Letter to the Editor

Evaluation of Protective Effects of Bioactive Phytochemicals Against Methotrexate in *Salmonella typhimurium* TA1535/pSK1002 Coupled with Micronucleus Assay*

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We evaluated the antimutagenic effects of 10 kinds of bioactive phytochemicals and some phytochemical combinations against methotrexate (MTX)-induced genotoxicity by the umu test in *Salmonella typhimurium* TA1535/pSK1002 combined with a micronucleus assay. We observed that allicin, proanthocyanidins, polyphenols, eleutherosides, and isoflavones had higher antimutagenic activities than the other five types of bioactive phytochemicals. At the highest dose tested, MTX-induced genotoxicity was inhibited by 25%-75%. Kunming mice treated by MTX along with bioactive phytochemical combinations showed significant reduction in micronucleus induction and sperm abnormality rate (P<0.01). These results indicate that bioactive phytochemical combinations can be potentially used as new cytoprotectors.

Methotrexate (MTX) is one of the most intensively investigated and effective chemotherapeutic agents. Moreover, the genotoxic effects of MTX have already been reported in somatic cells by using chromosome aberration and micronucleus test as the end points of evaluation of MTX chemotherapy for gestational trophoblastic tumors and acute lymphoblastic leukemia, which are responsible for the increasing risk of second tumors. A recent study has confirmed that MTX induces cytotoxic and genotoxic effects in the germ cells of mice (Padmanabhan et al., 2008). Thus, the development of efficient protective agents that could reduce the risk of second cancers caused by MTX cytogenotoxicity has attracted more attention.

In recent years, the interest in using natural plant products for their medicinal value is increasing continually. However, most of the previous studies have focused on the cytoprotection of individual bioactive phytochemicals or in combination with other agents, such as β-carotene and quercetin. There are also only few cytoprotective studies of various bioactive phytochemicals, particularly bioactive phytochemical combinations. In the present study, the antimutagenic potential of 10 different bioactive phytochemicals (chlorogenic acid, allicin, gingerols, ginkgo flavone, ginsenosides, proanthocyanidins, polyphenols, polysaccharides, eleutherosides, and isoflavones) and some bioactive phytochemical combinations (green tea polyphenols, eleutherosides from Siberian ginseng, and grape seed proanthocyanidins) against MTX-induced genotoxicity was evaluated by the umu test in *Salmonella typhimurium* TA1535/pSK1002 combined with a micronucleus assay. The results demonstrated that the umu test was an effective assay to evaluate the antimutagenic potential of bioactive phytochemicals. Moreover, the data suggested that the bioactive phytochemical combination of grape seed and Siberian ginseng extracts can be used as new cytoprotectors.

S. typhimurium TA1535/pSK1002 was kindly provided by Dr. Yoshimitsu Oda. 4-NQO was used as a positive control. Dimethyl Sulphoxide (DMSO) served as the control and solvent. All bioactive phytochemicals were purchased from Changsha Active Ingredients Group Inc. (China). Kunming specific pathogen-free mice (4-6 weeks old, average body weight 19±2 g) were provided by the Laboratory Animal Center (LAC) of Henan University of Science and Technology. The umu test was performed according to a previously described method (Oda et al., 1985).

doi: 10.3967/bes2016.017

*This study was supported by the National Natural Science Foundation of China (Grant No. U1304307); and the Young Core Instructor Foundation from the Education Commission of Henan Province, China (Grant No. 2014GGJS-056).

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A total of 32 mice were randomly divided into four groups with eight mice in each group (four males and four females). The combination of bioactive phytochemicals was prepared by dissolving two bioactive phytochemicals in DMSO, followed by diluting the mixture with distilled water to an effective component concentration of 50 mg/L for each bioactive phytochemical. The combination of bioactive phytochemicals was administered 1 week prior to MTX exposure. Treatment group I: mice were administered a combination of green tea polyphenols and eleutherosides from Siberian ginseng [0.2 mL/10 (g·w), i.g. once daily] for 15 d, and a single dose of MTX (2 mg/kg, i.p. once daily) was given on the 8th day. Treatment group II: mice were administered a combination of grape seed proanthocyanidins and eleutherosides from Siberian ginseng for 15 d, and MTX was administered on the 8th day in a similar manner. Model group: animals received distilled water instead of bioactive phytochemical combinations for 15 d, and the same MTX protocol was applied to this group on the 8th day. Control group: mice received distilled water through 15 days, and physiological saline instead of MTX was administered on the 8th day in a similar manner. After 12 h of the final doses, the animals were euthanized by cervical dislocation. The micronucleus assay was performed according to the method of Schmid (Schmid, 1975). The thymus and spleen indices were assayed according to a previously described method (Zhang et al., 2003).

A total 24 male mice were randomly divided into four groups with six mice in each group. MTX and the combination of bioactive phytochemicals were administered to the animals following the aforementioned methods. After 12 h of the final doses, the mice were sacrificed by cervical dislocation and both the epididymides were isolated. Sperm deformity test was conducted according to the method of Wyrobek et al. Two sperm suspensions were prepared from the caudal end of each testis by mincing the caudal ends in physiological saline. The sperm was spread on a slide glass and stained with 1% eosin Y for 45 min after which the slides were air-dried. A total of 1000 sperm cells of mice were assessed for morphological abnormalities under oil immersion at 1000× magnification. Sperm head morphology was scored under the categories of normal, sperm without hook, amorphous head, banana head, and triangular head essentially as described.

Data were analyzed for statistical significance using *t*-test (SPSS 13.0 for Windows). The values are expressed as mean±standard deviation (SD). $P<0.05$ was deemed as statistically significant.

Table 1 shows the effects of 10 types of bioactive phytochemicals on *umu* gene expression in *S. typhimurium* TA1535/pSK1002 treated with 50 mg/L MTX. It was observed that allicin, proanthocyanidins, polyphenols, eleutherosides, and isoflavones had strong antimutagenic effects against MTX, and the dose-response relationships were quite significant ($P<0.01$). Subsequently, pairwise combinations of the five bioactive phytochemicals possessing significant antimutagenic activities were prepared by dissolving two bioactive phytochemicals in DMSO solvent. The concentration of effective components of each bioactive phytochemical was made up to 1 g/L. The antimutagenic potential of bioactive phytochemical combinations was illustrated in Figure 1. All the bioactive phytochemical combinations showed strong antimutagenic effects as

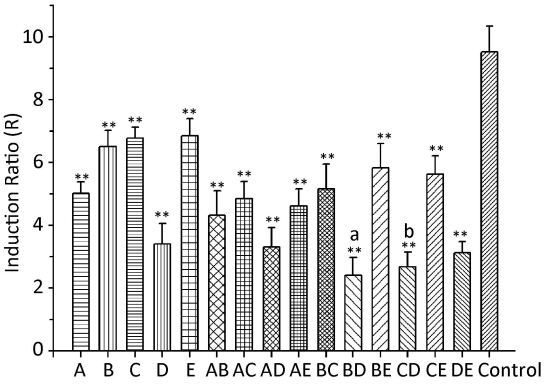

Figure 1. Effects of extract combinations on *umu* gene expression in *S. typhimurium* TA1535/pSK1002 exposed to MTX (50 mg/L). A: allicin (1 g/L), B: grape seed proanthocyanidins (1 g/L), C: green tea polyphenols (1 g/L), D: eleutherosides (1 g/L), E: soybean isoflavones (1 g/L), AB: allicin+grape seed proanthocyanidins; AC: allicin+green tea polyphenols, AD: allicin+eleutherosides, AE: allicin+soybean isoflavones, BC: grape seed proanthocyanidins+green tea polyphenols, BD: grape seed proanthocyanidins+eleutherosides, BE: grape seed proanthocyanidins+soybean isoflavones, CD: green tea polyphenols+eleutherosides, CE: green tea polyphenols+soybean isoflavones, DE: eleutherosides+soybean isoflavones. a: $P<0.01$ as compared to D, b: $P<0.05$ as compared to D, $^{**}P<0.01$ as compared to control.

compared to that of individual components (P<0.01). Combinations of polyphenols and eleutherosides and proanthocyanidins and eleutherosides showed higher cytoprotective activity than other combinations (P<0.05). Therefore, these two bioactive phytochemical combinations were selected as cell protective agent candidates. In addition, the results indicated that polyphenols, eleutherosides, and proanthocyanidins could have synergistic antimutagenic effects against MTX.

Micronucleus assay is internationally recognized as the standard method to detect the mutagenicity of chemicals. To assess the protective effects of the candidate cytoprotectors (based on the SOS/umu test) against MTX-induced genotoxicity, the micronucleus assay was performed in the subsequent experiments. Data on the bone marrow polychromatic erythrocyte (PCE) micronucleus test in mice exposed to MTX after administration of the candidate bioactive phytochemical combinations are shown in Table 2. Irrespective of the gender, MTX induced a statistically significant number of MN per thousand PCEs and the ratio of PCEs to normochromatic erythrocytes (NCEs) as compared to the control group (P<0.01). However, the treatment of the combinations of bioactive phytochemicals markedly decreased the incidence of mouse bone marrow micronucleus and improved the ratio of PCEs and NCEs. Significant differences were also observed between the treatment and model group

Table 1. Effects of Bioactive Phytochemicals on *umu* Gene Expression by MTX (50 μg/mL) in *Salmonella typhimurium* TA1535/pSK1002

Phytochemicals	Extracts Effective Components Concentration (mg/mL)	*R*	Inhibition (%)	Phytochemicals	Extracts Effective Components Concentration (mg/mL)	*R*	Inhibition (%)
Control	0	9.52±0.82					
Chlorogenic acid (Chrysanthemum)	0.0001	9.11±0.58	4.31	Proanthocyanidins (Grape seed)	0.001	9.33±0.48	2.00
	0.001	9.02±0.65	5.25		0.01	8.73±0.69	8.30
	0.01	8.9±0.49	6.51		0.1	7.57±0.52**	20.48
	0.05	9.33±0.51	2.00		1	6.50±0.53**	31.72
	0.25	8.67±0.57	8.93		5	5.76±0.61**	39.50
Allicin (Garlic)	0.001	7.33±0.54**	23.00	Polyphenols (Green tea)	0.01	9.16±0.50	3.78
	0.01	6.23±0.62**	34.56		0.1	8.02±0.55**	15.76
	0.05	5.85±0.66**	38.55		1	6.78±0.46**	28.78
	0.25	5.36±0.54**	43.70		10	5.52±0.52**	42.02
	1.25	4.84±0.71**	49.16		50	4.23±0.66**	55.57
Gingerols (Ginger root)	0.001	9.27±0.52	2.63	Polysaccharides (Reishi mushroom)	0.001	10.21±0.62	-
	0.01	9.81±0.66	-		0.01	9.35±0.56	1.79
	0.1	8.98±0.54	5.67		0.1	8.79±0.48	7.67
	1	9.04±0.59	5.04		1	8.32±0.49*	12.61
	5	8.86±0.58	6.93		5	7.06±0.57**	25.84
Ginkgo flavone (Ginkgo leaf)	0.01	8.95±0.53	5.99	Eleutherosides (Siberian ginseng root)	0.001	8.29±0.76*	12.92
	0.1	9.43±0.43	0.95		0.01	7.43±0.71**	21.95
	1	9.21±0.49	3.26		0.1	4.86±0.60**	48.95
	5	8.58±0.58	9.87		0.5	3.93±0.77**	58.72
	25	7.54±0.60**	20.80		1.5	3.13±0.87**	67.12
Ginsenosides (Ginseng root)	0.01	9.76±0.48	-	Isoflavones (Soybean)	0.001	8.91±0.72	6.41
	0.1	9.82±0.57	-		0.01	8.19±0.70*	13.97
	0.5	8.59±0.55	9.77		0.1	7.43±0.57**	21.95
	2.5	8.76±0.49	7.98		1	6.85±0.55**	28.05
	12.5	8.97±0.56	5.78		5	6.28±0.77**	34.03

Note. Inhibition (%)=100×($R_{control}$-R_{sample})/$R_{control}$; *P<0.05, **P<0.01.

(P<0.01). Furthermore, in terms of the inhibition of micronucleus formation, the combination of proanthocyanidins and eleutherosides was obviously superior to the combination of polyphenols and eleutherosides. Meanwhile, the two treatment groups showed no differences between male and female mice. Thus, it could be considered that the two combinations of bioactive phytochemicals had a marked effect on the suppression of MTX-induced micronuclei in mouse bone marrow cells. In contrast to allicin, proanthocyanidins, polyphenols, and isoflavones, the cytoprotective effect of eleutherosides from Siberian ginseng has been rarely reported. Our results showed that individual eleutherosides and their combinations with proanthocyanidins and polyphenols had strong antimutagenic effects.

To investigate whether MTX combined with the combinations of bioactive phytochemical treatment could strengthen or weaken the reproductive toxicity induced by MTX, sperm tests were performed. As shown in Figure S1, the incidence of mouse sperm head deformity in the treatment groups was not significantly different from that of the control (P>0.05). Significant differences could be observed between the treatment and model groups (P<0.01). The observation of no hooks and amorphous heads in the model group accounted for up to 90% of the total sperm head morphology, which was significantly higher than that of the control and treatment group. An earlier study had confirmed that MTX could induce reproductive toxicity (Padmanabhan et al., 2008). MTX treatment significantly reduced the sperm count and increased the occurrence of sperm head abnormalities. However, the administration of bioactive phytochemical combinations to Kunming specific pathogen-free mice resulted in obvious decreases in the sperm abnormality rate in the case of MTX exposure. Akram et al. (2012) reported that American ginseng extract treatment exhibited therapeutic effects on sperm parameters in rats treated with cyclophosphamide (CP), which is an antineoplastic agent and immunosuppressive medicine in the treatment of various types of tumors and autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (Tripathi and Jena, 2009). Recent studies have confirmed that green tea and soybean extracts showed protective effects against reproductive toxicity induced by CP. Fahmy et al. (2014) found a significant decrease in the percentage of sperm abnormalities upon the oral administration of soybean extracts. Our results illustrated that the two combinations of bioactive phytochemicals may alleviate the reproductive toxicity induced by MTX.

In conclusion, we developed an effective and quick method for screening the potential antimutagenic effects of bioactive phytochemicals based on the umu assay coupled with a micronucleus test. Allicin, proanthocyanidins, polyphenols, eleutherosides, and isoflavones showed higher antimutagenic activity than the other five types of bioactive phytochemicals. Moreover, of all the bioactive phytochemical combinations, the combinations of proanthocyanidins and eleutherosides and polyphenols and eleutherosides showed higher cytoprotective effects. In the case of MTX exposure,

Table 2. Effect of Cytoprotector Candidate Agents on Incidence of Micronucleated Polychromatic Erythrocytes in Bone Marrow Cells and Thymus and Spleen Indices of Mice Treated with Methotrexate

Groups	Number of Mice Treated/Sex	Total Number of PCE Observed	Number of PCE with MN	Total Number of MN	Average MN per 1000 PCE±SD	PCEs/NCEs
Control	4 F	6246	16	16	2.56±0.68	0.92±0.13
	4 M	6098	25	27	4.43±0.85	0.89±0.18
Model	4 F	6158	109	115	$18.58\pm3.15^{\Delta\Delta}$	$0.65\pm0.21^{\Delta\Delta}$
	4 M	6212	124	131	$21.09\pm4.18^{\Delta\Delta}$	$0.59\pm0.14^{\Delta\Delta}$
Treatment I	4 F	6176	79	82	$13.27\pm3.56^{**}$	$0.78\pm0.17^{**}$
	4 M	6138	81	86	$14.83\pm4.92^{**}$	$0.75\pm0.23^{**}$
Treatment II	4 F	6143	58	63	$10.26\pm2.89^{**,\#\#}$	$0.81\pm0.15^{**}$
	4M	6112	65	69	$11.28\pm3.74^{**,\#\#}$	$0.79\pm0.22^{**}$

Note. 1) Control group=saline; Model group=MTX+normal saline; Treatment group I=MTX+combination of polyphenols and eleutherosides; Treatment group II=MTX+combination of proanthocyanidins and eleutherosides. 2) F=female mice; M=male mice; 3) $^{\Delta\Delta}P$<0.01 *vs.* control. 4) $^{**}P$<0.01 *vs.* Model group. 5) $^{\#\#}P$<0.01 *vs.* Treatment group I.

administration of the two combinations of bioactive phytochemicals to Kunming mice resulted in significant decreases in micronucleus induction and sperm abnormality rate. These results imply that bioactive phytochemical combinations of proanthocyanidins, eleutherosides, and polyphenols could be used as new cytoprotectors.

ACKNOWLEDGMENT

We are grateful to Dr. Yoshimitsu Oda for the generous gift of *S. typhimurium* TA1535/pSK1002.

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Received: September 15, 2015;
Accepted: January 10, 2016

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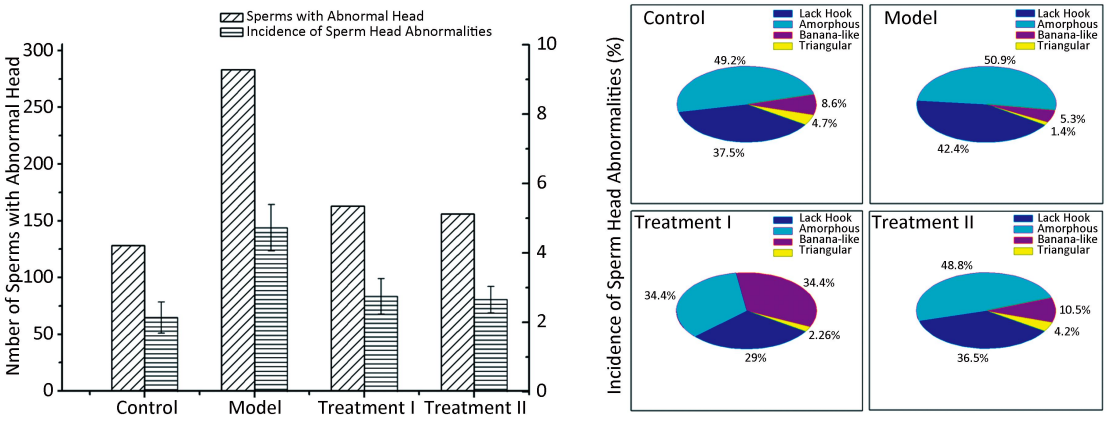

Figure S1. Effect of cytoprotector candidate agents on sperm head abnormalities of male Kunming mice after 7 consecutive days of MTX treatment. Control group=saline; Model group=MTX+normal saline; Treatment group I=MTX+combination of polyphenols and eleutherosides; Treatment group II=MTX+combination of proanthocyanidins and eleutherosides.