

Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline

R. J. AITKEN,* L. E. BENNETTS,* D. SAWYER,* A. M. WIKLENDT* and B. V. KING†

*ARC Centre of Excellence in Biotechnology and Development, Discipline of Biological Sciences, and Hunter Medical Research Institute, Newcastle, and †School of Mathematical and Physical Sciences, The University of Newcastle, Newcastle, NSW, Australia

Summary

Concern has arisen over human exposures to radio frequency electromagnetic radiation (RFEMR), including a recent report indicating that regular mobile phone use can negatively impact upon human semen quality. These effects would be particularly serious if the biological effects of RFEMR included the induction of DNA damage in male germ cells. In this study, mice were exposed to 900 MHz RFEMR at a specific absorption rate of approximately 90 mW/kg inside a waveguide for 7 days at 12 h per day. Following exposure, DNA damage to caudal epididymal spermatozoa was assessed by quantitative PCR (QPCR) as well as alkaline and pulsed-field gel electrophoresis. The treated mice were overtly normal and all assessment criteria, including sperm number, morphology and vitality were not significantly affected. Gel electrophoresis revealed no gross evidence of increased single- or double-DNA strand breakage in spermatozoa taken from treated animals. However, a detailed analysis of DNA integrity using QPCR revealed statistically significant damage to both the mitochondrial genome ($p < 0.05$) and the nuclear β -globin locus ($p < 0.01$). This study suggests that while RFEMR does not have a dramatic impact on male germ cell development, a significant genotoxic effect on epididymal spermatozoa is evident and deserves further investigation.

Keywords: male germline, DNA damage, radio frequency electromagnetic radiation, germline mutation, whole-animal irradiation, quantitative PCR

Introduction

Exposure to radio frequency electromagnetic radiation (RFEMR) fields of the type generally employed in cellular phone communication have been held to exert a range of genotoxic effects on mammalian cells including aneuploidy, sister chromatid exchange, impaired intrachromosomal recombination and enhanced micronuclei formation (Sykes *et al.*, 2001; d'Ambrosio *et al.*, 2002; Tice *et al.*, 2002; Mashevich *et al.*, 2003). In addition, RFEMR has been found to enhance proto-oncogene expression, increase

DNA synthesis and stimulate intracellular mitogenic second messenger formation (Maes *et al.*, 1996; Goswami *et al.*, 1999; Harvey & French, 2000; Pacini *et al.*, 2002). However, these data are controversial and several investigators have concluded that the cellular damage induced by RFEMR, when heating effects are set aside, is minimal (Brusick *et al.*, 1998; Black & Heynick, 2003; Jauchem, 2003; Meltz, 2003). Thus microbial mutation assays have returned largely negative results, as have *Drosophila melanogaster* (fruit fly) *in vivo* mutation assays (Brusick *et al.*, 1998). Furthermore, RFEMR does not appear to induce DNA strand breaks in murine fibroblasts (Malyapa *et al.*, 1997; Li *et al.*, 2001), human blood cells (Garson *et al.*, 1991; Maes *et al.*, 1997) or the yeast, *Saccharomyces cerevisiae* (Gos *et al.*,

Correspondence: R. J. Aitken, ARC Centre of Excellence in Biotechnology and Development, Discipline of Biological Sciences, and Hunter Medical Research Institute, Newcastle, Australia.
E-mail: jaitken@mail.newcastle.edu.au

2000). RFEMR has also been shown to have no efficacy as a tumour promoter in major tissues in mice (Heikkinen *et al.*, 2001; Mason *et al.*, 2001) or rats (Bartsch *et al.*, 2002). In addition, there are no convincing data to suggest that RFEMR has an impact on long-term survival or the incidence of cancer in laboratory animals (Elder, 2003).

The clinical data are equally confusing. An increased risk of cancer in exposed individuals has been detected in several epidemiological studies but the effects are inconsistent (Blettner & Berg, 2000; Breckenkamp *et al.*, 2003). An increase in lipid peroxidation, and a decrease in the activity of the antioxidants, superoxide dismutase and glutathione peroxidase, in human erythrocytes has been reported in human volunteers exposed to RFEMR (Moustafa *et al.*, 2001). Similarly, an increase in chromosomal aberrations has been detected in individuals as a consequence of occupational RFEMR exposure (Lalic *et al.*, 2001). However, other studies failed to find cytogenetic abnormalities in workers chronically exposed to this type of radiation (Garson *et al.*, 1991; Maes *et al.*, 1997).

If RFEMR does exert genotoxic effects on human cells, one of the sensitive cell types is likely to be the post-meiotic male germ cell. By the time these cells have developed into spermatozoa they have lost their capacity for DNA repair and jettisoned most of their cytoplasm containing the antioxidant enzymes that protect most cell types from oxidative stress. They have also separated from the Sertoli cells that have nursed and protected them up to the point of spermiation and differentiated to the point that they can no longer undergo apoptosis in response to severe genetic damage. In this isolated state, spermatozoa must spend several days maturing within the male reproductive tract; throughout this time, they are extremely vulnerable to the induction of DNA damage.

The induction of DNA damage in spermatozoa has been associated with male infertility, early pregnancy loss and morbidity in the offspring, including childhood cancer (Ji *et al.*, 1997; Aitken, 1999). The possibility that mobile phone RFEMR might be one of the many environmental factors having an impact on the male reproductive system, was recently raised by a preliminary report in which significant correlations were found between mobile phone use and semen quality (Fejes *et al.*, 2004). Given the vulnerability of these cells to genotoxic damage, and the clinical significance of this damage in terms of fertility, pregnancy and childhood health (Aitken, 1999; Crow, 2000; Sawyer & Aitken, 2000; Morris *et al.*, 2002; Bungum *et al.*, 2004), studies are urgently needed on the impact of RFEMR on DNA damage in the male germline.

Materials and methods

Animal handling and treatment

Male CD1 Swiss mice (30–35 g) bred at The University of Newcastle, NSW, were used in all experiments. The animals, acclimatized for at least 1 week prior to any

experimental procedures, were housed, monitored and treated in accordance with institutional animal ethics requirements. Food and water was provided *ad libitum*. Once acclimatized, groups of mice were randomly placed into standard plastic mouse cages with modified plastic lids, instead of metal lids, in order to avoid interference with the radiation.

The irradiation equipment

A rectangular waveguide was constructed measuring 300 × 300 × 1000 mm (Fig. 1). In the following discussions, the long dimension is assumed to be in the longitudinal direction, while the short dimensions are assumed to be in the vertical and horizontal transverse directions. The waveguide dimensions were chosen such that 900 MHz radiation could propagate longitudinally along the waveguide and also so that two small (130 × 160 × 330 mm) polycarbonate mouse cages could be accommodated within the waveguide. The waveguide was fabricated from 1 × 1 mm brass mesh glued to an aluminium frame and the ends were filled with 15 cm thick carbon-impregnated foam (RFI Industries, Bayswater, Victoria, Australia), which absorbs RF radiation, minimizing the reflection of radiation back into the waveguide. To produce the radiation, a 3 GHz function generator (E4431B; Agilent, Palo Alto, CA, USA) was used to generate a pure tone of 900 MHz at +10 dBm, corresponding to an output power of 10 mW.

A value of around 900 MHz was selected for these experiments because it corresponds to the RFEMR

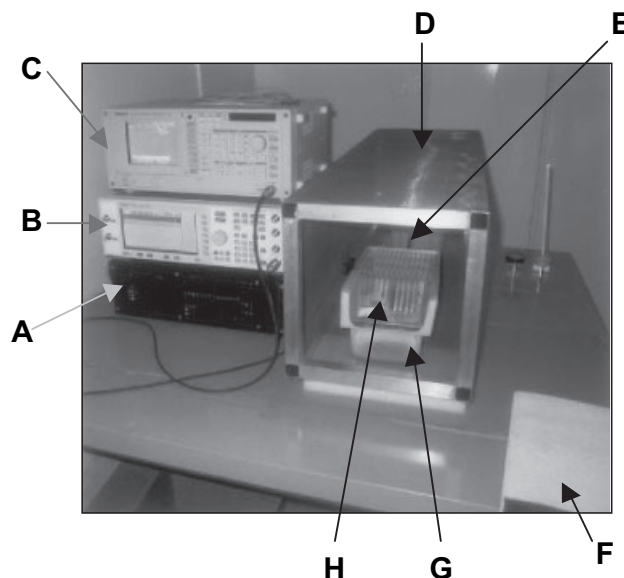


Figure 1. The waveguide and associated apparatus. (A) power supply; (B) function generator (allows manipulation of wave length and intensity); (C) spectrum analyser (measures intensity of wave when connected to a probe); (D) brass-meshed wave-guide; (E) mobile phone antenna; (F) carbon impregnated foam; (G) mouse cage; (H) custom-made Perspex mouse box lid.

employed by a large range of electrical devices including cordless phones, GSM (Global Systems for Mobile) network mobile phones and wireless devices such as speaker systems, remote presentation devices, baby monitors and headphones. It is also the radio frequency that has been used in previous studies on the health risks associated with mobile phone use (Utteridge *et al.*, 2002). This signal was amplified by a linear RF amplifier (800–950 MHz, nominal 16 W maximum output) to an output power of 8.8 W, as measured by an Oskerblock SWR435 power/SWR meter (Electronic Engineering Co., Tokyo, Japan). The amplifier output was connected through a matching network into a quarter wave mobile phone antenna placed transverse and vertical in the centre of the waveguide. The antenna matching circuit was tuned for maximum energy transfer to the antenna using the Oskerblock meter. An Advantest spectrum analyzer (Advantest, Tokyo, Japan) and a Hameg HZ530 E-field probe (Hameg GmbH, Mainhausen, Germany) were used to check radiation levels during the irradiation of the mice.

Prior to commencing the experiments on live mice, a calibrated single axis probe, utilizing an electrically short dipole and diode detector diode, was used to obtain an indication of the spatial variation of the RF fields inside the waveguide and to calibrate the Hameg probe. The dipole probe was inserted into the cavity through the foam RF absorber to measure the electric field in the two transverse polarizations over a grid of points separated by 6 cm in the transverse directions and 10 cm in the longitudinal direction.

Irradiation of the mice

For each experiment, four or five mice were placed inside the waveguide for 7 days, housed in a polycarbonate cage, and were considered the treated mice. The same number of mice were placed outside the waveguide in an identical polycarbonate cage for a corresponding 7 days and were considered controls. As the waveguide was made of an open brass mesh, the environment for both sets of mice was the same except for the presence or absence of irradiation 900 Mhz, which commenced from 19:00 hours each day and concluded at 07:00 hours the next day. Daily measurements included room temperature and humidity readings, as well as the internal temperature of the waveguide. The mice were also weighed and inspected for clinical signs of illness daily. After the irradiation schedule, mice were killed by CO₂ asphyxiation, and testes and cauda epididymides were dissected.

Harvesting and analysis of mouse spermatozoa

In the centre of a Petri dish, a 100 µL droplet of Biggers-Whitten-Whittingham (BWW) medium (Biggers *et al.*, 1971) was overlaid with 3 mL of water-saturated mineral oil. An additional 200 µL of BWW was then added to the 100 µL BWW droplet and the dish transferred to a 37 °C humidified chamber for 10 min. The Petri dish was subsequently placed on a paper-covered styrofoam tile on the bench to prevent heat loss and one freshly dissected cauda epididymis

was placed into each BWW droplet. The cauda epididymis was dissected from the vas deferens, fat and other epididymal tissue to minimize contamination by blood and epithelial cells. A small cut was then made at the tip of the cauda epididymis and the spermatozoa were teased out under gentle positive pressure. The Petri dish was then returned to the humidified 37 °C chamber for 10 min to allow spermatozoa to swim out. Subsequent to this incubation, the spermatozoa in BWW were removed from the water-saturated mineral oil and transferred to 1.5 mL microcentrifuge tubes. Standard sperm analysis was conducted on all samples including counts, morphology, motility and vitality; the last parameter being assessed by propidium iodide staining (Willoughby *et al.*, 1996).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed using the BioRad CHEF-DR III Pulsed Field Electrophoresis System (Bio-Rad, Hercules, CA, USA). Spermatozoa were resuspended to a concentration of 1×10^5 cells in 50 µL PBS. Subsequently, 50 µL of 2% low-melt agarose (Bio-Rad) in phosphate-buffered saline (PBS) was added to the sperm suspension, placed in gel plug moulds, and left to set at 4 °C overnight before being transferred to individual 1.5 mL microcentrifuge tubes. To each tube, 1 mL of gel lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% SDS, 1% Triton X-100, pH 10.0) was added and left at 4 °C overnight. Plugs were rinsed three times with 1 mL/plug of proteinase K buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0) for 10 min each, before adding proteinase K to a final concentration of 1 mg/mL to each plug and incubating at 37 °C for 1–4 days. The gel plugs were then rinsed three times with 1 mL of gel plug storage buffer (50 mM EDTA, 20 mM Tris, pH 8.0) for 10 min each wash. Plugs were stored in gel plug storage buffer at 4 °C until required. The PFGE profiles used for these studies allowed for the separation of DNA fragments up to 225 kb in length. A 1% pulsed-field agarose gel was made in 0.5X TBE (1X TBE is 100 mM Tris base, 100 mM boric acid, 2 mM EDTA, pH 8.0) and allowed to set for 1 h. Gel plugs of approximately 5 mm × 3 mm were slipped into the wells of the gel and sealed with 2% pulsed-field agarose. TBE (0.5X) electrophoresis buffer was cooled to 14 °C prior to, and for the duration of, the electrophoretic run. Parameters for the electrophoretic run were set to 6 V/cm for 15 h at an angle of 120°. Pulsed-field parameters included initial and final switch times of 0.1 and 10 s, respectively. Gels were stained in 250 mL of 1 µg/mL ethidium bromide (EtBr) for 15 min, rinsed gently with double-distilled H₂O for two changes of 15 min each, viewed under UV using an imaging system (Gel Doc 1000, Bio-Rad), and the image processed using Multi-Analyst software (version 1.1; Bio-Rad).

DNA extraction and quantification

Aliquots of 5×10^6 spermatozoa were washed twice in PBS, pelleted by centrifugation, and frozen at –80 °C over

night. Frozen spermatozoa were then thawed and resuspended in 1 mL STE buffer (50 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) plus 1% SDS, 1% 2-mercaptoethanol, and 2 mg/mL proteinase K. The cells were incubated at 55 °C for 6 h with periodic agitation. Samples were then transferred to 15 mL polypropylene conical tubes and the DNA purified by standard phenol/chloroform extractions. After ethanol precipitation overnight, the DNA was washed with 70% ethanol, air-dried for 15 min and re-dissolved in 100 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

PicoGreen (Molecular Probes, Eugene, OR, USA) was used to quantify double-stranded DNA as described (Sawyer *et al.*, 2001, 2003). Briefly, a 1:10 dilution in TE (10 mM Tris, 1 mM EDTA, pH 8.0) of the DNA samples was made, and 5 and 10 µL aliquots of each sample placed into a 96-well plate, with 0–75 ng of lambda DNA to generate a standard curve. PicoGreen solution was added to each sample according to the manufacturer's instructions, and incubated in the dark for 5 min before reading in a fluorescent plate reader (Fluoromark; Bio-Rad). Values obtained from the read-out [given in relative fluorescence units (RFU)] were used to estimate the concentration of DNA in the samples, and 10 ng/µL solutions were generated. The 10 ng/µL samples were subsequently re-quantified, and 9 and 3 ng/µL DNA stocks were generated from these.

Alkaline gel electrophoresis

Alkaline gel electrophoresis was performed as described (Sutherland *et al.*, 1999). Briefly, a 0.8% agarose gel was prepared in 50 mM NaCl, 4 mM EDTA. Cold alkaline electrophoresis solution (30 mM NaOH, 2 mM EDTA) was poured into the gel tank once the gel was completely set, and was allowed to equilibrate for 1 h. DNA (500 ng) was treated with alkaline loading buffer (430 mM NaOH, 36 mM EDTA, 20% glycerol) for 20 min and then loaded into the gel. The gel was run at a constant voltage of 1 V/cm for 18 h. Following electrophoresis, the gel was removed from the tank, rinsed with double distilled H₂O, then neutralized with two changes of 250 mL 0.4 M Tris-HCl, pH 8.0 for 1 h. After neutralization, the gels were stained with 1 µg/mL EtBr in water for 15 min, rinsed gently with double distilled H₂O for two changes of 15 min each, and viewed under UV using an imaging system (Gel Doc 1000, Bio-Rad).

Quantitative PCR

Quantitative PCR (QPCR) was performed following the protocols described by Ayala-Torres *et al.* (2000) as reported in earlier publications (Sawyer *et al.*, 2001, 2003). Briefly, aliquots of DNA (7.5–50 ng) were subjected to PCR under quantitative conditions, control reactions containing half the concentration of control template DNA being included in each set of PCR reactions. These samples were prepared by diluting control DNA 1 : 1 with TE buffer. For these quantitative controls, the actual values recorded for '50%'

samples were 50 ± 2% for the β-globin gene and 47 ± 4% for the large mitochondrial fragment. Primers specific for a 10 kb fragment of murine mtDNA, and for a 9 kb fragment of the nuclear β-globin gene were used in the PCR reactions (Ayala-Torres *et al.*, 2000). The respective genes were chosen based on our ability to reproducibly and consistently amplify the target of interest under quantitative conditions. Details regarding primer sequences and PCR reaction conditions have been published (Ayala-Torres *et al.*, 2000). All PCR reactions were performed in Hybaid PCR Express 96-well thermocyclers as previously described (Ayala-Torres *et al.*, 2000; Sawyer *et al.*, 2003). After thermocycling, PCR products were quantified by PicoGreen fluorescence using a fluorescence microplate reader (Bio-Rad). PCR reaction mixtures (10 µL) were measured in duplicate. PCR reactions containing no template DNA were performed to provide blanks. To ensure specificity of the PCR reactions, PCR products were also visualized after electrophoresis through a 0.8% agarose gel and stained with EtBr as described (Sawyer *et al.*, 2003).

Quantification of DNA lesions

The QPCR assay is based on the observation that any DNA lesion which inhibits strand synthesis by a thermostable polymerase can be detected as a decrease in amplification of the target amplicon. Based on this premise, lesion frequencies were calculated as previously described (Yakes *et al.*, 1996; Ayala-Torres *et al.*, 2000). Briefly, amplification of treated samples was normalized to controls to produce an amplification ratio. Assuming a random distribution of lesions along the target amplicon, and employing the Poisson expression $f(x) = e^{-\lambda} \lambda^x / x!$, where λ is equal to the average lesion frequency, lesion frequencies of the treated samples were calculated. In order to use this expression to calculate lesion frequencies, the control samples are assumed to have no lesions. Thus, control samples were set to $x = 0$, reducing the Poisson expression to $f(0) = e^{-\lambda}$. The lesion frequency per amplicon was then calculated as $\lambda = -\ln(A_D/A_0)$, where A_D/A_0 is the ratio of the treated samples (A_D) to the amplification of the control samples (A_0). To determine lesions per 10 kb (10^4 base pairs), the λ -value obtained was divided by the amplicon fragment length, and then multiplied by 10. It should be emphasized that this analytical approach provides an estimation of lesion frequency *relative to the control value* and does not indicate the absolute frequency of lesions in the treated sample. For a detailed description of the rationale behind these calculations, readers are referred to the papers published by Yakes *et al.* (1996) and Ayala-Torres *et al.* (2000).

Statistical analyses

Quantitative PCR data values are reported as means, normalized to control values, ±SEM. For the β-globin target, valid QPCRs were secured on samples from 17 animals from four separate experiments. Valid mitochondrial

target QPCRs were obtained on nine animals from three experiments. Statistical analysis was performed using the Superanova program following square root transformation of the data (Abacus Concepts, Berkeley, CA, USA) and for all experiments, a $p \leq 0.05$ was considered significant.

Results

The rate of absorption of energy from EMR is the specific absorption rate (SAR) given in watts per kilogram (W/kg). The Federal Communications Commission in the USA requires wireless phones to have an SAR of <1.6 W/kg and mobile phones are typically associated with SAR values around 0.3–1.5 W/kg. An SAR value of more than 4 W/kg is associated with heating effects that will adversely affect biological systems irrespective of the energy source. An SAR well below this threshold is therefore essential if the effects observed are to be clearly ascribed to the EMR and not a consequence of secondary heating effects.

The transverse electric (E) field within the waveguide averaged over nine points corresponding to the volume of the cage area was 85 ± 10 V/m along the vertical axis and 30 ± 10 V/m along the horizontal axis. Given that the vertically oriented antenna will launch radiation with vertical polarization, we would expect the vertically polarized component of the total electric field to be large, as found. In addition, we would expect the longitudinal and transverse horizontal components of the E field to be small. This has been shown for the transverse horizontal component as it is one-third of the vertical component. Now, for propagation in a lossless waveguide, the intensity of the radiation, I , is related to the electric field, E , by

$$E^2 = 377I$$

so the major contribution to the radiation intensity comes from the vertical component of the E field. Neglecting the horizontal component of the E field, an average intensity within the volume of the waveguide occupied by the mice is found to be 19 ± 10 W/m², equivalent to 1.9 ± 1 mW/cm². As the mice are mainly moving in the cage with their bodies aligned in a horizontal plane, the radiation is dominantly H polarized with respect to the long axis of the mouse. The whole-body SAR was then estimated using a value for the ratio of the SAR to irradiation intensity of 0.045 (W per kg/mW per cm²) for a prolate spheroid model of a mouse (Allen & Hurt, 1977; Durney *et al.*, 1986) in the H orientation. The SAR found from the above ratio and measured average intensity was then approximately 90 mW/kg.

A major concern that has plagued many studies in the past is the thermal effect of the irradiation on the animals (Brusick *et al.*, 1998). However, the above SAR is considerably less than the value of 4 W/kg where thermal effects are exhibited. We confirmed that thermal effects were unimportant, by measuring the temperature of a saline solution irradiated within the waveguide at the same intensity used

for our mice irradiations. Any increases in temperature on both short (minute) and long (hour) timescales were found to be less than the 0.1 K sensitivity of the temperature recording instrument. This is to be expected, as the SAR of a body with specific heat, c_p , is related to its rise in temperature, dT , in a short time period, dt , by

$$\text{SAR} = c_p dT/dt$$

so a temperature rise of <0.1 K over a 60 sec time period would correspond to a SAR for the solution (assuming c_p is 4.184 kJ/K/kg) of <7 W/kg. Given that the SAR of the mice in our experiment is only 90 mW/kg any temperature rises during the irradiation would then be much <0.1 K.

Animal and tissue parameters

The mice exhibited no overt signs of stress as a consequence of RFEMR irradiation and no differences in body weight were noted between irradiated and control animals. Similarly exposure to RFEMR had no effect on the weights of the testes and epididymides of treated animals. Analysis of sperm parameters also showed no significant effects of the irradiation on treated animals. In all exposure groups, sperm counts remained unchanged, as did the percentage of motile, morphologically normal and live cells (Fig. 2).

In terms of DNA fragmentation, no evidence of a gross increase in the incidence of single or double strand breaks

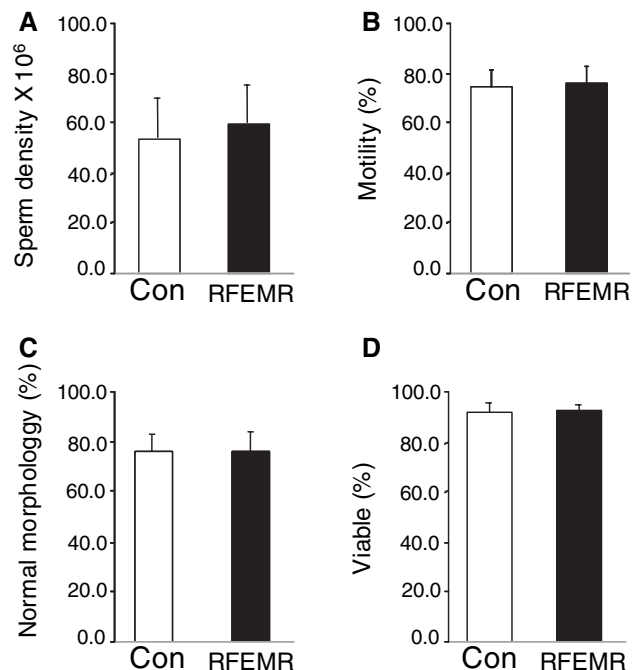


Figure 2. Sperm parameters in control and treated mice. After irradiation, epididymides were excised, and cauda epididymal spermatozoa were isolated as described in Materials and Methods. (A) caudal sperm reserves, (B) percentage motile spermatozoa, (C) percentage of spermatozoa with normal morphology, (D) percentage of viable spermatozoa.

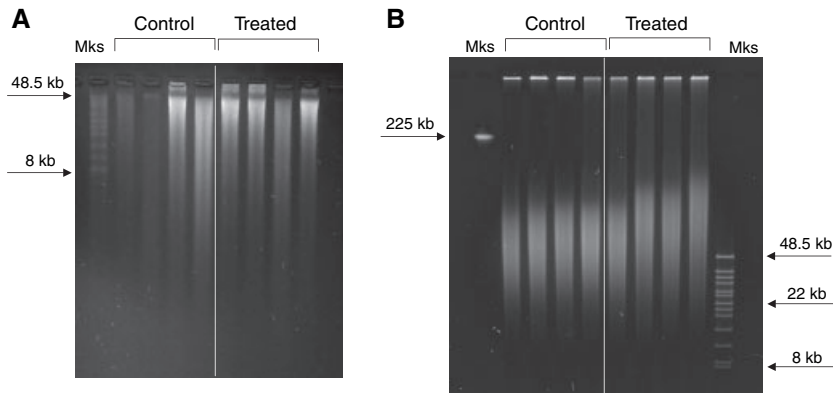


Figure 3. Alkaline and pulsed-field gel electrophoresis of mouse sperm DNA. Sperm DNA was prepared as described in Materials and Methods, and subjected to (A) alkaline and (B) pulsed-field gel electrophoresis. Representative gels of five samples from control and treated groups are shown. Mks = molecular size markers.

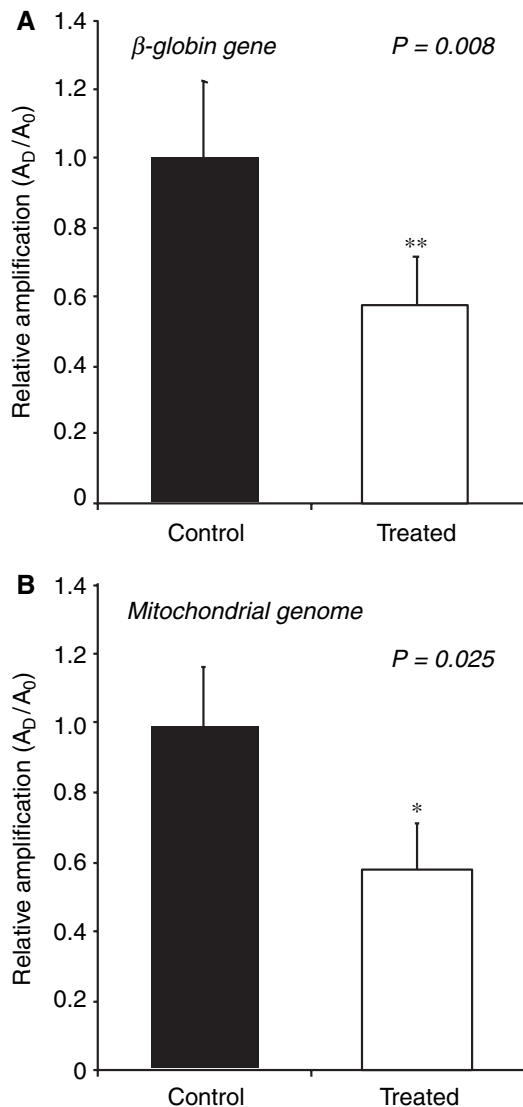


Figure 4. Quantitative PCR analysis of gene-specific DNA damage. Sperm DNA was prepared for QPCR as described in Materials and Methods. Individual QPCR profiles of (A) a 9 kb fragment of the β -globin gene and (B) a 10 kb fragment of the mitochondrial genome. * $p < 0.05$; ** $p < 0.01$.

was evident using alkaline and PFGE respectively (Fig. 3). However evidence for the induction of DNA damage was found in the QPCR data. ANOVA revealed a significant decrease in the amplification of the β -globin gene in association with exposure to RFEMR ($p = 0.008$; Fig. 4a) equating with an estimated lesion frequency of 0.755 lesions/10 kb DNA. Similarly, analysis of the integrity of the mitochondrial genome by QPCR revealed a significant decrease in DNA amplification in the irradiated mice ($p = 0.025$; Fig. 4b) equating with an estimated lesion frequency of 0.529 lesions/10 kb DNA.

Discussion

Although data supporting a possible genotoxic effect of RFEMR has been obtained in certain cell types (Maes *et al.*, 1996; Tice *et al.*, 2002; Mashevich *et al.*, 2003) impacts on the whole organism remain controversial. Thus while some evidence has been obtained to suggest that irradiation of lymphoma prone mice with EMR at 898.4 MHz significantly increases the incidence of non-lymphoblastic lymphomas (Repacholi *et al.*, 1997), this result has not been replicated (Utteridge *et al.*, 2002). Similarly, while there is some evidence to suggest that RFEMR exposure might lead to peripheral neurophysiological changes in some individuals (Westerman & Hocking, 2004), there is considerable uncertainty as to whether these effects are clinically significant (Hossmann & Hermann, 2003). The possibility that RFEMR exposure might have adverse impacts on the reproductive system has not extensively addressed for either sex; indeed, this is the first study to search for such an effect in the male germline.

It is well known that exposure of men to a multitude of chemical agents and ionizing radiation increases mutation frequency in the offspring, making them more prone to genetic disease, birth defects and childhood cancer (Aitken, 1999; Sawyer *et al.*, 2001). This phenomenon has been confirmed in both epidemiological and animal studies, and has been labelled 'male-mediated developmental toxicity'. The present study was designed to determine if any potential adverse effects of RFEMR might be mediated through the

male germline, employing a system wherein precise wavelengths and field intensities could be set and independently verified and the calculated SAR was well below the thermal threshold of 4 W/kg (Weisbrot *et al.*, 2003). It should also be noted that the SAR value recorded in this study is significantly lower than the 0.3–1.5 W/kg associated with mobile phone use. As a consequence, the reproductive health risks associated with RFEMR exposure may be even more serious than those reported in this study. Clearly a range of SAR values should be assessed in future investigations.

The QPCR technique has been used to detect a wide variety of DNA lesions in a gene-specific manner (Ayala-Torres *et al.*, 2000). The fundamental premise of this assay is that DNA lesions, including base adducts and strand breaks, impede the progress of the polymerase used in the PCR reactions. Hence, when DNA damage is present, the amplification of the target sequence will be decreased. The mitochondrial genome is known to be particularly susceptible to DNA damage and provides a sensitive biomarker for genotoxicity (Sawyer *et al.*, 2001). Lesion frequencies as low as 1–2 lesions per 10^5 base pairs can be detected with this assay (Yakes *et al.*, 1996; Sawyer *et al.*, 2001, 2003). For the sake of comparison, the pulsed field electrophoretic technique can detect 1 lesion per 10^3 base pairs (Sutherland *et al.*, 2003) while alkaline gel electrophoresis can detect down to 2 lesions per 5×10^3 base pairs (Sutherland *et al.*, 1999). Thus, while the gel electrophoretic data revealed no dramatic evidence of single or double DNA strand breaks, the more sensitive QPCR analysis detected significant DNA damage in both the nuclear and mitochondrial genomes of spermatozoa recovered from the cauda epididymis. Previous studies have also observed an impact of RFEMR on single and double DNA strand breaks in other cell types (Lai & Singh, 1996), although this is not a consistent finding (Meltz, 2003; Hook *et al.*, 2004) and the mechanisms are not clear. Impairment of DNA repair mechanisms has been proposed (Lai & Singh, 1996), but seems unlikely in the case of spermatozoa as these terminally differentiated cells have no capacity for detecting or repairing DNA damage. A direct effect of RFEMR on DNA integrity has also been suggested (Lai & Singh, 1996) but the underlying mechanisms remain unresolved.

The 7-day exposure period employed in this study means that spermatozoa recovered from the cauda epididymis at autopsy would have been exposed to the RFEMR during epididymal transit. This exposure regime was deliberately selected, because at this point in their life history, spermatozoa have lost all capacity for DNA repair and are very vulnerable to factors that might affect the integrity of their DNA (Aitken, 1999). It is presumably this vulnerability that accounts for the particular sensitivity of spermatozoa to the genotoxic effects of RFEMR. DNA damage arising in this way would have to be repaired by the oocyte in the few hours that lapse between fertilization and initiation of the first cleavage division. Mistakes at this point have the potential to create mutations that could disrupt the normality of embryonic development and the health and well being of the offspring (Aitken & Krausz, 2001). This proposed mechanism might explain why the linkages between EMR and childhood disease suggested by epidemiological studies have been so difficult to replicate in the laboratory. It is possible that exposure of the child to EMR was less of a factor in the genesis of disease than exposure of the father. Whether this hypothesis is biologically or clinically tenable will have to be established in follow-up studies employing a range of SAR values and exposure times. In addition, more refined analytical techniques will have to be used to determine the precise nature of the genetic damage induced in spermatozoa exposed to RFEMR, and the mechanisms responsible for its induction. In the meantime, these preliminary data, together with recent reports suggesting an impact of mobile phone use on human semen quality (Fejes *et al.*, 2004), should alert us to the possible adverse effects of RFEMR on genetic integrity in the male germline and stimulate more extensive, detailed investigations of this association.

Acknowledgements

This project was funded by a collaboration of international and local agencies: RMC small grant no. 2521229, NIEHS Grant (NRSA, USA) no. F32HM 08557-01, ARC large grant no. AO9918038 and the ARC Centre of Excellence in Biotechnology and Development.

References

- d'Ambrosio, G., Massa, R., Scarfi, M. R. & Zeni, O. (2002) Cytogenetic damage in human lymphocytes following GMSK phase modulated microwave exposure. *Bioelectromagnetics* **23**, 7–13.
- Aitken, R. J. (1999) The human spermatozoon – a cell in crisis? (The Amoroso Lecture). *Journal of Reproduction and Fertility* **115**, 1–7.
- Aitken, R. J. & Krausz, C. G. (2001) Oxidative stress, DNA damage and the Y chromosome. *Reproduction* **122**, 497–506.
- Allen, S. J. & Hurt, W. D. (1977) Development and use of a calorimeter to measure specific absorption rates in small laboratory animals. In: *URSI International Symposium on the Biological Effects of Electromagnetic Waves*, Airlie, VA, Abstract 61.
- Ayala-Torres, S., Chen, Y., Svoboda, T., Rosenblatt, J. & Van Houten, B. (2000) Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction. *Methods* **22**, 135–147.
- Bartsch, H., Bartsch, C., Seebald, E., Deerberg, F., Dietz, K., Vollrath, L. & Mecke, D. (2002) Chronic exposure to a GSM-like signal (mobile phone) does not stimulate the development of DMBA-induced mammary tumors in rats: results of three consecutive studies. *Radiation Research* **157**, 183–190.

- Biggers, J. D., Whitten, W. K. & Whittingham, D. G. (1971) The culture of mouse embryos in vitro. In: *Methods in Mammalian Embryology* (ed. J. C. Daniels), pp. 86–116. Freeman, San Francisco.
- Black, D. R. & Heynick, L. N. (2003) Radiofrequency (RF) effects on blood cells, cardiac, endocrine, and immunological functions. *Bioelectromagnetics Supplement* **6**, S187–S195.
- Blettner, M. & Berg, G. (2000) Are mobile phones harmful? *Acta Oncologica* **39**, 927–930.
- Breckenkamp, J., Berg, G. & Blettner, M. (2003) Biological effects on human health due to radiofrequency/microwave exposure: a synopsis of cohort studies. *Radiation and Environmental Biophysics* **42**, 141–154.
- Brusick, D., Albertini, R., McRee, D., Peterson, D., Williams, G., Hanawalt, P. & Preston, J. (1998) Genotoxicity of radiofrequency radiation. *Environmental and Molecular Mutagenesis* **32**, 1–16.
- Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L. & Giwercman, A. (2004) The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Human Reproduction* **19**, 1401–1408.
- Crow, J.F. (2000) The origins, patterns and implications of human spontaneous mutation. *Nature Reviews Genetics* **1**, 40–47.
- Durney, C. H., Massoudi, H. & Iskander, M. (1986) *Radiofrequency Radiation Dosimetry Handbook*, 4th edn (USAFSAM-TR-85-73). <http://www.brooks.af.mil/AFRL/HED/hedr/reports/handbook/preface.htm> (date of last access: 28 January 2005).
- Elder, J. A. (2003) Survival and cancer in laboratory mammals exposed to radiofrequency energy. *Bioelectromagnetics Supplement* **6**, S101–S106.
- Fejes, I., Závaczki, Z., Szöllösi, J., Koloszar, S., Kovács, L. & Pál, A. (2004) Relationship between regular cell phone use and human semen quality. Abstracts of the 20th Annual Meeting of the ESHRE, Berlin. *Human Reproduction Supplement* **11** Abstract O-162.
- Garson, O. M., McRobert, T. L., Campbell, L. J., Hocking, B. A. & Gordon, I. (1991) A chromosomal study of workers with long-term exposure to radio-frequency radiation. *Medical Journal of Australia* **155**, 289–292.
- Gos, P., Eicher, B., Kohli, J. & Heyer, W. D. (2000) No mutagenic or recombinogenic effects of mobile phone fields at 900 MHz detected in the yeast *Saccharomyces cerevisiae*. *Bioelectromagnetics* **21**, 515–523.
- Goswami, P. C., Albee, L. D., Parsian, A. J., Baty, J. D., Moros, E. G., Pickard, W. F., Roti Roti, J. L. & Hunt, C. R. (1999) Proto-oncogene mRNA levels and activities of multiple transcription factors in C3H 10T 1/2 murine embryonic fibroblasts exposed to 835.62 and 847.74 MHz cellular phone communication frequency radiation. *Radiation Research* **151**, 300–309.
- Harvey, C. & French, P. W. (2000) Effects on protein kinase C and gene expression in a human mast cell line, HMC-1, following microwave exposure. *Cell Biology International* **23**, 739–748.
- Heikkinen, P., Kosma, V. M., Hongisto, T., Huuskonen, H., Hyysalo, P., Komulainen, H. *et al.* (2001) Effects of mobile phone radiation on X-ray-induced tumorigenesis in mice. *Radiation Research* **156**, 775–785.
- Hook, G. J., Zhang, P., Lagroye, I., Li, L., Higashikubo, R., Moros, E. G., Straube, W. L., Pickard, W. F., Baty, J. D. & Roti Roti, J. L. (2004) Measurement of DNA damage and apoptosis in Molt-4 cells after in vitro exposure to radiofrequency radiation. *Radiation Research* **161**, 193–200.
- Hossmann, K. A. & Hermann, D. M. (2003) Effects of electromagnetic radiation of mobile phones on the central nervous system. *Bioelectromagnetics* **24**, 49–62.
- Jauchem, J. R. (2003) A literature review of medical side effects from radio-frequency energy in the human environment: involving cancer, tumors, and problems of the central nervous system. *Journal of Microwave Power and Electromagnetic Energy* **38**, 103–123.
- Ji, B. T., Shu, X. O., Linet, M. S., Zheng, W., Wacholder, S., Gao, Y. T., Ying, D. M. & Jin, F. (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of non-smoking mothers. *Journal of the National Cancer Institute* **89**, 238–244.
- Lai, H. & Singh, N. P. (1996) Single- and double-strand DNA breaks in rat brain cells after acute exposure to radiofrequency electromagnetic radiation. *International Journal of Radiation Biology* **69**, 513–521.
- Lalic, H., Lekic, A. & Radosevic-Stasic, B. (2001) Comparison of chromosome aberrations in peripheral blood lymphocytes from people occupationally exposed to ionizing and radiofrequency radiation. *Acta Medica Okayama* **55**, 117–127.
- Li, L., Bisht, K. S., LaGroye, I., Zhang, P., Straube, W. L., Moros, E. G. & Roti Roti, J. L. (2001) Measurement of DNA damage in mammalian cells exposed in vitro to radiofrequency fields at SARs of 3–5 W/kg. *Radiation Research* **156**, 328–332.
- Maes, A., Collier, M., Slaets, D. & Verschaeve, L. (1996) 954 MHz microwaves enhance the mutagenic properties of mitomycin C. *Environmental and Molecular Mutagenesis* **28**, 26–30.
- Maes, A., Collier, M., Van Gorp, U., Vandoninck, S. & Verschaeve, L. (1997) Cytogenetic effects of 935.2-MHz (GSM) microwaves alone and in combination with mitomycin C. *Mutation Research* **393**, 151–156.
- Malyapa, R. S., Ahern, E. W., Straube, W. L., Moros, E. G., Pickard, W. F. & Roti Roti, J. L. (1997) Measurement of DNA damage after exposure to electromagnetic radiation in the cellular phone communication frequency band (835.62 and 847.74 MHz). *Radiation Research* **148**, 618–627.
- Mashevich, M., Folkman, D., Kesar, A., Barbul, A., Korenstein, R., Jerby, E. & Avivi, L. (2003) Exposure of human peripheral blood lymphocytes to electromagnetic fields associated with cellular phones leads to chromosomal instability. *Bioelectromagnetics* **24**, 82–90.
- Mason, P. A., Walters, T. J., DiGiovanni J., Beason, C. W., Jauchem, J.R., Dick, E. J., Jr *et al.* (2001) Lack of effect of 94 GHz radio frequency exposure in an animal model of skin carcinogenesis. *Carcinogenesis* **22**, 1701–1708.
- Meltz, M. L. (2003) Radiofrequency exposure and mammalian cell toxicity, genotoxicity, and transformation. *Bioelectromagnetics Supplement* **6**, S196–S213.
- Morris, I. D., Ilott, S., Dixon, L. & Brison, D. R. (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (cometassay) and its relationship to fertilization and embryo development. *Human Reproduction* **17**, 990–998.
- Moustafa, Y. M., Moustafa, R. M., Belacy, A., Abou-El-Ela, S. H. & Ali, F. M. (2001) Effects of acute exposure to the radiofrequency fields of cellular phones on plasma lipid peroxide and antioxidase activities in human erythrocytes. *Journal of Pharmaceutical and Biomedical Analysis* **26**, 605–608.
- Pacini, S., Ruggiero, M., Sardi, I., Aterini, S., Gulisano, F. & Gulisano, M. (2002) Exposure to global system for mobile communication (GSM) cellular phone radiofrequency alters

- gene expression, proliferation, and morphology of human skin fibroblasts. *Oncological Research* **13**, 19–24.
- Repacholi, M. H., Basten, A., Gebiski, V., Noonan, D., Finnie, J. & Harris, A. W. (1997) Lymphomas in E mu-Pim1 transgenic mice exposed to pulsed 900 MHz electromagnetic fields. *Radiation Research* **147**, 631–640.
- Sawyer, D. E. & Aitken, R. J. (2000) Male-mediated developmental defects and childhood disease. *Reproductive Medicine Reviews* **8**, 107–126.
- Sawyer, D. E., Roman, S. D. & Aitken, R. J. (2001) Relative susceptibilities of mitochondrial and nuclear DNA to damage induced by hydrogen peroxide in two mouse germ cell lines. *Redox Reports* **6**, 182–184.
- Sawyer, D. E., Mercer, B. G., Wiklendt, A. M. & Aitken, R. J. (2003) Quantitative analysis of gene-specific DNA damage in human spermatozoa. *Mutation Research* **529**, 21–34.
- Sutherland, B. M., Bennett, P. V. & Sutherland J. C. (1999) DNA damage quantitation by alkaline gel electrophoresis. *Methods in Molecular Biology* **113**, 183–202.
- Sutherland, B. M., Bennett, P. V., Georgakilas, A. G. & Sutherland, J. C. (2003) Evaluation of number average length analysis in quantifying double strand breaks in genomic DNAs. *Biochemistry* **42**, 3375–3384.
- Sykes, P. J., McCallum, B. D., Bangay, M. J., Hooker, A. M. & Morley, A.A. (2001) Effect of exposure to 900 MHz radiofrequency radiation on intrachromosomal recombination in pKZ1 mice. *Radiation Research* **156**, 495–502.
- Tice, R. R., Hook G. G., Donner, M., McRee, D. I. & Guy, A.W. (2002) Genotoxicity of radiofrequency signals. I. Investigation of DNA damage and micronuclei induction in cultured human blood cells. *Bioelectromagnetics* **23**, 113–126.
- Utteridge, T. D., Gebiski, V., Finnie, J. W., Vernon-Roberts, B. & Kuchel, T. R. (2002) Long-term exposure of E-mu-Pim1 transgenic mice to 898.4 MHz microwaves does not increase lymphoma incidence. *Radiation Research* **158**, 357–364.
- Weisbrot, D., Lin, H., Ye, L., Blank, M. & Goodman, R. (2003) Effects of mobile phone radiation on reproduction and development in *Drosophila melanogaster*. *Journal of Cell Biochemistry* **89**, 48–55.
- Westerman, R. & Hocking, B. (2004) Diseases of modern living: neurological changes associated with mobile phones and radiofrequency radiation in humans. *Neuroscience Letters* **361**, 13–16.
- Willoughby, C. E., Mazur, P., Peter, A. T. & Critser, J. K. (1996) Osmotic tolerance limits and properties of murine spermatozoa. *Biology of Reproduction* **55**, 715–727.
- Yakes, F. M., Chen, Y. & Van Houten, B. (1996) PCR-based assays for the detection and quantitation of DNA damage and repair. In: *Technologies for Detection of DNA Damage and Mutations* (ed. G. P. Pfeifer), pp. 171–184. Plenum Press, New York and London.

Received 23 July 2004; revised 1 October 2004; accepted 1 October 2004