In vitro effect of 900 MHz GSM radiation on mitochondrial membrane potential and motility of human spermatozoa

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Abstract and Key words

Ejaculated, density purified, human spermatozoa were exposed to 900 MHz GSM mobile phone radiation at two specific absorption rate levels (SAR 2.0 and 5.7 W/kg) and examined at various time points post exposure. Change in sperm mitochondrial membrane potential was analyzed using flow cytometry. Sperm motility was determined by computer assisted sperm analysis (CASA). There was no effect of 900MHz GSM radiation on mitochondrial membrane potential. This was also the case for all kinematic parameters assessed at SAR of 2.0 W/kg. However, two kinematic parameters (VSL and BCF) were statistically significantly altered after the exposure at SAR 5.7 W/kg. Effects seen cannot be ascribed to heating, as the temperature did not increase by more than 0.3ºC. A thorough investigation at lower SAR levels is required to determine the extent of the influence of RF-EMF on human sperm motility.

Key words: human spermatozoa, mobile phone radiation, mitochondrial membrane potential, CASA.

INTRODUCTION

Mobile phone technologies have innervated our society over the past decade with an estimated 1.6 billion worldwide users today [European Commission, Community Research 2005]. This widespread use has brought about public concern regarding the safety and possible health effects associated with mobile phone use [Heynick *et al*., 2003; ICNIRP, 2004]. More recently, attention has been drawn to the possibility that RF-EMF from mobile phones could be added to the growing list of environmental factors that contribute to the decline in male fertility [Derias et al., 2006].

Several recent studies have highlighted the possibility that RF-EMF could influence sperm motility [Davoudi et al., 2002; Fejes et al., 2005; Kilgallon and Simmons, 2005; Erogul et al., 2006]. Motility is a pre-requisite in the journey of spermatozoa to the oocyte eventuating in hyperactivated motility, which is required for sperm penetration of the zona pellucida [Yanagimachi, 1994; de Lamirande et al., 1997; Ho and Suarez, 2001]. Sperm motility is

defined as the observation of spontaneous sperm movement and is a significant factor when evaluating fertilising potential [Mortimer and Mortimer, 1999; Jeyendran, 2003]. The WHO [1999] recommends a simple grading system for the manual evaluation of motility. However, due to extreme variability in operator determined sperm motility, it is important to develop objective measurements in motility assessment such as computer aided sperm analysis (CASA). In addition, CASA assessment of motility affords the opportunity to evaluate a magnitude of different sperm motion characteristics. Motility assessment should not be based on CASA observations alone, but further sperm function tests must be done to confirm the energetic state of the sperm.

Inner mitochondrial membrane potential is a sensitive indicator of the energetic and functional state of mitochondria and the cell [Ly et al, 2003]. In human spermatozoa a decrease in mitochondrial membrane potential is highly correlated with diminished sperm motility and fertilisation potential [Donnely et al., 2000; Marchetti et al., 2002; Piasecka and Kawiak, 2003; Wang et al., 2003]. Furthermore, a recent report by Aitken et al. [2005] showed that RF-EMF caused significant damage to the mitochondrial genome in epididymal sperm of male mice. This, as well as the correlation that exists between reduced mitochondrial membrane potential and diminished motility and fertility, has prompted the investigation of the effect of RF-EMF exposure on mitochondrial membrane potential and motility in human spermatozoa.

The current study was designed to examine the effect of 900 MHz RF-EMF exposure on fully differentiated, highly motile human spermatozoa that have the potential to fertilize the human oocyte. Control and RF-EMF exposed human spermatozoa were examined for changes in mitochondrial membrane potential ($\Delta\psi m$) using MitoTracker fluorescent stain (MitoTracker® CMX-Ros) and flow cytometry. The changes in sperm motility parameters were determined by CASA.

MATERIALS AND METHODS

Chemicals

Ham's F10 medium, bovine serum albumin (BSA) and Dulbecco's phosphate buffered saline (DPBS) were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, Missouri, USA). MitoTracker® Red CMX-Ros was purchased from Molecular Probes (Eugene, Oregon, USA).

Experimental set-up and dosimetry

The exposure system previously described by Leszczynski et al. [2002] was installed at the Reproductive biology laboratory (University of Pretoria, South Africa) and calibrated by technicians from STUK (NIR Laboratory, Finland). Mobile phone microwave radiation (900 MHz pulse modulated RF) was simulated in a specially constructed exposure system, based on the use of a high Q waveguide resonator operating in TE_{10} mode. The irradiation chamber (Figure I) was placed vertically inside a Nu-Aire CO_2 incubator (NuAir Corp., Plymouth, MN, USA). Two 55 mm-diameter glass petri-dishes (Schott dishes, Merck Chemicals (Pty) Ltd, South Africa) were placed inside the irradiation chamber, with the plane of the culture medium aligned parallel to the E-field vector. Temperature controlled water was circulated through a thin (9 mm) rectangular glass-fibre-moulded waterbed underneath the petri-dishes. The RF-EMF signal was generated with an EDSG-1240 signal generator and modulated with a pulse duration of 0.577 ms and repetition rate of 4.615 ms to match the GSM signal modulation scheme. The signal was amplified with a RF-EMF Power Labs R720F amplifier and fed to the exposure waveguide via a monopole type feed post.

Cells were exposed for 1 hr to a 900 MHz GSM-like signal at an average SAR of either 2.0 or 5.7 W/kg. The SAR distribution in the cell culture was determined using SEMCAD 1.8 software (SPEAG, Switzerland) with a graded simulation grid. More than 70% of the cells were within ± 3 dB of the average SAR. A total of 440 000 voxels were used to simulate the medium with the largest grid size in the culture medium being 0.1 x 0.1 x 0.1 mm^3. Simulation results were verified with temperature rise based SAR measurements using a calibrated Vitek- type temperature probe (BSD-Medical). Temperature measurements were also performed to assure that the cells remained at a constant temperature level during the exposures. Results indicated that at the higher SAR level (5.7 W/kg) the temperature of cells ranged between 36.7ºC and 37.3ºC, while at the lower SAR level (2.0 W/kg) temperature ranged from 36.8 ºC to 37.2 ºC.

Collection and preparation of semen samples

Semen samples were collected from healthy, non-smoking donors (n = 12) by masturbation after 2 to 3 days of sexual abstinence. The study was conducted according to guidelines established for research on human subjects (Ethics Committee application no. 163/2003, University of Pretoria). The semen samples were allowed to liquefy for 30 min at 37°C, after which standard semen parameters were evaluated according to the World Health Organisation (WHO) criteria [1999]. All semen parameters were within the normal range as defined by WHO criteria, but variable morphology was observed with the average normal morphology 8.85 $\pm$ 1.2 % according to Tygerberg strict criteria [Kruger et al., 1986]. From the time of specimen collection and throughout all procedures and tests, spermatozoa were maintained under capacitating conditions (37°C in a humidified 6% CO_2 incubator, pH of media 7.3).

To purify spermatozoa, a three-step discontinuous Percoll gradient (95-70-50%) diluted in Ham's F10 medium supplemented with 0.5% BSA was used. After the processing step the purified population of highly motile spermatozoa (from the 95% layer) was washed in 3 ml of the same media by centrifugation (300 g for 10 min), recovered and re-suspended in 1 ml 0.5% BSA supplemented Ham's F10 medium before preparation for RF-EMF exposure. The motile sperm concentration of the total sample (total ejaculate) after Percoll density centrifugation was $\geq$ 40 x 10^6/ml in order to provide sufficient number of cells for experimentation.

The presence of leukocytes in the purified highly motile fraction of spermatozoa could contribute to the inhibition of sperm movement and ATP production [Marchetti et al., 2002; Henkel et al., 2005] as well as reactive oxygen species (ROS) generation [Armstrong et al., 1999; Henkel et al., 2005]. Therefore, a leukocyte specific antibody (CD45) was used to exclude these cells from the sperm population. The total percentage of $CD45^+$ cells was less than 0.3% of the total sperm population after density separation and, according to the WHO [1999] not of pathological significance.

Processed spermatozoa were counted (improved Neubauer Haemocytometer) and concentrations adjusted to 20x10^6 sperm/ml. Of this sperm suspension, 1 ml was seeded into sterile glass petri-dishes containing 2 ml of 0.5% BSA supplemented Ham's F10 medium. Control and RF-EMF exposed dishes (2 each) were simultaneously prepared and exposed for one hour inside the RF-EMF chamber (RF-EMF exposed samples) and next to the chamber (control exposed samples) inside a humidified CO_2 incubator. Exposure to the different SAR levels, were performed for each of the donors at two separate occasions.

Directly after the control/RF-EMF exposure, sperm were gently recovered from the petri-dishes, transferred to separate conical test tubes (Lasec, USA) and concentrations adjusted to 20x10^6/ml by centrifugation (300 g for 5 min). Spermatozoa were then incubated under capacitating conditions and $\Delta\psi$m was assessed immediately (T_1) as well as 2 h (T_2) and 24 h (T_3) post-exposure while aliquots taken from each tube at the different time points were used to assess sperm motility. All tests were run in duplicate.

Flowcytometric analysis - Assessment of mitochondrial membrane potential

Flow cytometric analysis was performed on a Coulter Epics® XL.MCL flow cytometer equipped with an air cooled argon laser (Beckman Coulter, Miami, Florida, USA) for the analysis of all SAR 2.0 W/kg samples, while a Coulter Epics® Altra flow cytometer equipped with a water cooled coherent enterprise laser (Beckman Coulter) was used for analysis of all SAR 5.7 W/kg samples. Appropriate controls were used to confirm the results from the different flow cytometers. The sperm population was identified using forward-angle light scatter, while side-angle light scatter was used to exclude electronic noise and debris. A total of 10 000 events were acquired for each endpoint. Analysis was done with System II software when using the XL.MCL and EXPO 32 software when using the Altra. The results are expressed as the mean cell number (cells/channel vs. % CMXros).

Baseline $\Delta\psi$m was determined by the technique adapted from Marchetti et al., [2004]. MitoTracker® Red CMX-Ros, stored as a stock concentration of 1 mM at - 20°C, was added to sperm at a final concentration of 150 nM/10^6 sperm/ml in Ham's F10 supplemented with 0.5% BSA medium. Sperm suspensions were incubated for 15 min in a humidified incubator. After incubation, sperm were washed with warm medium (kept at 37°C), the supernatant removed and the sperm re-suspended in 1 ml of this medium before flow cytometry analysis (using the XL.MCL for all SAR 2.0 W/kg samples and the Altra for all SAR

5.7 W/kg samples). MitoTracker® Red CMX-Ros is excited at a frequency of 579 nm and emits at 599 nm and fluorescence was detected in FL3.

Carbamoylcyanide m-chlorophenylhydrazone (mClCCP) previously described by Marchetti et al., [2002] was used to provide a positive control for the abolishment of the mitochondrial membrane potential of spermatotozoa. Spermatozoa (10^6) were incubated in the presence of 50 μmol/l mClCCP for 15 min in a humidified incubator before proceeding with the MitoTracker® Red CMX-Ros staining procedure to determine $\Delta\psi m$.

Motility Assessment

Sperm motility after RF-EMF irradiation was determined using the Hamilton Thorne Integrated Visual Optical System (IVOS 10, 60 Hz; Hamilton Thorne Research, Danvers, MA, USA). At each time point post RF-EMF exposure, 5 μl of the sperm suspension from the RF-EMF exposed and control sperm were loaded into two 20 μm Microcell chambers (2X-CEL, Hamilton Thorne Research, Danvers, MA, USA). The chambers were then placed on a heated microscope plate (Nikon, Optiphot, Japan), which was maintained at 37°C and video recordings were made of at least 10 random fields per chamber. Each field was recorded for 30 seconds. The pre-recorded video was analysed using the Hamilton Thorne Integrated Visual Optical System. The Hamilton Thorne computer calibrations were set at 30 frames at a frame rate of 30 images/second. Data from each individual cell track were recorded and analysed. At least 200 sperm were analysed per field of the 10 fields recorded for each aliquot sampled.

Sperm kinetic parameters evaluated included progressively (PRG) motile as well as non-progressive and immotile sperm, curvilinear velocity (VCL; a measure of the total distance travelled by a given sperm during the acquisition divided by the time elapsed); average path velocity (VAP; the spatially averaged path that eliminates the wobble of the sperm head); straight line velocity (VSL; the straight-line distance from beginning to end of track divided by time taken); beat-cross frequency (BCF; frequency of lateral head displacement), ALH (the mean width of sperm head oscillation) and the derivatives, straightness (STR = VSL divided by VAP x 100) and linearity (LIN = VSL divided by VCL x 100, departure of sperm track from a straight line). To be classified as hyperactivated (HYPA), a trajectory had to meet all of the 60 Hz SORT criteria [Mortimer et al., 1998], *i.e.*, VCL ≥ 150 μm/s, LIN ≤ 50% and ALH ≥ 7μm.

Statistical analysis

Data were analysed using Stata Statistical Software Release 8.0 (Stata Corporation, 2003, College Station, Texas, USA). A within subject design considering two treatments, control vs. RF-EMF (SAR 2.0 and 5.7 W/kg) respectively, at three time points (T_1- directly after exposure, T_2- 2 hours after exposure and T_3-24 hours after exposure), for a total of 12 donors was analysed by means of time series regression under the random effect option. Similarly the exposure levels 2.0 and 5.7 W/kg were compared over time after confirmation of equivalent experimental conditions reflected by the homogeneity of the results from controls during the two experiments. Data is presented as mean values ± standard deviation (SD) for all twelve donors with each test run in duplicate. Correlations were computed using the Pearson's correlation coefficient. All statistical tests were two-sided and statistical significance was considered when $p < 0.05$. Repeatability of duplicate tests was confirmed with the intraclass correlation coefficient for assays (CASA and mitochondrial membrane potential assessment) ≥ 0.92.

RESULTS

Mitochondrial membrane potential

The abolishment of the mitochondrial membrane potential of human spermatozoa by treatment with mClCCP and detection thereof using MitoTracker® Red CMX-Ros is demonstrated in Figure II. Depolarisation of the mitochondrial membrane potential by mClCCP caused a significant decrease in CMX-Ros fluorescence. RF-EMF exposure, on the other hand, did not result in a significant $\Delta\psi m$ for either of the SAR values (2.0 and 5.7 W/kg) and assessment times: directly after exposure (T_1), at 2 hours (T_2) and at 24 hours (T_3) after exposure (Figure III A and B). However, mitochondrial membrane potential decreased significantly as a function of time only (RF-EMF exposure independent) and this time-dependent decline was seen in all sperm used in both 2.0 and 5.7 W/kg exposure experiments (Figure III A and B).

To determine the statistical significance of the changes in mitochondrial membrane potential, RF-EMF exposed sperm at SAR 2.0 W/kg were compared (time series regression) to sperm exposed at SAR 5.7 W/kg at all three time points. The differences were found not to be statistically significant (T_1, p = 0.163; T_2, p = 0.485; T_3, p = 0.272).

Motility

(i) Progressive Motility

CASA assessment of progressive motility (type "a+b" motility, rapid + slow progressive motility) [WHO, 1999] in RF-EMF exposed compared to control spermatozoa for SAR levels 2.0 and 5.7 W/kg determined directly (T_1), 2 (T_2) and 24 (T_3) hours post exposure are summarised in Table I. Linear regression analysis showed no statistically significant effect of RF-EMF exposure on progressive motility of human spermatozoa at either of the SAR levels ($[p = 0.899]_{2\ W/kg}$ and $[p= 0.935]_{5.7\ W/kg}$). When comparing progressive motility in RF-EMF exposed sperm at SAR 2.0 W/g to SAR 5.7 W/kg as a function of time (Mann-Whitney U-test), we noted no significant effect of SAR level on motility at T_1 (p = 0.910), T_2 (p = 0.675) or T_3 (p = 0.312).

Furthermore, a summary of the linear regression analysis results comparing rapid (type a)- slow (type b)-, non-progressive (type c) and immotile (type d) categories are given in Table II. There was no statistically significant effect on rapid progressive, slow progressive, non-progressive or immotile categories comparing RF exposed spermatozoa at either of the SAR levels with controls. In addition, an increase in SAR had no effect on any of the motility categories.

(ii) Velocity parameters

For SAR 2.0 W/kg (Figure IV A): No statistical difference (linear regression analysis) was noted in any of the velocity parameters over the three time points comparing RF-EMF exposed sperm at SAR 2.0 W/kg with controls. Directly after exposure both RF-EMF exposed and controls exhibited similar velocities, however two hours after exposure, RF-EMF exposed sperm displayed more rapid movement in all parameters compared to the controls. However, the differences were not statistically significant. This situation was reversed 24 hours after exposure with RF-EMF exposed sperm showing a decrease in all velocity parameters compared to controls.

For SAR 5.7 W/kg (Figure IV B): Linear regression analysis comparing RF-EMF exposed sperm to controls over the three different times noted a statistical significant difference in VSL (p = 0.05), the other two velocity parameters were border line significant [VAP (p = 0.062) & VCL (p = 0.093)]. At all three time points, RF-EMF exposed spermatozoa showed a decrease in all velocity parameters compared to controls. Furthermore an increase in SAR resulted in a dose related decrease in all velocity parameters with $Velocity_{5.7W/kg} < Velocity_{2.0W/kg}$.

(iii) Motion parameters

For SAR 2.0 W/kg (Figure V A): Linear regression analysis between RF-EMF exposed sperm and controls over the three time intervals, noted no statistical difference in ALH, BCF, STR, LIN or hyper-activated motility (data not shown).

For SAR 5.7 W/kg (Figure V B): There was no statistically significant difference (linear regression analysis) in ALH, STR, LIN or hyper-activated motility (data not shown) between RF-EMF exposed sperm and controls, however BCF was significantly lower (p = 0.04) in exposed sperm, compared to controls.

Correlation between mitochondrial membrane potential and progressive motility

To assess the relationship between the change in mitochondrial membrane potential and progressive motility, the latter was introduced as a co-variate in analysis of progressive motility using a "within subject" design. Two treatments (RF-EMF and control) were considered at three time points, for a total of 12 donors. At both SAR levels, $\triangle\psi m$ significantly correlated with progressive motility (p = 0.009 for SAR 2.0 W/kg and p = 0.002 for SAR 5.7 W/kg).

DISCUSSION

The National Radiation Protection Board (NRPB) [2003] recently reviewed the influence of RF-EMF radiation on reproduction and concluded that there was no convincing evidence

suggesting an effect. Furthermore, effects reported could be attributed to thermal insult induced by RF exposure. However, this conclusion as far as an effect of RF-EMF on male sexual function and fertility was drawn from a very limited number of studies (some of doubtful scientific basis) and exposure levels considered were orders of magnitude higher than that found in the mobile telephone range [NRBP, 2003]. What can be said with certainty about current knowledge concerning the influence of RF-EMF on male germ cells is that it is extremely limited.

Various studies have recently expounded on the possible influence of RF-EMF on DNA integrity of male germ cells [Aitken et al., 2005] as well as on sperm motility [Davoudi et al., 2002; Fejes et al., 2005; Erogul et al., 2006]. As with so many studies conducted in this field, some of the findings were criticized due to a lack of dosimetry and not taking confounding risk factors into account [European fast response team on EMF and Health, 2004]. Another possible weakness in the assessment of sperm motility as used by these studies is the lack of using an automated system such as CASA to eliminate operator bias in manual motility assessment.

Therefore, in the present study dosimetry was based on numerical simulations, which were validated by temperature rise based SAR measurements. In addition, heating would not be a likely factor in this study due to the cooling methods employed in the RF chamber and temperature control within the incubator. Furthermore, to avoid the effect of intra- and inter-observer variations in the assessment of sperm kinematic parameters, a computer assisted sperm analysis system for the quantification of sperm velocity- and motion-parameters was used. Since sperm motion is highly correlated with a high mitochondrial membrane potential [Marchetti et al, 2002, 2004], $\Delta\psi m$ was determined by flow cytometry as an additional measure of the energetic state of the sperm cell.

RF exposure at neither of the two SAR levels (2.0 and 5.7 W/kg) had any effect on progressive motility assessed by CASA, a finding in agreement with Fejes *et al*. [2005] who also noted no change in overall progressive motility after RF exposure. However, Fejes *et al*. [2005] did observe a decrease in rapid progressive and an increase in slow progressive spermatozoa after RF exposure. On the other hand, Erogul *et al*. [2006] found a decrease in both rapid and slow progressive spermatozoa, while non-progressive and immotile sperm populations increased after exposure. Davoudi *et al*. [2002] only noted a decrease in the proportion of rapid progressive sperm after prolonged exposure (1 month, 6 h/day). One commonality noted in all the studies is a reduction in rapid progressive motility, a result not observed in the present study. Not only did RF exposure at either 2.0 or 5.7 W/kg not affect rapid progressive motility; no effect was seen on slow progressive, non-progressive or immotile spermatozoa. Apart from the use of manual techniques of motility assessment in all of the above studies, motility was assessed in unprocessed semen samples. It is well known that leukocyte contamination significantly contributes to ROS generation [Whittington and Ford, 1999; Henkel *et al*., 2005] leading to amongst others a reduction in sperm motility [Armstrong *et al*., 1999]. It is thus possible that ROS generation due to leukocyte contamination and not RF-exposure could have contributed to the decreased progressive motile population observed in these studies, explaining the lack of an effect observed in the processed semen sample used in the present study.

During capacitation (which occurs *in vitro* after 2-3 hours of incubation leading to our choice of setting T_2 at 2 hours after exposure), spermatozoa become hyperactivated in preparation of oocyte penetration. As a result the sperm's velocity increases (determined by VAP, VSL and VCL) and the motion (determined by ALH BCF, STR and LIN) becomes more erratic. A decrease in any of these parameters could lead to a decrease in sperm fecundity. At a SAR of 2.0 W/kg no statistically significant effect on any of the kinematic-parameters were noted at any of the time points post irradiation. However, at a SAR of 5.7 W/kg a significant decrease in VCL and BCF parameters were observed. This result should not be ascribed to thermal effects, as the temperature rise during the exposure did not exceed 0.3ºC. Therefore, the observed statistically significant decline in motility parameters is either an artefact or it is possible that an alternative mechanism, such as intrinsic ROS generation, could be accountable for this effect. We have observed in subsequent experiments that the decrease in sperm motility parameters observed at the higher SAR level significantly correlated with an increase in intrinsic ROS generation noted the at the same SAR level [Falzone et al., 2006].

In experiments, mitochondrial membrane potential decreased over time, but no significant difference between RF-EMF exposed and control spermatozoa were noted. The lack of an effect on mitochondrial membrane potential observed directly, 2 hours and 24 hours after RF-EMF irradiation for both SAR 2.0 W/kg and SAR 5.7 W/kg correlates well with a recent observation by Capri and co-workers [2004]. These authors noted that, *in vitro* exposure of human lymphocytes to 900 MHz had no effect on the mitochondrial membrane potential assessed at different time points after the exposure. The high mitochondrial membrane potential observed in this study in sperm exposed at both SAR 2.0 W/kg and 5.7 W/kg, was statistically significantly correlated with progressive motility.

In conclusion, the exposure of sperm to RF-EMF had no effect on mitochondrial membrane potential. However, the observed changes in some of the sperm motility parameters should be examined further in order to determine whether other factors in addition to ROS generation but not mitochondrial membrane potential could be responsible for the observed effect. Furthermore, considering the recent reports noting an effect on sperm motility at lower SAR levels than that employed in the present study [Erogul et al., 2006; Fejes et al., 2005], the effect of RF-EMF on human spermatozoa motility, using the expanded analysis criteria set in this study, should be replicated at lower SAR levels that would simulate the radiation absorption from carrying the cell phone in a pocket close to the testes.

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Tables:

Table I. Mean percentage progressive motility ± SD after RF-EMF exposure in exposed and control spermatozoa (n = 12) determined directly- (T_1), 2h- (T_2) and 24h- (T_3) after exposure.

	Progressive motility (a + b)	RF-EMF	Control	p
A (SAR 2.0 W/kg)	T_1	86.8 ± 9.33	87.2 ± 7.32	p= 0.899
	T_2	86.2 ± 7.69	84.6 ± 9.18	
	T_3	62.7 ± 15.14	65.7 ± 19.15	
B (SAR 5.7 W/kg)	T_1	86.5 ± 7.44	86.8 ± 5.34	p= 0.935
	T_2	87.5 ± 8.56	86.1 ± 8.36	
	T_3	70.0 ± 14.51	65.0 ± 16.45	

Table II. Linear regression results of percentage rapid-, slow-, non-progressive and immotile spermatozoa after RF-EMF (2.0 and 5.7 W/kg) exposure compared to control spermatozoa.

Motile category	SAR 2.0 W/kg	SAR 5.7 W/kg	SAR 2.0 vs. 5.7 W/kg
Rapid - a	p = 0.401	p = 0.961	p = 0.821
Slow - b	p = 0.518	p = 0.477	p = 0.974
Non-progressive - c	p = 0.765	p = 0.961	p = 0.819
Immotile - d	p = 0.446	p = 0.946	p = 0.856

Legends to figures:

I. **Front view and set-up of RF-EMF exposure chamber [Leszczynski et al., 2002]**. Two glass petri dishes are placed inside the chamber on top of a temperature regulated waterbed. The RF-EMF signal is fed into the chamber placed inside a CO_2 incubator via a monopole type feed post.

II. Cytofluorometric analysis of the depolarisation of the mitochondrial membrane potential showing a frequency histogram of processed spermatozoa (blue) stained with, 150 nM MitoTracker® Red CMX-Ros before treatment (green) with the mitochondrial membrane potential abolisher mClCCP (red-brown).

III. **$\Delta\psi$m:** The mean percentage ± SD of MitoTracker® Red CMX-Ros (polarised population - $\Delta\psi m^{high}$) staining in RF-EMF (**A:** SAR 2.0 W/kg and **B:** SAR 5.7 W/kg) exposed compared to control sperm cells (n = 12) determined directly (T_1), 2 (T_2) and 24 hours post exposure (T_3).

IV. Velocity parameters comparing RF-EMF exposed spermatozoa for SAR 2.0 W/kg (A) and 5.7 W/kg (B) with controls directly (T_1), 2 (T_2) and 24 (T_3) hours after a 1 hour 900 MHz GSM exposure (*p<0.05).

V. Motion parameters comparing RF-EMF exposed spermatozoa for SAR 2.0 W/kg (A) and 5.7 W/kg (B) with controls directly (T_1), 2 (T_2) and 24 (T_3) hours after a 1 hour 900 MHz GSM exposure (*p<0.05).

Figure I

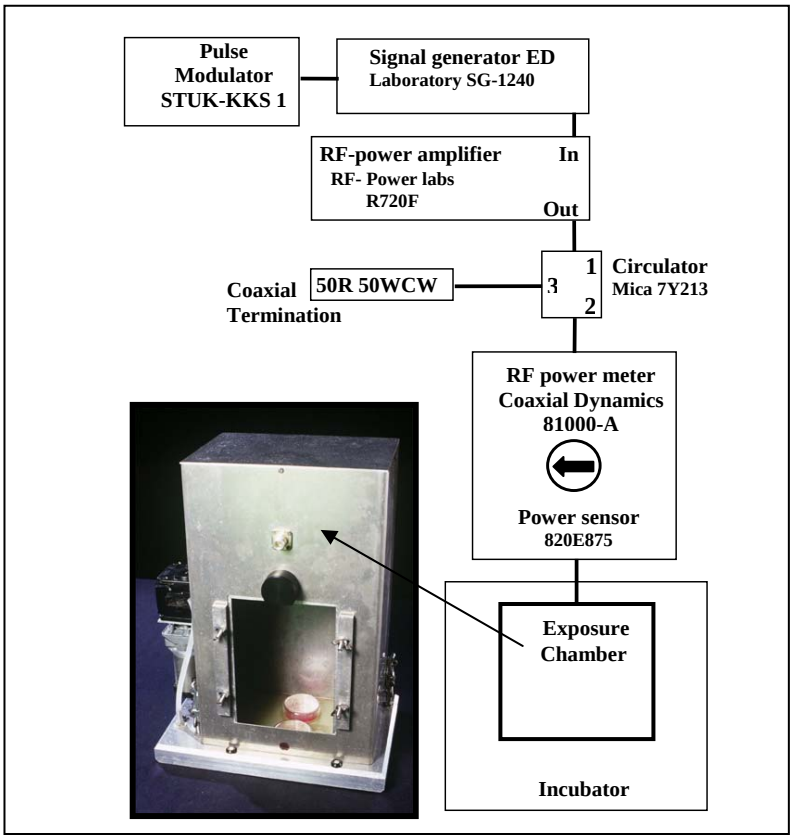

Pulse
Modulator
STUK-KKS 1
Signal generator ED
Laboratory SG-1240
RF-power amplifier
RF- Power labs
R720F
In
Out
Coaxial
Termination
50R 50WCW
3
1
2
Circulator
Mica 7Y213
RF power meter
Coaxial Dynamics
81000-A
Power sensor
820E875
Exposure
Chamber
Incubator

Figure II.

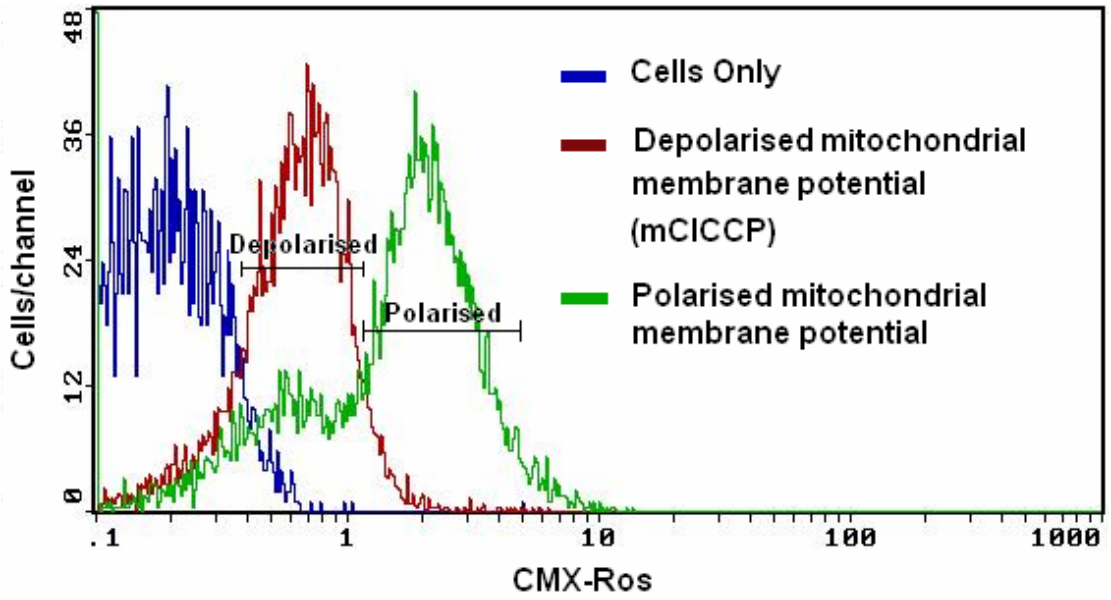


Figure III.

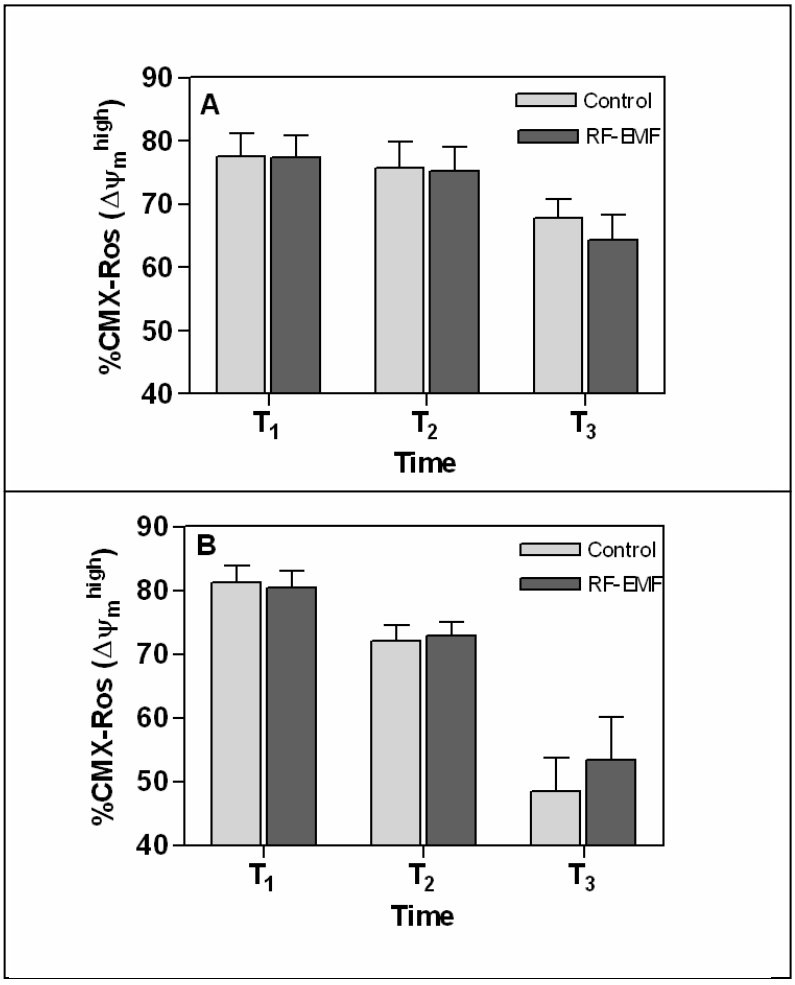


Figure IV.

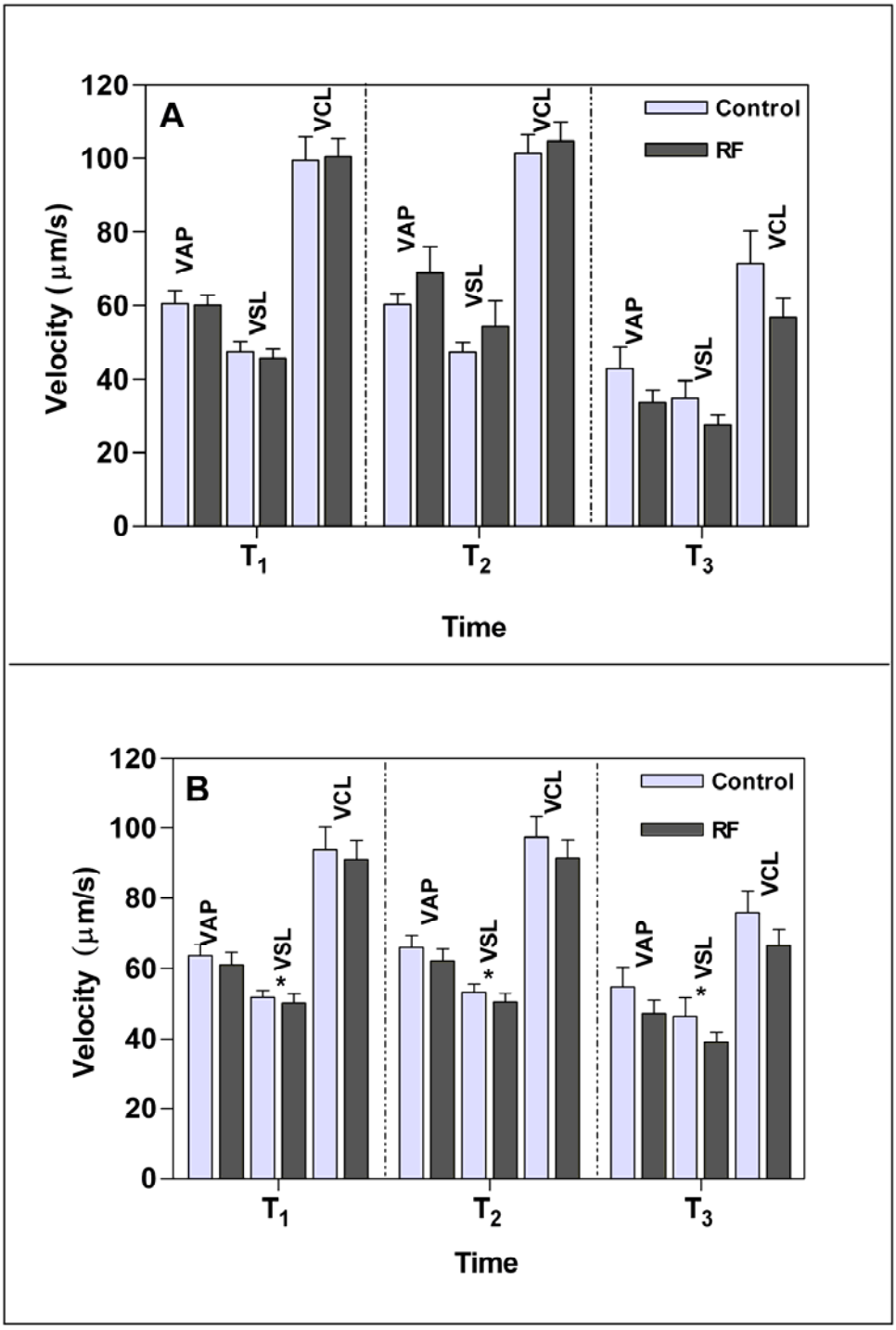
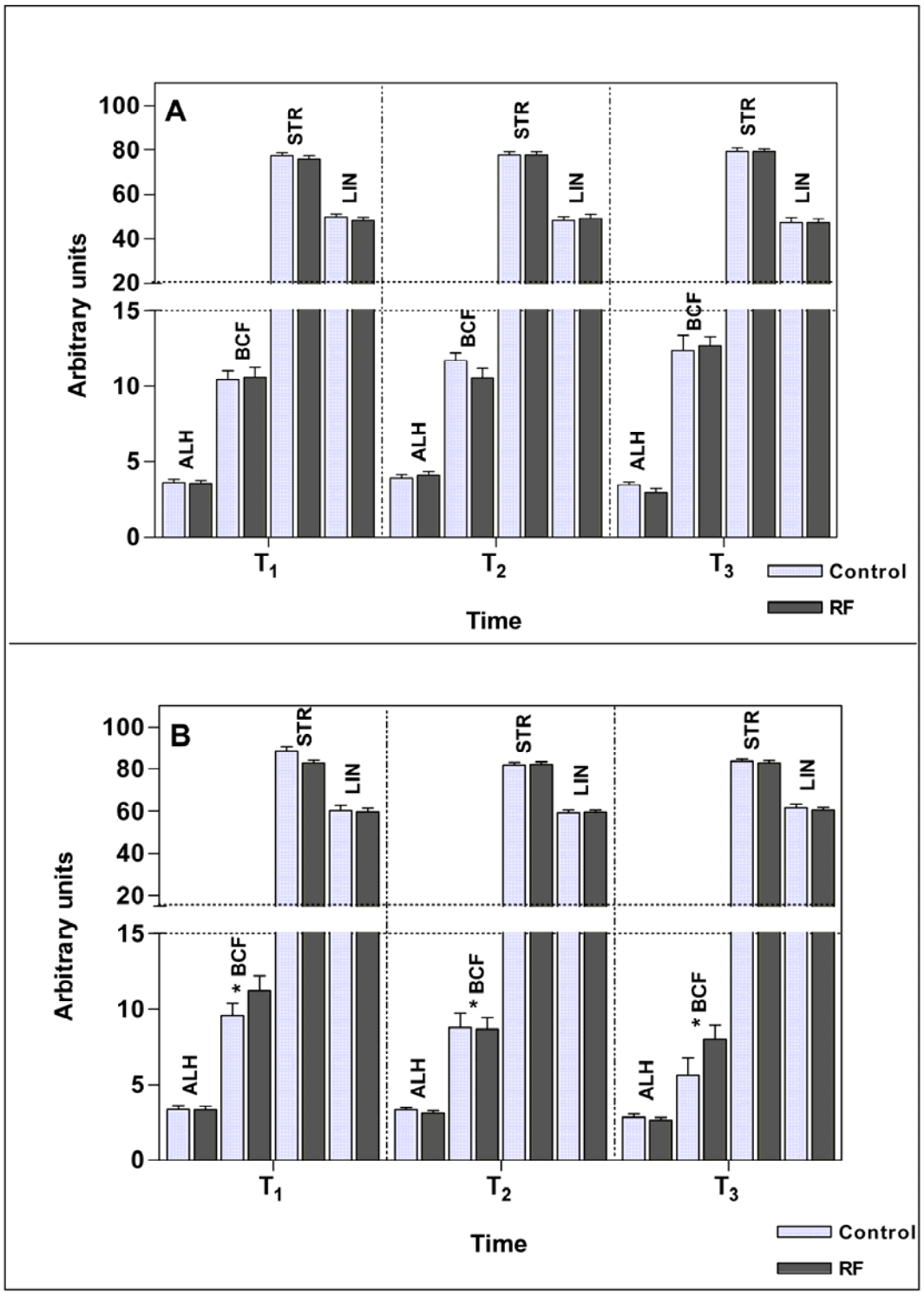

A
Control
RF
Velocity (μm/s)
120
100
80
60
40
20
0
VAP
VSL
VCL
T1
T2
T3
Time
B
Control
RF
Velocity (μm/s)
120
100
80
60
40
20
0
VAP
*VSL
VCL
T1
T2
T3
Time

Figure V.


A
B
Arbitrary units
Time
T1
T2
T3
ALH
BCF
* BCF
STR
LIN
Control
RF