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Transport of folic acid across Caco-2 cells is more effective than 5-methyltetrahydrofolate following the *in vitro* digestion of fortified bread

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ABSTRACT

Folic acid is the fortificant of choice mainly because of its stability. The mandatory fortification program was implemented based on the evidence that increased intake of folic acid in the periconceptional period reduced the prevalence of neural tube defects affected pregnancies. This study aimed to determine the transport of folic acid from fortified bread across Caco-2 cells, and to compare this with the transport of 5-methyltetrahydrofolate (5-MTHF). Optimised *in vitro* digestion involved the addition of ascorbic acid at physiological concentration to preserve folate. Quantitative analyses were performed using the previously developed ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method with a slight modification to the UPLC gradient. Our study found that in the *in vitro* digested bread, the measured 5-MTHF concentrations were significantly higher in the presence of ascorbic acid than in its absence. The transport of folic acid across Caco-2 cells from fortified bread was approximately 14% and not significantly different from the transport of folic acid solution. The experimental data also revealed that transport of 5-MTHF from fortified bread was approximately 1.7%, whilst in control experiments 5% of 5-MTHF standard solution was transported across the cells. The results of the current study suggest that transport of folic acid across Caco-2 cells was greater than transport of 5-MTHF.

1. Introduction

Folate or vitamin B_9 is an essential water soluble vitamin, which is involved in one-carbon unit transfer reactions during DNA synthesis, DNA methylation and amino acid metabolism. Folate exists in many diverse forms and the term folate generally refers to all the naturally occurring and synthetic forms of folate. Pteroylglutamic acid or folic acid (FA) is commonly used to fortify food and dietary supplements because of its stability and low production cost (Wright, Dainty, & Finglas, 2007). Naturally occurring folate in food is predominantly present as 5-methyltetrahydrofolate (5-MTHF). Addition of 5-MTHF to food has been suggested to have advantages; the potential risk of 5-MTHF for masking the haematological symptoms of vitamin B_{12} deficiency is particularly lower than FA (Pietrzik, Bailey, & Shane, 2010). Also, the cellular uptake of circulating 5-MTHF is subject to tight cellular control (Pietrzik et al., 2010). Evidence to date shows that intake of FA in periconceptional period is inversely related to neural tube defects (NTD) affected pregnancies (Czeizel & Dudas, 1992; Wald, Sneddon, Densem, et al., 1991). On this basis some types of staple food are mandatorily fortified with FA to increase the dietary intake of FA in women capable of becoming pregnant.

It has been suggested that FA from fortified food is 85% bioavailable (Pfeiffer, Rogers, Bailey, et al., 1997) compared to naturally occurring folate which is 50% bioavailable (Sauberlich, Kretsch, Skala, et al., 1987). However, recent studies do not support the findings of previous research. It has been argued that the previous methods had some limitations and suggested that naturally occurring folate is 80% bioavailable (Winkels, Brouwer, Siebelink, et al., 2007). An accurate determination of the relative bioavailability of vitamins requires carefully controlled human studies, but *in vivo* human studies are labour intensive, time-consuming, expensive and complex (Rodriguez-Amaya, 2010). Using an *in vitro* approach, this study aimed to predict the concentrations of FA and 5-MTHF from fortified bread following intestinal absorption. As an absorption/transport model, a static *in vitro* digestion (Fernandez-Garcia, Carvajal-Lerida, & Perez-Galvez, 2009) in combination with Caco-2 cells was used to simulate the absorption in enterocytes (epithelial cells of the small intestine).

The Caco-2 cell line was isolated from a well differentiated human tumour, a primary adenocarcinoma of the colon. In culture, Caco-2

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cells proliferate indefinitely and after 21 days they differentiate into enterocytes (Said, 2011; Simon-Assmann, Turck, Sidhoum-Jenny, et al., 2007). The Caco-2 cell absorption model has been commonly used in drug development, particularly to identify lead compounds with desired pharmacological and biopharmaceutical/pharmacokinetic properties (D'Souza, Shertzer, Menon, et al., 2003). The cells form tight junctions and express many brush border enzymes (Delie & Rubas, 1997; Tavelin, Gråsjö, Taipalensuu, et al., 2002), which are important for drug metabolism studies (Tavelin et al., 2002). The use of Caco-2 cells coupled with *in vitro* digestion has been validated as a model that is predictive of the *in vivo* permeability and absorption (Delie & Rubas, 1997; Fernandez-Garcia et al., 2009; Simon-Assmann et al., 2007) and used for predicting the bioavailability of nutrients (Netzel, Netzel, Zabaras, et al., 2011; O'Callaghan & O'Brien, 2010; Öhrvik, Öhrvik, Tallkvist, et al., 2010; Rodriguez-Amaya, 2010; Sy, Gleize, Dangles, et al., 2012; Verwei, Arkbåge, et al., 2005). The objective of this study was to determine the bioaccessibility of FA and 5-MTHF following the static *in vitro* digestion of fortified bread, as well as transport of FA and 5-MTHF across Caco-2 cells used as an absorption model.

2. Materials and methods

All cell culture reagents were sourced from Invitrogen (Sydney, Australia) α-amylase, hog bile extract and porcine pancreatin were purchased from MP Biomedicals (Solon, OH) and porcine pepsin from Sigma Aldrich (Sydney, Australia). Pepsin (15.2 mg/mL) was prepared in 0.1 M hydrochloric acid (Univar, Ajax-Finechem, Sydney, Australia). Transwell 24-well plates including permeable supports (12 mm insert diameter) with polycarbonate membranes were purchased from Corning (Sydney, Australia). A Millicell voltohmmeter, used to measure trans-epithelial electrical resistance (TEER) was obtained from Millipore (Billerica, MA). Caco-2 cells (sourced from American Type Culture Collection) were a gift from the Lowy Cancer Research Centre (University of New South Wales, Sydney, Australia). Standard FA and [6S] 5-MTHF in powder form were supplied by Schircks Laboratories (Jona, Switzerland) and stored at −20 °C. Stable isotope labelled ($^{13}C_5$) FA and ($^{13}C_5$) 5-MTHF, used as internal standards (IS), were purchased from Merck-Eprova (Schaffhausen, Switzerland) and stored at −85 °C.

2.1. Bread samples

Commercial unfortified flour, white and wholemeal flour were obtained from supermarkets in the Sydney metropolitan area in June 2012. Bread was made from the following ingredients: bakers' flour (1000 g), salt (15 g), bread improver (15 g), oil (20 g), instant dry yeast (13 g) and water (600 g). To each flour type, either FA or 5-MTHF was added. Initially, the fortificant (250 μg/100 g flour) was dissolved in tap water. Dough was proved for 60 min before baking at 200 °C for 30 min. Bread loaves were cooled down, ground and stored in the freezer for folate extraction that was processed following our previous paper (Chandra-Hioe, Bucknall, & Arcot, 2011).

2.2. In vitro digestion

The *in vitro* digestion was carried out according to Netzel et al. (2011) with some modifications. To the ground bread samples (5 g, in duplicate) lukewarm tap water (2.5 mL) and 5 mL α-amylase (20 mg/mL, pH 6.56) were added into the centrifuge tubes, vortexed and placed in a 37 °C shaking water bath for 5 min. The pH of the samples was then adjusted to 2.1–2.2 with 0.1 M hydrochloric acid. Optimised *in vitro* gastric digestion involved the addition of ascorbic acid to the samples to create a similar concentration of ascorbic acid (50 μM) present physiologically in the gastric juice (Ng, Lucock, & Veysey, 2008). Following gastric and intestinal digestion, the samples were centrifuged at 10,000 *g* (4 °C) for 20 min to obtain clear supernatants, referred to as the digesta. Samples without bread (nil digesta) were also prepared alongside the bread digesta as an experimental control, used to correct the amount of endogenous folate present in the digestive enzymes. Digesta samples were assessed for cell toxicity using CellTiter 96® AQueous non-radioactive cell proliferation assay (Promega, Sydney, Australia).

2.3. Transport study

Caco-2 cell suspensions (200 μL) containing 8×10^4 cells were seeded on the Transwell polycarbonate membrane (0.33 cm^2 growth area) and incubated in a humidified 5% CO_2 atmosphere, at 37 °C for 21 days. The TEER was measured on day 21 using a Millicell voltohmmeter, to assess the integrity of the cell monolayers (Verwei, Arkbåge, et al., 2005; Verwei, Van Den Berg, et al., 2005). In order to enhance the transport of added FA and 5-MTHF, a starvation protocol was introduced, where the cells were incubated in HBSS for 4 h at 37 °C and 5% CO_2 humidified atmosphere. The cells were then rinsed twice with pre-warmed HBSS before the transport study was started.

Each sample of the bread digesta (200 μL) was then applied into the apical chambers (n = 3) and incubated for 1 h at 37 °C in a humidified 5% CO_2 atmosphere. The TEER was re-measured, "transport" samples from each apical and each basolateral chambers were collected separately into Eppendorf tubes containing ascorbic acid and stored at −20 °C until extraction and analysis (within 1–2 days). After the transport study, growth medium was added to the apical and basolateral chambers, the cells were maintained in the humidified incubator (37 °C, 5% CO_2) and TEER was measured at 24 and 48 h. The transport studies were carried out using Caco-2 cells between passages 25 and 40.

The Statistical Package for the Social Sciences (SPSS) version 20 (SPSS Inc., Chicago, IL) was used to analyse the experimental data. Mean values were compared using one-way ANOVA and P value of 0.05 was chosen to report the level of significance.

2.4. Microscope imaging

To image the integrity of the cell monolayer using microscopy, cells on the Transwell membrane were fixed immediately after the sample collection. Initially, cells were washed, fixed and repeatedly rinsed prior to staining (Tavelin et al., 2002). The cells were incubated with Alexa Fluor® 488 phalloidin (1:400) conjugate (Life Technologies, Mulgrave, Australia) for 1 h (D'Souza et al., 2003). Subsequently, the nuclei were stained using Hoechst 33342 (Life Technologies, Mulgrave, Australia) for 15 min. The Transwell membrane was removed from the insert, placed on a microscope slide, mounted using ProLong Gold mounting media (Life Technologies, Mulgrave, Australia) and covered using glass coverslip n 1.5. Confocal microscope Leica SP5 MultiPhoton (Wetzlar, Germany) was employed for imaging the monolayers, where a 20 × oil immersion objective (NA 0.7) was selected. To image Alexa Fluor® 488 phalloidin conjugate 488 nm argon-ion laser was used and bandwidth of 495–600 nm was collected. To image Hoechst 33342 Spectra-Physics Mai Tai Ti Sapphire laser was used. Nuclei were imaged at a 740 nm wavelength while bandwidth of 405–450 nm was collected.

2.5. Sample preparation for quantitative analysis

For the purpose of quantitative analysis, stable isotopes IS (34 ng/mL $^{13}C_5$ FA and 34 ng/mL $^{13}C_5$ 5-MTHF) were spiked into the digesta, apical and basolateral samples. All samples were then equilibrated using a rotary shaker at room temperature for 10 min before solid phase extraction (SPE) (Chandra-Hioe et al., 2011).