, K. A., Lee, H. C., Lee, J. J., et al. (2013). Effects of combined radiofrequency radiation exposure on levels of reactive oxygen species in neuronal cells. *J. Radiat. Res. (Published online)*:rrt116.
- Kerbacher, J. J., Meltz, M. L., Erwin, D. N. (1990). Influence of radiofrequency radiation on chromosome aberrations in CHO cells and its interaction with DNA-damaging agents. *Radiat. Res.* 123: 311–319.
- Kerman, M., Senol, N. (2012). Oxidative stress in hippocampus induced by 900 MHz electromagnetic field emitting mobile phone: Protection by melatonin. *Biomed. Res.* 23:147–151.
- Kesari, K. K., Kumar, S., Behari, J. (2010). Mobile phone usage and male infertility in Wistar rats. *Indian J. Exp. Biol.* 48:987–992.
- Kesari, K. K., Kumar, S., Behari, J. (2011). 900-MHz microwave radiation promotes oxidation in rat brain. [Research Support, Non-U.S. Gov't]. *Electromagn. Biol. Med.* 30:219–234.
- Kesari, K. K., Meena, R., Nirala, J., et al. (2013). Effect of 3G cell phone exposure with computer controlled 2-D stepper motor on non-thermal activation of the hsp27/p38MAPK stress pathway in rat brain. *Cell Biochem. Biophys.* 68:347–358.
- Khalil, A. M., Abu Khadra, K. M., Aljaberi, A. M., et al. (2014). Assessment of oxidant/antioxidant status in saliva of cell phone users. *Electromagn. Biol. Med.* 32:92–97.
- Khalil, A. M., Gagaa, M. H., Alshamali, A. M. (2012). 8-Oxo-7, 8-dihydro-2'-deoxyguanosine as a biomarker of DNA damage by mobile phone radiation. *Hum. Exp. Toxicol.* 31:734–740.
- Kim, J. Y., Hong, S. Y., Lee, Y. M., et al. (2008). In vitro assessment of clastogenicity of mobile-phone radiation (835 MHz) using the alkaline comet assay and chromosomal aberration test. [Research Support, Non-U.S. Gov't]. *Environ. Toxicol.* 23:319–327.
- Kismali, G., Ozgur, E., Guler, G., et al. (2012). The influence of 1800 MHz GSM-like signals on blood chemistry and oxidative stress in non-pregnant and pregnant rabbits. *Int. J. Radiat. Biol.* 88: 414–419.
- Koc, A., Unal, D., Cimentepe, E. (2013). The effects of antioxidants on testicular apoptosis and oxidative stress produced by cell phones. *Turk. J. Med. Sci.* 43:131–137.
- Koylu, H., Mollaoglu, H., Ozguner, F., et al. (2006). Melatonin modulates 900 MHz microwave-induced lipid peroxidation changes in rat brain. *Toxicol. Ind. Health* 22:211–216.
- Koyu, A., Ozguner, F., Yilmaz, H., et al. (2009). The protective effect of caffeic acid phenethyl ester (CAPE) on oxidative stress in rat liver exposed to the 900 MHz electromagnetic field. *Toxicol. Ind. Health* 25:429–434.
- Kumar, S., Nirala, J. P., Behari, J., et al. (2014). Effect of electromagnetic irradiation produced by 3G mobile phone on male rat reproductive system in a simulated scenario. *Indian J. Exp. Biol.* 52: 890–897.
- Lai, H., Singh, N. P. (1996). Single- and double-strand DNA breaks in rat brain cells after acute exposure to radiofrequency electromagnetic radiation. *Int. J. Radiat. Biol.* 69:513–521.
- Lai, H., Singh, N. P. (1997). Melatonin and a spin-trap compound block radiofrequency electromagnetic radiation-induced DNA strand breaks in rat brain cells. *Bioelectromagnetics* 18:446–454.
- Lantow, M., Lupke, M., Frahm, J., et al. (2006a). ROS release and Hsp70 expression after exposure to 1.800 MHz radiofrequency electromagnetic fields in primary human monocytes and lymphocytes. *Radiat. Environ. Biophys.* 45:55–62.
- Lantow, M., Schuderer, J., Hartwig, C., et al. (2006b). Free radical release and HSP70 expression in two human immune-relevant cell lines after exposure to 1800 MHz radiofrequency radiation. *Radiat. Res.* 165:88–94.
- Litovitz, T. A., Krause, D., Penafiel, M., et al. (1993). The role of coherence time in the effect of microwaves on ornithine decarboxylase activity. *Bioelectromagnetics* 14:395–403.
- Litovitz, T. A., Penafiel, L. M., Farrel, J. M., et al. (1997). Bioeffects induced by exposure to microwaves are mitigated by superposition of ELF noise. *Bioelectromagnetics* 18:422–430.
- Liu, C., Duan, W., Xu, S., et al. (2013a). Exposure to 1800 MHz radiofrequency electromagnetic radiation induces oxidative DNA base damage in a mouse spermatocyte-derived cell line. *Toxicol. Lett.* 218: 2–9.
- Liu, C., Gao, P., Xu, S.-C., et al. (2013b). Mobile phone radiation induces mode-dependent DNA damage in a mouse spermatocyte-derived cell line: A protective role of melatonin. *Int J Radiat Biol.* 89: 993–1001.

- Liu, Y., Fiskum, G., Schubert, D. (2002). Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* 80:780–787.
- Low, H., Crane, F. L., Morre, D. J. (2012). Putting together a plasma membrane NADH oxidase: a tale of three laboratories. *Int. J. Biochem. Cell Biol.* 44:1834–1838.
- Lu, Y. S., Huang, B. T., Huang, Y. X. (2012). Reactive oxygen species formation and apoptosis in human peripheral blood mononuclear cell induced by 900 MHz mobile phone radiation. *Oxid. Med. Cell Longev.* 2012:740280.
- Luo, Y.-p., Ma, H.-R., Chen, J.-W., et al. (2014). [Effect of American Ginseng Capsule on the liver oxidative injury and the Nrf2 protein expression in rats exposed by electromagnetic radiation of frequency of cell phone]. *Zhongguo Zhong xi yi jie he za zhi Zhongguo Zhongxiyi jiehe zazhi = Chin. J. Integr. Tradit. Western Med.* 34: 575–580.
- Luukkonen, J., Hakulinen, P., Maki-Paakkanen, J., et al. (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.
- Maes, A., Collier, M., Verschaeve, L. (2000). Cytogenetic investigations on microwaves emitted by a 455.7 MHz car phone. *Folia Biol.* 46: 175–180.
- Maes, W. (2005). *[Stress Caused by Electromagnetic Fields and Radiation]*. Neubeuern, Germany: IBN.
- Mailankot, M., Kunnath, A. P., Jayalekshmi, H., et al. (2009). Radio frequency electromagnetic radiation (RF-EMR) from GSM (0.9/1.8GHz) mobile phones induces oxidative stress and reduces sperm motility in rats. *Clinics* 64:561–565.
- Manta, A. K., Stravopodis, D. J., Papassideri, I. S., et al. (2013). Reactive oxygen species elevation and recovery in Drosophila bodies and ovaries following short-term and long-term exposure to DECT base EMF. *Electromagn. Biol. Med.* 33:118–131.
- Marino, A. A., Carrubba, S., Frilot, C., et al. (2009). Evidence that transduction of electromagnetic field is mediated by a force receptor. *Neurosci. Lett.* 452:119–123.
- Marjanovic, A. M., Pavicic, I., Trosic, I. (2014). Cell oxidation–reduction imbalance after modulated radiofrequency radiation. *Electromagn. Biol. Med. (Published online)*. 13:1–6.
- Marzook, E. A., Abd El Moneim, A. E., Elhadary, A. A. (2014). Protective role of sesame oil against mobile base station-induced oxidative stress. *J. Radiat. Res. Appl. Sci.* 7:1–6.
- Meena, R., Kumari, K., Kumar, J., et al. (2013). Therapeutic approaches of melatonin in microwave radiations-induced oxidative stress-mediated toxicity on male fertility pattern of Wistar rats. *Electromagn. Biol. Med.* 33:81–91.
- Megha, K., Deshmukh, P. S., Banerjee, B. D., et al. (2012). Microwave radiation induced oxidative stress, cognitive impairment and inflammation in brain of Fischer rats. *Indian J. Exp. Biol.* 50:889–896.
- Meral, I., Mert, H., Mert, N., et al. (2007). Effects of 900-MHz electromagnetic field emitted from cellular phone on brain oxidative stress and some vitamin levels of guinea pigs. *Brain Res.* 1169: 120–124.
- Motawi, T., Darwish, H., Moustafa, Y., et al. (2014). Biochemical modifications and neuronal damage in brain of young and adult rats after long-term exposure to mobile phone radiations. *Cell Biochem. Biophys.* 70:845–855.
- Moustafa, Y. M., Moustafa, R. M., Belacy, A., et al. (2001). Effects of acute exposure to the radiofrequency fields of cellular phones on plasma lipid peroxide and antioxidant activities in human erythrocytes. *J. Pharm. Biomed. Anal.* 26:605–608.
- Nagata, M. (2005). Inflammatory cells and oxygen radicals. *Curr. Drug Targets* 4:503–504.
- Naziroglu, M., Celik, O., Ozgul, C., et al. (2012a). Melatonin modulates wireless (2.45 GHz)-induced oxidative injury through TRPM2 and voltage gated Ca(2+) channels in brain and dorsal root ganglion in rat. *Physiol. Behav.* 105:683–692.
- Naziroglu, M., Cig, B., Dogan, S., et al. (2012b). 2.45-Gz wireless devices induce oxidative stress and proliferation through cytosolic Ca(2+)(+) influx in human leukemia cancer cells. *Int. J. Radiat. Biol.* 88:449–456.
- Naziroglu, M., Gumral, N. (2009). Modulator effects of L-carnitine and selenium on wireless devices (2.45 GHz)-induced oxidative stress and electroencephalography records in brain of rat. *Int. J. Radiat. Biol.* 85: 680–689.
- Nguyen, H. L., Zucker, S., Zarrabi, K., et al. (2011). Oxidative stress and prostate cancer progression are elicited by membrane-type 1 matrix metalloproteinase. *Mol. Cancer Res.* 9:1305–1318.
- Ni, S., Yu, Y., Zhang, Y., et al. (2013). Study of oxidative stress in human lens epithelial cells exposed to 1.8 GHz radiofrequency fields. *PLoS One.* 8:e72370.
- Okayama, Y. (2005). Oxidative stress in allergic and inflammatory skin diseases. *Curr. Drug Targets* 4:517–519.
- Oksay, T., Naziroglu, M., Dogan, S., et al. (2014). Protective effects of melatonin against oxidative injury in rat testis induced by wireless (2.45 GHz) devices. *Andrologia* 46:65–72.
- Oktem, F., Ozguner, F., Mollaoglu, H., et al. (2005). Oxidative damage in the kidney induced by 900-MHz-emitted mobile phone: Protection by melatonin. *Arch. Med. Res.* 36:350–355.
- Oral, B., Guney, M., Ozguner, F., et al. (2006). Endometrial apoptosis induced by a 900-MHz mobile phone: Preventive effects of vitamins E and C. *Adv. Ther.* 23:957–973.
- Oshino, N., Jamieson, D., Sugano, T., et al. (1975). Optical measurement of catalase-hydrogen peroxide intermediate (compound-i) in liver of anesthetized rats and its implication to hydrogen-peroxide production in situ. *Biochem. J.* 146:67–77.
- Ott, M., Gogvadze, V., Orrenius, S., et al. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis* 12:913–922.
- Ozguner, F., Altinbas, A., Ozyaydin, M., et al. (2005a). Mobile phone-induced myocardial oxidative stress: Protection by a novel antioxidant agent caffeic acid phenethyl ester. *Toxicol. Ind. Health.* 21:223–230.
- Ozguner, F., Bardak, Y., Comlekci, S. (2006). Protective effects of melatonin and caffeic acid phenethyl ester against retinal oxidative stress in long-term use of mobile phone: A comparative study. *Mol. Cell Biochem.* 282:83–88.
- Ozguner, F., Oktem, F., Ayata, A., et al. (2005b). A novel antioxidant agent caffeic acid phenethyl ester prevents long-term mobile phone exposure-induced renal impairment in rat. Prognostic value of malondialdehyde, N-acetyl-beta-D-glucosaminidase and nitric oxide determination. *Mol. Cell Biochem.* 277:73–80.
- Ozguner, E., Guler, G., Seyhan, N. (2010). Mobile phone radiation-induced free radical damage in the liver is inhibited by the antioxidants N-acetyl cysteine and epigallocatechin-gallate. *Int. J. Radiat. Biol.* 86: 935–945.
- Ozguner, E., Kismali, G., Guler, G., et al. (2013). Effects of prenatal and postnatal exposure to gsm-like radiofrequency on blood chemistry and oxidative stress in infant rabbits, an experimental study. *Cell Biochem. Biophys.* 67:743–751.
- Özorak, A., Naziroglu, M., Çelik, Ö., et al. (2013). Wi-Fi (2.45 GHz)- and mobile phone (900 and 1800 MHz)-induced risks on oxidative stress and elements in kidney and testis of rats during pregnancy and the development of offspring. *Biol. Trace Elem. Res.* 156: 221–229.
- Panagopoulos, D. J., Karabarbounis, A., Margaritis, L. H. (2002). Mechanism for action of electromagnetic fields on cells. *Biochem. Biophys. Res. Commun.* 298:95–102.
- Panagopoulos, D. J., Messini, N., Karabarbounis, A., et al. (2000). A mechanism for action of oscillating electric fields on cells. *Biochem. Biophys. Res. Commun.* 272:634–640.
- Paulraj, R., Behari, J., Rao, A. R. (1999). Effect of amplitude modulated RF radiation on calcium ion efflux and ODC activity in chronically exposed rat brain. *Indian J. Biochem. Biophys.* 36:337–340.
- Pavicic, I., Trosic, I. (2010). Interaction of GSM modulated RF radiation and macromolecular cytoskeleton structures. *Paper presented at the 6th International Workshop on Biological Effects of Electromagnetic Fields.*
- Pilla, A. A. (2012). Electromagnetic fields instantaneously modulate nitric oxide signaling in challenged biological systems. *Biochem. Biophys. Res. Commun.* 426:330–333.
- Qin, F., Yuan, H., Nie, J., et al. (2014). [Effects of nano-selenium on cognition performance of mice exposed in 1800 MHz radiofrequency fields]. *Wei sheng yan jiu = J. Hygiene Res.* 43:16–21.
- Ragy, M. M. (2014). Effect of exposure and withdrawal of 900-MHz-electromagnetic waves on brain, kidney and liver oxidative stress and some biochemical parameters in male rats. *Electromagn. Biol. Med. (Published online)*:1–6.
- Ralph, S. J., Rodríguez-Enríquez, S., Neuzil, J., et al. (2010). The causes of cancer revisited: “Mitochondrial malignancy” and ROS-induced oncogenic transformation – Why mitochondria are targets for cancer therapy. *Mol. Aspects Med.* 31:145–170.

- Rao, V. S., Titushkin, I. A., Moros, E. G., et al. (2008). Nonthermal effects of radiofrequency-field exposure on calcium dynamics in stem cell-derived neuronal cells: Elucidation of calcium pathways. *Radiat. Res.* 169:319–329.
- Repacholi, M. H., Basten, A., Gebiski, V., et al. (1997). Lymphomas in E mu-Pim1 transgenic mice exposed to pulsed 900 MHz electromagnetic fields. *Radiat. Res.* 147:631–640.
- Ruediger, H. W. (2009). Genotoxic effects of radiofrequency electromagnetic fields. *Pathophysiology* 16:89–102.
- Sadetzki, S., Chetrit, A., Jarus-Hakak, A., et al. (2008). Cellular phone use and risk of benign and malignant parotid gland tumors – A nationwide case-control study. *Am. J. Epidemiol.* 167:457–467.
- Saikhedkar, N., Bhatnagar, M., Jain, A., et al. (2014). Effects of mobile phone radiation (900 MHz radiofrequency) on structure and functions of rat brain. *Neurol. Res.* 36:1072–1079.
- Santini, R., Santini, P., Danze, J. M., et al. (2002). Study of the health of people living in the vicinity of mobile phone base stations: 1. Influences of distance and sex. *Pathol. Biol.* 50:369–373.
- Sato, Y., Akiba, S., Kubo, O., et al. (2011). A case-case study of mobile phone use and acoustic neuroma risk in Japan. *Bioelectromagnetics* 32:85–93.
- Sefidbakht, Y., Moosavi-Movahedi, A. A., Hosseinkhani, S., et al. (2014). Effects of 940 MHz EMF on bioluminescence and oxidative response of stable luciferase producing HEK cells. *Photochem. Photobiol. Sci.* 13:1082–1092.
- Shahin, S., Singh, V. P., Shukla, R. K., et al. (2013). 2.45 GHz microwave irradiation-induced oxidative stress affects implantation or pregnancy in mice, *Mus musculus*. *Appl. Biochem. Biotechnol.* 169:1727–1751.
- Sharma, V. P., Singh, H. P., Kohli, R. K., et al. (2009). Mobile phone radiation inhibits *Vigna radiata* (mung bean) root growth by inducing oxidative stress. *Sci. Total Environ.* 407:5543–5547.
- Sies, H. (2014). Role of metabolic H₂O₂ generation: Redox signalling and oxidative stress. *J. Biol. Chem.* 289:8735–8741.
- Singh, H. P., Sharma, V. P., Batish, D. R., et al. (2012). Cell phone electromagnetic field radiations affect rhizogenesis through impairment of biochemical processes. *Environ. Monitor. Assess.* 184: 1813–1821.
- Sokolovic, D., Djindjic, B., Nikolic, J., et al. (2008). Melatonin reduces oxidative stress induced by chronic exposure of microwave radiation from mobile phones in rat brain. *J. Radiat. Res. (Tokyo)*. 49: 579–586.
- Sokolovic, D., Djordjevic, B., Kocic, G., et al. (2013). Melatonin protects rat thymus against oxidative stress caused by exposure to microwaves and modulates proliferation/apoptosis of thymocytes. *Gen. Physiol. Biophys.* 32:79–90.
- Suleyman, D., M. Zulkuf, A., Feyzan, A., et al. (2004). Does 900 MHz GSM mobile phone exposure affect rat brain? *Electromagn. Biol. Med.* 23:201–214.
- Szmigielski, S., Szudzinski, A., Pietraszek, A., et al. (1982). Accelerated development of spontaneous and benzopyrene-induced skin cancer in mice exposed to 2450-MHz microwave radiation. *Bioelectromagnetics* 3:179–191.
- Tice, R. R., Hook, G. G., Donner, M., et al. (2002). Genotoxicity of radiofrequency signals. I. Investigation of DNA damage and micronuclei induction in cultured human blood cells. *Bioelectromagnetics* 23:113–126.
- Tkalec, M., Malaric, K., Pevalek-Kozlina, B. (2007). Exposure to radiofrequency radiation induces oxidative stress in duckweed Lemna minor L. *Sci. Total Environ.* 388:78–89.
- Tkalec, M., Stambuk, A., Srut, M., et al. (2013). Oxidative and genotoxic effects of 900 MHz electromagnetic fields in the earthworm *Eisenia fetida*. *Ecotoxicol. Environ. Saf.* 90:7–12.
- Tök, L., Nazıroğlu, M., Doğan, S., et al. (2014). Effects of melatonin on Wi-Fi-induced oxidative stress in lens of rats. *Indian J. Ophthalmol.* 62:12–15.
- Toler, J. C., Shelton, W. W., Frei, M. R., et al. (1997). Long-term, low-level exposure of mice prone to mammary tumors to 435 MHz radiofrequency radiation. *Radiat. Res.* 148:227–234.
- Tomruk, A., Guler, G., Dincel, A. S. (2010). The influence of 1800 MHz GSM-like signals on hepatic oxidative DNA and lipid damage in nonpregnant, pregnant, and newly born rabbits. *Cell. Biochem. Biophys.* 56:39–47.
- Tsybulin, O., Sidorik, E., Brieva, O., et al. (2013). GSM 900 MHz cellular phone radiation can either stimulate or depress early embryogenesis in Japanese quails depending on the duration of exposure. *Int. J. Radiat. Biol.* 89:756–763.
- Tsybulin, O., Sidorik, E., Kyrlyenko, S., et al. (2012). GSM 900 MHz microwave radiation affects embryo development of Japanese quails. *Electromagn. Biol. Med.* 31:75–86.
- Türedi, S., Hancı, H., Topal, Z., et al. (2014). The effects of prenatal exposure to a 900-MHz electromagnetic field on the 21-day-old male rat heart. *Electromagn. Biol. Med. (Published online)*.1–8.
- Turker, Y., Nazıroğlu, M., Gumral, N., et al. (2011). Selenium and L-carnitine reduce oxidative stress in the heart of rat induced by 2.45-GHz radiation from wireless devices. *Biol. Trace Elem. Res.* 143: 1640–1650.
- Vaks, V. L., Domrachev, G. A., Rodygin, Y. L., et al. (1994). Dissociation of water by microwave radiation. *Radiophys. Quant. Electron.* 37:85–88.
- Valko, M., Leibfritz, D., Moncol, J., et al. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39:44–84.
- Valko, M., Rhodes, C. J., Moncol, J., et al. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Interact.* 160:1–40.
- Wang, X., Sharma, R. K., Gupta, A., et al. (2003). Alterations in mitochondria membrane potential and oxidative stress in infertile men: A prospective observational study. *Fertil. Steril.* 80:844–850.
- Wolf, R., Wolf, D. (2007). Increased incidence of cancer near a cell-phone transmitted station. In F. Columbus (Ed.), *Trends in Cancer Prevention* New York: Nova Science Publishers, Inc. pp. 1–8.
- Xu, S., Zhou, Z., Zhang, L., et al. (2010). Exposure to 1800 MHz radiofrequency radiation induces oxidative damage to mitochondrial DNA in primary cultured neurons. *Brain. Res.* 1311:189–196.
- Yakymenko, I., Sidorik, E., Kyrlyenko, S., et al. (2011). Long-term exposure to microwave radiation provokes cancer growth: Evidences from radars and mobile communication systems. *Exp. Oncol.* 33: 62–70.
- Yakymenko, I., Sidorik, E., Tsybulin, O., et al. (2011). Potential risks of microwaves from mobile phones for youth health. *Environ. Health* 56: 48–51.
- Yurekli, A. I., Ozkan, M., Kalkan, T., et al. (2006). GSM base station electromagnetic radiation and oxidative stress in rats. *Electromagn. Biol. Med.* 25:177–188.
- Zhao, T. Y., Zou, S. P., Knapp, P. E. (2007). Exposure to cell phone radiation up-regulates apoptosis genes in primary cultures of neurons and astrocytes. *Neurosci. Lett.* 412:34–38.
- Zmyślony, M., Politanski, P., Rajkowska, E., et al. (2004). Acute exposure to 930 MHz CW electromagnetic radiation in vitro affects reactive oxygen species level in rat lymphocytes treated by iron ions. *Bioelectromagnetics* 25:324–328.
- Zotti-Martelli, L., Peccatori, M., Maggini, V., et al. (2005). Individual responsiveness to induction of micronuclei in human lymphocytes after exposure in vitro to 1800-MHz microwave radiation. *Mutat. Res.* 582:42–52.

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Increased blood–brain barrier permeability in mammalian brain 7 days after exposure to the radiation from a GSM-900 mobile phone

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Abstract

Microwaves were for the first time produced by humans in 1886 when radio waves were broadcasted and received. Until then microwaves had only existed as a part of the cosmic background radiation since the birth of universe. By the following utilization of microwaves in telegraph communication, radars, television and above all, in the modern mobile phone technology, mankind is today exposed to microwaves at a level up to 10^{20} times the original background radiation since the birth of universe.

Our group has earlier shown that the electromagnetic radiation emitted by mobile phones alters the permeability of the blood–brain barrier (BBB), resulting in albumin extravasation immediately and 14 days after 2 h of exposure.

In the background section of this report, we present a thorough review of the literature on the demonstrated effects (or lack of effects) of microwave exposure upon the BBB.

Furthermore, we have continued our own studies by investigating the effects of GSM mobile phone radiation upon the blood–brain barrier permeability of rats 7 days after one occasion of 2 h of exposure. Forty-eight rats were exposed in TEM-cells for 2 h at non-thermal specific absorption rates (SARs) of 0 mW/kg, 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg. Albumin extravasation over the BBB, neuronal albumin uptake and neuronal damage were assessed.

Albumin extravasation was enhanced in the mobile phone exposed rats as compared to sham controls after this 7-day recovery period (Fisher's exact probability test, $p = 0.04$ and Kruskal–Wallis, $p = 0.012$), at the SAR-value of 12 mW/kg (Mann–Whitney, $p = 0.007$) and with a trend of increased albumin extravasation also at the SAR-values of 0.12 mW/kg and 120 mW/kg. There was a low, but significant correlation between the exposure level (SAR-value) and occurrence of focal albumin extravasation ($r_s = 0.33$; $p = 0.04$).

The present findings are in agreement with our earlier studies where we have seen increased BBB permeability immediately and 14 days after exposure. We here discuss the present findings as well as the previous results of altered BBB permeability from our and other laboratories. © 2009 Elsevier Ireland Ltd. All rights reserved.

Keywords: Albumin; Blood–brain barrier; Mobile phone; Rat

1. Introduction: radiofrequency radiation and the blood–brain barrier

Today about half of the world's population owns the microwave-producing mobile phones. An even larger number is exposed to the radiation emitted from these devices through “passive mobile phoning” [1]. Life-long exposure to the microwaves (MWs) from mobile phones, with start already at a young age, is becoming increasingly common

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; CW, continuous wave; EMF, electromagnetic field; GSM, global system for mobile communication; ICNIRP, International Commission of Non-ionizing Radiation Protection; MRI, magnetic resonance imaging; RF, radio frequency; SAR, specific absorption rate; TEM-cell, transverse electromagnetic transmission line chamber.

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among the new generations of mobile phone users. The question is: to what extent are living organisms affected by these radio frequency (RF) fields?

The mobile phones are held in close proximity to the head, or within a metre of the head when hands-free sets are used. The emitted microwaves have been shown to have many effects upon the mammalian brain; e.g. alterations of cognitive functions [2,3], changes of neurotransmitter levels such as decrease of cholinergic activity [4], gene expression alterations in cerebellum [5], cortex and hippocampus [6], and impact upon the brain EEG activity [7]. Also, the human brain EEG beta rhythms energies were increased by exposure to 450 MHz MWs modulated at different low frequencies [8]. Recent epidemiological studies also indicate that long-term exposure increases the risk of not only for benign vestibular schwannoma (previously named acoustic neurinoma) [9], but also malignant glioblastoma multiforme [10] for mobile phone use longer than 10 years and with cumulative exposure from mobile phones exceeding 2000 h.

It has been shown that electromagnetic fields (EMFs) increase the permeability of the blood–brain barrier (BBB) (for reference see [11]). The BBB is a hydrophobic barrier, formed by vascular endothelial cells of the capillaries in the brain, with tight junctions between these endothelial cells. It protects the mammalian brain from potentially harmful compounds in the blood. Also, perivascular structures such as astrocytes and pericytes as well as a bi-layered basal membrane help maintaining the BBB.

The current recommendations for limits of exposure to the general public for EMF radiation [12] are set in order to avoid thermal effects upon the brain parenchyma.

In our previous studies we have seen that non-thermal RF fields cause significantly increased leakage of the rats' own albumin through the BBB of exposed rats sacrificed immediately after the exposure, as compared to sham exposed control animals [11,13–18]. Two hours of exposure to the radiation from a global system for mobile communications (GSM) phone at 915 MHz, at non-thermal specific absorption rates (SAR) values of 0.12 mW/kg, 12 mW/kg and 120 mW/kg, gives rise to focal albumin extravasation and albumin uptake into neurons also 14 days after exposure, but not after 28 days [19]. Significant neuronal damage is present 28 days [19] and 50 days after exposure [20], but not after 14 days [19]. Also, in experiments from other laboratories, BBB permeability is increased in connection to mobile phone exposure [21–23] and other kinds of EMF such as magnetic resonance imaging (MRI) exposure [24–26]. In other studies, no such BBB alterations have been demonstrated in connection to mobile phone exposure [27–29] or other kinds of EMF exposure [30,31].

1.1. The blood–brain barrier

An intact BBB is necessary for the protection of the mammalian brain from potentially harmful substances circulating in the blood. In the normal brain, the passage of compounds over the BBB is highly restricted and homeostasis within

the sensitive environment of the brain parenchyma can be maintained.

The BBB is formed by the vascular endothelial cells of the capillaries of the brain and the glial cells wrapped around them. The tight junctions, that seal the endothelial cells together, limit paracellular leakage of molecules. A bi-layered basal membrane supports the abluminal side of the endothelial cells. The glial astrocytes, surrounding the surface of the basal membrane cells, are important for the maintenance, functional regulation and repair of the BBB. The protrusions of the astrocytes, called end feet, cover the basal membrane on the outer endothelial surface and thus form a second barrier to hydrophilic molecules and connect the endothelium to the neurons. Twenty-five per cent of the abluminal membrane of the capillary surface is covered by pericytes [32], which are a type of macrophages. Seemingly, they are in the position to significantly contribute to the central nervous system (CNS) immune mechanisms [33].

The physiological properties of the CNS microvasculature are different from those of peripheral organs. The numbers of pinocytotic vesicles for nutrient transport through the endothelial cytoplasm are low and there are no fenestrations. Also, there is a fivefold higher number of mitochondria in the BBB endothelial cells as compared to muscular endothelial cells [34].

In a functioning BBB, the membrane properties control the bidirectional exchange between the general circulation and the CNS. Water, most lipid-soluble molecules, oxygen and carbon dioxide can diffuse from the blood to the nerve cells. The barrier is slightly permeable to ions such as sodium, potassium and chloride, but large molecules, such as proteins and most water-soluble chemicals only pass poorly. However, when this barrier is damaged, in conditions such as tumours, infarcts or infections, also the normally excluded molecules can pass through, possibly bringing toxic molecules out into the brain tissue. The selective permeability is disrupted temporally in cases of epileptic seizures [35,36] and severe hypertension [37]. The result of this can be cerebral oedema, increased intracranial pressure and irreversible brain damage. Also, toxic substances from the blood circulation now reach out to the neurons. Even transient openings of the BBB can lead to permanent tissue damage [37].

1.2. The earliest studies on the effects of microwave exposure

The first studies on the MW effects upon the BBB were reported in the 1970s, when the radiation from radars and MW ovens were considered to be possible health threats. Increased leakage of fluorescein after 30 min of pulsed and CW exposure [38] and passage of ^{14}C -mannitol, inulin and dextran at very low energy levels [39] were reported. The permeation of mannitol was found to be a definite function of exposure parameters such as power density, pulse width, and the number of pulses per second. Also, the BBB permeability depended on the time between the EMF exposure and the

sacrifice of the animals, with more pronounced effects seen in the animals sacrificed earlier after the EMF exposure. In attempts to replicate the findings of Oscar and Hawkins [39], however, these results were not found [40,41]. Similar lack of MW induced BBB effects, was reported by Ward et al. [42] after exposure of rats to CWs at 2450 MHz; Ward and Ali [43] after exposure at 1.7 GHz; and Gruenau et al. [44] after exposure to pulsed or CW waves at 1.8 GHz (including totally 31 rats). On the other hand, Albert and Kerns [45] observed EMF-induced BBB permeability after exposure at 2450 MHz CWs, with an increase in the number of pinocytotic vesicles among the irradiated animals, but after a recovery time of 1–2 h, the permeation was hardly detectable anymore. For details concerning the EMF exposure parameters in these studies, see [11].

1.3. MRI exposure

MRI entails a concurrent exposure to a high-intensity static field, a RF field and a time-varying magnetic field. In connection to the introduction of the MRI technique, the effects of exposure to these kinds of fields upon the BBB permeability were investigated.

As mentioned above, Shivers et al. [24] observed that the EMF exposure of the type emitted during a MRI procedure resulted in a temporarily increased BBB permeability in the brains of rats. Through transendothelial channels, a vesicle-mediated transport of horseradish peroxidase (HRP) took place. Replications of the initial findings by Shivers et al. [24] were made by Garber et al. [46], whereas Adzamlı et al. [30] and Preston et al. [31] could not repeat the findings.

After some years, quantitative support of the findings by Shivers et al. [24] was presented by the same group [25,26]. In rats exposed to the MRI, the BBB permeability to DTPA (diethylenetriaminepentaacetic acid) increased. A suggested mechanism explaining the increased permeability was a stimulation of endocytosis, made possible through the time-varying magnetic fields.

Also our studies supported the findings of the Shiver–Prato group; seeing that BBB permeability to albumin was increased after exposure to MRI radiation [13]. The most significant effect was observed after exposure to the RF part of the MRI.

1.4. Studies on mobile phone exposure

The mobile phone induced effects upon the BBB permeability is a topic of importance for the whole society today. We have previously found an increased BBB permeability immediately after 2 h of mobile phone exposure [14], and also after 14 days [19] and 50 days [20].

Repetitions of our findings of increased BBB permeability after mobile phone exposure have been made [47,21,22]. Four hours of GSM-900 MHz exposure at brain power densities ranging from 0.3 to 7.5 W/kg resulted in significantly increased albumin extravasation both at the SAR-value of

7.5 W/kg, which is a thermal effect, but also at 0.3 W/kg and 1.3 W/kg [47] (statistical evaluations discussed by Salford et al. [1]). Albumin extravasation was also seen in rats exposed for 2 h to GSM-900 MHz at non-thermal SAR-values of 0.12, 0.5 and 2 W/kg using fluorescein-labelled proteins [21,22]. At SAR of 2 W/kg a marked BBB permeabilization was observed, but also at the lower SAR-value of 0.5 W/kg, permeabilization was present around intracranial blood vessels. However, the extravasation at 0.5 W/kg was seen at a lesser extent as compared to that seen at 2 W/kg. Subgroups of the rats included in these studies were sympathectomised, which means that they were in a chronic inflammation-prone state with increased BBB opening due to changes in the structures of the blood vessels. Interestingly, the sympathectomised rats exposed to GSM radiation had a remarkable increase of the BBB leakage as compared to their sympathectomised sham controls. From these findings it seems likely that an already disrupted BBB is more sensitive to the RF fields than an intact BBB.

In another study, the uptake of rhodamine–ferritin complex through the BBB was investigated [23]. In this study, increased BBB permeability was clearly seen at exposure levels of 2 W/kg and durations of 30–120 min. When the rats were pre-treated with colchicine, the EMF-induced rhodamine–ferritin uptake was however blocked. Colchicine is well-known for its inhibition of microtubular function. It was concluded that the microtubules seemed to play an important role for the BBB opening.

Lack of EMF-induced BBB alterations has also been reported [27–29,48]. In a small study including only 12 EMF exposed animals, no albumin extravasation was seen, neither after 2 nor 4 weeks of 1 h of daily exposure (average whole-body exposure at 0.25 W/kg) [27]. In a study including 40 animals, Kuribayashi et al. [28] concluded no BBB alterations was seen after 90 min of daily EMF exposure for 1–2 weeks at SAR-values of 2 or 6 W/kg. Finnie et al. [29] exposed mice for 1 h daily. However, only the SAR-value of 4 W/kg, which is above the ICNIRP limit [12], was included. In a further study by Finnie et al. [48] 207 mice were exposed for 104 weeks at SAR-values of 0.25–4 W/kg, however without any observable effects upon the BBB permeability. The same group also reported that the immature BBB was insensitive to mobile phone exposure, seen after GSM-900 radiation exposure of pregnant mice from day 1 to day 19 of gestation (SAR of 4 W/kg, exposure for 60 min daily). No increased albumin extravasation was seen in the new-born mice immediately after parturition [49] and the same lack of GSM-900 radiation effects upon the BBB permeability was reported for young rats by Kumlin et al. [50], however, in this case only 12 out of totally 48 exposed rats were analyzed histopathologically. The remaining rats were subject to memory tests, where an improved learning and memory was seen in the EMF exposed rats as compared to the sham controls. Notably, in all these studies, the SAR-values for exposure are relatively high; never including the low SAR-values below 200 mW/kg.

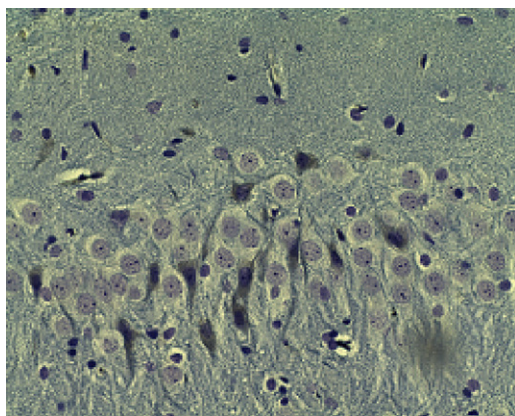


Fig. 1. Albumin neuronal uptake and early neuronopathy in the hippocampal pyramidal cell row among normal neurons. Albumin: cresyl violet, $\times 20$.

In more recent years, *in vitro* models have been increasingly applied to investigate the BBB; in one of these, it was shown that EMFs at 1.8 GHz increase the permeability to sucrose [51]. After modifications of the BBB model to one with higher tightness, however, the same group could not replicate their initial findings [52]. With application of the EMF of the kind emitted from 3G mobile phones, the same group further concluded that their *in vitro* BBB model also did not alter its tightness or transport behaviour in connection to this type of exposure [53].

1.5. Neuronal damage in connection to mobile phone exposure

In our previous studies of animals surviving a longer period after the exposure, we have evaluated the occurrence of neuronal damage extensively [19,20]. This neuronal damage is seen as condensed dark neurons. Dark neurons have been proposed to have three main characteristics [54]: (i) irregular cellular outlines, (ii) increased chromatin density in the nucleus and cytoplasm and (iii) intensely and homogeneously stained nucleus. Twenty-eight days after 2 h of mobile phone exposure, the neuronal damage was significantly increased in the exposed rats as compared to the sham exposed controls [19]. Also 50 days after the same kind of mobile phone exposure, there was an increased occurrence of neuronal damage [20].

In our studies, normal neurons have been shown to have increased uptake of albumin [19] (Fig. 1). Also, in dark neurons this neuronal albumin uptake can be seen (Fig. 2). In our previous studies, damaged neurons were seen in all locations, intermingled with normal neurons especially in the cortex, hippocampus and basal ganglia. The damaged neurons were often shrunken and dark staining, homogenized with loss of discernable internal cell structures (Fig. 3). Some damaged neurons showed microvacuoles in the cytoplasm (Fig. 4). These vacuoles are a sign of severe neuronopathy, indicating an active pathological process. There was no evidence of haemorrhages or glial reaction.

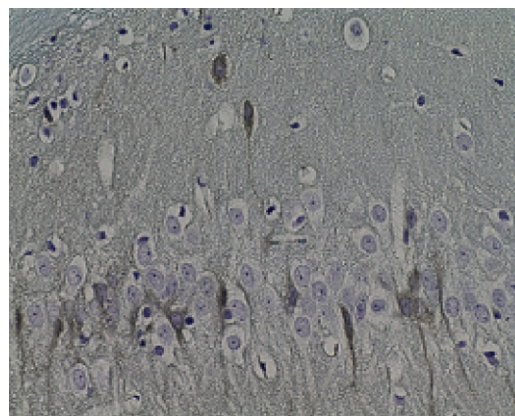


Fig. 2. Shrunken homogenized dark neurons with brownish discoloration due to uptake of albumin, interspersed among normal neurons in the hippocampal pyramidal cell row. Albumin: cresyl violet, $\times 20$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

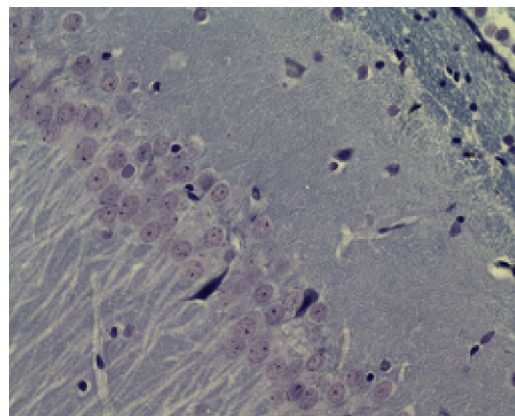


Fig. 3. Two dark neurons in the hippocampal pyramidal cell row. Albumin: cresyl violet, $\times 20$.

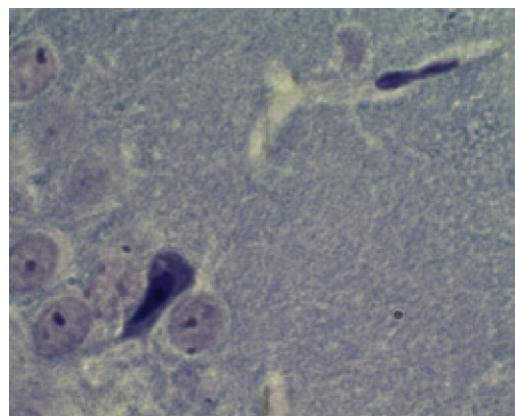


Fig. 4. Dark neuron in the hippocampal pyramidal cell row, with homogenized nucleus and cytoplasm with a vacuole. Higher magnification of part of the figure. Albumin: cresyl violet, $\times 40$.

Dark neurons are reported in clinical and experimental neuropathology from living tissues, but not in autopsy material unless the post-mortem period is short. This could indicate that the formation of dark neurons is an active process that requires living neurons and that these cells must be reasonably intact [55]. This could be in accordance with our findings from the 50-days survival animals, where apoptosis could not be detected in any of the RF EMF exposed brains with application of Caspase-3 [56].

Dark neurons occur not only after GSM exposure [19,20] but also in connection to experimental ischemia [57], hypoglycemia [58] and epilepsy [59]. Possibly, dark neurons could be artefacts, having a pressure-derived mechanical origin, as has been shown for cortical biopsies (this is less likely considering the atraumatic method of dissection used here including fixation before handling and in view of the deep location of the dark neurons). However, dark neurons also appear as a result of other, and not fully clarified, mechanisms, as seen in the case of GSM exposure, ischemia, hypoglycemia and epilepsy. A pharmacologic origin, such as depolarization related to tissue glutamate release in injury, could explain the pathogenetic mechanism for dark neurons in these cases, rather than the pressure-derived mechanical origin. Indeed, the formation of dark neurons can be prevented using pharmacologic forms of glutamate antagonism [55]. In the case of our studies, our technique for the resection of the rat brains is chosen to avoid mechanical pressure.

Findings of dark neurons in connection to mobile phone exposure have been reported by Ihan et al. [60] (GSM exposure of rats for 7 days, 1 h daily). Also, an increase of oxidative damage was seen in the exposed rats as a significant increase in malondialdehyde (MDA) (an index for lipid peroxidation), nitric oxide (NO) levels, brain xanthine oxidase (XO) and adenosine deaminase (ADA) activities, as compared to the controls. With treatment of the anti-oxidant *Ginkgo biloba*, the EMF induced increments of XO, ADA, MDA and NO were prevented. The anti-oxidant activity of *G. biloba* is attributed to its flavinoid glycosides, which are the active compounds in the leaves. The action of these flavinoids is to destroy free radicals, such as NO and lipid peroxide radicals. Also the formation of dark neurons was reported to be prevented when the rats had been treated with *G. biloba*. Other attempts to repeat our findings of dark neurons after mobile phone exposure have been performed in a collaborative effort with Bernard Veyret's group in Bordeaux [61]. In this study, the situation 14 days and 50 days after 2 h of GSM-900 radiation exposure at average brain SAR-values of 0.14 W/kg and 2.0 W/kg was evaluated. No increased amount of dark neurons was reported.

It has been suggested that BBB leakage is the major reason for nerve cell injury, such as dark neurons in stroke-prone spontaneously hypertensive rats [62]. Albumin leaks into the brain and neuronal degeneration is seen in areas with BBB disruption in several circumstances: after intracarotid infusion of hyperosmolar solutions in rats [63]; in the stroke prone hypertensive rat [65]; in acute hypertension by aor-

tic compression in rats [37]. The linkage between albumin extravasation over the BBB and neural damage might be a potentiating effect of albumin upon the glutamate-mediated neurotoxicity [64]. Indeed, both albumin- and glutamate-induced lesions have the same histopathological appearance with invasion of macrophages and absence of neuronal cell bodies and axons in the lesion areas [65]. The glutamate itself can also increase the BBB opening [66], leading to further albumin extravasation out into the brain parenchyma. From our previous findings of albumin extravasation 14 days after exposure [19] and dark neurons not until after 28 days [19] and 50 days [20], it could be hypothesized that albumin extravasation into the brain parenchyma, is the first observable effect of the mobile phone exposure. The albumin leakage precedes and possibly could be the cause of, the damage to the neurons seen as the dark neurons later on. In this connection, the findings of [37] that transient openings of the BBB can result in permanent tissue damage, can also be mentioned. Hypertensive opening of the BBB resulted in albumin extravasation after 2 h, but the effects remained, although to a lesser extent, also after 7 days. Many neurons with cytoplasmatic immunoreactivity for albumin appeared shrunken. Seven days after the BBB opening, there was a neuronal loss in these areas and a vigorous glial reaction, indicating that some neurons were irreversibly damaged [37].

2. Aims of the present study

In the present study we have continued to investigate the effects of EMFs upon the rat brain, now with focus on what happens 7 days after GSM exposure at 915 MHz for 2 h at non-thermal energy levels of 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg. The main questions to be answered were: whether the same increase of the BBB permeability is seen 7 days after exposure as that showed previously immediately after exposure and after 14 days, and whether different exposure levels result in a different response.

In order to compare to our previous findings, we have used the same exposure system, GSM signal, animal model and histopathological methods as in our previous studies.

3. Materials and methods

3.1. GSM exposure

The animals were exposed to RF EMFs in the same transverse electromagnetic transmission line cell (TEM-cells) as previously described and used by [1,2,5,13–19]. The TEM-cells were designed by dimensional scaling from previously constructed cells at the National Bureau of Standards [67]. TEM-cells are known to generate uniform EMFs for standard measurements.

A genuine GSM mobile phone, operating at the 900 MHz frequency band, with programmable power output, was con-

nected via a coaxial cable to the TEM-cells. Through a power splitter, the power was divided into equal parts fed into the four TEM-cells used (TEM-cell A, B, C and D). No voice modulation was applied. Each of the four TEM-cells is connected to a 50 Ω dummy load, into which the output is terminated. By using these TEM-cells, the pulse modulated exposure fields can be accurately generated without the distortion that is typically introduced when conventional antennas are used to establish impulse test fields. Thus, a relatively homogeneous exposure of the animals is allowed [68].

The TEM-cell is enclosed in a wooden box (inner dimensions of 15 cm $\times$ 15 cm $\times$ 15 cm) that supports the outer conductor, made of brass net, and central conducting plate. The central plate separates the top and bottom of the outer conductor symmetrically. Eighteen holes (diameter 18 mm) in the sidewalls and top of the wooden box make ventilation possible. Air is circulated through the holes of the TEM-cells using four fans, each placed next to the outer walls of its respective TEM-cell. The holes are also used for examination of the interior during exposure. For a further description of the TEM-cell, see [68].

The rats were placed in plastic trays (14 cm $\times$ 14 cm $\times$ 7 cm) to avoid contact with the central plate and outer conductor. The bottom of the tray was covered with absorbing paper to collect urine and faeces. Each TEM-cell contained two plastic trays, one above and one below the centre septum. Thus two rats could be kept in each TEM-cell simultaneously. All the animals could move and turn around within the TEM-cells.

For the actual experimental situation with one rat in each compartment of the TEM-cell, the conversion factor K for SAR per unit of input power could be fitted to the data as

$$K = (1.39 \pm 0.17) - (0.85 \pm 0.22)w \quad (1)$$

with w the sum of weights in kilograms of the 2 rats in the cell and the variance given as SEM. For a more detailed description, see [2].

Whole-body SAR and brain SAR vary with orientation. In our present set-up, the average of SAR for the brain grey matter was 1.06 times the average whole-body SAR, with a standard deviation of 56% around the average value for the different orientations, as estimated by us previously [19].

3.2. Animals

All animal procedures were performed according to the practices of the Swedish Board of Animal Research and were approved by the Animal Ethics Committee, Lund-Malmö.

Forty-eight inbred male and female Fischer 344 rats (the rats were supplied by Scanbur AB, Stockholm, Sweden) were 2–3 months of age at the initiation of the EMF exposure. Male and female rats weighed 225 g $\pm$ 56 g (standard deviation) and 233 g $\pm$ 60 g (standard deviation) respectively.

The rats were housed in rat hutches, two in each cage, under standard conditions of 22 °C room temperature, artificial daylight illumination and rodent chow and tap water *ad libitum*.

The 48 rats were divided into four exposure groups, each group consisting of 8 rats, and one sham exposed group with 16 animals.

The peak power output from the GSM mobile phone fed into the TEM-cells was 1 mW, 10 mW, 100 mW and 1000 mW per cell respectively for a period of 2 h. This resulted in average whole-body SAR of 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg for the four different exposure groups.

All animals were kept in the animal facilities for a recovery period of 7 days after exposure. At the end of this period they were anaesthetized and sacrificed by perfusion-fixation with 4% formaldehyde.

3.3. Histopathology and methods

The brains were fixed *in situ* through saline perfusion through the ascending aorta for 3 min followed by 4% formaldehyde for 10 min and immersion in 4% formaldehyde for 24 h. They were then removed from the skulls by a non-traumatic technique (resection of bone structures at the skull base, followed by a midline incision from the foramen magnum to the nose) and immersion fixed in 4% formaldehyde for more than 24 h. Whole coronal sections of the brains were dehydrated and embedded in paraffin, sectioned at 5 μ m with a microtome and stained for RNA/DNA with cresyl violet to visualize damaged neurons. Albumin was demonstrated with the IgG fraction of rabbit anti-rat albumin (Dakopatts, Helsingborg, Sweden) diluted 1:8,000. This reveals albumin as brownish spotty or more diffuse discolorations. Biotinylated swine anti-rabbit IgG was used as a secondary antibody. Then avidin, peroxidase conjugated, was coupled to the biotin and visualized with DAB (diaminobenzidine).

All brains were examined histopathologically by our neuropathologist (A.B.). All microscopic analyses were performed blind to the test situation.

Regarding albumin extravasation, the number of immunopositive extravasates (foci) were recorded under a microscope. None or occasional minor leakage was rated as normal, whereas one larger or several leakages were regarded as pathological. Immunopositive sites were, however, disregarded when localized in the hypothalamus, above the median eminence and laterally including the lateral hypothalamic nuclei, in the immediate vicinity of the third ventricle and just beneath the pial membrane. These structures are well known for their insufficient BBB. Also the presence and distribution of albumin uptake into neurons was judged semi-quantitatively.

Regarding neuronal damage, this were judged semi-quantitatively as no or occasional (score 0), moderate (score 1) or abundant occurrence (score 2) of dark neurons.

3.4. Statistics

As an initial discriminative test, the occurrence of an effect of exposure (score 1 or higher for albumin foci; score 0.5 or higher for neuronal albumin uptake and dark neurons) was tested against sham exposed controls using Fisher's exact probability test.

The Kruskal–Wallis one-way analysis of variance by ranks was used for a simultaneous statistical test of the score distributions for the five conditions of sham or EMF exposure. When the null hypothesis could be rejected, the non-parametric Mann–Whitney *U*-test for independent samples was used to compare each of the groups of GSM exposed and sham exposed animals.

The occurrence of covariates such as gender, the position of the rat in the TEM-cell (upper/lower compartment) and the TEM-cell used (TEM-cell A, B, C or D) was evaluated using linear regression analysis.

Spearman's non-parametric correlation analysis was used for evaluation of correlation between exposure level, albumin foci, neuronal albumin and dark neurons.

4. Results

In exposed animals there were albumin positive foci around capillaries in the white and grey matter (Fig. 5). The albumin had diffused into the neuropil between the cell bodies, surrounding the neurons, which either contained no albumin or contained albumin in some foci. Scattered neurons were albumin positive. Regarding the dark neurons, cresyl violet staining showed that these were scattered and sometimes grouped within the brain parenchyma.

The occurrence of albumin outside brain vessels was characterized as albumin foci around vessels. After the 7 days recovery time, albumin foci were found significantly more often among exposed rats (25%) than among sham exposed

rats (0%) (Fisher's exact probability test, $p = 0.04$). There was a low, but significant correlation between the exposure level (SAR-value) and the occurrence of albumin foci (Spearman analysis, $r_s = 0.33$; $p = 0.04$). Taking the level of exposure and quantification of neuropathological effects into account it could be concluded from a simultaneous non-parametric comparison of all 5 exposure levels with the Kruskal–Wallis test, that the distribution of albumin foci differed significantly (Kruskal–Wallis, $p = 0.012$).

Pair-wise comparisons between the different exposure levels and sham exposed animals revealed statistically significant differences for SAR of 12 mW/kg (Mann–Whitney, $p = 0.007$), whereas a trend of increased albumin extravasation could be seen for 0.12 mW/kg (Mann–Whitney, $p = 0.1$) and 120 mW/kg (Mann–Whitney, $p = 0.1$).

Also, the occurrence of neuronal albumin was evaluated. A simultaneous analysis for all exposure levels revealed a significant difference between the five groups (Kruskal–Wallis, $p = 0.03$, however Fisher's exact probability, $p = \text{ns}$). A pair-wise comparison revealed that albumin uptake occurred more frequently at 1.2 mW/kg as compared to sham exposed (Mann–Whitney, $p = 0.02$). No difference was found for the occurrence of neuronal damage (Kruskal–Wallis, $p = \text{ns}$; Fisher's exact probability test, $p = \text{ns}$).

Linear regression analysis did not reveal any influence of gender, position of the animals in the TEM-cell (upper/lower compartment) or the TEM-cell used (TEM-cell A, B, C or D) on the frequency of albumin foci, neuronal albumin or occurrence of dark neurons.

5. Discussion

The present study provides evidence that GSM exposure results in disruption of the BBB permeability, with remaining, observable effects 7 days after the exposure occasion. Only non-thermal SAR-levels, below the limits of allowed exposure for humans [12] are considered. This finding of increased albumin extravasation after 7 days (Kruskal–Wallis, $p = 0.012$ with all animals included in the analysis, which is also in agreement with the Fisher's exact probability test, $p = 0.04$) is in line with our earlier findings of albumin leakage both immediately following 2 h of GSM exposure [16] and 14 days [19] after 2 h of GSM exposure. Also, the increased occurrence of neuronal albumin 7 days after the exposure is in line with the findings 14 days after exposure [19].

In our previous study, where the animals have been sacrificed immediately after the EMF exposure, we have seen albumin extravasation only at the most in 50% of the identically exposed animals, although all animals are inbred Fischer 344 rats [16]. Among the rats exposed to the pulse modulated EMFs at 915 MHz, 35% showed albumin extravasation. Also in the sham exposed animals, albumin leakage was present (in 17% of the animals). When the animals have survived 7 days after the EMF exposure, albumin extravasation is seen in a lesser proportion (25% of the exposed

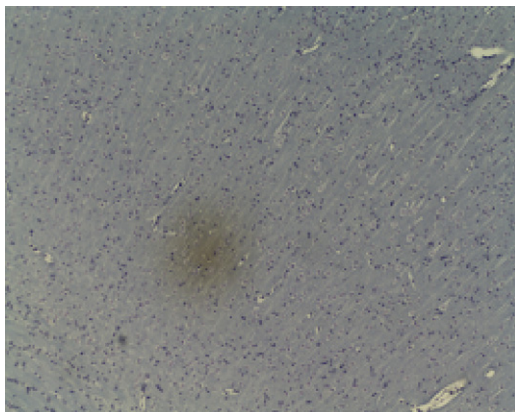


Fig. 5. Focal leakage of albumin shown in brown in the cortex. Albumin: cresyl violet, $\times 10$. GSM-900 EMF exposure at 12 mW/kg. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

animals) and in none of the sham controls. This could be due to a rapid diffusion of extravasated albumin down to, and beyond concentrations possible to demonstrate immunohistochemically. Numerous routes of clearance of extravasated molecules out from the brain tissue are present in the living brain and compounds can also become sequestered intracellularly, become protein bound or metabolized. After 14 days, albumin extravasation is seen in a somewhat larger proportion of the EMF exposed animals (29% of the exposed animals) and none of the sham controls. Thus, a secondary BBB opening might have started at some time point after the initial opening, leading to a vicious circle of albumin leakage.

The mechanism for the passage of albumin over the BBB is not clear. Extravasation might occur through paracellular routes, including alterations of tight junctions between the vascular endothelial cells, or transcellular routes with induction of pinocytosis or transcytosis, formation of transendothelial channels or disruption of the endothelial cell membrane. In connection to EMF exposure, amplified vesicle-mediated transport across the microvessel endothelium occurs, including also transendothelial channels, but no passage through disrupted inter-endothelial tight junctions [24].

One remarkable observation is that exposure at very low whole-body average power densities gives rise to a pronounced albumin leakage. In the present study, significant effects could be seen already at 12 mW/kg, although the different animal groups included a relatively small number of animals. Most certainly, the trends seen for exposure levels of 0.12 mW/kg and 120 mW/kg would have reached statistical significance if more animals had been included in the different exposure groups.

The phenomenon with increased BBB permeability already at very low energy levels might represent a U-curve response. In our other studies, we have seen that the rats in several of the groups with different SAR-levels of EMF exposure have a significant BBB opening [16,19]. The U-response curve occurs also in connection with other kinds of MW exposure, where cerebral vessel permeability after an initial rise decreased with increasing MW power [39].

Further investigation of BBB permeability in connection to EMF exposure is important not only in order to reduce the potentially harmful effects, but also to use possible beneficial effects [69]. The transport of drugs over the BBB might be regulated, so that targets within the brain can be reached. For example, steering of BBB passage of the antiretroviral agent saquinavir has been accomplished in an *in vitro* model of the human BBB, where a frequency of 915 MHz generated the highest BBB permeability [69]. This could be extremely important in order to reduce the HIV replication in the brain of HIV-infected individuals.

6. In conclusion

The time between EMF exposure and sacrifice of the animals is of great importance for the detection of albumin foci.

Seven days after 2 h of GSM mobile phone exposure, there is still an increased permeability of the BBB of exposed rats. This is in concordance with earlier findings of albumin extravasation out into the brain parenchyma immediately and 14 days after 2 h of mobile phone exposure.

7. General conclusion

Taken together, it can be concluded that in a number of studies MW effects upon the BBB permeability have been observed. Increased permeability can be seen both immediately after exposure, but also 7 days after the exposure, as reported in this primary report, and after 14 days. It seems that the effects of the MW radiation might result in persistent changes, such as those seen in our own studies with neuronal damage both 28 and 50 days after 2 h of mobile phone exposure. In a future perspective, with increasing number of active mobile phone users, passive mobile phoning, radiation emitted from base stations and also MWs emitted from other communication sources, effects of low non-thermal levels of exposure must be considered further. The effects seen in the rat studies give some clues about what might possibly happen in the human brain, with a BBB very similar to that of rats. While awaiting latency periods long enough for adequate epidemiological interpretations, further studies on both animals and cells are of utmost importance.

Acknowledgements

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References

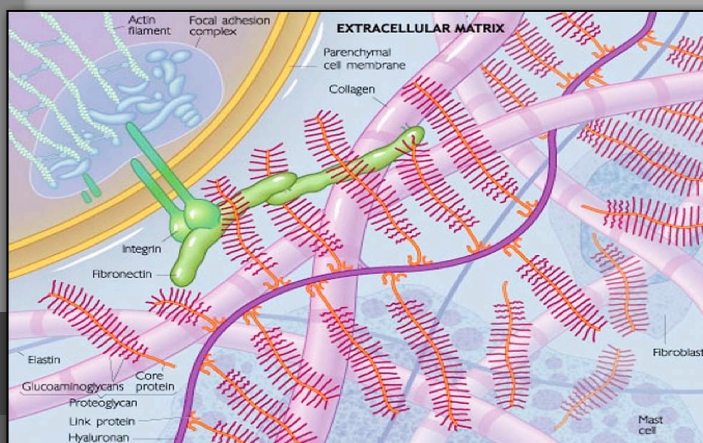
- [1] L.G. Salford, B. Persson, L. Malmgren, A. Brun, in: P. Marco (Ed.), *Téléphonie Mobile et Barrière Sang-Cerveau. Téléphonie mobile—effets potentiels sur la santé des ondes électromagnétiques de haute fréquence*, Emburg, Belgium, 2001, pp. 141–152.
- [2] H. Nittby, G. Grafström, D. Tian, A. Brun, B.R.R. Persson, L.G. Salford, J. Eberhardt, Cognitive impairment in rats after long-term exposure to GSM-900 mobile phones, *Bioelectromagnetics* 29 (2008) 219–232.
- [3] V. Keetly, A.W. Wood, J. Spong, C. Stough, Neuropsychological sequelae of digital mobile phone exposure in humans, *Neuropsychologia* 44 (2006) 1843–1848.
- [4] H. Lai, M.A. Carino, A. Horita, A.W. Guy, Opioid receptor subtypes that mediate a micro-wave induced decrease in central cholinergic activity in the rat, *Bioelectromagnetics* 13 (1992) 237–246.
- [5] I.Y. Belyaev, C. Bauréus Koch, O. Terenius, K. Roxström-Lindquist, L.O.G. Malmgren, W.H. Sommer, L.G. Salford, B.R.R. Persson, Exposure of rat brain to 915 MHz GSM microwaves induces changes in gene expression but not double stranded DNA breaks or effects on chromatin conformation, *Bioelectromagnetics* 27 (2006) 295–306.

- [6] H. Nittby, B. Widegren, M. Krogh, G. Grafström, G. Rehn, H. Berlin, J.L. Eberhardt, L. Malmgren, B.R.R. Persson, L.G. Salford, Exposure to radiation from global system for mobile communications at 1800 MHz significantly changes gene expression in rat hippocampus and cortex, *Environmentalist* (2008b) (published online ahead of print 15 April 2008).
- [7] F. Vecchio, C. Babilono, F. Ferreri, G. Curcio, R. Fini, C. Del Percio, Maria Rossini P., Mobile phone emission modulated interhemispheric functional coupling of EEG alpha rhythms, *Eur. J. Neurosci.* 25 (2007) 1908–1913.
- [8] H. Hinrikus, M. Bachmann, J. Lass, D. Karai, V. Tuulik, Effect of low frequency modulated microwave exposure on human EEG: individual sensitivity, *Bioelectromagnetics* 29 (2008) 527–538.
- [9] L. Hardell, M. Carlberg, Hansson Mild F K., Case–control study on cellular and cordless telephones and the risk for acoustic neuroma or meningioma in patients diagnosed 2000–2003, *Neuroepidemiology* 25 (2005) 120–128.
- [10] L. Hardell, M. Carlberg, K. Hansson Mild, Pooled analysis of two case–control studies on use of cellular and cordless telephones and the risk for malignant brain tumours diagnosed in 1997–2003, *Int. Arch. Occup. Environ. Health* 79 (2006) 630–639.
- [11] H. Nittby, G. Grafström, J.L. Eberhardt, L. Malmgren, A. Brun, B.R.R. Persson, L.G. Salford, Radiofrequency and extremely low-frequency electromagnetic field effects on the blood–brain barrier, *Electromagn. Biol. Med.* 27 (2008) 103–126.
- [12] ICNIRP, Guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields (up to 300 GHz), *Health Phys.* 74 (1998) 494–522.
- [13] L.G. Salford, A. Brun, J. Eberhardt, L. Malmgren, B.B. Persson, Electromagnetic field-induced permeability of the blood–brain barrier shown by immunohistochemical methods. Interaction mechanism of low-level electromagnetic fields, in: B. Nordén, C. Ramel (Eds.), *Living Systems*, Oxford University Press, Oxford, UK, 1992, pp. 251–258.
- [14] L.G. Salford, A. Brun, J.L. Eberhardt, B.R.R. Persson, Permeability of the blood–brain-barrier induced by 915 MHz electromagnetic-radiation continuous wave and modulated at 8, 16, 50 and 200 Hz, *Bioelectrochem. Bioenerg.* 30 (1993) 293–301.
- [15] L.G. Salford, A. Brun, K. Stureson, J.L. Eberhardt, B.R.R. Persson, Permeability of the blood–brain-barrier induced by 915 MHz electromagnetic-radiation continuous wave and modulated at 8, 16, 50 and 200 Hz, *Microsc. Res. Technol.* 27 (1994) 535–542.
- [16] B.R.R. Persson, L.G. Salford, A. Brun, Blood–brain barrier permeability in rats exposed to electromagnetic fields used in wireless communication, *Wireless Networks* 3 (1997) 455–461.
- [17] L.G. Salford, H. Nittby, A. Brun, G. Grafström, J.L. Eberhardt, L. Malmgren, B.R.R. Persson, Non-thermal effects of EMF upon the mammalian brain: the Lund experience, *Environmentalist* 27 (2007) 493–500.
- [18] L.G. Salford, H. Nittby, A. Brun, G. Grafström, L. Malmgren, M. Sommarin, J. Eberhardt, B. Widegren, B.R.R. Persson, The mammalian brain in the electromagnetic fields designed by man—with special reference to blood–brain barrier function, neuronal damage and possible physical mechanisms, *Prog. Theoret. Phys. Suppl.* 174 (2008) 283–309.
- [19] J.L. Eberhardt, B.R. Persson, A.E. Brun, L.G. Salford, L.O. Malmgren, Blood–brain barrier permeability and nerve cell damage in rat brain 14 and 28 days after exposure to microwaves from GSM mobile phones, *Electromagn. Biol. Med.* 27 (2008) 215–229.
- [20] L.G. Salford, A. Brun, J.L. Eberhardt, L. Malmgren, B.R.R. Persson, Nerve cell damage in mammalian brain after exposure to microwaves from GSM mobile phones, *Environ. Health. Perspect.* 111 (2003) 881–883.
- [21] F. Töre, P.E. Dulou, E. Haro, B. Veyret, P. Aubineau, Two-hour exposure to 2-W/kg 900-MHz GSM microwaves induces plasma protein extravasation in rat brain and dura mater, in: *Proceedings of the 5th International Congress of the EBPA*, Helsinki, Finland, 2001, pp. 43–45.
- [22] F. Töre, P.E. Dulou, E. Haro, B. Veyret, P. Aubineau, Effect of 2 h GSM-900 microwave exposures at 2.0, 0.5 and 0.12 W/kg on plasma protein extravasation in rat brain and dura mater, in: *Proceedings of the 24th Annual Meeting of the BEMS*, 2002, pp. 61–62.
- [23] C. Neubauer, A.M. Phelan, H. Kues, D.G. Lange, Microwave irradiation of rats at 2.45 GHz activates pinocytotic-like uptake of tracer by capillary endothelial cells of cerebral cortex, *Bioelectromagnetics* 11 (1990) 261–268.
- [24] R.R. Shivers, M. Kavaliers, G.C. Teskey, F.S. Prato, R.M. Pelletier, Magnetic resonance imaging temporarily alters BBB permeability in the rat, *Neurosci. Lett.* 76 (1987) 25–31.
- [25] F.S. Prato, R.H. Frappier, R.R. Shivers, M. Kavaliers, P. Zabel, D. Drost, T.Y. Lee, Magnetic resonance imaging increases the BBB permeability to 153-gadolinium diethylenetriaminepentaacetic acid in rats, *Brain Res.* 523 (1990) 301–304.
- [26] F.S. Prato, J.M. Wills, J. Roger, H. Frappier, D.J. Drost, T.Y. Lee, R.R. Shivers, P. Zabel, BBB permeability in rats is altered by exposure to magnetic fields associated with magnetic resonance imaging at 1.5 T, *Microsc. Res. Technol.* 27 (1994) 528–534.
- [27] G. Tsurita, H. Nagawa, S. Ueni, S. Watanabe, M. Taki, Biological and morphological effects on the brain after exposure of rats to a 1439 MHz TDMA field, *Bioelectromagnetics* 21 (2000) 364–371.
- [28] M. Kuribayashi, J. Wang, O. Fujiwara, Y. Doi, K. Nabae, S. Tamano, T. Ogiso, M. Asamoto, T. Shirai, Lack of effects of 1439 MHz electromagnetic near field exposure on the BBB in immature and young rats, *Bioelectromagnetics* 26 (2005) 578–588.
- [29] J.W. Finnie, P.C. Blumbergs, J. Manavis, T.D. Utteridge, V. Gebbski, J.G. Swift, B. Vernon-Roberts, T.R. Kucher, Effect of global system for mobile communication (GSM)-like radiofrequency fields on vascular permeability in mouse brain, *Pathology* 33 (2001) 338–340.
- [30] I.K. Adzamlı, E.A. Jolesz, M. Blau, An assessment of BBB integrity under MRI conditions: brain uptake of radiolabelled Gd-DTPA and In-DTPA-IgG, *J. Nucl. Med.* 30 (1989) 839–840.
- [31] E. Preston, K. Buffler, N. Haas, Does magnetic resonance imaging compromise integrity of the BBB? *Neurosci. Lett.* 101 (1989) 46–50.
- [32] R.N. Frank, S. Dutta, M.A. Mancini, Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat, *Invest. Ophthalmol. Vis. Sci.* 28 (1987) 1086–1091.
- [33] W.E. Thomas, Brain macrophages: on the role of pericytes and perivascular cells, *Brain. Res. Rev.* 31 (1999) 42–57.
- [34] W.H. Oldendorf, M.E. Cornford, W.J. Brown, The large apparent work capability of the BBB: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat, *Ann. Neurol.* 1 (1977) 409–417.
- [35] A. Mihály, B. Bozöky, Immunohistochemical localization of serum proteins in the hippocampus of human subjects with partial and generalized epilepsy and epileptiform convulsions, *Acta Neuropathol.* 127 (1984) 251–267.
- [36] A. Mihály, B. Bozöky, Immunohistochemical localization of extravasated serum albumin in the hippocampus of human subjects with partial and generalized and epileptiform convulsions, *Acta Neuropathol.* 65 (1984) 471–477.
- [37] T.E.O. Sokrab, B.B. Johansson, A transient hypertensive opening of the BBB can lead to brain damage, *Acta Neuropathol.* 75 (1988) 557–565.
- [38] A.H. Frey, S.R. Feld, B. Frey, Neural function and behaviour: defining the relationship, *Ann. NY. Acad. Sci.* 247 (1975) 433–439.
- [39] K.J. Oscar, T.D. Hawkins, Microwave alteration of the BBB system of rats, *Brain Res.* 126 (1977) 281–293.
- [40] J.H. Merritt, A.F. Chamness, S.J. Allen, Studies on BBB permeability, *Radial. Environ. Biophys.* 15 (1978) 367–377.
- [41] E. Preston, R.J. Vavasour, H.M. Assenheim, Permeability of the BBB to mannitol in the rat following 2450MHz microwave irradiation, *Brain Res.* 174 (1979) 109–117.
- [42] T.R. Ward, J.A. Elder, M.D. Long, D. Svendsgaard, Measurement of BBB permeation in rats during exposure to 2450-MHz microwaves, *Bioelectromagnetics* 3 (1982) 371–383.

- [43] T.R. Ward, J.S. Ali, BBB permeation in the rat during exposure to low-power 1.7-GHz microwave radiation, *Bioelectromagnetics* 6 (1985) 131–143.
- [44] S.P. Gruenau, K.J. Oscar, M.T. Folker, S.I. Rapoport, Absence of microwave effect on blood–brain-barrier permeability to [C-14]-labeled sucrose in the conscious rat, *Exp. Neurol.* 75 (1982) 299–307.
- [45] E.N. Albert, J.M. Kerns, Reversible microwave effects on the BBB, *Brain Res.* 230 (1981) 153–164.
- [46] H.J. Garber, W.H. Oldendorf, L.D. Braun, R.B. Lufkin, MRI gradient fields increase brain mannitol space, *Magn. Reson. Imag.* 7 (1989) 605–610.
- [47] K. Fritze, C. Sommer, Effect of global system for mobile communication (GSM) microwave exposure on BBB permeability in rat, *Acta Neuropathol.* 94 (1997) 465–470.
- [48] J.W. Finnie, P.C. Blumbergs, J. Manavis, T.D. Utteridge, V. Gebiski, R.A. Davies, B. Vernon-Roberts, T.R. Kuchel, Effect of long-term mobile communication microwave exposure on vascular permeability in mouse brain, *Pathology* 34 (2002) 244–347.
- [49] J.W. Finnie, P.C. Blumbergs, Z. Cai, J. Manavis, T.R. Kuchel, Effect of mobile telephony on blood–brain barrier permeability in the fetal mouse brain, *Pathology* 38 (2006) 63–65.
- [50] T. Kumlin, H. Livonen, P. Miettinen, A. Juvonen, T. van Groen, L. Paranen, R. Pitkäaho, J. Juutilainen, H. Tanila, Mobile phone radiation and the developing brain: behavioral and morphological effects in juvenile rats, *Radiat. Res.* 168 (2007) 471–479.
- [51] A. Schirmacher, S. Winters, S. Fischer, J. Goeke, H.J. Galla, U. Kullnick, E.B. Ringelstein, F. Stögbauer, Electromagnetic fields (1.8 GHz) increase the permeability to sucrose of the BBB in vitro, *Bioelectromagnetics* 21 (2000) 338–345.
- [52] H. Franke, E.B. Ringelstein, F. Stögbauer, Electromagnetic fields (GSM1800) do not alter BBB permeability to sucrose in models in vitro with high barrier tightness, *Bioelectromagnetics* 26 (2005) 529–535.
- [53] H. Franke, J. Streckert, A. Bitz, J. Goeke, V. Hansen, E.B. Ringelstein, H. Nattkämper, H.J. Galla, F. Stögbauer, Effects of universal mobile telecommunications system (UMTS) electromagnetic fields on the BBB in vitro, *Radiat. Res.* 164 (2005) 258–269.
- [54] T. Sugimoto, G.J. Bennett, K.C. Kajander, Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: effects of a chronic constriction injury, transaction and strychnine, *Pain* 42 (1990) 205–213.
- [55] Z.S. Kherani, R.N. Auer, Pharmacologic analysis of the mechanism of dark neuron production in cerebral cortex, *Acta Neuropathol.* 116 (2008) 447–452.
- [56] D. Bexell, No neuronal apoptosis after EMF microwave exposure from mobile phones. Report supervised by Leif G. Salford at the Rausing Laboratory.
- [57] E. Kövesdi, J. Pál, F. Gallyas, The fate of “dark” neurons produced by transient focal cerebral ischemia in a non-necrotic and non-excitotoxic environment: neurobiological aspects, *Brain Res.* 1147 (2007) 272–283.
- [58] F. Gallyas, A. Csordás, A. Schwarcz, M. Mázló, “Dark” (compacted) neurons may not die through the necrotic pathway, *Exp. Brain Res.* 160 (2005) 473–486.
- [59] B. Söderfeldt, H. Kalimo, Y. Olsson, B.K. Siesjö, Bicuculline-induced epileptic brain injury. Transient and persistent cell changes in rat cerebral cortex in the early recovery period, *Acta Neuropathol.* 62 (1983) 87–95.
- [60] A. İlhan, A. Gurel, F. Armutcu, S. Kamisli, M. Iraz, O. Akyol, S. Ozen, *Ginkgo biloba* prevents mobile phone-induced oxidative stress in rat brain, *Clin. Chim. Acta* 340 (2004) 153–162.
- [61] F. Poulletier de Gannes, E. Haro, M. Taxile, E. Ladevze, L. Mayer, M. Lascau, P. Levêque, G. Ruffie, B. Billaudel, I. Lagroye, B. Veyret, Do GSM-900 signals affect blood–brain barrier permeability and neuron viability? in: Abstract at the 28th Annual Meeting of the Bioelectromagnetics Society, Cancun, Mexico, 2006, pp. 164–165.
- [62] K. Fredriksson, H. Kalimo, C. Norberg, B.B. Johansson, Y. Olsson, Nerve cell injury in the brain of stroke-prone spontaneously hypertensive rats *Acta Neuropathol. (Berl)* 76 (1988) 227–237.
- [63] T.S. Salahuddin, H. Kalimo, B.B. Johansson, Y. Olsson, Observations on exudation of fibronectin, fibrinogen and albumin in the brain after carotid infusion of hyperosmolar solutions. An immunohistochemical study in the rat indicating longlasting changes in the brain microenvironment and multifocal nerve cell injuries, *Acta Neuropathol. (Berl)* 76 (1988) 1–10.
- [64] S. Eimerl, M. Scramm, Acute glutamate toxicity in cultured cerebellar granule cells: agonist potency, effects of pH, Zn²⁺ and the potentiation by serum albumin, *Brain Res.* 560 (1991) 282–290.
- [65] B. Hassel, E.G. Iversen, F. Fonnum, Neurotoxicity of albumin in vivo, *Neurosci. Lett.* 167 (1994) 29–32.
- [66] W.D. Dietrich, O. Alonsi, M. Halley, R. Busto, M.Y.-T. Globus, Intraventricular infusion of N-methyl-D-aspartate: 1. Acute blood–brain barrier consequences, *Acta Neuropathol.* 84 (1992) 621–629.
- [67] M.L. Crawford, Generation of Standard EM using TEM transmission cells, *IEEE Trans. Electromagn. Comput.* 16 (1974) 189–195.
- [68] L. Malmgren, Radio frequency systems for NMR imaging: coil development and studies of non-thermal biological effects. PhD Thesis. Lund, Sweden. Department of Applied Electronics, Lund University, 1998.
- [69] Y.-C. Kuo, C.-Y. Kuo, Electromagnetic interference in the permeability of saquinavir across the blood–brain barrier using nanoparticulate carriers, *Int. J. Pharm.* 351 (2008) 271–281.

Mechanisms of Harm; DNA Damage; Microwave RF Interacts
with Molecular Structures; Dr. Paul Dart MD.; 2013

Microwave RF Interacts with Molecular Structures



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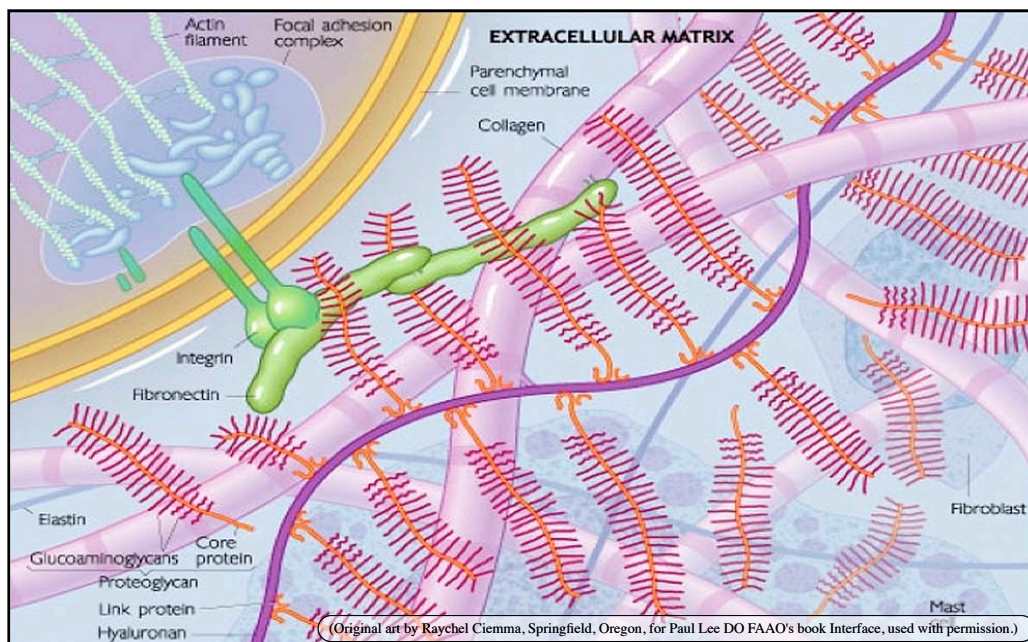
The molecules in our bodies vary in size and total electric charge.

These molecular structures of our body can resonate with fluctuating electromagnetic fields.

Any charged particle has a resonant frequency.

This frequency varies depending on the total mass and charge of the particle.

Molecules resonate in fluctuating electromagnetic fields.



(Original art by Raychel Ciemma, Springfield, Oregon, for Paul Lee DO FAAO's book [Interface](#), used with permission.)

The molecules in our bodies vary in size and total electric charge.

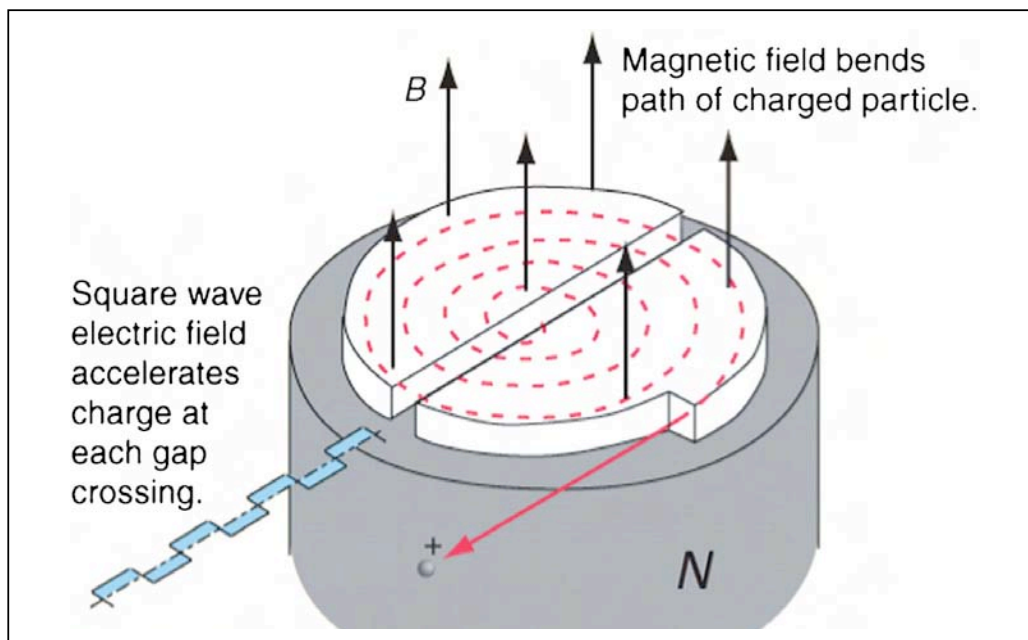
These molecular structures of our body resonate with fluctuating electromagnetic fields.

Resonance Frequency



When you push something at its resonant frequency, a small force can produce a lot of motion.

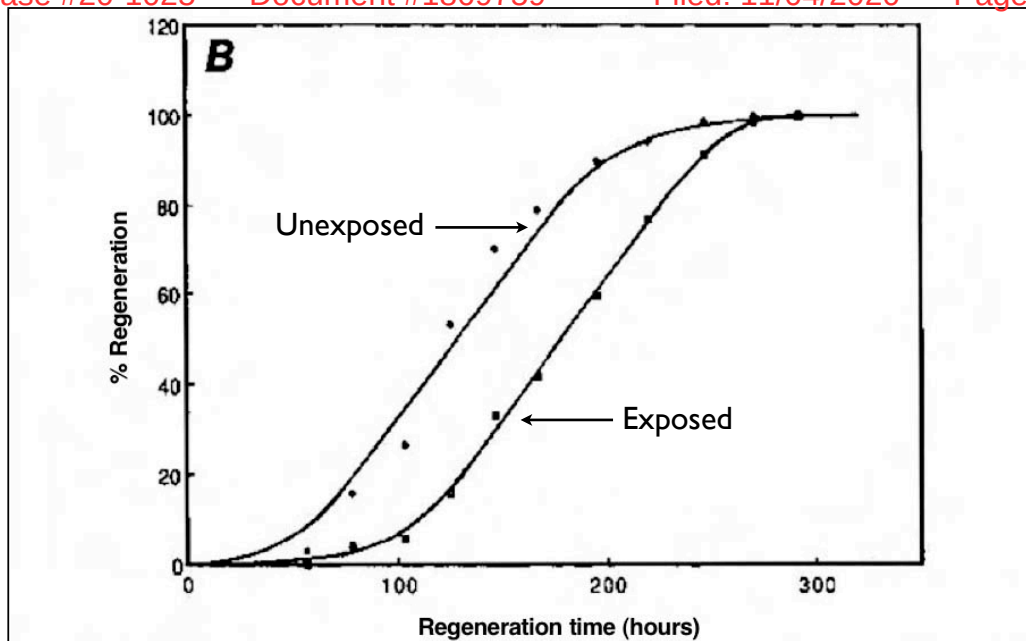
Resonance Frequency



Placing the particle in an electromagnetic field that fluctuates at the resonant frequency will amplify the motion of the particle.

This is how a cyclotron works, and the frequency is often referred to as the "Ion Cyclotron Resonance" or ICR frequency.

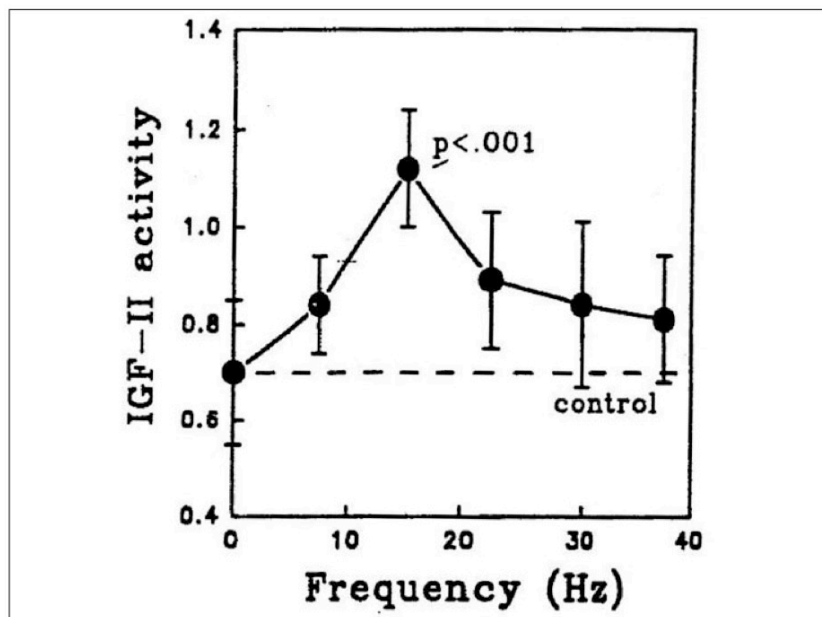
Magnetic fields that fluctuate at the resonant frequency of an ion like calcium, or of a specific enzyme, can have dramatic effects on biochemical processes in the body.



Planaria exposed to a magnetic field fluctuating at the calcium ion's ICR frequency take far longer (48 hours) to regenerate than those that are not exposed.

Liboff A. Weak low-frequency electromagnetic fields are biologically interactive. In: Giuliani L, Soffritti M, eds. Non-Thermal Effects and Mechanisms of Interaction Between Electromagnetic Fields and Living Matter -- An ICEMS Monograph. Fidenza, Italy: Mattioli, (2010): 51-61. <http://www.ramazzini.it/ricerca/publications.asp>

“frequency window”

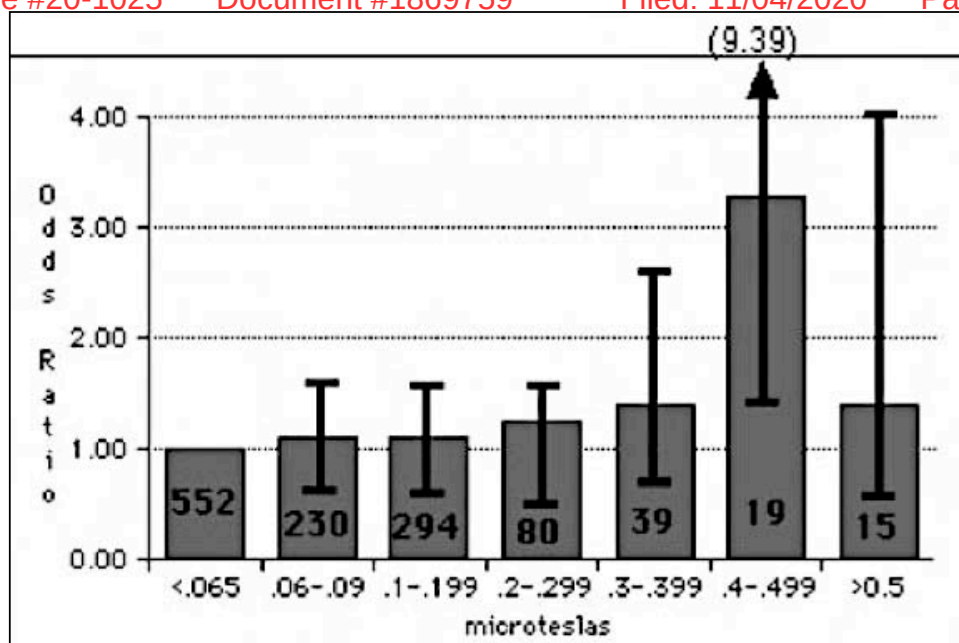


Some effects of fluctuating EMF occur at specific frequencies, called “frequency windows”.

The peak in IGF-II expression for human osteosarcoma bone cells exposed to combined magnetic fields occurs when the field is tuned to the calcium ion's ICR frequency

Liboff A. Weak low-frequency electromagnetic fields are biologically interactive. In: Giuliani L, Soffritti M, eds. Non-Thermal Effects and Mechanisms of Interaction Between Electromagnetic Fields and Living Matter -- An ICEMS Monograph. Fidenza, Italy: Mattioli, (2010): 51-61. <http://www.ramazzini.it/ricerca/publications.asp>

"power window"



At a given frequency, some power levels may have a different effect than others. This is a "power window"

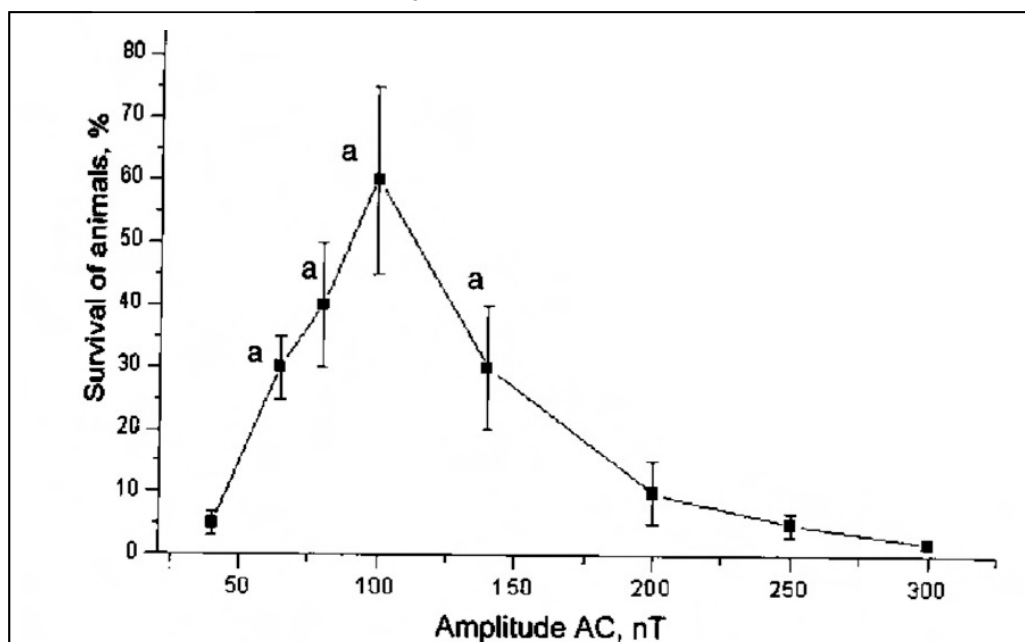
In this illustration, the odds ratio for childhood onset of Acute Lymphoblastic Leukemia is significantly higher if they are exposed to 60 cycle magnetic fields at a magnitude of 0.4 to 0.499 microtesla.

Lower and higher field magnitudes do not show the same effect.

Liboff A. Weak low-frequency electromagnetic fields are biologically interactive. In: Giuliani L, Soffritti M, eds. Non-Thermal Effects and Mechanisms of Interaction Between Electromagnetic Fields and Living Matter -- An ICEMS Monograph. Fidenza, Italy: Mattioli, (2010): 51-61. <http://www.ramazzini.it/ricerca/publications.asp>

Fig. 1. Odds ratios for childhood ALL, determined by Linet et al 6, as a function of residential magnetic field. The large ratios seen for fields between .4 and .499 μ T, although having many less participants, are nevertheless statistically significant

"power window"

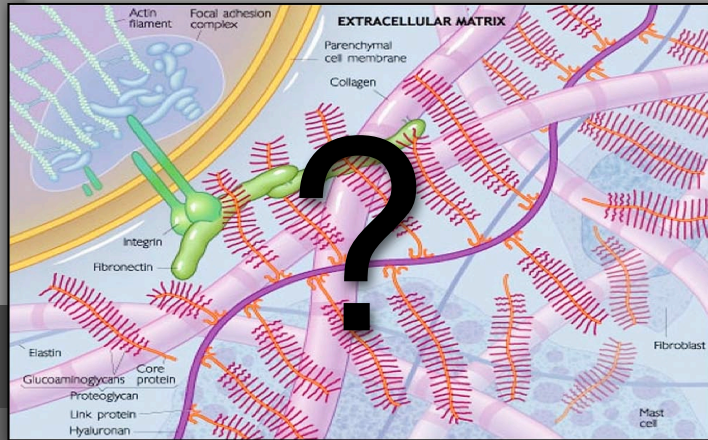


Mice with Ascites Ehrlich carcinoma 33, exposed to a fluctuating EM field tuned to the ICR frequency for aspartic acid and glutamic acid ions. Survival varies with the AMPLITUDE (magnitude) of the field.

Liboff A. Weak low-frequency electromagnetic fields are biologically interactive. In: Giuliani L, Soffritti M, eds. Non-Thermal Effects and Mechanisms of Interaction Between Electromagnetic Fields and Living Matter -- An ICEMS Monograph. Fidenza, Italy: Mattioli, (2010): 51-61. <http://www.ramazzini.it/ricerca/publications.asp>

Fig. 5. Survival curve for mice infected with Ascites Ehrlich carcinoma33, under ICR conditions corresponding to mean tuning (4.4 Hz) for aspartic acid and glutamic acid ions. In contrast to Fig. 2 where the frequency is varied, a resonance (or window) peak is observed as the AC magnetic field intensity is varied

What does this
mean?



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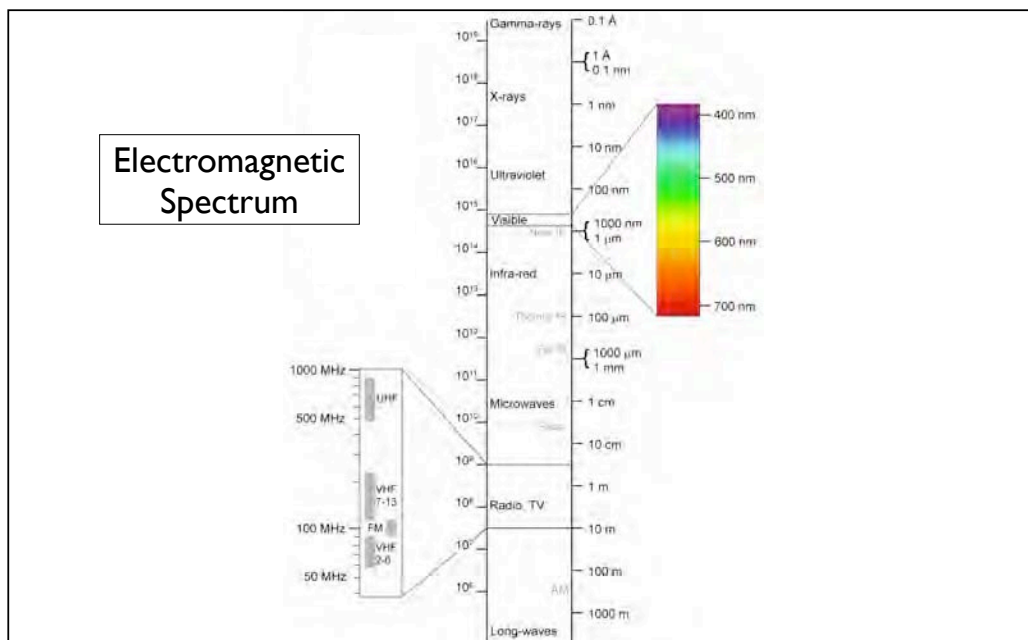
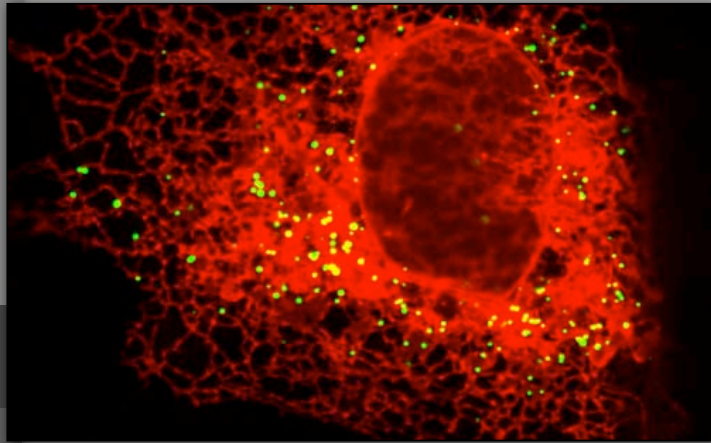
There are thousands of enzymes and other molecules in the human body.

Each has its own mass, charge, and resonant frequency.

This means that different electromagnetic frequencies will resonate with different molecules.

Which means that the biological effects of EMF on molecular physiology are probably much more complex than is generally assumed to be the case.

Microwave RF Produces Oxidative Stress in Cells



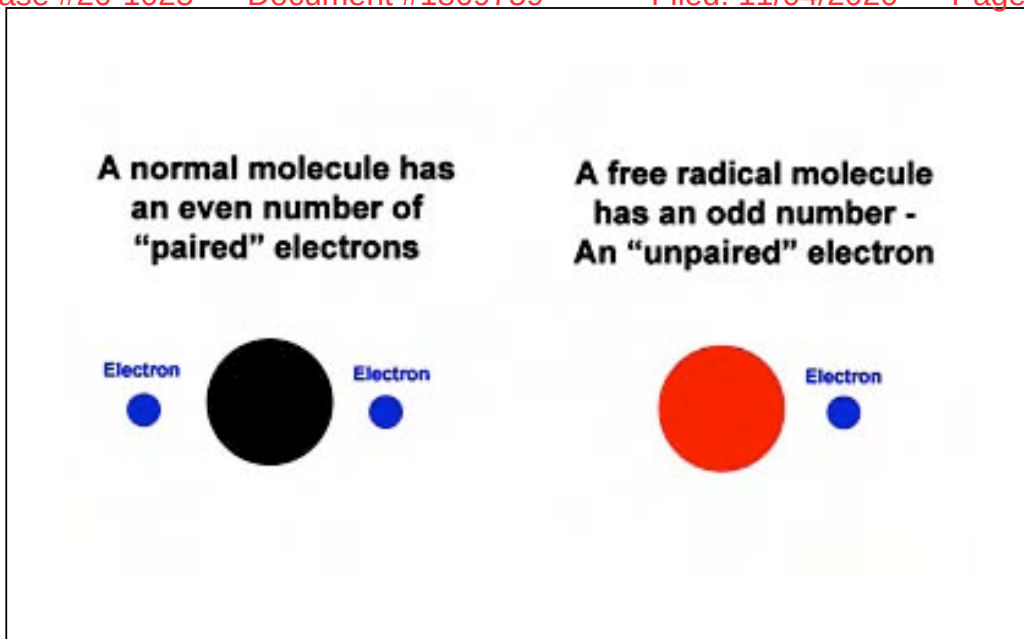
Ionizing radiation from the high energy end of the electromagnetic spectrum can directly break DNA molecular bonds, causing mutations.

But photons of microwave RF do not have enough energy to directly break covalent molecular bonds.

Industry advocates often make the statement that since RF cannot break molecular bonds, there is no way that it can cause cancer.

Such statements sound like good physics. But they reflect a poor understanding of biology.

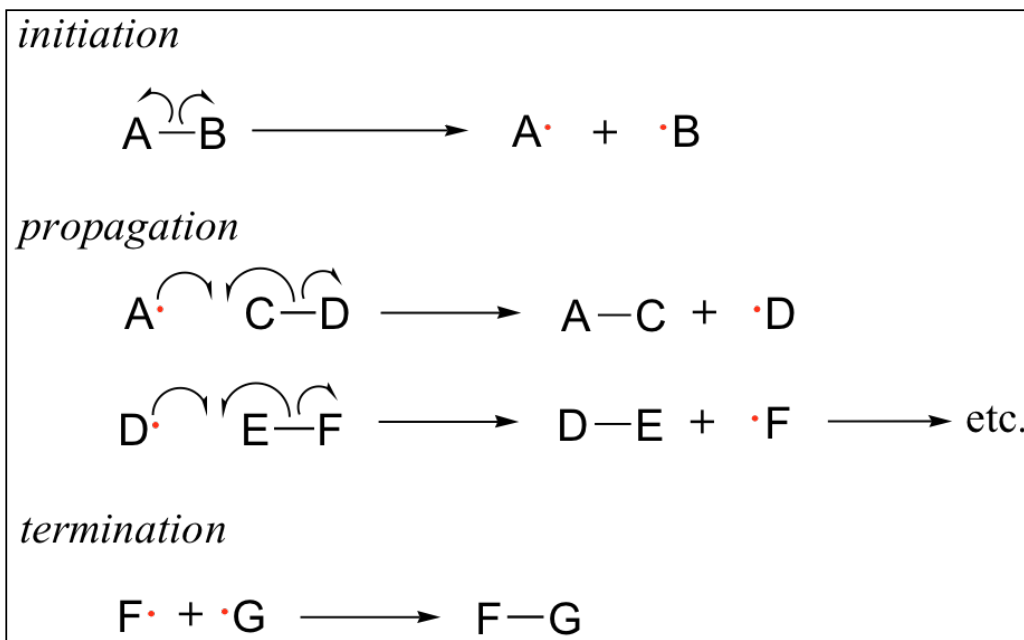
Tobacco can cause cancer. Genital warts can cause cancer. Asbestos can cause cancer. There are many ways to cause cancer besides ionizing radiation.



Free radicals are oxidizing agents.

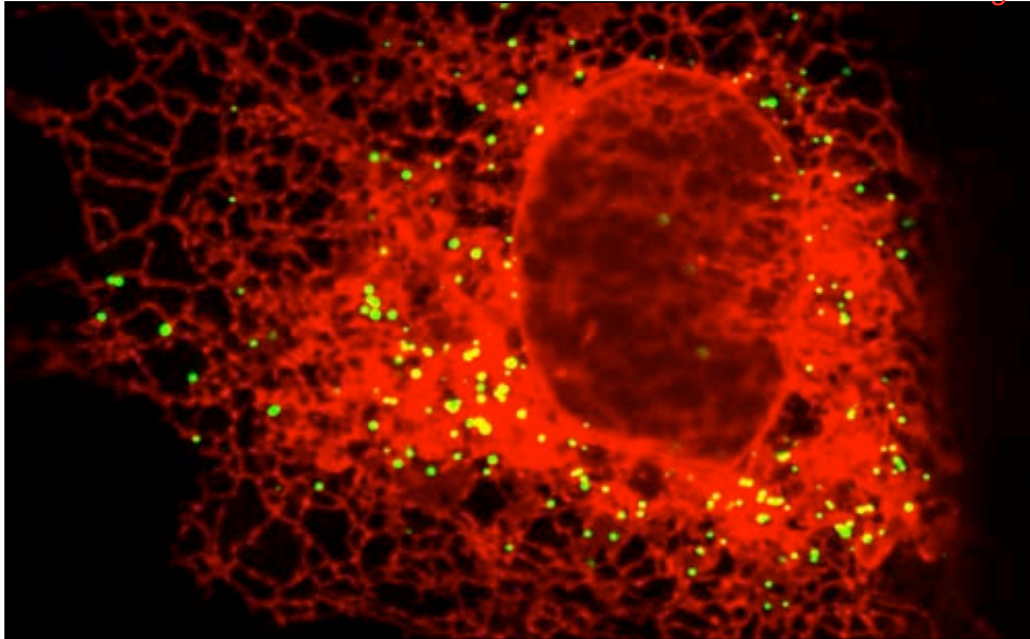
They take electrons from other atoms or molecules, which can break molecular bonds.

Life cycle of a free radical.



Precursor molecule (AB) splits to form two free radicals.

Free radicals then can produce chain reactions, causing oxidative damage.



Peroxisomes (yellow) in a cell – packages of free radicals stored in cells.

Cells are making free radicals all the time.

Our bodies release them in inflammation to combat bacteria, remove diseased tissue, etc.

The free radicals release by the inflammatory process can break covalent bonds and fragment macromolecules.

Review Article

J. Cell. Mol. Med. Vol XX, No X, 2013 pp. 1-9

Guest Editor:

Electromagnetic fields act *via* activation of voltage-gated calcium channels to produce beneficial or adverse effects

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- Introduction
- Possible modes of action following voltage-gated calcium channel stimulation
- Therapeutic bone-growth stimulation *via* Ca^{2+} /nitric oxide/cGMP/protein kinase G

- Ca^{2+} /nitric oxide/peroxynitrite and pathophysiological responses to EMF exposures: the example of single-strand DNA breaks
- Discussion and conclusions

Abstract

The direct targets of extremely low and microwave frequency range electromagnetic fields (EMFs) in producing non-thermal effects have not been clearly established. However, studies in the literature, reviewed here, provide substantial support for such direct targets. Twenty-three studies have shown that voltage-gated calcium channels (VGCCs) produce these and other EMF effects, such that the L-type or other VGCC blockers block or greatly lower diverse EMF effects. Furthermore, the voltage-gated properties of these channels may provide biophysically

This recently published article reviews published evidence that EMF can produce physiologic effects by altering the function of voltage gated calcium channels in cell walls.

Pall ML. Electromagnetic fields act via activation of voltage-gated calcium channels to produce beneficial or adverse effects. *J Cell Mol Med* (2013);

Table 1 EMF responses blocked or lowered by calcium channel blockers				
Ref. no.	EMF type	Calcium channel	Cell type or organism	Response measured
2	Pulsed magnetic fields	L-type	Human lymphocytes	Cell proliferation; cytokine production
3	Static magnetic field (0.1 T)	L-type	Human polymorphonuclear leucocytes	Cell migration; degranulation
5	ELF	L-type	Rat chromaffin cells	Differentiation; catecholamine release
6	Electric field	L-type	Rat and mouse bone cells	Increased Ca^{2+} , phospholipase A2, PGE2
7	50 Hz	L-type	Mytilus (mussel) immunocytes	Reduced shape change, cytotoxicity
8	50 Hz	L-type	AtT20 D16V, mouse pituitary corticotrope-derived	Ca^{2+} increase; cell morphology, premature differentiation
9	50 Hz	L-type	Neural stem/progenitor cells	<i>In vitro</i> differentiation, neurogenesis
10	Static magnetic field	L-type	Rat	Reduction in oedema formation
11	NMR	L-type	Tumour cells	Synergistic effect of EMF on anti-tumour drug toxicity
12	Static magnetic field	L-type	Myelomonocytic U937 cells	Ca^{2+} influx into cells and anti-apoptotic effects
13	60 Hz	L-type	Mouse	Hyperalgesic response to exposure
14	Single nanosecond electric pulse	L-type	Bovine chromaffin cells	Very rapid increase in intracellular Ca^{2+}

These are some of the 23 published studies documenting that EMF can increase flow through these calcium channels, producing biological effects.

In all these studies, the effects of EMF on increased cellular calcium levels could be blocked by calcium channel blocking drugs.

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15	Biphasic electric current	L-type	Human mesenchymal stromal cells	Osteoblast differentiation and cytokine production
16	DC & AC magnetic fields	L-type	β -cells of pancreas, patch clamped	Ca^{2+} flux into cells
17	50 Hz	L-type	Rat pituitary cells	Ca^{2+} flux into cells
18	50 Hz	L-type, N-type	Human neuroblastoma IMR32 and rat pituitary GH3 cells	Anti-apoptotic activity
19	Nanosecond pulse	L-type, N-type, P/Q-type	Bovine chromaffin cells	Ca^{2+} dynamics of cells
20	50 Hz	Not determined	Rat dorsal root ganglion cells	Firing frequency of cells
21	700–1100 MHz	N-type	Stem cell–derived neuronal cells	Ca^{2+} dynamics of cells
22	Very weak electrical fields	T-type	Sharks	Detection of very weak magnetic fields in the ocean
23	Short electric pulses	L-type	Human eye	Effect on electro-oculogram
24	Weak static magnetic field	L-type	Rabbit	Baroreflex sensitivity
25	Weak electric fields	T-type	Neutrophils	Electrical and ion dynamics
26	Static electric fields, 'capacitive'	L-type	Bovine articular chondrocytes	Agrican & type II collagen expression; calcineurin and other Ca^{2+} /calmodulin responses

EMF: electromagnetic field; ELF: extremely low frequency.

In all these studies, the effects of EMF on increased cellular calcium levels could be blocked by calcium channel blocking drugs.

Pall ML. Electromagnetic fields act via activation of voltage-gated calcium channels to produce beneficial or adverse effects. *J Cell Mol Med* (2013);

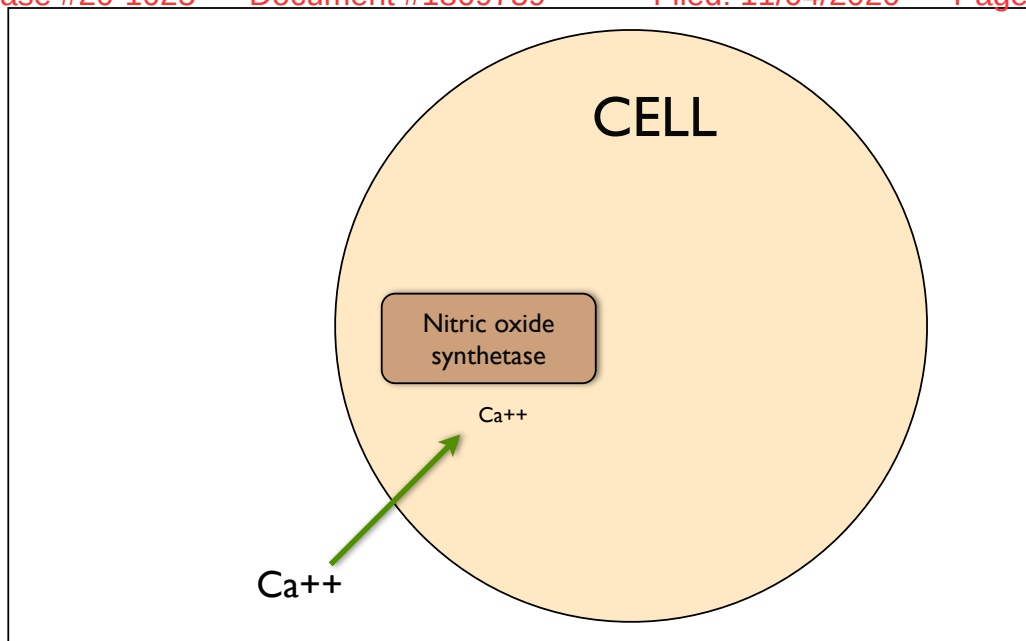
EMF Activation of VGCCs Increases Free Radical Production

USCA Case #20-1025

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Normally, Calcium concentrations are much higher outside of cells than inside them.

Influxes of calcium into cells act as chemical signals to alter cellular physiologic activity.

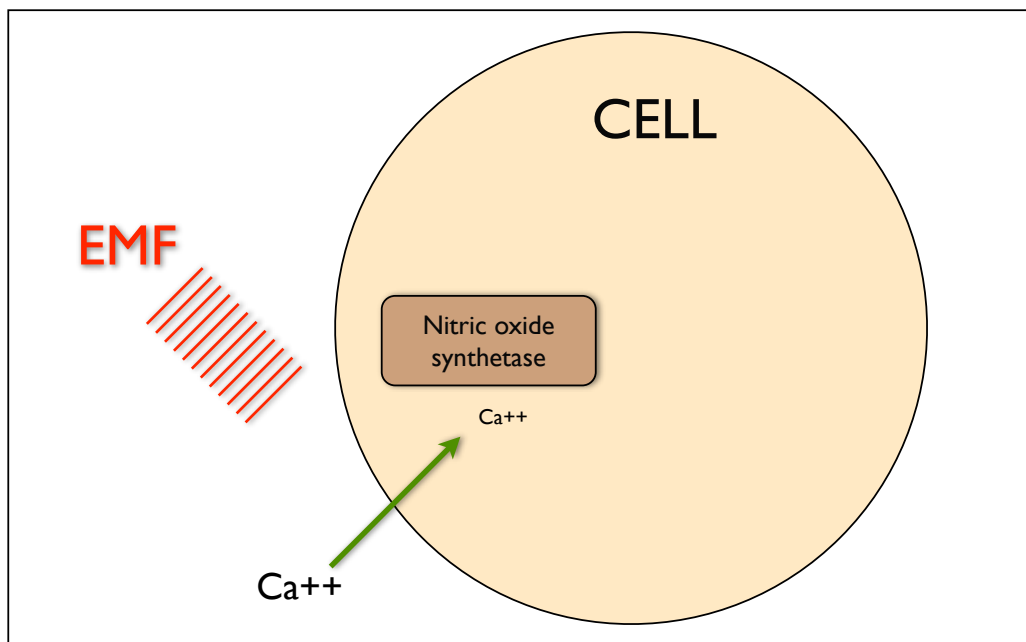
Here we have a diagram of a cell, with high levels of calcium outside, and lower levels of calcium inside.

The green arrow is a voltage-gated calcium channel, that can open to allow more calcium to enter the cell.

Inside the cell, we can see an enzyme (nitric oxide synthetase).

As discussed by Pall ML. Electromagnetic fields act via activation of voltage-gated calcium channels to produce beneficial or adverse effects. *J Cell Mol Med* (2013);

EMF Activation of VGCCs Increases Free Radical Production



An electromagnetic field arrives at the cell wall.

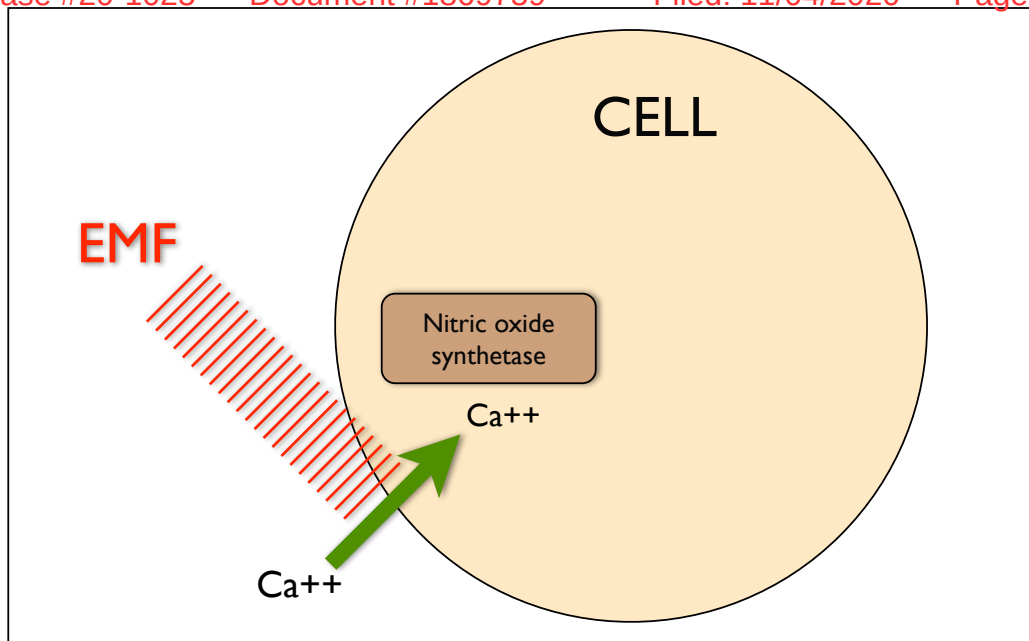
EMF Activation of VGCCs Increases Free Radical Production

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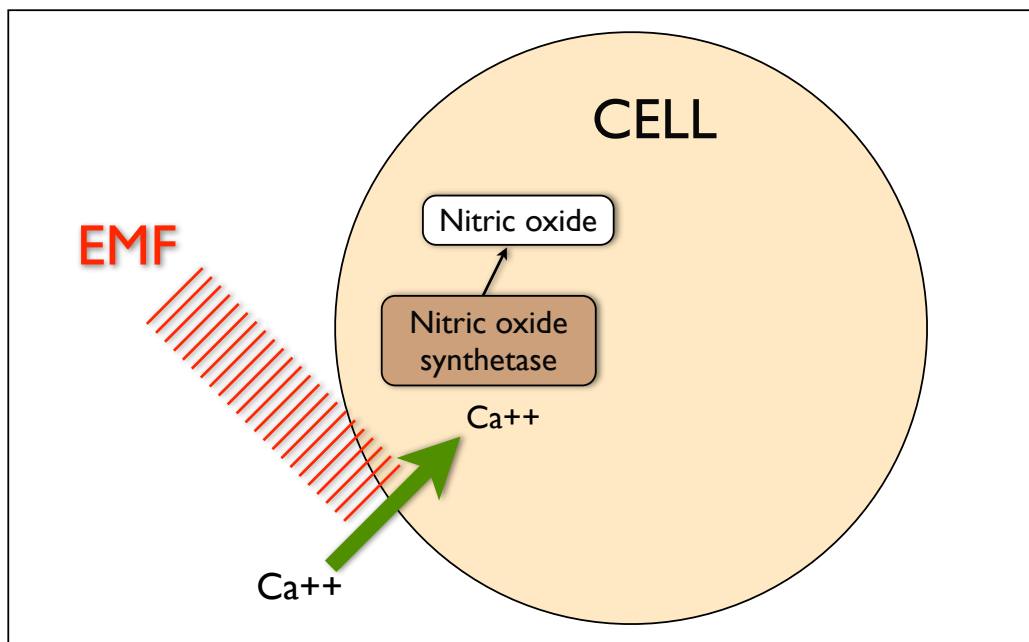
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The electromagnetic field stimulates opening of voltage-gated calcium channels (VGCCs) in the cell membrane.

This increases Ca++ entry into the cell.

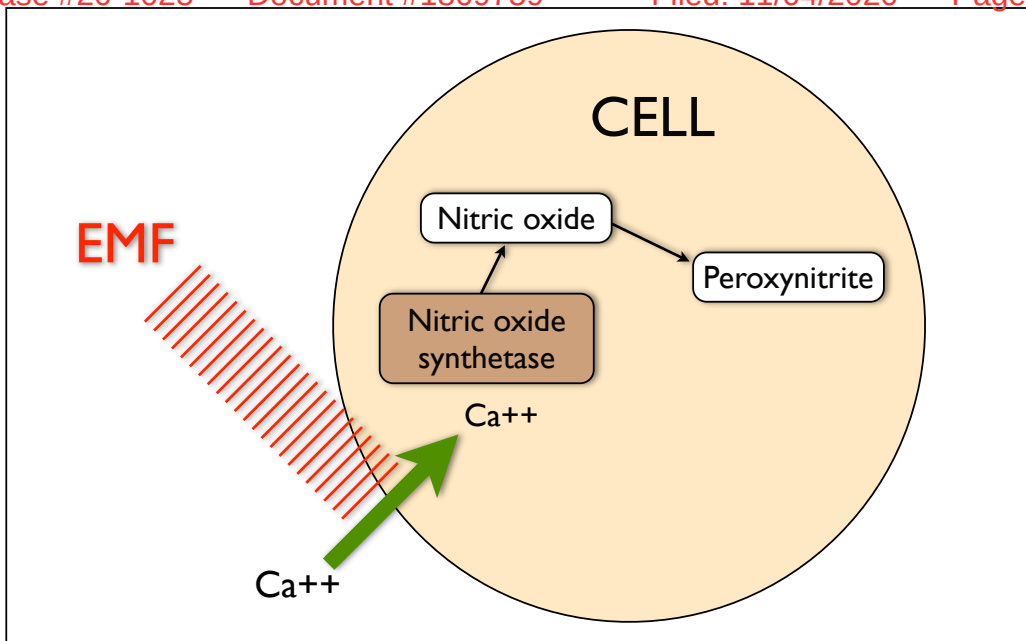
EMF Activation of VGCCs Increases Free Radical Production



Increased intracellular calcium levels stimulate the activity of nitric oxide synthetase,

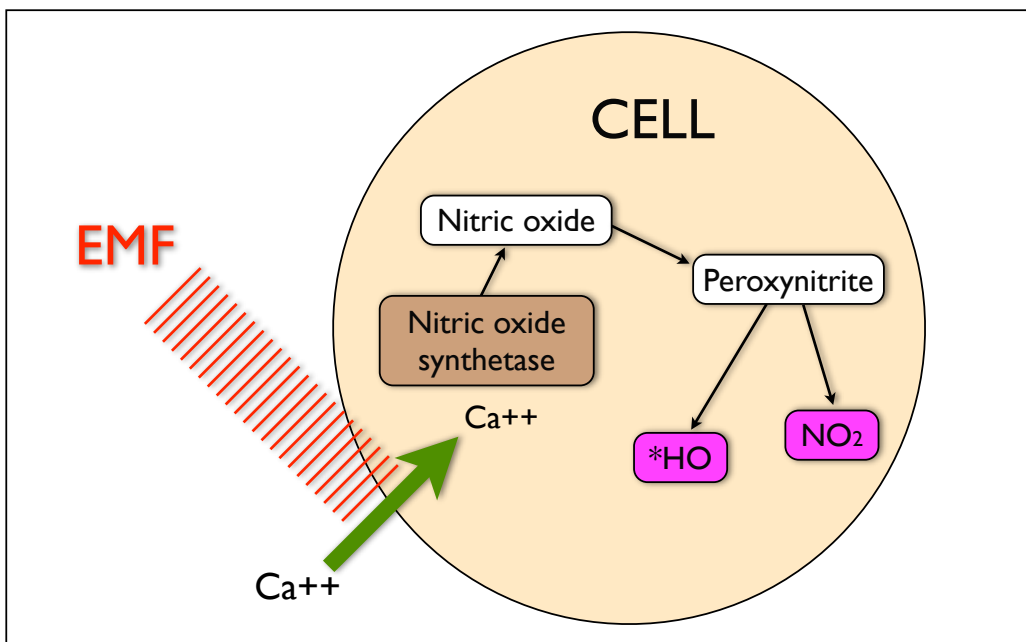
Which leads to increased production of nitric oxide in the cell.

EMF Activation of VGCCs Increases Free Radical Production



Increased nitric oxide leads to increase in peroxynitrite, a potent non-radical oxidant.

EMF Activation of VGCCs Increases Free Radical Production



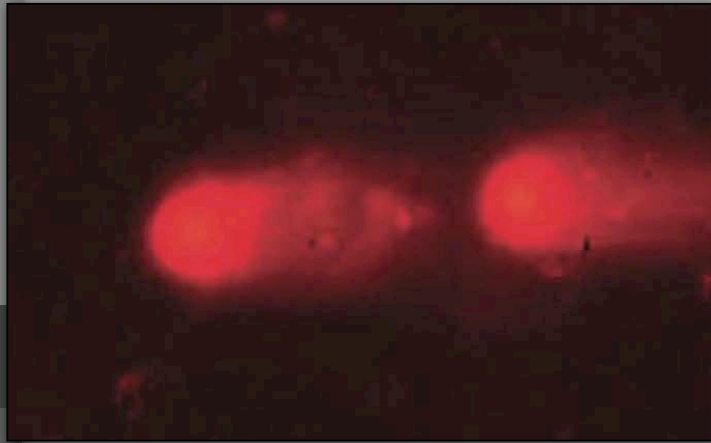
Peroxynitrite produces free radicals, including hydroxyl radical and NO_2 .

This increase in free radicals then leads to inflammation, oxidant stress, and damage to cell structures, including DNA.

The EMF doesn't directly damage the cell. It just deranges cellular metabolism.

The free radicals that are produced by this change in metabolism are what causes the damage.

Oxidative Stress From Microwave RF Damages DNA



The mechanisms of **how** RF increases free radical activity and oxidative stress are still being explored.

But the fact that RF **does do this** has been CLEARLY ESTABLISHED by many research studies.

This increase in free radical levels can and does lead to DNA damage.

Comet assay: Unexposed control



The Comet assay is one way to measure DNA damage.

This is a study of DNA extracted from normal rat brain cells (unexposed controls).

Electrophoresis: DNA molecules of given mass and charge placed in a diffusion medium.

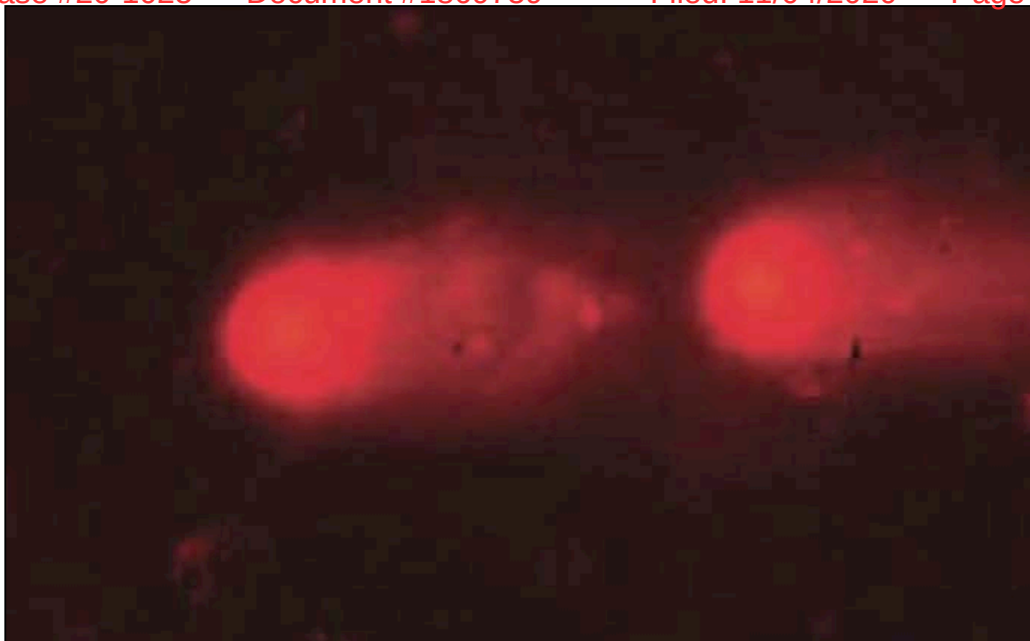
Preparation placed in a static electric field.

DNA molecules migrate towards a charged pole.

DNA molecules that are the same size, so they migrate at the same rate, will stay in a clump.

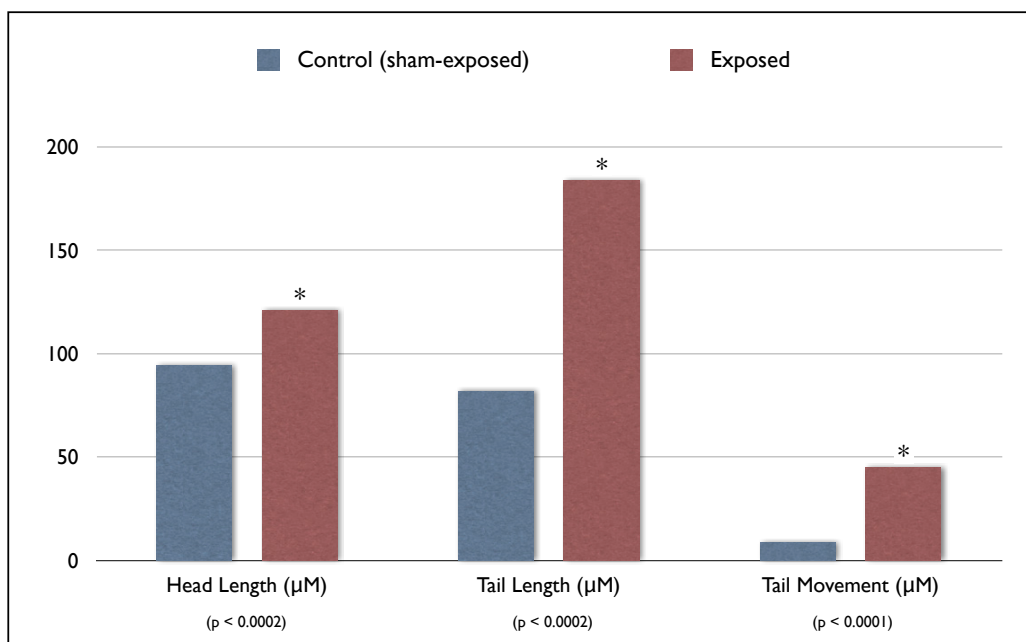
RF exposure: 2.45 GHz @ 0.34 mW/cm², 2 hours per day x 35 days

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DNA from brain cells of exposed rats. Here, some of the DNA molecules are broken. The broken parts vary in mass and total charge, so they migrate through the gel at different rates. This leaves a "comet tail" of lighter fragments behind the main body of intact DNA. The length of the tail can be measured. This is a **very sensitive** assay for DNA damage.

Kesari KK, Behari J, Kumar S. Mutagenic response of 2.45 GHz radiation exposure on rat brain. *Int J Radiat Biol* (2010a); 86(4):334-343.

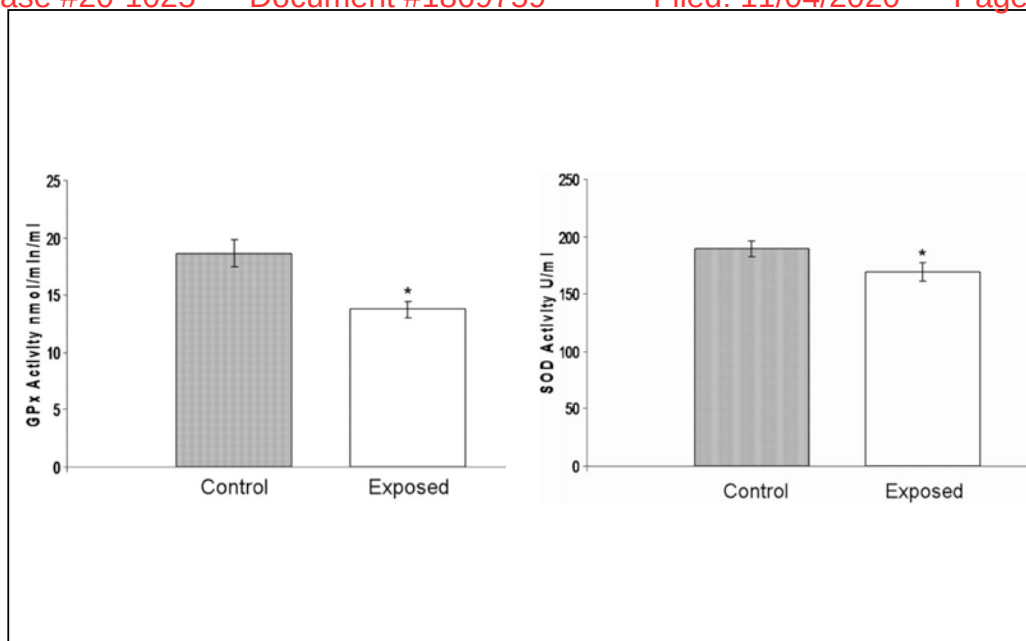
RF exposure: 2.45 GHz @ 0.34 mW/cm², 2 hours per day x 35 days

Comet Assay: **Measure of DNA fragmentation** in rat brains, produced by prolonged exposure to microwave RF.

In this study, exposure was 2 h a day for 35 days an exposure level of one third of the FCC exposure limit.

FCC exposure limit = 1 mW/cm²

Kesari KK, Behari J, Kumar S. Mutagenic response of 2.45 GHz radiation exposure on rat brain. *Int J Radiat Biol* (2010a); 86(4):334-343.

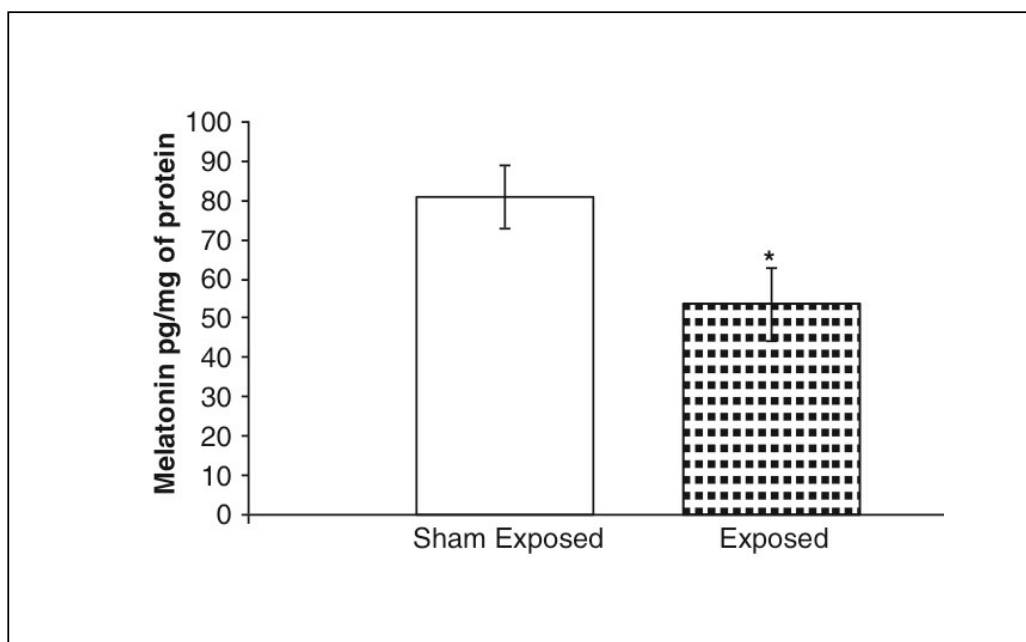
RF exposure: 2.45 GHz @ 0.34 mW/cm², 2 hours per day x 35 days**Depletion of antioxidants in RF-exposed rat brains.**

This consumption of anti-oxidants is **evidence of increased oxidant stress**, due to excess free radical production.

Kesari KK, Behari J, Kumar S. Mutagenic response of 2.45 GHz radiation exposure on rat brain. *Int J Radiat Biol* (2010a); 86(4):334-343.

Abstract

Purpose: To investigate the effect of 2.45 GHz microwave radiation on rat brain of male wistar strain. **Material and methods:** Male rats of wistar strain (35 days old with 130 ± 10 g body weight) were selected for this study. Animals were divided into two groups: Sham exposed and experimental. Animals were exposed for 2 h a day for 35 days to 2.45 GHz frequency at 0.34 mW/cm² power density. The whole body specific absorption rate (SAR) was estimated to be 0.11 W/Kg. Exposure took place in a ventilated Plexiglas cage and kept in anechoic chamber in a far field configuration from the horn antenna. After the completion of exposure period, rats were sacrificed and the whole brain tissue was dissected and used for study of double strand DNA (Deoxyribonucleic acid) breaks by micro gel electrophoresis and the statistical analysis was carried out using comet assay (IV-2 version software). Thereafter, antioxidant enzymes and histone kinase estimation was also performed. **Results:** A significant increase was observed in comet head (P50.002), tail length (P50.0002) and in tail movement (P 5 0.0001) in exposed brain cells. An analysis of antioxidant enzymes glutathione peroxidase (P 5 0.005), and superoxide dismutase (P50.006) showed a decrease while an increase in catalase (P50.006) was observed. A significant decrease (P 5 0.023) in histone kinase was also recorded in the exposed group as compared to the control (sham-exposed) ones. One-way analysis of variance (ANOVA) method was adopted for statistical analysis. **Conclusion:** The study concludes that the chronic exposure to these radiations may cause significant damage to brain, which may be an indication of possible tumour promotion (Behari and Paulraj 2007).

RF exposure: 2.45 GHz @ 0.21 mW/cm², 2 hours per day x 45 days**Suppression of melatonin secretion by 2.45 GHz RF:**

Bad news, since melatonin is also a potent antioxidant.

Kesari KK, Kumar S, Behari J. Pathophysiology of microwave radiation: effect on rat brain. *Appl Biochem Biotechnol* (2012); 166(2):379-388.

10 MINUTE BREAK HERE

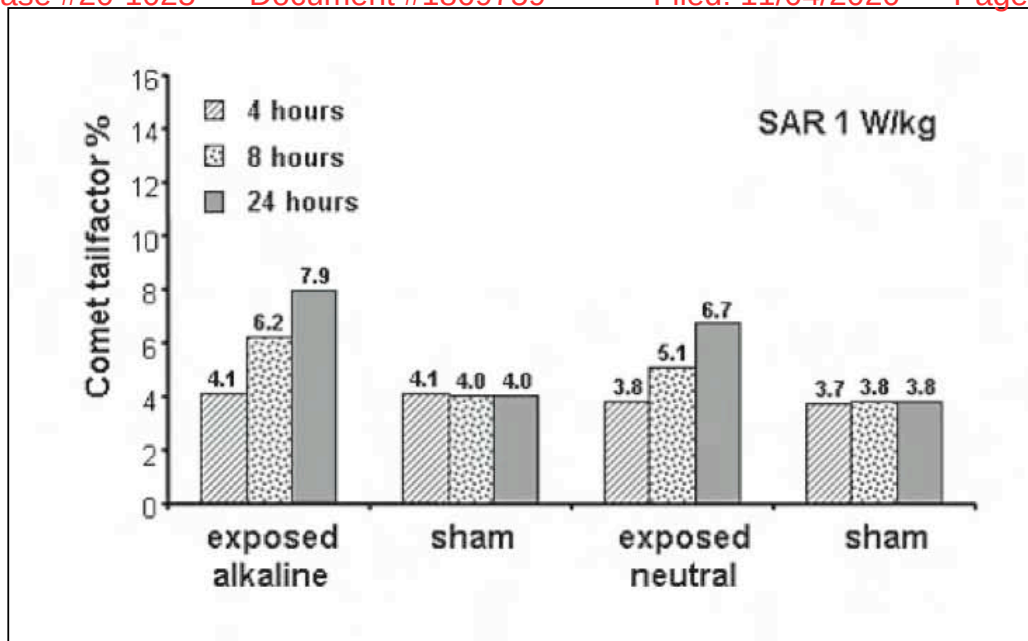
Evidence of DNA damage by microwave RF

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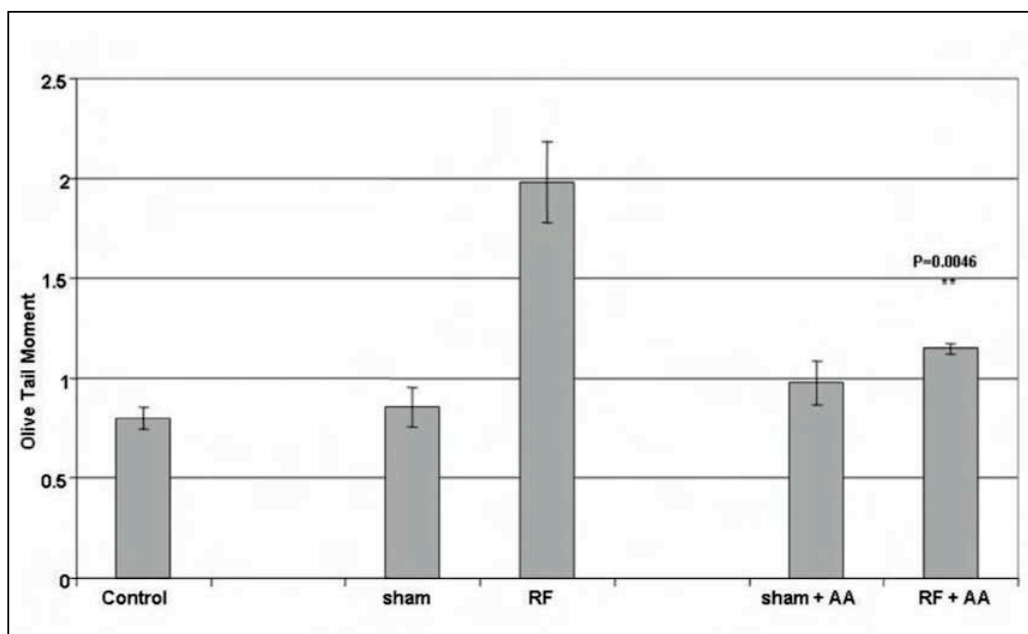


Another study, using Human fibroblasts.
 1950 MHz, 5 minutes on/10 minutes off.
 Total exposure for 4, 8, or 24 hours.
 DNA fragmentation measured by Comet Assay.

Figure 9. Intermittent RF-EMF exposure (1950 MHz, 5 minutes on/10 minutes off, 1 and 2 W/kg, 4, 8 and 24 hours) increases the DNA strand break frequency in human fibroblasts dependent on the duration of exposure as measured with the alkaline and neutral Comet assay (H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Adlkofer F. Risk Evaluation of Potential Environmental Hazards from Low Energy Electromagnetic Field Exposure Using Sensitive In Vitro Methods. *Bioelectromagnetics* (2006); 331-354.

DNA damage blocked by anti-oxidants



A cell study, with human fibroblasts, exposed to 1950 MHz RF, 5 minutes on/10 minutes off.

(right hand columns => **DNA damage blocked by anti-oxidant effect of vitamin C (ascorbic acid).**

The research group of Prof. Tauber, Berlin, investigated the effect of RF-EMF on HL-60 cells, i.e. a human promyelocytic cell line. After continuous exposure to RF-EMF of 1800 MHz and a SAR value of 1.3 W/kg they observed a highly significant increase in the number of single and double DNA strand breaks as measured by the alkaline Comet assay and of micronuclei as measured with the micronucleus test, thus fully confirming the findings obtained in the Vienna laboratory. Additionally, as clearly shown in Figures 12 and 13, the generation of DNA strand breaks and micronuclei can be prevented, when the radical scavenger ascorbic acid is added to the culture medium before exposure.

Figure 12, from: Adlkofer F. Risk Evaluation of Potential Environmental Hazards from Low Energy Electromagnetic Field Exposure Using Sensitive In Vitro Methods. *Bioelectromagnetics* (2006); 331-354.

Medical Treatments & Modulation; Treatment of advanced hepatocellular carcinoma with very low levels of amplitude-modulated electromagnetic fields. British Journal of Cancer. (Costa et al); 2011

Treatment of advanced hepatocellular carcinoma with very low levels of amplitude-modulated electromagnetic fields

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BACKGROUND: Therapeutic options for patients with advanced hepatocellular carcinoma (HCC) are limited. There is emerging evidence that the growth of cancer cells may be altered by very low levels of electromagnetic fields modulated at specific frequencies.

METHODS: A single-group, open-label, phase I/II study was performed to assess the safety and effectiveness of the intrabuccal administration of very low levels of electromagnetic fields amplitude modulated at HCC-specific frequencies in 41 patients with advanced HCC and limited therapeutic options. Three-daily 60-min outpatient treatments were administered until disease progression or death. Imaging studies were performed every 8 weeks. The primary efficacy end point was progression-free survival ≥ 6 months. Secondary efficacy end points were progression-free survival and overall survival.

RESULTS: Treatment was well tolerated and there were no NCI grade 2, 3 or 4 toxicities. In all, 14 patients (34.1%) had stable disease for more than 6 months. Median progression-free survival was 4.4 months (95% CI 2.1–5.3) and median overall survival was 6.7 months (95% CI 3.0–10.2). There were three partial and one near complete responses.

CONCLUSION: Treatment with intrabuccally administered amplitude-modulated electromagnetic fields is safe, well tolerated, and shows evidence of antitumour effects in patients with advanced HCC.

British Journal of Cancer (2011) **105**, 640–648. doi:10.1038/bjc.2011.292 www.bjcancer.com

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Keywords: hepatocellular carcinoma; phase II study; radiofrequency electromagnetic fields; tumour-specific modulation frequencies; 27.12 MHz

Treatment of inoperable or metastatic solid tumours is a major challenge in oncology, which is limited by the small number of therapeutic agents that are both well tolerated and capable of long-term control of tumour growth. Hepatocellular carcinoma (HCC) is the second most common cause of cancer death in men and the sixth in women worldwide (Jemal *et al*, 2011). Hepatocellular carcinoma is the most common tumour in certain parts of the world, particularly in East Asia, Africa, and certain countries of South America. This tumour is less frequent in Europe and in the United States, but has become the fastest rising cancer in the United States (Jemal *et al*, 2011). In the United States alone, it is estimated that 24 120 new cases were diagnosed and there were 17 430 deaths from HCC in 2010 (Jemal *et al*, 2010), a 27% increase in the number of new cases since 2004 (Jemal *et al*, 2004). The

prognosis of patients suffering from advanced HCC is poor with an average survival of fewer than 6 months (Kassianides and Kew, 1987; Jemal *et al*, 2011).

Therapies for HCC are limited. Resections of the primary tumour or liver transplantation are the preferred therapeutic approaches in patients who are surgical candidates (Bruix and Sherman, 2005). Although these interventions result in long-term survival for some patients, only a minority benefit from them because of limitations due to tumour size, patient's overall condition, and presence of hepatic cirrhosis (Cance *et al*, 2000). Only a small number of randomised trials show a survival benefit in the treatment of HCC. Chemoembolisation has been shown to confer a survival benefit in selected patients with unresectable HCC (Llovet *et al*, 2002). Data from two phase III randomised placebo-controlled studies demonstrate improved survival in patients with advanced HCC receiving the multikinase inhibitor sorafenib (Llovet *et al*, 2008b; Cheng *et al*, 2009). Additional therapies for this disease are sorely needed, especially for the large number of patients with advanced disease who cannot tolerate

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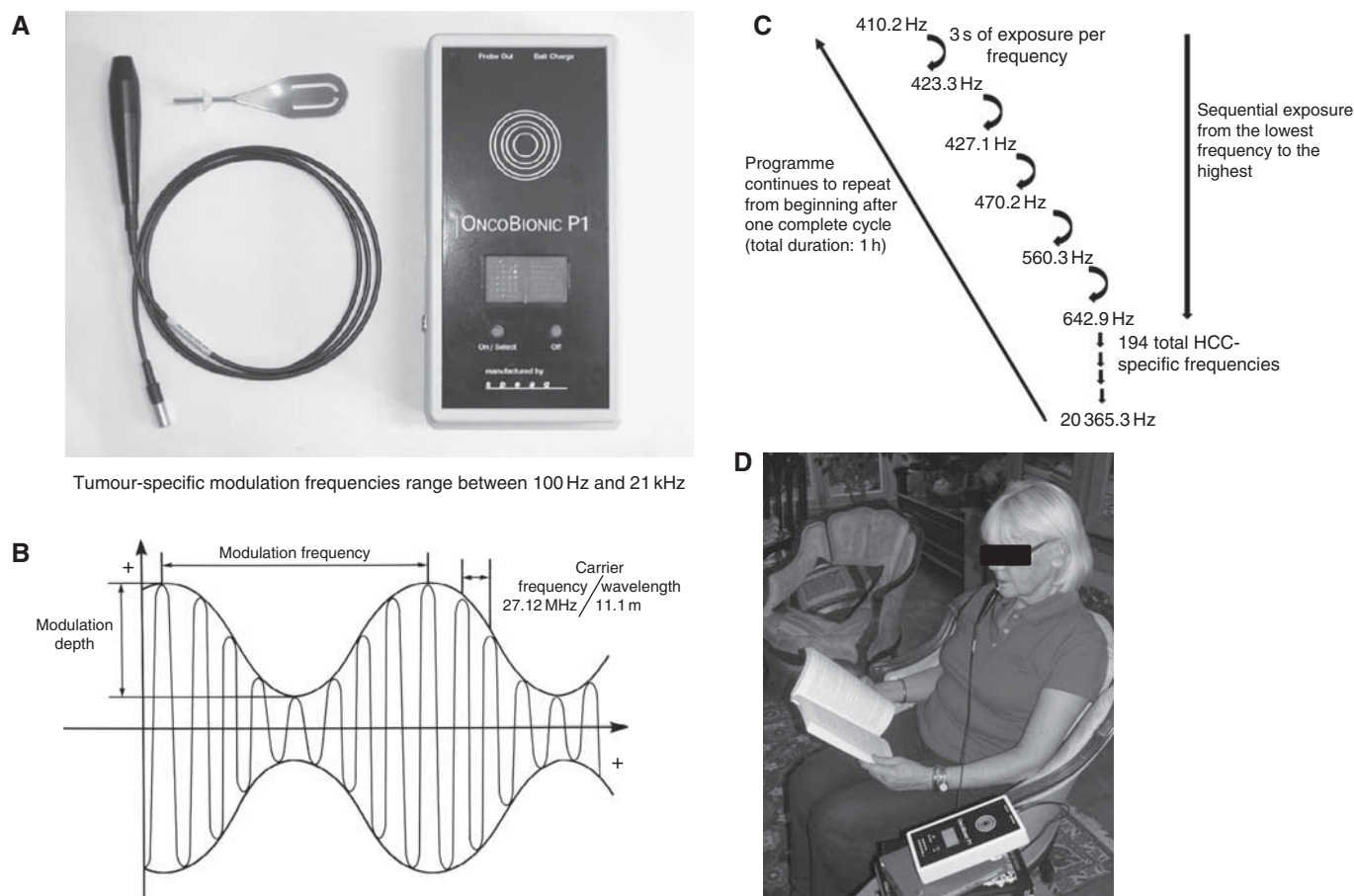


Figure 1 Delivery of HCC-specific modulation frequencies. **(A)** The generator of AM EMFs is a battery-driven RF EMF generator connected to a spoon-shaped mouthpiece. **(B)** Schematic description of AM EMFs. The carrier frequency (27.12 MHz) is sinusoidally modulated at specific frequencies. **(C)** Patient receiving treatment with RF AM EMF. **(D)** HCC treatment programme consisting of sequential emission of 194 modulation frequencies for 60 min.

chemotherapy or intrahepatic interventions because of impaired liver function (Thomas and Zhu, 2005).

The intrabuccal administration of low and safe levels of electromagnetic fields, which are amplitude-modulated at disease-specific frequencies (RF AM EMF) (Figure 1), was originally developed for the treatment of insomnia (Pasche *et al*, 1990). The highest levels of EMFs encountered during treatment are found at the interface between the tongue and the mouth probe and are compliant with international safety limits (ICNIRP, 1998; Pasche and Barbault, 2003). Tumour-specific modulation frequencies have been identified for several common forms of cancer and one report suggests that this novel therapeutic approach is well tolerated and may be effective in patients with a diagnosis of cancer (Barbault *et al*, 2009). However, the safety and potential efficacy of this treatment approach in the treatment of advanced HCC are unknown. We designed this single-group, open-label, phase I/II study to assess the feasibility of this treatment in patients with advanced HCC and limited therapeutic options.

PATIENTS AND METHODS

Patients

The study was aimed at offering treatment to patients with Child–Pugh A or B advanced HCC and limited therapeutic options. Patients were classified as having advanced disease if they were not

eligible for surgical resection or had disease progression after surgical or locoregional therapies or had disease progression after chemotherapy or sorafenib therapy. Patients with measurable, inoperable HCC were eligible for enrolment. Previous local or systemic treatments were allowed as long as they were discontinued at least 4 weeks before enrolment. Inclusion criteria included Eastern Cooperative Oncology Group performance status of 0, 1, or 2 and biopsy-confirmed HCC. Also allowed were patients with no pathological confirmation of HCC with a level of α -fetoprotein higher than 400 ng ml^{-1} and characteristic imaging findings as assessed by multislice computer tomography (CT) scan or intravenous contrast ultrasound (US). As per the University of São Paulo Department of Transplantation and Liver Surgery guidelines, liver biopsies are avoided in patients eligible for transplant or with severely impaired liver function. Exclusion criteria included confirmed or suspected brain metastasis, Child–Pugh C, previous liver transplant, and pregnancy.

Study design

This was an investigator-initiated, single centre, uncontrolled phase I/II trial in patients with advanced HCC. The trial was approved by the local human investigation committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The protocol was registered: clinicaltrial.gov identifier no. NCT00534664.

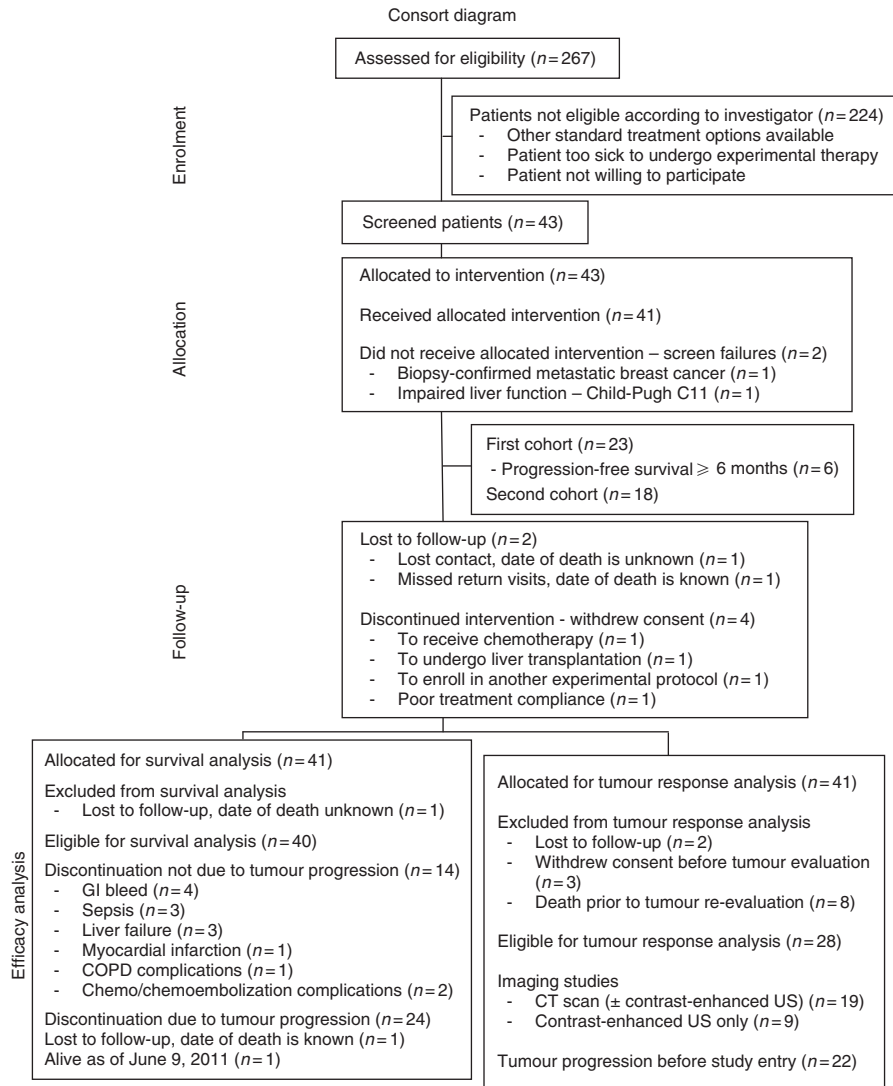


Figure 2 CONSORT diagram.

Administration of AM EMFs

The generator of AM EMFs consists of a battery-driven radio-frequency (RF) EMF generator connected to a 1.5 m long 50 Ω coaxial cable, to the other end of which a stainless-steel spoon-shaped mouthpiece is connected via an impedance transformer (Figure 1A). The RF source of the device corresponds to a class C amplifier operating at 27.12 MHz. The carrier frequency is AM (Figure 1B) with a modulation depth of $85 \pm 5\%$, whereas the modulation frequency is generated by a digital direct synthesiser with a resolution of 10^{-7} . The treatment sequence is controlled by a microcontroller (Atmel AT89S8252, Fribourg, Switzerland), that is, duration of session, sequence of modulation frequencies and duration of each sequence can be programmed via PC over a RS232 interface. The RF output is adjusted to 100 mW into a 50 Ω load, which results in an emitting power identical to that of the device used for the treatment of insomnia (Pasche *et al*, 1990; Reite *et al*, 1994; Pasche *et al*, 1996). The United States Food and Drug Administration has determined that such a device is not a significant risk device and it has been used in several studies conducted in the United States (Reite *et al*, 1994; Pasche *et al*, 1996; Kelly *et al*, 1997). A long-term follow-up survey of 807 patients who have received this therapy in the United States, Europe and

Asia showed that the rate of adverse reactions was low and was not associated with increases in the incidence of malignancy or coronary heart disease (Amato and Pasche, 1993). The maximum specific absorption rate (SAR) of the applied RF averaged over any 10 g of tissue has been estimated to be less than 2 W kg^{-1} , and the maximum temperature increase is significantly lower than 1°C anywhere in the body owing to RF absorption. The induced RF field values within the primary and metastatic tumours are significantly lower. In contrast, the RF fields induced during RF ablation of tumours cause hyperthermia and result in SAR in the range of $2.4 \times 10^5 \text{ W kg}^{-1}$ (Chang, 2003), that is, more than 100 000 times higher than those delivered by the device used in this study.

We have previously reported the discovery of HCC-specific modulation frequencies in 46 patients with HCC using a patient-based biofeedback approach and shown the feasibility of using AM EMFs for the treatment of patients with cancer (Barbault *et al*, 2009). The treatment programme used in this study consisted of three-daily outpatient treatments of 1 h duration, which contained HCC-specific modulation frequencies ranging between 100 Hz and 21 kHz administered sequentially, each for 3 s (Figure 1C and Supplementary Table S1).

The treatment method consists of the administration of AM EMFs by means of an electrically conducting mouthpiece, which is

in direct contact with the oral mucosa (Figure 1D). The patients were instructed on the use of the device and received the first treatment at the medical centre's outpatient clinic. A device was provided to each patient for the duration of the study. The patients were advised to self-administer treatment three times a day. Treatment was administered until tumour progression was objectively documented. At that time, treatment was discontinued. Treatment compliance was assessed at every return visit by recording the number of treatments delivered in the preceding 2 months.

Efficacy end points and disease assessment

The primary end point of this trial was the proportion of patients progression-free at 6 months. Secondary end points were progression-free survival (PFS) (first day of treatment until progression of disease or death) and overall survival (OS) (first day of receiving treatment to death). Objective response was assessed using the Response Evaluation Criteria in Solid Tumours group classification for patients with disease assessed by either helical multiphasic CT (Therasse *et al*, 2000). Whenever contrast-enhanced US radiological assessment was used, it was performed and reviewed by the same radiologist specialised in HCC (MCC) as this imaging modality is investigator dependent. Tumour measurements were performed at baseline and every 8 weeks. Only patients with at least one repeat tumour measurement during therapy were considered for response analysis. Throughout the study, lesions measured at baseline were evaluated using the same technique (CT or contrast-enhanced US). Overall tumour response was scored as a complete response (CR), partial response (PR), or stable disease (SD) if the response was confirmed at least 4 weeks later. Alpha-fetoprotein (AFP) levels were measured every 8 weeks in all patients throughout the study, but changes in AFP were not an end point for assessment of response. Pain was assessed according to the NCI-CTCAE v.3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcae3.pdf).

Statistical analyses and efficacy assessment

All eligible patients who began treatment were considered assessable for the primary and secondary end points. A Simon two-stage phase II minimax design was used (Simon, 1989) to evaluate the rate of progression-free survival at 6 months. The interim analysis was performed once enrolment into the first stage was completed. In the first stage, 23 patients were observed. If two or fewer patients had progression-free survival ≥ 6 months, the trial would be terminated early for lack of efficacy. If the progression-free survival of 3 or more of the first 23 patients was equal or greater than 6 months, then an additional 18 patients would be enrolled to a maximum of 41 patients. If eight or more of the 41 had PFS of at least 6 months, we would conclude that the treatment was efficacious. This design had a Type I error rate of 5% and a Type II error rate of 10% for the null hypothesis of a 6-month PFS rate of 10% vs the alternative of 27.5%. Kaplan–Meier estimates of survival, PFS, and duration of response were calculated with standard errors based on Greenwood's formula. These calculations were performed using the Proc Lifetest in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient recruitment and follow-up

From October 2005 to July 2007, 267 patients were assessed for eligibility (Figure 2). In all, 43 patients with advanced HCC and Child–Pugh A or B were enrolled in this study. The date of last patient follow-up is 9 June 2011. Of these, 20 patients (46.5%) had histological confirmation of HCC; 23 patients (53.5%) were

Table 1 Treatments received by patients with advanced HCC before enrolment (n = 41)

No previous treatment	7
Chemoembolisation	25
¹³¹ I-Lipiodol	1
Octreotide	1
Percutaneous alcohol injection therapy	1
Surgery	9
Systemic chemotherapy or sorafenib	5

Abbreviation: HCC = hepatocellular carcinoma. Two patients had surgery and chemoembolisation, two patients had surgery and systemic chemotherapy, one patient had surgery and chemoembolisation and systemic chemotherapy, one patient had surgery and percutaneous alcohol injection, one patient had surgery and sorafenib, one patient had chemoembolisation and systemic chemotherapy and one patient had surgery and octreotide.

Table 2 Patients' baseline characteristics

	No.	%
Age (years)		
Median age	64	
Range	18–85	
≥ 65	19	46.3
< 65	22	53.6
Sex		
Female	6	14.6
Male	35	85.4
ECOG performance status		
0	5	12.2
1	28	68.3
2	8	19.5
Child–Pugh status		
A5	15	36.6
A6	2	4.9
B7	6	14.6
B8	5	12.2
B9	11	26.8
No cirrhosis	2	4.9
BCLC status		
B	6	14.6
C	35	85.4
AFP > ULN		
Yes	28	68.3
No	13	16.7
Aetiology		
ETOH	2	4.9
Hepatitis B	6	14.6
Hepatitis B+C	1	2.4
Hepatitis C	22	53.7
ETOH+hepatitis C	1	2.4
NOS	9	22.0
Portal thrombosis	10	24.3
Extrahepatic disease		
Yes	16	39.0
Location		
Lung	6	14.6
Bone	3	7.3
Lymph nodes	4	9.8
Peritoneal carcinomatosis	1	2.4
Adrenal gland	1	2.4

Abbreviations: AFP = α -fetoprotein; BCLC = Barcelona Clinic Liver Cancer; ECOG = Eastern Cooperative Oncology Group; ETOH, ethyl alcohol; ULN, upper limit of normal.

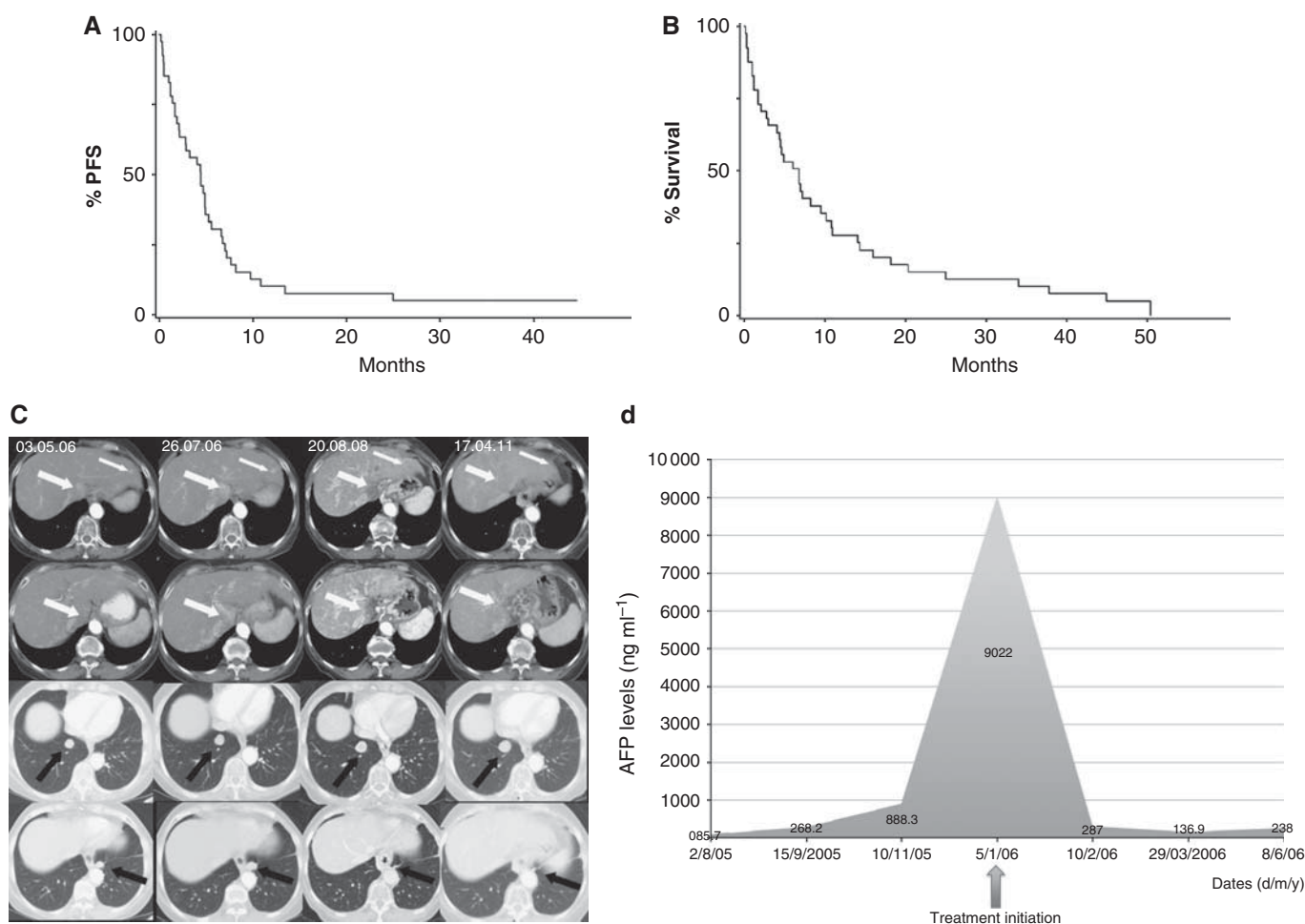


Figure 3 Progression-free and overall survival. **(A)** Median progression-free survival was 4.4 months (95% CI 2.1–5.3). **(B)** Median overall survival was 6.7 months (95% CI 3.0–10.2). **(C)** Long-term partial response in a patient with biopsy-proven hepatocellular carcinoma. A 76-year-old woman with hepatitis C and Child–Pugh A5, BCLC C, biopsy-proven hepatocellular carcinoma with bilateral pulmonary metastases, who had evidence of disease progression (+36% by Response Evaluation Criteria in Solid Tumours (RECIST) criteria) between 3 May 2006 (first column) and 26 July 2006 (second column) while enrolled in the SHARP study (Llovet *et al*, 2008b). Treatment with AM EMFs was initiated on 9 August 2006. Subsequent restaging multiphase contrast-enhanced computed tomographies (CTs) with images from corresponding levels (across rows) are demonstrated in the third and fourth columns over the course of 57 months. Note that the hypervascularity of the focal hepatic lesions (arrows in first two rows) became relatively hypoenhancing on arterial phase (20 August 2008). The patient developed main portal vein thrombosis with cavernous transformation as a complication of her cirrhosis. However, the intrahepatic lesion size is stable regardless of enhancement pattern. Note also that the left lung base lesion resolved (4th row), and the right lung base lesion remained stable (3rd row) over the duration of treatment. **(D)** Alpha-fetoprotein response in a 67-year-old patient with Child–Pugh A5, BCLC C HCC and hepatitis C (hepatitis B negative).

diagnosed based on elevated levels of α -fetoprotein and characteristic imaging findings such as vascular invasion and characteristic differences in tumour blood flow. One patient was excluded because liver biopsy established the diagnosis of metastatic breast cancer. Another patient was excluded because of severely impaired liver function (Child–Pugh C11). These two patients who did not meet the inclusion criteria were registered as screening failures. Hence, a total of 41 patients were eligible to receive experimental therapy (Figure 2).

Two patients were lost to follow-up as they did not come back for their scheduled appointments. Repeated efforts were made to reach the patients and their families. The date of death of only one patient is known, and no information on response to treatment is available for either patient. Four patients withdrew consent while receiving therapy after 8.0, 9.3, 20.3, and 21.0 months, respectively (Figure 2). One patient elected to receive chemotherapy, one patient had poor treatment compliance as defined by administration of less than 50% of planned treatments at two consecutive return visits, one patient

elected to enrol in another experimental protocol, and one patient requested to be considered for liver transplantation as part of an extended indication, which does not fulfil the Milan criteria (Mazzaferro *et al*, 1996). This latter patient experienced disease progression and was ultimately not eligible for liver transplantation. Of the 35 patients who discontinued experimental therapy, four died of gastrointestinal bleeding, three of sepsis, three of hepatic failure, one of chronic obstructive pulmonary disease, two of chemotherapy- and chemoembolisation-related complications, and one of myocardial infarction (Figure 2). The remaining 24 patients discontinued because of disease progression assessed by imaging or significant clinical deterioration as assessed by the investigator (Figure 2). Estimated 60-day mortality was 27.8%; seven of 10 deaths were directly related to progression of disease. They were caused by liver failure in association with significant hepatic tumour involvement, without other cause of death, other than tumour involvement. Two deaths were secondary to gastrointestinal bleeding. One death was due to liver failure.

A total of 31 patients (75.6%) had radiological evidence of disease progression at the time of enrolment as defined by comparison of baseline imaging studies, with imaging studies obtained within the previous 6 months; 34 (82.9%) patients had received therapy before enrolment, five (14.6%) of them systemic chemotherapy or sorafenib (Table 1). Seven (17.1%) patients had not received therapy before enrolment for the following reasons: (1) severely impaired liver function in five cases; and (2) two patients refused to receive chemotherapy for metastatic disease. As shown in Table 2, the majority of patients had severely impaired liver function as demonstrated by the fact that 22 (53.7%) patients had Child–Pugh B disease and 35 (85.4%) BLCL stage C disease.

Table 3 Independently reviewed best response (N = 41)

Best response	No.	%
Partial response ^a	4	9.8
Stable disease ^b	16	39.0
Progressive disease	8	19.5
Not available for response assessment	13	31.7

^aDuration of the partial responses were +58.0, 46.9, 14.5 and 5.3 months (patient withdrew consent to undergo liver transplant). ^bTo be classified as a stable disease, patients needed to have stable disease for ≥ 12 weeks.

Table 4 Characteristics of patients with either PR and/or long-term survival in excess of 24 months

Age at enrolment and sex	Race	Cause/cirrhosis (Child–Pugh)	Previous treatment/resection	AFP $\uparrow$ /pathology confirmation	Extra hepatic metastasis/portal thrombosis	BCLC	Okuda	CLIP	MELD	Progression before study entry/response	Treatment duration/overall survival (months)	Cause of death	Treatment received after completion of experimental therapy
62 M	Caucasian	Hep C/yes (A5)	Yes/no	Yes/yes	No/no	B	I	0	6	Yes/N/A	2.0/32.0	Tumour progressed	Systemic chemotherapy
67 F	Caucasian	Hep C/yes (B9)	Yes/no	Yes/yes	No/no	C	2	2	11	Yes/PR	11.7/11.7	GI bleed	None
30 M	Black	NOS/no	Yes/es	No/yes	No/no	B	N/A	N/A	N/A	No/PR	13.5/37.6	Tumour progressed	Chemoembolisation and systemic chemotherapy
61 M	Caucasian	Hep C/yes (A5)	Yes/no	No/no	No/no	C	I	I	6	Yes/SD	26.8/26.8	COPD	None
56 M	Caucasian	Hep B/C/yes (A5)	No/no	Yes/no	No/no	B	I	0	10	Yes/SD	4.9/50.3	Tumour progressed	Chemoembolisation
63 M	Caucasian	Hep C/yes (A5)	Yes/no	Yes/no	No/no	C	I	I	4	Yes/PR	4.9/14.3	Tumour progressed	None
76 F	Caucasian	Hep C/yes (A5)	No/no	No/no	No/yes	C	I	I	6	Yes/SD	44.6/44.6	Tumour progressed	None
76 F	Caucasian	Hep C/yes (A5)	No/yes	No/yes	Yes/yes	C	I	I	6	Yes/PR	+58.0/+58.0	On therapy	Still receiving experimental treatment

Abbreviations: AFP = α -fetoprotein; BCLC = Barcelona Clinic Liver Cancer; CLIP = Cancer Liver Italian Programme; GI = gastrointestinal; MELD = Model for end-stage liver disease; N/A = not applicable; PR = partial response; SD = stable disease.

Table 5 Changes in AFP levels

Patient age and gender	AFP 6 months (ng ml ⁻¹)	Baseline AFP (ng ml ⁻¹)	8-week AFP (ng ml ⁻¹)	AFP variation (%)	Treatment duration (months)	End treatment status	Virus status
65 M	4.31	9.76	5.95	–39.0	3.0	Progression-death	HepC
67 F	888.3	9022.0	238.0	–97.3	11.7	GI bleed-death	HepC
64 M	4.7	4.5	2.6	–42.2	8.8	AMI-death	HepB
18 M	6.7	35.7	16.4	–55.7	7.8	Revoked consent-death	NOS

Abbreviations: AFP = α -fetoprotein; AFP 6 months = AFP measured within 6 months before enrolment; AMI = acute myocardial infarction; baseline AFP = AFP at treatment initiation; GI = gastrointestinal; HepB = hepatitis B virus; HepC = hepatitis C virus; NOS = not otherwise specified; 8-week AFP = AFP at 8 weeks during treatment.

patients without biopsy-proven disease subsequently withdrew consent after 4.9 months to undergo liver transplantation. The patient died of progression of disease 9.4 months later before undergoing liver transplantation. One patient with Child–Pugh B disease had a partial response lasting 11.7 months and died of gastrointestinal bleeding. One patient died of disease progression at 44.6 months. Overall, there were six long-term survivors with an OS greater than 24 months and four long-term survivors with an OS greater than 3 years. Importantly, five of the six (83%) long-term survivors had radiological evidence of disease progression at the time of study enrolment (Table 4). Two of three patients with the longest survival (44.6 and +58 months) had radiological evidence of disease progression at the time of enrolment, BLCL stage C disease, as well as portal vein thrombosis, three predictors of short survival (Llovet *et al*, 2003). Serial AFP measurements, which predict radiological response and survival in patients with HCC (Chan *et al*, 2009; Riaz *et al*, 2009), were available for 23 patients. AFP decreased by 20% or more in four (9.8%) patients following initiation of therapy (Table 5). Figure 3D shows the time course of a 37-fold decrease in AFP in a patient who had a long-lasting (11.7 months) partial response as assessed by CT.

In all, 11 patients reported pain before treatment initiation, 3 patients reported grade 3, 5 patients reported grade 2, and 3 patients grade 1. Five patients reported complete disappearance of pain and two patients reported decreased pain shortly after treatment initiation. Two patients reported no changes and two patients reported increased pain. There were no treatment-related grade 2, 3, or 4 toxicities. The only treatment-related adverse events were grade 1 mucositis (one patient) and grade 1 somnolence (one patient) over a total of 266.8 treatment months.

DISCUSSION

Treatment with AM EMFs did not show any significant toxicity despite long-term treatment. The lack of toxicity experienced by

the 41 patients presented in this report as well as the 28 patients from our previous report (Barbault *et al*, 2009) can be readily explained by the very low and safe levels of induced RF EMFs, which are more than 100 000 times lower than those delivered during RF ablation procedures (Chang, 2003). Hence, the putative mechanism of action of this novel therapeutic approach does not depend on temperature changes within the tumour.

These data are comparable to recent phase II studies evaluating the effectiveness of standard chemotherapy as well as novel targeted therapies in HCC (Abou-Alfa *et al*, 2006; Boige *et al*, 2007; Chuah *et al*, 2007; Cohn *et al*, 2008; Dollinger *et al*, 2008; Siegel *et al*, 2008). In a large phase II study assessing the effects of sorafenib in patients with HCC and Child–Pugh A and B who had not received previous systemic treatment, Abou-Alfa *et al* (2006) observed partial responses using the WHO criteria in 2.2% of patients. Investigator-assessed median time to progression was 4.2 months, and median OS was 9.2 months. Of note, all 137 patients from that study had evidence of disease progression after 14.8 months (Abou-Alfa *et al*, 2006), whereas, at the same time point, four (9.8%) of the patients enrolled in this study did not have evidence of disease progression. These findings suggest that RF AM EMF may increase the time to radiological progression in advanced HCC.

The majority of patients enrolled in this study had either failed standard treatment options or had severely impaired liver function that limited their ability to tolerate any form of systemic or intrahepatic therapy. Indeed, 16 patients (39.0%) had Child–Pugh B8 or B9 disease. Among these patients, the median progression-free survival was 4.4 months (95% CI 1.6–7.6 months), which is identical to that of the entire group. Five of these 16 patients (31.3%) received therapy for more than 7.5 months, which indicates that this therapy is well tolerated even in patients with severely impaired liver function.

Previous treatment with standard chemotherapy or sorafenib does not seem to impact the effectiveness of AM EMFs in the treatment of HCC. Indeed, three of the four patients who had a

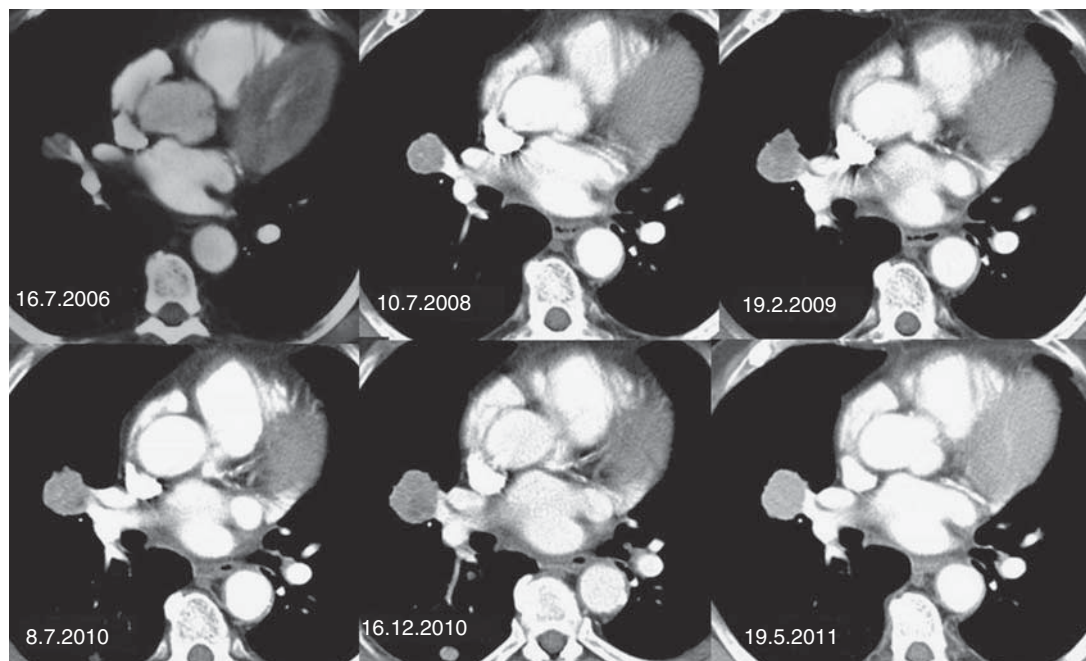


Figure 4 A 70-year-old man with recurrent thyroid cancer metastatic to the lungs: stable disease at 57.5 months. Long-term stable disease in a 70-year-old man with recurrent biopsy-proven thyroid carcinoma metastatic to the lungs enrolled in the previously published feasibility study (Barbault *et al*, 2009). Treatment with AM EMFs was initiated on 20 August 2006. As of 9 June 2011, the patient is asymptomatic and still receiving treatment with no evidence of disease progression. Images through the target metastatic lesion in the right hilum demonstrate minimal size change over the 4 years, given differences in computed tomography acquisition techniques over that time interval.

partial response while receiving AM EMFs had received previous systemic therapies (chemotherapy and sorafenib) and one had received intrahepatic therapy with ^{131}I -lipiodol.

Tumour shrinkage as assessed by radiological imaging as well as changes in AFP levels were documented in patients with advanced HCC receiving RF EMF modulated at HCC-specific frequencies administered by an intrabuccal probe. Antitumour activity in patients with advanced HCC was exemplified by partial responses observed in four patients (9.8%) and decreases in AFP levels greater than 20% in four patients. A total of 18 patients (43.9%) either had objective response or SD ≥ 6 months.

Importantly, this therapeutic approach has long-lasting therapeutic effects in several patients with metastatic cancer. Two of these patients, one with recurrent thyroid cancer metastatic to the lungs (Figure 4) enrolled in our feasibility study (Barbault *et al*, 2009) and the patient shown in Figure 3C, are still receiving treatment without any evidence of disease progression and without side effects almost 5 years after being enrolled in these studies. These findings suggest that, in some patients, this therapeutic approach may achieve permanent control of advanced cancer with virtually no toxicity.

Our phase I/II study has several limitations. First, only 19 of the 41 patients had biopsy-proven HCC, and the others were diagnosed by clinical criteria, an approach similar to that used in a recently reported phase II trial evaluating the clinical and biological effects of bevacizumab in unresectable HCC (Siegel *et al*, 2008). Importantly, analysis restricted to these 19 patients shows rates of progression-free survival at 6 months, median progression-free survival and OS that are similar to those without biopsy-proven HCC (Supplementary Figures 1C and D). Furthermore, three of the four partial responses were observed in patients with biopsy-proven HCC. Hence, these findings strongly suggest that treatment with AM EMFs yields similar results in patients with

and without biopsy-confirmed HCC. Another potential limitation of our study consists in the use of contrast-enhanced ultrasound for the monitoring of some patients with HCC. It should be pointed out that recent studies indicate that the use of this imaging technique is comparable to that of CT scan with respect to the measurement of HCC tumours (Choi, 2007; Maruyama *et al*, 2008).

Antitumour response is considered the primary end point for phase II studies to proceed to further investigations. Studies applying Cox proportional hazards analysis indicate that this end point is consistently associated with survival in trials of locoregional therapies for HCC (Llovet *et al*, 2002) and a recent consensus article suggests that randomised studies are necessary to capture the true efficacy of novel therapies in HCC (Llovet *et al*, 2008a). In summary, the encouraging findings from this study warrant a randomised study to determine the impact of AM EMFs on OS and time to symptomatic progression.

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Conflict of interest

AB and BP have filed a patent related to the use of electromagnetic fields for the diagnosis and treatment of cancer. AB and BP are founding members of TheraBionic LLC.

Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

REFERENCES

- Abou-Alfa GK, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, De GJ, Douillard JY, Lathia C, Schwartz B, Taylor I, Moscovici M, Saltz LB (2006) Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 24(26): 4293–4300
- Amato D, Pasche B (1993) An evaluation of the safety of low energy emission therapy (published erratum appears in *Compr Ther* 1994;20(12):681. *Compr Ther* 19: 242–247
- Barbault A, Costa F, Bottger B, Munden R, Bomholt F, Kuster N, Pasche B (2009) Amplitude-modulated electromagnetic fields for the treatment of cancer: discovery of tumor-specific frequencies and assessment of a novel therapeutic approach. *J Exp Clin Cancer Res* 28(1): 51
- Boige V, Raoul JL, Pignon JP, Bouche O, Blanc JF, Dahan L, Jouve JL, Dupouy N, Ducreux M (2007) Multicentre phase II trial of capecitabine plus oxaliplatin (XELOX) in patients with advanced hepatocellular carcinoma: FFCD 03-03 trial. *Br J Cancer* 97(7): 862–867
- Bruix J, Sherman M (2005) Management of hepatocellular carcinoma. *Hepatology* 42(5): 1208–1236
- Cance WG, Stewart AK, Menck HR (2000) The National Cancer Data Base Report on treatment patterns for hepatocellular carcinomas: improved survival of surgically resected patients, 1985–1996. *Cancer* 88(4): 912–920
- Chan SL, Mo FK, Johnson PJ, Hui EP, Ma BB, Ho WM, Lam KC, Chan AT, Mok TS, Yeo W (2009) New utility of an old marker: serial alpha-fetoprotein measurement in predicting radiologic response and survival of patients with hepatocellular carcinoma undergoing systemic chemotherapy. *J Clin Oncol* 27(3): 446–452
- Chang I (2003) Finite element analysis of hepatic radiofrequency ablation probes using temperature-dependent electrical conductivity. *Biomed Eng Online* 2: 12
- Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z (2009) Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 10(1): 25–34
- Choi BI (2007) Doppler and harmonic ultrasound for hepatocellular carcinoma. *Hepatol Res* 37(Suppl 2): S172–S177
- Chuah B, Lim R, Boyer M, Ong AB, Wong SW, Kong HL, Millward M, Clarke S, Goh BC (2007) Multi-centre phase II trial of Thalidomide in the treatment of unresectable hepatocellular carcinoma. *Acta Oncol* 46(2): 234–238
- Cohn AL, Myers JW, Mamus S, Deur C, Nicol S, Hood K, Khan MM, Ilegbodu D, Asmar L (2008) A phase II study of pemetrexed in patients with advanced hepatocellular carcinoma. *Invest New Drugs* 26(4): 381–386
- Dollinger MM, Behrens CM, Lesske J, Behl S, Behrmann C, Fleig WE (2008) Thymostimulin in advanced hepatocellular carcinoma: a phase II trial. *BMC Cancer* 8: 72
- ICNIRP (1998) Guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields (up to 300 GHz). *Health Phys* 74: 494–522
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *Cancer J Clin* 61(2): 69–90
- Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. *Cancer J Clin* 60(5): 277–300
- Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ (2004) Cancer statistics, 2004. *Cancer J Clin* 54(1): 8–29
- Kassianides C, Kew MC (1987) The clinical manifestations and natural history of hepatocellular carcinoma. *Gastroenterol Clin North Am* 16(4): 553–562
- Kelly TL, Kripke DF, Hayduk R, Ryman D, Pasche B, Barbault A (1997) Bright light and LEET effects on circadian rhythms, sleep and cognitive performance. *Stress Med* 13: 251–258
- Llovet JM, Burroughs A, Bruix J (2003) Hepatocellular carcinoma. *Lancet* 362(9399): 1907–1917
- Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ, for the Panel of Experts in HCC-Design Clinical Trials (2008a) Design and endpoints of

- clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 100(10): 698–711
- Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Sola R, Rodes J, Bruix J (2002) Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 359(9319): 1734–1739
- Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J, the SHARP Investigators Study Group (2008b) Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 359(4): 378–390
- Maruyama H, Yoshikawa M, Yokosuka O (2008) Current role of ultrasound for the management of hepatocellular carcinoma. *World J Gastroenterol* 14(11): 1710–1719
- Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L (1996) Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 334(11): 693–700
- Pasche B, Erman M, Hayduk R, Mitler M, Reite M, Higgs L, Dafni U, Amato D, Rossel C, Kuster N, Barbault A, Lebet JP (1996) Effects of low energy emission therapy in chronic psychophysiological insomnia. *Sleep* 19: 327–336
- Pasche B, Erman M, Mitler M (1990) Diagnosis and management of insomnia. *N Engl J Med* 323: 486–487
- Pasche B, Barbault A (2003) Low-energy emission therapy: current status and future directions. In *Bioelectromagnetic Medicine*, Rosch PJ, Markov MS (eds) pp 321–327. Marcel Dekker Inc.: New York, NY
- Reite M, Higgs L, Lebet JP, Barbault A, Rossel C, Kuster N, Dafni U, Amato D, Pasche B (1994) Sleep inducing effect of low energy emission therapy. *Bioelectromagnetics* 15: 67–75
- Riaz A, Ryu RK, Kulik LM, Mulcahy MF, Lewandowski RJ, Minocha J, Ibrahim SM, Sato KT, Baker T, Miller FH, Newman S, Omary R, Abecassis M, Benson III AB, Salem R (2009) Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: oncologic marker of radiologic response, progression, and survival. *J Clin Oncol* 27(34): 5734–5742
- Siegel AB, Cohen EI, Ocean A, Lehrer D, Goldenberg A, Knox JJ, Chen H, Clark-Garvey S, Weinberg A, Mandeli J, Christos P, Mazumdar M, Popa E, Brown RSJ, Rafii S, Schwartz JD (2008) Phase II trial evaluating the clinical and biologic effects of bevacizumab in unresectable hepatocellular carcinoma. *J Clin Oncol* 26(18): 2992–2998
- Simon R (1989) Optimal two-stage designs for phase II clinical trials. *Control Clin Trials* 10(1): 1–10
- Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 92(3): 205–216
- Thomas MB, Zhu AX (2005) Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 23(13): 2892–2899



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Cancer cell proliferation is inhibited by specific modulation frequencies

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BACKGROUND: There is clinical evidence that very low and safe levels of amplitude-modulated electromagnetic fields administered via an intrabuccal spoon-shaped probe may elicit therapeutic responses in patients with cancer. However, there is no known mechanism explaining the anti-proliferative effect of very low intensity electromagnetic fields.

METHODS: To understand the mechanism of this novel approach, hepatocellular carcinoma (HCC) cells were exposed to 27.12 MHz radiofrequency electromagnetic fields using *in vitro* exposure systems designed to replicate *in vivo* conditions. Cancer cells were exposed to tumour-specific modulation frequencies, previously identified by biofeedback methods in patients with a diagnosis of cancer. Control modulation frequencies consisted of randomly chosen modulation frequencies within the same 100 Hz–21 kHz range as cancer-specific frequencies.

RESULTS: The growth of HCC and breast cancer cells was significantly decreased by HCC-specific and breast cancer-specific modulation frequencies, respectively. However, the same frequencies did not affect proliferation of nonmalignant hepatocytes or breast epithelial cells. Inhibition of HCC cell proliferation was associated with downregulation of *XCL2* and *PLP2*. Furthermore, HCC-specific modulation frequencies disrupted the mitotic spindle.

CONCLUSION: These findings uncover a novel mechanism controlling the growth of cancer cells at specific modulation frequencies without affecting normal tissues, which may have broad implications in oncology.

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Treatment of hepatocellular carcinoma (HCC) is a major challenge given the limited number of therapeutic options available (Thomas and Zhu, 2005). We have developed a novel approach to treat advanced HCC, consisting of intrabuccal administration of very low levels of radiofrequency electromagnetic fields (RF EMF), amplitude-modulated at specific frequencies, and identified using biofeedback methods in patients with cancer (Barbault *et al*, 2009). The encouraging findings from a feasibility study (Barbault *et al*, 2009) led to the design of a phase I/II trial in patients with advanced HCC, and objective responses assessed by CT-scan and changes in alpha-fetoprotein levels were observed in several patients with biopsy-proven HCC (Costa *et al*, 2011). These findings prompted us to initiate reverse translational experiments to investigate the mechanism of action of amplitude-modulated electromagnetic fields. Two different *in vitro* exposure systems operating at 27.12 MHz were used to expose cells in culture, replicating patient-treatment conditions.

Proliferation of both HepG2 and Huh7 HCC cells was significantly decreased upon exposure to radiofrequency electromagnetic

fields, which were modulated at HCC-specific modulation frequencies. To determine how such frequencies modulate cancer cell growth, we assessed differential gene expression with RNA-seq and found that the expression of several genes was significantly downregulated by HCC-specific modulation frequencies. Previous reports have shown that low intensity, intermediate frequency electric fields are capable of inhibiting cancer growth by interfering with the proper formation of the mitotic spindle (Kirson *et al*, 2004; Kirson *et al*, 2007). Similarly, we found that electromagnetic fields that are amplitude-modulated at HCC-specific frequencies disrupt the mitotic spindle of HCC cells. Thus, we provide novel evidence that very low level of amplitude-modulated electromagnetic fields block the growth of HCC cells in a tumour- and tissue-specific fashion.

MATERIALS AND METHODS

In vitro exposure devices

The design and construction of the two *in vivo* exposure devices (Figure 1) used to conduct these experiments is described in the Supplementary Information.

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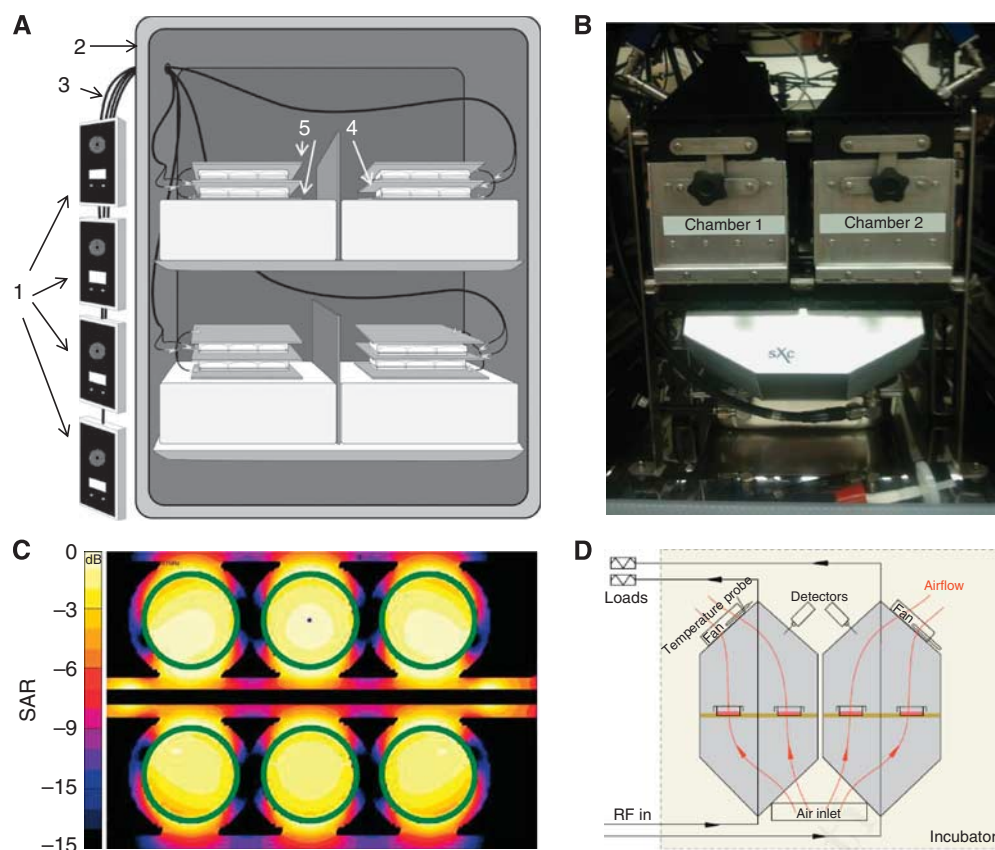


Figure 1 *In vitro* exposure experimental setups. **(A)** Parallel plate capacitor. Emitting devices (1) are placed outside the incubator (2). Each device is connected to a coaxial cable (3), which is connected to a set of brass plates inside the incubator. The centre brass plate (4) is connected to the inner conductor of the emitting device coaxial cable. The outer two brass plates (5) are connected to the outer conductor of the emitting device coaxial cable. Plates containing cells are placed in between the brass plates. **(B)** TEM cell. The system contains two identical TEM cells placed in an incubator. **(C)** Distribution of the specific absorption rate (SAR) of cell monolayer in the TEM cell (1 dB per contour). **(D)** Schematic representation showing the air flow through the TEM cell.

Cell lines

HepG2 and Huh7 cells, both of Biosafety Level 1, were used as representative HCC cell lines. HepG2 cells were obtained from ATCC (Manassas, VA, USA), and Huh7 cells were a gift from Dr Nareej Saxena (Emory University). Normal hepatocytes, THLE-2 cells, were also obtained from ATCC. The breast adenocarcinoma cell line MCF-7 was used as a representative non-HCC malignant cell line (ATCC). The breast epithelial cell line MCF-10A (ATCC) was used to represent normal breast cells. Lymphoblastoid cell lines from healthy individuals enrolled in IRB-approved protocols were provided by Dr Jeff Edberg (UAB).

[³H]thymidine incorporation assay

Growth inhibition (GI) was assessed in HCC cells exposed to HCC-specific modulation frequencies as previously described (Rosman *et al*, 2008).

Luminescent cell viability assay

Cell proliferation was quantitated using the Promega CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA), a method to determine the number of viable cells in culture based on ATP quantitation.

RNA-seq

We performed RNA-seq as previously described (Reddy *et al*, 2009). We used HepG2 cells exposed to either HCC-specific modulation frequencies or to randomly chosen frequencies. We double-selected polyA-containing mRNA from 3 µg of total RNA by using oligo-dT magnetic beads. We fragmented the mRNA with RNA fragmentation buffer and removed free-ions with a G-50 Sepharose spin column. Fragmented mRNA was used as a template to synthesise single-stranded cDNA with SuperScript II reverse transcriptase with random hexamer primers in the presence of RNaseOUT (Invitrogen by Life Technologies Corporation, Carlsbad, CA, USA). We synthesised double-stranded DNA (dsDNA) for sequencing by ligating Illumina (Illumina, San Diego, CA, USA) sequencing adaptors to blunted and dA-extended dsDNA, and size-selected fragments of 200–300 bp from a 2% Invitrogen gel and purified with a Qiagen Gel Extraction kit (Qiagen, Valencia, CA, USA). Lastly, we amplified the dsDNA library with 15 rounds of PCR with Illumina sequencing primers. Sequencing was performed on an Illumina GenomeAnalyzer IIX and the paired 36 bp reads were mapped to the hg18 reference genome by using ELAND (Illumina), allowing up to two mismatches per read and 10 or fewer map locations. By using the ERANGE software package (<http://woldlab.caltech.edu/rnaseq>), we placed uniquely mapped reads against 29 673 transcripts from NCBI build 36.1 of the human genome. After placing unique reads, ERANGE assigned multiple mapping reads and reads mapping to

splice junctions according to the number of unique reads in potential transcripts. Once all reads were mapped, ERANGE reported gene expression in units of reads per kilobase of exon and per million tags sequenced (RPKM).

Quantitative PCR

At the conclusion of the AM-EMF exposure portion of the experiment, RNA extraction (Qiagen) and reverse transcription (TaqMan, Applied Biosystems by Life Technologies Corporation) were performed to generate cDNA. Experiments comparing gene expression in HCC cells receiving HCC-specific AM-EMF with gene expression in HCC cells not receiving any exposure were conducted using Applied Biosystems pre-designed TaqMan Gene Expression Assays (*PLP2*, cat#Hs01099969_g1; *XCL2*, cat#Hs00237019_m1; Applied Biosystems by Life Technologies Corporation). Real-time quantitation was completed in quadruplicate according to the manufacturer's instructions using an ABI 7900HT Real-Time PCR System (ABI by Life Technologies Corporation), with analysis performed using ABI SDS2.2 software. Quantitative values of gene expression were determined by comparing PCR amplification curves to a known standard curve generated in tandem with the experimental samples. Each sample was individually normalised to the average corresponding to endogenous expression of *GAPDH* (*GAPDH*, cat#Hs99999905_m1, TaqMan, Applied Biosystems by Life Technologies Corporation). Averages of the normalised values from each condition were then used to compare the relative gene expression between the experimental groups. The s.e.m. was determined for each experimental condition.

Confocal laser scanning microscopy

Cells undergoing mitosis were imaged using the Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY, USA). For imaging experiments, 22 mm square microscope cover glass (Corning Life Sciences, Lowell, MA, USA, cat#2865–22) were flame-sterilised with 200-proof ethanol and placed in 6-well or 35 mm Falcon tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA). Approximately 300 μ l of cell suspension/growth media was added directly to the top of the cover slips, and cells were plated at varying concentrations (4×10^5 – 5×10^5 cells per ml) on separate cover slips for each assay to control for variability in antibody affinity between different experiments. Once the cells were given 8–18 h to attach to the cover slips, 3 ml of complete growth media was added to each well containing a cover slip. Following RF EMF exposure, indirect immunofluorescent microscopy compared the cells receiving HCC-specific modulation frequencies with cells not receiving any exposure (Microtubule Marker (AE-8) sc-73551, Fluorescent Secondary Alexa Fluor 488 goat anti-mouse IgG (H + L): A-11001; Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Karyotype analysis

To determine whether these changes were associated with karyotypic changes, HepG2 cells exposed to HCC-specific modulation frequencies or unexposed were harvested, slides prepared, and metaphase chromosomes G-banded using standard methods. The chromosomes were analysed and the karyotype described according to the International System for Cytogenetic Nomenclature (Brothman *et al*, 2009).

Statistical analyses

One sample two-sided *t*-test was performed to test the significance of cell proliferation exposed to RF EMF amplitude-modulated at tumour-specific or randomly chosen frequencies. ANCOVA analysis: For the long-term (7 weeks) GI analysis and the GI analysis for varying SAR values (0.05, 0.1, 0.4, and 1.0 W kg⁻¹), data were fit to a linear model, and time point and dosage level were considered as covariates in determining significance.

RESULTS

Assessment of cell proliferation in the presence of RF EMF

Cell proliferation assays were conducted after 7 days, that is, 21 h of exposure to amplitude-modulated RF EMF. Treatment with HCC-specific modulation frequencies (Supplementary Table 1) significantly reduced the proliferation of HepG2 and Huh7 cells using both the parallel plate capacitor and the transverse electromagnetic (TEM) setups (Figure 1). The observed growth-inhibitory effect on HepG2 cells was of the same magnitude when using a tritium incorporation assay and a bioluminescence assay based on ATP consumption (Figure 2A). Having shown similar results with two different assays, the remainder of the cell proliferation experiments were conducted with the more commonly used tritium incorporation assay. Cell proliferation of HepG2 and Huh7 cells exposed to HCC-specific modulation frequencies was significantly lower than the proliferation of cells exposed either to randomly chosen frequencies (Supplementary Table 2) or not exposed to RF EMF (Figure 2A, columns 1–3). When HepG2 cells were exposed for only 1 h daily, we did not observe any significant inhibition of cell proliferation (Figure 2B). Daily exposure for 6 h instead of 3 h resulted in the same level of cell-proliferation inhibition (Figure 2B). To determine when HCC-specific modulation frequencies begin to exert anti-proliferative effects on HepG2 cells, we assessed cell proliferation following 3 days (9 h) of exposure and did not find any significant difference between cells exposed to HCC-specific modulation frequencies and unexposed cells (Figure 2B).

Further, to determine whether the growth-inhibitory effect of HCC-specific modulation frequencies persists over time and results in a decrease in the total number of tumour cells, we counted the number of HepG2 cells following treatment with HCC-specific modulation frequencies and that of untreated HepG2 cells weekly for up to 7 weeks. Cells that were either exposed to HCC-specific modulation frequencies or not exposed were split weekly at the same ratio over a period of 7 weeks. As shown in Figure 2C, when compared with unexposed HepG2 cells, the number of HepG2 cells following exposure to HCC-specific modulation frequencies decreased steadily over 7 weeks, resulting in a cumulative loss of 1.71×10^6 cells per ml at week 7.

The average specific absorption rate (SAR) for cells exposed in the parallel capacitor plate system is 0.03 W kg⁻¹ (Supplementary Information). All initial experiments conducted with the TEM system were conducted at a SAR of 0.4 W kg⁻¹. To determine the range of SARs within which significant GI was observed, additional cell proliferation experiments were performed at 0.05, 0.1 and 1.0 W kg⁻¹. A significant anti-proliferative effect was observed at all SARs ranging from 0.05 to 1.0 W kg⁻¹ ($P = 0.0354$). All subsequent assays with the TEM system were conducted at an SAR of 0.4 W kg⁻¹.

Inhibition of cell proliferation is tumour and tissue specific

Our previous clinical observations revealed that patients with HCC had biofeedback responses to specific modulation frequencies that were different from those identified in patients with other types of cancer, such as breast cancer (Barbault *et al*, 2009). To experimentally assess the relevance of these findings on the proliferation of tumour cells, we determined the specificity of frequencies identified in patients with these two tumour types given the documented objective clinical responses that included one complete and one partial response in two patients with metastatic breast cancer (Barbault *et al*, 2009) and three partial and one near-complete responses in four patients with HCC (Costa *et al*, 2011). A total of 194 breast cancer-specific modulation frequencies ranging in the same modulation frequency band from

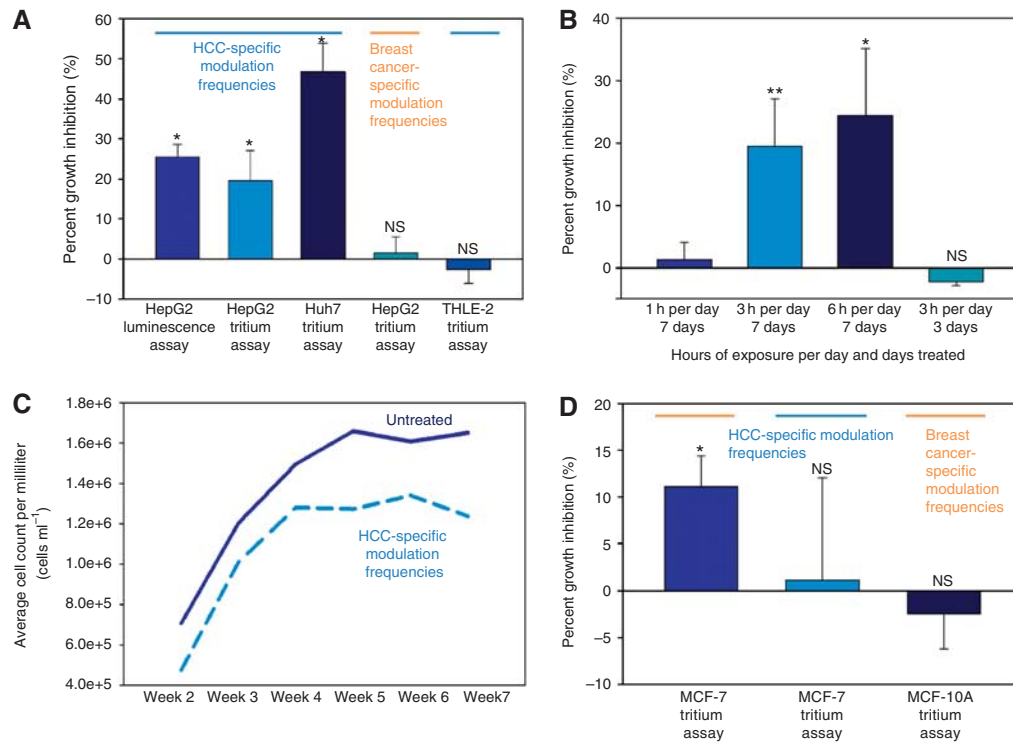


Figure 2 Cell proliferation assays of cell lines exposed to HCC-specific or breast cancer-specific modulation frequencies. **(A)** Cells were not split after initial seeding; medium was exchanged every 48 h. Experiments were performed with both equipment setups. Left to right columns: (1) HepG2 cells exposed to HCC-specific modulation frequencies with GI evaluated with a luminescence assay, $25.46 \pm 3.22\%$ GI ($P = 0.0009$). (2) HepG2 cells exposed to HCC-specific modulation frequencies with GI evaluated using tritium incorporation, $19.44 \pm 7.60\%$ GI ($P = 0.00993$). (3) Huh7 cells exposed to HCC-specific modulation frequencies, $47.73 \pm 7.14\%$ GI ($P = 0.018$). (4) HepG2 cells are not significantly inhibited when exposed to breast cancer-specific modulation frequencies, $1.49 \pm 3.99\%$ GI ($P = 0.8815$). (5) THLE-2 cells are not affected by HCC-specific modulation frequencies, $-2.54 \pm 3.54\%$ GI ($P = 0.6550$). Values represent average percent GI ($n = 6$) $\pm$ %STERR. **(B)** Cell proliferation assays exposing cells for varying hours per day. Left to right: 1 h per day $1.36 \pm 2.77\%$ ($P = 0.8508$); 3 h per day $19.44 \pm 7.60\%$ ($P = 0.0099$); 6 h per day $24.46 \pm 10.75\%$ ($P = 0.0301$); 3 h per day for 3 days $-2.12 \pm 0.66\%$ ($P = 0.4067$). Values represent average percent GI ($n = 6$) $\pm$ %STERR. **(C)** Cumulative decrease in cell counts over time when HepG2 cells are exposed to HCC-specific modulation frequencies. Samples were subcultured by volume every 7 days (1:20 split by volume). Average total cells mL⁻¹ per week: week 2: 7.07×10^5 , 4.75×10^5 ; week 3: 1.20×10^6 , 1.01×10^6 ; week 4: 1.50×10^6 , 1.28×10^6 ; week 5: 1.66×10^6 , 1.22×10^6 ; week 6: 1.61×10^6 , 1.34×10^6 ; week 7: 1.65×10^6 , 1.24×10^6 for untreated and treated samples, respectively. For the duration of the 7-week experiment with time considered as a covariate: $P = 0.005751$. **(D)** Left to right columns: (1) MCF-7 cells exposed to breast tumour-specific modulation frequencies, $11.08 \pm 3.30\%$ GI ($P = 0.0230$). (2) MCF-7 cells are not significantly inhibited when exposed to HCC-specific modulation frequencies, $1.49 \pm 3.99\%$ ($P = 0.8815$) GI, respectively. (3) MCF-10A cells are not affected by breast tumour-specific modulation frequencies, $-2.46 \pm 3.75\%$ GI ($P = 0.8579$). Values represent average percent GI ($n = 6$) $\pm$ %STERR.

100 Hz to 21 kHz have been identified in patients with a diagnosis of breast cancer (Supplementary Table 3). In all 9 (4.6%) of the HCC-specific modulation frequencies are identical to breast cancer-specific modulation frequencies.

The two patients with metastatic breast cancer who had experienced an objective response to breast cancer-specific modulation frequencies had tumours that over-expressed oestrogen receptor (ER+) and progesterone receptor (PR+), but did not over-express ERBB2 (ERBB2-) (Barbault *et al*, 2009). We therefore chose the MCF-7 cell line as it represents the same tumour phenotype, that is, ER+, PR+, ERBB2-. Although the growth of MCF-10A breast cells was unaffected by exposure to breast cancer-specific modulation frequencies, exposure of MCF-7 breast cancer cells to breast cancer-specific modulation frequencies significantly inhibited cell proliferation (Figure 2D). However, exposure of HepG2 cells to the same breast cancer-specific modulation frequencies did not affect cell proliferation (Figure 2A). Similarly, the proliferation of MCF-7 cells was not affected by exposure to HCC-specific modulation frequencies (Figure 2D). Consequently, the observed anti-proliferative effect on HCC and breast cancer cells was observed only upon exposure to tumour-specific modulation frequencies previously identified in patients with a

diagnosis of HCC and breast cancer, respectively, despite the fact that more than 57% of the modulation frequencies only differed by <1% (Supplementary Tables 1 and 3).

Having demonstrated that the anti-proliferative effect of amplitude-modulated frequencies was tumour specific, we sought to determine whether the HCC-specific modulation frequencies have an effect on the proliferation of THLE-2 normal hepatocytes. As shown in Figure 2A, exposure of THLE-2 cells to HCC-specific modulation frequencies did not have any measurable effect on cell proliferation. These findings provide strong support for the novel notion that a combination of narrowly defined, specific modulation frequencies identified in a group of patients with the same type of cancer is capable of inhibiting cell proliferation in a tumour- and tissue-specific fashion.

Tumour-specific modulation frequencies and gene regulation

To study the mechanism by which tumour-specific modulation frequencies inhibit cell proliferation, we assessed the gene expression profile of HepG2 cells exposed to HCC-specific modulation frequencies using RNA-seq, as it provides a more

comprehensive assessment of differential gene expression across a broader range of expression levels than microarray-based analysis (Wang *et al*, 2009). Overall, we did not observe statistically significant differences in transcript levels when comparing two HepG2 cultures exposed for 1 week, 3 h a day to HCC-specific modulation frequencies with two HepG2 cultures exposed to randomly chosen modulation frequencies (Supplementary Figure 1). However, we did observe a small number of genes with an absolute fold-change >1.5 and a minimum mean RPKM of 1.5 following exposure to HCC-specific modulation frequencies. Two genes with an absolute fold-change >1.8 appeared to be down-regulated in HepG2 cells exposed to HCC-specific modulation frequencies, *PLP2* and *XCL2*, and were considered to be candidates worthy of further experiments. We validated the downregulation of *PLP2* and *XCL2* with quantitative PCR in both HepG2 as well as Huh7 cells exposed to HCC-specific modulation frequencies (Figures 3A and B). There was no significant downregulation of *PLP2* and *XCL2* in MCF-7 breast cancer cells (Figure 3C). Similarly, there was no downregulation of *PLP2* and *XCL2* in nonmalignant cells, that is, in THLE-2 normal hepatocytes (Figure 3D), or in lymphoblastoid cell lines from healthy individuals (Figures 3E and F). These findings support the novel notion that the demodulation effects of RF EMF amplitude-modulated at specific frequencies inhibit cell proliferation and affect the expression of several genes in a tumour- and tissue-specific fashion.

Tumour-specific modulation frequencies and disruption of the mitotic spindle

There is evidence that the proliferation of several rodent and human cancer cell lines is arrested by exposure to sinusoidal electric fields of $100\text{--}200\text{ V m}^{-1}$ at a frequency of $100\text{--}300\text{ kHz}$

(Kirson *et al*, 2004). This approach has also shown efficacy in animal and human tumour models as well as promising results in the treatment of patients with cancer (Kirson *et al*, 2004; Kirson *et al*, 2007; Salzberg *et al*, 2008; Kirson *et al*, 2009). The anti-tumour effect of this therapeutic approach appears to be caused by disruption of the mitotic spindle mediated by interference of spindle tubulin orientation and induction of dielectrophoresis (Kirson *et al*, 2004; Kirson *et al*, 2007). In contrast to the sinusoidal signals (Kirson *et al*, 2004), the carrier frequency of the signal applied in our experiments is more than 100 times higher; the peak E-field amplitude of the carrier at 0.4 W kg^{-1} corresponds to approximately 35 V m^{-1} inside the cell medium when the signal is sinusoidally amplitude-modulated at specific frequencies with 85% modulation depth (Kirson *et al*, 2004).

Despite these significant differences, confocal laser scanning microscopy revealed pronounced disruption of the mitotic spindle in more than 60% of HepG2 cells exposed for 1 week, 3 h per day to HCC-specific modulation frequencies whereas there was no disruption of the mitotic spindle in unexposed HepG2 cells (Figures 4A and B). Specifically, the observed cytoskeletal disruption in cells exposed to HCC-specific modulation frequencies was apparent in cells in mitosis, in which we saw centrosomal distortion and poor chromosomal separation at anaphase (Figure 4D). We found no evidence of karyotypic differences between HepG2 cells exposed to HCC-specific modulation frequencies and unexposed HepG2 cells.

DISCUSSION

By exposing HCC cells to 27.12 MHz RF EMF sinusoidally amplitude-modulated at specific frequencies, which were previously

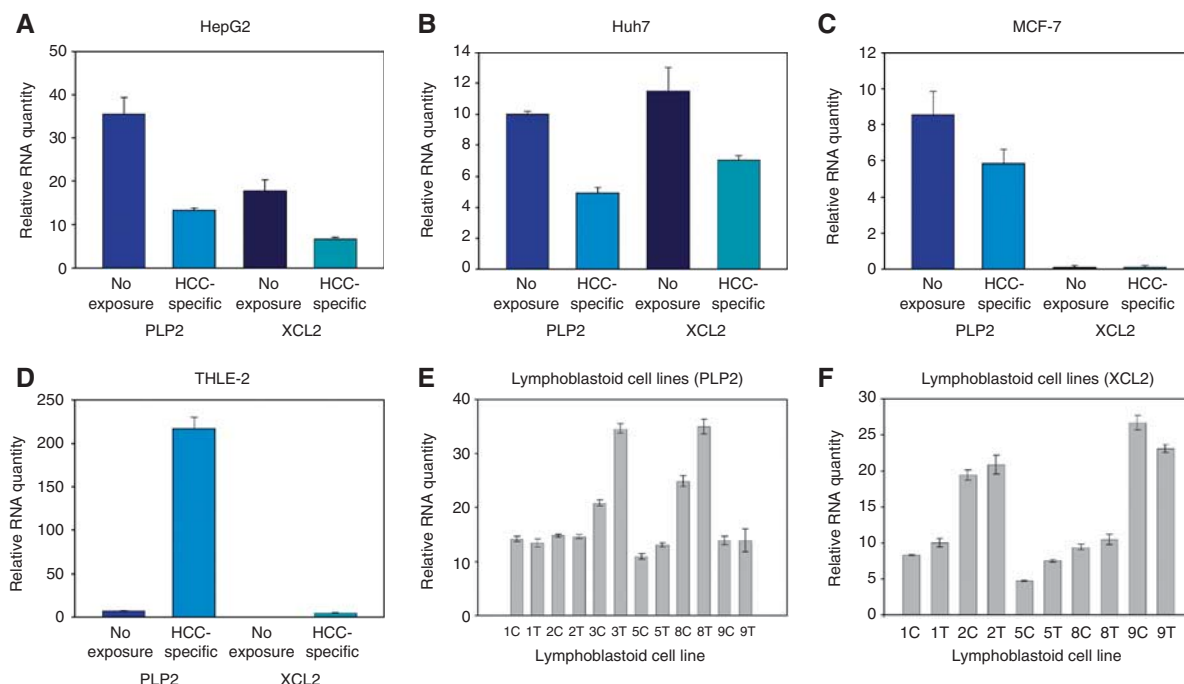


Figure 3 Expression of *XCL2* and *PLP2* receiving HCC-specific RF EMF compared with cells not receiving exposure. **(A)** HepG2: *PLP2* (35.46 ± 3.85 ; 13.17 ± 0.70) and *XCL2* (17.87 ± 2.49 ; 6.52 ± 0.48) ($P = 9.0371 \times 10^{-3}$ and $P = 0.0179$, respectively). **(B)** Huh7: *PLP2* (10.02 ± 0.19 ; 4.95 ± 0.35) and *XCL2* (11.52 ± 1.49 ; 7.02 ± 0.29) ($P = 9.4981 \times 10^{-5}$ and $P = 0.0536$, respectively). **(C)** MCF-7: *PLP2* (8.52 ± 1.30 ; 5.84 ± 0.77) and *XCL2* (levels not detectable). **(D)** THLE-2: *PLP2* (7.11 ± 0.14 ; 216.89 ± 13.18) and *XCL2* (0.03 ± 0.01 ; 4.55 ± 1.04) in THLE-2 cells exposed to HCC-specific modulation frequencies ($P = 5.5108 \times 10^{-4}$ and $P = 0.0221$, respectively). **(E)** Expression levels of *PLP2* in lymphoblastoid cell lines (C = unexposed; T = HCC-specific exposure) (for all cell lines compiled $P = 0.418$), LCL 3 expression was significant $P = 0.0021$ as was LCL 8 $P = 0.0159$ **(F)** Expression levels of *XCL2* in lymphoblastoid cell lines (for all cell lines compiled ($P = 0.899$), LCL 1 expression difference was significant $P = 0.0002$. Values represent average relative RNA expression ($n = 4$) $\pm$ s.e.m. Levels were normalised to levels of GAPDH.

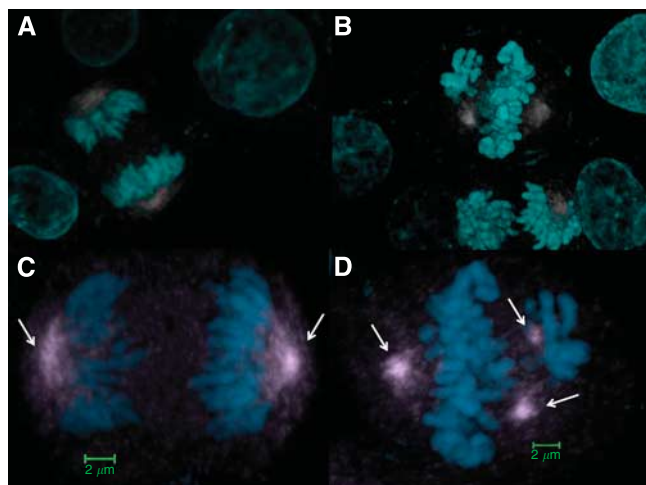


Figure 4 Mitotic spindle disruption in cells receiving HCC-specific RF EMF compared with cells not receiving exposure. (A) HepG2 efficiently assembles a bipolar mitotic spindle, allowing cells to pass through the mitotic assembly checkpoint and successfully progress from metaphase to anaphase. (B) >60% of dividing HepG2 cells exposed to HCC-specific modulation frequencies exhibit microtubule-associated anomalies, (C) high magnification of unexposed HepG2 cells in mitosis (D) high magnification of HepG2 cells exposed to HCC-specific modulation frequencies shows errors such as tripolar spindle formation (Cyan = DAPI; Gray = Microtubules; Arrows = mitotic spindle).

identified in patients with a diagnosis of HCC (Barbault *et al*, 2009) and result in therapeutic responses in patients with HCC (Costa *et al*, 2011), we demonstrate a robust and sustained anti-proliferative effect. This effect was seen within SARs ranging from 0.03 to 1.0 W kg⁻¹, that is, within the range of exposure in humans receiving treatment administered intrabuccally (Barbault *et al*, 2009; Costa *et al*, 2011). HCC-specific modulation frequencies began to hinder cell proliferation after 7 days of exposure and the anti-proliferative effect increased over a 7-week period. The anti-proliferative effect HCC-specific modulation frequencies were observed only in HCC cells, but not in breast cancer cells or normal hepatocytes.

The specificity of modulation frequencies is exemplified by the fact that two sets of similar modulation frequencies (breast cancer-specific and randomly chosen) within the same range, that is, from 100 Hz to 21 kHz, did not affect the proliferation of HCC cells. Similarly, the proliferation of breast cancer cells was affected only by breast cancer-specific modulation frequencies, but neither by HCC-specific nor by randomly chosen modulation frequencies. The fact that >50% of the modulation frequencies from these three programs differed by <1%, provides strong experimental evidence that the biological effects are only mediated by a combination of narrowly defined, tumour-specific modulation frequencies.

The modulation-frequency specific laboratory findings are consistent with the clinical observation of a complete response in a patient with breast cancer metastasis to the adrenal gland and the bone while a primary malignancy of the uterus continued to grow (Barbault *et al*, 2009). This suggests that a combination of precise tumour-specific modulation frequencies is needed to block cancer growth *in vitro* and in patients with a diagnosis of cancer. The clinical results reported by Barbault *et al*, (2009) and Costa *et al*, (2011) as well as laboratory evidence included in this report provide support for the novel and transformational concept that the growth of human tumours arising from the same primary tissue may be effectively blocked by identical modulation

frequencies. While receiving treatment with HCC-specific modulation frequencies, one black and three white patients with advanced carcinoma had partial responses (Costa *et al*, 2011). Furthermore, proliferation of the Huh7 HCC cell line, which is derived from a Japanese patient's tumour (Nakabayashi *et al*, 1982), exhibited the most pronounced response to HCC-specific modulation frequencies (Figure 2A). This indicates that the frequency signature and biological effects of HCC-specific modulation frequencies are likely independent of ethnic status.

There is no known biophysical mechanism accounting for the effect observed in these experiments; however, other modulation-frequency dependent effects have been observed in biological systems at similarly low exposure levels. Documented effects have occurred in cellular processes controlling cell growth, proliferation, and differentiation (Blackman, 2009). Further, modulation of the signal appears to be a critical factor in the response of biological systems to electromagnetic fields (Blackman, 2009). The amount of electromagnetic energy delivered is far too low to break chemical bonds or cause thermal effects, necessitating alternative mechanistic explanations for observed biological outcomes. Several theories have been put forth to explain biological responses to electromagnetic fields. Some reports have shown that low levels of electromagnetic fields can alter gene expression and subsequent protein synthesis by interaction of the electromagnetic field with specific DNA sequences within the promoter region of genes (Blank and Goodman, 2008; Blank and Goodman, 2009). Such changes have been demonstrated in the family of 'heat shock' proteins that function in the cell stress response (Blank and Goodman, 2009).

To thoroughly interrogate gene expression changes in cells exhibiting decreased cell proliferation, we used high-throughput sequencing technologies to sequence the cells' cDNA, a technique that has become invaluable in the study of cancer (Maher *et al*, 2009). Tumour cell G1 was associated with downregulation of *PLP2* and *XCL2* as well as with disruption of the mitotic spindle. *PLP2* encodes an integral membrane protein that localises to the endoplasmic reticulum in epithelial cells. The encoded protein can multimerise and may function as an ion channel (Breitwieser *et al*, 1997). *PLP2* enhances chemotaxis of human osteogenic sarcoma cells (Lee *et al*, 2004) and *PLP2* downregulation is associated with decreased metastasis in a mouse model of cancer (Sonoda *et al*, 2010). *XCL2* encodes for a protein that enhances chemotactic activity for lymphocytes and downregulation of *XCL2* has been shown to be associated with good prognosis in patients with breast cancer (Teschendorff *et al*, 2007; Teschendorff and Caldas, 2008). The pronounced disruption of the mitotic spindle seen in the majority of HepG2 cells exposed to HCC-specific modulation frequencies undergoing mitosis is not associated with karyotypic changes, but may be a major determinant of the anti-tumour effects of HCC-specific modulation frequencies accounting for the therapeutic responses seen in patients receiving the same modulation frequencies (Costa *et al*, 2011).

Exposure of HCC cells to the same RF EMF modulated at slightly different modulation frequencies did not result in changes in gene expression, which demonstrates that inhibition of cell proliferation is associated with changes in gene expression levels.

In conclusion, we show that very low levels of 27.12 MHz radiofrequency electromagnetic fields, which are comparable to the levels administered to patients, inhibit tumour cell growth when modulated at specific frequencies. The exciting findings presented in this report suggest that the anti-proliferative effect of modulation frequencies is both tumour- and tissue-specific, and is mediated by changes in gene expression as well as disruption of the mitotic spindle. These findings uncover a new alley to control tumour growth and may have broad implications for the treatment of cancer.

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Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

REFERENCES

- Barbault A, Costa F, Bottger B, Munden R, Bomholt F, Kuster N, Pasche B (2009) Amplitude-modulated electromagnetic fields for the treatment of cancer: discovery of tumor-specific frequencies and assessment of a novel therapeutic approach. *J Exp Clin Cancer Res* **28**(1): 51
- Blackman C (2009) Cell phone radiation: evidence from ELF and RF studies supporting more inclusive risk identification and assessment. *Pathophysiology* **16**: 205–216
- Blank M, Goodman R (2008) A mechanism for stimulation of biosynthesis by electromagnetic fields: charge transfer in DNA and base pair separation. *J Cell Physiol* **214**: 20–26
- Blank M, Goodman R (2009) Electromagnetic fields stress living cells. *Pathophysiology* **16**: 71–78
- Breitwieser GE, McLenithan JC, Cortese JF, Shields JM, Oliva MM, Majewski JL, Machamer CE, Yang VW (1997) Colonic epithelium-enriched protein A4 is a proteolipid that exhibits ion channel characteristics. *Am J Physiol* **272**(3 Pt 1): C957–C965
- Brothman AR, Persons DL, Shaffer LG (2009) Nomenclature evolution: changes in the ISCN from the 2005 to the 2009 edition. *Cytogenet Genome Res* **127**: 1–4
- Costa FP, de Oliveira AC, Meirelles R, Machado MCC, Zanesco T, Surjan R, Chammas MC, de Souza Rocha M, Morgan D, Cantor A, Zimmerman J, Brezovich I, Kuster N, Barbault A, Pasche B (2011) Treatment of advanced hepatocellular carcinoma with very low levels of amplitude-modulated electromagnetic fields. *Br J Cancer* **105**(5): 640–648
- Kirson ED, Dbaly V, Tovarys F, Vymazal J, Soustiel JF, Itzhaki A, Mordechovich D, Steinberg-Shapira S, Gurvich Z, Schneiderman R, Wasserman Y, Salzberg M, Ryffel B, Goldsher D, Dekel E, Palti Y (2007) Alternating electric fields arrest cell proliferation in animal tumor models and human brain tumors. *PNAS* **104**(24): 10152–10157
- Kirson ED, Giladi M, Gurvich Z, Itzhaki A, Mordechovich D, Schneiderman RS, Wasserman Y, Ryffel B, Goldsher D, Palti Y (2009) Alternating electric fields (TTFields) inhibit metastatic spread of solid tumors to the lungs. *Clin Exp Metastasis* **26**(7): 633–640.
- Kirson ED, Gurvich Z, Schneiderman R, Dekel E, Itzhaki A, Wasserman Y, Schatzberger R, Palti Y (2004) Disruption of cancer cell replication by alternating electric fields. *Cancer Res* **64**(9): 3288–3295
- Lee SM, Shin H, Jang SW, Shim JJ, Song IS, Son KN, Hwang J, Shin YH, Kim HH, Lee CK, Ko J, Na DS, Kwon BS, Kim J (2004) PLP2/A4 interacts with CCR1 and stimulates migration of CCR1-expressing HOS cells. *Biochem Biophys Res Commun* **324**: 768–772
- Maher CA, Kumar-Sinha C, Cao X, Kalyana-Sundaram S, Han B, Jing X, Sam L, Barrette T, Palanisamy N, Chinnaiyan AM (2009) Transcriptome sequencing to detect gene fusions in cancer. *Nature* **458**(7234): 97–101, doi:10.1038/nature07638
- Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J (1982) Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res* **42**: 3858–3863
- Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ, Myers RM (2009) Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res* **19**(12): 2163–2171
- Rosman DS, Phukan S, Huang CC, Pasche B (2008) TGFBR1*6A enhances the migration and invasion of MCF-7 breast cancer cells through RhoA activation. *Cancer Res* **68**(5): 1319–1328
- Salzberg M, Kirson E, Palti Y, Rochlitz C (2008) A pilot study with very low-intensity, intermediate-frequency electric fields in patients with locally advanced and/or metastatic solid tumors. *Onkologie* **31**(7): 362–365.
- Sonoda Y, Warita M, Suzuki T, Ozawa H, Fukuda Y, Funakoshi-Tago M, Kasahara T (2010) Proteolipid protein 2 is associated with melanoma metastasis. *Oncol Rep* **23**: 371–376
- Teschendorff AE, Caldas C (2008) A robust classifier of high predictive value to identify good prognosis patients in ER-negative breast cancer. *Breast Cancer Res* **10**(4): R73.
- Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C (2007) An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol* **8**(8): R157.
- Thomas MB, Zhu AX (2005) Hepatocellular carcinoma: the need for progress. *J Clin Oncol* **23**(13): 2892–2899
- Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**: 57–63

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Medical Treatments & Modulation; Treating cancer
with amplitude-modulated electromagnetic fields: a
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Editorial

Treating cancer with amplitude-modulated electromagnetic fields: a potential paradigm shift, again?

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The Zimmerman *et al* (2012) study published here, coupled with the group's two preceding papers (Barbault *et al*, 2009; Costa *et al*, 2011), identify a potential modality for treating tumours at a dramatic reduction in trauma and cost. This set of clinical and explanatory laboratory results should be understood in the context of the history of research into the biological effects of electromagnetic fields (EMFs).

The most successful clinical application is the use of EMF to initiate fusion in fractured long bones that would not otherwise heal. Pulsed fields were designed to simulate the natural piezoelectric signals generated from bones under varying stress while walking (e.g., Bassett, 1985). There are also other reports that EMF can reduce pain and stimulate wound healing after surgery.

The group's two previous clinical reports were critical to the design of this new Zimmerman *et al* study. Barbault *et al* (2009) described how they obtained the specific frequencies for different tumour diagnoses, which are then used in the amplitude-modulated (AM)-EMF treatment of those patients to stabilise the disease beyond normal expectations. Costa *et al* (2011) reported surprising clinical benefits from using the specific AM-EMF signals to treat advanced hepatocellular carcinoma, stabilising the disease and even producing partial responses up to 58 months in a subset of the patients. Now Zimmerman *et al* have examined the growth rate of human tumour cell lines from liver and breast cancers along with normal cells from those tissues exposed to AM-EMF. Reduced growth rate was observed for tumour cells exposed to tissue-specific AM-EMF, but no change in growth rate in normal cells derived from the same tissue type, or in tumour or normal cells from the other tissue type. The growth rate inhibitory response was field-strength (SAR) and exposure-time dependent. In ancillary tests, they observed reduction in gene expression and increases in mitotic spindle dysfunction only for the AM-EMF exposure that reduced the cell growth rate.

The work of Zimmerman *et al*, Costa *et al* and Barbault *et al* was not done in a vacuum. More than 30 years ago, Suzanne Bawin working in Ross Adey's lab (Bawin *et al*, 1975), with independent replication by my group (Blackman *et al*, 1979), demonstrated that biological effects could be caused by certain AM frequencies on a carrier wave but not other frequencies, similar to the current work. Subsequent reports in the 1980s by several groups continued to support and extend the initial findings (Adey, 1992; Blackman, 1992).

This growing collection of reports demonstrating AM-EMF-induced biological effects led to recognition by national and international authorities that this modality needed to be

considered in hazard evaluation, in addition to field-induced heating as a cause for health concern. The National Council on Radiation Protection and Measurements (1986) recommended a reduction in the allowable exposure intensity limits for AM radiation above a certain level, and the World Health Organization (1993) explicitly acknowledged AM as a future issue to be examined in setting exposure guidelines. Unexpectedly, research funding for this area dried up around 1990 and scientific advances dramatically slowed. A promising area of research fell by the wayside.

The Zimmerman *et al* paper, providing essential laboratory data to support the two previous clinical treatment papers, has resurrected the promising AM-EMF paradigm. It should lead to a major reevaluation of this novel and potentially effective treatment for cancer and possibly other conditions. This study demonstrates the fundamental requirement for a biological 'information content' code (i.e., the AM spectral profile, much like different AM radio stations with different content – e.g., all news, or music) that can affect tumour cells from the tissue of origin, while apparently being ignored by normal cells from various tissues and tumour cells from different tissues of origin. The correspondence between AM-EMF-induced effects on cell proliferation, gene expression, and mitotic spindle dysfunction provide some clues to a possible biological mechanism of action.

The tools developed in Barbault *et al* (2009) to identify relevant treatment frequencies can be seen to have direct clinical and medical relevance in determining the characteristics of a new modality that may prove useful in cancer treatment. The precision of the frequency definitions, down to 1 mHz, is very unusual, but it is reminiscent of the biological effects reported for 40–48-GHz frequencies by Grundler *et al* (1982), and may represent a true effective frequency limitation that most studies would have missed, because of the lack of available, precise generation equipment or lack of the investigator knowledge.

The Zimmerman *et al* study raises a number of issues to be resolved. First, a more detailed elucidation of AM-EMF-induced genomic pathway changes is needed in order to put the results on a firmer mechanistic basis. Second, more information is needed on the nature of the growth inhibition, for example, is it persistent or do resistant cells emerge from continued treatment? Third, will cells from liver or breast tissues in different stages of transformation reduce or enhance sensitivity to AM-EMF exposure? Fourth, will tissue-specific AM-EMF tumour treatments for humans have similar effects on cells from animal tumours? For example, if rodent liver tumour cells respond similarly to the treatments, this may open a new, more rapid investigation of the therapeutic efficacy of the technique.

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When the three studies are taken together, it is apparent that there are gaps in knowledge that can limit the acceptance of this treatment for cancer. How do the biofeedback endpoints (skin electrical resistance, pulse amplitude and blood pressure) engage with the disease state to provide an indication of effective frequencies to treat patients, and most surprisingly, to directly affect tumour cells *in vitro* from the same tissue type? The issue of frequency precision in the AM-EMF signal also needs to be examined and characterised as a function of different physiological growth conditions. Equally mysterious is the mechanism by which AM-EMF administered via a spoon-shaped antenna placed in the mouth can influence cancer cells in the liver or breast of patients. Finally, these patients had advanced cancer and were in palliative care when EMF testing began. Would earlier intervention in breast cancer or liver cancer cases with AM-EMF prove to be more effective?

Funding is needed for further medical and basic science research to identify and characterise the biological influence that amplitude-modulated EMFs have on the body, in its normal state, when recovering from disease or injury, and when initially affected

by disease. As a caution, 'information content' EMF signals may not always have beneficial consequences for humans or their environment, so research should examine potential detrimental biological outcomes as well.

The group of three papers demonstrate a new, potentially important modality in the treatment of cancer that could lead to a paradigm shift in disease treatment. I hope that this medical application of AM-EMF will not be allowed languish without funding, as happened with its previous, ill-fated emergence.

Disclaimer

This commentary has been subjected to review by the National Health and Environmental Effects Research Laboratory, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

REFERENCES

- Adey RW (1992) Collective properties of cell membranes. In *Interaction Mechanisms of Low-level Electromagnetic Fields in Living Systems*, Norden B, Ramel C (eds) pp 47–77. Oxford University Press: Oxford; New York
- Barbault A, Costa FP, Bottger B, Munden RF, Bomholt F, Kuster N, Pasche B (2009) Amplitude-modulated electromagnetic fields for the treatment of cancer: discovery of tumor-specific frequencies and assessment of a novel therapeutic approach. *J Exp Clin Cancer Res* 28: 51–60
- Bassett CA (1985) The development and application of pulsed electromagnetic fields (PEMFs) for ununited fractures and arthrodeses. *Clin Plast Surg* 12: 259–277
- Bawin SM, Kaczmarek LK, Adey WR (1975) Effects of modulated VHF fields on the central nervous system. *Ann N Y Acad Sci* 247: 74–81
- Blackman CF (1992) Calcium release from nervous tissue: experimental results and possible mechanisms. In *Interaction Mechanisms of Low-Level Electromagnetic Fields in Living Systems*, Norden B, Ramel C (eds), pp 107–129. Oxford University Press: Oxford; New York
- Blackman CF, Elder JA, Weil CM, Benane SG, Eichinger DC, House DE. (1979) Induction of calcium ion efflux from brain tissue by radio-frequency radiation: effects of modulation-frequency and field strength. *Radio Sci* 14(6S): 93–98
- Costa FP, de Oliveira AC, Meirelles R, Machado MC, Zanesco T, Surjan R, Chammas MC, de Souza Rocha M, Morgan D, Cantor A, Zimmerman J, Brezovich I, Kuster N, Barbault A, Pasche B. (2011) Treatment of advanced hepatocellular carcinoma with very low levels of amplitude-modulated electromagnetic fields. *Br J Cancer* 105: 640–648
- Grundler W, Keilmann F, Putterlik V, Strube D (1982) Resonant-like dependence of yeast growth rate on microwave frequencies. *Br J Cancer Suppl* 5: 206–208
- National Council on Radiation Protection and Measurements (1986) Biological Effects and Exposure Criteria for Radiofrequency Electromagnetic Fields. pp 382, NCRP Report No. 86, NCRP: Bethesda, MD
- World Health Organization (1993) Environmental Health Criteria 137. "Electromagnetic Fields (300Hz to 300 GHz)". pp 290, Geneva
- Zimmerman ZW, Pennison MJ, Brezovich I, Nengun Y, Yang CT, Ramaker R, Absher D, Myers RM, Kuster N, Costa FP, Barbault A, Pasche B (2012) Cancer cell proliferation is inhibited by specific modulation frequencies. *Br J Cancer* 106: 307–313

Modulation; Dr. Alan Frey PhD., Comments, Feb. 7, 2013

FCC 12-152

**Before the
Federal Communications Commission
Washington, D.C. 20554**

In the Matter of

Notice of Proposed Rulemaking

18 FCC Rcd 13187, 13188 ¶1 (2003)

And

Service Rules for the Advanced Wireless Services

H Block---Implementing Section 6401 of the

Middle Class Tax Relief and Job Creation Act of

2012 Related to the 1915-1920 MHz and

1995-2000 MHz Bands ¶53 footnote 95

ET Docket No. 03-137

WT Docket No. 12-357

To: Office of the Secretary
Federal Communications Commission
Washington, DC 20554

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February 5 , 2013

AFFIDAVIT OF Allan H. Frey

I Allan H. Frey, attest that my statements are true to the best of my knowledge.

1. My name is Allan H. Frey

Comment round for ET Docket No. 03-137 and WT Docket No. 12-357.

2. My address is 11049 Seven Hill Lane, Potomac, MD 20854 USA
Email: allan@freys.us
3. I am a scientist with a considerable amount of experience in research on the biomedical effects of the non-ionizing radiation being considered here by the FCC.
4. The issue is not whether cell phones are safe; it is whether the particular frequencies and modulations that the FCC assigned to cell phones, based on faulty assumptions, are safe.
5. The FCC made assumptions about physiology and about available biological data with non-ionizing radiation. It then assigned frequencies and modulations for use with cell phones, in part, based on those assumptions; assumptions which are not valid.
6. The two attached articles that have been published in the scientific literature indicate the nature of the problem with the FCC's decision making regarding cell phones ("Cell phone health risk" and "On the safety of cell phone radiation"). There are many more articles, by me and others, that are available in the scientific literature that show that the FCC's assumptions are not justified. Thus, the FCC has placed the Public at risk in what amounts to a grand experiment with their health, without their informed consent.


Allan H. Frey

February 5, 2013

Modulation; Real Versus Simulated Mobile Phone Exposures in
Experimental Studies. Biomed Res Int. (Prof. Panagopoulos et al); 2015

Review Article

Real versus Simulated Mobile Phone Exposures in Experimental Studies

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We examined whether exposures to mobile phone radiation in biological/clinical experiments should be performed with real-life Electromagnetic Fields (EMFs) emitted by commercially available mobile phone handsets, instead of simulated EMFs emitted by generators or test phones. Real mobile phone emissions are constantly and unpredictably varying and thus are very different from simulated emissions which employ fixed parameters and no variability. This variability is an important parameter that makes real emissions more bioactive. Living organisms seem to have decreased defense against environmental stressors of high variability. While experimental studies employing simulated EMF-emissions present a strong inconsistency among their results with less than 50% of them reporting effects, studies employing real mobile phone exposures demonstrate an almost 100% consistency in showing adverse effects. This consistency is in agreement with studies showing association with brain tumors, symptoms of unwellness, and declines in animal populations. Average dosimetry in studies with real emissions can be reliable with increased number of field measurements, and variation in experimental outcomes due to exposure variability becomes less significant with increased number of experimental replications. We conclude that, in order for experimental findings to reflect reality, it is crucially important that exposures be performed by commercially available mobile phone handsets.

1. Introduction

Determination of realistic exposures from mobile phones and other wireless devices of modern telecommunications remains an important scientific challenge, especially since it is key to defining public health protection. The situation is further complicated by divergent results reported in the related literature that very well could be due to unrealistic exposure conditions, which in turn lead to ineffective and misdirected interventions.

The International Agency for Research on Cancer (IARC), while still classifying Radio Frequency (RF) Electromagnetic Fields (EMFs) as possibly carcinogenic, criticized and excluded from consideration experimental studies that used commercially available mobile phone handsets in exposing biological samples, as having “unreliable dosimetry” [1],

without further scientific rationale. Similarly the Health Protection Agency (HPA) criticized this exposure methodology reporting that the exposure is “highly variable” with “lack of control” due to network reasons (number of subscribers each moment) and movement of the animals within the vials/boxes in case of freely moving animals but recognizes that restriction of the animals during the exposures will result in additional stress. Their critique recommended that exposures should be performed by devices or handsets set to produce emissions at fixed frequency and output power by use of engineering or hardware controls [2]. In both reports the criticisms were based on the fact that real mobile phone emissions always include significant variations in their intensity, frequency, and other parameters, especially in the near-field of the antenna.

But billions of mobile phone users are daily exposed for increasing periods to real emissions from their handsets in the near-field of the antenna in contact with their ears/bodies, not to any simulated emissions with fixed parameters. Is it then scientifically correct to study the effects of a “highly variable” field by using fields with fixed parameters? In our opinion, it is not, especially in the case when the varying nature of the field seems to be an important reason for its increased biological activity.

The aim of the present study is to review biological and clinical experimental studies on mobile phone radiation effects which have employed exposures with real mobile phone emissions, as opposed to the mainstream studies which employ simulated mobile phone emissions produced by generators or test phones, and seek an explanation for the divergent results reported in the literature. In case that we find a significant conflict in the results between the two types of experimental exposures (real versus simulated), our aim is to attempt giving an explanation based on the differences between the two types of EMF-emissions.

We note that the issue of the present study applies also for every other type of RF/microwave emitting devices used in modern telecommunications, such as Internet connection wireless devices and local wireless networks (Wi-Fi), domestic cordless phones (DECT, Digitally Enhanced Cordless Technology), and baby monitors. The emissions from all these devices, although differing in specific frequencies and modulation types, are very similar. The reason that we concentrate on studies with mobile phone radiation (either real or simulated) is only the fact that they constitute the vast majority of the published studies testing the biological activity of RF/microwave EMFs.

2. Adaptation of Living Organisms to EMFs

Living organisms have been constantly exposed throughout evolution to terrestrial static electric and magnetic fields of average intensities ~ 130 V/m and ~ 0.5 G, respectively. While no adverse health effects are connected with usual exposure to these natural ambient fields, variations in their intensities on the order of 20% during “magnetic storms” or “geomagnetic pulsations” due to changes in solar activity with an average periodicity of about 11 years are connected with increased rates of animal/human health incidents, including nervous and psychic diseases, hypertensive crises, heart attacks, cerebral accidents, and mortality [3, 4].

It is clear that living organisms perceive EMFs as environmental stressors [4–7]. But since man-made EMFs constitute a very new stressor for living organisms within the billions of years of biological evolution, the cells have not developed defensive mechanisms, for example, special genes to be activated for protection against electromagnetic stress of man-made EMFs. This can be the reason why in response to man-made EMFs cells are found to activate heat-shock genes and produce heat-shock proteins very rapidly (within minutes) and at a much higher rate than for heat itself [6]. It seems to be for the same reason that mobile phone radiation is found to induce DNA damage and cell death in insect reproductive cells at a higher degree than other types of

external stressors examined before like food deprivation or chemicals [8–10]. Thus it appears that cells are much more sensitive to man-made EMFs than to other types of stress previously experienced by living organisms such as heat, cold, starvation, or chemicals. But repetitive stress leading to continuous expression of heat-shock genes or DNA damage may lead to cancer [1, 11].

One reason for the increased biological activity of man-made EMFs can be that cells/organisms adapt more easily to any external stressor, and to EMFs, when this stressor is not of significantly varying type, in other words when its parameters are kept constant or vary only slightly. Since living organisms do not have defense mechanisms against variations on the order of 20% of natural EMFs as explained above, it is realistic to expect that they do not have innate defenses against unnatural (man-made) EMFs, which are mostly not static but varying (alternating, pulsed, modulated fields, including simultaneously several different frequencies, etc.) and totally polarized in contrast to natural EMFs. [We note that even though the polarities and intensities of the static terrestrial electric and magnetic fields do not change significantly (except during specific periods as explained) there are always small changes and local variations in the direction of the field lines that make these natural static fields only partially and never totally polarized [3, 4]. This is in contrast to all man-made EMFs which are totally and invariantly polarized due to the invariant geometry of their electric circuits.]

Indeed, pulsed or modulated electromagnetic signals (radiation) are found in numerous studies published since the midseventies to be more bioactive than continuous signals of identical other parameters (intensity, frequency, duration, waveform, etc.) [12–24]. Moreover, intermittent exposure to mobile phone radiation (real or simulated) with short intermittence durations (which makes the field even more variable) is repeatedly found to be more bioactive than the corresponding continuous exposure [25, 26]. This experimental evidence further supports the argument that the more complicated and variable the field/stressor is, the more difficult it is for a living organism to adapt to it.

3. The Increased Variability of EMFs Emitted by Mobile Telephony Antennas

All types of digital mobile telephony radiation, except for their RF carrier signal, employ Extremely Low Frequencies (ELF) necessary for the modulation and for increasing the capacity of transmitted information by pulsing the signal. The combination of the RF carrier and the ELF pulsing frequencies has been found to be more bioactive than the RF carrier alone [16, 21]. Moreover, according to a plausible suggested mechanism [27], (a) the ELF frequencies included in any pulsed or modulated RF signal are more responsible for the biological effects, (b) changes in field intensity play a major role, and (c) the pulsing of the signal makes it twice more bioactive. A constant carrier RF wave modulated by a constant ELF field can certainly be simulated but this is not the case in real mobile telephony signals, in which both the carrier and the modulation are constantly and

unpredictably varying in intensity, frequency, and waveform during a phone-conversation [7, 28–30].

The intensity of radiation varies significantly each moment during a usual phone-conversation depending on signal reception, number of subscribers sharing the frequency band each moment, air conductivity, location within the wireless infrastructure, presence of objects and metallic surfaces, “speaking” versus “nonspeaking” mode, and so forth. These variations are much larger than 20% of the average signal intensity (as opposed to the periodical variations in the terrestrial fields known to cause health effects). Moreover the phase of the carrier signal varies continuously during a phone-conversation, and the RF frequency constantly changes between different available frequency channels, especially in third generation (3G) radiation. The wave shape is also constantly changing depending on how the changing information transmitted each moment modulates the carrier wave. Thus, the parameters of this radiation change constantly and unpredictably each moment and large, sudden, unpredictable variations in the emitted EMF/radiation take place constantly during a usual phone-conversation. The more the amount of carried information is increased (by adding text, speech, pictures, music, video, internet, etc.) in more recent phone generations (G)/types (2G, 3G, 4G, etc.), the more complicated and unpredictably varying the cell phone signals become [2, 7, 28–30].

Thus, real digital mobile phone (and other wireless communication devices) emissions change constantly and unpredictably. As a consequence, living organisms cannot adapt to such a highly varying type of stress. Moreover, due to the unpredictably varying type of the real emissions, it is impossible to simulate them by EMFs of fixed parameters.

4. Real Exposure Studies as Opposed to Studies with Simulated Exposures

A significant number of studies have already been published which employed commercially available mobile phones during connection (“talk”, “listen”, or “call” modes) for exposure to a wide variety of animals (including humans)/biological samples, including *Drosophila* [6, 8, 26, 31–37], ants [38], chicken eggs [39], quails [40], human sperm *in vitro* [41, 42], human volunteers *in vivo* [43–52], mice or rats or guinea-pigs or rabbits *in vivo* [53–69], mouse cells *in vitro* [70], bees [71–73], protozoa [74], and even purified proteins *in vitro* [75]. An impressive percentage (95.8%) of these studies (46 out of 48 studies with real-life exposures) have recorded significant adverse biological or clinical effects, ranging from loss of orientation, kinetic changes, and behavioral or electroencephalographic (EEG) changes to decrease in male and female reproductive capacity, reproductive declines, molecular changes, changes in enzymatic activity, DNA damage and cell death, and histopathological changes in the brain. It was found that during “talk” mode (voice modulation) the exposure is significantly more bioactive than during “listen” mode due to the voice modulation and associated increased intensity of the emissions [7, 31]. From the remaining two studies, one reported no effect [55] and one reported an

increase in short-term memory of children [47] which we do not count as an adverse effect although it may be.

On the contrary, more than 50% of the studies performed with simulated signals have showed no effects [1, 2, 76], even though several recent review studies suggest an overall predominance of studies showing effects regardless of real or simulated exposures [7, 77–80]. A recent meta-analysis of 88 studies published during 1990–2011 investigating genetic damage in human cells from RF radiation, 87 of which did not employ real telecommunication EMFs, reported no overall association with genotoxicity [81].

Although we may have missed a few more studies with real mobile phone exposures, it becomes evident that there is a strong conflict between the overall results of studies performed with real mobile phone emissions and the overall results of studies with simulated emissions from generators and “test” phones. Moreover, while within the group of studies with simulated emissions there is also a conflict between studies that find effects and studies that do not, the group of studies with real exposures demonstrates an impressive consistency in showing effects almost at 100%. Moreover, this impressive consistency is corroborated by increasing epidemiological evidence, especially during the last years, for an association between (real-life) mobile phone use and brain tumors [82–84], by statistical studies reporting symptoms of unwellness among people residing around mobile telephony base station antennas or among mobile phone users [85–90], and by open field studies reporting declines in bird and amphibian populations around mobile telephony base station antennas [91–95].

This apparent consistency of results in the laboratory studies with real emissions and their additional corroboration with recent epidemiological/statistical and open field studies’ evidence seems to be unnoticed by health agencies and public health authorities which simply disregard these studies despite their important findings which imply the urgent establishment of much more stringent exposure limits than the current ones [96].

Although in most studies employing real mobile phone emissions the biological samples were exposed in close proximity (within the near-field up to approximately 5 cm) with the mobile phone handset, in several studies the samples/animals were exposed at greater distances in the far-field up to 1 m [32, 34, 35, 39, 51, 53, 56–58] where the intensity variations are much smaller and the dosimetry is absolutely “reliable” as is generally accepted for far-field antenna measurements [97]. In one of these studies it was found that at 20–30 cm distance from the mobile phone the biological effect (DNA damage) was even more intense than at zero distance [32].

A mobile phone antenna’s near-field extends to a distance of 5.2 or 2.6 cm, for 900 or 1800 MHz, respectively (most commonly employed carrier frequencies in 2G mobile telephony radiation), according to the relation $r = \lambda/2\pi$, (r is the distance of near-field far limit from the antenna when the length of the antenna is smaller than the wavelength λ of the emitted radiation) [98].

In studies with real mobile phone emissions investigating the dependence of observed effects on dose (radiation

intensity and/or exposure duration) [8, 31–35, 39, 40, 62], the effects have been found to be dose dependent. The dependence on dose was in most cases nonlinear, although in two studies the dependence of certain effects on exposure duration was approximating linearity [35, 62].

The results of experiments with real-life (variable) mobile phone EMFs are indeed not identically reproducible, since between successive exposures at any specific location the exact characteristics of the emitted signal are always different. But the average field values over a few minutes' (or more) period are close to each other, and thus the results of different replicate experiments with real emissions as the independent variable, although not identical quantitatively, are qualitatively similar. Statistical significance in the results can be increased by increasing the number of experimental replications while keeping rigorous control of all other parameters (animal/sample conditions, temperature, humidity, light, stray EMFs within the lab, etc.). Then, as the number of replications increases, field variability becomes less significant [99].

5. Discussion

In the present study we showed that the percentages of positive results differ significantly between studies with real mobile phone exposures and studies with simulated exposures, regardless of biological samples or other procedure details. The basic difference between real and simulated mobile telephony EMFs is the inherent significant variability of the first which we believe is the reason for the strong divergence in the experimental results.

In spite of the criticism on the studies employing real exposures by health agencies [1, 2] (the different aspects of which we extensively addressed) and the consequent difficulty in the publication process, the number of studies with real mobile phone emissions is increasing rapidly in the peer-reviewed literature, especially during the last years. An increasing number of scientists realize that real exposures by commercially available mobile phone handsets are the only way to represent conditions experienced by users in real-life, since they are very different and considerably more bioactive than the exposures made by simulated fields.

Any variability in the field and correspondingly in the dosimetry does not change the fact that people are actually exposed daily for increasing periods to this “highly variable” field in contact with their heads/bodies and at different distances. The presented scientific data show that this constant variation in the field makes it considerably more active biologically.

In order to have a measure of this variability, RF and ELF measurements of average intensity $\pm$ standard deviation (SD) of the emitted real EMFs should be included in the studies, in addition to the Specific Absorption Rate (SAR) information supplied by the manufacturer (referring to a simulated human head [100]). With increasing number of measurements the SD decreases enough for the dosimetry to be judged as reliable [8, 26, 31–36, 99].

If we accepted that the real EMFs emitted by commercially available mobile phones are so much variable and their

dosimetry is so much unreliable that the studies employing real EMF-emissions are not to be taken into account because of “unknown” dosimetry, then these devices should not be approved by the public authorities to be available in the market, since unpredictable unmeasurable signal changes can result in unpredictable biological alterations. Once these devices are approved for the market (a fact that we do not challenge) the definition of the exposure is the *exposure to a user's head during a usual phone-conversation*, and this, in our opinion, should be enough for the studies to be taken into account by health agencies and authorities. Nevertheless, the measurements of the emitted EMFs suggested above are important to better quantify real-life exposures, in addition to verifying that the average emissions by the handsets used in the experiments do not transcend the existing limits [96].

It is useful to create simulations in order to study in the lab conditions of specific environments which are not accessible for laboratory work (outer space, underwater high depths, etc.). The simulations in such cases should be as close as possible to the real conditions. However, using nonrealistic simulations, especially when real conditions are easily accessible to be studied in the lab with well-controlled other parameters, is, in our opinion, a serious scientific flaw that is pervading the mobile phone bioeffects literature. The employment of simplified nonrealistic simulations may be useful for specific purposes, for example, to study what the effects would be if the signal characteristics were different, in order to improve them.

Experiments comparing the biological activity between real and simulated mobile telephony EMFs with similar average parameter values should urgently be conducted in order to test the validity of our presented arguments. Studies performed with simulated fields/exposures, especially those that did not show any effects, should, in our opinion, be repeated with real exposures of similar average signal parameters while keeping all the remaining experimental variables identical. In case that these experiments verify our arguments, health agencies should immediately revise their guidelines in regard to which studies should be considered most important and on whether the available data are indeed conflicting or not. Moreover, according to the precautionary principle, the existing exposure criteria should drastically be revised, since the effects reported in all studies with real mobile phone emissions have been recorded with EMF-intensities well below (up to thousands of times below) the existing exposure limits [8, 26, 31–75, 96].

Without account for real exposure parameters, studies suffer from imprecision that likely biases results toward null hypotheses, increasing the probability that true health risks among consumers are being missed. Simulated signals with fixed parameters bear little, if any, resemblance to what mobile phone users actually experience, even when they employ combinations of simulated signals [101–103].

In order for the biological/clinical studies testing the bioactivity of mobile telephony radiation to account for real conditions, we conclude that exposures should be performed by real EMFs as these are emitted by commercially available mobile phones. The same holds for experiments with other types of EMFs employed in modern telecommunication

systems such as DECT phones and Wi-Fi. In addition to that, simulated emissions may be used to study, for example, the effects of separate parameters of the real EMFs, but in no way should simulated emissions substitute the real ones.

As the scientific database regarding the biological effects of EMFs emitted by modern telecommunications continues to grow, it is important for experimental study designs to grow in rigor and provide a more informed basis for interpretation. One important step is to employ real-life exposures.

To investigate the biological/health effects from a widely accessible device exposing daily billions of humans we should not try to simulate the device but simply use the device itself. In particular, we should not try to simulate its real varying emissions with totally unrealistic invariant ones. This is a serious scientific flaw that may lead to totally devious results with enormous adverse consequences for public health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] IARC, *Non-Ionizing Radiation, Part 2: Radiofrequency Electromagnetic Fields*, vol. 102, World Health Organization, 2013.
- [2] Health Protection Agency, *Health Effects from Radiofrequency Electromagnetic Fields*, 2012.
- [3] A. P. Dubrov, *The Geomagnetic Field and Life*, Plenum Press, New York, NY, USA, 1978.
- [4] A. S. Presman, *Electromagnetic Fields and Life*, Plenum Press, New York, NY, USA, 1977.
- [5] E. M. Goodman, B. Greenebaum, and M. T. Marron, "Effects of electromagnetic fields on molecules and cells," *International Review of Cytology*, vol. 158, pp. 279–338, 1995.
- [6] D. Weisbrot, H. Lin, L. Ye, M. Blank, and R. Goodman, "Effects of mobile phone radiation on reproduction and development in *Drosophila melanogaster*," *Journal of Cellular Biochemistry*, vol. 89, no. 1, pp. 48–55, 2003.
- [7] D. J. Panagopoulos, "Biological impacts, action mechanisms, dosimetry and protection issues of mobile telephony radiation," in *Mobile Phones: Technology, Networks and User Issues*, M. C. Barnes and N. P. Meyers, Eds., Nova Science Publishers, New York, NY, USA, 2011.
- [8] D. J. Panagopoulos, E. D. Chavdoula, I. P. Nezis, and L. H. Margaritis, "Cell death induced by GSM 900-MHz and DCS 1800-MHz mobile telephony radiation," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 626, no. 1-2, pp. 69–78, 2007.
- [9] D. Drummond-Barbosa and A. C. Spradling, "Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis," *Developmental Biology*, vol. 231, no. 1, pp. 265–278, 2001.
- [10] I. P. Nezis, D. J. Stravopodis, I. Papassideri, M. Robert-Nicoud, and L. H. Margaritis, "Stage-specific apoptotic patterns during *Drosophila* oogenesis," *European Journal of Cell Biology*, vol. 79, no. 9, pp. 610–620, 2000.
- [11] P. W. French, R. Penny, J. A. Laurence, and D. R. McKenzie, "Mobile phones, heat shock proteins and cancer," *Differentiation*, vol. 67, no. 4-5, pp. 93–97, 2001.
- [12] S. M. Bawin, L. K. Kaczmarek, and W. R. Adey, "Effects of modulated VMF fields, on the central nervous system," *Annals of the New York Academy of Sciences*, vol. 247, pp. 74–81, 1974.
- [13] S. M. Bawin and W. R. Adey, "Sensitivity of calcium binding in cerebral tissue to weak environmental electric fields oscillating at low frequency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 73, no. 6, pp. 1999–2003, 1976.
- [14] S. M. Bawin, W. R. Adey, and I. M. Sabbot, "Ionic factors in release of $^{45}\text{Ca}^{2+}$ from chicken cerebral tissue by electromagnetic fields," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 75, no. 12, pp. 6314–6318, 1978.
- [15] C. F. Blackman, S. G. Benane, J. A. Elder, D. E. House, J. A. Lampe, and J. M. Faulk, "Induction of calcium-ion efflux from brain tissue by radiofrequency radiation: effect of sample number and modulation frequency on the power-density window," *Bioelectromagnetics*, vol. 1, no. 1, pp. 35–43, 1980.
- [16] S. Lin-Liu and W. R. Adey, "Low frequency amplitude modulated microwave fields change calcium efflux rates from synaptosomes," *Bioelectromagnetics*, vol. 3, no. 3, pp. 309–322, 1982.
- [17] Z. Somosy, G. Thuroczy, T. Kubasova, J. Kovacs, and L. D. Szabo, "Effects of modulated and continuous microwave irradiation on the morphology and cell surface negative charge of 3T3 fibroblasts," *Scanning Microscopy*, vol. 5, no. 4, pp. 1145–1155, 1991.
- [18] B. Veyret, C. Bouthet, P. Deschaux et al., "Antibody responses of mice exposed to low-power microwaves under combined, pulse-and-amplitude modulation," *Bioelectromagnetics*, vol. 12, no. 1, pp. 47–56, 1991.
- [19] M. A. Bolshakov and S. I. Alekseev, "Bursting responses of *Lymnea* neurons to microwave radiation," *Bioelectromagnetics*, vol. 13, no. 2, pp. 119–129, 1992.
- [20] G. Thuroczy, G. Kubinyi, M. Bodo, J. Bakos, and L. D. Szabo, "Simultaneous response of brain electrical activity (EEG) and cerebral circulation (REG) to microwave exposure in rats," *Reviews on Environmental Health*, vol. 10, no. 2, pp. 135–148, 1994.
- [21] L. M. Penafiel, T. Litovitz, D. Krause, A. Desta, and J. M. Mullins, "Role of modulation on the effect of microwaves on ornithine decarboxylase activity in L929 cells," *Bioelectromagnetics*, vol. 18, no. 2, pp. 132–141, 1997.
- [22] A. Höytö, J. Luukkonen, J. Juutilainen, and J. Naarala, "Proliferation, oxidative stress and cell death in cells exposed to 872 MHz radiofrequency radiation and oxidants," *Radiation Research*, vol. 170, no. 2, pp. 235–243, 2008.
- [23] S. Franzellitti, P. Valbonesi, N. Ciancaglini et al., "Transient DNA damage induced by high-frequency electromagnetic fields (GSM 1.8 GHz) in the human trophoblast HTR-8/SVneo cell line evaluated with the alkaline comet assay," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 683, no. 1-2, pp. 35–42, 2010.

- [24] A. Campisi, M. Gulino, R. Acquaviva et al., "Reactive oxygen species levels and DNA fragmentation on astrocytes in primary culture after acute exposure to low intensity microwave electromagnetic field," *Neuroscience Letters*, vol. 473, no. 1, pp. 52–55, 2010.
- [25] E. Diem, C. Schwarz, F. Adlkofer, O. Jahn, and H. Rüdiger, "Non-thermal DNA breakage by mobile-phone radiation (1800 MHz) in human fibroblasts and in transformed GFSH-R17 rat granulosa cells in vitro," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 583, no. 2, pp. 178–183, 2005.
- [26] E. D. Chavdoula, D. J. Panagopoulos, and L. H. Margaritis, "Comparison of biological effects between continuous and intermittent exposure to GSM-900 MHz mobile phone radiation: detection of apoptotic cell-death features," *Mutation Research*, vol. 700, no. 1-2, pp. 51–61, 2010.
- [27] D. J. Panagopoulos, A. Karabarounis, and L. H. Margaritis, "Mechanism for action of electromagnetic fields on cells," *Biochemical and Biophysical Research Communications*, vol. 298, no. 1, pp. 95–102, 2002.
- [28] J. Tisal, *GSM Cellular Radio Telephony*, John Wiley & Sons, West Sussex, UK, 1998.
- [29] F. Hillebrand, *GMS and UMTS. The Creation of Global Mobile Communication*, John Wiley & Sons, Chichester, UK, 2002.
- [30] P. Curwen and J. Whalley, "Mobile communications in the 21st century," in *Mobile Telephones: Networks, Applications and Performance*, A. C. Harper and R. V. Bures, Eds., pp. 29–75, Nova Science Publishers, 2008.
- [31] D. J. Panagopoulos, A. Karabarounis, and L. H. Margaritis, "Effect of GSM 900-MHz mobile phone radiation on the reproductive capacity of *Drosophila melanogaster*," *Electromagnetic Biology and Medicine*, vol. 23, no. 1, pp. 29–43, 2004.
- [32] D. J. Panagopoulos, E. D. Chavdoula, and L. H. Margaritis, "Bioeffects of mobile telephony radiation in relation to its intensity or distance from the antenna," *International Journal of Radiation Biology*, vol. 86, no. 5, pp. 345–357, 2010.
- [33] D. J. Panagopoulos, E. D. Chavdoula, A. Karabarounis, and L. H. Margaritis, "Comparison of bioactivity between GSM 900 MHz and DCS 1800 MHz mobile telephony radiation," *Electromagnetic Biology and Medicine*, vol. 26, no. 1, pp. 33–44, 2007.
- [34] D. J. Panagopoulos and L. H. Margaritis, "The identification of an intensity 'window' on the bioeffects of mobile telephony radiation," *International Journal of Radiation Biology*, vol. 86, no. 5, pp. 358–366, 2010.
- [35] D. J. Panagopoulos and L. H. Margaritis, "The effect of exposure duration on the biological activity of mobile telephony radiation," *Mutation Research*, vol. 699, no. 1-2, pp. 17–22, 2010.
- [36] D. J. Panagopoulos, "Effect of microwave exposure on the ovarian development of *Drosophila melanogaster*," *Cell Biochemistry and Biophysics*, vol. 63, no. 2, pp. 121–132, 2012.
- [37] L. H. Margaritis, A. K. Manta, K. D. Kokkaliaris et al., "*Drosophila oogenesis* as a bio-marker responding to EMF sources," *Electromagnetic Biology and Medicine*, vol. 33, no. 3, pp. 165–189, 2014.
- [38] M.-C. Cammaerts and O. Johansson, "Ants can be used as bio-indicators to reveal biological effects of electromagnetic waves from some wireless apparatus," *Electromagnetic Biology and Medicine*, vol. 33, no. 4, pp. 282–288, 2014.
- [39] F. Batellier, I. Couty, D. Picard, and J. P. Brillard, "Effects of exposing chicken eggs to a cell phone in 'call' position over the entire incubation period," *Theriogenology*, vol. 69, no. 6, pp. 737–745, 2008.
- [40] O. Tsybulin, E. Sidorik, O. Brieieva et al., "GSM 900 MHz cellular phone radiation can either stimulate or depress early embryogenesis in Japanese quails depending on the duration of exposure," *International Journal of Radiation Biology*, vol. 89, no. 9, pp. 756–763, 2013.
- [41] A. Agarwal, N. R. Desai, K. Makker et al., "Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study," *Fertility and Sterility*, vol. 92, no. 4, pp. 1318–1325, 2009.
- [42] I. Gorpichenko, O. Nikitin, O. Banyra, and A. Shulyak, "The influence of direct mobile phone radiation on sperm quality," *Central European Journal of Urology*, vol. 67, no. 1, pp. 65–71, 2014.
- [43] A. S. Yadav and M. K. Sharma, "Increased frequency of micronucleated exfoliated cells among humans exposed in vivo to mobile telephone radiations," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 650, no. 2, pp. 175–180, 2008.
- [44] S. T. Çam and N. Seyhan, "Single-strand DNA breaks in human hair root cells exposed to mobile phone radiation," *International Journal of Radiation Biology*, vol. 88, no. 5, pp. 420–424, 2012.
- [45] Q. Luo, Y. Jiang, M. Jin, J. Xu, and H.-F. Huang, "Proteomic analysis on the alteration of protein expression in the early-stage placental villous tissue of electromagnetic fields associated with cell phone exposure," *Reproductive Sciences*, vol. 20, no. 9, pp. 1055–1061, 2013.
- [46] M. Mandalà, V. Colletti, L. Sacchetto et al., "Effect of bluetooth headset and mobile phone electromagnetic fields on the human auditory nerve," *Laryngoscope*, vol. 124, no. 1, pp. 255–259, 2014.
- [47] M. M. Movvahedi, A. Tavakkoli-Golpayegani, S. A. Mortazavi et al., "Does exposure to GSM 900 MHz mobile phone radiation affect short-term memory of elementary school students?" *Journal of Pediatric Neurosciences*, vol. 9, no. 2, pp. 121–124, 2014.
- [48] H. D'Costa, G. Trueman, L. Tang et al., "Human brain wave activity during exposure to radiofrequency field emissions from mobile phones," *Australasian Physical and Engineering Sciences in Medicine*, vol. 26, no. 4, pp. 162–167, 2003.
- [49] F. Ferreri, G. Curcio, P. Pasqualetti, L. De Gennaro, R. Fini, and P. M. Rossini, "Mobile phone emissions and human brain excitability," *Annals of Neurology*, vol. 60, no. 2, pp. 188–196, 2006.
- [50] F. Vecchio, C. Babiloni, F. Ferreri et al., "Mobile phone emission modulates interhemispheric functional coupling of EEG alpha rhythms," *European Journal of Neuroscience*, vol. 25, no. 6, pp. 1908–1913, 2007.
- [51] F. Vecchio, C. Babiloni, F. Ferreri et al., "Mobile phone emission modulates inter-hemispheric functional coupling of EEG alpha rhythms in elderly compared to young subjects," *Clinical Neurophysiology*, vol. 121, no. 2, pp. 163–171, 2010.
- [52] F. Vecchio, M. Tombini, P. Buffo et al., "Mobile phone emission increases inter-hemispheric functional coupling of electroencephalographic α rhythms in epileptic patients," *International Journal of Psychophysiology*, vol. 84, no. 2, pp. 164–171, 2012.
- [53] A. İlhan, A. Gurel, F. Armutcu et al., "Ginkgo biloba prevents mobile phone-induced oxidative stress in rat brain," *Clinica Chimica Acta*, vol. 340, no. 1-2, pp. 153–162, 2004.
- [54] M. A. Elhag, G. M. Nabil, and A. M. Attia, "Effects of electromagnetic field produced by mobile phones on the oxidant and antioxidant status of rats," *Pakistan Journal of Biological Sciences*, vol. 10, no. 23, pp. 4271–4274, 2007.

- [55] S. Dasdag, M. Z. Akdag, F. Aksen et al., "Whole body exposure of rats to microwaves emitted from a cell phone does not affect the testes," *Bioelectromagnetics*, vol. 24, no. 3, pp. 182–188, 2003.
- [56] A. R. Ferreira, T. Knakievicz, M. A. de Bittencourt Pasquali et al., "Ultra high frequency-electromagnetic field irradiation during pregnancy leads to an increase in erythrocytes micronuclei incidence in rat offspring," *Life Sciences*, vol. 80, no. 1, pp. 43–50, 2006.
- [57] J.-G. Yan, M. Agresti, T. Bruce, Y. H. Yan, A. Granlund, and H. S. Matloub, "Effects of cellular phone emissions on sperm motility in rats," *Fertility and Sterility*, vol. 88, no. 4, pp. 957–964, 2007.
- [58] M. Balci, E. Devrim, and I. Durak, "Effects of mobile phones on oxidant/antioxidant balance in cornea and lens of rats," *Current Eye Research*, vol. 32, no. 1, pp. 21–25, 2007.
- [59] M. Mailankot, A. P. Kunnath, H. Jayalekshmi, B. Koduru, and R. Valsalan, "Radio frequency electromagnetic radiation (RF-EMR) from GSM (0.9/1.8GHZ) mobile phones induces oxidative stress and reduces sperm motility in rats," *Clinics*, vol. 64, no. 6, pp. 561–565, 2009.
- [60] A. Gul, H. Çelebi, and S. Uğraş, "The effects of microwave emitted by cellular phones on ovarian follicles in rats," *Archives of Gynecology and Obstetrics*, vol. 280, no. 5, pp. 729–733, 2009.
- [61] E. B. Imge, B. Kilicoğlu, E. Devrim, R. Çetin, and I. Durak, "Effects of mobile phone use on brain tissue from the rat and a possible protective role of vitamin C—a preliminary study," *International Journal of Radiation Biology*, vol. 86, no. 12, pp. 1044–1049, 2010.
- [62] T. S. Aldad, G. Gan, X.-B. Gao, and H. S. Taylor, "Fetal radiofrequency radiation exposure from 800–1900 mhz-rated cellular telephones affects neurodevelopment and behavior in mice," *Scientific Reports*, vol. 2, article 312, 2012, Erratum in: *Scientific Reports*, vol. 3, article 1320, 2013.
- [63] M. A. Al-Damegh, "Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E," *Clinics*, vol. 67, no. 7, pp. 785–792, 2012.
- [64] O. Koca, A. M. Gökçe, M. I. Öztürk, F. Ercan, N. Yurdakul, and M. I. Karaman, "Effects of intensive cell phone (Philips Genic 900) use on the rat kidney tissue," *Urology Journal*, vol. 10, no. 2, pp. 886–891, 2013.
- [65] S. A. Meo and K. A. Rubaan, "Effects of exposure to electromagnetic field radiation (EMFR) generated by activated mobile phones on fasting blood glucose," *International Journal of Occupational Medicine and Environmental Health*, vol. 26, no. 2, pp. 235–241, 2013.
- [66] T. K. Motawi, H. A. Darwish, Y. M. Moustafa, and M. M. Labib, "Biochemical modifications and neuronal damage in brain of young and adult rats after long-term exposure to mobile phone radiations," *Cell Biochemistry and Biophysics*, vol. 70, no. 2, pp. 845–855, 2014.
- [67] I. Meral, H. Mert, N. Mert et al., "Effects of 900-MHz electromagnetic field emitted from cellular phone on brain oxidative stress and some vitamin levels of guinea pigs," *Brain Research*, vol. 1169, no. 1, pp. 120–124, 2007.
- [68] I. Meral, Y. Tekintangac, and H. Demir, "Effects of 900 MHz electromagnetic field emitted by cellular phones on electrocardiograms of guinea pigs," *Human and Experimental Toxicology*, vol. 33, no. 2, pp. 164–169, 2014.
- [69] M. K. Irmak, E. Fadilloğlu, M. Güleç, H. Erdoğan, M. Yağmurca, and Ö. Akyol, "Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits," *Cell Biochemistry and Function*, vol. 20, no. 4, pp. 279–283, 2002.
- [70] C. Liu, P. Gao, S.-C. Xu et al., "Mobile phone radiation induces mode-dependent DNA damage in a mouse spermatocyte-derived cell line: a protective role of melatonin," *International Journal of Radiation Biology*, vol. 89, no. 11, pp. 993–1001, 2013.
- [71] V. P. Sharma and N. R. Kumar, "Changes in honeybee behaviour and biology under the influence of cellphone radiations," *Current Science*, vol. 98, no. 10, pp. 1376–1378, 2010.
- [72] N. R. Kumar, S. Sangwan, and P. Badotra, "Exposure to cell phone radiations produces biochemical changes in worker honey bees," *Toxicology International*, vol. 18, no. 1, pp. 70–72, 2011.
- [73] D. Favre, "Mobile phone-induced honeybee worker piping," *Apidologie*, vol. 42, no. 3, pp. 270–279, 2011.
- [74] M.-C. Cammaerts, O. Debeir, and R. Cammaerts, "Changes in *Paramecium caudatum* (Protozoa) near a switched-on GSM telephone," *Electromagnetic Biology and Medicine*, vol. 30, no. 1, pp. 57–66, 2011.
- [75] M. Barteri, A. Pala, and S. Rotella, "Structural and kinetic effects of mobile phone microwaves on acetylcholinesterase activity," *Biophysical Chemistry*, vol. 113, no. 3, pp. 245–253, 2005.
- [76] L. Verschaeve, J. Juutilainen, I. Lagroye et al., "In vitro and in vivo genotoxicity of radiofrequency fields," *Mutation Research/Reviews in Mutation Research*, vol. 705, no. 3, pp. 252–268, 2010.
- [77] L. Verschaeve, "Genetic damage in subjects exposed to radiofrequency radiation," *Mutation Research*, vol. 681, no. 2-3, pp. 259–270, 2009.
- [78] S. La Vignera, R. A. Condorelli, E. Vicari, R. D'Agata, and A. E. Calogero, "Effects of the exposure to mobile phones on male reproduction: a review of the literature," *Journal of Andrology*, vol. 33, no. 3, pp. 350–356, 2012.
- [79] S. Cucurachi, W. L. M. Tãmis, M. G. Vijver, W. J. G. M. Peijnenburg, J. F. B. Bolte, and G. R. de Snoo, "A review of the ecological effects of radiofrequency electromagnetic fields (RF-EMF)," *Environment International*, vol. 51, pp. 116–140, 2013.
- [80] A. Balmori, "Electrosmog and species conservation," *Science of the Total Environment*, vol. 496, pp. 314–316, 2014.
- [81] P. T. J. Vijayalaxmi, "Genetic damage in human cells exposed to non-ionizing radiofrequency fields: a meta-analysis of the data from 88 publications (1990–2011)," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 749, no. 1-2, pp. 1–16, 2012.
- [82] M. Kundi, "Mobile phone use and cancer," *Occupational and Environmental Medicine*, vol. 61, no. 6, pp. 560–570, 2004.
- [83] V. G. Khurana, C. Teo, M. Kundi, L. Hardell, and M. Carlberg, "Cell phones and brain tumors: a review including the long-term epidemiologic data," *Surgical Neurology*, vol. 72, no. 3, pp. 205–214, 2009.
- [84] L. Hardell, M. Carlberg, F. Söderqvist, and K. H. Mild, "Case-control study of the association between malignant brain tumours diagnosed between 2007 and 2009 and mobile and cordless phone use," *International Journal of Oncology*, vol. 43, no. 6, pp. 1833–1845, 2013.
- [85] E. A. Navarro, J. Segura, M. Portolés, and C. Gómez-Perretta, "The microwave syndrome: a preliminary study in Spain," *Electromagnetic Biology and Medicine*, vol. 22, no. 2-3, pp. 161–169, 2003.
- [86] O. E. Salama and R. M. Abou El Naga, "Cellular phones: are they detrimental?" *The Journal of the Egyptian Public Health Association*, vol. 79, no. 3-4, pp. 197–223, 2004.