# The effect of pulsed 900 MHZ GSM mobile phone radiation on the acrosome reaction, head morphometry and zona binding of human spermatozoa

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**Running Title**

Effect of RF-EMF exposure on human sperm

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**Abstract**

Several recent studies have indicated that radiofrequency electromagnetic fields (RF-EMF) have an adverse effect on human sperm quality, which could translate to an effect on fertilization potential. The present study evaluated the effect of RF-EMF on sperm-specific characteristics in order to assess the fertilizing competence of sperm. Highly motile human spermatozoa, were exposed for one hour to 900 MHz mobile phone radiation at a specific absorption rate (SAR) of 2.0 W/kg and examined at various times after exposure. The acrosome reaction was evaluated using flow cytometry. The radiation did not affect sperm propensity for the acrosome reaction. Morphometric parameters were assessed by computer assisted sperm analysis (CASA). Significant reduction in sperm head area ($9.2 \pm 0.7~\mu m^2$ vs. $18.8 \pm 1.4~\mu m^2$) and acrosome percentage of the head area ($21.5 \pm 4\%$ vs. $35.5 \pm 11.4\%$) were reported among exposed sperm compared with unexposed controls. Sperm–zona binding was assessed directly after exposure using the hemizona assay (HZA). The mean number of zona-bound sperm of the test hemizona and controls was $22.8 \pm 12.4$ and $31.8 \pm 12.8$ ($p<0.05$), respectively. This study concludes that while RF-EMF exposure did not adversely affect the acrosome reaction, it had a significant effect on sperm morphometry. In addition a significant decrease in sperm binding to the hemizona was observed. These results could indicate a significant effect of RF-EMF on sperm fertilisation potential.

**Introduction**

Infertility affects approximately 15% of couples of reproductive age worldwide. At infertility clinics, more than 30% of couples present with no clear diagnosis of a female cause and semen analysis typically show no gross abnormalities, such couples are classified as having idiopathic infertility (Liu *et al.*, 2004). Idiopathic infertility is often attributed to a so-called male factor such as abnormal morphology (Kruger *et al.*, 1986), delayed or no acrosome response (Liu *et al.*, 2004) or impaired sperm-zona binding (Franken *et al.*, 1993).

The reported decline in male fertility (Andersen *et al.*, 2000; Joffe, 2000; Skakkebaek *et al.*, 2001; Swan *et al.*, 2000) can be ascribed to several environmental factors (Claman, 2004; Sheiner *et al.*, 2003) amongst which RF-EMF emitted by mobile phones is one of the reported possible causes (Agarwal *et al.*, 2008a; Erogul *et al.*, 2006).

There are several studies available that show an effect of mobile phone irradiation on human spermatozoa. Aitken *et al.* (2005) reported on a genotoxic effect of RF-EMF, whereas other studies indicated an effect on sperm motility (Agarwal *et al.*, 2008a; Agarwal *et al.*, 2008b; Erogul *et al.*, 2006; Wdowiak *et al.*, 2007) as well as morphology (Agarwal, 2007). However, in contradiction with previous reports, our recent study revealed that pulsed 900 MHz mobile phone radiation operated at a SAR of 2.0 W/kg, the maximal permissible transmission power under current standards (ICNIRP, 1998) did not adversely affect specific sperm motion parameters as determined by CASA (Falzone *et al.*, 2008). Nonetheless a decline in motility alone does not render spermatozoa incapable of fertilization.

Among the array of sperm diagnostics test recommended by ESHRE's Andrology Special Interest Group (Mortimer & Fraser, 1996) for clinically relevant functional testing are the acrosome reaction and sperm-zona binding assays. Apart from these diagnostic assays, sperm morphology has also been shown to be a clinically reliable predictor of fertilization rates *in vitro* (Kruger *et al.*, 1986) and *in vivo* (Eggert-Kruse *et al.*, 1995).

The objective of this two-part study was therefore, (i) to investigate the effect of RF-EMF exposure on sperm head morphometry and the acrosome response, and (ii) to evaluate the consequence of RF-EMF on the spermatozoa's zona pellucida binding capacity. Morphometric evaluations were done using CASA, while the acrosome reaction was assessed using flow cytometry. These automated techniques were chosen to avoid operator bias in assessment. The HZA was evaluated by two independent observers under blinded conditions.

The study revealed a significant reduction of morphometric parameters in exposed sperm, however surprisingly the acrosome reaction was not affected. In addition, sperm zona binding decreased significantly in exposed samples. These findings were indeed unexpected considering that we previously noted no effect on sperm motility (Falzone *et al.*, 2008). Given the significance of these results, RF-EMF could potentially affect male fertilisation potential.

**Materials and Methods**

*Material*

Ham's F10 medium, bovine serum albumin (BSA), Dulbecco's phosphate buffered saline (DPBS), and fluorescein-labelled *Pisum sativum* agglutinin (PSA-FITC), were all obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). The fluorescent stain 7-amino-actinomycin D (7-AAD) was purchased from BD Biosciences (Pharmingen, NJ, USA).

*Irradiation Equipment*

Mobile phone microwave radiation (900 MHz pulse modulated RF) was simulated in a specially constructed exposure system formerly described (Leszczynski *et al.*, 2002). Characterisation of the chamber and dosimetry was as reported previously (Falzone *et al.*, 2008). The irradiation chamber was placed vertically inside a Forma $CO_2$ incubator (Labotec (Pty) Ltd, Johannesburg, South Africa). Two 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 Electric-field vector, while controls were kept at 37 ºC next to the irradiation chamber. Temperature controlled water was circulated through a thin rectangular glass-fibre-moulded waterbed underneath the petri-dishes. Cells were exposed for 1 h to a 900 MHz Global Systems of Mobile (GSM) communication-like signal at an average SAR of 2.0 W/kg. During the exposure, 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 mean age of the study population was $23 \pm 5$ years ($\pm$ SD). The study was conducted according to the Declaration of Helsinki for medical research and institutional approval was also granted (Ethics Committee application no. 163/2003, University of Pretoria). The semen samples were allowed to liquefy at 37 °C, after which standard semen parameters were evaluated (Word Health Organisation, 1999). All semen samples conformed to the WHO criteria and sperm morphology assessments were performed according to Tygerberg strict criteria (Kruger *et al.*, 1986).

Motile sperm fractions were retrieved by a three-step discontinuous Percoll gradient (95%, 70% & 50%) diluted in Ham's F10 medium supplemented with 0.5% BSA. These fractions were washed (300 g for 10 min), recovered and re-suspended ($20 \times 10^6$ sperm/ml) in 1 ml 0.5% BSA supplemented Ham's F10 medium. Duplicate control and RF-EMF exposed (test) dishes were simultaneously prepared. Controls were placed in a humidified $CO_2$ incubator maintained at 37 °C and 6% $CO_2$, while test dishes were exposed for one hour inside the RF-EMF chamber placed inside the same incubator.

Directly after the control/RF-EMF exposure, sperm were gently recovered from the petri-dishes and transferred to separate conical test tubes. Spermatozoa were then incubated under capacitating conditions (6% $CO_2$ at 37°C) and assessed at different times, directly ($T_1$) as well as 2 h ($T_2$) and 24 h ($T_3$) h after exposure (unless otherwise stated).

*Computerized Morphometric Assessment*

Duplicate slides were prepared for each donor directly ($T_1$), and 2h ($T_2$) after RF-exposure. Slides were left to air-dry until staining at a later time. A rapid staining procedure for sperm morphology assessment (Hemacolor, Merck Chemicals, Darmstadt, Germany) was used to stain cells (Cooper *et al.*, 1992).

The prepared cells were examined with the Hamilton Thorne IVOS computer aided sperm analysis (CASA) system (IVOS 10, Hamilton Thorne Research, Danvers, MA). A total of 200 sperm were assessed per slide. Sperm cells were displayed at equivalent brightness on the monitor and only cells that did not present any overlap with debris or other cells were considered for analysis. Morphometry values were evaluated with the Hamilton Thorne IVOS using Metrix Software. In the case where the original image and the vetted image did not correspond, the image was discarded. The following parameters were determined; major and minor axis ($\mu$m), area ($\mu m^2$), perimeter ($\mu$m) and the acrosomal region (%).

*Assessment of the acrosomal status*

For assessment of acrosomal status directly after exposure as well as 2 and 24 h after exposure, duplicate samples taken from the RF-EMF exposed and control sperm were stained with the viability probe 7-AAD in order to assess the acrosome reaction in live sperm only. The acrosome was assessed with PSA-FITC according as previously described (Falzone *et al.*, 2009). Specimens were gated by light scatter properties (size and granularity) of spermatozoa and analysed for dual colour fluorescence using flow cytometry. Flow cytometry was performed on a Coulter Epics® XL.MCL flow cytometer

(Beckman Coulter, Miami, Florida, USA) equipped with an air-cooled argon laser. The sperm population was identified using forward-angle light scatter, while side-angle light scatter was used to exclude electronic noise and debris. Analysis was done with System II software and the results were expressed as the mean cell number (cells/channel vs. % stain). A total of 10 000 events were acquired for each endpoint.

*Sperm-oocyte interaction*

Institutional Ethics approval was obtained for the use of non-fertilized oocytes from patients with access oocytes undergoing IVF treatment (University of Pretoria, no S. 233/2003). A total of 5 oocytes (metaphase II) were used per donor. Non-fertilized oocytes were prepared for cryostorage as reported earlier (Hammitt *et al.*, 1993). On the day of use, the straw's content was emptied by pushing a steal plunger gently through the straw expelling the oocytes into a petri dish. The oocytes were transferred to a droplet containing thawing medium (2.5 M Sucrose, 3% BSA in Hams F10 medium). Oocytes were then washed three times before commencing with bisection. Cutting was performed under an inverted phase contrast microscope (Axiovert200, Carl Zeiss (Pty)Ltd, Germany) with a micromanipulation system (Narishige, Japan). Once bisected, the ooplasm was dislodged by mouth micro-pipetting. The hemizonae were kept at room temperature until addition of spermatozoa.

*Hemizona assay*

After RF-EMF exposure, sperm concentrations were adjusted to 0.5 x $10^6$ cells/ml, of this 50 µl droplets of RF-EMF exposed and control spermatozoa were placed into

separate Petri dishes. The hemizonae were then transferred to the droplets, the droplets were covered with mineral oil and incubated for a further 4 h until assessment. By calculating the ratio of spermatozoa bound to the two halves, an indication of the binding capacity of the control sperm sample versus the sample exposed to RF-EMF was obtained.

*Statistical Analysis*

A sample of 12 donors were chosen to have a 95% power to detect a clinical relevant difference of 5% in mean increase over 24 h between RF-EMF exposed and control samples using a paired t-test at the 0.05 level of significance.

To determine the effect of RF-EMF on morphometry, data from all 12 donors were analysed with Stata Statistical Software Release 8.0 (Stata Corp. 2003, USA). A within subject design considering two treatments, control vs. RF-EMF (SAR 2.0 W/kg), at two times ($T_1$- directly after exposure and $T_2$- 2 h after exposure) was used and results were analysed using a time series regression analysis under the random effects option. Similarly acrosomal status was assessed at three times ($T_1$- directly after exposure, $T_2$- 2 h after exposure and $T_3$-24 h after exposure). Data are presented as mean values $\pm$ SD.

To determine the effect of RF-EMF on the hemizona binding assay, data (from 10 donors) were analysed using a Wilcoxon signed ranks test (Stata Statistical Software). All statistical tests were two-sided and statistical significance was considered when $p < 0.05$.

**Results**

*Morphometric analysis*

Time series regression analysis of all morphometric parameters comparing RF exposed sperm with controls revealed a statistically significant reduction in all the morphometric parameters (Table 1). RF-EMF exposed sperm were also compared directly ($T_1$) and 2 h ($T_2$) after exposure, but no difference was noted in any of the sperm morphometric parameters as a function of time.

*Acrosome Reaction*

Flow cytometric results from all donors are summarised in Fig. 1. The number of non-viable cells (7-AAD positive) assessed at the three different time points (Fig. 1A), did not differ significantly between RF-EMF exposed and control cells ($p = 0.38$). The number of acrosome reacted live (FITC positive, 7-AAD negative) spermatozoa increased with time (Fig. 1B). However, there was no statistical difference ($p = 0.84$) between RF-EMF exposed and control sperm at any of the time points. The number of acrosome intact live (FITC negative, 7-AAD negative) spermatozoa decreased with time (Fig. 1C), but there was no statistical difference between exposed sperm and controls ($p = 0.92$).

Although not statistically significant, the biggest difference between exposed and control sperm when considering the number of acrosome reacted as well as intact live sperm were noted at time point $T_2$ (2 h post exposure). At this time point, RF-EMF exposure caused a 15% increase in acrosome reacted (decrease in acrosome intact) live sperm compared to control cells.

**Table 1** Mean ± SD results of morphometric data of sperms exposed to 2.0 W/kg vs. control and $p$-values of the time-series regression analysis of sperm morphometry

| Parameter | Control (mean ± SD) | RF ($T_1$) (mean ± SD) | RF ($T_2$) (mean ± SD) | RF vs. control $p$-values | $T_1$ vs. $T_2$ $p$-values |
|---|---|---|---|---|---|
| Major axis (μm) | 5.93 ± 0.60 | 3.53 ± 0.17 | 4.85 ± 0.33 | 0.002** | 0.842 |
| Minor axis (μm) | 4.45 ± 0.23 | 2.39 ± 0.10 | 3.3 ± 0.31 | 0.022* | 0.380 |
| Area (μm$^2$) | 18.83 ± 1.37 | 9.24 ± 0.66 | 11.12 ± 1.45 | 0.001** | 0.810 |
| Perimeter (μm) | 16.67 ± 1.19 | 9.36 ± 0.37 | 13.23 ± 0.79 | 0.004** | 0.531 |
| Acrosome (%) | 35.45 ± 11.37 | 21.48 ± 3.98 | 19.9 ± 2.47 | 0.006* | 0.140 |

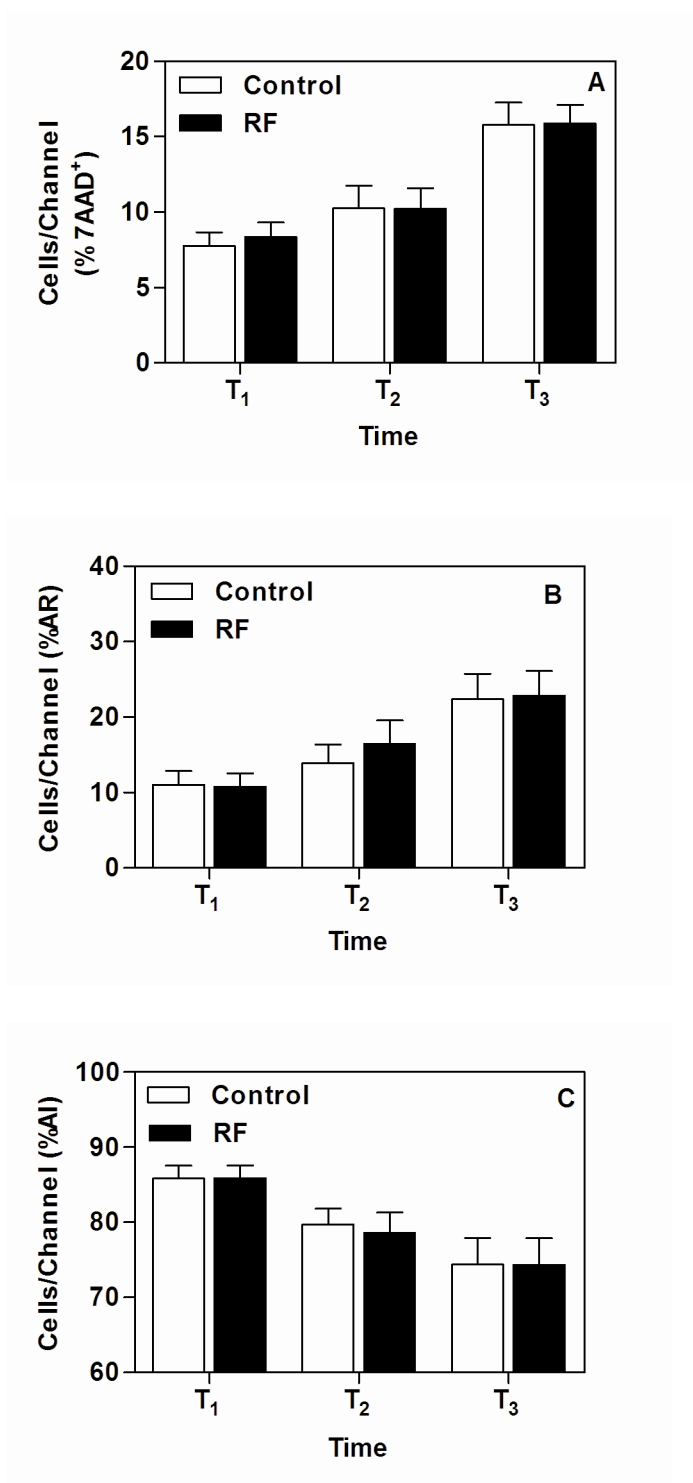*$p < 0.05$, **$p < 0.005$.
RF, radiofrequency.

**Figure 1:** Summary of FCM results from donors (n = 12) noting non-viable cells, (A) % 7-AAD positive stained spermatozoa, (B) % acrosome reacted live, and (C) % acrosome intact live cells.

*Sperm-oocyte interaction*

Results of the Wilcoxon signed ranks test are illustrated in Fig. 2. There was a statistically significant (p = 0.02) reduction in zona binding in RF-EMF exposed sperm compared to controls. Controls bound an average of 31.8 ± 12.8 sperm / hemizona compared to 22.8 ± 12.4 sperm / hemizona of RF-EMF exposed spermatozoa. Sperm samples from all donors used in this assay bound on average more than 20 control sperm/hemizona, the minimum criterion for limiting false-negative results with the HZA (Oehninger *et al.*, 1991).
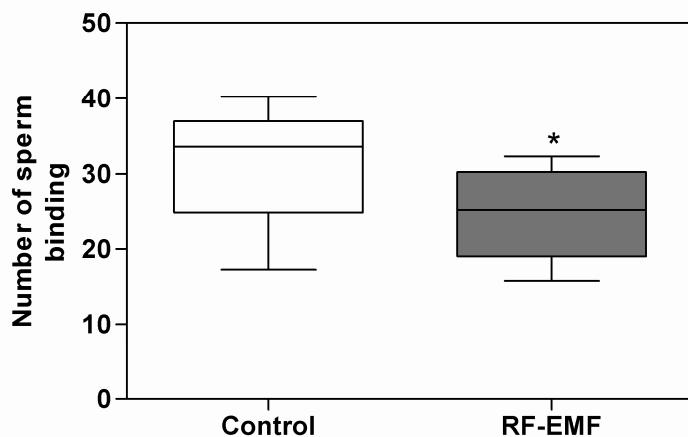


**Figure 2:** Box and whiskers plot of sperm-zona binding after an hour exposure to RF-EMF (control vs. RF-EMF, p=0.02)

**Discussion**

In the current study, highly motile spermatozoa were pre-selected from density purified sperm fractions as these sperm have the potential to fertilize the human oocyte. Furthermore, spermatozoa were exposed to the highest permissible power output (SAR of

2.0 W/kg) from mobile phones to elicit a worse case scenario. The same principles were followed in our previous study reporting no effect on sperm motility parameters (Falzone *et al.*, 2008). It was therefore interesting to note that although sperm motility under these exposure conditions remained unaffected, a significant decline in sperm morphometry parameters were noted. In the same study we also investigated the effect of RF-EMF on mitochondrial membrane potential (mmp) which is a sensitive indicator of the energetic and functional state of mitochondria and the cell (Ly *et al.*, 2003). In particular, a decrease in mmp is highly correlated with diminished sperm motility and fertilisation potential (Marchetti *et al.*, 2002; Wang *et al.*, 2003). Mitochondrial membrane potential remained unaffected by RF-EMF, thus we could conclude that the current results were either an artefact or the mechanism by which RF-EMF affects sperm cells caused a change in morphology that did not affect the sperm motility or the energetic state of the cell. Both Agarwal *et al.* (2008a) and Wdowiak *et al.* (2007) noted an increase in abnormal sperm morphology which was dependent on the duration of daily exposure to mobile phone radiation. However it was not noted in their assessment of sperm morphology, if RF-EMF affected sperm head size. Agarwal *et al.*, (2008b) concluded from their study that the decline in sperm vitality could be the result of reactive oxygen species (ROS) generation. Since they evaluated the effect of RF-EMF on neat semen, and the current study was designed to assess the effect on purified spermatozoa, it could be that leukocyte contamination could have affected their results as they did not exclude these cells during their evaluation.

From all the morphometric parameters evaluated, the acrosomal region was also significantly reduced after RF-irradiation. To evaluate if this observation could have an

effect on the spermatozoa's ability to undergo the acrosome reaction, acrosomal status was assessed in RF exposed spermatozoa using flow cytometric analysis.

In current experiments no significant difference was noted in the ability of capacitated spermatozoa to initiate the acrosome reaction after RF exposure. On the other hand CaI induction led to a significant increase in acrosome reacted spermatozoa using the same protocol (Falzone *et al.*, 2009). Flow cytometric results cannot explicate if RF exposure had an effect on the acrosomal region. This is a main limitation of assessing acrosomal status by flow cytometry as detailed information regarding the stage of AR cannot be provided as with the visual method (Nikolaeva *et al.*, 1998). Therefore it is possible that RF exposure could result in a reduction of the acrosomal region as was observed with CASA evaluation of the morphometric features of the sperm cells. To confirm if these observations could affect sperm fecundity we subsequently evaluated the binding potential of spermatozoa to the human zona pellucida after RF exposure.

RF-EMF exposure resulted in a statistically significant reduction in sperm binding at a SAR of 2.0 W/kg compared with controls. Taking into consideration that the number of spermatozoa bound to the ZP correlates with fertilisation rate (Oehninger *et al.*, 2000), RF exposure therefore has the potential to affect male fertility and possibly impair fertilisation rates.

However before RF-EMF can be added to the list of external factors affecting male fertility a clearer understanding of the possible mechanism responsible for this effect should be elucidated. The decreased acrosomal region after RF exposure could be responsible for the reduction in zona binding observed in the HZA. If a stress pathway is activated in spermatozoa as a result of RF exposure it is possible that Hsp27 activation,

known to be involved in stress fibre stabilisation due to increased actin polymerisation (Landry & Huot, 1995) could result in the shrinkage noted in exposed spermatozoa. This would confirm an observation by Leszczynski *et al.* (2002) who ascribed the shrinkage of endothelial cells, after 900 MHz GSM irradiation, to Hsp27 activation and phosphorylation. In addition, if a stress response is initiated in spermatozoa, heat shock protein activation and phosphorylation could potentially interfere with signalling pathways in preparation of capacitation and sperm- zona pellucida interactions. It has been shown that Hsp 70 and 90 are involved in various processes of sperm capacitation as well as zona pellucida recognition and oolemma fusion (Bohring & Krause, 2003; Ecroyd *et al.*, 2003; Huang *et al.*, 2000a; Huang *et al.*, 2000b; Huszar *et al.*, 2003; Matwee *et al.*, 2001). In particular, antibodies to Hsp70 significantly reduced tight binding of spermatozoa to the zona pellucida (Neuer *et al.,* 1998). Therefore our laboratory also investigated the activation of a stress pathway as a result of RF-EMF in human spermatozoa.

In conclusion, an *in vitro* result cannot and should not be extrapolated to an *in vivo* situation, without additional research and ultimately epidemiological confirmation. It is however of concern that RF exposure affected the outcome of the hemizona assay, a test that has clinical significance to predicting male fertilising potential.

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