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Radioprotective Effect of Lifukang, a Chinese Medicinal Plants Prescription

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Abstract – We have examined *in vitro* and *in vivo* radioprotective effect of a Chinese medicinal plants prescription, Lifukang. Micronucleus assay was employed to evaluate *in vitro* radioprotective effect of Lifukang. In the presence of Lifukang, the frequencies of miconuclei were greatly reduced from 7.2 to 2.9, 1.6 and 1.6% at the concentrations of Lifukang from 0 to 2, 10 and 50 μg/ml, respectively. For *in vivo* assay, we monitered the incidences of apoptotic cells in mouse small intestine crypts and endogeneous spleen colonies. When Lifukang was administered to mice P.O. or I.P. at doses of 1 mg/ml in drinking water for 7 days or 0.3 mg/mouse 24 hrs prior to irradiations, respectively, the average numbers of apoptotic cells were reduced to 3.1 or 2.3, respectively, as compared to 4.4 acquired from untreated control experiments. In addition, in spleen colony assay, Lifukang increased the number of hematopoietic spleen colonies. When samples were administered after irradiation, better results were obtained. The numbers of spleen colonies were increased from 14 colonies to 18.3 or 19.6 colonies when Lifukang was given through P.O. (1 mg/ml in drinking water for 11 days) or I.P. (0.3 mg/mouse) after irradiation, respectively.

Key words – Natural product, Lifukang, *in vitro* and *in vivo* radioprotective effect.

Introduction

The possibilities of human beings being exposured to various radiations become increased because of the increase in use of nuclear power as a major energy source, and use of radioactive compounds in medical applications, industries, and research laboratories. However, studies on the effect of radiations on the living organisms, and research and develpoment of radioprotectors still need more attention and progress. Since the radioprotective effect of thiols were demonstrated by Patt *et al.* (1949), many chemical compound have been tested for possible radioprotectors. Among them a synthetic aminothiol, WR-2721 [S-2-(3-aminopropylamino) ethylphosphorothioic acid], and its derivative, WR-1065 (the dephosphorylated form of WR-2721), have been shown to decrease radiation-induced damages most effectively (Grdina *et al.*, 1985; Grdina and Nagy, 1986; Meyn and

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Jenkins, 1983). In addition, some of non-thiol containing compound, such as 16,16-dimethyl prostaglandin E_2 (dm PGE_2) were reported to have radioprotective ability (Hans and Thomas, 1983; Berk *et al.*, 1990). Combined use of radioprotective agents showed synergistic effect. When WR-2721 and dmPGE_2 were used together, increased cell survival was observed compared to the effect on cell survival of each agent alone (Hanson, 1987). These results implies that either the radioprotection mechanisms of these two agents are different or they act on different subcellular targets. However, most of radioprotective compound developed so far are restricted in their use because of their toxic side effects.

Natural products have relatively recently begun to be evaluated as a possible radioprotector. Because natural products have been used for centuries in Asian counturies, it is widely accepted that they have relatively low toxicity. Ginseng extracts and other fractions of ginseng extracts are reported to have radioprotective effects (Takeda *et al.*, 1981; Yonezawa *et al.*, 1981; Takeda *et al.*, 1982; Zang *et al.*, 1987; Kim *et al.*, 1993; Cho *et al.*, 1995). Our laboratories have focused on the response of cells to radiation damages and the mechanism of radioprotection (Kim *et al.*, 1995a; Kim *et al.*, 1995b; Kim *et al.*, 1996a; Kim *et al.*, 1996b; Hyun *et al.*, 1997; Kim *et al.*, 1997a; Kim *et al.*, 1997b; Nam *et al.*, 1997). Also, we are trying to find radioprotective agents and to understand their mechanisms of radioprotection. Recently, We showed that the radioprotective effects of ginseng extracts (Kim *et al.*, 1993; Cho *et al.*, 1995). In this study, we evaluated *in vitro* and *in vivo* radioprotective effect of a Chinese medicinal plants prescription, Lifukang.

Experimental

Cell culture and irradiation – Chinese hamster lung (V79-4, ATCC CCL-93) cells were cultured in DMEM medium containing 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics in a humidified 5% CO_2/95% air incubator at 37℃. Log phase cells were used for micronucleus assay. Cells were irradiated with 0, 1.5, 3, 6, or 8 Gy of ^{60}Co γ-rays (Theratron-780 teletherapy unit) at a dose rate of 1.395 Gy/min at room temperature.

Drug – Lifukang, a Chinese medicinal plants prescription, was prepared by Academy of Traditional Chinese Medicine, Beijing, China.

Animals and irradiation – N:GP(s) mice of either sex were caged and allowed free access of pellets of standard food and water. All experiments were performed with 7-8 weeks old mice. Animals were irradiated with 2 Gy for radiation-induced apoptotic cell assay, or with 6.5 Gy for speen colony assay of ^{60}Co γ-rays (Gammacell 3000 Elan, Nordion international Co., Canada) at a dose rate of 1.09 Gy/min.

Drug treatment – Lifukang was dissolved in DMSO to a final concentration of 100 μg/μl and stored at -20℃ as a stock solution. Before use, stock solution was routinely diluted to 1,000 times concentrated solutions of desired final concentrations in media and 0.001 vol. of cell culture media was added to media. DMSO concentrations were kept below 0.1% in media. Dithiotreitol (DTT) was prepared in PBS as 100 mM stock solution and aliquarts were stored at -20℃. Diluted Lifukang or DTT stock solution were added to media about 30-60 min prior to irradiation.

For *in vivo* assays, drugs were administerated as the followings: For oral administeration, Lifukang was dissolved in drinking water to a concentration of 1 mg/ml and was administered to mice for 7 days before irradiation or for 11 days after irradiation. For intraperitoneal injection, Lifukang was dissolved in PBS and 0.3 mg/head were injected 24 hrs prior to irradiation or 30 min after irradiation. DTT were dissolved in PBS and 1.5 mg/head were injected intraperitoneally 30 min before or after irradiations.

Micronucleus assay – Cytochalasin-B (Aldrich Chemical Co.) was added to media at a final concentration of 3 μg/ml immediately after irradiation. After 20 hr incubation in the presence of cytochalasin-B, cells were trypsinized and fixed in methanol:acetic acid (3:1) solution. Fixed cells were spread onto slide glasses, air-dried and stained with Giemsa. We used Almássy's method (Almássy *et al.*, 1987) to identify the micronucleus in cytokinesis-blocked binucleated cells and the frequencies of micronuclei were scored in 500 binucleated cells at 400X magnification.

Radiation-induced apoptosis in small intestine – Six hours after irradiation, small intestines were removed and fixed in Carnoy's solution for at least 30 min. Histological transverse sections (4 μm thick) were prepared and stained with hematoxylin and eosin. Good longitudinal sections of crypts were selected. With these crypt sections, apoptotic cells were counted as follows. When several apoptotic fragments were, from their size and clustering, thought to represent the remains of a single cell, they were recorded as a single event. Fifty crypts sections were scored for each mouse.

Endogenous spleen colony assay – Ten days after irradiation, mice were sacrificed and spleens were removed surgically and weighed. Spleens were fixed in Bouin's solution and hematopoietic colonies on the surface were counted with naked eyes.

Results and Discussion

***In vitro* radioprotective effect of Lifukang** – We used Chinese hamster cells (V 79-4) in this study. This cell line is a normal-drived cell line and this cell line and its derivatives have been used for many studies in radiobiology (Smoluk *et al.*, 1988; Stap and Aten, 1990; Sölen *et al.*, 1990; Hahn *et al.*, 1992). V79-4 cells in log phase were irradiated with 0, 1.5, 3, 6, and 8 Gy of γ-rays and the frequencies of micronucli were scored. Table 1 shows the distribution and the frequency of micronuclei in irradiated V79-4 cells. Fig. 1 shows best linear fitting of the data shown in Table 1. The dose response relationship have been described as linear or linear-quadratic expressions (Almássy *et al.*, 1987). We tried to fit both linear and linear-quadratic models and the linear fitting gave us better results. There was a significant relationship between the micronucleus frequency and the irradiated γ-ray doses (Fig. 1).

Table 1. Frequencies of micronucli in Chinese hamster (V79-4) cells irradiated with γ-rays

Dose (Gy)	Micronuclei in 500 cells	Micronucleus distribution			%
		1	2	3	
0	0	0			0
1.5	36	36			7.2
3	48	48			9.6
6	69	65	2		13.8
8	117	98	8	1	24.0

In order to investigate the radioprotective effect of Lifukang, we added various concentrations of Lifukang (0, 2, 10, and 50 μg/ml) to culture media at least 30 min before irra-

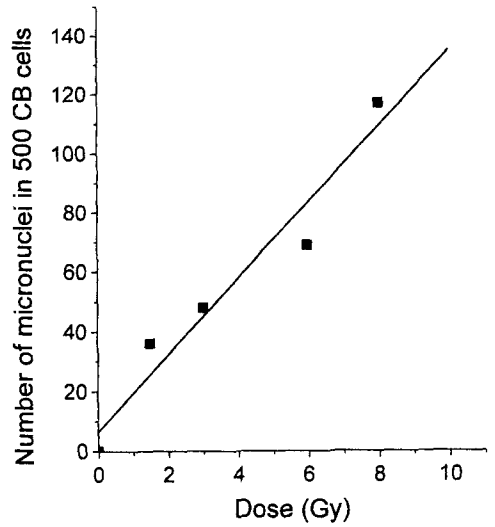

Fig. 1. Dose-response curve for γ-ray induced micro nuclei in Chinese hamster (V79-4) cells. CB cytokinesis-blocked.

Table 2. Frequencies of micronuclei in 1.5 Gy of γ-ray-irradiated Chinese hamster (V79-4) cells in the presence of Lifukang or DTT

Treatment		Micronuclei in 500 binucleated cells	%
Unirradiated control		15	3.4
Lifukang,	0 μg/ml	36	7.2
	2 μg/ml	14	2.9
	10 μg/ml	8	1.6
	50 μg/ml	8	1.6
DTT,	1 mM	15	3.4

diation. Then, cells were irradiated with 1.5 Gy of γ-ray in the presence or absence of Lifukang. Frequencies of micronucleus were scored as shown in Table 2. When there was no treatment, we observed 36 micronuclei (7.2 %) in 500 cytokinesis-blocked (CB) cells. At concentrations of 2 μg/ml of Lifukang, the numbers of micronuclei in 500 CB cells was reduced to 14 (2.9%). At higher concentrations of Lifukang (10 and 50 μg/ml), the numbers of micronuclei in 500 CB cells was even lower, only 8 micronuclei (1.6%) were observed in both cases. These reduction in frequencies of micronuclei was even lower than that of the positive control dithiotreitol (DTT), which is 15 micronuclei in 500 CB cells (3.4%). These data suggest that Lifukang shows radioprotective effect *in vitro*, and the effect is comparable to or even higher than that of DTT. Therefore, we continued to investigate *in vivo* radioprotective effect of Lifukang.

***In vivo* radioprotective effect of Lifukang** – We measured the frequencies of apoptotic cells in small intestinal crypts of 2 Gy of γ-ray irradiated mice as biological endpoint of radiation damage (Fig. 2) and the results are shown in Table 3. When there was no treatment, average 4.4 apoptotic cells were observed in each crypt of irradiated mice. When Lifukang preparation was administered P.O., the average number of apoptotic cells was reduced to 3.1. When Lifukang preparation was administered I.P., the

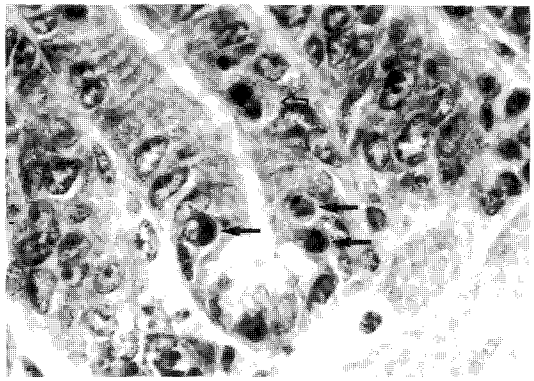

Fig. 2. Apoptotic cells in mouse small intestine 6 hrs after 2 Gy of γ-ray irradiation. Three apoptotic cells (⬆) and a mitotic cell (⇧) lain in crypt epithelium are indicated.

Table 3. Effect of Lifukang on radiation-induced apoptosis in small intestinal crypt of γ-ray-irradiated mice

Treatment	Numbers of apoptotic cells
Unirradiated control	0.094 ± 0.02
Irradiated	4.4 ± 0.5
Lifukang (P.O.)	3.1 ± 0.3*
Lifukang (I.P.)	2.3 ± 0.5*
DTT (I.P.)	2.1 ± 0.4#

Lifukang was administered at doses of 1 mg/ml in drinking water for 7 days (P.O.) or 0.3 mg/mouse 24 hr prior to 2 Gy of γ-ray irradiations. Two hundred crypts were scored in 4 mice. Mean ± S.D., (*, $P<0.05$), #, $P<0.01$.

reduction in the average numbers of apoptotic cell were even lower, 2.3 apoptotic cells in each crypt were scored. These results are comparable with the effects of positive control, DTT which showed 2.1 apoptotic cells in each crypt.

In addition, we used the formation of endogenous spleen colonies to analyze *in vivo* radioprotective effect of Lifukang (Table 4). Irradiation control group showed average 14 endogenous spleen colonies. Administration of Lifukang through P.O. either before or after irradiation increased the formation of endogeneous spleen colonies to 15.3 or 18.3, respectively. In the case of I.P. injection either before or after irradiation, the numbers of the endogenous spleen colony were increased to 14.7 or 19.6, respectively. Our data

Table 4. Effect of Lifukang on the formation of spleen colonies in γ-ray-irradiated mice

Treatment (n=7)	Numbers of endogenous spleen colonies
Unirradiated control	0
Irradiated	14.0±2.7
Lifukang before IR (P.O.)	15.3±2.5
Lifukang before IR (I.P.)	14.7±4.5
Lifukang after IR (P.O.)	18.3±5.2
Lifukang after IR (I.P.)	19.6±3.4*

For P.O., Lifukang was administered at doses of 1 mg/ml in drinking water for 7 days before irradiation or for 11 days after irradiation. For I.P., 0.3 mg/mouse of Lifukang was administered 24 hr before or 30 min after irradiations. IR; irradiation of 6.5 Gy of γ-ray. Mean ± S.D., (*, P<0.01).

showed that there are better endogenous spleen colony formation effect when Lifukang was administered after irradiation. These data suggest that Lifukang may be involved in protection of the cells from the damage and also in recovery from radiation-induced damages of the cell.

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