Original Article

In Vitro Effect of Cell Phone Radiation on Motility, DNA Fragmentation and Clusterin Gene Expression of Human Sperm

Running Title: Cell Phone Radiation Effects on Sperm Parameters

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Abstract

**Background:** Use of cellular phones that emits radiofrequency electromagnetic field (RF-EMF) has been increased exponentially and became a part of everyday life. This study aimed to investigate the effects of RF-EMF radiation emitted from cellular phones on sperm motility variables, sperm DNA fragmentation and clusterin (CLU) gene expression.

**Materials and Methods:** 124 semen samples were grouped into; normozoospermia (N, n=26), asthenozoospermia (A, n=32), asthenoteratozoospermia (AT, n=31) and oligoasthenoteratozoospermia (OAT, n=35). Semen samples were divided into two aliquots; samples not exposed to cell phone and samples exposed to cell phone radiation for 1 hr. Before and immediately after exposure both aliquots were subjected to assessment of sperm motility, acrosin activity, sperm DNA fragmentation and CLU gene expression. Statistical differences were analyzed using paired t-student test for comparisons where \( P < 0.05 \) was set as significant.

**Results:** There was significant decrease in sperm motility, sperm linear velocity, sperm linearity index, sperm acrosin activity and significant increase in sperm DNA fragmentation percent, CLU gene expression and CLU protein levels in the exposed semen samples to RF-EMF compared with non-exposed samples in OAT > AT > A > N groups \( (P<0.05) \).

**Conclusions:** Cell phone emissions have a negative impact on exposed sperm motility indices, sperm acrosin activity, sperm DNA fragmentation and CLU gene expression especially in OAT cases.

**Key words:** Cell phone; spermatozoa; electromagnetic radiation; sperm motility.

**Introduction**

Nowadays, cell phone technology is an integral part of everyday life, and its use will continue to grow as their providers proceed to offer more liberal services and newer, better products. Generally, a growing
concern for cell phones possible adverse effects on human health evoked flurry of scientific activities. Several studies had associated between human health and exposure to radiofrequency electromagnetic field (RF-EMF), emphasizing on clinical conditions as childhood leukemia, brain tumors, neurodegenerative diseases and genotoxicity (1).

RF energy is a type of non-ionizing radiation, including EMR that is produced by cell phones but is not strong enough to cause ionization of atoms or molecules. Cellular phones emit low levels of RF in the micro-wave range while being used. Although high levels of RF can produce health effects by heating tissues, exposure to low-level RF does not produce such effects. Several experimental studies demonstrated that exposure to electromagnetic or static magnetic fields had adverse effects on the reproductive system (2).

De Iuliis et al. (3) demonstrated that RF-EMF in both the power density and frequency range of mobile phones enhances mitochondrial reactive oxygen species (ROS) generation by human spermatozoa, decreasing sperm motility, vitality while stimulating DNA base adduct formation and, ultimately sperm DNA fragmentation. Kang et al. (4) showed that cell phone radiation may cause structural and functional injuries of the testis, altered semen parameters and reduced epididymal sperm concentration. In May 2011, the international agency for research on cancer (IARC) at WHO categorized the RF-EMF from mobile phones and from other devices that emit similar non-ionizing electromagnetic fields as a group 2B (possible) human carcinogen (5).

Sperm DNA fragmentation in the male germ line has been associated with impaired fertilization, poor embryonic development and high rates of miscarriage (6). Of course, the attention has been focused on the environmental and genetic factors that might be involved in the etiology of sperm DNA damage. One of these factors that had potentially risen is the increased exposure to RF-EMF emitted from cell phones (7).
Clusterin (CLU), a 70-80 kDa heterodimeric, disulfide-linked glycoprotein is expressed in a variety of tissues being over-expressed in tissues undergoing stress. CLU appeared to be a potential pathophysiologically gene having multiple functions related to apoptosis, inflammation, proliferation, and differentiation (8, 9).

This study aimed to assess the possible relationship of in vitro RF-EMF exposure from cell phones on sperm motility variables, sperm DNA fragmentation and sperm CLU gene expression.

**Materials and Methods**

Semen samples were collected from 124 individuals presented to Mansoura University Hospital, Egypt after Institutional review Board approval. Exclusion criteria were; smoking, leucocytospermia, varicocele and abnormal karyotyping. Semen samples were collected by masturbation after an abstinence period of 4-5 days according to WHO guidelines (10). According to their semen analysis; they were grouped into normozoospermia (N, n=26), asthenozoospermia (A, n=32), asthenoteratozoospermia (AT, n=31) and oligoasthenoteratozoospermia (OAT, n=35).

Semen samples were separated into two groups of non-exposed (control) and exposed (experimental). The exposure was performed for 60 min. Exposed semen samples were exposed to EMW emitted from a commercially available cellular phone (850 MHz frequency; maximum power <1 W; specific absorption rates 1.46 W/kg) kept at 10 cm distance for 60 min. Unexposed semen aliquots were kept under the same conditions without RF-EMW exposure at room temperature to avoid the effect of temperature or reactive oxygen species (ROS) formation on semen parameters. After elapsed time, both aliquots were evaluated in terms of sperm motility variables, acrosin activity, sperm DNA fragmentation and CLU gene expression before and immediately after exposure.

**Sperm acrosin activity**

It was assessed by gelatinolysis where gelatin-covered slides were prepared by spreading 20μl 5% gelatin (Merck, Darmstadt, Germany) in distilled water on the slides. The slides were air-dried, stored at 4°C overnight, fixed and washed in phosphate-buffered saline (PBS). Purified spermatozoa were diluted 1:10
in PBS containing 15.7mMol α-D-glucose. Semen samples were incubated in a moist chamber at 37°C for 2 h. The halo diameter around any 10 sperm was measured in phase contrast with an eyepiece micrometer. The halo formation rate was calculated/slide as the percentage of spermatozoa showing a halo after evaluating 100 spermatozoa (acrosin activity index = halo diameter x halo formation rate) (11).

**Sperm DNA Fragmentation analysis**

It was performed in fresh semen using flowcytometry (DAKO-Cytomation, Glostrup, Denmark) by the kit supplied by Coulter (Beckman Coulter, Fulterton, CA, USA) based on the fluorescence emission from individual sperm stained with propidium iodide (PI) and excitation with 488 nm argon laser. Semen samples were diluted with phosphate buffered saline (PBS) (pH 7.4) to 2 x10⁶ sperm/ml where 50 µl were incubated with 100 µl lysing reagent for 15 sec then 2 ml of PI were added and mixed and immediately after staining, tube acquisition was done by flowcytometry where the intensity of its fluorescence emission corresponds to the DNA content. The analysis displays a constant and characteristic bimodal non-artifactual DNA pattern confirming the existence of two distinct populations. The main population is represented by a peak followed by a shoulder which is the marginal population representing a sperm group altered in the nuclear condensation (DNA damage), yielding unstable chromatin appearing more stainable. The Percentage of sperm cells with DNA damage was automatically calculated by the flowcytometer after acquisition of 5000 sperm (12).

**CLU gene expression**

Simultaneous total RNA and total proteins isolation were done using Tri-Fast reagent kit (PeqLab Biotechnologie GmbH, Germany). The remaining DNA was removed by digestion with DNase I (Sigma-Aldrich, St. Louis, MO, USA). The concentration of isolated RNA was determined spectrophotometrically at optical density 260 nm. Ten µl of each sample was added to 990 µl diethylpyrocarbonate (DEPC) treated water and quantified by measuring the absorbance at 260nm
as RNA yield (µg/ml) = A260 X 40 X 100 (dilution factor). Purity of RNA was assessed by gel electrophoresis through formaldehyde agarose gel electrophoresis and ethidium bromide staining to show 2 sharp purified bands representing 28S and 18S ribosomal RNA.

RT-PCR for extracted RNA

Semiquantitative RT-PCR was performed using ready-to-go RT-PCR beads for first cDNA synthesis and PCR reaction (Amersham Biosciences, Piscataway, NJ, USA) utilizing Moloney murine leukemia virus reverse transcriptase and Taq polymerase to generate PCR product from RNA template. Each bead is optimized to allow the first strand cDNA synthesis and PCR reaction to proceed sequentially as a single tube, single step reaction.

Synthesis of cDNA

The following was added to the tube containing the beads, the first strand primer (2µl), 3 µl (30 pmol) PCR gene-specific primer (sense), 3µl (30 pmol) PCR gene-specific primer (anti-sense), 25 µl total template RNA containing 1 µg and 17µl DEPC-treated water till 50 µl were added. One tube was prepared as a negative control reaction to test DNA contamination. The dehydrated bead (without template and primers) was incubated at 95°C for 10 min to inactivate M-MuLV reverse transcriptase where 50 µl mineral oil was added to overlay the reaction. The reactions were transferred to the thermal cycler, incubated at 40°C for 30 min for synthesis of cDNA followed by incubation at 95°C for 5 min to inactivate reverse transcriptase and denature the template. The sequence of oligonucleotide primers of clusterin gene were designed from GenBank sequences 5′-CTTGATGCCCCTCTCTCCGTA-3′ (sense) and 5′-AACGTCGAGTCAGAAGTGTG-3′ (antisense), located at nucleotides 684 to 704 and 1194 to 1214 of CLU cDNA. Thermal cycling reaction was performed, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min then final extension at 72°C for 10 min. The products were subjected to agarose gel electrophoresis using 2% agarose stained with ethidium bromide and visualized via light UV transilluminator and photographed.
Western blotting of CLU protein by immunoblotting technique was done using rabbit anti-human CLU polyclonal unconjugated primary antibody antibody against β- tubulin as a control. Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) was used as secondary antibody. Colorimetric immunodetection of the protein used an enzyme substrate (tetramethylbenzidine) that reacted with the reporter enzyme (HRP) and precipitated onto the conjugated antibodies. The bands on the membrane were digitally photographed and were analyzed with Scion image release alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA) performing bands detection and conversion to peaks. Area under each peak was calculated in square pixels and used for quantification. CLU gene expression and CLU protein levels were determined by calculating the ratio between the square pixel values of the target bands in relation to the control bands.

Statistical Analysis

It was performed using SPSS program version 17 (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± standard deviation (SD). The statistical differences were analyzed using paired t-student test for comparison between two groups. P< 0.05 was set as significant.

Results

The mean sperm concentration in the N, A, AT and OAT groups was 54.34 ± 5.0, 38.85 ± 4.04, 23.52 ± 8.94 and 8.00 ± 3.77 (10^6/ml) respectively. The mean sperm abnormal forms percent in the investigated groups was 11.42 ± 2.61, 10.04 ± 3.7, 30.80 ± 7.22, 39.68 ± 5.6 respectively. Sperm motility, sperm linear velocity, sperm linearity index and sperm acrosin activity were significantly decreased (P <0.05). However, there were significant increase in sperm DNA fragmentation percent, CLU gene expression and CLU protein levels in the exposed semen samples to RF-EMR compared with non-exposed samples in OAT > AT > A > N groups (P <0.05). Semen samples of N group demonstrated minimal nonsignificant decrease in sperm motility, sperm linear velocity, sperm linearity index, sperm acrosin activity and
demonstrated significant increase in sperm DNA fragmentation percent, sperm CLU gene expression and CLU protein levels (P <0.05) compared with the non-exposed samples (Table 1-Figures 1, 2).

Discussion

In the current study, semen exposure to RF-EMF led to a significant decrease of sperm motility variables compared to non-exposed semen samples. Previously, Fejes et al. (7) pointed to cell phone use and male infertility by an epidemiological study observing negative correlation between its use and various attributes of semen quality, particularly sperm motility. This was followed by an experimental study involving exposure of male mice to RF-EMF that revealed a significant impact on the integrity of both the mitochondrial and nuclear genomes (13). Kilgallon and Simmons (14) demonstrated that storage of mobile phones close to the testes can decrease semen quality. Similarly, Erogul et al. (15) reported decreased rapid and slow sperm motility in semen samples exposed to 900 MHz cell phone for 5 min where non-progressive and immotile sperm populations were increased after exposure. Agarwal et al. (16) confirmed such negative impact on semen quality correlating defects in sperm count, motility, viability and normal morphology with longer duration of usage independent of the initial semen quality. Agarwal et al. (17) added that exposed spermatozoa to mobile phone radiation for 1 hr leads to significant declines in sperm motility and vitality associated with increased cellular ROS generation coupled with decreased ROS-Total antioxidant capacity score.

Such significant decline in sperm motility indices was explained by intrinsic ROS generation reinforced with the significant increase of sperm DNA fragmentation in the exposed semen samples compared to the unexposed one in vitro (18). Several lines of evidence suggested that oxidative stress (OS) plays a key role in the underlying etiology where spermatozoa are sensitive to such stress as they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack in the form of unsaturated fatty acids and DNA (19). Moskovtsev et al. (20) showed that EMF of cell phones may cause DNA breakage in spermatozoa in a low-frequency EMF possibly due to ROS production that stimulated sperm plasma membrane redox system. De Iuliis et al. (3) added that RF-EMF in both the
power density and frequency range of mobile phones enhances mitochondrial ROS generation by human spermatozoa, decreasing its motility and vitality while stimulating DNA base adduct formation and, ultimately sperm DNA fragmentation.

It has been suggested that cohorts of spermatozoa are particularly vulnerable to the induction of OS by RF-EMF where a decrease in sperm motility and viability is expected to be linked to concentration of superoxide anion in semen that can oxidize sperm membrane phospholipids. In addition, these reported effects could be attributed to thermal insult induced by RF exposure (18). Aitken et al. (13) observed a significant impact on the integrity of both the mitochondrial and nuclear genomes after exposure of male mice to RF-EMF. In contrast, McNamee et al. (21), Tice et al. (22) and Stronati et al. (23) demonstrated nonsignificant DNA damage in human cells exposed to RF-EMF.

De Iuliis et al. (3) suggested that one of the key environmental factors involved in the stimulation of sperm mitochondria to produce high levels of ROS, might be excess exposure to RF-EMF from mobile phones. Moreover, such stress is known to induce functional and structural lesions including loss of motility mediated by peroxidative damage to the sperm plasma membrane as well as formation of DNA base adducts in the sperm nucleus leading to DNA fragmentation (24). Agarwal et al. (25) concluded that DNA damage due to EMW is significant but this damage may be of cumulative effect of repeated exposure not revealed after short term exposures.

Exposure to emitted radiation from mobile phones was demonstrated to have an up-regulation of both CLU mRNA and its full length protein in infertile semen samples compared with the normozoospermic samples. Strocchi et al. (26) hypothesized that increased levels of CLU mRNA in morphologically normal cells were due to cells attempting to protect themselves from local stress conditions. Therefore, increased CLU expression could be explained by the physiological defense to reduce cell damage and maintain cell viability during periods of stress exposure exerted through CLU ability to act as a scavenger. Trougakos et al. (8) proposed that CLU has antioxidant properties and is capable of protecting cells from apoptosis induced by ROS. Strocchi et al. (27) supported the notion that an increase in CLU expression
may be a physiological defense mounted to reduce cell damage and maintain cell viability during periods of increased OS.

Therefore, increased CLU expression was associated in parallel with increased sperm DNA fragmentation and decreased sperm acrosin activity being triggered by OS (28). It is suggested that OS plays a key role in the underlying sperm DNA fragmentation as well as acrosin activity. When ROS production by the sperm mitochondria is excessive, then the gamete's limited endogenous antioxidant defenses are rapidly overwhelmed and oxidative damage is induced that led to peroxidation of the sperm acrosomal membrane, diminished acrosin activity (29-32).

A point of limitation in this study is assessing exposed multiple duration effects in addition to reversibility effect to know if sperm affection is time related or not that is needed for further work. Also, a question is raised about the popular current use of iPads with cellular access that emits RF radiation, is its RF dose greater than with cell phones and therefore potentially more damaging.

**Conclusions**

Cell phone emissions have a negative impact on sperm motility indices, sperm acrosin activity, sperm DNA fragmentation and CLU gene expression especially in OAT cases.

**Conflict of interest**

No conflict of Interest

**References**


Figure 1. CLU gene expression of non-exposed groups (right) and exposed group (left) to mobile phone radiation;

Lane1: DNA marker, Lane 2: N group, Lane 3: A group, Lane 4: AT group, Lane 5: OAT group, Lane 6: negative control.
Figure 2. CLU protein expression by Western blotting (40kd) in non-exposed groups (upper right) and non-exposed groups (upper left). Internal control, tubulin expression by Western blotting (50 kd) in the non-exposed groups (lower right) and non-exposed groups (lower left). Lane 1 N group, Lane 2: A group, Lane 3: AT group, Lane 4: OAT group.
Table 1. Estimated data in the exposed semen groups vs non-exposed groups (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>N (n=26)</th>
<th>A (n=32)</th>
<th>AT (n=31)</th>
<th>OAT (n=35)</th>
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<tbody>
<tr>
<td></td>
<td>Unexposed</td>
<td>Exposed</td>
<td>Unexposed</td>
<td>Exposed</td>
</tr>
<tr>
<td>Sperm motility</td>
<td>60.8 ± 4.5</td>
<td>56.5 ± 4.2</td>
<td>26.5 ± 9.4</td>
<td>18.4 ± 7.9</td>
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<tr>
<td></td>
<td>4.2</td>
<td>5.0*</td>
<td>11.9*</td>
<td>7.9*</td>
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<td>Sperm linear</td>
<td>59.6 ± 8.0</td>
<td>56.0 ± 4.2</td>
<td>39.1 ± 11.7</td>
<td>26.5 ± 5.0</td>
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<td>velocity %</td>
<td>8.4</td>
<td>12.8*</td>
<td>9.5*</td>
<td>9.4*</td>
</tr>
<tr>
<td>Sperm linearity</td>
<td>79.0 ± 7.0</td>
<td>76.7 ± 10.2</td>
<td>65.6 ± 11.4</td>
<td>52.3 ± 16.3</td>
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<tr>
<td>index</td>
<td>6.8</td>
<td>8.9*</td>
<td>9.7*</td>
<td>11.4*</td>
</tr>
<tr>
<td>Sperm acrosin</td>
<td>13.2 ± 3.3</td>
<td>12.6 ± 2.4</td>
<td>8.3 ± 5.0</td>
<td>4.05 ± 2.5</td>
</tr>
<tr>
<td>activity</td>
<td>3.2</td>
<td>2.0*</td>
<td>2.5*</td>
<td>1.9*</td>
</tr>
<tr>
<td>CLU - RNA</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>1.5 ± 1.2</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>expression</td>
<td>0.1*</td>
<td>0.6</td>
<td>0.8*</td>
<td>1.1*</td>
</tr>
<tr>
<td>CLU - protein</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>1.4 ± 1.9</td>
<td>4.1 ± 3.2</td>
</tr>
<tr>
<td>expression</td>
<td>0.5*</td>
<td>0.4*</td>
<td>0.8*</td>
<td>2.1*</td>
</tr>
<tr>
<td>Sperm DNA</td>
<td>11.5%</td>
<td>30.8%**</td>
<td>18.8%***</td>
<td>56.3%**</td>
</tr>
<tr>
<td>fragmentation %</td>
<td></td>
<td>29%</td>
<td>71.0%**</td>
<td>40.0%</td>
</tr>
</tbody>
</table>

*aSignificant difference compared with unexposed semen samples (P<0.05)

*bSignificant difference compared with unexposed semen samples (P<0.01)

*cSignificant difference compared with unexposed semen samples (P<0.001)