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Quercetin tests negative for genotoxicity in transcriptome analyses of liver and small intestine of mice

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ABSTRACT

Given the positive results of quercetin in *in vitro* genotoxicity studies, the *in vivo* genotoxic properties of this important dietary flavonoid warrant testing, especially considering possible high intake via widely available food supplements. Here, this was done by transcriptome analyses of the most relevant tissues, liver and small intestine, of quercetin supplemented mice.

Quercetin (0.33%) supplemented to a high-fat diet was administered to mice during 12 weeks. Serum alanine aminotransferase and aspartate aminotransferase levels revealed no indications for hepatotoxicity. Microarray pathway analysis of liver and small intestine showed no regulation of genotoxicity related pathways. Analysis of DNA damage related genes also did not point at genotoxicity. Furthermore, a published classifier set of transcripts for identifying genotoxic compounds did not indicate genotoxicity. Only two transcripts of the classifier set were regulated, but in the opposite direction compared with the genotoxic compounds 2-acetylaminofluorene (2-AAF) and aflatoxin B1 (AFB1).

Based on the weight of evidence of three different types of analysis, we conclude that supplementation with quercetin at ~350 mg/kg bw/day for 12 weeks in mice showed no up-regulation of genotoxicity related pathways in liver and small intestine.

1. Introduction

Flavonoids present in foods of plant origin like fruits, vegetables, and tea, are thought to play a role in the beneficial effects of these foods (Peterson et al., 2012). Apples, onion and tea are major dietary sources of one of these flavonoids, quercetin, and in addition quercetin is widely available as a dietary supplement in Western countries. However, the safety of quercetin has been under discussion for several decades, since also possible adverse effects of quercetin, including genotoxicity, have been reported (National Toxicology, 1992; Pamukcu et al., 1980; Resende et al., 2012).

Genotoxicity results in DNA damage and when not repaired, this will result in lasting mutations, which can ultimately lead to cancer. Quercetin tested positive in various *in vitro* genotoxicity tests. For example, genotoxic properties of quercetin were shown in bacterial systems including the Ames test, with and without metabolic activation (MacGregor and Jurd, 1978; Nagao et al., 1981; Resende et al., 2012; Silva et al., 2000; Vrijsen et al., 1990). This genotoxicity has been related to the quinone–quinone methide chemistry of quercetin (MacGregor and Jurd, 1978). Furthermore, in several cultured human and rodent cells, formation of micronuclei, DNA single strand breaks and chromosomal aberrations were observed after quercetin exposure (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007; Caria et al., 1995; Carver et al., 1983; Rueff et al., 1986; Silva et al., 2000). It was also shown that quercetin can generate DNA adducts in different cell types *in vitro* (van der Woude et al., 2005).

However, genotoxicity was not shown in rodents after oral quercetin administration measured by, among others, micronuclei, chromosomal aberrations and unscheduled DNA synthesis in bone marrow, liver, and gastric mucosa cells (Aeschbacher et al., 1982; Cierniak et al., 2004; Ngomuo and Jones, 1996; Taj and Nagarajan, 1996; Utesch et al., 2008). Several long-term (ranging from 64 weeks to lifespan) *in vivo* studies showed no carcinogenicity after quercetin exposure ranging from 0.2% to 10% (providing ~60–3500 mg/kg bw/day in rodents) (Hirono et al., 1981; Ito et al., 1989; Saito et al., 1980; Stoewsand et al., 1984). Other *in vivo* studies have shown, however, an indication for higher incidence of tumours after quercetin exposure. In rats fed a 0.1% (providing ~50 mg/kg bw/day)

Abbreviations: 2-AAF, 2-acetylaminofluorene; AFB1, aflatoxin B1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMD_{10}, benchmark dose.

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quercetin diet for 58 weeks, tumours in intestine and bladder were found (Pamukcu et al., 1980). A 2-year study with 0.1–4% (providing ~50–2000 mg/kg bw/day) quercetin within the National Toxicology Program reported carcinogenicity in kidneys of male rats induced by the highest dose of quercetin (National Toxicology, 1992). These indications of genotoxicity *in vitro* and the non-conclusive results on carcinogenicity *in vivo*, have raised concerns regarding the safety of quercetin. The genotoxicity of quercetin, even without metabolic activation, may specifically be of concern for organs of first contact, especially upon intake of highly dosed food supplements. Supplements with intakes as high as 1000–1500 mg/day represent dose levels far beyond average dietary intake (20 mg/day). At these high dose levels detoxification of quercetin via metabolism may be saturated resulting in exposure of the intestine and possibly liver to un-metabolised quercetin. This implies that *in vivo* genotoxicity studies in these first pass tissues could provide further insight in the possible hazards of high dose quercetin supplements.

Nowadays, transcriptome analysis has been shown to be an informative tool to predict genotoxicity (Melis et al., 2014; Thomas et al., 2007). Therefore, the aim of the present study was to characterise the potential genotoxic properties of quercetin in the small intestine as well as in the liver of mice by transcriptome analysis, in order to provide new additional information to evaluate the safety of quercetin.

2. Materials and methods

2.1. Animals and treatment

The experiment was performed according to the Dutch Animal Experimentation Act (1996) and the experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC 2011079). In brief, 24 male C57BL/6JOlaHsd mice (Harlan Laboratories, Horst, The Netherlands) were individually housed and maintained under environmentally controlled conditions (temperature 21 °C, 12 h/12 h light-dark cycle, 55 ± 15% humidity), with *ad-libitum* access to food and water. At arrival, the mice were 9 weeks old and after adaptation for 3 weeks the mice (n = 12) received a standardised high-fat diet (40en% fat; Hoevenaars et al., 2012) without or with supplementation of 0.33% *(w/w)* quercetin (~325 mg/kg bw/day) (Sigma, Zwijndrecht, The Netherlands). Effects on body weight and lipid and energy metabolism have been reported before (Hoek-van den Hil et al., 2014). One quercetin fed mouse was excluded from all analyses, because a nasal abscess developed in week 6. After 12 weeks of intervention, all mice were fasted for 2–4 hours during the light phase and anesthetised by inhalation of 5% isoflurane using O_2 as a carrier. Blood was sampled via orbital extraction in collect serum tubes (Greiner Bio-one, Longwood, FL, USA), kept on ice for max 2 hours, and centrifuged for 10 min at 3000 *g* at 4 °C to obtain serum, aliquoted, and stored at –80 °C. After blood collection, mice were killed by cervical dislocation, and liver was dissected and weighted, and small intestinal scrapings were snap frozen in liquid nitrogen and stored at –80 °C.

2.2. ALT and AST measurements

Possible hepatotoxicity was analysed by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using enzymatic assay kits (Bioo Scientific Corporation, Austin, TX, USA; MaxDiscovery™ ALT and AST Enzymatic Assay, resp.), according to the manufacturer's instructions. Five microlitre of fresh serum was used per well and samples were measured in duplicate and averaged. Students' *t*-test was used to compare the two normal distributed groups (GraphPad Prism version 5.03, San Diego, CA, USA).

2.3. Microarrays

RNA from liver and small intestinal scrapings was isolated using RNeasy columns (Qiagen, Venlo, The Netherlands) and used for microarray analysis. For global transcriptome analysis liver and small intestinal samples of individual mice and 8x60K Agilent whole-mouse genome microarrays (G4852A, Agilent Technologies Inc., Santa Clara, CA, USA) were used according to the manufacturer's protocol with a few modifications as described previously (van Schothorst et al., 2007). cDNA was synthesised for each animal from 200 ng RNA. Normalisation and data analysis were performed as published (Pellis et al., 2003) using Feature Extraction version 10.7.3.1 (Agilent Technologies). Based on visual inspection three arrays of liver were excluded in which hybridisation was not homogenous, and no arrays of small intestine were excluded. Microarray data have been deposited in NCBI Gene Expression Omnibus (GEO) under accession number GSE51343 (liver) and GSE63227 (small intestine).

2.4. Microarray data analysis

First, Student's *t*-tests were used with false discovery rate (FDR) adjustment for multiple testing correction according to Benjamini–Hochberg (Hochberg and Benjamini, 1990). In total 34,373 and 31,718 probes were expressed in liver and small intestine, respectively. Of these, no significant differentially expressed genes were found between the quercetin and the control group when FDR $p < 0.05$ was used as cut-off. Follow-up analyses were performed based on a *t*-test without FDR adjustment ($p < 0.05$) to prevent that data would be discarded that could possibly indicate genotoxicity due to the use of too stringent criteria. Fold changes were expressed as ratio of intervention group versus control group.

The first analysis was general pathway analysis using MetaCore (Thomson Reuters, New York, NY, USA). Secondly, regulated genes ($p < 0.05$) present in 'DNA damage' specific marked pathways in Metacore were studied. For comparison with known genotoxic compounds, published microarray data (NCBI GEO, accession number GSE43977) of genotoxic compounds 2-acetylaminofluorene (2-AAF) and aflatoxin B1 (AFB1) in C57BL/6J mice were taken along. These specific gene sets were selected based on availability of microarray data of studies in mice with known genotoxic compounds given via the diet for at least 7 days and a sufficient number of arrays per group ($n > 6$). These mice received 2-AAF (300 ppm, ~40 mg/kg bw/day) and AFB1 (1 ppm, ~0.15 mg/kg bw/day) via feed for 7 days, afterwards microarrays were performed with RNA from liver tissue (Melis et al., 2014). The doses were identified as suitable sub toxic doses to identify gene expression patterns (Melis et al., 2014). In addition, these doses were at least 5 times higher than the benchmark dose (BMD_{10}), the dose that induces 10% extra tumour incidence above background levels (Paini et al., 2011).

This was followed by the third analysis, where regulation of a published classifier set of transcripts, reported to predict the genotoxic potential of compounds (Melis et al., 2014), was used to investigate the possible genotoxic properties of quercetin. Nineteen out of 20 transcripts of the classifier set were used since one transcript (LOC75771) was not present on the Agilent arrays. Here, we also made the comparison with 2-AAF and AFB1.

3. Results and discussion

3.1. Hepatotoxicity

General hepatotoxicity markers being ALT and AST levels in serum revealed a significantly lower level of ALT in serum of quercetin fed mice and no significant differences for AST between quercetin and control mice (Fig. 1). Higher levels of ALT and AST are indications for hepatotoxicity, but all observed values in quercetin mice were within the normal range for ALT and AST levels (Boehm et al., 2007). Overall, this indicates that the tested dose of quercetin did not cause hepatotoxicity.

3.2. General microarray analysis

Significantly expressed genes ($p < 0.05$) resulted in 1648 regulated probes in liver and 1230 in small intestine. Previous pathway analysis in Metacore with these regulated transcripts in liver revealed no pathways regulated by quercetin (FDR < 0.01) (Hoek-van den Hil et al., 2014). This previous study showed beneficial health effects of quercetin on a high fat diet. In the present study, pathway analysis of the small intestine revealed several pathways regulated

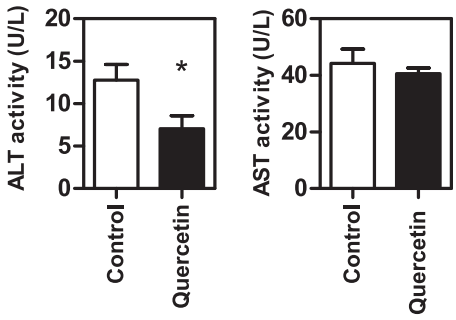

Fig. 1. ALT and AST activity in serum of mice exposed to ~350 mg/kg bw/day quercetin for 12 weeks as compared with controls. Data are presented as mean ± SEM. Alanine aminotransferase; ALT, aspartate aminotransferase; AST.