



Mitochondrial DNA damage and oxidative damage in HL-60 cells exposed to 900 MHz radiofrequency fields



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ABSTRACT

HL-60 cells, derived from human promyelocytic leukemia, were exposed to continuous wave 900 MHz radiofrequency fields (RF) at 120 $\mu\text{W}/\text{cm}^2$ power intensity for 4 h/day for 5 consecutive days to examine whether such exposure is capable of damaging the mitochondrial DNA (mtDNA) mediated through the production of reactive oxygen species (ROS). In addition, the effect of RF exposure was examined on 8-hydroxy-2'-deoxyguanosine (8-OHdG) which is a biomarker for oxidative damage and on the mitochondrial synthesis of adenosine triphosphate (ATP) which is the energy required for cellular functions. The results indicated a significant increase in ROS and significant decreases in mitochondrial transcription factor A, mtDNA polymerase gamma, mtDNA transcripts and mtDNA copy number in RF-exposed cells compared with those in sham-exposed control cells. In addition, there was a significant increase in 8-OHdG and a significant decrease in ATP in RF-exposed cells. The response in positive control cells exposed to gamma radiation (GR, which is also known to induce ROS) was similar to those in RF-exposed cells. Thus, the overall data indicated that RF exposure was capable of inducing mtDNA damage mediated through ROS pathway which also induced oxidative damage. Prior-treatment of RF- and GR-exposed cells with melatonin, a well-known free radical scavenger, reversed the effects observed in RF-exposed cells.

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1. Introduction

Wireless communication devices which emit non-ionizing radiofrequency fields (RF) in the frequency range 800–2600 MHz have gone through phenomenal development and deployment worldwide during the last several decades. Their widespread use has raised concerns about the potential human health hazards of increased and chronic exposure to RF. Researchers have been using several different parameters to evaluate the biological and health effects of in vitro and in vivo exposure to RF in animals and humans. Among these, genetic damage had received the attention of several researchers since such damage in somatic cells can lead to the development of cancer or cell death while the same in germ cells can lead to mutations that can be transmitted to subsequent generations. Thus far, studies on the genotoxic potential of RF have been focused mainly on the large amount of DNA present in the

nucleus of cells [1–3] while the small amount of DNA located in mitochondria (mtDNA or mitogenome) in cells did not received much attention.

Mitochondria are dynamic, double membrane bound organelles located in the cytoplasm of eukaryote cells and their number varies widely by cell type, tissue and organism [4]. They have been described as 'power house' of cells [5] and utilizes oxygen from respiration to synthesize adenosine triphosphate (ATP) which is the cells' energy used for various functions including cellular metabolism, calcium signaling, regulation of membrane potential, cell cycle, cell growth, apoptosis, etc. [6–9]. Each human cell contain ~100 mitochondria and each mitochondrion is composed of 1 to 15 copies of circular, closed, double-stranded mtDNA composed of 16,569 base pairs. The 2 strands of mtDNA are differentiated by their nucleotide content, guanine-rich heavy strand (H) and cytosine-rich light strand (L). The H-strand codes for 9 genes while L-strand codes for 28 genes. Thus, mtDNA encodes a total of 37 genes: 13 of these encodes essential polypeptides/proteins of the respiratory electron transport chain to generate ATP, 22 mitochondrial transfer RNA (tRNA) which serve as the link between the messenger RNA (mRNA) and the amino acid sequence of proteins

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and 2 ribosomal RNA (rRNA, one large 16S and one small 12S) which are essential for translation of mRNAs into protein [10].

In human cells, the genes for mtDNA copy number (ND1) and ATP overlap each other. The 2 rRNAs (16S and 12S) are essential for translation of mRNA into protein. It has been reported that damage to mtDNA may start at transcription and replication levels [11]. The mitochondrial transcription factor A (mt-TFA) is the key activator of mitochondrial transcription as well as a participant in mtDNA replication, mitochondrial biogenesis and cellular metabolism in cells. Aberrant transcription of rRNAs (16S) may accumulate in the mitochondria and interfere with protein folding by modifying their secondary structure which could change the function of their enzymes or initiate unfolded protein response resulting in cellular apoptosis [12]. Since the transcription is coupled with replication, alteration in mt-TFA can have an effect in maintaining mtDNA copy number [13–15]. Furthermore, mitochondrial polymerase gamma protein (poly- γ) is reported to be involved in the repair of mtDNA damage and fidelity in replication [16]. In addition, mtDNA polymerase is the target of oxidative damage induced by reactive oxygen species (ROS, see below) which set forth a cascade of events leading to mitochondrial dysfunction and subsequent energy decline [17]. Thus, all these different components are important for the fidelity in replication and integrity of mtDNA in a cell.

Mitochondria utilizes oxygen during ATP synthesis. This process is efficient but, may prematurely reduce oxygen resulting in the formation of ROS. There is well-documented evidence that excess ROS induces oxidative stress/damage leading to mitochondrial dysfunction and also damages mtDNA: both have been implicated in ageing and in a number of human diseases including autism, cancer, cardiovascular diseases, diabetes, nervous system disorders, etc. [18–26]. Because of the location inside the mitochondria, mtDNA may be potentially at risk for damage by ROS and other similar ROS-generating agents such as ionizing radiation [27,28].

To the best of our knowledge, there has been only one peer-reviewed scientific publication in which ROS and mtDNA damage were examined in cells exposed to RF. Xu et al. [29] have isolated cortical neuronal cells from Sprague Dawley rats and primary cultured cells were exposed *in vitro* to modulated 1800 MHz RF at an average specific absorption rate (SAR) of 2 W/kg for 24 h. The results showed that 1800 MHz RF exposure induced a significant increase in ROS and 8-hydroxy-2'-deoxyguanine (8-OHdG), a biomarker of oxidative damage and also decreased mitochondrial tRNA transcripts as well as mtDNA copy number in mitochondria. Each of these effects were reversed when the cells were pre-treated with melatonin (Mel), an efficient free radical scavenger and a wide-spectrum antioxidant [30,31]. In this investigation, we have used cultured human promyelocytic leukemia cells (HL-60) to 900 MHz RF at 120 $\mu\text{W}/\text{cm}^2$ power intensity for 4 h/day, for 5 consecutive days and examined: (1) ROS, (2) mitochondrial transcription factor A (mt-TFA) and mtDNA polymerase gamma (poly- γ), (3) mtDNA transcripts, ND1 and 16S, (4) mtDNA copy number, (5) 8-OHdG and, (6) intracellular ATP. In addition, HL-60 cells were treated with Mel to determine if such treatment reduces ROS levels and protects the cells from mtDNA damage. The observations in RF-exposed cells were compared with those which were unexposed (kept in incubator) and sham-exposed (SH, without RF transmission) as well as those subjected to gamma radiation (GR, positive control cells).

2. Materials and methods

2.1. HL-60 cell cultures

HL-60 cells were gift from the Department of Toxicology, School of Public Health, Soochow University, Suzhou city, China (orig-

inal source – American Type Culture Collection, VA, USA). They were cultured in RPMI 1640 medium (Hyclone, Suzhou, China) supplemented with 2 mM L-glutamine, 2.5 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated FBS (Gibco, Shanghai, China) in an incubator which maintained $37 \pm 0.5^\circ\text{C}$ and humidified atmosphere of 5% carbon dioxide and 95% air. Sub-culturing was done every 2–3 days when the cell density reached $>10^6$ cells/ml. The entire study was repeated 3 times.

2.2. RF and sham (SH) exposure

The exposure system was built in-house at Soochow University, Suzhou (Jiangsu Province, China) and described in detail earlier [32]. Briefly, it consisted of GTEM chamber (Giga-hertz Transverse Electro-Magnetic chamber, 5.67 m length, 2.83 m width and 2.07 m height), a signal generator (PMM, Cisano sul Neva, Italy) and a power amplifier (HD Communication, Ronkonkoma, NY). The continuous wave 900 MHz RF signal was generated, amplified and fed into the GTEM chamber through an antenna (Southeast University, Nanjing, Jiangsu, China). The RF field inside the GTEM was probed using a field strength meter (PMM, Cisano sul Neva, Italy) to determine the precise position which provided the required 120 $\mu\text{W}/\text{cm}^2$ power intensity. The rationale for using this power intensity was our earlier observation of a significant survival advantage of lethally irradiated mice which were exposed to 900 MHz RF at 120 $\mu\text{W}/\text{cm}^2$ power intensity compared to those exposed at 12 $\mu\text{W}/\text{cm}^2$ or 1200 $\mu\text{W}/\text{cm}^2$. The power was monitored continuously and recorded every 5 min in a computer controlled data logging system which indicated $120.178 \pm 0.003 \mu\text{W}/\text{cm}^2$ during the RF exposure. The GTEM was installed in a room which maintained $37 \pm 0.5^\circ\text{C}$ with 87% relative humidity (without CO_2). The temperature inside the GTEM was also similar during RF exposure of the cells for 4 h/day for 5 consecutive days. For sham exposure, cells in petri dishes were kept in the same GTEM chamber and exposed, without RF transmission, for the same duration each day. The medium was changed once during 5 days of exposure.

The set-up used to expose the cells was similar and described earlier [33]. On the day of the experiment, cells were washed and re-suspended in fresh medium at a concentration of $5 \times 10^5 \text{ ml}^{-1}$. Aliquots of 8 ml medium were placed in several separate 100 mm petri dishes and they were assigned to the following groups: (1) unexposed control cells kept in the incubator, (2) sham exposure (SH), (3) RF exposure, (4) 6 Gy gamma radiation (GR), (5) Mel, (6) SH+Mel, (7) RF+Mel and (8) GR+Mel. Mel was prepared fresh, dissolved in minimum volume of ethanol, diluted with medium and added to the cells (1.0 M final concentration) 4 h prior to each RF, SH exposure and GR exposure (cells in groups 5, 6, 7 and 8). For RF/SH exposure, the cells in 8 separate petri dishes (arranged in two rows of 4 each and all dishes touching each other) were placed on a non-conductive table/platform in GTEM at a height of 100 cm at the precise location where the required power intensity of 120 $\mu\text{W}/\text{cm}^2$ was measured. The distance between petri dishes and the exposure unit (probe) was 18 cm. The cells were allowed to settle at the bottom of each petri dish before RF exposure. The SAR was calculated using was calculated using Burkhardt's formula [34]. At the bottom of the petri-dish, at the input power intensity of 120 $\mu\text{W}/\text{cm}^2$ (the direction of propagation of the incident field parallel to the plane of the medium), the peak and average SARs estimated were extremely low: they were 4.1×10^{-4} and $2.5 \times 10^{-4} \text{ W/kg}$, respectively.

2.3. Gamma radiation (GR)

The positive control cells were exposed to an acute dose of 6 Gy γ -radiation from ^{60}Co source (Nordion, Ottawa, ON, Canada, dose rate 0.5 Gy/min). The source was at another location few min

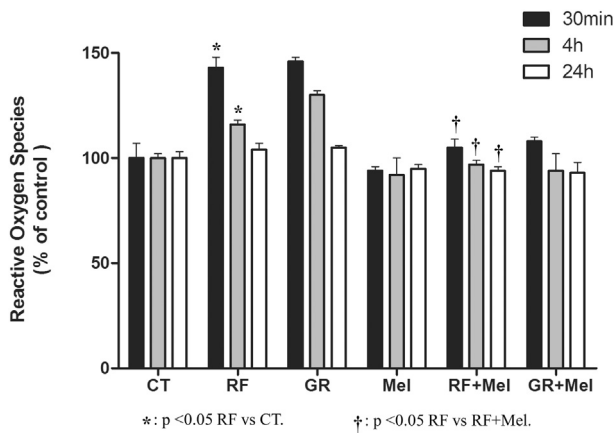


Fig. 1. Relative levels of reactive oxygen species in HL-60 cells at 30 min, 4 h and 24 h after exposure. Control (CT), RF-exposed (RF), Gamma-irradiated (GR), Melatonin-treated (Mel), RF + Mel and GR + Mel. RF versus CT: $p < 0.05$; RF versus RF + Mel: $p < 0.05$; See text for other details.

away from our laboratory. Hence, there was an interval of ~10 min between GR exposure of the cells and their transport to the laboratory to perform different assays.

2.4. Laboratory protocols

Immediately after different exposures, cells were kept in an incubator maintaining $37 \pm 0.5^\circ\text{C}$ and humidified atmosphere of 5% carbon dioxide and 95% air, and collected at 30 min, 4 h and 24 h, washed in phosphate buffered saline (PBS, Gibco, Shanghai, China) and used in different assays described below.

2.4.1. Reactive oxygen species (ROS)

The basis for the assay used is as follows. The 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a nonpolar dye which measures the activity of ROS within a cell. Following its diffusion into the cell, the dye is de-acetylated by cellular esterases to a non-fluorescent compound. However, when oxidized by intracellular ROS (hydroxyl and peroxyl radicals, peroxides and others), the dye rapidly becomes highly fluorescent DCF which gets trapped inside the cell. The fluorescence intensity of DCF is positively correlated with the activity of ROS inside the cells [35]. For ROS measurement, the cells were incubated with DCFH-DA ($10 \mu\text{mol/L}$) at $37 \pm 0.5^\circ\text{C}$ for 20 min and washed twice. Then, the intracellular fluorescence intensity of DCF was measured at excitation wavelength of 488 nm and the emission wavelength of 525 nm.

2.4.2. mtTranscription factor A (mt-TFA) and poly- γ (Western Blot Analysis)

Protein extracts were prepared by lysing the cells in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1 mM phenyl-methyl-sulfonyl fluoride (all obtained from Beyotime, Shanghai, China). The cell lysates were centrifuged at $14,000 \times g$ for 5 min at 4°C and the supernatant containing solubilized proteins was collected. The protein concentration in all samples was determined by the BCA protein assay kit (Beyotime, Shanghai, China). From each sample, equal amount of protein ($40 \mu\text{g}$ per lane) was loaded, separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). The membranes were blocked for 2 h in 5% fat-free dry milk (Yili Industrial, Inner Mongolia, China) containing Tween 20-Tris-buffered saline (TTBS). After blocking, the membranes were incubated with primary antibodies (Abcam, Cam-

bridge, USA), viz., mouse monoclonal anti-mt-TFA antibody, rabbit monoclonal anti-DNA poly- γ antibody and mouse monoclonal anti- β actin overnight at 4°C and washed three times in TTBS. The membranes were further incubated with horseradish peroxidase-conjugated antibodies for mt-TFA, poly- γ , β -actin (Beyotime, Shanghai, China) for 1.5 h at room temperature. This was following by washing the membranes three times with TTBS. The immunoreactive proteins on the membranes were detected with an enhanced chemiluminescence reagents (Millipore Corporation) using G-BOX Chemi XRQ (Syngene, UK). The blots were quantified by densitometry and normalized for β -actin to correct for differences in loading of the proteins in treated cells [36].

2.4.3. mtDNA transcription (RT-PCR)

Total RNA was extracted by using trizol agent (Tiangen Biotech, Beijing, China). The purity was assessed from OD260/OD280 ratios and they were in the range of 1.7 to 2.0. The cDNA was synthesized from the messenger RNA (mRNA) using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. This was followed by RT-PCR amplification of ND1 and 16S as mtDNA markers and GAPDH as a marker for house-keeping gene.

The primer sequences used for ND1 were:

Forward: 5'-CCCTAAAACCCGCCACATCT-3' sense and

Reverse: 5'-GAGCGATGGTGAGAGCTAAGGT-3' antisense;

The primer sequences used for 16S were:

Forward: 5'-CTTAGCCAAACCATTTACCCAAA-3' sense and

Reverse: 5'-CATCTTTCCTTGCGGTACTATATC-3' antisense,

The primer sequences used for GAPDH were:

Forward: 5'-CGACCACTTTGTCAAGCTCA-3' sense and

Reverse: 5'-AGAGTTGTCAGGGCCCTTTT-3' antisense.

The relative transcription levels of mtDNA ND1 and 16S in RT-PCR products in different exposure groups were normalized by subtracting that of GAPDH-Ct (ΔCt). The fold change value was calculated using the expression $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct}$ represents $\Delta\text{Ct}_{\text{exposed}} - \Delta\text{Ct}_{\text{control}}$ [37].

2.4.4. mtDNA copy number (RT-PCR)

DNA was extracted using the TIANamp Genomic DNA kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. The real-time PCR (RT-PCR) technique utilized ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA) and SYBR Green detection method. Each RT-PCR reaction mixture (25 μl total volume) contained 1 μl of template DNA (10 ng), 1 μl of each of the forward and reverse primers for mtDNA (ND1 is the target gene), 12.5 μl of SYBR Green Master (Roche Group, Basel, Switzerland) and 9.5 μl ultrapure water. All samples were denatured by heating at 50°C for 2 min and at 95°C for 10 min, followed by 30 cycles of 15 s at 95°C and 1 min at 60°C . The ND1 mtDNA primers were designed to minimize amplification of mtDNA pseudogenes embedded in the nuclear DNA and were:

Forward: 5'-CCCTAAAACCCGCCACATCT-3' and

Reverse: 5'-GAGCGATGGTGAGAGCTAAGGT-3'

The primers for a house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Good Science, Shanghai, China) were also included as internal standards and they were:

Forward: 5'-CGACCACTTTGTCAAGCTCA-3' and

Reverse: 5'-AGAGTTGTCAGGGCCCTTTT-3'.

The relative transcription levels of mtDNA ND1 and 16S in RT-PCR products in different exposure groups were normalized by subtracting that of GAPDH-Ct (ΔCt). The fold change value was calculated using the expression $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct}$ represents $\Delta\text{Ct}_{\text{exposed}} - \Delta\text{Ct}_{\text{control}}$ [37].

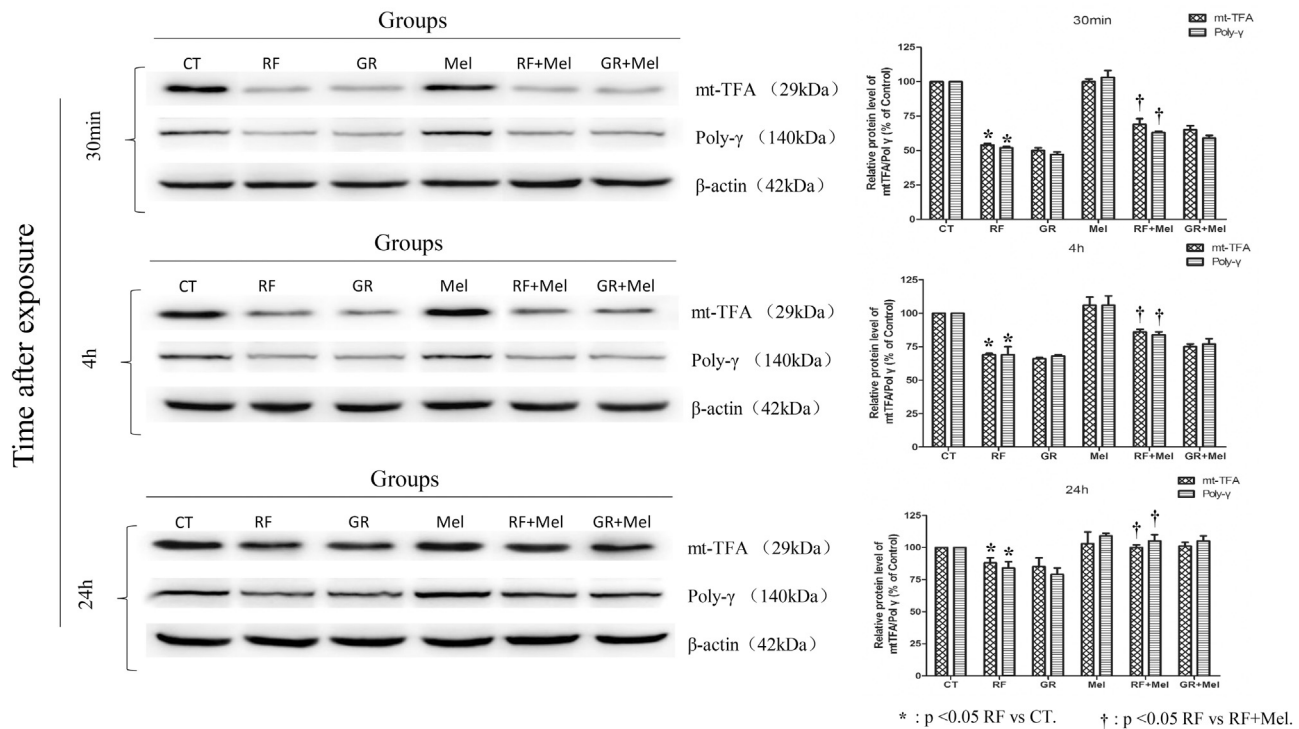


Fig. 2. Protein levels of mtDNA transcription factor A (mt-TFA) and mtDNA polymerase gamma (poly-γ) in HL-60 cells at 30 min, 4h and 24h after exposure. Control (CT), RF-exposed (RF), Gamma-irradiated (GR), Melatonin-treated (Mel), RF + Mel and GR + Mel. RF versus CT: $p < 0.05$; RF versus RF + Mel: $p < 0.05$; See text for other details.

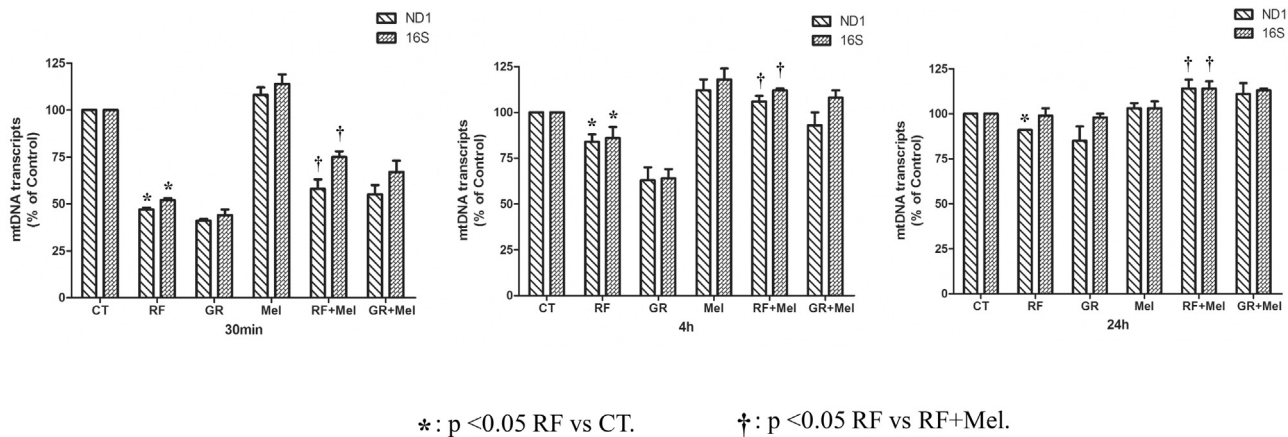


Fig. 3. Expression levels of mtDNA ND1 and 16S transcription in HL-60 cells at 30 min, 4h and 24h after exposure. Control (CT), RF-exposed (RF), Gamma-irradiated (GR), Melatonin-treated (Mel), RF + Mel and GR + Mel. RF versus CT: $p < 0.05$; RF versus RF + Mel: $p < 0.05$; See text for other details.

2.4.5. 8-Hydroxy-2'-deoxyguanosine (8-OHdG)

Mitochondria in cells were isolated using mitochondria isolation kit (Solarbio Science & Technology, Beijing, China). Then, they were split/dissociated to determine the level of 8-OHdG using sandwich enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, Wuhan, China). The microtiter plate provided in the kit was pre-coated with purified anti-8-OHdG antibody. Any 8-OHdG in the test sample would conjugate with the anti-8-OHdG antibody. Avidin conjugated horseradish peroxidase (HRP) anti-8-OHdG antibody was added to each microplate well and incubated at $37 \pm 0.5^\circ\text{C}$ for 30 min. Excess conjugate and unbound antibody were washed from the plate. Then, TMB (3-3',5,5'-tetramethylbenzidine) substrate solution was added to each well which was converted by HRP into blue color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid stop solution when the color changes from blue to yellow. The absorbance (optical density) of the color

change was measured in spectrophotometer at the wavelength of 450 nm. The concentration of 8-OHdG in the samples is then determined by comparing the OD of the samples to the standard curve, and converted into pg/mg protein (described above) [38].

2.4.6. Intra-cellular adenosine triphosphate (ATP)

ATP content was determined using an ATP Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer recommended protocol. Briefly, the cells were lysed in the lysis buffer, centrifuged at 4°C at $13,000 \times g$ for 10 min and $50 \mu\text{l}$ of supernatant was added to $100 \mu\text{l}$ of ATP detection solution, incubated at room temperature for 5 min, mixed and the luminescence was determined immediately using a multifunctional microplate reader with luminometer (FilterMax F5, Molecular Devices, US). The concentration of ATP was calculated from the standard curve and expressed as nmol/ μg protein (described above) [39].

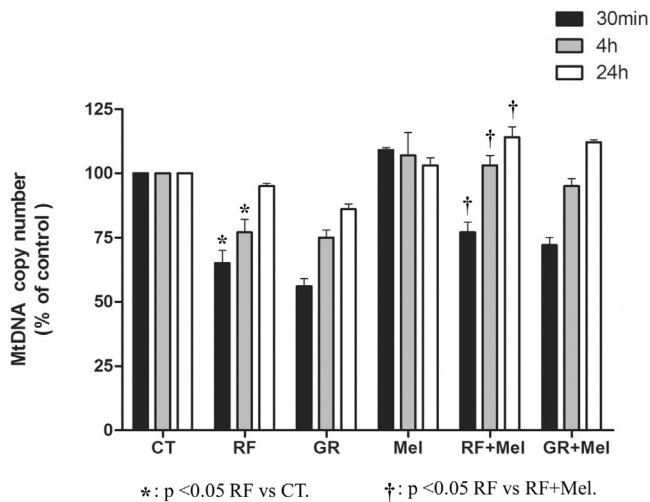


Fig. 4. Expression levels of mtDNA copy number in HL-60 cells at 30 min, 4 h and 24 h after exposure. Control (CT), RF-exposed (RF), Gamma-irradiated (GR), Melatonin-treated (Mel), RF + Mel and GR + Mel. RF versus CT: $p < 0.05$; RF versus RF + Mel: $p < 0.05$; See text for other details.

2.5. Statistical analysis

All data were analyzed using statistical analysis system software for windows version 9.4 [40]. A two-way analysis of variance was performed for ROS, ATP, 8-OHdG and β -actin data (average of 3 experiments) to compare different treatment groups, time and their interaction. Pairwise Fisher's least significant difference multiple comparisons were also used to make comparisons. Residuals were confirmed to have the bell shaped curve indicating near normal distribution. For the data on transcriptions and mtDNA copy number, several steps were undertaken prior to analysis. For each of the three triplicate experiments, the difference (delta) of mt-TFA, poly- γ , ND1, 16S, GADPH and β -actin was calculated. Then, the three control triplicates were averaged. Then, the delta score of each treatment triplicate had the average control delta subtracted from it. Finally, 2 was raised to the negative power of this change. These were analyzed in a two-way analysis of variance with treatment (un-exposed control, SH, RF, GR, SH + ML, RF + ML, and GR + ML) and time (30 min, 4 h and 24 h) and their interaction. Finally, Fisher's least significant difference pairwise comparison procedure was used and the residuals were also verified to have a near normal distribution. The statistical significance between groups was set at $p < 0.05$.

3. Results

There were no significant differences between un-exposed cells kept in the incubator and SH-exposed cells in any of the parameters/end-points investigated at 30 min, 4 h and 24 h. Since SH-exposed cells were handled the same way as RF-exposed cells, they were considered more appropriate for comparison and hence, referred below as control cells (CT). The results in RF- and GR-exposed ($\pm$ Mel treatment) were compared with those in CT and expressed as % observed in CT. The data presented in Figs. 1–6 were average $\pm$ standard deviation from 3 independent experiments (the statistical differences between RF and CT and, between RF and RF + Mel were only represented in the Figures).

3.1. ROS

(i) Cells exposed to RF showed significantly increased in ROS at 30 min and 4 h after exposure (43% and 16% increase over CT,

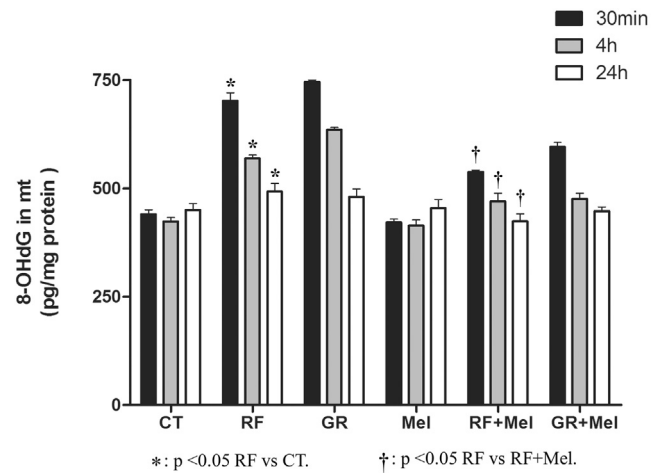


Fig. 5. The 8-OHdG content of mitochondria in HL-60 cells at 30 min, 4 h and 24 h after exposure. CT (control), RF, GR, Melatonin (Mel), RF + Mel and GR + Mel at different times after exposure. RF versus CT: $p < 0.05$; RF versus RF + Mel: $p < 0.05$; See text for other details.

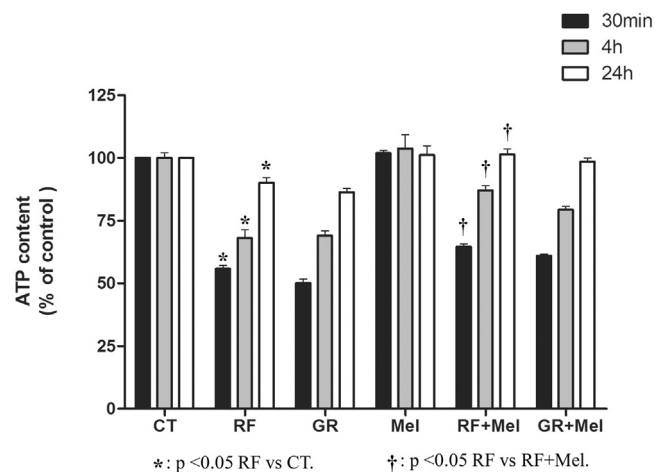


Fig. 6. Relative levels of intracellular adenosine triphosphate (ATP) in HL-60 cells at 30 min, 4 h and 24 h after exposure. Control (CT), RF-exposed (RF), Gamma-irradiated (GR), Melatonin treated (Mel), RF + Mel and GR + Mel at different times after exposure. RF versus CT: $p < 0.05$; RF versus RF + Mel: $p < 0.05$; See text for other details.

respectively) ($p < 0.05$). The level decreased gradually and returned to nearly those in CT cells at 24 h after RF exposure (only 4% increase over CT) ($p > 0.05$). (ii) Compared with the cells exposed to RF alone, those treated with Mel for 4 h and then exposed to RF showed significant decrease in ROS at 30 min, 4 h and 24 h (38%, 19% and 10% decrease over RF alone, respectively) ($p < 0.05$) (Fig. 1).

3.2. Mitochondrial transcription factor A (mt-TFA) and poly- γ protein

(i) There were significant decreases in mt-TFA and poly- γ levels in RF exposed cells at 30 min, 4 h and 24 h: the decreases were 46%, 31% and 12% and, 49%, 31% and 16%, respectively, over CT ($p < 0.05$). (ii) Compared to the cells exposed to RF alone, those treated with Mel for 4 h and then exposed to RF showed a gradual and significant increases in both mt-TFA and poly- γ levels at 30 min, 4 h and 24 h (15%, 17% and 12% and 13%, 14% and 21% increase over RF alone, respectively) ($p < 0.05$) (Fig. 2).

3.3. mtDNA transcripts (ND1 and 16S)

(i) Cells exposed to RF showed significant decreases in ND1 and 16S transcripts at 30 min and 4 h (53%, 16% and 48%, 14% decrease over CT, respectively) ($p < 0.05$) and the most decrease was observed at 30 min after exposure. At 24 h after RF exposure, the ND1 transcripts in RF-exposed cells were still lower while 16S transcripts were similar to those in CT (only 9% and 1% lower, respectively). (ii) Compared with the cells exposed to RF alone, those treated with Mel for 4 h and then exposed to RF showed significantly higher ND1 and 16S levels at 30 min, 4 h and 24 h (11–23%, 18–26% higher over RF alone) ($p < 0.05$) (Fig. 3).

3.4. mtDNA copy number

(i) Cells exposed to RF showed significantly decreased mtDNA copy number at 30 min and 4 h (35%, 23% decrease over CT, respectively) and, the most decrease was observed at 30 min after exposure ($p < 0.05$). At 24 h, mtDNA copy number was similar to that in CT (5% over CT) ($p > 0.05$). (ii) Treatment with Mel for 4 h followed by RF exposure resulted in a significant and gradual increase in mtDNA copy number at 30 min, 4 h and 24 h compared to those exposed to RF alone and the increases were 12%, 26%, 19%, respectively ($p < 0.05$) (Fig. 4).

3.5. 8-OHdG

(i) Cells exposed to RF showed significant increases in 8-OHdG levels at 30 min, 4 h and 24 h (59%, 34% and 10%) over CT and the most increase was observed at 30 min after RF exposure ($p < 0.05$). (ii) Compared with cells exposed to RF alone, those treated with Mel for 4 h and then exposed to RF showed a gradual and significant attenuation of 8-OHdG at 30 min, 4 h and 24 h (37%, 24% and 15% lower than in RF alone) ($p < 0.05$) (Fig. 5).

3.6. Intracellular ATP

(i) A significant decrease in intracellular ATP was observed in cells exposed to RF cells at 30 min, 4 h and 24 h (44%, 32% and 10% decrease, respectively over CT,) and the most decrease was observed at 30 min ($p < 0.05$). (ii) Compared with cells exposed to RF alone, those treated with Mel for 4 h followed by RF exposure showed gradual increase in ATP levels at 30 min, 4 h and 24 h (9%, 19%, 11%) increase respectively, over RF alone ($p < 0.05$) (Fig. 6).

For all parameters/end-points investigated, at 30 min, 4 h and 24 h, the response in positive control cells exposed to GR ($\pm$ Mel treatment) was similar to those in RF-exposed cells (Figs. 1–6).

4. Discussion

Biological and health effects of RF exposure from wireless communication devices have been under investigation in cells from animals and humans since several decades. Whether or not RF exposure increases ROS in animal and human cells has been the focus for many researchers because of their ability to induce oxidative damage/stress which lead to several adverse health effects [18–26]. Thus far, the observations reported on the effect of RF exposure on ROS were controversial: the results from some studies indicated increased ROS while others did not [1–3]. Furthermore, even when the same experimental design was used, the response was different in different cell types. Hence, the discrepancies may be due to differences in RF signals which include frequency, modulation, SAR, power density, duration of exposure, etc. In the current investigation, a significant increase in ROS was observed in HL-60 cells exposed to continuous wave 900 MHz RF at 120 μ W/cm² power intensity (average SAR of 25×10^{-4} W/kg) for 4 h/day for 5

consecutive days. However, the initial increase observed at 30 min gradually decreased and returned to those in CT at 24 h after RF exposure. It is possible that the ROS were degraded/inactivated during the time interval.

In the present investigation, we have examined several parameters related to the stability of mtDNA in RF-exposed cells. There were significantly decreased levels of mt-TFA and poly- γ at 30 min, 4 h and 24 h and these which might have affected mtDNA nucleoid structure and stability of ribosomal mtRNA (16S) resulting in damage to mtDNA which was reflected in decreased mtDNA copy number in RF-exposed cells compared to those in CT. It is conceivable that these changes might have been mediated through ROS which showed corresponding increases and decreases at 30 min, 4 h and 24 h.

The observations in RF-exposed cells in the current study also indicated increased levels of 8-OHdG, a biomarker for oxidative damage, at 30 min, 4 h and 24 h. Although the levels decreased gradually but still higher than in CT at 24 h after RF exposure. Perhaps, inefficient mtDNA proofreading and damage repair systems [41] might have prevented the repair of all 8-OHdG. Nonetheless, the ELISA method used in the study has limitations which include specificity and artifacts. Hence, a more specific method such as the application of mass spectrometry-based analysis [42–44] would provide more reliable 8-OHdG levels.

Since mitochondria play a predominant role in the production of energy/ATP required for cellular functions [5], we have investigated whether RF exposure has an effect in this process. The results indicated that the ATP levels were significantly decreased at 30 min after RF exposure (with a concomitant increase in ROS) but, gradually increased over time (concomitant with the decrease in ROS) and still significantly lower than those in CT at 24 h. Perhaps, mitochondria required more time to return to its original functional capacity to synthesize ATP up to the levels in CT. In this context, it is worth mentioning that in our earlier investigation using HL-60 cells exposed to 900 MHz RF at 12 μ W/cm² power for 1 h/day for 3 days, there were significant increases intracellular free Ca²⁺ and apoptosis and, significant decreases in Ca²⁺–Mg²⁺ ATPase activity, mitochondrial membrane potential and viability compared with those in SH-exposed cells [33]. All these observations suggested the potential effect of ROS in causing oxidative damage leading to mitochondrial dysfunction. It is not clear whether mtDNA damage mediated through ROS resulted in mitochondrial dysfunction or ROS-induced oxidative damage in mitochondria led to mtDNA damage. It is possible that both were dependent on each other and occurred simultaneously since the data showed positive correlation in increased and decreased levels at 30 min, 4 h and 24 h. Nonetheless, the vicious cycle could repeat itself ultimately contributing to mitochondria-related adverse health effects mentioned in the introduction (Fig. 7).

The results from the current investigation also suggested that treatment of the cells with Mel for 4 h before RF exposure was able to effectively attenuate mitochondrial dysfunction as well as mtDNA damage. This was evident from decreased ROS levels, increased ATP, mt-TFA and poly- γ levels, ND1 and 16S transcripts which might have restored mtDNA copy number. All these observations were consistent with the reports that Mel improves oxidative phosphorylation, decreases ROS and protects mtDNA against oxidative damage at the mitochondrial level [30,31,45,46]. Similar observations have been reported in cultured rat cortical neuronal cells [29].

There are limitations in this study. First, the observations were made in HL-60 cells which were derived from human promyelocytic leukemia and such cancer cells are known to differ from normal healthy cells in terms of cell proliferation control systems, metabolism and physiology. It is also important to mention that the power density used in the study is very low. Perhaps, cancer cells

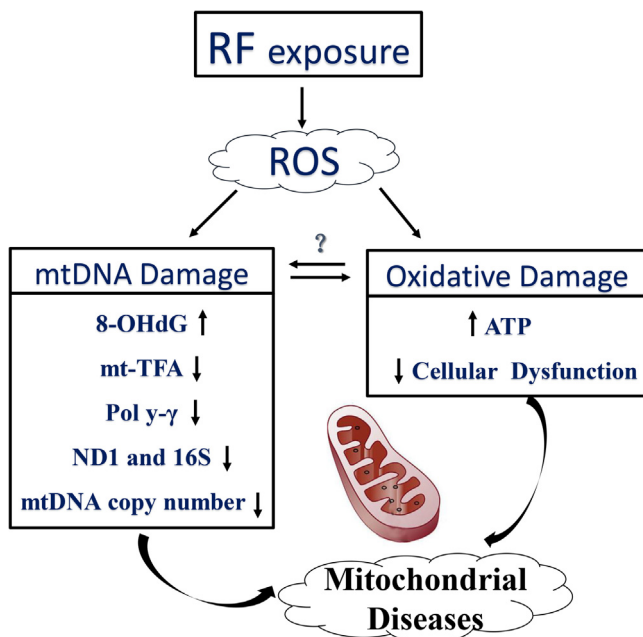


Fig. 7. Summary of events in HL-60 cells after RF exposure.

may be more sensitive to low intensity RF exposure. Hence, the data may or may not be applicable to healthy human cells and also to other RF exposure situations such as different frequencies, modulations, power densities, SAR, etc. Second, it is not known whether the similar observations can be made in female reproductive cells where mtDNA follows maternal inheritance pattern. In general, an egg contains several more mtDNA molecules than a healthy sperm. This is due to degradation of sperm mtDNA in the male genital tract. Also, mitochondria are present at the base of the tail of the sperm which is used for motility to propel them towards egg during fertilization and, either the tail is lost or the sperm may fail to enter the egg. In addition, sperm is destroyed by the egg cell after fertilization. It was also reported that paternal sperm mitochondria (containing mtDNA) are marked with ubiquitin to select them for later destruction inside the embryo [47,48]. Similar results have been observed in non-targeted effects of exposure to ionizing radiation [49–51]. Two recent reviews and meta-analyses indicated that the pooled results from in vitro and in vivo studies suggested that RF exposure reduced sperm motility and viability [52,53]. Further studies are required to determine the effect of RF exposure in both ROS and mtDNA in normal healthy cells as well as in reproductive cells.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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