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Original article

Radiation effects and radioprotection by Thai medicinal plants in mouse macrophage cell line

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Abstract

Objective: To investigate the effects of radiation on growth-arrested (GA) and micronucleus-production (MP) rates, and the radioprotective properties of Thai medicinal plants in mouse macrophage cell line RAW264.7 *in vitro*. **Methods**: Mouse macrophage cell line (RAW264.7) was cultured *in vitro*. Various radiation exposures, growth-arrested rate assay, micronucleus production assay, and radioprotection by Thai medicinal plants were performed. **Results**: The results showed that GA and MP rates for γ-rays and UV were dose-dependent. The 50%-affected dose of γ and UV radiation for the GA rate was 10 Gy and 159 microwatt/cm^2 for 0.5 seconds, respectively. After X-ray exposure, there was no apparent effect on RAW264.7 cells, even with a forty-fold human diagnostic dose. Two exposures to γ radiation at 20 Gy resulted in a significantly higher MP rate than 20 Gy single exposure or control ($P<0.05$). The Thai medicinal plants (Kamin-chun capsules, Curcuma longa Linn; Hed lingeu, Ganoderma lucidum; Ya Pakking capsule, *Murdannia loriformis*) could not prevent cell damage, but epigallocatechin gallate and L-cysteine could provide protection from 2 Gy γ-ray exposure. **Conclusion**: γ radiation caused chromosomal damage during cell division and UV caused cell death, while X-ray radiation was safe. The radioprotective effects of Thai medicinal plants, Kamin-chun, Hed lingeu, and Ya Pakking, could not prevent cell damage in this study.

Keywords: Radiation; RAW264.7; Growth-arrested rate; Micronucleus production; Radioprotection; Thai medicinal plants

INTRODUCTION

Ionizing radiation e.g. gamma (γ) rays, X-rays, and ultraviolet (UV) radiation, can ionize oxygen-containing molecules into reactive oxygen species (ROS), which can damage lipids, proteins, carbohydrates, and nucleic acid in living cells. Ionizing radiation is a mutagenic and carcinogenic agent. Since these forms of radiation have been used widely in medical diagnosis and treatment, laboratory research, and in the food-preservation industry, natural and accidental exposure may be a health hazard to humans. At relatively low doses, ionizing radiation can cause double-strand DNA breaks, which contribute to chromosomal aberrations and affect cell viability[1-3] in mitosis and meiosis, X-rays have been reported to cause chromosomal fragmentation

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and aberration[4]. Ionizing radiation can also react with DNA and cause primary lesions, e. g. single-strand breaks, double-strand breaks, DNA-DNA and DNA-protein crosslinks, and subsequently cause purine and pyrimidine base damage[5]. The causality of disease due to radiation has not been fully elucidated, but it has been suggested that radiation-induced oxidative stress is a cause of genetic damage in mammals[6, 7]. Some chemicals and anti-cancer drugs can reduce cell damage from oxidative stress[8, 9]. Recently, some plant-derived drugs have shown potential as radioprotectors, e. g. " Brahma Rasayana ", which contains *Emblica officinalis* Gaertn, has been reported as an excellent non-toxic radioprotector in animal models and human volunteers undergoing radiotherapy[10, 11]. Several compounds, of natural or synthetic origin, have been evaluated for possible radioprotective effects. We evaluated the effects of radiation and the radioprotective properties of some Thai medicinal plants *in vitro* using mouse macrophage cells.

MATERIALS AND METHODS

Cell culture

The mouse macrophage cell line (RAW264.7) was purchased from ATCC (American Type Culture Collection, VA, USA). Cells were propagated in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 4 mM L-glutamine, 0.15% $NaHCO_3$, 0.45% glucose, 4 mg% Gentamycin and 10% heat-inactivated fetal bovine serum in a 6 mm Petri dish (Falcon) at 37℃, 5% CO_2 incubation. The medium was changed every 2-3 days. Cells were harvested by flushing or scraping with a cell scraper (Falcon), avoiding air bubbles, then centrifuged at 1 200 rpm for 5 min. Cell viability was assessed by counting unstained cells by 1 / 10 volume in 0.4% trypan blue on a hemacytometer under a microscope. A suspension of viable cells (1×10^4 cells per chamber of a 4-chamber slide (Falcon) per 1 mL) was prepared and incubated overnight. Cells were washed twice with DMEM before irradiation or drug exposure.

Preparation of medicinal plants

Epigallocatechin gallate (MP Biomedicals Inc.), L-cysteine (Sigma) and 3 Thai medicinal plants (Kamin-chun capsules, *Curcuma longa* Linn. (Ouayun's quality herbal formula), Hed lingeu capsule, *Ganoderma lucidum* (Royal Chitralada Project), and Ya Pakking capsule, *Murdannia loriformis* (Chaophraya Abhaibhubejhr Hospital Foundation), were prepared as a 10 mg/mL suspension in water for injection. The drug suspension was ultrasonicated for 30 min at room temperature, filtered, and kept refrigerated for no longer than 2 weeks before use.

Radiation exposure

Cells were exposed to UV, X-rays, or γ-rays 3 times. A UV lamp (intensity 159 microwatts / cm^2) was used to expose cells for 2-10 seconds, 2 or 3 times, at intervals of 3 days. The X-ray machine (DH-158 HM, Hitachi) dosages were 80 kV, 630 mA, 20 mAs, 0.03 seconds; 1, 2, 4, 8, 10, 16, or 20 times; the distances between the objects and the radiation source varied between10-90 cm. The γ-radiation source was Cobalt-60 (Gamma cell 220 Excel), and the exposure intensities were 2, 6, 8, 10, 20, 40, 80, and 160 Gy; 1, 2, or 3 times, at intervals of 3 days. After radiation exposure, cells were further cultured for 4 hours and washed twice with DMEM before adding cDMEM. Then, 1 μg/mL cytochalacin B (Sigma) was added to the cells and incubated for 24-28 hrs. The cultured supernatant was removed. The cells were washed with phosphate buffer saline twice and 80 mM KCl added for 5 seconds to enlarge cells, then removed. The cells were dried in a dessicator overnight and kept dry until use.

Drug exposure

10 to 250 μg of Thai medicinal plants, epigallocatechin and L-cysteine, were added to the cell culture without FBS. The process was triplicated for 4 or 8 hours. After radiation exposure, cells were washed and dried using the same method described above.

Giemsa staining

Slides were fixed in methanol and stained with Giemsa solution (5% Giemsa stock in phosphate buffer pH 7.2 (72 mL of Na_2HPO_4 9.5 g or $Na_2HPO_4 . 2H_2O$ 11.876 g in distilled water 1 000 mL and 28 mL of KH_2PO_4 9.02 g in distilled water 1 000 mL and topped up with distilled water 900 mL) for 15 min at room temperature. Slides were washed with water and excess water removed with fiber-free paper. The slides were dried in a desiccator, covered with cover slips and mounted with permount.

Radioprotective properties of the Thai medicinal plants

10 to 100 μg of drug suspension were added to the cells in DMEM in triplicate for 16 hours before each radiation exposure. After exposure, the cells were washed twice with DMEM and cultured with cDMEM until the next exposure. The dosages and times of radiation exposure varied as previously described; the time interval of exposure was 3 days.

Growth-arrested (GA) rate assay

The GA rates of the cells exposed to the drugs, radiation, or medicinal plants prior to exposure to radiation, were assessed by counting binuclear cells (M) and mononuclear cells (N), a total of 1 000 cells, and comparing the differences between the exposed cells and the unexposed control.

Growth-arrested rate = $M/(M+N)_{control} - M/(M+N)_{treatment}$

Micronucleus production (MP) assay

Micronucleus production was assayed using the previous method[12], with slight modification. The micronucleus production rates of the cells exposed to drugs, radiation, or medicinal plants prior to radiation exposure, were assessed by counting 1 000 binuclear cells. The micronuclei, which separated from the nuclei, were round or oval, stained similar to those of the nuclei, and less than one-quarter of a nucleus in size. The numbers of cells with or without micronuclei in the experimental and control groups were compared.

A drug concentration that could not retard cell growth was used and the minimum dose of radiation that could produce micronuclei in mouse macrophage cells was used in the radioprevention study.

Statistical analysis

The statistical package SPSS version 11.0 (SPSS, Inc., Chicago, IL) was used for statistical analysis. The differences between, and associations of, growth-arrested rates and micronucleus production rates of the exposure groups were compared using the Wilcoxon rank sum or Kruskal Wallis tests, and the chi square test. Correlations between dosage and exposure-related GA and MP rates were assessed by Spearman method.

RESULTS

Effects of radiation exposure

High doses of ionizing radiation affected the viability, growth-arrested rates, and micronucleus production rates of the mouse macrophage cells (Table 1). Only γ radiation caused micronuclei to develop in cells. γ radiation at doses < 20 Gy did not cause cell death, but inhibited growth rates and induced micronucleus production. Cell death was observed after exposure to > 40 Gy of γ radiation. γ-ray exposure of 80 Gy caused 80% cell death. Repeated exposures to γ and UV radiation showed significantly more cell death than a single exposure, and UV exposure caused more cell deaths than γ radiation ($P<0.05$). With UV radiation, cell death increased in relation to duration of exposure. Two UV exposures of 5 seconds caused 20% ~22% cell death, while 10 seconds caused 51% ~55%. Repeating γ-ray exposures 3 times at 6 Gy caused micronucleus production, but not 2 times at 20 Gy. With 2 exposures of γ radiation at 40 Gy, and high dosages (80 and 160 Gy), there were statistically significant differences in cell viability, growth-arrested rates, and micronucleus production rates, from the unexposed control ($P<0.05$). Higher radiation of 10 Gy γ-ray caused significantly higher GA rates ($P<0.05$). Cells exposed to γ radiation of 10 Gy or less showed no significant differences in MP rates from the control. Micronucleus production was significantly higher in cells exposed to 80 and 160 Gy γ radiation ($P<0.005$). X-rays at a diagnostic dose used for humans did not cause cell death, inhibit cell growth, or cause chromosomal disturbance, even after 40-fold normal human diagnostic exposure. UV radiation at 159 microwatts/cm^2 had a very strong ionizing effect, and caused the most cell death. Cell damage from UV radiation was time-dependent. Increased exposure time to UV caused more cell death and inhibited more cell growth, but did not produce more micronuclei. UV exposure for 15 minutes caused 40% cell death, 45 minutes 85%, and 60 minutes 100%. Repeated doses of UV caused significantly more cell deaths and higher GA rates than the unexposed control ($P<0.05$).

UV radiation caused serious damage to the mouse macrophage cells, as shown in Figure 1. The degenerating cells showed loss of cell membrane and cytoplasm, cell enlargement and cell lysis after UV exposure for 2 seconds.

Table 1. Viability, growth-arrested rates, and micronucleus-production rates in mouse macrophage cell line (RAW264.7) after different types of radiation exposure *in vitro*.

#	Radiation	Exposure dose	Amount of cells / mL	Viability (%)	Growth-arrested rate	Micronucleus production rate
1	Gamma ray	No exposure	2×10^5	99.2 ± 0.727	0.00 ± 0.002	0.02 ± 0.014
		2 Gy	4×10^5	100.0 ± 5.661	0.05 ± 0.001	0.07 ± 0.0143
		2 Gy ×2	4×10^5	99.8 ± 4.331	0.02 ± 0.001	0.14 ± 0.038
		2 Gy ×3	4×10^5	95.2 ± 7.211	0.05 ± 0.003	0.17 ± 0.018
		6 Gy ×3	2×10^5	98.4 ± 3.228	0.10 ± 0,005	0.22 ± 0.074 *
		10 Gy	4×10^5	98.2 ± 6.663	0.07 ± 0.006	0.15 ± 0.087
		10 Gy ×3	2×10^5	89.5 ± 5.532	0.60 ± 0.045 *	0.37 ± 0.037 *
		20 Gy	2×10^5	99.5 ± 0.274	0.47 ± 0.007 *	0.14 ± 0.058
		20 Gy ×2	2×10^5	73.2 ± 2.117 *	0.66 ± 0.044 *	0.15 ± 0.033
		40 Gy	2×10^5	97.9 ± 0.231	0.57 ± 0.027 *	0.15 ± 0.009
		40 Gy ×2	2×10^5	80.0 ± 2.097 *	0.81 ± 0.078 *	0.25 ± 0.045 *
		80 Gy	2×10^5	81.2 ± 3.543 *	0.59 ± 0.025 *	0.54 ± 0.165 *
		160 Gy	2×10^5	72.1 ± 5.122 *	0.89 ± 0.223 *	0.77 ± 0.231 *
2	X-ray(80 kV,	No exposure	2×10^5	95.8 ± 2.852	0.05 ± 0.008	0.00 ± 0.065
	630 mA, 20 mAS)	10 ×	1×10^5	99.3 ± 4.333	0.04 ± 0.007	0.01 ± 0.008
		20 ×	1×10^5	100.0 ± 0.984	0.00 ± 0.005	0.01 ± 0.021
		40 ×	2×10^5	98.1 ± 4.336	0.00 ± 0.008	0.03 ± 0.004
3	Ultraviolet	No exposure	2×10^5	99.2 ± 0.367	0.02 ± 0.007	0.00 ± 0.002
	(159 microwatts/cm^2)	2sec.	1×10^5	87.9 ± 6.321	0.14 ± 0.025	0.05 ± 0.041
		2 sec ×3	2×10^5	70.3 ± 3.442 *	0.56 ± 0.003 *	0.10 ± 0.049
		4 sec.	4×10^5	89.5 ± 9.332	0.16 ± 0.072	0.04 ± 0.039
		5 sec.	2×10^5	99.4 ± 0.122	0.45 ± 0.005 *	0.15 ± 0.004
		5 sec. ×2	2×10^5	78.2 ± 1.275 *	0.61 ± 0.065 *	0.13 ± 0.051
		8 sec.	4×10^5	70.5 ± 4.753 *	0.19 ± 0.037	0.00 ± 0.042
		10 sec.	2×10^5	98.8 ± 0.539	0.21 ± 0.090	0.03 ± 0.031
		10 sec. ×2	2×10^5	46.6 ± 2.809 *	0.54 ± 0.034 *	0.05 ± 0.001
		15 sec.	2×10^5	30.6 ± 1.445 *	0.70 ± 0.082 *	0.01 ± 0.002

The values are presented as mean ± SD.

* Represents statistically significant differences from the un-exposed control (without radiation exposure) ($P<0.05$).

Radiation protection by Thai medicinal plants

All Thai medicinal plants, Kamin-chun, Hed lingeu, Ya Pakking, L-cysteine and epigallocatechin gallate, at therapeutic dosages, were *assessed as* safe for mouse macrophage cells *in vitro*. They had no effects on viability, growth-arrested rate, or micronucleus production rate, except at higher doses (> 300 mg/mL), which could inhibit cell growth. The radioprotection results of the Thai medicinal plants, and some chemicals, are shown in Figure 2. The

minimum level of γ radiation (2 Gy) produced micronuclei in the current study. Repeated gamma exposure caused a significant increase in micronucleus production, compared with a single exposure ($P < 0.05$). At 2 Gy single γ radiation exposure, epigallocatechin gallate (50 μg/mL) and L-cysteine (100 μg/mL) could reduce the MP rate, compared with the unexposed control ($P < 0.05$). With two exposures, only L-cysteine could reduce the MP rate, compared with the unexposed control ($P < 0.001$). With three exposures, neither epigallocatechin gallate nor L-cysteine could reduce the MP rate. No Thai medicinal plant (100 μg/mL) could prevent micronucleus production due to γ radiation.

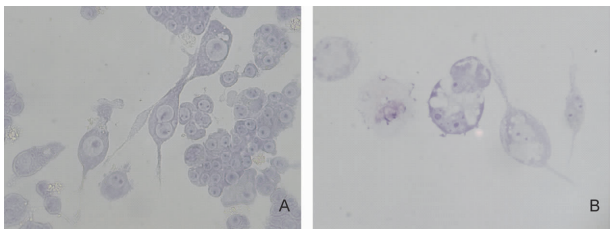

Figure 1. Morphology of mouse macrophage cells (RAW264.7) before and after UV radiation exposure at 159 microwatts / cm^2 for 2 seconds.
(A) Cells before UV exposure, showing normal cells with intact cell membrane, cytoplasm and nucleus. (B) cells after UV exposure, showing loss of cell membrane and cytoplasm, cell enlargement, and cell lysis. Giemsa stain at 200x magnification.

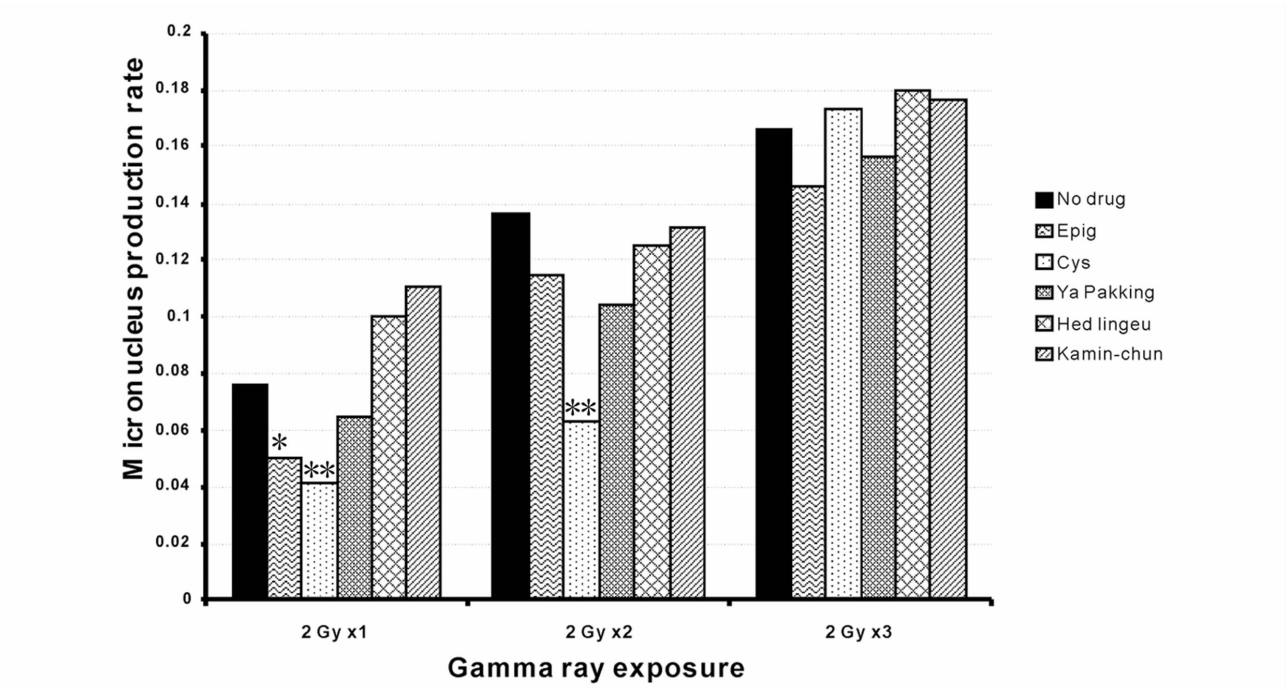

Figure 2. Mean micronucleus-production rate in mouse macrophage cell line (RAW264.7) after radiation exposure alone, or radiation exposure and subsequently adding the Thai medicinal plant (Ya Pakking, Hed Lingeu, and Kamin-chun), 100 μg/mL once, twice or thrice *in vitro*, $n = 4$. Significant ($*P < 0.05$, $**P < 0.001$) differences were compared between micronucleus production rate in experimental groups and unexposed control. Epig represents epigallocatechin gallate (50 μg/mL); cys represents L-cysteine (100 μg/mL).

DISCUSSION

The micronucleus production rate, determined by cytokinesis-blocked micronucleus assay, is a rapid, reliable, and sensitive method for determining abnormal nuclear cell division post-radiation exposure. Measurement of micronucleus production rate is used as a biological indicator for cellular radiosensitivity; because of its speed and simplicity, it has been widely used in cytotoxicity assays. Micronucleus production and apoptosis are indicators of a particular mode of cell death. Several authors have reported a meaningful quantitative relationship between micronucleus production rate and clonogenic survival[13-15]. A combination of micronucleus production and apoptosis assays should be used to predict cellular radiosensitivity[5]. Abend et al[6] (1995) described the possibility of micronucleus production

and apoptosis assays being used together to measure total cell damage, and to predict cellular radiosensitivity.

The degree of cell damage from radiation depends on dosage, number of exposures, stage of the cell cycle, the cellular antioxidant system, and the oxygen content in the tissue[17, 18]. Gamma rays, X-rays, and UV radiation cause different degrees of cell damage. Biological damage is mostly indirect, and mediated by reactive oxygen species (ROS), such as hydroxyl radical, superoxide radical, hydrogen peroxide, single oxygen, etc., generated by the radiolysis of water[19, 20]. These reactive species are known to cause degradation of important macromolecules, including DNA and cell membranes[21]. ROS significantly induces lipid peroxidation of biological membranes, thereby producing cell damage[22]. The growth of the affected cells may be retarded, leading to cell dysfunction and death, or cause cell lysis directly.

In the present study, X-rays at a human diagnostic dose (80 kV, 630 mA, 20 mAs) are very safe for cells. Radiation exposure with UV 159 microwatts/cm^2 had the strongest ionizing effect, resulting in cell-membrane damage and lysis within seconds. Radiation can cause cellular and chromosomal damage during cell division. γ radiation three times at 6 Gy caused chromosomal aberration, but did not cause immediate cell death. Therefore, UV caused cell death, while γ radiation caused nuclear fragmentation, leading to abnormal genetic transformation.

Ionizing radiation can cause human cell damage. Radiation exposure may be accidental, such as natural exposure, or deliberate, such as radiotherapy/radiodiagnosis. The past 60 years have seen an increased clinical need for more effective radioprotective agents and less toxic drugs to treat radiation exposure. The initial attempts focused on synthetic thiol compounds, which reduce mortality due to radiation exposure very effectively. Recently, there has been great interest in the potential of radioprotective herbs. Many drug extracts from ginseng, *spirulina*, garlic, *ocimum*, *mentha*, and *Rajgira* have been reportedly radioprotective[23-27]. In the present study, epigallocatechin gallate and cysteine protected against abnormal cell division and cell damage caused by radiation. Epigallocatechin gallate, a component of green tea, has been reported to inhibit the growth of cancer cells[28]. Cysteine can minimize pathological changes in tissues due to irradiation[29]. Kaminchan, Hed lingeu, and Ya Pakking all possess indications for the relief of cancer. Even though mouse macrophage cells develop abnormal chromosomal aberration after -irradiation, only epigallocatechin gallate and cysteine can reduce micronucleus production; however, they cannot protect against higher levels of -irradiation (> 2 Gy). These results suggest that higher doses of radiation cause irreversible abnormal cell division, but that epigallocatechin gallate and cysteine can protect macrophage cells against damage from low doses of radiation. However, tissue culture of mouse macrophage cells is still a valuable tool for elucidating clinical problems, especially drug toxicity testing, cell susceptibility screening, studies of the mechanisms of cell toxicity/cell protection, and using the micronucleus production assay as a parameter of chromosomal damage from ionizing radiation. Further radioprotection studies of other Thai medicinal plants should be undertaken.

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