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Study of Low-intensity 2450-MHz Microwave Exposure Enhancing the Genotoxic Effects of Mitomycin C Using Micronucleus Test and Comet Assay *in vitro*[1]

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Objective To determine the interaction between 2450-MHz microwaves (MW) radiation and mitomycin C (MMC). **Methods** The synergistic genotoxic effects of low-intensity 2450-MHz microwave and MMC on human lymphocytes were studied using single cell gel electrophoresis (SCGE) assay (comet assay) and cytokinesis-blocked micronucleus (CBMN) test *in vitro*. The whole blood cells from a male donor and a female donor were either only exposed to 2450-MHz microwaves (5.0 mW/cm^2) for 2 h or only exposed to MMC (0.0125 μg/mL, 0.025 μg/mL, 0.05 μg/mL and 0.1 μg/mL) for 24 h; and the samples were exposed to MMC for 24 h after exposure to MW for 2 h. **Results** In the comet assay, the comet lengths (29.1 μm and 25.9 μm) of MW were not significantly longer than those (26.3 μm and 24.1 μm) of controls (P>0.05). The comet lengths (57.4 μm, 68.9 μm, 91.4 μm, 150.6 μm and 50.6 μm, 71.7 μm, 100.1 μm, 145.1 μm) of 4 MMC groups were significantly longer than those of controls (P<0.01). The comet lengths (59.1 μm, 92.3 μm, 124.5 μm, 182.7 μm and 57.4 μm, 85.5 μm, 137.5 μm, 178.3 μm) of 4 MW plus MMC groups were significantly longer than those of controls too (P<0.01). The comet lengths of MW plus MMC groups were significantly longer than those of the corresponding MMC doses (P<0.05 or P<0.01) when the doses of MMC were ≥0.025 μg/mL. In the CBMN, the micronucleated cell (MNC) rates of MW were 5‰ and 6‰, which showed no difference compared with those (4‰ and 4‰) of controls (P>0.05). The MNC rates of 4 MMC groups were 8‰, 9‰, 14‰, 23‰ and 8‰, 8‰, 16‰, 30‰ respectively. When the doses of MMC were ≥0.05 μg/mL, MNC rates of MMC were higher than those of controls (P<0.05). MNC rates of 4 MW plus MMC groups were 12‰, 13‰, 20‰, 32‰ and 8‰, 9‰, 23‰, 40‰. When the doses of MMC were ≥0.05 μg/mL, MNC rates of MW plus MMC groups were much higher than those of controls (P<0.01). MNC rates of 4 MW plus MMC groups were not significantly higher than those of the corresponding MMC doses. **Conclusion** The low-intensity 2450-MHz microwave radiation can not induce DNA and chromosome damage, but can increase DNA damage effect induced by MMC in comet assay.

Key words: Microwaves; Mitomycin C; Comet assay; Micornucleus test; Synergistic effects

INTRODUCTION

As radiofrequency fields (RF), especially microwaves (300 KHz-300GHz) have been

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applied in various areas including industry, traffic, medicine, radio and television communication as well as domestic life, people are exposed to some non-ionizing electromagnetic wave everyday. These sources of non-ionizing radiation cause atmospheric pollution similar to the pollution from various industrial sources. Consequently possible harmful effects of RF radiation to the general population and occupationally exposed persons are of great concern. According to a great majority of papers, microwaves did not induce genetic effects *in vitro* and *in vivo*, at least under non-thermal exposure conditions, and seemed not to be teratogenic or cancinogenic[1]. However, people are often exposed to mixed environmental factors, though single microwave exposure can not result in genetic effects, and this exposure might enhance the mutagenicity, carcinogenicity or teratogenicity of chemical or physical factors. Therefore, the synergistic effects of microwave radiation with other mutagens or carcinogens deserve special attention.

Environmental carcinogens and mutagens (both physical and chemical ones) can induce DNA and chromosome damage. Assessment of DNA and chromosome damage can be performed at different steps of the interaction as well as the effect of mutagen on DNA. DNA damage includes DNA single and double strand breaks, alkali labile sites (apurinic, apyrimidinic, alkylation and phosphotriester formation), base damage and modification, DNA-DNA and DNA-protein crosslinks and so on. DNA single-strand break is an important marker for DNA damage, and can lead to carcinogenicity, cell death and aging. The alkaline single cell gel electrophoresis (SCGE, comet assay) is the most sensitive method to assay DNA single-strand breaks and can detect one break per 2×10^{10} daltons of DNA in lymphocytes[2-4]. Also the micronucleus test has been widely used to measure chromosome damage effects of environmental mutagens and carcinogens on cells *in vivo* and *in vitro*. The presence of micronuclei is taken as evidence of the appearance of chromatid/chromosome fragments or lagging chromosomes, or of effects on the mitotic spindle and the appearance of numerical chromosome aberrations[5,6]. So both of the comet assay and the CBMN test have been utilized to detect physical and chemical mutagens[4,5].

The fact that microwave lacks a direct DNA damaging action does not exclude its indirect DNA damage mechanism. It may especially be questioned whether the applied electromagnetic fields are able to "sensitize" cells to the effects of chemical mutagens or carcinogens. Up to now only a few studies were performed in this respect; they covered several frequencies and gave contradictory results[7-9]. In the present study we focused on the combined effects of 2450 MHz microwaves and a chemical mutagen. Mitomycin (MMC) is an antineoplastic drug and a DNA-crosslinking agent which can induce DNA and chromosome damage. In this paper, SCGE and CBMN were used to detect the synergistic effects of 2450 MHz microwaves and MMC *in vitro*.

MATERIALS AND METHODS

Blood Samples

Blood samples were taken from two non-smoking healthy donors (a male and a female), with no recent diagnostic or occupational exposure to ionizing or chemicals. Heparinized blood was divided into 10 dose groups (1) control group; (2) microwave radiation group (MW); (3) 4 MMC (Sigma) groups, the blood was exposed to MMC for 24 h at the final concentrations 0.0125 μg/mL, 0.025 μg/mL, 0.05 μg/mL, 0.1 μg/mL respectively[10]; (4) 4 MW plus MMC groups (MW+MMC), the preceded-exposure to MW was used, according to the study reported by Maes[9], the blood was first exposed to microwave radiation for 2 h and

then treated with MMC for 24 h. 1 mL blood was used for each dose group in the SCGE and 0.4 mL was used for each dose group in CBMN test. In CBMN test, the blood was exposed to microwave radiation for 2 h before harvesting cells, then exposed to MMC for 24 h.

Microwave Radiation

The exposure system was a mechanically modified version of the system described by Garaj[11]. The radiation source was a magnetron, with central frequency 2450MHz.The power density of the blood sample was measured by a power meter (Model 8712 survey meter) so that the power density of the blood could be controlled at 5.0 mW/cm^2. The exposure system was kept under controlled temperature conditions at 24℃ in a dark room. A digital contact thermometer was used to continuously monitor the sample surface temperature. The mean increasing temperature of blood sample was <0.5℃.

SCGE

Human lymphocytes were isolated with the procedure described by He[12], and were resuspended in PBS. Following isolation, the cells were mixed with 0.4% trypan blue solution. After 15 min, cells were counted and checked for viability. The remaining cells were immediately used for SCGE. The assay was performed basically according to Singh *et al.*[13]. Roughened slides were cleaned with 100% ethanol and air-dried. Two solutions, 0.5% normal melting agarose (NMA) and 0.5% low melting agarose (LMA), were prepared in Ca^{2+}, Mg^{2+}- free PBS. 100 μL of NMA was used for the first layer, while 75 μL LMA+10 μL PBS cell suspension (10000 cells) was used for the second layer. Finally, 75 μL LMA was added to the third layer. Slides were immersed in freshly prepared lysis solution (1% sodium sarcosinate, 2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris-HCl pH10, 1% Triton X-100 and 10% DMSO) at 4℃ for 1 h. Then slides were placed in a horizontal electrophoresis unit covered with fresh buffer (1 mmol/L Na_2EDTA, 300 mmol/L NaOH pH13) for 30 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 30 min at 20 V and 300 mA. The above treatments were performed in an ice bath. Subsequently, the slides were washed gently 3 times to remove alkali and detergent in a neutralization buffer (0.4 mol/L Tris-HCl, pH7.5). Each slide was stained with 50 μL ethidium bromide (2 μg/mL). All the above steps were conducted under yellow lamp in the dark to prevent additional DNA damage.

Observations were made at 200×magnification using a confocal microscope (Leica Company) equipped with an 530 nm excitation filter and a 590 nm emission filter. Comet image length of 100 cells per dose-sample was measured for DNA migration using photoshop software. Statistical analysis was made using Wilcoxon's rank-sum test after finding the heteroscedasticity of the data.

Micronucleus Test

0.4 mL of the whole-blood samples were added to 4.6 mL of RPMI 1640 (Sigma) containing 20% fetal calf serum, 0.2 mg/mL phytohaemagglutinin independent cultures were set up for each radiation-dose. The cultures were incubated at 37℃ for 72 h. 4.5 μg/mL cytochalasin B (Sigma) was added to each culture at 28 h before harvesting cells. Cells were then processed according to the method described by Fenech[14] and modified to enable the use of whole blood culture. 1 000 binucleated (BN) lymphocytes were scored for the presence of micronucleated cells (MNC). Meanwhile, nuclear division index (NDI)[15] was calculated for every group, according to the following formula:

$$NDI=\{1N+(2\times 2N)+(4\times >2N)\}/400 \text{ cells.}$$

Statistical analyses were made using the Chi-square test.

RESULTS

Comet Assay

Tables 1 and 2 showed the results of the comet assay in a male donor and a female donor respectively. The cell viability of all dose-samples was > 95%. The comet lengths of MW groups were 29.1 μm and 25.9 μm respectively, which were not longer than those (26.3 and 24.1 μm) of controls ($P>0.05$). The four doses of MMC (0.0125 μg/mL, 0.025 μg/mL, 0.05 μg/mL, 0.1 μg/mL) induced significant DNA migration which was 57.4μm, 68.9μm, 91.4 μm, 150.6 μm and 50.6 μm, 71.7 μm, 100.1 μm, 145.1 μm respectively, and longer than those of controls ($P<0.01$). The comet lengths of MW plus MMC groups were 59.1 μm, 92.3 μm, 124.5 μm, 182.7 μm and 57.4 μm, 85.5 μm, 137.5 μm, 178.3 μm respectively, also significantly longer than those of controls ($P<0.01$). Meanwhile, the comet lengths of MW plus MMC groups were significantly longer than those of corresponding MMC doses ($P<0.05$ or $P<0.01$) when doses of MMC were $\geqslant$0.025 μg/mL.

Micronucleus Test

Tables 3 and 4 showed the results of the micronucleus test in a male donor and a female donor respectively. The rates of micronucleated cells (MNC) in MW groups were 5‰ and 6 ‰, which were not significantly higher than those (4‰ and 4‰) of control groups ($P>0.05$). The MNC rates of MMC groups were 8‰, 9‰, 14‰, 23‰ and 8‰, 8‰, 16‰, 30‰ respectively. When MMC doses were $\geqslant$0.05 μg/mL, the MNC rates of MMC groups were significantly higher than those of controls ($P<0.05$ or $P<0.01$) in both male and female donors. The MNC rates of 4 MW plus MMC groups were 12‰, 13‰, 20‰, 32‰ and 8‰, 9‰, 23‰, 40‰ respectively. When MMC doses were $\geqslant$0.025 μg/mL in the male donor and $\geqslant$0.05 μg/mL in the female donor, the MNC rates of MW plus MMC groups were significantly higher than those of controls ($P<0.05$ or $P<0.01$). Although the MNC rates of MW plus MMC groups were higher than those of corresponding MMC doses, the differences were not significant ($P>0.05$). NDI of MMC groups and MW plus MMC groups decreased with MMC doses. Moreover, NDI of MW plus MMC group was less than that of MMC group, suggesting that the division of human lymphocytes was reduced.

DISICUSSION

The possible effects of MW and MMC on DNA or chromosome structure in somatic cells are very important because these changes could be associated with cell death and development of cancer. A lot of investigations of RF-induced genetic effects in somatic or germ cells have already been conducted in many different cell and animal systems. Different frequencies were studied with the emphasis on the 2450-MHz microwave and mobile telephone frequencies. In the investigations using different eukaryotic cell systems *in vitro*, the both positive and negative results were obtained[1]. When some evidence for a genetic effect was reported, this could be ascribed to hyperthermia. G. d'Ambrosio *et al.*[16] reported

an increased incidence of micronuclei in human lymphocytes exposed to microwaves. However, the exposure was accompanied by a thermal increase of 5℃. Maes *et al.*[17] reported their investigation, which clearly suggested that the observed chromosome abnomalities in lymphocytes were obtained under thermal exposure conditions. Some non-thermal positive responses were reported[18], but the responses could be sporadic positive results. So a number of positive results remain puzzling.

TABLE 1

DNA Migration (μm) Induced by MW, MMC and MW Plus MMC in Human Lymphocytes From a Male Donor *in vitro*

Group	Intensity (mW/cm^2)	Doses (μg/mL)	No. of Cells Analyzed	CL (μm) $\bar{x} \pm s$	Mean Rank	P^a	P^b
Control	0	0	100	26.3±6.6	101.9		
MW	5.0		100	29.1±8.1	120.3	>0.05	
MMC		0.0125	100	57.4±18.2	348.7	<0.01	
		0.025	100	69.9±22.0	425.5	<0.01	
		0.05	100	91.4±22.7	572.7	<0.01	
		0.1	100	150.6±31.1	836.8	<0.01	
MW+ MMC	5.0						
		0.0125	100	59.1±20.2	359.1	<0.01	>0.05
		0.025	100	92.3±21.9	579.2	<0.01	<0.05
		0.05	100	124.5±21.6	746.7	<0.01	<0.01
		0.1	100	182.7±37.5	914.2	<0.01	<0.01

Note. CL: comet length; [a]compared with control; [b]compared with corresponding MMC doses.

TABLE 2

DNA Migration (μm) Induced by MW, MMC and MW Plus MMC in Human Lymphocytes From a Female Donor *in vitro*

Group	Intensity (mW/cm^2)	Doses (μg/mL)	No. of Cells Analyzed	CL (μm)[a] $\bar{x} \pm s$	Mean Rank	P^a	P^b
Control	0	0	100	24.1±4.3	105.5		
MW	5.0		100	25.9±7.5	119.6	>0.05	
MMC		0.0125	100	50.6±18.6	328.5	<0.01	
		0.025	100	71.7±27.9	456.3	<0.01	
		0.05	100	100.1±26.0	613.3	<0.01	
		0.1	100	145.1±24.9	809.8	<0.01	
MW+ MMC	5.0						
		0.0125	100	57.4±25.1	370.4	<0.01	>0.05
		0.025	100	85.5±29.7	534.5	<0.01	<0.05
		0.05	100	137.5±39.8	762.9	<0.01	<0.01
		0.1	100	178.3±31.8	904.2	<0.01	<0.01

Note. CL: comet length; [a]compared with control; [b]compared with corresponding MMC doses.

TABLE 3

Rates of Micronucleated Cells Induced by MW, MMC and MW Plus MMC in Human Lymphocytes From a Male Donor *in vitro*

Group	Intensity (mW/cm^2)	Doses (μg/mL)	No. of Cells Analyzed	Rates of Micronucleated Cells (‰)	P^a	P^b	NDI
Control	0	0	1000	4			2.65
MW	5.0		1000	5	>0.05		2.84
MMC		0.0125	1000	8	>0.05		2.72
		0.025	1000	9	>0.05		2.54
		0.05	1000	14	<0.05		2.36
		0.1	1000	23	<0.01		2.19
MW+ MMC	5.0	0.0125	1000	12	>0.05	>0.05	2.58
		0.025	1000	13	<0.05	>0.05	2.26
		0.05	1000	20	<0.01	>0.05	2.16
		0.1	1000	32	<0.01	>0.05	1.88

Note. NDI: Nuclear Division Index; [a]compared with control; [b]compared with corresponding MMC doses.

TABLE 4

Rates of Micronucleated Cells Induced by MW, MMC and MW Plus MMC in Human Lymphocytes From a Female Donor *in vitro*

Group	Intensity (mW/cm^2)	Doses (μg/mL)	No. of Cells Analyzed	Rates of Micronucleated Cells(‰)	P^a	P^b	NDI
Control	0	0	1000	4			2.59
MW	5.0		1000	6	>0.05		2.54
MMC		0.0125	1000	8	>0.05		2.28
		0.025	1000	8	>0.05		2.56
		0.05	1000	16	<0.05		2.33
		0.1	1000	30	<0.01		2.05
MW+ MMC	5.0	0.0125	1000	8	>0.05	>0.05	2.50
		0.025	1000	9	>0.05	>0.05	2.44
		0.05	1000	23	<0.01	>0.05	2.15
		0.1	1000	40	<0.01	>0.05	1.47

Note. NDI: Nuclear Division Index; [a]compared with control; [b]compared with corresponding MMC doses.

As positive findings were almost invariably connected with thermal exposure conditions or with RF-independent situations and many negative findings (in bacteria, algae, mammalian or human cells) were reported, it could be concluded that *in vitro* RF-exposure appears not

to induce any genetic damage under non-thermal conditions[1]. In this study, the results showed that low-intensity (5.0 mW/cm^2) 2450-MHz microwaves alone for 2 h could not induce DNA single-strand break and increase micronucleated cells rate on human lymphocytes *in vitro*. Therefore, the results of our experiment support the above conclusion. Since *in vitro* RF-exposure under non-thermal condition seems not to result in genetic damage, the synergistic effects of RF-exposure deserve special attention. Many investigations showed that in most cases no synergistic effect was found between the applied field and a number of known mutagens (for example, mitomycin C, adriamycin, proflavin) when the exposures were simultaneous[1]. But when the RF-exposure preceded the mutagen, a synergistic effect was sometimes found (MMC with 954-MHz waves emitted by the antenna of a GSM base station)[9]. However, when cells were exposed to 935.2 MHz microwaves (4.5W) followed by MMC, synergistic effect was less evident[19]. The results of our experiment indicated that low-intensity 2450-MHz microwave radiation (5.0 mW/cm^2) for 2 h could increase the DNA damage effect induced by MMC on human lymphocytes in comet assay *in vitro*, when microwave exposure preceded MMC exposure. But in micronucleus test, the synergistic effect was not evident, which may be due to the different end-points and sensitivity between the comet assay and micronucleus test[12].

MMC is a DNA-crosslinking agent. It causes DNA crosslinking lesions that inhibit DNA unwinding rather than induce DNA migration just after short-time treatment of MMC. These lesions were detected in the SCGE by their inhibition of bleomycin-induced DNA migration just after treatment[20]. In this study, it was discovered that the comet lengths of MMC groups and MW plus MMC groups were significantly longer than those of controls ($P<0.05$ or $P<0.01$) after long-time treatment of MMC for 24 h. It is possible that incomplete DNA repair sites of crosslinkings contributed to the delayed response[21].

The conclusions reached in this study are as fellows: (1) continuous exposure to microwave for 2 h, at a frequency of 2450 MHz, a power density of 5.0 mW/cm^2, could not induce DNA and chromosome damage in human lymphocytes; (2) in the present condition, microwaves could increase the DNA damage effect induced by MMC on human lymphocytes in comet assay *in vitro*, when microwave exposure preceded MMC exposure. In the present exposure conditions, the meaning increasing temperature of blood sample was <0.5℃. It could be considered that the synergistic effect on MMC genotoxicity did not belong to thermal effect. Up to now, the synergistic mechanism of microwaves with chemical or physical agents has not been understood. Therefore, it should be stressed that the mechanism studies need to be conducted, in order to explain the reported synergism more clearly.

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