In vivo Genotoxicity Assessment of Matrine and the Water Extract of Sophorae Radix Using a Comet Assay

Seongmoo Heo, Jiyeon Lee, Hyojeong Jeon, Myoungjun Kim, Young-Shin Chung*
Department of Biotechnology and The Research Institute for Basic Sciences, Hoseo University, Asan, Korea

(Received January 25, 2021/Revised February 27, 2021/Accepted April 1, 2021)

ABSTRACT - The genotoxic potentials of the water extract of Sophorae Radix (WSR) and matrine as a major compound were investigated in murine hepatocytes and blood cells using single-cell gel electrophoresis. According to the test guidelines of OECD TG 489, an in vivo alkaline Comet assay was performed using male mice. The treatment doses of the WSR were 1,000 and 2,000 mg/kg body weight to include the limiting dose. The three test doses for matrine were 50, 100, and 200 mg/kg, and the maximum dose was estimated from a dose range-finding test. The negative control and test articles were administered three times by oral gavage, and the positive control (ethyl methane-sulfonate, EMS) was administered twice. Within 3-4 h after the last treatment, the liver tissue and blood were collected, and single-cell electrophoresis was performed under alkaline conditions. The in vivo Comet assay showed no changes in body weights during the treatments with the test articles. The WSR and matrine did not induce significant DNA damage in the liver and blood cells of mice compared to the vehicle treatment. In conclusion, matrine and the WSR may not have genotoxic potential.

Key words : Sophorae Radix, Matrine, in vivo Genotoxicity, Comet assay

Sophorae flavescens Aiton is an important medicinal plant with antitumor and antimicrobial properties that has been used in traditional medicine to treat stomach problems, inflammation, and skin diseases (such as psoriasis and eczema) with severe itching. The dried root of Sophorae flavescens Aiton (Sophorae Radix), also known as Kushen in China, has been used in decoction and powder forms in East Asia, including Korea. Recently, many studies have investigated the pharmacology of the genus Sophorae (Fabaceae) with its chemical constituents.

The chemical constituents of Sophorae Radix include alkaloids (3.3%), flavonoids (1.5%), alkylxanthones, quinones, triterpene glycosides, fatty acids, and essential oils. Matrine and oxymatrine are the major active alkaloid components in Sophorae Radix. Kurarinone and sophorafalvanone G are the active prenylated flavonoids. A large number of studies have reported that matrine has antitumor activity against various cancer tissues, such as lung, breast, liver, pancreas, stomach, colon, prostate, osteosarcoma, and leukemia. In addition, matrine has pharmacological activity in neurological diseases (Alzheimer’s disease and cerebral ischemia), asthma, cardiac fibrosis, ischemia, liver fibrosis, rheumatoid arthritis, and mental diseases.

Although Sophorae Radix and its components have been applied widely in the health area, side effects and toxicity have been reported. An oral subchronic study for 13 weeks suggested that Sophorae Radix induced liver injury and anemia. Exposure to matrine had adverse effects, such as developmental toxicity, neurotoxicity, and hepatotoxicity.

Recently, the genotoxic potential of Sophorae Radix was demonstrated from several reports. A bacterial reverse mutation assay showed that Sophorae Radix had no mutagenic property, but an in vitro chromosomal aberration assay revealed weak clastogenic potential with an S9 mix. A micronucleus study using the bone marrow erythrocytes of mice did not show genotoxicity. Certain natural products showed genotoxic potential, especially from a bacterial reverse mutation assay, or chromosomal aberration test. The components of natural products, such as alkaloids or flavonoids from natural products, were reported to induce chromosomal aberrations, sister chromatid exchanges, and DNA damage. Therefore, the genotoxic potential of Sophorae Radix may be related to such alkaloids.
or flavonoids.

In this study, the genotoxic potential of *Sophorae Radix* and matrine (as an alkaloid component) were investigated by performing an *in vivo* alkaline Comet assay using murine hepatocytes and blood cells.

**Materials and Methods**

**Chemicals and materials**

Matrine was purchased from Tokyo Chemical Industry (Tokyo, Japan), and oxymatrine was obtained from Cayman (Ann Arbor, MI, USA). Ethyl methanesulfonate (EMS), dimethyl sulfoxide (DMSO), and Triton X-100 were supplied by Sigma-Aldrich (St. Louis, MO, USA). SYBR Gold, comet slides, and low-melting agarose were obtained from TREVIGEN Co. (Gaithersburg, MD, USA). Ethyl methanesulfonate (EMS), (Tokyo, Japan), and oxymatrine was obtained from Cayman (Ann Arbor, MI, USA). SYBR Gold, comet slides, and low-melting agarose were obtained from TREVIGEN Co. (Gaithersburg, MD, USA).

**Preparation of the water extract of Sophora Radix (WSR) and HPLC analysis**

*Sophora Radix* was purchased from Seoul Yangnyeong Si (Seoul, Korea), and the WSR was prepared by boiling twice with distilled water for 40 minutes at 120°C. After filtration, the water extract was freeze-dried. The WSR was characterized by analyzing matrine and oxymatrine on an Agilent 1260 high performance liquid chromatography (HPLC) instrument equipped with a UV detector (Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic conditions were modified from the previous reports and presented in detail below. A C<sub>18</sub> bonded INNO Column (5 μm particle size, 4.6×250 mm, Young Jin Biochrom Co. Ltd., Seongnam, Korea) was used to separate matrine and oxymatrine. The mobile phase was composed of methanol-acetonitrile-5 mM potassium phosphate buffer (pH 6.0) at a ratio of 5:3:2 (v/v/v). The flow rate was 1.0 mL/min, and the UV wavelength was set to 220 nm. The amounts of matrine and oxymatrine in the WSR were estimated based on the analytic results of the standard chemicals.

**Animals, treatments, and liver & blood cells harvest**

All animal studies were approved independently by the Animal Care and Use Committees of the participating institutes and conducted in accordance with the most current version of the Korean Association of Laboratory Animal Science Guidelines (approval numbers: HSIAUC-17-051, HSIAUC-18-124). ICR male mice (six weeks old, SPF) were purchased from Nara Biotech (Pyeongtac, Korea) and acclimated for approximately five days before the treatments. Five mice were housed per a cage, and food and water were provided *ad libitum*. An ambient temperature, relative humidity, and photoperiod of 22±3°C, 50±10%, and 12 hours, respectively, were maintained throughout the study.

The mice were administered the test materials daily for three consecutive days (the first two at 24-hour intervals and the last treatment at a 21-hour interval). The maximum dose for the WSR treatment was selected as a limit dose because the previous micronuclei study did not report any death of mice administered 2,000 mg/kg<sup>27</sup>, and the maximum tolerated dose for matrine was determined from a dose range-finding test. The two experimental groups for the WSR received an oral gavage with 1,000 or 2,000 mg/kg, while the three exposure groups for matrine were 50, 100, and 200 mg/kg. For both studies, the vehicle and EMS were used as the negative and positive control, respectively. The body weights were measured daily prior to the administration of the test materials. Blood samples were collected at 3-4 hours after the last administration (on day 3) using K<sub>3</sub>EDTA-coated microtubes, and the liver tissues were extracted.

**Single-cell gel electrophoresis**

An analysis of DNA damage was performed using the whole blood and liver tissue, as described in OECD TG 489<sup>39</sup>. Briefly, the post lateral liver tissue was cut and minced with a mincing buffer. The blood cell or liver cell suspension was mixed with low melting agarose and loaded onto comet slides. After the membranes of the single cells and nuclei on the slides were lysed in cold lysis buffer, the DNA was unwound in an alkaline solution. Electrophoresis of the processed slides was conducted using an alkaline buffer solution (pH>13) at a constant voltage of 0.7 V/cm (300 mA) for 30 min. After staining the DNA with SYBR Gold, the % tail DNA and tail moment were determined. A digital (CCD) camera linked to a fluorescence microscope (×400, Leica DMI8, Buffalo Grove, IL, USA) was used to image the comets, and analyzer software (Comet Assay IV<sup>TM</sup>, Perceptive Instruments, Suffolk, UK) was used to integrate the images. The percentage of tail DNA in a single nucleus on the slide was estimated, and 150 cells per animal were analyzed.

**Data analysis**

The body weight and % tail DNA were analyzed by analysis of variance (ANOVA), followed by Dunnett’s multiple comparison *t*-tests (two-tailed) (SPSS Ver. 18.0, Chicago, IL, USA) for comparisons between the treated animals and the vehicle control group. The level of significance was set to *P*<0.05 or *P*<0.01.

**Results**

**Analysis of WSR on HPLC**

HPLC chromatograms for the standard chemicals of matrine and oxymatrine showed retention times at 5.729 and
The amounts of those components in the WSR were estimated using calibration curves to be 9.5 and 39.9 mg/g, respectively.

### Results of the Comet assays

Table 1 lists the results of the Comet assay for the WSR. The body weights among the experimental groups were similar on days 1 and 3 of administration, and no significant changes were observed during the treatment period. The administration of the WSR at two different dosages, including the limit dose, did not increase the % tail DNA and tail moment in the liver and blood cells of mice significantly. The percent tail DNA from the negative control was within the range of less than 6% allowed by the guidelines of the OECD TG 489, while the positive control, EMS, induced a significant increase in the % tail DNA compared to the negative control ($P<0.01$).

Table 2 lists the results of the Comet assay for matrine. The maximum dose of matrine for oral gavage was determined to be 200 mg/kg from the dose range-finding test, and two more dosages, 50 and 100 mg/kg were used.
The body weights of the animals were similar in the groups on exposure days 1 and 3. The test materials did not cause changes in body weights for three days. The percent tail DNA and tail moment estimated from the single cell gel electrophoresis were not induced significantly by matrine. The Comet assay for matrine was performed appropriately because the percent tail DNA of the negative control was within the acceptable ranges, and EMS induced a significant increase in the % tail DNA \( (P<0.01) \).

**Discussion**

The genotoxicities of the water extract of *Sophorae Radix* (WSR) and its component, matrine, were investigated in vivo by single-cell gel electrophoresis using murine hepatocytes and blood cells. The present study showed that the administration of matrine and WSR did not induce DNA damage in the liver and blood cells of mice.

The genotoxicities of *Sophorae Radix* had been investigated in several reports. Chromosomal aberration and micronucleus assays were performed using the bone marrow cells of mice administered intraperitoneally with the water extract of *Sophorae Radix*. The results showed significant increases in the frequencies of the chromosomal aberrant cells and micronuclei-producing cells. Mutagenicity was not observed in the bacterial reverse mutation tests. Cho et al. reported that the *Sophorae Radix* extract did not cause reverse mutations with Salmonella Typhimurium TA98, TA1535, and TA1537 in the absence or presence of the S9 mix. On the other hand, a suspicious positive result was observed at chromosomal aberration with Chinese hamster lung (CHL) cells in the absence or presence of the S9 mix. An in vitro micronucleus assay with CHL cells showed no genotoxicity in CHL cells in the absence or presence of the S9 mix. Recently, three battery tests for an evaluation of the genotoxicities showed that *Sophorae Radix* might be a weak clastogen against CHL cells in vitro, only in the presence of the S9 mix. On the other hand, it did not cause micronuclei formation of bone marrow cells from mice administered via oral gavage. Overall, *Sophorae Radix* showed clastogenic potential in mice in vivo with intraperitoneal administration and in the in vitro chromosomal aberration test and no mutagenic potential in the bacterial mutation test. Furthermore, *Sophorae Radix* did not induce micronuclei formation in the bone marrow cells of animals with an oral treatment and in vitro CHL cells.

In general, the Comet assay identifies a material inducing DNA damage. Under alkaline conditions (pH=13), the Comet assay can detect single and double-stranded breaks resulting from direct interactions with DNA, alkali labile sites, or a consequence of transient DNA strand breaks caused by the DNA excision repair. These strand breaks may lead to chromosomal damage, which is also associated with many human diseases, including cancer (OECD TG 489). The International Conference of Harmonization (ICH) suggested that an in vivo Comet assay with in vivo micronucleus test could be performed to confirm the clastogenic potential resulting from in vitro chromosomal aberration test. In the present study, the clastogenic potential of WSR was examined using a Comet assay of murine blood cells and hepatocytes. DNA damage was not detected in both cells; hence, the DNA in the liver cells and bone marrow cells may not be the targets of WSR.

Matrine was reported to have adverse effects and pharmaceutical efficacy. Matrine caused hepatotoxicity in male C57BL/6 mice and exerted oxymatrine-induced hepatotoxicity in a subchronic toxicity study. The matrine treatment induced ROS-dependent mitochondrial apoptosis by inhibiting the Nrf2-mediated antioxidant response by forming a Keap1/Nrf2 protein complex and arrested the cell cycle at the S phase, resulting in hepatotoxicity in the H7702 cell line. The pro-oxidant capacity of matrine may indicate some induction of DNA oxidation.

The acute toxicity test of matrine showed that the tolerable dose of matrine was above 80 mg/kg body weight in Kunming mice, and the LD\(_{50}\) was 157.13 mg/kg. In the current study, the maximum dose range-finding test of matrine using ICR mice administered daily for three consecutive days showed that the maximum tolerable dose was 200 mg/kg. The body weights were not changed significantly during the treatment periods, and no significant differences were observed between the treatment and control animals. The difference in the tolerable doses could be due to differences in the administration route and mouse strain. In a previous report, the Kunming mice were treated with matrine by an intraperitoneal injection, while an oral gavage was used in the present study.

In summary, WSR and matrine were administered to the mice via oral gavage, and the blood cells and liver cells were collected and loaded to single-cell gel electrophoresis. A significant increase in the percent tail DNA was not observed in the images of the single-cell nuclei. These results show that WSR and matrine may not have genotoxic potential.

**Acknowledgments**

This research was supported by the Academic Research Fund of Hoseo University in 2017 (2017-0350).

국문요약
본 연구는 고삼 물추출물과 주 성분인 마트린의 유전 독성 유발능을 마우스 간세포 및 혈구세포에서 측정 단계
Conflict of interests

The authors declare no potential conflict of interest.

ORCID

Seongmoo Heo https://orcid.org/0000-0002-9799-0922
Jiyeon Lee https://orcid.org/0000-0002-7479-4080
Hyounwoon Jeon https://orcid.org/0000-0002-9514-014X
MyoungJun Kim https://orcid.org/0000-0001-8082-7267
Young-Shin Chung https://orcid.org/0000-0002-7774-1304

References


18. Vardari, A.T., Duzgun, Z., Erdem, C., Kaymaz, B.T., Ergul, Z., Cetintas, V.B., Matrine induced G0/G1 arrest and apoptosis in human acute T-cell lymphoblastic leukemia (T-
Genotoxicity Tests of Matrine and Sophorae Radix


