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).

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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

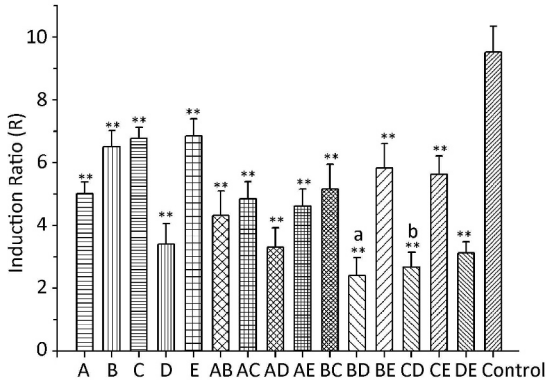

Figure 1. Effects of extract combinations on *umu* gene expression in *S. typhimurium* TA1535/pSK1002 exposed to MTX (50 mg/L). A: allicin (1 g/L), B: grape seed proanthocyanidins (1 g/L), C: green tea polyphenols (1 g/L), D: eleutherosides (1 g/L), E: soybean isoflavones (1 g/L), AB: allicin+grape seed 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.