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).

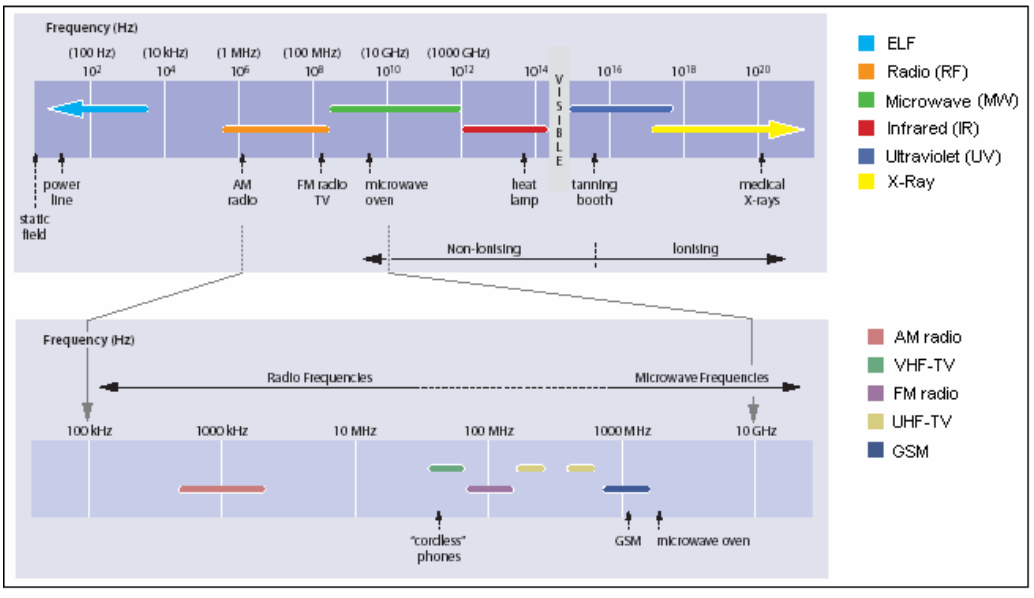

Figure 1.1 Triage of the Electromagnetic spectrum indicating the position of mobile phone emissions (Adapted with permission from the EU Commission, Health and Electromagnetic fields, 2005).

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.

1.6 REFERENCES

Adair, R.K. 2002. Vibrational resonances in biological systems at microwave frequencies. *Biophys J*., 82, 1147-52.

Adair, R.K. 2003. Biophysical limits on athermal effects of RF and microwave radiation. *Bioelectromagnetics*., 24, 39-48.

Aitken, R.J., Bennetts, L.E., Sawye, R. D., Wiklendt, A.M., King, B.V. 2005. Impact of radio-frequency electromagnetic radiation on DNA integrity in the male germline. *Int J Androl*., 28, 171-9.

Antipenko, E.N., Koveshnikova, I.V. 1987. Cytogenetic effects of microwaves of non-thermal intensity in mammals. *Dokl Akad Nauk SSSR*., 296, 724.

Apollonio, F., D'Inzeo, G., Tarricone, L. 1998. Modelling of neuronal cells exposed to RF fields from mobile telecommunication equipment. *Bioelectrochem Bioenerget*., 47, 199-205.

Balode, Z. 1996. Assessment of radio-frequency electromagnetic radiation by the micronucleus test in Bovine peripheral erythrocytes. *Sci Total Environ*., 180, 81-7.

Beechey, C.V., Brooker, D., Kowalczuk, C.I., Saunders, R.D., Searle, A.G. 1986. Cytogenetic effects of microwave irradiation on male germ cells of the mouse. *Int J Radiat Biol*., 50, 909-16.

Beere, H.M. 2005. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Invest*., 115, 2633-9.

Berman, E., Carter, H.B., House, D. 1980. Tests for mutagenesis and reproduction in male rats exposed to 2450 MHz CW microwaves. *Bioelectromagnetics*., 1, 65-9.

Bisht, K.S., Moros, E.G., Straube, W.L., Baty, J.D., Roti Roti, J.L. 2002. The effect of 835.62 MHz FDMA or 847.74 MHz CDMA modulated radiofrequency radiation on the induction of micronuclei in C3H 10T1/2 cells. *Rad Res*., 157, 506-15.

Bit-Babik, G., Chou, C.K., Faraone, A., Gessner, A., Kanda, M., Balzano, Q. 2003. Estimation of the SAR in the human head and body due to radiofrequency

radiation exposure from handheld mobile phones with hands-free accessories. *Rad Res.*, 159, 550-7.

Blanchard, J., Blackman, C.F. 1994. Clarification and application of an ion parametric resonance model for magnetic field interactions with biological systems. *Bioelectromagnetics.*, 15, 205- 16.

Blank, M. (ed.) 1995a. *Electromagnetic fields: Biological interactions and mechanisms.* Washington DC: American Chemical Soc.

Blank, M. 1995b. Na/K-adensine-triphosphatase. *Adv Chem.*, 250, 339-48.

Bray, F., Richiardi, L., Ekbom, A., Pukkala, E., Cuninkova, M., Moller, H. 2006. Trends in testicular cancer incidence and mortality in 22 European countries: continuing increases in incidence and declines in mortality. *Int J Cancer.*, 118, 3099-11.

Capri, M., Scarcella, E., Fumelli, C., Bianchi, E., Salvioli, S., Mesirca, P., Agostini, C., Antonili, A., Schiavoni, A., Catellani, G., Bersani, F., Franceschi, C. 2004. In vitro exposure of human lymphocytes to 900 MHz CW and GSM modulated radiofrequency: studies of proliferation, apoptosis and mitochondrial membrane potential. *Rad Res.*, 162, 211-8.

Caraglia, M., Marra, M., Mancinelli, F., D'Ambrosio, G., Massa, R., Giordano, A., Budillon, A., Abbruzzese, A., Bismuto, E. 2005. Electromagnetic fields at mobile phone frequency induce apoptosis and inactivation of the multi-chaperone complex in human epidermoid cancer cells. *J Cell Physiol.*, 204, 539-48.

Chiabrera, A., Bianco, B., Moggia, E., Kaufman, J.J. 2000. Zeeman-stark modeling of the RF EMF interaction with ligand binding. *Bioelectromagnetics.*, 21, 312-24.

COST 244. 1996. *Design of exposure systems for in vitro and in vivo RF experiments.* Position document. P. Raskmark, B. Veyret, eds. Brussels: European Union (DG XIII).

COST 281. 2003. *Potential health implications from mobile telecommunication systems.* European co-operation in the field of scientific and technological research. Available from: http://www.COST281.org. [Accessed 25/02/2004].

Cotgreave, I.A. 2005. Biological stress responses to radio frequency electromagnetic radiation: are mobile phones really so (heat) shocking? *Arch Biochem Biophys.*, 435, 227-40.

Creagh, E.M., Sheehan, D., Cotter, T.G. 2000. Heat shock proteins – modulators of apoptosis in tumour cells. *Leukemia*., 14, 1161-73.

Czyz, J., Guan, K., Zeng, Q., Nikolova, T., Meister, A., Schonborn, F., Schuderer, J., Kuster, N., Wobus, A.M. 2004. High frequency electromagnetic fields (GSM signals) affect gene expression levels in tumour suppressor p53-deficient embryonic stem cells. *Bioelectromagnetics*., 25, 296-307.

D'Ambrosio, G., Massa, R., Scar., M.R., Zeni, O. 2002. Cytogenetic damage in human lymphocytes following GMSK phase modulated microwave exposure. *Bioelectromagnetics*., 23, 7-13.

Dasdag, S., Ketani, M.A., Akdag, Z., Ersay, A.R., Sari, I., Demirtas, O.C., Celik, M.S. 1999. Whole-body microwave exposure emitted by cellular phones and testicular function of rats. *Urol Res*., 27, 219-23.

Davoudi, M., Brössner, C., Kuber, W. 2002. The influence of electromagnetic waves on sperm motility. *J Urol Urogynaco*., 9, 18-22.

DiCarlo, A., White, N., Guo, G., Garrett, P., Litovitz, T. 2002. Chronic electromagnetic field exposure decreases HSP70 levels and lowers cytoprotection. *J Cell Biochem*., 84, 447-54.

Diem, E., Schwarz, C., Adlkofer, F., Jahn, O., Rüdiger, H. 2005. Non-thermal DNA breakage by mobile phone radiation (1800 MHz) in human fibroblasts and in transformed GFSH-R17 rat granulosa cell in vitro. *Mutat Res*., 583, 178-83.

Donnellan, M., McKenzie, D.R., French, P.W. 1997. Effects of exposure to electro-magnetic radiation at 835 MHz on growth, morphology and secretory characteristics of a mast cell analogue, RBL-2H3. *Cell Biol Int*., 21, 427-39.

Duesberg, P., Rasnick, D. 2000. Aneuploidy, the somatic mutation that makes cancer a species of its own. *Cell Motil Cyto.*, 47, 81-107.

Duesberg, P., Stindl, R., Hehlmann, R. 2000. Explaining the high mutation rates in cancer cells to drug and multi-drug resistance by chromosome reassortment that are catalyszed by aneuploidy. *Proc Natl Acad Sci*., 97, 14295-300.

Erogul, O., Oztas, E., Yildirim, I., Kir, T., Aydur, E., Komesli, G., Irkilata,, H.C., Irmak, M.K., Peker, A.F. 2006. Effects of electromagnetic radiation from cellular phone on human sperm motility: an in vitro study. *Arc Med Res*., 37, 840-3.

European Commission - Research directorate - European Communities. 2005. Health and electromagnetic fields. Available from: http://www.Publications.eu.int. [Accessed 17/10/2006].

Fejes, I., Závaczki, Z., Szöllõsi, J., Koloszár, S., Daru, J., Kovács, L., Pal, A. 2005. Is there a relationship between cell phone use and semen quality? *Arch Androl*., 51, 385-93.

Forgacs, Z., Kubinyi, G., Sinay, G., Bakos, J., Surjan, A., Revesz, C., Thuroczy, G. 2005. Effects of 1800MHz GSM-like exposure on the gonadal function and haematological parameters of male mice. *Magy Onkol*., 49, 149-51.

French, P.W., Donnellan, M., McKenzie, D.R. 1997. Electromagnetic radiation at 835 MHz changes the morphology and inhibits proliferation of a human astrocytoma cell line. *Bioelectrochem Bioenerget*., 43, 13-8.

French, P.W., Penny, R., Laurence, J.A. 2000. Mobile phones, heat shock proteins and cancer. *Differentiation*., 67, 93-7.

Fritze, K., Wiessner, C., Kustner, N., Sommer, C., Gass, P., Hermann, D.M., Kiessling, M., Hossman, K.A. 1997. Effect of global system for mobile communication microwave exposure on the genomic response of the rat brain. *Neuroscience*., 81, 627-39.

Fröhlich, H. 1980. The biological effects of microwaves and related questions. *Adv Electronics Electron Phys*., 53, 85.

Fröligh, H. (ed.). 1988. *Biological coherence and response to external stimuli*. Berlin: Springer-Verlag.

Garaj-Vrhovac, V., Horvat, D., Koren, Z. 1990a. The effect of microwave radiation on the cell genome. *Mutat Res*., 243, 8.

Garaj-Vrhovac, V., Horvat, D., Koren, Z. 1990b. Comparison of chromosome aberration and micronucleus induction in human lymphocytes after occupational exposure to vinyl chloride monomer and microwave radiation. *Periodicum Biologorium*., 92, 411-8.

Garrido, C.P., Ottavi, A., Fromentin, A., Hammann, A., Arrigo, A.P., Chauffert, B., Mehlen, P. 1997. Hsp27 as a mediator of confluence-dependent resistance to cell death induced by anticancer drugs. *Cancer Res*., 57, 2661-7.

Garson, O.M., McRobert, T.L., Campbell, L.J., Hocking, B.A., Gordon, I. 1991. A chromosomal study of worked with long-term exposure to radio-frequency radiation. *Med J Aust*., 155, 289-96.

Goodman, R., Blank, M. 2002. Insights into electromagnetic mechanisms. *J Cell Physiol*., 192, 16-22.

Goswami, P.C., Albee, L.D., Parsian, A.J., Baty, J.D., Moros, E.G., Pickard, W.F., Roti Roti, J.L., Hunt, C.R. 1999. Protooncogene mRNA levels and activities of multiple transcription factors in C3H10T½ murine embryonic fibroblasts exposed to 835.62 and 847.74 MHz cellular phone communication frequency radiation. *Rad Res*., 151, 300-9.

Goud, G.N., Usha Rani, M.U., Reddy, P.P., Reddi, O.S., Rao, M.S., Saxena, V.K. 1982. Genetic effects of microwave radiation in mice. *Mutat Res*., 103, 39-47.

Grundler, W., Kaiser, F. 1992. Experimental evidence for coherent excitations correlated with cell growth. *Nanobiology*., 1, 163-76.

Grundler, W., Kaiser, F., Keilman, F. 1992. Mechanisms of electromagnetic interaction with cellular systems. *Naturwissenschaften*., 79, 551-9.

Hardell, L., Hallquist, A., Mild, K.H., Carlberg, M., Pahlson, A., Lilja, A. 2002. Cellular and cordless telephones and the risk for brain tumours. *Eur J Cancer Prev*., 11, 377-86.

Hardell, L., Mild, K.H., Calberg, M. 2003. Further aspects on cellular and cordless telephones and brain tumours. *Int J Oncol*., 22, 399-407.

Hardell, L., Carlberg, M., Hansson Mild, K. 2005a. Case-control study on cellular and cordless telephones and the risk for acoustic neuroma or meningioma in patients diagnosed 2000-2003. *Neuroepidemiology*, 25, 120-8.

Hardell, L., Carlberg, M., Hansson Mild, K. 2005b. Case-control study of the association between the use of cellular and cordless telephones and malignant brain tumours diagnosed during 2000-2003. *Environ Res*., 100, 232-41.

Hardell, L., Carlberg, M., Ohlson, C.G., Westberg, H., Eriksson, M., Hansson Mild, K. 2006. Use of cellular and cordless telephones and risk of testicular cancer. *Int J Androl*., 1-8.

Harvey, C., French, P.W. 1999. Effects on protein kinase C and gene expression in a human mast cell line, HMC-1, following microwave exposure. *Cell Biol Int*., 23, 739-48.

Hocking, B. 2003. Update on mobile phones and health. *Int Med J*., 33, 235-6.

Hook, G.J., Zhang, P., Lagroye, J., Li, L., Higashikubo, R., Moros, H.G., Straube, W.L., Picard, W.F., Baty, J.D., Roti Roti, J.L. 2004. Measurement of DNA damage and apoptosis in Molt-4 cells after in vitro exposure to radiofrequency radiation. *Rad Res*., 161, 193-200.

Huot, J., Houle, F., Spitz, D.R., Landry, J. 1996. HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer Res*., 56, 273-9.

Hyland, G.J. 2000. Physics and biology of mobile telephony. *Lancet*., 356, 1833-36.

Hyland, G.J. 2001. *The physiological and environmental effects of non-ionising electromeagnetic radiation*. European Parliament, Directorate General for Research, Directorate A, The STOA Programme. PE 297.574/Fin.St.

IARC (International Agency for Research on Cancer). 2002. *Monographs on the Evaluation of carcinogenic Risks to Humans*, Volume 80. Non-Ionizing Radiation, Part 1: Static and extremely low frequency (ELF) electric and magnetic fields. Lyon: IARC Press.

ICNIRP (International Commission on Non-Ionizing Radiation Protection). 1998. Guidelines for limiting exposure in time-varying electric, magnetic, and electromagnetic fields (up to 300 GHz). *Health Phys.*, 74, 494-522. Available from: http://www.icnirp.de. [Accessed 22/08/2003].

IEGMP. 2000. *Mobile Phones and Health. Report of the Independent Expert Group on Mobile Phones*, Chairman: Sir. William Stewart, National Radiation Protection Board (NRPB), London: Chilton,. Available from: http://www.iegmp.org.uk. [Accessed 09/01/2001].

IEGEMF. 2007. Recent research on EMF and health risks. Fourth annual report from SSI's Independent Expert Group on Electromagnetic Fields. Stockholm: Statens Strålskyddsinstitut.

Irmak, M.K., Fadillioglu, E., Guleg, M., Erdogan, H.M., Yagmurca, M., Akol. 2002. Effects of electromagnetic radiation from a cellular telephone: the oxidant and antioxidant levels in rabbits. *Cell Biochem Funct*., 20, 279-83.

Ivaschuck, O.I., Jones, R.A., Ishida-Jones, T., Haggren, W., Adey, W.R., Phillips, J.I. 1997. Exposure of nerve growth factor-treated PC-12 rat pheochromocytoma cells to a modulated radiofrequency field at 836.55 MHz: effects on *c-jun* and *c-fos* expression. *Bioelectromagnetics*., 18, 223-41.

Jokela, K., Leszczynski, D., Paile, W., Salomaa, S., Puranen, L., Hyysalo, P. 1999. Radiation safety of handheld mobile phones and base stations. STUK-A161 Report. OY Helsinki: Edita Ab.

Killgallon, S.J., Simmons, L.W. 2005. Image content influences men's semen quality. *Biol Lett*., 1, 253-5.

Kotnik, T., Miklavcic, D. 2000a. Second-Order model of membrane electric field induced by alternating external electric fields. IEEE transactions on biomedical engineering., 47, 1074-81.

Kotnik, T., Miklavcic, D. 2000b. Theoretical evaluation of the distributed power dissipation in biological cells exposed to electric fields. *Bioelectromagnetics*., 21, 385-94.

Kwee, S., Raskmark, P., Velizarov, S. 2001. Changes in cellular proteins due to environmental non-ionizing radiation. I Heat shock proteins. *Electro-Magnetobiology*., 20, 1061-72.

Lagroye, I., Hook, G.J., Wettring, B.A., Baty, J.D., Moros, E.G., Straube, W.L., Roti Roti, J.L. 2004a. Measurements of alkali-labile DNA damage and protein –DNA crosslinks after 2450 MHz microwave and low-dose gamma irradiation *in vitro*. *Rad Res*., 161, 201-14.

Lagroye, I., Anane, R., Wettring, B.A., Moros, E.G., Straube, W.L., La Regina, M., Niehoff, M., Pickard, W.F., Baty J., Roti Roti, J.L. 2004b. Measurement of DNA damage after acute exposure to pulsed-wave 2450 MHz microwaves in rat brain cells by two alkaline comet assay methods. *Int J Rad Biol*., 80, 11-20.

Lai, H., Singh, N.P. 1995. Acute low-intensity microwave exposure increases DNA single-strand breaks in rat brain cells. *Bioelectromagnetics*., 16, 207-10.

Lai, H., Singh, N.P. 1996. Single- and double-strand DNA breaks in rat brain cells after acute exposure to radiofrequency electromagnetic radiation. *Int J Rad Biol.*, 69, 513-21.

Lantow, M., Viergutz, T., Weiss, D.G., Simkó, M. 2006a. Comparative study of cell cycle kinetics and induction of apoptosis or necrosis after exposure of human mono mac 6 cells to radiofrequency radiation. *Radiat Res.*, 166, 539-43.

Lantow, M., Schuderer, J., Hartwig, C., Simkó, M. 2006b. Free radical release of Hsp70 expression in two human immune-relevant cell lines after exposure to 1800MHz radiofrequency radiation. *Radiat Res.*, 165, 88-94.

Lednev, V.V. 1991. Possible mechanisms for influence of weak magnetic fields on biosystems. *Bioelectromagnetics*., 12, 71-6.

Leszczynski, D., Joenväärä, S., Reivinen, J., Kuokka, R. 2002. Non-thermal activation of the hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanism for cancer- and blood brain barrier-related effects. *Differentiation*, 70, 120-9.

Li, L., Bisht, K.S., LaGroye, I., Zhang, P., Straube, W.L., Moros, E.G., Roti Roti, J.L. 2001. Measurement of DNA damage in mammalian cells exposed in vitro to radiofrequency fields at SARs of 3-5 W/kg. *Radiat Res*., 156, 328-32.

Liboff, A.R., Rozek, R.J., Sherman, M.L., McLeod, B.R., Smith, S.D. 1987. Ca^{2+} cyclotron resonance in human lymphocytes. *J Bioelectricity.*, 6, 12-22.

Lim, H.B., Cook, G.G., Barker, A.T., Coulton, L.A. 2005. Effects of 900MHz electromagnetic fields on nonthermal induction of heat shock proteins in human leukocytes. *Radiat Res*., 163, 45-52.

Linet, M.S., Hatch, E.E., Kleinerman, R.A., Robinson, L.L., Kaune, W.T., Friedman, D.R., Severson, R.K., Haines, C.M., Hartsock, C.T., Niwa, S., Wacholder, S., Tarone, R.E. 1997. Residential exposure to magnetic fields and acute lymphoblastic leukemia in children. *New Eng J Med*., 337, 1-7.

Lui, D.Y., Baker, H.W. 1992. Tests of human sperm function and fertilization *in vitro*. *Fertil Steril*., 58, 465-82.

Maes, A., Verschaeve, L., Arroyo, A., De Wagter, C., Vercruyssen. L. 1993. *In vitro* cytogenetic effects of 2450 MHz waves on human peripheral blood lymphocytes. *Bioelectromagnetics*., 14, 495-503.

Maes, A., Collier, M., Slaets, D., Verschaeve, L. 1995. Cytogenetic effects of microwaves from mobile communication frequencies 954 MHz. *Electro-Magnetobiology*., 14, 91-101.

Malyapa, R.S., Ahern, E.W Straube, W.L., Moros, E.G., Pickard, W.F., Roti Roti, J.L. 1997a. Measurement of DNA damage following exposure to 2450 MHz electromagnetic radiation. *Radiat Res*, 148, 608-17.

Malyapa, R.S., Ahern, E.W., Straube, W.L., Moros, E.G., Pickard, W.F., Roti Roti, J.L. 1997b. Measurement of DNA damage following exposure to electromagnetic radiation in the cellular communications frequency band (835.62 and 847.74 MHz). *Radiat Res*, 148, 618-25.

Malyapa, R.S., Ahern, E.W., Bi, C., Straube, W.L., LaRegina, M., Pickard, W.F., Roti Roti, J.L. 1998. DNA damage in rat brain cells after *in vivo* exposure to 2450 MHz electromagnetic radiation and various methods of euthanasia. *Radiat Res*, 149, 637-46.

Manikowska-Czerska, E., Czerski, P., Leach, W.M. 1985. Effects of 2.45 GHz microwaves on meiotic chromosomes of male CBA/CAY mice. *J Hered.*, 76, 71.

Marinelli, F., La Sala, D., Cicciotti, G., Cattini, L., Trimarchi, C., Putti, S., Zamparelli, A., Giuliani, L., Tomassetti , G., Cinti, C. 2004. Exposure to 900 MHz electromagnetic field induces an unbalance between pro-apoptotic and pro-survival signals in T-lymphoblastoid leukaemia CCRF-CEM cells. *J Cell Physiol*., 198, 324-32.

Mashevich, M., Folkman, D., Kesar, A., Barbul, A., Korenstein, R., Jerby, E., Avivi, L. 2003. Exposure to human peripheral blood lymphocytes to electromagnetic fields associated with cellular phones leads to chromosomal instability. *Bioelectromagnetics*., 24, 82-90.

Mehlen, P., Schulze-Osthoff, K., Arrigo, A.P. 1996. Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. *J Biol Chem*., 271, 16510-4.

Michaelis, J., Schüz, J., Meinert, R., Menger, M., Grigat, J.P., Kaatsch, P., Kaletsch, U., Miesner, A., Stamm, A., Brinkmann, K., Kärner, H. 1997. Childhood leukemia and electromagnetic fields: results of a population-based case-control study in Germany. *Cancer Causes Control*., 8, 167-74.

Mickley, G.A., Cobb, B.L., Mason, P.A., Farrell, S. 1994. Disruption of a putative working memory task and selective expression of brain *c-fos* following microwave-induced hyperthermia. *Physiol Behav*., 55, 1029-37.

Miyakoshi, J., Takemasa, K., Takashima, Y., Ding, G.R., Hirose, H., Koyama, S. 2005. Effects of exposure to a 1950 MHz radio frequency field on expression of Hsp70 and Hsp27 in human glioma cells. *Bioelectromagnetics*., 26, 251-7.

MMF (Mobile Manufacturers Forum). 2001. Mechanisms for interactions of Radiofrequency Energy with Biological systems: Principal conclusion from a seminar held in Washington DC.

Morrissey, J.J., Raney, S., Heasley, E., Rathinavelu, P., Dauphnee, M., Fallon, J.H. 1999. Iridium exposure increases c-*fos* expression in the mouse brain only at levels which likely result in tissue heating. *Neuroscience*., 92, 1539.

Moulder, J.E., Erdreich, L.S., Malyapa, R.S., Merritt, J., Picard W.F., Vijayalaxmi, D.Z. 1999. Cell phones and cancer: what is the evidence for a connection? *Rad Res*., 151, 513-31.

Moulder, J. E., Foster, K. R., Erdreich, L. S., Mcnamee, J. P. 2005. Mobile phones, mobile phone base stations and cancer: a review. *Int J Rad Biol*., 81, 189-203.

Nikolova, T., Czyz, J., Rolletschek, A., Blyszczuk, P., Fuchs, J., Jovtchev, G., Schuderer, J., Kuster, N., Wobus, A.M. 2005. Electromagnetic fields affect transcript levels of apoptosis-related genes in embryonic stem cell derived neural progenitor cells. *FASEB J*., 19, 1686-8.

NRPB (National Radiological Protection Board). 1993. Restrictions on human exposure to static and time varying electromagnetic fields and radiation: scientific basis and recommendations for the implementation of the Board's Statement. *Doc NRPB*, 45, 7. Available from http://www.nrpb.org.

NRPB. 2003. Health Effects from Radiofrequency Electromagnetic Fields. Report of an Independent Advisory Group on Nonionising Radiation. Chilton, Didcot, UK: National Radiation Protection Board. *Doc NRPB*, 14. Available: http://www.nrpb.org/ publications/documents_of_nrpb/pdfs/doc_14_2.pdf.

NRPB. 2005. A summary of recent reports on mobile phones and health (2000-2004). *Doc NRPB*, W65, 1-34. Available from http://www.nrpb.org.

Nylund, R., Leszczynski, D. 2006. Mobile phone radiation causes changes in gene and protein expression in human endothelial cell lines and the response seems to be genome- and proteome-dependent. *Proteomics*., 6, 4769-80.

Oehninger, S., Franken, D.R., Sayed, E., Barroso, G., Kolm, P. 2000. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. *Hum Repro Update.*, 6, 160-8.

Pacini, S., Ruggiero, M., Sardi, I., Aterini, S., Gulisano, F., Gulisano, M. 2002. Exposure to global system for mobile communication (GSM) cellular phone radiofrequency alters gene expression, proliferation and morphology of human skin fibroblasts. *Oncol Res.*, 13, 19-24.

Panagopoulos, D., Karabarbounis, A., Margaritis, L.H. 2002. Mechanism for action of electromagnetic fields on cells. *Biochem. Biophys Res Commun*., 298, 95-102.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S.M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E.S., Kufe, D., Kharbanda, S. 2000. Negative regulation of cytochrome c-mediated oligomerization of apaf-1 and activation of procasapse-9 by heat shock protein 90. *EMBO J*., 19, 4310-22.

Pederson, G.F., Anderson, J.B. 1999. RF and ELF exposure from cellular phone handsets: TDMA and CDMA systems. *Rad Prot Dosim*., 83, 131-9.

Pokorny, J., Wu T.M. (eds.). 1998. *Biophysical Aspects of Coherence and Biological Order*. Prague: Springer.

Punyiczki, M., Fésüs, L. 1998. Heat Shock and Apoptosis. The two defence systems of the organism may have overlapping molecular elements. *Ann NY Acad Sci*., 851, 67-74.

Qutob, S.S., Chauhan, V., Bellier, P.V., Yauk, C.L., Douglas, G.R., Berndt, L., Williams, A., Gajda, G.B., Lemay, E., Thansandote, A., McNamee, J.P. 2006. Microarray gene expression profiling of a human glioblastoma cell line exposed *in vitro* to a 1.9 GHz Pulsed-modulated radiofrequency field. *Rad Res.*, 165, 636-44.

REFLEX *(Risk evaluation of potential environmental hazards from low frequency electromagnetic field exposure using sensitive in vitro methods)*. 2004. Funded by the European Union, Final report, 31 May.

Remondini, D., Nylund, R., Reivinen, J., Poulletier de Gannes, F., Veyret, B., Lagroye, I., Haro, E., Trillo, M.A., Capri, M., Franceschi, C., Schlatterer, K., Gminski, R., Fitzner, R., Tauber, R., Schuderer, J., Kuster, N., Leszczynski, D., Bersani, F., Maercker, C. 2006. Gene expression changes in human cells after exposure to mobile phone microwaves. *Proteomics*., 6, 4745-54.

Rephacholi, M.H., Basten, A., Gebski, V., Noonan, D., Finnie, J., Harris, A.W. 1997. Lymphomas in Eμ-Pim1 transgenic mice exposed to pulsed 900 MHz electromagnetic fields. *Rad Res.*, 147, 631-40.

Rephacholi, M.H. 1998. Low-Level Exposure to Radio-frequency Electromagnetic Fields: Health Effects and Research needs. *Bioelectromagnetics*., 19, 1-19.

Rotkovska, D., Moc, J., Kautska, J., Bartonicknov, A., Keprtova, J., Hofer, M. 1993. Evaluation of the biological effects of police radar RAMER 7E. *Environ Health Perspect*., 101, 134-43.

Sanchez, S., Milochau, A., Ruffie, G., Poulletier de Gannes, F., Lagroye, I., Haro, E., Surleve-Bazeille, J-E., Billaudel, B., Lassegues, M., Veyret, B. 2006. Human skin cell stress response to GSM-900 mobile phone signals. In vitro study on isolated primary cells and reconstructed epidermis. *FEBS J*., 273, 5491–07.

Sarkar, S., Ali, S., Behari, J. 1994. Effect of low power microwave on the mouse genome: a direct DNA analysis. *Mutat Res.*, 320, 141-9.

Saunders, R.D., Darby, S.C., Kowalcazuk, C.I. 1983. Dominant lethal studies in male mice after exposure to 2450 MHz microwave radiation. *Mutat Res.*, 117, 345-52.

Saunders, R.D., Kowalczuk, C.I., Beechey, C.V., Dunford, R. 1988. Studies of the induction of dominant lethals and translocations in male mice after chronic exposure to microwave radiation. *Int J Radiat Biol.*, 53, 983-92.

Schoemaker, M.J., Swerdlow, A.J., Ahlbom, A., Auvinen, A., Blaashaas, K.G., Cardis, E., Christensen, H.C., Feychting, M., Hepworth, S.J., Johansen, C., Klaeboe, L., Lonn, S., McKinney, P.A., Muir, K., Raitanen, J., Salminen, T., Thomsen, J., Tynes, T. 2005. Mobile phone use and risk of acoustic neuroma: results of the Interphone case-control study in five North European countries. *Br J Cancer*., 93, 842-8.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2006. *Preliminary Opinion on Possible effects of Electromagnetic Fields (EMF) on Human Health.* Luxembourg: European Commission Health & Consumer Protection Directorate General - Public Health and Risk Assessment.

Shallom, J.M., Di Carlo, A.L., Ko, D., Penafiel, L. M., Nakai, A., Litovitz T.A. 2002. Microwave exposure induces Hsp70 and confers protection against hypoxia in chick embryos. *J Cell Biochem*., 86, 490–496.

Sobel, E., Davanipour, Z. 1996. EMF exposure may cause increased production of amyloid beta and eventually lead to Alzheimers disease. *Neurology*., 47, 1594-600.

Stronati, L., Testa, A., Moquet, J., Edwards, A., Cordelli, E., Villani, P., Marino, C., Fresegna, A.M., Appolloni, M., Lloyd, D. 2006. 935 MHz cellular phone radiation. An *in vitro* study of genotoxicity in human lymphocytes. *Int J Radiat Biol.*, 82, 339-46.

Stuchly, M.A. 1998. Biological concerns in wireless communications. *Crit Rev Biomed Eng*., 26, 117-51.

Sukcharoen, N., Keith, J., Irvine, D.S., Aitken, R.J. 1996. Prediction of the in vitro fertilisation (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Hum Rep*., 11, 1020-34.

Tian, F., Nakahara, T., Wake, K., Taki, M., Miyakoshi, J. 2002. Exposure to 2.45 GHz electromagnetic fields induces hsp70 at a high SAR of more than 20 W/kg but not at 5W/kg in human glioma MO54 cells. *Int J Rad Biol*., 78, 433-440.

Tibbles, L.A., Woodgett, J.R. 1999. The stress activated protein kinase pathways. *Cell Mol Life Sci*., 55, 1230-54.

Tice, R.R., Hook, G.G., Donner, M., McRee, D.I., Guy, A.W. 2002. Genotoxicity of radio-frequency signals. I. Investigation of DNA damage and micronuclei induction in cultured human blood cells. *Bioelectromagnetics*., 23, 113-26.

Van Leeuwen, G.M.J., Lagendijk, J.J.W., Van Leersum, B.J.A.M., Zwamborn, A.P.M., Hornsleth, S.N., Kotte, A.N.T. 1999. Calculation of brain temperatures due to exposure to a mobile phone. *Phys Med Biol.*, 44, 2367-73.

Van Zandt, L.L. 1986. Resonant microwave absorption by dissolved DNA. *Phys Rev Letts.*, 5716, 2085-91.

Vanderwaal, R.P., Cha, B., Moros, E.G., Roti Roti, J.L. 2006. HSP27 phosphorylation increases after 45ºC or 41ºC heat shocks but not after non-thermal TDMA or GSM exposures. *Int J Hypertherm*., 22, 507–19.

Varma, M.M., Traboulay, E.A., Jr. 1977. Evaluation of dominant lethal test and DNA studies in measuring mutagenicity caused by non-ionizing radiation. In: *Biological Effects of Electromagnetic Waves*. Selected papers of the UNSC/URSI Annual Meeting, Boulder, Colorado, October 1975. C.C. Johnson and M.L. Shore, eds. Rockville, Maryland: US Department of Health, Education and Welfare, Volume 1, p 386.

Varma, M.M., Dage, E. L., Joshi, S.R. 1977. Comparison of native and microwave irradiated DNA. *Experiention.*, 33/12, 1649.

Velizarov, S., Raskmark, P., Kwee, S. 1999. The effects of radiofrequency fields on cell proliferation are non-thermal. *Bioelectrochem Bioenergetics*., 48, 177-80.

Vijayalaxmi, D.Z., Frei, M.R., Dusch, S.J., Guel, V., Meltz, M.L., Jauchem, J.R. 1997. Frequency of micronuclei in the peripheral blood and bone marrow of cancer-prone mice chronically exposured to 2450-MHz radiofrequency radiation. *Rad Res.*, 147, 495-500.

Vijayalaxmi, D. Z., Frei, M.R., Dusch, S.J., Guel,V., Meltz, M., Jauchem, J.R. 1998. Correction of an error in calculation in the article “Frequency of micronuclei in the peripheral blood and bone marrow of cancer prone mice chronically exposed to 2450 MHz radiofrequency radiation. *Rad Res*., 149, 308-13.

Vijayalaxmi, D.Z., Seaman, R.L., Belt, M.L., Doyle, J.M., Mathur, S.P., Prihodas, T.J. 1999. Frequency of micronuclei in the blood and bone marrow cells of mice exposed to ultra-wideband electromagnetic radiation. *Int J Rad Biol*., 751, 115-20.

Vijayalaxmi, M.R., Bisht, K.S., Pickard, W.F., Meltz, M.L., Roti Roti, J.L., Moros, E.G. 2001a. Chromosome damage and micronucleus formation in human blood lymphocytes exposed in vitro to radiofrequency radiation at a cellular telephone frequency (847.74 MHz, CDMA). *Rad Res.*, 156, 430-3.

Vijayalaxmi, M.R., Pickard, W.F., Bisht, K.S., Leal, B.Z., Meltz, M.L., Roti Roti, J.L., Straube, W.L., Moros, E.G., 2001b. Cytogenetic studies in human blood lymphocytes exposed in vitro to radiofrequency radiation at a cellular telephone frequency (835.62 MHz, FDMA). *Rad Res*., 155, 113-21.

Vijayalaxmi, M.R., Sasser, L.B., Morris, J.E., Wilson, B.W., Anderson, L.E. 2003. Genotoxic potential of 1.6 GHz wireless communication signal: In Vivo two-year bioassay. *Rad Res.*, 159, 558-64.

Wainwright, P.R. 1999. Localized specific absorption rate calculations in a realistic phantom leg at 1-30 MHz using a finite element method. *Phys Med Biol.*, 44, 1041-52.

Walters, T.J., Mason, P.A., Sherry, C.J., Stevens, C., Merritt, J.H. 1995. No detectable bioeffects following acute exposure to high peak power ultra-wide band electromagnetic radiation in rats. *Aviat Space Environ Med.*, 666, 562.

Weaver, J.C. 2002. Understanding conditions for which biological effects of non-ionising electromagnetic fields can be expected. *Bioelectrochem.*, 56: 207-9.

Weisbrot, D., Lin, H., Ye, L., Blank, M., Goodman, R. 2003. Effects of mobile phone radiation on reproduction and development in Drosophila melanogaster. *J Cell Biochem.*, 89, 48-55.

Whitehead, T.D., Moros, E.G., Brownstein, B.H., Roti Roti, J.L. 2006. The number of genes changing expression after chronic exposure to Code Division Multiple Access or Frequency DMA radiofrequency radiation does not exceed the false-positive rate. *Proteomics.*, 6, 4739-44.

Winker, R., Ivancsits, S., Pilger, A., Adlkofer, F., Rüdiger, H.W. 2005. Chromosomal damage in human diploid fibroblasts by intermittent exposure to extremely low-frequency electromagnetic fields. *Mutat Res.*, 585, 43-9.

Zeng, O., Chen, G., Weng, Y., Wang, L., Chiang, H., Lu, D., Xu, Z. 2006. Effects of Global System for Mobile Communications 1800 MHz radiofrequency electromagnetic fields on gene and protein expression in MCF-7 cells. *Proteomics*, 6, 4732-8.

Zeni, O., Chiavoni, A.S., Sannino, A., Antolini, A., Forgio, D., Bersani, F., Scarfi, M.R. 2003. Lack of genotoxic effects (micronucleus induction) in human lymphocytes exposed *in vitro* to 900 MHz electromagnetic fields. *Rad Res.*, 160, 152-8.

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).

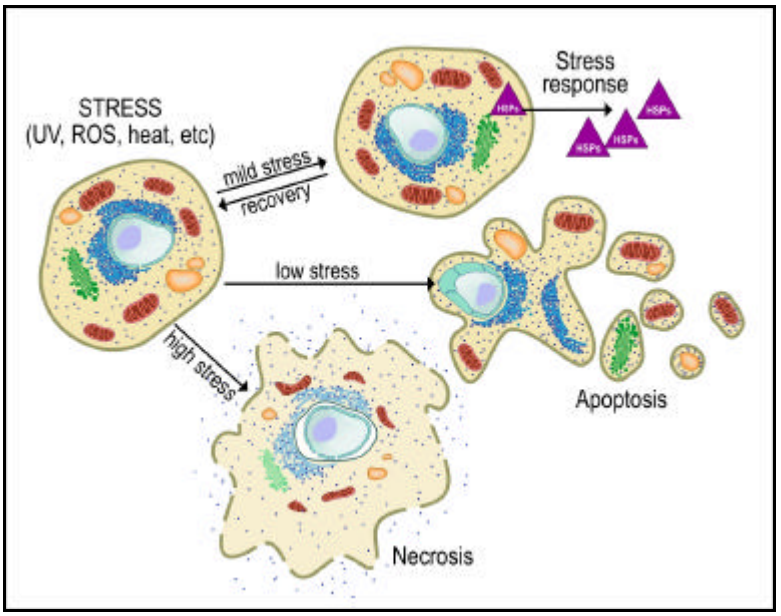

Figure 2.1 ***The stress response***: Exposure to damaging stimuli can trigger a cellular stress response resulting either in recovery or activation of the apoptotic program. Severe exposure could initiate cellular necrosis.

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).

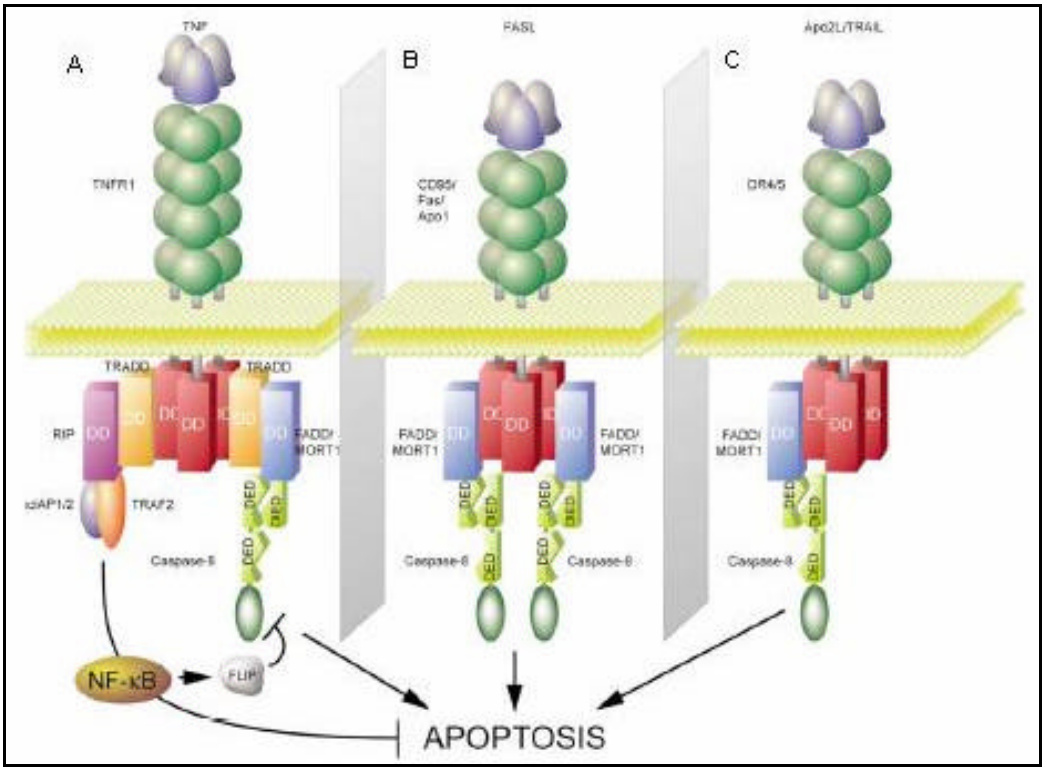

Figure 2.2 ***Extrinsic Apoptotic Pathway*** (Danial and Korsmeyer, 2004 - with permission). Binding of the various trimeric ligands to death receptors, **(A)** TNF to TNRF-1, **(B)** FasL to Fas (APO-1/CD95) and **(C)** APO2L/TRAIL to DR4/5 triggers the downstream assembly of the DISC.

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.

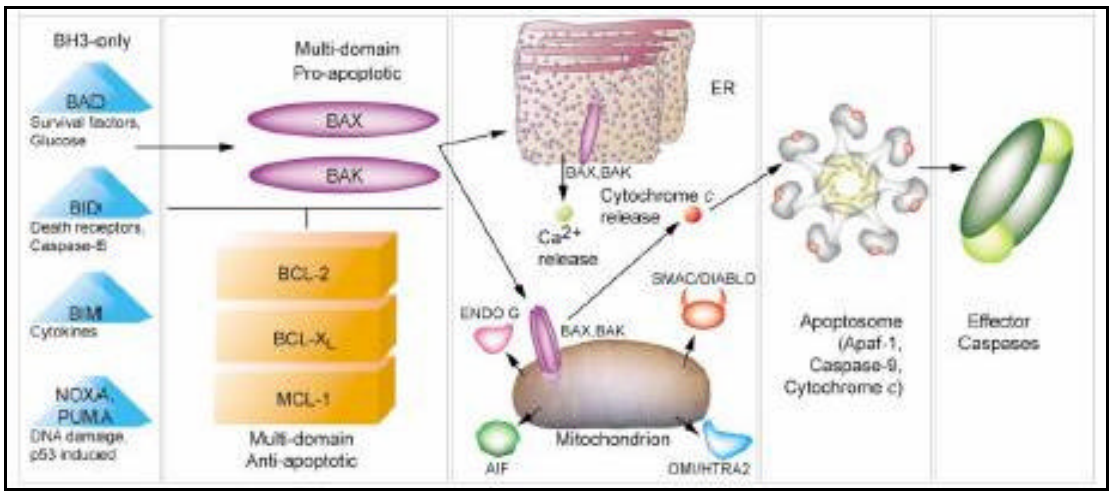

Figure 2.3 ***Intrinsic Apoptotic Pathway*** (Danial and Korsmeyer, 2004 - with permission). BH3 protein activation lead to Bax, Bak activation resulting either in the assembly of the apoptosome or ER induced apoptosis.

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).

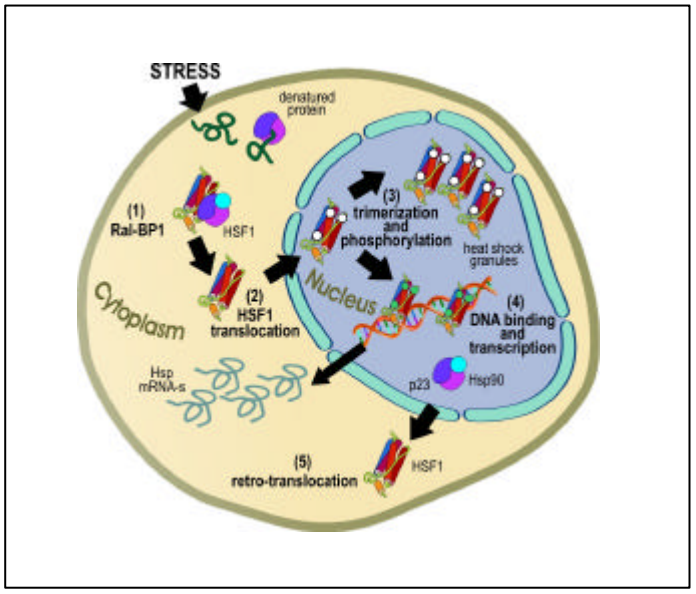

Figure 2.4 ***Major transcription factors leading to the induction of Hsp synthesis***: **(1)** cytoplasmic complex of HSF-1 and Hsp90; **(2)** HSF-1 translocation to the nucleus; **(3)** intra-nuclear distribution of HSF-1; **(4)** nuclear complex of HSF-1 and Hsp90; **(5)** retro translocation of HSF-1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

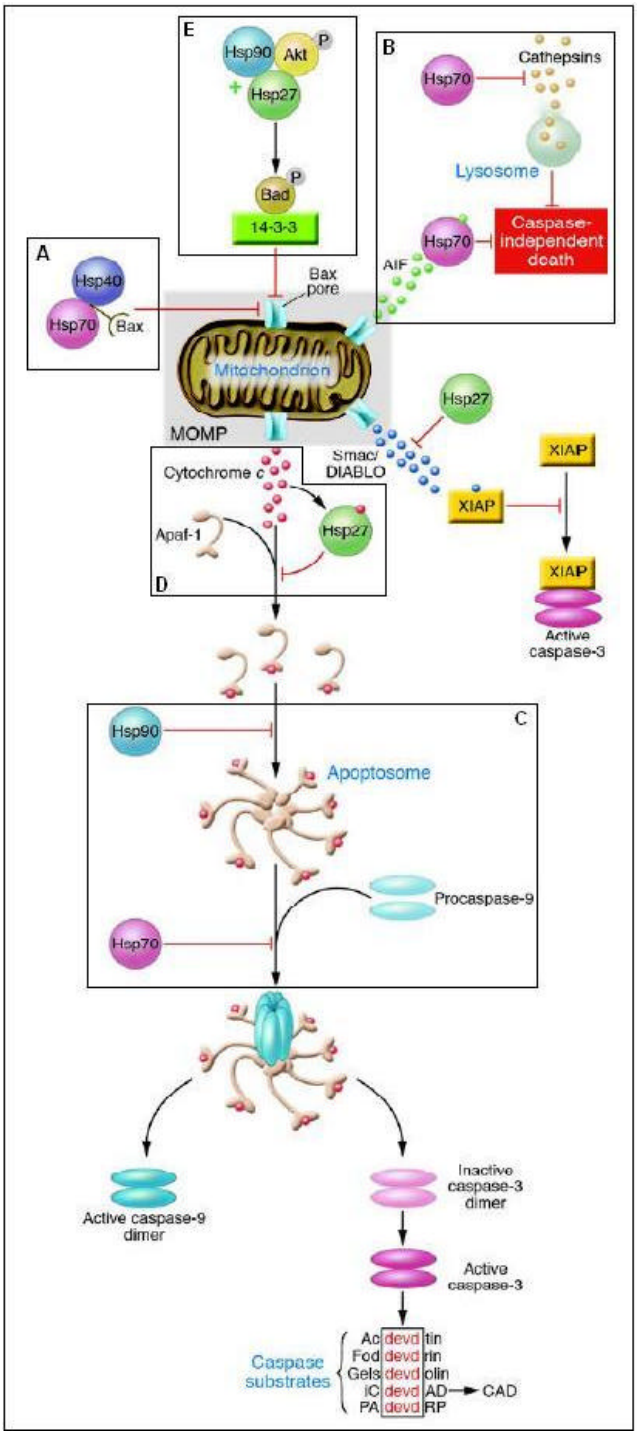

Figure 2.5 ***Regulation of the intrinsic apoptotic pathway by Hsps*** (with permission, Beere 2005); **(A)** inhibiting BAX translocation to the mitochondrion, **(B)** suppression of AIF activity, **(C)** inhibiting the activation of pro-caspase-9 by the apoptosome, **(D)** sequestering cytocrome-c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

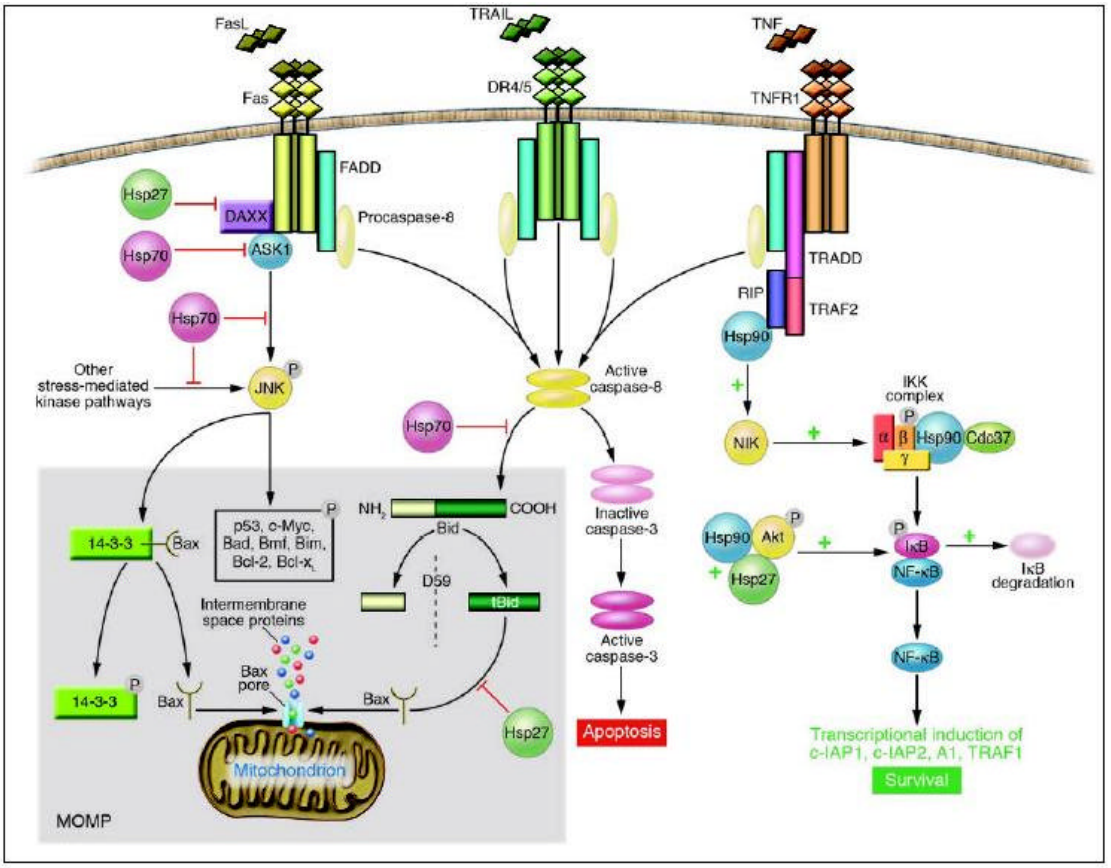

Figure 2.6 ***Regulation of the extrinsic apoptotic pathway by Hsps*** (with permission, Beere, 2005). Hsps regulate both death receptor signalling (Hsp27 - JNK and Bax pathway; Hsp70 – Bid pathway) and cell survival pathways (Hsp90 - NF-κB pathway).

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.

2.5 REFERENCES

ASRM practice committee. 2004. Report on optimal evaluation of the infertile male. *Fertil Steril*., 82, S123-30.

Ahlers, A., Belka, C., Gaestel, M., Lamping, N., Sott, C., Herrmann, F., Brach, M.A. 1994. Interleukin-1 induced intracellular signalling pathways converge an the activation of mitogen-activated protein kinase and mitogen-activated protein kinase-activated protein kinase 2 and the subsequent phosphorylation of the 27 kilo Dalton heat shock protein in monocytic cells. *Mol Pharmacol*., 46, 1077-83.

Aitken R.J., Gordonm, E., Harkissm, D., Twiggm, J.P., Milnem, P., Jenningsm, Z. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod*., 59, 1037-46.

Aitken R.J., Bakerm M.A. 2002. Reactive oxygen species generation by human spermatozoa: a continuing enigma. *Int J Androl*., 25, 191-4.

Aitken, R.J., Bennetts, L.E., Sawyer, D., Wiklendt, A.M., King, B.V. 2005. Impact of radiofrequency electromagnetic radiation on DNA integrity in the male germline. *Int J Androl*., 28, 171-9.

Allen, R.L., O'Brien, D.A., Eddy, E.M. 1988. A novel hsp70-like protein (P70) is present in mouse spermatogenic cells. *Mol Cell Biol.*, 8, 828-32.

Almeida, C., Cardoso, M.F., Sousa, M., Viana, P., Goncalves, A., Sliva, J., Barros, A. 2005. Quantitative study of caspase-3 activity in semen and after swim-up preparation in relation to sperm quality. *Hum Reprod*., 20, 1307-13.

Arrigo, A.P., Landry, J. 1994. Expression and function of the low-molecular-weight heat shock proteins. *In:* R.I. Morimoto, A. Tissières, C. Georgopoulos, eds. *The biology of heat shock proteins and molecular chaperones*. Cold spring Harbour Laboratory Press, Cold Spring Harbour, NY, 335-73.

Arrigo, A.P. 1998. Small stress proteins: chaperonens that act as regulators of intracellular redox state and programmed cell death. *Biol Chem.*, 379, 19-26.

Ashkenazi, A.M., Dixit, V.M. 1998. Death receptors: signalling and modulation. *Science*., 281, 1305-8.

Baccetti, B., Collodel, G., Piomboni, P. 1996. Apoptosis in human ejaculated sperm cells. *J Submicrosc Cytol Pathol*., 28, 587-96.

Baltz, J.M., Williams, P.O., Cone, R.A. 1990. Dense fibres protect mammalian sperm against damage. *Biol Reprod*., 43, 485-91.

Bar-Chama, N., Lamb, D. 1994. Urologic Clinics. *North Am*., 21, 433-46.

Barrientos, A., Moraes, C.T. 1999. Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J Biol Chem*., 274, 16188-97.

Barroso, G., Morshedi, M., Oehninger, S. 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod*., 15, 1338-44.

Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M., Green, D.R. 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1. *Nat Cell Biol*., 2, 469-75.

Beere, H.M. 2004.'The stress of dying': the role of heat shock proteins in the regulation of apoptosis. *J Cell Science*., 117, 2641-51.

Beere, H.M. 2005. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Invest*., 115, 2633-39.

Benndorf, R., Haye, K., Ryazantsev, S., Wieske, M., Behkle, J., Lutsch, G. 1994. Phosphorylation and supramolecular organisation of murine small heat shock protein hsp25 abolish its actin polymerisation-inhibiting activity. *J Biol Chem*., 269, 207-14.

Blanc-Layrac, G., Bringuier, A-F., Guillot, R. and Feldmann, G. 2000. Morphological and biochemical analysis of cell death in human ejaculated spermatozoa. *Cell Mol Biol*., 46, 187-97.

Bohring, C., Krause, W. 2003. Characterisation of spermatozoa surface antigens by antisperm antibodies and its influence on acrosomal exocytosis. *Am J Reprod Immunol*., 50, 411-9.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., Thompson, C.B. 1993. Bcl-x, a Bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*., 74, 597-608.

Bruey, J.M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S.A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A.P., Kroemer, G., Solary, E., Garrido, C. 2000. Hsp27

negatively regulates cell death by interacting with cytochrome-c. *Nat Cell Biol*., 2, 645-52.

Buzzard, K.A., Giaccia, A.J., Killender, M., Anderson, R.L. 1998. Heat Shock Protein 72 Modulates Pathways of Stress-induced Apoptosis. *J Biol Chem*., 273, 17147-53.

Cano, E., Mahadevan, L. 1994. Parallel signal processing among mammalian MAPKs Trends. *Biochem Sci.*, 20, 117-22.

Carlsen, E., Giwercman, A., Keiding, N., Skakkebaek, N.E. 1992. Evidence for decreasing quality of semen during past 50 years. *Br Med J*., 305, 609-13.

Castro, A., Parodi, D., Morales, I., Madariaga, M., Rios, R. and Smith, R. 2004. Absence of Fas protein detection by flow cytometry in human spermatozoa. *Fertil Steril*, 81, 1019-25.

Cayli, S., Jakab, A., Ovari, L., Delpiano, E., Celik-Ozenci, C., Sakkas, D., Ward, D., Huszar, G. 2003. Biochemical markers of sperm function: male fertility and sperm selection for ICSI. *Reprod Biomed Online*., 7, 462-8.

Cedeno, A.P., Lima, S.B., Cenedez, M.A., Spaine, D.M., Ortiz, V., Oehninger, S. 2006. Oligzoospermia and heat shock protein expression in ejaculated spermatozoa. *Hum Reprod*., 21, 1791-4.

Chapman, R., Sidrauski, C., Walter, P. 1998. Intracellular signalling from the endoplasmic reticulum to the nucleus. *Ann Rev Cell Dev Biol*., 14, 459-85.

Chen, G., Cao, P., Goeddel, D.V. 2002. TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell*., 9, 401–10.

Chrétien, P., Landry, J. 1988. Enhanced constitutive expression of the 27 kDa heat shock proteins in heat-resistant variants from Chinese hamster cells. *J Cell Physiol*., 137, 157-66.

Clemons, N.J., Buzzard, K., Steel, R., Anderson, R.L. 2005. Hsp72 Inhibits Fas-mediated Apoptosis Upstream of the Mitochondria in Type II Cells. *J Biol Chem*., 28, 9005-12.

Cobb, M., Hepler, J., Cheng, M., Robbins, D. 1994. The mitogen-activated protein kinases, ERK1 and ERK2. *Semin Cancer Biol*., 5, 261-8.

Cobb, M. Goldstein, E., 1995. How MAP kinases are regulated. *J Biol Chem*., 270, 14843-6.

Craig, E.A., Cross, C.A. 1991. Is Hsp70 the cellular thermometer? *Trends Biochem Sci*., 16, 135-40.

Creagh, E.M., Sheehan, D., Cotter, T.G. 2000. Heat shock proteins – modulators of apoptosis in tumour cells. *Leukemia*., 14, 1161-73.

Cummins, J.M., Jequier, A.M., Kan, R. 1994. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? *Mol Reprod Dev*., 37, 345-62.

Dadoune, J.P., Pawalak, A., Alfonsi, M.F., Siffroi, J.P. 2005. Identification of transcripts by microarrays, RT-PCR and in situ hybridisation in human ejaculate spermatozoa. *Mol Hum Reprod*., 11, 133-40.

Danial, N.N., Korsmeyer, S.J. 2004. Cell death: critical control points. *Cell*, 116, 205-19.

Daniel, P.T. 2000. Dissecting the pathways to death. *Leukaemia*., 14. 2035-44.

Davis, R. J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell* 103, 239-252.

de Vries, K.J., Wiedmer, T., Sims, P.J., Gadella, B.M. 2003. Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. *Biol Reprod*., 68, 2122-34.

Desagher, S., Osen-Sand, A., Nochols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., Martinou, J. 1999. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome-c release during apoptosis. *J Cell Biol*., 144, 891-901.

Deschesnes, R.G., Huot, J., Valerie, K., Landry, J. 2001. Involvement of p38 in apoptosis –associated membrane blebbing and nuclear condensation. *Mol Biol Cell*., 12, 1569-82.

Dix, D.J., Allen, J.W., Collins, B.W., Mori, C., Nakamura, N., Poorman-Allen, P., Goulding, E.H., Eddy, E.M. 1996. Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis and male infertility. *Proc Natl Acad Sci*., 93, 3264-8.

Dix, D.J. 1997. Hsp70 expression and function during gametogenesis. *Cell Stress Chaperones*., 2, 73-7.

Dix, D.J., Allen, J.W., Collins, B.W., Poorman-Allen, P., Mori, C. Blizard, D.R., Brown, P.R., Goulding, E.H., Strong, B.D., Eddy, E.M. 1997. Hsp70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. *Development*., 124, 4595-603.

Dokras, A., Giraldo, J.L., Habana, A., Erel, T., Kovanci, E., Huszar, G. 1999. Sperm cellular maturity and the treatment choice of IVF or intracytoplasmic sperm injection: the contributions of sperm creatine kinase m-isoform ratio measurements. In: *Program of ASRM Annual Meeting*, Abstract 199. 25-30 September 1999. Toronto: ASRM.

Donnelly, E.T., O'Connell, M., McClure, N., Lewis, S.E. 2000. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Hum Reprod*., 15, 1552-61.

Dorion, S., Landry, J. 2002. Activation of the mitogen-activated protein kinase pathways by heat shock. *Cell Stress Chaperonens*., 792, 200-6.

Duru, N.K., Morshedi, M.S., Schuffner, A., Oehninger, S. 2000. Semen treatment with progesterone and/or acetyl-l-carnitine does not improve sperm motility or membrane damage after cryopresentation-thawing. *Fertil Steril*., 74, 715-20.

Duru, N.K., Morshedi, M.S., Schuffner, A., Oehninger, S. 2001. Cryopreservation-thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. *J Androl*., 22, 646-51.

Earnshaw, W.C., Martins, L.M., Kaufmann, S.H. 1999. Mammalian caspases: structure, activation, substrates and functions during apoptosis. *Annu Rev Biochem*., 68, 383-424.

Ecroyd, H., Jones, R.C., Aitken, R.J. 2003. Tyrosine Phosphorylation of HSP-90 during mammalian sperm capacitation. *Biol Reprod*., 69, 1801-7.

Eddy, E.M., O'Brein, D.A., Welch, J.E. 1991. Mammalian sperm development *in vivo* and *in vitro*. *In*: P.M. Wassarman, ed. *Elements of mammalian fertilization*. Boca Raton, FL, USA: CRC Press, 1-28.

Edinger, A.L., Thompson C.B. 2004. Death by design: apoptosis, necrosis and autophagy. *Current Opinion Cell Biol*., 16, 663–9.

Eguchi, Y., Shimizu, S., Tsujimoto, Y. 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res*., 57, 183-40.

Eguchi, J., Koji, T., Nomata, K., Yoshii, A., Shin, M., Kanetake, H. 2002. Fas-fas ligand system as a possible mediator of spermatogenic cell apoptosis in human maturation-arrested testes. *Hum Cell*., 15, 61-8.

Ergur, A.R., Dokras, A., Giraldo, J.L. Habana, A., Kovanci, E., Huszar, G. 2002. Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. *Fertil Steril*., 77, 910-8.

Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., Henson, P.M. 2000. A receptor for phospatidylserine-specific clearance of apoptotic cells. *Nature*., 405, 85-90.

Fontaine, J-M., Rest, J.S., Welsh, M.J., Benndorf, R. 2003. The sperm outer dense fibre protein is the 10th member of the super family of mammalian small stress proteins. *Cell Stress Chaperonens*., 8, 62-9.

Francavilla, S., D'Abrizio, P., Rucci, N., Silvano, G., Properzi, G., Straface, E., Cordeschi, G., Necozione, S., Gnessi, L., Arizzi, M., Ulisse, S. 2000. Fas and Fas-ligand expression in foetal and adult human testis with normal or deranged spermatogenesis. *J Clin Endocrinol Metabol*., 85, 2692-700.

Franklin, R.A., McCubery, J.A. 2000. Review: Spotlight on apoptosis. Kinases: positive and negative regulators of apoptosis. *Leukemia*., 14, 2019-34.

Freeman, M.L., Borrelli, M.J., Meredith, M.J., Lepock, J.R. 1999. On the path of the heat shock response: destabilisation and formation of partially folded protein intermediates, a consequence of protein thiol modification. *Free Rad Bio Med*., 26, 737-45.

Gabai, V.L., Meriin, A.B., Mosser, D.D., Caron, A.W., Rits, S., Shifrin, V.I., Sherman, M.Y. 1997. Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *J Biol Chem*., 272, 18033-7.

Gabai, V.L., Mabuchi, K., Mosser, D.D., Sherman, M.Y. 2002. Hsp72 and stress kinase c-jun N-terminal kinase regulate the bid-dependent pathway in tumour necrosis factor-induced apoptosis. *Mol Cell Biol*., 22, 3415-24.

Galea-Lauri, J., Richardson, A.J., Latchman, D.S., Katz, D.R. 1996. Increased heat shock protein 90 (Hsp90) expression leads to increased apoptosis in the monoblastoid cell line U937 following induction with TNF-a and cyclohexeimide. A possible role in immunopathology. *J Immunol*., 157, 4109-18.

Gandini, L., Lombardo, F., Paoli, D., Caponecchia, L., Familiari, G., Verlengia, C., Dondero, F., Lenzi, A. 2000. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod*., 15, 830-9.

Garrido, C.P., Ottavi, A., Fromentin, A., Hammann, A., Arrigo, A.P., Chauffert, B., Mehlen, P. 1997. Hsp27 as a mediator of confluence-dependent resistance to cell death induced by anticancer drugs. *Cancer Res*., 57, 2661-7.

Glander, H.J., Schaller, J. 1999. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. *Mol Hum Reprod*., 5, 109-15.

Gotoh, T., Terada, K., Oyadomari, S., Mori, M. 2004. hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. *Cell Death Differ*., 11, 390–402.

Green, D., Kroemer, G., 1998. The central executioners of apoptosis: caspases and mitochondria? *Trends Cell Biol*., 8, 267-71.

Green, D.R., Reed, J.C. 1998. Mitochondria and apoptosis. *Science*., 281, 1309-12.

Grunewald, S., Paasch, U., Glander, H.J., Anderegg, U. 2005. Mature human spermatozoa do not transcribe novel RNA. *Andrologia*., 37, 69-71.

Guo, Y.L., Baysal, K, Kang, B., Yang, L.J., Williamson, J.R. 1998. Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. *J Biol Chem*., 273, 4027-34.

Gurbuxani, S., Schmitt, E., Cande, C., Parcellier, A., Hammann, A., Daugas, E., Kouranti, E., Spahr, C., Pance, A., Kroemer, G., Garrido, C. 2003. Heat shock protein 70 binding inhibits nuclear import of apoptosis-inducing factor. *Oncogene*., 22, 669-78.

Hayashi, T., Kageyama, Y., Ishizaka, K., Kihara, K., Oshima, H. 2002. Involvement of apoptosis in the control of Sertoli and pre-meiotic germ cell numbers in the developing rabbit testis. *Andrologia*., 34, 34-40.

Hendrick, J.P., Hart, F.U. 1993. Molecular chaperone function of heat shock proteins. *Annu Rev Biochem*., 62, 349-84.

Hengartner, M.O. 2000. The biochemistry of apoptosis. *Nature*., 407, 770-6.

Hightower, L.E. 1991. Heat shock, stress proteins, chaperones and proteotoxicity. *Cell*., 66, 191-7.

Host, E., Lindenberg, S., Ernst, E., Christensen, F. 1999. DNA strand breaks in human spermatozoa: a possible factor to be considered in couples suffering from unexplained infertility. *Acta Obstet Gynecol Scand*., 78, 622-5.

Huang, S.Y., Kuo, Y.H., Tsou, H.L., Lee, Y.P., King, Y.T., Huang, H.C., Yang, P.C., Lee, W.C. 2000a. The decline of Porcine sperm motility by glendanamycin, a specific inhibitor of heat shock protein 90 (Hsp90). *Theriogenology*., 53, 1177-84.

Huang, S.Y., Kuo, Y.H., Lee, Y.P., Tsou, H.L., Lin, E.C., Ju, C.C., Lee, W.C. 2000b. Association of heat shock protein 70 with semen quality of boars. *Animal Reprod Sci*., 63, 231-40.

Huot, J., Lambert, H., Lavoie, J.N., Guimond, A., Houle, F., Landry, J. 1995. Characterisation of 45/54 kDa Hsp27 kinase, a stress kinase which may activate the phosphorylation-dependant protective function of mammalian heat-shock protein 27. *Eur J Biochem*., 227, 416-27.

Huszar, G., Vigue, L., Morshedi, M. 1992. Sperm creatine phosphokinase m-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. *Fertil Steril.*, 57, 882-8.

Huszar, G., Vigue, L., Oehninger, S. 1994. Creatine kinase (CK) immuno cytochemistry of human hemizona-sperm complexes: selective binding of sperm with mature CK-staining pattern. *Fertil Steril.*, 61, 136-42.

Huszar, G., Sbracia, M., Vigue, L., Miller, D., Shur, B. 1997. Sperm plasma membrane remodelling during spermiogeneticy maturation in men: relationship among plasma membrane ß-1, 4, galactosyl transferase, cytoplasmic creatine phosphokinase and creatine phosphokinase isoform ratios. *Biol Reprod*., 56, 1020-4.

Huszar, G., Stone, K., Dix, D., Vigue, L. 2000. Putative creatine kinase m-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. *Biol Reprod*., 63, 925-32.

Huszar, G., Ozenci, C.C., Cayli, S., Zavaczki, Z., Hansch, E., Vigue, L. 2003. Hyaluronic acid binding by human sperm indicates cellular maturity: viability and un-reacted acrosomal status. *Fertil Steril*., 79, 1616-24.

Jäättelä, M. 1993. Over expression of major heat shock proteins Hsp70 inhibits tumour necrosis factor-induced activation of phospholipase A_2. *J Immunol*., 151, 4286-94.

Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T., Egebald, M. 1998. Hsp70 exerts its anti-apoptotic function downstream of caspase 3-like pro-caspases. *EMBO J*., 17, 6124-34.

Jindal, S., Dudani, A.K., Singh, B., Harley, C.B., Gupta, R.S. 1989. Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. *Mol Cell Biol*., 9, 2279-83.

Jindal, S., Young, R.A. 1991. Immune recognition of stress proteins in infection and surveillance of stressed cells. *In*: B. Maresca, S. Lindquist, eds. *Heat Shock*. Berlin: Springer-Verlag, 193-202.

Kamaruddin, M.B. 1998. *Heat shock protein 70 in barine spermatogenesis and fertilization*. PhD thesis. University of Guelph, Ontario, Canada.

Kamradt, M.C., Lu, M., Werner, M.E., Kwan, T., Chen, F., Strohecker, A., Oshita, S., Wilkinson, J.C., Yu, C., Oliver, P.G., Duckett, C.S., Buchsbaum, D.J., LoBuglio, A.F., Jordan, V.C., Cryns, V.L. 2005. The small heat shock protein alpha B-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. *J Biol Chem*., 280, 11059–66.

Karmann, K., Min, W., Fanslow, W., Pober, J. 1996. Activation and homologous desensitizing of human endothelial cells by CD40 ligand, tumour necrosis factor and interleukin 1. *J Exp Med*., 184, 173-82.

Kerr, J.F.R., Wyllie, A.H., Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implication in tissue kinetics. *Br J Cancer*., 26, 239-57.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., Newmeyer, D.D. 1997. The release of cytochrome-c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*., 275, 1132-326.

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pols, S.T., Van Oers, M.H. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*., 84, 1415-20.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P., Craig, R.W. 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci*., 90, 3516-20.

Krammer, P.H. 1999. CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol*., 71, 163-210.

Kroemer, G., Zamzami, N., Susin, S.A. 1997. Mitochondrial control of apoptosis. *Immunol Today*., 18, 44-51.

Kroemer, G., Dallaporta, B., Resche-Rigon, M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis. *Ann Rev Physiol*., 60, 619-42.

Kroemer, G., Reed, J.C. 2000. Mitochondrial control of death. *Nature Med*., 6, 513-9.

Kruidering, M., van de Water, B., Zhan, Y., Baelde, J.J., Heer, E., Mulder, G.J., Stevens, J.L., Nagelkerke, J.F. 1998. Cisplatineffects on F-actin and matrix proteins precede renal tubular cell detachment and apoptosis in vitro. *Cell Death Diff*., 5, 601-14.

Lachaud, C., Tesarik, J., Canada, M.L., Mendoza, C. 2004. Apoptosis and necrosis in human ejaculated spermatozoa. *Hum Reprod*., 19, 607-10.

Lambard, S., Galeraud-Denis, I., Martin, G., Levy, R., Chocat, A., Carreau, S. 2004. Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. *Mol Hum Reprod*., 10, 535-41.

Landry, J., Lambert, H., Zhou, M., Lavoi, J.N., Hickey, E., Weber, L.A., Anderson, C.W. 1992. Human Hsp27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognise the same amino acid motif as S6 kinase II. *J Biol Chem*., 267, 794-803.

Lee, J., Richburg, J.H., Younkin, S.C., Boekelheide, K. 1997. The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology*., 138, 2081-8.

Lee, J., Richburg, J.H., Shipp, E.B., Meistrich, M.L., Boekelheide, K. 1999. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. *Endocrinology*., 140, 852-8.

Lee, H.C., Yin, P.H., Lu, C.Y., Chi, C.W., Wei, Y.H. 2000. Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochem J*., 348, 425-32.

Leszczynski, D., Joenväärä, S., Reivinen, J., Kuokka, R. 2002. Non-thermal activation of the hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanism for cancer-and blood brain barrier-related effects. *Differentiation*., 70, 120-9.

Levy, R., Seifer-Aknin, I. 2001. Apoptosis during spermatogenesis and ejaculated spermatozoa: importance for fertilization. *Ann Biol*., 59, 531-45.

Li, H., Zhu, H., Xu, C., Yuan, J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the fas pathway of apoptosis. *Cell*., 94, 491-501.

Li, L., Luo, X., Wang, X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*., 412, 95-9.

Liossis, S.N., Ding, X.Z., Kiang, J.G., Tsokos, G.C. 1997. Over expression of the heat shock protein 70 enhances the TCR/CD3- and Fas/Apo-1/CD95-mediated apoptotic cell death in Jurkat T cells. *J Immunol*., 158, 5668-75.

Loeffler, M., Kroemer, G. 2000. The mitochondrion in cell death control certainties and incognita. *Exp Cell Res*., 256, 18-26.

Luconi, M., Barni, T., Vanelli, G.B., Krausz, C., Marra, F., Benedetti, P.A., Evangelista, V., Fancavilla, S., Properzi, G., Forti, G., Baldi, E. 1998. Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biol Reprod*., 58, 1476-89.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., Wang, X. 1998. Bid, a Bcl-2 interacting protein, mediates cytochrome-c release in response to activation of cell surface death receptors. *Cell*., 94, 481-90.

Ly, J.D., Grubb, D.R., Lawen, A. 2003. Failed fertilization: is it predictable? *Curr Opin Obstet Gynecol*., 15, 211-8.

Maekawa, M., O' Brein, D.A., Allen, R.L., Eddy, E.M. 1989. Heat-shock cognate protein (hsc71) and related proteins in mouse spermatogenic cells. *Biol Reprod*., 40, 843-52.

Mallouk, Y., Vayssier-Taussat, M., Bonventre, J.V., Polla, B.S. 1999. Heat shock protein 70 and ATP as partners in cell homeostasis. *Int J Mol Med*., 4, 463-74.

Marchetti, C., Obert, G., Deffosez, A., Formstecher, P., Marchetti, P. 2002. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod*., 17, 1257-65.

Marchetti, C., Gallego, M.A., Defossez, A., Formstecher, P., Marchetti, P. 2004. Staining of human sperm with fluorochrome-labelled inhibitor of caspases to detect activated caspases: correlation with apoptosis and sperm parameters. *Hum Reprod*., 19, 1127-34.

Martin, G., Sabido, O., Durand, D., Levy, R. 2005. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. *Hum Reprod*., 20, 3459-68.

Matsumoto, M., Fujimoto, H. 1990. Cloning of a hsp70-related gene expressed in mouse spermatids. *Biochem Biophys Res Comm*., 166, 43-9.

Matwee, C., Kamaruddin, M., Betts, D.H., Basur, P.K., King, W.A. 2001. The effects of antibodies to heat shock protein 70 in fertilization and embryo development. *Mol Hum Reprod*., 7, 829-37.

McKenna, W.G. 2001. *Apoptosis, radiosensitivity and the cell cycle*. OncoLink. University of Pensylvania Cancer Centre. Online: wysiwyg:///16/http://oncolink.upenn.edu/speciality/mol_bio/apopt.html. [Accessed 21/11/2001].

McPherson, S., Longo, F.J. 1992. Localization of DNase 1-hypersensitive regions during rat spermatogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. *Mole Reprod Dev*., 31, 268-79.

McVicar, C.M., McClure, N., Williamson, K., Dalzell, L.H., Lewis, S.E. 2004. Incidence of Fas positivity and deoxyribonucleic acid double-stranded breaks in human ejaculated sperm. *Fertil Steril*., 81, 767-74.

Mehlen, P., Schulze-Osthoff, K., Arrigo, A.P. 1996. Small stress proteins as novel regulators of apoptosis. *J Biol Chem*., 271, 16510-4.

Meinhardt, A., Wilhelm, B., Setz, J. 1999. New aspects of spermatogenesis. Expression of mitochondrial marker proteins during spermatogenesis. *Hum Reprod Update.*, 592, 108-19.

Meriin, A.B., Yaglom, J.A., Gabai, V.L., Zon, L., Ganiastas, S., Mosser, D.D., Sherman, M.Y. 1999. Protein-damaging stresses activate c-JunN-terminal kinases via inhibition of its dephosphorylation: a novel pathway controlled by Hsp72. *Mol Cell Biol*., 19, 2547-55.

Micheau, O., Tschopp, J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell.*, 114, 181-190.

Miller, D., Brough, S., Al-Harib, O. 1992. Characterization and cellular distribution of human spermatozoal heat shock proteins. *Hum Reprod*., 7, 645-37.

Miller, D., Briggs, D., Snowden, H., Hamilton, J., Rollinson, S., Lilford, R., Krawetz, S.A. 1999. A complex population of RNAs exists in human ejaculate spermatozoa: implications for understanding molecular aspects of spermiogenesis. *Gene*., 237, 385-92.

Miller, D. 2000. Analysis and significance of messenger RNA in human ejaculated spermatozoa. *Mol Reprod Dev*., 56, 259-64.

Morimoto, R.I. 1993. Cells in stress: transcriptional activation of heat shock genes. *Science*., 259, 1409-10.

Morimoto, R.I., Jurivich, D.A., Kroeger, P.E., Mathur, S.K., Murphy, S.P., Nakai, A., Sarge, K., Abravaya, K., Sistonen, L.T. 1994. Regulation of heat shock gene transcription by a family of heat shock factors. *In*: R.I. Moromoto, A. Tissieres, C. Georgopoulus, eds. *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press, 417-55.

Morimoto, R.I., Kline, M.P., Bimston, D.N., Cotto, J.J. 1997. The heat shock response: regulation and function of heat shock proteins and molecular chaperones. *Essays Biochem*., 32, 17-29.

Mosser, D.D., Caron, A.W., Bourget, L., Meriin, A.B., Sherman, M.Y., Morimoto, R.I., Massie, B. 2000. The chaperone functions of Hsp70 is required for protection against stress induced apoptosis. *Mol Cell Biol*., 20, 7146-59.

Moustafa, M.H., Sharma, R.K., Thornton, J., Mascha, E., Abdel-Hafez, M.A., Thomas, A.J., Agarwal, A. 2004. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod*., 19, 129-38.

Muratori, M., Piombi, P., Baldi, E. Filimberti, E., Pecchioli, P., Moretti, E., Gambera, L., Baccetti, B., Biagiotti, R., Forti, G., Maggi, M. 2000. Functional and ultrastructural features of DNA-fragmented human sperm. *J Androl*., 21, 903-12.

Muzio, M., Stockwell, B.R., Steinnicke, H.R., Savesen, G.S., Dixit, V.M. 1998. An induced proximity model for caspase-8 activation. *J Biol Chem*., 273, 2926-30.

Nair, S.C., Toran, E.J., Rimerman, R.A., Hjermstad, S., Smithgall, T.E., Smith, D.F., 1996. A pathway of multi-chaperone interactions common to diverse regulatory proteins: Estrogen receptor, Fas tyrosine kinase, heat shock transcription factor Hsf1 and the aryl hydrocarbon receptor. *Cell Stress Chaperones*., 1, 237-250.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., Yuan, J. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-ß. *Nature*., 403, 98-103.

Neuer, A., Spandorfer, S.D., Giraldo, P., Dieterle, S., Rosenwaks, Z., Witkin, S.S. 2000. The role of heat shock proteins in reproduction. *Hum Reprod Update.*, 6, 149-59.

Nonoguchi, K., Tokuchi, H., Okuna, H., Watanabe, H., Egawa, H., Saito, K., Ogawa, O., Fujita, J. 2001. Expression of Apg-l a member of the Hsp110 family, in the human testis and sperm. *Int J Urol*., 8, 308-14.

Oltvai, Z.N., Milliman, C.L., Korsmeyer, S.J. 1993. Bcl-2 hetero-dimerizes in vivo with a conserved homologue, Bax that accelerates programmed cell death. *Cell*., 74, 609-19.

Oosterhuis, G.J., Mulder, A.B., Kalsbeek-Batenburg, E., Lambalk, C.B., Schoemaker, J., Vermes, I. 2000. Measuring apoptosis in human spermatozoa: a biological assay for semen quality? *Fertil Steril*., 74, 245-50.

Ostermeier, G.C., Dix, D.J., Miller, D., Kathri, P., Krawetz, S.A. 2002. Spermatozoal DNA profiles of normal fertile men. *Lancet*., 360, 772-7.

Paasch, U., Grunewald, S., Fitzl, G., Glander, H.J. 2003. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. *J Androl*., 24, 246-52.

Paasch, U., Grunewald, S., Agarwal, A., Glander, H.J. 2004. Activation pattern of caspases in human spermatozoa. *Fertil Steril*., 81, 802-9.

Pahl, H.L. 1999. Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol Rev*., 79, 683-701.

Pan, G., Humke, E.W., Dixit, V.M. 1998. Activation of caspases triggered by cytochrome-c *in vitro*. *FEBS Lett*., 426, 151-4.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S.M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E.S., Kufe, D., Kharbanda, S. 2000. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procasapse-9 by heat shock protein 90. *EMBO J*., 19, 4310- 22.

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantome, A., Plenchette, S., Khochbin, S., Solary, E., Garrido, C. 2003. HSP27 is a ubiquitin-binding protein involved in I-κBα proteasomal degradation. *Mol Cell Biol*., 23, 5790-802.

Park, H.S., Lee, J.S., Huh, S.H., Seo, J.S., Choi, E.J. 2001. Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. *EMBO J*., 20, 446-56.

Park, J.Y., Ahn, H.J., Gu, J.G., Lee, K.H., Kim, J.S., Kang, H.W., Lee, J.H. 2003. Molecular identification of Ca2+ channels in human sperm. *Exp Mol Med*., 35, 285-92.

Parsell, D.A., Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Ann Rev Genet*., 27, 437-96.

Paul, A., Wilson, S., Belham, C.M., Robinson, C.J., Scott, P.H., Gould, G.W., Plevin, R. 1997. Stress-activated protein kinases: activation, regulation and function. *Cell Signal*., 9, 403-10.

Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S., Arrigo, A.P. 2002. Hsp27 as a negative regulator of cytochrome-c release. *Mol Cell Biol*., 22, 816-34.

Pelham, H.R.B. 1984. Hsp70 accelerates the recovery of nucleolar morphology after heat shock. *EMBO J.*, 3, 3095-100.

Pentikainen, V, Erkkila, K., Dunkel, L. 1999. Fas regulates germ cell apoptosis in the human testis in vitro. *Am J Physiol.*, 276, E310-6.

Piasecka, M., Kawiak, J. 2003. Sperm mitochondria of patients with normal sperm motility and with asthenozoospermia: morphological and functional study. *Folia Histochem Cytobiol*., 41, 125-39.

Prohászka, Z., Füst, G. 2004. Immunological aspects of heat-shock proteins- the optimum stress for life. *Mol. Immunol*., 41, 29-44.

Punyiczki, M., Fésüs, L. 1998. Two defence systems of the organism may have overlapping molecular elements. *Annals New York Acad Sci*., 30, 67-74.

Ravagnan, L., Gurbuxani, S., Susin, S.A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jaattela, M., Penninger, J.M., Garrido, C., Kroemer, G. 2001. Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol.*, 3, 839-43.

Reed, J.C. 2000. Mechanisms of apoptosis. *Am J Pathol*., 157, 1415-30.

Ritossa, F. 1962. A new puffing pattern induced by heat shock and DNP in Drosophila. *Experientia*., 18, 571-3.

Robbins, D., Zheng, E., Cheng, M., Xu, S., Ebert, D., Cobb, M. 1994. MAP kinases ERK1 and ERK2: pleiotropic enzymes in a ubiqiotous signalling network. *Adv Cancer Res*., 63, 93-116.

Rodriguez, I., Ody, C., Araki, K., Garcia, I., Vassalie, P. 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J*., 16, 2262-70.

Roulston, A., Reinhard, C., Amiri, P., Williams, L.T. 1998. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumour necrosis factor alpha. *J Biol Chem*., 273, 10232-9.

Said, T.M., Paasch, U., Glander, H-S., Agarwal, A. 2004. Role of caspases in male infertility. *Hum Reprod Update.*, 10, 39-51.

Sailer, B.L., Jost, L.K., Everson, D.P. 1995. Mammalian sperm DNA susceptibility to *in situ* denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl*., 16, 80-7.

Sakkas, D., Mariethoz, E., St. John, J.C. 1999. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp Cell Res.*, 251, 350-5.

Sakkas, D. 1999. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod.*, 4, 31-7.

Sakkas, D., Moffatt, O., Manicardi, G.C., Mariethoz, E., Tarozzi, N., Bizzaro, D. 2002. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod.*, 66, 1061-7.

Sakkas, D., Seli, E., Bizzaro, D., Tarozzi, N., Manicardi, G.C. 2003. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online.*, 7, 428-32.

Sakkas, D., Seli, E., Manicardi, G.C., Nijs, M., Ombelet, W., Bizzaro, D. 2004. The presence of abnormal spermatozoa in the ejaculate: did apoptosis fail? *Hum Fertil.*, 7, 99-103.

Sakkas, D., Seli, E. 2005. Spermatozoal nuclear determinants of reproductive outcome: implications for ART. *Hum Reprod Update.*, 11, 337-49.

Saxena, N., Peterson, R.N., Sharif, S., Saxena, N.K., Russell, L.D. 1986. Changes in the organisation of surface antigens during in vitro capacitation of boar spermatozoa as detected by monoclonal antibodies. *J Reprod Fertil.*, 78, 601-14.

Scheibel, T., Buchner, J. 1998. The Iisp90 complex – a super-chaperone machine as a novel drug target. *Biochem Pharmacol.*, 56, 675-82.

Schmitz, I., Walczak, H., Krammer, P.H., Peter, M.E. 1999. Differences between CD95 type I and II cells detected with the CD95 ligand. *Cell Death Differ.*, 6, 821-2.

Schrader, S.M., Kanitz, M.H. In: Gold E, Schenker M, Lesley B, Eds. 1994. *State of the art reviews in occupational medicine, reproductive hazards*. Philadelphia, PA, Hanley & Belfus, Inc., 405-14.

Schuffner, A., Morshedi, M, Oehninger, S. 2001. Cryopreservation of fractionated highly motile human spermatozoa: effect on membrane phosphatidylserine externalization and lipid peroxidation. *Hum Reprod.*, 16, 2148-53.

Schuffner, A., Morshedi, M., Vaamonde, D., Duran, E.H., Oehninger, S. 2002. Effect of different incubation conditions on phosphatidylserine externalization and

motion parameters of purified fractions of highly motile human spermatozoa. *J Androl*., 23, 194-201.

Schulte, T.W., Blagosklonny, M.V., Romanova, L., Mushinski, J.F., Monia, B.P., Johnston, J.F., Nguyen, P., Trepel, J., Neckers, L.M. 1996. Destabilization of Raf-1 by geldanamycin leads to disruption of the Raf-1-MEK-mitogen-activated protein kinase signalling pathway. *Mol Cell Biol*., 16, 5839-45.

Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S.A., Mannella, C.A., Korsmeyer, S.J. 2003. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome-c during apoptosis. *Dev Cell*., 2, 55-67.

Shen, H.M., Dai, J., Cha, S.E., Lim, A., Ong, C.N. 2002. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod*., 17, 1266-73.

Sikka, S.C. 2001. Relative impact of oxidative stress on male reproductive function. *Curr Med Chem*., 8, 851-62.

Sinha Hikim, A.P., Wang, C., Lue, Y., Johnson, L., Wang, X.H., Swerdloff, R.S. 1998. Spontaneous germ cell apoptosis in humans: evidence for ethnic differences in the susceptibility of germ cells in programmed cell death. *J Clin Endocrinol Metab*., 83, 152-6.

Son, W.Y., Hwang, S.H., Han, C.T., Lee, J.H., Kim, S., Kim, Y.C. 1999. Specific expression of heat sick protein HspA2 in human male germ cells. *Mol Hum Reprod*., 12, 1122-6.

Sõti, C., Nagy, E., Giricz, Z., Vigh, L., Csermely, P., Ferdinandy, P. 2005. Heat shock proteins as emerging therapeutic targets. *B J Pharmacol*., 146, 769-80.

Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., Alnemri, E.S. 1998. Auto-activation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell*., 1, 949-57.

Steel, R., Doherty J.P., Buzzard, K., Clemons, N., Hawkins, C.J., Anderson, R.L. 2004. Hsp72 inhibits apoptosis upstream of the mitochondria and not through interactions with Apaf-1. *J Biol Chem*., 279, 51490-9.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothdrome, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., Kroemer, G. 1999.

Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*., 397, 441-6.

Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., Kroemer, G 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med*., 184, 1331-42.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., Takahashi, R. 2001. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell.*, 8, 613–21.

Tamar, M.S., Paasch, U., Glander, H-S., Agarwal, A. 2004. Role of caspases in male infertility. *Hum Reprod Update.*, 10, 39-51.

Tesarik, J., Martinez, F., Rienzi, L., Iacobelli, M., Ubaldi, F., Mendoza, C., Greco, E. 2002. In vitro effects of FSH and testosterone withdrawal on caspase activation and DNA fragmentation in different cell types of human seminiferous epithelium. *Hum Reprod*., 17, 1811-9.

Tesarik, J., Ubaldi, F., Rienzi, L., Martinez, F., Iacobelli, M., Mendoza, C., Greco, E. 2004. Caspase-dependent and independent DNA fragmentation in Sertoli and germ cells from men with primary testicular failure: relationship with histological diagnosis. *Hum Reprod*., 19, 254-61.

Tissieres, A., Mitchell, H.K. and Tracy, U.M. 1974. Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol Biol*., 84, 389-98.

Tomlinson, M.J., Moffatt, O., Manicardi, G.C., Bizzaro, D., Afnan, M., Sakkas, D. 2001. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Hum Reprod.*, 16, 2160-5.

Troiano, L., Granata, A.R., Cossarizza, A., Kalashnikova, G., Bianchi, R., Pini, G., Tropea, F., Carani, C., Franceschi, C. 1998. Mitochondrial membrane potential and DNA stainability in human sperm cells: a flow cytometry analysis with implications for male infertility. *Exp Cell Res*., 241, 384-93.

Tsujimoto, Y. 1997. Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. *Cell Death Differ*., 4, 427-8.

Tulsiani, D.R.P., Yoshida-Komiya, H., Araki, Y. 1997. Mammalian fertilization: a carbohydrate event. *Biol Reprod*., 57, 487-94.

Van Der Heiden, M.G., Thompson, C.B. 1999. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis. *Nat Cell Biol.*, 1, E209-16.

Van Molle, W., Wielockx, B., Mahieu, T., Takada, M., Taniguchi, T., Sekikawa, K., Libert, C. 2002. HSP70 protects against TNF-induced lethal inflammatory shock. *Immunity*., 16, 685-95.

Vayssier, M., Polla, B.S. 1998. Heat shock proteins chaperoning life and death. *Cell Stress Chaperonens*., 3, 221-227.

Veis, D.J., Sorenson, C.M., Shutter, J.R., Korsmeyer, S.J. 1993. Bcl-2 deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. *Cell*., 75, 229-40.

Verhagen, A.M., Ekert, PG., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J, Vaux, D.L. 2000. Identificatin of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*., 102, 43-53.

Villaroya, S., Scholer, R. 1986. Regional heterogeneity of human spermatozoa detected with monpclonal antibodies. *J Reprod Fertil*., 76, 435-47.

Villaroya, S., Scholer, R. 1987. Lateral diffusion of a sperm-head antigen during incubation in a capacitation medium and the induction of the acrosome reaction in vitro. *J Reprod Fertil*., 80, 545-62.

Wang, K., Yin, X.M., Chao, D.T., Milliman, C.L., Korsmeyer, S.J. 1996. Bid: a novel BH3 domain-only death agonist. *Genes Dev*., 10, 2859-69.

Wang, X., Sharma, R.K., Sikka, S.C., Thomas, A.J. Jr., Falcone, T., Agarwal, A. 2003. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril*., 80, 531-5.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., Mac Gregor, G.R., Thompson, C.B., Korsmeyer, S.J. 2001. Pro-apoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*., 292, 727-30.

Weng, S.L., Taylor, S.L., Morshedi, M., Schuffner, A., Duran, E.H., Beebe, S., Oehninger, S. 2002. Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod*., 8, 984-91.

Westerheide, S.D., Morimoto, R.I. 2005. Heat shock response modulators as therapeutic tools for diseases of protein conformation. *J Biol Chem*., 280, 3097-100.

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.

Wyllie, A.H., Kerr, J.F., Currie, A.R. 1980. Cell death: the significance of apoptosis. *Int Rev Cytol*., 68, 251-306.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R., Greenberg, M. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*., 270, 1326-31.

Yanagimachi, R. 1994. Mammalian Fertilisation. *In:* E. Knobil, J. Neill, eds. *The physiology of reproduction*. 2nd ed., New York: Raven Press. 189-317.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D. P., Wang, X. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome-c from mitochondria blocked. *Science*., 275, 1129-32.

Zakeri, Z.F., Wolgemuth, D.J. 1987. Developmental-stage-specific expression of the hsp70 gene family during differentiation of the mammalian germ line. *Mol Cell Biol.*, 7, 1791-6.

Zakeri, Z.F., Wolgemuth, D.J., Hunt, C.R. 1988. Identification and sequence analysis of a new member of the mouse Hsp70 gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. *Mol Cell Biol*., 8, 2925-32.

Zou, J., Guettouche, T., Smith, D.F., Voellmy, R. 1998. Repression of heat shock transcription factor HSF1 activation by HSP90 (Hsp90 complex) that forms a stress-sensitive complex with HSF1. *Cell.*, 94, 471-80.