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THE EFFECT OF NON THERMAL 900 MHZ GSM IRRADIATION ON HUMAN SPERMATOZOA

by

NADIA FALZONE

Submitted in fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR
(REPRODUCTIVE BIOLOGY)

in the

DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF PRETORIA

Supervisor: Dr. C. Huyser
Co-Supervisors: Profs. D. R. Franken and D. Leszczynski

April 2007

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To Paolo

Thank you for your support in reaching this goal.

"I can do everything through Him who gives me strength"

Phil 4: 13.

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DECLARATION BY CANDIDATE

" I hereby declare that the thesis submitted for the degree Philosophiae Doctor, at the University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references".

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ABSTRACT

Several studies have highlighted the possibility that radio-frequency electromagnetic fields (RF-EMF) used in mobile phone technology could influence DNA integrity of male germ cells as well as sperm motility. Current knowledge concerning the influence of RF-EMF on male germ cells is extremely limited. In the present study the hypothesis that 900 MHz GSM radiation could induce the activation of stress response in human spermatozoa was investigated.

Ejaculated, density purified, human spermatozoa from donors were exposed to 900 MHz GSM mobile phone radiation at specific absorption rate (SAR) levels of 2.0 and 5.7 W/kg and examined at various time points post exposure. Sperm motility and morphology were evaluated by computer-aided sperm analysis (CASA). The ability of RF-EMF exposed sperm to undergo the acrosome reaction was evaluated by flow cytometry. Sperm binding to the zona pellucida of human oocytes was determined by the hemi-zona (HZA) assay. Apoptotic markers, phosphatidylserine (PS) externalization, change in mitochondrial membrane potential ($\Delta\psi m$), reactive oxygen species (ROS) generation, caspase activation and DNA fragmentation were analysed using flow cytometry. Heat shock protein (Hsp) 27 and 70 expression and activity were analyzed using specific antibodies with flow cytometry and Western blot methods. Stress fibre stabilization (F-actin polymerization) was visualized using fluorescent dye labelled phalloidin.

No effect was seen on kinematic parameters assessed at SAR 2.0 W/kg, however straight line velocity (VSL) and beat cross frequency (BCF) were significantly altered after exposure at SAR 5.7 W/kg. Sperm shrinkage (decrease in surface area) was observed at both exposure levels. RF-EMF did not influence exposed spermatozoa's ability to undergo the acrosome reaction. A significant decrease in sperm-zona binding was observed at both exposure levels. RF radiation did not have an effect on any apoptotic markers. ROS generation increased significantly with an increase in SAR (5.7 W/kg). RF-EMF did not induce a stress response in exposed sperm (no activation of Hsp70 and 27 activity).

These results cannot be ascribed to heating, as the temperature did not increase by more than 0.2 - 0.3ºC during exposure. The decrease in sperm-zona binding is the result of an alternative non-stress inducible pathway. 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.

KEY WORDS

human spermatozoa, mobile phone radiation, sperm functionality, stress response, apoptosis.

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STRUCTURE AND SCOPE OF THE THESIS

SECTION A: REVIEW OF LITERATURE

Chapter 1 provides an overview of the operating principles of mobile phones, in addition the biological mechanism/s of radio-frequency electromagnetic fields (RF-EMF) are discussed, and scientific evidence is presented of *in-vitro* biological effects. Based on evidence provided that RF-EMF could interact with biological systems by inducing a stress response, a review on current literature elucidating the stress response phenomenon was conducted. **Chapter 2** therefore addresses the molecular basis for cellular stress in somatic cells. The occurrence of a stress pathway in human spermatozoa and the implications thereof for male fertility were considered.

SECTION B: THE EFFECT OF NON-THERMAL 900 MHz GSM MOBILE PHONE RADIATION ON HUMAN SPERMATOZOA

In **Chapters 3**, **4** and **5** the effect of 900 MHz mobile phone radiation on human spermatozoa was evaluated at two specific absorption rates (SAR) of 2.0 and 5.7 W/kg. RF-EMF exposure of spermatozoa was firstly assessed using sperm specific assays (sperm capacitation and sperm-zona binding – **Chapter 3**). Based on these findings the hypothesis that a stress pathway as a result of RF-EMF insult is operational in human spermatozoa was tested. The induction of apoptosis (**Chapter 4**) and activation of heat shock proteins (**Chapter 5**) after RF-EMF were evaluated.

SECTION C: CONCLUSIONS

In **Chapter 6** a summary of the findings of this study are given, conclusions are drawn, and recommendations for future research are provided.

SECTION D: ANNEXURES

Annexure A addresses the characterisation of the vertical RF chamber used in this research. Dosimetric analysis results are also summarised**.** Dosimetric evaluations were conducted at STUK, Radiation and Nuclear Safety Authority, Helsinki, Finland

Annexure B contains a summary of the macroscopic and microscopic sperm parameters used in the evaluation of the sperm donors.

Annexure C summarises the results of the macroscopic and microscopic sperm assessment of each donor.

OBJECTIVES OF THE STUDY

Human semen parameters could serve as valuable indicators of toxic and genotoxic effects of occupational and environmental factors. Furthermore, spermatozoa are terminally differentiated cells that are unable to repair DNA damage, which make sperm an extremely sensitive model to use in the investigation of the effect of environmental stressors such as RF-EMF.

To determine:

1. if and by which mechanism RF radiation from mobile phone emissions affect human spermatozoa and what implication these findings have on male fertility,
2. the effect of RF exposure on sperm capacitation and sperm-zona binding,
3. if a stress response is operational in spermatozoa as a result of RF-EMF, by investigating;
    i. the induction of apoptosis,
    ii. heat shock protein phosphorylation and expression,
4. the suitability of using human spermatozoa as a reproductive model to indicate effects/influences of mobile phone radiation.

SUMMARY

Several studies have highlighted the possibility that radio-frequency electromagnetic fields (RF-EMF) used in mobile phone technology could influence DNA integrity of male germ cells as well as sperm motility. Current knowledge concerning the influence of RF-EMF on male germ cells is extremely limited. The main objective of this research was directed at determining the effect of non-thermal 900 MHz GSM modulated RF-exposure on human sperm fecundity by assessing sperm specific functions, sperm functionality and induction of a stress response.

Ejaculated, density purified, human spermatozoa obtained from donors were exposed to RF-EMF at two specific absorption rate levels (SAR 2.0 and 5.7 W/kg) and examined at various time points post exposure. To determine the influence of RF exposure on sperm specific functions, sperm propensity for acrosomal exocytosis was assessed using a new technique developed to evaluate the acrosome reaction (AR) by flow cytometry. Sperm motility and morphometry were determined by computer aided sperm analysis (CASA) and sperm binding potential was evaluated by the hemi-zona assay (HZA). 900 MHz GSM exposure had no effect on the AR however some motility parameters (straight line velocity and beat cross frequency) were significantly altered. Sperm surface area and acrosomal region were also significantly reduced as a result of RF-EMF. The ability of RF-exposed sperm to bind the human oocyte evaluated with the HZA was significantly impeded.

Sperm functionality was assessed using flow cytometry; (i) the percentage of Annexin-V positive and, propidium iodide (PI) negative spermatozoa, (ii) the change in spermatozoa's mitochondrial membrane potential ($\Delta\psi m$), (iii) caspase activation, (iv) the percentage of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) positive spermatozoa and (v) generation of reactive oxygen species (ROS) were examined. No effect on any of the examined parameters after 900 MHz GSM exposure at either SAR level was noted. These results show that mobile phone radiation does not induce apoptosis.

The ability of 900 MHz GSM radiation to induce a stress response was evaluated by heat shock protein (Hsp) activation. Hsp27 and Hsp70 expression as well as activity were analyzed using specific antibodies with flow cytometry and Western Blot methods. Stress fibre stabilisation (F-actin polymerization) was visualized using fluorescent dye labelled phalloidin. RF-EMF had no effect on Hsp27 expression and phosphorylation nor Hsp70 expression as determined by flow cytometry and Western blot analysis. Visual assessment of stress fibre stabilization after RF exposure in sperm cells did not show any increased F-actin accumulation in the cells. RF-EMF exposure did not induce an Hsp27- or Hsp70-dependent stress response in human spermatozoa.

The effect of RF-EMF on sperm-zona binding and cell shrinkage observed at exposures of 2.0 and 5.7 W/kg, seems to be the result of an alternative non-Hsp dependent mechanism. Additional studies investigating the effect of RF-EMF on sperm-zona binding should be conducted, specifically exploring the ligand-receptor effector systems involved in sperm-zona binding. It is also suggested that electron microscopy be used to investigate conformal and structural changes as a result of RF-EMF. Considering recent reports noting an effect on sperm motility at lower SAR levels than that employed in the present study, 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.

PUBLICATIONS

Submitted for publication:

1) Falzone, N., Huyser, C., Fourie, F le R., Leszczynski, D., Franken, D.R. *In vitro* effect of 900 MHz GSM radiation on mitochondrial membrane potential and motility of human spermatozoa. *Bioelectromagnetics*., submitted February 2007. (Approved for publication April 2007).
2) Falzone, N., Huyser, C., Becker, P., Fourie, F le R., Leszczynski, D., Franken, D.R. The effect of non-thermal 900 MHz GSM mobile phone radiation on the acrosome reaction, head morphometry and zona binding of human spermatozoa. *Hum Reprod*., submitted July 2007.

In preparation:

1) Falzone, N., Huyser, C., Franken, D.R. Leszczynski, D. Lack of activation of Hsp27- and Hsp70-dependent stress response in human spermatozoa exposed to 900 MHz GSM radiation. *Bioelectromagnetics*., submittion September 2007.
2) Falzone, N., Huyser, C., Fourie, F le R., Cockeran, R., Leszczynski, D., Franken, D.R. *In vitro* exposure of human spermatozoa to 900 MHz GSM radiation: effect on apoptosis. *Rad Res*., submittion November 2007.
3) Falzone, N., Huyser, C., Cockeran, R., Becker, P., Franken, D.R. Significance of somatic apoptosis markers in human spermatozoa.

Conference papers:

1) Falzone, N., Huyser, C., Fourie, F le R., Franken, D.R., Leszczynski, D., 2005. Pilot study: Effects of 900 MHz GSM radiation on human sperm function. Annual Bioelectromagnetics Society meeting, Dublin, Ireland, June, 2005.
2) Falzone, N., Huyser, C., Fourie, F le R., Franken, D.R., Leszczynski, D., 2006. Effect of 900 mhz gsm radiation on apoptotic status and functionality of human spermatozoa. Annual Bioelectromagnetics Society meeting, Cancun, Mexico, June, 2006.

3) Falzone, N., Huyser, C., Fourie, F le R., Leszczynski, D., Franken, D.R., 2007. Effect of 900 MHz GSM radiation on human sperm functionality. IFFS conference, Durban, South Africa, May, 2007.

4) Falzone, N., Huyser, C., Fourie, F le R., Franken, D.R., Leszczynski, D., 2007. Lack of activation of Hsp27- and Hsp70-dependent stress response in human spermatozoa exposed to 900 MHz GSM radiation. Annual Bioelectromagnetics Society meeting, Kanazawa, Japan, June, 2007.

LIST OF ABBREVIATIONS

7-AAD - 7-amino-actinomycin D

? ψ_m - Change in the mitochondria membrane potential

aCp - Active caspase

AIF - Apoptosis inducing factor

ALH - Amplitude of lateral head displacement

AR - Acrosome reaction

ART - Artificial reproductive technologies

ATP - Adenosine triphosphate

BCF - Beat cross frequency

BSA - Bovine serum albumin

CASA - Computer aided sperm analysis

CDMA - Code Division Multiple Access

Cp - Caspase

CW - Continuous wave

DNA - Deoxyribonucleic acid

DISC - Death-inducing signalling complex

DPBS - Dulbecco's phosphate buffered saline

DMSO - Dimethyl sulfoxide

ELF - Extremely low frequencies

EM - Electromagnetic

EMF - Electromagnetic field

ER - Endoplasmic reticulum

ERK - Extracellular regulated kinases

FCM - Flow cytometry

FDMA - Frequency Division Multiple Access

FDTD - Finite Difference Time Domain

FITC - Fluorescein isothiocyanate

HSE - Heat shock element

HSF - Heat shock transcriptional factor

Hsc - Heat shock cognate protein

Hsp - Heat shock protein

HYPA - Hyper activated motility

HZA - Hemi zona assay

IVF - *In vitro* fertilization

kD - kilo Dalton

MAPK - Mitogen activated protein kinases

MMP - Mitochondrial membrane potential

MOMP - Mitochondrial outer membrane permiabilisation

PBS - Phosphate buffered saline

PCD - Programmed cell death

pCp - Pro-caspase

PBS - Phosphate buffered saline

PS - Phosphatidylserine

PI - Propidium iodide

PSA - *Pisum sativum agglutinin*

PTPC - Permeability pore complex

RF - Radio frequency

RF-EMF - Radio frequency electromagnetic field

ROS - Reactive oxygen species

SAR - Specific absorption rate

SD - Standard deviation

STR - Straightness

TDMA - Time Division Multiple Access

TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

VAP - Average path velocity

VCL - Curvilinear velocity

VSL - Straight line velocity

ZP - Zona pellucida

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SECTION A

LITERATURE REVIEW

CHAPTER 1

BIOLOGICAL EFFECTS OF MOBILE PHONE RADIATION

CHAPTER 2

MOLECULAR BASIS FOR CELLULAR STRESS:
OCCURRENCE IN HUMAN SPERMATOZOA AND
IMPLICATIONS FOR MALE FERTILITY

CHAPTER 1

BIOLOGICAL EFFECTS OF MOBILE PHONE RADIATION

1.1 INTRODUCTION

It is estimated that there are currently 1.5 billion mobile phone users (hand-held cellular phones as well as newer personal communication services that deliver voice, data and images) globally (SCENIHR, 2006). The widespread use of mobile phones has brought about public concern regarding possible health effects of these devices, especially concerns of brain cancer (Moulder *et al*., 1999; IEGMP, 2000; Hardell *et al*., 2003; Moulder *et al*., 2005), as the antennas of these devices lie along the head during use (Stuchly, 1998; Bit-Babik *et al*., 2003). An increasing incidence of testicular cancer reported by several Western countries (Bray *et al*., 2006) has sparked the concern that electromagnetic fields (EMFs) emitted by mobile phones may be a risk factor for testicular cancer (Hardell *et al*., 2006).

What is troubling is that scientific knowledge gleaned from research conducted over the past 50 years on possible biological effects of EMFs is at most contradictory or inconsistent and therefore unreliable (REFLEX report, 2004). This current uncertainty has not helped to appease public concern and has resulted in increased allegations in the media and in the courts that cellular phones and other types of hand-held transceivers are a cause of cancer (Moulder *et al*., 1999; IEGMP, 2000). The scientific community base risk assessment of mobile phone use on epidemiological studies. Although these studies are needed to ultimately validate the extent of any potential health hazard of EMF (REFLEX report, 2004), this research has to be supported by data from animal and *in vitro* studies until essential exposure metrics have been established based on mechanisms of field interactions in tissues. The present study therefore focuses on the *in vitro* assessment of biological effects elicited by radio-frequency modulated electromagnetic fields (RF-EMFs).

In the following paragraphs the operating principles of mobile phones will be elucidated, the biological mechanism discussed, and scientific evidence presented of *in-vitro* biological effects.

1.2 RADIO-FREQUENCY FIELDS FROM MOBILE PHONES – PHYSICS AND DOSIMETRY

Mobile phones and base stations transmit and receive information (voice messages, fax, computer data, images, etc.) by radio-communication (IEGMP, 2000). Information is sent using electromagnetic waves (also referred to as radio waves, electromagnetic radiation or fields). The electromagnetic (EM) spectrum extends from direct current to the ionising radiation realm and is divided into ill-bound sub-regions according to the state of technology and the precise phenomenon under consideration (Moulder *et al*., 1999). One such a division illustrating the position of mobile phone communications is shown in Figure 1.1. Cellular and personal communication systems (PCS) reside in the wave realm, specifically in the ultra high frequency (UHF) region from 300 to 3000 MHz (Moulder *et al*., 1999).

The RF wave used for radio-communication is referred to as the carrier wave, by itself it carries no information and communicates nothing (Moulder *et al*., 1999). Information (speech, data, images etc.) has to be imposed upon the wave by a process known as modulation (Moulder *et al*., 1999; IEGMP, 2000).

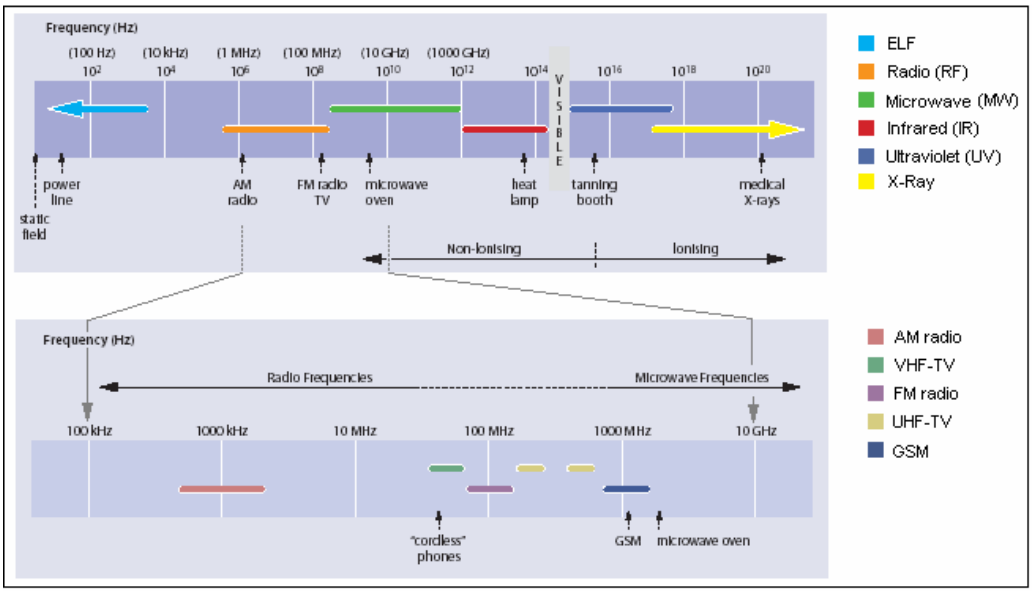

Figure 1.1 Triage of the Electromagnetic spectrum indicating the position of mobile phone emissions (Adapted with permission from the EU Commission, Health and Electromagnetic fields, 2005).

1.2.1 Modulation

Modulation alters the carrier wave by changing its frequency either by pulsing (digital modulation, DM), by varying its amplitude (amplitude modulation, AM) or by varying its phase (phase modulation, FM) (Moulder *et al*., 1999). To increase the number of users that can communicate with a base station at the same time, encoding strategies are used. There are various strategies to encode information. Three of the basic coding strategies currently used are:

1.2.1.1 FDMA (Frequency Division Multiple Access)

The section of the EM spectrum is divided into 30 kHz slots, with one subscriber having access to the slot at a time. The carrier information is either modulated by amplitude modulation, frequency modulation or digital modulation.

1.2.1.2 TDMA (Time Division Multiple Access)

This technique allows the communication channel to be used by eight phones, thus the channel is divided into eight time slots. By compressing 4,6 ms portions of information and transmitting it in bursts or pulses of 576 μs, a pulse modulation rate of 217 Hz (1/4.6 ms) is achieved (IEGMP, 2000; Hyland, 2000). The eight time slots together form a frame with a repetition rate of 217 Hz; the data transmitted is further compressed as the frames are grouped into "multi-frames" of 25 pulses by omitting every 26^{th} frame. This results in a lower frequency signal of 8.34 Hz (217 Hz/26), which is a permanent feature of the emission since it is not affected by the call density (Hyland, 2000).

1.2.1.3 CDMA (Code Division Multiple Access)

This modulation scheme allows for 5 MHz bandwidths simultaneously accessible by a number of users, with each transmission labelled by a coding scheme unique to that user. All transmissions occur simultaneously thus the changes in amplitude of the carrier wave are essentially random (noise-like) (IEGMP, 2000). Two types of CDMA are currently used, FDD (Frequency Division Duplex) and TDD (Time Division Duplex), each using pulse modulation. The pulse frequency with FDD is 1600 Hz and for TDD it can vary between 100 Hz and 800 Hz (Pederson and Anderson, 1999).

1.2.2 Cellular phone technologies

The first generation cellular system employed was TACS (Total Access Communication System), which is currently being phased out and bandwidth is being allocated to more recent systems like GSM (Global System for Mobile Communications). GSM (second generation) is the European digital phone standard and operates mostly in either the 900 MHz or 1800 MHz band (IEGMP, 2000; SCENIHR, 2006). This standard is the most widely used worldwide and the digital processing uses phase modulation (IEGMP, 2000). The maximum power that GSM mobile phones are permitted to transmit at under current standards, are 2 W (900 MHz) and 1 W (1800 MHz) (ICNIRP, 1998). However, because TDMA is used, the average power transmitted by a phone never exceeds one-eighth of these maximum values (0.25 W and 0.125 W, respectively) (IEGMP, 2000; Hyland, 2000). Furthermore, by applying an energy saving discontinuous transmission mode (DTX), which allows for the power to be switched off when the user stops speaking, the power is further reduced. This results in an even lower frequency pulsing at 2 Hz (Hyland, 2000).

The third generation of mobile telecommunications technology is called UMTS (Universal Mobile Telecommunications System) and is known worldwide as IMT-2000 (International Mobile Telecommunications – 2000) (IEGMP, 2000). This system is currently being introduced and will operate in the 1885 – 2010 MHz and 2110 – 2200 MHz frequency bands.

1.2.3 Output from mobile phones

RF power from a mobile phone is mostly transmitted by the antenna and the circuit elements inside the handset (IEGMP, 2000). Depending on what the position of the antenna is in relation to the head, the average field values can be calculated. The IEGMP (2000) notes maximum electric field strengths of 400 V/m for a 2 W, 900 MHz phone and about 200 V/m for a 1 W, 1800 MHz phone with the maximum magnetic field calculated for these phones approximately 1 μT. These values are appreciably lower when the phone is in close contact to the head. Exposure thus results from the RF fields that are pulsed at 8.34 Hz and 217 Hz as well as the

magnetic fields near the phone that oscillate at the same frequencies due to the TDMA modulation scheme.

1.2.4 RF radiation dose and measurement

Dosimetry is the critical component of any scientific study designed to assess the effects of RF fields on biological systems (COST 244, 1996). The fundamental RF dosimetry parameter that specifies the metric for internal exposure is the specific absorption rate (SAR), expressed in watts per kilogram (W/kg) (Repacholi, 1998; Moulder *et al.*, 1999). Radio-frequency fields penetrate the body in a manner that decreases with increasing frequency (IEGMP, 2000). To assess the effects that the RF field could have on biological tissue, it is necessary to determine the magnitude of the fields within the various body parts exposed (IEGMP, 2000). In order to do this, knowledge of the electrical properties of the different types of tissue is required, which once determined, makes it possible to calculate the electric (E) and magnetic (B) fields at every part of the body caused by a particular source of radiation, such as a mobile phone (IEGMP, 2000). The rate at which the energy is absorbed by a particular mass of tissue m, is $m\sigma E^2/\rho$, where σ and ρ are respectively the conductivity and density of the tissue and E is the root mean square (rms) value of the electric field (IEGMP, 2000).

For typical biological tissue the SAR is given by equation (1):

$$SAR = (E_{local})^2 \times \frac{\sigma_{eff}}{\rho} \quad (1)$$

where E_{local} is the rms electric field (in V/m) in the organism at the point of interest, σ_{eff} (S/m) is the effective conductivity, and ρ (kg/m^3) is the local mass density (Moulder *et al.*, 1999).

SAR varies from point to point in the body due to the change in conductivity of different tissues and fluctuations in the electric field with position (IEGMP, 2000). The ANSI/IEEE limits the spatial average SAR to 0.08 W/kg whole body and to 1.6 W/kg averaged over any 1 g of tissue. The ICNIRP (1998) limits on SAR (e.g. 0.4 W/kg occupational and 0.08 W/kg public whole body exposures) are similar to that quoted by the ANSI/IEEE (Moulder *et al.*, 1999). According to the ICNIRP (1998),

the threshold for biological effects is seen at SAR values above 4 W/kg. However, this limit has been derived from the thermal effects observed from SAR values of 1 – 4 W/kg. A SAR value of 4 W/kg is associated with a temperature increase of more than 1°C, and although the sensitivity of various types of tissue to thermal damage varies widely, irreversible effects occur above this value (ICNIRP, 1998). The rise in body temperature is the cause of the biological effects observed. Thus, current exposure limits do not take into account non-thermal effects or the threshold at which these effects occur.

Specific absorption rate can be estimated by using any of three methods: (i) *micro-antennas to measure E-fields*, (ii) *miniature thermal probes to determine the increase in temperature*, and (iii) *numerical modelling*. In the current study, SAR was determined using methods (ii) and (iii) (Annexure A).

(i) Micro-antennas:

Small antennas can be used to determine the local electric field in the tissue and SAR can be calculated by equation (1) if σ_{eff} is known. However, it is difficult to place the antenna in the exact position. Technically suitable small antennas with sub-millimetre characteristic dimensions have yet to be developed (Moulder *et al.*, 1999). Furthermore, σ_{eff} must be known for the tissue and frequency of interest.

(ii) Miniature thermal probes:

RF radiation causes heating of tissue, therefore thermal probes placed in the vicinity are able to detect the change in temperature and could be used to determine the SAR (Moulder *et al.*, 1999). In a medium with spatially homogeneous SAR,

$$SAR = c_\tau \frac{\partial T}{\partial t} \qquad (2)$$

where c_τ is the specific heat at constant pressure in J/kgK and ?T is the change in tissue temperature over a time ?t.

In principle, SAR could thus be measured by switching on the radiation source and measuring the temperature change as a function of time (Moulder *et al.*, 1999). The problem with this type of calculation of SAR is that heat diffuses with time and

spatially non-uniform SAR could over the time required to produce a measurable temperature offset be significantly confounded by thermal diffusion.

(iii) Numerical modelling:

Numerical modelling of macroscopic bodies is a well-developed method that offers an alternative to determining SAR. Finite difference time domain (FDTD) simulations can be used to predict SAR in a given organism and well-characterised irradiation geometry (Moulder *et al*., 1999).

1.2.5 Biological basis for limiting exposure to mobile phones

The previous paragraphs broadly addressed the physics and dosimetry of GSM telephony. Though understanding that mobile phones operate at power levels appreciably lower than those associated with biological effects, it still does not explain why there is such a social impression that RF-EMFs pose a health risk. Part of this is certainly the result of inadequate risk communication between stakeholders and the public (COST 281, 2003). It is not only complicated to establish exposure limits due to the dual nature of the energy under consideration but also the biological effects elicited by this radiation could possibly be ascribed to either the electric or magnetic component of the field. Nonetheless, there is a biological basis for the interaction of GSM radiation with living tissue.

Electric Field - The human organism supports a variety of oscillatory electrical activities, each of these characterised by a specific frequency. As it so happens, some of these frequencies correspond to those utilized by the microwave carrier (900 and 1800 MHz) and also to those frequencies arising from the TDMA strategy (8.34 Hz), as well as to the 2 Hz pulsing associated with the DTX mode of the phone (Hyland, 2001). The biological processes vulnerable to the interference of RF-EMF include highly organised electrical activities at a cellular level that are the result of active metabolism (Fröhlich, 1988; Grundler and Kaiser, 1992). The latter two frequencies (8.34 Hz and 2 Hz) correspond to frequencies of electrical oscillations in the EEG pattern of the human brain, specifically in the ranges of the alpha and delta brain waves.

Magnetic Field **-** What is interesting to note is that the internal circuitry of mobile phones also generate low frequency magnetic fields (Hocking, 2003). Furthermore, the International Agency for Research on Cancer (IARC) has declared 50 Hz magnetic fields as a possible carcinogen (IARC, 2002) due to evidence supporting an increase in childhood leukaemia (Michaelis *et al*., 1997; Linet *et al*., 1997).

Heating **-** It has long been established that tissue heating due to electromagnetic radiation can lead to various biological effects (Repacholi, 1998; ICNIRP, 1998; IEGMP, 2000). Evidence (van Leeuwen *et al*., 1999; Wainwright, 1999) suggests that the highest temperature rise found in the brain determined for mobile phone emissions for frequencies of 900 and 1800 MHz is around 0.1ºC. Mobile phones, according to present standards (NRPB, 1993; ICNIRP, 1998), are permitted to transmit at maximum specific absorption rates of 2 W/kg (900 MHz) and 1 W/kg (1800 MHz). Operational powers of 2 W/kg and less do not cause a significant increase in body temperature (Jokela *et al*., 1999; van Leeuwen *et al*., 1999; IEGMP, 2000; Wainwright, 1999).

If RF-EMF emitted by mobile phones elicits biological effects solely due to thermal processes, then no biological effects should be observed from mobile phone use operated under these safety standards. However, there are a number of studies conducted under isothermal conditions where significant changes were reported after RF-EMF exposure (Donnellan *et al*., 1997; French, *et al*., 1997; Harvey and French, 1999; Repacholi, *et al*., 1997) such as, altered cell growth (French, *et al*., 1997; Velizarov *et al*., 1999), exocytosis (Donnellan *et al*., 1997), gene expression (Harvey and French, 1999; Pacini *et al*., 2002), chromosomal instability (Mashevich *et al*., 2003), cancer development in animals (Repacholi *et al*., 1997) and the expression of heat shock proteins (French *et al*., 2000; Leszczynski *et al*., 2002, Weisbrot *et al*., 2003; Czyz *et al*., 2004).

This seems to indicate that biological effects due to RF radiation cannot solely be attributed to temperature changes. Up until now, no satisfactory mechanism has been proposed to explain the biological effects observed due to isothermal RF exposure.

1.3 BIOPHYSICAL INTERACTION OF RF-EMF WITH BIOLOGICAL SYSTEMS

The energy deposited in tissue by a 900 MHz GSM mobile phone (4 x 10^{-6}eV) or by an 1800 MHz GSM mobile phone (7 x 10^{-6}eV) is orders of magnitude lower than that required in breaking a chemical bond (1eV) (IGEMP, 2000). For this reason, some consider it questionable whether the low energy arising from mobile phones could be able to induce biological effects. The lack of a comprehensive biophysical mechanism is often cited as justification that RF-EMF radiation has no effect and is therefore safe. When the scientific community initially embarked on research investigating the effects of mobile phone radiation, research was associated with effects seen from ionising radiation. In this field of research, physical interactions bring about chemical processes which then manifest as biological changes. This progression of events is neither clearly defined nor well understood for weak electromagnetic fields (Weaver, 2002).

1.3.1 Biophysical mechanisms

Although much research has been done to explore the physical interaction process of EM fields (Fröhlich, 1980; Liboff *et al.*, 1987; Lednev, 1991; Blanchard and Blackman, 1994; Adair, 2002, 2003; Goodman and Blank, 2002; Weaver, 2002; Panagopoulos *et al.*, 2002) the interaction mechanism/s is still uncertain. Several experimental areas (RF-EMF produced temperature gradients; interactions at a cellular level; interactions at a molecular/chemical level; many-body interactions and magnetic dipole interactions) have been emphasized in literature (reviewed in MMF, 2001). However, none of the above mentioned mechanisms afford a comprehensive answer. A list of the most plausible mechanisms and interaction processes are summarised in Table 1.1.

Table 1.1 Summary of biophysical mechanisms of RF-EMF.

Mechanism	Proposed model	Initial physical interaction	References
RF-EMF produced temperature gradients	Periodic temperature changes could affect biochemical processes.	Localised heating caused by RF-EMF.	MMF, 2001.
Interactions at a cellular level - membrane associated changes	RF-EMF interactions affect ion channel flux rates - a trans-membrane voltage of 1 μV required for initial physical change.	Not clear	Apollonio *et al*. 1998.
	Altered ligand binding to receptor proteins as a result of pulsed and modulated RF-EMF.	Not clear	Chiabrera *et al*., 2000.
	Mobile Charge Interaction (MCI) model - magnetic fields interact with ion movement in cells changing their velocities. Could affect enzyme reaction rates.	Not clear – field strengths < than GSM range required.	Blank, 1995a, b.
	Polarisation of ions in the counter-ion layer adjacent to the outer cell membrane surface - a protein teetering between two energy states could change state as a result of polarisation energy or polarisation could effect cell/protein aggregation.	No sharp cut-off frequency – may be plausible at GHz frequencies.	Kotnik and Miklavcic, 2000a, b.
Interactions at a molecular/chemical level	Resonant absorption of RF energy - not plausible as H_20 molecules would damp effects and resonances occur at frequencies where the quantum energy is thousands of times greater than for a 1 GHz quantum.	Frequencies in the THz range required.	MMF, 2001; Adair, 2002.
	Magnetic field of RF-EMF induces weak electric fields throughout cell – influences DNA-mediated charge transport, activating DNA and initiation of transcription.	EM field exposure induces Hsp70 synthesis at 60Hz, 80mG for 20 min.	Goodman and Blank, 2002.

continue……

Table 1.1 (Continue) Summary of biophysical mechanisms of RF-EMF.

Mechanism	Proposed model	Initial physical interaction	References
Many-body interactions ***(Cooperativity; Coherence; Non-linear Dynamics and Stochastic resonance)***	Existence of a band of frequencies absorbent to EM fields leading to a coherent state of vibration (Fröhlich, 1980, 1988) - DNA polymers and elements of fibre structures (cytoskeletons), including microtubules and actin filaments could have resonant vibrational modes in GSM range - however H_20 molecules would damp effects.	Frequencies >> 40GHz required - not plausible in GSM range, but some cellular structures have vibrational modes in GSM range.	van Zandt, 1986; Pokorny and Wu, 1998.
	ELF's generated in mobile phones by pulsed EMF, exert an oscillating force on every free ion causing the oscillating ions to undergo a periodical displacement of electric charge consequently upsetting the electrochemical balance of the membrane by gating the channels - severe damping by aqueous environment would negate the effect.	Not plausible, requires un-damped resonant absorption at microwave frequencies for effects to occur.	Panagopoulos *et al.*, 2002.
Magnetic field interactions - radical pair recombination	Radical pair recombination is altered by static magnetic fields - unlikely that energy transfer by RF-EMF with electric field strength of 200 V/m could modify radical pair recombination.	Electric field strength >> than found in GSM range required.	Adair, 2003.

1.4 BIOLOGICAL EFFECTS OF RADIO-FREQUENCY FIELDS FROM MOBILE PHONES

1.4.1 Evidence of biological effects of RF-EMF fields

Epidemiological studies provide the most direct evidence of health risks associated with a suspected environmental hazard. However, laboratory studies can contribute meaningfully to understanding the mechanism of such a hazard. More than 10 years ago, Grundler *et al*. (1992) predicted that epidemiological surveys would probably not provide an answer regarding the modifying potential of EMFs, but rather that careful cellular studies designed to take the underlying bio-mechanism into account would do so. For this reason, review of scientific evidence was limited in this study to cellular studies. Epidemiological findings related to cellular studies were broadly addressed at the end of each section.

The Stewart Report (IEGMP, 2000) provides to date the most comprehensive review on possible effects of mobile phones on health. This report takes into account all publications dated prior to 2000. The bulk of studies dealt with the possible cancer induction (genotoxic effect) properties of RF-EMFs, while very little research was presented on other possible effects (i.e. stress response - gene/protein expression) of RF-EMFs. A summary of the IEGMP (2000) findings on cellular studies focussing on genotoxic effects and gene and protein expression is presented in Table 1.2. In addition, the National Radiological Protection board (NRPB, 2005), recently reviewed twenty-six reports produced between 2000 and 2004 by international committees, expert groups, and agencies on the possible health effect of mobile phone use.

These findings as well as subsequent cellular studies conducted after 2000 and not included in the IEGMP (2000) report, are summarised in Tables 1.3 A-D. Scientific evidence is presented of biological effects in each of the following categories, genotoxic effects, apoptosis, gene/protein expression, and effect on male germ cells. Each category specifies whether the study was conducted on human or animal subjects/cells, and *in vitro* or *in vivo*. Only studies employing GSM signals in the 900 MHz, 1.8 GHz and 2.4 GHz range, with RF intensities as prescribed by current safety standards (ICNIRP, 1998), were reviewed. Many studies have been criticized

for their design of using high power intensities that are associated with thermal effects (COST 281, 2003). Therefore, these studies have been omitted.

1.4.2 Health risks associated with genotoxic effects from RF-EMF exposure

It is generally accepted that the energy emitted by mobile phones is too low to directly induce DNA damage. However, it is possible that cellular constituents affected by RF-EMF, for instance, free radicals, may indirectly target DNA. Alterations in the chromosome compliment (aneuploidy) are one of the most relevant changes associated with carcinogenesis (Duesberg *et al*., 2000). Aneuploidy results either from chromosome mal-segregation or from chromosome fragmentation and constitutes the "somatic mutation (itself) that makes cancer" (Duesberg and Rasnick, 2000).

The REFLEX (REFLEX report, 2004) study on *in vitro* effects of RF-EMFs performed by twelve different research groups in seven European countries noted some genotoxic effects (Diem *et al*., 2005; Nikolova *et al*., 2005) after prolonged RF exposure. In addition, several other studies have reported RF fields induced genotoxic effects (D'Ambrioso *et al.*, 2002; Tice *et al*., 2002; Irmak *et al*., 2002; Mashevich *et al*., 2003; Czyz *et al.*, 2004). However, the majority of investigations report no genotoxic effect of RF-exposure (Malyapa *et al*., 1997; Vijayalaxmi *et al*., 2001a,b, 2003; Li *et al*., 2001; Bisht *et al*., 2002; Zeni *et al*., 2003). Conclusions drawn from studies reviewed in the SSI report (IEGEMF, 2007) also conclude that there is no evidence to support any genotoxic effect of RF fields. A summary of recent reports on the genotoxic effects of RF-EMFs is given in Table 1.3A.

Epidemiological evidence of genotoxic effects does not support the *in vitro* data of some of these studies. To date, the Interphone study (a multinational case-control study coordinated by IARC) provides the most comprehensive report on epidemiological findings (summarized in SCENIHR, 2006). Although the relatively short latency period associated with the less than 10-year use of mobile phones should be taken into account, none of the Interphone studies reported an increased risk of any cancer. In contrast, Hardell *et al*. (2005a, b), a group not forming part of the Interphone study, has consistently reported an increase in incidence of acoustic neuroma. Schoemaker *et al.* (2005) reviewed the Interphone data and also concluded

that there may be an association between mobile phone use and risk of acoustic neuroma. Epidemiological evidence of genotoxic effects of RF-EMF is far from conclusive and prospective long-term follow up studies have been proposed to fill the current gaps in knowledge (SCENIHR, 2006).

1.4.3 Health risks associated with the induction of apoptosis as a result of RF-EMF exposure

Apoptosis is a physiological process of programmed cell death occurring in development and cell differentiation, and in response to mild damaging stimuli. It is also an important protection mechanism against cancer, as it eliminates potential tumour cells. Several reports have investigated the possibility of RF induced apoptosis in human peripheral blood mononuclear cells (Capri *et al.*, 2004), lymphoblastoid cells (Marinelli *et al.*, 2004), epidermis cancer cells (Caraglia *et al.*, 2005), human Mono Mac 6 cells (Lantow *et al.*, 2006a), and in Molt4 cells (Hook *et al.*, 2004). None of these studies reported an increase in apoptosis as a result of RF-EMF.

On the other hand, Marinelli *et al.* (2004) reported better survival rate of T-lymphoblastoid leukaemia cells, and Caraglia *et al.* (2005) found apoptosis induction in human epidermoid cancer cells. In addition, participants of the REFLEX-study (Bersani-, Tauber-, and Wobus- groups) found no effects of RF fields on cell cycle, cell proliferation, cell differentiation, apoptosis induction, DNA synthesis, and immune cell functionality. Some studies did note certain findings after RF field exposure on the transcript level of genes related to apoptosis and cell cycle control. However, these responses were not associated with detectable changes of cell physiology (Nikolova *et al.*, 2005). These results are noted in Table 1.3B.

Table 1.2 Summary of IEGMP (2000) report on biological effects of mobile phone exposure in cellular systems.

Effect	Findings	References	Recommendations
Genotoxic	*Mutations:* At non-thermal temperatures, RF fields do not induce mutation in either somatic or germ cells.	Varma *et al*., 1976; Varma and Traboulay, 1977; Berman *et al*., 1980; Goud *et al*., 1982, Saunders *et al*., 1983, Saunders *et al*., 1988.	*Mutations:* No convincing evidence that mutations occur.
	DNA damage: Some *in vivo* studies show increased DNA damage but this is not supported by *in vitro* studies.	Sarkar *et al*., 1994; Rotkovska *et al*., 1993; Malyapa *et al*., 1998; Lai and Singh, 1995, 1996.	*DNA damage*: Further research is required.
	Chromosomal aberrations and sister chromatid exchange: No conclusive evidence that RF radiation causes genotoxic effects at non-thermal levels.	Maes *et al*., 1993, 1995; Saunders *et al*., 1988; Beechey *et al*., 1986; Manikowska-Czerska *et al*., 1985; Garson *et al*., 1991; Garaj-Vrhovac *et al*., 1990a.	*Chromosomal aberrations and Sister chromatid exchange:* No convincing evidence that aberrations/exchanges occur.
	Micronucleus formation: Several studies seem to indicate that RF fields cause an increase in micronucleus frequency.	Balode, 1996; Vijayalaxmi *et al*., 1997, 1998; Vijayalaxmi *et al*., 1999; Antipenko and Koveshinkova, 1987; Garaj-Vrhovac *et al*., 1990b.	*Micronucleus formation:* Results are not simple to interpret and should be confirmed with additional experiments.
Gene / Protein Expression	Possibility that RF-EMF causes a stress response judged by elevated gene expression.	Ivaschuck *et al*., 1997; Goswami *et al*., 1999; Mickley *et al*., 1994; Walters *et al*., 1995; Morrissey *et al*., 1999; Fritze *et al*., 1997.	To define genetic responses to RF radiation, cellular models should be used with cells carrying transgenes linked to important genes.

Table 1.3A Genotoxic effects from mobile phone exposure.

Model	Dosimetry	Result	Reference	Relevance to current study
In vivo				
Fischer rats.	1600 MHz, 2 h/day, 7 days/week, SAR 0.036 - 0.077 W/kg - whole body.	No increase in micronucleus frequency in bone marrow smears taken from chronically exposed rats.	Vijayalaxmi*et al*., 2003.	RF-EMF did not exert a genotoxic effect *in vivo.*
Rabbits.	900 MHz, GSM, 30 min/day, 7 days, power density 0.02 $mWcm^{-2}$.	Indirect genotoxic effect – increase in serum superoxide dismutase (SOD) activity and decrease in serum nitric oxide (NO) levels. Elevated SOD levels could indicate elevated reactive oxygen species (ROS) generation. Decreased NO as result of increased ROS generation.	Irmak *et al*., 2002.	RF-EMF induces an oxidative stress (increased ROS production) measured by elevated SOD and decreased NO levels.
Mice - embryonic stem (ES) cells.	1710 MHz GSM, 6 h & 48 h, SAR 1.5 W/kg.	Increased DNA double strand breaks in ES cells after 6 h exposure, but not for 48 h exposure.	Czyz *et al*., 2004.	RF-EMF (1710 MHz GSM-217Hz) exerted a genotoxic effect.

continue……

Table 1.3A (Continue) Genotoxic effects from mobile phone exposure.

Model	Dosimetry	Result	Reference	Relevance to current study
In vitro				
Rat brain cells.	2450 MHz, continuous wave (CW).	No DNA damage observed (comet assay).	Lagroye, 2004a, b.	No genotoxic effect observed.
Mouse C3H 10T½ fibroblasts & human glioblastoma U87MG cells.	835.62 MHz (FM-CW), 847.74 MHz (CDMA), 24 h, SAR 0.6 W/kg.	RF exposure did not induce DNA damage (determined by comet assay).	Malyapa *et al*., 1997a.	No genotoxic effect observed.
Mouse C3H 10T½ fibroblasts & human glioblastoma U87MG cells.	2450 MHz (CW) 2 h, 4 h & 24 h, SAR 0.7 - 1.9 W/kg.	RF exposure did not induce DNA damage (determined by comet assay).	Malyapa *et al*., 1997b.	No genotoxic effect observed.
Mouse C3H 10T½ fibroblasts.	835.62 MHz (FDMA), 847.74 MHz (CDMA), 2 h, 4 h & 24 h, SAR 3.2 - 5.1 W/kg.	RF exposure did not induce DNA damage (determined by comet assay).	Li *et al*., 2001.	No genotoxic effect observed.
Mouse C3H 10T½ fibroblasts.	835.62 MHz (FDMA), 847.74 MHz (CDMA), 3 h, 8 h, 16 h & 24 h, SAR 3.2 - 4.8 W/kg.	RF exposure did not induce micronuclei formation.	Bisht *et al*., 2002.	No genotoxic effect observed.

continue……

Table 1.3A (Continue) Genotoxic effects from mobile phone exposure.

Model	Dosimetry	Result	Reference	Relevance to current study
In vitro				
Human promyelocytic cell line HL-60 cells.	1800 MHz, Continuous wave (CW) & different RF fields (CW, CW on/off, GSM-217Hz, GSM talk), 2 h, 6 h, 24 h & 72 h, SAR 0.2, 1.0, 1.3, 1.6, 2.0 & 3.0 W/kg.	Increased micronucleus (MN) frequency and DNA strand breaks (SAR 1.3, 1.6 & 2.0 W/kg) for CW and different RF modulated fields. Caused indirect genotoxic effect by reactive oxygen species (ROS) generation (1800 MHz, CW, 24h, SAR 1.3 W/kg).	REFLEX, 2004. (Participant 2 - Tauber group).	RF-EMF (CW & different RF modulated fields) exerted a genotoxic effect, most notably at SAR levels of 1.3-2.0 W/kg. RF-EMF (1800 MHz, CW, 24h, SAR 1.3 W/kg) increased ROS generation.
Human fibroblasts & SV 40 transformed GFSH-R17 rat granulosa cells.	1800 MHz (CW, CW on/off, GSM-217Hz, GSM-talk), 4 h, 16 h & 24 h, SAR 1.2 – 2.0 W/kg.	Increased DNA damage in fibroblasts and granulosa cells (single and double strand breaks), all exposure conditions. Increased chorosomal aberrations in exposed (1950 MHz, GSM, SAR 1.0 W/kg, 15 h) fibroblasts. Increased micronucleus formation in exposed (1950 MHz, GSM, SAR 2.0 W/kg, 15 h) fibroblasts.	REFLEX, 2004. (Participant 3 - Rüdiger group. Winker *et al*., 2005).	RF-EMF (CW, CW on/off, GSM-217Hz, GSM-talk) exerted a genotoxic effect, most notably at SAR levels of 1.2-2.0 W/kg.

continue……

Table 1.3A (Continue) Genotoxic effects from mobile phone exposure.

Model	Dosimetry	Result	Reference	Relevance to current study
Human peripheral blood lymphocytes.	900 MHz (CW & GSM), 6 min, 1 h/day for 3 days, SAR 0.2 - 1.6 W/kg	No increase in micronucleus frequency at any exposure schedule.	Zeni *et al*., 2003.	RF-EMF had no genotoxic effect.
Human peripheral blood lymphocytes.	830 MHz CW, 72 h, SAR 2.6 - 8.8 W/kg.	Increase in aneuploidy as a function of SAR. RF fields caused chromosomal instability.	Mashevich *et al*., 2003.	Thermal induction did not induce genotoxic effects.
Human peripheral blood lymphocytes.	1748 MHz (GMSK & CW), 15 min, SAR 2.25 W/kg.	Slight but not statistically significant increase in micronucleus frequency with GMSK phase modulated radiation, this effect disappeared with CW exposure.	D'Ambrioso *et al*., 2002.	GMSK phase modulation but not CW exposure results in MN frequency increase.
Human peripheral blood lymphocytes.	837 (analogue signal, TDMA, CDMA) & 1909.8 (GSM) MHz, 3 h & 24 h, SAR 1.0, 5.0 & 10 .0 W/kg	No increase in DNA damage or micronucleus frequency at any RF signal modulation scheme for SAR 1.0 W/kg and 3 & 24h exposure. Significant increase in MN frequency at all RF signal modulation schemes for SAR 5.0 & 10.0 W/kg after 24h exposure.	Tice *et al*., 2002.	Genotoxic effect of RF-EMF is dependant on SAR and independent on RF signal modulation schemes.

continue……

Table 1.3A (Continue) Genotoxic effects from mobile phone exposure.

Model	Dosimetry	Result	Reference	Relevance to current study
Human peripheral blood lymphocytes.	847.74 MHz (CDMA), 24 h, SAR 4.9 & 5.5 W/kg.	No increase in micronucleus frequency or chromosomal aberrations at any exposure schedule.	Vijayalaxmi *et al*., 2001a,b.	RF-EMF had no genotoxic effect.
Human peripheral blood lymphocytes.	935 MHz GSM, 24 h, SAR 1.0 or 2.0 W/kg.	No increase in DNA strand breaks, chromosomal aberrations, sister chromatid exchange and micronuclei.	Stronati *et al*., 2006.	No genotoxic effects.
Human diploid fibroblasts & rat granulosa cells.	1800 MHz (intermittent; CW), 4 h, 16 h, 24 h, SAR 1.2 or 2.0 W/kg.	Increased DNA damage after 16 h RF exposure (determined by comet assay).	Diem *et al*., 2005.	RF-EMF at 1800 Hz exerted a genotoxic effect.

Table 1.3B Effect of mobile phone exposure on apoptosis.

Model	Dosimetry	Result	Reference	Relevance to current study
In vitro				
Rat brain - (astrocytes) & neuronal - (granule cells) cells.	900 MHz, GSM, exposure 1 h & 24 h, SAR 2.0 W/kg.	No effect on apoptosis in rat brain or neuronal cells (determined by Annexin V and $DiOC_6$ assay).	REFLEX, 2004. (Participant 9 - Bersani group).	900 MHz GSM at 20 W/kg did not induce apoptosis.
Mouse embryonic stem cells.	1710 MHz GSM, 6 h & 48 h exposure, SAR 1.5 W/kg.	Possible influence on Bcl-2 mediated apoptotic pathway.	Nikolova *et al*., 2005. REFLEX, 2004. (Participant 4 - Wobus group).	1710 MHz GSM had effect on Bcl-2 apoptotic pathway.
Human promyelocytic cell line - HL-60 cells.	1800 MHz, CW, exposure 24 h, SAR 1.3 W/kg.	No effect on apoptosis (determined by Annexin V and TUNEL assay).	REFLEX, 2004. (Participant 2 - Tauber group).	SAR of 1.3 W/kg (1800 MHz, CW) did not induce apoptosis.
Human neuroblastoma (SH-SY5Y) & glioblastoma cell line (U87), monocytes (U937) & microglial cells (CHME5) & lymphocytes.	900 MHz, GSM, exposure 1 h & 24 h, SAR 2.0 W/kg.	No effect on apoptosis in human brain or neuronal cells (determined by Annexin V and $DiOC_6$ assay).	REFLEX, 2004. (Participant 9 - Bersani group).	900 MHz GSM at 2.0 W/kg did not induce apoptosis.
Human skin cells, keratinocytes.	900 MHz, GSM, exposure 48 h, SAR 2.0 W/kg.	No effect on apoptosis in skin cells.	Sanchez *et al*., 2006	900 MHz GSM at 2.0 W/kg did not induce apoptosis.

continue……

Table 1.3B (Continue) Effect of mobile phone exposure on apoptosis.

Model	Dosimetry	Result	Reference	Relevance to current study
Human endothelial cell line.	900 MHz GSM, exposed 1 h, SAR 2 W/kg.	Caused down-regulation of Fas/TNFα suggesting an anti-apoptotic pathway in exposed cells.	Leszczynski *et al*., 2002.	900 MHz GSM at 2.0 W/kg inhibited apoptosis.
Molt-4 T lymphoblastoid cells.	847.74 MHz, CDMA, SAR 3.2 W/kg, 2 h, 3 h & 21 h, 835.62 MHz FDMA, SAR 3.2 W/kg, 2 h, 3 h & 21 h, 813.56 MHz iDEN, SAR 2.4 or 24 mW/kg, 2 h, 3 h & 21 h, 836.55 MHz TDMA SAR 2.6 or 26 mW/kg, 2 h, 3 h & 21 h.	No effect on DNA damage (determined by comet moment & length) or apoptosis (determined by Annexin V assay) determined for any frequency, modulation or exposure time.	Hook *et al*., 2004.	900 MHz GSM > 2.0 W/kg did not induce apoptosis.
Human lymphocytes.	900 MHz (GSM or CW) exposure 1 h/d for 3 days, SAR 70-76 mW/kg.	GSM modulated radiation showed a slight decrease in cell proliferation and slight increase in Apoptotic cells (determined by Annexin V assay). CW exposure had no effect on apoptosis.	Capri *et al*., 2004.	900 MHz GSM < 2.0 W/kg induced apoptosis.

1.4.4 Health risks associated with gene/protein expression as a result of RF-EMF exposure

The synthesis of heat shock proteins (Hsps) in cells is part of a normal defence mechanism to protect against various stressors (i.e. temperature elevation, heavy metals, toxic chemicals, and oxidative stress). RF-EMF radiation has been shown to stimulate the synthesis of stress proteins, which indicates that cells recognise mobile phone radiation as potentially harmful (Fritze *et al*., 1997; French *et al*., 2000; Kwee *et al*., 2001; Leszczynski *et al*., 2002; Di Carlo *et al*., 2002). On the other hand, a review by Cotgreave (2005) found no evidence of Hsp induction due to RF exposure.

Although present evidence is equivocal, one proposed hypothesis suggests that RF-EMFs act as a stress inductor, causing changes in protein conformation leading to the synthesis of Hsps (French *et al*., 2000; Leszczynski *et al*., 2002). RF field exposure is not directly carcinogenic but could potentially alter cell function in a way that increases the risk of cancer. Furthermore, there is increasing evidence that links Hsps with cancer, based on the alteration of their expression in most classes of tumours (French *et al*., 2000).

Stress proteins are known regulators of a broad spectrum of physiological processes (Tibbles and Woodgett, 1999) including apoptosis (Mehlen *et al*., 1996; Creagh *et al*., 2000; Pandey *et al*., 2000). Leszczynski *et al*. (2002) postulated that increased expression and phosphorylation of Hsp27 by RF-EMF exposure might facilitate the development of brain cancer by inhibiting the cytochrome-c/caspase-3 apoptotic pathway, and cause an increase in blood-brain barrier permeability through stabilization of endothelial cell stress fibres.

The implication of this study has far reaching effects. Activated Hsp27 could support RF-EMF exposed brain cells induced to undergo either spontaneous or external factor-provoked transformation damage. In favourable circumstances, this could facilitate clonal expansion of the transformed/damaged cells which is a prerequisite for tumour development. Furthermore, Hsp27 has been shown to be responsible for the induction of resistance of tumour cells to death induced by anti-cancer drugs (Huot *et al*., 1996; Garrido *et al*., 1997). It would thus appear from the study of Leszczynski *et al*. (2002), that RF-EMF induced changes in Hsp27

phosphorylation/expression might not only promote tumour development but could also contribute to drug resistance.

Continuous use of mobile phones could potentially lead to a down-regulation of innate stress protective mechanisms, which could corroborate recent findings of epidemiological studies where enhanced probability of cancer (Hardell *et al.*, 2002) and Alzheimer's disease (Sobel *et al.*, 1996) were found as a result of chronic mobile phone use (Di Carlo *et al.*, 2002).

A summary of the most recent reports on the ability of RF-EMFs to induce a stress response or alter gene/protein expression is reviewed in Table 1.3C.

Table 1.3C Effect of mobile phone exposure on gene and protein expression.

Model	Dosimetry	Result	Reference	Relevance to current study
In vivo				
Genomic response in rat brains.	900 MHz GSM, 4 h exposure, SAR 0.3 & 1.5 W/kg.	No effect on Hsp70 mRNA, *c-fos*, *c-jun or GFAP* mRNA, measured directly, 24 h and 7 days post exposure for either SAR.	Fritze *et al*., 1997.	No effect on Hsp70 transcription.
Drosophila melangoster.	900 MHz, GSM, 2 h/day for 10 days, SAR 1.4 W/kg.	RF exposure increased number of offspring, Hsp70 levels increased serum response element (SRE) DNA binding and induced phosphorylation of nuclear transcription factor RLK-1.	Weisbrot *et al*., 2003.	Increased transcriptional activity of genes could have a direct effect on growth and development.
Mouse C3H10T½ fibroblasts.	835.62 MHz (FDMA) & 847.74 MHz (CDMA), 24 h, SAR 5 W/kg.	No change in gene expression after RF-EMF.	Whitehead *et al*., 2006.	No effect on gene transcription.
Chick embryos.	915 MHz GSM, 30 min (once off) or 30-60 min/day for 4 days, SAR 1.7 W/kg.	Single 30 min exposure induced hypoxia protection, while repeated exposure inhibited Hsp70 levels and lowered cyto-protection.	Di Carlo *et al*., 2002.	Decreased Hsp70 levels as a result of RF-EMF.
Chick embryos.	915 MHz GSM, 30 min, SAR 1.7 & 2.5 W/kg.	Elevated levels of Hsp70 after 2 reaching maximum after 3 h.	Shallom *et al*., 2002.	Increase in Hsp70 expression, protected against hypoxic stress.

continue……

Table 1.3C (Continue) Effect of mobile phone exposure on gene and protein expression.

Model	Dosimetry	Result	Reference	Relevance to current study
In vitro				
Mouse embryonic stem (ES) & p53-/- ES cells.	1710 MHz GSM, 6 h & 48 h exposure, SAR 1.5 W/kg.	A 6 h exposure did not result in any gene modification, after 48 h loss of p53 function rendered pluripotent ES cells sensitive to GSM modulated EMF. Prominent induction of Hsp70 levels in p53-/- cells was observed.	Czyz *et al*., 2004. (Participant 4 - Wobus group).	Cellular response is determined by p53 function. RF-EMF exposure may effect the Bcl-2 mediated apoptotic pathway.
Human neuroblastoma cells (NB69) and neural stem cells (NSC).	1800 MHz, GSM-CW & GSM-217Hz, 24 h, SAR 0.2, 1.0, 1.3, 1.6, 2.0 & 3.0 W/kg.	RF-EMF (GSM-CW & GSM-217Hz) interfered with the expression of fibroblast growth factor receptors in NB69 and NSC cells.	REFLEX, 2004. (Participant 5 - Trillo group).	Interference with receptors.
Human glioblastoma cell line (U87MG).	1900 MHz (GSM), 4 h, SAR 0.1, 1.0 & 10.0 W/kg.	Pulsed modulated RF fields did not affect gene expression in U87MG cells.	Qutob *et al*., 2006.	No effect on gene expression/transcription.
Human promyelocytic cell line (HL-60 cells).	1800 MHz, CW, 24 h, SAR 1.3 W/kg.	RF-EMF exposure resulted in altered protein expression (41 proteins were up- and 1 down- regulated, with 14 proteins expressed *de-novo*).	REFLEX, 2004. (Participant 2 - Tauber group).	Up-regulation and *de*-novo synthesis of genes.

continue……

Table 1.3C (Continue) Effect of mobile phone exposure on gene and protein expression.

Model	Dosimetry	Result	Reference	Relevance to current study
Human lymphocytes.	900 MHz (GSM or CW), 1 h/d for 3 days, SAR 70-76 mW/kg.	GSM modulated radiation had no effect on gene expression in human lymphocytes.	Capri *et al*., 2004.	No effect on gene expression/transcription.
Human skin cells, keratinocytes.	900 MHz GSM, 48 h exposure, SAR 2.0 W/kg.	No change in Hsp70 & 27 expression in keratinocytes, slight but significant increase of Hsp70 in reconstructed epidermis, decrease of Hsc70.	Sanchez *et al*., 2006.	Adaptive cell behaviour in response to RF.
Human lymphocytes and monocytes.	900 MHz (GSM or CW), 20 min, 1 h & 4 h, SAR 0.4, 2.0 & 3.6 W/kg.	Thermal exposure but not RF exposure caused an increase in Hsp70 & 27 expression.	Lim *et al*., 2005.	No effect on Hsp70 & 27. Used non-proliferating cells.
Human skin fibroblasts.	900 MHz GSM, 1 h exposure, SAR 0.6 W/kg.	Morphological (genes coding for structural proteins were highly expressed) and functional (high expression in mitogenic signal transduction genes [MAP-kinase family], cell growth inhibitors & genes regulating apoptosis) changes.	Pacini *et al*., 2002.	Stress response proteins highly expressed.

continue……

Table 1.3C (Continue) Effect of mobile phone exposure on gene and protein expression.

Model	Dosimetry	Result	Reference	Relevance to current study
Human endothelial cell line (EA.hy926).	900 MHz (GSM-217 Hz), 1 h, SAR 2.0 W/kg.	RF-EMF exposure increased phosphorylation of Hsp27.	Leszczynski *et al*., 2002.	Up-regulation of Hsp27.
Human endothelial cell line (EA.hy926 & EA.hy926v1).	900 MHz (GSM-217 Hz), 1 h, SAR 2.8 W/kg.	RF-EMF altered gene and protein expression in both cell lines differently.	Nylund and Leszczynski, 2006.	Cell response to RF exposure might be genome-and proteome dependant.
Human endothelial cell line (EA.hy926), lympho-blastoma cells (U937), leukaemia cells (HL-60), neuroblastoma cells (NB69), T lymphocytes and CHME5 cells.	900 & 1800 MHz (GSM-217 Hz & GSM talk), 1 h or 24 h, SAR 1.0 - 2.5 W/kg.	EA.hy926, U937 and HL-60 cells showed significant up or down regulation of genes, this was not seen in NB69, T-lymphocytes or CHME5 cells.	Remondini *et al*., 2006.	Investigation of affected gene families did not implicate a stress response but up-regulation of cellular metabolism.
Human HeLa, S3 and EA.hy926 cells.	847 MHz (TDMA), 1 h, 2 h or 24 h, SAR 5 W/kg, 900 MHz (GSM), 1 h, 2 h or 5 h, SAR 3.7 W/kg.	RF exposure did not alter Hsp27 expression.	Vanderwaal *et al*., 2006.	Thermal exposure induced Hsp27 expression but not RF-EMF.
Human breast cancer cell line (MCF-7).	1800 MHz (GSM), 24 h, SAR 3.5 W/kg.	RF-EMF had no effect on gene or protein expression in MCF-7 cells.	Zeng *et al*., 2006.	No effect on gene/protein expression.

continue……

Table 1.3C (Continue) Effect of mobile phone exposure on gene and protein expression.

Model	Dosimetry	Result	Reference	Relevance to current study
Rat glioma (C6) and human nerve (U87-astrocytoma; SH-SY5Y - neuroblastoma) cells; human endothelial cells (EA-hy926) and immune cells.	900 MHz (GSM), 48 h, SAR 0.2 – 2.0 W/kg.	RF-EMF did not affect expression and activation of inducible nitric oxide (*iNOS or NOS2*) or heat shock protein expression in nerve cells. No up-regulation of Hsp27 in EA-hy926 cells. Inconclusive results on Hsp27 expression in rat brain. Weak effect of gene expression in immune cells.	REFLEX, 2004. (Participant 9 - Bersani group).	No up-regulation of Hsp27.
Human glioma cells.	1950 GSM.	No up-regulation of Hsp27 in human glioma cells.	Miyakoshi *et al*., 2005.	No up-regulation of Hsp27.
Human Mono Mac 6 and KS62 immune cells.	1800 MHz (GSM), SAR 0.5, 1.0, 1.5 and 2.0 W/kg.	No up-regulation of Hsp70 was noted in either of the cell lines.	Lantow *et al*., 2006b.	No up-regulation of Hsp70.
MO54 cells.	2.45GHz, SAR 5, 20, 50 100 W/kg.	Increase in Hsp70 expression with increase in exposure time except at SAR 5 W/kg.	Tian *et al*., 2002.	Hsp70 expression related to time of exposure and SAR level.

1.4.5 Health risks associated with effects on male germ cells from RF-EMF exposure

The 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. This conclusion, as far as an effect of RF-EMF on male sexual function and fertility is concerned, 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. In addition, an epidemiological study by Hardell *et al*. (2006) reported no association between the use of cellular or cordless telephones and testicular cancer although they did not investigate the correlation with decreased sperm function.

Current evidence, reviewed in Table 1.3D, suggests effects around a central theme of reduced motility in RF-EMF exposed spermatozoa. Reduced motility in spermatozoa is directly correlated with reduced fertilizing potential (Lui and Baker, 1992; Sukcharoen *et al*., 1996; Oehninger *et al*., 2000). However, due to the highly specialised nature of the spermatozoon, an accurate prediction of an effect on fertilisation cannot be concluded from motility alone but should also include assessment of sperm - oocyte fusion, sperm capacitation, nuclear DNA normality, and biochemical assays monitoring sperm fecundity. What can be said with certainty regarding the current knowledge concerning the influence of RF-EMF on male germ cells is that it is extremely limited.

Table 1.3D Effect of mobile phone exposure on male germ cells.

Model	Exposure conditions/ Dosimetry	Findings	Reference	Relevance to current study
In vivo				
Testicular function – mice.	1800 MHz, 1 month, 2 h/day SAR 0.141 W/kg.	Significant decrease in seminiferous tubular diameter, increased rectal temperature.	Dasdag *et al*., 1999.	Increased temperature could result in DNA damage.
Gonadal function – mice.	1800 MHz, 10 times, 2 h/day, 100 $\mu W/cm^2$, SAR 8.1-2.3 mW/kg.	Significant increase in red blood cell count, volume, and serum testosterone levels.	Forgacs *et al*., 2005.	Could influence hormone levels.
Spermatozoa – mice.	900 MHz, 7 days, 12 h/day SAR 90 mW/kg.	Significant damage to mitochondrial genome and β-globin locus.	Aitken *et al*., 2005.	Effect on fertilization.
Spermatozoa – human.	GSM-mobile phone, 5 days, 6 h/day (no dosimetry provided).	Significant decrease in rapid progressive sperm, increase in slow progressive sperm	Davoudi *et al*., 2002.	Possible effect on male fertilizing potential.
Spermatozoa – human.	GSM-mobile phone, prolonged use (duration not specified), (no dosimetry provided).	Significant decrease in rapid progressive sperm, increase in slow progressive sperm.	Fejes *et al*., 2005.	Possible effect on male fertilizing potential.
Spermatozoa – human.	GSM-mobile phone (no dosimetry provided).	Decrease in semen quality.	Kilgallon and Simmons, 2005.	Possible effect on male fertilizing potential.

continue….

Table 1.3D (Continue)	Effect of mobile phone exposure on male germ cells.			
Model	**Exposure conditions/ Dosimetry**	**Findings**	**Reference**	**Relevance to current study**
Human – *in vitro*				
Spermatozoa - normospermic men.	900 MHz, 5 min, 0.02 mW/cm^2.	Significant decrease in rapid progressive sperm, increase in slow progressive sperm, significant increase in immotile sperm.	Erogul *et al*., 2006.	Possible effect on male fertilizing potential.

1.5 STRESS RESPONSE AS A POSSIBLE PATHWAY FOR RF-EMF EXPOSURE

There are a growing number of studies noting a change in gene and protein expression as a result of RF-EMF. In particular, the up-regulation of Hsps (as part of a stress response) have been cited as a possible target for RF fields. If this could be confirmed, it would be important for a mechanistic understanding of the interaction of RF-EMF on cellular systems. In particular, these findings could support two plausible biophysical interaction mechanisms proposed by Kotnik and Miklavcic (2000a,b), and Goodman and Blank (2002).

The stress response and apoptosis are interrelated. It is well documented that treatment of mammalian cells with different apoptosis inducing factors (serum deprivation, growth factor withdrawal, or agents such as calcium ionophores, topoisomerase inhibitors, protein kinase C inhibitors, as well as chemicals producing free radicals, drugs, heat shock, anoxia, and ionising radiation) are able to induce the stress response (Punyiczki and Fésüs, 1998). On the other hand over-expression of Hsps as a result of heat stress or environmental stimuli have been shown to enhance resistance to apoptosis (Beere, 2005; Punyiczki and Fésüs, 1998), while certain small heat shock proteins (sHsps) act as regulators of the apoptotic pathway (Mehlen, *et al.*, 1996).

It is clear from the evidence presented in this chapter that biological effects arising from RF-EMF exposure may be based on different biophysical mechanisms. Consensus will only be reached in the scientific community regarding the possible health hazard that mobile phones pose when a better understanding of the physical regulation of biological mechanisms at the atomic level is gained.

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CHAPTER 2

MOLECULAR BASIS FOR CELLULAR STRESS: OCCURRENCE IN HUMAN SPERMATOZOA AND IMPLICATIONS FOR MALE FERTILITY

2.1 INTRODUCTION - GENERAL ASPECTS OF CELLULAR STRESS

Cellular damage due to various stress factors (e.g. heat, reactive oxygen species chemicals, radiation, etc.) could entail one of two opposing responses, namely, cellular death (apoptosis or necrosis), or recovery (initiation of the stress response). These events are illustrated in Figure 2.1. Apoptosis, also known as programmed cell death (PCD), is a mechanism of removing damaged cells to prevent inflammation. The stress response, on the other hand, prevents damage or facilitates recovery to maintain cell survival. "Interactions between these two paradoxical pathways determine the fate of a cell and, as such, have a profound effect on the biological consequences of stress" (Beere, 2004).

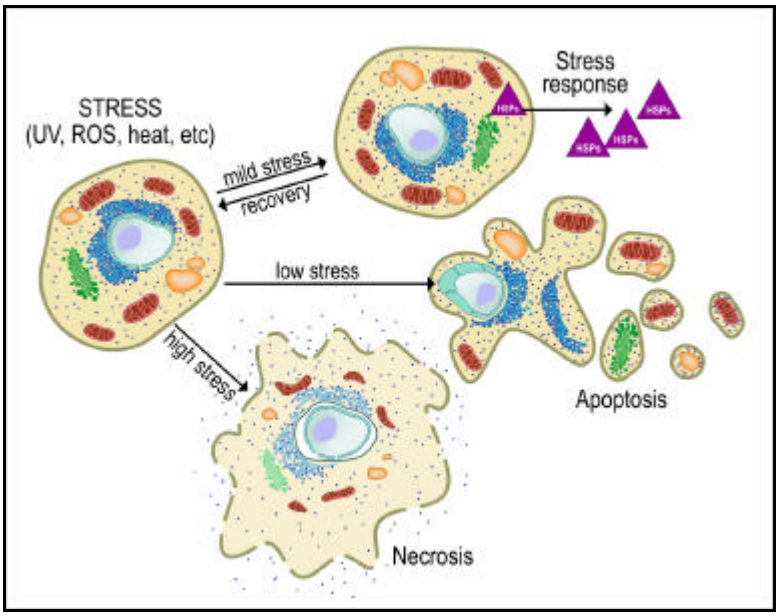

Figure 2.1 ***The stress response***: Exposure to damaging stimuli can trigger a cellular stress response resulting either in recovery or activation of the apoptotic program. Severe exposure could initiate cellular necrosis.

2.2 BIOCHEMICAL CHARACTERIZATION OF APOPTOSIS

In 1972, Kerr *et al*. distinguished between two classes of cellular death, apoptosis and necrosis. Apoptosis is an orderly cell-intrinsic process regulated by a variety of cellular signalling pathways and is a central part of normal development (Danial and Korsmeyer, 2004). It is involved in organogenesis, tissue homeostasis, and editing of the immune system to remove autoreactive clones (McKenna, 2001). Programmed cell death depends on the induction and action of specific genes that bring about the controlled disassembly of the cell. Cells induced to undergo apoptosis shrink, exhibit mitochondrial break down, develop bubble like blebs on the cell surface, undergo nuclear chromatin degradation, and break into small membrane-wrapped fragments (Wyllie *et al.*, 1980). The phospolipid phosphatidylserine (PS), which is normally hidden within the plasma membrane, is exposed on the surface. PS is then bound by receptors on phagocytic cells like macrophages or neighbouring cells that engulf the cell (Fadok *et al*., 2000). The phagocytic cells in turn secrete cytokines that inhibit inflammatory damage to surrounding cells.

In contrast to apoptosis, necrosis is not seen in normal development but is invariably the response to cellular injury or toxic damage. This form of unorganised death results from cellular metabolic collapse when a cell can no longer maintain ionic homeostasis. During necrosis, cells undergo a series of distinctive changes, characterized morphologically by vacuolation of the cytoplasm, breakdown of the plasma membrane resulting in the release of intercellular contents, and pro-inflammatory molecules that induces inflammation of surrounding tissues (Kroemer *et al*., 1998; Edinger and Thompson, 2004).

2.2.1 The effectors of apoptosis

The effectors of apoptosis are a family of aspartic acid-directed cysteine proteases, better known as caspases (cysteinyl aspartate-specific proteinases - Cp) (Reed, 2000). Caspases are able to cleave after an aspartic acid residue. They are expressed as inactive proenzymes (pCp), which are cleaved into active forms (aCp) in cells where PCD has been initiated and as such play a crucial role in the transduction of apoptotic signals (Tamar *et al*., 2004). As part of the apoptotic process, caspases act

both as initiators (Cp2, Cp8, Cp9, and Cp10) of cellular disassembly in response to pro-apoptotic signals, and as effectors (Cp3, Cp6, and Cp7) thereof.

Caspases can be activated by two distinct mechanisms during apoptosis (Hengartner, 2000). Firstly, molecules (death activators) binding to specific receptors on the cell surface can trigger apoptosis – referred to as the extrinsic apoptotic pathway. Secondly, signals arising from within the cell can initiate the response called the intrinsic apoptotic pathway. Whether apoptosis results from extra-cellular ligand binding or intracellular, from the release of organelle apoptotic factors, all these pathways converge on a caspase cascade.

The Bcl-2 (B cell leukaemia) family of proteins constitute a cardinal part in the regulation of both the intrinsic- and extrinsic- apoptotic pathways. Bcl-2 is a membrane-associated protein, which in the intact cell is largely found in the nuclear envelope, endoplasmic reticulum, and mitochondria. It belongs to a growing family of apoptosis-regulatory proteins that may be either death antagonists (anti-apoptotic) e.g. Bcl-2, Bcl-X_L (Boise *et al.*, 1993), and Mcl-1 (Kozopas *et al.*, 1993) or death agonists (pro-apoptotic), e.g. Bax, Bak (Oltvai, 1993) and Bid (Wang *et al.*, 1996).

Activation of different damage pathways or stimulation of pro-apoptotic signal transduction cascades converge into a common death pathway either by perturbation of mitochondrial membrane integrity through the activation of the permeability pore complex (PTPC or mega-channel) and/or Bcl-2-Bax complex in the mitochondrial outer membrane contact site or by primary activation of caspase cascades (Jäättelä *et al.*, 1998).

2.2.2 Extrinsic regulation of apoptosis

When an apoptotic promoter such as a death ligand binds to specific death receptors on the cell surface, a death-inducing signalling complex (DISC) is activated (Daniel, 2000; Danial and Korsmeyer, 2004). There are three death receptor pathways regulated by ligand binding (Figure 2.2). Firstly, (Figure 2.2 A) the tumour necrosis factor receptor (TNFR-1) family, which is activated by TNF binding (Danial and Korsmeyer, 2004). Secondly, (Figure 2.2 B) the Fas (APO-1/CD95) death receptor activated by Fas ligand (FasL) binding (Krammer, 1999), and lastly, (Figure 2.2 C)

the DR4/5 death receptors that share a different death ligand, namely, Apo2L/TRAIL (TNF-Related Apoptosis Inducing Ligand) (Ashkenazi and Dixit, 1998). Once the DISC is activated by ligand binding the adaptor protein FADD/MORT, bearing both death domains (DD) and death effector domains (DED) motifs, binds the DD of the death receptor while the DED domain recruits pCp8.

High concentrations of pCp8, is believed to lead to its autoproteolytic activation following the "induced proximity" model (Muzio *et al*., 1998). The activated inducer caspase, Cp8, then amplifies the apoptotic signal by cleaving and activating effector caspases, Cp3, Cp6, and Cp7. This process culminates in the degrading of hundreds of regulatory proteins and activation of endonucleases and other proteases resulting in apoptotic cell death (Krammer, 1999).

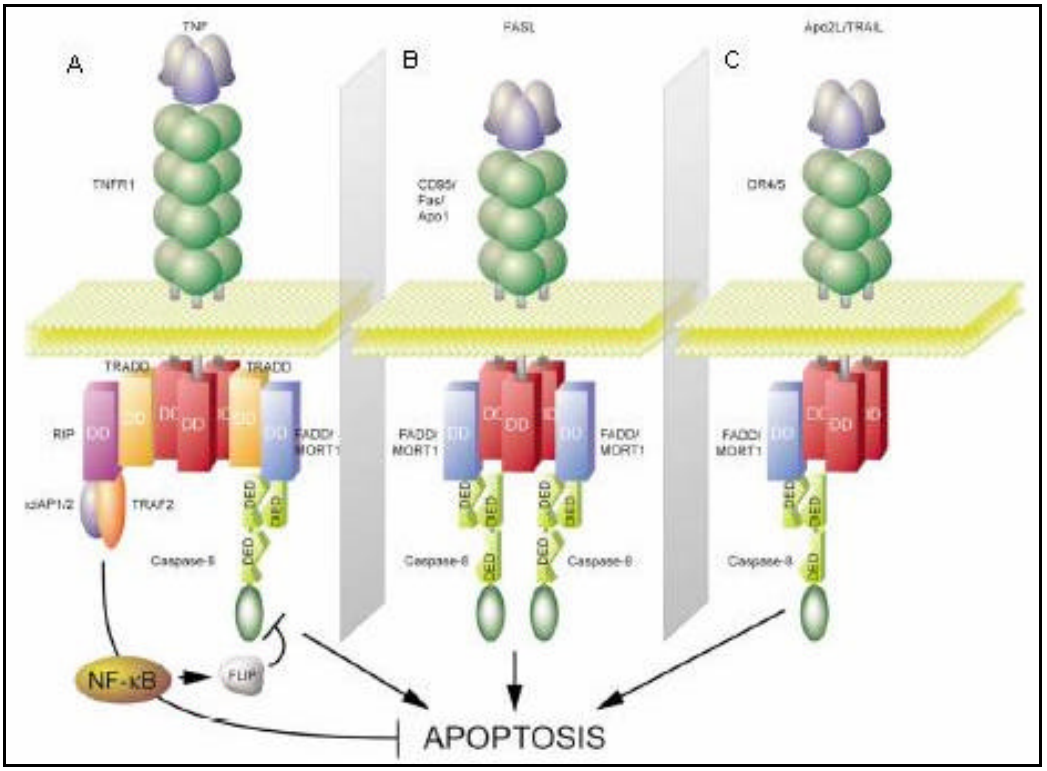

Figure 2.2 ***Extrinsic Apoptotic Pathway*** (Danial and Korsmeyer, 2004 - with permission). Binding of the various trimeric ligands to death receptors, **(A)** TNF to TNRF-1, **(B)** FasL to Fas (APO-1/CD95) and **(C)** APO2L/TRAIL to DR4/5 triggers the downstream assembly of the DISC.

In addition to cell death, death receptor signalling also (in select circumstances) mediates the decision between apoptosis and survival (Danial and Korsmeyer, 2004). The formation of differential complexes between various DD or DED proteins (Micheau and Tschopp, 2003) leads to either nuclear factor (NF)-κB activation or Cp8 inhibition. Once activated NF-κB has the ability to inhibit apoptosis.

It has been noted that in some cellular systems, death receptor mediated apoptosis involves the activation of mitochondrial cytochrome-c release and mitochondrial permeability shift transition (Schmitz *et al*., 1999). This has been explained by the recent discovery of Bid cleavage by Cp8, effectively connecting the death receptor to the mitochondrial apoptosis pathway (Li *et al*., 1998; Luo *et al*., 1998). Caspase-8 activation is achieved through death receptor DISC recruitment which leads to Bid cleavage and subsequent binding to Bax. Once activated, Bax facilitates the release of cytochrome-c from the mitochondria leading to the apoptosome assembly and mitochondrial apoptosis (Desagher *et al*., 1999).

2.2.3 Intrinsic regulation of apoptosis

Stress signalling pathways arising from within the cell can also initiate apoptosis. Both the mitochondria and the endoplasmic reticulum (ER) are known orchestrators of apoptosis. Similar to the DISC formation following ligand death receptor binding, a complex, better known as the apoptosome, is formed during mitochondrial apoptosis (Figure 2.3). In both mitochondrial and ER apoptosis, caspases are the central executioners of the apoptotic pathway, while the Bcl-2 family of proteins play a critical regulatory role in the execution of the intrinsic apoptotic pathway.

Members of the Bcl-2 family, Bad, Bid, Bim, and NOXA/PUMA (known as BH3-only proteins) serve as upstream sentinels that selectively respond to specific, proximal death and survival signals (Figure 2.3). Pro-apoptotic Bax and Bak activation by these proteins facilitates the release of cytochrome-c from mitochondria leading to the formation of the apoptosome and effector caspase activation, while the Cp12 induced apoptosis pathway in ER results in the disruption of Ca^{2+} homeostasis (Figure 2.3). Anti-apoptotic members Bcl-2, Bcl-X_L, and Mcl-1 bind and sequester specific BH3 molecules preventing Bax, Bak activation.

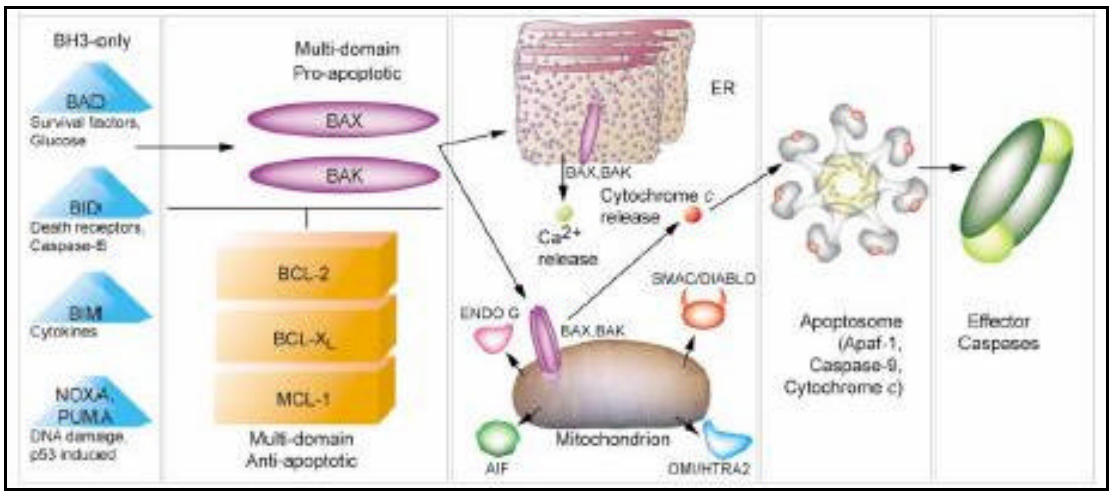

Figure 2.3 ***Intrinsic Apoptotic Pathway*** (Danial and Korsmeyer, 2004 - with permission). BH3 protein activation lead to Bax, Bak activation resulting either in the assembly of the apoptosome or ER induced apoptosis.

2.2.3.1 The role of mitochondria in apoptosis

There has been a generally accepted paradigm shift from the nucleus that determines the apoptotic process to the mitochondria that constitutes the centre of death control (Loeffler and Kroemer, 2000). Mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become evident. These changes occur to the inner and outer mitochondrial membranes leading to mitochondrial outer membrane permiabilisation (MOMP) and release of pro apoptotic inter membrane space proteins. Mitochondrial outer membrane permeabilization is achieved by a direct action on the permeability transition pore complex, a multi-protein ensemble that can interact with pro- and anti-apoptotic members of the Bcl-2 family (Desagher *et al*., 1999; Wei *et al*., 2001).

The leakage of inter-membrane mitochondrial space proteins cytochrome-c (Kluck *et al*., 1997; Yang *et al*., 1997), Smac/Diablo (Verhagen *et al*., 2000), OMI/HTRA2 (Suzuki *et al*., 2001), endonuclease-G (Li *et al*., 2001), and apoptosis inducing factor (AIF) (Susin *et al*., 1999) from the cytosol leads to their participation in the activation of caspases and endonucleases. Cytochrome-c interacts with Apaf-1 (apoptotic protease activating factor) a protein released from Bcl-2. The released

cytochrome-c and Apaf-1 bind to molecules of pro-caspase 9, a protease (protein cleaver). The resulting complex of cytochrome-c, Apaf-1, pCp9, and ATP is known as the apoptosome (Van Der Heiden, 1999; Kroemer and Reed, 2000). This complex aggregates within the cytosol, which together activates Cp3 leading to an expanding cascade of proteolytic activity (Pan *et al*., 1998). The release of AIF from the mitochondria triggers chromatin condensation and large scale DNA fragmentation (Susin *et al*., 1999).

It would thus appear that the death-life decision is closely linked to the status of the mitochondria membranes (Green and Reed, 1998; Lee *et al*., 2000). Dissipation of the mitochondrial membrane potential resulting from irreversible mega-channel opening, constituting changes in the mitochondria membrane potential (?ψ_m), is incompatible with the vital role of mitochondria in bioenergetics, redox, and ion homeostasis (Green and Kroemer, 1998). The release and/or inactivation of cytochrome-c causes disruption in electron transport, resulting in the generation of ROS and a drop in ATP production (Barrientos and Moraes, 1999). Both could potentially contribute to PCD in the absence of caspases. The choice between apoptotic and necrotic cell death is, however, dictated by the activation of downstream caspases. Since ATP is required for the function of the apoptosome and concomitant caspase activation, it seems plausible that apoptosis can occur as long as ATP levels are sufficient. It thus follows that when ATP levels are depleted, necrotic cell death will occur (Eguchi *et al*., 1997; Tsujimoto, 1997).

2.2.3.2 The endoplasmic reticulum regulation of apoptosis

This novel apoptotic pathway is mediated through Cp12 activation (Nakagawa *et al*., 2000). Caspase-12 activation occurs independent from death receptor signalling or mitochondrial signal pathways. It is initiated by the “unfolded protein response”, which is detected when protein (exported from the ER) folding is inhibited (Chapman, *et al*., 1998; Pahl, 1999). Caspase-12 is localised at the cytosolic side of the ER membrane and is activated by ER stress apoptotic signals, for instance, disruption of ER calcium (Ca^{2+}) homeostasis and excess protein accumulation in the ER. Furthermore, a combination of oxygen and glucose deprivation also triggers Cp12 activation and induces unfolded protein response markers (Daniel, 2000). Pro-

apoptotic homologues Bax and Bak together comprise a vital gateway for the initiation of the ER apoptotic pathway (Scorrano *et al*., 2003).

2.2.4 The role of kinases in the regulation of apoptotic signal transduction

Stress activated protein kinases (SAPKs) play a pivotal role in the mediation of extracellular signal transduction to the nucleus by regulating the events that determine the functional outcome of the cell in response to the stress-stimuli (Paul *et al*., 1997). The SAPKs form part of a greater family of mitogen-activated protein kinases (MAPK) that consist of the ERK (extracellular regulated kinases) (Robbins *et al*., 1994; Cobb *et al*., 1994), JNK/SAPK1 (jun N-terminal kinases/stress activated protein kinases) (Cano and Mahadevan, 1994; Davis, 2000), and the p38 kinases (Cano and Mahadevan, 1994; Cobb and Goldstein, 1995). The activation of JNK and p38 signalling pathways are generally seen as promoting apoptosis (Guo *et al*., 1998; Roulston *et al*., 1998), while the ERK signalling pathway tends to be apoptotic inhibiting (Xia *et al*., 1995).

Activation of the JNK and p38 kinases results from the ligation of a variety of receptors including the TNF- and FAS-receptors (Karmann *et al*., 1996). Once activated, JNK potentiates apoptosis by caspase activation (Franklin and McCubery, 2000). While p38 pathway activation, among others, leads to heat shock protein phosphorylation, specifically Hsp27 (Chrétien and Landry, 1988; Landry *et al*., 1992; Huot *et al*., 1995).

2.3 THE HEAT SHOCK RESPONSE: HEAT SHOCK PROTEINS

Changes in the environment, injury, disease, even growth and differentiation places organisms under stress. Organisms have evolved to cope with many different forms of stress by means of the heat shock- or cellular stress- response (Punyiczhi and Fésüs, 1998; Creagh *et al*., 2000; Beere, 2005). Ritossa first described the stress response in 1962, when *Drosophila* salivary glands exposed to heat shock, un-couplers of oxidative phosphorylation or anoxia, produced a new chromosomal puffing pattern (Ritossa, 1962). Further investigation revealed that heat shock activated the synthesis of only a few polypeptides while inhibiting the synthesis of

most others (Tissieres *at el*., 1974). These polypeptides were then aptly named heat shock proteins.

2.3.1 Heat shock protein families

Heat shock proteins comprise a large super-family of ubiquitously expressed highly conserved proteins present in all major cellular compartments in every organism from bacteria to human beings (Punyiczhi and Fésüs, 1998; Creagh *et al*., 2000). The Hsps are classified according to their molecular masses (kD) into different families including: Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsp-family (Hsp28, Hsp27, and Hsp25). Some Hsps are not only stress inducible but are constitutively expressed, indicating a cellular function under normal conditions (Creagh *et al*., 2000). These include important housekeeping functions, for example, many stress proteins are molecular chaperones that help nascent polypeptides assume their proper conformation (Mallouk *et al*., 1999). Other essential functions include intracellular trafficking, antigen presentation, nuclear receptor binding, and regulating apoptosis (Jindal and Young, 1991). Furthermore, Hsps play important roles in the cell cycle, as well as in cellular differentiation and growth (Prohászka and Füst, 2004). The major Hsp families and their corresponding functions are summarised in Table 2.1.

2.3.2 Induction and regulation of the heat shock response

In eukaryotes, Hsp synthesis is regulated at the transcriptional level by the activation of the heat shock transcriptional factor (HSF-1) (Morimoto *et al*., 1994). In the unstressed cell, a chaperone complex (Hsp90, Hsp70, p60, FKB52, FKB51, CyP40 and p23) binding HSF-1 (Figure 2.4 - (1)), maintains it in an inactive form (Nair *et al*., 1996; Dorion and Landry, 2002).

During stress, Hsps become pre-occupied with the stress-induced unfolding and denaturation of native proteins resulting in the dissociation of the cytoplasmic chaperone/HSF-1 complex (Morimoto, 1993; Zou *et al*., 1998). In the process HSF-1 trimerizes and its nuclear localization signal is uncovered allowing its translocation and accumulation in the nucleus (Figure 2.4 - (2 & 3)). There it binds to a specific DNA sequence (-nGAAn-), referred to as the heat shock element (HSE) (Morimoto *et al*., 1994; Mallouk *et al*., 1999; Westerheide and Morimoto, 2005). This results in the activation of HSF-1 and Hsp gene transcription leading to an accumulation of

Hsp expression (Freeman *et al.*, 1999; Mallouk *et al.*, 1999; Dorion and Landry, 2002) (Figure 2.4 - (4)).

Elevated levels of Hsps result in termination of Hsp transcription, and HSF-1 deactivation (Figure 2.4 - (5)), demonstrating that Hsps negatively regulate heat shock gene transcription via an auto regulatory loop (Craig and Cross, 1991; Hightower, 1991).

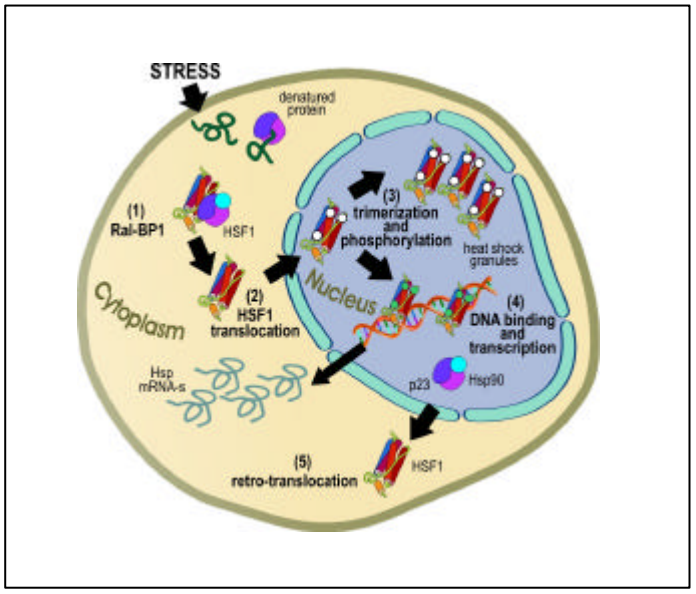

Figure 2.4 ***Major transcription factors leading to the induction of Hsp synthesis***: **(1)** cytoplasmic complex of HSF-1 and Hsp90; **(2)** HSF-1 translocation to the nucleus; **(3)** intra-nuclear distribution of HSF-1; **(4)** nuclear complex of HSF-1 and Hsp90; **(5)** retro translocation of HSF-1 to the cytoplasm. (Adapted from Sõti *et al.*, 2005).

Table 2.1 Heat shock protein families, their expression and functions.

Hsp Family	Function	References
Hsp110 - constitutive and inducible forms.	▪ Chaperone activity - prevents protein aggregation and help keep denatured proteins in a folding-competent state, ▪ Plays a role in thermo-tolerance.	Creagh *et al*., 2000.
Hsp90 - consists of Hsp 90 and Grp94, abundantly expressed.	▪ Grp94 are involved in protein secretory pathways including binding to newly synthesised, unfolded, and/or incompletely glycosylated proteins in the lumen of the ER, ▪ Chaperone activity especially after stress, ▪ Protection against apoptosis.	Hendrick and Hart, 1993; Creagh *et al*., 2000; Sõti *et al*., 2005; Beere, 2005.
Hsp70 - highly conserved, constituting both constitutive and inducible forms.	▪ Cytosolic forms of Hsp70 are essential in post-translational translocation of newly synthesized proteins from the cytosol into the ER in an ATP-dependant manner, ▪ Plays critical role in the folding of complex proteins like immunoglobulins, ▪ During a stress response, plays an important role in thermo-tolerance, ▪ Inhibits apoptosis.	Mallouk *et al*., 1999; Creagh *et al*., 2000; Westerheide and Morimoto, 2005; Beere, 2005.
Hsp60 - also known as GroEL proteins.	▪ Plays a role in the folding of monomeric proteins and/or catalysing the assembly of olicomeric complexes in a highly ordered manner, ▪ Serves as a scaffold on which newly synthesised proteins are correctly folded and assembled into their final form.	Hendrick and Hart, 1993; Creagh *et al*., 2000.
Hsp 40 - consists of about 100 members.	▪ Functions as a co-chaperone for Hsp70, ▪ Interacts with Hsp70 in an ATP-dependant fashion.	Gotoh *et al*., 2004.
Small Hsps - including the α-crystallin family consists of 12- to 43-kDa proteins.	▪ Expressed at low levels in unstressed cells, upon a stress response exhibit a 10-20 fold increase, ▪ Plays an important role during growth and development, ▪ Anti-apoptotic properties.	Mehlen *et al*., 1996; Creagh *et al*., 2000; Paul *et al*., 2002; Beere, 2005.

Apart from heat shock, the cellular stress response is activated by a myriad of different stressors, summarised briefly as physiological, pathological, and environmental conditions in Table 2.2. Irrespective of the type of stress triggering Hsp activation, one aspect common to all these stress signal pathways is the activation of HSF-1 following extensive protein aggregation caused by the stressor (Morimoto *et al*., 1997). Hsp activation can also be achieved through MAPK phosphorylation. Stress stimuli resulting in activating the p38 stress pathway in turn activates the MAP kinases, which leads to the phosphorylation of Hsp27 (Dorion and Landry, 2002).

Table 2.2 Conditions that lead to the activation of heat shock proteins (adapted from Prohászka and Füst, 2004).

Physiological	Pathological	Environmental
Cycle of cell division	Viral infection	Thermal changes
Growth factors	Bacterial infection	Heavy metals
Cytokines (TNF, FAS)	Parasitic infection	Ethanol
Cell differentiation	Fever	Antibiotics
Tissue development	Inflammation	Chemotherapeuticals
Hormonal stimulation	Ischemia	Radiation (UV)
	Hypertrophy	Chemical toxicants
	Oxidative stress	
	Malignancy	
	Autoimmunity	
	Toxins	
	Lipopolysaccharide	

2.3.3 Hsps as mediators of apoptosis

Upon exposure to stressors, especially those associated with the protein machinery, certain Hsps are activated and expressed at high levels to protect the cell by maintaining protein homeostasis. It is this protective function of Hsps that leads to the suppression of several forms of cell death, including apoptosis (Beere, 2005). On the other hand, it has also been reported that Hsps do not exert the same effect on cell death under all conditions and in all models; it may either have no effect, inhibit, or

enhance apoptosis (Vayssier and Polla, 1998). Activation of Hsps represents the functional endpoints of upstream apoptotic signals, making it extremely difficult to pinpoint the mechanisms that mediate the survival-/death-promoting effects of Hsps. Certain Hsps namely, Hsp 27, Hsp70, and Hsp90, have been implicated as modulators of PCD (Vayssier and Polla, 1998; Beere, 2005).

2.3.3.1 Hsp regulation of the intrinsic apoptotic pathway

Both Hsp70 and Hsp27 are able to modulate Bid-dependant apoptosis (Paul *et al.*, 2002; Gabai *et al.*, 2002). By suppressing caspase-8 mediated cleavage and activation of Bid, Hsp70 inhibits TNF-death receptor induced release of cytochrome-c (Mosser *et al.*, 2000; Paul *et al.*, 2002; Steel *et al.*, 2004). Hsp27 prevents the translocation of Bid to the mitochondria; this action could be related to Hsp27's ability to stabilize the cytoskeleton (Beere, 2005).

During Nitiric Oxide induced apoptosis, Hsp70 and its co-chaperone, Hsp40, prevent translocation of Bax to the mitochondria, thereby inhibiting MOMP and cytochrome-c release (Gotoh *et al.*, 2004) (Figure 2.5 A). Furthermore, through MOMP, a caspase independent cell death factor, AIF, is released (Susin *et al.*, 1996,1999). Hsp70 does not directly regulate the release of AIF from mitochondria, but prevents the nuclear translocation and pro-death signalling of AIF (Ravagnan *et al.*, 2001; Gurbuxani *et al.*, 2003) (Figure 2.5 B).

In addition to AIF release as a result of MOMP, cytocrome-c is also released which initiates the apoptosome assembly. A key factor in this assembly relies on the interaction between Apaf-1 and pCp9 to generate aCp9 (Srinvasula *et al.*, 1998). Hsp70 and Hsp90 can halt the apoptosome formation by direct interaction with Apaf-1 to prevent its association with pro-caspase-9 (Beere *et al.*, 2000; Pandey *et al.*, 2000) (Figure 2.5 C). Interestingly, Hsp27 may also disrupt the apoptosome formation by sequestering cytochrome-c upon its release from the mitochondria (Bruey *et al.*, 2000) (Figure 2.5 D). Hsps are also involved in the regulation of various pro-and anti-apoptotic signalling cascades, for instance, Hsp27 and Hsp90 help stabilise the effector protein kinase (PBB/Akt) to promote cellular survival (Figure 2.5 E).

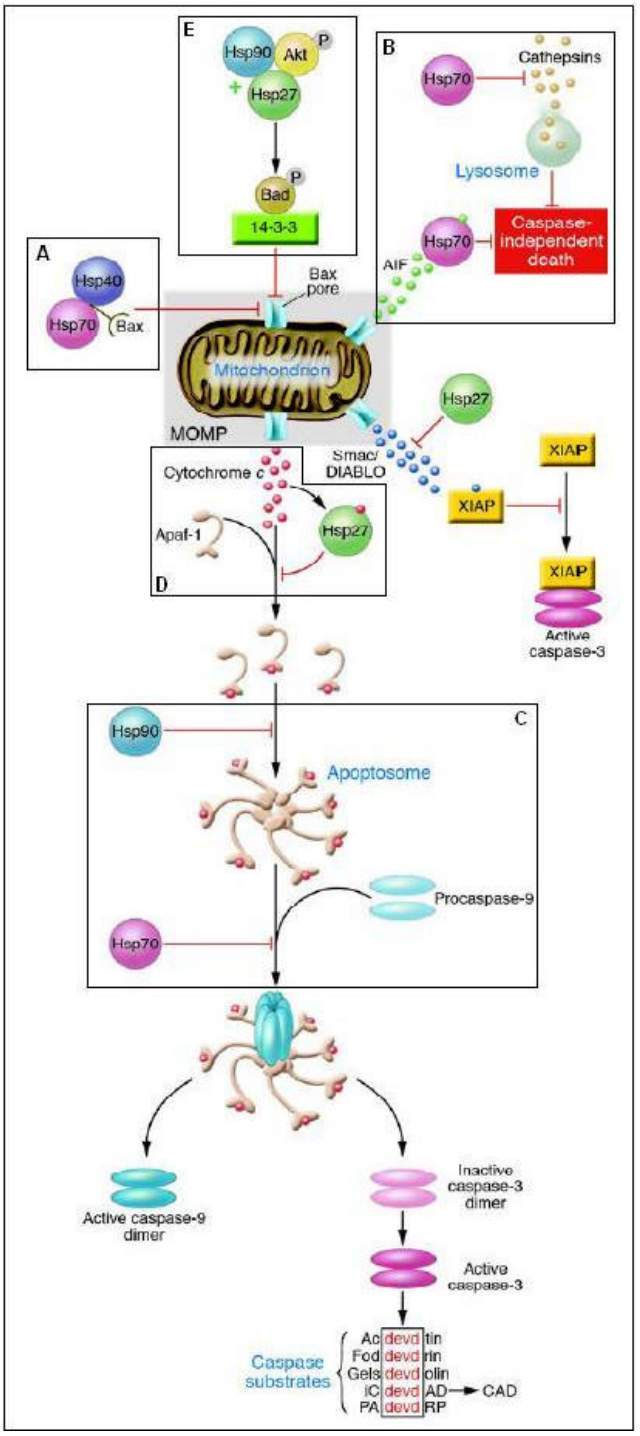

Figure 2.5 ***Regulation of the intrinsic apoptotic pathway by Hsps*** (with permission, Beere 2005); **(A)** inhibiting BAX translocation to the mitochondrion, **(B)** suppression of AIF activity, **(C)** inhibiting the activation of pro-caspase-9 by the apoptosome, **(D)** sequestering cytocrome-c release as a result of MOMP, **(E)** inhibiting Akt/BAD activation of MOMP.

2.3.3.2 Hsp regulation of the extrinsic apoptotic pathway

Several studies have noted the ability of Hsps to regulate Fas- (Mehlen, *et al*., 1996; Liossis *et al*., 1997; Clemons *et al*., 2005), TRAIL- (Kamradt *et al*., 2005), and TNF- (Jäättelä *et al*., 1993; Galea-Lauri *et al*., 1996, Mehlen *et al*., 1996; Van Molle *et al*., 2002) dependant apoptotic pathways. In particular, Hsp27 and Hsp90 have been noted to regulate TNF-mediated cellular survival (Chen *et al*., 2002; Park *et al*., 2003; Parcellier *et al*., 2003). The exact mechanism of action by which Hsps regulate these pathways are likely to be extremely complex due to the diversity of death receptors and ligands and the complexity of their signalling pathways (Figure 2.6). Hsps can directly bind the DD resulting in either cells survival signalling (Hsp90 – TRAF/RIF: recruitment NF-κB survival pathway) or regulate elements of the death receptor–mediated pathways (Hsp27 regulating the recruitment of DAXX which leads downstream to JNK apoptotic kinase pathway, Hsp70 regulating Bid which leads to MOMP, and Hsp27 regulating Bax that leads to MOMP). What further compound matters is that some Hsps are able to mediate cell death or promote survival (Vayssier and Polla, 1998; Beere, 2005).

2.3.3.3 Hsp regulation of the MAPK activated apoptotic pathways

The regulation of MAP kinase signalling pathways in response to stress stimuli is achieved in some cases through Hsp mediation. Stress inducible Hsp70 has been noted to regulate JNK activity (Buzzard *et al*., 1998) through a mechanism involving either the direct binding of Hsp70 to JNK (Park *et al*., 2001) or by Hsp70-mediated protection of a JNK phosphatase from heat denaturation (Meriin *et al*., 1999) (Figure 2.6). The inhibition of JNK activity by Hsp70 therefore results in the inhibition of JNK mediated apoptosis. This is also seen in p38 MAPK regulation of apoptosis, where induced Hsp70 could inhibit p38 MAPK activity (Gabai *et al*., 1997). Furthermore, Hsp70 has also been implicated in the protection of TNF induced apoptosis by inhibiting caspase conversion and suppressing downstream proteolitic events (Jäättelä *et al*., 1998). Mallouk *et al*. (1999) reported that Hsp70 protected cells against necrosis thereby potentiating apoptosis.

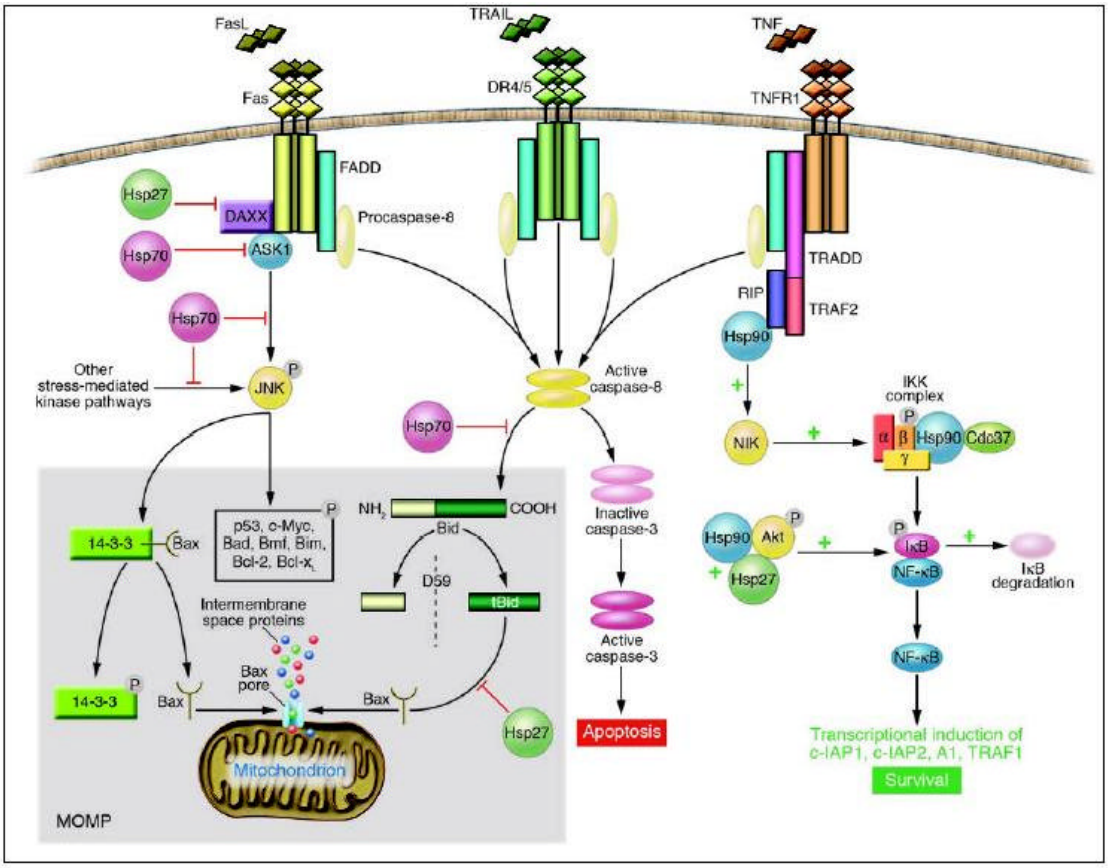

Figure 2.6 ***Regulation of the extrinsic apoptotic pathway by Hsps*** (with permission, Beere, 2005). Hsps regulate both death receptor signalling (Hsp27 - JNK and Bax pathway; Hsp70 – Bid pathway) and cell survival pathways (Hsp90 - NF-κB pathway).

Hsp90 constitutes various fundamental cellular processes, such as hormone signalling and cell cycle control with several signal transduction pathways interacting with Hsp90 (Scheibel and Buchner, 1998). Binding of Hsp90 with the drug geldanamycin causes disruption in MAPK signalling (Schulte *et al*., 1996).

Hsp27 is able to modulate apoptosis through the FAS kinase pathway (Arrigo, 1998). This protective action of Hsp27 can be attributed to its interference with upstream signalling from cytochrome-c release and/or direct interaction with cytochrome-c (Paul *et al*., 2002). Specifically, it has been noted that degradation of F-actin by the F-actin depolymerising agent cytochalasin D (Paul *et al*., 2002), the apoptosis promoting agents staurosporine (Paul *et al*. 2002), and cisplatin (Garrido *et al*., 1997;

Kruidering *et al*., 1998) are all able to activate cytochrome-c release from mitochondria and initiate apoptosis. The depolymerisation of cytosolic F-actin can be inhibited by Hsp27 expression, confirming its status as a negative regulator of apoptosis.

In contrast, activation of the p38 kinase pathway under stress conditions results in the phosphorylation of Hsp27 (Ahlers *et al*., 1994). Hsp27 phosphorylation has been proposed as a key determinant in actin microfilament stabilisation during stress (Arrigo and Landry, 1994; Benndorf *et al*., 1994). However, this action of Hsp27 can also mediate inappropriate actin polymerisation activity, which can lead to extensive cell blebbing and apoptosis (Dorion and Landry, 2002; Deschesnes *et al*., 2001). Hsp27 can thus either protect against MAP kinase activated apoptosis signalling or enhance it.

Hsp90, Hsp70, and Hsp27 play very important roles in the regulation of apoptosis through their interaction with a myriad of signalling pathways, and as such, they can either act as pro- or anti-apoptotic agents.

2.4 CELLULAR STRESS IN HUMAN SPERMATOZOA

In the past years, many environmental, physiological, and genetic factors have been implicated in poor sperm function and unexplained male factor infertility (Carlsen *et al*., 1992; Schrader *et al*., 1994; Cummins *et al*., 1994). Assisted reproductive techniques like *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) offer an alternative to such male factor patients (Bar-Chama and Lam, 1994; WHO, 1999; ASRM Practice committee, 2004). However, indiscriminate use of such assisted reproductive approaches is not warranted, especially when the aetiology of sperm dysfunction is poorly understood (Sikka, 2001; Sakkas *et al*., 2004; Sakkas and Seli, 2005).

In view of the current findings that RF-EMF could affect male fertility, it is necessary to understand how, and for that matter, if human spermatozoa are able to launch a stress response when exposed to stress stimuli and if this could lead to apoptosis. To answer this, a better understanding of sperm biochemistry is required

to firstly address the issue of whether a stress pathway is functional in sperm, and, secondly, to determine if sperm are able to undergo apoptosis.

2.4.1 Heat shock protein expression during spermatogenesis

The production of heat shock proteins are essential for cell survival and recovery from stress (Parsell and Lindquest, 1993). During spermatogenesis, dramatic transformations and cellular differentiation take place. It is therefore not surprising that spermatogenesis is accompanied by the expression of different heat shock proteins (Dix *et al.*, 1996; Meinhardt *et al.*, 1999; Neuer *et al.*, 2000). What is interesting to note is that each of the three distinct developmental phases during spermatogenesis, namely mitotic proliferation of spermatogonia, meiotic development of spermatocytes, and post-meiotic maturation of spermatids into spermatozoa (Eddy *et* al. 1991), are marked with the expression of specific Hsp's (Son *et al.*, 1999; Neuer *et al.*, 2000).

In mouse germ cells, two unique members of the Hsp70 family are expressed only during spermatogenesis; Hsp70-2 and Hsc70t (Dix *et al.*, 1996). Spermatocyte specific Hsp70-2 is expressed at high levels in pachytene spermatocytes during the meiotic phase of development (Allen *et al.*, 1988; Zakeri *et al.*, 1988) while testis specific Hsc70t is expressed in post-meiotic spermatids (Maekawa *et al.*, 1989; Matsumoto and Fujimoto, 1990). The expression Hsp70-2 is not heat inducible in murine germ cells, indicating a restricted role in germ cell maturation (Allen *et al.*, 1988; Zakeri *et al.*, 1988; Matsumoto and Fujimoto, 1990).

As with murine germ cells, Hsp's are expressed during human spermatogenesis. The most notable of the stress proteins implicated in human spermatogenesis are Hsp60 and 70 (Meinhardt *et al.*, 1999; Neuer *et al.*, 2000). Both of these Hsp families consist of proteins that are highly expressed in a constitutive manner. However, Hsp60 acts predominantly as a chaperone and is only mildly stress inducible (Meinhardt *et al.*, 1999), whereas Hsp70 is stress inducible and both the constitutively expressed Hsc70 and Hsp70 relocate to the nucleus upon stress induction (Pelham, 1984). The Hsp60 family is mainly located in the mitochondria (Jindal *et al.*, 1989) while the Hsp70 family is localized in distinct cellular

compartments (nucleus, mitochondria, and cytosol) (Meinhardt *et al.*, 1999; Neuer *et al.*, 2000).

In addition to the 70-kD (Hsp70) super-family of proteins, a male germ-cell specific protein, HspA2, homologous to the mouse Hsp70-2 protein has been identified in human germ cells and in sperm (Huszar *et al.*, 2000). In human spermatogenic cells, HspA2 is the predominant Hsp70. The HspA2 chaperone plays a prominent role during both meiosis and spermatogenesis (Dix *et al.*, 1996, 1997; Dix, 1997). Furthermore, the expression of HspA2 coincides with major protein translocation during cytoplasmic extrusion and remodelling of the sperm plasma membrane (Huszar *et al.*, 2000). This remodelling of the plasma membrane facilitates the development of the zona pellucida binding site (Huszar *et al.*, 1994, 1997). Not surprisingly, diminished HspA2 expression in immature sperm is directly correlated to a decreased fertilizing potential (Dokras *et al.*, 1999; Huszar *et al.*, 1992). Furthermore, Cedeno *et al.* (2006) recently noted that HspA2 is down regulated in men with idiopathic oligoteratozoospermia, confirming the importance of this heat shock for normal spermatogenesis.

In the male testes, Hsp70 and 60 along with Hsp90 assist in the post-translational maturation and translocation of newly synthesized polypeptides (Meinhardt *et al.*, 1999; Ergur *et al.*, 2002; Cayli *et al.*, 2003). They also afford a mechanism for heat protection in the male germ cells by binding unfolded or partially folded proteins to prevent their aggregation or irreversible thermal denaturation (Zakeri and Wolgemuth, 1987; Meinhardt *et al.*, 1999). Spermatids/spermatogonia are thus well equipped to launch a stress response following a stress assault. However, spermatozoa are terminally differentiated cells in which transcription and/or translation of nuclear encoded mRNAs are unlikely (Miller, 2000; Aitken, 2005; Grunewald *et al.*, 2005). It is therefore questionable whether mature spermatozoa would be able to react to a stress stimulus.

2.4.2 Heat shock proteins: presence and functionality in spermatozoa

When Miller *et al.* (1992) first investigated the expression of Hsp's in ejaculate human spermatozoa, he found that human male gametes contain a complex repertoire of Hsp's, which may include sperm-specific variants. Multiple isoforms of Hsp70

and one isoform of Hsp90 were identified. Hsp70 was noted as an abundant surface antigen whose presence could be attributed to adsorption from seminal plasma as Hsp70 is a profuse component of seminal plasma. This protein was also located in the midpiece, neck, and equatorial segments (Miller *et al*., 1992), as well as throughout the tail and on the surface of the tail plasma membrane (Huszar *et al*., 2003). Heat shock protein 90, on the other hand, is localized mainly in the neck, tail, and equatorial segments in human spermatozoa.

Since then, numerous Hsp's have been identified in human spermatozoa, including the sperm outer dense fibre protein (ODPF), a member of the small heat shock (sHsp's) super family (Fontaine *et al*., 2003) thought to be involved in cytoskeletal organisation as well as maintenance of elastic structures and the elastic recoil of sperm tails (Baltz *et al*., 1990). Nonoguchi *et al*. (2001) also reported the presence of a member of the Hsp110 family, Apg-1, in human spermatozoa.

Miller and co-workers (1992) hypothesized that "spermatozoa are pre-prepared for adverse environmental conditions by virtue of their existing complement of Hsp's." Further investigation by the same author revealed the presence of transcripts for Hsp 70 and 90 in ejaculated human spermatozoa (Miller, 2000). This is not a new finding as it is well known that an abundance of mRNAs are present in human ejaculated spermatozoa (Miller *et al*., 1999; Ostermeier *et al*., 2002; Lambard *et al*., 2004; Dadoune *et al*., 2005). However, it is generally believed that these mRNAs are remnants of untranslated stores during spermatogenesis (Miller *et al*., 1999) and that *de novo* synthesis of RNA in mature ejaculated human spermatozoa is not possible (Grunewald *et al*., 2005). Nonetheless, the mRNAs present in spermatozoa are still functional and can be reverse transcribed for putative translational or regulatory events during or after conception (Miller, 2000; Grunewald *et al*., 2005).

It would thus seem that spermatozoa are not only equipped with Hsps but are also able to up-regulate Hsp expression by transverse scriptase, a mechanism known to be functional in spermatozoa (Miller, 2000). What remains to be confirmed is the ability of the Hsp's to produce a shift in their sub cellular localization to facilitate a change in substrate specificity. Other researchers reported on the translocation and rearrangement of spermatozoal proteins during capacitation and acrosome reaction

(Saxena *et al*., 1986; Villaroya and Scholer 1986, 1987). In particular, Hsp70 undergoes re-location to the equatorial segment during induced capacitation and acrosome reaction in ejaculated bovine spermatozoa (Kamaruddin, 1998).

Considering the evidence provided, ejaculated spermatozoa appear to be equipped with the necessary Hsp machinery to act as chaperonens and perform stress-induced related functions on their journey through the female reproductive tract and the ultimate task of fertilisation. However, these functions may be sperm-specific and not inducible by conventional stress stimuli. Numerous studies have since expounded on the role of Hsp's in fertilization.

2.4.2.1 Role of heat shock proteins in fertilization

The fertilisation process is a complex mechanism that requires several biochemical and biophysical events to occur in ejaculated spermatozoa (Luconi *et al*., 1998). These events culminate in the sperm membrane fusion with the oocyte plasma membrane (oolemma) and subsequent penetration of the oocyte (Yanagimachi, 1994). Proteins found on the sperm plasma membrane and zona pellucida are known mediators of many of these recognition/fusion actions (Tulsiani *et al*., 1997).

It has been postulated that the surface antigen Hsp70 plays a role in the regulation of the acrosome reaction (Miller *et al*., 1992; Bohring and Krause, 2003) as well as sperm-zona pellucida interaction (Huszar *et al*., 2003). This could explain why Matwee *et al*. (2001) found that the presence of anti-Hsp70 antibody significantly reduced tight binding of spermatozoa to the zona pellucida of bovine oocytes and interrupted completion of meiosis II and pronuclear formation. The presence of anti-Hsp70 in culture medium from day 3 to 9 of development increased apoptosis and reduced the number of embryos reaching blastocyte stage. They therefore concluded that Hsp70 functions in the prevention of apoptosis during early blastocyte development. Mallouk *et al*. (1999) also reported a strong supportive relationship between Hsp70 expression, maintenance of ATP levels, and apoptosis.

Furthermore, the equatorial site of Hsp90 could be involved in oocyte-plasma membrane recognition (Miller *et al*., 1992). Huang *et al*. (2000a) reported that inhibition of Hsp90 by geldanamycin affected the sperm motility of ejaculates from

mature boars. In a further study (Huang *et al*., 2000b), it was found that the semen quality of boars significantly declined with decreased levels of Hsp70. Recently, Ecroyd *et al*. (2003) reported that Hsp90 becomes tyrosine phosphorylated during capacitation, confirming the role of this chaperone in the signalling pathway leading to human sperm capacitation. Although a member of the Hsp110 family, Apg-1 has been identified in human spermatozoa, its role in fertilisation has not been elucidated (Nonoguchi *et al*., 2001).

If exposure of ejaculated spermatozoa to RF-EMF could initiate an up-regulation of Hsp expression, it has the potential to interfere with the acrosome reaction and sperm-oocyte penetration. As these proteins remain activated for up to an hour post exposure (Leszczynski *et al*. 2002), an *in vivo* exposure prior to intercourse could potentially affect fertilisation.

2.4.2.2 Apoptosis in spermatozoa

Apoptosis has been demonstrated to be the underlying mechanism of germ cell death during normal spermatogenesis and is required for the control and development of normal spermatogenesis (Rodriguez, 1997, Sinha Hikim *et al*., 1998; Eguchi *et al*., 2002, Francavilla *et al*., 2000; Hayashi, 2002). To better understand the fertilisation process and to improve fertility diagnostics for ART (artificial reproductive technologies), scientists and clinicians alike have recently started investigating the role of apoptosis in spermatogenesis and the spermatozoon. However, many contradicting theories exist about the role (and presence) of apoptosis in human spermatozoa (pro apoptotic pathway: Host *et al*., 1999; Gandini *et al*., 2000; Barosso *et al*., 2000; Blanc-Layrac *et al*., 2000; Levy and Seifer-Aknin, 2001; Shen *et al*., 2002; Weng *et al*., 2002; Almeida *et al*., 2005 and against apoptotic pathway: Baccetti *et al*., 1996; Muratori *et al*., 2000; Sakkas *et al*., 2003; Tesarik *et al*., 2004; Moustafa *et al*., 2004; Lachaud *et al*., 2004; Martin *et al*., 2005).

One possible explanation for these contradictory reports are the diverse methods and techniques used to determine apoptotic status in the spermatozoon. When revisiting the pathway of apoptosis activation in somatic cells, the activation of the Fas/FasL receptor protein could trigger the activation of caspases. A drop in mitochondrial membrane potential has also been associated with triggering apoptosis. Furthermore,

an early occurrence in the apoptotic process is the translocation of phosphatidylserine, a membrane bound protein to the outer leaflet, whereas DNA fragmentation occurs later during the apoptotic process. In reviewing literature, the following aspects regarding apoptosis in human spermatozoa were investigated.

2.4.2.3 The Fas mediated pathway in sperm cell apoptosis

During spermatogenesis, germ cell maturation is supported by Sertoli cells, and as such, there exists a symbiotic relationship between the capacity of Sertoli cells to support a limited number of germ cells (Pentikäinen *et al.*, 1999). It has been demonstrated that, in mice and rats, Sertoli cells express FasL and signal the commencement of PCD in Fas expressing germ cells to limit the number of the germ cells they can support (Lee *et al.*, 1997; Rodriguez *et al.*, 1997). This role of Fas/FasL was also confirmed to be functional in the human testis in regulating germ cell apoptosis (Lee *et al.*, 1997, 1999; Pentikäinen *et al.*, 1999; Francavilla *et al.*, 2000; Levy and Seifer-Aknin, 2001).

When the expression of Fas in ejaculated human spermatozoa was investigated, it was found that spermatozoa do express Fas (Sakkas, 1999; McVicar *et al.*, 2004). However, the percentage of Fas-positivity seemed to be correlated with abnormal sperm parameters, with the result that sub-fertile men expressed higher concentrations of Fas-positivity in their ejaculate compared to normal men (Sakkas, 1999; McVicar *et al.*, 2004). Sakkas *et al.* (1999) therefore postulated that the presence of Fas in ejaculated spermatozoa is indicative of an abortive apoptosis program during spermatogenesis and does not arise from a functional death receptor apoptosis pathway in mature sperm. Then again, a more recent study by Castro *et al.* (2004) reported the absence of Fas expression on the surface of normozoospermic and nonnormozoospermic men questioning Sakkas and co-worker's (1999) theory of an abortive apoptotic program.

2.4.2.4 Apoptosis promotion by caspase activation in spermatozoa

Caspase activation occurs when the precursor is converted to the active enzyme by proteolytic processing either by another protease or by autocatalysis in response to the binding of cofactors or removal of inhibitors (Reed, 2000). One factor implicated in the activation of PCD is the binding of FasL to Fas, triggering the activation of

caspase-8. In turn, Cp8 transduces a signal to effector caspases, such as Cp3, Cp6 and Cp7. This action leads to the degradation of cellular substrates including cytoplasmic structural proteins such as actin or nuclear proteins such as poly-ADP-ribose polymerase (PARP) (Said *et al*., 2004).

Caspase-3 is considered to be the major executioner caspase, as its activation signals the point of no return in apoptotic signalling (Earnshaw *et al*., 1999). An inactive form of Cp3 was identified as a membrane bound antigen in human spermatozoa (Bohring and Krause, 2003), while activated Cp3 was localized exclusively to the sperm midpiece region (Weng *et al*., 2002). In human spermatozoa, Cp3 enzymatic activity has been correlated with low motility (Veis *et al*., 1993; Weng *et al*., 2002; Marchetti *et al*., 2004). Weng *et al.* (2002) found that the high motility fractions of fertile men exhibited no activated Cp3 activity. In addition, it has also been demonstrated that infertile men have higher percentages of activated caspases (Paasch *et al*., 2003) with Cp3 activity significantly associated with teratozoospermia and asthenozoospermia (Almeida *et al*., 2005).

To better understand the role of caspases in human spermatozoa, Marchetti *et al.* (2004) and Paasch *et al.* (2004) investigated the relationship between caspase activation and other death cell markers (DNA fragmentation, mitochondrial membrane potential, and externalisation of phosphatidylserine). Paasch *et al.* (2004) found that Cp9 activity was closely associated to membrane damage. They attributed this activation to the close proximity of mitochondria to the outer membrane as Cp9 is located in the midpiece of spermatozoa. However, Cp3 activity was not significantly correlated with membrane changes. Marchetti *et al.* (2004) found that caspase activity significantly correlated with DNA fragmentation and that viable cells with a high mitochondrial membrane potential (intact membrane) negatively correlated with caspase activity.

2.4.2.5 Association of mitochondrial membrane potential with sperm apoptosis

The reduction of mitochondrial membrane potential is a general feature of PCD; it precedes other death cell markers such as DNA fragmentation, ROS production, and the late increase of membrane permeability (Kroemer *et al*., 1997). In human spermatozoa, analysis of the mitochondrial membrane potential offers a means, not

only to assess apoptosis, but also motility in sperm (Marchetti *et al.*, 2004). The ?ψ_m is a sensitive indicator of the energetic state of mitochondria as it can be used to assess mitochondrial respiratory chain activity, electron transport systems, and activation of the mithochondrial permeability transition leading to the release of cytochrome-c (Ly *et al.*, 2003).

In human spermatozoa, a positive correlation exists between a reduction in mitochondrial membrane potential, diminished motility, and low fertilization rates after IVF (Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Marchetti *et al.*, 2002; Piasecka and Kawiak, 2003; Wang *et al.*, 2003). A decrease in mitochondrial membrane potential could lead to the release of cytocrome–c, resulting in the activation of Cp9, which in turn initiates the proteolytic cascade that culminates in apoptosis (Green and Reed, 1998).

2.4.2.6 Externalisation of Phosphatidylserine as an indication of apoptosis in spermatozoa

One of the earliest events during apoptosis occurs with the asymmetry disturbance of the lipid bilayer resulting in the externalisation of the PS (Koopman *et al.*, 1994). The exposure of PS on the outside of the cell surface therefore serves as an early marker for apoptosis. In a study by Blanc-Layrac *et al.* (2000), very little PS externalisation was noted 4 hours after density gradient centrifugation of human spermatozoa, but it increased significantly after 24 hours indicating that these cells committed PCD. In contrast, washed sperm showed a significant increase of PS externalisation after only 4 hours, which decreased to insignificant levels after 24 hours. These authors therefore conclude that two forms of cell death had occurred in the two groups namely apoptosis and necrosis. However, the presence of PS was not concurrent with other apoptotic markers, such as membrane blebbing and loss in mitochondrial membrane potential.

In another study conducted by Oosterhuis *et al.* (2000), a strong correlation was seen between PS externalisation and DNA fragmentation in washed spermatozoa, therefore leading the authors to believe that apoptosis occurred just before or just after ejaculation. This finding contradicts that of Blanc-Layrac *et al.* (2000) who ascribed the presence of PS in washed sperm to necrosis. Numerous other studies

have since reported conflicting reports regarding the accuracy of using PS externalisation as a positive marker for apoptosis (de Vries *et al*., 2003; Martin *et al*., 2005) or the correlation of PS externalisation with other apoptotic markers (Barroso *et al*., 2000; Duru *et al*., 2001; Moustafa *et al*., 2004).

Furthermore, Glander and Schaller (1999) reported that PS externalisation after cryopreservation was the result of membrane deterioration, a finding that has been corroborated by others (Duru *et al*. 2000, 2001; Schuffner *et al*., 2001). Recent reports related the externalisation of PS as a consequence of capacitation and the acrosome reaction rather than apoptosis (Schuffner *et al*., 2002; de Vries *et al*., 2003; Martin *et al*., 2005). These findings suggest that phosphatidylserine externalisation could be an indicator for membrane integrity rather than apoptosis and that its presence in sperm should be confirmed with other positive apoptosis markers, for instance DNA fragmentation.

2.4.2.7 DNA fragmentation in spermatozoa as a consequence of apoptosis

DNA fragmentation results from the activation of endonucleases during the apoptotic program. DNA fragmentation in sperm samples has significant clinical relevance as increased rates of DNA fragmentation has been associated with decreased fertilisation (Marchetti *et al*., 2002) and pregnancy (Tomlinson *et al*., 2001). The question that has been raised often is, whether DNA damage in ejaculated spermatozoa is the consequence of 1) *problems in the nuclear remodelling process* (Sakkas *et al*., 2002, 2003, 2004) resulting from DNA packing during the transition of the histone to protamine complex during the late spermatid stage of spermatogenesis (McPherson and Longo, 1992; Sailer *et al*., 1995), 2) *the result of free-radical induced damage* (Aitken *et al*., 1998; Aitken and Baker, 2002; Castro *et al*. 2004), or 3) *the consequence of apoptosis* (Gandini *et al*., 2002; Shen *et al*., 2002; Weng *et al*., 2002)?

An investigation by Muratori *et al*. (2000) suggested that sperm DNA fragmentation did not have a significant correlation with other characteristics supportive of apoptosis, such as nuclear shape, chromatin packaging, or presence of translucent vacuoles in cytoplasmic residues, but was, however, correlated with ultra structural features suggestive of impaired motility. Apoptosis-like phenomena in spermatozoa

could be the result of various pathologies, which could be triggered by changes in hormone levels or by abnormal exposure to death-promoting signals such as reactive oxygen species (Castro *et al.*, 2004). Castro *et al.* (2004) further noted that this apoptotic process could have been initiated in the testes or along the male genital track, but was, at least not in their study, a consequence of the apoptotic process operating in ejaculated spermatozoa. In contradiction, Host *et al.* (1999), Muratori *et al.* (2000), Levy and Sefer-Aknin (2001), Gandini *et al.* (2002), Shen *et al.* (2002) and Weng *et al.* (2002) all reported that different apoptotic markers, including morphological characteristics, DNA fragmentation, PS externalisation, and caspase activation are present in human spermatozoa and indicative of an apoptotic process present in spermatozoa.

Barroso *et al.* (2000) attempted to clarify whether DNA fragmentation is a consequence of apoptosis by demonstrating a significant and positive correlation between the detection of DNA fragmentation associated with late apoptosis and monoclonal anti-body binding to single stranded DNA detecting earlier stages of apoptosis. They found that phosphatidylserine binding showed similar staining in fractions with increased- (high motility) and low- (low motility) DNA damage. Raising another question, is positive PS staining an indication of early stages of apoptosis, or a reflection of sperm capacitation, or the result of processing techniques? Research by other groups, have reported that caspases were rarely detected in human post meiotic germ cells and that their presence was not correlated with DNA fragmentation (Tesarik *et al.*, 2002; Lachaud *et al.*, 2004).

It is still open for debate whether the human spermatozoon is capable of initiating apoptosis or not, or whether the presence of apoptosis markers (phosphatidylserine, caspase-3, and Fas/FasL) are due to failed apoptosis during spermatogenesis or activated by the spermatozoon itself due to ROS activation.

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SECTION B

THE EFFECT OF NON-THERMAL 900 MHz GSM MOBILE PHONE RADIATION ON HUMAN SPERMATOZOA

Chapter 3

Capacitation & Zona Pellucida Binding

Chapter 4

Apoptosis

Chapter 5

Heat Shock Protein & Stress Fibre Activation

CHAPTER 3

CAPACITATION AND ZONA PELLUCIDA BINDING OF HUMAN SPERMATOZOA

3.1 INTRODUCTION - MOLECULAR BASIS FOR CAPACITATION IN HUMAN SPERMATOZOA

Human spermatozoa are unable to fertilize oocytes immediately after ejaculation even when they are brought into close contact with the oocyte surface. Only after transit through the female genital tract do they undergo a physiological change called *capacitation* that renders them competent for fertilization (Visconti *et al*., 2002; Breitbart, 2003). Sperm capacitation involves multiple metabolic, biochemical, membrane and ionic changes (de Lamirande *et al*., 1997; Yanagimachi, 1994). Intracellular changes known to occur in the capacitated spermatozoon include increases in membrane fluidity, cholesterol efflux, changes in intracellular calcium (Ca^{2+}) and 3', 5'-cyclic adenosine monophosphate (cAMP) concentrations, protein tyrosine phosphorylation and changes in the swimming pattern and chemotatic motility (Breitbart, 2002; Visconti *et al*., 2002). Capacitation is marked by two distinct processes namely hyperactivated motility and the acrosome reaction (AR).

Hyperactivated motility is described as a deviation from the relatively linear and progressive swimming pattern associated with spermatozoa in seminal plasma (De Jonge and Barratt, 2006). It is characterised by an almost frantic, whiplash movement of the flagellum (Yanagimachi, 1994) and is designed to assist spermatozoa in penetrating highly viscous and dense oviductal fluids (Ho and Suárez, 2001). The AR on the other hand is an exocytotic event that occurs immediately prior to fertilisation (Jeyendran, 2000). In this reaction the outer acrosomal membrane fuses with the surrounding plasma membrane, cumulating in the release and dispersal of the acrosomal content (Yanagimachi, 1994).

3.1.1 Hyperactivated motility

Intracellular Ca^{2+} plays a significant role in the regulation of sperm hyperactivation and is responsible for the increased asymmetrical flagellar movement (Darszon *et al.*, 1999, 2001; Ho and Suarez, 2001). It acts on the axoneme thereby increasing the principal flagellar bend curvature. However, the source of the Ca^{2+} required for hyperactivation is still uncertain, some report it arises from an extracellular influx (Ho and Suarez, 2001) or release from intracellular stores (Harper *et al.*, 2004). Ionic changes represent only a small part in the complex process of sperm hyperactivation. The anion, bicarbonate also regulates hyperactivation (Burkman, 1991).

Bicarbonate increases intracellular pH, thereby promoting the alkaline environment required for hyperactivation (Burkman, 1991). Furthermore, this anion activates adenylyl cyclase (Okamura *et al.*, 1985) resulting in cAMP production, but in insufficient amounts for the hyperactivation of whole spermatozoa (Mujica *et al.*, 1994). It is proposed that the influx of Ca^{2+} activates cyclic nucleotide-gated channels, which could indicate a possible role by which cAMP is involved in hyperactivation (Ho and Suarez, 2001). In addition, sperm are known to produce low and controlled amounts of extracellular ROS, which promotes hyperactivation (de Lamirande and Gagnon, 1993a,b; de Lamirande *et al.*, 1997; Herrero and Gagnon, 2001).

The sperm enzyme responsible for the ROS generation has as yet not been identified (de Lamirande and Gagnon, 1995; Ford, 2004), but it is presumed that the sulfhydryl/disulfide pair on sperm proteins could be the targets for ROS (de Lamirande *et al.*, 1998; de Lamirande and Gagnon, 2003). Sperm capacitation is also associated with an increase in tyrosine phosphorylation, however, it is unclear what role, if any this plays in hyperactivation (de Lamirande *et al.*, 1997; Leclerc *et al.*, 1996, 1997). Several substances found in seminal plasma can either promote or inhibit hyperactivation. Semenogelin (a main protein of the semen coagulum) (de Lamirande *et al.*, 2001), as well as other constituents of seminal plasma, including cholesterol (Purdy and Graham, 2004), zinc (Andrews *et al.*, 1994; de Lamirande *et al.*, 1997), and ROS scavengers (de Lamirande and Gagnon, 1993a), all play a preventative role in premature hyperactivation of spermatozoa. Other factors also

found in seminal plasma can induce sperm hyperactivation (de Lamirande *et al.*, 1993b). On the whole, a fine balance of inhibitory and stimulatory factors that prevent premature hyperactivation regulates this process in spermatozoa.

3.1.2 The human acrosome reaction

The acrosome is a secretory vesicle derived from the Golgi apparatus and is located in the anterior region of the sperm head (Brucker and Lipford, 1995). Austin and Bishop (1958) first reported on the AR in mammals and described it as (a) the multiple fusions between the outer acrosome membrane of the spermatozoa and the overlaying plasma membrane; and (b) exposure and release of the acrosomal contents.

During fertilisation, the acrosome intact sperm penetrates the cumulus oophorus and corona radiata of the oocyte and binds to the surface of the zona pellucida (ZP) by the plasma membrane overlying the acrosome (Baker *et al.*, 2000). The AR is triggered by this binding process with the ZP and continues until the sperm reaches the inner third of the ZP. The sperm then traverses the perivitelline space and binds the oocyte oolemma. Initially, the contact is between the microvillous processes of the oocyte oolemma and the plasma membrane overlying the equatorial segment of the sperm. When fusion is completed, the sperm is consequently engulfed by the oocyte which then triggers the cortical granule reaction. This causes changes in the inner third of the ZP and the oolemma thereby inhibiting further sperm penetration and preventing polyspermia (Yanagimachi, 1994). These events are illustrated in Figure 3.1.

3.1.2.1 Signal transduction between the zona pellucida and the spermatozoon

When capacitated spermatozoa come into contact with the ZP of the oocyte, ion channels on the sperm plasma membrane are mobilized and facilitate the rapid diffusion of extracellular Ca^{2+} (Darszon *et al.*, 1999; Tosti and Boni, 2004). Darszon *et al.* (2001) further reported that Ca^{2+} influx is involved in the dehiscence of the acrosomal vesicle and in membrane fusion. This is probably accomplished due to the massive influx of Ca^{2+} inactivating Na^{+}-K^{+} ATPase (pumping Na^{+} out of and K^{+} into the cell), resulting in a rapid increase in intracellular Na^{+}.

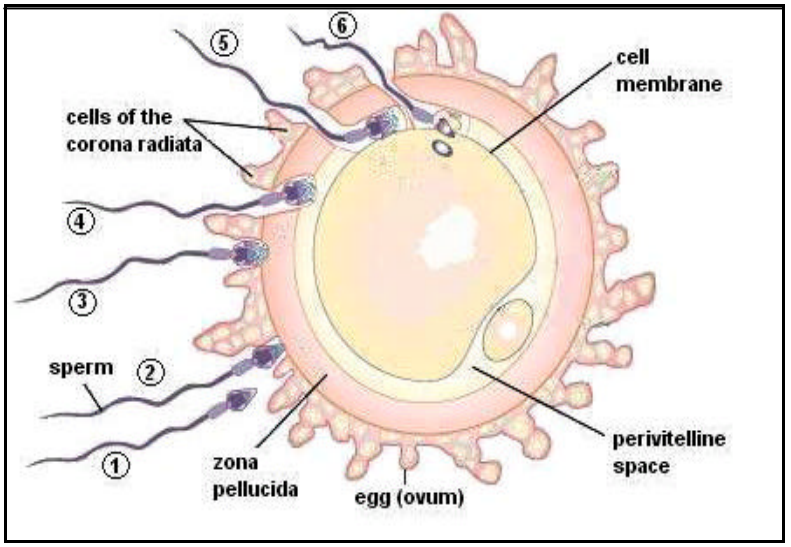

Figure 3.1 The human fertilisation process (Adapted from "fertilization." Encyclopædia Britannica Online, 2007): **(1)** Chemoattraction, **(2)** specific recognition – loose association, **(3)** acrosomal exocytosis, **(4)** penetration – sperm-egg binding, **(5)** membrane fusion, **(6)** sperm invagination.

In turn this causes an efflux of H^+ (through the Na^+/ H^+ antiport), which eventuates in the increase of intracellular pH (Lièvano *et al*., 1985; González-Martínez and Darszon, 1987; Yanagimachi *et al*., 1990; González-Martínez *et al*., 1992). A change in trans-membrane resting potential (RP) occurs due to the K^+ channel induced Na^+/H^+ exchange and pH rise causing a fast and transient hyperpolarisation followed by a Ca^{2+} mediated depolarisation (Florman *et al*., 1998; Arnoult *et al*., 1999; Patrat *et al*., 2000 and Darszon *et al*., 2001). In addition, the intracellular rise in Ca^{2+} results in the dispersion of F-actin, facilitating the close contact between the outer acrosomal and plasma membranes which eventuates in membrane fusion and completion of the AR (Breitbart, 2003).

Ion channels on the head of the sperm cell responsible for the Ca^{2+} entry and cytosolic increase include: low and high voltage-activated channels, receptor operated Ca^{2+} channels, and store-operated Ca^{2+} channels (Benoff, 1998). T-Type voltage gated Ca^{2+} channels play an important part in mediating the acrosome reaction (Florman *et al*., 1998; Darszon *et al*., 2001). Gating of these channels have

been demonstrated by AR inducers such as progesterone (Garcia and Meizel, 1999). A mechanism for gating plasma membrane Ca^{2+} channels in human sperm was proposed by Rossato *et al.* (2001). They demonstrated the existence of intracellular Ca^{2+} stores whose depletion could bring forth gating of Ca^{2+} activated K^+, with K^+ efflux causing a hyperpolarisation and the capacitative gating of voltage gated Ca^{2+} channels resulting in depolarisation of the plasma membrane.

Anion Cl^- channels have also been indicated to play a possible role in the AR (Morales *et al.*, 1993). However, the mechanism by which these channels are gated by the ZP or other agents during fertilization, have not yet been elucidated (Tosti and Boni, 2004). Electrophysiological studies found different types of Cl^- channels with dissimilar conductance on the sperm head (Bai and Shi, 2001). Since sperm have been shown to contain a high intracellular Cl^- concentration, it is plausible that a Cl^- efflux could cause the depolarisation of the RP (Sato *et al.*, 2000). Furthermore, progesterone, while inducing the AR, also seems to induce a Cl^- efflux (Meizel, 1997).

It is important to note that hyperactivated movement, although associated with capacitation, is not a prerequisite for sperm-egg fusion; a live acrosome-reacted spermatozoon has the potential to fertilize. The actual mechanism that triggers AR and ZP binding is also not well understood. Franken (1998) elucidated on the possible role of G-proteins, molecular modifications of the sperm plasma membrane that result in areas with “high- fluidity”. They may present the sites for sperm binding to the ZP and AR as well as the involvement of a selectin-like ligand structure on the ZP that recognise selectin-like receptors on the sperm membrane surface (Oehninger *et al.*, 1998). It has since been established that sperm binding is mediated by means of O-linked carbohydrate side chains of the glycoproteins ZP1/ZPB, ZP2/ZPA, ZP3/ZPA comprising the zona of various species (Florman and Wassarman, 1985; Harris *et al.*, 1994; Wassarman and Litscher, 1995). In particular, the ZP protein-3 (ZP3) serves as a primary receptor for spermatozoa (Miller *et al.*, 1992).

Furthermore, although the identity and unambiguous function of the components serving as putative sperm receptors have not been identified (Töpfer-Petersen, 1999),

Oehninger (2001) provided evidence of the presence of specific carbohydrate moieties on human spermatozoa that recognise selectin ligands on the ZP. In addition, multiple concerted and collaborative interactions between ZP3 and various surface components of sperm, possibly involving receptor aggregation and phosphorylation, may be required to achieve AR (Darzon *et al*., 1995).

It has previously been reported that the ability of spermatozoa to undergo induced AR following exposure to different effectors including chemical components, such as calcium ionophore (Aitken *et al*., 1993) and pentoxifylline (Tesarik and Mendoza, 1993), as well as physiological inducers, such as oocyte-cumulus complexes (Stock *et al*., 1989), follicular fluid (Siegel *et al*., 1990), progesterone-binding protein corticoid-binding globulin (Baldi *et al*., 1998), and ZP (Cross *et al*., 1988; Coddington, 1990; Hoshi *et al*., 1993) are of clinical value in predicting the fertilizing capacity of men (Cummins *et al*., 1991; Henkel *et al*., 1993). In addition, capacitated sperm that have acrosome reacted prior to reaching the ZP show poor penetration and reduced viability (Corselli and Talbot, 1986; Cummins and Yanagimachi, 1986; Lui and Baker, 1990). If an external source, like RF-EMF, could influence sperm specific functions, such as the acrosome reaction prior to the sperm reaching the ZP, it could have a far-reaching effect on male fertilizing potential.

The objective of the first part of the study was therefore to investigate the effect of RF-EMF exposure on sperm capacitation. Sperm motility parameters, including hyperactivation, were evaluated by computer aided sperm analysis (CASA) while the AR was assessed by flow cytometry. If RF-EMF irradiation of human sperm could in any way affect the AR, then investigating the physiological action of sperm-zona binding could confirm this. The second part of the study therefore focused on the consequence of RF-EMF on the spermatozoa's binding ability to the human ZP.

3.2 RF-EMF EXPOSURE SYSTEM AND EXPERIMENTAL PROTOCOL

3.2.1 Experimental set-up

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 (Finland). Mobile phone microwave radiation (900 MHz pulse modulated RF) was simulated in a specially constructed exposure system (described in Annexure A), based on the use of a high Q waveguide resonator operating in TE_{10} mode. The irradiation chamber (Figure 3.2) was placed vertically inside a Forma CO_2 incubator (Class 100, Labothech, SA). 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 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 an 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, Salt Lake City, UT, USA). 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 W/kg) temperature ranged from 36.8 ºC to 37.2 ºC.

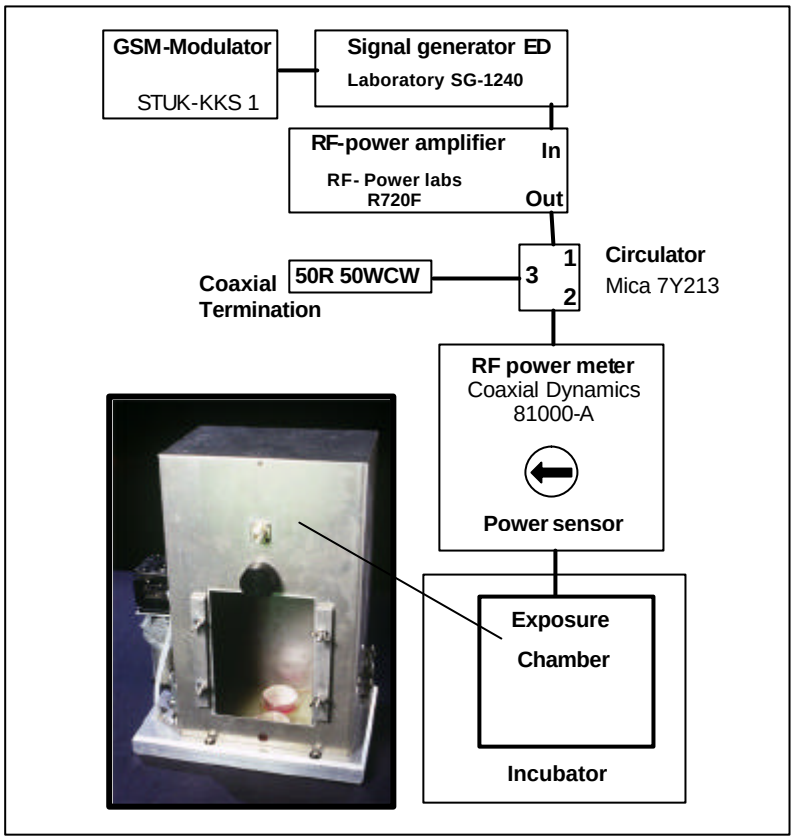

Figure 3.2 Front view and set-up of RF-EMF exposure chamber. Two glass petri dishes are placed inside the chamber on top of a temperature regulated waterbed. The RF-EMF signal is fed into the chamber placed inside a CO_2 incubator via a monopole type feed post.

3.2.2 Collection of semen

Semen samples were collected from healthy, non-smoking donors (n = 12) by masturbation after 2 to 3 days of sexual abstinence (mean age 23 ± 5 years). The study was conducted according to the Declaration of Helsinki for medical research and institutional approval was also granted (Faculty of Health Sciences Research Ethics Committee, University of Pretoria, no. 163/2003). The semen samples were allowed to liquefy at 37°C, after which standard semen parameters were evaluated according to the World Health Organisation (WHO, 1999) criteria (outlined in Annexure B). All semen samples conformed to the WHO criteria and sperm morphology assessments were performed according to Tygerberg criteria (Kruger *et al.* 1986) (mean sperm parameters, motility >50%, morphology 7.85 ± 4.2 % - results

are summarised in Annexure C). 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). Samples used had less than 1 x 10^6 leukocytes/ml (CD45 staining) and were antibody-free (mixed antiglobulin reaction - MAR negative).

3.2.3 Density gradient purification and preparation of spermatozoa

Sperm washing is routinely performed before IVF and intrauterine insemination (Zini *et al.*, 2000). The purpose of using a sperm processing technique is to recover a highly functional sperm population. To purify spermatozoa, a three-step discontinuous Percoll gradient (95-70-50%) diluted in Ham's F-10 medium, supplemented with penicillin G and calcium and further supplemented with 0.5% BSA was used (all reagents were from Sigma Chemical Co., St Louis, MO, USA). 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 F-10 medium before preparation for RF-EMF exposure. The motile sperm concentration of the total sample (total ejaculate), after Percoll density centrifugation, was ≥ 40 x 10^6/ml, which provided sufficient number of cells for experimentation.

Processed spermatozoa were counted (improved Neubauer Haemocytometer) and concentrations adjusted to 20 x 10^6 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 F-10 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 maintained at 37°C. 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, SA) and concentrations adjusted to 20 x 10^6 /ml by centrifugation (300 g for 5 min). Spermatozoa were then

incubated under capacitating conditions and assessed at different time points, directly (T_1), 2 (T_2) and 24 (T_3) hours post-exposure. All tests were run in duplicate.

3.3 CAPACITATION: ASSESSMENT OF THE HUMAN MOTILITY AND THE ACROSOME REACTION

A graphic presentation of the exposure protocol is given in Figure 3.3. The AR was evaluated using flow cytometry (FCM) (methods outlined in paragraph 3.3.1) while the motion characteristics were determined using CASA (method outlined in paragraph 3.3.2). Acrosomal status and sperm motility were all assessed on the same day at three different time points over a period of 4 weeks for all twelve donors. In addition, during experimentation, slides were prepared for morphology assessment pre- and post- RF exposure using the same computer assisted sperm analysis software as for motility assessment. The hemi-zona assay designed to determine the binding ability of spermatozoa was assessed for all twelve donors at a later date.

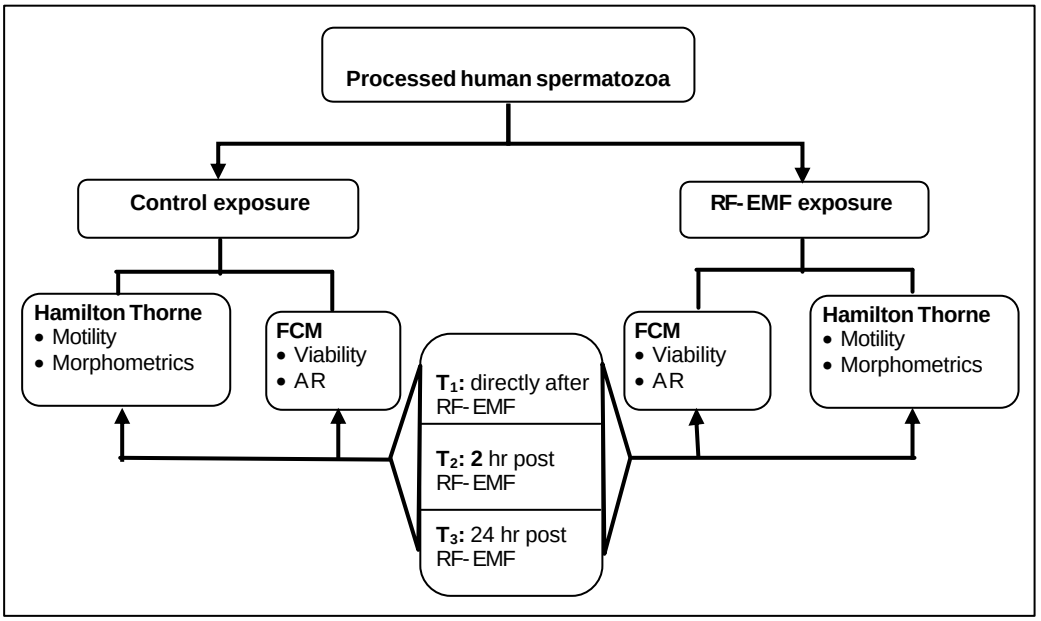

Figure 3.3 RF-EMF exposure protocol: Assessment of the acrosome reaction and motility characteristics post RF-EMF and control exposure.

3.3.1 Capacitation: assessment of motility

To determine the effect of RF-EMF (2.0 and 5.7 W/kg) radiation on sperm motility, computer aided sperm analysis was performed. At each time point, post RF-EMF exposure, 5 µl of the sperm suspension from the RF-EMF, and control exposed sperm were loaded into two 20 µml Microcell chambers (2X-CEL, Hamilton Thorne Research, Danvers, MA). The chambers were then placed on a heated microscope plate (Nikon, Optiphot, Japan), which was maintained at 37°C. Video recordings were made of at least 10 random fields per chamber. Each field was recorded for 30 seconds. At a later time, the pre-recorded video was analysed using the Hamilton Thorne Integrated Visual Optical System (IVOS) (Hamilton Thorne Research, Danvers, MA). 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.

The sperm kinetic parameters evaluated included: motile (MOT) and progressively (PRG) motile 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 at which the sperm's curvilinear path crosses the average path), amplitude of lateral head displacement (ALH - the mean width of sperm head oscillation), and the derivatives; straightness (STR = VSL / VAP x 100) and linearity (LIN = VSL / VCL x 100, departure of sperm track from a straight line) as well as hyperactivated motility (HYPA, to be classified as hyperactivated, a trajectory had to meet all of the 60 Hz SORT criteria, *i.e.*, VCL = 150 µm/s, LIN = 50% and ALH = 7 µm (Mortimer *et al.*, 1998)). Data from each individual cell track were recorded and analysed.

3.3.1.1 Morphometric assessment

Slides from each donor (n = 12) were prepared in duplicate prior to exposure and at two time points after exposure (T_1-directly after and T_2 - 2h after RF-exposure) and 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).

Morphology was evaluated with the Hamilton Thorne IVOS using Metrix Software (Hamilton Thorne Research). The following parameters were evaluated: major and minor axis (µm), elongation (%), area (μm^2), perimeter (µm) and the acrosomal region (%). All samples were divided into 3 morphological categories (Annexure C). A typical Metrix evaluation of morphometric parameters is depicted in Figure 3.4 A and B.

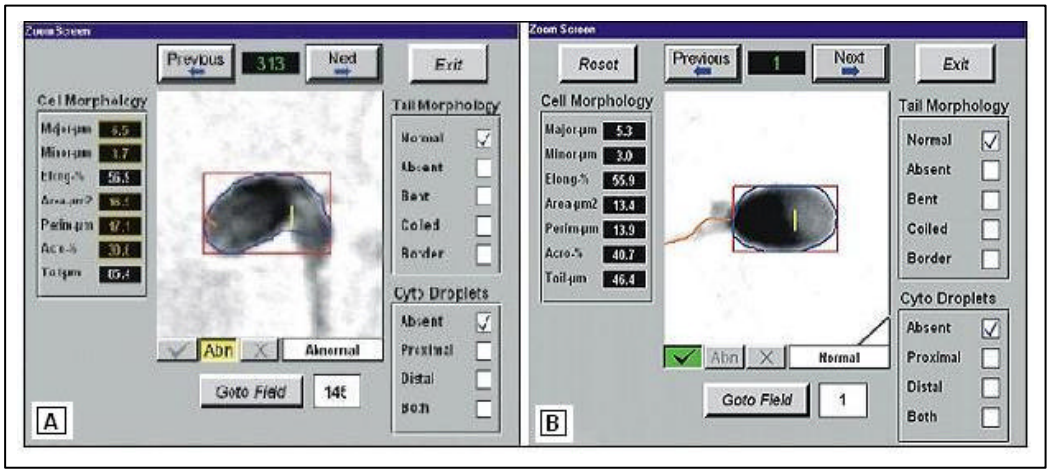

Figure 3.4 Metrix calculation of sperm morphometric parameters: (A) an abnormal spermatozoon, (B) a normal spermatozoon.

3.3.2 Capacitation: Assessment of the acrosomal status

The AR is associated with the release of proteolitic enzymes from the acrosomal vesicle. These enzymes, specifically acrosin, are targeted by the fluorescent-labelled lectin *Pisum sativum* agglutinin conjugated with fluoro-isothiocyanate (PSA-FITC) (Cross *et al*., 1986). The proportion of spermatozoa undergoing the AR can then be evaluated by the decrease in the fluorescence intensity of PSA-FITC (Nikolaeva *et al*., 1998). Acrosome intact sperm will have intensely fluorescent acrosomes while acrosome reacted spermatozoa will bind significantly less PSA. In order to determine acrosomal status, the acrosomal membrane has to be permeabilised to facilitate lectin

binding of the acrosomal contents. However, since the AR is only evaluated in viable sperm, dead cell discrimination is very important to accurately quantify acrosomal status. Therefore, the staining ability of two fluorescent viability probes compatible with flow cytometry were compared.

The first part of this study focussed on the evaluation of viability probes used in conjunction with PSA-FITC to determine acrosomal status in live spermatozoa. Secondly the AR was assessed by flow cytometry after RF-EMF and ionophore challenge.

3.3.2.1 Viability probes used in the acrosome reaction

It is important to be able to differentiate between live acrosome reacted and dead reacted sperm to accurately assess the effect of an acrosome promoter. Therefore a viability probe is used for dead cell exclusion. Traditional viability probes used include non-fluorescent trypan blue (triple stain, Talbot and Chancon, 1981) and fluorescent probes including bisbenzimide (Hoechst 33258) (Cross *et al.*, 1986), ethidium bromide (Carver-Ward *et al.*, 1996), and propidium iodide (Miyazaki *et al.*, 1990). However, as the fluorescence emission of some of these probes overlap with those used for combined surface and intracellular staining, it is difficult to accurately assess acrosomal status and viability at the same time. Propidium iodide (PI) is normally used as the viability probe of choice in flow cytometry.

Propidium iodide's spectral emission extensively overlaps with FITC and phyco-erythrin (PE) (Schmid *et al.*, 1992). In addition, it has also been shown to "leach" from permeabilised sperm over time due to weak or reversible binding (Trestappen *et al.*, 1988; Riedy *et al.*, 1991). Furthermore, for single argon laser instruments, the UV excitable Hoechst 33258 stain cannot be used. Evaluation of the AR entails the fixation and permeabilisation of sperm, therefore another viability probe, 7-amino-actinomycin D (7-AAD) was compared to PI's staining post fixation at different time points (for 1 hr) to optimise the detection of dead sperm. This study was performed to confirm the suitability of using 7-AAD for dead cell exclusion with the flow cytometry AR protocol.

(i) 7-Amino-Actinomycin D

The viability stain 7-AAD (λ_{ex} = 499 nm and λ_{emm} = 655 nm) is a fluorescent DNA-binding agent that intercalates between cytosine and guanine bases (Philpott *et al.*, 1996) and has a high DNA binding constant and a slow post fixation and permeabilisation dissociation rate (O'Brien and Bolton, 1995; Philpott *et al.*, 1996). The emission spectra of 7-AAD, allows it to be used in conjunction with other flourocromes (PE and FITC) with minimal fluorescence emission overlap (O'Brien and Bolton, 1995; Nunez *et al.*, 2004). In addition, 7-AAD has previously been used to discriminate between dead and live sperm (Bronson *et al.*, 1999; Nunez *et al.*, 2004).

The exact mechanism of 7-AAD is not known, but it has been proposed that the chromophore intercalates between G-C bases and the large peptide moiety upon binding to DNA interacts with the minor groove of the double helix (Evenson *et al.*, 1986).

(ii) Propidium Iodide

Propidium Iodide (λ_{ex}= 488 nm and λ_{emm} = 655 nm) is a supravital stain that rapidly penetrates non-viable sperm through disrupted plasma membranes (Riedy *et al.*, 1991) and strongly intercalates DNA depending on the degree of unwinding (Darzynkiewicz *et al.*, 1997).

(iii) Comparison between 7-AAD and PI as viability probes used in the acrosome reaction

Spermatozoa recovered from the 95% motile sperm suspension after the density gradient technique (described previously), were re-suspended at a cell density of 1 x 10^6/ml in Ham's F-10 (supplemented with 0.5% BSA). Two sets of triplicate tubes were respectively labelled with 10 µl 7-AAD (50 µg/ml, BD PharMingen, BD Bioscience, NJ, USA) and 10 µl of PI (50 µg/ml, BD Biosciences, USA). The sperm were then gently vortexed and 7-AAD and PI labelled sperm were left to incubate for 20 min at room temperature (25°C) in the dark.

Cytofix/Cytoperm^{TM} (BD Biosciences, USA) solution, containing neutral pH-buffered saline, saponin and 4% (w/v) paraformaldehyde, was used for the

simultaneous fixation and permeabilisation of sperm. After addition of 250 µl Cytofix/Cytoperm^{TM} solution, sperm were incubated for 20 min on ice, pelleted (300 g for 5 min) and the supernatant discarded after which sperm were washed twice (300 g for 5 min) with 2 ml PBS (Sigma Chemical Co.). Directly following, and at specific time points post fixation and permeabilisation, viability staining was assessed on a Beckman Coulter Epics ALTRA flow cytometer. Fluorescence emission from 7-AAD was detected in the far-red range of the spectrum (650 nm long-pass filter) and uptake of 7-AAD was measured in PMT 5. PI fluorescence was detected in the orange range of the spectrum (562-588 nm band pass filter) (Schmid *et al*., 1992; O'Brien and Bolton, 1995) and PI-labelled sperm were measured in PMT 4. A flow chart presenting the protocol used in assessing viable spermatozoa is given in Figure 3.5.

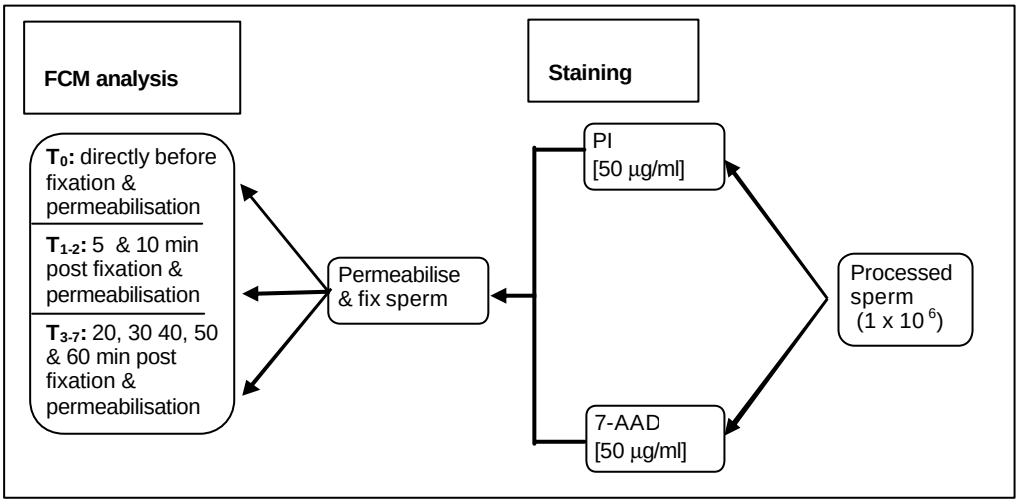

Figure 3.5 Flow cytometric assessment of 7-AAD and PI as viability probes after fixation and permeabilisation of human spermatozoa.

3.3.2.2 Evaluation of the acrosome reaction by flow cytometry

The techniques described by Uhler *et al*. (1993) and Henley *et al*. (1994) were adapted. Spermatozoa recovered from the 95% motile sperm suspension after density gradient purification was re-suspended (20 x 10^6 /ml in Ham's F-10, 5% BSA) in glass petri dishes (two dishes each for RF-EMF and control exposure). The sperm were then exposed for 1 hour to 900 MHz RF-EMF (2.0 W/kg).

After irradiation, spermatozoa were incubated under capacitating conditions (5% CO_2 at 37° C) and assessed directly (T_1), 2 (T_2) and 24 (T_3) hours post exposure. At the same time, points aliquots were taken to assess motility using the Hamilton Thorne Integrated Visual Optical System (Hamilton Thorne Research, Danvers, MA, USA).

For assessment of acrosomal status, duplicate tubes taken from the RF-EMF exposed and control sperm (1 x 10^6 /ml) were stained with the viability probe 7-AAD (50 µg/ml) and incubated for 20 min at room temperature in the dark. Remaining sperm were kept in a humidified CO_2 incubator at 37°C for assessment at later time points. Sperm were then pelleted at 300 g for 5 min. The supernatant was discarded and the sperm washed twice (300 g for 5 min.) with 2 ml PBS. Sperm were re-suspended in 250 µl Cytofix/Cytoperm™ solution and incubated for 20 min on ice.

After the fixation and permeabilisation procedure, sperm were again washed twice by centrifugation (300 g for 5 min) with 2 ml PBS before the human acrosome was assessed with fluorescein-labelled *Pisum sativum* agglutinin (PSA-FITC, Sigma Chemical Co.). PBS dissolved PSA-FITC was added at a final concentration of 1 mg/ml and samples were incubated at room temperature in the dark for 15 min. After PSA-FITC staining, spermatozoa were finally washed by centrifugation with 2 ml PBS before re-suspending the pellet in 1 ml PBS. Specimens were gated by light scatter properties (size and granularity) of spermatozoa and analysed for dual colour fluorescence. Dead sperm (7-AAD-stained) were excluded and PSA-FITC staining was assessed only among live sperm and detected on PMT 2. Flow cytometric analysis was performed with a Coulter Epics® XL.MCL flow cytometer (Beckman Coulter, Miami, Florida, USA) using System II software. Results are expressed as the mean cell number with a total of 10 000 events acquired for each endpoint.

(i) Visual assessment of the acrosome reaction

Loading of the stains was visually inspected (Zeiss confocal microscope, LSM 510 META, Carl Zeiss (Pty) Ltd, Germany) by preparing smears from small aliquots taken of the sperm suspension after addition of PSA-FITC. Slides were left to air-dry after which they were mounted and evaluated under oil immersion and 100x

magnification. Acrosome intact sperm have intensely green fluorescent acrosomal regions while acrosome reacted sperm could be differentiated by patchy fluorescence (undergoing AR) a fluorescent equatorial segment (almost completed AR) or no fluorescence (acrosome reacted).

(ii) Induction of the acrosome reaction by calcium ionophore

For a positive control, sperm following 3 hr capacitation were re-seeded in sterile 10 ml conical test tubes at a final concentration of 1 x 10^6 sperm/ml. A frozen stock solution (5 mM) of calcium ionophore (A23187, Sigma Chemical Co.) in dimethyl sulphoxide (DMSO, Sigma Chemical Co.) was diluted in Ham's F-10 medium (0.5% BSA) and 10 µl was added to 1 ml of the capacitated sperm suspension making a final concentration of 10 µM A23187. The samples were incubated for 30 min at 37 °C in a 5% CO_2, 95% humidity incubator. A duplicate aliquot was treated with 10 µl of 1:10 diluted DMSO. Detection of the acrosome after calcium ionophore (A23187) challenge was assessed with PSA-FITC staining, using FCM analysis as described previously.

3.4 HEMI-ZONA ASSAY

3.4.1 Zona pellucida binding - mechanism:

Tight binding of human spermatozoa to the outer surface of the human ZP is an essential event in gamete interaction leading to fertilization (Burkman *et al.*, 1988; Mortimer and Mortimer, 1999; WHO, 1999). Spermatozoa penetrate through the cumulus oophorus to bind tightly to the zona protein ZP3; this in turn triggers the spermatozoa AR. Only acrosome reacted spermatozoa will bind to the zona protein (ZP3) thereby facilitating penetration of the zona matrix and progression into the perivitelline space (Franken, 1998; Mortimer and Mortimer, 1999).

The hemi-zona assay was originally designed as a diagnostic test for male factor infertility (Burkman *et al.*, 1990; Franken *et al.*, 1990; Oehninger *et al.*, 1991; Franken and Oehninger, 2006) and has since been utilized to investigate the effect of various substances on human sperm-ZP binding. The assay (illustrated in Figure 3.6) utilizes non-viable, non-fertilizable human oocytes (obtained from *in vitro*

fertilization programmes). The bisected oocyte provides two matched halves (hemi-zonae) with functionally equal zona surfaces, yielding a controlled comparison of sperm binding (Franken *et al.*, 1990). By calculating the ratio of spermatozoa bound to the two halves, an indication of the binding capacity of the control sperm sample versus the stressor (RF-EMF) exposed sample can be obtained.

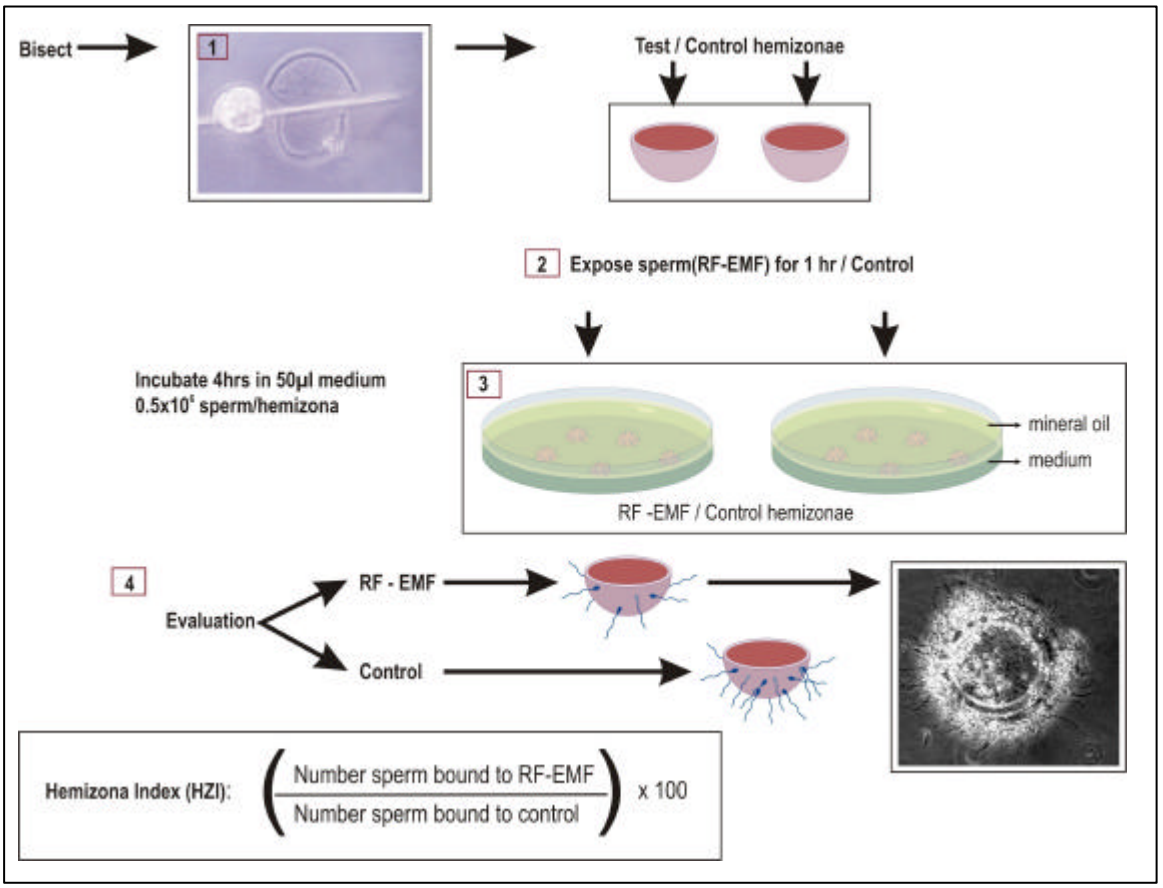

Figure 3.6 The hemi-zona assay: (1) Oocytes from IVF were bisected and kept at room temperature while **(2)** spermatozoa were exposed for 1 hour to RF-EMF (SAR 2.0 and 5.7 W/kg). **(3)** After exposure hemi-zonae were added to 50 µl spermatozoa droplets (0.5 x 10^6 cells/ml). **(4)** Spermatozoa and hemi-zonae were co-incubated for an additional 4 h before visual assessment of binding.

3.4.2 Source and preparation of human zonae pellucidae

Institutional Ethics approval was obtained for the use of non-fertilized oocytes from patients with access oocytes undergoing IVF treatment (Faculty of Health Sciences

Research Ethics Committee, University of Pretoria, no. 5/2006). A total of 5 oocytes (metaphase II) were used per donor (unless otherwise indicated). Non-fertilized oocytes were prepared for cryostorage using the technique described by Hammitt *et al.* (1993). Oocytes were incubated for 3 min in a solution of 2.5 M Sucrose, 3% BSA, 3.5 M DMSO in Ham's F-10 medium (all reagents were from Sigma Chemical Co.), transferred to 0.25 ml cryo- straws and kept in liquid nitrogen until further use.

On the day of use, the straw's content was emptied by pushing a steel plunger gently through the straw, thus expelling the oocytes into a petri dish. The oocytes were transferred to a droplet containing thawing medium (2.5 M Sucrose, 3% BSA in Ham's F-10 medium). Oocytes were then washed three times before commencing with bisection. Cutting was performed under an inverted phase contrast microscope (Axiovert 200, Carl Zeiss (Pty) Ltd, Germany) with a micromanipulation system (Narishige, MM-89, Japan). Once bisected, mouth micro pipetting dislodged the ooplasm. The hemi-zonae were kept at room temperature until addition of spermatozoa.

3.4.3 Sperm-oocyte interaction

The procedure is schematically illustrated in Figure 3.6. Spermatozoa (from 10 donors) were collected from the 95% layer after a 3-step Percoll density gradient separation (as described previously), concentrations were adjusted to 20 x 10^6 cells/ml and cells seeded into glass petri dishes (one dish per donor) already containing 2 ml Ham's F-10 medium supplemented with 3% BSA. Control samples were prepared simultaneously. Spermatozoa were then exposed for an hour to RF-EMF (2.0 and 5.7 W/kg), while control dishes were kept at 37ºC in the same incubator.

After exposure, 50 µl droplets containing 0.5 x 10^6 cells/ml of RF-EMF exposed and control spermatozoa were placed into separate petri dishes. The hemi-zonae were then transferred to the droplets, the droplets were covered with mineral oil and incubated for a further 4 hours 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.

3.5 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 and control exposed samples using a paired t-test at the 0.05 level of significance. A standard deviation of 4.95% was assumed.

To analyse the effect of RF-EMF on motility and acrosomal status results from a within subject design considering two treatments, control vs. RF-EMF (SAR 2.0 and 5.7 W/kg), at three time points (T_1- directly after exposure, T_2- 2 hours after exposure and T_3- 24 hours after exposure) were analysed as a mixed linear model (time series regression) with Stata Statistical Software Release 8.0 (Stata Corp. 2003, USA). Similarly morphometry, was assessed at two time points (T_1- directly after exposure and T_2- 2 hours after exposure). Throughout the analysis no interaction between time and dose was present therefore the p-values associated with dose only was reported. Data is presented as mean values ± SD (standard deviation). Correlations between routine semen parameters (morphology), motility, and the results obtained from flow cytometry analysis were computed using the Spearman rank correlation coefficient.

Comparison between PI and 7-AAD staining, post fixation/permeabilisation over time was accomplished using linear regression analysis. Slopes were considered significantly different if $p < 0.05$. For comparison of two groups (RF-EMF and control exposed sperm), a two-tailed Mann-Whitney U-test was performed.

To determine the effect of RF-EMF on the hemi-zona binding assay, data were analysed using a Wilcoxon signed ranks test (Stata Statistical Software) while the correlation between morphology and number of cells bound to the hemi-zona were calculated using a Spearman's rank correlation coefficient (GraphPadPrism® version 3.00 software, GraphPadSoftware, San Diego, CA, USA). 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 = 0.92.

3.6 RESULTS

3.6.1 Motility and morphology: CASA

Computer aided sperm analysis was used in the assessment of sperm motility and morphology after RF-EMF exposure. The progressive motility (type "a + b" motility, rapid + slow motility) of RF-EMF exposed compared to control spermatozoa for SAR levels 2.0 and 5.7 W/kg respectively were determined directly after (T_1), 2 hours (T_2) and 24 hours (T_3) post exposure (Table 3.1 A and B). Table 3.2 notes the time series regression results of percentage rapid-, slow-, non-progressive and immotile spermatozoa comparing RF-EMF (2.0 and 5.7 W/kg) exposure with their respective controls. Since no interaction between time and dose was present in Tables 3.1 and 3.2 the p-values associated with dose only are reported. Sperm kinetic results are summarized in Figures 3.7 and 3.8. Morphometric results are summarised in Table 3.3, while results comparing morphometric parameters for SAR 2.0 W/kg and SAR 5.7 W/kg are summarized in Tables 3.4 and 3.5 respectively.

3.6.1.1 Percentage progressive motility

Time series regression analysis (Table 3.1) showed no statistically significant effect of RF-EMF exposure on progressive motility of human sperm compared to controls at either of the SAR values ($[p = 0.764]_{2.0\ W/kg}$ and $[p = 0.856]_{5.7\ W/kg}$). When comparing progressive motility in RF exposed sperm for SAR 2.0 W/kg to SAR 5.7 W/kg as a function of time (Mann-Whitney U-test), no significant effect of SAR level on motility was noted at any of the time points; T_1 ($p = 0.910$), T_2 ($p = 0.675$) or T_3 ($p = 0.312$).

Furthermore, a summary of the time series regression analysis results comparing rapid- slow-, non-progressive and immotile categories are given in Table 3.2. There was no statistically significant effect on rapid progressive - a, slow progressive - b, non-progressive - c or immotile categories - d 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.

Table 3.1 Percentage progressive motility after exposure to **(A)** RF-EMF (2.0 W/kg) and **(B)** RF-EMF (5.7 W/kg) in exposed and control spermatozoa determined directly (T_1), 2 h (T_2) and 24 h (T_3) after exposure.

A	Progressive motility	Progressive motility	
Time	**RF-EMF**	**Control**	**p-value***
T_1	86.5 ± 7.44	86.8 ± 5.34	p= 0.764
T_2	87.5 ± 8.57	86.1 ± 8.36	
T_3	70.0 ± 14.51	65.0 ± 16.45	
B	**RF-EMF**	**Control**	**p-value***
T_1	86.6 ± 9.33	87.2 ± 7.32	p = 0.856
T_2	86.2 ± 7.69	84.6 ± 9.18	
T_3	62.71 ± 15.14	65.7 ± 19.15	

* p-value supplied for the time series regression analysis comparing RF exposed with control samples.

Table 3.2 Time series regression results of percentage rapid-, slow-, non-progressive and immotile spermatozoa respectively after RF-EMF (2.0 or 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 = 0.809	p = 0.834
Slow - b	p = 0.518	p = 0.477	p = 0.771
Non-progressive - c	p = 0.765	p = 0.537	p = 0.433
Immotile - d	p = 0.446	p = 0.946	p = 0.469

***** 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.

3.6.1.2 Velocity parameters

Comparison between VAP, VSL, and VCL (µm/s) of spermatozoa exposed to RF-RMF (2.0 and 5.7 W/kg) and controls are shown in Figure 3.7 A and B respectively.

For SAR 2.0 W/kg (Figure 3.7 A): No statistical difference (time series 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. 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 3.7 B): Time series 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 ($p = 0.06$) & VCL ($p= 0.09$)]. 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 $Velocity_{5.7W/kg} < Velocity_{2.0W/kg}$.

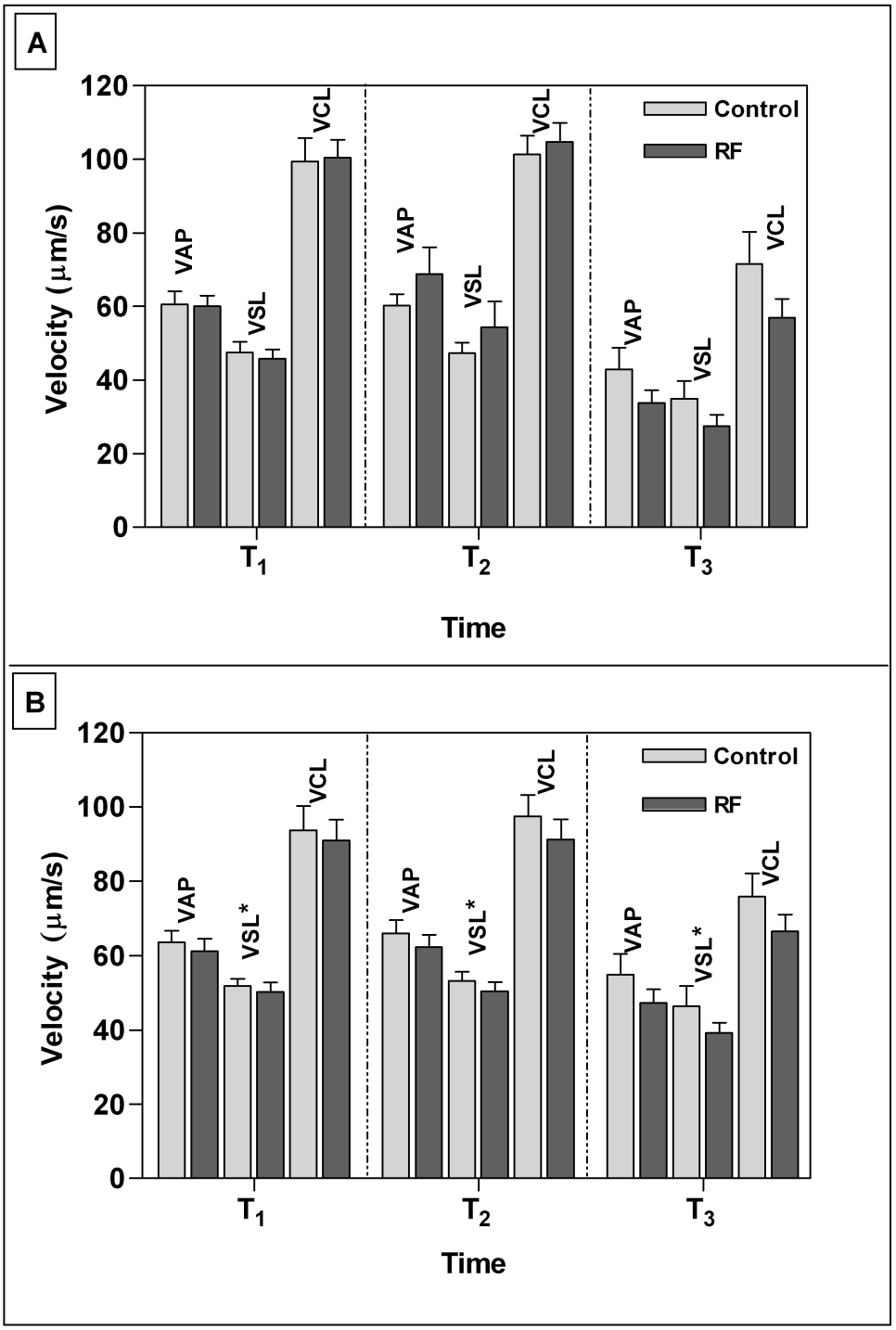

Figure 3.7 Velocity parameters comparing RF-EMF (dark-grey) exposed spermatozoa for SAR 2.0 W/kg (A) and 5.7 W/kg (B) with controls (light-grey) directly (T_1), 2 (T_2), and 24 (T_3) hours after a 1 hour 900 MHz GSM exposure (*$p < 0.05$).

3.6.1.3 Motion parameters

Additional motion kinetics measured by CASA included ALH (µm), BCF (beats/sec), STR (%), LIN (%), and hyper-activated motility. Area (μm^2) and elongation (%), although not part of motion parameters, were also determined. The effect of RF-EMF (SAR 2.0 and 5.7 W/kg) exposure on ALH, BCF, STR, and LIN is illustrated in Figure 3.8 A and B respectively. Time series regression analysis of the effect of RF-EMF on area and elongation is noted separately.

For SAR 2.0 W/kg (Figure 3.8 A): Time series regression analysis between RF-EMF exposed sperm and controls over the three time intervals noted no statistical difference ($p > 0.05$) in ALH, BCF, STR, LIN, or hyper-activated motility. This was also the case for area ($p = 0.08$) and elongation ($p = 0.40$).

For SAR 5.7 W/kg (Figure 3.8 B): There was no statistically significant difference (time series regression analysis) in ALH, STR, LIN, or hyper-activated motility between RF-EMF exposed sperm and controls. However, BCF was significantly higher ($p = 0.04$) in exposed sperm compared to controls. Elongation in exposed sperm did not differ from controls at any of the time points determined ($p = 0.15$), although the area in exposed sperm decreased significantly ($p = 0.38$) as a function of time compared to controls.

3.6.1.4 Morphometric analysis

There was no statistically significant difference in any of the morphology parameters determined by CASA comparing sperm evaluated pre exposure with sperm evaluated directly after a 1 hour RF-EMF exposure. Average morphometric values ± SD of each parameter measured comparing RF exposed (2.0 and 5.7 W/kg) sperm with controls for both normal and abnormal sperm at two time points are given in Tables 3.3 A and B respectively. Time series regression analysis of morphometric parameters comparing RF exposed sperm with controls at both SAR values noted a statistically significant reduction in all the morphometric parameters except elongation (Table 3.4).

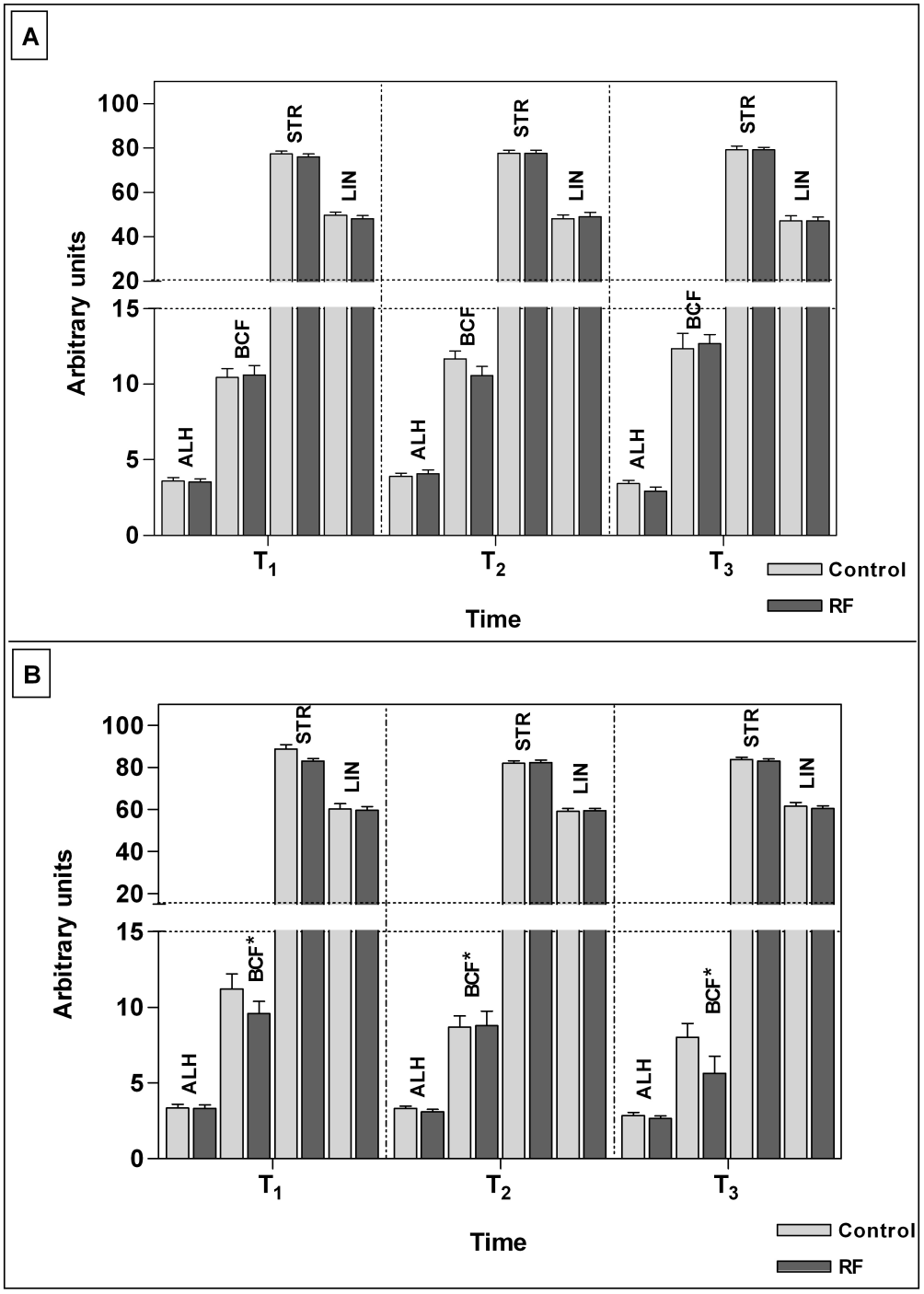

Figure 3.8 Motion parameters comparing RF-EMF (dark grey) exposed spermatozoa for SAR 2.0 W/kg (A) and 5.7 W/kg (B) with controls (light grey) directly (T_1), 2 (T_2), and 24 (T_3) hours after a 1 hour 900 MHz GSM exposure (*$p<0.05$).

Table 3.3 Summary of morphometric results of sperm exposed to **(A)** 2.0 W/kg and **(B)** 5.7 W/kg compared to control values.

A	SAR 2.0 W/kg		Control	
Parameter	**Normal**	**Abnormal**	**Normal**	**Abnormal**
Major axis (μm)	3.53 ± 0.17	5.56 ± 0.73	5.93 ± 0.60	6.02 ± 0.90
Minor axis (μm)	2.39 ± 0.10	3.67 ± 0.38	4.45 ± 0.23	4.08 ± 0.63
Elongation (%)	45.26 ± 2.70	67.22 ± 10.26	75.07± 7.24	68.9 ± 13.31
Area (μm^2)	9.24 ± 0.66	14.80 ± 2.07	18.83 ± 1.37	17.35 ± 3.50
Perimeter (μm)	9.36 ± 0.37	14.82 ± 1.36	16.67± 1.19	16.28 ± 1.97
Acrosome (%)	21.48 ± 3.98	23.53 ± 11.84	22.75 ± 4.85	22.43 ± 9.14
B	**SAR 5.7 W/kg**		**Control**	
Parameter	**Normal**	**Abnormal**	**Normal**	**Abnormal**
Major axis (μm)	3.39 ± 0.38	5.61 ± 0.72	4.125 ± 0.53	5.125 ± 0.735
Minor axis (μm)	2.25 ± 0.24	3.79 ± 0.38	2.875 ± 0.348	3.5 ± 0.573
Elongation (%)	42.66 ± 6.10	68.5 ± 10.61	52.8 ± 7.89	69.93 ± 16.35
Area (μm^2)	9.11 ± 1.61	15.18 ± 1.91	10.85 ± 1.448	12.75 ± 2.45
Perimeter (μm)	9.08 ± 0.93	15.25 ± 1.39	11.28 ± 0.848	13.88 ± 1.48
Acrosome (%)	23.70 ± 4.99	29.56 ± 11.58	26.85 ± 11.01	38.6 ± 14.15

Table 3.4 Summary of p values of the time series regression analysis results of sperm morphology determined by CASA. RF exposed sperm were firstly compared to controls, morphometric parameters were compared directly (T_1) and 2 hours (T_2) after exposure and the number of normal and abnormal forms were compared.

	SAR 2.0 W/kg			SAR 5.7 W/kg		
Parameter	**RF vs. Control**	**T_1 vs. T_2**	**Normal vs. Abnormal**	**RF vs. Control**	**T_1 vs. T_2**	**Normal vs. Abnormal**
Major axis	0.002**	0.842	0.081	0.010*	0.989	0.845
Minor axis	0.022*	0.380	0.857	0.062*	0.865	0.832
Elongation	0.185	0.205	0.592	0.830	0.478	0.963
Area	0.001**	0.810	0.585	0.003**	0.813	0.879
Perimeter	0.004**	0.531	0.243	0.015*	0.882	0.752
Acrosome	0.006*	0.140	0.086	0.011*	0.083	0.218

*$p < 0.05$, **$p < 0.005$

When morphometric parameters of RF exposed sperm at SAR 2.0 W/kg were compared with sperm exposed at SAR 5.7 W/kg, a statistically significant reduction was noted in area and acrosomal region (Table 3.5). There was no statistically significant difference in any of the parameters evaluated comparing T_1 and T_2 as well as number of normal and abnormal forms except when comparing acrosomal region (Table 3.5). Both area and acrosomal region decreased as SAR levels increased.

Table 3.5 Time series regression analysis comparing sperm morphometric parameters of sperm exposed at 2.0 W/kg to sperm exposed at 5.7 W/kg are summarised as p values.

Parameter	2.0 vs. 5.7 W/kg	T_1 vs. T_2	Normal vs. Abnormal
Major axis	0.068	0.784	0.097
Minor axis	0.085	0.350	0.835
Elongation	0.485	0.179	0.620
Area	0.020*	0.616	0.720
Perimeter	0.067	0.465	0.253
Acrosome	0.001**	0.008*	0.051*

*$p < 0.05$, **$p < 0.005$

3.6.2 Acrosome reaction

The flow cytometric evaluation of the two viability dyes (7-AAD and PI) post fixation and permeabilisation is illustrated in Figure 3.9. To ensure the correct loading of the dyes (7-AAD and PSA-FITC), for flow cytometric analysis, stained sperm were visually inspected (Figure 3.10), while positive controls were used to confirm both 7-AAD (Figure 3.11) and PSA-FITC (Figure 3.12) staining. Figure 3.13 (A) depicts flow cytometry fluorescence histograms showing AR as a function of time and Figure 3.13 (B) shows flow cytometry dot plots and projections used to gate acrosome reacted sperm. Flow cytometric results of RF-EMF and control exposed sperm assessed directly, 3 and 24 hr after exposure, are given in Figure 3.14.

3.6.2.1 Comparison between 7-AAD and PI as viability probes for flow cytometry post fixation and permeabilisation

Flow cytometric evaluation of 7-AAD and PI staining post fixation and permeabilisation showed that both the % gated sperm and mean fluorescent intensity of 7-AAD and PI stained spermatozoa decreased exponentially with time (Figure 3.9). Data is presented as log (mean fluorescence) and log (% gated) sperm versus time. Each data point represents the mean of three experiments done in duplicate. In each, 10 000 sperm were analysed by flow cytometry. The difference in slopes between PI and 7-AAD log (mean fluorescence) as well as log (% gated) was shown to be highly significant ($p < 0.0001$).

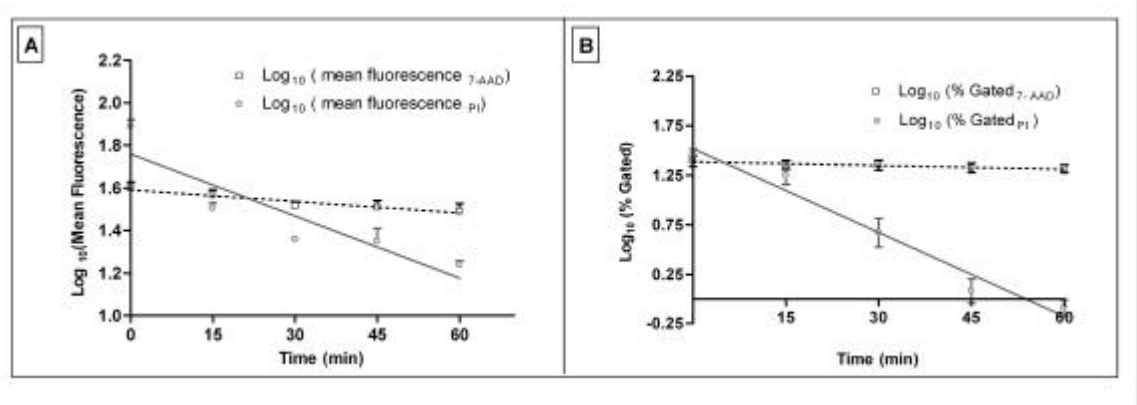

Figure 3.9 **(A)** Linear regression of log (mean fluorescence)$_{7\text{-AAD}}$ ($R^2 = 0.93$) and log (mean fluorescence)$_{PI}$ ($R^2 = 0.83$). **(B)** Linear regression of log (%gated) $_{7\text{-AAD}}$ fluorescence ($R^2 = 0.93$) and log (%gated) $_{PI}$ fluorescence ($R^2 = 0.97$).

3.6.2.2 Visual assessment of the acrosome reaction

Slides made of aliquots taken of spermatozoa stained with PSA-FITC and 7-AAD for flow cytometric evaluation were visually inspected via fluorescence microscopy (Zeiss Confocal Microscope, Germany). PSA-FITC stained the acrosomal contents of acrosome intact spermatozoa bright green (Figure 3.10 A) while no fluorescence was noted in acrosome reacted spermatozoa (Figure 3.10 D). Sperm undergoing the AR, either demonstrated patchy fluorescence in the acrosomal area (Figure 3.10 B) or equatorial staining (Figure 3.10 C). Non-viable spermatozoa stained red (7-

AAD$^+$), as 7-AAD could penetrate their compromised plasma membrane (not shown). For flow cytometric analysis, cells staining 7-AAD$^+$ were excluded in the determination of acrosome status.

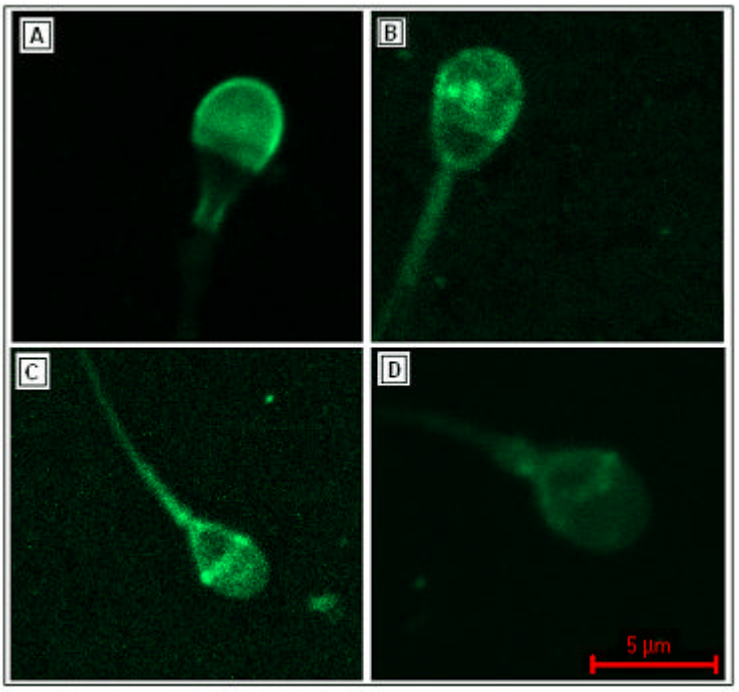

Figure 3.10 ***Fluorescent staining (PSA-FITC) of the human acrosome reaction***: (**A)** Acrosomal cap intact, (**B)** Patchy fluorescence, **(C)** Equatorial staining and **(D)** Acrosome reacted.

3.6.2.3 Assessment of the acrosome reaction by flow cytometry

(i) Evaluation of 7-AAD staining

Figure 3.11 illustrates the staining of human spermatozoa with the viability stain 7-AAD. Unstained cells (background fluorescence) are depicted by the red-brow population, while addition of 7-AAD shifted the population to the right (blue curve). Treatment of cells with Triton X-100 (2 µl / 1 x 10^6 sperm/ml) prior to staining with 7-AAD caused an increase in 7-AAD$^+$ fluorescence (green curve).

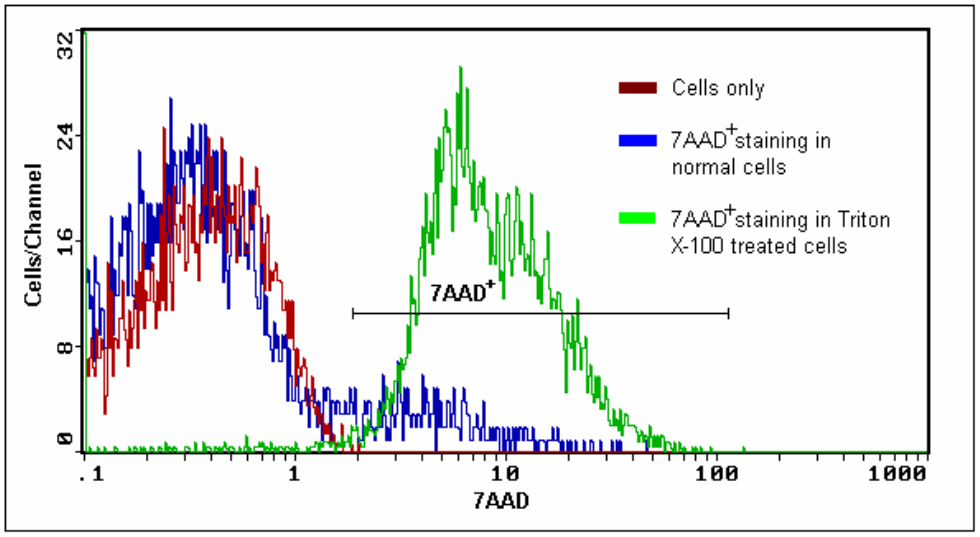

Figure 3.11 7-AAD Viability staining - Flow cytometric histograms showing overlay plots of unstained sperm (red-brown), sperm labelled with 10µl 7-AAD (blue) and sperm treated with 2 µl of Triton x-100 (green) for 15 min. prior to 7-AAD staining.

(ii) Induction of the acrosome reaction

The induced AR assay described by the WHO (1999), was adapted for flow cytometric assessment of the acrosomal status. A typical Log fluorescence histogram of DMSO treated spermatozoa (green curve) versus calcium ionophore (A23187)-induced acrosome reacted sperm (red-brown curve) is illustrated in Figure 3.12. Induction of the AR by calcium ionophore caused a decrease in PSA-FITC fluorescence, shifting the fluorescence intensity to the left. Acrosomal status was assessed in all twelve donors (samples run in duplicate) using 7-AAD to exclude non-viable cells. The mean percentage of spermatozoa that had undergone the AR following calcium ionophore induction was 13.48% ± 3.32.

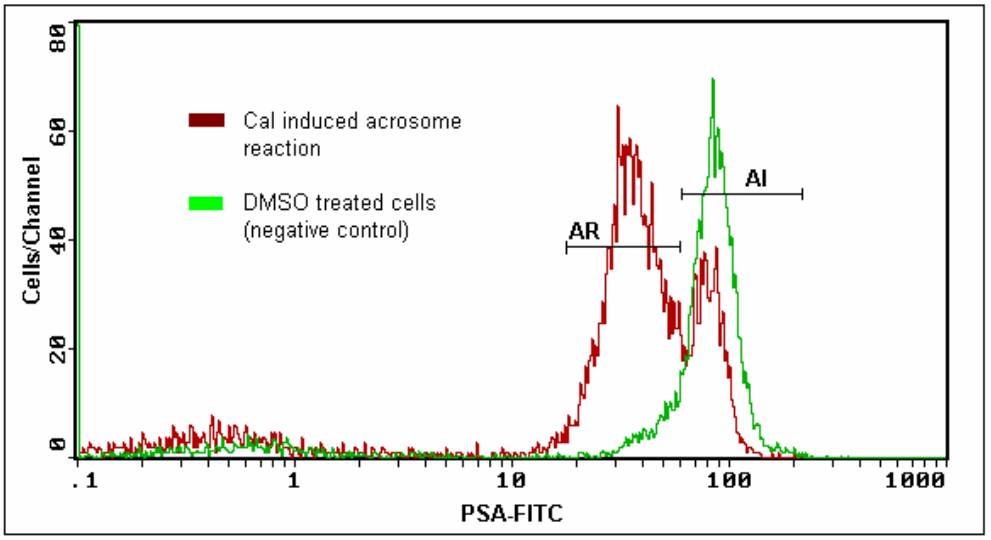

Figure 3.12 PSA-FITC staining - Flow cytometric histograms showing overlay plots of capacitated sperm (24 h) induced to undergo the AR by pre-incubation with A23187 (positive control - red-brown) and sperm treated with DMSO (negative control for calcium ionophore (CaI) stimulation - green) prior to PSA-FITC staining. Fluorescence peaks note the acrosome reacted (AR) and acrosome intact (AI) sperm populations.

The natural progression of the AR in sperm incubated under capacitating conditions is demonstrated in Figure 3.13 A. After 3 hours incubation under capacitating conditions (orange curve) 13.89% ± 8.34 of the cells had spontaneously undergone the AR, this number increased to 27.37% ± 3.48 after 24 hours incubation (green curve). Ionophore challenge (induced AR assay, WHO, 1999) after 24 h capacitation increased the number of acrosome reacted spermatozoa (red-brown curve) to 48.50% ± 11.45.

Typical dot plots and fluorescence histograms of PSA-FITC and 7-AAD staining are illustrated in Figure 3.13 B. The cell population was gated to exclude 7-AAD$^+$ cells, while regions F (acrosome intact) and H (acrosome reacted) were set to gate the differences in PSA-FITC staining.

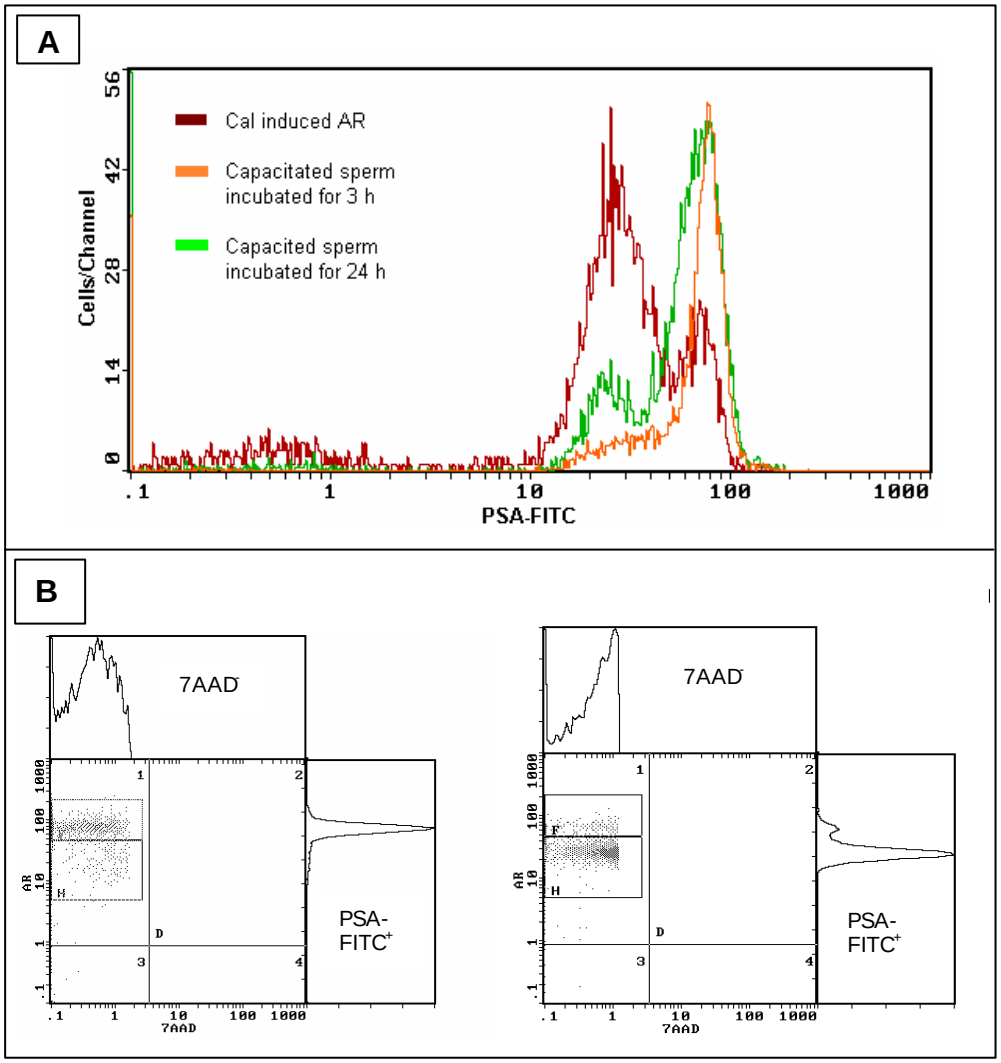

Figure 3.13 PSA-FITC staining - **(A)** Flow cytometric histograms showing overlay plots of capacitated sperm, sperm induced to undergo the AR by pre-incubation with CaI (positive control-red-brown); sperm incubated for 3 hours (orange) and sperm incubated for 24 hours (green) prior to PSA-FITC staining. Gating shows acrosome reacted (AR) and acrosome intact (AI) spermatozoa. **(B)** Flow cytometric dot plots and projections showing gating of PSA-FITC staining in 7-AAD$^-$ (live cells) only, region F denotes acrosome intact spermatozoa and region H acrosome reacted sperm.

(iii) Evaluation of the acrosome reaction post RF-EMF

When evaluating the number of non-viable cells (7-AAD$^+$) at the three different time points (Figure 3.14 A), no statistical difference was seen between RF-EMF exposed (SAR 2.0 W/kg) and control cells ($p = 0.397$). The number of acrosome reacted live (FITC$^+$7-AAD$^-$) spermatozoa increased with time (Figure 3.14 B). However, there was no statistical difference ($p = 0.839$) between RF-EMF exposed and control sperm at any of the time points. In a similar manner, the number of acrosome intact live (FITC$^-$7-AAD$^-$) spermatozoa decreased with time, but again, there was no statistical difference ($p = 0.920$) between exposed sperm and controls. 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 hours 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.

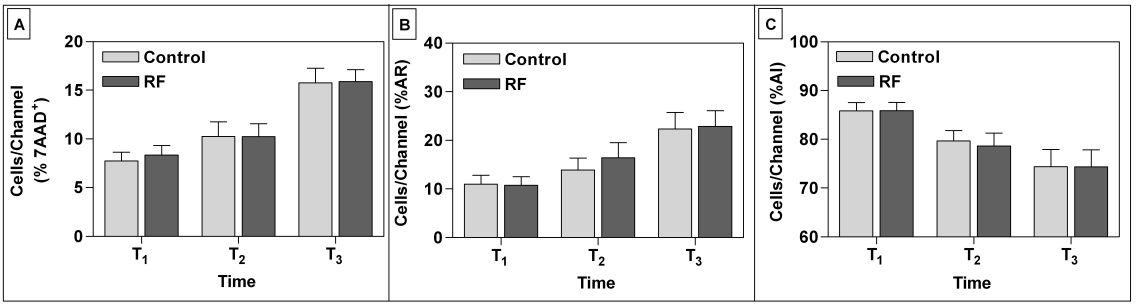

Figure 3.14 Comparison between RF-EMF (dark grey) and control (light grey) sperm assessed by FCM directly, 2 - and 24 - hours after a 1 hour 900 MHz GSM exposure (SAR 2.0 W/kg) **(A)** % 7-AAD$^+$ staining spermatozoa, **(B)** % acrosome intact live cells, **(C)** % acrosome reacted live.

3.6.3 Sperm-oocyte interaction - hemi-zona assay

There was a statistically significant ($p = 0.02$) reduction in zona binding in RF-EMF exposed sperm at SAR 2.0 W/kg compared to controls. Controls bound an average of 31.77 sperm / zona compared to 25.83 sperm / zona of RF-EMF exposed spermatozoa. All donors used in this assay bound on average more than 20 control

sperm/hemi-zona, the minimum criterion for limiting false-negative results with the HZA (Oehninger *et al.*, 1991). Results of the Wilcoxon signed ranks test are illustrated in Figure 3.15.

For comparison, spermatozoa from the two donors that demonstrated the highest difference in binding potential after RF exposure (at 2.0 W/kg) were exposed at the higher SAR of 5.7 W/kg. A total reduction of 27% in binding was noted, with controls binding an average of 32.10 sperm / zona compared to the 23.43 sperm / zona of the RF exposed group.

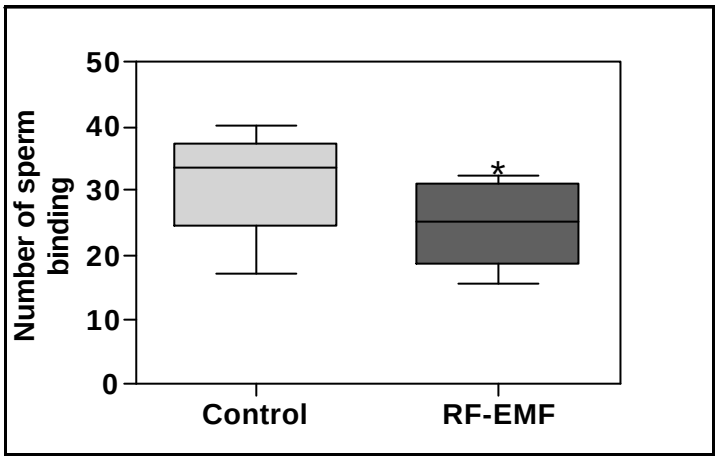

Figure 3.15 Box and whiskers plot (showing medians) of number of sperm binding non-fertilized metaphase II oocytes (5 oocytes per donor, n = 10) after an hour exposure to 900 MHz GSM radiation. RF-EMF exposure caused a significant reduction in sperm bound to the hemi-zonae compared to controls (*p = 0.02).

Morphology was also correlated with the number of sperm binding (Figure 3.16). Although morphology was not significantly correlated with number of sperm bound after RF-exposure (r_s = 0.51, p = 0.13), the greatest reduction (22.4%) in binding ability compared to controls was seen in the group of sperm with the lowest morphology (refer to Annexure C for morphology classification). Category A spermatozoa (5 - 10% normal morphology) showed a reduction of 22.4% in binding ability after RF exposure compared to 16.9% in category B (> 10% normal morphology) and 21.0% in category C (> 15% normal morphology) spermatozoa.

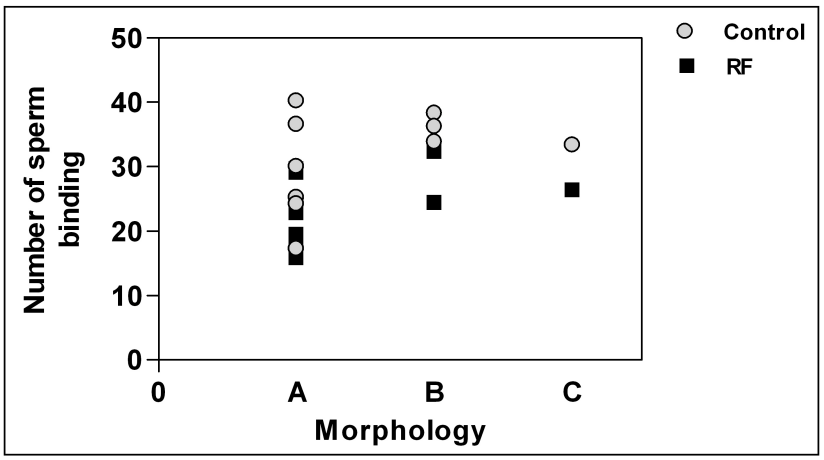

Figure 3.16 Correlation of morphology and number of spermatozoa binding the hemi-zonae after an hour exposure to 900 MHz GSM radiation (■). Number of control (●) spermatozoa is also shown for comparison.

3.7 DISCUSSION

Various studies have recently expounded on the possible influence of RF-EMF 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 (EFCP6 2005). Another possible weakness in the assessment of sperm motility as used by these studies is the lack of the use of an automated system such as CASA to eliminate operator bias in manual motility assessment.

Therefore, in the present study, dosimetry was verified by simulation and temperature rise calculations. In addition, temperature during exposure was regulated at 37°C and did not increase by more than 0.2°C during exposure at SAR 2.0 W/kg, while at SAR 5.7 W/kg the temperature did not increase more than 0.3°C. 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 motility categories, velocity and motion parameters were used.

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 (Plante *et al.*, 1994; Whittington and Ford, 1999; Henkel *et al.*, 2003) leading to amongst others a reduction in motility (Plante *et al.*, 1994; Armstrong *et al.*, 1999). It is thus possible that ROS generation and not RF-exposure could have contributed to the decreased progressive motile population observed, 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 – therefore our choice of assessing at T_2 - 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 change in any of these parameters could thus lead to a decrease in sperm fecundity. In the present study, RF-EMF at both SAR levels had no effect on hyperactivated motility. At a SAR of 2.0 W/kg, RF-EMF did not affect any of the kinematic-parameters (velocity or motion), at any of the time points post irradiation. However, at a SAR of 5.7 W/kg a significant change in VCL and BCF parameters were observed. This result cannot be ascribed to thermal effects, as the temperature rise during the exposure did not exceed 0.3ºC

(confirmed by temperature probe measurements - Annexure A). Therefore, the observed statistically significant change in motility parameters at SAR 5.7 W/kg could possibly be the result of an alternative mechanism, such as a decrease in mitochondrial fecundity or reactive oxygen species generation resulting in depleted ATP levels.

CASA evaluation (Metrix Software) of morphology revealed a statistically significant decline in all morphometric parameters except elongation. Of specific interest was the decrease in area and acrosomal region parameters noted after exposure at both SAR levels. A significant decrease in area was also observed after RF exposure at 5.7 W/kg when computer aided sperm analysis was used in the assessment of sperm motility, which serves as confirmation that RF-exposure causes a decrease in spermatozoa area. Although RF exposure at SAR 2.0 W/kg did not cause a statistically significant decrease in area, shrinkage of spermatozoa with time was none the less observed. 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 and 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, 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 AR, acrosomal status was assessed in RF exposed spermatozoa.

Several methods have been developed to evaluate the acrosomal status of human sperm, including lectin binding protocols - first described by Aitken *et al.* (1984), the triple stain technique (Talbot and Chacon, 1981), indirect immunofluorescence using monoclonal (Wolf *et al.*, 1985) or polyclonal antibodies (Töpfer-Petersen *et al.*, 1986 and Sanchez *et al.*, 1991), chlortetracycline fluorescence assays (Lee *et al.*, 1987), and fluorescein-labelled lectins (Cross *et al.*, 1986). More recently, flow cytometric analysis of human sperm using fluorescein-labelled lectins (Miyazaki *et al.*, 1990; Purvis *et al.*, 1990; and Uhler *et al.*, 1993; Nikolaeva *et al.*, 1998) or monoclonal antibodies (Bronson *et al.*, 1999) have been described as a more accurate, faster,

simpler, dynamic, and more objective method than fluorescence microscopy for the assessment of acrosome-reacted human sperm. The main limitation of assessing acrosomal status by flow cytometry is that detailed information regarding the stage of AR cannot be provided as with the visual method (Nikolaeva *et al*., 1998).

The suitability of using 7-AAD as viability probe for dead cell discrimination during FCM evaluation of the AR evaluation was confirmed. Unlike PI, 7-AAD did not leech from permeabilised, fixed spermatozoa and fluorescence remained stable for up to 1 h post staining. The presence of two distinct fluorescence populations after PSA-FITC labelling of capacitated spermatozoa correspond to acrosome reacted and acrosome intact spermatozoa. These findings are similar to those of Miyazaki *et al*. (1990), Uhler *et al*. (1993), Henley *et al*. (1994), and Nikolaeva *et al*. (1998). In addition, the PSA-FITC staining protocol described here, provided a rapid and precise assessment of acrosome reacted as well as acrosome intact spermatozoa with flow cytometry.

In experiments, no significant difference was noted in the competence of capacitated spermatozoa to initiate the AR after RF exposure. Acrosome reacted sperm increased as a function of time and, similarly, the acrosome intact spermatozoa decreased with time. However no difference was noted between the RF exposed and control populations. On the other hand, calcium ionophore induction lead to a significant increase in acrosome reacted spermatozoa. Flow cytometric results cannot explicate if RF exposure had an effect on the acrosomal region, which can only be confirmed by visual evaluation. Therefore, it is possible that RF exposure could have resulted in a reduction of the acrosomal region as was observed with CASA evaluation of morphology. The only telling way to confirm if these observations could affect sperm fecundity is by evaluating the binding potential of spermatozoa to the human ZP after RF exposure.

The hemi-zona assay plays an essential role in the diagnosis of male factor infertility (Burkman *et al*., 1988; Franken *et al*., 1993; Franken and Oehninger, 2006) and has a high predictive power for fertilisation outcome (Oehninger *et al*., 2000; Franken and Oehninger, 2006). In current experiments, 900 MHz RF-EMF exposure resulted in a statistically significant reduction in sperm binding at a SAR of 2.0 W/kg compared

with controls, a result even more pronounced at the higher SAR level of 5.7 W/kg. Taking into consideration that the number of spermatozoa bound to the ZP correlates with fertilisation rate (Lui *et al.*, 1989; Oehninger *et al.*, 2000; Bastiaans *et al.*, 2003), RF exposure therefore has the potential to affect male fertility and disrupt fertilisation.

However, before RF-EMF can be added to the list of environmental factors affecting male fertility a clearer understanding of the possible mechanism responsible for this effect should be elucidated. It has been noted in literature that RF exposure resulted in an efflux of intracellular calcium $[Ca^{2+}]_i$ in neurological cells (Bawin *et al.*, 1975; Blackman *et al.*, 1985), a process that could potentially affect sperm capacitation where an influx of Ca^{2+} is required in both sperm hyperactivation and the AR. It is unlikely that RF-EMF had an effect on Ca^{2+}, as RF-exposure had no effect on either hyperactivation or the AR. The decreased acrosomal region after RF exposure could be responsible for the reduction in binding observed in the HZA. Alternatively, it is possible that RF exposure could affect the gating of receptors (Chiabrera *et al.*, 2000) on the sperm head, which would explain why fewer exposed sperm bound to the hemi-zona, while no effect was seen on sperm propensity for the AR. If on the other hand, a stress response is initiated in spermatozoa, Hsp activation and phosphorylation could potentially interfere with signalling pathways in preparation of capacitation and sperm-zona pellucida interactions. It has been shown that Hsp70 and 90 are involved in various processes of sperm capacitation as well as zona recognition and fusion (Huang *et al.*, 2000a,b; Matwee *et al.*, 2001; Bohring and Krause, 2003; Huszar *et al.*, 2003; Ecroyd *et al.*, 2003). In particular, antibodies to Hsp70 significantly reduced tight binding of spermatozoa to the zona pellucida (Neuer *et al.*, 1998).

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

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CHAPTER 4

APOPTOTIC STATUS OF HUMAN SPERMATOZOA

4.1 INTRODUCTION

The ability of fully differentiated human spermatozoa to initiate apoptosis is not conclusive at this point (Oehninger *et al*., 2003; Taylor *et al*., 2004). Some attribute the presence of apoptotic markers such as morphological changes (Baccetti *et al*., 1996; Gandini *et al*., 2000), loss of asymmetric distribution of PS at the plasma membrane (Barroso *et al*., 2000; Oosterhuis *et al*., 2000), FAS positivity (Sakkas, 1999; Pentikainen *et al*., 1999), and DNA fragmentation (Barroso *et al*., 2000; Gandini *et al*., 2000) to failed apoptosis during spermatogenesis (Sakkas *et al*., 1999, 2002, 2003; Said *et al*., 2004; Almeida *et al*., 2005). On the other hand, other studies hypothesise a sperm specific apoptotic pathway (Weng *et al*., 2002) independent of caspase activation (Weng *et al*., 2002; Tesarik *et al*., 2004).

Regardless of whether human spermatozoa are able to initiate apoptosis or not, many physiological processes in spermatozoa are mediated by organelles/molecules that also serve as biomarkers for apoptosis. For instance, loss of mitochondrial membrane potential could result in the release of cytochrome-c into the cytosol triggering the caspase-9 dependant apoptotic pathway (Kroemer and Reed, 2000). However, in human spermatozoa, loss of mitochondrial membrane potential has a direct effect on sperm motility (Marchetti, 2002). Therefore, change in mitochondrial membrane potential as a result of RF-EMF exposure could provide a sensitive marker for sperm fecundity.

Another process known to occur in the early stages of apoptosis is the externalisation of the phospholipid, phosphatidylserine. PS externalisation is governed by scramblase activity (Vermes *et al*., 1995), and it is also associated with bicarbonate-mediated phospholipid scrambling during capacitation of human spermatozoa (Gadella and Harrison, 2002; de Vries *et al*., 2003; Martin *et al*., 2005). Thus, PS externalisation not only indicates the initiation of early apoptosis, but it also signifies that membrane changes required for capacitation are operational in human spermatozoa. Capri *et al*. (1995) reported on the RF-field effects on scramblase

activity, therefore necessitating the investigation of the effect of RF-EMF on PS externalisation in human spermatozoa.

Furthermore, Leszczynski *et al*. (2002) reported that RF-EMF inhibits apoptosis through the caspase-3 dependant apoptotic pathway. In human sperm, the role for caspases is still questionable. However, Weng *et al*. (2002), de Vries *et al*. (2003) Paasch *et al*. (2003) and Taylor *et al*. (2004) recently reported the presence of active and inactive forms of caspases in low and highly motile fractions of human sperm. The possibility that RF-EMF could influence caspase activity therefore had to be investigated.

Lastly, it has been proposed that a caspase independent pathway could be operational in sperm; therefore if RF-EMF could initiate apoptosis in spermatozoa it should be confirmed by investigating DNA fragmentation. Various studies have claimed a genotoxic effect of RF exposure (Lai and Singh, 1995, 1996, 1997; Maes *et al*., 1996; Tice *et al*., 2002; Mashevich *et al*., 2003). In addition, ROS generation as a consequence of RF-EMF could also contribute to DNA fragmentation. Irmak *et al*., (2002) reported that RF exposure lead to an increase in oxidative stress. Hence, the generation of ROS and DNA fragmentation required investigation.

This section therefore focused on the detection of cellular stress in RF-EMF exposed human spermatozoa by determining changes in various apoptotic markers (PS externalisation, mitochondrial membrane potential changes, caspase activation, and DNA fragmentation, as well as reactive oxygen species generation).

4.2 EXPERIMENTAL PROTOCOL

To determine the effect of RF-RMF on early- (PS externalisation, $\triangle\psi m$, and caspase activation) and late- apoptotic markers (DNA fragmentation), as well as factors that could affect apoptotic markers (endogenous generation of ROS), RF-EMF exposed and control spermatozoa were subjected to a variety of assays. A diagram outlining the experimental approach is shown in Figure 4.1.

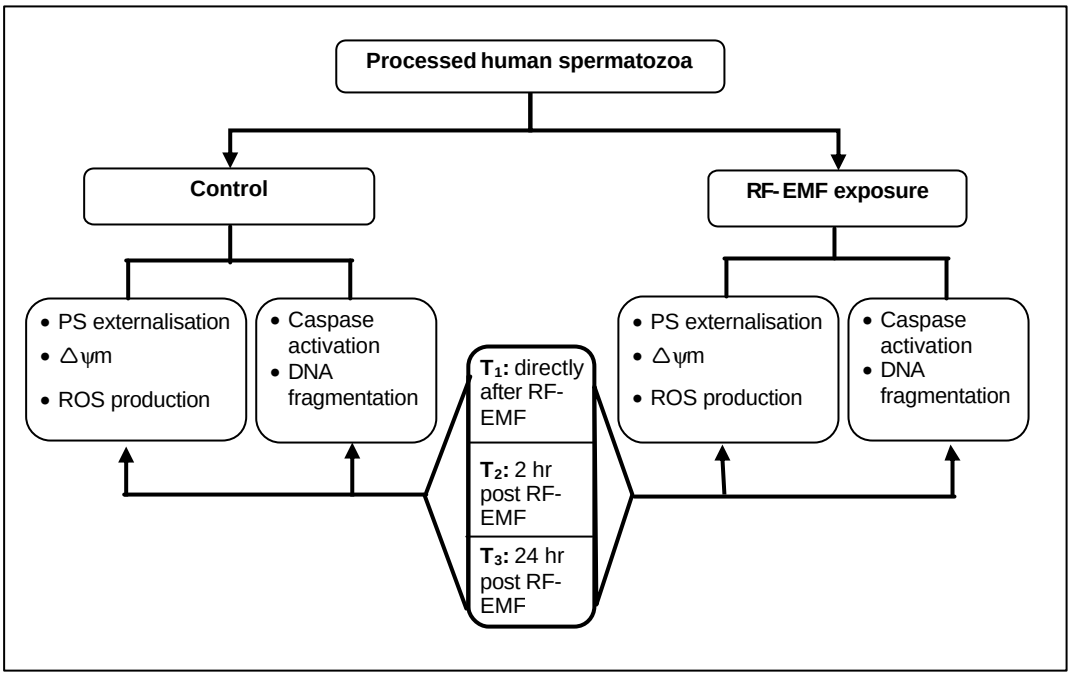

Figure 4.1 RF-EMF exposure protocol: Assessment of the apoptotic status post RF-EMF and control exposure.

Apoptosis detected by Annexin V staining (PS externalisation), Δψm by MitoTracker® Red uptake and ROS generation by conversion of hydroethidine (HE) to ethidium were all assessed on the same day at three different time points over a period of 4 weeks for all twelve donors. Similarly, caspase activation and DNA fragmentation were assessed at a later date, again on the same day at three different time points over a period of 4 weeks for all twelve donors. This was necessary due to the fact that it was not practicably possible to simultaneously evaluate all apoptotic parameters at each time point. Appropriate solvent controls were used as positive and negative controls for each biomarker.

Semen collected from the donors were purified by density gradient centrifugation (as described in Chapter 3) and seeded at a concentration of to 20 x 10^6 sperm/ml in petri dishes marked for RF-EMF exposure or controls (2 petri dishes each). Directly after the control/RF-EMF exposure, spermatozoa were gently recovered from the petri dishes, transferred to separate conical test tubes (Lasec) and concentrations were adjusted to 20 x 10^6/ml by centrifugation (300 g for 5 min). Spermatozoa were then incubated under capacitating conditions (5% CO_2 at 37°C) and assessed at different

time points (directly (T_1), 2 (T_2) and 24 (T_3) hours post-exposure) for apoptosis. All tests were run in duplicate.

Flow cytometric analysis was performed on a Coulter Epics® XL.MCL flow cytometer (XL.MCL) equipped with an air cooled argon laser (Beckman Coulter) for the analysis of all SAR 2.0 W/kg samples, while a Coulter Epics® Altra flow cytometer (Altra) equipped with a water cooled coherent enterprise laser (Beckman Coulter) was used for analysis of all SAR 5.7 W/kg samples unless otherwise stated. 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 with SD.

4.3 ASSESSMENT OF THE APOPTOTIC STATUS IN SPERMATOZOA

4.3.1 Phosphatidylserine externalisation determined by the Annexin V assay

Early phases of apoptosis are marked by the loss in plasma membrane asymmetry resulting in the translocation of the membrane phospholipid PS from the inner to the outer plasma membrane leaflet, thereby exposing PS to the external environment. Annexin V is 35-36 kDa Ca^{2+}-dependant phospholipid-binding protein with a high affinity for PS, therefore it serves as a sensitive probe for detecting sperm within a population that are actively undergoing apoptosis (Koopman *et al*., 1994; Vermes *et al*., 1995; Martin *et al*., 1995). Annexin V is conjugated with FITC for easy detection.

PI is a standard flow cytometric viability probe and can be used to distinguish viable from non-viable sperm (Koopman *et al*., 1994; Vermes *et al*., 1995; Martin *et al*., 1995; O'Brein and Bolton, 1995). Viable sperm with intact membranes will exclude PI whereas damaged or dead sperm will be permeable to PI. Using Annexin V-FITC in conjunction with PI, one can distinguish between viable sperm (Annexin V-FITC and PI negative), sperm that are in early apoptosis (Annexin V-FITC positive and PI

negative), sperm that are in late apoptosis or already dead (Annexin V-FITC and PI positive), and nonviable sperm (sperm that have already undergone apoptotic death and those that have died as a result of a necrotic pathway).

To determine PS externalisation, BD Bioscience's Annexin V-FITC apoptosis detection kit II (BD Biosciences) was used. The Annexin V-FITC staining protocol of the manufacturer was followed, with minor adjustments.

4.3.1.1 Annexin V-FITC staining protocol

Aliquots (100 µl) taken from the control and RF exposed spermatozoa were re-suspended in 2 ml cold Dulbecco's PBS (Sigma Chemical Co.) and washed by centrifugation (300 g for 5 min). Spermatozoa were re-suspended in 400 µl Annexin V binding buffer (BD Biosciences) (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 5 x 10^6 sperm/ml. A 100 µl of the solution was then transferred to a 5 ml flow tube to which 5 µl Annexin V-FITC (BD Biosciences) and 5 µl of PI (BD Biosciences) were added. The sperm were gently vortexed and left to incubate for 15 min at room temperature (25°C) in the dark.

After the incubation time, 400 µl of the Annexin V binding buffer was added to each tube and the spermatozoa were analysed by flow cytometry within 20 min. A minimum of 10 000 spermatozoa were examined for each test. The sperm population was gated by using forward-angle light scatter, while side-angle light scatter was used to exclude electronic noise and debris. The FITC-labelled Annexin V positive sperm were measured in the FL1 channel. PI fluorescence is detected in the far-red range of the spectrum (650 nm long pass filter) (Schmid *et al*., 1992; O'Brein and Bolton, 1995) and PI-labelled sperm were measured in the FL2 channel of the flow cytometer. All tests were run in duplicate.

To ensure specificity of Annexin V-FITC binding, appropriate controls were used. Recombinant Annexin V provided a negative control while, staurosporine used to induce apoptosis, provided a positive control for Annexin V-FITC labelling.

4.3.1.2 Annexin V blocking by recombinant Annexin V

Recombinant Annexin V was provided as part of the BD Bioscience Annexin V-FITC apoptosis detection kit II. Pre-incubation of cell samples with purified recombinant Annexin V serves to block Annexin V-FITC binding sites, thereby demonstrating the specificity of Annexin V-FITC staining. The protocol provided by the manufacturers was followed.

Sperm were washed twice by centrifugation (300 g for 5 min) with cold Dulbecco's PBS and then re-suspended in Annexin V binding buffer at a concentration of 1 x 10^6 sperm/ml. Of this cell suspension, 100 µl was transferred to a 5 ml flow tube. Purified recombinant Annexin V (10 µg) was then added to the suspension and the sperm were gently mixed and left to incubate for 15 min at room temperature. Annexin V-FITC (5µl) was added and the sperm incubated for a further 15 min in the dark after which 400 µl of the Annexin V binding buffer was added to each tube and the spermatozoa were analysed by flow cytometry within 20 min.

4.3.1.3 Induction of apoptosis by staurosporine

Cells induced to undergo PCD by staurosporine die with the characteristic morphological features of apoptosis followed by caspase activation (Weil *et al.*, 1998). Although the exact activation mechanism is still unknown, staurosporine acts as a protein kinase inhibitor, and induced cell death can be repressed by either the over-expression of the PCD-suppressor gene Bcl-2 or petide caspase inhibitors (Jacobson *et al.*, 1993, 1994, 1996; Bertrand *et al.*, 1994). It has previously been demonstrated that staurosporine induces apoptosis in human spermatozoa (Weng *et al.*, 2001; Morshedi *et al.*, 2003) and specifically increases PS translocation (Morshedi *et al.*, 2003).

Staurosporine (Sigma Chemical Co.) was stored as aliquots at a stock concentration of 1 mM at -20ºC. To establish optimal agonist concentrations, induction of apoptosis assessed by the Annexin V assay was performed for a range of concentrations for staurosporine. Directly before experimentation, aliquots were diluted in BSA free Ham's F-10 medium and added to sperm (1 x 10^6/ml) at final concentrations of 1 µM, 5 µM, and 10 µM. Sperm were incubated for 15 min with

the different staurosporine concentrations after which 0.5% BSA was added to the sperm suspension. The sperm were left to incubate for a further 2 hours in a humidified CO_2 incubator at 37°C, washed twice by centrifugation (300 g for 5 min) with cold PBS before proceeding with Annexin V-FITC staining protocol and flow cytometric analysis.

4.3.2 Mitochondrial membrane potential

Baseline $\Delta\psi m$ was determined by the method described by Marchetti *et al*. (2004a) with minor adjustments. Mitochondria were stained with the mitochondrial selective stain MitoTracker® Red CMXRos (Molecular Probes, Eugene, USA). The MitoTracker® Red probe passively diffuses across the plasma membrane and accumulates in active mitochondria. MitoTracker® Red CMXRos is excited at a frequency of 579 nm and emits at 599 nm. Mitochondrial membrane depolarisation is thus associated with a decrease in fluorescence.

4.3.2.1 MitoTracker® Red CMXRos staining procedure

MitoTracker® Red CMXRos was stored as a stock concentration of 1 mM at -20°C, dilutions were made directly before staining and MitoTracker® was added to sperm at a final concentration of 150 nM per 1 x 10^6 sperm/ml in 0.5% BSA supplemented Ham's F-10 medium. Sperm suspensions were then incubated for 15 min in a humidified (5% CO_2) incubator at 37°C. After incubation, sperm were washed (5 min at 300 g), the supernatant removed and the sperm re-suspended in 1 ml warm medium (37°C) before being analysed by flow cytometry. MitoTracker® Red CMXRos fluorescence was detected in FL3.

4.3.2.2 Abolishment of $\Delta\psi m$ by Carbamoylcyanide m-chlorophenylhydrazone

Carbamoylcyanide m-chlorophenylhydrazone (mClCCP) has previously been used to abolish the mitochondrial membrane potential of spermatozoa (Marchetti *et al.*, 2002). Spermatozoa (1 x 10^6/ml) were incubated in the presence of 50 µmol/l mClCCP for 15 min at 37°C in a humidified incubator (5% CO_2) before proceeding with the MitoTracker® Red CMXRos staining procedure to determine $\Delta\psi m$.

4.3.3 Detection of superoxide

ROS in semen are either produced by the infiltration of leukocytes as a result of genitourinary inflammation (Wolff, 1995) or is a consequence of damaged and immature spermatozoa (Aitken *et al.*, 1988). Spermatozoa are highly susceptible to peroxidative damage due to the abundance of polyunsaturated fatty acids in their plasma membrane (Aitken *et al.*, 1989). The primary product of ROS production in human spermatozoa is the superoxide anion radical ($O_2^{-\cdot}$) which subsequently dismutases to H_2O_2 in the presence of intracellular superoxide dismutase (Aitken and Fisher, 1994). Intrinsic ROS production has been linked to DNA damage in spermatozoa (Griveau and Le Lannou, 1997; Henkel *et al.*, 2004). For this reason, ROS production was evaluated not only as a possible consequence of RF-EMF exposure but also to determine its effect on DNA damage. Furthermore, the presence of leucocytes in the ejaculate also contributes to ROS production therefore a leukocyte specific antigen (CD45) was used to firstly identify and quantify leukocytes in the sperm population of all donors and secondly to correctly gate the sperm population.

4.3.3.1 Detection of $O_2^{-\cdot}$ with hydroethidine

The methods described by Marchetti *et al.* (2002) and Henkel *et al.* (2004) for the detection of the superoxide anion radical in human spermatozoa was adapted. Hydroethidine (Molecular Probes Inc.) an uncharged cell-permeant compound is converted to cationic ethidium by superoxide oxidation. Ethidium (E^+) is cell impermeant and intercalates with the DNA.

Hydroethidine was stored as stock solutions of 1 mM in DMSO at -20ºC. Directly prior to use, HE (2 µmol/l) was added to spermatozoa in BSA free Ham's F-10 medium recovered from the RF exposed and control petri-dishes (1 x 10^6 sperm/ml). Sperm suspensions were then incubated for 15 min at 37°C in a humidified incubator (5% CO_2), washed by centrifugation (300 g for 5 min) with 2 ml PBS, and re-suspended in 1 ml PBS before FCM analysis. Ethidium fluorescence was detected in FL1.

It has previously been shown that cytofluorometric analysis of white blood cells (WBC) and seminal leukocytes are comparable (Ricci *et al*., 2000). Therefore, to correctly identify and gate different WBC subpopulations a known number (2 x 10^6 white blood cells / 1 x 10^6 sperm/ml) of peripheral blood leukocytes (obtained from heparinized blood of healthy donors), were added to normal semen samples devoid of leukocytes. CD 45 labelling as outlined above was then used to correctly gate the different leukocyte regions.

4.3.3.2 Determination of leukocyte contamination in processed spermatozoa

The presence of leukocytes in seminal plasma contributes to the inhibition of both sperm movement and ATP production (Armstrong *et al*., 1999; Henkel *et al*., 2004). Therefore, to eliminate the effect of leukocytes on spermatozoa, leukocyte presence in processed sperm were detected by labelling leukocytes with a monoclonal CD45 antibody to enable the correct gating of the sperm population for flow cytometry.

The CD45 leukocyte antigen conjugated with FITC (BD Bioscience) reacts with the 180, 190, 205, and 220 KDa isoforms of the leukocyte antigen present on all human leukocytes including lymphocytes, monocytes, granulocytes, eosinophils, and thymocytes (Knapp *et al*., 1989). The antibody was stored at 4°C.

The percentage leukocyte contamination present in each of the twelve donor's processed sample was determined by adding 20 µl of the CD45 solution to 1 x 10^6 sperm/ml suspensions and incubating samples for 20 min at room temperature in the dark. After incubation, samples were analysed by flow cytometry. The FITC signal was measured on FL 1 through a 530/30 nm band pass filter.

4.3.4 Caspase-3 activation.

Caspase-3 is a key protease that is activated during the early stages of apoptosis. Active caspase-3 (aCp3), is a marker for cells that are actively undergoing apoptosis, and once activated, proteolytically cleaves and activates other caspases as well as relevant targets within the cytoplasm, namely D4-GDI, Bcl-2, and poly-ADP-ribose polymerase (PARP) in the nucleus (D'Amours *et al*., 1999).

Active caspase-3 consists of a heterodimer of 17 and 12 kDa subunits derived from the 32 kD proenzyme. BD Pharmingen (BD Bioscience) supplies a PE-conjugated monoclonal rabbit-anti active caspase-3 antibody apoptosis kit containing essential reagents for measuring apoptosis by flow cytometric analysis. The PE-conjugated monoclonal rabbit-anti active caspase-3 antibody recognises both human and mouse aCp3. The antibodies were raised against a sequence corresponding to active human caspase-3 and are affinity purified using Protein G and conjugated with PE under optimal conditions (contains no unconjugated antibody). The antibodies are solubilized in phosphate buffer (pH 7.2) with 500 mM NaCl, 0.09% (w/v) sodium azide and 0.2% (w/v) BSA and are stored at 4°C.

Reagents supplied with the kit include, Cytofix/Cytoperm™ Solution and Perm/Wash™ Buffer. The Cytofix/Cytoperm™ Solution (neutral pH-buffered saline, saponin and 4% (w/v) paraformaldehyde) was used for the simultaneous fixation and permeabilization of sperm prior to intracellular staining and was stored at 4°C. Perm/Wash™ Buffer was used to permeabilise sperm for intracellular staining, and also serves as an antibody diluent and cell wash buffer. The buffer contains foetal bovine serum (FBS), sodium azide and saponin and is stored at 4°C.

4.3.4.1 Active caspase-3 PE staining protocol

Sperm recovered from the RF exposed petri dishes were first labelled with MitoTracker® Red CMXRos before being washed twice with cold PBS, re-suspended in Cytofix/Cytoperm™ solution at a concentration of 1 x 10^6 sperm/0.5 ml, and incubated for 20 min on ice. Sperm were then pelleted (centrifuge at 300 g for 5 min) and the supernatant discarded after which sperm were washed twice (300 g for 5 min) with Perm/Wash™ buffer at a volume of 0.5 ml buffer/1 x 10^6 sperm at room temperature. Next, sperm were re-suspended in a 100 µl Perm/Wash™ buffer per 20 µl antibody containing solution and incubated for 30 min at room temperature. After incubation, spermatozoa were again washed in 1 ml Perm/Wash™ buffer and finally re-suspended in 0.5 ml Perm/Wash™ buffer before flow cytometric analysis.

4.3.4.2 Caspase inhibition by CaspACE™ FITC-VAD-FMK

CaspACE™ FITC-VAD-FMK (Promega Corporation, Madison, USA) is used as an *in situ* marker for apoptosis. The marker is a FITC conjugate of the cell permeable caspase inhibitor VAD-FMK. This structure allows delivery of the inhibitor into the cells where it binds to activated caspases. CaspACE™ FITC-VAD-FMK *in situ* marker is supplied as a 5 mM solution in DMSO.

RF-EMF and control cells (1 x 10^6/ml) were washed (300 g for 5 min) with 2 ml PBS before the pellet was re-suspended in 1 ml PBS and 5 µM CaspACE™ FITC-VAD-FMK was added to the suspension. Cells were then incubated for 20 min at room temperature in the dark before being washed twice, re-suspended in 1 ml PBS, and analysed by FCM. FITC fluorescence was detected in FL1.

4.3.5 DNA fragmentation

One of the later steps of apoptosis resulting from the activation of endonucleases during PCD is DNA fragmentation (Enari *et al*., 1998; Sakahira *et al*., 1998). These nucleases degrade the higher order chromatin structure into fragments of ~300 kb and subsequently into even smaller fragments of about 50 bp in length (Walker *et al*., 1993). The APO-DIRECT™ kit (BD Bioscience) is a single step method for labelling DNA breaks with FITC-dUTP (TUNEL – Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling).

The kit comprises of two parts. Part A contains the PI/RNase staining buffer (5 µg/ml PI, 200 µg/ml RNase), reaction buffer (containing cacodylic acid [dimethylarsenic]), rinsing buffer (containing 0.05% sodium azide), and wash buffer (containing 0.05% sodium azide). Part B contains FITC-dUTP (0.25 nMol/reaction containing 0.05% sodium azide), negative control cells and positive control cells (both containing 70% (v/v) ethanol) as well as TdT enzyme ([S.A. = 100,000 U/mg] 200 µg/ml in 50% (v/v) glycerol solution). The control cells were derived from a human lymphoma cell line and have already been fixated.

4.3.5.1 Fixation protocol for APO-Direct™ samples

Spermatozoa recovered from the RF exposed and control petri dishes were re-suspended at a concentration of 2 x 10^6 sperm/ml in 1% (w/v) paraformaldehyde

(Sigma Chemical Co.) in PBS (pH 7.4). The cell suspension was incubated on ice for 30 min before the sperm were centrifuged for 5 min at 300 g and the supernatant discarded. Sperm were washed in 5 ml of PBS and again pelleted by centrifugation before the wash procedure was repeated. The cell pellet was then re-suspended in the residual PBS by gentle vortexing and adjusted to 1 x 10^6 sperm/ml in 70% ice-cold ethanol. Sperm were frozen at -20°C until assessment at a later stage.

4.3.5.2 APO-Direct™ staining protocol

Frozen sperm were left to thaw at room temperature before commencing with the labelling step. The sperm were re-suspended by gently swirling the tubes after which the cell suspensions were centrifuged for 5 min at 300 g. The supernatant containing the 70% (v/v) ethanol was removed by gentle aspiration and the sperm re-suspended in 1 ml of the wash buffer. The cell suspension was washed twice more by centrifuging (300 g, 5 min) before the supernatant was discarded and the sperm re-suspended in 50 µl of the staining solution (10 µl reaction buffer, 0.75 µl TdT Enzyme, 8 µl FITC-dUTP and 32.25 µl distilled H_2O/ assay).

The samples suspended in the staining solution were incubated for 60 min at 37°C after which they were washed twice in 1 ml of the rinse buffer (300g, 5 min). After removing the supernatant by gentle aspiration, the cell pellet was re-suspended in 0.5 ml of the PI/RNase staining buffer. The sperm were incubated for 30 min at room temperature in the dark after which DNA fragmentation was assessed by flow cytometry. For each determination, at least 10 000 spermatozoa were examined by using flow cytometry. The FITC-labelled dUTP-positive spermatozoa were measured in the FL 1 channel of the flow cytometer.

4.3.5.3 Induction of DNA fragmentation by DNase

A positive control sample was prepared by the additional treatment of each sample with RQ1 RNase-Free DNase (Promega Corporation, Madison, WI, USA). RQ1 (RNA-Qualified) RNase-Free DNase is a DNase 1 (endonuclease) that degrades both double-stranded and single-stranded DNA producing 3′-OH oligonucleotides (Moore, 1981). After fixation and permeabilization of spermatozoa in preparation of the APO-Direct™ protocol, sperm (1 x 10^6 sperm/ml) were pelleted and re-

suspended in 10 µl of the 10x reaction buffer (40 mM Tris-HCL [pH 8.0], 10 mM $MgSO_4$ and 1 mM $CaCl_2$ – Promega Corp.) to which 2 IU RQ1 RNase-Free DNase was added. Spermatozoa were incubated for 15 min at 37°C. After incubation, the reaction was stopped by incubating the samples in 10 µl (20 mM) RQ1 DNase stop solution (pH 8.0) for 10 min at 65°C. Samples were washed twice with PBA (PBS + 0.5% BSA) before commencing with the labelling step of the APO-Direct™ protocol described above.

4.4 STATISTICAL ANALYSIS

To determine the effect of different concentrations of staurosporine on sperm viability assessed using the Annexin V-FITC and PI assay, data were analysed using a two-tailed paired t-test with GraphPadPrism® version 4.01 software (GraphPadSoftware, San Diego, CA, USA).

The effect of RF exposure on various apoptotic parameters was determined by analysing the data with Stata Statistical Software Release 8.0 (Stata Corp.) using a within subject design considering two treatments, control vs. RF-EMF (SAR 2.0 or 5.7 W/kg), 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. Results were analysed using time series regression under the random effect option. Data is presented as mean values ± SD for all twelve donors with each test run in duplicate. Repeatability of duplicate tests was confirmed with the intraclass correlation coefficient for assays = 0.92. Correlations between different apoptotic markers were computed using the Pearson's correlation coefficient. All statistical tests were two-sided and statistical significance was considered when $p < 0.05$.

4.5 RESULTS

4.5.1 Phosphatidylserine externalisation determined by the Annexin V assay

Results of Annexin V staining specificity using recombinant Annexin V and induction of PS externalisation using staurosporine are shown in Figures 4.2 and 4.3 respectively. A typical flow cytometric dot plot and fluorescence histograms after

Annexin V and PI staining are shown in Figure 4.4. The effect of 900 MHz GSM (SAR of 2.0 and 5.7 W/kg) irradiation on PS externalisation are summarised in Figures 4.5 and 4.6 respectively.

4.5.1.1 Annexin V blocking by recombinant Annexin V

Figure 4.2 shows a typical fluorescence histogram of the total Annexin V (16.5%) staining (red) in a density purified sperm sample. After addition of 10 µg purified recombinant Annexin V to 1 x 10^6 /ml sperm suspensions, Annexin V staining was completely blocked (blue).

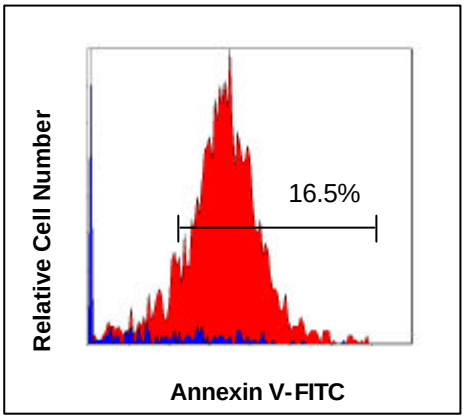

Figure 4.2 Flow cytometric histograms of total Annexin V before (red) and after (blue) addition of recombinant Annexin V- demonstrating the specificity of Annexin V staining.

4.5.1.2 Induction of apoptosis by staurosporine

The percentage PI^+, Annexin V^+, and apoptotic (Annexin V^+ PI^-) cells after addition of different concentrations of staurosporine are depicted in Figure 4.3. The percentage PI positive stained cells increased from 20.65 ± 0.35 for control cells to 92.380 ± 0.09 after 2 hour exposure to 10 µM staurosporine. Using a lower concentration (1 µM), no statistical significant difference in viability was noted between control cells and staurosporine treated cells after 2 hours. The total amount of PS externalised after a 2 hour exposure to 10 µM staurosporine was highly significant ($p < 0.0001$) compared to control cells. When comparing PS

externalisation (Annexin V^+) after staurosporine treatment in viable cells (PI^-), there was no statistical significant difference in $\%AV^+/PI^-$ control and 1 µM staurosporine treated cells. However $\%AV^+/PI^-$ control cells differed significantly from 5 µM ($p < 0.05$) and 10 µM ($p < 0.005$) staurosporine treated cells.

Spermatozoa treated with 1 µM staurosporine were again analysed by flow cytometry after a further incubation period of 2 hours (4 hours after addition of staurosporine). The number of non-viable cells (PI^+) differed significantly ($p < 0.005$) as well as the total amount of PS externalised (Annexin V^+) ($p < 0.005$) compared with the 2 hour viability and PS assessment. However, no statistically significant difference in percentage Annexin V^+/PI^- sperm after the additional 2 hour assessment was noted.

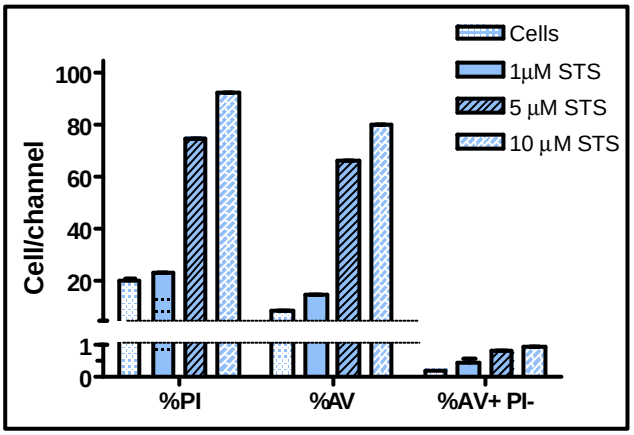

Figure 4.3 The percentage PI, Annexin V and Annexin V^+/PI^- sperm after a 2 hour exposure to 1 µM, 5 µM and 10 µM staurosporine (STS), each datum represents the mean ± SD of three determinations.

4.5.1.3 The effect of 900 MHz GSM irradiation on phophatidylserine externalisation

Figure 4.4 shows a typical dot-plot with fluorescence histograms of the flow cytometric analysis of PS externalisation in spermatozoa. PI fluorescence is used to distinguish viable and non-viable cells in the sperm population. The dot-plot regions

respectively correspond to necrotic cells - $AV^- PI^+$, dead cells - $AV^+ PI^+$, unlabelled sperm population - $AV^- PI^-$, and apoptotic cells - $AV^+ PI^-$. The population gated by C in histogram A notes the total Annexin V fluorescence. PI fluorescence, shown in histogram B and gated by E, gives the total population of dead cells in the sample. In some samples, a secondary peak of weaker fluorescence (gated by D in histogram B), were noted.

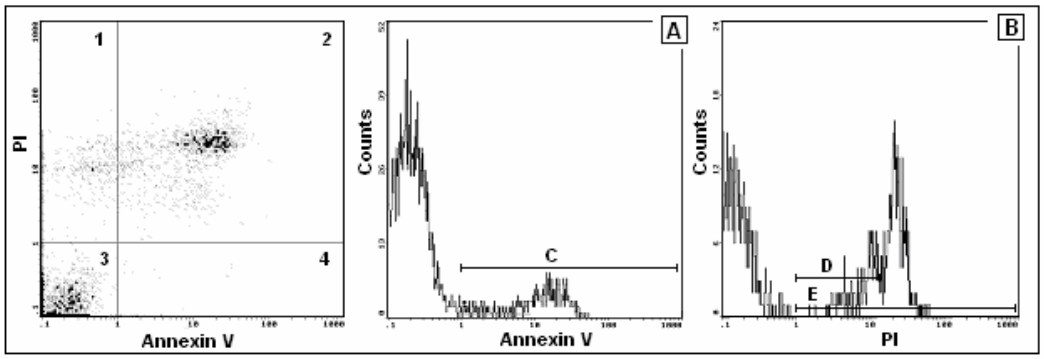

Figure 4.4 Dot plot and fluorescence histograms of **(A)** Annexin V and **(B)** PI staining of human spermatozoa.

When considering the total Annexin V staining after 1 hour RF-EMF exposure at a SAR value of 2.0 W/kg (Figure 4.5 A_1) no difference ($p = 0.406$) was seen between RF exposed and control cells assessed directly after exposure (T_1), 2 hours after exposure (T_2), or 24 hours after exposure (T_3). Similarly, no difference was seen in total PI fluorescence ($p = 0.144$) (Figure 4.5 A_2) and number of apoptotic cells ($p = 0.784$) (Annexin $V^+ PI^-$ Figure 4.5 A_3). There was also no difference in the number of apoptotic necrotic ($p = 0.407$) (Annexin $V^+ PI^+$ Figure 4.6 A_1) and dead cells ($p = 0.651$) (Annexin $V^- PI^+$ Figure 4.6 A_2).

For a SAR of 5.7 W/kg, no difference ($p = 0.448$) between RF exposed and control cells determined directly (T_1), 2(T_2) and 24 hours (T_3) after exposure were noted when total Annexin V staining (Figure 4.5 B_1) was assessed. This was also the case with total PI fluorescence ($p = 0.323$) (Figure 4.5 B_2) and number of apoptotic cells ($p = 0.711$) (Annexin $V^+ PI^-$) (Figure 4.5 B_3). The total number of apoptotic necrotic

($p = 0.457$) (Annexin V^+ PI^+ Figure 4.6 B_1) and dead cells ($p = 0.841$) (Annexin V^- PI^+ Figure 4.6 B_2) were also not significantly affected. Interestingly, for both SAR values Annexin V^+ PI^- cells decreased at T_2 (Figure 4.6 A_3 and B_3).

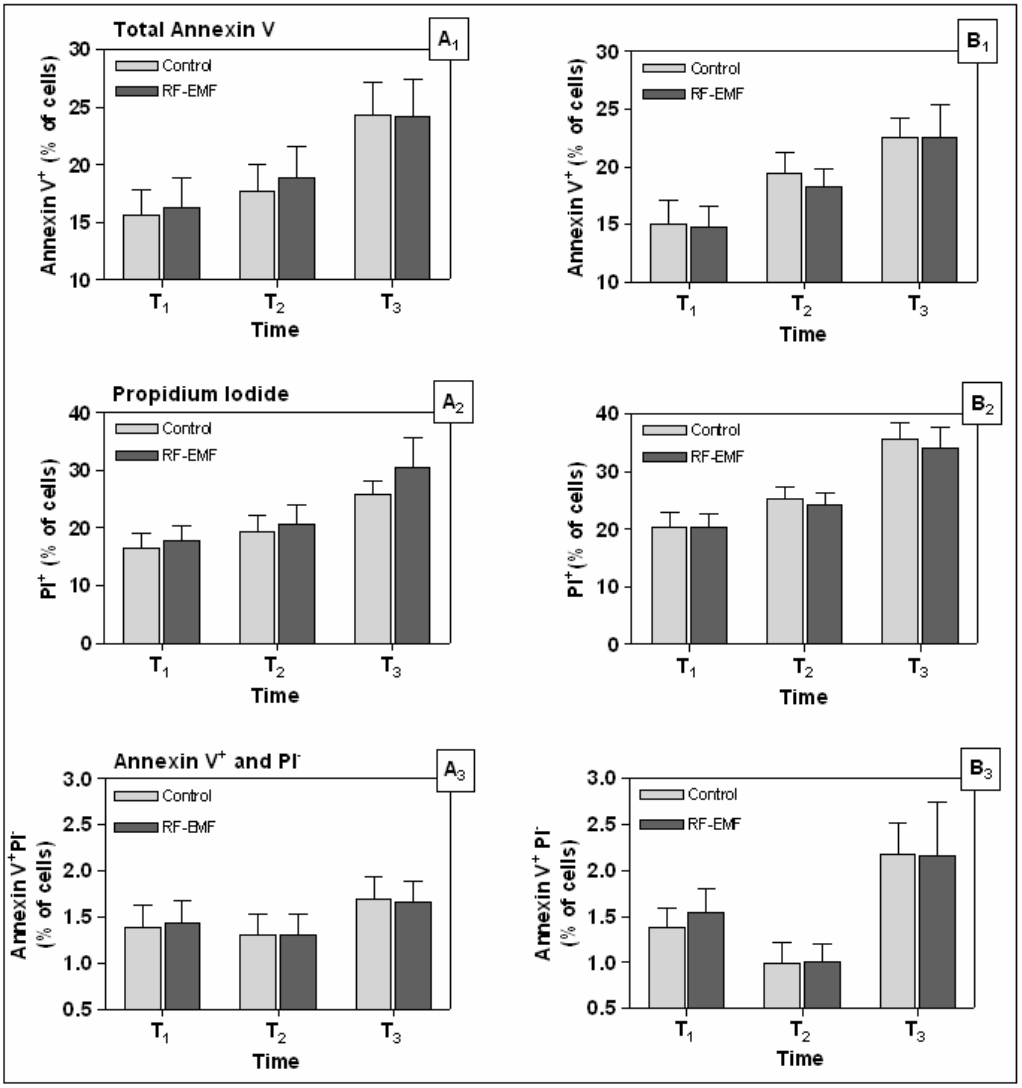

Figure 4.5 In **A_1** (2.0 W/kg) and **B_1** (5.7 W/kg) the total percentage Annexin V staining is noted as a function of time. **A_2** (2.0 W/kg) and **B_2** (5.7 W/kg) depict the total percentage of non-viable cells (PI^+) as a function of time. **A_3** (2.0 W/kg) and **B_3** (5.7 W/kg) show the total percentage Annexin V^+ viable cells (PI^-) as a function of time.

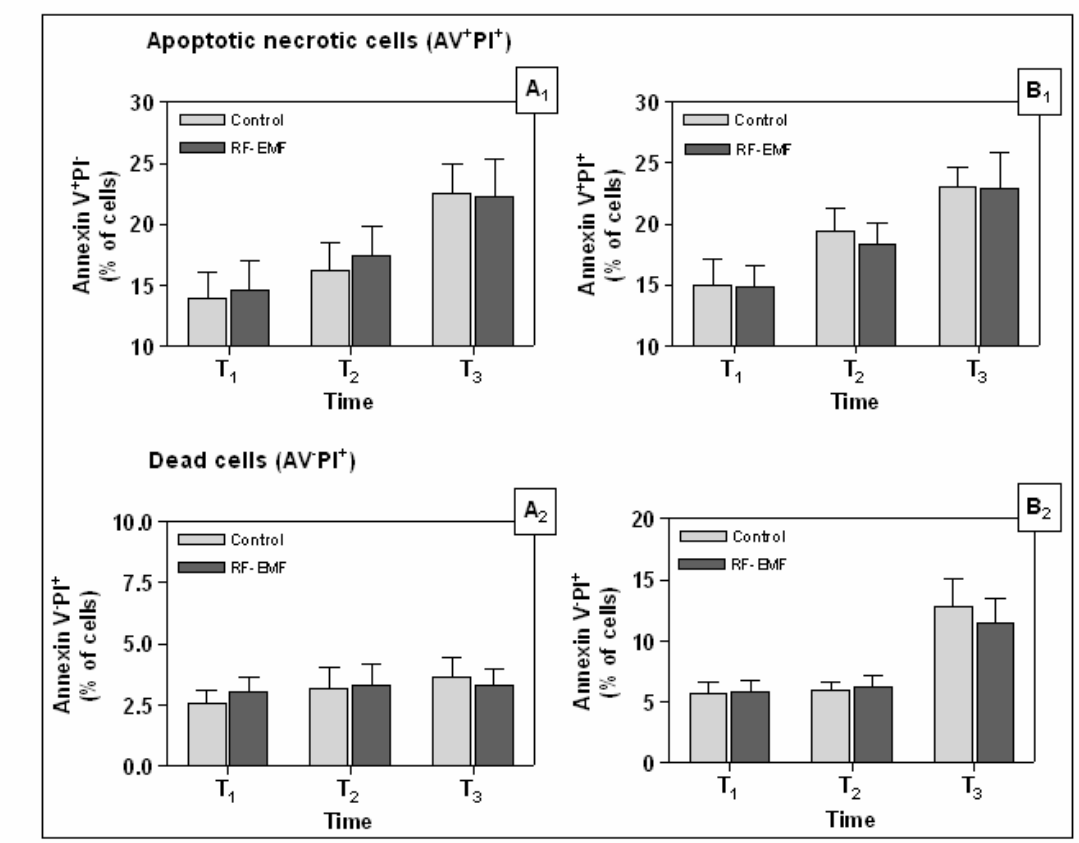

Figure 4.6 In Figures **A_1** (2.0 W/kg) and **B_1** (5.7 W/kg) the total number of apoptotic necrotic (Annexin V^+ PI^+) staining is noted as a function of time. **A_2** (2.0 W/kg) and **B_2** (5.7 W/kg) depict the total percentage of dead cells (Annexin V^- PI^+) as a function of time.

To determine the effect of the increased exposure dose on cell viability (PI fluorescence) and PS externalisation (Annexin V fluorescence), total Annexin V fluorescence, total PI fluorescence, and percentage apoptotic cells (Annexin V^+ PI^-) of RF-exposed sperm at all three time points at 2.0 W/kg were also compared with sperm exposed at 5.7 W/kg. This was possible, as control values for the different SAR levels did not differ significantly between experiments. Using Stata statistical software a paired t-test was performed at each time point for each SAR level. No statistical difference in total Annexin V fluorescence, total PI fluorescence, or percentage apoptotic cells determined at any of the time points were observed (Table 4.1).

Table 4.1 Summary of paired t-test results comparing viability (PI-fluorescence) and PS externalisation (Annexin V fluorescence) of RF-exposed spermatozoa at SAR 2.0 W/kg with SAR 5.7 W/kg.

Biomarker	T_1	T_2	T_3
Propidium Iodide	p = 0.391	p = 0.241	p = 0.885
Annexin V	p = 0.673	p = 0.798	p = 0.504
Annexin V^+ PI^-	p = 0.767	p = 0.297	p = 0.985

Investigating the progression of apoptosis in RF-EMF exposed cells (SAR 2.0 W/kg), a Pearson's correlation test was used to correlate the percentage early apoptotic cells (Annexin V^+ PI^-) with late apoptotic-necrotic cells (Annexin V^+ PI^+), as well as percentage dead cells (Annexin V^- PI^+) (Table 4.2). Similar correlations and statistical significance were observed for control cells (data not shown) as well as RF-EMF exposed cells (SAR 5.7 W/kg).

Table 4.2 Comparison between Annexin V^+ PI^-, Annexin V^+ PI^+ and Annexin V^- PI^+ staining cells as a progression of time.

	Biomarker	Annexin V^+ PI^-	Annexin V^+ PI^+	Annexin V^- PI^+
T_1				
	Annexin V^+ PI^-		r = 0.523, p = 0.121	r = 0.496, p = 0.145
	Annexin V^+ PI^+			r = 0.857, p < 0.005**
T_2				
	Annexin V^+ PI^-		r = 0.520, p =0.101	r = 0.698, p < 0.05*
	Annexin V^+ PI^+			r = 0.863, p < 0.005**
T_3				
	Annexin V^+ PI^-		r = 0.718, p < 0.05	r = 0.464, p = 0.129
	Annexin V^+ PI^+			r = 0.794, p < 0.005**

* significant, ** highly significant

There was a significant positive correlation between late apoptotic necrotic cells (Annexin V^+ PI^+) and early apoptotic cells (Annexin V^+ PI^-) after 24 hours (Table 4.2). However no correlation was seen between early apoptotic (Annexin V^- PI^+) and dead cells (Annexin V^- PI^+), except at T_2 - 2 hours after exposure. This corresponds

with the decrease seen in Annexin V^+ PI^- staining. A highly significant correlation existed at all three time points between apoptotic necrotic (Annexin V^+ PI^+) and dead spermatozoa (Annexin V^- PI^+).

4.5.2 Mitochondrial membrane potential

A typical flowcytometric representation of MitoTracker® Red CMXRos staining is shown in Figure 4.7. Depolarisation of the mitochondrial membrane potential is associated with a shift in fluorescence. The effect of 900 MHz GSM irradiation on mitochondrial membrane potential with SAR values of 2.0 and 5.7 W/kg respectively are summarised in Figure 4.8.

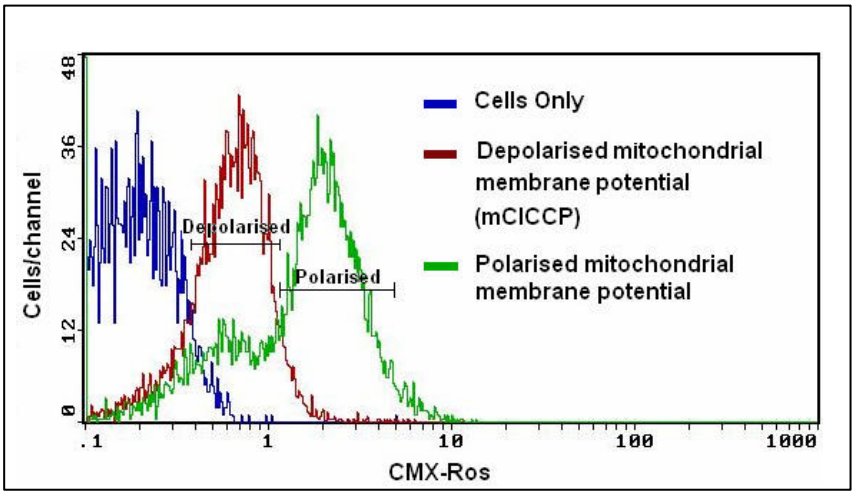

Figure 4.7 Cytofluorometric analysis of the depolarisation of the mitochondrial membrane potential showing a frequency histogram of processed spermatozoa (blue) stained with, MitoTracker® Red CMXRos before treatment (green) with the mitochondrial membrane potential abolisher mClCCP (red-brown).

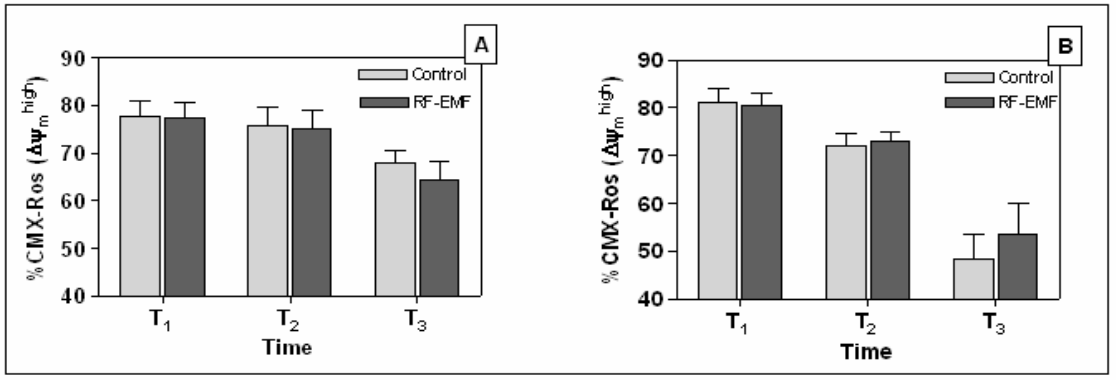

Figure 4.8 The percentage of MitoTracker® Red CMXRos (150 nM) staining in RF-EMF exposed sperm at a SAR of (A) 2.0 W/kg and (B) 5.7 W/kg compared to control samples determined as a function of time.

For both 2.0 W/kg and 5.7 W/kg the mitochondrial membrane potential decreased significantly ($p < 0.05$) over time (Figure 4.8 A and B). However this decrease was not significant ($p = 0.272$ and $p = 0.751$) between RF exposed compared with control sperm at SAR values 2.0 and 5.7 W/kg respectively (Figure 4.8 A and B). Furthermore there was no interaction observed between exposed (SAR 2.o ơ 5.7 W/kg) and control spermatozoa.

From Figure 4.8 it is evident that a dose (SAR 2.0 vs. 5.7 W/kg) effect was not present since at all the respective time points control and SAR values displayed similar polarised mitochondrial membrane percentages ($\Delta\psi_m^{high}$).

4.5.3 Detection of ROS

4.5.3.1 Detection of leukocyte contamination in processed spermatozoa

Cytofluorometric dot plots of gated white blood cells in a processed sperm sample (population C) are depicted in Figure 4.9. The population of cells gated by D note the total population of lymphocytes, E notes the population of monocytes and F notes the population of granulocytes. After addition of 2 x 10^6 white blood cells/ml to the processed sperm sample (Figure 4.9 B), the percentage of $CD45^+$ labelled white blood cells normalized to the gated population increased 50 fold for lymphocytes and monocytes and 96 fold for granulocytes.

When analysing the total number of white blood cell (population D + E + F) contamination in the processed sample of each of the twelve donors, the percentage $CD45^+$ labelled cells normalised to the total sperm population was less than 0.62%.

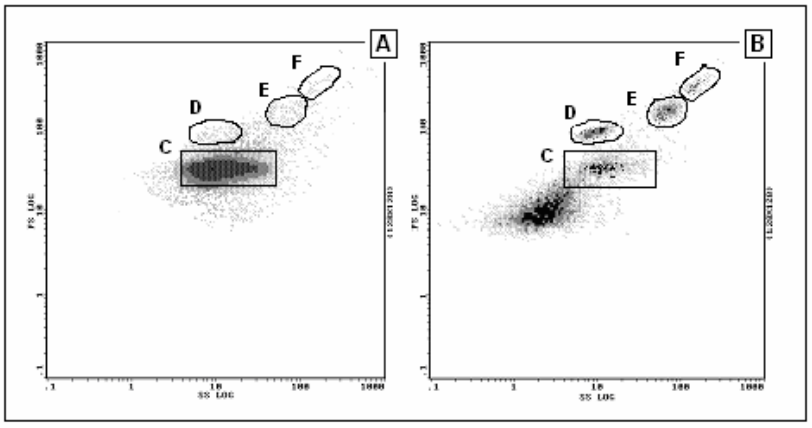

Figure 4.9 Cytofluorometric dot plot showing **(C)** gated sperm population, **(D)** lymphocytes, **(E)** monocytes and **(F)** granulocytes in **(A)** a processed sperm population and **(B)** a processed sperm population spiked with 2 x 10^6 white blood cells.

4.5.3.2 Detection of O_2^- with hydroethidine

A typical fluorescence histogram of HE staining is depicted in Figure 4.10. ROS generation, specifically O_2^- production, increased with the addition of white blood cells, shown in Figure 4.10 as an increase in HE fluorescence.

A statistical significant increase ($p < 0.05$) in O_2^- production was noted after 24 hours in both RF exposed and control sperm for both SAR values (Figure 4.11). However, there was no difference in O_2^- production in spermatozoa exposed at SAR 2.0 W/kg ($p = 0.641$) or 5.7 W/kg ($p = 0.402$) compared to control cells determined directly (T_1), 2 (T_2) and 24 hours (T_3) after exposure.

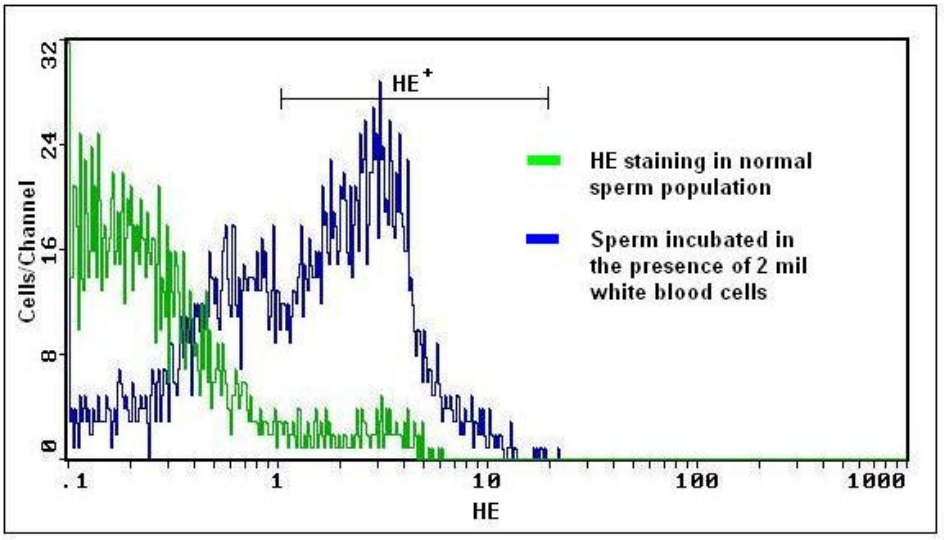

Figure 4.10 Cytofluorometric analysis of O_2^- production in human spermatozoa using hydroethidine (HE). Frequency histogram notes the increased production of ethidium (E^+) due to superoxide oxidation in processed spermatozoa incubated in the presence of 2 x 10^6/ml white blood cells (blue) compared to normal sperm (green).

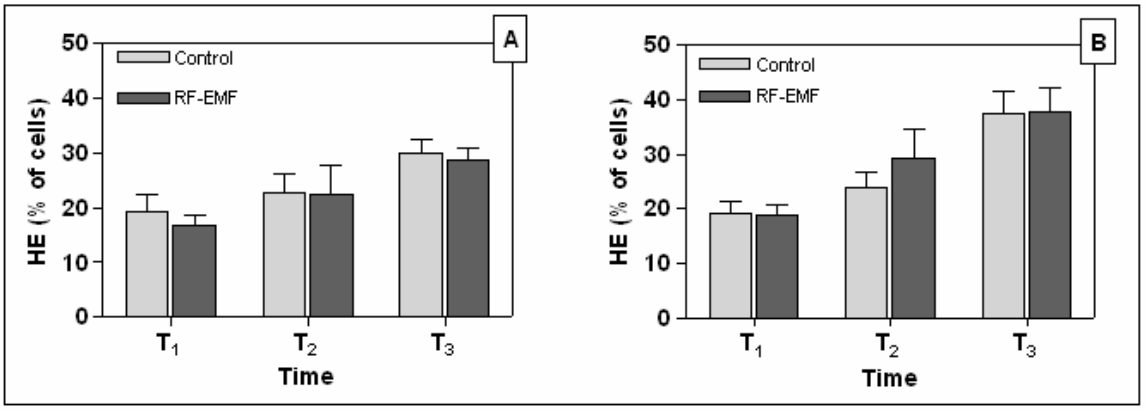

Figure 4.11 The percentage of hydroethidine staining in sperm exposed to RF-EMF at **(A)** SAR 2.0 W/kg and **(B)** SAR 5.7 W/kg compared to control samples determined directly after exposure (T_1), 2 hours after exposure (T_2) and 24 hours after exposure (T_3).

4.5.4 Caspase activation

4.5.4.1 Detection of active caspase-3

In all samples (n =12) analysed, active caspase-3 activity was less than 1% after density gradient purification (Figure 4.12). There was also no statistically significant difference in time series regression analysis (p = 0.320) assessing caspase-3 activity after RF-EMF exposure at 2.0 W/kg compared to controls (Figure 4.12). Due to the low yield in active caspase-3 activity, a biomarker FITC-VAD-FMK, directed against all active caspase activity, was used to assess the effect of RF fields at SAR's 2.0 and 5.7 W/kg on total caspase activation.

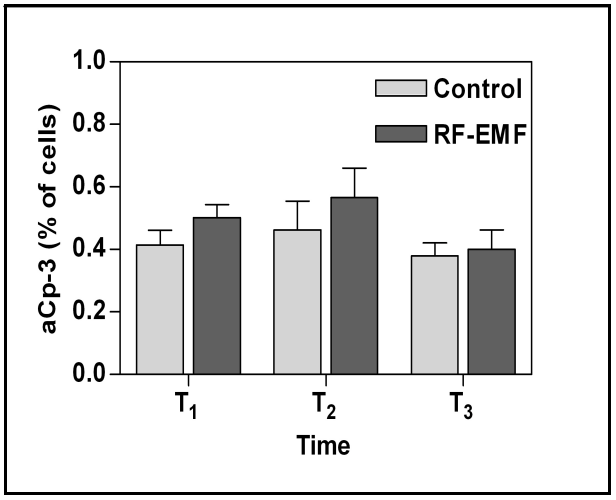

Figure 4.12 The percentage of cells staining positive for activated caspase-3 determined for control and RF-EMF (SAR 2.0 W/kg) exposed cells detected at T_1 (directly after exposure), T_2 (2 hours after exposure) and T_3 (24 hours after exposure).

4.5.4.2 FITC-VAD-FMK detection of activated caspases

Figure 4.13 shows the fluorescent intensity profile over time of a processed sperm sample labelled with FITC-VAD-FMK measured by flow cytometry. The cytofluorometric profiles show a major population presenting FITC-VAD^- (or unlabeled cells), while a smaller sub-population denotes the FITC-VAD^+ cell population. The unstained population (brown) shifted after FITC-VAD-FMK staining, the secondary population (smaller peak) shows the positive FITC-VAD-FMK staining.

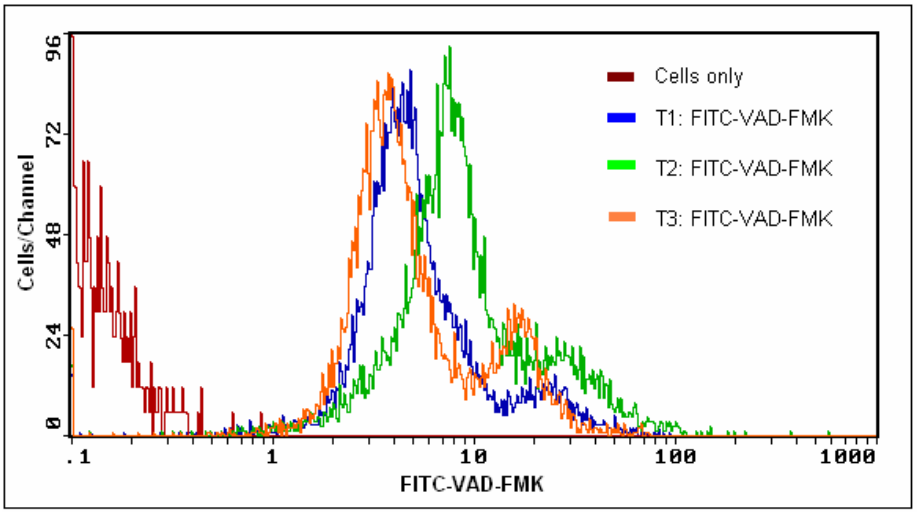

Figure 4.13 Cytofluorometric analysis of the frequency histogram of processed spermatozoa (red-brown) stained with, FITC-VAD-FMK directly after RF-EMF exposure (blue), 2 hours after exposure (green) and 24 hours after exposure (orange).

Statistical analysis of active caspase activation after 1 hour RF-EMF irradiation (SAR 2.0 W/kg) determined for both RF exposed and control cells at T_1 (directly after exposure), T_2 (2 hours after exposure) and T_3 (24 hours after exposure) note a decrease in activated caspases (Figure 4.14 A). However, the decrease in activated caspases in RF-exposed sperm did not differ statistically from control sperm ($p = 0.395$) as determined over the 24 hour period. Similarly, at a SAR level of 5.7 W/kg, there was no difference in caspase activation comparing RF exposed and control sperm ($p = 0.537$) (Figure 4.14 B).

However, caspase activation determined in RF exposed sperm (SAR 2.0 W/kg) decreased over time, whereas an increase over time was noted in RF exposed sperm at SAR 5.7 W/kg. Bearing in mind that only three donors were used at the higher SAR level, it is quite plausible that the differences in caspase activation noted for the different SAR values could be due to the relatively small sample size and large variances at the higher SAR value and not a true reflection of caspase activation for all twelve donors as noted at the SAR of 2.0 W/kg.

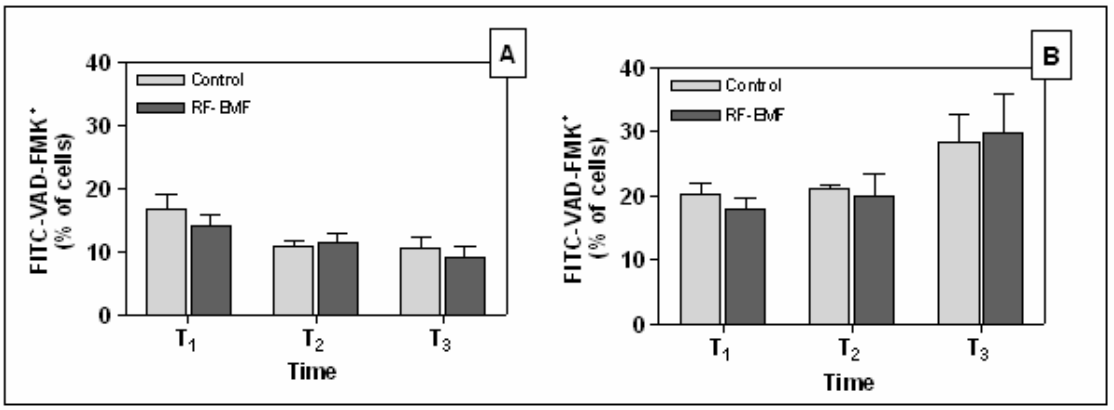

Figure 4.14 The percentage of cells staining positive for FITC-VAD-FMK determined for control and RF-EMF **(A)** SAR 2.0 W/kg and **(B)** SAR 5.7 W/kg exposed cells detected at T_1 (directly after exposure), T_2 (2 hours after exposure) and T_3 (24 hours after exposure).

4.5.5 DNA fragmentation

Figure 4.15 illustrates the fluorescent staining of fragmented DNA detected by TUNEL (FITC-dUTP) labelling. After DNase treatment, the unlabeled cell population (red-brown) shifted completely to the right demonstrating an increase of $TUNEL^+$ staining. A smaller fraction of fragmented DNA (green population) is noted in normal processed spermatozoa.

In Figure 4.16 A, the percentage TUNEL positive stained cells after 1 hour RF-EMF (SAR 2.0 W/kg) exposure is compared to control cells at different time points (directly after exposure - T_1, 2 hours after exposure - T_2 and 24 hours after exposure - T_3). The percentage $TUNEL^+$ control cells increased more rapidly over time compared to RF exposed cells, however, this difference was not statistically significant ($p = 0.298$). There was also no statistical difference ($p = 0.122$) in percentage $TUNEL^+$ sperm at the higher SAR level (5.7 W/kg) noted between RF exposed and control sperm shown in Figure 4.16 B.

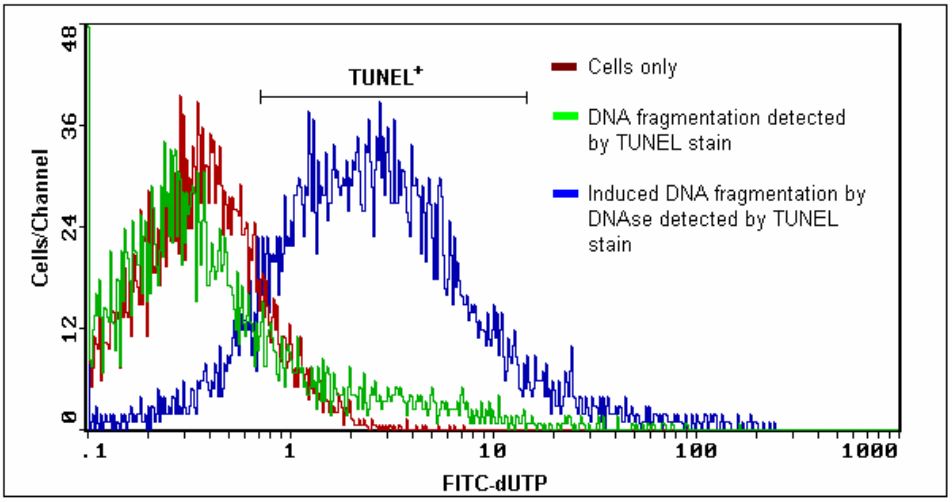

Figure 4.15 Cytofluorometric analysis of the frequency histogram of processed unstained spermatozoa (red-brown), before TUNEL staining (green) and induction of DNA damage using DNAse (blue).

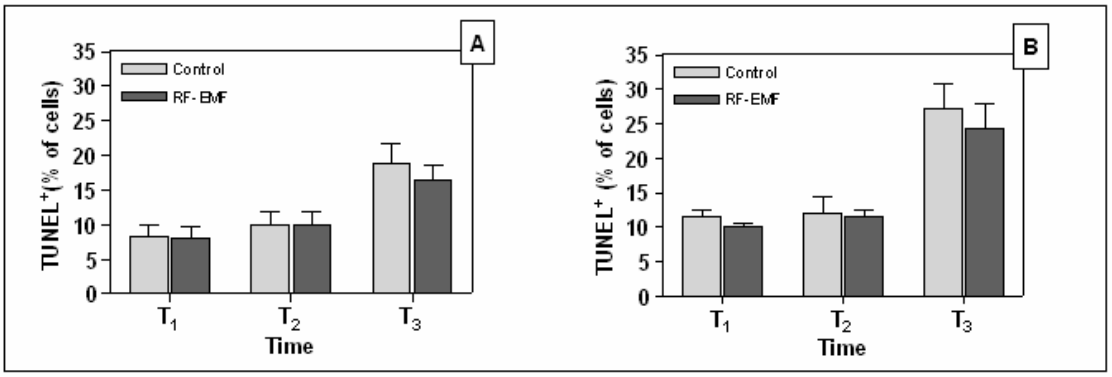

Figure 4.16 The percentage of cells staining positive for TUNEL determined for control and RF-EMF **(A)** SAR 2.0 W/kg and **(B)** SAR 5.7 W/kg exposed cells detected at T_1 (directly after exposure), T_2 (2 hours after exposure) and T_3 (24 hours after exposure).

4.5.6 Correlation between apoptotic markers and ROS

Table 4.3 A and B summarises the correlation coefficients (Pearson's) of apoptotic markers after RF-EMF exposure at SAR 2.0 W/kg and SAR 5.7 W/kg respectively.

Table 4.3 A Correlations between apoptotic biomarkers of RF-EMF (SAR – 2.0 W/kg) exposed spermatozoa evaluated directly (T_1), 2 hours (T_2) and 24 hours (T_3) after exposure.

T_1	$AV^+ PI^-$	CMXRos	F-VAD-FMK	TUNEL	HE
$AV^+ PI^-$		r = -0.618; p < 0.05*	r = -0.775; p < 0.005**	r = 0.111; p = 0.732	r = 0.466; p = 0.175
CMXRos			r = 0.470; p = 0.123	r = -0.157; p = 0.626	r = -0.015; p = 0.968
F-VAD-FMK				r = -0.347; p = 0.269	r = 0.018; p = 0.962
TUNEL					r = -0.100; p = 0.783
HE					
T_2					
$AV^+ PI^-$		r = -0.660; p < 0.05*	r = -0.327; p = 0.326	r = 0.065; p = 0.849	r = -0.399; p = 0.374
CMXRos			r = 0.319; p = 0.312	r = -0.261; p = 0.412	r = -0.374; p = 0.408
F-VAD-FMK				r = -0.107; p = 0.739	r = -0.442; p = 0.321
TUNEL					r = -0.489; p = 0.266
HE					
T_3					
$AV^+ PI^-$		r = -0.283; p = 0.428	r = -0.009; p = 0.984	r = -0.158; p = 0.684	r = -0.449; p = 0.225
CMXRos			r = -0.552; p = 0.199	r = -0.361; p = 0.339	r = -0.032; p = 0.935
F-VAD-FMK				r = -0.264; p = 0.492	r = -0.192; p = 0.680
TUNEL					r = -0.635; p = 0.066
HE					

AV = Annexin V; PI = Propidium iodide; F-VAD-FMK = FITC-VAD-FMK; TUNEL = Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; HE = hydroethidine.

Table 4.3 B Correlations between apoptotic biomarkers of RF-EMF (SAR – 5.7 W/kg) exposed spermatozoa evaluated directly (T_1), 2 hours (T_2) and 24 hours (T_3) after exposure.

T_1	$AV^+ PI^-$	CMXRos	F-VAD-FMK	TUNEL	HE
$AV^+ PI^-$		r = 0.652; p = 0.548	r = 0.784; p = 0.427	r = -0.317; p = 0.795	r = 0.955; p = 0.191
CMXRos			r = 0.982; p = 0.121	r = -0.926; p = 0.247	r = -0.398; p = 0.739
F-VAD-FMK				r = -0.838; p = 0.368	r = 0.565; p = 0.618
TUNEL					r = -0.837; p = 0.368
HE					
T_2					
$AV^+ PI^-$		r = -0.863; p = 0.337	r = 0.974; p = 0.144	r = -0.051; p = 0.967	r = 0.969; p = 0.157
CMXRos			r = -0.727; p = 0.481	r = -0.460; p = 0.696	r = -0.960; p = 0.180
F-VAD-FMK				r = -0.274; p = 0.823	r = 0.890; p = 0.301
TUNEL					r = -0.194; p = 0.876
HE					
T_3					
$AV^+ PI^-$		# correlations not possible because TUNEL and F-VAD data only collected for 3 donors, not 12			
CMXRos					
F-VAD-FMK					
TUNEL					
HE					

AV = Annexin V; PI = Propidium iodide; F-VAD-FMK = FITC-VAD-FMK; TUNEL = Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; HE = hydroethidine

4.6 DISCUSSION

In recent years, flow cytometry has become the method of choice for analysis of apoptosis in a variety of cell systems as it provides a rapid, accurate, and quantitative analysis of a high number of cells (Telford *et al*., 1994; Darzynkiewicz *et al*., 1997). This application is also currently widely used in assessing apoptosis in human spermatozoa (Ricci *et al*., 2000; D'Cruz *et al*., 2000; Oosterhuis *et al*., 2000; Sakkas *et al*., 2002; Marchetti *et al*., 2004a,b; Erenpreiss *et al*., 2004; Eley *et al*., 2005).

Although there are many conflicting reports on whether apoptosis is possible in fully differentiated spermatozoa, the classical approach of investigating the effect of a possible "toxic" agent entails the study of cell proliferation, apoptosis, and the possibility of a dose related effect. Using this approach we embarked on a comprehensive study of the effect of 900 MHz RF-EMF on early and late apoptotic markers in human spermatozoa.

PS externalisation is an early upstream indicator of apoptosis as its presence on the outer leaflet of the plasma membrane precedes other alterations such as DNA fragmentation, changes in nuclear and cytoplasmic organisation, and cellular fragmentation into apoptotic bodies (van Blerkom and Davis, 1998). In an attempt to induce apoptosis in spermatozoa using staurosporine, concentrations of 5 µM and 10 µM, although significantly increasing the amount of PS externalisation, also effectively killed the spermatozoa. A lower concentration (1 µM) did not cause a marked increase in cell death (PI^+), but even though the total amount of PS (Annexin V^+) increased significantly, this was not accompanied by a significant increase in apoptotic cells (Annexin V^+ PI^-). Bearing in mind that apoptosis can take place in a few hours or even minutes (Wyllie *et al*., 1980; Majno and Joris, 1995; Uchiyama, 1995; Vaux and Korsmeyer, 1999), and that it progresses rapidly, it is difficult to detect cells in early stages of apoptosis (Schwartzman and Cidlowski, 1993). This was also demonstrated in a study of Ramos and Wetzels (2001), were Annexin V binding of sperm following addition of H_2O_2 increased in conjunction with an increase in dead cells (PI^+), resulting in almost all cells being in apoptotic necrosis (Annexin V^+ PI^+) after a 60 min incubation with H_2O_2. Thus, very few cells were

detected in early apoptosis, which is in agreement with findings of the current study after staurosporine treatment. Eley *et al*. (2005) recently reported that staurosporine (1 mM) did significantly induce early apoptosis in human spermatozoa, although this was accompanied by a substantial increase in necrotic cells. These results should be interpreted with caution as other biomarkers for apoptosis have to confirm a positive result.

The percentage of early apoptotic live (Annexin V^+ PI^-) cells reported is closer in agreement with that found by Ricci *et al*. (2002) than what was previously published (Glander and Schaller, 1999; Oosterhuis *et al*., 2000; Barosso *et al*., 2000). This is probably due to the method employed to accurately select the sperm population. Similarly to Ricci and co-workers (2002), a leukocyte specific antibody (CD45) was used to accurately gate the sperm population for flow cytometry. At no time during experimentation (i.e. treatment with staurosporine or exposure to RF-EMF), was a significant increase in early apoptotic cells seen. There were also no statistical difference between RF-EMF exposed and control cells comparing number of dead cells (PI^+) and total PS externalisation (Annexin V^+) assessed directly, 2 and 24 hours after exposure. Hence it can be concluded that an hour exposure of human spermatozoa *in vitro* to 900 MHz GSM radiation does not adversely effect PS externalisation. This finding is consistent with results from a recent paper by Hook *et al*. (2004) that also found no evidence of an effect on Molt-4 cells after *in vitro* exposure to RF-EMF.

If PS externalisation in human spermatozoa were an exclusive marker for early apoptosis, a linear response of Annexin V^+ PI^- staining with time could be expected. This was not seen in the current results, with PS externalisation decreasing for both exposed and control cells 2 hours post RF-EMF exposure while increasing again 24 hours after exposure irrespective of the SAR level used. It is thus possible that PS externalisation accompanied by Annexin V staining changes with alterations in the plasma membrane fluidity due to capacitation and is not an exclusive marker of apoptosis. Several studies have reported conflicting results with Annexin V staining (Glander and Schaller, 1999; Osterhuis *et al*., 2000; Marchetti *et al*., 2004). Interestingly, Ricci *et al*. (2002) reported that mature sperm are indeed able to

undergo apoptosis, leading to the exposure of PS on the outside of the plasma membrane. They hypothesised that Annexin V^- PI^+ cells are unable to bind Annexin V due to the high degree of membrane disorganisation in this group of cells, and that these cells are actually in the latter stage of apoptosis. In accordance with Ricci and co-workers (2002), two populations staining PI^+, one fraction staining Annexin V^+, while the other Annexin V^- were observed in the present study. The natural progression of cells in apoptosis would entail an increase of cells in early apoptosis (Annexin V^+ PI^-) followed by an accumulation of cells in apoptotic necrosis (Annexin V^+ PI^+) with time, whereas cells staining Annexin V^- PI^+ are generally considered dead. If the cells in this latter group were actually in late apoptosis, this would mean that Annexin V^+ PI^- sperm should inversely correlate with Annexin V^- PI^+ sperm over time. The current data does not support this hypothesis. In fact, at no time point was a negative correlation between early apoptotic and dead cells noted.

Taking all this evidence into consideration, it would seem that Annexin V staining on its own as an indicator of apoptosis, at least for human spermatozoa, is not an unambiguous gauge of apoptosis. Recent studies concluded that PS externalisation in human spermatozoa does not correlate with apoptosis but is rather associated with plasma membrane changes after cryostorage (Glander and Schaller, 1999), as well as capacitation (Gadella and Harrison, 2002; de Vries *et al*., 2003; Martin *et al*., 2005).

Analyses of the mitochondrial function by means of determining changes in the inner mitochondrial membrane potential can serve as a sensitive indicator for the energetic state of the mitochondria and the cell. It can also be used in the assessment of mitochondrial respiratory chain activity, electron transport systems, and the activation of the mitochondrial permeability transition (Ly *et al*., 2003). In human spermatozoa reduced mitochondrial membrane potential correlates with diminished motility and fertility (Donnely *et al*., 2000; Marchetti *et al*., 2002; Piasecka and Kawiak, 2003; Wang *et al*., 2003). Recently, Marchetti *et al*. (2004a) compared different fluorochromes for the detection of $\Delta\psi m$ in human spermatozoa, the authors noted that Mitotracker® RedCMX-Ros provides a simple, sensitive method for the assessment of $\Delta\psi m$.

Although, the mitochondrial membrane potential decreased over time, 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 5.7 W/kg correlates well with that reported by Capri and co-workers (2004) who also found that *in vitro* exposure of human lymphocytes to 900 MHz had no effect on the mitochondrial membrane potential assessed at different time points. It is thus unlikely that RF-EMF could have an effect on the electron transport chain and the proton pump machinery in mitochondria.

Disruption of the mitochondrial membrane potential could result in the release of cytochrome c into the cytosol where it binds apoptotic protease activating factor-1 (Apaf-1) resulting in the activation of a family of cysteine proteases, called caspases. Caspases exist in cells as inactive zymogens and become proteoliticly activated by apoptotic signals from the cell (Sinha Hikim *et al*., 2003). Caspase activation entails the activation of initiator caspase 9 followed by executioner caspases 3, 6, and 7 activation. Activation of caspase-3 is seen as the point of no return in the execution of apoptosis. However, we failed to detect significant levels of activated caspase-3 activity in the highly motile fraction of mature spermatozoa. This result is in agreement with what has been reported by others (Weil *et al*., 1998; de Vries *et al*., 2003). On the other hand, Almeida *et al*. (2005) recently reported caspase-3 activity in human spermatozoa after swim-up preparation. This finding could be explained because swim–up prepared sperm contain more sperm with DNA damage compared with sperm separated by density-gradient (Host *et al*., 2000). Caspases are not completely removed during undisturbed spermatogenises (Paasch *et al*., 2003) but are present in cytoplasmic droplets in spermatids and immature spermatozoa (Weil *et al*., 1998; Blanco-Rodríguez and Martínez-García, 1999) as well as in the cytoplasm localised in the midpiece region (Weng *et al*., 2002; Paasch *et al*., 2003; Marchetti *et al*., 2004b). It is thus possible that there would be more sperm presenting caspases after swim-up preparation compared to density gradient separation. Furthermore, Marchetti *et al*. (2004b) also detected active caspase-3 by western blot but only in semen samples with the highest percentage of FITC-VAD-FMK^{+} cells. In addition, Taylor *et al*. (2004) observed higher caspase catalytic activity in low motility

fractions compared to high motility sperm fractions. It is thus not surprising that such low levels of active caspase-3 were detected in the highly motile density gradient purified spermatozoa used in the present study.

Noting that Weng *et al.* (2002) postulated a sperm-specific apoptotic pathway that may not include caspase-3 activation, a cell-permeable fluorescent derivative of the inhibitor peptide VAD-FMK able to detect the overall caspase activation status in human spermatozoa was used in experiments. First described by Paasch *et al.* (2003), Marchetti and co-workers (2004b) confirmed that FITC-VAD-FMK could be utilized for the flow cytometric analysis of activated caspases in human sperm. Current results are comparable with that reported by Marchetti *et al.* (2004b). When considering the total caspase activation (FITC-VAD-FMK^{+}) after RF-EMF exposure, no difference between RF exposed and control cells at any time point irrespective of the SAR level was observed.

Leszczynski *et al.* (2002) reported that RF-EMF inhibited apoptosis through the caspase-3 dependant apoptotic pathway. It is thus not surprising that this effect was not observed in the highly motile fraction of density gradient separated spermatozoa. Firstly as negligible amounts of caspase-3 activity were observed and furthermore it has not conclusively been demonstrated that caspases are involved in the execution of apoptosis in human sperm.

One of the final phases of apoptosis is the degradation of DNA through the activation of an endonuclease that cleaves DNA between regularly spaced nucleosomal units (Boatright *et al.*, 2003; Said *et al.*, 2004). There are many conflicting reports regarding the ability of RF-EMF to induce DNA damage. Whereas genotoxic effects were observed in certain cell types (Maes *et al.*, 1996; Tice *et al.*, 2002; Mashevich *et al.*, 2003), in other cell types RF-EMF did not appear to induce DNA damage (Maes *et al.*, 1997; Malyapa *et al.*, 1997; Li *et al.*, 2001, Zeni *et al.*, 2003). If on the other hand RF-EMF does exert a genotoxic effect on human cells, post-meiotic male germ cells would be particularly sensitive to DNA damage, as they have lost their capacity for DNA repair. During spermatogenesis male germ cells are supported by Sertoli cells. However, once these cells mature they lose most of their cytoplasm containing antioxidants that protect them from oxidative stress making them highly

susceptible to any genotoxic agent (Aitken, 1999). Aitken *et al*. (2005) reported that a 7-day exposure of male mice to RF-EMF significantly induced DNA damage in both the nuclear and mitochondrial genomes of spermatozoa recovered from the cauda epididymis.

Using the TUNEL assay, we examined the induction of DNA fragmentation in spermatozoa exposed to SAR levels of 2.0 and 5.7 W/kg. The percentage of DNA fragmentation in density gradient purified fractions reported here corresponds with previous findings where flow cytometry and the TUNEL assay were used to assess DNA damage (Ramos and Wetzels, 2001; Marchetti *et al*., 2004b). Although DNA fragmentation increased over time, there were no significant differences detected between RF-EMF exposed and control sperm.

Aitken and co-workers (2005) assessed DNA damage after an *in vivo* exposure period of 7 days, whereas in the present study, DNA damage was assessed directly after a one-hour exposure period. It is thus possible that DNA damage is cumulative as was suggested by Fejes *et al*. (2005) and not observable after an exposure time of one hour. The current approach on the other hand was to expose sperm *in vitro* to a worst-case scenario to see of RF-EMF could elicit a genotoxic response after an hour RF-EMF exposure to SAR levels of 2.0 and 5.7 W/kg. Based on results shown here, RF exposure at either of the SAR levels did not induce DNA fragmentation. Similar findings reporting a lack of DNA damage after RF-EMF exposure of Molt-4-T-lymphoblastoid cells (Hook *et al*., 2004) as well human lymphocytes (Meas *et al*., 1997; Vijayalaxmi *et al*., 2000) have been reported.

Reactive oxygen species generation by human spermatozoa as a result of aerobic metabolism is not just of significance because of peroxidative damage caused to the sperm plasma membrane due to the relative lack of antioxidant protection by cytoplasmic antioxidant enzymes (Aitken and Baker, 2002), but also due to the structural and chemical damage they cause to proteins and target DNA (Riley and Behrman, 1991; Aitken *et al*., 1998; Ramos and Wetzels, 2001; Henkel *et al*., 2004). The presence of leukocytes in the purified highly motile fraction of spermatozoa could contribute significantly to increased ROS generation. Therefore, a leukocyte specific antibody (CD45) was used to gate the sperm population correctly to enable

the quantification of intrinsic ROS generation only. The total percentage $CD45^+$ cells were less than 0.62% of the total sperm population after density separation and, according to the WHO (1999), not pathological.

To determine intrinsic ROS production within the spermatozoa, a redox probe hydroethidine, which is specifically oxidized by O_2^- to form the membrane impermeant polynucleotide stain ethidium (Gallop *et al*., 1984), was used. Unlike current ROS probes (luminol and lucigenen) that decompose with the omission of light (Aitken *et al*., 2003), hydroethidine intercalates with the DNA (Rothe and Valet, 1990), which makes it ideal for flow cytometric detection. Recently, Henkel *et al*. (2004) and Marchetti *et al*. (2002) used hydroethidine to evaluate ROS production in human spermatozoa. RF-EMF had no effect on ROS generation in human spermatozoa when comparing RF-EMF exposed sperm with control sperm, directly after RF-EMF exposure as well as 2 and 24 hours after exposure for both SAR 2.0 and 5.7 W/kg.

When assessing the correlation between apoptotic indicators, a high mitochondrial membrane potential inversely, though not significantly, correlated with ROS generation and DNA fragmentation at all time points after initial RF exposure (2.0 W/kg). This result was consistent with previous reports regarding the correlation of these apoptotic markers in human spermatozoa (Marchetti *et al*., 2002). A negative correlation also existed between ROS generation and DNA fragmentation as was expected (Aitken *et al*., 1998). In addition, the correlation between DNA damage and PS externalisation, as well as caspase activation, was investigated. Results indicate that these markers were not significantly correlated. Considering that Marchetti *et al*. (2002) found that sperm prepared by density gradient removed most of the damaged cells resulting in the lack of correlation between ROS, DNA fragmentation, and loss of viability, it confirms what was observed in the current study. However, the objective of this study was to establish if RF-EMF could induce apoptosis in human spermatozoa *per se*, irrespective of the current dispute in academia questioning the ability of fully differentiated spermatozoa to initiate apoptosis.

Sakkas *et al*. (2003, 2004) and others (Gandini *et al*., 2002; Tesarik *et al*., 2004) are of the opinion that the presence of apoptotic markers in human spermatozoa are due to an abortive apoptotic program during spermatogenesis and the failure of elimination of these cells before ejaculation. These authors further hypothesise that the presence of DNA damage could be the result of incorrect nuclear remodelling due to problems arising directly from protamine deposition during spermatogenesis. Based on the results presented here, we are in agreement with these authors as apoptotic markers in human spermatozoa did not correlate with apoptosis.

In conclusion, the study encompassed a wide variety of both early and late apoptotic markers as well as considering ROS generation as a function of time. This study not only provides evidence that an hour RF-EMF exposure of human spermatozoa *in vitro* does not significantly affect any of the apoptotic indicators investigated here, but also sheds light on the apoptotic behaviour of fully differentiated sperm over time.

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CHAPTER 5

ACTIVATION OF HEAT SHOCK PROTEINS IN HUMAN SPERMATOZOA

5.1 INTRODUCTION

The activation of a cellular stress response is designed to prevent and to repair damage incurred by exposure to a stressor. This stress response is immediately detectable in changes in the phosphorylation level of various proteins. Stress proteins have been widely studied as biomarkers of effect (Kreps *et al*., 1997) and under this category the highly conserved heat-shock proteins (Hsps) have received much attention. Hsps are expressed or phosphorylated due to a variety of cellular injuries and as such their activation can be used as an indicative response of cellular stress.

Several recent studies have indicated that RF-EMF increases expression or phosphorylation of certain stress response proteins (Daniells *et al*., 1998; Leszczynski *et al*., 2002, 2004; Czyz *et al*., 2004; Lee *et al*., 2005; Nylund and Leszczynski, 2006). Therefore, by examining changes in protein synthesis and phosphorylation levels in reply to a cellular stress response, it is possible to determine whether cells can respond to the very low energy emitted by mobile phones.

Various heat shock proteins have been identified in mature human spermatozoa (discussed in Chapter 2). Whether these Hsps are up-regulateable is uncertain, as mature sperm cells lack the operative mechanism for *de novo* protein synthesis (Díez-Sánchez *et al*., 2003; Moustafa *et al*., 2004; Grunewald *et al*., 2005; Lalancette *et al*., 2006). However, the presence of mRNAs in ejaculate human spermatozoa are well established (Kramer and Krawetz, 1997; Ostermeier *et al*., 2002), and it was initially thought that these mRNAs are remnants of untranslated stores during spermatogenesis (Miller, 2000; Grunewald *et al*., 2005). Grunewald *et al*. (2005) concluded that although no novel RNA transcription in human spermatozoa is possible, mRNAs present in spermatozoa are still functional. This finding was supported by Ostermeier *et al*. (2004), who demonstrated that paternal mRNAs were

transferred to the oocyte at fertilization, as transcripts coding for specific proteins were detected in early embryos after fertilization, while they were not found in the oocyte. Grunewald *et al.* (2005) also noted that RNA from spermatozoa could be reverse transcribed which is in agreement with data of Giordano *et al.* (2000). Furthermore, transcripts for Hsp70 and 90 have recently been found in ejaculate human spermatozoa (Miller, 2000; Dadoune *et al.*, 2005).

Hsp70 is up-regulateable as a result of stress (Hahn and Li, 1990; Senisterra *et al.*, 1997) whereas Hsp90 is activated by phosphorylation (serine/threonine/tyrosine) (Lees-Miller and Anderson, 1989a, b; Harris *et al.*, 2000; Brouet *et al.*, 2001). Hsp27 exists as an oligomeric structure and is also activated by phosphorylation after serine residues (Ser82, Ser78, and Ser15) (Rogalla *et al.*, 1999) through the p38 mitogen-activated protein kinase (MAPK) stress pathway (Landry *et al.*, 1992; Rouse *et al.*, 1994; Huot *et al.*, 1995). In human spermatozoa, MAPKs, also known as extracellular signal regulated kinases (ERKs) involved in the phosphorylation of specific proteins during capacitation, have been identified (Luconi *et al.*, 1998). In addition, several papers have identified sequentially activated kinase cascades in human spermatozoa that phosphorylate proteins after tyrosine, serine and, threonine residues (Naz, 1999; Baldi, *et al.*, 2002; Visconti *et al.*, 2002; Lalancette *et al.*, 2006; Martinez-Heredia *et al.*, 2006). In particular, Ficarro *et al.* (2003) noted that Hsp90 becomes tyrosine phosphorylated during human sperm capacitation.

In addition, Hsp70 plays an important role in fertilisation and early embryo development (Matwee *et al.*, 2001) since the presence of antibodies to Hsp70 significantly reduced tight binding of spermatozoa to the ZP and decreased blastocyte development (Neuer *et al.*, 1998, 2000). Hsp70 is thought to be involved in protein folding and translocation of proteins across the membrane during acrosomal excocytosis (Bohring and Krause, 2003) and prevents apoptosis during early development (Bloom *et al.*, 1998; Muscarella *et al.*, 1998). Consequently, Hsp70 transcripts, amongst others, could be the male gametes contribution to early embryogenesis. If RF-EMF could cause the premature translation of these transcripts, this could lead to disruption of fertilisation and early embryonic development.

Hsp27 is a known regulator of F-actin polymerisation (Huot *et al*., 1996; Rosseau *et al*., 1997). Furthermore, actin has been identified in various areas in human sperm, most notably the acrosome, post-acrosomal area, neck, and principal tail-piece (Fouquet and Kann, 1992). It is thought that actin polymerisation plays a role in acrosomal exocytosis as the sperm plasma membrane undergoes complex modifications during sperm-ZP interaction including changes in plasma membrane fluidity (Liu *et al*., 1999, Brener *et al*., 2003). Liu *et al*. (2002) confirmed this by demonstrating that an anti-actin mAb prevented actin polymerisation resulting in the inhibition of the AR of ZP bound spermatozoa. Thus, Hsp27 phosphorylation as a result of RF-EMF could affect F-actin polymerisation, which in turn could potentially have an effect on the human sperm AR. This would justify studying the status of F-actin polymerisation in human spermatozoa after RF-EMF.

Spermatozoa posses the ability not only to translate mRNA by means of transverse scriptase but could also activate proteins through kinase activity. Whether Hsps present in spermatozoa would recognise RF-EMF as a stressor and launch a stress response remains to be demonstrated. Therefore, this section focuses on the detection of cellular stress in RF-EMF exposed human spermatozoa by investigating the expression and/or phosphorylation of Hsps 70 and 27, in particular, as well as F-actin polymerisation

5.2 EXPERIMENTAL PROTOCOL

Ejaculated density gradient purified human spermatozoa were exposed for an hour to 900 MHz GSM (SAR 2.0 W/kg) irradiation inside a specially constructed waveguide (characterised in Chapter 3), while controls were kept at 37ºC in a humidified CO_2 incubator. Depending on the experiment, spermatozoa were heat shocked for up to an hour at 43ºC in a water bath to provide a positive control for Hsp expression/phosphorylation or F-actin activation.

The ability of RF-RMF and/or heat shock to elicit a cellular stress response in human spermatozoa was examined by determining phosphorylation and expression status of different heat shock proteins. A diagram outlining the experimental approach is shown in Figure 5.1. Hsp70 and 27 expression and/or phosphorylation were assessed

by flow cytometric analysis while slides were prepared for visualisation (immunofluorescence) and F-actin activation on the same day at three different time points or directly after exposure over a period of 4 weeks for all twelve donors. As an additional control, cellular stress due to RF-EMF and heat shock was determined in a breast cancer cell line (MCF-7). F-actin accumulation in MCF-7 cells was visually inspected after an hour exposure to either RF-EMF or 43ºC.

Western Blot analysis (WB) analysis of Hsps 70 and 27 directly after RF-EMF or heat shock were assessed at a later date using two donors (category A and B morphology as outlined in Annexure C) only. In addition, the expression of Hsps 110, 90, 75, 60, and 40 in the same two donors were analysed to determine if other Hsps could be affected by RF-EMF. The human epithelial cell line AE.hy926 heat shocked for 1 hour at 43ºC provided a positive control for the WB analysis of Hsps 70 and 27.

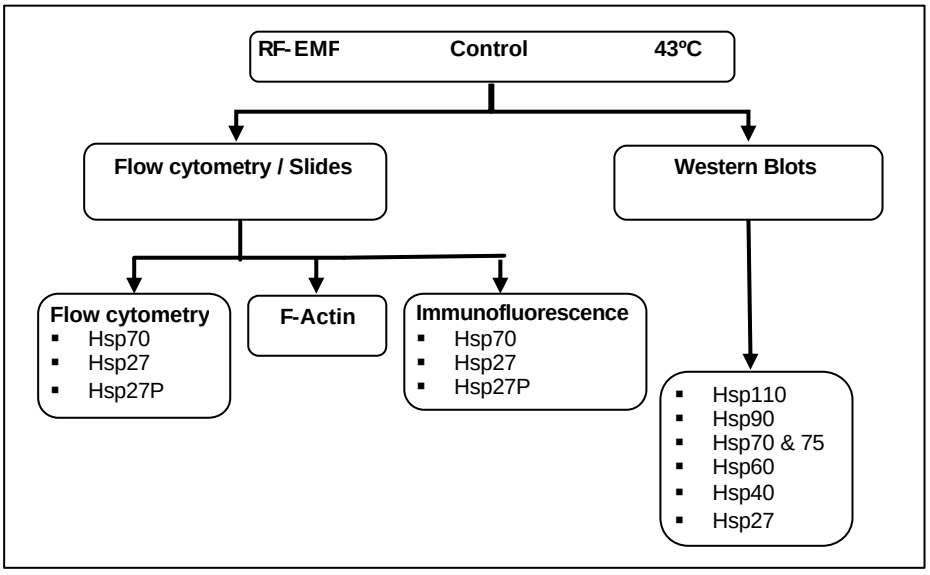

Figure 5.1 RF-EMF exposure protocol. Assessment of cellular stress in human spermatozoa post RF-EMF exposure and heat shock at 43ºC.

5.3 DETERMINATION OF HEAT SHOCK PROTEIN EXPRESSION AND PHOSPHORYLATION

5.3.1 Flow cytometric analysis of Hsp70 and Hsp27 expression and/or phosphorylation

Fixation, permeabilisation, Hsp detection, and staining were adapted from the method described by Bachelet *et al.* (1998). Fixation was performed with a freshly prepared paraformaldehyde solution: 0.37 g paraformaldehyde (Sigma Chemical Co.) solubilised in 10 ml PBS by heating the mixture at 70°C. After cooling the solution to room temperature, the pH was adjusted to 7.4. Cell extracts equivalent to 1-2 x 10^6 spermatozoa per sample were re-suspended in 100 µl paraformaldehyde solution, incubated for 10 min on ice and washed twice by centrifugation (300 g for 5 min) in 1 ml PBS. Cell membranes were permeabilised with 0.5% Triton X-100 in PBS for 10 min at room temperature.

Spermatozoa were then washed (300 g, 5 min) with PBA (PBS supplemented with 0.5% BSA) to block non-specific binding. Primary monoclonal antibody staining was performed with three different heat shock proteins, Hsp70-directed against its inducible form (BD Biosciences), Hsp27 (Calbiochem, CA, USA) and Hsp27P-phosphorylated Hsp27 at Ser 82 (Calbiochem). Spermatozoa were incubated for 30 min at room temperature with anti-Hsp70, anti-Hsp27 and anti-Hsp27P diluted to 1:100 in PBA, after which the cells were washed in PBA (300 g, 5 min). Secondary antibody staining was performed by incubating cells for 30 min at room temperature with FITC-conjugated rat anti-mouse IgG_1 (for Hsp70 and 27) and IgG_{2a} (for Hsp27P), (BD Biosciences) diluted to 1:100 in PBA. Cells were then washed (300 g for 5 min) in PBA, re-suspended in 1 ml PBS/BSA, and kept on ice directly after labelling until flow cytometric analysis. Cells kept on ice remained stable, as no difference in fluorescence intensity was noted in cells kept on ice for up to 4 hours after initial labelling. To determine non-specific binding of the secondary antibody (FITC-conjugated rat anti-mouse IgG_1 or IgG_{2a}), spermatozoa were prepared as described above but the primary antibody labelling was omitted.

Flow cytometric analysis was performed on a Coulter Epics® XL.MCL flow cytometer (XL.MCL) equipped with an air cooled argon laser and System II software

(Beckman Coulter). A total of 10 000 events were acquired for each endpoint and all tests were run in duplicate. The results are expressed as the mean cell number. Aliquots were also taken directly after exposure to visually assess Hsp distribution within the sperm cell (for visual assessment primary and secondary antibodies were diluted to 1:50 in PBA). Spermatozoa were visualised under 100x oil magnification and images were acquired using a Zeiss fluorescence microscope (Axioskop40, Axiocam camera and Axiovision AC software V4.5, Carl Zeiss (Pty)Ltd.).

5.3.2 Western Blot analysis of Hsps 110, 90, 75, 70, 60, 40, and 27 expression

5.3.2.1 Protein extraction

Following 900 MHz GSM exposure, petri dishes containing control samples and RF-EMF exposed samples (20 x 10^6 cells) were placed directly onto ice and the cells were transferred to sterile test tubes (Greiner Bio-One, Germany) by gently washing the petri dishes with 3 ml of ice cold PBS containing 1 mM sodium orthovanadate (Sigma Chemical Co.) and 1 % protease inhibitors (Sigma Chemical Co.). Cells were then centrifuged for 10 min at 300 g, the supernatant removed, and the pellet re-suspended in 1 ml ice-cold PBS mixture (containing 1 mM sodium orthovanadate and 1% protease inhibitors). Sperm-suspensions were then transferred to eppendorph tubes (Sigma Chemical Co.) and centrifuged (230 g) at 4ºC for 10 min after which the supernatant was removed and the pellet stored at -80ºC until further analysis.

After thawing, pellets were lysed with 50 µl lysis buffer (containing 2% SDS - sodium dodecyl sulphate; 1% 0.1 M sodium orthovanadate (Sigma Chemical Co.), and 1% Protease inhibitor cocktail (Sigma Chemical Co.)) for 60 min on ice. Cells were then boiled on a heater block (100°C) for 5 minutes. Cell extraction was accomplished by aspiration through a needle (22G, at least 10 times). Non-soluble debris was removed by centrifugation (10 min at 300 g). Protein content was determined using the Bradford method (Bio-Rad's DC protein Assay) (Bradford 1976). In addition, results were confirmed using the LOWRY method (results not shown) with a cell concentration of 20 x 10^6 cells/ml producing approximately 50 µg of proteins.

5.3.2.2 Electrophoresis

Sperm extracts containing 50 µg of proteins were loaded onto Invitrogen Nupage 10% Bis-Tris gel 1.0 mm x 10 wells. Running conditions were 200 V for 55 minutes using MOPS/SDS running buffer (1.0 M MOPS, 1.0 M TrisBase, 69.3 mM SDS, 20.5 mM EDTA, free acid).

5.3.2.3 Blotting

Directly after electrophoresis, blots of the 1 D-gels were performed. Proteins were blotted on PVDF membranes (Bio-Rad, UK) using a 10% Towbin buffer as a transfer buffer (25 mM Tris, 192 mM glycine, 10% MeOH) with Bio-Rad's mini wet-blot equipment. Running conditions were 80 V for 60 min. Membranes were blocked at room temperature in TBS (Tris- buffered Saline, 20mM TrisBase, 137mM NaCl, adjusted with concentrated HCl to pH 7.6) containing 3% BSA for 1 hour. Hsps were detected in the membranes using appropriate antibodies (Stress Gen, Canada). All antibodies used were rabbit polyclonal and Hsp27 and Hsp70 were diluted 1/3000 while all other antibodies were diluted 1/2000 in 3% BSA. Membranes with antibodies were left overnight in 4ºC. The following day membranes were washed 3 times with TBS containing 0.05% Tween for 5-10 minutes each wash. Directly afterwards the secondary antibody was added, Goat-anti-rabbit (DAKO), diluted 1/30 000 in 3% BSA and membranes were left for 2 hours. Membranes were then washed 3 times with TBS (0.05% Tween), where after Hsps were detected by enhanced chemiluminescence (Millipore Immobilon western Chemilumenescent HRP- substrate). Analysis of Hsps, were performed using Non-linear Dynamics Phoretics 1D version 2003.02 software.

5.3.2.4 Western Blot analysis of Hsp70 and Hsp27 in EA.hy926 cells

EA.hy926 cells (kindly donated by Dr. Cora-Jean S. Edgell, North Carolina University, NC, USA) were grown in Dulbecco's MEM, supplemented with antibiotics, 10% foetal bovine serum, L-glutamine and HAT-supplement (all supplements were from GIBCO). For experiments, cells were trypsinated (GIBCO, 0.25% trypsin 1mM EDTA) and reseeded at a density of 400 000 cells per petri dish (NUNC). Cells were left to adhere overnight before confluent monolayers of EA.hy926 were heat shocked for 1 hour at 43°C in a CO_2 incubator. Control samples

were kept at 37°C for the duration of the exposure. Protein extraction, electrophoresis and blotting were performed as described for spermatozoa.

5.4 PHYSIOLOGICAL EFFECTS OF HSP ACTIVATION

5.4.1 Detection of stress fibres in human spermatozoa

Directly after RF-EMF exposure, cells (1 x 10^6/ml) were washed twice with PBS (300 g, 5 min) and fixed in 3.7% paraformaldehyde (Sigma Chemical Co.). These cells were then washed a further three times by centrifugation (300 g, 5 min) in PBS before being permeabilised with 0.1% Triton X-100 (Sigma Chemical Co.). Droplets were then made on a slide and the cells allowed to air dry. The dry slides were rinsed three times with PBS and incubated for 30 min at room temperature in a 1% BSA-PBS solution. Cells were then overlaid with 5 µl Alexa Fluor 488 Phalloidin (BD Biosciences) in 200 µl PBS and incubated for 30 min at room temperature. The stained slides were rinsed three more times with PBS and once with distilled water, before mounting the slides with mounting medium (Sigma Chemical Co.). Once mounted, slides were analysed using a Zeiss fluorescence microscope (Axioskop40, Carl Zeiss (Pty) Ltd.) and images were acquired with an AxioCam digital camera using Axiovision AC V4.5 software.

5.4.2 Detection of stress fibres in MCF-7 cells

To confirm the methodology of stress fibre detection, a human breast cancer cell line MCF-7, exposed to RF-EMF as well as heat shock (43ºC) for 1 hour, was used. MCF-7 cells were grown to confluency in tissue culture flasks and were trypsinated (Trypsin/Versene, Highveld Biological, SA) the day before exposure. Cells were counted and seeded at a concentration of 350 x 10^3 cells/coverslip which was placed at the bottom of a glass petri dish overlaid with 3 ml complete medium (Dulbecco's-MEM, Gibco, Scientific Group, USA) supplemented with 10% foetal bovine serum (Highveld Biological) and Penstrep-Fungizone (Highveld Biological) and left to adhere overnight. The following day, the medium was replaced with 3 ml complete medium (kept at 37ºC) and cells were exposed for a period of 1 hour to 900 MHz GSM radiation or heat shocked at 43ºC in a warm oven, while control cells were maintained at 37ºC in a humidified CO_2 incubator. F-actin accumulation was

detected with Alexa Fluor-Phalloidin using the same method described for visual assessment of stress fibre stabilisation in sperm, except that staining was performed on cells adherent to cover-slips.

5.5 STATISTICAL ANALYSIS

Statistical analysis was done with Stata Statistical Software Release 8.0 (Stata Corp., 2003, USA) using a within subject design considering two treatments, control and RF-EMF (SAR 2.0 W/kg), at three time points for a total of 12 donors. Flow cytometric results were analysed using time series regression under the random effect option. Western Blot data (for two donors only) was analyzed using a Wilcoxon matched pairs signed ranks test due to the relative small sample size (n = 6 per donor). All data is presented as mean values ± SD.

5.6 RESULTS

5.6.1 Flow cytometric analysis and visualisation of Hsp70 and 27 expression and phosphorylation

Baseline expression of Hsp27 in human spermatozoa as determined by flow cytometry and the phosphorylation thereof detected by the monoclonal antibody anti-Hsp27P are noted in Figure 5.2 A, while indirect immunofluorescence detection of Hsp27P in sperm is depicted in Figure 5.2 B. Hsp70 expression determined by flow cytometry, and indirect immunofluorescence detection thereof is shown in Figure 5.3 A and B.

5.6.1.1 Flow cytometric analysis of Hsp27 expression and phosphorylation

Hsp27 fluorescence produced a stronger fluorescence signal compared to phosphorylated Hsp27 (Figure 5.2 A). In flow cytometric analysis of Hsp27 phosphorylation, non-specific binding of the secondary antibody (FITC) resulted in a background fluorescence of 1.12 ± 0.92 cells/channel. Hsp27 phosphorylation detected by the anti-Hsp27P antibody decreased significantly ($p < 0.05$) as a function of time (Figure 5.2). However no significant difference in Hsp27 phosphorylation after RF-EMF exposure at any of the time points was detected compared to control

spermatozoa. Hsp27P increased slightly in 43°C exposed spermatozoa, but this increase was not significantly different from spermatozoa maintained at 37°C (controls). Figure 5.2 B depicts typical Hsp27P staining in control (I) and RF-EMF exposed (II) spermatozoa, Hsp27P fluorescence was mainly located in the neck and principle tail-piece of spermatozoa. No significant increase in fluorescence intensity after RF exposure was noted.

5.6.1.2 Flow cytometric analysis of Hsp70 expression

In flow cytometric analysis of Hsp70 expression, non-specific binding of the secondary antibody (FITC) resulted in a background fluorescence of 1.80 ± 0.91 cells/channel. Hsp70 expression decreased as a function of time (Figure 5.3 A) with fluorescence decreasing below background fluorescence after 24 hours (data not shown). Hsp70 expression after RF-exposure was slightly elevated, however, no significant difference was noted at any of the time points compared to control spermatozoa. There was also no difference in Hsp70 expression between RF-EMF exposed, control, or heat shocked spermatozoa determined respectively after 1 hour RF-EMF or 43°C exposure. Hsp70 fluorescence (Figure 5.3 B) was detected mainly in the neck area of spermatozoa, and no difference was noted in fluorescence intensity between control (I) and RF-exposed (II) sperm.

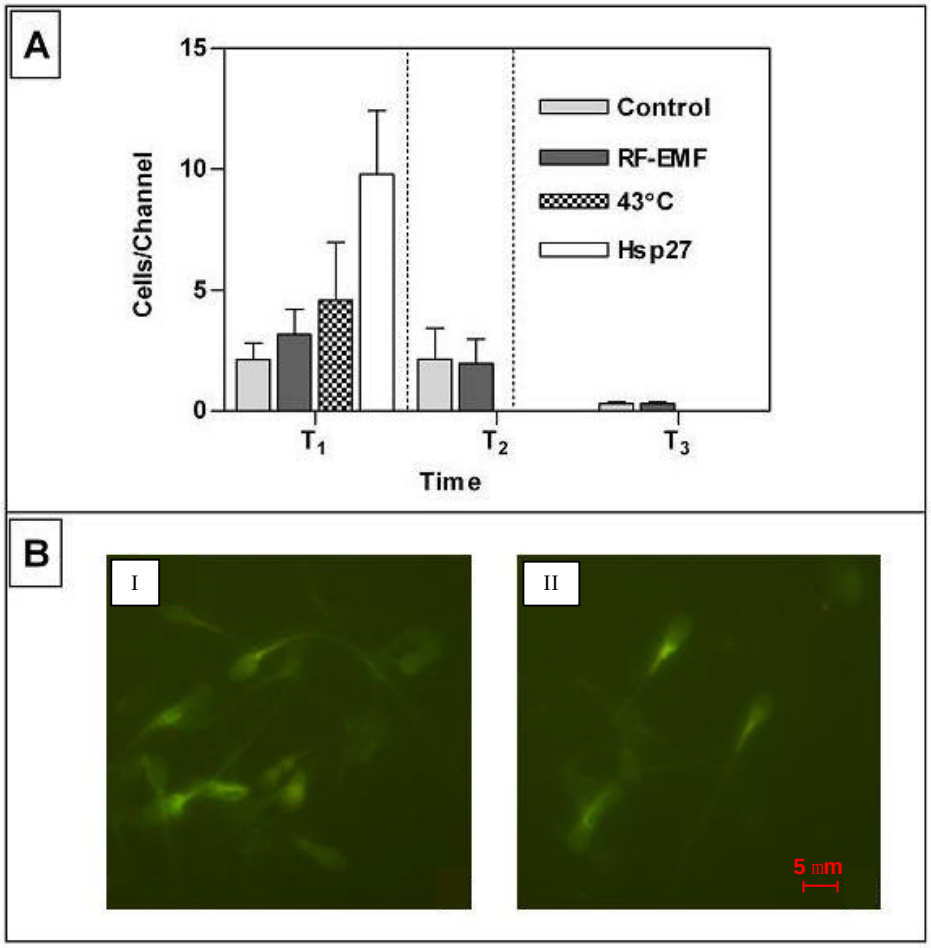

Figure 5.2 **(A)** Flow cytometric analysis of Hsp27 phosphorylation (dark grey) detected by anti-Hsp27P directly (T_1), 2 (T_2) and 24 hours (T_3) after an hour RF-EMF exposure at SAR 2.0 W/kg (n = 12). Baseline Hsp27 expression (white) as well as Hsp27 phosphorylation after a 1 hour heat shock at 43°C (black and white) are given at time 1. Control (light grey) samples were maintained at 37°C during the exposures. **(B)** Detection of Hsp27P expression by immunofluorescence staining directly after exposure (T_1), in (I) control and (II) RF-exposed sperm, Hsp27P fluorescence was mainly located in the neck area of the sperm.

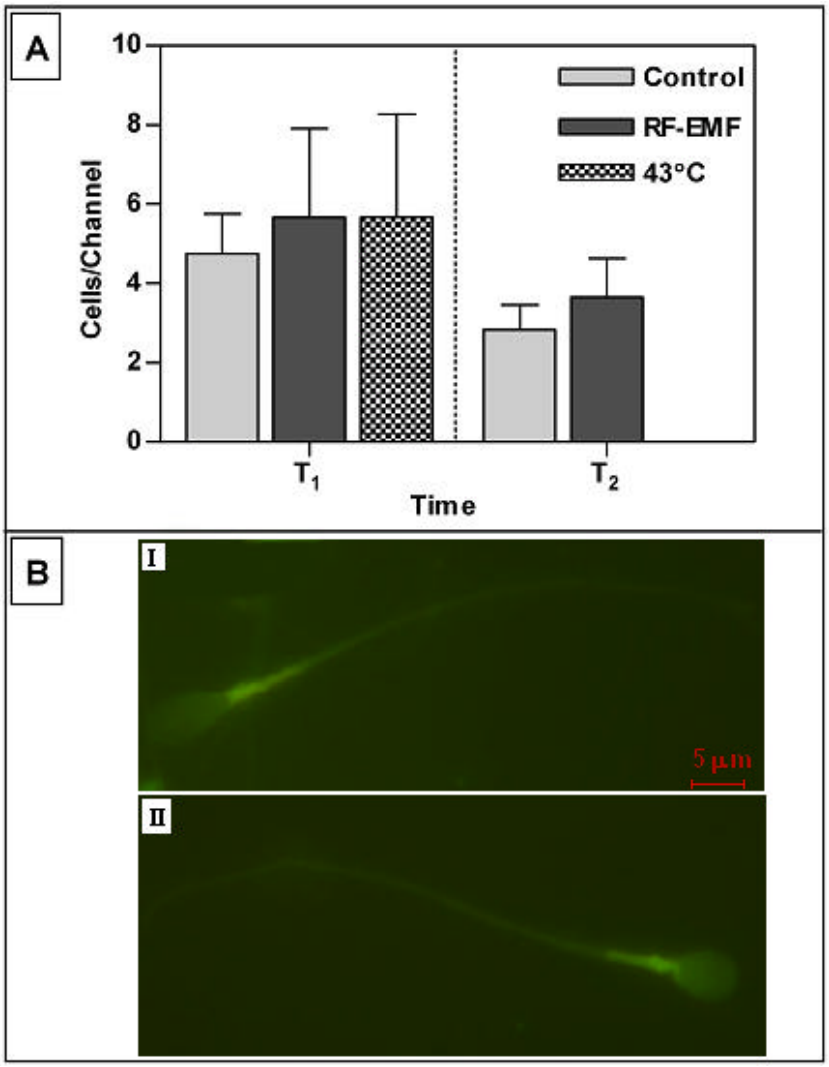

Figure 5.3 **(A)** Flow cytometric analysis of Hsp70 expression detected directly (T_1) and 2 (T_2) after an 1 hour RF-EMF exposure at SAR 2.0 W/kg or heat shock at 43°C (n = 12). Control samples were maintained at 37°C during the exposures. **(B)** Detection of Hsp70 immunofluorescence staining directly after exposure (T_1), in (I) control and (II) RF-exposed sperm, Hsp70 fluorescence was mainly located in the neck area of the sperm.

5.6.2 Western Blot analysis of Hsps 110, 90, 70, 75, 60, 40, and 27 expression

5.6.2.1 Western Blot analysis of Hsp27

A typical autoradiogram of Hsp27 expression in donor 2 (D_2)-I and donor 12 (D_{12})-II as well as in the epithelial cell line (AE.hy926)-III is illustrated in Figure 5.4 A. The 27 kD band corresponding to Hsp27 was confirmed by anti-Hsp27 antibody staining. Densitometric analysis of the Western Blots represents the results of six experiments (Figure 5.4 B). Statistical analysis (Wilcoxon matched pairs signed ranks test) between control, RF-EMF exposed or heat shocked sperm revealed no significant difference in Hsp27 expression determined for either of the two donors. The EA.hy926 cells expressing Hsp27 significantly differed ($p < 0.05$) from that of sperm cells.

5.6.2.2 Western Blot analysis of Hsp70

A strong 70 kD signal was observed in Western blots of SDS-PAGE resolved sperm proteins probed with an anti-Hsp70 polyclonal antibody (Figure 5.5 A). The densitometric analysis of the Western Blots (Figure 5.5 B), represent the results of six experiments. Hsp70 expression in sperm cells were significantly greater ($p < 0.05$) compared to EA.hy926 cells. For donor 2 (D_2) there was no difference in Hsp70 expression for any of the conditions (control, RF-EMF or 43°C). However, there was a marginally significant difference ($p = 0.067$) in Hsp70 expression in RF-EMF exposed sperm compared to control sperm for donor 12 (D_{12}). The expression of Hsp70 in heat shocked (43°C) sperm and RF-EMF exposed sperm for D_{12} did not differ significantly ($p > 0.05$) and this was also the case between control and heat shocked (43°C) sperm.

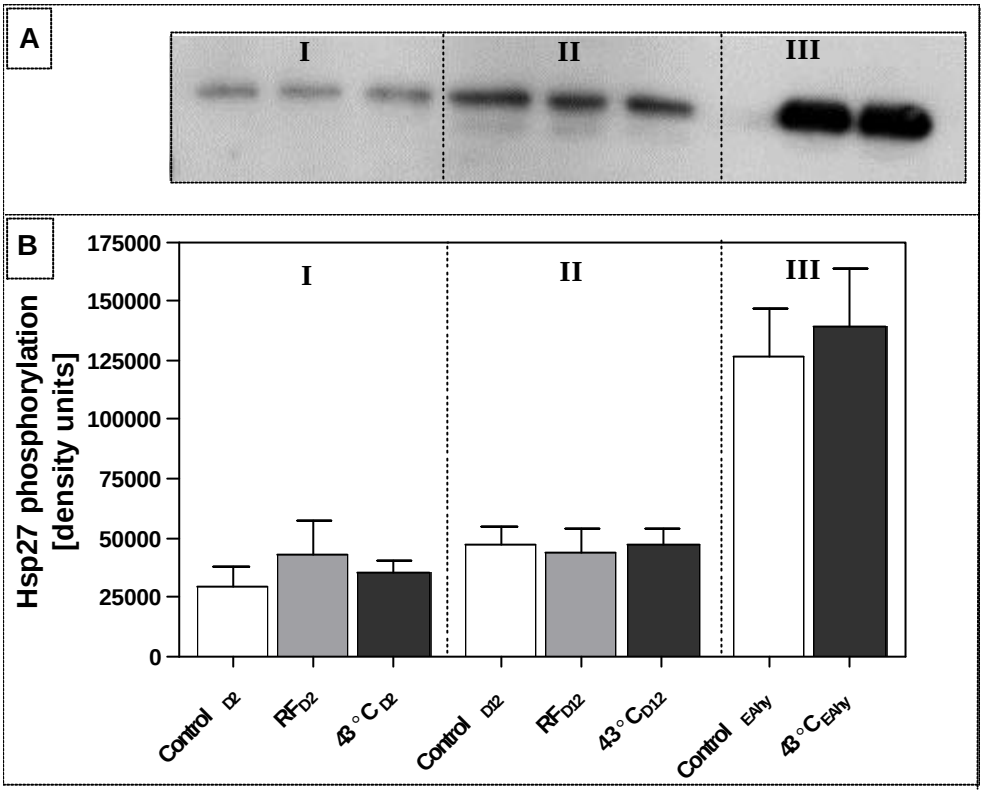

Figure 5.4 Western Blot analysis of Hsp27: (A) Autoradiogram of SDS-PAGE resolved proteins for donor 2 (I), donor 12 (II) and EA.hy926 cells (III). The position of the bands correspond to heat shock proteins of M_r 27 kD, specific for Hsp27. **(B)** Densitometric analysis of Hsp27 phosphorylation status in spermatozoa directly after an hour exposure to RF-EMF at SAR 2.0 W/kg or 43°C. Control samples were maintained at 37°C for the duration of the exposure. As a control Hsp27 expression in EA.hy926 cells (III) after heat shock at 43°C is also noted.

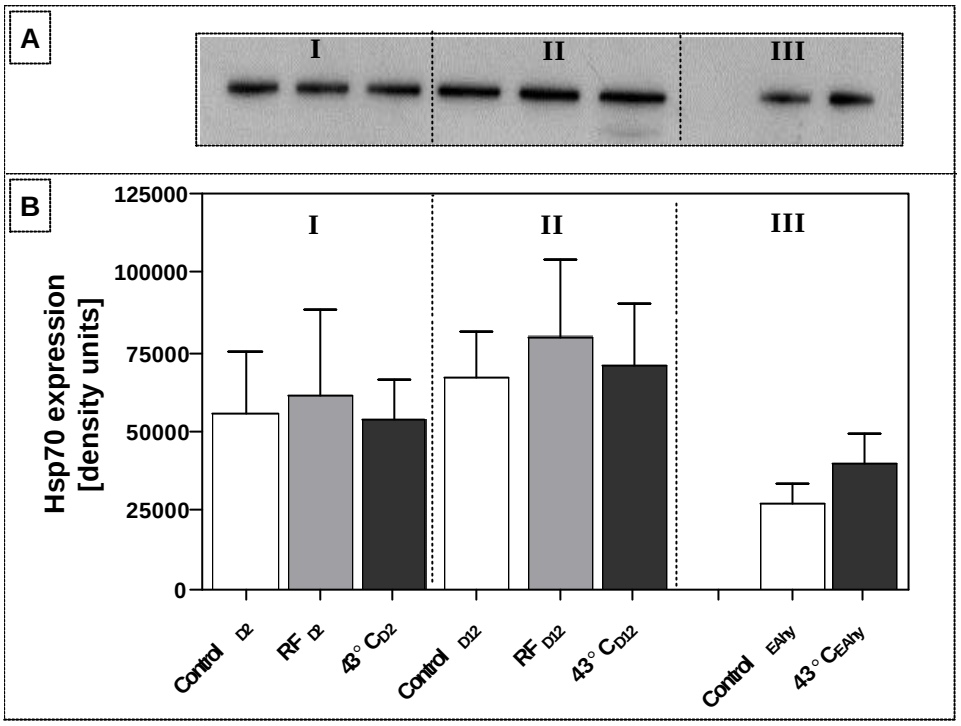

Figure 5.5 Western blot analysis of Hsp70: (A) Autoradiogram of SDS-PAGE resolved proteins for donor 2 (D_2- I), donor 12 (D_{12} - II) and EA.hy926 cells (III). The position of the bands correspond to heat shock proteins of M_r 70 kD, specific for Hsp70. **(B)** Densitometric analysis of Hsp70 expression in spermatozoa directly after an hour exposure to RF-EMF at SAR 2.0 W/kg or 43°C. Control samples were maintained at 37°C for the duration of the exposure. As a control Hsp70 expression in EA.hy926 cells (III) after heat shock at 43°C is also noted.

5.6.2.3 Western Blot analysis of Hsps 110, 90, 75, 60, and 40

It is not known if other spermatozoal heat shock proteins would respond to RF-EMF, therefore additional heat shock proteins were probed in cellular extracts from donor 2 and 12. The SDS-PAGE results and corresponding densitometric analysis of Hsps 110, 90, 75, 60, and 40 expression are presented in Figures 5.6 and 5.7 and are the result of single experiments conducted in each of the two donors. Densitometric values are the results of single Western blot analysis per donor. Hsps 60, 75, and 110 expression in donor 2 after RF-EMF was lower than that found in control samples (Figure 5.6 B-I), only Hsp75 expression showed a possible increase after heat shock. Interestingly, Hsp60 and 75 expression decreased after RF-EMF compared to control and 43°C exposed samples. However this effect was not observed in donor 12, on the contrary Hsp60 and 75 expression after RF-EMF compared well to control samples (Figure 5.6 B-II). The only possible effect noted in Hsp expression measured in donor 12 is the down regulation of Hsp60 after 43°C exposure – this was measured twice in donor 12 to confirm the low Hsp60 expression.

A very strong 40 kD signal was observed for both donors in WB analysis of SDS-PAGE resolved Hsp40 (Figure 5.7 A). Densitometric analysis demonstrated a marginally greater expression in Hsp40 for 43°C exposed samples in both donors. However, no difference in expression was noted between control and RF-EMF exposed samples (Figure 5.7 B-I and II). Hsp90 expression was unaffected by either RF-EMF and heat shock, with expression in control samples for both donors similar to that of the stressed (RF-EMF and 43°C) samples (Figure 5.7 B I and II).

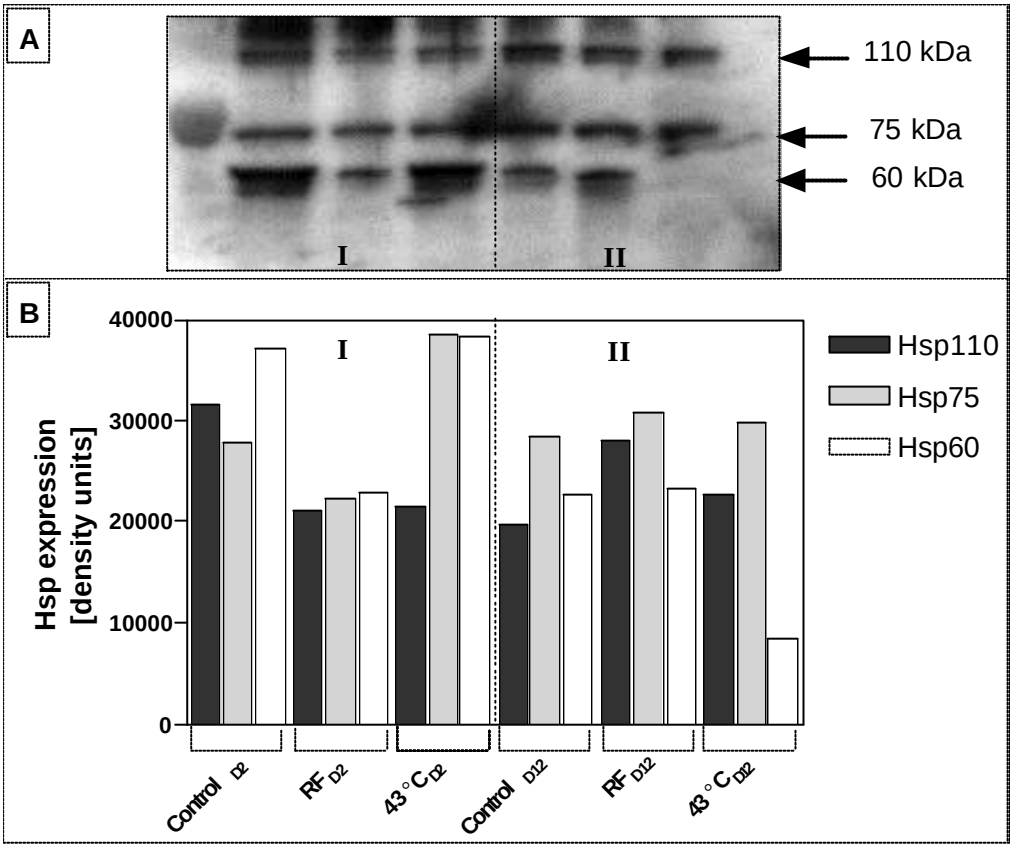

Figure 5.6 Western blot analysis of Hsps 110, 75 and 60: **(A)** Autoradiogram of SDS-PAGE resolved proteins for donor 2 (D2- I), and donor 12 (D12 - II). The position of the bands corresponds to heat shock proteins of M_r 110 kD, 75 kD and 60 kD specific for Hsp110, Hsp75 and Hsp60 respectively. **(B)** Densitometric analysis of Hsp110, Hsp75 and Hsp60 expression in spermatozoa directly after an hour exposure to RF-EMF at SAR 2.0 W/kg or 43°C. Control samples were maintained at 37°C for the duration of the exposure.

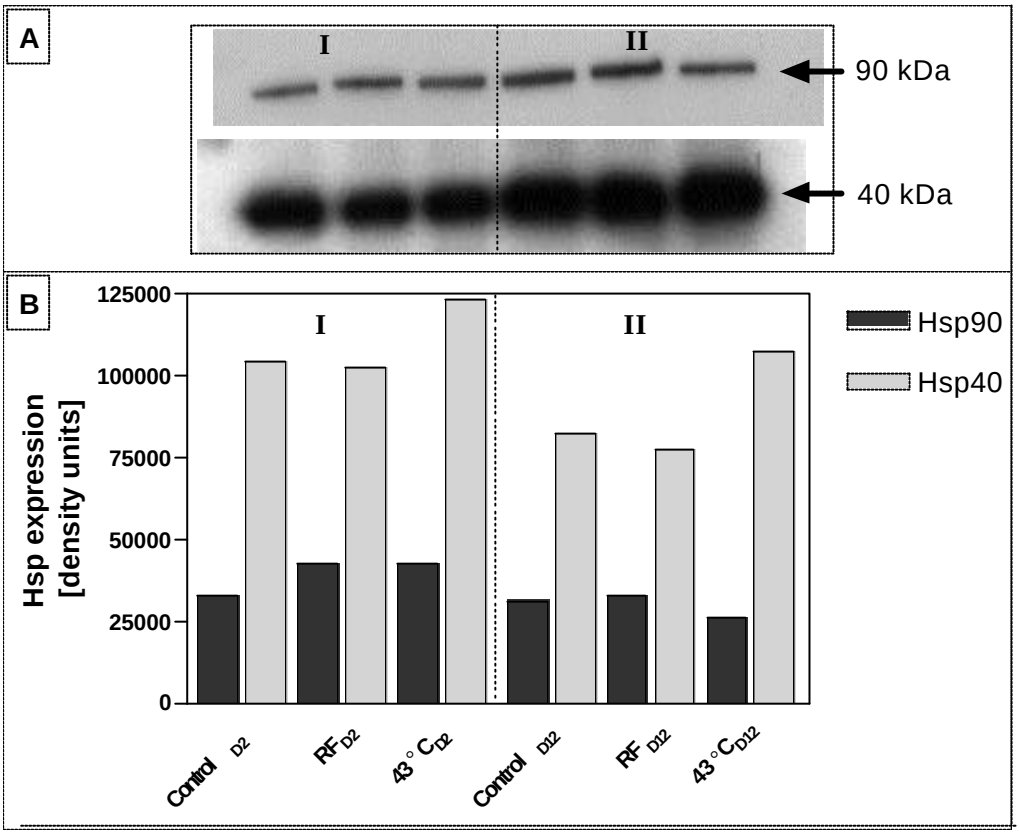

Figure 5.7 Western Blot analysis of Hsps 90 and 40: (A) Autoradiogram of SDS-PAGE resolved proteins for donor 2 (D_2- I), and donor 12 (D_{12} - II). The position of the bands corresponds to heat shock proteins of M_r 90 kD, and 40 kD specific for Hsps 90 and 40. **(B)** Densitometric analysis of Hsps 90 and 40 expression in spermatozoa directly after an hour exposure to RF-EMF at SAR 2.0 W/kg or 43°C. Control samples were maintained at 37°C for the duration of the exposure.

5.6.3 Detection of stress fibres

In order to establish if heat shock protein phosphorylation has a physiological affect on F-actin polymerisation, RF-EMF exposed cells were labelled with phalloidin, an inhibitor of F-actin depolimerisation. The distribution of F-actin in spermatozoa and MCF-7 cells are depicted in Figures 5.8 and 5.9 respectively.

5.6.3.1 F-actin polymerisation in RF-EMF exposed human spermatozoa

To determine the effect of heat shock on F-actin polymerisation, spermatozoa were exposed for a period of one hour to 37°C in a CO_2 incubator, 39°C in a warm oven and 43°C in a water bath. The temperatures were specifically chosen as it was previously reported that sperm cells subjected to a temperature increase from 35°C to 39°C did express Hsps whereas this effect was negated by a further temperature increase to 43°C (Nononguchi *et al.*, 2001). Hsp27 regulates F-actin polymerisation, therefore heat shock was applied at different temperatures to optimise the possible effect that Hsp27 expression/phosphorylation could have on F-actin polymerisation. Special care was taken in keeping the temperature of ejaculated spermatozoa constant at 35°C when processing the sperm. Directly after processing, sperm were subjected to the different temperature exposure regimes (Figure 5.8 A and C). No notable difference in F-actin concentration was seen in spermatozoa exposed to 37°C, 39°C and 43°C.

Slides made of fixed and permeabilised spermatozoa were frozen away at -20°C, until phalloidin staining on the day of analysis. Slides were prepared for all twelve donors, at each time point after exposure (T_1, T_2, T_3), for both control and RF-EMF exposed cells. Typical staining is represented in Figure 5.8 B. No difference in staining was noted between control and RF-EMF exposed spermatozoa. There were also no changes in phalloidin staining as a function of time.

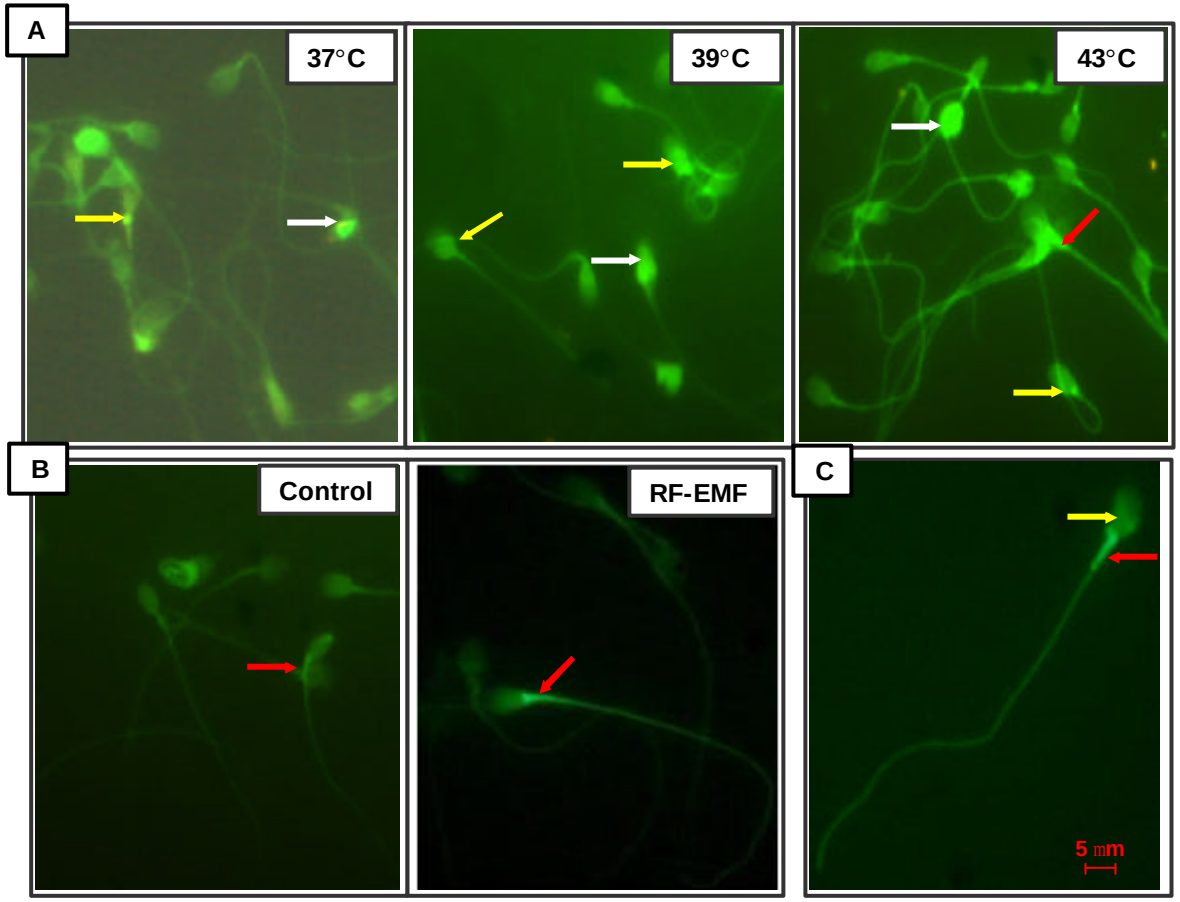

Figure 5.8 Immunolocalization of F-actin. (A) AlexaFluor-labelled phalloidin stained spermatozoa predominantly in the acrosome (white arrow), post-acrosomal area (yellow arrow), as well as neck and principal tail-piece areas (red arrow). **(B)** Cellular response of spermatozoa exposed to RF-EMF, control cells were maintained at 37°C for the duration of the exposure. **(C)** Typical staining of AlexaFluor-labelled phalloidin.

5.6.3.2 F-actin polymerisation in RF-EMF exposed MCF-7 cells

In control and RF-EMF exposed MCF-7 cells (Figure 5.9), the highest F-actin content was localised at the perimeters of the cell (yellow arrows), with a network of stress fibres staining over the whole cytoplasm of the cell (orange arrows). However, no marked difference in stress fibre stabilisation determined by increased phalloidin-AlexaFluor staining was seen between control cells and RF-EMF exposed cells. Interestingly, MCF-7 cells heat shocked at 43°C for one-hour show an increase in F-actin concentration localised in the nuclear mass of the cell. Rounding up of the cells was also clearly visible, while edge ruffling was noticed in some cells (white arrows). F-actin aggregation resulting in distinct pockets of high phalloidin concentration was distributed throughout entire cells (red arrows).

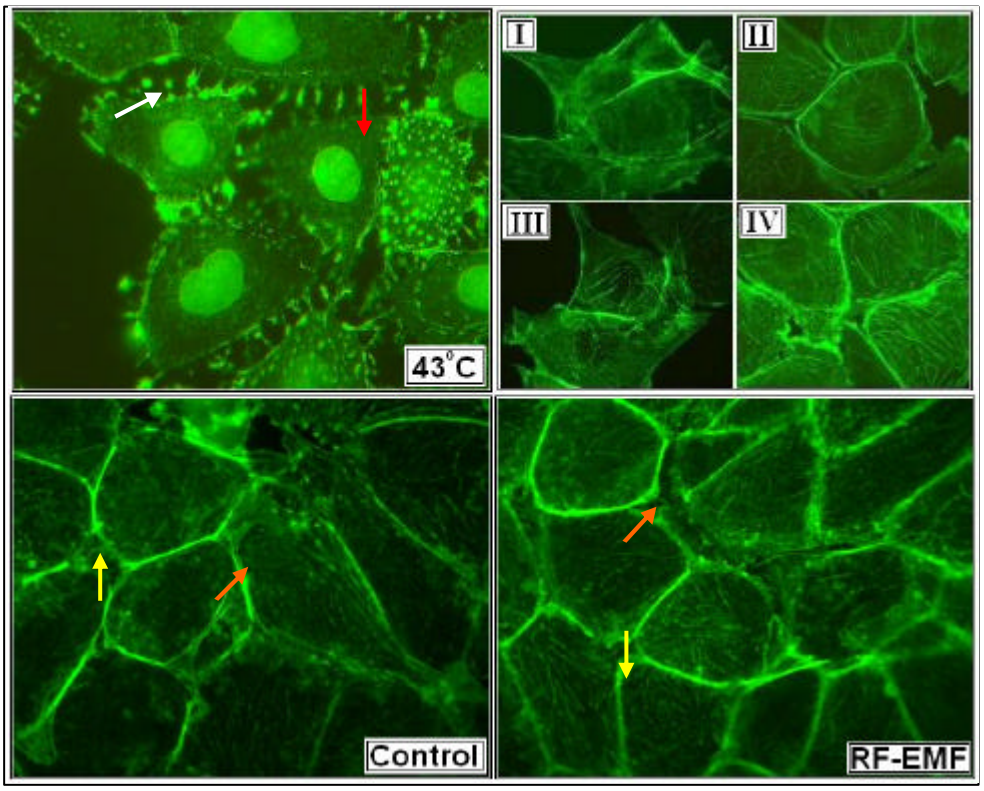

Figure 5.9 Cellular response of MCF-7 cells to RF-EMF and heat shock, control cells were maintained at 37°C for the duration of the exposures. Stress fibres are detected by AlexaFluor-labelled phalloidin. Inserts I and II show the distribution of F-actin in control cells compared to cells exposed to RF-EMF for 1 hour (inserts III and IV).

5.7 DISCUSSION

In the present study, we have identified the presence of Hsp27, a member of the small heat shock protein family (sHsp), in ejaculated density purified human spermatozoa by WB analysis and specific antibody staining. Hsp27 is a classically heat inducible Hsp, but also occurs in the absence of stress in various cells in the body (Pittenger *et al*., 1992; Sugiyama *et al*., 2000). As no *de novo* synthesis of proteins in fully differentiated spermatozoa is possible, the effect of RF-EMF on the phosphorylation status of Hsp27 was investigated.

Hsp27 phosphorylation was determined by flow cytometry using a FITC conjugated anti-Hsp27P antibody. No increased phosphorylation of Hsp27 in human spermatozoa was noted after RF-EMF exposure. This is in agreement with several other studies that have reported no increased Hsp27 phosphorylation after RF exposure in human lymphocytes (Lim *et al*., 2005), glioblastoma cells (Miyakoshi *et al*., 2005; Qutob *et al*., 2006), and HeLa and EA.hy926 cells (Vanderwaal *et al*., 2006). Heat shock, on the other hand, resulted in a mild (though not significantly different from controls) increase of Hsp27P fluorescence in spermatozoa. In contrast a high expression of Hsp27 was noted in heat shocked EA.hy926 cells. Immunomicroscopy probing using antibodies against phosphorylated Hsp27 revealed that Hsp27P was mainly confined to the neck area of spermatozoa. However, no difference in fluorescence in RF-exposed samples were noted compared to controls maintained at 37°C.

The possibility that Hsp70 could be up-regulated after RF-exposure via transverse scriptase was also investigated. Western blot probing with an Hsp70 polyclonal antibody resulted in a strong 70 kD signal compared to Hsp70 expression in EA.hy926 cells. Multiple isoforms of Hsp70 have been identified in male germ cells and spermatozoa including the sperm specific variant HspA2 (Miller *et al*., 1992; Huzar *et al*., 2000; Bohring and Krause, 2003). In addition, Hsp70 was reported to be an abundant surface protein, explaining the strong 70 kD signal observed. However, no up-regulation of cytoplasmic Hsp70 in response to RF exposure was observed. Using an antibody directed against the stress inducible form of Hsp70, we also noted no significant increase in Hsp70 fluorescence after RF exposure determined by flow

cytometric analysis. Several other studies have reported a lack in up-regulating Hsp70 expression after RF exposure in human lymphocytes (Capri *et al*., 2004; Lim *et al*., 2005), human glioblastoma cells (Miyakoshi *et al*., 2005), and mammalian cells (Laszlo *et al*., 2005) supporting these results.

In addition, Hsp70 in human sperm was not up-regulated by hyperthermia in contrast to EA.hy926 cells. This result was not unexpected, as it has previously been demonstrated that the murine Hsp70 gene is not heat inducible (Zakeri *et al*., 1988; Rosario *et al*., 1992; Son, 1999). Immunomicroscopy evaluation of Hsp70 staining was in accordance with Miller *et al*. (1992) who also noted Hsp70 staining located in the neck, mid-piece, and equatorial segment of fixed spermatozoa. In addition, RF exposure did not increase stress inducible Hsp70 fluorescence intensity.

One possible limitation of the data presented is that an anti-Hsp70 antibody was used for Western blotting and an antibody directed against the stress inducible form of Hsp70 was used for flow cytometric analysis, whereas HspA2, the male germ cell specific homologue for Hsp70, is the most abundant member of the heat shock protein 70 family in human germ cells. HspA2 has < 90% amino acid homology to other members of the human Hsp70 gene family and 83.3% to the stress inducible Hsp70 gene (Bonnycastle *et al*., 1994). Therefore, it is uncertain whether HspA2 or other members of the Hsp70 family of proteins was detected in the present study. However, Bohring and Krause (2003) concluded that it is unlikely that Hsp70 and HspA2 are involved in a stress response but rather have chaperone functions during capacitation, acrosome reaction, and oocyte binding.

Besides Hsp27 and 70, the expression of Hsps 40, 60, 90, and 110 were also investigated by Western blotting after RF exposure. In addition, the expression of another member of the Hsp70 family of proteins - Hsp75, a moderately stress inducible glucose regulated stress protein, present within the mitochondrial matrix (Creagh *et al*., 2000), was also probed. Bearing in mind that WB analysis of heat shock proteins 110, 90, 75, 60, and 40 were the result of single experiments in each of two donors, it cannot be concluded that these proteins are unaffected by RF-EMF. However, taking the bulk of evidence presented here, it seems unlikely that RF-EMF has an effect on the sperms ability to launch a stress response via the heat shock

protein pathway. None of the Hsps found in spermatozoa and probed using Western Blots or flow cytometric analysis showed a significant increase in expression or phosphorylation after RF-EMF. Furthermore, Hsp expression after heat shock (43°C) analysed with flow cytometry and Western Blots did not unequivocally show that heat stress can up-regulate Hsp expression in human spermatozoa.

The actin network plays an important role in regulating exocytosis in endocrine and secretory cells (Burgoyne *et al*., 1991; Dudani and Ganz, 1996). In spermatozoa, the AR is regarded as equivalent to exocytosis and is therefore regulated by similar processes (Liu *et al*., 2002). In the sperm head, actin occurs mainly in its monomeric form but polymerises to form filamentous F-actin during sperm capacitation (Rogers *et al*., 1989; Castellani-Ceresa *et al*., 1993; Spungin *et al*., 1995; Liu *et al*., 1999, 2005). In particular, F-actin increases during capacitation leading to the establishment of an F-actin network between the plasma and outer acrosomal membrane (Spungin *et al*., 1995; Breitbart and Spungin, 1997; Liu *et al*., 2005; Breitbart *et al*., 2005) and its depolymerisation is essential for the AR (Liu *et al*., 1999; Breitbart *et al*., 2005).

Liu *et al*. (1999) noted the preferential staining of actin, using an anti-actin antibody in the acrosomal region of acrosome intact spermatozoa, as well as the post acrosomal, mid-piece and tail sections of both acrosome reacted and intact spermatozoa. A similar staining pattern for F-actin was observed in the current study. We did not observe an increase in actin polymerisation in sperm exposed to different temperatures or in RF exposed spermatozoa. In contrast, heat stress in MCF-7 cells induced accumulation of F-actin, demonstrated by the aggregation of F-actin and their concentration around the nucleus of the cell. RF exposure, however, did not induce an increase in stress fibre stabilisation in MCF-7 cells. Zeng *et al*. (2006) recently reported that RF exposure of MCF-7 cells did not lead to any change in protein expression. Considering that actin polymerisation is modulated by Hsp27 phosphorylation, and that we noted no increased Hsp27P expression in RF exposed spermatozoa, it is thus plausible that no increase in F-actin was noted after RF exposure in either spermatozoa or MCF-7 cells.

Actin polymerisation in human spermatozoa also depends on tyrosine phosphorylation associated with capacitation (Brener, 2003). Hsp90 has the potential to mediate serine/threonine and/or tyrosine kinase signalling in the pathway to a global increase in protein tyrosine phosphorylation during capacitation (Ecroyd, 2003). If RF-EMF could have affected Hsp90 activity it would have indirectly affected actin polymerisation. Since neither increase in Western Blot expression of Hsp90 nor changes in actin polymerization was seen, it could be concluded that RF-EMF had no effect on protein tyrosine phosphorylation.

These results, taken together, seem to indicate that fully mature spermatozoa are unable to launch a stress response when exposed to high temperatures or RF-EMF exposure. Noting the specific roles of some Hsps in fertilisation (Eddy, 1999; Neuer *et al*., 2000), it would seem likely that the complement of heat shock proteins that spermatozoa are equipped with are functional mainly as chaperonens during fertilisation. This finding suggests that mobile phone radiation is not a stressor of human spermatozoa.

5.8 REFERENCES

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SECTION C

CONCLUSIONS

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

CHAPTER 6

6.1 CONCLUSION AND RECOMMENDATIONS

Several studies have highlighted the possibility that RF-EMF affects sperm motility (Davoudi *et al*., 2002; Fejes *et al*., 2005; Kilgallon and Simmons, 2005; Erogul *et al*., 2006), or that it exerts a genotoxic effect (Aitken *et al*., 2005). This lead others to conclude that mobile phone exposure along with other environmental factors could decrease semen quality (Kilgallon and Simmons, 2005). However, none of these studies directly assessed the effect of RF-EMF on sperm physiology, sperm specific, or sperm functional assays. Nor did they elucidate a possible pathway for the effects observed taking the initial biophysical electromagnetic interaction into consideration. Therefore, when embarking on the current study, the possibility that RF-EMF could have an effect on sperm fertilizing potential by focussing on sperm capacitation (AR and hyperactivated motility) and the binding of exposed spermatozoa to the ZP (using the HZA) of human oocytes was first investigated. In assessing the effect of RF-EMF on sperm motility, morphometry and acrosomal status automated systems such as CASA and FCM were used to eliminate operator bias in manual assessment. This approach was also taken throughout the study to eliminate possible operator partiality since the study was not conducted under blinded conditions.

RF-EMF proved to have no effect on the ability of spermatozoa to undergo the AR, although an effect on various sperm kinetic parameters was observed while hyperactivation of spermatozoa was not affected (summarised in Table 6.1). In addition, spermatozoa were analysed morphometrically after RF-EMF exposure and a significant decrease in most of the morphometric parameters were observed. Most notably, exposed sperm decreased in area and the acrosomal region also decreased in size. To confirm if any of these observations could have an effect on sperm fertilizing potential, exposed spermatozoa were co-incubated with human oocytes and their binding capacity was evaluated. Not only did fewer exposed sperm bind,

but this effect also became more pronounced when sperm were exposed at a higher SAR level.

A reasonable explanation for the decrease in sperm-zona binding observed could be ascribed to the decrease in sperm acrosomal region noted after RF exposure. The workable hypothesis supported by many studies, that RF-EMF elicits a stress response by activating a heat shock protein pathway (Fritze *et al*., 1997; French *et al*., 2000; Kwee *et al*., 2001; Leszczynski *et al*., 2002; Di Carlo *et al*., 2002; Goodman and Blank, 2002), could potentially provide an explanation for this finding. During a stress response, Hsps are activated and some Hsps (Hsp27) are involved in stabilizing stress fibres (F-actin polymerisation), resulting in an overall decrease in cell size. Since F-actin de-polymerisation is an acquired event leading to the fusion of the inner and outer acrosomal membranes, thereby facilitating acrosomal release and sperm penetration (Liu *et al*., 2002, 2005; Breitbart *et al*., 2005), badly timed actin polymerisation could interfere with this process. Besides Hsp27, Hsp70 plays a significant role in sperm capacitation as well as zona fusion. Premature activation of Hsp70 as a result of RF-EMF could potentially have an effect on sperm-zona binding ability. Thus, an operational stress pathway in spermatozoa as a result of RF exposure could potentially account for the reduction in sperm binding observed. Therefore the activation of a stress pathway (looking both at apoptosis and heat shock protein activation/phosphorylation) in RF-EMF exposed spermatozoa was investigated.

Table 6.1 Summary of effects of RF-EMF on sperm specific and functional assays in highly motile human spermatozoa.

Assay	2.0 W/kg (RF vs. C)	5.7 W/kg (RF vs. C)	2.0 W/kg vs. 5.7 W/kg	Comments
Capacitation				
Acrosome reaction[1]	No effect	-	-	No effect in number of dead ($7AAD^+$)-, AI- or AR -sperm.
Motility[2]	**No effect**	**No effect**	**NS**	**Progressive motility (a + b) - No effect**
i) Velocity (VAP, VCL, VSL)	No effect	$\downarrow^\dagger$	S (area & acrosome)	i) VSL† (VAP & VCL borderline S), $Velocity_{5.7W/kg} < Velocity_{2.0W/kg}$
ii) Motion (BCF, ALH, STR, LIN)	No effect	$\downarrow^\dagger$		ii) BCF† (ALH, STR, LIN - NS)
iii) Morphometric analysis	$\downarrow^\dagger$	$\downarrow^\dagger$		iii) $Area_{RF\text{-}EMF} < Area_{Control}{}^\dagger$, $Acrosome_{RF\text{-}EMF} < Acrosome_{Control}{}^\dagger$
iv) Hyperactivation	No effect	No effect		
Sperm-zona binding				
HZA	$\downarrow^\dagger$	-	-	For SAR 2 W/kg: $HZI_{RF\text{-}EMF} < HZI_{Control}{}^\dagger$
Apoptosis[1]				
PS externalisation	No effect	No effect	NS	Including no effect on number of dead cells (PI^+)
MMP	No effect	No effect	-	
Caspase activation	No effect	No effect	-	Including active caspase-3 & all activated caspases
ROS generation	No effect	No effect	-	
DNA fragmentation	No effect	No effect	-	
Stress response				
Hsp 70	No effect[3]	-	-	Slight but NS increase assessed by FCM and WB.
Hsp 27	No effect[3]	-	-	
Hsp 27P	No effect[3]	-	-	
F-actin polymerisation	No effect[4]	-	-	Also assessed in MCF-7 cells - No effect

Assessed by FCM[1], CASA (Hamilton Thorne) [2], FCM and WB analysis[3], indirect immunofluorescence[4].

$^\dagger p < 0.05$ (time series regression analysis between exposed and control spermatozoa determined over all three time points).

*$p < 0.05$ (student t-test comparing SAR 2.0 W/kg with SAR 5.7 W/kg).

NS = Not Significant, S = significant. $\downarrow$ = Reduction.

RF exposure did not induce apoptosis in spermatozoa (summarized in Table 6.1), but the increase in ROS generation noted at the higher SAR level in both RF exposed and control spermatozoa significantly correlated with the decrease in motility observed at the same SAR level. According to Duru *et al*. (2000), reactive oxygen species have been found to have a dual effect on human spermatozoa. Physiologically, ROS are required for capacitation and the AR. However, ROS at high concentrations induce motility loss and lead to sperm dysfunction such as poor sperm-ZP binding and sperm oocyte fusion. An increase in ROS could thus have been responsible for the reduced sperm-zona binding, but we did not see any significant generation of ROS (at any of the SAR levels) after RF-exposure (results summarised in Table 6.1). Furthermore, from experimental results, it became clear that some of the biomarkers used (i.e. Annexin V) to investigate apoptosis could be the result of normal physiological processes occurring in spermatozoa and not necessarily an indicator for apoptosis. In addition, results further seem to indicate that fully differentiated human spermatozoa lack the ability to initiate apoptosis, thus leaving the possibility that Hsp activation could be responsible for the observed reduction in sperm-zona binding.

Although the presence of both Hsp27 and Hsp70 were identified in human spermatozoa, neither of these Hsps were significantly up-regulated as a result of RF-EMF. In addition, heat shock (43°C) also did not have any effect on Hsp27 phosphorylation and expression, nor did it have any affect on Hsp70 expression. In somatic cells, Hsp27 phosphorylation regulates actin polymerisation that in spermatozoa plays a significant role in sperm membrane fusion and initiation of the AR (Breitbart *et al*., 2005). The lack of an effect of RF-EMF on Hsp27 phosphorylation was clearly demonstrated by the lack of stress fibre activation in exposed sperm; thus, the ability of spermatozoa to launch a stress response under these circumstances has to be questioned. Therefore, we have to conclude that for spermatozoa, at least, RF-EMF does not result in the activation of a stress pathway and that the reduced binding observed must be the result of an alternative pathway.

Considering the processes that regulate sperm-zona binding (Figure 6.1), two alternative electromagnetic interaction mechanisms supported by theory could be

responsible for the decrease in sperm-egg binding. Firstly, RF exposure could lead to the irregular gating of electro-sensitive channels on the plasma membrane resulting in the disruption of the cell's electrochemical balance and function (Panagopoulus *et al*., 2002) and secondly, RF-EMF could have an effect on ligand binding to receptor proteins (Chiabrera *et al*., 2000).

Several types of ion channels have been identified in spermatozoa consisting of high and low voltage-activated channels, receptor-operated channels, and store operated channels (Benoff, 1998; Tosti and Boni, 2004). In addition, human spermatozoa express multiple ligand receptors on their head plasma membranes (Benoff, 1998) involved in a multitude of processes including species recognition and initiation of the AR (Dietl and Rauth, 1989; Jovine *et al*., 2005).

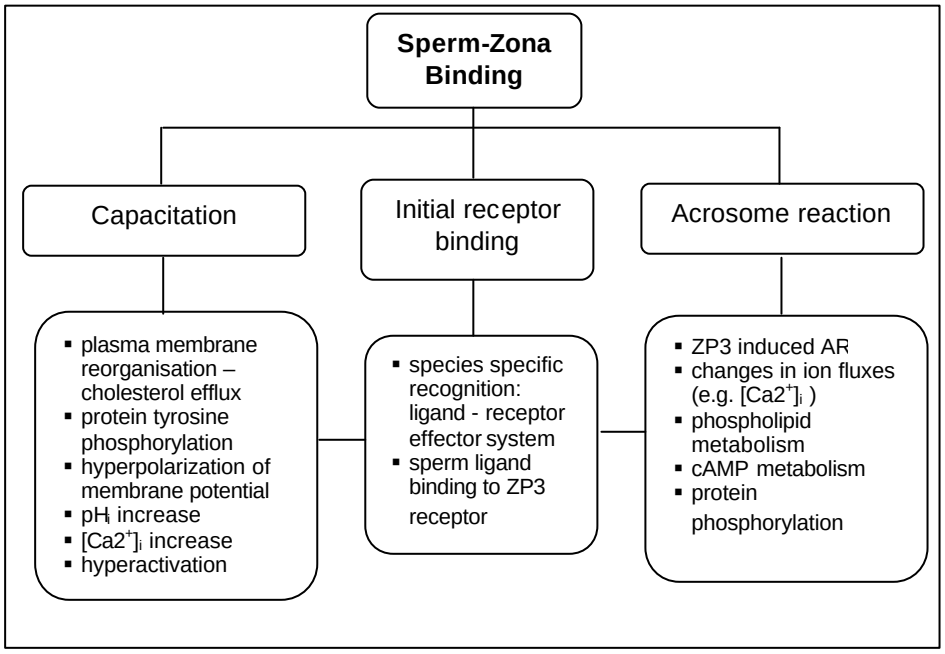

Figure 6.1 The processes of homologous sperm-zona binding and penetration.

One of the most significant ions regulating sperm capacitation, hyperactivation, and the AR is Ca^{2+} (Darszon *et al*., 2006). If RF-EMF could, either through ligand-

receptor mediated Ca^{2+} channel activation, or directly through gating Ca^{2+} channels have affected sperm biding, then an effect on RF exposed sperm's hyperactivated motility and acrosomal exocytosis would have been observed. Neither of these two processes were affected by RF-EMF; thus RF exposure did not affect sperm binding through Ca^{2+} flux changes. In addition, Cl^- channels have been indicated in the activation process of the AR. Bearing in mind that different types of Cl^- channels with dissimilar conductance have been observed on the sperm head (Bai and Shi, 2001), it is possible that RF induced fields could in some manner have influenced the gating of these channels resulting in decreased sperm-zona binding.

Alternatively, RF-EMF could have affected the gating of receptors on the sperm head, which would explain why fewer exposed sperm bound to the hemizona, while no effect was seen on sperm propensity for the acrosome reaction or hyperactivated motility. However, if this biophysical mechanism was responsible for the decrease in exposed sperm binding ability, it remains unclear why RF-EMF would selectively affect some receptors (involved in binding) and not others.

Another possibility is that scramblase activity could be the target of RFexposure. It has previously been reported that RF-fields could possibly affect scramblase activity (Capri *et al*., 2004). Scramblase is a key enzyme that governs the translocation of phospholipids across the membrane lipid bylayer (Gadella and Harrison, 2002; Martin *et al*., 2005). Its collapse is generally associated with a loss in asymmetry and the exposure of phospholipids PS and phosphatidylethanolamine at the outer membrane surface. This process is often considered to be an early indicator for apoptosis (Sakkas *et al*., 1999; Pentikainen *et al*., 1999). However, changes in the transverse distribution of membrane phospholipids as a result of scrambling is observed in a variety of cellular phenomena, including cell adhesion (Barroso *et al*., 2000) and exocytosis (Oosterhuis *et al*., 2000). PS externalisation as a result of RF exposure investigated in the current study was unaffected by RF exposure, but it is possible that scramblase activation of alternative phospholipids involved in adhesion were influenced, thereby affecting sperm-zona binding.

Apart from the effect on sperm binding, significant changes in sperm area and acrosomal size were also observed. It is possible that RF exposure could have an

effect on the cytoskeleton and/or remodelling of the acrosomal cap. However, no affect on stress fibre activation that could have accounted for the decrease in size observed, was noted. Furthermore, the acrosomal content was not affected as flow-cytometry results noted no difference in fluorescence intensity between control and exposed sperm. Another possible explanation, as a result of an effect on ion transport, could have been attributed to changes in membrane structure resulting from cytoskeletal changes (Tosti and Boni, 2004). However, it seems unlikely that RF-EMF could have affected ion transport systems as these effects would have directly affected sperm capacitation, hyperactivation, and the AR.

Besides the biological evidence presented, here that an hour exposure of spermatozoa to a 900 MHZ GSM modulated radio-frequency field has the potential to disrupt sperm-zona binding, the capacitated human spermatozoon affords a unique cellular system to study the mechanism of RF-EMF. Unlike somatic cells, capacitated spermatozoa must maintain a state of hyperpolarisation during sperm-zona fusion. Low voltage Ca^{2+} channels present in mature sperm are inactivated at holding potentials between –80 and –60 mV and cannot be readily activated from more positive holding potentials (Lievano *et al*., 1996; Visconti *et al*., 2002). In addition, ion channels can catalyse the flow of millions of ions through the lipid bylayer resulting in huge changes within a small cell, like the sperm, within milliseconds (Darszon *et al*., 2006). Thus, if the biophysical mechanism of RF-EMF involves induction of weak electric fields arising from the mobile phone's magnetic field (Goodman and Blank, 2002), studying ion flux rates and membrane electrophysiology using patch-clamp technology could elucidate this mechanism.

In conclusion, additional studies investigating the effect of RF-EMF on sperm-zona binding should be conducted, specifically investigating the ligand-receptor effector systems involved in sperm-zona binding. Through the judicious use of specific lectins, insights into the molecular mechanism responsible for the decrease in binding could be gained. Given that this biophysical mechanism also resulted in sperm structural changes (decreased area and acrosomal size), it is suggested that electron microscopy be used to investigate conformal and structural changes as a result of RF-EMF. Furthermore, considering the recent reports noting an effect on

sperm motility at lower SAR levels than that employed in the present study (Fejes *et al*., 2005; Erogul *et al*., 2006), the effect of RF-EMF on human spermatozoa, 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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SECTION D

ANNEXURES

ANNEXURE A

SAR SIMULATION RESULTS

ANNEXURE B

MACROSCOPIC AND MICROSCOPIC SPERM PARAMETERS

ANNEXURE C

DONOR SPERM PARAMETERS - RESULTS

ANNEXURE A

SAR SIMULATION RESULTS

A.1 VERTICAL 900MHZ EXPOSURE CHAMBER

The irradiation system consists of a rectangular wave-guide placed vertically inside a cell culture incubator (Figure A.1). The chamber is made from aluminium and is fitted on top of a waterbed that has place for two petri dishes (outer diameter of 55 mm). Water is circulated via a pump (Figure A.1 [B-I]) from the waterbed under the petri dishes to the cooling tower behind the chamber (Figure A.1 [B-II]).

The RF-EMF signal is generated with an EDSG-1240 signal generator (Figure A.1 [C-III]) and modulated (Figure A.1 [C-V]) to match the GSM signal modulation scheme. The generated signal is amplified with RF Power Labs R720F amplifier (Figure A.1 [C-IV]) and conducted through a circulator (Mica 7Y213, Figure A.1 [C-VI]) to a power meter (Coaxial Dynamics 81000-A, Figure A.1 [C-VII]). The signal from the power meter is fed to the wave-guide chamber via a monopole type feed post. The waveguide is short-circuited at both ends, thus generating a resonant field with two electric field maximums. The power is absorbed mainly by the circulating water and cell culture medium.

A.1.1 Temperature control unit

The temperature control unit is attached to the chamber. It consists of a water pump, a waterbed (underneath the petri dishes), a water-cooling plate (behind the chamber), and a DC-power supply unit. The power supply unit provides power to the pump, which in turn circulates water from the waterbed to the water-cooling plate. The cooling plate is in direct contact with the inner wall of the incubator to provide maximal thermal exchange. Water from the cooling plate is then pumped back to the waterbed under the petri dishes.

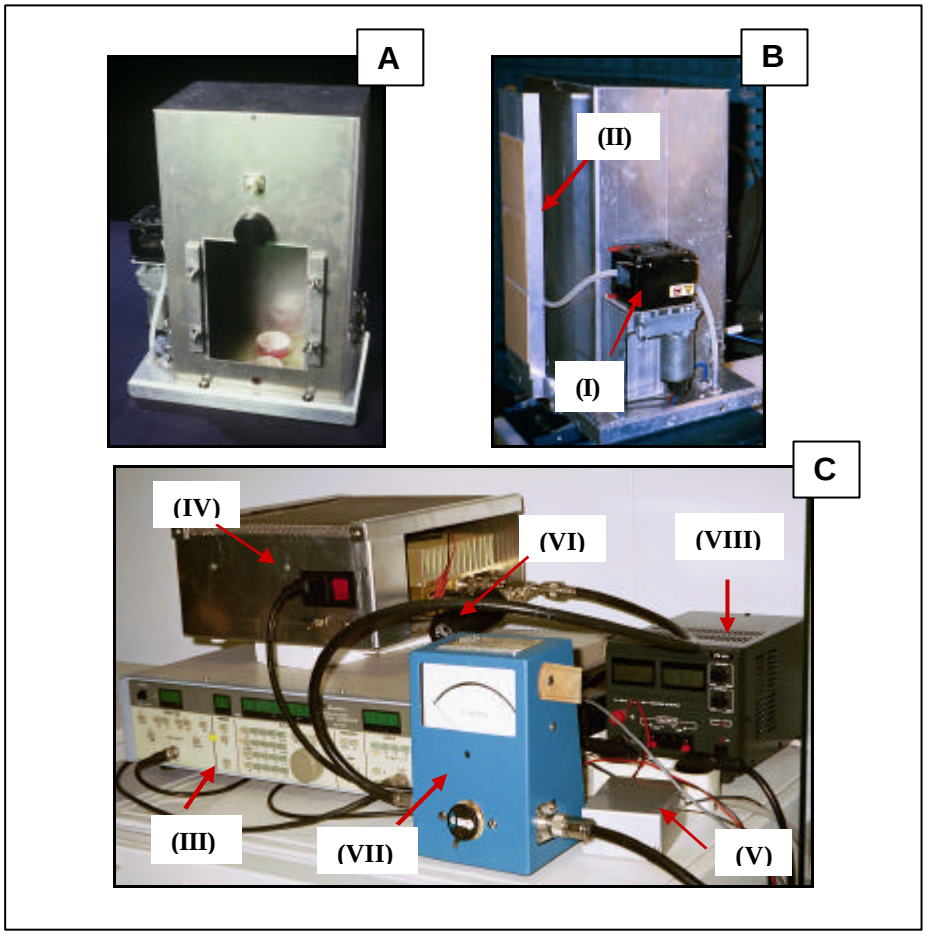

Figure A.1 **(A)** Front view of vertical exposure chamber showing the position of the petri dishes with the hatch open. **(B)** Side view of the chamber with the water pump housing (I), water is circulated from the waterbed below the petri dishes to the aluminium cooling plate (II) behind the chamber (thermal conducting tape attached to back of plate). The cooling plate is placed in contact with the inside wall of the incubator to allow for thermal exchange. **(C)** Peripheral equipment include; Signal generator (III), Amplifier (IV), GSM modulator (V), Coaxial terminator (VI), Power meter and sensor (VII), DC power supply (VIII).

The waterbed is covered with a thin layer (8 mm) of epoxy laminate. The petri dishes are positioned inside the chamber into two 'cups' (seen in Figure A.2). The height of the waterbed was designed (with APLAC simulations) so that it effectively is a quarter wavelength long, thus giving maximal E-field coupling to the cells. The temperature rise in the cells, caused by the absorbed RF-radiation, is compensated

for by the circulating water underneath the petri dishes. The local energy absorbed by the cells is transferred away in much the same way as heat is dissipated in the human body by blood circulation.

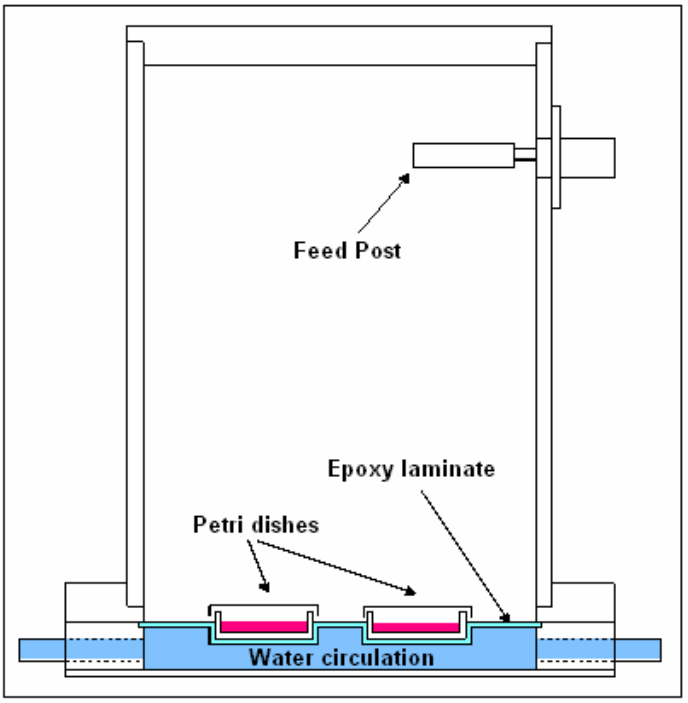

Figure A.2 Schematic drawing of the irradiation chamber. The petri dishes (diameter 55 mm) are placed in special 'cups' moulded into the epoxy laminate above the cooling water so that the medium (3 ml) is at same level than the cooling water. The water is covered with a 0.8 mm thick epoxy laminate.

A.1.2 Signal generator

The signal generator (ED-Laboratory SG1240) is used to generate the carrier signal. The frequency fed to the irradiation chamber depends on the loading of the chamber and is set so that the minimum amount of power is reflected back from chamber.

A.1.3 GSM-Modulator

The modulator in turn is connected to the signal generator. The GSM-modulator (STUK-KKS1) is used to modulate the carrier signal so that the output of the generator will match the GSM 900 MHz pulse sequence. A pulse repetition

frequency of 217 Hz (GSM modulation scheme: 0.577 ms pulse duration repeated every 4.615 ms) was used. The modulated signal is on for 1/8 of the time.

A.1.4 RF-power amplifier

The modulated signal (from the GSM modulator) generated by the signal generator is amplified by the RF-power amplifier (RF Power Labs R720F).

A.1.5 Circulator and coaxial termination

The circulator (MICA 7Y213) is used to protect the amplifier from overheating if too much power is reflected back from the chamber in the case of malfunctioning or a loose cable connection. The circulator guides the reflected power from the chamber to the Coaxial termination (50R50WCW) which turns the power to heat.

A.1.6 RF-power meter and power sensor

The RF-power meter (Coaxial Dynamics 81000-A,) is used to monitor the power fed into the chamber and power reflected back from the chamber. The power meter's sensor (820E875) shows the direction of the measured power with the arrow in front of the sensor. This means that the power fed to the chamber is measured when the arrow points to the cable connected to the chamber. When turning the sensor 180 degrees it measures the reflected power. The power meter is equipped with 2.5 W power sensor (820E875) which can measure both the forward power fed to the chamber and reflected power from chamber to the coaxial termination (50R50WCW).

A.2 SAR CALCULATION

The dosimetric evaluation of the irradiation chamber was performed at STUK (Radiation and Nuclear Safety Authority, Helsinki, Finland) and could be divided into three parts, namely, measurements, electromagnetic simulations, and thermodynamic simulations.

A.2.1 Field and Temperature measurements

SAR can be determined either by E-field measurements or by temperature rise measurements (Figure A.3) when the specific heat constant of the liquid is known. In

experiments, a total volume of 3 ml of medium per petri dish was used. E-field probes that can accurately measure electric fields in such a small volume were not commercially available at the time of measurement, thus E-field measurements were made in the air above the medium.

E-Field measurements: The E-field was measured in the air above the petri dishes. Measurement points were in the centreline of the chamber. The E-field was measured with a calibrated miniature E-field probe (Narda 8021B). E-field measurements were done at room temperature (22.5°C) and the corresponding computer simulations were done with permittivity values determined at the same temperature (measurements were also repeated with the SPEAG ET3DV6 probe and DASY4 software). Results are shown in Figure A.4.

Temperature Measurements: The absolute SAR-determination was based on temperature rise measurements done with a small Vitek-type temperature sensor. The sensor was calibrated with a calorimeter against a calibrated glass-capillar thermometer. During measurements, the sensor was placed at the centre of the petri dish at a height 1 mm from the bottom. The measurement set-up is shown in Figure A.3. Long-term temperature rise measurements were also done to evaluate the change in temperature of the cultivating medium during the exposure. Results are shown in Figure A.5.

A.2.2 Electromagnetic simulations

Computer simulations were done to evaluate the SAR-distribution inside the cultivating liquid, E-field distribution in cultivating liquid, and air and return loss of the chamber. The simulations were made using a finite difference time domain method (FDTD), with commercial SEMCAD 1.8 software (SPEAG, Switzerland). The simulation grid was graded and consisted of over 24 million cells. 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. Results are shown in Figures A.6.

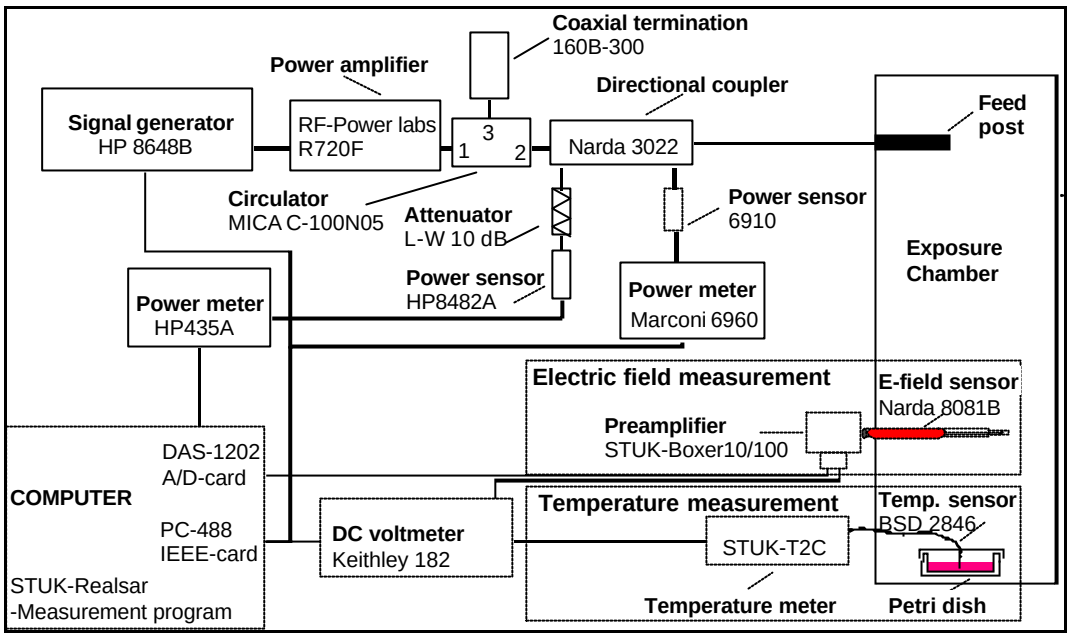

Figure A.3 Schematic diagram of temperature and electric field measurement set-up. In upper part is shown the RF-power generation and measurement part and below that, the electric field and temperature measurement parts.

A.2.3 Thermodynamic simulations

In order to investigate the accuracy of the thermal SAR measurements and to evaluate the thermal dose given to the cells, the temperature increase in the medium during irradiations was calculated using a thermal model. It was based on the conventional 3D heat transfer equation for non-homogeneous isotropic medium:

$$r\, c_p \frac{\partial T}{\partial t} = \nabla K \nabla T + r\, SAR \quad \text{, where}$$

ρ = 1000 m^3 kg^{-1} is the density, c_p = 4180 J kg^{-1} $^oC^{-1}$ is the specific heat capacity,

T is temperature (^oC), t is time (s), K= 0.56 W m^{-1} K^{-1} s^{-1} is the thermal conductivity.

Temperature measurements were performed with a thermistor sensor. The simulation and temperature measurement results are shown in Figure A7. There was a close agreement between the measured results compared to the computed results, which

indicates that the thermal model realistically describes the thermal properties of the system and increases confidence on thermal SAR assessments.

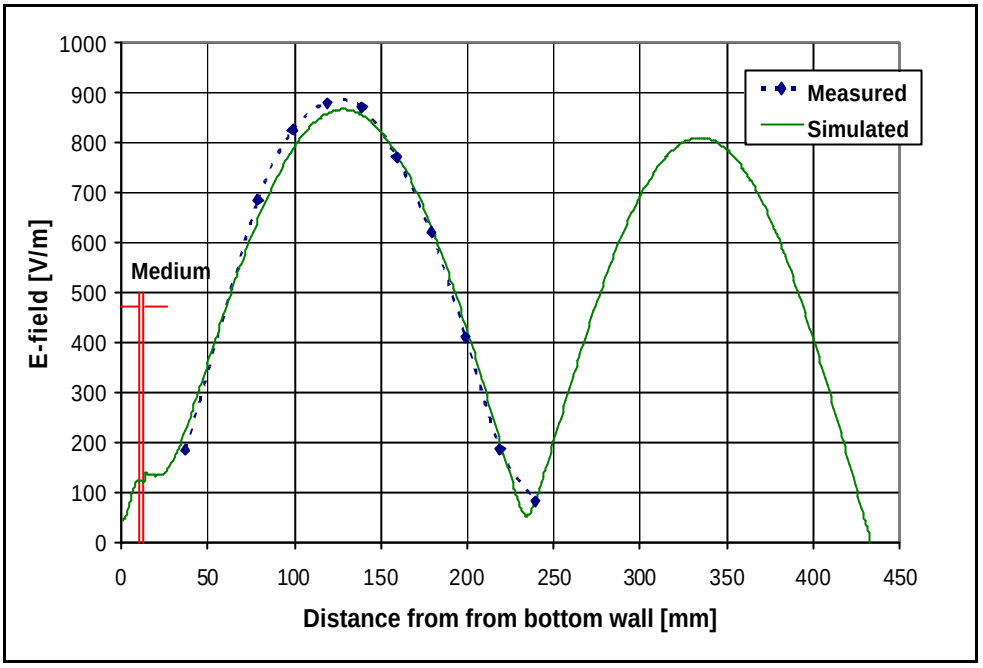

Figure A.4 Measured and simulated E-field in chamber. Measurements (blue diamonds) were made in air using the SPEAG ET3DV6 probe.

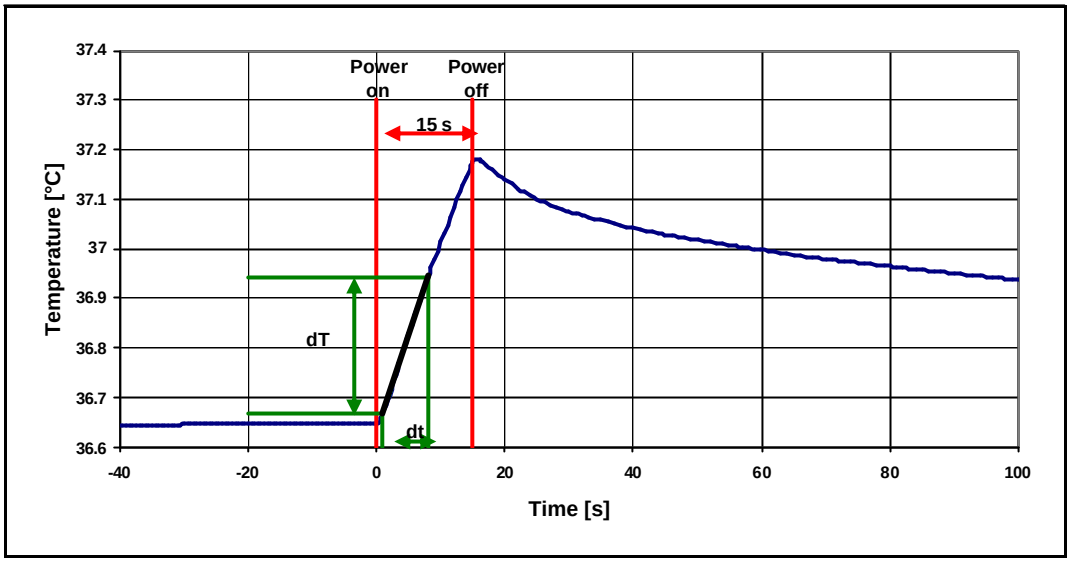

Figure A.5 Typical SAR measurements based on temperature increase using a Vitek type sensor. The SAR is evaluated from linearised temperature increase (dT) between the 1 to 8 seconds time (dt) after power on.

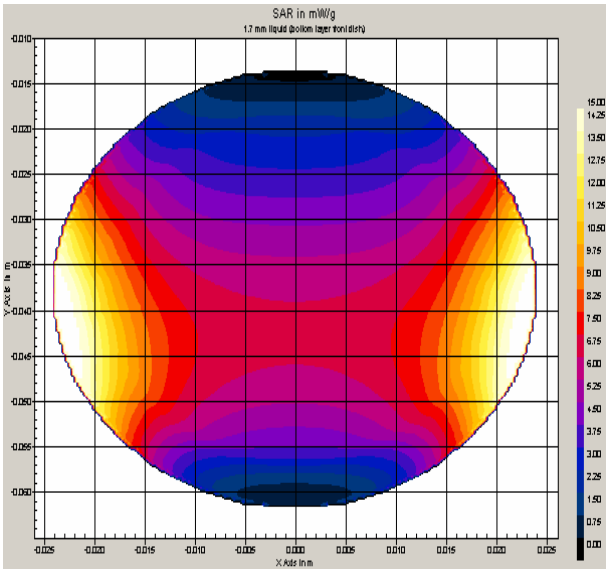

Figure A.6 Simulated (XFDTD) relative SAR distribution shown in one petri dish placed in the vertical chamber. The scale is given from 0 mW/g (black) to 15 mW/g (yellow). The inner diameter of petri dish is 50 mm.

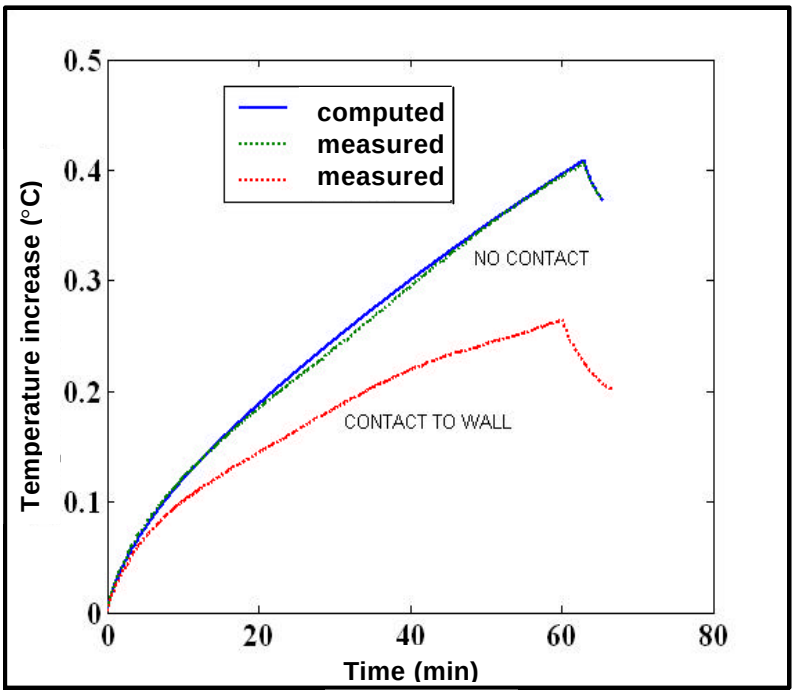

Figure A.7 Temperature increase measurements. The thermistor was either placed in the middle of the field or in contact with the petri dish wall.

The final SAR simulation and calculation results are summarized in Table A.1.

Table A.1. SAR distribution results used in experimentation.

dBm	Average SAR (W/kg)	Temperature increase during experiments (°C)
-17.6	5.6 W/kg	± 0.3°C
-13.2	2.0 W/kg	± 0.2 °C

A.3 MAGNETIC FIELD MEASUREMENTS

During experiments, two sets of petri dishes used for control exposures were simultaneously placed inside the CO_2 incubator while an additional two sets of petri dishes were exposed to 900 MHz GSM radiation. To determine the exact placing of the control petri dishes inside the CO_2 incubator without any magnetic interference arising from the RF-exposure or the magnetic field generated by the water-pump, a magnetometer (LEMI-011, Hermanus Magnetic Observatory) was used. The results are illustrated in Figure A.8. Placing of control petri dishes directly in front of the

vertical RF-chamber hatch closely resembled conditions when the same dishes were placed inside the RF-chamber during exposures.

Magnetic field determined by magnetometer:

(1) Magnetic Field > Earth's magnetic Field

(2) Magnetic Field ˜ Earth's magnetic Field

(3) RF power on and off-Magnetic Field ˜ Earth's Magnetic Field

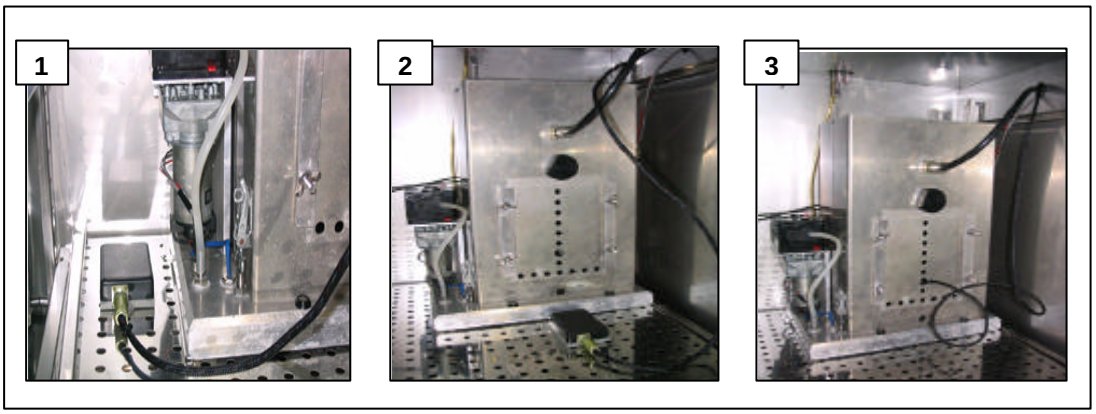

Figure A.8. Magnetometer measurement set-up. Placement of the magnetometer inside the CO_2 incubator in positions 2 and 3 closely resembled that of the earth's magnetic field.

A.4 REFERENCES

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ANNEXURE B: MACROSCOPIC AND MICROSCOPIC SPERM PARAMETERS

B.1 EVALUATION OF SPERMATOZOA

The initial step in the evaluation of spermatozoa is the determination of semen parameters followed by the assessment of sperm functionality and finally functionality is demonstrated with bioassays. The evaluation process of spermatozoa that was followed in this study is illustrated in Figure B.1. In addition to the initial macroscopic and microscopic evaluation of donors, morphology and motility were determined by computer aided sperm analysis (Chapter 3). In the following paragraphs the macroscopic and microscopic evaluation procedures followed in this study are briefly outlined.

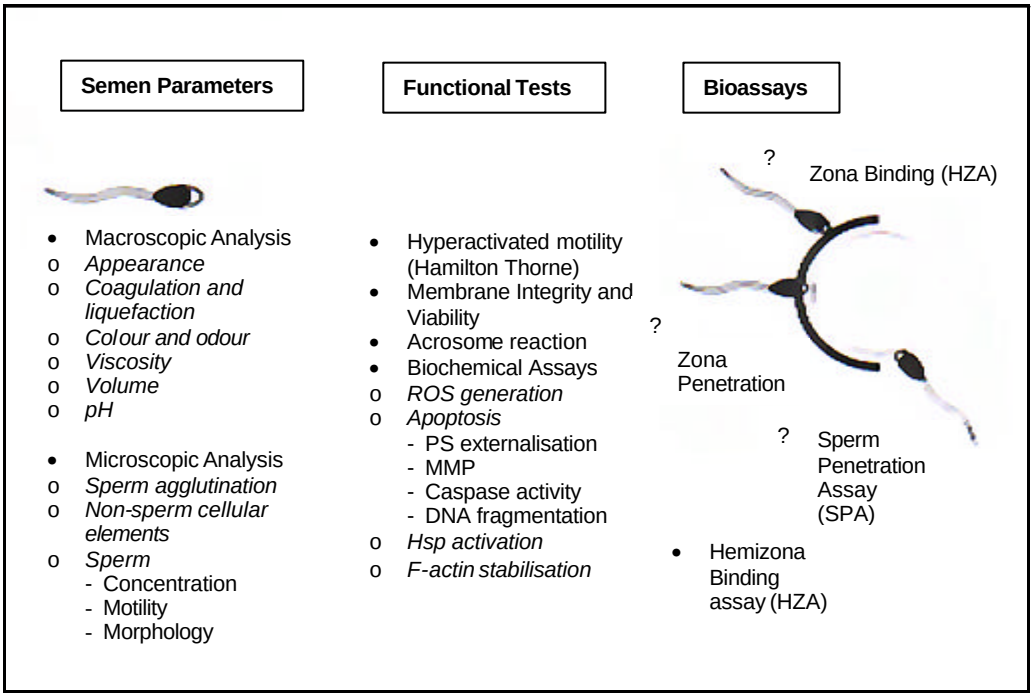

Figure B.1 Diagram illustrating different semen assessment parameters, functional tests and bioassays in the evaluation of human spermatozoa. (Adapted with permission from Oehninger *et al.*, 1991).

B.1.1 Semen parameters – macroscopic and microscopic evaluation

According to the World Health Organisation (WHO, 1999), standard human semen analysis generally assesses the semen volume, sperm concentration, the percentage of sperm showing forward progression (motility), and the percentage of sperm with normal morphology as analysed by light microscopy. There is no single sperm parameter that can accurately predict fertilisation potential, therefore a large number of diagnostic tests have been designed to determine the capability of a given individual's semen specimen to achieve fertilization of a human oocyte. These parameters as defined by the WHO (1999) are summarised in the following paragraphs.

After collection of a semen sample, semen parameters including macroscopic analysis (*appearance, coagulation and liquefaction, colour and odour, viscosity, volume and pH*) as well as microscopic analysis (*sperm agglutination - SpermMar IgG test, non-sperm cellular elements, concentration - Neubauer haemocytometer, motility - differential count, and morphology (Papanicolaou stain using Tygerberg strict criteria)*) were evaluated.

B.1.1.1 Macroscopic analysis

(i) Appearance

Normal semen consists of a mixture of spermatozoa suspended in secretions from the testis and epididymis, which at the time of ejaculation are combined with secretions from the prostate, seminal vesicles and bulbourethral glands (WHO, 1999). The appearance of normal semen when held against a light source is opaque (Jeyendran, 2003; Mortimer, 1994). Semen appearance has no apparent clinical value in human ejaculate analysis.

Method: The appearance (transparency) of the semen sample was noted. Semen was considered normal if it was opaque in appearance.

(ii) Coagulation and liquefaction

Immediately following ejaculation the specimen normally coagulates into a gelatinous mass and then liquefies (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994).

Normal specimens will require less that 60 min to liquefy at 37°C. A sample is considered abnormal if it takes longer than 120 min to liquefy at 37°C.

Method: Semen samples were left for 30 min to liquefy in an incubator at 37°C.

(iii) Colour and odour

Normal ejaculate colour ranges between whitish-grey, pearl white and yellowish (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994). A highly concentrated sample can be observed by the high degree of cloudiness, while a low concentrated sample will appear transparent and watery. Abnormal samples are pink, reddish, brown or yellow in colour.

Semen odour is distinctly pungent and has been compared to that of the flowers of the chestnut or St. John's beard tree. The odour is due to secretions from the prostate gland and a change in odour could be indicative of inflammation (Menkveld and Kruger, 1995). Semen colour and odour have no specific significance to sperm fertility evaluation but could be symptomatic of non-functioning accessory sexual glands (Jeyendran, 2003).

Method: The colour of the semen sample could indicate possible infection, therefore samples were considered normal when they were whitish–grey to light yellow in appearance.

(iv) Viscosity

Viscosity is a measure of the friction between various seminal fluid components as they slide by each other. This parameter is determined by appraising the length of thread formed when liquefied semen is dropped from a pipette (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994). A normal sample would form small, discrete droplets, whereas in abnormal cases a tread of more than 2 cm long could form when the sample leaves the pipette (WHO, 1999; Jeyendran, 2003). Furthermore, increased viscosity could lead to the inability to determine sperm quality objectively as it can interfere with the determination of sperm motility, concentration and antibody coating of spermatozoa (WHO, 1999; WHO Seminology workshop manual, 2001).

The relationship between viscosity and fertility is unknown; however high viscosity combined with poor sperm motility could lead to a decrease in fertilisation capacity (Jeyendran, 2003; Mortimer, 1994).

Method: Semen was dropped from a wide bore disposable pipette. Semen falling in small discrete droplets was considered normal, while semen forming a thread of more than 2 cm long was noted as very viscous.

(v) Volume

Volume is defined as the quantity of ejaculate a person produces. Semen volume is primarily determined by accessory gland secretion contributions (Seminal vesicle: 75%, Prostrate: 20%, Vas deferens, epididymis and testicles: 5% and bulbourethral gland: minuscule amount to coat urethra) (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994). The volume is measured to the nearest 0.1 ml with a 3-5 ml disposable syringe, graduated pipette or centrifuge tube (Jeyendran, 2003). The normal volume of an ejaculate is 2-6 ml after a sexual abstinence period of 3-5 days.

Method: The total volume of the semen sample was determined by a disposable graduated pipette.

(vi) pH

The pH of the semen sample is measured within one hour of collection using a special indicator paper (range 6.4-8.0). A drop of semen is placed on the indicator strip and after 30 seconds compared against a colour scale. Normal pH values range from 7.6 to 8.6, whereas pH values less than 7.2 or greater than 9.4 are noted as abnormal (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994).

Method: The pH of the sample was determined by placing 11.5 µl of semen on a strip of litmus paper (Merck, Germany, # 9557). The colour was then immediately compared against a colour scale.

B.1.1.2 Microscopic analysis

To evaluate sperm quality microscopic analysis assesses the agglutination of spermatozoa, the presence of cellular elements other than spermatozoa, concentration, motility and morphology.

(i) Sperm agglutination - SpermMar IgG test

The term agglutination is used to describe the clumping of sperm into aggregates. There are basically two types of agglutination, non-specific agglutination and site-specific agglutination. Non-specific agglutination describes the adherence of sperm to various seminal debris, leukocytes or mucus threads and various other non-sperm elements (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994). Site-specific agglutination on the other hand is used to indicate the adherence of sperm to each other in a site-specific manner, such as head-to-head, head-to-tail, tail-to tail, or any such combination (Jeyendran, 2003).

To determine agglutination status, wet microscopic smears are prepared and observed under a high-power field as described by Jeyendran (2003). Sperm is considered normal if no agglutination is observed. The sample is deemed equivocal if one or two clusters are observed and is regarded as abnormal when more than two clusters are observed per high-power field. The clinical significance of extensive agglutination is diminished availability of “free” sperm that could potentially inhibit fertilisation potential. Furthermore, site-specific agglutination could suggest immunological pathology and should be investigated by analysing anti-sperm antibodies. The SpermMar IgG test is designed indicate the presence of sperm antibodies reacting with antigens on spermatozoa. Non-specific agglutination may suggest an accessory gland infection.

Method: Site-specific agglutination of human spermatozoa was determined by the Sperm Mar IgG test (FertiproNV, Harrilabs, SA). The direct SpermMar test is performed by mixing 5 µl fresh untreated semen with 5 µl human IgG coated latex particles on a micro slide. Monospecific antihuman IgG antiserum (5 µl) was then immediately added to this mixture. The slide was then covered by a cover slip and observed under a light microscope using a 40x magnification. Agglutination will occur between IgG antibody positive spermatozoa and latex particles. If latex

particles attached to motile sperm were observed, a total of 100 sperm were counted and the percentage IgG antibody positive sperm scored.

(ii) Non-sperm cellular elements

Cellular elements such as leukocytes, erythrocytes, epithelial cells, microorganisms, and sperm precursors may be present in seminal fluid (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994). These elements are observed by investigating wet microscopic smears.

A high presence of either leukocytes, microorganisms and sperm precursors (spermatids, spermatocytes and spermatogonia) are usually associated with below normal sperm count and abnormal sperm morphology and may imply an overall reduction in fertility potential (Jeyendran, 2003).

Method: A wet smear was prepared by pipetting 11.5µl of the semen sample on a microscope slide the slide was then covered by a cover slip. The wet smear was evaluated under a light microscope at 40x magnification for the presence of non-sperm cellular elements.

(iii) Concentration - Neubauer haemocytometer

Sperm concentration is measured as the number of sperm per millimetre of seminal fluid, and the sperm count is the total number of sperm in the ejaculate (Jeyendran, 2003). The most accurate measurement method is by haemocytometer. This method requires the dilution of the ejaculate with media after which two separate preparations of the semen are evaluated on each side of the counting chamber (Mortimer, 1994; WHO, 1999; Jeyendran, 2003). The WHO (1999) recommends the Neubauer Haemocytometer (100 µm depth) above other counting chambers like the Microcell (20 µm depth) and Makler (10µm depth).

The sperm count is calculated as the product of the sperm concentration and ejaculate volume. A normal ejaculate contains more than 20.0 x 10^6 sperm per ml of semen, abnormal counts are noted if there are less than 10.0 x 10^6 sperm per ml of semen (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994).

Method: To count the concentration of sperm cells/ml, 50 µl of the semen was added to 950 µl trypan blue stain (Sigma Chemicals Co.) in a press top tube. The sperm were allowed to absorb the stain before both counting chambers of the Neubauer were loaded and left in a humid chamber for 10 min before scoring sperm counts in each chamber.

(iv) Motility - differential count

Sperm motility is defined as the observation of spontaneous sperm movement and is a significant factor when evaluating fertility potential (Mortimer and Mortimer, 1999; Jeyendran, 2003). It is determined by noting the ratio of motile progressive sperm to total sperm number (in a given volume) and is expressed as a percentage (Jeyendran, 2003). The WHO (1999) recommends a simple grading system for the manual evaluation of motility, however other methods such as the computer aided sperm analysis (CASA) method, although requiring more complex equipment affords a more accurate assessment. Both these methods were used in the study to evaluate sperm motility.

Sperm motility as recommended by the WHO (1999) is estimated by microscopic examination. A wet preparation is made by placing approximately 10 µl of thoroughly mixed, liquefied semen on a warm microscope slide and covering it with a warm 22 x 22 mm cover-slip (sperm motility is temperature dependent and is therefore assessed under controlled thermal conditions) (Mortimer and Mortimer, 1999; WHO, 1999). At least four to five microscopic fields are systematically examined by classifying motility parameters of no less than 200 spermatozoa (WHO, 1999; Jeyendran, 2003). Each spermatozoon is graded according to:

- Rapid progressive motility (a)
- Slow or sluggish progressive motility (b)
- Non-progressive motility (c)
- Immotility (d)

Within 60 min of ejaculation a normal sample contains 50% or more motile sperm (grades a + b) or 25% or more progressive motility (grade a) (WHO, 1999). Abnormal samples will show less than 35% overall sperm motility.

Method: The motility of unprocessed semen was determined by pipetting 11.5 µl of semen unto on a microscope slide, which was then covered by a cover slip. The slide was evaluated under a light microscope at 40x magnification and a total of 100 sperm counted with a differential counter by categorising the sperm according to WHO (1999) criteria (grades a-d). Percentages were scored and the total motility of the sample taken as the sum of the percentages from grade a and b motility.

(v) Morphology (Papanicolaou stain using Tygerberg strict criteria)

Sperm morphology defines the shape, size and surface appearance of sperm (Jeyendran, 2003). Sperm morphology is assessed by microscopic evaluation of a stained semen smear on a dry slide at high magnification and is by its very nature, highly subjective (Jeyendran, 2003; Jequier, 2000; Mortimer, 1994). The Papanicolaou stain is the preferred method used in andrology laboratories and is also recommended by the WHO (1999). Wet smears are prepared and air-dried on a warm plate before being stained (Mortimer, 1994; Jeyndran, 2003). After staining no less than 200 sperm are counted at high magnification (100x) under oil immersion using bright field optics (Mortimer and Mortimer, 1999; WHO, 1999). Sperm morphology is assessed by the Tygerberg strict criteria (Menkveld *et al*. 1990; Mortimer and Mortimer, 1999).

Method: The evaluation of sperm morphology was made according to the method described by Kruger *et al*. (1986). To determine the percentage of morphologically normal spermatozoa a wet smear was prepared by pipetting 11.5 µl of the semen sample onto a micro slide. The smear was made by pulling the semen droplet evenly along the slide (if the sample was very concentrated the droplet was smeared at a 45° angle, low concentrations were smeared at an angle of 10-30°). The slide was then allowed to air dry at room temperature. Slides were stained using a modified Papanicolau staining procedure before being mounted with DABCO.

The staining procedure is briefly summarized:

The slides were left for one day to allow the DABCO (Sigma Chemical Co.) to set, before being analysed under a light microscope by using a 100x oil immersion lens. A total of 200 spermatozoa were counted and classified as normal according to the Tygerberg strict criteria when; the head had a smooth oval configuration, the acrosome comprised 40-70% of the sperm head, no head, neck, midpiece or tail abnormalities were noted. All other sperm were scored as abnormal.

After noting all the semen parameters for each individual donor, the semen samples were processed using a density gradient centrifugation procedure. Since components from seminal fluid have been reported to influence sperm motility (Somlev *et al.*, 1997; Katkov and Mazur, 1998) experiments were conducted using processed sperm.

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ANNEXURE C

DONOR SPERM PARAMETERS - RESULTS

C.1 MACROSCOPIC SEMEN PARAMETERS

All samples were taken from healthy donors and no deviations in appearance, colour, and odour were noted. In addition, no abnormal coagulation was noted in semen samples and samples did not require an extended period to liquefy. Furthermore, viscosity of samples were considered normal (none of the samples were extremely viscous). The total volume and pH of the ejaculate for each of the twelve donors are illustrated in Figure C.1, while averages are summarised in Table C.1.

C.2 MICROSCOPIC SEMEN PARAMETERS

According to the WHO criteria (1999), normal semen parameters are defined as 'a+b' type motility or progressive motility ≥ 50% and 'a' type motility or forwards motility ≥ 25%; sperm cell concentration ≥ 20 x 10^6 cells/ml; and sperm cells with morphology < 15% abnormal forms. Based on these criteria, semen profiles were classified into three categories namely: **A** (n = 4) - normal motility and concentration with < 5% normal morphology, **B** (n = 5) - normal motility and concentration with between 5% - 10% normal morphology, and **C** (n = 3) - normal motility and concentration with > 10% normal morphology. All samples used had less than 1 x 10^6 leukocytes/ml (this was also confirmed with CD45 staining – for reference see Chapter 3) and were MAR negative. The motile sperm concentration after density purification of the total sample (total ejaculate) was ≥ 40 x 10^6 /ml in order to provide sufficient number of cells for experimentation. This formed the criteria by which donors were selected for participation in the study.

Sperm parameters determined for each of the 12 donors are presented in Figure C.1 A-E.

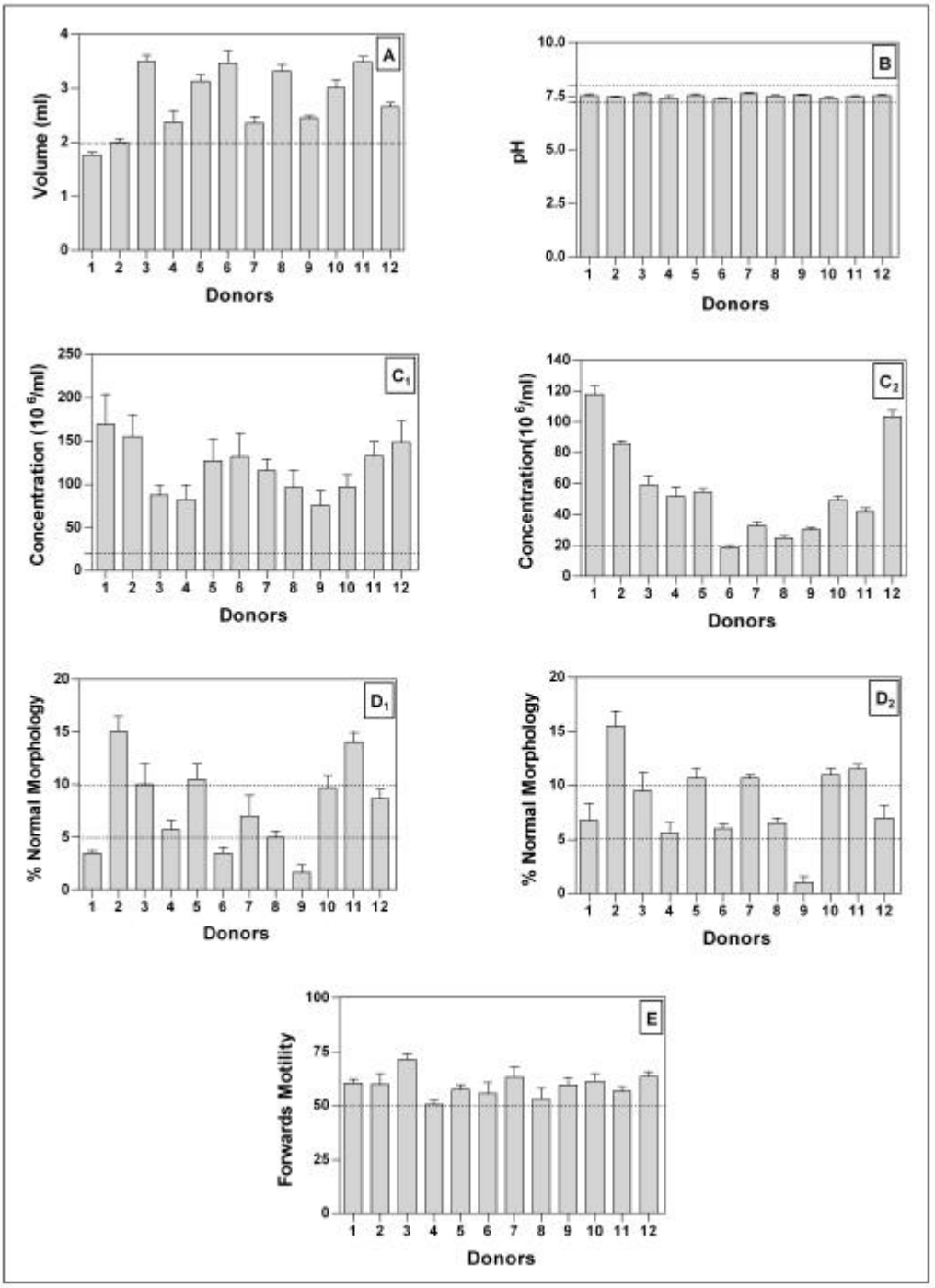

Figure C.1 Sperm parameters determined for each donor. Each end point represents the mean and standard deviation (SD) of at least 5 replicates. In all graphs WHO reference values are noted with dotted lines. **(A)** Total volume; **(B)** pH; **(C1)** Concentration pre-processing; **(C2)** Concentration post-processing; **(D1)** Morphology pre-processing; **(D2)** Morphology post-processing and **(E)** Forwards motility, of each semen sample.

A summary of the average sperm parameters obtained for all 12 donors (volume, pH, concentration, morphology, and motility) are noted in Table C.1. Average values for the volume, pH, and motility were obtained from the total semen sample pre density gradient purification. Both the concentration and morphology averages are supplied for pre- and post- density gradient purification.

Table C.1 Average sperm parameters: Volume, pH, and motility averages ± SD of the semen sample is noted. Concentration and morphology averages ± SD are given both pre- and post- density gradient purification.

Parameter	Pre-Average ± SD	Post-Average ± SD
Volume (ml)	2.83 ± 0.34	-
pH	7.49 ± 0.08	-
Motility (%)	59.38 ± 5.43	-
Concentration (10^6/ml)	118.21 ± 30.75	55.32 ± 30.65
Morphology	7.85 ± 4.20	8.5 ± 3.80

C.3 REFERENCES

WHO (World Health Organisation) 1999. *Laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. Cambridge, UK: Cambridge Univ Press, 4th Edition.