Original Article

In vitro screening of relative bioaccessibility of carotenoids from foods

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Carotenoids are lipophilic pigments in plant foods that are of particular interest as precursors of vitamin A, a nutrient required for vision, cell differentiation, and the immune system. In order to mediate such activities, carotenoids and their metabolites must be absorbed for delivery to tissues. Unlike many other dietary lipids, the efficiency of carotenoid absorption is typically inefficient, being affected by food matrix, style of processing, other dietary components, and nutritional and physiological status. Thus, reliable prediction of carotenoid bioavailability is problematic. We have developed a relatively simple and cost effective procedure to study the potential bioavailability, *i.e.*, the bioaccessibility, of carotenoids. The method involves simulated oral, gastric and small intestinal digestion of test samples to access the efficiency of incorporation into micelles, an obligatory step for absorption of lipophilic compounds. The model can be further expanded by adding micelles generated during small intestinal phase of digestion to monolayers of Caco-2 human intestinal epithelial cells to investigate apical uptake, cellular metabolism and transepithelial transport of carotenoids. Recent work by Borel and associates has demonstrated that the relative bioaccessibility of carotenoids observed *in vitro* is highly correlated with *in vivo* observations and results from bioavailability trials with human subjects. Results from recent studies using the *in vitro* model to screen relative bioaccessibility of *β*-carotene in various cultivars of cassava, impact of amount and types of fatty acyl groups in triglycerides on micellarization of carotenoids, and the mechanism of digestion and intestinal cell uptake of xanthophyll esters are presented.

Key Words: carotenoids, bioavailability, bioaccessibility, in vitro digestion, Caco-2 cells

INTRODUCTION

Carotenoids are a family of isoprene-derived lipophilic pigments that typically have 40-carbon molecules and multiple conjugated unsaturated carbon-carbon bonds in the *trans*-configuration. Carotenoids generally are classified as hydrocarbon carotenoids or carotenes (*e.g.*, *β*-carotene, *β*C; α-carotene, *α*C; and lycopene, LYC) and oxy-carotenoids or xanthophylls (lutein, LUT; zeaxanthin, ZEA and *β*-cryptoxanthin, *β*CX). These compounds are synthesized by vascular plants and some bacteria and fungi, but not by animals. Their functions in photosynthetic organisms include facilitation of the light harvesting process, protection against light-induced generation of damaging free radicals, and attraction of pollinators and seed dispersers. It has been estimated that approximately 60 of the more than 600 carotenoids in nature are present in the human diet, and about one third of those ingested have been detected in human plasma. These plant compounds and some of their metabolites have been shown to promote animal and human health by preventing oxidative damage, quenching singlet oxygen, modulating transcriptional activity and perhaps gap junction communication, and serving as precursors of vitamin A (VA). VA and its metabolites are necessary for embryonic development, immunocompetence and vision.[1]

In order to mediate the above activities, carotenoids and their metabolites have to be bioavailable, *i.e.*, they must be absorbed and delivered to the target tissues for utilization or storage. Carotenoid absorption requires release from the food matrix, transfer to lipid droplets and incorporation into mixed bile salt micelles during digestion, uptake and possible metabolism by enterocytes, and incorporation into chylomicrons for secretion into lymph.[2] The bioavailability of carotenoids is affected by numerous factors that include the following: 1) physicochemical properties (chemical speciation; crystalline vs. liquid state; *trans* vs. *cis* isomers; and, free vs. esterified vs. protein bound complexes); 2) food sources and matrix (localization in chloroplasts vs. chromoplasts; leaf vs. flower vs. seed; particle size (*i.e.*, puree vs. chopped vs. intact, food vs. supplements); 3) processing (*e.g.*, raw vs. processed food); and 4) interaction with other dietary compounds such as lipids, fiber, phytosterols and other carotenoids during digestion and absorption. Gut health, nutritional status, and genotype also affect digestion and absorption of carotenoids.[2,3] Thus, accurate prediction of carotenoid bioavailability from a particular food or meal is a significant challenge.

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IN VIVO EXAMINATION OF CAROTENOID BIOAVAILABILITY

The absorption of carotenoids and their esterified retinyl metabolites has been the subject of numerous investigations. Whereas direct evaluation of carotenoid absorption in human subjects represents the "gold" standard for assessing bioavailability, limitations associated with experimental design and the high cost of labor and equipment preclude systematic screening of various food sources, styles of processing, and other factors in the meal.[4]

Animal models often offer a number of advantages compared to human subjects for examining the bioavailability of ingested compounds.[5] These advantages include the ability to induce dietary deficiency and excess, use of radioisotopes as tracers, and collection of tissues of interest. However, no animal model accurately mimics the absorption and metabolism of carotenoids in humans. For example, enterocyte conversion of *βC* to VA in rodent intestine is much more efficient than for humans. Likewise, although gerbils, ferrets and pre-ruminant calves can absorb *β*C intact, only gerbils and pre-ruminant calves hydrolyze ingested *β*C to VA with efficiency similar to humans.[5,6]

IN VITRO APPROACHES TO ASSESSING RELATIVE BIOAVAILABILITY OF CAROTENOIDS

In vitro models have the potential to provide useful insights about the relative bioavailability of carotenoids from various cultivars of plant foods, and the effects of different styles of processing foods and other components of the meal on the potential bioavailability, *i.e.*, the bioaccessibility of ingested carotenoids. *In vitro* methods that have been utilized for the investigation of carotenoid stability and partitioning during digestion include simulated gastric digestion and small intestinal digestion, isolated intestinal segments, isolated brush-border and basolateral membrane vesicles, isolated enterocytes and transformed intestinal cell lines, and particularly the Caco-2 human cell line.

Our laboratory developed a model that coupled the simulated gastric and small intestinal digestive processes with highly differentiated cultures of Caco-2 cell to address problems related to digestive stability, micellarization, and cellular uptake of carotenoids from foods and supplements.[7] The *in vitro* digestion procedure is graphically displayed in **Figure 1.** Since its development, the model has been modified by us and others to better reflect physiological conditions. Changes have included the inclusion of an oral digestion phase,[8] the addition of lipase and carboxyl ester lipase (CEL) along with pancreatin to enhance lipolysis,[9] and the substitution of high speed for ultra-centrifugation to isolate the fraction containing carotenoids incorporated into micelles during small intestinal digestion (**Figure 2**). Caco-2 cells readily accumulate carotenoids from micelles generated during digestion, confirming their accessibility for cellular metabolism and absorption. The model has facilitated the evaluation of the effects of chemical speciation, food matrix, style of food processing, and various dietary factors on digestive stability, micellarization, and intestinal cell uptake and transport of carotenoids from foods and supplements.[7-14] The advantages and disadvantages of the coupled model are discussed in **Table 1**. Most important, Borel and associates have validated the use of micellarization during *in vitro* digestion as a reliable estimate of the *in vivo* bioaccessibility of carotenoids.[10] They recently reported that bioaccessibility as determined by *in vitro* digestion is highly correlated with data derived by sampling small intestinal luminal contents from human subjects fed carotenoid rich vegetables and bioavailability data from published human studies.

Thus, simulated digestion has proved to be an economical and efficient model for screening the relative bioaccessibility of carotenoids from fruits and vegetables, meals and supplements.

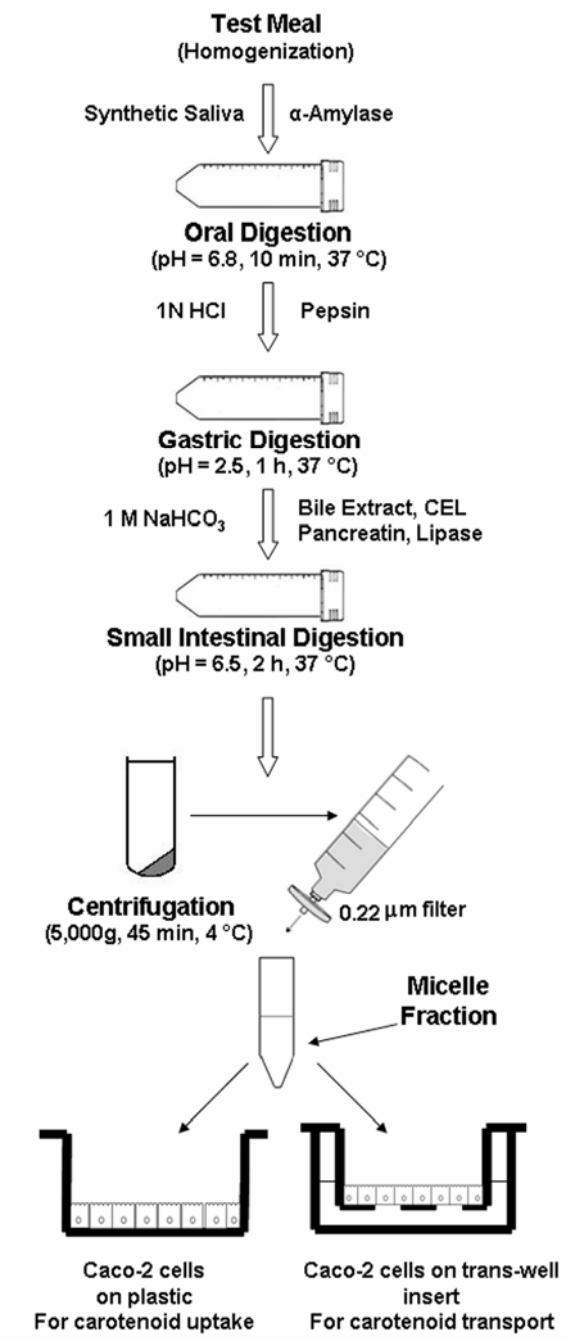

Figure 1. Graphical representation of coupled *in vitro* digestion/Caco-2 cell uptake model to assess carotenoid bioaccessibility.

Table 1. Characteristics and limitations of standard *in vitro* digestion method and Caco-2 cell model.

Advantages

In vitro digestion

- Relatively inexpensive and technically simple
- Equipment in standard laboratory is sufficient
- High throughput potential facilitates screening of numerous samples
- Readily controlled for investigation of mechanisms

Caco-2 cells

- Exhibit similar phenotype to normal absorptive epithelial cells
- Growth on dish surface and on membrane inserts to investigate uptake of compounds from luminal compartment or uptake and transport across monolayer, respectively
- Cells synthesize and secrete chylomicrons in response to prandial state

Disadvantages

In vitro digestion

- Closed system that is not responsive to composition and quantity of test foods
- Some enzymatic activities present in exocrine pancreas secretions are absent in pancreatin
- Oral phase often not included although it can readily be added
- Large intestinal phase of digestion generally absent

Caco-2 cells

- Cell line orginates from human colonic adencarcinoma
- Caco-2 cultures lack mucin, biofilms and presence of other epithelial cell types and humoral factors in small intestine that can affect enterocyte activities

SELECTIVE APPLICATIONS

Screening the effects of cultivars, processing and other dietary factors on bioaccessibility of carotenoids from foods

For many decades, VA deficiency continues to remain an important public health issue in developing countries. Infants and women of child bearing age are the most vulnerable populations. Clinical deficiency of VA can result in the permanent loss of vision, immunosupression and mortality if untreated.[2] Planned strategies to combat micronutrient deficiency in developing countries involve biofortification of staple crops by genetic engineering and by traditional breeding of nutrient dense cultivars with varieties possessing optimal agroeconomic characteristics.[15] Cost-effective, high throughput approaches are required to screen multiple genotypes produced during a planting season for timely decisions on appropriate accessions for further breeding. Recently, we screened the bioaccessibility of βC in 10 cultivars of boiled cassava containing less than detectable to 6.9 µg βC /g wet wt.[8] The post digestive recovery and the efficiency of micellarization of βC after simulated oral, gastric, and small intestinal digestion were independent of genotype, averaging 70% and 30%, respectively. We also found that the uptake of all-*trans* βC in Caco-2 cells was directly proportional to the amounts present in micelles generated during small intestinal digestion ($R^2 = 0.99$, $p < 0.001$).

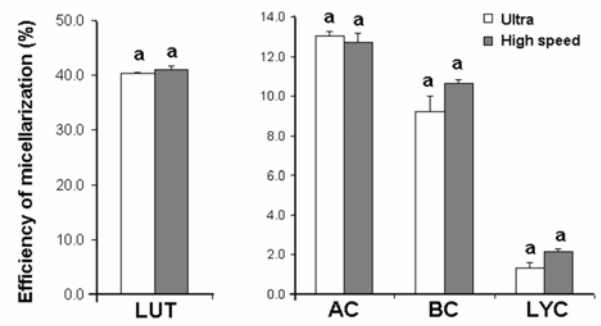

Figure 2 Impact of speed of centrifugation on measurement of micellarization of Carotenoids from Salad Meal with 1% Canola Oil. The salad purée (3 g) was digested *in vitro* with 1.0 % (v/w) canola oil. After digestion, 10 mL aliquots of chyme were subjected to either ultra centrifugation (167,000g, 20min) or high speed centrifugation (5,000g, 45min) at 4ºC. Aqueous fractions were isolated and filtered (0.22µm pores). Data (means ± SEM) are the relative (%) efficiency of micellarization of indicated carotenoids during *in vitro* digestion. Data were examined with three independent observations (n = 3). Identical letters above the error bars denote that the mean percentages of micellarization of the indicated carotenoid are not significantly different ($p > 0.05$)

These results suggests that all-*trans* βC content represents a key marker for selecting cultivars to cross with high yield varieties to generate novel varieties of cassava enriched in bioavailable pro-VA.

Investigating mechanisms involved in digestion and intestinal cell uptake of carotenoids

The coupled *in vitro* digestion/Caco-2 cell model also provides a tool to study the mechanisms associated with the micellarization and intestinal cell uptake and transport of carotenoids. For example, we have investigated the effects of amounts and structure of triglycerides (TG) on micellarization of carotenoids.[12] A carotenoid-rich salad meal with varying amounts and types of TG was digested using simulated gastric and small intestinal conditions. Consistent with *in vivo* studies,[10] micellarization of LUT (+ZEA) exceeded αC and βC which were greater than that of LYC. Partitioning of carotenes into micelles was enhanced ($p < 0.05$) by addition of TG (2.5% v/w) to the meal and dependent on chain length of fatty acyl groups in the TG (C18:1 > C8:0 > C4:0). However, the degree of unsaturation of C18 fatty acyl chains in TG added to the salad purée did not significantly alter the efficiency of micellarization of carotenoids. We also exposed Caco-2 cells to micelles generated during simulated digestion of salad purée with either triolein or trioctanoin. Apical uptake of βC was independent of fatty acyl composition of micelles, whereas LUT uptake was slightly, although significantly ($p < 0.05$), increased from samples with digested triolein compared to trioctanoin. These results suggest that micellarization of αC, βC and LYC during digestion requires minimal lipid content (0.5-2.5% v/w) in the meal, and that TG composition may affect efficiency of this process.

In another application of the model, we addressed the mechanism of digestion of xanthophyll esters.[9] Free and fatty acyl esters of xanthophylls are present in many fruits and vegetables. Human studies have showed similar bioavailability of xanthophylls ingested as either free or

esterified forms.[2] However, esterified xanthophylls are not present in chylomicrons. We examined pre-absorptive metabolism and transport of free vs. esterified xanthophyll to determine the relative roles of digestion vs. intestinal cells on the hydrolysis of esterified xanthophylls. Free, mono- and diesters of ZEA and LUT were measured in several plant foods, including wolfberry, orange pepper, red pepper and squash. Wolfberry, a small fruit used to promote ocular health in traditional Chinese medicine, contains the greatest concentration of ZEA with the diester derivative as the predominant species. Free ZEA was more efficiently incorporated into mixed micelles (81.8 ± 8%) than mono- and diesters (44 ± 5% and 11 ± 4%, respectively). CEL enhanced micellarization of ZEA from the plant food by partially hydrolyzing ZEA esters during small intestinal digestion. Free ZEA and a small amount of ZEA mono-ester, but no ZEA diester, were present in cells after exposure to the micelle fraction for 4h. Addition of CEL to medium with micelles further enhanced cellular accumulation of ZEA ($p < 0.05$) by hydrolyzing ZEA esters present in micelles. In contrast, ZEA mono-esters taken up by Caco-2 cells were not hydrolyzed by cellular esterases. Thus, results with the simulated digestion/Caco-2 cell model suggest that xanthophyll esters are hydrolyzed by CEL prior to transport of the xanthophyll into enterocytes.

CONCLUSION

The coupled *in vitro* digestion/Caco-2 cell model provides a cost effective tool for screening the bioaccessibility of carotenoids from foods, meals and supplements. Results provide testable hypotheses of the appropriate design of the more labor intensive but highly focused and necessary human studies. The coupled model also facilitates the investigation of characteristics and regulation of gastrointestinal metabolism and transport of dietary carotenoids.

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ABBREVIATIONS

*α*C, *α*-carotene; *β*C, *β*-carotene; *β*CX, *β*-cryptoxanthin; CEL, Carboxyl Ester Lipase; LUT, lutein; LYC, lycopene; TG, triglyceride; ZEA, zeaxanthin.

AUTHOR DISCLOSURES

Mark L Failla, Tianyao Huo, Sagar K Thakkar, no conflicts of interest.

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