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Safety evaluation of nano/sub-microsized lignan glycosides from sesame meal

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ABSTRACT

Nanotechnology is an emerging field in the food industry, but the potential hazards and toxicities of nano-sized food ingredients are still not clear. The aim of this study was to evaluate the mutagenicity and oral toxicity of nano/submicro-sized ingredient, lignan glycosides from sesame meal (N-LGSM), prepared by a wet-milling method to reduce the average particle size to 189 nm. Genotoxicity assays including Ames test, chromosome aberration assay, micronuclei assay and a 28-day subacute oral toxicity assay were employed to evaluate the mutagenicity and oral toxicity. Results of the Ames test showed that neither lignan glycosides from sesame meal (LGSM) nor N-LGSM increased mutagenicity toward *Salmonella typhimurium* strains TA97, TA98, TA100, TA102 and TA1535. Furthermore, LGSM and N-LGSM were found to have no mutagenic effects on increasing chromosome aberrations in treated Chinese Hamster Ovary (CHO) cells and the number of micronuclei in mouse erythrocytes. Most importantly, in the 28-day subacute oral toxicity trial, the "no observed adverse effect levels" (NOAELs) of LGSM and N-LGSM were determined to be 1000 and 200 mg/kg/day, respectively for both male and female mice. In conclusion, the nano/submicrosizing process did not cause the mutagenicity and oral toxicity for LGSM, and therefore it is a safe processing method for sesame lignan glycosides.

1. Introduction

Sesame (*Sesamum indicum* L.), one of the most important oilseed crops in the world, has been used extensively as a traditional functional food in India and East Asian countries (Namiki, 1995). The major bioactive components found in defatted sesame meal are lignan glycosides, including sesaminol glucosides, pinoresinol glucosides and sesamolinol glucosides (Katsuzaki, Kawakishi, & Osawa, 1994). Sesame products containing these hydrophilic compounds have been reported to possess antioxidative, anti-carcinogenic and neuroprotective activities (Kang, Kawai, Naito, & Osawa, 1999; Sheng et al., 2007; Um, Ahn, Kim, Kim, & Ha, 2009).

Nanotechnology, as defined by the U.S National Nanotechnology Initiative (NNI), is science, engineering, and technology conducted at the nanoscale, which is about 1–100 nm (http://www.nano.gov/nanotech-101/what/definition). Nano-scaled particles may have unusual physicochemical properties that differ from bulk materials of the same chemical composition due to their small size, surface structure activity, solubility, shape and aggregation (Nel, Xia, Madler, & Li, 2006). Because of the advantage of small size and high surface reactivity of nanoparticles, nanotechnology plays an important role in developing medical and pharmaceutical applications (Jain, 2008).

Among many methods used to produce nanoparticles, wet-milling technology has been successfully applied in pharmaceutical production. Poor water-soluble drugs such as phenytoin, indomethacin, nifedipine, danazol, naproxen were successfully prepared as nanosuspensions by using a media-milling technique with Yttria-stabilized zirconia beads as a milling media (Takatsuka, Endo, Jianguo, Yuminoki, & Hashimoto, 2009). Previous studies in our laboratory also demonstrated that the nano/submicro-sized lignan glycosides from sesame meal (N-LGSM) could be successfully prepared by using a wet milling technology with 0.3 mm zirconia beads as the milling media. The nano-sized LGSM exhibited higher transport and absorption efficiency in Caco-2 cell monolayer, and it was more bioavailable in rats (Liao, Hung, Jan, et al., 2010; Liao, Hung, Lu, et al., 2010).

Nano-sized particles could also result in undesirable biological effects. Several studies suggested that nanoparticles can enter the body through skin, lungs or intestinal tract, depositing in several organs, potentially causing adverse effects by modifying the physiochemical properties of living matter (Lanone & Boczkowski, 2006). Therefore, the aim of this study was to evaluate whether the nano/submicrosizing process would increase the mutagenicity or genotoxicity of a bioactive ingredient, in this case sesame lignan glycosides. A bacterial reverse mutation assay (Ames test), chromosome aberration assay and mouse micronucleus assay were

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applied to evaluate the mutagenicity. A 28-day subacute oral toxicity assay using ICR mice was also carried out to obtain the "no observed adverse effect levels" (NOAELs) of LGSM and N-LGSM.

2. Materials and methods

2.1. Materials and chemicals

Black sesame meal was a gift from Yuan-Shun Food Co. (Yun-Ling County, Taiwan). F-12K medium, trypsin-EDTA, fetal bovine serum and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). L-histidine, D-(+)-glucose, sodium chloride, magnesium chloride and sodium ammonium phosphate were purchased from Merck (Darmstadt, Germany). Acridine orange, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (β-NADP), colchicine, benzo[a]pyrene, S9, 9-aminoacridine, 2-aminoanthracene, 4-nitro-*o*-pheylenediamine and sodium azide, were purchased from Sigma Chemical Co (St. Louis, MO, USA).

2.2. Preparation of the crude extract of lignan glycosides from sesame meal

The preparation method was previously described (Shyu & Hwang, 2002). Briefly, black sesame meal was extracted with n-hexane (1:10, g/mL) by stirring for 8 h at room temperature, repeated the extraction two more times. After removing the supernatant by filtration, the defatted sesame meal was collected and extracted with 80% methanol (1:10, g/mL) for 8 h at room temperature, repeated two more times. The methanolic extract of lignan glycosides from sesame meal (LGSM) was obtained by evaporating methanol under reduced pressure, and this crude extract contained mainly lignan glycosides.

2.3. Preparation of nano/sub-microsuspension of lignan glycosides from sesame meal

The nano/sub-microsuspension of lignan glycosides from sesame meal (N-LGSM) was prepared in a media-milling machine (MiniPur; Netzsch-Feinmahltechnik GmbH, Staufen, Germany) as previously described (Liao, Hung, Jan, et al., 2010). Briefly, 8 g of LGSM was suspended in 400 mL of deionized water, and the suspension of LGSM was agitated and circulated in the milling system, which contained Yttria-stabilized zirconia beads with a diameter of 0.3 mm. The following conditions were applied: rotation speed 3600 rpm, feed speed 360 mL/min, milling time 30 min, volume of milling medium 140 mL, and temperature 10 °C. Prior to the Ames test and chromosome aberration test, LGSM and N-LGSM were sterilized by autoclave at 121 °C for 20 min. The particle size of N-LGSM was confirmed by using a light scattering particle size analyzer (Nanotrac 150, Microtrac Inc., Largo, FL, USA).

2.4. Bacterial reverse mutation assay (Ames test)

Ames test was performed as previously described (Mortelmans & Zeiger, 2000). The *Salmonella typhimurium* strains, including TA 97, TA 98, TA100, TA102 and TA1535 were obtained from Culture Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). Prior to the assay, the bacteria were amplified at 37 °C for 16 h on a shaker, 200 rpm. Briefly, the mixture of 0.1 mL of LGSM or N-LGSM, 0.1 mL of bacteria, 0.5 mL of S9 mix or PBS, and 2 mL of heated top agar, were gently mixed and poured onto glucose minimal agar plates. The S9 liver extracts contain enzymes that may activate potential mutagens. Diagnostic mutagens, comprising 9-aminoacridine, 4-nitro-*o*-phenylenediamine, sodium azide, mitomycin C (MMC) and 2-aminoanthracene, were used as positive control chemicals. After incubation at 37 °C for 48 h, the number of revertant colonies in the plates was scored. Results were shown as the mean number of revertants ± standard deviations from a triplicate per dose. A mutagen is defined as a compound or mixture causing a two-fold increase in the number of revertants compared with spontaneous revertants (negative control), or a dose-related increase in the number of revertants in one or more strains. The only exception is strain TA102, which has a relatively high spontaneous revertant number, where an increase by 1.5-fold above the control level is taken as an indication of a mutagenic effect (Suter et al., 2002).

2.5. Chromosome aberration assay in CHO-K1 cells

The assay was performed as previously described (Ishidate & Yoshikawa, 1980). The Chinese hamster ovary epithelial cells (CHO-K1) were purchased from the Culture Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). CHO-K1 cells were cultured in F-12K medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and grown in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were first seeded into 6-cm culture dishes at 5×10^5 cells/plate for 24 h, and then treated with LGSM or N-LGSM at the concentrations of 1.5, 0.75 and 0.1 mg/mL for 3 h in the absence or presence of S9 mix. The positive control was treated with MMC (1 μg/mL) without S9 mix or banzo [a]pyrene (BaP, 5 μg/mL) with S9 mix. After treatment, the cells were treated with 0.1 μg/mL of colchicine for 3 h and harvested into centrifuge tubes by trypsinization and centrifugation, 5 min at 200 × g. After decanting the culture medium, the cells were resuspended in the remaining medium. Eight mL of 0.075 M KCl hypotonic solution was added to the cell suspension and incubated in 37 °C for 10 min. The cells were collected from the hypotonic solution by centrifuging 5 min at 200 × g, and fixed by adding 5 mL of ice-cold Carnoy's solution (methanol:acetic acid, 3:1). The cells were spread on glass slides; air-dried and stained with 3% Giemsa solution in Sorenson's phosphate buffer (pH 6.8) for 30 min. Residual stain was removed by distilled water. A total of 100 well-spread, intact metaphase cells were scored for each treatment under microscope at ×1000 magnification. Structural chromosome aberrations were classified into six types: chromosome-type gap (G), chromosome-type break (B), chromosome-type ring (R), chromosome-type dicentric (D), chromatid-type gap (g) and chromatid-type break (b).

2.6. In vivo rodent erythrocyte micronucleus assay

The micronucleus assay was carried out as previously described (Hayashi, Morita, Kodama, Sofuni, & Ishidate, 1990). Healthy male Crl:CD-1(ICR) BR mice (5-week-old) from BioLASCO Co (Taipei, Taiwan) were used in the micronucleus assay to evaluate the *in vivo* clastogenic activity by scoring micronuclei in reticulocytes (RETs). Briefly, mice were treated with a single dose of LGSM (5, 2.5 and 1.25 g/kg body weight) or N-LGSM (0.2, 0.1, 0.05 g/kg body weight) by oral gavage ($n = 5$). The positive control group received a single dose of mitomycin C (1 mg/kg body weight) via intraperitoneal injection. After 24, 48 and 72 h, blood samples of mice were collected from the tail vein, and approximately 10 μL of blood was smeared onto slides coated with acridine orange. The number of micronucleated cells was scored in 1000 RETs in each mouse. RETs on the slides were observed by using a fluorescent microscope equipped with a blue excitation (480 nm wavelength) and a yellow-to-orange barrier filter (515–530 nm wavelength).