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Availability and dose response of phytophenols from a wheat bran-rich cereal product in healthy human volunteers

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Absorption and excretion of phytophenols from a wheat bran-rich cereal product in healthy human volunteers

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Abstract

Scope: Phytophenols present in cereals are metabolised to compounds that could be partly responsible for the reduced risk of chronic diseases and all-cause mortality associated with fibre-rich diets. The bioavailability, form and *in vivo* concentrations of these metabolites requires to be established.

Materials/Methods: Eight healthy volunteers consumed a test meal containing a recommended dose (40 g) and high dose (120 g) of ready-to-eat wheat bran cereal and the systemic and colonic metabolites determined quantitatively by LC-MS.

Conclusion: Analysis of the systemic metabolomes demonstrated that a wide range of phytophenols were absorbed/excreted (43 metabolites) within five hours of consumption. These included 16 of the 21 major parent compounds identified in the intervention product and several of these were also found to be significantly increased in the colon. Not all of the metabolites were increased with the higher dose, suggesting some limitation in absorption due to intrinsic factors and/or the food matrix. Many compounds identified (e.g. ferulic acid and major metabolites) exhibit anti-inflammatory activity and impact on redox pathways. The combination of postprandial absorption and delivery to the colon, as well as hepatic recycling of the metabolites at these concentrations is likely to be beneficial to both systemic and gut health.

1. Introduction

Diets containing whole-grain (WG) cereals can be protective against several cancers and metabolic disorders such as type 2 diabetes (T2DM), cardiovascular disease (CVD) and coronary artery disease (CAD). This is clearly demonstrated through both epidemiological evidence and intervention studies [1-8]. There are many factors, which may contribute to disease development, but subclinical inflammation is recognised as an important aspect [6]. WG cereals have been shown to impact on inflammatory markers, but there is a gap in the knowledge as to the bioactive dietary factors responsible [7,8]. The biological effects are mainly attributed to the fibre and the non-nutrient phytochemical content of WG cereals. Phytochemicals, and in particular phytophenols are of particular interest, as these are located in the outer layer of the grain and in the germ fraction; the bran fraction playing the more important role due to the germ being released during cereal processing [9,10]. The biological activity of the phytophenols depends on bioaccessibility from the food matrix, as well as bioavailability, metabolism and catabolism *in vivo* [11]. It is important that the biological effects should not just be attributed to the parent compounds present in foods, but also to the various metabolites produced in the body [12]. The small intestine plays a major role in absorption and metabolism of dietary phytophenols. However, many of these compounds, especially those attached to other cellular components such as fibre are not absorbed early in the gastrointestinal tract and are delivered to the colon relatively intact, where they are extensively metabolised by the gut microbiota prior to being absorbed and/or excreted [13]. Additionally, the microbial species can be influenced by dietary intake with consequences for impacting on human health [14]. When absorbed from the colon, microbial metabolites can be transported via the hepatic portal system, where they can generate numerous circulating metabolites [15]. In order to comment on potential health benefits of dietary constituents, it is important to determine the bioavailabilty and structure of these potentially bioactive metabolites [16]. The aim of this study is to identify and quantify the main phytophenol metabolites delivered to both the colon and to the systemic circulation after consumption of ready-to-eat wheat bran cereal and additionally to determine whether their absorption is concentration dependant.

2. Materials and Methods

2.1 Study Design

Volunteers recruited undertook a general health screen to check they meet the study criteria and complete food frequency and physical activity questionnaires. The volunteers recruited were asked to follow their normal diet until three days before the intervention at which time they consumed a low phytochemical diet and completed food diaries. Volunteers were fasted for twelve hours prior to commencing the intervention and were not permitted to drink or eat anything within the first five hours following consumption. For the remaining intervention period (24 hours), volunteers could consume the low phytochemical diet. Each volunteer consumed wheat bran-rich cereal at a recommended serving (40 g) along with semi-skimmed milk (125 mL) and the high-fibre serving (120 g) with semi-skimmed milk (375 mL). These serving provided approximately 10.8 g and 32.4 g of wheat bran fibre respectively (data provided by the manufacturer). There was a washout period of at least two week between interventions. Baseline faecal, blood and urine sample were collected prior to beginning the intervention and blood and urine samples were collected at 1, 3 and 5 hours during the intervention period (Figure 1A) Additionally, urine was collected throughout the twenty four hour study period and a fasted blood sample at 24 hours (data not shown). The first faecal sample after completion of the study was also collected. Containers, cool bags and ice packs, along with clear guidance as to storing the urine and faecal samples were provided. The studies followed all the principles of Declaration of Helsinki and good clinical practice, and all procedures involving human subjects were reviewed and approved by the Human Studies Management Committee of the Rowett Institute of Nutrition and Health, University of Aberdeen, UK and the Rowett Institute Ethics Panel. The study was carried out from February 2012 until June 2012.

2.2 Volunteer Recruitment

Eight healthy volunteers; seven females and one male; Age 38.62 ± 10.8 (23-54) years; BMI 23.39 ± 2.63 (19.94-26.85) kg m^{-2} were recruited for the study. Local ethical committee approval was received and written informed consent was obtained from each subject. Selection took place following a general medical screening made by a qualified medical doctor and confirmation of adherence to inclusion/exclusion criteria. ***Inclusion Criteria***: healthy, non-smokers, between 18-55 years old, BMI between 18 and 30 kg m^{-2}. ***Exclusion Criteria***: Taking any prescribed medicines, analgesics, antipyretic, anti-inflammatory compounds or nutritional supplements. Volunteers who had taken antibiotics or who had

given a large blood donation in last three months prior to taking part in the study were also excluded. Volunteers were asked to complete the validated Scottish Collaborative Group Food Frequency questionnaire to assess habitual fibre intake. Volunteers were provided with information on which foods to avoid for the three days prior to and during the intervention were asked to complete a four-day food diary during this period to check for compliance. All eight volunteers took part in the acute intervention to assess bioavailability (Figure 1).

2.3 Intervention Product

The intervention product used for the study was commercially available All Bran original cereal (Kellogg's). The intervention product was analysed as described previously [17]. In brief; samples (approx. 0.1 g dry weight; n=3) were suspended in HCl (0.2 mol dm^{-3}; 3 cm^3) and the free phenolic acids extracted into ethyl acetate (EtOAc; 5 cm^3). The layers were separated by centrifugation (5 min; 1800 x g; 4 ºC), the extraction repeated three times and the EtOAc extracts combined. To liberate both the acid- and alkali-labile esterified phenolic acids, the aqueous fraction was sequentially hydrolysed by both alkali (NaOH; 1 mol dm^{-3}; room temperature) and acid (HCl; 6 mol dm^{-3}; 95 °C), extracting into EtOAc as described above. All extracts were left to stand over sodium sulphate (anhydrous) for one hour, filtered and the combined organic layers evaporated to dryness under reduced pressure at temperature not exceeding 40 °C. The extracts were then re-dissolved in methanol (0.5 cm^3) and prepared for LC-MS analysis as described below.

2.4 General Reagents

The methanol used was LC-MS grade from Fluka, hydrochloric acid was analytical grade from Fisher, sulphatase (S9626) and glucuronidase (G7017) from Sigma; 13C- benzoic acid (ISOTEC TJ2365, 277746-250 mg), 4,7,8- tri-MelQx (TRC A630000 12AZC-165-1).

2.5 Human Sample Processing

Blood was collected directly into heparinised tubes at the 0, 1, 3, 5 and 24 hour time points. The samples were centrifuged (4 °C, 1500 x g, 15 min) within 45 minutes to separate the plasma. Urine samples were measured by weight and volume at time same points up to five

hours and then the total 24-hour sample collected. The harvested plasma and the urine was aliquoted and stored at –80 °C. The faecal samples were weighed, mixed and immediately frozen at –80 °C. Faecal samples were thawed, mixed once again, weighed and the faecal waters (FW) separated using a high speed centrifuge (50,000 x g; 2 hours; 10 ^{o}C). The FW (supernatant) was carefully removed, aliquotted into eppendorfs and immediately stored at -80^{o}C for LC-MS analyses. Surplus of all the human samples was retained for use in future related research under signed consent by volunteers before participating in the study.

2.6 Human Samples Analysis

2.6.1 Conjugated systemic metabolites

Urine and plasma samples were thawed to 4 ºC and 150 µl was transferred to an eppendorf and sulphatase (H-1 *Helix pomatia*; 0.5 units in citrate phosphate buffer 0.3 mol dm^{-3}; pH6; 120 µl) and glucuronidase (HP-2 *Helix pomatia* 100390 units/ml; 30 µl) were added. Samples were incubated for 18 hours at 37 ºC. IS1 (60 µl), IS2 (60 µl) and acidified (0.4 mol dm^{-3} HCl) methanol (180 µl) were added. Vortexed and centrifuged (12,500 x g; 5 mins; 4 ºC).

2.6.2 Faecal metabolites

Faecal water samples were thawed to 4 ºC and 80 µl transferred to an eppendorf. Internal IS1 (80 µl), IS2 (80 µl) and methanol (160 µl) were added Vortexed and centrifuged (12,500 x g; 5 mins; 4 ºC).

2.7 LC-MS Analysis

Liquid chromatography separation of the metabolites produced was performed on an Agilent 1100 HPLC system (Agilent Technologies, Wokingham, UK) using a Zorbax Eclipse 5µm, 150 mm x 4mm column (Agilent Technologies). Three different gradients (1-3) were used to separate the different categories of metabolites and the mobile phase solvents in each case were water containing 0.1% acetic acid and acetonitrile containing 0.1% acetic acid. In all cases the flow rate was 300 µL/min with an injection volume of 5 µL. The LC eluent was directed into, without splitting, an ABI 3200 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) fitted with a Turbo Ion Spray™ (TIS) source. For LC methods 1 and 2, the mass spectrometer was run in negative ion mode with the following source

settings: ion spray voltage -4500 V, source temperature 400 ºC, Gases 1 and 2 set at 15 and 40 respectively and the Curtain Gas set to 10. For LC method 3, the mass spectrometer was run in positive ion mode with the following source settings; ion spray voltage 5500, source temperature 400 °C , Gases 1 and 2 set at 14 and 40 respectively and the Curtain Gas set at 10. All the metabolites were quantified using multiple reaction monitoring. Standard solutions (10 ng/µL) for all analytes were prepared and pumped directly via a syringe pump. The ion transitions for each of the analytes were determined based upon their molecular ion and a strong fragment ion. For several categories of compounds, it was inevitable that their molecular ion and fragment ion would be the same, but this was overcome by their differing elution times. Their voltage parameters; declustering potential, collision energy and cell entrance/exit potentials were optimised individually for each analyte.

2.8 Statistical analysis

All data is expressed as mean ± standard deviation. Metabolic data sets were analysed by multivariate analysis PCA (SIMPCA-P+). The effect of treatment over time (plasma and urine metabolites) was assessed by two-sided post-hoc t-tests and the faecal metabolites by log-scale Anova with post-hoc least significant different tests for each of the interventions using the volunteers as their own controls. Genstat version 13 (VSN International) was used for these calculations and there were no indications of departures from equal variances.

3. Results

Principal component analysis (PCA) showed that the overall metabolite profiles in urine differed at 0, 1, 3, and 5 hours following consumption of high (Figure 2A) and low (Figure 2B) amounts of wheat bran. The urinary metabolome within the first five hours of consumption of the intervention product gives the best indication of the metabolites that have been absorbed early in the gastrointestinal tract. As concentrations are generally higher in the urine compared to plasma, changes in metabolites present at lower amounts can be determined. The data showed some inter-individual variation, but despite this, quantitative analysis of the individual metabolites present in urine showed that around 43 metabolites were significantly modulated by the intervention. Of the 21 major parent metabolites identified to be present in wheat bran (Table 1), 16 were significantly increased in urine

following consumption (Table 2). In general, most metabolites were excreted at higher concentrations during the acute intervention with a high dose of wheat bran. For some metabolites the increase was less than three-fold suggesting that the amount of metabolites absorbed early in the gastrointestinal tract may be limited by intrinsic factors, such as gut transporters, enzymes and food matrix effects [18]. Absorption is also likely to be influenced by other components in the plant matrix or in the milk. However, the ratio of cereal to milk was kept constant to standardise these effects. As observed previously the recovery of the hydroxylated benzoic acids in the urine was substantially higher than the recovery of hydroxycinnamic acids, suggesting that these compounds are more easily or competitively absorbed early in the gastrointestinal tract [19].

The overall profiles of the metabolites in plasma for both the high and low wheat bran interventions (Figure 3A and 3B) were not as clearly discriminated when compared to the PCA of the urinary metabolites (Figure 2A and 2B). This is likely to reflect the lower concentration of metabolites present in plasma, as well as high variability due to dynamic exchange. However, the major metabolites found in wheatbran; ferulic acid and the ferulic 5-5 dimer were significantly ($p < 0.05$) increased in the plasma, as were 2,6-dihydroxybenzoic acid, 3,5-dihydroxycinnamic acid, syringic acid and phenylacetic acid (Tables 5 and 6). It should be noted that certain metabolites, particularly; phenylacetic acid, 4-hydroxyphenylacetic acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, phenyllactic acid, 4-hydroxyphenyllactic acid, indole-3-acetic acid, indole-3pyruvic acid, indole-3-lactic acid and p-cresol were present at high amounts. These were consistant throughout and not significantly affected by consumption of the intervention product. These have been shown previously to be gut metabolites of the aromatic amino acids and could be a consequence of the low phytochemical diet on which the volunteers were placed prior to the intervention [20].

PCA shows discrimination between the faecal metabolites produced prior to consuming the wheat bran (control) and at 24 hours after consuming both the high (120 g) and low (40 g) doses of wheat bran (Figure 4). The colonic metabolites have been analysed with each volunteer as their own control. This is necessary for work concerning the gut microbiota, as there is generally large inter-individual variation in the species present [21]. Of the metabolites quantitatively analysed, 16 were significantly modulated by the intervention. The metabolites significantly increased by consumption of the high dose of wheat bran were

ferulic acid, the hydrogenated product of the 5-5 linked ferulic acid dimer, the 8-5 linked ferulic acid dimer and 2-hydroxyphenylpropionic acid. Enterolactone and 3,5-dihydroxybenzoic acid were significantly increased on the intervention with the low dose of wheat bran only. Again, high amounts of metabolites likely to be derived from metabolism of protein were present in the faecal samples collected throughout the intervention. Some of these (p-cresol, phenylacetic acid, 4-hydroxyphenylacetic acid, phenyllactic acid, 4-hydroxyphenyllactic acid, indole-3-acetic, indole-3pyruvic and indole-3-lactic acid) were found to be substantially lower in the colon 24 hours following consumption of the large dose of the wheat bran intervention product, although these decreases were not significant.

4. Discussion

Consumption of ready-to-eat wheat bran cereal resulted in significant absorption of phytophenols early in the gastrointestinal tract and urinary excretion within five hours of consuming the intervention product. Many of the phytophenols identified (vanillic acid, 4-hydroxy-3-methoxyacetophenone, ferulic acid and sinapic acid) were previously shown to have anti-inflammatory activity [22], to inhibit peroxidase and lipoxygenase enzymes (salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, 2,5- and 3,5-dihydroxybenzoic acids) [23,24], and to be redox active [25]. The metabolites that were found to be significantly increased in the colon (as measured by their presence in faecal samples) 24 hours following the intervention were ferulic acid, the hydrogenated product of the 5-5 linked dimer and the 8-5 linked ferulic dimer. Wheat bran is a particularly rich in the 5-5-linked dimer of ferulic acid and the hydrogenated product of this dimer is the major form produced by the gut bacteria at physiological concentrations [26]. Monomeric and dimeric ferulic acid and the major gut metabolites of these compounds are potent anti-inflammatory compounds, at low concentrations, which easily achievable *in vivo* [22,26]. Bioavailability of certain classes of phytophenols have been extensively reviewed [27]. Several studies have reported the availablity of free ferulic acid early in the GI tract, but there are fewer studies reporting the availability of ferulic acid bound to other cell wall components such as arabinoxylans [28-30]. Increased bioavailabilty of ferulic acid and its metabolites was observed following consumption of processed whole wheat bread, this was associated with significantly lower anti-inflammatory cytokines in LPS-stimulated blood following consumption [31]. There have been very few human studies reporting the bioavailability of the ferulic dimers, which

are major components of wheat bran in either the gut or systemic circulation. Coffee-derived ferulic acid dimers were shown to be bioavailable in both plasma and urine [32]. Interestingly a trend toward lower plasma plasminogen activator inhibitor 1 with higher excretion of ferulic acid and dehydrodiferulic acid was also reported [33]. Of the many factors considered to contribute to the development of diseases such as CVD, CAD and T2DM, there is substantial evidence for the chronic low-grade stimulation of inflammatory mediators [34]. Diets rich in fibre, such as wheat bran have potential to deliver anti-inflammatory compounds directly to the gut and through hepatic circulation in a sustained manner to the vascular system, as well as delivering an acute postprandial dose. Indeed, whole-grain wheat consumption reduced inflammation in a randomised controlled trial on overweight and obese individuals, with concomitant increases in ferulic acid and metabolites [35]. Inflammatory status and lipid profiles were also improved by ferulic acid supplementation in hypercholesterolemic subjects [36]. In summary, this data demonstrates that several phytochemicals and their metabolites present in wheat bran were found to be significantly increased in urine, plasma and faecal water samples obtained from volunteers after consuming wheat bran based ready-to-eat cereal. Many of the metabolites, and in particular, ferulic acid and its derivatives are known to exhibit anti-inflammatory activity, impact on redox pathways [22-25], with potential to deliver additional neurological benefits [38,39]. This may, in part, explain the beneficial effects of wholegrain consumption on systemic, gut and general health.

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Author Contribution

Initial concept (JM, RF, TH, BdR, GD, WRR); Study Design (MN, GD, WRR); Study Co-ordination and Analysis (MN, SHD, LS, WRR); Mass Spectrometry (GJD, LC), Statistical Analysis (GH), Data Interpretation (MN, WRR), Manuscript Preparation (MN, WRR), Manuscript Approval (All)

Figure Legends

Figure 1: Human intervention study design.

Figure Principal component analysis plot (unweighted) for all metabolites measured in urine obtained from volunteers (n=8) at 0,1,3, and 5 hours after consuming a high (A; 120 g) and low (B; 40 g) dose of wheat bran.

Figure 3: Principal component analysis plot (unweighted) for all metabolites measured in plasma obtained from volunteers (n=8) at 0,1,3, and 5 hours after consuming a high (A; 120 g) and low (B; 40 g) dose of wheat bran.

Figure 4: Principal component analysis plot (unweighted) for all metabolites measured in faecal waters obtained from volunteers (n=8) before (control) and 24 hours after consuming high (Intervention A; 120 g) and low (Intervention B; 40 g) doses of wheat bran.

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