Low power density microwave radiation induced early changes in rabbit lens epithelial cells

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Objective To determine whether low power density microwave radiation can induce irreversible changes in rabbit lens epithelial cells (LECs) and the mechanisms of the changes.

Methods One eye of each rabbit was exposed to 5 mW/cm^2 or 10 mW/cm^2 power density microwaves for 3 hours, while the contralateral eye served as a control. Annexin V-propidium iodide (PI) two-color flow cytometry (FCM) was used to detect the early changes in rabbit lens epithelial cells after radiation.

Results Lots of rabbit LECs were in the initial phase of apoptosis in the 5 mW/cm^2 microwave radiation group. A large number of cells became secondary necrotic cells, and severe damage could be found in the group exposed to 10 mW/cm^2 microwave radiation.

Conclusion Low power densities of microwave radiation (5 mW/cm^2 and 10 mW/cm^2) can induce irreversible damage to rabbit LECs. This may be the non-thermal effect of microwave radiation.

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Cataracts are the major cause of partial or total blindness in the world. It is well established that various factors, including microwave radiation can induce cataract formation.[1] In our society, mobile phones, laptops and microwave ovens have become a part of our daily life. All of these electronic devices emit microwave radiation. This has caused a resurgence of interest and concern for the biological effects and potential hazards of exposure to microwaves. The ocular lens of vertebrates is a unique organ, in which it is non-vascularized and non-innervated and contains a high percentage of water.[2] Since the ocular lens is sensitive to microwave radiation, it has become the focus of our concern. Large doses of microwave radiation can cause cataract formation. Since environmental exposure to microwave radiation usually involves low doses, until 1998, 10 mW/cm^2 was recommended as the exposure limit.[3,4] This limit was based primarily on two lines of reasoning. First, exposure of humans to this level would result in a maximum temperature rise of 1℃, a level without risk of irreversible damage. Second, a number of experimental investigations of the effects of microwave exposure in animals had indicated that irreversible tissue damage resulted from exposures at power densities of approximately 100 mW/cm^2. Thus, by applying a safety factor of 10, the level of 10 mW/cm^2 was suggested. In 1998, the International Non-Ionizing Radiation Committee (INIRC) developed guidelines setting a limit of microwave exposure to the eyes of 5 mW/cm^2.[5] Due to the lack of strict experimental evidence, INIRC did not nullify the old guidelines of 10 mW/cm^2. Neither standard, however, has been scientifically proven. Even now, the exact nature of the biological effects of microwaves is not completely understood, and leads to many questions: Is exposure to microwave radiation at the power density of 10 mW/cm^2 or 5 mW/cm^2 really safe? Will such exposure cause early irreversible damage to the lens? What are the effects of low dose microwave radiation? We are in the speculative stage, and lots of study should be done to test the standard and elucidate the mechanisms.

In our study, rabbits were exposed to microwave radiation with a power density of 5 mW/cm^2 or 10 mW/cm^2. The early microwave radiation damage to rabbit lens epithelial cells (LECs) was detected using fluorescein isothiocyanate

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(FITC)-labeled annexin Ⅴ/propidium iodide (PI) double staining flow cytometry (FCM) as a quantitative assay. We sought to verify the safety of exposure guidelines and investigate the mechanisms of microwave damage to lenses that had become cataractogenous. These results are expected to provide the basis for effective early protection.

METHODS

Animal preparation

Twenty healthy New Zealand white rabbits (2.0 – 2.5 kg) were selected randomly. Before the experiment, they were all carefully examined under a slip-lamp using mydriatic drops. No opacity of the lenses was detected. The 20 rabbits were randomly divided into 2 groups. One group (10 rabbits) was radiated with a power density of 5 mW/cm^2, while the other group (10 rabbits) with 10 mW/cm^2.

Microwave radiation assay

Microwave at a frequency of 2450 MHz (the frequency used most commonly) was emitted from a WKZ-Ⅱ microwave emission machine. The Narda Model 8700 (8721D Sensor) System (USA) was used to monitor the exact power density of microwave radiation.

Preparation of specimens

Rabbits were kept at a room temperature of 25℃. To receive a microwave power density of exactly 5 mW/cm^2 or 10 mW/cm^2, the heads of the rabbits were fixed. The distance between the rabbits' eyes and the microwave emission machine was 43 cm. The time of exposure was 3 h. One eye of each rabbit served as the experimental eye, while the contralateral eye served as the self-control. The control eyes were covered tightly by copper grid cloth, which seemed to be the most effective shielding material. The microwave radiation was 0 mW/cm^2 when we placed the probe under the shielding case. The rabbits were kept conscious and eyes open throughout the whole experiment. Rabbits were killed by aero-embolism right after the experiment. Eyes were enucleated and transferred to 0.9% saline. Continuous curvilinear capsulorhexis was also used here to obtain the anterior capsules about 8 mm in diameter. These specimens then underwent the FCM assay.

Flow cytometry

Single cell suspension of lens epithelia adhering to their capsules was made on 300-mesh copper grids. Double staining for FITC-annexin Ⅴ binding and for cellular DNA using PI was performed according to the manufacturer's protocol (Immunotech, USA). In brief, samples were washed with ice-cold PBS and centrifuged for 5 min at 500 × g at 4℃. The supernatant was discarded, and the pellets were resuspended in ice-cold binding buffer by diluting them to a concentration of 10^5 – 10^6 cells/ml. The tubes were kept on ice. Five microliter of diluted annexin Ⅴ FITC and 5 μl of dissolved PI were added to 490 μl of the cell suspension. After gently mixing, the suspensions were incubated for 10 minutes. Finally, the cells were analyzed by flow cytometry (Becton-Dickinson, USA).

Statistical analysis

The paired-sample t test was used to analyze the data. Data are reported as $\bar{x} \pm s$. $P \leqslant 0.05$ was considered significant.

RESULTS

To determine the effects of low power density microwaves at the cellular level, rabbits were irradiated by 5 mW/cm^2 and 10 mW/cm^2 for 3 hours. Damage to rabbit LECs was detected by FCM (Figs. 1 – 4). A, B and C represent different cell groups: area A, cells destroyed during the course of the experiment (annexin $Ⅴ^-/PI^+$); area B, the secondary necrotic cells (annexin $Ⅴ^+/PI^+$); area C, the early apoptotic cells (annexin $Ⅴ^+/PI^-$); the unlabelled area represents viable cells (annexin $Ⅴ^-/PI^-$). The figures show the percentages represented by each different cell group: B_1 = 7.06%; C_1 = 7.14%; B_2 = 9.66%; C_2 = 13.82%; B_3 = 14.07%; C_3 = 14.63%; B_4 = 28.35% and C_4 = 16.31%.

In order to find out whether low power density microwaves can induce cell apoptosis or secondary necrosis, we compared the experimental eye to the contralateral control eye in the 5 mW/cm^2 and 10 mW/cm^2 groups. In the group irradiated with microwaves of 5 mW/cm^2, significant differences in the percentage of early apoptotic cells could be observed between the experimental eyes and the contralateral control eyes by the paired-sample t test ($t = 3.793$, $P \leqslant 0.01$). There was no statistical difference in the percentage of secondary necrotic cells ($t = 1.731$, $P > 0.05$). The difference of damaged cells (whole percentage of early apoptotic cells and necrotic cells) between the experimental eyes and the control eyes was also significant ($t = 2.775$, $P \leqslant 0.05$; Table 1). The other experimental group was irradiated with 10 mW/cm^2 microwaves for 3 hours. The difference in the percentage of early apoptotic cells between the experimental eyes and the control eyes was not statistically significant ($t = 2.213$, $P > 0.05$). LEC secondary necrosis was significantly enhanced by microwave radiation ($t = 2.259$, $P \leqslant 0.05$). The results also showed that microwave radiation at a power density of 10 mW/cm^2 obviously induced LEC damage ($t = 5.357$, $P \leqslant 0.001$; Table 2).

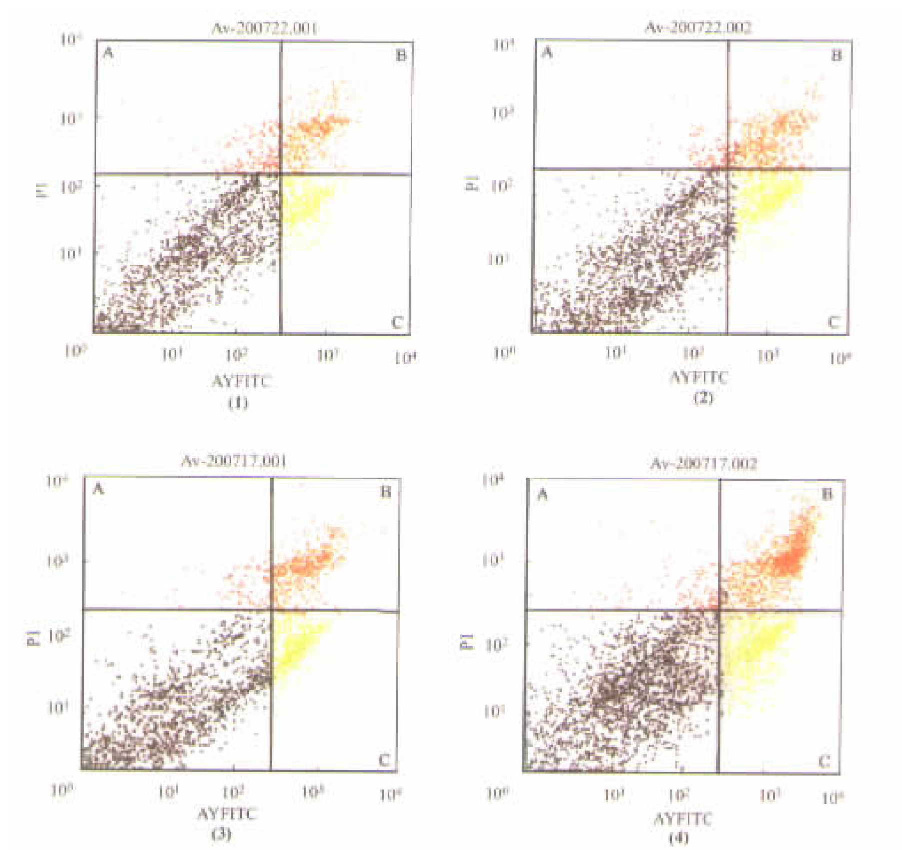

Fig. 1. Changes in LECs of the control eye in group Ⅰ (5 mW/cm²).
Fig. 2. Changes in LECs of the experimental eye in group Ⅰ (5 mW/cm²).
Fig. 3. Changes in LECs of the control eye in group Ⅱ (10 mW/cm²).
Fig. 4. Changes in LECs of the experimental eye in group Ⅱ (10 mW/cm²).

Table 1. Experimental eyes and control eyes of group Ⅰ (5 mW/cm²) ($\bar{x} \pm s$, %)

	Early apoptotic cells	Secondary necrotic cells	Damaged cells
Experimental eye	16.0060 ± 6.7780	10.3440 ± 6.7782	26.3490 ± 13.0181
Self-control eye	8.5670 ± 5.0384	6.4820 ± 4.0529	15.0490 ± 8.7769
t value	3.793	1.731	2.775
P value	≤0.01	> 0.05	≤0.05

Table 2. Experimental eyes and control eyes of group Ⅱ (10 mW/cm²) ($\bar{x} \pm s$, %)

	Early apoptotic cells	Secondary necrotic cells	Damaged cells
Experimental eye	20.4580 ± 7.1586	16.1870 ± 5.6147	36.6450 ± 6.7342
Self-control eye	14.8020 ± 7.0369	11.3470 ± 3.8778	26.1490 ± 8.5569
t value	2.213	2.259	5.357
P value	> 0.05	≤0.05	≤0.001

In order to detect the degree of damage caused by different dosages of radiation, statistical analysis was also performed between the two groups by the independent-sample t test. The results showed that there was no difference in the percentage of apoptotic cells after irradiation between the two groups ($t = 1.428$, $P > 0.05$). Significant differences were found between the groups in the percentage of secondary necrotic cells ($t = -2.099$, $P \leqslant 0.05$) and damaged cells ($t = -2.221$, $P \leqslant 0.05$; Table 3).

Table 3. Differences between groups Ⅰ and Ⅱ ($\bar{x} \pm s$, %)

	Early apoptotic cells	Secondary necrotic cells	Damaged cells
Group Ⅰ	16.0060 ± 6.7780	10.3440 ± 6.7782	26.3490 ± 13.0181
Group Ⅱ	20.4580 ± 7.1586	16.1870 ± 5.6147	36.6450 ± 6.7342
t value	1.428	− 2.099	0.039
P value	> 0.05	≤0.05	≤0.05

DISCUSSION

Cell death is the irreversible accumulation of the loss of cell activity. The two versions of cell death are apoptosis and

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necrosis. Lens epithelial cell apoptosis may be a common cellular basis for the initiation of non-congenital cataract development in humans and animals.[6] If normal physiological conditions are altered or disturbed by factors such as radiation, the viability of the LECs may be jeopardized, resulting in opacification of the lens.

Recently it was shown that structural changes in the plasma membrane of apoptotic cells are functional in signaling the process of cell death to the environment.[7] The early transverse redistribution of plasma membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the outer is one of the well-documented hallmarks of cells undergoing apoptosis.[8,9] Annexin Ⅴ is a Ca^{2+} dependent phospholipid-binding protein with a high affinity for PS, and it can be conjugated to fluorochrome FITC without impairing its phospholipid binding properties. Hence, this protein can be used as a sensitive probe for PS exposure on the cell membrane.[10] Translocation of PS to the external cell surface is not unique to apoptosis, but also occurs during cell necrosis. The difference between these 2 forms of cell deaths is that during the initial stage of apoptosis the cell membrane remains intact, while at the very moment of necrosis the cell membrane loses its integrity and becomes leaky. Therefore, annexin Ⅴ assay offers the possibility of detecting the early phases of apoptosis. It has to be performed in conjunction with a dye exclusion test to establish the integrity of the cell membrane. Using FCM, cell staining was evaluated with fluorescein isothiocyanate (FITC)-labeled annexin Ⅴ (green fluorescence), coupled simultaneously with dye exclusion of propidium iodide (PI) (negative for red fluorescence). In cells with damaged cell membrane, PI induces a red fluorescence of the DNA, while it is excluded by cells with preserved membrane. Hence, during the initial phase of apoptosis, cells are still able to exclude PI and therefore do not show any red fluorescence signal, similar to living cells. The assays presented here discriminate intact cells (annexin $Ⅴ^-/PI^-$), early apoptotic cells (annexin $Ⅴ^+/PI^-$), and secondary necrotic cells (Annexin $Ⅴ^+/PI^+$) after microwave irradiation. The two-parameter FCM method we used permits the detection of the early phases of apoptosis and is sensitive in comparison with existing traditional tests.[11] Apoptosis is generally described as a rapidly occurring process, completed within a period of about 6 hours.[12] In our study, we ran the FCM assay right after a 3-hour microwave radiation and detected irreversible changes in the LECs of rabbits at the cellular level. Increasing the radiation power density resulted in significant differences in the percentage of apoptotic and secondary necrotic cells. Controlled and irradiated samples could be distinguished in a statistically significant fashion. The microwave radiation of 5 mW/cm^2 induced LEC apoptosis, while 10 mW/cm^2 caused LEC necrosis. Radiation at 10 mW/cm^2 caused greater damage than radiation at 5 mW/cm^2 in both apoptotic and necrotic cells. These cell changes were all irreversible.

Microwave is a subdivision of radiowaves usually defined as the portion of the electromagnetic spectrum between 300 MHz and 300 GHz. A power of density under 10 mW/cm^2 to which people are exposed while living in an environment is considered to be low dose of microwave radiation. The adequacy of the originally proposed standard, 10 mW/cm^2, has been questioned from time to time, but as in the past, there has been little reason to require modification of this standard.[13] The later safety standard of 5 mW/cm^2 also lacks sufficient evidence. Guy et al[14] reported in 1975 that 80 – 100 mW/cm^2 microwave exposure for 2 – 3 hours led to an increase of the lenticular temperature to 41 – 43℃ and resulted in cataract induction. In our study, rabbits were also exposed to microwave radiation for 3 hours. The results showed that radiations at both 5 mW/cm^2 and 10 mW/cm^2 induced irreversible damage to rabbit LECs at the cellular level. This may lead to a loss of the normal function of LECs and cause lens opacification. The microwave power densities of 5 mW/cm^2 and 10 mW/cm^2 are cataractogenic, though they have not caused visible damage. The safety of these two standards is to be questioned.

The exact nature of the biological effects of microwaves is not completely understood. Various explanations have been offered for the mechanism of the formation of microwave induced lenticular opacities, and attempts have been made to delineate the biochemical changes that occur during cataractogenesis. Originally, it was believed that microwave induced lens pathology is the consequence of average temperature elevation which occurs as a function of the power absorbed by the lens.[15] The standard of 5 mW/cm^2 was provided to prevent excessive heating in tissues at or near the body surface, as temperature rises of more than 1 – 2℃ can have adverse health effects. At the beginning of the 1970s, studies tended to suggest that exposures at power densities of less than 10 mW/cm^2 affected intracellular moieties to alter metabolic and/or genetic processes. Interpretation of the significance of these findings is difficult because no objective statistical analysis is available. In our study, we detected early cell apoptosis and early cell necrosis by FCM assay, a quantitative and sensitive method for evaluating cellular level damage. The microwave radiations of 5 mW/cm^2 and 10 mW/cm^2 did not destroy the integrity of the cells. These are the characteristic cellular level change of "non-thermal" microwave radiation of power densities less than 10 mW/cm^2. Cellular research on the effects of non-thermal radiation on metabolic and genetic mechanisms is now being carried out.

The results of our study are valuable in determining whether

low power density microwave radiation (5 mW/cm^2 and 10 mW/cm^2) in the general environment is safe to humans. We also found that the non-thermal mechanisms of low power density microwaves induced cataracts. Further investigations are required to elucidate the exact mechanism of microwave-induced cataractogenesis.

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