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Red palm oil-supplemented and biofortified cassava gari increase the carotenoid and retinyl palmitate concentrations of triacylglycerol-rich plasma in women

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Abstract

Boiled biofortified cassava containing β -carotene can increase retinyl palmitate in triacylglycerol-rich plasma. Thus, it might alleviate vitamin A deficiency. Cassava requires extensive preparation to decrease its level of cyanogenic glucosides, which can be fatal. Garification is a popular method of preparing cassava that removes cyanogen glucosides. Our objective was to compare the effectiveness of biofortified gari to gari prepared with red palm oil. The study was a randomized cross-over trial in 8 American women. Three gari preparations separated by 2 wk washout periods were consumed. Treatments (containing 200 – 225.9 g gari) were: biofortified gari (containing 1 mg β -carotene); red palm oil-fortified gari (1 mg β -carotene), and unfortified gari with a 0.3 mg retinyl palmitate reference dose. Blood was collected six times from –0.5 – 9.5 h post-ingestion. Triacylglycerol-rich plasma was separated by ultracentrifugation and analyzed by HPLC with diode array detection. Area under the curve for β -carotene, α -carotene, and retinyl palmitate increased after the fortified meals were fed ($P < 0.05$), though the retinyl palmitate increase induced by the red palm oil treatment was greater than that induced by the biofortified treatment ($p < 0.05$). Vitamin A conversion was 2.4 ± 0.3 and 4.2 ± 1.5 μ g pro-vitamin A carotenoid:1 μ g retinol (means $\pm$ SEM) for red palm oil and biofortified gari, respectively. These results show that both treatments increased β -carotene, α -carotene, and retinyl palmitate in triacylglycerol-rich plasma concentrations in healthy well-nourished adult women, supporting our hypothesis that both interventions could support efforts to alleviate vitamin A deficiency.

Keywords

biofortified cassava; red palm oil; vitamin A; beta-carotene; human

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1. Introduction

Vitamin A deficiency is a leading cause of morbidity and mortality, especially in young children and pregnant and lactating women [1]. Food-based interventions focused on alleviating vitamin A deficiency in susceptible populations are especially useful in rural areas, because they may provide vitamin A-forming carotenoids as well as income to farmers [2].

Cassava is an inexpensive and easy to grow tuber that can survive droughts and is resistant to many pests [3]. Common varieties of cassava are poor sources of pro-vitamin A carotenoids [4,5], but recently multi-national non-governmental organizations such as HarvestPlus have developed biofortified cassava with relatively high concentrations of β -carotene (BC) [2,6–8]. Several yellow-fleshed cassava cultivars with moderately high concentrations of β -carotene have been released to rural populations in Nigeria, where cassava is a staple crop [9].

Previous studies showed that biofortified cassava enriched with β -carotene successfully maintained vitamin A status in Mongolian gerbils [10], and increased β -carotene and retinyl palmitate (RP) concentrations in the triacylglycerol (TAG)-rich plasma of healthy adult women [11]. Results from the previous human study [11] were used to simulate the amount of vitamin A which would be consumed if all native white cassava were replaced with biofortified cassava. In a model based on these previous results, replacing native with biofortified cassava would provide over 90% of the recommended dietary intake of vitamin A [12].

However, this study used boiled cassava, which is not the preferred cassava-based food in Africa. Cassava contains cyanogenic compounds that must be decreased to make it safe to consume [13,14]. Several food processing methods have been developed in Sub-Saharan Africa to remove cyanide: the most popular being sun drying and garification [15–18]. In garification cassava is minced, fermented, dewatered, and then roasted to form coarse flour. More information is required on the effects of garification on the bioavailability and bioconversion of carotenoids from biofortified cassava.

The concentration of carotenoids in cassava-based foods can either be increased by substituting biofortified cassava for common unfortified cassava, or by adding a food with a very high concentration of β -carotene or vitamin A to the cassava. Red palm oil is an extremely rich source of β -carotene and α -carotene, two common pro-vitamin A carotenoids. As such it has been used to prevent vitamin A deficiency in individuals and small-scale human intervention trials for decades [19–28].

In this study, we compare the effect of biofortified gari (BFG) to an equal amount of red palm oil-fortified cassava gari (ROG), containing equivalent amounts of β -carotene, on the β -carotene, α -carotene, and retinyl palmitate concentrations of triacylglycerol-rich plasma. Treatments were fed to 8 healthy well-nourished American women in a cross-over double-blind study. Our objectives were to compare the effectiveness of BFG and ROG containing similar amounts of β -carotene for increasing β -carotene, α -carotene and retinyl palmitate concentrations in healthy adult women, and to calculate the retinyl equivalency of cassava

prepared as BFG or ROG. Our hypothesis was that both interventions would be effective at increasing β -carotene and retinyl palmitate in triacylglycerol-rich plasma.

2. Methods and materials

2.1. Subjects

Twelve healthy, nonsmoking, non-pregnant women aged 19 – 43 y participated in the study. Women were eligible for the study if they had a BMI of 18 – 35 kg/m², blood pressure less than 135/90 mm Hg, and triacylglycerol, cholesterol, total protein, electrolytes, kidney and liver function tests (such as blood urea nitrogen), hemoglobin, hematocrit, red blood cells, and white blood cells within clinically normal ranges. Exclusion criteria included the use of medications that affect retinoid, carotenoid, or cholesterol absorption from food such as fat, triacylglycerol, or cholesterol-lowering medications, medicines containing high dosages of retinoids, vitamin A or carotenoid supplements, illegal drugs, or tobacco. In addition, subjects could not be allergic to cassava or palm oil. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of California, Davis Institutional Review Board, #48011, renewed yearly. Written informed consent was obtained from all subjects. The clinical trial registry name was Beta-carotene absorption and bioconversion to vitamin A from biofortified cassava gari, registration ID number NCT02210507, and the registry website ClinicalTrials.gov.

Four women left the study prior to completion, one for scheduling conflicts and three because they disliked either the taste or the large portion of the gari served in the study. The demographic and physiological data of the participants who dropped the study were not different than those for the participants who completed the study. A recruiting diagram is shown in Figure 1.

2.2. Chemicals

Methanol, isopropanol, and ammonium acetate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The calibration standard apo-8-carotenal, β -carotene, retinol, and retinyl palmitate were purchased from Sigma-Aldrich (St. Louis, MO, USA) and β -cryptoxanthin and α -carotene standards were purchased from Santa Cruz Biochemicals (Santa Cruz, CA, USA).

2.3. Gari

2.3.1 Gari preparation—Biofortified cassava, cross-bred to contain high amounts of β -carotene (Genotype 07/0593), was grown by the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria). IITA also provided the TME-419 unfortified cassava used for the red palm oil-added and unfortified white cassava gari. IITA prepared the three batches of gari (from biofortified, red palm oil-added, and typical unfortified white cassava). Gari was made from 9 – 15 month old cassava plants. Processing was begun within 24 hours of harvesting. Cassava was peeled, washed, and grated with a motorized cassava grater. The mash was put into a dark plastic trough and fermented for 2 d at ambient temperature. The fermented mash was put into sacks and a hydraulic press was used to dewater the mash. The

moist cake was pulverized by passing through a grater a second time, then roasted. The roasted gari was cooled to room temperature then sieved to obtain uniform granules. Then the gari was packed into air-tight containers and air-freighted to the Western Human Nutrition Research Center by DHL (Plantation, FL, USA), import exemption P587-110919-009.

After the gari preparations were received at the Western Human Nutrition Research Center they were packaged under minimal fluorescent lighting into meal-sized portions, blown with food-grade nitrogen gas, and then heat-sealed into air-tight grey Mylar bags. Food grade nitrogen was blown over each package, using a food-grade nitrogen regulator (Ready Reserve Inc., Twin Falls, ID, USA). Gari preparations were stored at -20°C in a food safe freezer until use.

2.3.2 Gari analysis—Gari preparations were analyzed for cyanogenic glycosides by two methods. Cyanide was measured by our lab using a La Motte colorimetric assay (LaMotte, Chestertown, MD, USA), according to previously published procedures [11]. For confirmation cyanide was also analyzed by GC-MS by the Carlson Company LLC, Colorado Springs, CO, USA), a certified lab tester. In addition, the gari preparations were tested for food safety (aerobic and anaerobic bacterial plate counts, tests for *Staphylococcus aureus*, *Salmonella*, *Escherichia coli* and total coliform bacteria; mold counts; aflatoxins B1, B2, G1 and G2; and heavy elements (arsenic, cadmium, lead, and mercury) by ICP-MS. Food safety tests were conducted by Covance (Madison, WI, USA) according to their standardized methods.

2.4. Dietary protocols

2.4.1. Meals provided prior to gari interventions—Each subject consumed three randomized dietary interventions of gari separated by 2wk washout periods. Before each intervention, subjects were required to eat a diet low in polyphenols, retinol, and carotenoids for 7d. For the first 4d subjects were given instructions by the study dietitian on how to reduce their dietary intake of polyphenols, retinoids, and carotenoids [11]. Subjects were also required to complete a 4d food record to assess their adherence with the dietary restrictions. On the last 3d before each intervention, subjects were provided with nutrient-controlled research meals calculated to be low fat, low protein, low retinoid (4 µg retinyl activity equivalents), low carotenoid, and to be low in polyphenols (by restricting food groups rich in polyphenols). Five aliquots were collected from each of the test meals and analyzed for retinoid and carotenoid content [11]. The diet consumed on the 3d prior to the test day contained 108 µg β-carotene, 182 µg β-cryptoxanthin, and 0 µg α-carotene and retinoids by analysis. Total calculated calories were 2,190 kcal with 69.6 g protein (12.4% of kcals), 24.6 g fat (9.8% of kcals), and 428.1 g carbohydrate (77.6 % of kcals).

Subjects were required to eat only what was provided to them, but they were not required to eat everything. The nutrient-controlled meals were designed by a Registered Dietitian and were prepared in the Metabolic Kitchen of the Western Human Nutrition Research Center, then given to the subjects for retherming. Nutrient data were calculated using the Nutrition

Data System for Research software, version 2014 (The Nutrition Coordinating Center, University of Minnesota, MN, USA).

2.4.2. Gari test meals—On each test day, overnight-fasted subjects consumed one of the following randomized gari meals for breakfast: 1) BFG, 225.9 g biofortified gari to contain approximately 1 mg β -carotene, 2) ROG, 205.6 g unfortified white gari mixed with red palm oil to contain approximately 1 mg β -carotene, and 3) WG+RP, 200.0 g unfortified white gari with a retinyl palmitate tracer.

On the day prior to each test day a Mylar bag containing the appropriate gari was removed from the -20°C storage and thawed at 25°C for 16 hours. The Mylar bag of gari was opened, weighed, and then mixed with deionized water and a small amount of gravy to improve its palatability. Approximately 200 g gari was mixed with 200 g deionized water and 200 g gravy made from water, flour, beef broth concentrate, salt and white pepper and microwaved to an internal temperature of 71°F (165°F). The gari meal recipes with actual weights of ingredients are given in Table 2. When subjects were fed the WG+RP meal they were also given a capsule containing a reference dose of 0.3 mg pure food-grade retinyl palmitate (kindly donated by DSM, Heerlen, Netherlands) diluted in canola oil. The reference dose was provided in blue capsules, kindly donated by Marjorie Haskell of the University of California Davis. When subjects were fed the BFG and ROG meals they received empty blue capsules. The test meals were consumed under supervision by the Metabolic Kitchen and Human Feeding Lab staff. A proximate analysis was performed on the gari preparations (Covance, Madison, WI, USA). The results of the analysis can be seen in Table 3. The all-*trans*- β -carotene, *cis*- β -carotene, total β -carotene and α -carotene concentrations of the different garis are shown in Table 4.

Subjects could drink water *ad libitum* on test days, but did not eat again until after their last (9.5 hour) blood collection. After this blood collection their catheters were removed and they were fed dinner (a chicken burrito made with chicken, black bean, cabbage, cheddar, and Mexican style sauce; potato chips, and chocolate chip, peanut butter, and sugar cookies).

2.5. Analytical procedures

2.5.1. General laboratory procedures—Laboratory workers were blinded to the dietary treatment received by subjects through the blood processing, lipoprotein isolation, and chromatography analysis. All plasma handling and sample processing was carried out under gold fluorescent lights in order to protect light sensitive compounds, except triacylglycerol and cholesterol measurement was conducted under regular white light.

2.5.2. Carotenoid and retinoid analysis in food—Food samples and plasma triacylglycerol fractions were analyzed by reversed-phase chromatography on an Agilent 1260 system (Santa Clara, CA, USA). Food samples were analyzed using a YMC Carotenoids column, $3\mu\text{m}$, $3 \times 150\text{ mm}$ (YMC America, Inc., Allentown, PA, USA) with an isocratic mobile phase of 80:20 (v/v) methanol: methyl-tert-butyl ether, at a flow rate of $0.75\mu\text{L}/\text{min}$ for 26 min, modifying the method of Kimura [29].

2.5.3 Isolation of triacylglycerol-rich plasma—Throughout all blood processing procedures and analyses, lab workers were blinded to the dietary treatment received by the subject. Blood processing procedures have been described elsewhere [11]. Briefly, blood was collected by a catheter inserted into an antecubital vein. Approximately 30 min before the gari meals, the catheter was inserted and a baseline sample of 13 mL blood was collected. Baseline blood was used for C β -carotene analysis as well as for carotenoid and retinoid tests. Postprandial blood samples (10 mL) were collected at 2, 3.5, 5, 7.25, and 9.5 h after the test meal. After the 9.5 h blood draw, the catheter was withdrawn. Blood was transferred into EDTA-vacutainers, and plasma separated by centrifugation at 1,300 X g for 10 min at 4°C on a Beckman Coulter Allegra™ 6R Centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA).

The triacylglycerol fractions, containing newly-absorbed carotenoids and retinyl palmitate, were separated from plasma by ultracentrifugation as previously described. These procedures resulted in a reproducible triacylglycerol fraction consisting of chylomicrons and large VLDL. Approximately 100 μ L of the triacylglycerol fraction was removed by slicing the ultracentrifuge tube and dispensed into a 15 mL test tube and deproteinised with 600 μ L ethanol (containing 0.1% butylated hydroxytoluene as antioxidant). 100 μ L β -apo-8-carotenal was added as an internal standard and 200 μ L propyl gallate (5% in ethanol) as an additional antioxidant. Sample then was extracted twice with 2 mL hexane. The hexane layers were dried under nitrogen and reconstituted in 100 μ L 70:20:10 (v/v/v) acetonitrile-dichloromethane-methanol. triacylglycerol and cholesterol concentrations were measured at each blood draw time point using a clinical chemistry analyzer (Integra 400 Plus, Roche Diagnostics, Indianapolis, IN, USA).

2.5.4 Carotenoid and retinoid analysis in triacylglycerol-rich plasma—

Triacylglycerol-rich plasma carotenoids and retinoids were separated with an YMC column (Carotenoid S-3 μ m, 50 $\times$ 2.1 mm) using an isocratic mobile phase: 70:20:10 (v/v/v) acetonitrile-dichloromethane-methanol, as previously described [30,31]. Triacylglycerol plasma extract size was 100 μ L, of which 20 μ L was injected onto the column. Injections were run in duplicate. The total run time was 12 min at 0.2 mL/min. Column temperature was maintained at 37°C throughout. α -Carotene, β -carotene and β -apo-8-carotenal were measured at 452 nm and retinyl palmitate at 325 nm. A pooled plasma sample purchased from UTAK (Valencia, CA, USA) was used to evaluate interassay precision of the plasma triacylglycerol fraction. Inter-assay precision ranged between 3 and 9% for carotenoids and retinoids. Peaks were identified by co-migration with authentic standards and by spectrograms. Unfortunately, we were not able to confirm peak identification by NMR, IR, or other rigorous methods because of the tiny amounts of material available.

2.6. Stability of gari preparations

The stability of the gari preparations was tested at room temperature (23°C), 4°C, –20°C and –70°C. Gari stored at 23°C and 4°C were measured approximately daily for 11 days. Gari stored at –20°C and –70°C were measured approximately once a month for 6 mos. Carotenoid and retinoid concentrations were measured by HPLC as described above.

2.7. Statistical analysis

The experimental group size was estimated using a one-tailed power calculation (SAS, Cary NC) with effect $1.3X \sigma$, $\alpha = 0.05$, $\beta = 0.20$) based on a study of the influence of biofortified cassava on the β -carotene and vitamin A concentrations in TRL plasma [11]. The power calculation suggested a minimum group size of 7. However, we enrolled 8 subjects.

Areas under the concentration-time curve (AUCs) were calculated using trapezoidal approximation after subtracting initial fasting concentrations. The AUC for β -carotene and α -carotene were calculated by subtracting the unfortified white cassava control group concentrations at each time point.

Most retinyl esters in the plasma triacylglycerol-rich fraction had concentrations that were too low to quantify accurately. However, retinyl palmitate, the most common retinyl ester, is often used to estimate total retinyl ester formation [32,33]. With retinyl palmitate absorption typically ranging between 75–99% [34], we assumed a mid-range recovery of 90% retinyl palmitate.

The bioavailability of carotenoids from gari was quantified as previously described [11]. Briefly, absorption was estimated using the assumption that the $t^{1/2}$ of β -carotene, retinyl palmitate, and chylomicrons were equivalent (at 0.192 h) [35]; and the plasma volume (mL) = $927 + (31.47 \times \text{body weight in kg})$ [36].

Retinyl equivalence was calculated as described by Li et al [33]. The retinyl palmitate AUC values were converted to mass (nmol) retinyl palmitate in the entire plasma pool by multiplying the TAG retinyl palmitate concentration (nmol/L plasma) by the estimated plasma volume [37].

Total plasma pool retinyl palmitate (nmol) = TAG-rich RP (nmol/L) $\times$ estimated plasma volume (L)

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(1)

Where estimated plasma volume (L) = $(927 + (31.47 \times \text{body weight in kg}))/1000$ [36]

Vitamin A (nmol) formed from fortified gari was calculated by comparing the AUC after ingestion of fortified gari to the AUC after ingestion of the WG+RP), which contained a retinyl palmitate reference dose of 0.3 mg (1047.1 nmol).

$$\text{RP formed from gari (nmol)} = (\text{RP}_{\text{ROG or BFG}} / \text{RP}_{\text{WG+RP}}) \times 1047.1 \text{ nmol} \quad (2)$$

The bioconversion factor for β -carotene in fortified gari was calculated by dividing the β -carotene equivalents in fortified gari by the retinyl palmitate formed.

$$\text{BC bioconversion} = (\text{BC equivalents (nmol)} \times 536.8 \text{ (ng/nmol)}) / (\text{retinyl palmitate formed (nmol)} \times 286.5 \text{ (ng/nmol)})$$

(3)

Differences in AUC and plasma triacylglycerol fractions were analyzed using repeated measures ANOVA while retinyl equivalence differences were analyzed using paired Student *t* tests. *P* values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Subject characteristics

The study participants were healthy premenopausal women with normal weight, blood pressure, BMI, cholesterol, and triacylglycerol. Select blood chemistries and demographic characteristics are shown in Table 4. These characteristics did not change during the study. BMI, cholesterol, and triacylglycerol concentrations did not change during the study.

3.2. Gari test meals

The gari test meals were very safe: cyanide concentrations were low (1.5 – 5 ppm). Aerobic and anaerobic plate counts, bacteria, mold, and aflatoxins were low and well within acceptable ranges for foods. Arsenic, cadmium, and mercury were very low (<10 ppb), and lead was 374 ppb.

Unfortunately, the gari test meals were very large, filling, and not especially well liked by our participants. Only 8 out of 12 participants who began the study completed it, with several leaving because they did not like the taste and/or size of the gari meals provided. Furthermore, most participants could not quite finish the gari meal. The mean percentage of consumption was 75.9 ± 18.7%. The percent consumed did not vary by treatment.

BFG and ROG were fairly well matched for *trans*- and total β -carotene equivalent concentration (Table 3). ROG also contained α -carotene, which is assumed to form vitamin A half as well as β -carotene. Both interventions were pale orange (Figure 2), and their appearance was considered more attractive than the non-fortified white gari (WG+RP). BFG and ROG were not distinguishable by color, taste, or particle size.

3.3. Carotenoid stability in gari meals

Carotenoid concentrations and compositions were stable in ROG and BFG gari stored under nitrogen in Mylar bags at –20°C and –70°C for at least 78d. No significant degradation or change in isomer composition was seen at these temperatures. Previous studies in our lab

showed that carotenoid concentrations were considerably less stable in biofortified gari stored at -20°C in an unsealed container, degrading within a week.

3.4. HPLC method

Previously, investigations of newly-formed carotenoids and retinoids in triacylglycerol-rich plasma have used a sensitive electrochemical detector such as the ESA Coularray [11, 38]. However, this detector has not been manufactured for years and its service and repairs are now expensive and problematic. Fortunately, HPLC columns, software and detectors have progressed, allowing for more sensitive detection. We developed a new method for measuring retinoids and carotenoids in triacylglycerol-rich plasma using a conventional HPLC system (the Agilent 1260) and reversed-phase column. The method uses a short reversed phase column with small particle packing, which decreases peak broadening, increasing peak height and improving sensitivity and specificity. This method is capable of resolving and detecting low concentrations of β -carotene, α -carotene, and retinyl palmitate. The chromatograms for a typical subject are included as Supplemental Material.

3.5. Postprandial triacylglycerol response

The triacylglycerol fraction appeared to contain both chylomicrons and VLDL [11,39,40], which is appropriate because recently consumed β -carotene and retinyl palmitate are found in both of these lipoprotein fractions [33,39,41]. After consuming the gari meals, plasma triacylglycerol and cholesterol concentrations increased in triacylglycerol-rich plasma within 2 h and remained high for 3 hours before decreasing.

3.6. Postprandial α -carotene, β -carotene and retinyl palmitate response

Triacylglycerol-rich-plasma concentrations of β -carotene rose in both BFG and ROG within 2 hr (Figure 3A). Triacylglycerol-rich plasma concentrations of α -carotene rose in ROG within 2 hr, and BFG within 5 hr after ingestion (Figure 3B). Triacylglycerol-rich concentrations of retinyl palmitate increased in all groups within 2 hr, especially in the ROG group. The ROG group was higher than the BFG or WG+RP at 3.5 hr, and remained high at 5 hr before rapidly decreasing at 7.25 hr (Figure 4).

Area-under-the-curve (AUC) for α -carotene and β -carotene increased for both the ROG and BFG treatments. However, the increase was greater after the ROG treatment ($p = 0.025$ for α -carotene, 0.007 for β -carotene), Figure 5. The AUC for the retinyl palmitate formed from ROG or BFG were both significantly greater than baseline, with BFG providing an amount of retinyl palmitate that was equivalent to that provided by the WG+RP treatment, which contained 0.3 mg pure retinyl palmitate (Figure 6). ROG provided more retinyl palmitate than the WG+RP or the BFG treatments ($p = 0.019$).

The mean retinyl equivalence values for ROG and BFG are shown in Table 5. The mean amount of β -carotene in the ROG with a retinyl activity equivalent of $1\text{ }\mu\text{g}$ retinol ranged from 1.1 to 4.1 for ROG and 0.3 to $12.1\text{ }\mu\text{g}$ for the BFG meal. The difference in retinyl equivalence values for the ROG and BFG meals was not significantly different ($P=0.23$). Retinyl equivalencies were highly variable in all groups, as is common for retinyl

equivalencies calculated for most foods and supplements [11,42–44], presumably because of genetic differences.

4. Discussion

This study measured the effect of meals containing similar amounts of red palm oil fortified and β -carotene-biofortified gari on the plasma triacylglycerol-rich fraction response of β -carotene and retinyl palmitate in healthy well-nourished women. The plasma triacylglycerol-rich fraction contains newly absorbed β -carotene and newly formed retinyl palmitate, thus allowing us to evaluate the bioavailability and bioconversion of β -carotene from red palm oil fortified gari in humans. Our primary hypothesis was that both gari interventions would increase β -carotene and retinyl palmitate in triacylglycerol rich plasma.

One important result from this study is that feeding BFG, or especially ROG, increased β -carotene in the plasma triacylglycerol-rich fraction of these women. A second important result is that BFG, and especially the ROG meal increased retinyl palmitate in triacylglycerol-rich plasma. Thus, our results support our hypothesis.

A third important result is that β -carotene from both ROG and BFG were efficiently converted into retinol, with average retinyl equivalence of 2.3 for ROG and 4.2 for BFG.

4.1 Methods

A methodological result of interest is that biofortified gari is stable for at least 8 months when protected from oxidation (by storing it under nitrogen at -20°C in a heat-sealed Mylar bag). This contrasts to results from our laboratory, where samples which were stored at -20°C but which were not protected from air lost carotenoids within a week. Further studies of the effects of oxygen on biofortified gari, such as the effect of storing gari in tightly sealed containers, would be useful.

Another important methodological improvement is that we developed a method to measure α -carotene, β -carotene, and retinyl palmitate concentrations in triacylglycerol-rich plasma by diode array detection with a standard HPLC. This method uses currently available short columns to decrease peak broadening, which increases peak height and improves separation and quantitation. The method allows separation and quantitation of major carotenoids and retinoids in triacylglycerol-rich plasma using the common diode array detection method.

4.2 β -Carotene and α -carotene

Both β -carotene and α -carotene increased in triacylglycerol-rich plasma after the ROG and BFG meals, although the increase of α -carotene after the BFG meal was small. These results were expected for the ROG meal, which contained substantial amounts of both α -carotene and β -carotene. The increase of α -carotene after the BFG was less expected, because BFG contained very little α -carotene. It may be worth noting that the α -carotene increase after the BFG feeding occurred only at the later time points, 5 to 7 hrs after the gari meal. This was not a meal effect, since subjects were not fed until 9.5 hr after their intervention meals. We have seen similar phenomena before in our human studies [43,44] We hypothesize that

the fat provided in the BFG meal may solubilize α -carotene that is stored in enterocytes from previous meals, providing a transient increase.

4.3. Biofortified versus red palm oil fortified gari

Although the ROG and BFG meals contained similar amounts of all-*trans*- β -carotene, β -carotene equivalents, and fat, ROG appeared to be a more effective source of both β -carotene and retinyl palmitate for humans than the biofortified gari made from cassava genotype 07/0593. There are a couple of differences between these treatments that might explain the greater effectiveness of ROG compared to the variety of BFG tested. First, the ROG contained slightly more β -carotene equivalents, more α -carotene, and less *cis*- β -carotene than BFG. Both α -carotene and *cis*- β -carotene are assumed to form about half as much retinyl palmitate as all-*trans*- β -carotene forms, because of differences in their chemical structures [45] and because they are poorer substrates for beta-carotene 15, 15'-oxygenase 1 (BCO1), the most important enzyme cleaving pro-vitamin A carotenoids to retinal [46,47]. However, there have been no comparisons of the effectiveness of α -carotene versus *cis* isomers of β -carotene at forming vitamin A in humans. It is possible that α -carotene is more effective at forming retinyl palmitate than the *cis*- β -carotene isomers in biofortified gari. However, α -carotene contributed only 23% of the β -carotene equivalents in red palm oil, so it is unlikely to be solely responsible for the difference in effectiveness between ROG and BFG.

Another difference that might be important is that while the ROG and BFG meals tested provided similar amounts of fat (9 % kcals) and similar amounts of β -carotene, the sources of the fat and β -carotene differed. The inclusion of fat in a meal usually increases carotenoid bioavailability [39,40]. However, there is considerable uncertainty about the concentrations of fat or oil that are required for this effect, and about whether the carotenoid must be emulsified in the fat or can benefit merely from the presence of fat in a meal [39,40,48–51]. ROG was made with red palm oil. Carotenoids are an integral part of red palm oil, so the carotenoids are completely emulsified in the fat. BFG, on the other hand, contained β -carotene as an integral part of the cassava gari, while the fat was mixed with the gari just before serving. Therefore, the β -carotene in the BFG meal was not necessarily emulsified in the fat. Thus, the β -carotene in red palm oil may have been more bioavailable than the β -carotene in biofortified cassava.

In our previous study with boiled cassava there was little difference between the carotenoid absorption and retinyl equivalency in our low fat meal (containing 6 g or fat) and higher fat meal (20 g). Specifically, there was no significant difference in the AUC for β -carotene or retinyl palmitate between these meals [11]. However, the gari meals we provided were very large, and contained a relatively small amount of fat (9 % kcals/meal). Thus, it may be that the BFG meal would have provided more carotenoids and retinoids if it had contained more fat.

4.4. Retinyl equivalence

The means of retinyl equivalence of the ROG and BFG meals were 2.3:1 and 4.2:1, respectively. These results are excellent, and are comparable to our previous results for

boiled cassava of 4.2 – 4.5 µg retinyl equivalents in humans [11] and 3.7:1 retinyl equivalents for biofortified cassava fed to vitamin A-depleted Mongolian gerbils [10]. Retinyl equivalence for ROG and BFG were also similar to those seen in other β-carotene biofortified foods, which seem to have higher bioconversion rates than natural sources of β-carotene [52,53]. The retinyl equivalence we calculated for the ROG meals is similar to that estimated for β-carotene in oil [54], although a previous human study measuring retinyl equivalence in red palm oil calculated a somewhat higher retinyl equivalency, of 5.7:1 [51].

4.5 Limitations

An important limitation of this study was that it used a single cultivar of biofortified cassava, and a single preparation of red palm oil-fortified gari. Results using other biofortified cassava cultivars, or other mixtures of red palm oil, may yield different and potentially less successful results. However, results might also be improved, as cultivars are bred with higher carotenoid concentrations. A second important limitation was that the study was conducted in healthy American women. Results might be different in African women because of differences in genetic background or dietary intakes, especially in those who have poorer health status or nutrient intake.

5. Conclusion

Both BFG and ROG were effective suppliers of vitamin A in this study, suggesting that both fortification strategies could be used to increase vitamin A status in people. Adding red palm oil to gari appears to be a good method to increase retinyl palmitate concentrations in the triacylglycerol-rich plasma of healthy adult women. Thus, increasing awareness of the usefulness of red palm oil as a source of vitamin A is desirable. BFG prepared using a common African cultivar (genotype 07/0593) also increased retinyl palmitate concentrations in triacylglycerol-rich plasma, although less effectively than ROG. Furthermore, our previous study of boiled cassava showed that it provided bioavailable β-carotene, and increased retinyl palmitate concentrations in triacylglycerol-rich plasma. This suggests that further development of biofortified cassava to increase its β-carotene concentrations or improve its absorption is warranted.

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Abbreviations

AC	α-carotene
AUC	area-under-the-curve

BC	β -carotene
BFG	biofortified cassava gari
IITA	International Institute of Tropical Agriculture
ROG	red-palm oil fortified gari
RP	retinyl palmitate
TAG	triacylglycerol
WG+RP	white cassava gari with retinyl palmitate tracer

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Figure 1.
Flow chart of subject recruitment and selection for study.

Figure 2.

Gari preparations: ROG, WG+RP, and BFG.

Appearance of red palm oil fortified (ROG), white gari (WG+RP), and biofortified (BFG).

Bowls of gari resting on mylar storage bags. Left = ROG, middle = WG+RP, Right = BFG.

Figure 3A

Figure 3B

Figure 3.

Figure 3A. β -Carotene concentrations in triacylglycerol-rich plasma after fortified gari ingestion

Figure 3B. α -Carotene concentrations in triacylglycerol-rich plasma after fortified gari ingestion

Means ($\pm$ SEM) change of carotenoid concentrations in triacylglycerol-rich plasma after subjects ingested either BFG or ROG. (n = 8). WG+RP, which contained negligible amounts of β -carotene and α -carotene, was used as a control; its carotenoid concentrations at each time point were subtracted from the ROG and BFG groups. 3A = β -carotene concentration; 3B = α -carotene concentrations. BFG = ____ ; ROG = - - -.

Figure 4. retinyl palmitate concentrations in triacylglycerol-rich plasma after fortified gari ingestion
Means ($\pm$ SEM) change of retinyl palmitate concentrations in triacylglycerol-rich plasma after subjects ingested BFG, ROG, or WG+RP. (n = 8). BFG = — ; ROG = - - -. WG+RP = ... * = significantly different from WG+RP.

Figure 5.

Comparison of AUC of α -carotene and BC = β -carotene concentrations in triacylglycerol-rich plasma

($n = 8$). WG+RP, which contained negligible amounts of β -carotene and α -carotene, was used as a control; its carotenoid concentrations at each time point were subtracted from the ROG and BFG groups. $\square$ = WG+RP, $\blacksquare$ = ROG, $\blacksquare$ = BFG.

Figure 6.

Comparison of retinyl palmitate in triacylglycerol-rich plasma for each treatment group

* = significantly different from WG+RP, (n = 8). ■ = ROG, ■ = BFG.

TABLE 1

Subject demographics and blood chemistries (n = 8)

	means	SEM
Age (y)	27.1	2.9
Body weight (kg)	65.7	3.4
BMI (cm ² /kg)	24.9	0.78
Glucose (mmol/L)	4.61	0.12
(mg/dL)	82.8	2.1
Total cholesterol (mmol/L)	4.81	0.22
(mg/dL)	186	8.6
HDL-cholesterol (mmol/L)	1.51	0.15
(mg/dL)	58.3	5.8
LDL-cholesterol (mmol/L)	2.97	0.18
(mg/dL)	115	6.8
Triacylglycerol (mmol/L)	0.72	0.096
(mg/dL)	63.4	8.5
Hemoglobin (mmol/L)	2.09	0.039
(mg/dL)	13.5	0.25
Hematocrit (%)	40.3	0.76
Chol:HDL	3.3	0.23

Table 2

Gari meal description

Meal component	WG+RP	ROG	BFG
Gari (gm)	200.0	205.6	225.9
Water, deionized (gm)	187.0	191.0	212.4
Beef gravy (gm)	187.0	191.0	212.4
Onions, Fried, Canned, Pulverized (gm)	7.7	7.3	10.8
Oil, Canola (gm)	0.7	0.0	0.0

Table 3

Calculated nutrient composition of meals

Meal Description		WG+RP	ROG	BFG
β-carotene				
Equivalents	mg	0.00	1.00	1.00
Energy	kcal	971	995	1093
Protein	gm	10.6	10.9	12.0
Total lipid	gm	10.4	9.6	11.3
Carbohydrate	gm	209.4	216.9	236.7
Total Fiber	gm %	9.6	10.0	10.9
Protein	kcal %	3.3	3.4	3.4
Fat	kcal %%	9.5	8.6	9.1
Carbohydrate	kcal	86.9	87.8	87.2
Meal Weight	gm	582	594.9	661.5

Table 4

Carotenoid concentrations of gari (means)

Diet	Genotype	All <i>trans</i> -BC		<i>Cis</i> -BC		AC		BC-equivalent*	
		(μg/g)	SEM	(μg/g)	SEM	(μg/g)	SEM	(μg/g)	SEM
ROG	TMS	3.21	0.26	3.11	0.35	2.84	0.22	6.18	0.59
WG	TME 419	0	0	0	0	0	0	0	0
BFG	07/0593	3.37	0.30	4.34	0.36	0	0	5.54	0.53

BC equivalents = all *trans*-BC + 1/2 *cis*-BC + 1/2 AC

TABLE 5

Vitamin A equivalence of BFG and ROG (by weight, n = 8)

ROG		BFG	
<i>by weight</i>		<i>by weight</i>	
(means)	(SEM)	(means)	(SEM)
2.3	0.3	4.2	1.5

* Calculated as nmol of BC = β -carotene equivalents in fortified gari/nmol of retinyl palmitate formed.