

## Short Communication

# New Zealand green-lipped mussels (*Perna canaliculus*) enhance non-haem iron absorption *in vitro*

Robin J. C. Stewart, Jane Coad, Gordon W. Reynolds and Kevin C. Pedley\*

*Institute of Food, Nutrition and Human Health, Massey University (Turitea), Tennent Drive, Palmerston North, New Zealand*

(Submitted 10 June 2011 – Final revision received 13 October 2011 – Accepted 29 October 2011 – First published online 3 January 2012)

### Abstract

Fe bioavailability can be manipulated by the nutritional composition of a meal. Ascorbic acid and unidentified components of meat, fish and poultry, but particularly beef, all appear to enhance the absorption of non-haem Fe. The aim of the present study is to identify whether extracts of green-lipped mussels (GLM; *Perna canaliculus*) enhance non-haem Fe absorption in Caco-2 cells and to compare the effect with that of beef. Raw GLM and raw beef homogenates were digested *in vitro* with pepsin at pH 2, and pancreatin and bile salts at pH 7. Tracer <sup>55</sup>Fe was used to measure cellular Fe uptake. Ascorbic acid was used as a positive control and egg albumin, exposed to the same *in vitro* digestion process, was used as a negative control. Caco-2 cell monolayers were incubated with treatments for 60 min. All values were standardised per µg of GLM, egg albumin, beef or ascorbic acid. The results showed that ascorbic acid enhanced non-haem Fe absorption to the highest degree. Beef and GLM digestates both significantly enhanced Fe absorption compared with egg albumin. In conclusion, GLM digestate significantly enhances non-haem Fe uptake in Caco-2 cells with a similar magnitude to that of beef.

**Key words:** Iron absorption: Mussels: Caco-2: Meat factor

Red meat, pork, poultry and fish all significantly enhance Fe absorption when consumed as part of a vegetable-based meal in human subjects<sup>(1)</sup>. Further investigation into the bioactive components of meat, poultry and fish factor suggests that cysteine-rich myofibrils<sup>(2)</sup>, glycosaminoglycans<sup>(3)</sup> and L-α-glycerophosphocholine<sup>(4)</sup> could be responsible for this enhancing effect, either individually or in combination<sup>(5)</sup>. Other dietary factors including PUFA<sup>(6)</sup> and non-digestible soluble carbohydrates including inulin and fructooligosaccharides<sup>(7)</sup> may also increase non-haem Fe absorption; however, the magnitude of enhancement and the mechanism remains elusive.

Of all meat sources, beef has been repeatedly reported to promote Fe absorption to the highest degree in human subjects<sup>(1,4,8)</sup>. However, addition of SFA to the diet via increased meat consumption or the replacement of dietary PUFA with SFA is strongly associated with negative cardiovascular outcomes, while the reverse is cardio-protective<sup>(9)</sup>. For this reason, promoting red meat consumption in order to improve Fe status at a population level may be problematic, and the identification of an alternative meat, poultry and fish factor

source rich in PUFA or low in SFA is warranted. The effect of oily fish on Fe uptake has been investigated *in vivo*; however, protocol inconsistencies have led to contradicting results<sup>(6,10,11)</sup>.

There has been little investigation into the effect of bivalve molluscs on non-haem Fe absorption both *in vitro* and *in vivo*. New Zealand green-lipped mussels (GLM, *Perna canaliculus*) are rich in both haem and non-haem Fe, myofibrillar proteins, low-molecular-weight aminoglycans and *n*-3 PUFA<sup>(12)</sup>, and may therefore provide an alternative source of meat factor. The aim of the present study is to investigate the effects of GLM digestate on non-haem Fe uptake in Caco-2 cell monolayers and compare its effects with that of ascorbic acid, egg albumin and beef.

### Materials and methods

#### Cell culture

Caco-2 cells were sourced from the American Type Culture Collection and cultured in T75 flasks (Nunc) in 15 ml of cell-culture medium (D10), adjusted to pH 7.4, containing

**Abbreviations:** GLM, green-lipped mussels; TEER, transepithelial electrical resistance.

\* **Corresponding author:** Associate Professor K. C. Pedley, fax +64 6 350 5657, email k.c.pedley@massey.ac.nz

Dulbecco's modified Eagle's minimal essential medium (Invitrogen), fetal bovine serum (10%) (Invitrogen), penicillin–streptomycin–neomycin (1%; Invitrogen), non-essential amino acids (1%; Invitrogen) and Glutamax (1%; Invitrogen) at 37°C with 5% CO<sub>2</sub> and 90% humidity. Cells were obtained at passage 20 and used between passages 30 and 35. Cells were seeded at a density of 50 000 cells/cm<sup>2</sup> on hydrated ThinCert chambers (Greiner Bio-One). The medium was replaced every 2 d and monolayers were used 21 d post-seeding after monitoring the formation of cell junctions using transepithelial electrical resistance (TEER) measurements. Only wells with a TEER value between 250 and 800 Ωcm<sup>2</sup> (after subtraction of the membrane resistance) were used. In parallel, ThinCert chambers were seeded for assessment of cell number to complement the TEER values. Cell nuclei were stained with bisbenzimidazole (Sigma B-2883; Sigma), imaged using a confocal microscope (Nikon C1; Nikon) at 405 nm excitation, and the number of cell nuclei per ThinCert was counted using ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, [imagej.nih.gov/ij/](http://imagej.nih.gov/ij/), 1997–2011).

### Treatments

Fresh GLM flesh (Countdown) was trimmed from the shell to a total weight of 400 g. Egg albumin (Zeagold) was reconstituted to the biological concentration of raw egg albumin by one part egg albumin to seven parts deionised water to a final weight of 400 g. Lean beef sirloin was trimmed of all subcutaneous fat and connective tissue to a final weight of 400 g. All samples were homogenised separately in a Sunbeam 4181 blender (Sunbeam) with 500 ml deionised water.

### In vitro digestion

**Gastric phase.** Porcine pepsin (Sigma P-7000; Sigma) was diluted in 0.1 M-HCl (BDH Chemicals) to a working solution containing 7812 units/ml. Aliquots of GLM, beef and egg albumin homogenates were incubated at 37°C for 30 min. Each 20 g aliquot was titrated with 1 M-HCl (BDH Chemicals) to pH 2.4 and combined with 0.67 ml of pepsin solution. Samples were incubated at 37°C for 2 h in a shaking water-bath.

**Intestinal phase.** Porcine pancreatin (2.4 mg/ml, activity 3 × US Pharmacopeia specifications; Sigma P-1625; Sigma) and porcine bile salts (6.25 mg/ml, glycine and taurine conjugates of hyodeoxycholic and other bile salts; Sigma B-8631; Sigma) were prepared immediately before use in 0.1 M-NaHCO<sub>3</sub>. Samples treated by peptic digest were immediately titrated to pH 6 with 1 M-NaHCO<sub>3</sub> (Sigma). A 5 ml aliquot of pancreatin/bile solution was added to each sample. Samples were incubated for 2 h at 37°C. The digests were immediately stored at –20°C.

The osmolarity of digestates was measured (OsmoLAB 16S; LLA Instruments) and diluted with deionised water to 300 mOsm. Each treatment was further diluted 3-fold with Hanks balanced salt solution (HBSS) to correct K concentration to about 5 mmol/l. The non-haem Fe concentration of each digestate was analysed by spectroscopy using a ferrozine method<sup>(13)</sup> and protein concentration was analysed by

the Bradford assay. Ascorbic acid (95.5 mM-HBSS) was prepared in an ascorbate:Fe molar ratio of 4:1. The beef, GLM and egg albumin digestates and ascorbic acid were combined with a pre-prepared Fe working solution containing 1:10 <sup>55</sup>Fe and <sup>56</sup>Fe, respectively (37 kBq), and titrated to pH 6.5.

**Experimental design.** Digestate treatment aliquots (400 μl) were applied to eight apical reservoirs, each in a randomised order. The uptake solution was aspirated and a 100 μl apical reservoir sample was removed. The Caco-2 cells were incubated for 60 min at 37°C with 5% CO<sub>2</sub> and 90% humidity. At 60 min, one 100 μl aliquot was taken from the basolateral reservoir of each well. The apical and basolateral reservoirs were rinsed twice with HBSS (pH 7.4), and removal solution (0.5 mM-EDTA in HBSS, pH 7.4). Monolayers were solubilised in 0.2 M-NaOH (Sigma) for 12 h at 4°C. The monolayers were aspirated and 100 μl aliquots of cell lysate were removed. All samples were analysed by scintillation counting (Wallac Trilux 1450 Microbeta; PerkinElmer).

All results were standardised per μg of beef, GLM, egg albumin or ascorbic acid. Briefly, the protein concentration of each 300 mOsm digestate was compared with the protein concentration values generated from the proximal analysis of whole sirloin beef, GLM and egg albumin (after subtraction of the protein contribution from digestive enzymes) in order to calculate digestate dilution factor for each treatment. This dilution factor was then used to standardise Fe uptake (%) per μg of whole beef, GLM, egg albumin or ascorbic acid. Fe absorption is then expressed as percentage of Fe absorbed compared with egg albumin digestate.

### Statistical analysis

Statistical analysis was performed using SAS 9.1 statistical software (SAS Institute, Inc.). Treatments were analysed by two-way ANOVA with the general linear model procedure. Where appropriate, *post hoc* analysis was carried out using least squares difference analysis.

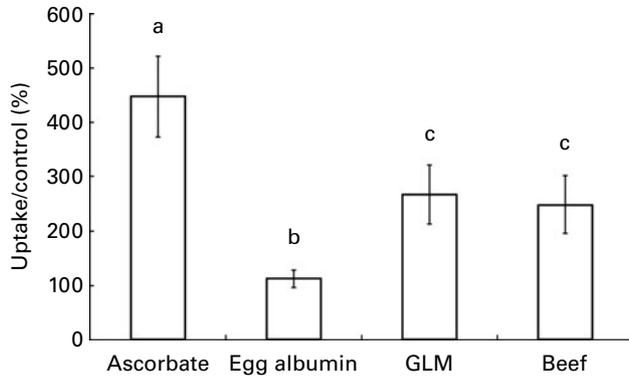
## Results

### Cell-culture viability

All Caco-2 cell monolayers had a TEER value between 250 and 650 Ωcm<sup>2</sup>. None of the monolayers was discarded. There was no significant effect of any treatments on the TEER values after the 60 min incubation (*P* > 0.05). There was no significant effect of treatments on the transport of Fe into the basolateral reservoir after the 60 min incubation. There was also no significant difference in Caco-2 cell numbers per ThinCert (*P* > 0.05).

### Treatment effects on cellular iron uptake

Caco-2 cell Fe uptake was significantly higher in the presence of ascorbic acid compared with the egg albumin (*P* < 0.0001), GLM (*P* = 0.016) and beef digestates (*P* < 0.01). The GLM digestate significantly enhanced Fe uptake compared with the egg albumin digestate (*P* = 0.038). Beef significantly enhanced Fe



**Fig. 1.** Caco-2 cell iron uptake in the presence of ascorbate, egg albumin digestate, green-lipped mussels (GLM) digestate or beef digestate. Caco-2 cell iron uptake was calculated after 60 min incubation with  $^{55}\text{Fe}$  and standardised per  $\mu\text{g}$  of undigested treatment. Results are mean percentage of iron uptake compared with an egg albumin negative control ( $n$  8), with standard errors represented by vertical bars. <sup>a,b,c</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ).

uptake compared with egg albumin ( $P < 0.05$ ), and there was no significant difference between the GLM and beef digestate treatments ( $P = 0.79$ ). The results are illustrated in Fig. 1.

## Discussion

Ascorbic acid consistently enhances Fe absorption approximately 2–6-fold compared with egg albumin in human subjects and cell cultures<sup>(14,15)</sup>. This effect is consistent with the present results clearly showing that when compared with the egg albumin digestate, ascorbic acid enhances Fe absorption by 4- to 5-fold.

Ascorbate has been proposed to enhance non-haem Fe absorption by maintaining ferrous Fe reduction<sup>(16)</sup> or by promoting Fe solubility in oxidising conditions<sup>(17)</sup>. In the present study, the enhancing effect appears to be associated with improving non-haem Fe reduction or solubility rather than competing for Fe chelation with inhibitory Fe chelators that were not present.

The enhancing effect of red meat on Fe absorption has been consistently reported<sup>(18,19)</sup>. The present study indicates that beef enhances Fe uptake 2–3-fold, a magnitude similar to that reported in human subjects<sup>(1)</sup> and cell-culture studies<sup>(20)</sup>.

Interestingly, GLM digestate also consistently enhanced non-haem Fe uptake in Caco-2 cell monolayers with a magnitude similar to that of beef. Although the factors released during GLM digestion remain elusive, it is expected that gastric digestion of GLM would yield cysteine-rich myofibrils from the dorsoventral and adductor muscles, and aminoglycans from the inner and outer tunics of the mantle<sup>(21)</sup>, which may enhance luminal Fe solubility by mechanisms similar to those of beef and fish<sup>(2,3)</sup>.

The GLM lipid fraction consists of  $> 50\%$   $n$ -3 PUFA including up to 15% EPA and 20% DHA<sup>(12)</sup>. As with oily fish, treatment with GLM oil at a moderate dose significantly reduces the pro-inflammatory response both *in vitro*<sup>(22)</sup> and *in vivo*<sup>(23)</sup>; therefore, the replacement of red meat with GLM within the diet may also provide cardioprotective properties.

In summary, we have shown that GLM digestate can enhance non-haem Fe uptake in Caco-2 cells to a similar degree to that of beef. These results suggest that supplementation of GLM may be a healthy alternative to that of red meat in order to improve non-haem Fe absorption. Further investigation into the mechanism of enhancement is warranted.

## Acknowledgements

This study was funded by a postgraduate scholarship from Massey University to R. J. C. S. The experimental work and data analysis were carried out by R. J. C. S. All authors participated equally in the experimental design, the interpretation of the results and the writing of the manuscript. All authors read and approved the final manuscript. The authors state that there are no conflicts of interest.

## References

1. Cook JD & Monsen ER (1976) Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. *Am J Clin Nutr* **29**, 859–867.
2. Mulvihill B, Kirwan FM, Morrissey PA, *et al.* (1998) Effect of myofibrillar muscle proteins on the *in vitro* bioavailability of non-haem iron. *Int J Food Sci Nutr* **49**, 187.
3. Huh EC, Hotchkiss A, Brouillette J, *et al.* (2004) Carbohydrate fractions from cooked fish promote iron uptake by Caco-2 Cells. *J Nutr* **134**, 1681–1689.
4. Armah CN, Sharp P, Mellon FA, *et al.* (2008) L-[alpha]-Glycerophosphocholine contributes to meat's enhancement of nonheme iron absorption. *J Nutr* **138**, 873–877.
5. Laparra JM, Tako E, Glahn RP, *et al.* (2008) Isolated glycosaminoglycans from cooked haddock enhance nonheme iron uptake by Caco-2 cells. *J Agric Food Chem* **56**, 10346–10351.
6. Seiquer I, Aspe T, Perez-Granados A, *et al.* (2002) Consumption of raw and fried sardine *Clupea pilchardus* as protein source of diets: effects on iron metabolism in rats. *J Sci Food Agric* **82**, 1497–1503.
7. Ohta A, Ohtsuki M, Baba S, *et al.* (1995) Effects of fructooligosaccharides on the absorption of iron, calcium and magnesium in iron-deficient anemic rats. *J Nutr Sci Vitaminol* **41**, 281.
8. Cook J & Monsen E (1975) Food iron absorption. I. Use of semisynthetic diet to study absorption of nonheme iron. *Am J Clin Nutr* **28**, 1289.
9. Astrup A, Dyerberg J, Elwood P, *et al.* (2011) The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: where does the evidence stand in 2010? *Am J Clin Nutr* **93**, 684–688.
10. Navas-Carretero S, Perez-Granados AM, Sarria B, *et al.* (2008) Oily fish increases iron bioavailability of a phytate rich meal in young iron deficient women. *J Am Coll Nutr* **27**, 96–101.
11. Rodriguez M, Saiz M, Muntane J, *et al.* (1996) Fatty acid composition of erythrocyte membranes affects iron absorption in rats. *J Nutr* **126**, 3109.
12. Murphy K, Mann N & Sinclair A (2003) Fatty acid and sterol composition of frozen and freeze-dried New Zealand green lipped mussel (*Perna canaliculus*) from three sites in New Zealand. *Asia Pac J Clin Nutr* **12**, 50.

13. Ahn D, Wolfe F & Sim J (1993) Three methods for determining nonheme iron in turkey meat. *J Food Sci* **58**, 288–291.
14. Teucher B, Olivares M & Cori H (2004) Enhancers of iron absorption: ascorbic acid and other organic acids. *Int J Vitam Nutr Res* **74**, 403–420.
15. Garcia MN, Flowers C & Cook JD (1996) The Caco-2 cell culture system can be used as a model to study food iron availability. *J Nutr* **126**, 251–258.
16. Kojima N, Wallace D & Bates G (1981) The effect of chemical agents, beverages, and spinach on the *in vitro* solubilization of iron from cooked pinto beans. *Am J Clin Nutr* **34**, 1392.
17. Conrad M & Schade S (1968) Ascorbic acid chelates in iron absorption: a role for hydrochloric acid and bile. *Gastroenterology* **55**, 35.
18. Kapsokefalou M & Miller D (1993) Lean beef and beef fat interact to enhance nonheme iron absorption in rats. *J Nutr* **123**, 1429.
19. Hurrell RF, Reddy MB, Juillerat M, *et al.* (2006) Meat protein fractions enhance nonheme iron absorption in humans. *J Nutr* **136**, 2808–2812.
20. Glahn R, Wien E, van Campen D, *et al.* (1996) Caco-2 cell iron uptake from meat and casein digests parallels *in vivo* studies: use of a novel *in vitro* method for rapid estimation of iron bioavailability. *J Nutr* **126**, 332.
21. Kier WM (1988) Arrangement and function of molluscan muscle. In *The Mollusca* [KM Wilbur, editor]. Durham, NC: Academic Press, Inc.
22. McPhee S, Hodges L, Wright P, *et al.* (2007) Anti-cyclooxygenase effects of lipid extracts from the New Zealand green-lipped mussel, *Perna canaliculus*. *Comp Biochem Physiol Part B: Biochem Mol Biol* **146**, 346–356.
23. Bui L & Bierer T (2003) Influence of green lipped mussels (*Perna canaliculus*) in alleviating signs of arthritis in dogs. *Vet Ther* **4**, 397–407.