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No evidence of genotoxic effect *in vivo* of the phytosterol oxidation products triols and epoxides

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Abstract

Phytosterols (PS) are naturally occurring compounds present in food products of plant origin. Due to reported positive health effects, some food products are also enriched with PS. In the same way as cholesterol is oxidised, PS also oxidise to a variety of oxidation products (POPs). The biological effects and safety aspects of POPs are still unclear. This study investigated whether POPs are genotoxic *in vivo*, using a flow cytometer-based micronucleus assay in mice. The highest dose of oxidation products administered was 67 mg/kg b.w. for epoxides and 9.4 mg/kg b.w. for triols. Synthesised and separated triols and epoxides from a mixture of sitosterol and campesterol were investigated. The frequency of micronucleated polychromatic erythrocytes (fMNPCE) in POP-exposed mice did not significantly differ from the control values in either of two experiments performed. The flow cytometer-based method also allows for restriction of the analysis to micronuclei with a high DNA content, indicating an aneugenic potency. Even with this approach, there was no significant increase in the frequency of micronucleated erythrocytes among POP-treated mice compared with control mice. Furthermore, no significant deviation in cell proliferation rate (%PCE) was observed.

Keywords: Micronucleus; Phytosterol oxides; Triols; Epoxides; Flow cytometer

1. Introduction

Modern consumers want food products to not only taste good, but also have beneficial effects on health. During recent years, the health effects of phytosterols (PS) have received a great deal of attention (for review see Awad and Fink, 2000) and daily intake is estimated to be between 100 and 450 mg/day (for review see Ostlund, 2002). An increased intake of phytosterols reduces the levels of total cholesterol and low-density lipoprotein cholesterol and possibly protects against cardiovascular disease (Jones et al., 1999; Hendriks et al., 1999; Hallikainen et al., 2000; Norman and Wong, 2001; Thomsen et al., 2004). However, the daily intake must be as high as 2 g PS to produce an approximately 10% reduction in serum cholesterol levels. To achieve this high level, some commercial food products are enriched with phytosterols, *e.g.* spreads, milk and mayonnaise (Normén et al., 2004; Conchillo et al., 2005). For example, some of the spreads on the Swedish market are enriched with 8 g phytosterols or stanols per 100 g spread. A high intake of PS is associated not only with positive effects against cardiovascular disease, but also reduced symptoms of benign prostatic hyperplasia (Berges et al., 1995). On the other hand, negative effects

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such as decreased bioavailability of beta-carotene and alpha-tocopherol have also been reported (Richelle et al., 2004).

The tendency of cholesterol to oxidise in the presence of heat and air is well known and has been reported in detail (Guardiola et al., 2002). Oxidation mechanisms and various oxidation products of cholesterol (COPs) have been isolated and characterised in detail in recent decades and many comprehensive reviews have been published (Schroepfer, 2000; Adcox et al., 2001; Guardiola et al., 2002). The toxic effects of COPs have been extensively studied and a relationship between high levels of COPs and coronary heart disease has been reported in many studies (Guardiola et al., 2002). Some COPs can be atherogenic, cytotoxic, mutagenic and carcinogenic, and may also inhibit cholesterol biosynthesis and membrane functions (Sevanian and Peterson, 1984; Peterson et al., 1988; Smith and Johnson, 1989; Cheng et al., 2005; Ryan et al., 2005). On the other hand, in a review by Björkhem and Diczfalusy (2002) they presented a more hesitant view on the impact of COPs as physiological regulators of atherogenic factors.

Much of the existing knowledge on the oxidation mechanisms for PS refers to studies on cholesterol due to similarities in their structure, but the biological effects and safety aspects of phytosterol oxidation products (POPs) are still rather unclear (Oehrl et al., 2001). Even though oxidation products of phytosterols occur in many different food products, the knowledge regarding levels of POPs is scarce (Dutta, 2004). To our knowledge, fortified spreads available on the market have only been investigated and the results published in two different studies (Grandgirard et al., 2004d; Johnsson and Dutta, 2006) and the levels of POPs present have been estimated to be about 50 μg/g spread. Data on the POP levels in some vegetable fats, corn and palm oil, are published in Johnsson and Dutta (2006). To verify the bioavailability of POPs, both hamsters and rats have been studied after oral administration of different doses of POPs (Grandgirard et al., 2004a,c). The results indicate a significant uptake of POPs in both species. In a monitoring study, human serum (healthy controls) has also been shown to contain noticeable levels of POPs (Grandgirard et al., 2004b). The quantified oxyphytosterol levels in humans varied between 5 and 57 ng/ml serum. However, the information about possible harmful effects of an increased intake of POPs is limited. Only a few studies have been published and usually a combination of many different oxidation products has been studied. Concerning possible genotoxic effects, Maguire et al. (2003) and Lea et al. (2004) published data from different *in vitro* studies. The results indicated no genotoxic potential of POPs.

Since the possible influence of uptake, distribution and metabolism is impossible to study *in vitro*, in the present study we investigated whether POPs are genotoxic *in vivo*, in a flow cytometer-based micronucleus assay in mice. Furthermore, both cholesterol epoxides and triols have been reported to display mutagenic potential (Peterson et al., 1988; Cheng et al., 2005), therefore pure compounds of triols and epoxides from sitosterol and campesterol were examined in the present study. These oxidation products are significant contributors to human exposure according to the few published papers available (Johnsson and Dutta, 2006; Grandgirard et al., 2004b).

2. Materials and methods

2.1. Reagents

All solvents and chemicals used in the production of the POPs were of P.A. grade and were purchased from VWR (Stockholm, Sweden), with the exception of 3-chloroperbenzoic acid, which was purchased from Sigma–Aldrich AB (Stockholm, Sweden), and the mixture of sito- and campesterol, which was purchased from Research Plus Inc. (Bayonne, NJ). The *in vivo* experiments used Hoechst 33342 (HO 342), Sodium Dodecyl Sulphate (SDS), and Colchicine (CAS 64-86-8) from Sigma–Aldrich AB (Stockholm, Sweden), fluothane from Astra Zeneca (Södertälje, Sweden), Percoll 65% from Amersham Bioscience (Sweden), glutaraldehyde 70% from TAAB laboratories, Reading (UK), Thiazole orange (TO) from Molecular Probes (Oregon) and phosphate buffer saline (PBS) from SVA (Uppsala, Sweden).

2.2. The formation of phytosterol oxidation products

The epoxides were synthesised according to a previously described method (Fieser et al., 1957). In brief, a mixture of sitosterol and campesterol was mixed with dichloromethane and *m*-chloroperbenzoic acid. The mixture was stirred for 4 h prior clean-up procedure and evaporation of the solvents. To produce the triols, the mixture of epoxides was dissolved in acetone. Periodic acid in water was added and the mixture was refluxed for 1.5 h. The sample was centrifuged and the pellet washed with acetone:water (1:1, v/v), dried under nitrogen and dissolved in chloroform. Both epoxides and tiols were further purified by preparative TLC as described elsewhere (Johnsson and Dutta, 2003).

The oxidation products (purity over 95%) were dissolved in corn oil and ethanol and used in the animals. The epoxide mixture consisted of (24*R*)-5α,6α-epoxy-24-methylcholestane-3β-ol (7.27 mg/ml), (24*R*)-5β,6β-epoxy-24-methylcholestane-3β-ol (0.97 mg/ml), (24*S*)-5α,6α-epoxy-24-ethylcholest-22-en-3β-ol (3.33 mg/ml), (24*R*)-5α,6α-epoxy-24-ethylcholestane-3β-ol (10.87 mg/ml) and