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Homogenization, lyophilization or acid-extraction of meat products improves iron uptake from cereal–meat product combinations in an *in vitro* digestion/Caco-2 cell model

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The effect of processing (homogenization, lyophilization, acid-extraction) meat products on iron uptake from meat combined with uncooked iron-fortified cereal was evaluated using an *in vitro* digestion/Caco-2 cell model. Beef was cooked, blended to create smaller meat particles, and combined with electrolytic iron-fortified infant rice cereal. Chicken liver was cooked and blended, lyophilized, or acid-extracted, and combined with $FeSO_4$-fortified wheat flour. In the beef–cereal combination, Caco-2 cell iron uptake, assessed by measuring the ferritin formed by cells, was greater when the beef was blended for the greatest amount of time (360 s) compared with 30 s ($P<0{\cdot}05$). Smaller liver particles (blended for 360 s or lyophilized) significantly enhanced iron uptake compared to liver blended for 60 s ($P<0{\cdot}001$) in the liver–flour combination. Compared to liver blended for 60 s, acid-extraction of liver significantly enhanced iron uptake ($P=0{\cdot}03$) in the liver–flour combination. Homogenization of beef and homogenization, lyophilization, or acid-extraction of chicken liver increases the enhancing effect of meat products on iron absorption in iron-fortified cereals.

Iron uptake: Meat: Caco-2 cells: Bioavailability

Anaemia, resulting from iron deficiency or other causes, affects an estimated two billion people around the world[1]. One approach to preventing iron deficiency and iron-deficiency anaemia is to increase individuals' intake of absorbable iron. Through food, this can be accomplished by increasing individuals' intake of haem iron[2], fortifying foods with bioavailable fortificants[3,4], coupling enhancers such as meat[5] and ascorbic acid[6] with the consumption of non-haem iron, or decreasing or removing inhibitors such as phytate from the food[7,8].

A recent study suggests an additional factor that can increase iron absorption: food particle size. In a trial with human adults, lyophilized beef powder increased by 85 % the non-haem iron absorbed from a whole-wheat gruel containing native iron and ferrous sulphate[9]. The authors speculated that the unusually large enhancing effect may have been due to the small particle size created by lyophilization and pulverization of the beef. While it has been established with iron fortificants that a smaller particle size is more bioavailable than a larger particle size[10–16], the effect of food particle size on iron bioavailability has not been studied.

Acid-extraction of meat is another method that can increase iron absorption. While contributing no significant amount of iron, fish extract added to $FeCl_3$ enhances iron uptake in an *in vitro* digestion/Caco-2 cell model[17]. The effect of acid-extraction of organ meats on iron bioavailability has not been studied.

A Caco-2 cell/*in vitro* model of iron bioavailability has been developed[18], validated[19–21] and applied for many purposes, including the identification of food conditions or combinations that enhance iron bioavailability[22–24]. The advantages of this iron bioavailability model are that it can quickly screen multiple foods or food combinations[25] and that it has yielded qualitatively similar results to human studies comparing the effect of different levels of ascorbic acid and tannic acid on iron bioavailability in semi-synthetic meals and wheat rolls, respectively[21]. In addition, it is a useful tool for generating hypotheses to be tested in human bioavailability studies[26].

The objective of the present study was to evaluate the effect of processing meat (homogenization or lyophilization as proxies for particle size, or acid-extraction) on iron uptake from meat combined with an iron-fortified cereal using an *in vitro* digestion/Caco-2 cell model.

Materials and methods

Chemicals, enzymes and hormones

Unless otherwise stated, all chemicals, enzymes and hormones were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The source of FeCl3 was a 1·04 g Fe/l solution in 1 % HCl (Sigma #1-9011).

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Food selection

The foods used in the present study had been evaluated by Peruvian mothers for their acceptable use as ingredients in an infant porridge[27]. These included locally available iron-fortified foods (Gerber infant rice cereal and Peruvian wheat flour) and meat products (beef, chicken liver and fish).

Experiment 1

Experiment 1 was conducted to evaluate the effect of cooked beef of different particle sizes on the uptake of iron from the beef and infant rice cereal mixture. The electrolytic iron-fortified infant rice cereal (529·34 μg Fe/g; Gerber Products Company, Fremont, MI, USA) and cube steak were purchased locally (P&C Supermarket, Ithaca, NY, USA). The cube steak was cut into approximately 40 g pieces, cooked in a microwave oven (Kenmore model 565·69400890, 1000 W; Hoffman Estates, IL, USA) for 2 min, and after cooling for ≥15 min placed in a blender sold for consumer use (Waring model 51BL31; Torrington, CT, USA) on low speed for the following time: 30, 60, 90, 120, 240 or 360 s. To 1·5 g uncooked infant rice cereal, 0·5 g cooked beef (70·6 μg Fe/g) was added; this combination contained 311–362 μg Fe/g. As a positive control, an ascorbic acid-containing juice was used because of its known enhancing effect on non-haem iron absorption[6]. Specifically, 0·5 g Tropicana orange juice (1·0 μg Fe/g; Tropicana Products Inc., Bradenton, FL, USA) was added to 1·5 g infant rice cereal.

Experiment 2

Experiment 2 was conducted to evaluate the effect of cooked chicken liver of different particle sizes on the uptake of iron from the liver and iron-fortified wheat flour mixture. The chicken liver was purchased locally (P&C Supermarket). The ferrous sulphate-fortified wheat flour (49·85 μg Fe/g) was purchased in Peru (S. Wong, Lima, Peru). The chicken liver was cut into approximately 40 g pieces, cooked in a microwave oven (Kenmore model 565·69400890) for 3 min, and after cooling for ≥15 min placed in a blender (Waring model 51BL31) on low speed for 60 or 360 s. Based on photographs of the blended beef used in Experiment 1, blending for 30 s resulted in coarse, non-homogeneous meat sizes which made pipetting difficult, therefore, the next time-point used in Experiment 1 (60 s) was used as the lowest time-point in Experiment 2. To 0·6 g Peru wheat flour, 0·2 g blended chicken liver (355·0 μg Fe/g) was added. Some cooked chicken liver was lyophilized (VirTis Benchtop Freeze Dryer; Gardiner, NY, USA) and ground to a powder (Krups Fast-touch Coffee-grinder; Peoria, IL, USA). Since lyophilized and ground chicken liver weighs approximately 51 % of unprocessed cooked chicken liver, 0·102 g lyophilized chicken liver was added to 0·6 g Peru wheat flour. The flour–liver combinations contained 42–77 μg Fe/g.

Since the acid extract of fish muscle has been found to enhance non-haem iron absorption[17], we wanted to explore if an acid extract of chicken liver had the same effect. Therefore, a low-pH extraction of 0·102 g lyophilized chicken liver was combined with 0·6 g Peru wheat flour. To prepare the extraction, lyophilized cooked chicken liver (0·102 g) was mixed with 3·4 ml 0·01 M-HCl, and brought to pH 2·0 with 5 M-HCl. It was incubated on a rocking platform shaker at 37°C in an incubator with 5 % CO_2 and 95 % air for 1 h. Following the incubation, the mixture was centrifuged (IEC Model HN-SII Centrifuge; Needham, MA, USA) at 700 ***g*** for 30 min. The supernatant was collected and mixed with the flour. As a positive control, the acid extract of 0·2 g lyophilized cooked haddock fillet fish powder (5·2 μg Fe/g) was added to 0·6 g Peru wheat flour. The acid extract of the fish powder was prepared like the extract of the chicken liver except that the fish powder was initially mixed with 6·66 ml 0·01 M-HCl.

In sum, four comparisons were evaluated in this experiment: flour–liver combination where the chicken liver was blended for 60 s *v.* flour–liver blended for 360 s (homogenization effect); flour–liver blended for 60 s *v.* flour–liver where the chicken liver was lyophilized (lyophilization effect); flour–liver blended for 60 s *v.* flour–liver using chicken liver extract (acid-extract effect); and flour–liver where the chicken liver was lyophilized *v.* flour–liver using chicken liver extract (lyophilization *v.* acid-extract effect).

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17, and used in experiments at passages 25–33. Cells were seeded at a density of 50 000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA, USA). The cells were grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) with 10 % (v/v) fetal calf serum (Gibco) and 25 mmol/l HEPES. The cells were maintained at 37°C in an incubator with 5 % CO_2 and 95 % air atmosphere at constant humidity. The medium was changed every 2 d. The cells were used in the iron uptake experiments at 13 d post-seeding.

In vitro *digestion*

The *in vitro* digestion protocol was performed as described previously[18]. Briefly, to each food condition, at pH 2 a pepsin solution was added, followed 1 h later by a pancreatin–bile acid solution at pH 5·5–6·0. A 1·5 ml portion of the resulting food digest (15 ml) was placed in the upper chamber of each cell-culture well created by attaching a dialysis membrane (15 kDa molecular weight cut-off) to an insert ring; Caco-2 cells had been grown on the bottom of the six-well plates. Iron from food digests placed in the upper chamber for 2 h dialyses through the membrane and becomes accessible for uptake by the Caco-2 cells. The dialysis membrane is necessary to protect the cells from the digestive enzymes (pepsin, pancreatin, bile), similar to the protection provided by the mucus layer in the human intestine. As is standard operating procedure for the laboratory, each food condition was sampled six times and each sample was independently subjected to the *in vitro* digestion/Caco-2 cell system. Ferritin formation by the Caco-2 cells, a marker for cell iron uptake, was used as the indicator of iron bioavailability.

Measurement of ferritin and total iron

All glassware used in the sample preparation and analyses was acid-washed. Caco-2 cell protein was measured on samples that had been solubilized in 0·5 mol/l NaOH, using a semi-micro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay; RAMCO Laboratories, Houston, TX, USA). A 10 μl sample of the sonicated Caco-2 cell monolayer, harvested in 2 ml water, was used for each ferritin measurement. An aliquot of the food digests, prior to being placed in the upper chamber of the culture well, was acid- and heat-digested to analyse its iron concentration using an inductively coupled plasma emission spectrometer (ICAP model 61E Trace Analyzer; Thermo Jarrell Ash Corporation, Franklin, MA, USA). The ferritin was expressed per unit of cell protein (ng ferritin/mg cell protein).

Statistical methods

Statistical analyses were completed with GraphPad Prism v4 (GraphPad Software, San Diego, CA, USA). Iron concentration and ferritin formation were log-transformed to better approximate a normal distribution and one-way ANOVA was performed. For specific *a priori* comparisons, an appropriate, limited number of single degree of freedom linear contrasts were run to compare the means of the experimental groups using Student's *t* tests[28]. Means were considered to be statistically significantly different if $P<0·05$.

Results

The iron concentration in the infant rice cereal with different beef particle sizes is shown in Fig. 1. Compared to the cereal with beef blended for 30 s, there was statistically more iron in the digests containing beef blended for 60 s ($P<0·01$), 90 s ($P<0·05$) and 360 s ($P<0·05$) and the same iron in the digests with beef blended for 120 or 240 s ($P\geq0·05$). To account for the unexpected differences in iron concentration among the cereal–beef combinations, likely due to technical difficulties in generating homogenous food samples, the ferritin formed by the Caco-2 cells was expressed as a percentage of the total iron in the food digest and compared (Table 1). Per unit iron, the percentage Caco-2 cell ferritin formed was statistically greater in the cereal–beef combination with beef blended for 360 s than in beef blended for 30 s ($P<0·05$).

The second experiment compared iron-fortified wheat flour combined with blended, lyophilized or acid-extracted chicken liver. The iron concentrations for the homogenization-effect pair and the acid-extract effect pair were comparable ($P>0·05$, Fig. 2); for the lyophilization-effect ($P<0·05$) and the lyophilization *v.* acid extract-effect ($P<0·01$) pairs, the iron concentrations were different. To account for the unexpected differences in iron concentration among the flour–liver combinations, the Caco-2 cell ferritin formation per unit iron was calculated and compared (Table 1). Per unit iron, the percentage Caco-2 cell ferritin formed was higher in the flour-containing digests with chicken liver blended for 360 s than with liver blended for 60 s ($P<0·001$), in the flour–liver combination where chicken liver was lyophilized compared to liver blended for 60 s ($P<0·001$), and in the flour–liver combination with acid-extract liver compared to liver blended for 60 s ($P=0·03$). There was no difference in the percentage Caco-2 cell ferritin formed between the lyophilized and acid-extract pairs ($P>0·05$).

Discussion

To our knowledge, this is the first evaluation of the effect of food particle size (as proxied by homogenization and lyophilization) on iron absorption. Using an *in vitro* digestion/Caco-2 cell method, we tested the hypothesis that smaller meat particles have a greater enhancing effect on iron absorption than larger meat particles. We found that beef blended for the most amount of time (360 s) significantly enhanced the iron uptake in the cereal–beef blend when compared with beef blended for 30 s, and that the smallest chicken liver particles (lyophilized or blended for 360 s) significantly enhanced the iron uptake in the flour–liver combinations compared to liver blended for 60 s. The present data suggest that as with iron fortificants, smaller meat particles obtained through homogenization or lyophilization have a greater enhancing effect on iron absorption than larger meat particles. Further, the 140 % enhancement of iron absorption using lyophilized chicken liver and wheat flour we observed is of the same magnitude as the 85 % enhanced absorption measured by Hallberg *et al.*[9] using lyophilized

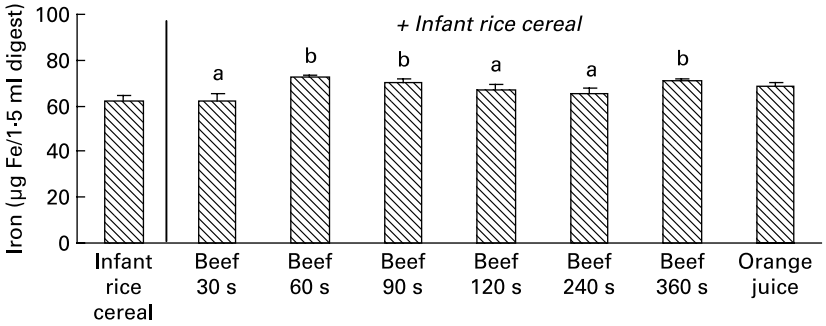

Fig. 1. The iron concentration of electrolytic iron-fortified infant rice cereal (1·5 g) combined with orange juice (0·5 g) or cooked beef (0·5 g) blended for different lengths of time. Using Student's *t* test, each cereal–beef combination was statistically compared to the cereal–beef combination where the beef was blended for 30 s. Values are means with their standard errors depicted by vertical bars (*n* 6). [a,b] Mean values with unlike letters were significantly different ($P<0·05$). Bars with no letters indicate that no statistical tests were carried out with these foods groups as these were not integral to the experiment hypothesis.

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Table 1. Ferritin formed per unit iron in the beef–cereal (Experiment 1) and liver–flour (Experiment 2) mixtures*
(Mean values with their standard errors)

Experiment 1			Experiment 2					
	Ferritin formed per unit iron (% of reference)			Ferritin formed per unit iron (% of reference)			Ferritin formed per unit iron (% of reference)	
Food condition	Mean	SEM	Food condition	Mean	SEM	Food condition	Mean	SEM
Infant rice cereal (reference)	100[a]	–	Flour + liver 60 s (reference)	100[a]	–	Flour + liver lyophilized (reference)	100[a]	–
Cereal + beef 30 s	102[a]	18	Flour + liver 360 s	466[b]	56	Flour + liver extract	129[a]	16
Cereal + beef 60 s	106[a]	11	Flour + liver lyophilized	207[b]	22			
Cereal + beef 90 s	106[a]	16	Flour + liver extract	267[b]	33			
Cereal + beef 120 s	101[a]	23						
Cereal + beef 240 s	112[a]	18						
Cereal + beef 360 s	140[b]	25						

[a,b] Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).
* For details of subjects and procedures, see Materials and methods.

beef and whole wheat gruel. In terms of acid extraction, we found that acid-extraction of chicken liver increased iron uptake and had the same effect on iron absorption in the flour–liver combinations as lyophilized liver.

Particle size

Several mechanisms have been suggested for the higher bioavailability of iron fortificants: size[10,11,13], solubility[14,16], surface area[14,29] and density[30]. A recent series of studies to evaluate the bioavailability of commercial elemental iron powders used in food fortification found that size (particle and subsieve), solubility and surface area but not density (pycnometric or apparent) of several fortificants were related with iron bioavailability as assessed by the relative bioavailability method in rats[31]. Fortificant size (particle and subsieve) was in turn inversely correlated with surface area and solubility, and the latter two were positively correlated, suggesting that these properties are all a function of the same phenomenon and do not independently contribute to greater bioavailability. The particle size (D_{50}) in these fortificants ranged from 6·78 μm in carbonyl fortificants to 35·99 μm in CO-reduced fortificants. Rohner *et al.* [32] found that by reducing the particle size of a poorly soluble iron fortificant to a nano scale (from 64·2 to 30·5 or 10·7 nm), solubility, surface area and rat relative bioavailability increased. In other words, at different size scales (i.e. μm and nm), the reduction in fortificant size led to greater solubility, surface area and iron bioavailability. The size of the homogenized or lyophilized meat products used in the current study was not assessed; however, based on photographs of the different meat products, there was a clear difference in meat size at the extremes of the blending time (i.e. size was larger at 30 or 60 s of blending compared with 360 s of blending) and these in turn were larger than the ground and lyophilized liver powder. The findings of the current study suggest that with food, as with fortificants, the principle holds that a relative reduction in size enhances iron bioavailability.

We cannot exclude that other factors which differed between the experiments could have influenced the uptake of iron in each experiment. For example, the predominant iron form in the two experiments differed; it was electrolytic iron in the first and a combination of ferrous sulphate, haem iron and non-haem liver iron in the second. Regardless of these differences between

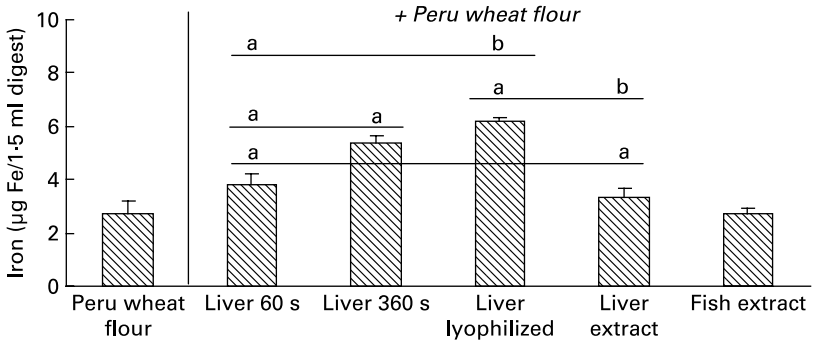

Fig. 2. The iron concentration of ferrous sulphate-fortified Peru wheat flour (0·6 g) combined with cooked chicken liver (0·2 g) blended for 60 or 360 s, lyophilized chicken liver (0·102 g), acid extract from 0·102 g lyophilized chicken liver or acid extract from 0·2 g lyophilized haddock fillet. Using Student's *t* test, four pairs were tested: flour–liver combination where the chicken liver was blended for 60 s *v.* flour–liver blended for 360 s (homogenization effect); flour–liver blended for 60 s *v.* flour–liver where the chicken liver was lyophilized (lyophilization effect); flour–liver blended for 60 s *v.* flour–liver using chicken liver extract (acid-extract effect); and flour–liver where the chicken liver was lyophilized *v.* flour–liver using chicken liver extract (lyophilization *v.* acid-extract effect). Values are means with their standard errors depicted by vertical bars (*n* 3). For each pair, values with unlike letters were significantly different ($P<0.05$). Bars with no letters above them indicate that no statistical tests were carried out with these foods groups as these were not integral to the experiment hypotheses.

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the experiments, it is noteworthy that the same trends were observed within each experiment: smaller meat sizes yielded more bioavailable iron than larger sizes.

The present data indicate that processing meat in a blender for a long period is as effective or more as lyophilizing and grinding the meat; this should be tested in a human bioavailability study. If the results are comparable, this has implications for the applicability of the results in resource-poor households at risk for iron deficiency. Specifically, as a means to increasing the bioavailability of non-haem iron consumed with meat, families could be recommended to reduce meat size through use of a blender or other locally available method. This, coupled with recommendations to cook in iron pots[33] and modifications to food selection and processing[34], could increase the bioavailable iron consumed.

Acid extracts

With respect to the meat factor that enhances non-haem iron absorption, many constituents have been studied: amino acids[35,36], animal protein[37–40] and carbohydrate fractions[17,41]. From acid extracts of fish, heparin-like glycosaminoglycans increased iron uptake in an *in vitro* digestion/Caco-2 cell model[17]; the active fractions were identified as the low-molecular-weight carbohydrate degradation products of the glycosaminoglycans. A human bioavailability study with purified glycosaminoglycans that were not acid-extracted found no improvements in iron bioavailability compared to a semi-synthetic meal with no glycosaminoglycans[41]. The key to glycosaminoglycans improving iron bioavailability may lie with their acid extraction from a food source[42]. In the current study, the enhancing effect of acid-extracted fish originally observed by Huh *et al.* [17] was replicated (data not shown), and in the same magnitude as for the acid-extracted chicken liver (131 % ferritin formed per unit iron compared with the reference in Table 1). The influence of the liver extract on iron uptake was comparable to the lyophilized liver, although the putative mechanism is not size but an as yet undetermined method presumably mediated by the carbohydrate fractions. Whether the active fractions of the liver and fish extracts are similar warrants investigation.

There were several technical difficulties encountered in the present study. First, though the iron concentration of the beef-containing samples in the first experiment and the chicken liver samples in the second experiment were designed to be the same, they were different. We believe that these differences are due to technical difficulties in generating homogenous food samples. Specifically, running the meat products through the blender resulted in non-homogeneous food sizes which in turn produced difficulties during pipetting of the samples through the *in vitro* digestion process. To overcome this difficulty, we expressed the results as percentage Caco-2 cell ferritin formed per unit iron in the digest. Second, we had no objective assessment of sizes of the different meat particles.

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