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Low power microwave radiation inhibits the proliferation of rabbit lens epithelial cells by upregulating $P27^{Kip1}$ expression

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Purpose: The goal of this study was to examine the effects of low power microwave radiation (<10 mW/cm^2) on the proliferation of cultured rabbit lens epithelial cells (RLEC).

Methods: Cultured RLEC were exposed to continuous microwave radiation at a frequency of 2,450 MHz and power densities of 0.10, 0.25, 0.50, 1.00, and 2.00 mW/cm^2 for 8 h. Cell morphologic changes were observed under a phase-contrast microscope. Cell viability was measured using the MTT assay and cell cycle analysis was measured using flow cytometry. After exposure to 2.00 mW/cm^2 microwave radiation for 4, 6, and 8 h, the expression of cell cycle-regulatory proteins, $P21^{WAF1}$ and $P27^{Kip1}$, was examined using western blot analysis. Finally, the levels of $P21^{WAF1}$ and $P27^{Kip1}$ mRNA were analyzed by reverse transcription-polymerase chain reaction (RT-PCR).

Results: After 8 h of radiation treatment, cells treated with 0.50, 1.00, and 2.00 mW/cm^2 microwave radiation exhibited decreased cell viability, increased cell condensation and an inhibition of DNA synthesis. RLEC showed significant G_0/G_1 arrest. No obvious changes could be detected in the 0.10 and 0.25 mW/cm^2 microwave treatment groups. Protein expression of $P27^{Kip1}$ was markedly increased after microwave radiation. However, the mRNA levels were unchanged. On the other hand, there were no detectable differences in $P21^{WAF1}$ protein expression and mRNA levels between microwave treatment and control groups.

Conclusions: This study suggests that low power microwave radiation higher than 0.50 mW/cm^2 can inhibit lens epithelial cell proliferation, and increase the expression of $P27^{Kip1}$. These effects may account for the decline of lens epithelial proliferation after exposure to microwave radiation.

Increased applications of electromagnetic fields are of great concern to public health. The character of the natural electromagnetic field has been altered significantly due to technological progress and numerous man-made sources such as industry, traffic, medicine, radio, television, microwaves, RTV apparatuses, etc. These sources of non-ionizing radiation cause atmospheric pollution similar to the pollution from various industrial sources [1].

The lens of the eye is derived from ectoderm and grows throughout life. This transparent organ consists of a single, cuboidal layer of epithelial cells on the anterior surface and elongated, terminally differentiated epithelial cells (fiber cells) in the interior. The lens epithelial layer is critical for lens physiology, and insults to this layer can play a role in lens pathology. Many studies have found that damage to the epithelium can be an early event in cataractogenesis. However, no systematic studies have investigated the biological effects of low power microwave radiation on lens epithelial cells.

It is well recognized that microwaves affect the biological functions of living organisms at both the cellular and molecular levels and can lead to the appearance of genotoxic effects [1-3]. However, the mechanisms by which electromagnetic fields exert their biological effects remain poorly characterized [4-6]. Special attention has been given to investigating the effects of low power microwave radiation (<10 mW/cm^2) on cell growth, cell cycle progression, and DNA synthesis. In human lymphocytes, 450 MHz fields at around 1.0 mW/cm^2 affected cAMP-independent protein kinase activity [7]. The exposure of glioma cells or lymphocytes to 27 or 2,450 MHz fields caused dose dependent effects on proliferation. DNA synthesis was increased in glioma cells and suppressed in lymphocytes [8]. Cell cycle alterations in CHO cells were induced by a 27 MHz exposure [9]. The major alteration was an increase in the number of cells in G_0/G_1, and a decrease of those in M phase. Cytogenetic damage has been reported in human lymphocytes from blood samples exposed to a 945 MHz field [10]. Very low power pulsed exposure of human amnion cells at 960 MHz have recently been reported to induce a decrease in cell growth rate with increased exposure time [11].

In our previous studies, we have demonstrated that low power microwave radiation can induce irreversible damage to rabbit lens epithelial cells (RLEC) in vivo [12,13]. The aim of this study was to determine the effect of microwave radiation on primary cultured RLEC viability and proliferation, and to determine if it inhibits RLEC from entering into the DNA synthesis phase in vitro. We also examined the expression levels

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of the cell cycle modulator proteins, $P21^{WAF1}$ and $P27^{Kip1}$, via western blot and RT-PCR analyses.

METHODS

Cell culture: The rabbits used in this investigation were handled in compliance with the "Guiding Principles in the Care and Use of Animals" (DHEW Publication, NIH 86-23) and according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. 12-week-old rabbits weighing 1 to to 1.5 kg were killed by CO_2 inhalation. The entire eye was removed and dipped in 75% ethanol for 30 s, then it was opened from the posterior segment. The dissection procedure was performed with sterile instruments under a laminar flow hood. Lenses were dissected carefully by a posterior approach and washed three times in phosphate-buffered saline to remove attached pigments and vitreous. The capsule epithelium was dissected by fine forceps and placed in a 35 mm^2 culture dish where it adhered to the plastic. Some drops of culture medium were applied to the epithelium specimens to prevent drying, and the dishes were put in a humidified CO_2 incubator (5% CO_2, 37 °C) for 6 h to allow firm attachment of the capsules. Another 2 ml of culture medium was then added, and the capsules were left for 3 or 4 days before the first trypsinization and subculturing. The medium, which was changed every 2 days, was modified Eagle's medium (MEM, GibcoBRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS), supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma, St. Louis MO). Cells used in subsequent experiments were generally from passages two to three.

Electromagnetic field exposure: To demonstrate the effect of low intensity microwave radiation on RLEC, cells were exposed to continuous microwave radiation with a frequency of 2,450 MHz and power densities of 0.10, 0.25, 0.50, 1.00, and 2.00 mW/cm^2 for 8 h under controlled temperature conditions (25 °C). Sham exposed cells were treated in an identical fashion to the exposed cells except that the power to the antenna was not activated. The exact microwave frequency was calculated using a frequency counter and the input power was accurately measured using an electromagnetic field detector (Narda Model 8700, Narda, Hauppauge, NY). There was less than a 0.6 °C difference between the temperature of the culture medium in the exposed and sham cells after 8 h of radiation. In order to detect $P21^{WAF1}$ and $P27^{Kip1}$ expression, cells were treated with 2 mW/cm^2 microwave radiation, collected from each culture dish and used for RT-PCR or stored at -80 °C until assayed by western blot.

Morphologic study: In order to detect the influence of microwave radiation on RLEC directly, treated cells were observed after 8 h of treatment with 0.10, 0.25, 0.50, 1.00, and 2.00 mW/cm^2 microwave radiation using a phase-contrast microscope (Nikon, Tokyo, Japan).

MTT assay: To examine cell viability, an MTT assay was used. Briefly, RLEC were seeded in 96-well cell culture plates at a density of $1x10^6$/ml cells in MEM supplemented with 10% fetal bovine serum. After microwave radiation, 20 μl MTT solution (5 mg/ml, Sigma) was added to each well for an additional 4 h. Then the MTT solution was removed, and the blue MTT formazan precipitate was dissolved in 150 μl DMSO. The optical density (*A* value) of samples was measured at 490 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad, Hercules, CA).

Cell cycle analysis: After treatment with different doses of microwave radiation for 8 h, cells were cultured in medium for another 16 h, then trypsinized and harvested by centrifugation, washed with PBS, and fixed in 70% ice cold ethanol. Cells ($1x10^5$) were stained with propidium iodide (0.5 ml/L in PBS, containing with 100 μg/ml RNase A) and subjected to flow cytometric analysis of DNA content using a Beckman Coulter (EPICS XL, Fullerton, CA) cytometer and Mcycle software (Beckman Coulter) was used for data analysis.

Gel electrophoresis and western blot analysis: After exposure to an 2.00 mW/cm^2 electromagnetic field for 4, 6, and 8 h, control and treated RLEC were collected, washed with cold PBS and lysed in buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 30 μg/ml Aprotinin, 1 mM Na_3VO_4, 0.3% SDS, 5 mM EDTA, 1% deoxycholate, and 1 mM PMSF. After centrifugation at 12,000 rpm for 5 min at 4 °C, protein concentrations were determined using the Lowry method. The samples (about 30 to 50 μg of total protein) were boiled for 3 min, separated by SDS-polyacrylamide gel electrophoresis at 150 V, and transferred to a polyvinylidene difluoride membrane (Hybond, Amersham, Arlington heights, IL). After transfer, membranes were blocked in TBST containing 5% nonfat dry milk for 1 h. Western immunoblotting was carried out using the primary antibodies anti-$P21^{WAF1}$, anti-$P27^{Kip1}$, or anti-β-action (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution. The membranes were then reacted with a horserad-

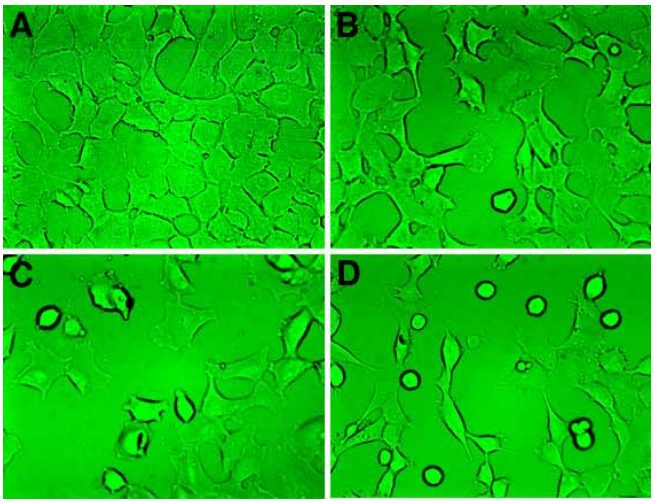

Figure 1. Phase-contrast microscopy analysis after microwave treatment. **A**: Control cells. **B**: Cells treated for 8 h with 0.50 mW/cm^2 microwave radiation. Cells were comparable to the control. **C**: Cells treated for 8 h with 1.00 mW/cm^2 microwave radiation. A few round, detached cells were visible in the microscopic field. **D**: Cells treated for 8 h with 2.00 mW/cm^2 microwave radiation. A considerable number of round cells were present, and the density of adherent cells was diminished when compared with the control. The original magnification was 400x.

ish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz) for 2 h. Signals were detected with an ECL Plus kit (Amersham) according to the manufacturer's instructions.

RNA isolation and semiquantitative RT-PCR: Total cellular RNA was isolated from RLEC using the TRIzol reagent (GibcoBRL) according to the manufacturer's protocol. Samples of RNA (about 1 to 2 μg of total RNA) were reverse transcribed and amplified with gene-specific primers using the ThermoScript RT-PCR System kit (GibcoBRL). Primer sequences used to amplify the target genes were; $P21^{WAF1}$ cDNA, 5'-CAG TGG ACA GCG AGC AGC TG (sense) and 5'-TAC AAG ACA GTG ACA GGT CC (antisense), 282 bp; $P27^{Kip1}$ cDNA, 5'-GAG GGC AAG TAC GAG TGG CAA (sense) and 5'-CTG CGC ATT GCT CCG CTA ACC (antisense), 238 bp; GAPDH cDNA, 5'-ATG GTG AAG GTC GGA GTC AAC G (sense) and 5'-GTT GTC ATG GAT GAC CTT GGC C (antisense), 495 bp. Expression of GAPDH was used as the internal standard. Amplification was performed in a thermal cycler at 94 °C for 30 s, at 56 °C for 20 s, and at 72 °C for 30 s over 30 cycles. Following PCR, 1 μl of PCR product was cloned and nucleotide sequencing of the amplified products was performed using a gene analyzer (mega Base 1000, Pharmacia, Peapack, NJ) to conform the identity of the fragment. PCR products were electrophoresized on a 2% agarose gel and visualized with ethidium bromide. The intensities of the amplified cDNA fragments were estimated using a video-densitometer. Samples without reverse transcriptase were used in the RT-PCR procedure as a negative control.

Statistical analysis: The data were analyzed by one way analysis of variance (ANOVA) and χ^2 analysis. A $p<0.05$ was assumed to be statistically significant.

RESULTS

Morphological changes induced by microwave radiation: Morphological changes in the RLEC were analyzed with a phase-contrast microscope. Compared with control groups (Figure 1A), the cell shape changed from a normal, flat, multiangle appearance to a slim, spindle-like one in the 0.50 mW/cm^2 microwave treatment groups (Figure 1B). Rounded cells were observed in the 1.00 mW/cm^2 microwave exposed groups (Figure 1C), and floating cells were detected in the 2.00 mW/cm^2 microwave exposed groups (Figure 1D). The density of adherent cells was decreased in the 0.50, 1.00, and 2.00 mW/cm^2 microwave treatment groups. There were no significant morphological changes in the 0.10 and 0.25 mW/cm^2 groups compared with control groups (data not shown).

Cell viability decreases after microwave radiation: After treatment with microwave radiation, RLEC were assayed for *A* value to detect cell viability compared to control groups. As shown in Figure 2, cell viability significantly decreased after 8 h of treatment with 0.50, 1.00, and 2.00 mW/cm^2 microwave radiation ($p<0.01$), but it was not modified in the 0.10 and 0.25 mW/cm^2 groups ($p>0.05$). Microwave radiation inhibited cell viability at power densities ranging from 0.50 mW/cm^2 to 2.00 mW/cm^2. Moreover, the high level (2.00 mW/cm^2) of microwave radiation inhibited cell viability prominently, the percent viability of cells was only (65.05±5.08%) compared to the control groups.

Microwave radiation blocks RLEC cell cycle in the G_0/G_1 phase: The effect of microwave radiation on the cell cycle was determined by flow cytometry. Figure 3 shows cell cycle alterations after microwave exposure. After 8 h of treatment with 0.50, 1.00, and 2.00 mW/cm^2 microwave radiation, the RLEC were arrested in the G_0/G_1 phase of the cell cycle. The percentage of RLEC in the G_0/G_1 phase were 71.95±2.12% ($p<0.01$), 75.68±3.35% ($p<0.01$) and 82.40±8.68% ($p<0.01$) respectively. However, the percentage of cells in the S phase of the cell cycle from the microwave treatment groups was significantly lower than controls ($p<0.01$). Thus, microwave radiation suppressed lens epithelial cell proliferation by in-

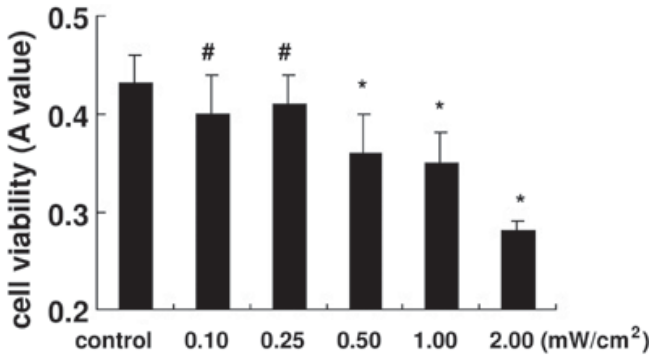

Figure 2. MTT analysis of cell viability after microwave treatment. A small decrease of cell viability could be seen after treatment for 8 h with 0.50 and 1.00 mW/cm^2 microwave radiation and was more prominent after treatment with 2.00 mW/cm^2 microwave radiation. The error bars represent the standard deviation. Results of statistical comparisons to the control group are denoted by a sharp ($p>0.05$) or asterisk ($p<0.01$).

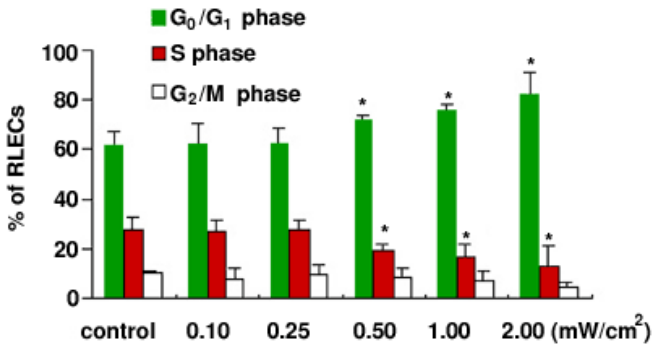

Figure 3. Flow cytometry analysis of cell cycle after microwave treatment. The number of cells in G_0/G_1 was increased after treatment with 0.50, 1.00, and 2.00 mW/cm^2 of microwave radiation. A significant reduction in the number of cells in the S phase was also observed. There were no modifications in cell cycle progression after 0.10 and 0.25 mW/cm^2 microwave treatment. All comparisons are to the control group; statistical significance is indicated by an asterisk ($p<0.01$).

hibiting and delaying the G_1/S transition of the cell cycle. There was no modification to the cell cycle in the 0.10 and 0.25 mW/cm^2 microwave treated cells, and no significant changes in the percentage of cells in the G_2/M phase between microwave treatment and control groups.

Effect of microwave radiation on cell cycle related gene expression of P21^{WAF1} and P27^{Kip1}: In order to determine possible alterations in gene expression after microwave radiation, genes involved in the cell cycle, such as P21^{WAF1} and P27^{Kip1}, were evaluated in the RLEC using western blot analysis (Figure 4A). β-Actin was used as a loading control. Both the microwave treated and the untreated cells exhibited P21^{WAF1} and P27^{Kip1} protein expression. However, there was a significantly increased expression of P27^{Kip1} protein in RLEC after 2.00 mW/cm^2 microwave radiation for 4, 6, and 8 h when compared to controls. There were no detectable differences in P21^{WAF1} protein levels between the microwave treatment and control groups (Figure 4B).

To confirm the expression levels of P27^{Kpi1} and P21^{WAF1} mRNA, RT-PCR was performed. The sequence of the PCR products was identical to sequences found in GenBank for the cDNA (Data not shown). Levels of individual PCR products were expressed as the ratio of individual product optical density to that of the internal standard GAPDH. As shown in Fig-

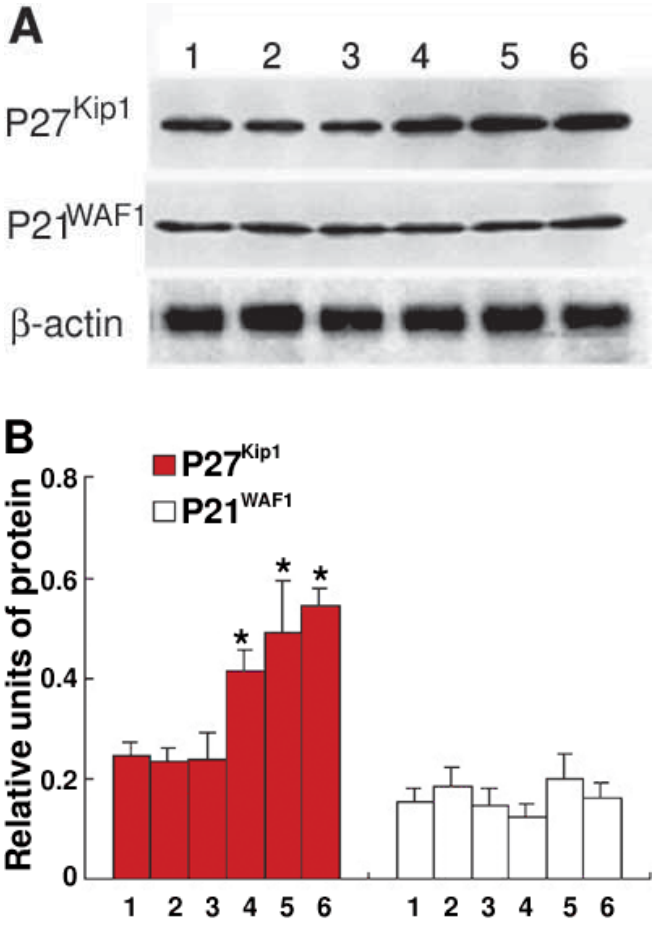

Figure 4. P21^{WAF1} and P27^{Kip1} protein levels examined by western blot analysis. **A**: Expression of P21^{WAF1} and P27^{Kip1} were assessed by western blot analysis. Lane 1 is sham treated cells for 4 h, lane 2 is sham treated cells for 6 h, lane 3 is sham treated cells for 8 h, lane 4 is cells treated with microwave for 4 h, lane 5 is cells treated with microwave for 6 h, and lane 6 is cells treated with microwave for 8 h. β-Actin was used as the loading control. **B**: Quantitative analysis of proteins by densitometric scanning of immunoblots (n=5). The error bars represent the standard deviation. All representations were as in **A**. Each displayed value was normalized against the density of the respective band of β-actin. There were statistically significant differences in P27^{Kip1} levels between the microwave groups and sham treatment groups ("*" indicates $p<0.01$ for comparison with sham treated cells).

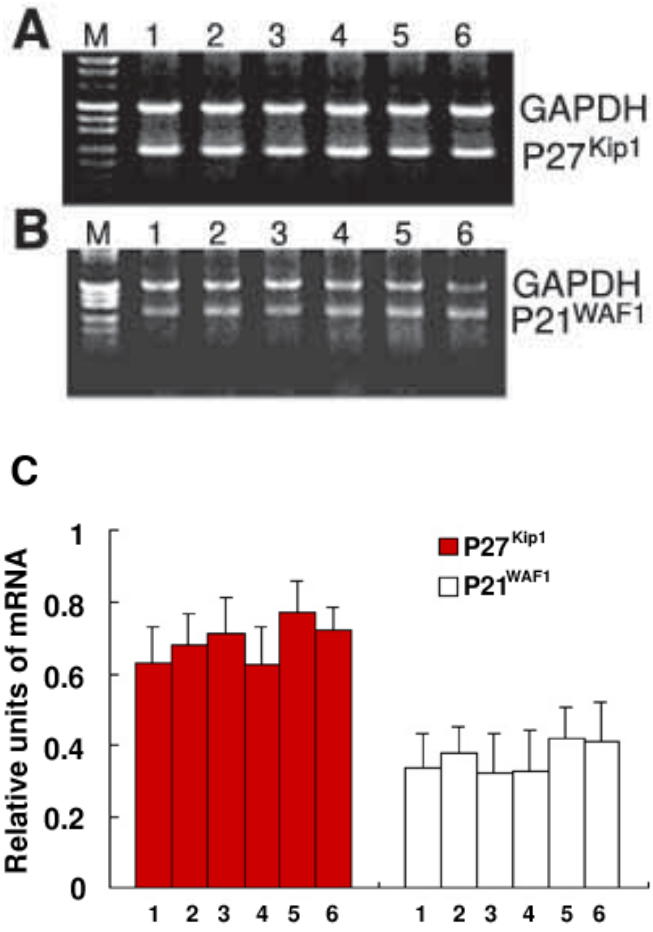

Figure 5. The levels of mRNA for P21^{WAF1} and P27^{Kip1} were examined by RT-PCR. Lane M is the DNA molecular size marker. Lane 1 is sham treated cells for 4 h, lane 2 is sham treated cells for 6 h, lane 3 is sham treated cells for 8 h, lane 4 is cells treated with microwave for 4 h, lane 5 is cells treated with microwave for 6 h, and lane 6 is cells treated with microwave for 8 h. Expression of GAPDH mRNA was used as the internal standard. **A**: Levels of P27^{Kip1} mRNA. **B**: Levels of P21^{WAF1} mRNA. **C**: Quantitative analysis of mRNA by densitometric scanning (n=4). The error bars represent the standard deviation. Each displayed value was normalized against the density of the respective band of GAPDH. There were no significant differences in P27^{Kip1} and P21^{WAF1} mRNA levels between microwave treatment and control groups ($p>0.05$).

ure 5A, the results of RT-PCR analyses showed that there were no detectable differences of $P27^{Kip1}$ mRNA expression in microwave treatment groups after 4, 6, and 8 h of radiation compared with controls ($p>0.05$). The expression levels of $P21^{WAF1}$ mRNA were also similar in all six groups which correlated with the western blot data (Figure 5B). This result suggests that microwave radiation cannot affect the levels of $P27^{Kip1}$ and $P21^{WAF1}$ mRNA expression (Figure 5C).

DISCUSSION

Data investigating the quantitative relationship between microwave exposure and bioeffects at both the cellular and molecular levels are scarce. In the present study, we used primary cultured RLEC to examine the effects of microwave radiation at 2,450 MHz on cellular functions, such as cell morphology, proliferation, cell cycle, and related gene expression. In correlation with the results of the morphologic analysis, MTT assays showed that microwave radiation could inhibit cell viability when the power density was higher than 0.50 mW/cm^2. Garaj-Vrhovac, et al. [14] have reported that microwave radiation inhihbited cultured Chinese hamster cells from entering the S phase of the cell cycle, which finally led to chromosome aberrations and other cytological damage. In agreement with their observations, the present study showed that RLEC cultured for 8 h in the presence of 0.50, 1.00, and 2.00 mW/cm^2 microwave radiation showed a significant effect on the cell cycle. Flow cytometry showed that microwave radiation inhibited DNA synthesis and arrested RLEC in the G_0/G_1 phase. Because DNA is most vulnerable to damage during replication, the cell cycle arrest caused by microwave radiation appeared to be a protective mechanism for preventing DNA damage.

Cell proliferation and progression through the cell cycle are regulated by the sequential activity of various cyclin-dependent kinases (cdks) [15-17]. Enzyme activity of cdks is dependent on physical interactions with one of the cyclin proteins, which are the regulatory subunits of these complexes. In addition, cdk activity can be negatively regulated by a group of proteins collectively termed cdk inhibitors (CKIs). CKI levels, similar to cyclin levels, vary during the cell cycle, thus contributing to the timing of cyclin-cdk activation. One family of CKIs includes $P21^{WAF1}$ and $P27^{Kip1}$. The N termini of these CKIs share homology and both bind to and inhibit cdks [18-20]. Overexpression of these inhibitors can attenuate the proliferative response, whereas a reduction in their expression increases proliferation. In this study, the protein level of $P27^{Kip1}$ was significantly upregulated by microwave radiation, suggesting that $P27^{Kip1}$ was involved in this process. There were no detectable differences in the $P21^{WAF1}$ protein levels between microwave treatment and control groups. We conclude that microwave radiation inhibits the proliferation of RELCs by arresting cells in G_0/G_1 by upregulating the protein expression of $P27^{Kip1}$, but not $P21^{WAF1}$.

There are multiple mechanisms that regulate the amount of $P27^{Kip1}$ available for interaction with cdks, which are dependent on both the cell type and the condition that leads to growth arrest. For example, Liu, et al. [21] have demonstrated that an increased accumulation of $P27^{Kip1}$ mRNA contributed to an increase in $P27^{Kip1}$ protein following exposure of U937 cells to vitamin D3. However, our data from RT-PCR showed that the regulation of the level of $P27^{Kip1}$ was not associated with its mRNA level. Therefore, the concentration of $P27^{Kip1}$ was regulated predominantly by a posttranslational mechanism, which is in agreement with some previous reports [22,23].

The effects of microwave radiation on cultured cells have been described by Kondo, et al. [24], Velizarov, et al. [6], Harvey, et al. [25], and Zotti-Martelli, et al. [26]. In these experiments, no increase in temperature above the permitted values was recorded, and the authors concluded that the microwave radiation had a specific non-thermal effect. During the exposure of our RLEC to microwave radiation, a slight increase in surface temperature was measured. However, it was not possible with the applied method to determine what the actual temperature changes were between the individual membranes of the live cells. The cytological effect described cannot be explained as the result of cell heating, but at the same time some thermal increase cannot be ruled out. It is considered that thermal interactions can cause a measurable increase in temperature. In addition, they can cause biological effects in which it is impossible to measure the rise in temperature, which depends on the electromagnetic field properties and the dielectric properties of the cells. Therefore, it can be concluded that the mechanism by which microwave radiation inhibits RLEC proliferation and upregulates $P27^{Kip1}$ includes both thermal and non-themal effects.

Lens epithelial cells proliferate and differentiate throughout the life span to produce highly organized and transparent lens fibers [27-29]. They are essential for the maintenance of lens transparency. Lens fiber cells obtain energy and nutrients, regulate water content, and maintain a proper physicochemical environment through the overlying lens epithelial layer [30]. The lens epithelium also serves as the first line of defense against oxidation damage to the whole lens. Any damage to lens epithelial cells will alter the permeability, cation transport, and biosynthesis of the lens epithelium. The propagation of those changes to the underlying fiber cells leads to the development of cataracts [31]. Investigations from Inaloz, et al. [32] indicated that low power microwave radiation could cause histological changes of RLEC, suggesting potential damage to the lens. This study and the results obtained in our work prove the presence of biological damage to the lens epithelial cells due to low power microwave radiation, and indicate the need for further research in both in vitro and in vivo systems.

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