Food and Chemical Toxicology 47 (2009) 2785–2789

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Spirulina maxima and its protein extract protect against hydroxyurea-teratogenic insult in mice

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ARTICLE INFO

Article history:
Received 13 January 2009
Accepted 19 August 2009

Keywords:
Spirulina maxima
C-phycocyanin
Hydroxyurea
Teratogenesis

ABSTRACT

Congenital malformations are one of the major causes of child mortality all over the world. In order to prevent them it is necessary to find substances that act as anti-teratogenic agents. In this study hydroxyurea (HU), an antineoplastic and teratogenic drug, was administered to pregnant mice because one of its major mechanisms of teratogenesis is the production of reactive oxygen species (ROS). The aim of this work was to determine if *Spirulina maxima* (SP) and its aqueous protein extract could protect against HU-teratogenic insult in mouse embryos. SP has been used for a long time because of its nutritional and pharmacological properties. The antioxidant activity, one of the most important, is related to the protein extract due to its content of phycobiliproteins. It was observed that neither SP nor its extract provoked teratogenic effects at any dose tested and even increased vitelline yolk sac circulation. Dams exposed to HU (30 mg/kg, i.p.) presented embryos with multiple alterations in their development. Groups treated with SP or its extract, before and after HU exposure, showed a protector effect in a dose-dependent manner. TBARS test confirmed that the protection effect was related to the antioxidant activity of both SP and its extract.

1. Introduction

Spirulina maxima is a cyanobacterium classified as blue-green algae. It has been used as a food (Dillon et al., 1995) because of its quantity of proteins, vitamins, essential amino acids, minerals and essential fatty acids (Campanella et al., 1999; Mendes et al., 2003). It has been reported in some reviews that SP have several pharmacological activities (Belay, 1993; Belay et al., 2002; Chamorro et al., 2002; Khan et al., 2005), of which antioxidant effect is one of the most important. Its antioxidant property is found in the protein extract, specifically some phycobiliproteins such as C-phycocyanin (CP) and allophycocyanin (Miranda et al., 1998; Wu et al., 2005; Lu et al., 2006). It was observed that this extract has the same antioxidant effect as pure phycocyanin at similar concentrations (Piñero et al., 2001; Bermejo et al., 2008).

There are a number of environmental, chemical and physical agents that have been identified as teratogenic substances (Brent, 2004), however congenital malformations are one of the major causes of child mortality all over the world. In the industrialized countries those agents are the leading cause of death, while in undeveloped countries they are the second or third (WHO, 2007).

In the development of any organism it has been observed that one of the most important periods is the organogenesis. At this stage some tissues could be affected by a xenobiotic, and possibly resulted in cellular death or a malformation. Wells et al. (2005) have proposed that some substances can cause congenital damage when they are enzymatically bioactivated within the embryo to highly toxic, electrophilic or free radical reactive intermediates. The latter can react directly or indirectly with molecular oxygen to initiate the formation of reactive oxygen species (ROS) which damage cellular macromolecules and interfere with embryonic and/or fetal development.

Hydroxyurea (HU), an antineoplastic drug employed in both clinical chemotherapy and the treatment of viral diseases (Hendricks and Mathews, 1998; Argiris et al., 2003), is a potent teratogen in animals such as mice, rats and rabbits (Scott et al., 1971; DeSesso, 1981; DeSesso et al., 1994; Yan and Hales, 2005; Woo

Abbreviations: HU, hydroxyurea; MDA, malondialdehyde; CP, C-phycocyanin; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate buffer solution; RNR, ribonucleotide reductase; SP, *Spirulina*; SPE, *Spirulina maxima* protein extract; TBARS, thiobarbituric reactive species.

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doi:10.1016/j.fct.2009.08.013

et al., 2006). Its principal mechanism involves the inhibition of ribonucleotide reductase (RNR) by its reaction with the catalytically essential tyrosyl radical in the enzyme (Hendricks and Mathews, 1998). It has been reported that within 2–4 h after maternal injection, this substance causes a rapid episode of cell death and profound inhibition of embryonic DNA synthesis (DeSesso and Goeringer, 1990). On the other hand, it has been proposed that HU may react within the embryo to produce ROS, which could be responsible for the early cellular death observed (DeSesso, 1979). Some antioxidants such as D-mannitol and propyl-gallate reduce HU toxicity, and others as vitamin E, sodium benzoate, catalase, peroxidase and superoxide dismutase protect against acute HU toxicity (Przybyszewski and Malec, 1982; Przybyszewski et al., 1987; DeSesso and Goeringer, 1990; DeSesso et al., 1994).

The aim of this study was to determine if SP, and specifically its protein extract, could protect against HU-induced teratogenicity in mouse embryos.

2. Materials and methods

The SP sample used in this study was from a bulk production batch (SDW-9714) of standardized quality (SP was analyzed and all its nutritional contents were determined and indicated in the label) supplied by Alimentos Esenciales para la Humanidad, S.A. de C.V., Mexico City.

2.1. Spirulina maxima protein extract (SPE)

One gram of SP was dissolved in 10 ml of phosphate buffer solution (PBS) pH 7.2 and stored in the dark. After 12 h the suspension was centrifuged for 15 min at 13000 rpm×2 (Sorvall RC-5B centrifuge Dupont Instruments) and for 30 min at 35000 rpm×2 (L-80 ultracentifuge Beckman). After each centrifuge cycle the precipitate (green color) was discarded and the supernatant was again centrifuged. The protein extract obtained was dialyzed against 0.01 M Na-phosphate solution in the dark with a 12 KDa membrane for 5 days and then lyophilized. All procedures were performed at 4 °C. In order to know if CP was the major component in the extract, SDS–PAGE and spectroscopic measurements were carried out.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

A 15% SDS–PAGE was carried out for SPE, CP (Sigma Chem. Co.) and a molecular weight marker (Sigmamarker-Sigma Chem. Co.) as described by Binder et al. (1972).

2.3. Spectroscopic measurements

UV–Vis spectra of protein samples were recorded on a BioSpec-mini spectrophotometer (Shimadzu). The maximum absorption wavelength for the extract was compared with the same reported for CP (620 nm). The purity was evaluated according to the absorbance ratio (A_{620}/A_{280} for C-phycocyanin), (Boussiba and Richmond, 1979).

2.4. Teratogenesis

Experimental animals were handled according to the Official Mexican regulations (NOM-062-ZOO-1999) related to laboratory animals, which are according to the NIH # 86-23. They were maintained at a constant temperature (24 ± 1 °C), humidity (50 ± 10%) and artificial illumination between 08.00 and 20:00 h.

Experimental design is according to the OECD regulations (414) for studies of evaluations of prenatal developmental toxicity with some protocol modifications, according to the study of Warner et al. (1983), as follows: CD-1 males (30 ± 2 g of body weight) were placed with females (25 ± 2 g of body weight) during 2 h before initiating illumination period. Dams which presented vaginal plug were separated, randomized in different groups through a classic random digit table (simple random sample) and their weight was registered. This was designated as day 1 of pregnancy. Group 1 and 2 were given water and tween 80 (0.005%), daily, respectively. Groups 4, 5 (1000 mg/kg), 6 (500 mg/kg) and 7 (100 mg/kg) were given SP, daily. Groups 8, 9 (400 mg/kg), 10 (200 mg/kg) and 11 (100 mg/kg) were given SPE, daily. In the morning of day 9 of gestation, groups 3, 5, 6, 7, 9, 10 and 11 received a single dose i.p. of 300 mg/kg of HU (Sigma Co.) in water as a vehicle. All groups were formed by 10 dams. Tween 80 was used to suspend SP. HU was given 1 h after SP or SPE. Water, tween 80, SP and SPE were given *per os* (intra gastric) in a maximum volume (ml) of 1% of the mouse body weight.

At day 11 of gestation dams were sacrificed and their uterine horns were taken out and put in phosphate buffer solution (PBS). Embryos were carefully explanted from uterine tissues and observed for yolk sac circulation and heartbeat. When no visible heart beat contraction was determined embryos were consider as dead and only that presented a rapid and uninterrupted beats were evaluated. Yolk sac circulation was observed in a similar way since no blow flow until rapid and uninterrupted flow. After that, embryos were cleaned of all their membranes and evaluated according to the Brown and Fabro (1981) morphological scoring system to qualify the embryos development (flexion, heart, neural tube, optic system, etc.). Food and water consumption and gravid uterine weight were not recorded since these variables were not affected in previous studies of SP and HU at the dose used in this protocol (Chamorro et al., 1989; Chamorro and Salazar, 1990; Chahoud et al., 2002).

2.5. Determination of lipid peroxidation

Five additional pregnant dams, to that used to the teratogenesis study, for groups 1, 2, 3, 4, 5, 8 and 9, were treated in order to determine lipid peroxidation by quantification of malondialdehyde (MDA). At day 11 of gestation dams were sacrificed and embryos were explanted as in the teratogenesis study. The embryos were cleaned of all their membranes. The lipid peroxidation was determined by quantification of thiobarbituric acid reactive species (TBARS), as described by Paniagua-Castro et al. (2008), as follows: embryos homogenates, prepared by pooling the tissue of three embryos, were mixed thoroughly with trichloroacetic acid. The suspension was heated for 1 h in a boiling water bath. After cooling, the flocculent was removed by centrifugation at 1000*g* for 10 min. The absorbance of the sample was measured at 535 nm (BioSpec-mini spectrophotometer Shimadzu) against an only reagent blank. Protein content was determined by the coomassie blue method (Bradford, 1976), with bovine serum albumin as a standard. All procedures were carried out with three embryos per dam in duplicate, for a total of 6 embryos per dam, which were randomly selected. Extinction coefficient of MDA ($1.56 \times 10^{-5}\ M^{-1}\ cm^{-1}$) was used to calculate MDA concentration in the samples.

2.6. Statistical analysis

All statistical analysis were performed with Microsoft® Office Excel 2007 and Sigma Stat® Statistical Software 2.03. The variation among groups in number of somite as well as protein and MDA content was determined by a one way ANOVA and the Tukey test when a significant difference was found. Differences among groups in the number of dams and embryos affected, dead implants and non viable embryos were determined by chi-square test or exact Fisher test when the former could not be performed. The difference was considered significant when $p \leqslant 0.05$.

3. Results

SDS–PAGE and spectroscopic measurements confirm the presence of CP as the major component in the extract. The maximum absorption wavelength for the extract was 619.88 nm, which corresponds to that reported for CP (620 nm), with an absorbance ratio (A_{620}/A_{280}) of 2.22.

The dams had an average of 25 ± 0.89 g body weight at day 1 of gestation and 34 ± 0.34 g at sacrifice, there were not differences among groups. Pre and post-implantation losses were similar in all groups, except in group 3, to those observed in controls. Table 1 summarizes the data found in the teratogenesis study. In the examination of a total of 152 embryos in group 1, only 5 displayed development alterations such as turning interruption and retarded hind and forelimb bud growth. All the other embryos exhibited a normal development pattern. In group 2 out of 161 embryos only 3 showed similar alterations as in the group 1. In both groups a normal vitelline yolk sac circulation was found.

In group 3 out of 152 embryos, 78 were viable, of these, 53 had abnormalities such as an open neural tube or nasal, maxillary and mandibular processes, optic and ear processes, turning interruption and retarded hind and forelimb buds growth. A diminished yolk sac circulation was also observed.

In group 4 no dead embryos were found and only 2 out of 148 had an abnormal hind and forelimb bud growth. In group 8, 4 embryos, of 119, displayed abnormalities in turning process and hind and forelimb bud growth. An increased yolk sac circulation was observed in both groups 4 and 8.

In groups 5, 6, 7, 9, 10 and 11, which were treated with SP and SPE, before and after exposure to HU, the number of dead embryos and development alterations was found to be decreased. The decrease was dose-dependent. In all those SP and SPE treated groups, the average number of somites was nearly the same as the control