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Mobile Phone Radiation Does Not Induce Pro-apoptosis Effects in Human Spermatozoa

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Recent reports suggest that mobile phone radiation may diminish male fertility. However, the effects of this radiation on human spermatozoa are largely unknown. The present study examined effects of the radiation on induction of apoptosis-related properties in human spermatozoa. Ejaculated, density-purified, highly motile human spermatozoa were exposed to mobile phone radiation at specific absorption rates (SARs) of 2.0 and 5.7 W/kg. At various times after exposure, flow cytometry was used to examine caspase 3 activity, externalization of phosphatidylserine (PS), induction of DNA strand breaks, and generation of reactive oxygen species. Mobile phone radiation had no statistically significant effect on any of the parameters studied. This suggests that the impairment of fertility reported in some studies was not caused by the induction of apoptosis in spermatozoa.

INTRODUCTION

The possibility of a health hazard posed by mobile phone radiation (RF EMFs) has been investigated for years, but the causality between this radiation and any disease remains unproven (*1, 2*). Recent reports that mobile phone radiation could affect male fertility (*3, 4*) have triggered interest in examining effects of radiation on human spermatozoa.

Aitken *et al.* (*4*) noted a significant genotoxic effect in epididymal spermatozoa after RF EMF exposure. It has been also reported that RF EMF exposure leads to oxidative stress (*5–7*) in human spermatozoa. A causal link between DNA damage in spermatozoa and the generation of reactive oxygen species (ROS) has been shown by Aitken and Baker (*8*).

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The role of RF EMFs in apoptosis is controversial (*9–14*). Also, it is not known whether apoptosis can be initiated in fully differentiated human spermatozoa (*15*). It has recently been reported that RF EMF exposure causes a dose-responsive increase in caspase 3 activity, a pro-apoptosis event, but does not cause apoptosis (*16*). Activation of caspase 3 is considered as an indicator of a cell's commitment to apoptosis (*17*), and activated caspases, in particular caspase 3, have previously been identified in human spermatozoa (*18–20*).

Another marker of apoptosis is active scramblase (*21*), a key enzyme governing translocation of phospholipids across the membrane lipid bilayer (*22, 23*). The inhibition of scramblase leads to exposure of phosphatydylserine (PS) on the outer surface of cell membrane surface, an event that is considered to be an early indicator of apoptosis (*23*). However, in human spermatozoa, it is still a matter of controversy whether PS externalization correlates with other markers of apoptosis (*20, 23–27*).

Regardless of whether human spermatozoa are able to initiate apoptosis, many physiological processes in spermatozoa are mediated by organelles/molecules that also serve as biomarkers for apoptosis. For instance, loss of mitochondrial membrane potential could result in the release of cytochrome c into the cytosol, triggering the caspase 9-dependent pathway to apoptosis (*28*). Conversely, in human spermatozoa, loss of mitochondrial membrane potential has a direct effect on sperm motility (*29*). Therefore, change in mitochondrial membrane potential as a result of RF EMF exposure could provide a sensitive marker for sperm fecundity. In a recent study (*30*) we found no evidence of such an interaction.

In the present study we examined the effect of a 1-h exposure to 900 MHz GSM radiation on the induction of pro-apoptosis events such as activity of caspases, externalization of phosphatydylserine, DNA strand breaks and activation of ROS in human spermatozoa.

MATERIALS AND METHODS

Reagents

Ham's F10 medium, bovine serum albumin (BSA), and Dulbecco's phosphate-buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO). CaspACE™ FITC-VAD-FMK (fluorescein isothiocyanate-valylalanyl aspartic acid-fluoromethylketone) was from Promega (Madison, WI). The Annexin V apoptosis detection kit I, APO-direct kit, 7-AAD and the TUNEL assay were from BD Biosciences (San Jose, CA). Dihydroethidine was from Molecular Probes (Eugene, OR).

Collection and Preparation of Spermatozoa

Semen samples were collected from healthy, nonsmoking donors ($n = 12$) by masturbation after 2 to 3 days of sexual abstinence. The study was conducted according to the Declaration of Helsinki for medical research, and institutional approval was also obtained (Ethics Committee application no. 163/2003, University of Pretoria). The semen samples were allowed to liquefy at 37°C, after which standard semen parameters were evaluated according to the World Health Organization (WHO) (*31*) criteria. Ejaculated human spermatozoa were purified by density gradient as described previously (*30*). A leukocyte-specific antibody (CD45) was used to exclude leukocytes in the processed highly motile fraction of spermatozoa because their presence could contribute to ROS generation (*32*). The total percentage of $CD45^+$ cells was less than 0.3% of the total spermatozoa population after density separation and, according to WHO (*31*), has no pathological significance.

Exposure System

Mobile phone RF EMF exposure (900 MHz pulse-modulated radiofrequency field) was simulated in a specially constructed exposure system based on the use of a high-Q waveguide resonator operating in TE_{10} mode described previously by Leszczynski *et al.* (*33*). Characterization of the chamber construction and radiation dosimetry were reported earlier (*30*). Briefly, two petri dishes containing the spermatozoa were placed inside the exposure system, which was placed inside a humidified incubator. Cells were exposed for 60 min to a 900 MHz GSM communication-like signal at an average SAR of 2.0 W/kg, which represents the recommended limit for local exposure to a mobile phone (*34*), or at 5.7 W/kg, which is almost three times the recommended limit. The RF EMF signal was generated with an EDSG-1240 signal generator and modulated with a pulse duration of 0.577 ms and repetition rate of 4.615 ms to match the GSM signal modulation scheme. The SAR distribution in the cell culture was determined using SEMCAD 1.8 software (Schmid & Partner Engineering AG-SPEAG, Zurich, Switzerland) with a graded simulation grid. Simulation results were verified with temperature rise-based SAR measurements using a calibrated Vitek-type temperature probe (BSD Medical, Salt Lake City, UT). During the exposure, temperature-controlled water was circulated through a glass fiber-moulded waterbed underneath the petri dishes, ensuring that the temperature of the spermatozoa remained at 37 ± 0.2°C.

Experimental Setup

Directly after the exposure, spermatozoa were carefully recovered from the petri dishes and transferred to conical test tubes. Spermatozoa were then incubated under capacitating conditions (6% CO_2 at 37°C) and examined at different times after exposure: immediately (T_1), after 2 h (T_2), and after 24 h (T_3).

Flow cytometry analysis was performed using a Coulter Epics® XL.MCL flow cytometer equipped with an air cooled argon laser (Beckman Coulter, Miami, FL). Data analysis was done using System II software. The spermatozoa population was identified using forward-angle light scatter, while side-angle light scatter was used to exclude electronic noise and debris. The results were expressed as the mean cell number (cells/channel vs. percentage stain). A total of 10,000 events were acquired for each end point.

Detection of Activated Caspases using CaspACE™ FITC-VAD-FMK

CaspACE™ FITC-VAD-FMK is used as an *in situ* marker for apoptosis and is supplied as a 5 m*M* solution in DMSO (Promega). The method described by Marchetti *et al.* (*35*) was adapted; i.e., RF EMF-exposed and control cells (1×10^6/ml) were washed with 2 ml PBS before the pellet (300*g* for 5 min) was resuspended in 1 ml PBS containing 5 μ*M* CaspACE™ FITC-VAD-FMK. Cells were then incubated for 20 min at room temperature in the dark before being washed twice, resuspended in 1 ml PBS, and analyzed by flow cytometry.

Phosphatidylserine Externalization Determined by the Annexin V Assay

Aliquots (100 μl) of control and RF EMF-exposed spermatozoa were diluted in 2 ml cold Dulbecco's PBS and washed by centrifugation at 300*g* for 10 min. Staining was performed according to the manufacturer's instructions (Annexin V apoptosis detection kit I, BD Biosciences). Briefly, the pellet containing spermatozoa was resuspended in 400 μl Annexin V binding buffer [10 m*M* Hepes/NaOH (pH 7.4), 140 m*M* NaCl, 2.5 m*M* $CaCl_2$] at a concentration of 5×10^6 spermatozoa/ml. Then 100 μl of the solution was transferred to a 5-ml flow tube to which 5 μl Annexin V-FITC and 5 μl of propidium iodide (PI) were added. The spermatozoa suspension was gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. After incubation, 400 μl of the Annexin V binding buffer was added to each tube and the spermatozoa were immediately analyzed by flow cytometry. The FITC-labeled Annexin V-positive spermatozoa were measured in the FL1 channel while PI-labeled spermatozoa were measured in the FL3 channel. All tests were run in duplicate.

Detection of DNA Fragmentation by the TUNEL Assay

Spermatozoa recovered from the RF EMF-exposed dishes were frozen at –20°C until the assay. For freezing and storage spermatozoa were suspended in 1% (w/v) paraformaldehyde (Sigma-Aldrich, SA) in PBS (pH 7.4) at a concentration of 2×10^6 spermatozoa/ml. The suspension was incubated on ice for 30 min before the spermatozoa were centrifuged for 5 min at 300*g* and the supernatant was discarded. Then the pellet of spermatozoa was resuspended and washed twice in 5 ml PBS. The cell pellet was then resuspended in the residual PBS by gentle vortexing and the volume was adjusted to 1×10^6 spermatozoa/ml in 70% ice-cold ethanol.

TUNEL staining was done according to the manufacturer's instructions (APO-direct kit). Frozen spermatozoa were thawed at room temperature, resuspended by gently swirling the tubes, and centrifuged for 5 min at 300*g*. The supernatant was removed by gentle aspiration and the spermatozoa pellet was resuspended in 1 ml of the wash buffer. The suspension was washed twice and the spermatozoa were resuspended in 50 μl of the staining solution (10 μl reaction buffer, 0.75 μl TdT Enzyme, 8 μl FITC-dUTP, and 32.25 μl distilled water/assay). The samples suspended in the staining solution were incubated for 60 min at 37°C, after which they were washed twice in 1 ml of the rinse buffer (5 min at 300*g*). The FITC-labeled dUTP-positive spermatozoa were measured in the FL1 channel of the flow cytometer.

Measurement of Reactive Oxygen Species

The method described previously (*29, 36*) for the detection of the superoxide anion radical ($O_2^{-\cdot}$) in human spermatozoa was adapted as follows. Dihydroethidine (stored as stock solutions of 1 m*M* in

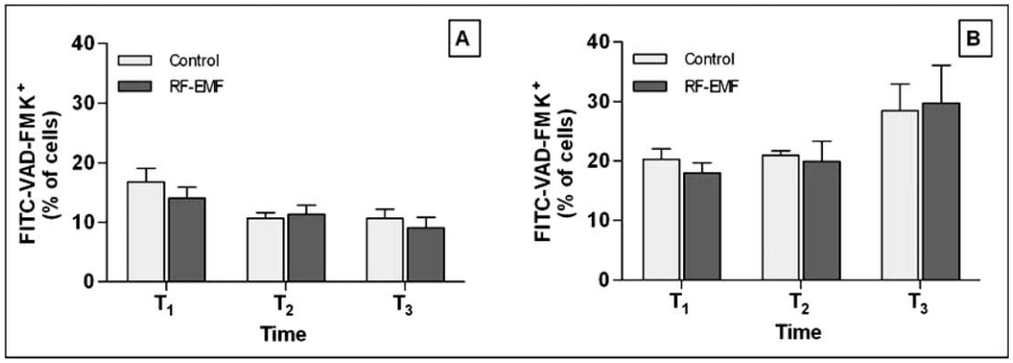

FIG. 1. Assessment of caspase activation (FITC-VAD-FMK$^+$) in spermatozoa exposed to an SAR of (panel A) 2.0 W/kg or (panel B) 5.7 W/kg compared to unexposed (control) spermatozoa. No significant differences in caspase activity between control and exposed spermatozoa were noted at any time or SAR.

DMSO at −20°C) was added directly to spermatozoa suspension: 1×10^6 spermatozoa/ml in BSA-free Ham's F10 medium. Spermatozoa suspensions were then exposed for 15 min at 37°C in a humidified incubator (5% CO_2) to 2 μmol/l dihydroethidine, washed with 2 ml PBS, and resuspended in 1 ml PBS before flow cytometry analysis of ethidium fluorescence.

Statistical Analysis

To determine the effect of RF EMFs on caspase activation, PS externalization, DNA fragmentation and ROS generation, results from a total of 12 donors were analyzed with Stata Statistical Software Release 8.0 (Stata Corporation, 2003, College Station, TX). A within-subject design based on time series regression under the random effect option considering two treatments, control and RF EMF (SAR 2.0 W/kg or 5.7 W/kg), at three times (T_1, directly after exposure, T_2, 2 h after exposure, and T_3, 24 h after exposure) was used. Data are presented as means ± SD for all 12 donors with each test run in duplicate. All statistical tests were two-sided, and statistical significance was considered when $P < 0.05$. Intraclass variability for assays was less than 8%.

RESULTS

FITC-VAD-FMK Detection of Activated Caspases

A slight decrease in activated caspase was observed in both control cells and cells exposed to the RF EMF for 60 min at an SAR of 2.0 W kg^{-1} (Fig. 1A). Caspase activation in RF EMF-exposed sperm did not differ significantly from that in control sperm over the 24-h period ($P = 0.4$). Similarly, at an SAR of 5.7 W kg^{-1}, there was no significant difference between caspase activation in RF EMF-exposed and control sperm ($P = 0.5$) (Fig. 1B); a slight increase over time was noted in both control and RF EMF-exposed sperm. Analyses comparing caspase activation for RF EMF-exposed cells at both SARs revealed no statistically significant differences over time. No caspase 3 activation was noted (results not shown).

Annexin V Assay: Phosphatidylserine Externalization and Cell Viability

No significant differences were seen in the total number of cells with externalized PS (Annexin V$^+$) (Fig. 2-A1), no significant difference ($P = 0.406$) control cells, and cells exposed at an SAR of 2.0 W kg^{-1} directly 2 h or 24 h after exposure. Similarly, no significant differences between RF EMF-exposed and control cells in nonviable (PI$^+$, Fig. 2-A2) ($P = 0.144$) or apoptotic (Annexin V$^+$; PI$^-$, Fig. 2-A3) cells ($P = 0.784$)) were seen at any of the three times.

For an SAR of 5.7 W kg^{-1}, no significant differences between RF EMF-exposed and control cells ($P = 0.448$) were noted in total Annexin V staining (Fig. 2-B1) directly, 2 h or 24 h after exposure. This was also the case for total PI fluorescence (Fig. 2-B2) and apoptotic cells (Annexin V$^+$ PI$^-$) (Fig. 2-B3) over time. For both SARs, Annexin V$^+$ PI$^-$ cells decreased at T_2 (Fig. 2-A3 and 2-B3). Considering that capacitation occurs after 3–4 h under capacitating conditions *in vitro*, it is possible that this decreased expression of PS on the outer membrane is associated with changes in the plasma membrane fluidity due to capacitation.

To determine the effect of the SAR level on cell viability (PI fluorescence) and PS externalization (Annexin V fluorescence), total Annexin V fluorescence, total PI fluorescence and the percentage of apoptotic cells (Annexin V$^+$ PI$^-$) in RF EMF-exposed sperm at the two SARs (2.0 and SAR 5.7 W kg^{-1}) were compared. A paired *t* test at each time found no significant differences.

DNA Fragmentation

The percentage of positive TUNEL-stained cells in control cells and in cells after 60 min RF EMF exposure at an SAR of 2.0 W kg^{-1} at the three different times is shown in Fig. 3A. The percentage of TUNEL$^+$ control cells appeared to increase slightly more rapidly over time compared to RF EMF-exposed cells, but this difference was not significant. There was also no significant difference in TUNEL$^+$ labeling between the RF EMF-exposed and control sperm at the higher SAR (5.7 W kg^{-1}), as shown in Fig. 3B. The possibility of a

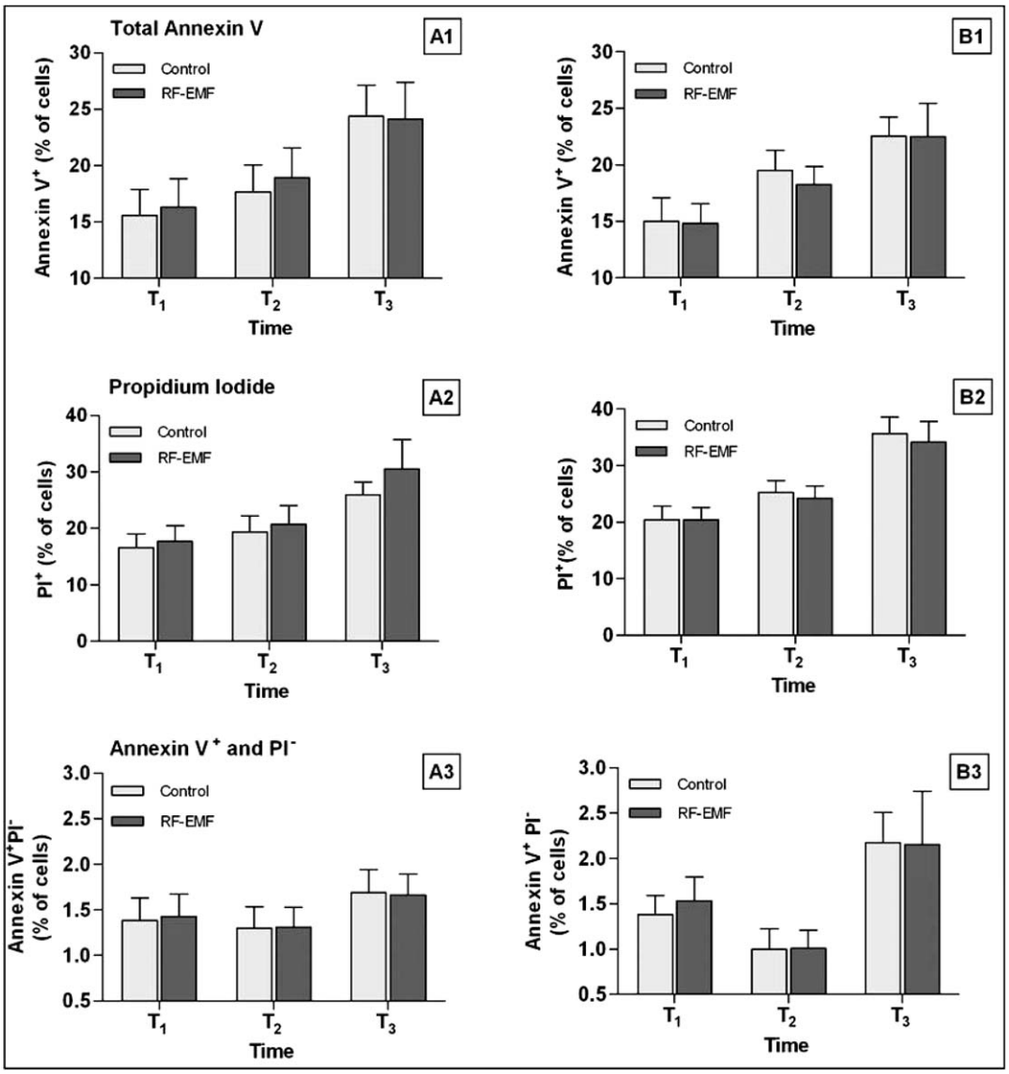

FIG. 2. Annexin V assay assessing PS externalization and cell viability in spermatozoa exposed to an SAR of (panel A) 2.0 W/kg or (panel B) 5.7 compared to unexposed (control) spermatozoa. Panels A1, B1: The mean percentages of cells stained with Annexin V. Panels A2, B2: The mean percentages of non-viable (PI^+) cells. Panels A3, B3: the mean percentages of viable apoptotic cells (Annexin V^+; PI^-).

dose-related effect on DNA fragmentation in RF EMF-exposed spermatozoa at SARs of 2.0 W kg^{-1} and 5.7 W kg^{-1} was also investigated; there were no statistically significant differences.

ROS Generation

A significant increase in O_2^- production was noted after 24 h in both RF EMF-exposed and control sperm for both SARs. No differences in O_2^- production between RF EMF-exposed and control cells were observed at either SAR at any time (Fig. 4A and B). Using a paired *t* test, the possibility of a dose-related effect on ROS production over time was also considered. At T_1 and T_2, there was no statistically significant difference in ROS production in cells exposed at the two SARs. However at T_3, ROS production in cells exposed at an SAR of 5.7 W kg^{-1} differed significantly from that in the same cells exposed at an SAR of 2.0 W kg^{-1}. When these data were compared to those for unexposed (control) sperm at the same time, there was no significant difference in ROS production in sperm exposed at the different SARs.

DISCUSSION

The objective of the present study was to determine whether 900 MHz GSM radiation can induce apoptosis in ejaculated, purified, highly motile human spermatozoa. If so, this could explain the mobile phone radiation-induced decline in human fertility reported in some

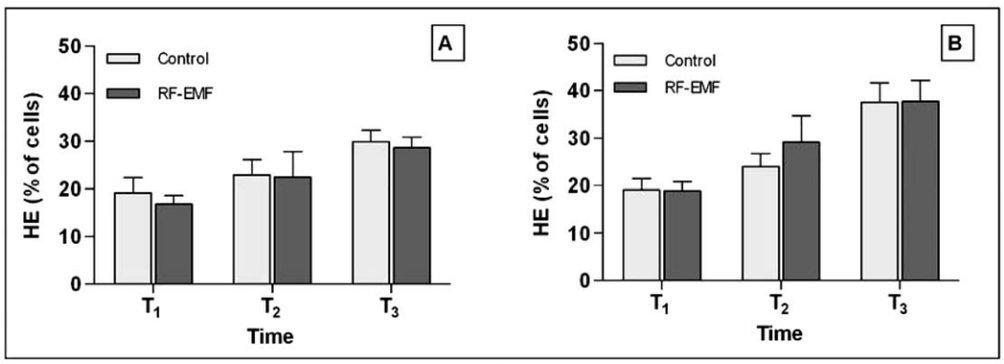

FIG. 3. The mean percentages of cells with fragmented DNA ($TUNEL^+$) in spermatozoa exposed to an SAR of (panel A) 2.0 W/kg or (panel B) 5.7 W/kg compared to unexposed (control) spermatozoa. No significant differences in TUNEL staining between control and exposed spermatozoa were noted at any time or SAR.

studies (*37*). It could also have a serious implications for assisted reproductive techniques because current data suggest that impaired sperm DNA integrity may have the greatest effect on IUI pregnancy rates and pregnancy loss by IVF and ICSI (*38*).

In human spermatozoa, the role of caspase activation is not clearly understood. Kotwicka *et al.* (*20*) noted that caspase activation and phosphatidylserine (PS) externalization (normally seen as an early indicator of apoptosis) in human spermatozoa were not directly related to other markers of apoptosis. This may suggest an independence of the mechanisms of PS translocation and caspase activation in the physiology of spermatozoa. In some studies an increase in caspase activity after RF EMF exposure was observed that did not correlate with expression of markers of apoptosis (*16, 39*).

In the current study, a cell-permeable fluorescent derivative of the inhibitor peptide VAD-FMK was used to detect the overall caspase activation in spermatozoa (*35, 40*). Caspase activities observed in control spermatozoa are comparable with those reported by Marchetti *et al.* (*35*). RF EMF exposure had no caspase-activating effect in human spermatozoa under our experimental conditions.

PS exposure to the outer leaflet of the plasma membrane has previously been observed in human spermatozoa (*40–43*). Consistent with results from previous studies showing a lack of PS externalization in various cell types after *in vitro* exposure to RF EMFs (*16, 44, 45*), no evidence of a change in PS externalization was found after a 1-h exposure of human spermatozoa to 900 MHz GSM radiation. The percentage of PS-externalizing living cells (Annexin V^+ PI^-) observed in the present study is in close agreement with the observations of Ricci *et al.* (*46*) but differs from those of other studies (*47, 48*). This is likely due to the similar method used by Ricci *et al.* (*46*) to isolate the spermatozoa population, where the use of leukocyte-specific antibody (anti-CD45) eliminated the leukocyte contamination.

There are several conflicting reports regarding the ability of RF EMFs to induce DNA damage. Genotoxic effects were observed in certain cell types (*49, 50*), while in other cells RF EMFs did not appear to induce DNA

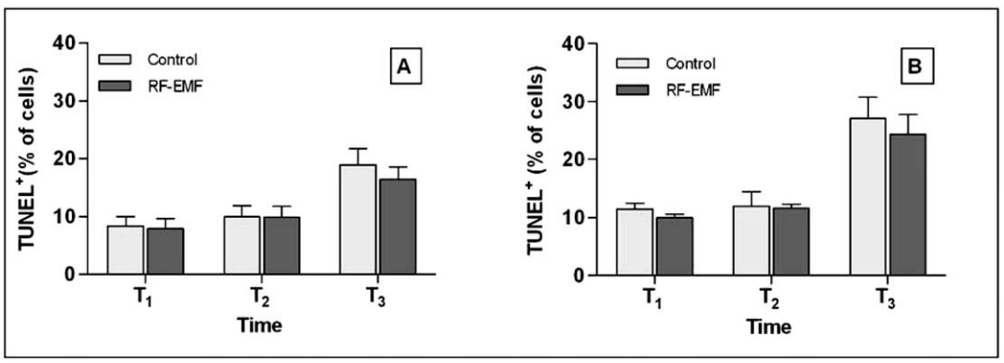

FIG. 4. The mean percentages of oxidative stress (O_2^-) determined by dihydroethidium (2 μM) staining in spermatozoa exposed to an SAR of (panel A) 2.0 W/kg or (panel B) 5.7 W/kg compared to unexposed (control) spermatozoa. No significant differences in ROS generation between control and exposed spermatozoa were noted at any time or SAR.

damage (*44, 51*). It has been reported (*52*) that RF EMF exposure may cause DNA damage due to the activation of a caspase-independent pathway to apoptosis. If RF EMFs indeed exert a genotoxic effect on human cells, then the post-meiotic male germ cells would be a particularly sensitive target (*53*).

Using the TUNEL assay, we examined DNA integrity in spermatozoa exposed to SARs of 2.0 and 5.7 W/kg. The DNA fragmentation levels observed in spermatozoa (control nonirradiated samples) in the present study correspond to those reported previously using flow cytometry and the TUNEL assay (*29, 54, 55*). However, significant differences in DNA fragmentation between RF EMF-exposed and control spermatozoa were observed. These results are in agreement with Agarwal *et al.* (*6*), who also noted no differences in DNA integrity after RF EMF exposure of unprocessed human spermatozoa. Aitken *et al.* (*4*), on the other hand, reported that a 7-day exposure of male mice to RF EMFs induced significant DNA damage in both the nuclear and mitochondrial genomes of spermatozoa recovered from the cauda epididymis. It is possible that DNA damage is a cumulative effect, as was suggested by Fejes *et al.* (*56*), and is not apparent after a 1-h exposure.

It has recently been suggested that oxidative stress may be the underlying mechanism responsible for the reported cellular effects of RF EMFs (*57, 58*). Spermatozoa are particularly susceptible to oxidative stress due to the high content of unsaturated fatty acids in their membranes and their limited stores of antioxidant enzymes (*8*). The presence of leukocytes in the purified highly motile fraction of spermatozoa could contribute to increased ROS generation. Therefore, we used a leukocyte-specific antibody (CD45) to remove leukocyte contamination. Our data demonstrated that the ability of human spermatozoa to generate ROS was not affected by RF EMF exposure. This observation agrees with the results of Lantow *et al.* (*11*), who used human monocytes and lymphocytes, but disagrees with Agarwal *et al.* (*6*), who noted a significant increase in oxidative stress in human spermatozoa after RF EMF exposure. It is possible that the RF EMF exposure-induced ROS production detected by Agarwal and coworkers (*6*) was due to the presence of leukocytes in the neat semen samples used in their study. Furthermore, ROS generation leads to the inhibition of sperm motility and ATP production (*29, 32, 54*). Therefore, the decreased motility parameters noted by Agarwal *et al.* (*6*) could be the result of specific effects of RF EMFs on leukocytes. We were unable to detect any changes in sperm motion characteristics after RF EMF exposure compared to controls (*30*) under experimental conditions where substantial ROS generation is unlikely to occur.

In conclusion, we found no evidence of any *in vitro* effect of RF EMF exposure on pro-apoptosis events such as caspase activation, phosphatydylserine expression, DNA fragmentation or ROS generation in human spermatozoa. These results appear to be reliable because we took great care to rule out any temperature rise-related effects and leukocyte contamination-related effects (ROS generation) in our experiments. The observed lack of apoptosis in human sperm exposed to mobile phone radiation suggests that the impairment of fertility that was reported in some *in vivo* studies was not caused by the induction of apoptosis of spermatozoa. The outcome of this *in vitro* study may suggest that alternative, non-apoptosis events that might affect fertility *in vivo*.

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