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Red clover isoflavone metabolite bioavailability is decreased after fructooligosaccharide supplementation

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ABSTRACT

Background: Red clover is an important source of isoflavones; which has been made commercially available as dietary supplements for the treatment of menopausal symptoms. Bioavailability and metabolism of these red clover isoflavones (RCI) have not been studied in detail. Fructooligosaccharides (FOS) stimulate the growth of intestinal bacteria and play an important role in the formation of certain isoflavone metabolites, such as equol and *O*-desmethylangolensin.
Objective: To determine the bioavailability of RCI metabolites and analyse whether FOS supplementation could influence their bioavailability.
Methods: Seventeen healthy adults were enrolled in the study carried out in two periods. In the first, compound bioavailability was determined after consumption of 80 mg of RCI (MF11RCE). In the second, a 6-day supplementation of 2 × 3000 mg/day of FOS was administered before isoflavone consumption.
Results: Biochanin A and formononetin were rapidly absorbed and both reached maximum concentrations at an average of 5–7 h. Demethylation was a major reaction in the metabolic pathway. Daidzein serum level peaked after about 12.6 h. Supplementation with FOS led to a significant decrease in the bioavailability of daidzein, dihydroformononetin, dihydrogenistein and dihydrodaidzein. An increase in equol production was also observed which did not reach statistical significance ($p > 0.05$).
Conclusion: This study is the first to provide detailed data on RCI bioavailability in humans and determine no influence of FOS yet a trend toward increased equol production. More research is warranted involving a greater sample size.

1. Introduction

Soy represents the richest source of isoflavone in the human diet. Red clover (*Trifolium pratense*), a member of the Fabaceae family, is also an important source of isoflavone, yet not frequently used as a food source.

Isoflavones are the best-known group of phytoestrogens that display various health promoting properties [1–7]. Epidemiological studies have associated high soy intake with a lower risk of presenting several conditions: osteoporosis, cardiovascular disease, menopausal symptoms and breast-, prostate- and colorectal cancer [8–11]. Isoflavone estrogenic actions were first discovered in the 1940s when sheep that grazed in clover pastures showed reproductive disorders [12,13]. Isoflavones are able to mimic oestrogen actions due to their structural similarity to 17β-estradiol and the ability to bind to oestrogen receptors (ERs). The observation of both estrogenic and anti-estrogenic isoflavone actions [14,15] led to their classification as natural selective oestrogen receptor modulators (SERMs) [16].

Over the past decade, isoflavones have been studied widely in humans due to their bioactivity and possible health effects, with focus on their metabolism and bioavailability. Most dietary isoflavones are extensively biotransformed in the intestine and the extent of intestinal metabolism may influence their potential physiological effects. Following ingestion and hydrolysis, aglycones are absorbed and transported to the liver, which plays an important role in their metabolism. Some isoflavones are further metabolised in the large intestine by the gut microflora before absorption. Equol and *O*-desmethylangolensin (O-DMA) are the most studied bacterial metabolites [17,18]. Kidney is the main route of isoflavone excretion; however, minimal excretion can also be observed through the bile and faeces [19,20].

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Several studies have addressed soy isoflavone bioavailability [21–23]. In contrast, only a few studies have focused on bioavailability of isoflavones from other sources (i.e. red clover). Red clover and soy both belong to the Fabaceae family, but they show quite different isoflavone profiles, which may impact bioavailability. While the primary isoflavones in soy and soy products are genistein and daidzein, predominant isoflavones in red clover are biochanin A and formononetin, respectively the methylated derivatives of genistein and daidzein [24]. Additionally, differences in glycosylation patterns may be observed according to the compound: glycoside conjugates (for soy isoflavones) [25] or aglycones (for red clover isoflavones: RCI) [24]. These distinct isoflavone profiles may translate in different health implications. Thus, it is not clear whether results observed from soy studies are applicable to red clover.

Isoflavone bioavailability is characterised by large inter-individual variations. These variations have been partly explained in studies showing that subjects were either "equol-producers" or "non-equol-producers" and that only about 30–50% of subjects produced equol [19,26–34]. Equol (Fig. 1A) does not occur in isoflavone containing plants yet produced from methylated isoflavones, mainly daidzein (Fig. 1B), by bacteria of the intestinal flora. Equol can also be found in the blood of humans with high soy consumption, has a higher affinity for both ERs alpha and beta than daidzein and its production can be disturbed by antibiotic treatment; although the exact involved mechanisms for the latter are not known [31].

Isoflavone bioavailability may also depend on both physiological and pathological factors influencing metabolism. Physiological factors include age, gender, genetic predisposition, enterohepatic circulation, nutrition and intestinal conditions. Pathological factors include liver, kidney, and heart diseases.

As mentioned, intestinal microflora has been suggested to play a key role in the large inter-individual variations of isoflavone bioavailability and metabolism [35,36] and thus explain the wide range of clinical results. It has been established that fructooligosaccharides (FOS) have prebiotic and growth stimulating properties that affect the beneficial intestinal bacteria [37] and therefore exert effects over isoflavone bioavailability. However, data regarding FOS influence over isoflavone bioavailability and metabolism is still controversial [38–40] and scarce.

Hence, new data is required to provide input on how to increase RCI bioavailability (i.e. in women experiencing menopausal disorders). Taking this into consideration, the present study was designed to investigate RCI metabolite bioavailability (mainly biochanin A and formononetin) after supplementation with 80 mg RCI and determine whether a 6-day FOS supplementation could modulate metabolite bioavailability and metabolism aspects.

2. Material and methods

2.1. Chemicals

Isoflavones (biochanin A, formononetin, daidzein, and genistein) and β-glucuronidase/sulfatase were purchased from Sigma Aldrich (St. Louis, MO, USA). Isoflavone metabolites (dihydrobiochanin A, dihydroformononetin, dihydrogenistein, dihydrodaidzein, angolensin, *O*-desmethylangolensin, 6-hydroxydaidzein, 6-hydroxydesmethylangolensin, 3′-hydroxygenistein, and equol) were synthesized by Plantech (Reading, UK). Buffer reagents (dimethylsulfoxide [DMSO]), acetonitrile, and formic acid, and water appropriate for liquid chromatography and mass spectrometry (LC-MS grade) were obtained from Sigma Aldrich (St. Louis, MO, USA). All reagents and chemicals were commercially available products of extra-pure grade.

2.2. Red clover preparation

RCI capsules contained exclusively aglycone isoflavones. Each capsule had approximately 80 mg isoflavones (0.28 mg daidzein, 1.72 mg genistein, 42.0 mg formononetin and 36.0 mg biochanin A) (MF11RCE, Lenus Pharma, Vienna) [41].

2.3. Fructooligosaccharide supplement

FOS supplement was administered orally in this study (Pfannenschmidt, Germany). It consisted of a soluble dietary fibre extracted from chicory roots. The dried substance was composed of: 99.7% total carbohydrates, 95–99% dietary fibre, 5% glucose and sucrose, and a maximum of 0.3% ash (composition of FOS supplement was provided by the supplier).

2.4. Subjects and study design

Seventeen healthy women and men (20 to 30 years) were enrolled in the study. Subjects were asked to abstain from eating isoflavone-rich foods for at least 2 weeks before the initiation of the study and throughout its duration. Exclusion criteria included: history of gastrointestinal disorders, hormone-dependent cancers, diabetes mellitus, isoflavone supplement intake within the past 3 months, hormonal contraceptive use within the past 12 months, current pregnancy or lactation, hormone replacement therapy (HRT) use, vegetarian dietary pattern, or pre-existing chronic renal, thromboembolic, thyroid, liver, pulmonary, or cardiovascular disease. Body weight, dietary habits, and physical activity remained constant throughout the study.

The present research was divided into two study periods. During the first, each subject consumed a single dose of 80 mg of RCI after an overnight fast. A blood and urine sample (as detailed in the next section) was collected before and after RCI intake. In the second study period, a 6-day-supplementation of 2 × 3000 mg of FOS daily preceded the consumption of a single dose of 80 mg of RCI after overnight fast. Blood and urine samples were collected in a similar fashion as in the first period.

Research protocol of the study was approved by the Ethics Committee of the Medical University of Vienna, Austria; and informed consent was obtained from each subject.

2.5. Sample collection

2.5.1. Blood samples

A baseline blood sample after an overnight fast was collected before ingestion of the red clover preparation. Further blood samples were

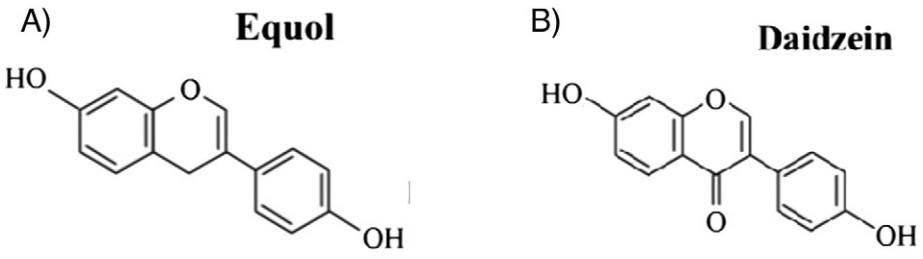

Fig. 1. Formula of equol (A) a metabolite produced mainly from daidzein (B) by intestinal bacteria.