



SECTION C

CONCLUSIONS

CHAPTER 6

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6.1 CONCLUSION AND RECOMMENDATIONS

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

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

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

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

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

Assay	2.0 W/kg (RF vs. C)	5.7 W/kg (RF vs. C)	2.0 W/kg vs. 5.7 W/kg	Comments
Capacitation				
Acrosome reaction ¹	No effect	-	-	No effect in number of dead (7AAD [†])-, AI- or AR -sperm.
Motility²	No effect	No effect	NS	Progressive motility (a + b) - No effect
i) Velocity (VAP, VCL, VSL)	No effect	↓ [†]	S (area & acrosome)	i) VSL [†] (VAP & VCL borderline S), Velocity _{5.7W/kg} < Velocity _{2.0W/kg}
ii) Motion (BCF, ALH, STR, LIN)	No effect	↓ [†]		ii) BCF [†] (ALH, STR, LIN - NS)
iii) Morphometric analysis	↓ [†]	↓ [†]		iii) Area _{RF-EMF} < Area _{Control} [†] , Acrosome _{RF-EMF} < Acrosome _{Control} [†]
iv) Hyperactivation	No effect	No effect		
Sperm-zona binding				
HZA	↓ [†]	-	-	For SAR 2 W/kg: HZI _{RF-EMF} < HZI _{Control} [†]
Apoptosis¹				
PS externalisation	No effect	No effect	NS	Including no effect on number of dead cells (PI [†])
MMP	No effect	No effect	-	Including active caspase-3 & all activated caspases
Caspase activation	No effect	No effect	-	
ROS generation	No effect	No effect	-	
DNA fragmentation	No effect	No effect	-	
Stress response				
Hsp 70	No effect ³	-	-	Slight but NS increase assessed by FCM and WB.
Hsp 27	No effect ³	-	-	
Hsp 27P	No effect ³	-	-	
F-actin polymerisation	No effect ⁴	-	-	Also assessed in MCF-7 cells - No effect

Assessed by FCM¹, CASA (Hamilton Thorne)², FCM and WB analysis³, indirect immunofluorescence⁴.

[†]p < 0.05 (time series regression analysis between exposed and control spermatozoa determined over all three time points).

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

NS = Not Significant, S = significant. ↓ = Reduction.

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

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

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

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

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

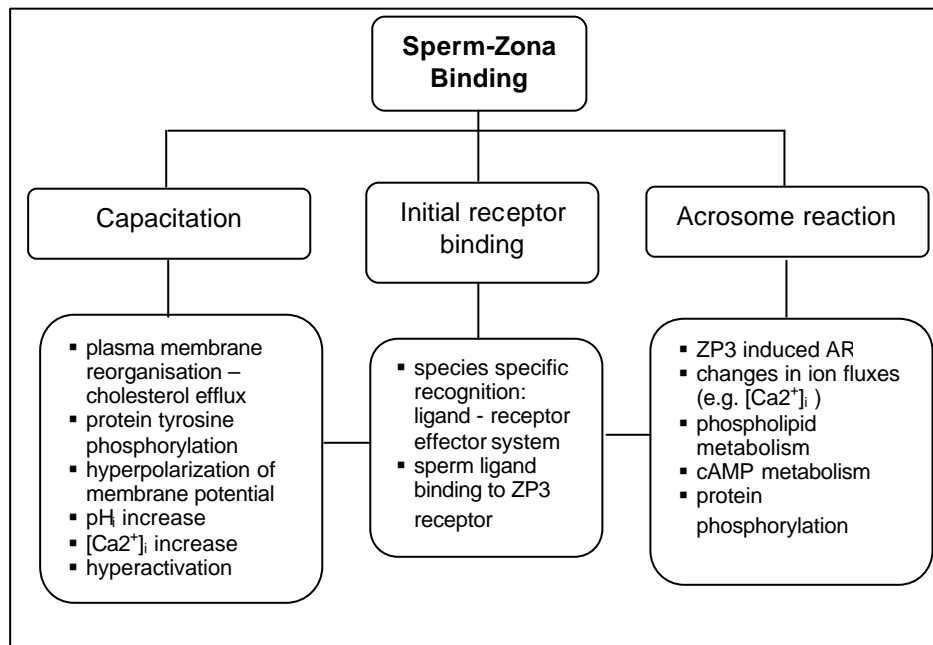


Figure 6.1 The processes of homologous sperm-zona binding and penetration.

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

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

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

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

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

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

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

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



sperm motility at lower SAR levels than that employed in the present study (Fejes *et al.*, 2005; Eroglu *et al.*, 2006), the effect of RF-EMF on human spermatozoa, using the expanded analysis criteria set in this study, should be replicated at lower SAR levels that would simulate the radiation absorption from carrying the cell phone in a pocket close to the testes.

6.2 REFERENCES

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