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A developmental toxicity study of 3S, 3′S-Astaxanthin in New Zealand white rabbits

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ABSTRACT

Astaxanthin, a naturally occurring pigment used to give the characteristic orange-pink colour to salmonid fish reared in aquaculture, is also marketed as a dietary supplement. Synthetic 3S, 3′S-Astaxanthin was tested for potential harmful effects on the *in utero* development of New Zealand white rabbits in a study according to international regulatory guidelines. There were two control groups, one being a placebo administration and three dose levels corresponding to 100, 200, and 400 mg of 3S, 3′S-Astaxanthin per kg body weight/day. The group sizes varied from 23 to 27 litters, providing approximately 200 fetuses per group for evaluation of developmental toxicity. There were no significant effects on the health of the does, nor on the size and viability of the litters. Malformations, both external and internal, were rare and occurred in all groups, including controls with no indication of a treatment relationship. Variations were much more common, being found in all litters. However, when examined by type and frequency, no pattern emerged indicating a relationship to administration of the test substance. It is concluded that administration of 3S, 3′S-Astaxanthin in a gelatin/carbohydrate powder formulation throughout pregnancy up to 400 mg/kg body weight/day is without harmful effects on reproduction or fetal development.

1. Introduction

Astaxanthins are carotenoids found naturally in algae, fish and crustaceans and are associated with the attractive pink colour of wild salmon flesh. This colour can only be obtained in aquaculture by using natural organisms containing these pigments (e.g. shrimp waste) or by mixing Astaxanthin with the diet. This is obtained either from microalgae (*Haematococcus pluvialis*) (Lorenz and Cysewski, 2000) or from fully synthetic sources. The three trans-isomers exist in nature in variable ratios whereas the synthetic material is either a racemic mixture 1:2:1 (3S,3′S:3R,3′S:3R,3′R) (Moretti et al., 2006) or a pure enantiomer.

Many carotenoids have significant biological activity, in particular their antioxidant properties (Lordan et al., 2011). Focus on this aspect has led to the characterisation of carotenoids as being generally beneficial (Rao and Rao, 2007). Some carotenoids, notably beta-carotene, are precursors for the synthesis of vitamin A in herbivores and omnivores.

Excessive dosing with vitamin A leads to a well known pattern of toxicity (hypervitaminosis). However, the cleavage of provitamin A carotenoids is highly regulated and vitamin A toxicity from this source is considered to be practically impossible (Penniston and Tanumihardjo, 2006). Preformed vitamin A toxicity includes a risk of teratogenicity (Teratology Society, 1987), but, while retinoic acid teratogenicity has been demonstrated at high doses in animal experiments, the evidence of harm in humans from vitamin A supplementation appears quite limited (Azaïs-Braesco and Pascal, 2000).

Of course, carotenoids are a chemical family with great structural and biological diversity (Britton, 1995) and no general assumptions can be made about potential for adverse developmental effects. In a recent EFSA-Opinion on the safety and efficacy of a synthetic, racemic Astaxanthin (EFSA, 2014) it was reported that there was no evidence of any embryotoxicity, fetotoxicity or teratogenicity in rats and rabbits in GLP-compliant developmental toxicity studies. For the pure S,S′-Astaxathin isomer toxicological information is limited. With regard to systemic toxicity, two recent sub-chronic studies, using as test materials either a natural astaxanthin-rich carotenoid extract (containing mainly the S,S′-

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Astaxanthin form; EFSA, 2007a) or a synthetic S,S′-Astaxanthin in a powder formulation, showed consistently no specific organ toxicity (Buesen et al., 2015; Katsumata et al., 2014). However, in the scientific literature no studies specifically on reproductive toxicity have been published so far. This is considered to be relevant information for the safety assessment of S,S′-Astaxanthin in food applications. Accordingly, an experimental study was designed in rabbits which is a well-accepted animal model for human developmental risk (Foote and Carney, 2000). For this purpose, 3S, 3′S-Astaxanthin was included into a gelatin/carbohydrate powder formulation in order to improve stability and bioavailability of the crystalline molecule. The formulation is intended for fortification of certain foods and for use in dietary supplements with an anticipated overall daily exposure of the consumer not above 10 mg Astaxanthin per day.

2. Materials and methods

This study was performed in compliance with the guidelines for testing of chemicals as prescribed by United States EPA OPPTS 870.3700 1998, OECD 414 (2001) and European Commission Regulation No. 440/2008. The study was performed in an AAALAC accredited laboratory and according to OECD good laboratory practice (OECD, 1997). It was notified to the local authority under the file number 23 177-07.

3S, 3′S-Astaxanthin (syn. 3,3′-Dihydroxy-β,β-carotene-4,4′-dione, CAS-no. 472-61-7) was obtained by chemical synthesis and had a purity of 95.6 g/100 g as determined by UV photometric analysis. Astaxanthin was formulated into powder beadlets due to the known low oral bioavailability of unprocessed carotenoids (Mercke Odeberg et al., 2003) and the limited stability of the pure crystalline substance during storage. The composition of the powder formulation was as follows: 3S, 3′S-Astaxanthin: 21.1%; Porcine gelatin: 23%; Sucrose: 47%; Sodium ascorbate: 2%; Mixed tocopherol (70%): 2.8%; Corn starch (native): 19%; Residual water: 6.2%. The content of Astaxanthin in the formulation was determined spectrophotometrically. In order to avoid any unspecific effects induced by formulation ingredients a placebo control group was included which was composed of all the formulation ingredients without the addition of Astaxanthin.

Groups of adult female New Zealand white rabbits (Charles River) were artificially inseminated and allocated at random to the various dose groups. The day of insemination was designated as Gestational Day 0 (GD 0, i.e. beginning of the study) and the following day as GD 1. Due to the large number of animals (150) involved this was done in six batches of animals over a period of nine days. They were dosed by gavage from gestational day 6–28 with 10 ml/kg body weight of deionised water (test group 0), blank formulation (placebo; test group 1) or 3S, 3′S-Astaxanthin at concentrations calculated to give 100, 200 or 400 mg/kg body weight of the test substance (test groups 2–4). These doses were selected based on information available for racemic Astaxanthin, which did not induce maternal toxicity or developmental toxicity at the highest tested dose of 400 mg/kg body weight/day in an unpublished study with rabbits (EFSA, 2014) and based on a previous maternal toxicity study with 3S, 3′S-Astaxanthin (BASF SE, 2014). The viscosity of the powdered formulation, when mixed with deionised water, precluded administration of more than 400 mg 3S, 3′S-Astaxanthin/kg body weight of in a volume of 10 ml/kg body weight. The lower doses were prepared by further dilution of the powdered formulation to give a standard volume. The test substance preparations were prepared at the beginning of the administration period and thereafter at maximum intervals of 11 days, which took into account the period of established stability. The content, the homogeneity and the stability of 3S, 3′S-Astaxanthin in the vehicle (deionised water) was investigated by HPLC analysis and photodiode array detection (DAD).

The food used was pelleted Kliba maintenance diet rabbit and guinea pig “GLP”, supplied by Provimi Kliba SA, Kaiseraugst, Switzerland. Food and drinking water (potable tap water in water bottles) were available ad libitum throughout the study.

The does were regularly examined during pregnancy and weighed on GD 0, 2, 4, 6, 9, 11, 14, 16, 19, 21, 23, 25, 28 and 29. Only does that survived and were pregnant were used in the subsequent calculations.

The does were killed by injection of pentobarbital (Narcoren®, 2 ml/animal) on gestational day 29 and the standard measures were taken: uterine weight, corpora lutea, live fetuses, dead fetuses and dead implantations (early and late).

Evaluations of all other parameters were performed by technicians who were unaware of the treatment group allocation in order to prevent bias. The following calculations were derived: conception rate, pre- and post-implantation loss.

After opening the uteri, all fetuses were examined for external abnormalities. Furthermore, the viability of the fetuses and the condition of placentas, umbilical cords, fetal membranes, and fluids were examined. Individual placental weights were recorded. Thereafter, the fetuses were sacrificed with pentobarbital. After the fetuses had been sacrificed, the abdomen and thorax were opened in order to examine the organs in situ before they were removed. The heart and the kidneys were sectioned in order to evaluate the internal structure. The sex of the fetuses was determined by examination of the gonads in situ. Half of the fetuses at random, plus any with relevant external abnormalities, had their heads removed for fixation in Bouin's solution and Wilson section (Wilson and Warkany, 1965).

After fixation in ethyl alcohol all skeletons (including those without heads), were cleared and double-stained to reveal the skeletal structure: bone and cartilage (Kimmel and Trammel, 1981).

In the present study, the internationally harmonized glossary of Wise et al. (1997) and the updated version Makris et al. (2009) was essentially used to describe findings in fetal morphology. Classification of these findings was based on the terms and definitions proposed by Chahoud and Solecki (Chahoud et al., 1999; Solecki et al., 2001, 2003): A malformation is regarded as a permanent structural change that is likely to adversely affect survival or health. A variation represents a change that also occurs in the fetuses of control animals and/or is unlikely to adversely affect survival or health. This includes delays in growth or morphogenesis that have otherwise followed a normal pattern of development. The term “unclassified observation” was used for those fetal findings, which could not be classified as malformations or variations.

Statistical analyses: All statistical evaluations for clinical, necropsy and fetal examinations compared treatment groups to the placebo control group. In addition, the placebo control group was compared to the vehicle control group. Parametric tests such as DUNNETT (two-sided), or non-parametric tests such as FISHER'S EXACT test (one-sided) or WILCOXON-test (one-sided) were used to test for the hypothesis either of equal means or equal proportions or medians.

Historical control data of the test facility have been utilized to enhance the assessment of the findings in the present study. The historical data were collected from 10 comparable studies conducted between January 2009 and January 2013, using the same strain of New Zealand white rabbits (Crl:KBL(NZW)) as in the present study.

3. Results

The stability of Astaxanthin in the application vehicle (deionized