An investigation into the effect of New Zealand green-lipped mussel (Perna *canaliculus*) on non-haem iron absorption

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Robin John Chrys Stewart

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Abstract

The bioavailability of dietary non-haem iron can be influenced by the nutritional composition of a meal. Ascorbic acid and components within meat, fish and poultry, but particularly red meat and pork, all appear to enhance the absorption of non-haem iron when consumed together within a meal compared to other protein sources. However the promotion of red meat or pork is problematic due to their high saturated fatty acid content and alternative foods to red meat or pork that may enhance iron absorption such as oily fish or shellfish require further investigation. The aims of the present study were to investigate the effects of short-term and prolonged supplementation of New Zealand green-lipped mussel (Perna *canaliculus*) (NZGLM) on mucosal iron transport and iron retention. The mechanism(s) by which mucosal iron transport is affected during NZGLM supplementation was also investigated. When investigated *in vitro,* NZGLM and beef both enhanced iron absorption by a similar magnitude compared to egg albumin. The enhancing effect of NZGLM on iron absorption was repeatedly observed using two separate *in vitro* models; Caco-2 cells and mouse small intestine mounted on Ussing chambers. When investigated in iron-deficient mice, mucosal iron transport and extra-intestinal iron retention were significantly enhanced when an iron supplement was combined with NZGLM compared to egg albumin. Conversely, an inhibitory effect was observed when mice were supplemented with NZGLM for a prolonged period of time prior to consuming an iron supplement. The inhibitory effect of NZGLM was not associated with the dietary iron load. Prolonged NZGLM supplementation inhibits mucosal iron absorption by reducing brush border

iron transport. The inhibitory effect of prolonged NZGLM supplementation was observed to be associated with its high calcium content; however other competitive nutrients such as copper, manganese or zinc may also contribute to the inhibitory effect. The main inhibitory effect is proposed to be calcium-stimulated DMT1 internalisation into cytosolic vesicles. This occurs after prolonged or repeated NZGLM supplementation. The findings of this study suggest that NZGLM enhances mucosal iron transport and distribution to extra-intestinal tissues when consumed as a single dose with an iron supplement. For this reason NZGLM may be an alternative iron absorption enhancer to red meat or pork with additional cardio-protective properties. Repeated NZGLM supplementation may reduce mucosal transport; therefore repeated NZGLM supplementation should be moderated in order to ensure that mucosal iron transport is not compromised.

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%	*Percent*
Δ	*Change*
°C	*Degrees Celsius*
µg	*Micrograms*
µL	*Microliter*
µM	*Micromole/Litre*
β-CPP	*β-casein phosphopeptide*
Ω	*Ohms*
Ω/cm^2	*Ohms per centimetre squared*
AA	*Ascorbic acid*
AHA	*American Heart Association*
ANOVA	*Analysis of variance*
AP	*Alkaline phosphatase*
Asc	*Ascorbate*
ATCC	*American Type Culture Collection*
ATP	*Adenosine triphosphate*
BMP (2,4,6)	*Bone morphogenetic proteins (2,4,6)*
$CaCl_2$	*Calcium chloride*
Caco-2	*Colorectal carcinoma cell line*
cm	*Centimetre*
cm^2	*Square centimetre*
CO_2	*Carbon dioxide*
cpm	*Counts per minute*
CS	*Chondroitin sulfate*
DcytB	*Duodenal cytochrome B*
DHA	*Dehydroascorbic acid*
DMEM	*Dulbecco's modified eagle medium*
DMSO	*Dimethyl sulfoxide*
DMT1	*Divalent metal transporter 1*
EDTA	*Ethylenediaminetetraacetic acid*
ELISA	*Enzyme-linked immunosorbent assay*
ERT	*Extrinsic radionuclide tracer*
FAK	*Focal Adhesion Kinase*
FBS	*Foetal bovine serum*
Fe	*Iron*
Fe^{2+}	*Ferrous iron*
Fe^{3+}	*Ferric iron*
$FeCl_3$	*Ferric chloride*
$FeSO_4$	*Ferrous sulphate*
FLVCR	*Feline leukemia virus subgroup C receptor-related protein*
FOS	*Fructooligosaccharide*

g	*Grams*
g/L	*Grams per litre*
GAG	*Glycosaminoglycan*
GLUT (1,2,3)	*Glucose transporter (1,2,3)*
H^+	*Hydrogen*
HA	*Hyaluronic acid*
HBSS	*Hanks balanced salt solution*
HCl	*Hydrochloric acid*
HFE	*High iron gene*
HHC	*Hereditary hemochromatosis*
HJV	*Hemojuvelin*
HO (1,2)	*Haem oxidase (1,2)*
ID	*Iron deficiency*
IDA	*Iron deficiency anaemia*
IL-6	*Interleuken-6*
IMP	*Integrin mobilferrin paraferritin*
IRE	*Iron response element*
IREG1	*Iron regulating protein 1*
IRP	*Iron response protein*
IRT	*Intrinsic radionuclide tracer*
K^+	*Potassium*
KCl	*Potassium chloride*
Kg	*Kilogram*
LIP	*Labile iron pool*
LPS	*Lipopolysaccharide*
LSD	*Least square difference*
L-α	*L-a-Glycerophosphocholine*
M	*Mole/litre*
MAPK	*Mitogen-activated protein kinase*
mg	*Milligram*
mg/L	*milligrams per litre*
min	*Minute*
mL	*Millilitre*
mm	*Millimetre*
mM	*Millimole/litre*
mOsm	*Milliosmole/kg*
MPF	*Meat poultry fish*
MPF factor	*Meat poultry fish factor*
mRNA	*Messenger ribonucleic acid*
MUFA	*Monounsaturated fatty acid*
mV	*Millivolts*
n	*Number*
n-3 PUFA	*Omega three polyunsaturated fatty acid*
NA	*Nitric acid*
Na^+	*Sodium*
Na^+/K^+ATPase	*Sodium/potassium ATPase pump*
NaCl	*Sodium chloride*

$NaHCO_3$	*Sodium bicarbonate*
NHE	*Sodium hydrogen exchanger*
nM	*Nanometres*
NRAMP	*Natural resistance-associated macrophage protein*
NZBS	*New Zealand blood service*
NZGLM	*New Zealand green-lipped mussel*
O_2	*Oxygen*
OH	*Hydroxide*
P	*Probability*
PBS	*Phosphate buffered saline*
PC	*Polycarbonate*
PCFT/HCP1	*Haem carrier protein 1*
PE	*Polyethylene*
PET	*Polyethylene terephthalate*
ppm	*parts per million*
PUFA	*Polyunsaturated fatty acid*
RBC	*Red blood cell*
rpm	*Revolutions per minute*
SCFA	*Short chain fatty acids*
SEM	*Standard error of the mean*
SFA	*Saturated fatty acid*
SGLT (1,2)	*Sodium-dependent glucose co-transporter (1,2)*
SI	*Sucrose isomaltase*
SS	*Semi-synthetic*
STAT	*Signal Transducer and Activator of Transcription*
STEAP3	*STEAP family member 3*
TCA	*Trichloroacetic acid*
TEER	*Trans-epithelial electrical resistance*
TfR (1,2)	*Transferrin receptor (1,2)*
UTR	*Un-translated region*
w/w	*Weight for weight*
WBC	*White blood cell*
WHO	*World Health Organization*
ZIP (8, 14)	*Zrt- and Irt-like protein (8, 14)*
Zn	*Zinc*

Chapter One: General introduction, aims and objectives

1.1. Thesis layout

The thesis is divided into ten chapters, including six experimental chapters. The experimental chapters of this thesis are based on manuscripts prepared for publication in scientific journals. Specific publication details are noted within their respective chapters.

Chapter One is a general introduction which describes the thesis layout. This chapter also introduces the importance of iron in human physiology, the problems associated with compromised iron status and nutritional interventions which may overcome these problems. These processes will be discussed in greater detail in Chapter Two which reviews the iron literature. Finally, the general introduction will introduce the aim of the thesis and the specific aims of the individual studies.

Chapter Two is a literature review of the physiological and chemical processes associated with iron absorption in humans and nutritional interventions which may be used to increase the fraction of iron absorbed from a meal. The role of this chapter is to provide an overview of the major gastro-intestinal, mucosal and systemic processes associated with iron absorption. The review will also appraise the role and mechanisms of action of specific nutrients in enhancing or inhibiting iron absorption.

Chapter Three describes the general methods used throughout the experimental chapters of the thesis. This includes basic analytical methods, treatments, sample preparation and general *in vitro* techniques. Two manuscripts based on the

development of the *in vitro* iron uptake assay are also included within Chapter 3. The first is a critical review of the Caco-2 cell culture literature. This manuscript proposes a standardised method for preparing Caco-2 cell cultures for investigating iron uptake *in vitro*. The second manuscript investigates the *in vitro* digestion protocol in order to optimise the digestion conditions and best prepare the treatment for subsequent analysis.

Chapter Four investigates the effects of red meat, NZGLM digestate, egg albumin digestate and ascorbic acid on non-haem iron absorption in Caco-2 cells. The aim of this study is to observe the effect of these treatments, once digested *in vitro,* on cellular iron absorption.

Chapter Five further investigates the effects of ascorbic acid, cooked/raw NZGLM digestate and cooked/raw egg albumin digestate on mucosal iron absorption in isolated sections of mouse small intestine. The aim of this study is to assess the repeatability of the results observed in Chapter Four using a second *in vitro* model. This chapter also investigates the effects of cooking the treatments prior to digestion.

Chapter Six investigates the effects of short-term and prolonged NZGLM supplementation on iron absorption in iron deficient mice. The aim of this study is to investigate the repeatability of the results observed *in vitro* and to investigate whether prolonged NZGLM supplementation further affects iron absorption and retention.

Chapter Seven investigates the effect of calcium supplementation on iron absorption in Caco-2 cells. The aim of the study is to differentiate the inhibitory effects of short-term and long-term calcium supplementation over a range of calcium concentrations in order to elucidate the mechanisms by which calcium supplementation reduces iron absorption.

Chapter Eight investigates the effect of endogenous calcium within NZGLM digestate on iron absorption in Caco-2 cells. The aim of this study is to identify whether the calcium present within NZGLM is associated with the inhibitory effect of prolonged NZGLM supplementation on cellular iron absorption or if other factors within NZGLM are responsible for this inhibition of iron absorption.

Chapter Nine describes a human trial which has been proposed in order to investigate the effect of NZGLM, ascorbic acid, red meat or egg albumin supplementation on iron repletion in iron-deficient human participants. The aim of this proposed study is to investigate the repeatability of the results observed *in vitro* and *in vivo* on systemic iron repletion in iron-deficient human participants.

Chapter Ten describes and summarises the general findings of the thesis and discusses the study limitations, recommendations and outlines future perspectives.

1.2. Background

Despite an abundance of iron in the earth's crust, iron deficiency affects up to two thirds of infants, children and women of a childbearing age in developing countries

(Scrimshaw 1991) and up to one fifth of infants, children and women of a child bearing age in developed countries (WHO 1993-2005). The severe form of iron deficiency, iron deficiency anaemia affects over 30% of the world's population.

Both iron deficiency and iron deficiency anaemia are associated with fatigue (Greig et al. 2013), reduced cognitive and physical performance (Leonard et al. 2014) and ultimately mortality (Rasmussen 2001). The consequence of anaemia-associated reduction in physical and cognitive productivity in South Asia alone has been estimated to cost approximately $32 billion per annum (Horton and Ross 2003). These estimates do not include the economic cost of death or hospitalisation.

Marginal iron deficiency occurs when iron stores can no longer sustain cellular iron requirements. Iron deficiency anaemia occurs when iron stores cannot sustain cellular requirements or haemoglobin synthesis, and therefore oxygen distribution is also compromised.

During iron deficiency and iron deficiency anaemia, iron-dependent enzyme synthesis is compromised and the ability of cells to undertake critical oxidation/reduction processes is reduced (Maguire et al. 1982). This severely affects the development and activity of cells which have a high demand for energy or require iron for undertaking specific oxidative reactions (Beard 2001). For this reason marginal iron deficiency and iron deficiency anaemia are associated with reduced energy metabolism, abnormal cognitive development (Batra and Sood 2005), reduced cognitive function (Leonard et al. 2014) and compromised immune function (Thibault et al. 1993).

Obligatory iron losses as a result of blood loss, sweating and sloughing of epithelia or augmented iron requirements during growth or expanding blood volume must be countered by the release of iron from body stores and by augmented iron absorption from the diet across the intestinal mucosa (Barrett et al. 1994; Coad and Conlon 2011). Iron deficiency develops when systemic iron recycling and mucosal iron transport cannot adequately replace iron requirements or losses.

Mucosal iron transport is affected by the type and concentration of iron within the meal and the presence of specific nutrients within the meal which interact with iron and affect its bioavailability (Hallberg 1981). Mucosal iron transport is also affected by the amount of iron stored within the mucosa (Eisenstein and Blemings 1998), systemic iron status (Nemeth et al. 2004) and systemic inflammation (Semrin et al. 2006). Inflammation simultaneously reduces iron absorption and decreases systemic iron availability from cellular iron stores (Weiss and Goodnough 2005).

In the absence of systemic inflammation, the transfer of iron from the digesta into the absorptive enterocyte is the limiting step for systemic iron repletion during iron deficiency. Improving enterocyte iron absorption by increasing the concentration of soluble iron within the diet is a strategy to protect against, or overcome iron deficiency (Røsvik et al. 2010). This is achieved by increasing the iron load within the diet (haem iron and non-haem iron), by improving the bioavailability of dietary iron (including iron absorption enhancers and excluding iron absorption inhibitors within meals) (Beck et al. 2011), or a combination of the two (Heath et al. 2001).

Haem iron is sourced exclusively from animal tissue. The iron molecule is incorporated within an organic porphyrin ring and is absorbed rapidly due to its high level of solubility (Conrad et al. 2000; Shayeghi et al. 2005). Haem iron absorption may be affected by the presence of a select number of nutrients consumed concurrently (Hallberg et al. 1991; Laftah et al. 2009).

Non-haem iron is present in both animal and plant food sources. Dietary non-haem iron can exist in two major chemical forms; either the reduced ferrous (Fe^{2+}) or the oxidised ferric (Fe^{3+}) form. The oxidative state of non-haem iron depends on the pH of the surrounding medium and the presence of reducing agents (Cook and Monsen 1977). The oxidation state of non-haem iron will influence its solubility (Jackman and Black 1951) and bioaccessibility (Gunshin et al. 1997). Non-haem iron absorption is also significantly influenced by the presence of a number of absorption-enhancing or inhibiting nutrients when consumed prior to (Rodríguez et al. 2003) or concurrently with this dietary iron source (Layrisse et al. 1969).

Non-haem iron absorption has been consistently reported to be up-regulated when consumed with red meat, poultry, pork or fish (MPF) (Cook and Monsen 1976; Kapsokefalou and Miller 1993; Baech et al. 2003). Select macronutrient fractions including cysteine rich myofibrils (Mulvihill et al. 1998), aminoglycans (GAG) (Huh et al. 2004), n-3 polyunsaturated fatty acids (PUFA) (Miret et al. 2003) and phosphatidylcholine (Armah et al. 2008) have been suggested as the potential bioactive components of MPF.

Red meat and pork have been reported to have the strongest iron absorption enhancing effect of all MPF factors in human participants (Cook and Monsen 1976) and have been repeatedly reported to enhance mucosal iron transport in animal models (Gordon and Godber 1989) and cell models (Glahn et al. 1996). These observations suggest that promoting red meat consumption with main meals may be a dietary strategy to improve iron status, and protect against iron deficiency.

Although red meat has beneficial effects on iron uptake, the saturated fatty acid (SFA) load associated with increased meat consumption (especially if consumed as an alternative to polyunsaturated fatty acids) is strongly associated with negative cardiovascular outcomes, while the reverse may be cardio-protective (Astrup et al. 2011). For this reason promoting red meat consumption in order to improve iron status at a population level may be problematic, and the identification of an alternative MPF factor source rich in polyunsaturated fatty acids or low in saturated fatty acids is required.

NZGLM meets the criteria for a potential alternative iron absorption enhancer. NZGLM contain a high concentration of haem iron, non-haem iron and nutrients which enhance iron absorption as described in Table 1. For this reason the hypothesis of this thesis was that NZGLM will promote non-haem iron absorption compared to egg albumin and may be used as a suitable alternative non-haem iron absorption enhancer to red meat.

Table 1.1. Iron load and potential MPF factors in NZGLM and beef.

Nutrient	NZGLM	Beef
Iron	10mg/100g	4mg/100g
GAGs	220mg/100g	100mg/100g
n-3 PUFA	800mg/100g	100mg/100g
SFA	900mg/100g	3300mg/100g
Cysteine	235mg/100g	237mg/100g

(Vlieg et al. 1991; Athar et al. 1999; Pedersen et al. 1999; Arumugam et al. 2009; USDA 2010)

1.3. Main objective

The main objective of this thesis was to investigate the effect of combining a non-haem iron supplement with NZGLM on non-haem iron absorption within the small intestine compared to a negative control egg albumin and a positive control ascorbic acid.

1.4. Specific objectives

- To investigate the effects of NZGLM supplementation on mucosal iron transport compared to egg albumin, ascorbic acid and red meat using independent *in vitro* models.

- To investigate the effects of NZGLM supplementation on mucosal iron transport and iron retention compared to egg albumin and ascorbic acid *in vivo*.
- To investigate the mechanisms by which NZGLM affects iron absorption
- To develop a method to investigate the effects of NZGLM supplementation on iron absorption and retention in iron-deficient human participants.

Chapter Two: Review of the literature

2.1. Introduction

Iron has the ability to undertake complex oxidation reactions. For this reason, iron is vital for facilitating energy metabolism (Zhang et al. 1998), carcinogen detoxification (de Montellano 2005), immune function (Kuvibidila et al. 1999) and multiple synthetic pathways (Youdim and Green 1978). When iron status is depressed, these processes are compromised and morbidity or mortality can result.

Although the majority of iron in tissues is recycled (Bonnet et al. 1960), iron losses during cell sloughing, tissue damage or haemoglobin loss/dilution must be replaced with dietary sources in order to maintain optimal iron status (Finch et al. 1977). Contralaterally, dietary iron absorption must not exceed tissue requirements because systemic iron cannot be excreted and iron accumulation in extra-intestinal tissues is associated with the generation of reactive oxygen species (Eaton and Qian 2002). In order to protect against excessive iron accumulation, internal iron absorption regulators control iron transfer across the small intestinal mucosa. The iron status of the absorptive enterocyte regulates brush border iron transport and mucosal iron storage (Frazer et al. 2003). Systemic iron status, hypoxia and inflammation regulate mucosal iron transfer and distribution (Nemeth et al. 2004).

Iron transport across the brush border membrane of the absorptive enterocyte is also determined by external factors which influence iron absorption. These factors enhance or inhibit dietary iron absorption by affecting the chemical nature of dietary iron or the function of brush border iron transporters (Hallberg 1981; Thompson et al. 2010).

Iron transport is ultimately controlled by both internal regulators and external factors of iron absorption. If the chemical nature of dietary iron does not promote brush border iron transport, then iron absorption is limited by external factors. If the chemical nature of dietary promotes brush border iron transport, then iron absorption is limited by internal iron regulatory factors.

The first aim of this literature review is to review iron transport mechanisms across the small intestinal mucosa, and iron distribution to extra-intestinal tissues. The mechanisms by which mucosal and extra-intestinal tissues regulate iron absorption are described. The second aim is to review foods and specific nutrients which have been reported to inhibit or enhance iron absorption, to describe their proposed mechanism and to discuss potential dietary strategies in order to promote iron repletion in iron deficient humans.

2.2. Iron absorption

In acidic conditions such as the stomach, dietary non-haem iron is reduced from the Fe^{3+} to the Fe^{2+} form. Ferrous iron forms a hydroxide shell, becomes soluble in aqueous solutions and is available for chelation by a range of negatively charged molecules (Conrad et al. 1991). As the acidic chyme moves into the duodenum, pancreatic bicarbonate oxidises ferrous hydroxide to ferric hydroxide (Spiro and Saltman 1969), the majority of which aggregates in aqueous solutions by the formation of long insoluble ferric hydroxide polymers which cannot access the brush border membrane (Terato et al. 1973).

Any remaining soluble ferric hydroxide may be transported across the absorptive enterocyte apical membrane by several proposed pathways. Ferric hydroxide aggregates are illustrated in Figure 2.1.

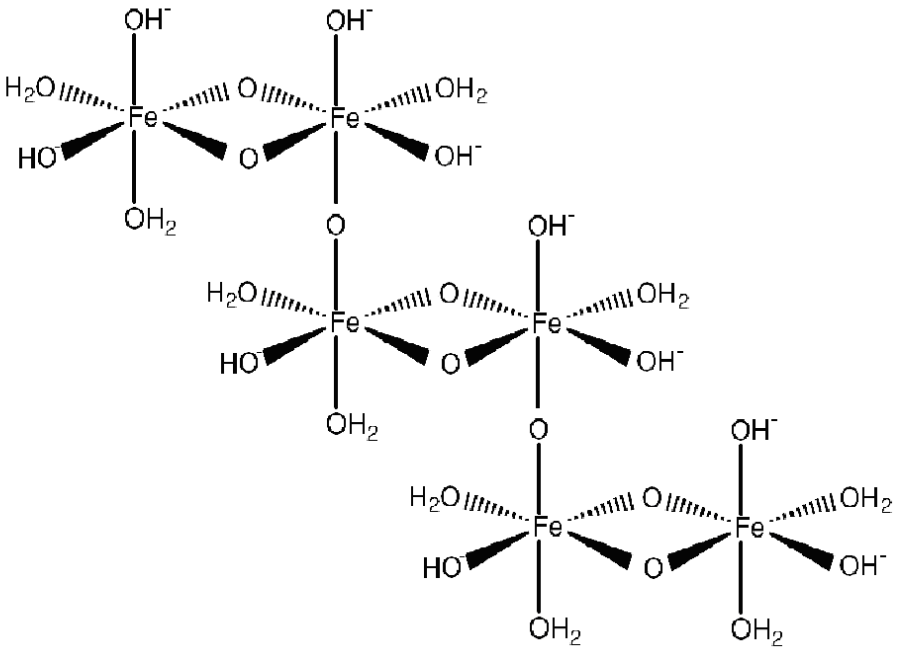

Figure 2.1. Proposed structure of ferric hydroxide aggregates

Ferric hydroxide molecules polymerise to form ferric hydroxide aggregates under oxidising conditions. Modified from Kiyama (1973) and Chipperfield (2003)

2.2.1. Non-haem iron transport

Divalent metal transporter 1 (DMT1) promotes cellular non-haem iron transport. This was first observed by measuring non-haem iron transport in *Xenopus* oocytes before and after transgenically expressing DMT1 (Gunshin et al. 1997). Missense

mutations within the trans-membrane domain of DMT1 are associated with decreased transport of iron across the enterocyte apical membrane, decreased iron import by erythroid precursor cells and severe microcytic anaemia in DMT1 knockout mice (Fleming et al. 1997) and rats (Fleming et al. 1998).

DMT1 transport is driven primarily by proton gradient across the plasma membrane typical of symport co-transport (Gunshin et al. 1997). The proton gradient is generated by a Na^+/H^+ antiport secondary active transport protein (NHE) (Hoogerwerf et al. 1996) which itself is driven by the influx of sodium ions down their concentration gradient. This sodium gradient is maintained by the basolateral Na^+/K^+ ATPase pump. The processes above are illustrated in Figure 2.2.

DMT1 is not specific for non-haem iron, and has a high affinity for other cations including copper, manganese, cobalt, cadmium, zinc, nickel and lead (Gunshin et al. 1997). Each divalent cation competes for a finite number of transporters when present as a combination within intestinal lumen. Competitive inhibition may occur if the concentration of one divalent cation far exceeds the others such as during mineral supplementation (Hill et al. 1963).

DMT1 transports ferrous, but not ferric iron (Gunshin et al. 1997). For this reason, if soluble ferric hydroxide is present within the small intestine, it must be reduced back to the ferrous form prior to transport by DMT1. Ferric iron reduction is undertaken in the small intestinal lumen by the membrane protein duodenal cytochrome B (DcytB) (McKie et al. 2001). DcytB is an integral protein within the apical membrane of duodenal and jejunal enterocytes. Acting primarily as a trans-

membrane electron shuttle, DcytB utilises the redox properties of two haem-bound moieties and the cofactor ascorbic acid to reduce ferric iron back to its ferrous form (Okuyama et al. 1998).

Transgenic expression of DcytB in *Xenopus* oocytes significantly enhances ferric reductase activity, an effect which is abolished after the application of anti–N terminal DcytB antibodies (McKie et al. 2001). The process of iron reduction by DcytB and subsequent iron import by DMT1 is illustrated in Figure 2.2.

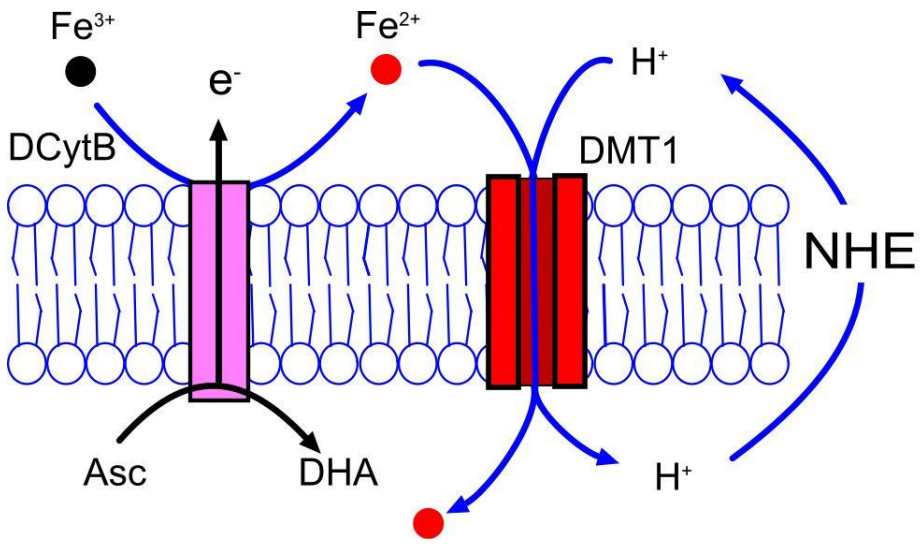

Figure 2.2. Iron transport across the brush border membrane

Soluble ferric iron is reduced to ferrous iron by DcytB, a process facilitated by the oxidation of cytosolic ascorbic acid to dehydroascorbic acid. Ferrous iron is transported across the brush border membrane by DMT1, a symport transporter driven by the proton gradient maintained by NHE.

Mice with point mutations within DcytB have haematological parameters similar to wild type mice (Gunshin et al. 2005). This observation contends the importance of DcytB in promoting iron uptake. However as noted by McKie (2008), additional compensatory changes in DMT1 expression may also occur if DcytB function is abnormal, in order to maintain iron status. Furthermore rodents, unlike humans, synthesise large quantities of ascorbic acid, therefore their requirements for a luminal ferrireductase may be less than that of humans. Nevertheless, the role of DcytB in iron metabolism in humans requires further investigation.

2.2.2. Integrin-mobilferrin-paraferritin pathway

Prior to DMT1 isolation, the proposed mechanism of non-haem iron absorption included transcytosis of transferrin across the intestinal mucosa. This mechanism was restricted by transferrin saturation and enterocyte ferritin concentration which were proposed to work synergistically together by an unknown mechanism (Granick 1946). However patients with congenital atransferrinemia absorb significantly more non-haem iron into the enterocyte than that of normal controls (Goya et al. 1972). For this reason although transferrin may be present within the intestinal mucosa (Huebers et al. 1983), it does not appear to be required for iron transport across the enterocyte brush border membrane.

Prior to the discovery of DMT1, the Integrin-mobilferrin-paraferritin pathway (IMP) was proposed as an alternative non-haem iron absorption mechanism to the transferrin transcytosis pathway. Ferrous iron has been reported to bind to mucin complexes within the stomach, a process which may reduce ferric hydroxide polymerisation within the small intestine (Conrad et al. 1991). Soluble mucin-bound ferric iron are suggested to interact with brush border-associated β_3 integrin proteins which binds ferric iron within the plasma membrane (Conrad et al. 1993; Conrad et al. 2000).

Ferric iron-bound integrin interacts with mobilferrin, a cytosolic protein which facilitates iron transfer the brush border membrane into the enterocyte cytosol (Conrad et al. 1994). Once within the cytosol mobilferrin-bound ferric iron interacts with cytosolic paraferritin, a large multi-domain protein consisting of mobilferrin, β-globulin and a novel flavin-containing monooxygenase. Paraferritin utilises NADPH to reduce ferric iron to ferrous iron (Conrad et al. 1996). Ferrous iron is then released into the labile iron pool (LIP). The Integrin-mobilferrin-paraferritin pathway (IMP pathway) is illustrated in Figure 2.3.

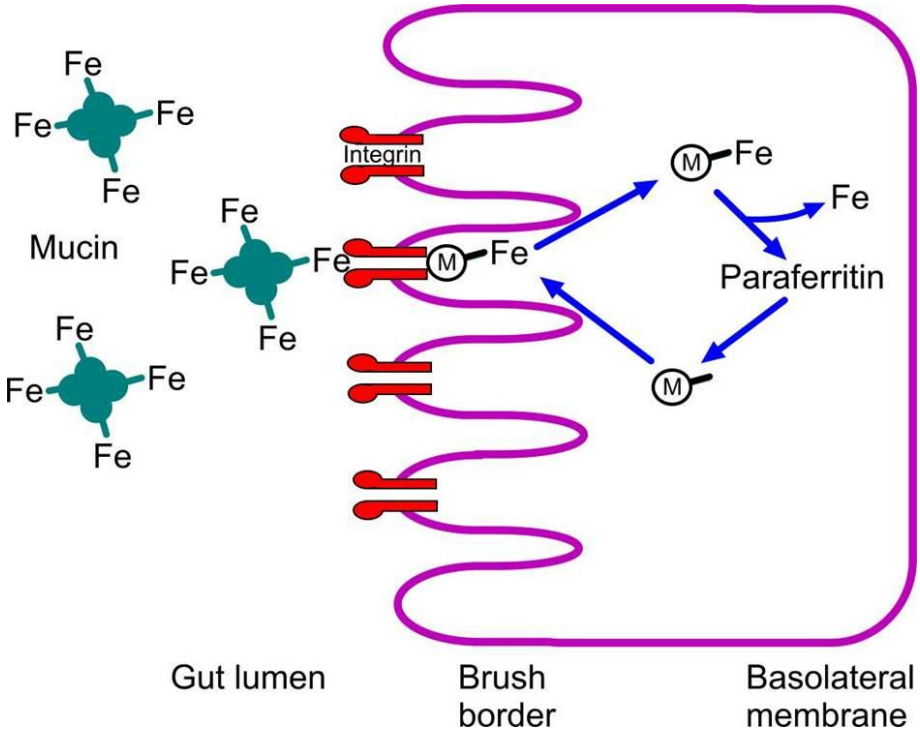

Figure 2.3. Proposed IMP pathway mechanism

Soluble ferric iron-mucin complexes bind with integrin within the brush border membrane of duodenal enterocytes. Integrin subsequently interacts with mobilferrin and ferric iron is transferred to an iron-binding site located within mobilferrin. Ferric iron is reduced to ferrous iron by paraferritin where it enters the cytosolic labile iron pool.

Despite an early proposal for the IMP pathway, the mechanism of iron transfer is still not well understood (Conrad and Umbreit 2006). Pulse chase experiments coupled with immuno-precipitation clearly show that mucin-bound iron interacts with β_3 integrin, and that iron-bound β_3 integrin interacts with mobilferrin (Conrad et al. 1994), however the exact mechanism remains unclear.

Recently ferric iron absorption was shown to be augmented in Caco-2 cells in the presence of ascorbic acid (Thumser et al. 2010). Although the authors could not explain the mechanism for this observation, they speculated that since Caco-2 cell DcytB expression is typically limited, a second pathway similar to that proposed by Conrad and Umbreit (2000) may be responsible for ferric iron uptake. No attempt has been made to further characterise this mechanism.

The generation of a suitable IMP-knockout mouse model to investigate iron absorption is lacking. Furthermore, the specificity of the antibody directed towards mobilferrin is unknown because unlike the anti-β_3 integrin antibody, the anti-mobilferrin antibody is not commercially available. Immunoprecipitation studies of mobilferrin have only been successfully reported by researchers using the antibody provided by Conrad's laboratory; thus further research into the validity of this absorption mechanism is required.

2.2.3. Haem iron absorption

Approximately 10% of total iron ingested in a "typical" Western diet per day is consumed as haem iron (Whitney et al. 2011). Due to its high level of bioavailability (compared to that of non-haem iron) up to 60% of iron absorbed by enterocytes may be derived from dietary haem iron (Bezwoda et al. 1983).

Haem iron is soluble under oxidising conditions (Conrad et al. 1966) and the porphyrin ring prevents iron chelation by dietary iron-absorption inhibitors (Wheby et al. 1970). Haem iron transport across the brush border membrane is mediated by two independent processes.

Haem-specific receptors have been identified within the duodenal microvilli of pigs (Gräsbeck et al. 1979) and cultured erythroleukemia cells (Galbraith et al. 1985). Pulse chase experiments suggest that haem iron is absorbed by receptor-mediated endocytosis, and iron is subsequently released from the porphyrin ring in the late endosome (Wyllie and Kaufman 1982). The mechanism of iron release from the porphyrin ring within the endosome requires further characterisation.

The folate transporter PCFT/HCP1 has been located on the apical membrane of mammalian enterocytes (Shayeghi et al. 2005). PCFT/HCP1 is an electrogenic proton-powered antiport transporter which transports both folate and haem across the apical membrane. Although PCFT/HCP1 has been repeatedly shown to transport haem iron (Shayeghi et al. 2005), the transport rate for folate is two orders greater than that of haem (Qiu et al. 2006), thus the actual contribution of PCFT/HCP1 to dietary haem iron uptake in a whole meal also containing folate is unknown.

Release of iron from the porphyrin ring occurs within the absorptive enterocyte. This process is thought to be undertaken by haem oxidase (Raffin et al. 1974), and appears to be the rate-limiting step in haem iron absorption (Wheby and Spyker 1981). Two isoforms of haem oxygenase have been identified; HO-1 and HO-2. HO-

1, but not HO-2 expression is up-regulated during iron deficiency (Collins et al. 2005). Interestingly the catalytic site of HO-1 is active exclusively within the cytosol (Barañano et al. 2000) and may therefore be ineffective for endosomal haem iron release.

More recently, HO-2 has been co-localised within the late endosome of duodenal enterocytes (West and Oates 2008), therefore two distinct pathways may be involved in haem catabolism. Furthermore the observed lack of haem iron absorption-enhancement during iron deficiency may well be associated with the rate limiting catalysis, or expression of HO-2. Further investigation in to the contribution of these two catalytic processes is required. Regardless of the absorption mechanism, once iron has been released from the porphyrin ring it is reduced to ferrous iron and joins the labile iron pool. The proposed models of haem iron absorption are illustrated in Figure 2.4.

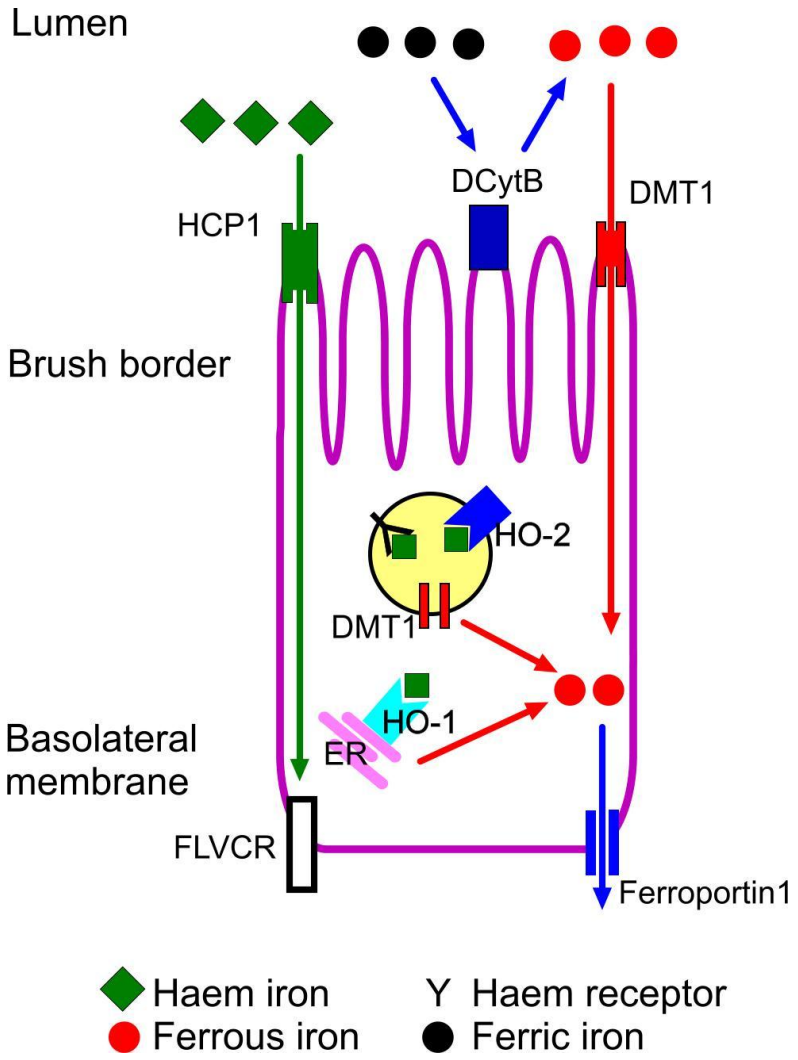

Figure 2.4. Proposed pathway for haem iron absorption

Haem iron is absorbed directly by HCP1 or by receptor-mediated endocytosis. Once transported across the apical membrane, cytosolic haem is catabolised by HO-1 and released into the labile iron pool or transported intact across the basolateral membrane. The mechanism of haem iron efflux from the absorptive enterocyte is unknown, but may include the transporter feline leukaemia virus subgroup C (FLVCR). Conversely endosomal haem is catabolised by HO-2 and iron is released into the cytosolic labile iron pool. Non-haem iron transport is also illustrated.

2.2.4. Ferritin (Fn) iron absorption

Non-haem iron may also be transported within intact ferritin proteins (Theil 2011). Compared to whole soybean flour, isolated soybean ferritin appears to significantly improve iron status (when standardised for iron load) in iron-deficient women (Murray-Kolb et al. 2003), anaemic rats (Beard et al. 1996) and cell cultures (San Martin et al. 2008). Ferritin may act as yet another dietary form of iron which may ultimately contribute to the labile iron pool.

Cell culture studies suggest that ferritin iron absorption does not compete with haem iron or non-haem iron absorption when included within the culture media (Kalgaonkar and Lönnerdal 2008). Furthermore the authors reported that ferritin iron absorption is not down-regulated by the co-supplementation of iron inhibitors tannic acid, phytate or calcium. This suggests that the mechanism of ferritin iron absorption is independent to that of non-haem iron.

Ferritin iron absorption is severely compromised if clathrin coat formation or vesicle budding is inhibited by cytosolic acidification, hyper-tonicity or by the introduction of small interfering RNA sequences specific for accessory protein 2 (San Martin et al. 2008) (essential for vesicle formation). This observation, along with the observations reported by Kalgaonkar and Lönnerdal (2008) suggests that ferritin, if present in the undigested form within the intestine, may be absorbed by the enterocyte by endocytosis.

The proposed endocytotic pathway may be similar to endocytosis of transferrin-bound iron within extra-intestinal tissues (Harding et al. 1983). This process has been investigated in Caco-2 cells where intact ferritin molecules were tracked from the apical reservoir across the apical membrane into the cytosol (San Martin et al. 2008). Using the fluorophore calcein, the authors observed that ferrous iron is subsequently released from the ferritin complex within the late endosome, contributing to the LIP. Receptor-mediated endocytosis of intact ferritin has been noted previously within the placenta (Liao et al. 2001) and liver (Chen et al. 2005).

For ferritin uptake to occur within the small intestinal mucosa, the ferritin protein shell must be protected from all gastrointestinal digestive processes in order to interact with ferritin receptors. Iron-replete ferritin appears to maintain a high level of integrity when treated with proteases compared to that of iron-deplete apoferritin *in vitro*. This suggests that the accumulation of iron within the ferritin molecule is associated with improved protein stability (Crichton 1969).

Gastric digestion of ferritin with pepsin at pH 2 prior to the addition of pancreatic proteases significantly reduces cellular iron loading compared to a gastric digestion at pH 4. Furthermore phytates and tannins significantly reduce cellular iron uptake when the ferritin molecule is treated with a gastric phase at pH 2, but not pH 4 (Kalgaonkar and Lönnerdal 2008). This suggests that iron is released from the ferritin structure under acidic conditions.

Gastric acidity varies significantly within adult humans depending on the timing, volume and type of meal consumed. Gastric pH ranges from approximately pH 1.7 during the fasting state to pH 5 immediately after consuming a meal (Dressman et al. 1990). The gastric pH will fluctuate depending on the size or composition of the meal (Richardson et al. 1976). This suggests that ferritin consumed in large, protein-rich meals may be protected from acidic conditions, increasing its availability within the small intestine. Further investigation into the impact of ferritin consumption on iron status in humans is required.

2.3. Enterocyte iron storage

Ferritin is a soluble protein complex which sequesters cytosolic ferrous iron (Harrison and Arosio 1996). Storage of iron within the ferritin complex inhibits free iron from catalysing the production of free radicals, which may otherwise promote widespread oxidative damage (Winterbourn 1995). Iron sequestration by ferritin also acts as a terminal iron storage site, maintaining enterocyte and systemic homeostasis by allowing the cell to sequester iron in a non-reactive complex until it is required for physiological processes within the enterocyte itself or for distribution to extra-intestinal tissues. If enterocyte or extra-intestinal iron requirements are reduced, ferritin-bound iron is simply lost when enterocytes are sloughed from the apex of the villus (Aisen et al. 2001).

When extra-intestinal iron requirements are increased, ferrous iron is mobilised from ferritin for iron export by ferroportin. This iron-shuttle mechanism by which iron is transported from ferritin to ferroportin is not well understood. Mobilferrin has been proposed as a potential candidate (Conrad et al. 1996) but confirmation of its role is required.

2.4. Enterocyte iron export

Ferroportin is a unidirectional export protein located within the basolateral membrane of enterocytes, and within the plasma membrane of hepatocytes and renal epithelia (Abboud and Haile 2000). Transgenic expression of ferroportin in *Xenopus* oocytes significantly increases the rate of iron export (Donovan et al. 2000). To date ferroportin is the only known mammalian iron export protein (Mayr et al. 2010).

Mutations to the ferroportin gene can lead to one of two disorders in iron absorption. The first is phenotypically similar to that of patients suffering from hereditary hemochromatosis (HHC). Mutations to the extracellular hepcidin-binding domain of ferroportin are associated with uncontrolled cellular iron release (Drakesmith et al. 2005). The second, commonly termed “ferroportin disease” is phenotypically dissimilar to that of HHC patients whereby patients present a combination of high serum ferritin concentrations and microcytic anaemia. Ferroportin disease directly reduces the ability of ferroportin to transport iron (Schimanski et al. 2005).

Once ferrous iron has been exported across the basolateral membrane it must be oxidised to the ferric form. Ferrous iron oxidation is undertaken by hephaestin, a homolog of the ferroxidase ceruloplasmin (Cianetti et al. 2010). Mutations within the hephaestin gene are associated with microcytic anaemia and iron loading within the absorptive enterocyte (Vulpe et al. 1999). The process of enterocyte iron export is illustrated in Figure 2.5.

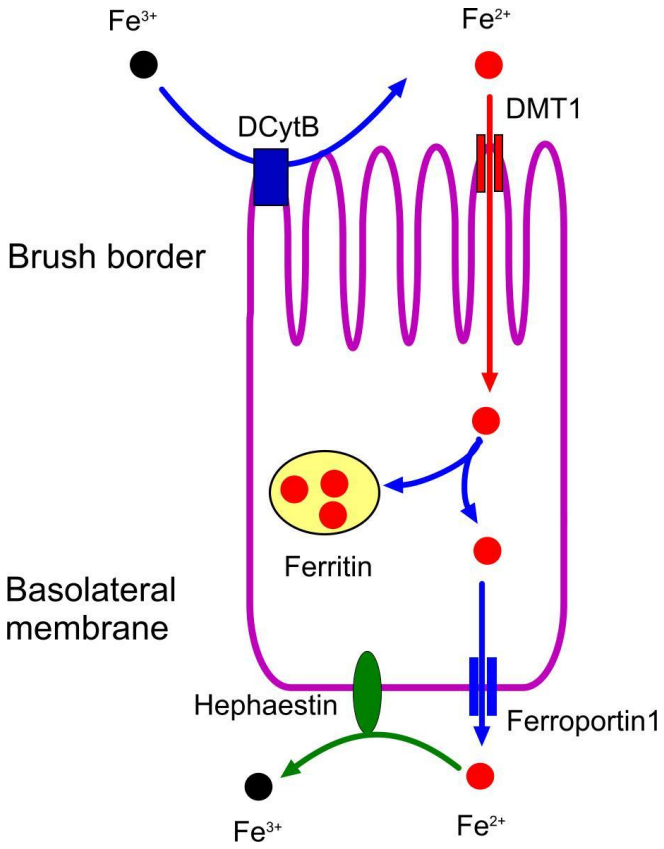

Figure 2.5. Iron transfer across the absorptive enterocyte.

Ferric iron is reduced to ferrous iron by Dcytb on the brush border membrane, which is subsequently transported across the apical membrane by DMT1. Cellular ferrous iron is stored in ferritin and/or released across the basolateral membrane by ferroportin, oxidised into the ferric form by hephaestin, chelated by transferrin and distributed to extra-intestinal tissues.

Ferric iron within the interstitial fluid is chelated by apo-transferrin (iron-deplete transferrin) molecules. Apo-transferrin has a high affinity for ferric iron but not ferrous iron at pH 7.4. Binding of two ferric iron molecules, one to each lobe, promotes a conformational change whereby each ferric iron molecule is tightly bound. Holo-transferrin (iron-replete transferrin) has a very high affinity for extra-intestinal transferrin receptors (TfRs) and a low affinity for intestinal TfRs (Hirose 2000).

2.5. Distribution of iron to extra-intestinal tissues:

Holo-transferrin docks onto TfRs located on the plasma membrane of target cells (Frazier et al. 1982). Once docked, transferrin, its associated ferric iron, extra-intestinal membrane-associated DMT1 and TfR are taken into the target cell by endocytosis (Harding et al. 1983).

Proton pumps present in the endosome lipid bilayer decrease the pH of the endosome lumen. This forces a domain-specific conformational change, decreasing the affinity of transferrin for ferric iron and extra-intestinal TfRs. Ferric iron dissociates from the transferrin molecule, is subsequently reduced to the ferrous state by the ferrireductase STEAP3 and exported from the endosome into the cytosolic LIP by DMT1. The empty transferrin molecule, its receptor and DMT1 are returned to the plasma membrane by exocytosis where transferrin disassociates into the interstitial fluid (de Jong et al. 1990). This process is illustrated in Figure 2.6.

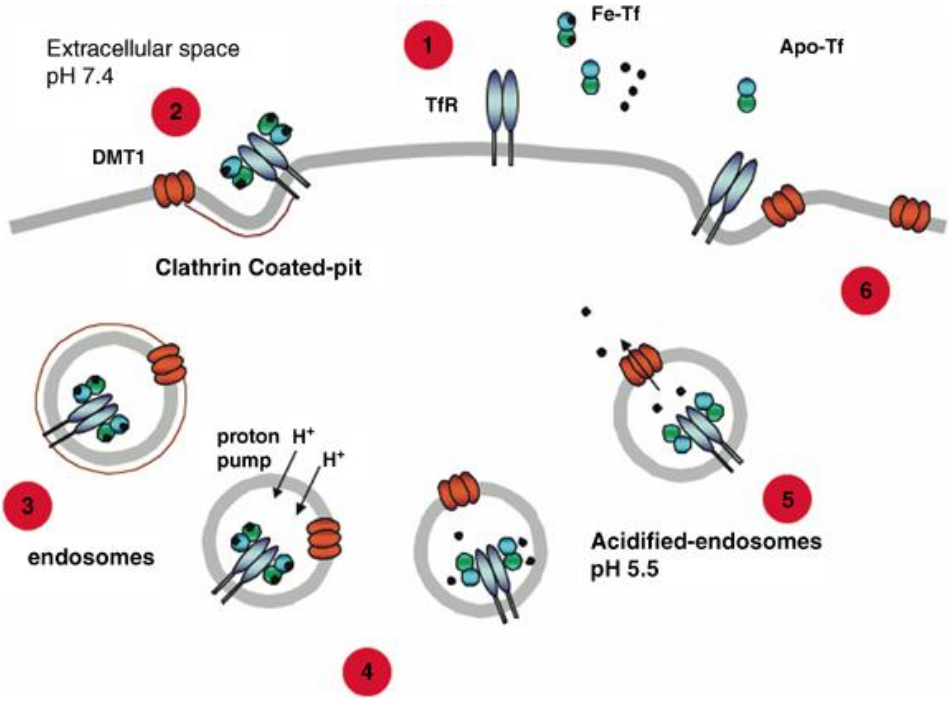

Figure 2.6. Endocytosis and recycling of transferrin in extra-intestinal tissues.

1) Holo-transferrin is circulated within the plasma. 2) Holo-transferrin docks to extra-intestinal transferrin receptors. 3) Holo-transferrin, TfR and DMT1 are internalised by clathrin-mediated endocytosis. 4) Luminal acidification within the late endosome promotes iron-transferrin disassociation. 5) Ferric iron is reduced and exported into the cytosol by DMT1. 6) The transferrin complex is then returned to the plasma membrane and transferrin is released. Modified from Callens et al. (2007).

2.6. Internal factors which regulate iron absorption

2.6.1 Iron regulatory elements

The enterocyte labile iron pool concentration regulates the expression of iron transport, iron storage and iron distribution proteins in order to ensure that excess dietary iron is not absorbed (Chen et al. 1998; Eisenstein and Blemings 1998).

Iron response elements (IREs), a conserved mRNA sequence, are located at either the 5’ or 3’ end of specific mRNA strands coding for proteins involved in enterocyte iron homeostasis (Rouault et al. 1997). IREs form a tight interaction with iron-regulatory proteins (IRPs), a family of cytosolic iron-sulphur proteins containing an IRE-binding motif.

Binding of IRPs to IREs can either inhibit or enhance mRNA translation depending on the specific location of the IRE (Eisenstein and Blemings 1998). If the IRP binds to mRNA sequences with an IRE sequence located on the 5’ end of the un-translated region (UTR) access to the ribosome is blocked and translation is inhibited. Translation of these sequences will only occur when the IRP is removed. If IRPs bind to IREs present on the 3’ UTR translation is not blocked, the mRNA sequence is protected from endonuclease activity and the expression of these proteins is enhanced. DMT1 and TfR have IREs on their 3’ mRNA UTR. Ferritin has an IRE located on its 5’ mRNA UTR.

Binding of an IRP to an IRE relies on the correct structure of the IRP – this is regulated by the concentration of the LIP (Pantopoulos 2004). When the enterocyte is iron replete, iron-IRP salt bridges are formed, facilitating the structural transformation of IRP and decreasing its affinity for 3’ or 5’ IREs. In this circumstance DMT1 and TfR mRNA will not be stabilised and translation will be down-regulated. The opposite occurs when the enterocyte is iron-deplete (Eisenstein and Blemings 1998). This process is illustrated in Figure 2.7.

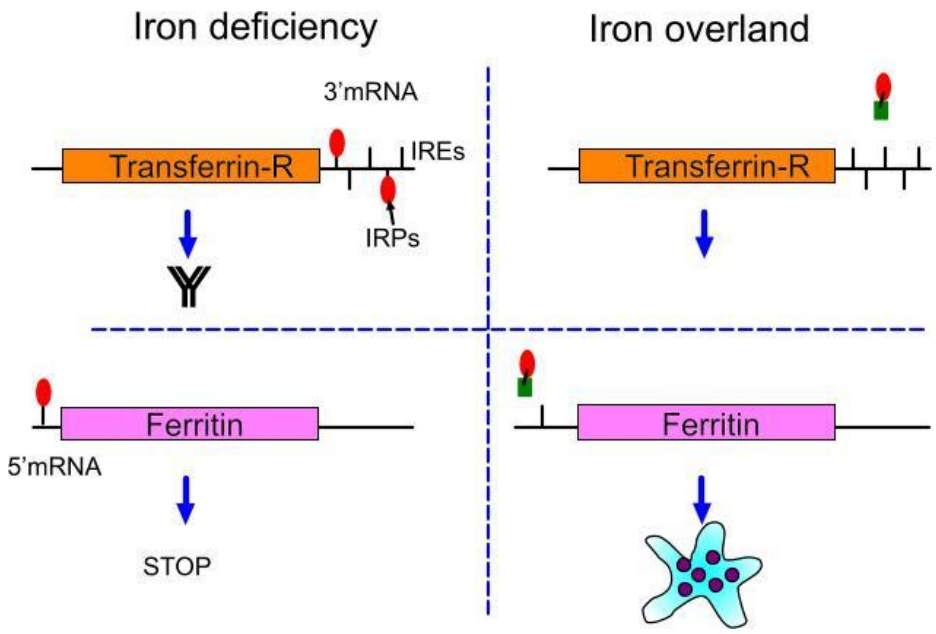

Figure 2.7. Changes in transferrin receptor and ferritin mRNA under low or high iron concentrations

When cytosolic iron concentrations are low (a), IRP has a high affinity for IRE and will stabilise 3' mRNA strands promoting the expression of iron transport proteins (e.g. transferrin receptor mRNA) and block 5' mRNA strands, inhibiting the expression of iron storage proteins (e.g. ferritin mRNA). When cytosolic iron concentrations are elevated (b), IRP will lose its affinity for IRE, therefore transport protein expression is reduced and ferritin expression is increased.

2.6.2 Hepcidin

Systemic iron homeostasis is regulated by the liver-synthesised protein hepcidin (Krause et al. 2000; Pigeon et al. 2001). Hepcidin is a small cationic peptide which is expressed in and released from hepatocytes as part of the innate immune response (Park et al. 2001) and under conditions of iron excess (Pigeon et al. 2001).

Once released, hepcidin binds to ferroportin located on the plasma membrane of enterocytes, hepatocytes and macrophages (Ganz 2005). Binding of hepcidin to its receptor enhances the internalisation and lysosomal degradation of ferroportin, therefore inhibiting iron export (Nemeth et al. 2004). Although hepcidin has long been associated with iron homeostasis, the regulation of its expression and actual role in iron status has only recently been established.

Hepcidin expression appears to be modulated by three specific signalling processes, the dysregulation of any of these three compromises iron homeostasis (van Dijk et al. 2008; Meynard et al. 2009). The first is an interaction between plasma bone morphogenetic proteins BMP2, BMP4 or BMP6 with hepatic BMP receptors and co-receptors as illustrated in Figure 2.8 (Babitt et al. 2006; Andriopoulos et al. 2009). The second is an interaction between holo-transferrin, transferrin receptor type 2 (TfR2) and the expression product of the high-Fe gene (Hfe) as illustrated in Figure 2.9 (Schmidt et al. 2008). The third is the activation of Janus kinases by the inflammatory cytokine interleukin 6 (IL6) (Wrighting and Andrews 2006).

Bone morphogenetic proteins belong to the transforming growth factor-β super-family of which around 20 have been characterised. To date, all BMPs share a high degree of homology and topology, and are involved in moderating embryonic development (Xiao et al. 2007) and post-natal development (Chen et al. 2004). Iron loading is associated with an increase in BMP-regulated hepcidin expression (Andriopoulos et al. 2009).

The BMP co-receptor hemojuvelin (HJV) (Babitt et al. 2006) and BMP receptors type 1 and 2 collectively bind extracellular BMPs and activate BMPR1 cytosolic kinase (Heldin et al. 1997). Activated BMPR1 cytosolic kinase phosphorylates cytosolic Smad transcription factors 1/5/8 (Babitt et al. 2006), greatly improving their affinity for Smad 4, a common modulator in the Smad signalling pathway (Wang et al. 2005). The formation of a heterogenic Smad complex promotes Smad translocation to the nucleus where it binds upstream of the hepcidin promoter and up-regulates hepcidin gene expression as illustrated in Figure 2.8.

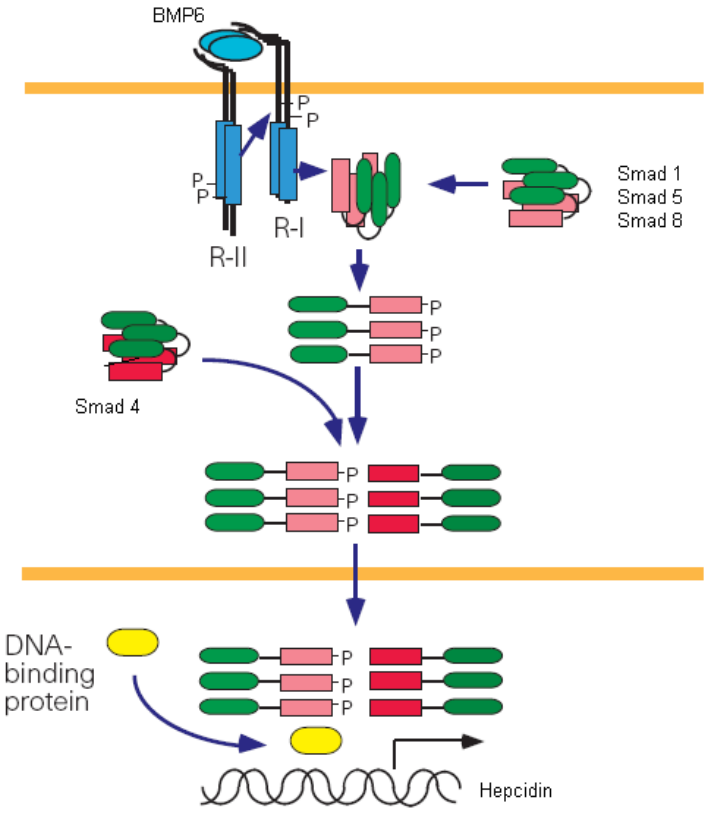

Figure 2.8. Hepcidin expression by BMP

Binding of BMP6 to BMPR1 and BMPR2 promotes Smad 1/5/8 phosphorylation and subsequent interaction with Smad 4, forming a heterometric complex which translocates to the nucleus and promotes hepcidin gene expression. Modified from Heldin et al. (1997).

Although many BMP ligands appear to promote hepcidin expression (Knutson 2010), BMP6 has been shown to have the most potent effect (Andriopoulos et al. 2009). $BMP6^{-/-}$ mice have a similar low-level of hepatic hepcidin expression to $HJV^{-/-}$ mice or $hepcidin^{-/-}$ mice (Meynard et al. 2009), whereby pathological hepatic iron overloading is paramount.

Interestingly, although BMP6 appears to be the main regulator of hepcidin expression, hepatic BMP6 expression is only increased 2-fold during hepatic iron loading compared to a 6-fold increase in iron-loaded enterocytes. Furthermore, enterocytes sourced from iron-replete $BPM6^{+/+}$ mice can promote hepcidin gene expression from iron-replete $BMP6^{-/-}$ hepatocytes ex vivo when co-cultured, while iron-replete $BMP6^{+/+}$ hepatocytes co-cultured with iron-replete $BMP6^{-/-}$ enterocytes have limited hepcidin expression (Arndt et al. 2009).

It is possible that BMP6 expression is initially stimulated during enterocyte iron loading in order to protect against systemic iron loading. The mechanism by which enterocyte iron loading promotes enterocyte BMP6 expression is not well characterised, however it may be proportional to enterocyte hydrogen peroxide generation as noted for BMP expression in endothelial cells (Csiszar et al. 2005).

Hepcidin expression is also regulated by TfR2, a transferrin receptor homologous to TfR1 (Levy et al. 1999). Both TfR1 and TfR2 appear to associate with the plasma membrane protein Hfe, however when serum holo-transferrin concentrations are low, the affinity of Hfe is higher for TfR1 (Schmidt et al. 2008). When serum holo-transferrin levels are high the affinity of Hfe for TfR1 decreases and the affinity of

Hfe for TfR2 increases. This promotes TfR2 phosphorylation and subsequent activation of mitogen-activated protein kinase (MAPK) transcription factors. Activated MAPK transcription factors translocate to the nucleus and up-regulate hepcidin expression (Calzolari et al. 2006) as illustrated in Figure 2.9.

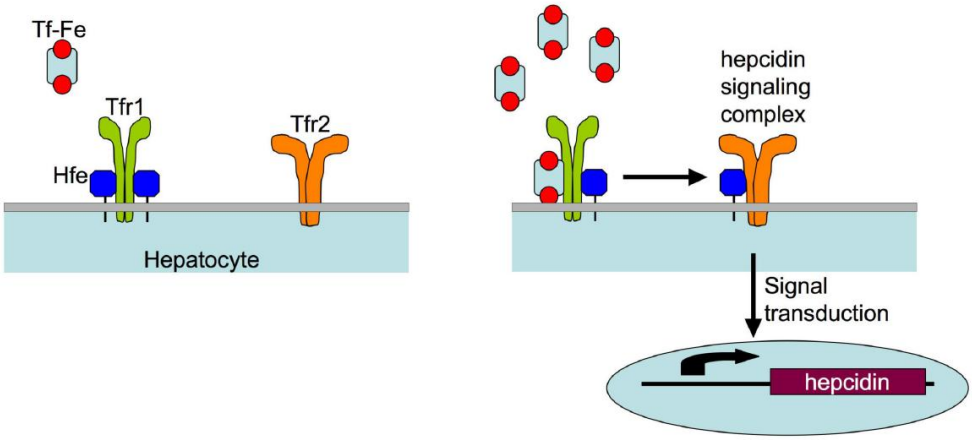

Figure 2.9. Proposed mechanism of hepcidin expression in the presence of holo-transferrin.

An increase in plasma holo-transferrin concentration increases the affinity of Hfe for TfR2. This interaction activates MERK transcription factors which promote hepcidin expression. Modified from Schmidt et al. (2008).

Pathological iron-loading can occur due to genetic mutations to BMP6 (Meynard et al. 2009) or Hfe (Feder et al. 1996). These separate signalling pathways appear to interact together within the hepatocyte cytosol whereby dysregulation of the BMP pathway will also inhibit the holotransferrin/TfR2 signalling pathway (Ramey et al. 2009). This suggests that dysregulation of either pathway may lead to pathological iron loading.

Hepcidin expression can also be stimulated by infection, tissue damage, chronic disease or any other condition which augments IL-6 expression by macrophage (Nemeth et al. 2004). Activated IL-6 receptors within the hepatocyte plasma membrane activate cytosolic Janus Kinases which in turn activate cytosolic Signal Transducer and Activator of Transcription (STAT) transcription factors. Activated STAT proteins translocate to the nucleus where they promote hepcidin gene expression (Heinrich et al. 1998; Wrighting and Andrews 2006).

2.7 Human iron requirements

The human body contains approximately 3-5g of iron in total. The actual value is dependent on age, sex and ethnicity (Zacharski et al. 2000). Approximately 80% of body iron is incorporated within haemoglobin and myoglobin, the remaining 20% is held in ferritin storage sites or utilised by proteins and various cytochromes involved in energy metabolism, carcinogen detoxification and nucleic acid synthesis (Fisher et al. 1973; Whitney et al. 2011).

Iron stores are maintained by recycling iron from senescent iron-containing cells by macrophage phagocytosis. This process is only approximately 90% efficient (Bonnet et al. 1960) and the remaining 10% must be replaced by dietary sources in order to maintain optimal iron status (Cook 1990; Hulten et al. 1995). Iron status can be compromised if iron requirements are rapidly increased or if mucosal iron absorption and distribution is insufficient.

Iron requirements may be increased by blood loss (during menstruation, phlebotomy or injury) or during periods of rapid erythropoiesis (such as the adolescent growth spurt or pregnancy) (Hallberg et al. 1989). Under these circumstances DMT1, DcytB, TfR and ferroportin expression are increased while hepcidin expression is decreased in order to promote iron absorption and distribution (Millard et al. 2004). Under these circumstances the bioavailability of iron within the diet has a major role in determining iron status.

Mucosal iron absorption and distribution is reduced during acute and chronic inflammation or infection. Pro-inflammatory cytokines and lipopolysaccharide (LPS) (a component of the bacteria cell wall) stimulate the expression of hepcidin, inhibit erythropoietin production and enhance phagocytosis by macrophage, significantly reducing systemic iron transport (Ganz and Nemeth 2012). Under these circumstances the bioavailability of iron within the diet has little role in determining iron status. This process is illustrated in Figure 2.10.

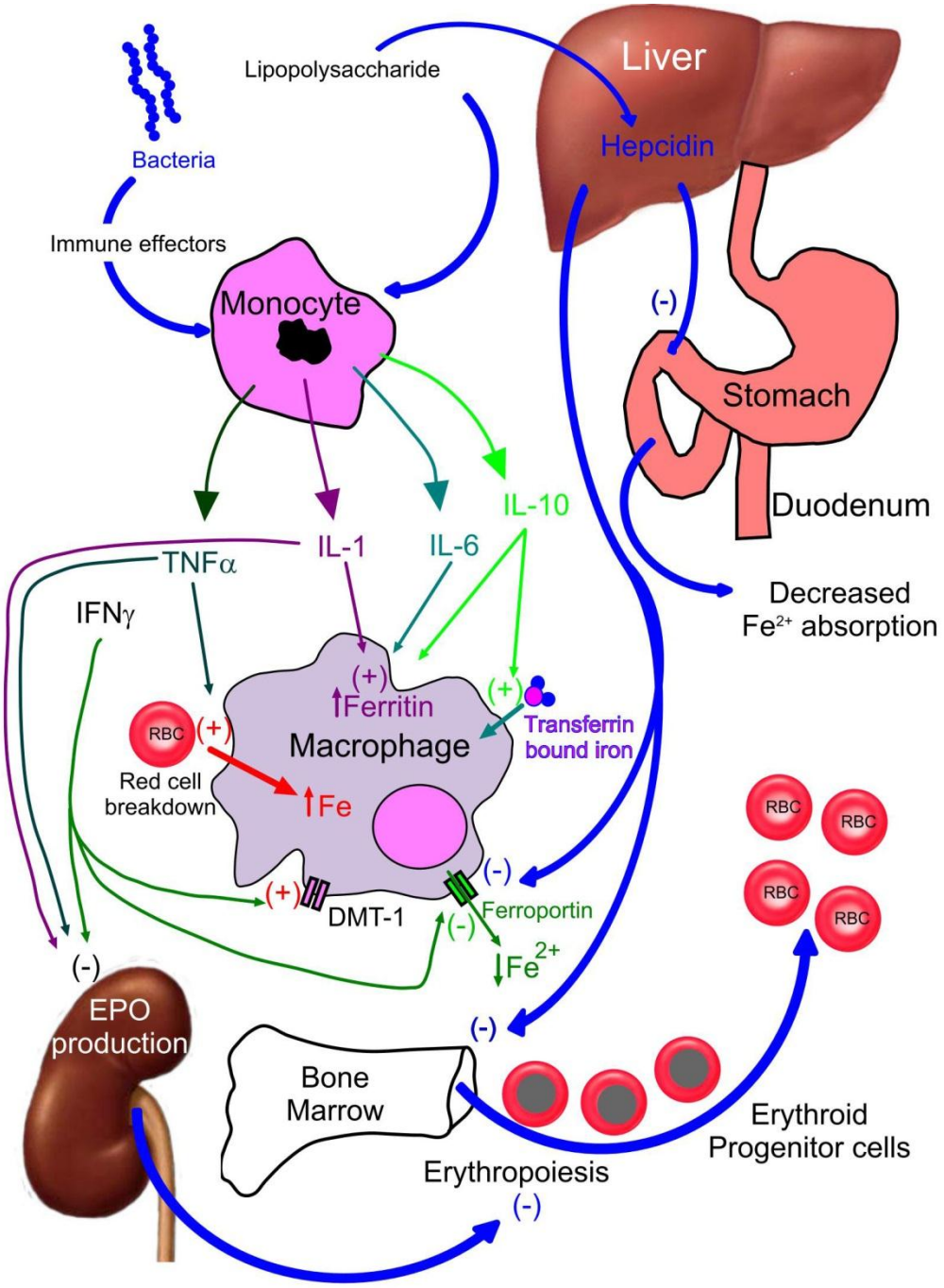

Figure 2.10. Iron homeostasis in the presence of pathogens or inflammation.

Inflammatory disease or pathogen derived lipopolysaccharide (LPS) simulates the release of pro-inflammatory cytokines. LPS promotes hepatic hepcidin release which inhibits iron export from body iron stores. Pro-inflammatory cytokines also inhibit the production of erythropoietin within the kidney, subsequent erythropoiesis, and promote both senescent RBC turnover and iron absorption by macrophage.

Iron status is routinely assessed by measuring blood haemoglobin concentration. However haemoglobin concentration is reduced only during severe iron deficiency and cannot be used to identify sub-optimal iron status. Other commonly used measures of iron status include serum ferritin concentration; which assesses systemic iron storage, and soluble transferrin receptor concentration, which assesses cellular iron requirements. Although serum ferritin measurements can be used to assess sub-optimal iron status, serum ferritin concentrations are also increased during systemic inflammation (Lipschitz et al. 1974). For this reason serum ferritin measurements should be measured in parallel with other inflammatory markers. Soluble transferrin receptor concentration has been suggested as an alternative marker of sub optimal iron status (Olivares et al. 2000); however the current assessment of plasma soluble transferrin receptor concentration has both a low sensitivity and poor specificity, in early-stage iron deficiency. Coupling measurements of serum ferritin and soluble transferrin receptor together and expressing these as a ratio may be a better method for diagnosis of early-stage iron deficiency (Leonard et al. 2013).

Iron deficiency can be divided into three subcategories as summarised in Table 2.1. If iron status is poor, processes such as oxidative metabolism, cognitive development, carcinogen detoxification and immune function can be severely compromised, possibly resulting in morbidity or mortality (Beard 2001; Clark 2008).

Table 2.1. Stages and biomarkers of iron deficiency. Modified from Cook and Finch (1979) and The World Health Organization (2001)

Type of deficiency	**Stage**	**Haematological biomarker**	**Haematological characteristic**
Mild iron deficiency	1	Serum ferritin <12µg/L	Absence of sustainable bone marrow
Marginal iron deficiency	2	Transferrin saturation <16%	Iron deficient erythropoiesis
Iron deficiency anaemia	3	Haemoglobin 80-129g/L males 80-119g/L females 70-110g/L pregnant females	Microcytic erythrocytes

2.8. External factors which influence iron absorption

Transport of iron across the brush border membrane of the absorptive enterocyte is influenced by the presence of foods or nutrients within the diet which alter the chemical properties of dietary iron or affect the function of brush border transporters.

The amount of non-haem iron transported across the brush border membrane in iron-deficient enterocytes (when the concentration of brush border membrane-associated DMT1 and DcytB is not reduced by internal factors) is influenced by the sum of iron absorption inhibitors and enhancers within the meal (Hallberg and Hulthen 2000). Since the majority of enhancers and inhibitors appear to compete against one another, dietary strategies can be developed in order to promote non-

haem iron absorption (Heath et al. 2001). This section of the literature review focuses on the main nutrients which influence dietary iron transport across the brush border membrane of the absorptive enterocyte.

2.8.1. Phytates

Highly phosphorylated inositol species, particularly inositol hexakisphosphate (phytate) and inositol pentakisphosphate significantly decrease the fraction of non-haem iron absorbed from a meal (Sharpe et al. 1950), and have been identified as a main potential contributor to iron deficiency anaemia (Haghshenass et al. 1972).

The inclusion of phytate-rich bran within iron-fortified white bread is associated with poor non-haem iron absorption (Hallberg et al. 1987). The addition of bran in a white bread roll (10% w/w) can significantly inhibit the absorption of non-haem iron from an entire meal (Bjorn-Rasmussen 1974). The inhibiting effect of phytate is dose dependent, with doses as low as 2mg per meal inhibiting non-haem iron absorption by 18% and 250mg inhibiting non-haem iron absorption by 82% (Hallberg et al. 1987).

The main sources of dietary phytate include cereals (especially maize, wheat and rice) and legumes (Maga 1982). Phytate is stored predominantly within the germ of maize, aleurone layer of wheat and the pericarp of rice (O'Dell et al. 1972). This phosphate/mineral store is released during germination by endogenous phytases (Beal and Mehta 1985). Legumes, seeds and nuts have a relatively equal spread of phytate throughout the endosperm.

Due to its localisation within cereals, processing methods which retain only the endosperm can reduce the phytate content of cereal by up to 90%. This process is less successful in legumes due to the high phytate content within the endosperm and hull. Heat treatment above 110°C, prolonged soaking or germination of cereals and legumes will reduce the concentration of phytate by up to 25% (Beal and Mehta 1985) however this alone may not be adequate to alleviate the inhibitory effect of phytate on non-haem iron absorption. The phytate content of foods may be reduced by up to 70% when incubated with phytase, a hydrolytic phosphatase which catabolises phytate into less phosphorylated inositol species (Leenhardt et al. 2005).

During gastric digestion, the majority of dietary phytate is reduced and solubilised allowing it to interact with soluble divalent cations (Grynspan and Cheryan 1983). Gastric emptying transfers the mineral-phytate salts from the reducing conditions of the stomach to the oxidising conditions of the proximal small intestine. Oxidation of mineral-phytate salts increases the affinity of phytate for pre-bound cations. This process is illustrated in Figure 2.11. Oxidised cation-bound phytate has been proposed to concurrently interact with other oxidised phytate molecules, forming insoluble phytate precipitates (Schlemmer et al. 2009).

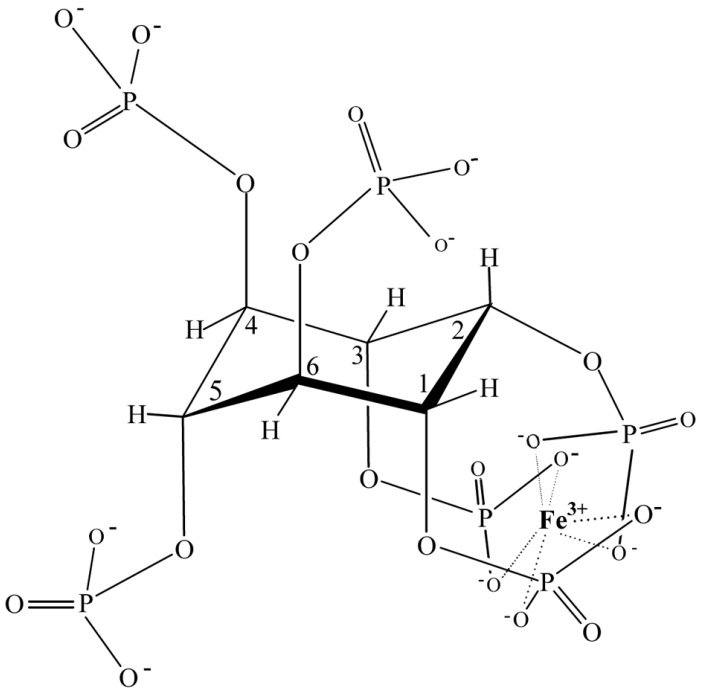

Figure 2.11. Interaction between phytate and iron at pH 6-7. Modified from Schlemmer et al. (2009)

Inositol species with few phosphate groups have a lower affinity for cations and are more soluble under oxidising conditions compared to phytate (Schlemmer et al. 2009). Interestingly, inositol trisphosphate and inositol tetrakisphosphate species may still inhibit non-haem iron absorption somewhat if hexakisphosphate and pentakisphosphate species are also present during the gastric phase of digestion. Inositol trisphosphate and tetrakisphosphate species appear to complex together with inositol hexakisphosphate, further augmenting the formation of insoluble precipitates (Brune et al. 1992).

Both microbial phytase (Türk and Sandberg 1992) and endogenous plant-derived phytase (Hallberg et al. 1987) will reduce the phytate content of cereals or legumes and improve non-haem iron absorption (Hurrell et al. 2003). Endogenous plant-derived phytase is activated by partial acidification (pH 5.5) during fermentation or when combined with organic acids prior to consumption.

Endogenous plant phytase may also be partially active within the acidic conditions of the stomach and have the ability to partially degrade phytate. Gastric phytase activity may be further enhanced with additional supplementation of microbial-sourced phytase (*Aspergillus* niger) during consumption of a phytate-rich meal. This treatment improves non-haem iron absorption in human trials (Sandberg et al. 1996), and is more effective than the consumption of activated plant phytases alone (Hallberg et al. 1987). Microbial-derived phytase is activated in acidic conditions below pH 5.5 and may better suit gastric conditions.

2.8.2. Polyphenols

Polyphenols are present in many cereals, vegetables and fruits (Vinson et al. 1998; Vinson et al. 2001). Like phytates, polyphenol-rich foods (Tuntawiroon et al. 1991), beverages (Hurrell et al. 1999) and polyphenol extracts (Brune et al. 1989) inhibit non-haem iron absorption from an entire meal. This relationship is dose dependent, with doses as low as 50mg tannic acid per meal decreasing non-haem iron bioavailability from a whole meal by almost 70% (Siegenberg et al. 1991).

The degree of hydroxylation of the polyphenol species is proportional to the compounds' affinity for non-haem iron (Brune et al. 1989). Polyphenols containing galloyl or catechol hydroxylation patterns have the highest affinity for non-haem iron (Towo et al. 2006). Polyphenol-mineral interactions are initiated under reducing conditions such as the gastric phase of digestion. The affinity of polyphenols for non-haem iron is further increased under oxidising conditions such as the intestinal phase of digestion. Oxidised, non-haem iron-bound polyphenols also aggregate (Salunkhe et al. 1983) removing iron from the soluble phase of the digesta. This is similar to oxidised phytic acid.

Salt bridge formation between phenolic hydroxyl groups and non-haem iron can be measured spectrophotometrically, thus interactions between phenols and other non-haem iron chelators have been studied extensively *in vitro* (Brune et al. 1991; South and Miller 1998). When looking at the competitive characteristics between phenolic compounds and chelators such as ascorbic acid, both appear to compete directly for non-haem iron.

If ascorbic acid and iron are combined prior to the addition of tannic acid, very little interaction between tannic acid and non-haem iron occurs. If both ascorbate and tannic acid are pre-combined prior to the addition of non-haem iron, both chelators appear to have a similar affinity for non-haem iron. When tannic acid and non-haem iron are pre-combined prior to the addition of ascorbic acid, iron-tannic acid complexes cannot be dissociated (South and Miller 1998). This suggests that interactions between tannic acid and non-haem iron cannot be reversed by subsequent supplementation of other dietary chelators.

Multiple processing methods have been established to reduce or remove the concentrations of polyphenols from foods in order to improve micronutrient availability. De-hulling, soaking, cooking and germination are associated with a moderate reduction in cereal polyphenol concentrations (Sinha and Kawatra 2003). The inhibiting effects of polyphenols on mineral bioavailability or protein digestibility may be further reduced by treatment with polyphenol oxidase (Matuschek and Svanberg 2005) or oxidising reagents (Chavan et al. 1979) however these processes are only partially effective. For this reason, avoiding polyphenol rich food or beverages is encouraged during main meals in order to best promote non-haem iron absorption.

2.8.3. Calcium

The consumption of bovine milk or bovine milk-based formula as an alternative to human milk has been strongly associated with iron deficiency in infants (Woodruff 1975). Iron status is not improved when the iron and protein concentration of bovine milk or bovine milk-based formula are altered to match human milk and is only improved if the iron load is significantly increased above that of human milk. This suggests that either nutrients within human milk enhance iron bioavailability, or that nutrients within bovine milk reduce iron bioavailability.

The main inhibitory effect of bovine milk on iron status has been attributed to its high calcium concentration which exceeds that of human milk by approximately four-fold (Hallberg et al. 1992). The bioavailability of non-haem iron within human

milk is also significantly reduced when the calcium concentration of human milk is increased to match the calcium concentration of bovine milk (Dalton et al. 1997). Additionally the inhibitory effects of dairy products on non-haem iron uptake from meal is further augmented when supplemented with calcium chloride (Reddy and Cook 1997). This clearly shows that calcium is an inhibitor of non-haem iron absorption.

The addition of calcium chloride to an non-haem iron-fortified bread roll significantly reduces iron uptake (Hallberg et al. 1991). The inhibitory effect of calcium added to a pre-cooked meal is dose-dependent from 5.65mM up to 33.8mM in humans. Calcium concentrations below 5.65mM have no inhibitory effect on non-haem iron uptake when added to a pre-cooked meal. No further inhibitory effect on non-haem iron uptake may be stimulated above 33.8mM (Hallberg and Hulthen 2000).

The inhibitory effect of supplementing iron-enriched foods with calcium has been repeatedly reported to reduce iron absorption in humans (Dawson-Hughes et al. 1986; Hallberg et al. 1991) and animals (Kletzten et al. 1940; Barton et al. 1983). Calcium chloride, calcium citrate and calcium phosphate all inhibit iron absorption when added to an iron-rich meal. Interestingly these calcium salts have no inhibitory effect on iron absorption when combined with iron in saline solution rather than within a meal (Cook et al. 1991; Gaitán et al. 2011). This suggests that the inhibitory mechanism of calcium may include an interaction between calcium, iron and other nutrients within a meal.

The inhibitory effect of calcium on non-haem iron absorption from a meal cannot be overcome by concurrent supplementation with ascorbic acid (Hallberg et al. 1992) or red meat (Monsen and Cook 1976). However non-haem iron absorption can be further reduced by the consumption of phytates or tannins (Hallberg et al. 1991). This suggests that the inhibitory mechanism of calcium is independent to other non-haem iron absorption-inhibitors which reduce iron solubility or dialisability.

Calcium directly reduces mucosal non-haem iron transport (Barton et al. 1983). Calcium is not transported by DMT1 and does not reduce iron uptake by competitive inhibition. Instead calcium attenuates DMT1-facilitated proton influx across the brush border membrane (Shawki and Mackenzie 2010). The influx of protons down their concentration gradient is proposed to drive non-haem iron transport by DMT1 (Gunshin et al. 1997). Attenuating DMT1 proton transport reduces the rate of non-haem iron transport across the brush border membrane.

In addition to reducing the rate of non-haem iron transport by DMT1, pre-incubating Caco-2 cells with calcium stimulates the removal of DMT1 from the plasma membrane by endocytosis and reduces Caco-2 cell non-haem iron uptake (Thompson et al. 2010). This mechanism is similar to iron-stimulated DMT1 internalisation which reduces the magnitude of non-haem iron transport across the enterocyte brush border membrane for up to eight hours (Johnson et al. 2005). The impact of DMT1 internalisation on non-haem iron uptake from a single meal is uncertain because the inhibitory effect requires a significant period of time to manifest.

Spacing the consumption of dietary calcium (400mg dose) from the consumption of dietary iron for one to four hours promotes iron retention compared to consuming both calcium and iron together within the same meal (Gleerup et al. 1995). This result suggests that calcium-stimulated internalisation of DMT1 may have little impact on iron status in humans and that inhibitory mechanisms other than DMT1 internalisation are paramount. The effect of higher calcium concentration preload doses requires further investigation.

Calcium has also been reported to reduce iron efflux from the absorptive enterocyte (Gaitán et al. 2012). Contrary to previous reports (Roughead et al. 2005; Kalgaonkar and Lönnerdal 2008; Shawki and Mackenzie 2010; Thompson et al. 2010), Gaitán et al. (2012) reported that brush border iron transport is not reduced in the presence of calcium. Instead the authors proposed that calcium reduces iron efflux across the basolateral membrane. However previous observations suggest that iron efflux is not affected in the presence of calcium (Roughead et al. 2005). Furthermore, ferroportin expression and the plasma membrane ferroportin concentration are not altered by increasing the calcium concentration of the small intestinal lumen (Thompson et al. 2010). For these reasons, this proposed calcium-stimulated reduction in iron efflux requires further investigation.

8.4. Zinc

The inhibiting effect of zinc on non-haem iron absorption or retention has been repeatedly reported in human trials (Rossander-Hulten et al. 1991), animal trials (Hamilton et al. 1978) and cell culture trials (Arredondo et al. 2006). The inhibitory effect of zinc on non-haem iron uptake is only observed when both minerals are consumed together in saline solution without the presence of other nutrients.

non-haem iron absorption is reduced by up to approximately 50% at Zn:Fe ratios ranging from 1.2:1 (w/w) to 5:1(w/w) in humans. This inhibiting effect is only reported at high mineral doses (11.71mg zinc and 10mg iron) but not at low mineral doses (0.57mg zinc and 0.5mg iron) (Olivares et al. 2007).

The mechanism by which zinc inhibits non-haem iron uptake from saline solution is yet to be determined. Although DMT1 has the ability to transport iron and zinc (Gunshin et al. 1997), the transport affinity of DMT1 for non-haem iron is several orders of magnitude higher (Garrick et al. 2006). This suggests that zinc does not compete with non-haem iron for DMT1 transport when both minerals are consumed at a similar concentration.

Interestingly DMT1 knockdown in Caco-2 cells reduces but does not eliminate cellular non-haem iron uptake across the brush border membrane (Espinoza et al. 2012). This suggests that other iron transporters may also be present within the brush border membrane. Iron absorption by these alternative transport pathways may be reduced by competitive inhibition.

Iron uptake is enhanced in cultured hepatocytes when engineered to overexpress the Zrt- and Irt-like protein 8 (Zip8) which may have a role in reducing the plasma concentration of non-transferrin-bound iron (Wang et al. 2012). Zip8 has been previously associated with zinc and cadmium transport (Cousins et al. 2006; He et al. 2006; Ramey et al. 2009).

Like Zip8, Zrt- and Irt-like protein 14 (Zip14) can also transport both non-haem iron and zinc (Pinilla-Tenas et al. 2011). Unlike Zip8, Zip14 is normally expressed at high concentrations within the duodenum and jejunum, where it localises specifically on the brush border membrane (Liuzzi et al. 2006). Liuzzi et al. (2006) have suggested that co-supplementation with zinc and non-haem iron may therefore reduce some iron transport through Zip14 by competitive inhibition. This may explain why zinc-iron supplementation may reduce iron absorption.

The inhibitory effect of zinc on non-haem iron absorption is abated when both zinc and iron are consumed within a complete meal in adults (Rossander-Hulten et al. 1991) or together with wheat flour products in children (Lopez de Romaña et al. 2005). These results suggest that additional nutrient interactions between minerals and the food matrix may interfere with competitive transport of non-haem iron and zinc. Further investigation into the mechanisms by which food abates the inhibiting effects of zinc on non-haem iron uptake is warranted.

2.8.5. Manganese

Like non-haem iron, manganese transport across the brush border membrane is significantly reduced in the DMT1-defective Belgrade rat (Chua and Morgan 1997) suggesting that the majority of non-haem iron and manganese transport is facilitated by DMT1. DMT1 transport kinetics for non-haem iron and manganese are similar: both are saturable, and regulated by pH and temperature (Forbes and Gros 2003) and transport of both is significantly reduced in the presence of calcium (Shawki and Mackenzie 2010). The affinity of DMT1 for non-haem iron is significantly higher than manganese (Illing et al. 2012) however the combination of both metals within a meal or saline solution still significantly reduces iron uptake *in vitro* by direct competitive inhibition (Garrick et al. 2003).

Manganese efflux across the basolateral membrane of the absorptive enterocyte *in vitro* is also undertaken by the iron exporter ferroportin. Manganese efflux is reduced in iron-loaded cells suggesting that the entire mucosal transfer of both iron and manganese is susceptible to competitive inhibition (Madejczyk and Ballatori 2012)

Significant inhibition of non-haem iron absorption has also been noted in human subjects when provided with manganese and iron in a 2.5:1 or 5:1 ratio (w/w) (Rossander-Hulten et al. 1991). This effect was reported when both manganese and non-haem iron were provided to human subjects either in saline solution, or when provided within a complete meal.

2.8.6. Ascorbic acid

Ascorbic acid and ascorbic acid-rich foods consistently enhance non-haem iron absorption when supplemented with iron alone (Layrisse et al. 1974) or combined within a complete meal in human trials (Hallberg et al. 1989), rodent trials (Reddy and Cook 1991) or cell culture trials (Glahn et al. 1999). The mechanism may include facilitating non-haem iron release from foods during the gastric phase of digestion (Kojima et al. 1981), ferrous iron reduction within the gastrointestinal tract (Kapsokefalou and Miller 1991), increasing non-haem iron solubility within the small intestine (Conrad and Schade 1968), competition with inhibitory iron chelators such as phytates and tannic acid for non-haem iron, or a combination of these and other effects (Hallberg et al. 1989; South and Miller 1998).

The optimal ratio of ascorbic acid required to promote iron absorption differs depending on the exact conditions of the treatment. When ascorbic acid is combined directly with an iron supplement, the highest rate of iron absorption is established in human participants at a 2.5:1 molar ratio of ascorbic acid to iron respectively (Cook and Monsen 1977). However when an iron-containing meal is enriched with ascorbic acid, the highest rate of iron absorption is only established in human participants at a 7.5:1 molar ratio of ascorbic acid to iron respectively (Brise and Hallberg 1962). Similar results have been observed in Caco-2 cell cultures (Garcia et al. 1996; Yun et al. 2004). The difference in ascorbic acid load required to promote the rate of iron absorption in food samples is suggested to reflect the competition between ascorbic acid and other inhibitory iron chelators (Lynch and Cook 1980).

Ascorbic acid may promote non-haem iron absorption in Caco-2 cells by both DMT1 and non-DMT1 dependent mechanisms. Thumser et al. (2010) noted that ascorbic acid-mediated ferric iron reduction under-predicted Caco-2 cell iron uptake. The authors proposed an additional ferric iron transport mechanism to account for this observation such as the IMP pathway or Zip14 transport.

Although ascorbic acid is undeniably the most effective enhancer of non-haem iron absorption, its use within meals may be limited somewhat due to its low level of stability under storage or processing conditions. As discussed by Teucher et al. (2004) ascorbate can be quickly degraded when exposed to light, oxygen or high temperatures. For this reason ascorbic acid must be stored under strict conditions and added to a meal subsequent to any heating procedure, in order to preserve its integrity. The stability of ascorbic acid in foods may be improved with additional processing such as microencapsulation; however this process will also include additional costs.

2.8.7. Effect of protein on iron absorption

Red meat, fish, offal, chicken, pork, and rabbit all enhance non-haem iron absorption when co-supplemented with an iron-enriched vegetable based meal or iron supplement in humans (Layrisse et al. 1969; Cook et al. 1974; Baech et al. 2003; Navas-Carretero et al. 2008), rodents (Seiquer et al. 2002) and *in vitro* (Glahn et al. 1996).

The enhancing effect of meat or fish appears to be associated with the specific amino acid composition of these foods (Layrisse and Martinez-Torres 1968). Of these amino acids, purified cysteine appears to be most influential on promoting non-haem iron absorption in humans (Martinez-Torres and Layrisse 1970).

The enhancing effect of purified, reduced amino acids has also been reported in animal trials (Van Campen and Gross 1969; Van Campen 1973). However these trials collectively suggest that lysine and histidine also enhance non-haem iron absorption. This data is somewhat conflicting to human trials.

The effects of individual amino acids on non-haem iron absorption are also conflicting *in vitro*. Cysteine, but not histidine or lysine, promotes non-haem iron uptake across the brush border membrane compared to an amino acid-free control (Glahn and Van Campen 1997). The enhancing effect is abated if cysteine is oxidised, or if the thoil group is replaced. Conversely, histidine-rich, low molecular weight peptides (released from beef during digestion *in vitro*) were observed to be most effective at promoting non-haem iron uptake (Swain et al. 2002). Again this is somewhat conflicting to human trials.

Cysteine will only up-regulate non-haem iron uptake in humans trials or cell cultures when present in the reduced form. Interestingly when meat proteins are oxidised (a process which removes all iron absorption-enhancing activity from purified cysteine) non-haem iron absorption is still enhanced, albeit to a lower degree (Taylor et al. 1986). These observations suggest that oxidation of cysteine to cystine is protected somewhat when cysteine is bound within a protein complex, or

that factors other than reduced cysteine present within beef, poultry and fish may also have the ability to enhance non-haem iron absorption.

Meat must be subjected to gastric digestion in order to promote non-haem iron absorption (Garcia et al. 1996). Interestingly enzymatic digestion is not strictly required to promote non-haem iron absorption, rather acid digestion alone is effective (Huh et al. 2004). Gastric digestion may promote iron solubilisation (Cotton et al. 1999) and cysteine reduction (Taylor et al. 1986).

The majority of cysteine-rich peptides derived from meat, poultry and fish are released from the bulk protein phase by gastric digestion alone (Storcksdieck et al. 2007). This allows reduced cysteine to interact with ferrous iron under reducing conditions. This may explain why cysteine-rich proteins within meat enhance intestinal non-haem iron solubility (Glahn and Van Campen 1997), intestinal non-haem iron reduction (Kapsokefalou and Miller 1991) and iron absorption (Hurrell et al. 1988).

Whey, casein and egg albumin all contain a complete amino acid profile, yet appear to inhibit non-haem iron absorption (Cook and Monsen 1976; Glahn et al. 1996). Peptides derived from egg and dairy products are hydrolysed to a lower degree during the gastric phase of digestion (Storcksdieck et al. 2007). For this reason cysteine-rich peptides within egg and dairy proteins may not be exposed until they arrive in the small intestine. Under these oxidising conditions the interaction between oxidised cysteine and ferric iron may be poor.

Furthermore, phosphorylated serine residues within whole dairy proteins have been proposed to have a high affinity for ferrous iron. High molecular weight iron-protein complexes reduce the ability of iron to interact with brush border transporters due to the size exclusion properties of the mucosal mucus and glycocalyx (West 1986).

Gastric digestion of casein is limited; therefore processing procedures such as casein hydrolysis prior to consumption may be an effective strategy to improve iron bioavailability in the presence of casein. Purified casein phosphopeptides (β-CPP) have been reported to significantly enhance non-haem iron absorption (Argyri et al. 2007) and retention (Ait-Oukhatar et al. 2002) compared to whole casein.

The mechanism of non-haem iron absorption-enhancement by β-CCP is not well defined, but may include exposure and reduction of β-CCP-associated cysteine residues during the gastric phase of digestion, promoting non-haem iron chelation (Bouhallab et al. 2002) and improving iron solubility under oxidising conditions (Cross et al. 2001).

Although β-casein hydrolysates have been reported to enhance iron absorption in animal trials and cell cultures (Argyri et al. 2007; García-Nebot et al. 2010), little enhancing effect has been reported in humans trials (Ait-Oukhatar et al. 2002).

2.8.8. Effects of lipids on iron absorption

Non-haem iron absorption is enhanced when consumed with dietary fatty acids (Hirooka et al. 1968; Amine and Hegsted 1975; Armah et al. 2008). This effect has been reported with polyunsaturated fatty acids (PUFA) (Seiquer et al. 2002), monounsaturated fatty acids (MUFA) (Pabón and Lönnerdal 2001) and saturated fatty acids (SFA) (Johnson et al. 1987) consumed as either triacylglycerides or free fatty acids.

Compared to MUFA, SFA enhance non-haem iron absorption when present at a low concentration (5% w/w) within the diet. Both SFA and MUFA promote non-haem iron absorption when supplemented at higher concentrations (15%, 20% or 30% w/w) (Amine and Hegsted 1975; Bowering et al. 1977; Johnson et al. 1987).

Fatty acids may enhance non-haem iron absorption by reducing and solubilising dietary iron for transport by DMT1 (similar to that of the protein meat factor) (Armah et al. 2008) or by DMT1-independent mechanisms (Simpson and Peters 1987).

The brush border membrane of the proximal small intestine has a high affinity for non-haem iron compared to other cell plasma membranes (Simpson and Peters 1987). The authors reported that the brush border affinity for and transport of non-haem iron is improved exponentially with membrane oleic acid enrichment (Simpson et al. 1988). The enhancing effect of MUFAs on non-haem iron uptake may not be restricted to membrane fatty acid enrichment. MUFAs also promote non-haem iron transport across the lipid bilayer when provided as a single dose

combined with iron. Combining non-haem iron with non-esterified fatty acids may promote the formation of lipid-soluble iron complexes. Lipid-soluble iron complexes appear to facilitate iron transport (Simpson and Peters 1987).

PUFAs enhance non-haem iron absorption when consumed as fish oil (Chang and Chen 1992; Pérez-Granados et al. 2001) or whole fish (Seiquer et al. 2002; Navas-Carretero et al. 2008). Contralaterally, repeated supplementation of PUFAs at a concentration which greatly exceeds the American Heart Association guidelines promotes mucosal non-haem iron absorption, yet compromises hepatic iron status (Rodriguez et al. 1996). This effect is specific for PUFA supplementation and not reported in rats supplemented with MUFA or SFA.

Long-term supplementation of a high-dose fish oil diet is associated with increased reticulocyte counts (indicative of augmented erythropoiesis), reduced plasma α-tocopherol concentrations (indicative of augmented antioxidant activity) and high plasma thiobarbituric acid (TBA) concentrations (indicative of augmented peroxidative damage) (Miret et al. 2003). These results suggest that excess enrichment of erythrocyte membranes with long chain PUFAs increases the rate of erythrocyte peroxidation, decreases the erythrocyte life span and increases the rate of erythrocyte turnover.

Increased erythropoiesis may augment mucosal iron transfer, and stimulate iron export from hepatocyte and macrophage iron stores. Enrichment of erythrocytes with MUFA or SFA may reduce the rate of membrane peroxidation and subsequent erythrocyte turnover rate.

The American Heart Association (AHA) recommends that healthy adults should consume approximately 2.9mg of oily fish per gram of bodyweight per week (AHA 2011). PUFAs provided at this dosage have been reported to improve iron status in humans (Navas-Carretero et al. 2008).

A novel, non-haem iron absorption-enhancing lipid fraction has been isolated in red meat (Armah et al. 2008). This fraction consists of a series of L-α-glycerophosphocholine (L-α) cleavage products which are formed by the digestion of the phospholipid phosphatidylcholine under standard *in vitro* digestion procedures. L-α (prepared by *in vitro* digestion or commercially available) improves non-haem iron uptake in Caco-2 cell cultures and human trials when combined within an inhibitory vegetarian meal.

Conversely L-α has been reported to have no enhancing effect on iron uptake from weaning formula in iron-replete women (Troesch et al. 2009). However this study may lack sensitivity due to the calcium load of the formula. Further investigation into the effects of L- α is required.

2.8.9. Effect of carbohydrates on iron absorption

The process of iron absorption is understood to occur almost entirely within the proximal small intestine (Wheby et al. 1963). Yet measuring iron absorption exclusively within the small intestine significantly underestimates iron retention from a single iron dose in rats (Bougle et al. 2002), dogs (Chernelch et al. 1970) or pigs (Blachier et al. 2007). Furthermore dogs are reported to significantly up-regulate colonic non-haem iron transport to a rate almost half that of the proximal small intestine during iron deficiency (Chernelch et al. 1970). This observation coincides with an increase in caecal and colonic DMT1 and DcytB expression in mice during iron deficiency (Takeuchi et al. 2005).

The colon has a significant role in mineral absorption, the magnitude of which can be increased by the consumption of non-digestible carbohydrates such as fructooligosaccharides (FOS) and resistant starch (Ohta et al. 1993; Rémésy et al. 1993; Morais et al. 1996; Shiga et al. 2003). Colonic fermentation of these products has been repeatedly reported to promote calcium and magnesium bioavailability by either increasing colonic mass (Sakai et al. 2000; Blachier et al. 2007), *Bifidobacterium* and *Lactobacillus* populace (Patterson et al. 2009), luminal short chain fatty acid (SCFA) production (Bougle et al. 2002; Shiga et al. 2003), luminal acidification (Lopez et al. 2000; Younes et al. 2001), mineral transporter expression (Ohta et al. 1998) or a combination. A similar mechanism may influence non-haem iron absorption.

FOS or resistant starch supplementation significantly promotes colonic non-haem iron absorption (Delzenne et al. 1995; Morais et al. 1996) and improves iron status in animals (Ohta et al. 1995; Yasuda et al. 2006). The enhancing effect appears to be largely associated with the process of fermentation which improves non-haem iron solubility (by decreasing the luminal pH (Ohta et al. 1995) and short-chain fatty acid (SCFA) iron chelation/solubilisation (Bougle et al. 2002)), undertakes ferric iron reduction (Yeung et al. 2005) and up-regulates the expression of DMT1 (Tako et al. 2008).

FOS supplementation significantly reduces post-operative anaemia by up to 70% in gastrectomized rats (Ohta et al. 1998). This protective effect of FOS supplementation is abated if the colon is removed concurrently during the gastrectomy procedure. If only the colon is removed rather than the stomach, the protective effect of FOS on iron status is abated (Sakai et al. 2000). This suggests that the stomach is important for iron absorption processes within the small intestine but not colon. If gastric function is impaired, or the subject is iron deficient, colonic iron absorption is up-regulated.

Non-haem iron absorption from yoghurt is significantly enhanced in Caco-2 cell monolayers if the yoghurt is incubated at 37º over a period of 48 hours prior to digestion (Laparra et al. 2008). This enhancing effect is not further augmented with additional FOS supplementation (4% w/w) or when FOS was added directly to the Caco-2 cell monolayers. This result indicates that FOS is not directly associated with iron absorption enhancement, but rather that the enhancement seen *in vivo* is associated with the by-products generated by bacterial fermentation.

The effects of the non-digestible carbohydrates on iron status in humans are conflicting. FOS supplementation has no effect on iron uptake in ileostomy patients (Ellegård et al. 1997) or healthy iron-replete adult men (Coudray et al. 1997; Van den Heuvel et al. 1998). However these studies were not designed to investigate iron uptake as their primary outcome and both the methodology and analysis was not designed to accurately quantify iron absorption. Further investigation into the effects of non-digestible carbohydrates on iron absorption in iron deficient adults is warranted.

Non-digestible carbohydrate supplementation may also improve iron uptake within the proximal small intestine. Glycosaminoglycans (GAGs), a carbohydrate species extracted during the gastric digestion of fish, significantly improves non-haem iron uptake in Caco-2 cell cultures (Huh et al. 2004). The enhancing effect of GAGs on iron uptake is less than that of whole digested fish (Laparra et al. 2008) suggesting that GAGs may improve iron uptake in combination with other meat factor components.

Interestingly the mechanism by which GAGs promote non-haem iron uptake in cell cultures may be independent of direct DMT1 transport. GAGs appear to be transported across the lipid bilayer by endocytosis and catabolised within the late endosome (Yanagishita and Hascall 1992). Sulfonated GAG residues have been proposed to act as divalent metal chelators, forming tight bonds with ferrous iron (Campo et al. 2003). This GAG-iron combination has also been reported to promote enterocyte non-haem iron uptake and utilization by a process of endocytosis across

the brush border membrane. Acidification within the late endosome may release iron from GAGs, allowing it to be exported into the cytosol (Laparra et al. 2009).

Despite successful absorption enhancement *in vitro*, the effects of GAG supplementation are less consistent in human trials. Commercially available aminoglycans in the form of chondroitin sulphate or hyaluronate appear to have no significant effect on non-haem iron absorption and utilisation in iron-replete humans (Storcksdieck et al. 2007) yet isolated myosin and actin proteins do have an effect using a similar experimental protocol (Hurrell et al. 2006).

The enhancing effect of aminoglycans may be species-specific. Chondroitin sulphate or hyaluronate have a much higher molecular weight compared to GAGs associated with non-haem iron absorption-enhancement (Huh et al. 2004; Laparra et al. 2008). These larger aminoglycan species also have no enhancing effect on iron absorption *in vitro* (Jin and Glahn 2007). Further investigation into the effects of low molecular weight aminoglycans on non-haem iron uptake in humans is required.

2.9 Summary and future directions

The enhancing effect of red meat and pork on non-haem iron absorption compared to other protein sources appears to be orchestrated by the combination of cysteine-rich myofibrils (Mulvihill et al. 1998), glycosaminoglycans (Pedersen et al. 1999) and fatty acids (Simpson and Peters 1987; Seiquer et al. 2002; Armah et al. 2008). When these factors are combined, iron absorption is significantly enhanced.

Foods, other than red meat, such as NZGLM, which are also rich in iron absorption-enhancers may also promote non-haem iron absorption while providing other health benefits. NZGLM contains a high concentration of cysteine-rich myofibrils (Shelud'ko et al. 1999), GAGs (Rubin et al. 2006), PUFAs and phospholipids (Murphy et al. 2002). The novel fatty acid and aminoglycan profile of NZGLM is also associated with potent anti-inflammatory properties (Whitehouse et al. 1997; Bui and Bierer 2003; Tenikoff et al. 2005; Clegg et al. 2006).

NZGLM is a rich in both haem and non-haem iron (Athar et al. 1999) and frequent consumption of NZGLM is currently recommended by the New Zealand Blood Service (NZBlood 2013) in order to replete iron stores. Although there is very limited robust data relating to domestic mussel consumption or acceptability in New Zealand, an estimate can be made based on the National Nutrition Survey results (Russell 1999) and domestic sales.

Approximately 113,000 tonnes of finfish (SeafoodNZ 2014) and 63,000 tonnes of shellfish (King and Lake 2013) are sold per year to the New Zealand domestic market and domestic sales of NZGLM make up 93% of all shellfish sold in New Zealand. Together with recreational fishing, this suggests that approximately one third of all seafood available for consumption in New Zealand is NZGLM.

Approximately 80% of the New Zealand population over the age of 15 years consumes seafood (finfish and shellfish) regularly (at least once per month) (Russell 1999). The authors reported that the highest consumers of shellfish within this population are within the lowest socioeconomic quintile.

Overall this data suggests that NZGLM may be a potential alternative to red meat as a source of iron absorption-enhancer. NZGLM is easily accessible in New Zealand, and NZGLM consumption is not restricted by cost. For this reason a comprehensive study is required in order to investigate the effects of NZGLM supplementation on non-haem iron absorption and retention.

Chapter Three: Materials and methods

3.1. Introduction

The materials and methods of core procedures used for multiple experiments are described in this chapter. Methods or materials which were used for specific assays are described in full within their respective chapters. The process of developing or validating specific methods such as the *in vitro* digestion and Caco-2 cell uptake assay will be described in full within this chapter.

3.2. General materials and methods

3.2.1. Treatments and chemicals

Fresh NZGLM (Perna *canaliculus*) and beef sirloin were purchased from Countdown (Palmerston North, New Zealand). Lyophilised egg albumin was purchased from Zeagold (Otago, New Zealand). All chemicals including enzymes and acids, unless otherwise stated, were purchased from Sigma (St. Louise, MO, USA). All hydrochloric acid (HCl) solutions were made from a concentrated HCl stock solution (H1758) in deionised water. All nitric acid (NA) solutions were made from NA stock solution (438073) in deionised water. All radionuclides and decontamination detergents were purchased from Perkin Elmer (Waltham, MA, USA). All cell culture media components unless otherwise stated were purchased from Invitrogen (Carlsbad, CA, USA).

3.2.2. Equipment

All glassware was immersed in 10% HCl for 12 hours, rinsed six times with deionised water and oven dried before use. When possible, new, sterile 15 and 50mL Falcon tubes (BD biosciences, NJ, USA) were used in place of glassware.

3.2.3. Treatment preparation

Fresh NZGLM flesh was trimmed from the shell (including adductor muscle) to give a total weight of 400g. Egg albumin was reconstituted by 1 part egg albumin to 7 parts de-ionised water as directed by the manufacturer to give a final weight of 400g. Lean beef sirloin was trimmed of all visible fat and connective tissue to give a final weight of 400g. All samples were homogenised separately in a Sunbeam 4181 blender (Sunbeam, Auckland, New Zealand) with 500mL deionised water.

A 200g portion of NZGLM and egg albumin homogenate were transferred into sealed 15mL Falcon tubes which were immersed in boiling water for 10 minutes. Samples were cooled immediately.

These samples were then lyophilised (Model FD18LT “ISLA” Massey University, Palmerston North, New Zealand) at -35°C for 36 hours, ground with a porcelain mortar and pestle and passed through an 800 micron sieve.

3.2.4. *In vitro* digestion

The initial *in vitro* digestion protocol was based on that previously undertaken within our laboratory by Walker (2003). Porcine pepsin (P7000, 800-2500 units/mg protein) was diluted in 0.1M HCl to a working solution containing 7812 units/mL. Aliquots of fresh NZGLM, beef and egg albumin homogenates described in Section 3.2.3 were incubated at 37°C for 30 minutes. Each 20g aliquot was titrated with 1M HCl to pH 2.4 and combined with 0.67mL of pepsin solution. Samples were incubated at 37°C for 2 hours in a shaking water bath.

Porcine pancreatin (P1625, activity 3 x USP specifications) and porcine bile salts (B8631) were combined with 0.1M sodium bicarbonate ($NaHCO_3$) (S5761) to a final concentration of 2.4mg/mL and 6.25mg/mL respectively. Samples treated by peptic digest were titrated to pH 6.0 with 1M $NaHCO_3$. Five millilitres of pancreatin/bile solution were added to each sample. Samples were incubated for 2 hours at 37°C.

The osmolarity of all digestates was measured (OsmoLAB 16S, LLA Instruments, Germany) and corrected with deionised water to 300mOsm. Each treatment was diluted a further 3 fold in Hank's buffered salt solution (HBSS) (H4641), to correct the potassium concentration to ~5mmol/L.

3.2.5. Total iron concentration assay

The total iron concentration of treatments or tissues was analysed after wet-ashing as described by Carpenter and Clark (1995). Briefly, 250mg-1000mg of fresh, cooked or lyophilised sample was digested by reflux in triplicate with 15mL 70% NA. The exact weight of each treatment was recorded. The remaining residue was combined with hydrogen peroxide (H3410) and heated further until dry. This hydrogen peroxide treatment was repeated three times. The final ash was reconstituted in 1M HCl at 20°C.

Iron concentration analysis was undertaken using a ferrozine method modified from Ahn et al. (1993). Briefly, aliquots of reconstituted ash were combined with 2.5mL citrate-phosphate buffer (0.906g citric acid monohydrate (C1909), 1.615g sodium phosphate dibasic (S7907) in 100mL in deionised water) and 1mL ascorbate solution (2% ascorbic acid (A0278) in 0.2N HCl) at 20°C in triplicate for 15 minutes. Samples were combined with 2mL of trichloroacetic acid (11% trichloroacetic acid (T6399) in deionised water) at 20°C for 10 minutes and 2mL of each sample were combined with 1.6mL ammonium acetate (10% ammonium acetate (A1542) in deionised water) and 0.2mL Ferroin colour reagent (75mg ferrozine (82950), 75mg neocuproine (N1501) in 25mL deionised water with 10µL 6M HCl) and developed at 20°C for 10 minutes. The colour of all samples was determined by spectroscopy at 562nm (Novaspec II, Amersham Pharmacia Biotech, UK). Iron concentrations were calculated from a 2.5ppm to 20ppm standard curve generated from a 1000 mg/L Fe stock solution (16596) in citrate phosphate buffer.

3.2.6. Haem iron assay

The total haem-iron concentration of treatments was analysed as described by Hornsey (1956), modified by Carpenter and Clark (1995). Briefly, 500mg of fresh homogenised treatment was combined with 20mL acetone (154598) and 0.5mL HCl and 4mL deionised water. The samples were combined by vortex for 1 minute and centrifuged at 300x *g* for 20 minutes. The supernatant of each sample was clarified by filtration through Grade 2 Whatman filter papers (Z240184). Filtrate samples were analysed for total haem pigments by spectroscopy at 640nm (Novaspec II, Amersham Pharmacia Biotech, UK). The concentration of haem iron was calculated from Equation 3.1. The concentration of non-haem iron present within a sample was calculated from Equation 2.

Equation 3.1. Calculation of haem iron

A_{640nm} x 680 = Total diluted haem pigments (ppm)

Total diluted haem pigments (ppm) * DF = Undiluted total haem pigments (ppm)

Total haem pigments (ppm) x 55.847/652 = Total haem iron (ppm).

Equation 3.1. Calculation of non-haem iron

Total iron concentration (ppm) - Total haem iron (ppm) = total non-haem iron

3.2.7. Bradford protein assay for digestates

The total protein concentration of each digestate was analysed as described by the Bradford Reagent Technical Bulletin (Sigma, St. Louis, MO, USA). Aliquots containing 100µL of diluted digestate were combined with 1.5mL Bradford reagent (B6916) in 2mL microcentrifuge tubes (Eppendorf T2795, Hamburg, Germany) in triplicate and developed for 10 minutes at 20°C. The protein concentration of each treatment was measured against a BSA standard curve (A4612) at 595nm (Novaspec II, Amersham Pharmacia Biotech, UK).

3.2.8. Cell culture media

The cell culture medium contained Dulbecco's modified Eagle's minimal essential medium (DMEM), foetal bovine serum (FBS) (10091130), antibiotic-antimycotic solution (Sigma A5955, St. Louis, MO, USA) (1%), non-essential amino acids (11140050) (1%) and Glutamax (35050061) (1%) at 37°C with 5% CO_2 and 90% humidity.

The cell culture medium used for normal cell growth and maintenance contained calcium enriched (1.8mM) DMEM media (11995073) and 10% FBS. The cell culture medium used for freezing or thawing cells contained calcium enriched (1.8mM) DMEM media and 20% FBS. The cell culture medium used for depleting cell iron stores contained calcium enriched (1.8mM) DMEM media without FBS. The cell culture medium used for depleting cell calcium stores contained calcium-free

DMEM media (21068-028) without FBS. When required, the culture media were supplemented with calcium chloride ($CaCl_2$) (Sigma, C1016 , St. Louis, MO, USA) dissolved in deionised water.

3.2.9. Cell thawing

Caco-2 cells (HTB37) were sourced frozen from American Type Culture Collection (ATCC) at passage 20. The thawing protocol was based on that described by the Caco-2 product sheet (ATCC® HTB-37™). Cells were rapidly defrosted in a water bath at 37°C. Cells were transferred to a 12mL Falcon tube containing 2mL 20% FBS defrosting media and cells were separated by centrifugation for 5 minutes at 125x *g*. The supernatant was discarded and the pellet was gently aspirated in 10mL 20% FBS media. Cells were seeded in T75 flasks (Nunc 178905, NY, USA) at a density of ~30,000 cells/cm^2 in 12mL of 20% FBS media.

3.2.10. Cell splitting

Cells were split every 7 days. The culture medium was removed, cells were rinsed twice with PBS and removed from the T75 flask using 1mL 10x trypsin-EDTA (Sigma T417, St. Louis, MO, USA). Cells were separated from the media by centrifugation for 5 minutes at 125x *g* and seeded to a concentration of approximately 30,000 cells/cm^2 in T75 flasks containing 15mL of 10% FBS-supplemented culture media.

Cells were maintained for 7 days at 37°C with 5% CO_2 and 90% humidity. Culture medium was replaced every four days.

3.2.11. Cell culture

Caco-2 cells between the passage numbers 28 and 35 were seeded at a density of 50,000cells/cm^2. The medium was replaced every 2 days and monolayers were used 21 days post-seeding after monitoring the formation of cell junctions using trans-epithelial electrical resistance (TEER) measurements. Only wells with a TEER value between 250Ωcm^2 and 800Ωcm^2 (after subtraction of the membrane resistance) were used. TEER recordings within this range represent differentiated Caco-2 cell monolayers (Behrens and Kissel 2003).

3.2.12. Cryopreservation

Caco-2 cells were cryopreserved over a range of passage numbers ranging from passage 22 to passage 30. The culture medium was removed, confluent cell monolayers were rinsed twice with PBS and removed from the T75 flask using 1mL 10x trypsin-EDTA. Cells were separated from the media by centrifugation for 5 minutes at 125x *g* and combined with 2mL of 20% FBS freezing media enriched with 5% (v/v) DMSO (472301). The cell suspension was further diluted to 2.25 million cells per mL with 20% FBS media. One millilitre aliquots of cell suspension were transferred into 2mL cryovials (Greiner GR126263, NC, USA) and frozen at -80°C in a

Nalgene freezing container (Thermo Fischer Scientific 5100-000, MA, USA) containing Isopropyl alcohol (Sigma I0398, St. Louis, MO, USA) for 48 hours. Frozen cultures were transferred to liquid nitrogen.

3.2.13. Iron uptake media

The iron uptake medium contained calcium-free DMEM, 25mM HEPES (H3375), 45.83µM $FeCl_3$ (157740) and 4.17µM ^{55}Fe (92.5kBq/mL) (NEZ043001MC), pH 6.7. Additional ascorbic acid and $CaCl_2$ were included as indicated in the following experimental procedures. Iron uptake media were always prepared immediately before use.

3.2.14. Calcium concentration assay

Digestate calcium concentrations were analysed by the cresol phenolphthalein-complexone method by the Nutrition Laboratory (Massey University, Palmerston North, NZ). Briefly the digestate was diluted in deionised water, equilibrated with ethanolamine and combined with a chromogen reagent containing o-cresolphthalein complexone, 8-hydroxyquinoline and HCl. The calcium concentration of all samples was measured using a Flexor EL150 analyser (ELITech Clinical Systems 6003-550, Puteaux, France).

3.2.15. Caco-2 cell preparation for analysis

On the completion of the radionuclide incubation period as indicated within the individual experimental chapters, the Caco-2 cell monolayers were rinsed twice with phosphate buffered saline (PBS) (P5493) and once with 0.5mM ethylenediaminetetraacetic acid (EDTA) (E6758)-supplemented PBS to remove any non-specifically bound iron on the monolayer or surrounding plastic scaffolding. Monolayers were incubated with 200mM sodium hydroxide (NaOH) (306576) for 12 hours at 4°C and samples of aspirated cell lysate were taken for ^{55}Fe radionuclide and cell protein analysis.

3.2.16. Bradford protein assay for Caco-2 cell monolayers

The total protein concentration of each solubilised Caco-2 cell monolayer was analysed as described by the Bradford Reagent Technical Bulletin (Sigma, St. Louis, MO, USA). Aliquots containing 5µL of cell lysate were combined with 250µL Bradford reagent (B6916) in 96 well plates (BD biosciences, NJ, USA) in duplicate and developed for 10 minutes at 20°C. The protein concentration of each monolayer was measured against a BSA standard curve (A4612) at 595nm (ELX 808 microplate reader, Bio-Tek Instruments, VT, USA).

Section 3.3: Optimising the preparation and use of Caco-2 cells in order to measure iron absorption: A review on the methodologies

Caco-2 cell culture techniques and methods of measuring iron transport vary greatly between laboratories. This chapter reviews how culture conditions may affect cellular differentiation and proposes a standardised culture protocol in order to improve the reproducibility of the iron uptake assay. This review also outlines and discusses important steps required during treatment preparation which may preserve cellular integrity and improve the accuracy of the assay.

3.3.1. Abstract

Caco-2 cells are frequently used as a pre-screening tool to investigate iron absorption. The methodologies used to culture Caco-2 cells and to quantify iron absorption vary greatly between laboratories. Standardising the Caco-2 cell culture protocol and the method of quantifying iron absorption may improve the repeatability of the Caco-2 cell iron absorption model. The objectives of this review are to discuss how culture conditions affect the morphological and biochemical properties of the Caco-2 cell, to discuss and standardise important steps during treatment preparation and to define the appropriate methods of quantifying iron absorption. Specifically, the culture conditions covered in this review include the Caco-2 cell passage number, the duration of culture and the use of support structures. Treatment preparation topics include quantifying iron absorption directly using radionuclides and quantifying iron absorption indirectly using cellular ferritin. In summary of this review, a standardised protocol for preparing the Caco-2 cell cultures is proposed.

3.3.2. Introduction

The Caco-2 cell (HTB37) is a colorectal adenocarcinoma cell of human origin. This cell line spontaneously differentiates into a heterogeneous, polarised monolayer bound at the apical-lateral junction by tight junction proteins (Fogh et al. 1977). Despite originating from the colon, the differentiated Caco-2 cell contains many similar morphological and biochemical properties to the duodenal absorptive enterocyte (Pinto et al. 1983), including a brush border membrane displaying a mosaic of nutrient transporters and brush-border-associated digestive enzymes.

Caco-2 cells have been used extensively to investigate mucosal transport of macronutrients (Hidalgo and Borchardt 1990), micronutrients (Wood et al. 2001), drugs (Per 1990) and toxins (Blanchfield et al. 2003). When combined with a digestion procedure, Caco-2 cells can be used to investigate iron absorption from foods, or to screen foods for iron absorption-enhancing properties (Glahn et al. 1996).

The Caco-2 cell system can be used to accurately predict the effect of nutrients on iron absorption in humans (Au and Reddy 2000; Garcia-Casal et al. 2000) and has been used as a pre-screening tool for human trials (Armah et al. 2008). The Caco-2 cell model can also be used to investigate the mechanisms of enterocyte iron transport (Thompson et al. 2010; Thumser et al. 2010).

Despite widespread use of Caco-2 cells within the scientific literature, culture methodologies are inconsistent (Sambuy et al. 2005). Furthermore, the experimental design and quantification of iron absorption is not standardised - this limits the comparison and interpretation of results between laboratories. Further standardisation of the methodology used to culture Caco-2 cells, prepare treatments and quantify iron absorption is required. The aim of this review was to evaluate the basic cell culture methodologies and suggest a standardised protocol for preparing Caco-2 cell cultures in order to investigate iron absorption.

3.3.3. Cell culture protocols

3.3.3.1. Cell passage

The Caco-2 cell monolayer growth rate, degree of differentiation, nutrient requirements, paracellular ion flux and transporter expression is dynamic and dependent on the culture passage number (Chantret et al. 1994; Yu et al. 1997; Behrens and Kissel 2003). The mechanism by which passage number influences cellular expression and metabolism has been proposed by Behrens et al. (2004). Repeated passage of the heterogeneous Caco-2 cell line may selectively promote the proliferation of fast-growing sub-populations within the monolayer. Over repeated passages the monolayer may become significantly more homogenous; altering both the monolayer's genotype and phenotype.

Compared to early-passage cultures, late-passage cultures have a significantly higher rate of mitosis, attainable TEER, and decreased monolayer permeability (Briske-Anderson et al. 1997; Yu et al. 1997; Behrens et al. 2004). These characteristics are unlike that of the small intestinal absorptive enterocyte *in vivo* and suggest that late-passage Caco-2 cell cultures may not be suitable for investigating iron absorption.

The expression of brush border hydrolases alkaline phosphatase (AP) and sucrose isomaltase (SI) (a marker of cell differentiation) by early-passage Caco-2 also poorly reflects that of absorptive enterocytes (Chantret et al. 1994; Briske-Anderson et al. 1997; Yu et al. 1997). This suggests that like late-cell passage caco-2 cell cultures, early-passage Caco-2 cell cultures may also not be suitable for investigating iron absorption.

The rate of proliferation and differentiation of Caco-2 cells remains relatively stable between passages 28 and 40 (Briske-Anderson et al. 1997; Rothen-Rutishauser et al. 2000). Caco-2 cell cultures within this passage range form polarised, differentiated monolayers with high levels of brush border hydrolase expression (Chantret et al. 1994), brush border nutrient transporter expression (Behrens et al. 2004) and tight junctions formation (Behrens and Kissel 2003). For these reasons, Caco-2 cell cultures within a passage range of 28-40 may best simulate the small intestinal enterocyte.

To date iron uptake assays have used Caco-2 cell cultures over a wide range of passage numbers. Although many laboratories use Caco-2 cells with a tight passage

range between passage 28 and 40 (Glahn et al. 1996; Glahn et al. 2002; Sharp et al. 2002), others are either unstated (Garcia et al. 1996) or outside this passage range (Garcia-Casal et al. 2000; Tallkvist et al. 2000). Standardisation of Caco-2 cell passage for investigating iron uptake is required.

3.3.3.2. Culture support structures

Caco-2 cells may be cultured on a range of support structures in order to investigate iron absorption. Non-porous supports such as culture plates (Glahn et al. 1998; Kalgaonkar and Lönnerdal 2008), coverslips (Tandy et al. 2000) or porous filters (Alvarez-Hernandez et al. 1991) have been previously used to analyse cellular iron absorption and the mechanism of cellular iron handling or transport.

Although commonly used for measuring ferritin production or imaging, culturing Caco-2 cells on non-porous supports is also associated with the formation of dome structures within the monolayer (Hara et al. 1993). Domes are formed approximately 5-10 days post-seeding due to basolateral ion efflux and its associated movement of water. Basolateral water movement forces patches of cells, held together by tight junctions, to detach from the membrane support (Ramond et al. 1985). Dome formation may be cytotoxic over prolonged culture durations depending on the composition and pH of the local environment within the sub-basolateral dome space.

Dome formation can be avoided by culturing cells on bicameral chamber containing filter supports with pores ≥ 0.03 microns (Ramond et al. 1985). This allows ions and water to be transported across the basolateral membrane and transferred into the basolateral media. The basolateral media metabolite concentrations and pH can then be maintained within a defined range.

The composition of the filter support also influences Caco-2 cell differentiation (Behrens and Kissel 2003). Polycarbonate (PC) filters are commonly used for investigating brush border membrane iron transport, however because PC filters are opaque, they cannot be used for fluorescent imaging. For this reason translucent filters such as polyethylene terephthalate (PET) or polyester (PE) have also been developed.

Although the brush border enzyme and transport properties are reportedly similar between cells cultured on PC, PE and PET filters, the morphology of the Caco-2 cells grown on PET or PE filters is strikingly different to Caco-2 cells cultured on PC filters. Caco-2 cells cultured on PET or PE membranes form short, cuboidal-like cells uncharacteristic of absorptive enterocytes with a distinct reduction in differentiation and polarity and a reduced level of monolayer organisation (Rothen-Rutishauser et al. 2000). These Caco-2 cells have a higher abundance of actin within their tight junction protein complexes, are less permeable to mannitol and have higher TEER values compared to Caco-2 cells grown on PC filters (Behrens and Kissel 2003). For this reason PC filters should be used for applications which do not require imaging in order to best simulate small intestinal absorptive enterocytes.

3.3.3.3. Use of Collagen or other adhesion molecules

Applying collagen (type 1 or type 4) or laminin to porous or non-porous supports promotes Caco-2 cell adhesion and the expression of brush border transporters and enzymes (Hidalgo et al. 1989; Basson et al. 1996; Behrens and Kissel 2003).

In vivo, enterocyte differentiation results from a complex interplay between basement membrane ligands such as collagen or laminin and integrin signalling proteins within the enterocyte basolateral membrane (Beaulieu 1992). The stimulation of integrin proteins activates cytosolic enzymes involved in cellular migration and cell-cell adhesion such as focal adhesion kinase (FAK) and mitogen activated protein kinase (MAPK) (Lévy et al. 1998).

Type-1 collagen-treated culture plates have been used repeatedly to promote monolayer development and differentiation (Glahn et al. 1999; Huh et al. 2004). Considering that the treatment of plastic supports with type-1 collagen promotes cellular differentiation and improves monolayer morphology, pre-treatment of PET filters supports with type-1 collagen is recommended (Behrens and Kissel 2003).

3.3.3.4. Seeding Density

The seeding density of Caco-2 cells for investigating iron absorption ranges within the literature between 40,000 cells/cm^2 to 100,000 cells/cm^2 (Salovaara et al. 2003; Thumser et al. 2010). Although seeding densities above 200,000 cells/cm^2 (Kibangou et al. 2005) and below 40,000 cells/cm^2 (Han et al. 1994) have been

published previously, higher seeding densities are reported to spontaneously form multi-layered cultures, while cultures less than 40,000 cells/cm^2 may form immature tight junctions (Behrens and Kissel 2003).

HTB37 cells (passage 33) seeded at 50,000 cells/cm^2 cultured to 21 days form a differentiated monolayer featuring a developed brush boarder membrane containing high levels of both sucrase isomaltase and alkaline phosphatase activity, tight junctions, low levels of paracellular permeability and a TEER value of 400-500 Ω/cm^2 (Briske-Anderson et al. 1997). For this reason, when using Caco-2 cells with a passage range of 28-40, a seeding density of 50,000 cells/cm^2 is recommended.

3.3.3.5. Culture time

Caco-2 cell development follows a strict pattern when cultured as described above. A confluent, undifferentiated monolayer is formed within 7 days (Behrens and Kissel 2003). At this time point tight junctions and an immature brush border membrane can be detected. However a mature monolayer featuring polarised expression of nutrient transporters, organised tight junctions and a brush border membrane is not developed until 21 days of culture (Briske-Anderson et al. 1997). Monolayer integrity is somewhat compromised after 30 days of culture (Behrens and Kissel 2003).

The Caco-2 cell gene expression profile is significantly altered after 14 days of culture (Mariadason et al. 2002). Caco-2 cells spontaneously undergo cell cycle arrest. At this point, the expression of genes associated with cellular proliferation and migration is down regulated. Contralaterally, the expression of genes involved in nutrient transport and metabolism are up-regulated, reflecting that of enterocytes *in vivo*. The authors proposed that this widespread change in gene expression is initiated predominantly by contact inhibition of the confluent monolayer, initiating a pre-programmed cascade of differentiation.

The Caco-2 culture time from seeding until use for investigating iron absorption ranges within the literature from 8 days (Garcia-Casal et al. 2000) to 21 days (Han and Wessling-Resnick 2002). Although monolayers have undergone differentiation by day 15 in culture, it is suggested that monolayers should be used at 21 days to insure monolayer uniformity (Briske-Anderson et al. 1997; Rothen-Rutishauser et al. 2000; Mariadason et al. 2002).

3.3.4. Correct treatment preparation

The differentiated Caco-2 cell monolayer is used to simulate the absorptive mucosa of the small intestine. Accordingly, the treatment should be digested mechanically, chemically and enzymatically *in vitro* to simulate the gastric and intestinal phases of digestion prior to exposing the Caco-2 cells to the treatment. The treatments should also be modified in order to preserve the integrity of the Caco-2 cell monolayer. These steps are discussed in Chapter 3, Section 3.4.

3.3.5. Measuring iron absorption

Iron transport across Caco-2 cells can be investigated by tracing the movement of an iron radionuclide from the apical reservoir media into the cell (Glahn et al. 1996), or across the cell into the basolateral reservoir (Gaitán et al. 2012). Iron absorption can also be investigated using indirect measures of iron absorption. This may include measuring the ferritin concentration after exposing the brush border membrane to iron (Glahn et al. 1998). Both measurements have been used to successfully predict iron absorption in humans (Glahn et al. 1996; Glahn et al. 1999). Since each method includes benefits and limitations, the choice of method should reflect the properties of the treatment.

3.3.5.1. Use of radionuclides to measure iron absorption

Iron radionuclides ^{55}Fe and ^{59}Fe have been used extensively within the literature to investigate iron absorption and distribution. The radionuclide may be added to a food item immediately prior to consumption (Björn-Rasmussen et al. 1972; Glahn et al. 1996) or incorporated intrinsically within the food item, grown, harvested and consumed (Layrisse et al. 1969).

The use of radionuclides is commonly used because the measurement of iron absorption is not restricted by cellular iron handling (e.g. cellular ferritin production). For this reason iron absorption can be analysed over multiple time points. This allows the rate of iron transport across the brush border membrane to be investigated (Thumser et al. 2010).

On the completion of a radionuclide uptake trial *in vitro*, the Caco-2 cell monolayer must be thoroughly rinsed with an iron chelator such as bathophenanthroline disulfonic acid or EDTA in order to remove non-specifically bound iron from the brush border membrane and surrounding plastic supports (Glahn et al. 1995). Failure to undertake this step may overestimate iron absorption.

3.3.5.2. Extrinsic nuclide tags

The use of an extrinsic radionuclide tag (ERT) is suitable for investigating iron absorption only if the radionuclide completely exchanges with the treatment's own endogenous iron pool. For this to occur, the entire non-haem iron content of the treatment must be released from the food in order to exchange with the ERT. The addition of an ERT to eggs, meat, refined wheat meal, refined maize meal or milk has been reported to completely combine and exchange and therefore accurately predict iron absorption (Schulz and Smith 1958; Björn-Rasmussen et al. 1972).

However ERTs may overestimate iron absorption from foods where non-haem iron release from the endogenous iron pool is restricted (such as un-processed grains) (Björn-Rasmussen et al. 1972). In this circumstance the exchangeable iron pool is significantly reduced, little exchange occurs between the ERT and the total endogenous iron pool and the proportion of ERT absorbed by the Caco-2 cell monolayer overestimates total non-haem iron absorption.

The accuracy of the ERT is improved if the radionuclide salt is of a similar chemical composition to the intrinsic iron which it exchanges with. For example, the combination of an ERT with an iron supplement or low-iron foods enriched with an iron supplement has been reported to have a high degree of accuracy at predicting total non-haem iron absorption (Layrisse et al. 1973).

3.3.5.3. Intrinsic nuclide tags

Foods may be intrinsically tagged by incorporating an iron radionuclide into plant or animal tissues. Intrinsic radionuclide tags (IRT) can be used as an alternative to ERT in order to investigate iron absorption (Layrisse et al. 1969). Unlike ERTs, the use of IRTs is not limited by the release of iron into the exchangeable iron pool and therefore lowers the risk of overestimating iron absorption. Like the use of ERTs, IRTs can be used to investigate the rate of iron absorption.

Although IRTs may be more accurate than ERTs, the use of IRTs has been somewhat limited within the recent literature. As discussed by Monsen (1974), the production of intrinsically tagged plant and animal products is expensive and time consuming, requiring specialised equipment and technical expertise. Furthermore the concentration of radionuclide present within the intrinsically tagged food sample may be too low to accurately measure iron absorption *in vitro* (Glahn et al. 1998).

3.3.5.4. Cellular ferritin production

The enterocyte LIP concentration regulates the expression of ferritin (Torti and Torti 2002). Ferritin production is directly proportional to brush border iron transport within the Caco-2 cell (Gangloff et al. 1996) therefore changes in cellular ferritin concentration can be used as an indirect measure of iron absorption (Glahn et al. 1998).

Like the use of IRTs, the measurement of serum ferritin is not limited by iron release into the exchangeable iron pool and therefore lowers the risk of overestimating iron absorption. The change in cellular ferritin concentration compared to a negative control represents iron transport across the brush border membrane. This measurement is not influenced by non-specifically bound iron within the brush border membrane or surrounding plastic supports. The cellular ferritin method can be used to measure iron absorption from single foods or whole meals.

Unlike the use of ERTs and IRTs which can be used to calculate iron transport immediately after the cessation of the uptake experiment, the ferritin assay requires a further 16-22 hours culture time post-treatment removal in order to stimulate ferritin production (Glahn et al. 1998; Thompson et al. 2010). For this reason the rate of iron absorption may be difficult to assess using indirect measures of iron absorption.

3.3.6. General conclusions

Although Caco-2 cells have been used extensively to investigate the effects of foods or specific nutrients on iron absorption, a standardised protocol has not yet been adopted. In-discrepancies in culture method can significantly affect the morphology, growth characteristics, cell differentiation and transport properties of the Caco-2 cell.

The Caco-2 cell line should be used no earlier than passage 28 and no later than passage 40 for investigating iron absorption. Cells should be seeded at a density of ~50,000 cells/cm^2 on PC filter supports where possible (unless required for fluorescence imaging) in order to promote optimal cell morphology and cultured for 21 days prior to uptake.

Treatments should be prepared under simulated gastrointestinal conditions and modified to maintain the integrity of the Caco-2 cell monolayer. Radionuclides should only be used if they will exchange completely with the exchangeable iron pool. If foods which have limited non-haem iron release are to be investigated (such as whole grains), intrinsic radionuclide techniques or the ferritin method should be used.

Section 3.4: Methods developed. Standardisation of an in vitro digestion method for investigating iron uptake in Caco-2 cells*

The objectives of this study were to investigate the activity of gastrointestinal proteases under simulated digestion conditions *in vitro* and to develop a method to remove the proteases post-digestion in order to maintain Caco-2 cell integrity. The following chapter describes the results of the study and discusses how the digestion procedure can be optimised to best simulate physiological conditions.

*Part of the material presented in this section has been previously presented at the NZNS Annual Conference: Stewart, R.J.C., Morton, H., Coad, J., Reynolds, G.W., Pedley, K. C. (2012). Standardisation of an in vitro digestion for investigating iron uptake in Caco-2 cells. *NSNZ Annual Conference*, 22-23 November, Auckland.

3.4.1. Abstract

The *in vitro* digestion/Caco-2 cell absorption model has been used extensively to investigate iron absorption; however the activity of gastric and pancreatic proteases is unknown during prolonged digestive phases. The objectives of this study were to investigate the activity of the digestive proteases during each digestion phase *in vitro* and to develop a protocol to remove or inactivate digestive enzymes post-digestion. Pepsin and pancreatic protease activity were measured by stop-rate spectroscopy at 30 or 60 minute intervals during the simulated gastric and intestinal digestion phases respectively. Proteases were removed or inactivated in the digestate by ultrafiltration, dialysis, heat treatment or pH reduction. The effectiveness of each method was assessed by measuring the protease activity of the digestate, measuring the trans-epithelial electrical resistance of differentiated Caco-2 cells and by fluorescence microscopy. The protease activity of pepsin and pancreatin was significantly decreased under simulated gastric and intestinal digestion conditions respectively suggesting that these proteases are being inactivated. Enzyme removal by ultrafiltration or dialysis and inactivation by pH treatment preserved monolayer integrity. Only ultrafiltration significantly reduced digestate protease activity and maintained monolayer integrity without incurring a significant dilution factor. Heat treatment and pH adjustment were less effective.

3.4.2. Introduction

Human trials are regarded as the gold standard for investigating factors which influence iron absorption (Casgrain et al. 2010) however these trials are limited by cost and/or ethical considerations. For this reason it is often necessary to pre-screen treatments of interest using an *in vitro* model.

In vitro models often include a digestion phase, developed to simulate gastrointestinal processes. This is then coupled with measurements of iron solubility and dialisability (Mulvihill et al. 1998). This may also be coupled with measurements of iron absorption by biological tissues such as ligated sections of small intestine (Van Campen 1973) or cultured enterocytes (Glahn et al. 1996).

Although post-digestion measurement of iron solubility or dialisability can be used to identify components which enhance or inhibit iron uptake in humans (Storcksdieck and Hurrell 2007), the use of biological tissues to measure iron absorption from a digest is more reliable (Au and Reddy 2000; Pynaert et al. 2006; Armah et al. 2008).

The validity of various *in vitro* digestion protocols has been reviewed extensively within the literature (Miller et al. 1981; Ekmekcioglu 2002). These reviews collectively suggest that for an *in vitro* system to accurately simulate digestive processes, the digestion protocol must contain both a gastric and intestinal digestion phase. Each phase must be based on its own specific physiological parameters. These parameters include pH, protease species and digestion time.

The reported time from when a protease is activated, until its respective digestion phase is completed *in vitro,* varies markedly within the literature (Miller and Berner 1989; Glahn et al. 2000). The stability and activity of pepsin or pancreatic proteases under these simulated gastrointestinal conditions for prolonged digestion times are unknown. Further investigation into the activity of pepsin and pancreatic proteases during simulated gastric and intestinal digestion phases is therefore required in order to ensure that protease activity is maintained for the duration of the experiment.

When incubating a digestate with absorptive enterocytes in order to measure iron absorption, the tissue preparation must be protected from any remaining proteases in order to maintain enterocyte integrity. Techniques such as digestate dialysis (Glahn et al. 1996) or protease dilution (Garcia et al. 1996) have been used previously, however both techniques significantly dilute the concentration of iron within the dialysate. This may be problematic when using an iron radionuclide to investigate iron absorption unless the treatment radionuclide concentration is considerably increased.

Two series of experiments are presented in this study. The first series was designed to investigate pepsin and pancreatin protease activity over a time range typically used within the literature for the simulated gastric and intestinal phases of digestion. The second series was designed to investigate the removal or inactivation of proteases within the completed digestate prior to incubation with Caco-2 cells.

3.4.3. Methods

3.4.3.1. In vitro digestion

Immediately before use, porcine pepsin (P7000, 800-2500 units/mg protein) was activated in 0.1M HCL (25mg/mL) for 5 minutes. For the simulated gastric digestion, 5mL saline solution (120mmol/L NaCl (S7653) and 5mmol/L KCl (P9333)) was titrated to pH 2.5 with 1M HCl prior to the addition of a 0.5mL activated pepsin aliquot. Samples were titrated to pH 2 and incubated at 37°C on a rocking shaker for 60 minutes.

Immediately before use, porcine pancreatin (P1625, activity 3 x USP specifications) and bile salts (B8631) were combined with 0.1M $NaHCO_3$ (S5761) (9.26mg/mL and 55.5mg/mL respectively). For the simulated intestinal digestion, whole gastric digests were titrated to pH 6.0 with 1M $NaHCO_3$ and combined with 0.5mL activated pancreatin/bile solution. All digests were adjusted to 300mOsm and made up to a final volume of 15mL with saline solution, pH 7.0, before incubation at 37°C on a rocking shaker for 120 minutes.

3.4.3.2. Pepsin activity assay

The gastric phase of the *in vitro* digestion system was set up in triplicate as described above. The pepsin activity of the gastric digest was analysed immediately after the addition of pepsin, and after 30, 60, 90 and 120 minutes incubation.

Pepsin activity was measured by spectrophotometric stop rate determination modified from Chow and Kassell (1968). Bovine haemoglobin (H2625) was combined with deionised water (2.5% w/w) and filtered through glass wool. The haemoglobin filtrate was diluted 4:1 with 0.3M HCl and incubated at 37°C for 5 minutes. Aliquots (1mL) of gastric digest were combined with 5mL acidified haemoglobin and the reaction was incubated for 10 minutes at 37°C. Pepsin activity was stopped by the addition of 5mL of 5% trichloroacetic acid solution (TCA) (T6399). For the blank, 5mL TCA solution was combined with 5mL of acidic haemoglobin prior to the addition of the gastric digest. All samples were separated by centrifugation at 450x *g* for 7 minutes and the supernatant was passed through 0.22µm Minisart SRP syringe filters (Sartorius AG, Goettingen Germany). The TCA-soluble peptide concentration of each sample was analysed at 280nm by spectrophotometer (Cary 300, Varian, CA, USA). The concentration of pepsin units per millilitre of gastric digest was calculated from Equation 3.4.1.

Equation 3.4.1. Calculation of pepsin activity

Pepsin units/mL gastric digest = (ABS_{280} test – ABS_{280} Blank) / (0.001 Δ ABS per unit of enzyme*10 min*1mL).

3.4.3.3. Pancreatic protease activity

The intestinal phase of the *in vitro* digestion system was set up in triplicate as described above. The protease activity of the intestinal digest was analysed in duplicate immediately after the addition of activated pancreatin, and after 60, 120 and 180 minutes incubation. The pancreatic protease activity assay was extended to 180 minutes in order to simulate prolonged *in vitro* simulations of the intestinal phase.

Protease activity was measured by spectrophotometric stop rate determination using the substrate casein, a method modified from Cupp-Enyard (2008). Bovine casein (C3400) was combined with potassium phosphate buffer (0.65%W/V) (50mM potassium phosphate dibasic, trihydrate (P5504) in deionised water) heated to 80°C ± 5°C for 10 minutes, cooled to 37°C and corrected to pH 7.5 with 1M NaOH. Aliquots of intestinal digest (1mL) were combined with 5mL casein solution in duplicate and the reaction was incubated for 10 minutes at 37°C.

Protease activity was stopped by the addition of 5mL of 1.6% TCA. For the blank, 5mL TCA solution was combined with 5mL of casein solution prior to the addition of the intestinal digest. All samples were separated by centrifugation at 335x *g* for 7 minutes. Supernatant aliquots (2mL) were combined with 5mL sodium carbonate (S7795) (5.3g/100mL deionised water) followed immediately by 1mL of Folin's reagent (F9252) (0.5M). All samples were incubated for 30 minutes at 37°C and passed through disposable 0.22µm Minisart SRP syringe filters (Sartorius AG, Goettingen Germany).

Samples and blanks were measured by spectrophotometry at 660nm and the concentration of TCA-soluble peptides were calculated from a tyrosine standard curve ranging from 0 to 0.5 µM tyrosine (W373605) in deionised water.

3.4.3.4. Removal or inactivation of digestive enzymes:

Five digestions as described above were undertaken in triplicate. Completed digestates were allocated to 1 of 5 treatments: control (enzymes not removed or inactivated), pH treatment, heat treatment, dialysis membrane or ultrafiltration. Additionally undigested HBSS was used as a negative control. The details of each treatment are summarised in Table 3.4.1.

Table 3.4.1. Protocols to remove or inactivate digestive enzymes from digestate

Treatment	*Sample*	*Protocol*
Positive control	Digestate	No intervention, positive control.
Negative control	HBSS	No intervention, negative control.
pH	Digestate	Titration to pH 2 with 1M HCl for 5 minutes. Titration back to pH 7 with 1M NaOH.
Heat	Digestate	Incubated at 65°C for 10 minutes. Immediately cooled to 37°C.
Dialysis	Digestate	1.5mL digestate placed inside dialysis membrane (12kD cut-off) (Sigma, St. Louis, Mo., U.S.A.). Inserted into 15mL Falcon tube (Grenier Bio-one) containing 1mL HBSS. Incubated at 37°C on a rocking shaker for 60 minutes. Dialysate collected.
Ultrafiltration	Digestate	Digestate filtered through a stirred ultrafiltration cell (Amicon, Danvers, MA) fitted with YM10 10KD molecular cut-off membrane (Amicon, Danvers, MA) under nitrogen (25psi). Filtrate collected.

3.4.3.5. Cell cultures

All cell cultures were maintained and split as described in Chapter 3, Sections 3.2.9 and 3.2.10. Caco-2 cells were cultured on 12-well bicameral Transwell chambers (Corning CLS3460, MA, USA) as described in Chapter 3, Section 3.2.11.

3.4.3.6. Caco-2 cell trans-epithelial electrical flux

All media was removed and replaced with pre-warmed HBSS (pH 7.4, 37°C). Caco-2 cell monolayer TEER measurements were recorded. HBSS within the apical reservoir was replaced with a 400µL aliquot of digestate or HBSS in a randomised order. The Caco-2 cells were incubated for 60 minutes at 37°C with 5% CO_2 and 90% humidity. At 60 minutes digestates were removed, replaced with HBSS (pH 7.4, 37°C) and TEER measurements were recorded.

Monolayers treated with HBSS, the control digestate and the digestate separated by ultrafiltration for 60 minutes were rinsed twice with HBSS and fixed with 4% paraformaldehyde (158127) for 30 minutes. Fixed samples were stained with bisbenzimide (Hoechst 33258), imaged by confocal fluorescence microscopy and analysed using ImageJ software (NIH).

3.4.3.7. Protease activity assay

The effect of ultrafiltration on enzyme removal was further analysed by assaying protease activity of the digestate immediately before and after filtration by stop-rate determination as described above for the analysis of pancreatic protease activity.

3.4.3.8. Calculations and Statistical analysis

Changes in pepsin or pancreatin activity per mL digestate at any time point during the experimental phase were compared to the initial protease activity at time zero. Changes in TEER and digestate protease activity after enzyme removal or inactivation were calculated as a percentage change compared to the untreated digestate.

Statistical analysis was performed using SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Pepsin and pancreatin activity during the digestion procedure was analysed by repeated measures ANOVA. Protease activity in the complete digestate and its effects on TEER and tissue integrity were analysed by 1-way ANOVA. All analyses were undertaken using the General Linear Model procedure. Where appropriate, post-hoc analysis was carried out using least square difference (LSD) analysis.

3.4.4. Results

Pepsin protease activity during the simulated gastric digestion phase is illustrated in Figure 3.4.1. Compared to the initial activity of pepsin at time zero, pepsin activity was significantly decreased by 10% after 30 minutes incubation (P=0.004), 18.5% by 60 minutes incubation (P<0.0001), 49% by 90 minutes incubation (P<0.0001) and 63% by 120 minutes incubation (P<0.0001).

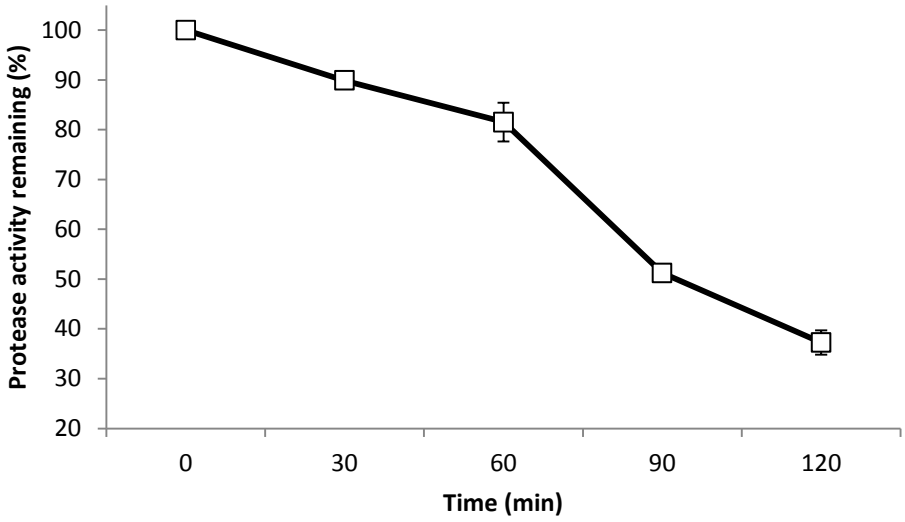

Figure 3.4.1. Pepsin activity during gastric digest.

Pepsin activity was measured by stop-rate determination in 1mL samples of gastric digestates. Samples were taken immediately after the addition of pepsin to the digest and after incubation at 37°C on a rocking shaker for 30, 60, 90 and 120 minutes. Data points represent mean values ± SEM (n = 6). All data points are significantly different from one another (P<0.05).

Pancreatin protease activity during the simulated intestinal digestion phase is illustrated in Figure 3.4.2. Compared to the initial activity of pancreatin protease at time zero, pancreatin protease activity significantly decreased by 6% after 60 minutes incubation ($P=0.015$), 21.5% by 120 minutes incubation ($P<0.0001$) and 32.5% by 180 minutes incubation ($P<0.0001$).

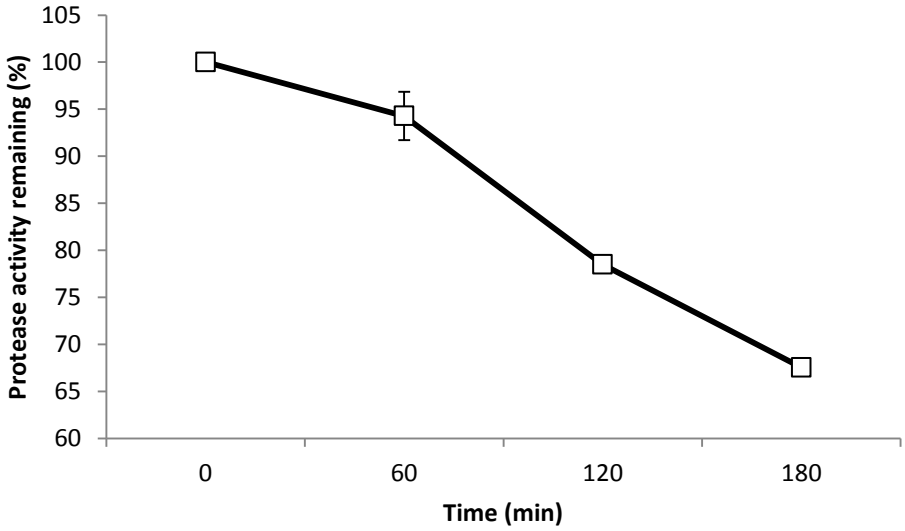

Figure 3.4.2. Pancreatin protease activity during intestinal digest.

Protease activity was measured by stop-rate determination in 1mL samples of intestinal digestates. Samples were taken immediately after the addition of pancreatin to the digest and after incubation at 37°C on a rocking shaker for 60, 120 and 180 minutes. Data points represent mean values ± SEM (n = 3). All data points are significantly different from one another (P<0.05).

Changes in Caco-2 cell monolayer TEER after 60 minutes incubation with digest treatments or HBSS are illustrated in Figure 3.4.3. TEERs were significantly reduced in the control digestate treatment compared to the HBSS treatment (P=0.001), the dialysis membrane treatment (P<0.001), the ultrafiltration treatment (P<0.001) and the pH treatment (P=0.002) but not the heat treatment (P=0.056). There was no significant difference between the heat and pH treatments (P=0.059). There was no statistical difference in TEER change between the HBSS control, dialysis membrane or ultrafiltration treatments (P>0.05).

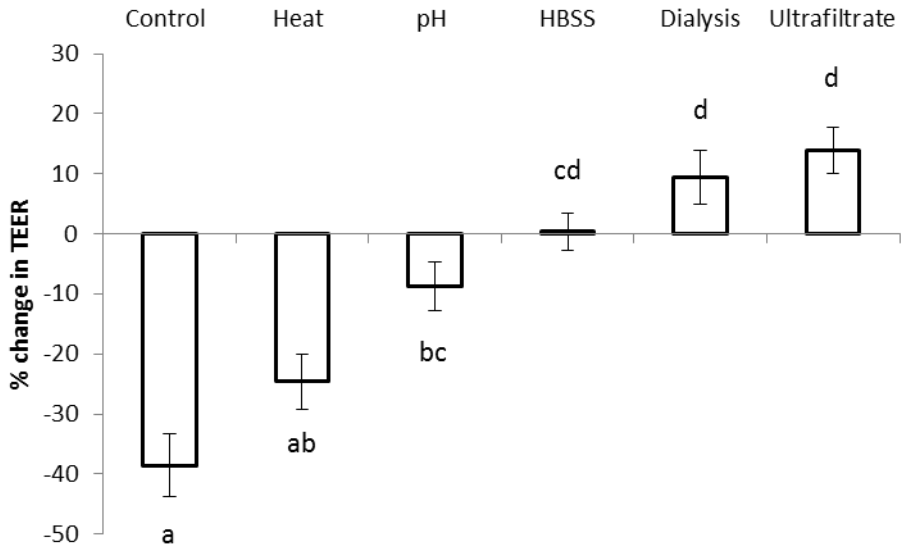

Figure 3.4.3. Percent change in trans-epithelial electrical resistance (TEER).

Caco-2 cell monolayers were incubated for 60 minutes with HBSS control, digestate control, digestate passed through 12KD dialysis membrane, digestate passed through a 10KD ultrafiltration membrane, digestate titrated to pH 2 post digestion for 5 minutes and then back pH 7 or HBSS digestate incubated at 65°C for 10 minutes. TEER values of Caco-2 cell monolayers were recorded before and after incubation with the digestate. Bars represent mean values ± SEM (n = 4-8). Values marked with dissimilar letters are significantly different from one another (P<0.005).

The effects of ultrafiltration and pH on digestate protease activity are illustrated in Table 3.4.2. Ultrafiltration significantly reduced digestate protease activity compared to the pH treatment (P<0.0001).

Table 3.4.2. Protease activity of digestate after ultrafiltration or inactivation by pH shock

Treat	% activity remaining	SE
Ultrafiltration	20.813	0.231
pH treatment	74.56	1.47

Values represent mean values ± SEM (n = 3). Values are significantly different from one another (P<0.0001).

The effects of incubating the HBSS control, ultrafiltrated digestate or control digestate on Caco-2 cell monolayer integrity are illustrated in Figure 3.4.4. The Caco-2 monolayer cell density was significantly reduced when incubated with the control digestate compared to monolayers incubated with the HBSS or digest treated with ultrafiltration (P<0.0001).

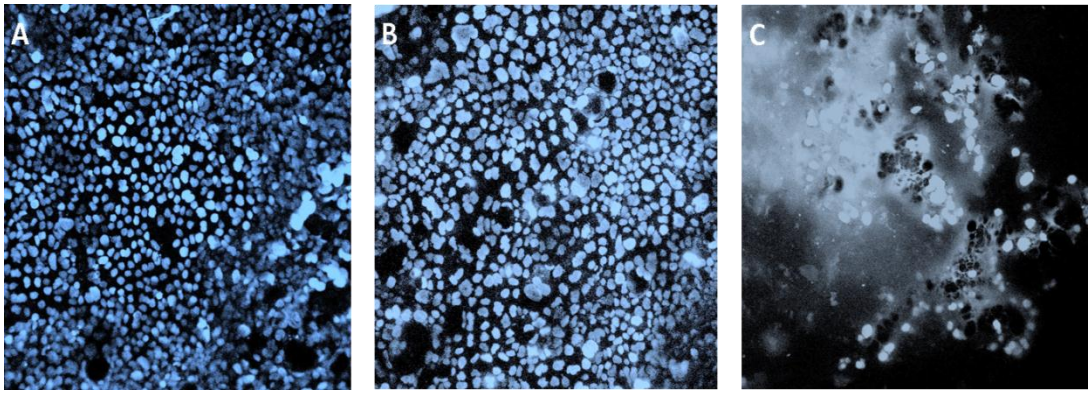

Figure 3.4.4. Caco-2 cell monolayer integrity.

Caco-2 cell monolayers were incubated for 60 minutes with HBSS control (A), digestate filtered through a 10KD ultrafiltration membrane (B) or control digestate (C). Caco-2 monolayers were treated with bisbenzimide, imaged by fluorescent microscopy (40X) and analysed by ImageJ software.

3.4.5. Discussion

3.4.5.1. Protease activity during digestion in vitro

During the gastric phase of digestion, the average adult stomach is calculated to produce approximately 0.8mg active pepsin/mL digesta (Vertzoni et al. 2005). The gastric mucosa constantly secretes pepsin into the acidic digesta to maintain this concentration in order to compensate for pepsin dilution, loss or inactivation during digestion.

In vitro digestion protocols typically combine activated pepsin to an acidified food slurry once at the initiation of the gastric phase (Miller et al. 1981). Although pepsin is neither removed nor diluted during this digestive process, it can be partially inactivated when pepsin inhibitors which may be present within the digestate (Vaintraub and Bulmaga 1991).

The current study suggests that pepsin activity is significantly reduced under gastric conditions when pepsin inhibitors are absent. The initial pepsin concentration of 1.2mg active pepsin/mL digesta was reduced to approximately 0.44mg active pepsin/mL digesta after 120 minutes incubation. The results suggest that either the simulated gastric conditions are not favourable for maintaining pepsin structure and function, or that pepsin may be inactivating itself. The latter seems likely since porcine pepsin has a pH optimum of pH 2 at 300mOsm (Sielecki et al. 1990) which were the exact conditions used in the current study.

Porcine pepsin A contains up to 90 potential autolytic cleavage sequences (PeptideCutter, ExPASy, Swiss Institute of Bioinformatics, 2012). Although many of these cleavage sequences will not be accessible for hydrolysis due to the enzymes tertiary structure, the significant reduction in pepsin protease activity observed during the gastric phase of digestion suggests that significant pepsin autolysis is likely.

Like the gastric mucosa, the human pancreas secretes pancreatic enzymes into the small intestine when digesta is present in order to maintain the enzyme/digestate ratio and compensate for protease loss or inactivation (Ekmekcioglu 2002). During the intestinal phase of the *in vitro* digestion, activated proteases and bile salts are typically combined with the neutralised gastric digesta once at the initiation of this digestive phase (Miller et al. 1981).

Although pancreatic enzymes are neither removed nor diluted during the digestion process *in vitro*, like pepsin they can be inactivated by protease inhibitors within the digestate (Singh and Krikorian 1982; Thiruvengadam and DiMagno 1988).

The current study showed that like pepsin, the protease activity of pancreatin was significantly reduced under *in vitro* conditions despite the digesta containing no known protease inhibitor. This result also suggests that either the simulated intestinal conditions are not favourable for maintaining pancreatin structure and function, or that the pancreatic proteases may be inactivating each other. Again, the latter seems likely since pancreatic proteases have pH optima close to pH 7 (Schwert and Takenaka 1955).

Porcine trypsin and chymotrypsin contain up to 14 potential autolytic cleavage sequences each (PeptideCutter, ExPASy, Swiss Institute of Bioinformatics, 2012). This suggests that like pepsin, pancreatic protease autolysis is also likely under simulated intestinal conditions, albeit to a lower degree.

The results of this study suggest that pepsin and pancreatic proteases are significantly inactivated under simulated gastrointestinal conditions. For this reason the concentration of active proteases must be accounted for during prolonged digestion protocols *in vitro*. Furthermore, proteases should be activated immediately prior to use, unless enzyme pre-treated is required (such as a 60 minute incubation with Chelex beads (Glahn et al. 2000)) in which case the protease concentration should be increased in order to account for a loss of active enzyme units.

3.4.5.2. Removal of digestive enzymes prior to measuring iron absorption in Caco-2 cells

The current study clearly shows that failing to remove the digestive enzymes prior to incubating the digestate with Caco-2 cells severely compromises the Caco-2 cell monolayer integrity compared to a protease-free control.

Removal of the digestive enzymes with techniques such as ultrafiltration significantly reduced the digestate protease activity and maintained Caco-2 cell monolayer TEER and morphology compared to the control digestate. Treatment dialysis was equally effective at maintaining Caco-2 cell monolayer TEER, however dialysis also introduced a significant dilution factor and was therefore discontinued.

Protease inactivation by heating the digestate to 65°C for 10 minutes was ineffective at maintaining Caco-2 monolayer TEER compared to the control digestate. Heat treatment was therefore discontinued. Titrating the digestate back to pH 2 after the intestinal phase of digestion for 5 minutes (prior to re-neutralisation) maintained Caco-2 cell monolayer TEERs compared to the control digestion however it only reduced protease activity by approximately 20% and was therefore discontinued. The results therefore suggest that ultrafiltration may be the most effective approach to maintain enterocyte integrity.

In summary, the results suggest that both pepsin and pancreatic proteases undergo significant inactivation under *in vitro* conditions. This may compromise the ability of the *in vitro* model to accurately simulate gastrointestinal conditions if these enzymes are not replaced. Once *in vitro* digestion is complete any remaining enzymes should be removed or inactivated prior to incubating the digestate with Caco-2 cells. Ultrafiltration may be the most effective method in order to maintain enterocyte integrity without accumulating a significant dilution factor.

Chapter Four: Effect of New Zealand green-lipped mussels on non-haem iron absorption in Caco-2 cells*

The primary objective of this study was to investigate the effect of New Zealand green-lipped mussel on non-haem iron absorption compared to ascorbic acid and beef (positive controls) and egg albumin (negative control) using Caco-2 cell cultures. The following chapter describes the results of the study and discusses the potential beneficial effect of New Zealand green-lipped mussel as an alternative iron absorption-enhancer to red meat.

*Part of the material presented in this section has been previously published as a peer reviewed journal article: Stewart, R. J. C., J. Coad, et al. (2012). New Zealand green-lipped mussels (Perna *canaliculus*) enhance non-haem iron absorption in vitro. British Journal of Nutrition **1**(1): 1-4 © reprinted with permission. Part of this material has also been presented at the NZNS Annual Conference: R.J.C. Stewart, J. Coad, G. Reynolds, K.C. Pedley (2010). Fishing for Iron. *NSNZ Annual Conference*, 6-8 December, Wellington.

4.1. Abstract

Iron 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 iron. The objectives of this study were to identify whether extracts of NZGLM enhance non-haem iron absorption in Caco-2 cells and to compare the effect to that of beef. Raw NZGLM and raw beef homogenate were digested *in vitro* with pepsin at pH 2, and pancreatin and bile salts at pH 7. ^{55}Fe was combined within the digestate in order to measure cellular iron uptake. Ascorbic acid was used as 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 minutes. All values were standardised per µg of NZGLM, egg albumin, beef or ascorbic acid. Ascorbic acid enhanced non-haem iron absorption to the greatest extent. Beef and NZGLM digestate both significantly enhanced iron absorption compared to egg albumin. In conclusion, NZGLM digestate significantly enhances non-haem iron uptake in Caco-2 cells with a similar magnitude to that of beef.

4.2. Introduction

Red meat, pork, poultry and fish all significantly enhance iron absorption when consumed as part of a vegetable-based meal in human participants (Cook and Monsen 1976). Further investigation into the bioactive components of the MPF factor suggests that cysteine-rich myofibrils (Mulvihill et al. 1998), GAGs (Huh et al. 2004) and L-α (Armah et al. 2008) could be responsible for this enhancing effect, either individually or in combination (Laparra et al. 2008). Other dietary factors including PUFAs (Seiquer et al. 2002) and non-digestible soluble carbohydrates including inulin and FOS (Ohta et al. 1995) may also increase non-haem iron absorption, however the magnitude of enhancement and the mechanism in humans remains elusive.

Of all MPF sources, red meat and pork have been repeatedly reported to promote iron absorption to the highest degree in human subjects (Cook and Monsen 1975; Cook and Monsen 1976; Armah et al. 2008). However, the addition of saturated fatty acids to the diet via increased meat consumption or the replacement of dietary polyunsaturated fatty acids with saturated fatty acids is strongly associated with negative cardiovascular outcomes, while the reverse is cardio-protective (Astrup et al. 2011).

Promoting red meat consumption in order to improve iron status at a population level may be problematic, and the identification of an alternative MPF factor source rich in PUFAs or low in SFAs would be valuable. The effect of oily fish on iron uptake has been investigated *in vivo*, however inconsistencies between protocols have led to contradicting results (Rodriguez et al. 1996; Seiquer et al. 2002; Navas-Carretero et al. 2008).

There has been little investigation into the effect of bi-valve molluscs on non-haem iron absorption either *in vitro* or *in vivo*. NZGLM are rich in both haem and non-haem iron, myofibrillar proteins, low molecular weight aminoglycans and n-3 PUFAs (Murphy et al. 2002) and may therefore be an alternative source of meat factor. The aim of this study was to investigate the effects of NZGLM digestate on non-haem iron uptake in Caco-2 cell monolayers and compare its effects to those of ascorbic acid, egg albumin and beef.

4.3. Materials and methods

4.3.1. Cell culture

All cell cultures were maintained as described in Chapter 3, Section 3.2.10. Cells were seeded on ThinCert chambers (Greiner Bio-One 665640, Germany) and cultured as described in Chapter 3, Section 3.2.11.

The number of cells attached to each ThinCert chamber was determined by fluorescence microscopy of cell nuclei stained with bisbenzimide (Hoechst 33258). Nuclei numbers were analysed using ImageJ software (NIH).

4.3.2. Treatments

Treatments were prepared as described in Chapter 3, Section 3.2.3. Fresh, but not lyophilised treatments were used for digestion.

4.3.3. In vitro digestion

The *in vitro* digestion was undertaken as described in Chapter 3, Section 3.2.4. The non-haem iron concentration of each digestate was analysed and calculated as described in Chapter 3, Section 3.2.5 and Section 3.2.6. The protein concentration was analysed as described in Chapter 3, Section 3.2.7.

Ascorbic acid (A0278) solution was prepared with Hank's buffered salt solution (HBSS) (H4641) at a concentration of 95.5µmol/L. Beef, NZGLM and egg albumin digestates and ascorbic acid were combined with a pre-prepared 23.87µM iron working solution containing 1:10 ^{55}Fe (NEZ043001MC) and ^{56}Fe (451649) respectively in 0.1M HCl. The final radioactivity of each digestate treatment was 92.5kBq/mL digestate. The final molar ratio of ascorbate:Fe was 4:1.

4.3.4. Experimental design

Digestate treated aliquots (400µL) were applied to eight apical reservoirs each in a randomised order. The uptake solution was aspirated and a 100µL apical reservoir sample was removed. The Caco-2 cells were incubated for 60 minutes at 37°C with 5% CO_2 and 90% humidity. At 60 minutes, one 100µL aliquot was taken from the basolateral reservoir of each well. Non-specifically bound iron on the Caco-2 cell brush border membrane was removed and cell monolayers were prepared for analysis. These processes are described in Chapter 3, Section 3.2.15.

All samples were combined with OptiPhase HiSafe 2 scintillation cocktail (PerkinElmer 1200-436, Waltham, MA, USA) and the concentration of radionuclide was determined by liquid scintillation counting (Wallac Trilux 1450 Microbeta PerkinElmer, Waltham, Massachusetts, USA).

All results were standardised per µg of beef, GLM, egg albumin or ascorbic acid present within the treatment aliquot. Briefly, the protein concentration of each 300mOsm digestate was compared to the protein concentration values generated from the proximal analysis of whole sirloin beef, NZGLM and egg albumin, less the digestive enzymes utilised for the *in vitro* digestion in order to calculate digestate dilution factor for each treatment. This dilution factor was then used to standardise iron uptake (%) per µg of whole beef, NZGLM, egg albumin or ascorbic acid based on the dose-dependent effect of iron absorption-enhancers (Hallberg and Hulthen 2000). Iron absorption was then expressed as percent iron absorbed compared to egg albumin digestate.

4.3.5. Statistical analysis

Statistical analysis was performed using SAS 9.1 statistical software (SAS Institute Inc., Cary, NC, USA). Treatments were analysed by 2-way ANOVA with the General Linear Model procedure. Where appropriate, post-hoc analysis was carried out using LSD analysis.

4.4. Results

4.4.1. Cell culture viability

All Caco-2 cell monolayers had a TEER value between 250Ωcm^2 and 800Ωcm^2. No monolayers were discarded. There was no significant effect of any treatments on TEER values after the 60 minute incubation ($P>0.05$). There was no significant effect of treatments on transport of iron into the basolateral reservoir after 60 minutes incubation. There was no significant difference in Caco-2 cell concentration between ThinCert chambers ($P>0.05$).

4.4.2. Treatment effects on cellular iron uptake

Caco-2 cell iron uptake was significantly higher in the presence of ascorbic acid compared to egg albumin digestate (3.9 fold; P<0.0001), NZGLM digestate (1.6 fold; P=0.016) and beef digestate (1.8 fold; P<0.01). NZGLM digestate significantly enhanced iron uptake compared to egg albumin digestate (2.3 fold; P=0.038). Beef significantly enhanced iron uptake compared to egg albumin (2.2 fold; P<0.05). There was no significant difference between NZGLM and beef digestate treatments (P=0.79). Results are illustrated in Figure 4.1.

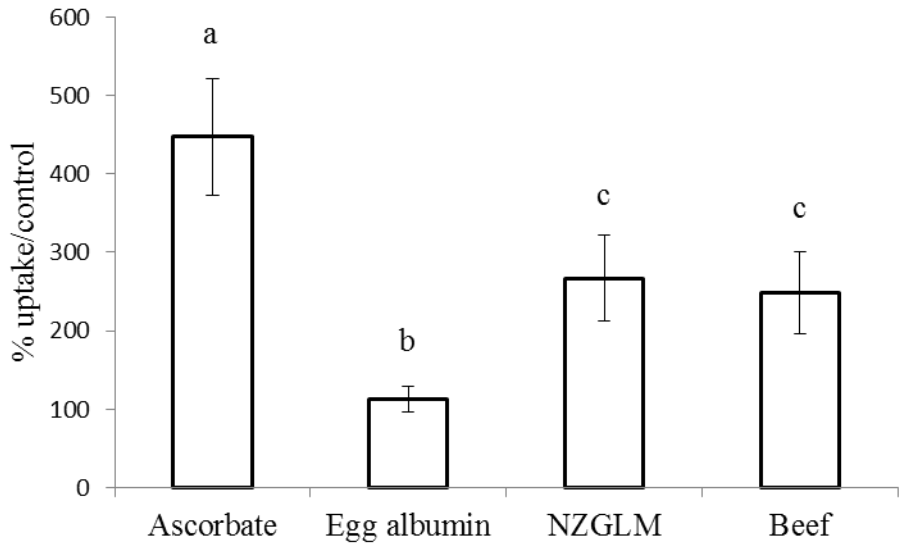

Figure 4.1. Caco-2 cell iron uptake in the presence of ascorbate, egg albumin digestate, NZGLM digestate or beef digestate.

Caco-2 cell iron uptake was calculated after 60 minutes incubation with ^{55}Fe and standardised per µg of undigested treatment. Results are expressed as mean percent iron uptake compared to an egg albumin negative control ± SEM, (n=8). Bars marked with dissimilar letters are significantly different from one another (P<0.05).

4.5. Discussion

Ascorbic acid has been reported to consistently enhance iron absorption by approximately 2-6 fold compared to egg albumin in human subjects and cell cultures (Garcia et al. 1996; Teucher et al. 2004). This effect is consistent with our results which show that when compared to egg albumin digestate, ascorbic acid enhances iron absorption by 4 to 5-fold.

Ascorbate has been proposed to enhance non-haem iron absorption by maintaining ferrous iron reduction (Kojima et al. 1981) or by promoting iron solubility in oxidising conditions (Conrad and Schade 1968). In the present study, because inhibitory iron chelators were not present, the enhancing effect appears to be associated with improving non-haem iron reduction or solubility rather than competing for iron chelation.

The enhancing effect of red meat on iron absorption has been consistently reported (Kapsokefalou and Miller 1993; Hurrell et al. 2006). The present study indicates that beef enhances iron uptake 2-3 fold, a magnitude similar to that reported in human subjects (Cook and Monsen 1976) and cell culture studies (Glahn et al. 1996).

Interestingly, NZGLM digestate also consistently enhanced non-haem iron uptake in Caco-2 cell monolayers with a magnitude similar to that of beef. Although the factors released during NZGLM digestion are unknown, gastric digestion of NZGLM is likely to yield cysteine-rich myofibrils from the dorso-ventral and adductor muscles, and aminoglycans from the inner and outer tunicas of the mantel (Kier 1988) which may enhance luminal iron solubility by mechanisms similar to that of beef and fish (Mulvihill et al. 1998; Huh et al. 2004).

The NZGLM lipid fraction consists of >50% n-3 PUFAs including up to 15% eicosapentaenoic acid and 20% docosahexaenoic acid (Murphy et al. 2002). Like oily fish, treatment with NZGLM oil at a moderate dose significantly reduces inflammation both *in vitro* (McPhee et al. 2007) and *in vivo* (Bui and Bierer 2003) therefore, the substitution of some red meat with NZGLM within the diet may also provide cardio-protective properties.

In summary, we have shown that NZGLM digestate can enhance non-haem iron absorption in Caco-2 cells by a similar magnitude to beef. These results suggest that incorporating NZGLM into a diet may enhance non-haem iron absorption. Further investigation into the mechanism of enhancement is recommended.

Chapter Five: Effect of raw and cooked New Zealand green-lipped mussels on iron absorption in sections of mouse intestine *in vitro*[*]

Chapter Five further investigates the effects of New Zealand green-lipped mussel on non-haem iron absorption compared to ascorbic acid and egg albumin in isolated sections of mouse small intestine. The following chapter describes the results of the study and further discusses the repeatability of New Zealand green-lipped mussel digestate as an iron absorption-enhancer.

*Part of the material presented in this section has been previously presented at the NZNS Annual Conference: Stewart, R.J.C., Coad, J., Reynolds, G.W., Pedley, K. C. (2011). New Zealand Green-Lipped Mussel (Perna *canaliculus*) Enhances Non-Haem Iron Absorption In-Vitro. Joint NSA/*NSNZ Conference*, 30 November to 2 December, Queenstown.

5.1. Abstract

Combining raw NZGLM with an iron supplement promotes non-haem iron absorption in Caco-2 cells. The repeatability of this observation and the effect of cooking NZGLM prior to digestion requires further investigation. The objectives of this study were to investigate the effect of combining raw and cooked NZGLM with an iron supplement on non-haem iron absorption in isolated sections of proximal small intestine. Cooked and raw NZGLM and egg albumin were digested *in vitro*. All digestates were extrinsically tagged with ^{55}Fe. Male Balb/c mice were maintained on a low-iron diet (≤5ppm) for two weeks to deplete iron stores. Sections of proximal small intestine from iron-deficient mice were mounted in Ussing chambers and the mucosal surface was incubated with digestates or ascorbic acid combined with an iron radionuclide for 60 minutes. The fraction of radionuclide absorbed by the mucosa was determined by liquid scintillation counting. Ascorbic acid and NZGLM significantly enhanced radionuclide absorption compared to egg albumin. Cooking NZGLM prior to digestion did not further enhance iron absorption. In summary the enhancing effect of NZGLM on iron absorption is repeatable *in vitro*. Cooking NZGLM does not further enhance iron absorption compared to raw NZGLM.

5.2. Introduction

Combining MPF with dietary non-haem iron has been reported to enhance iron absorption in cell cultures (Glahn et al. 1996), animals (Kapsokefalou and Miller 1993) and human participants (Hurrell et al. 1988). Like MPF, NZGLM contains many components which may enhance non-haem iron absorption (Mulvihill et al. 1998; Huh et al. 2004). When investigated *in vitro* using Caco-2 cell cultures, raw NZGLM enhanced iron absorption by a similar magnitude to that reported for raw beef. Beef is understood to be one of the most potent MPF factors in this respect (Cook and Monsen 1976).

Caco-2 cell cultures have been used to identify foods and individual nutrients which enhance non-haem iron absorption in humans (Au and Reddy 2000). However Caco-2 cell cultures are generally only used as a pre-screening tool because they have certain traits which are dissimilar to the small intestinal mucosa, such as a lack of mucus production, which may affect iron transport (Conrad et al. 1991). For this reason the repeatability of the results generated from Caco-2 cells often requires further assessment using a second model of iron transport in the small intestine (Kibangou et al. 2005) or iron retention in animals or humans (Armah et al. 2008).

The enhancing effect of raw NZGLM may be further augmented by cooking NZGLM prior to digestion which has been previously reported for pork (Sørensen et al. 2007). The enhancing effect of cooking meat prior to digestion may be a result of improved protein digestibility (Evenepoel et al. 1998) which in turn may improve MPF factor release and promote MPF factor-iron interactions during the gastric phase of digestion.

The aims of this study were to investigate the effect of NZGLM digestate on non-haem iron absorption in the proximal intestine of iron-deficient mice, and to investigate the effect of cooking NZGLM prior to digestion on iron absorption.

5.3. Methods

5.3.1. Treatments

Treatments were prepared as described in Chapter 3, Section 3.2.3. Fresh, and cooked (but not lyophilised) treatments were used for digestion.

5.3.2. In vitro digestion

The *in vitro* digestion was undertaken as described in Chapter 3, Section 3.2.4. The non-haem iron concentration of each digestate was analysed and calculated as described in Chapter 3, Section 3.2.5 and Section 3.2.6. The protein concentration was analysed as described in Chapter 3, Section 3.2.7.

Ascorbic acid (95.5mmol/L HBSS) was prepared in an ascorbate:iron molar ratio of 4:1. Raw and cooked NZGLM and egg albumin digestates and ascorbic acid were combined with a pre-prepared iron working solution (23.87mM) containing a 1:10 ratio of ^{55}Fe to ^{56}Fe. The final radioactivity of each digestate treatment was 92.5kBq/mL digestate.

5.3.3. Animals

Weaned male Balb/c mice were obtained from the Massey University Small Animal Production Unit (Palmerston North, New Zealand). Mice were maintained at 22°C ± 1°C on a 12 hour light/dark cycle with 50% ± 10% humidity on a standard commercial diet (Purina 5001, St. Louis, MO, USA) *ad libitum* for 21-35 days.

These iron-replete mice were randomly allocated into two treatment groups, one containing 18 mice which were allocated to a low-iron semi-synthetic (SS) diet (≤5ppm) and the other containing 8 mice which were allocated to an iron-replete (200ppm) SS diet. The composition of both of these diets are summarised in Table 5.1.

Table 5.1. Composition of low-iron SS diets

Ingredient	Diet composition	
	Iron-replete SS diet	Iron deplete SS diet
	g/Kg diet	
Lactic casein	200	200
Methionine	3	3
Wheaten starch	313.5	313.5
Sucrose	313.5	313.5
Microcrystalline cellulose	50	50
Corn oil	80	80
Vitamin premix[2]	10	10
Iron-free mineral premix[3]	30	30
Water	300	290
123mM Ferric chloride (in 0.1M HCl)	0	10

[1] NZGLM diet was formulated to provide approximately 1.5% total dietary energy from NZGLM
[2]Unitech Industries Limited, Auckland, New Zealand (formulation in Appendix 1)
[3]Plant and Food, Palmerston North, New Zealand (formulation in Appendix 1)

Diets were provided *ad libitum* with deionised water for 14 days. On day 15 all mice were euthanased by cervical dislocation and weighed. The entire small intestine was removed and placed in Ringers solution (pH 7.4, 37°C). The liver weight was measured and the liver was stored at -20°C for iron analysis.

All experimental procedures were approved by the Massey University Animal Ethics Committee (09/47). The experimental protocol is illustrated in Figure 5.1.

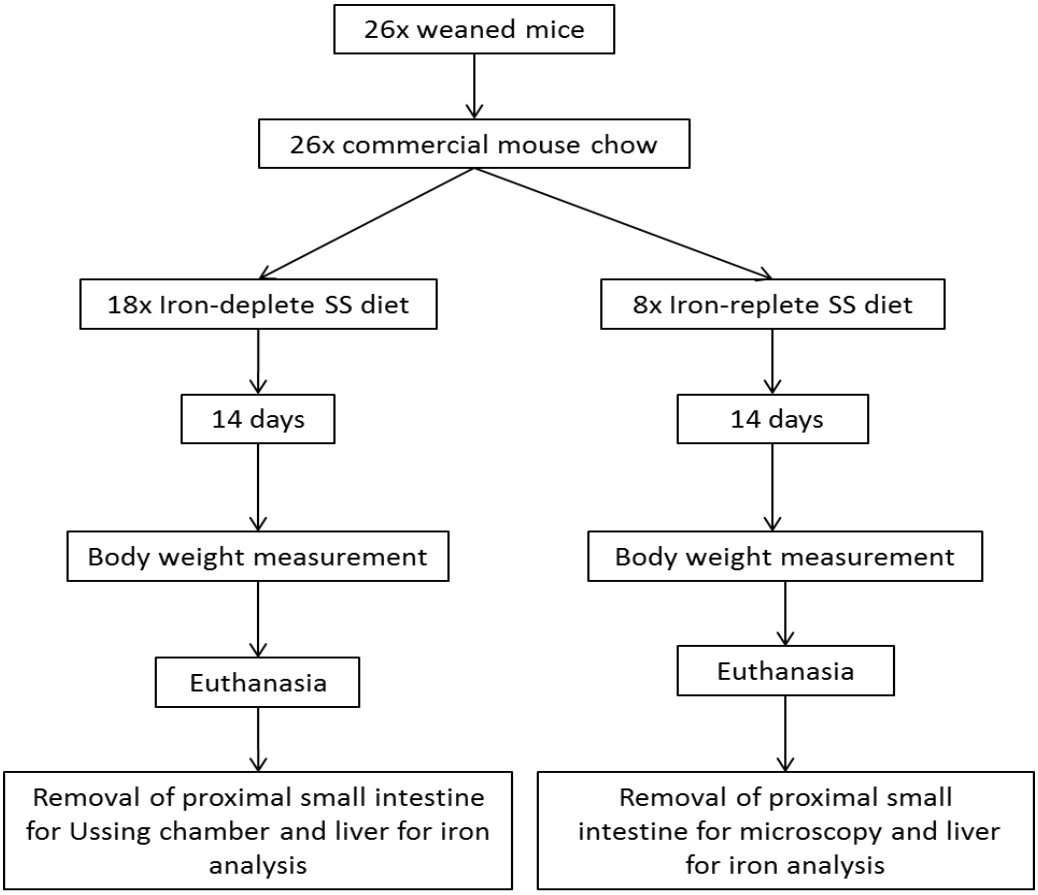

Figure 5.1. Mouse diet and dissection protocol flow chart.

5.3.4. Ussing chamber apparatus

Ussing Chambers (Physiologic instruments, CA, USA) were soaked in 1.6M NA for 1 hour and rinsed six times in deionised water prior to use.

Silver wire electrodes constructed within our laboratory were immersed in sodium hypochlorite (13440) for 30 minutes. Agar bridges were made in 1 mL slip-tip syringes (BD biosciences, 301025A, NJ, USA). Each syringe was filled with 750µL agar solution (3.5% agar (A5306) in deionised water) containing 2.95M KCl (P9541). Bridges were stored in saturated KCl at 4°C prior to use.

The Ussing chambers were assembled according to the manufactures instructions. The Tissue Clamp 3.0 unit (Campus Electronics, Dunedin, New Zealand) macroinstruction was programmed to provide a 0.5 second stimulation of 5.5mV every 300 seconds.

Silver chloride-electrodes were inserted into the agar bridges and the Tissue Clamp 3.0 unit respectively. The circuit between the bridges was connected with glucose-free Ringers solution (Table 5.2.) and the fluid resistance within the Ussing chamber was measured. Carbogen gas (95% O_2: 5% CO_2) was applied to the mucosal and serosal reservoir at a rate of approximately 5 bubbles per second.

The proximal small intestine of each mouse was cut into four individual segments. Each segment was 2cm long commencing 2cm distal of the pyloric sphincter. Each segment was dissected longitudinally and mounted onto the Ussing chamber tissue inserts in a randomised order. Both the mucosal and serosal reservoirs were rinsed twice with fresh Ringers solution and the tissue resistance and short circuit current was measured for 30 minutes until readings stabilised.

Table 5.2. Composition of Ringers solution

Ingredient	*Concentration (mM)*
Sodium chloride (S7653)	114
Potassium chloride (P9333)	5
Calcium chloride (C1016)	1.25
Magnesium chloride (M8266)	1.1
Sodium phosphate monobasic (S3139)	0.3
Sodium phosphate dibasic (S3264)	1.65
Sodium bicarbonate (S5761)	25
Glutamine (G3202)	2
Sodium pyruvate (P8574)	2

5.3.5. Experimental

The Ringers solution within the Ussing chamber was replaced with 4.7mL Ringers solution (pH 6.5) in the mucosal reservoir and 5mL Ringers solution (pH 7.5) in the serosal reservoir. The resistance of each tissue was recorded. A 300µL sample of radionuclide-free digestate was added to its respective mucosal reservoir. After 60 minutes duration 1mL of mucosal treatment was replaced with 1mL glucose solution (50mM glucose (G8270) in deionised water). Short circuit current was measured for 1 minute to ensure tissues were responsive to glucose.

The Ussing chamber apparatus was cleaned, acid washed and reset with fresh intestinal sections as described above. A 400µL sample of ^{55}Fe-containing digestate was added to its respective mucosal reservoir and circulated for 1 minute. A 100µL sample was taken from the mucosal and serosal reservoirs and each tissue was incubated with the treatment for 60 minutes.

At 60 minutes the tissue resistance was recorded (Ω/cm^2) and duplicate 100µL aliquots were taken from the mucosal and serosal reservoirs. The tissue inserts were removed, and the intestinal sections were rinsed with 1mL 0.1% NA and 100µL sample of this was taken. The mouse intestinal tissue was solubilised in 2mL NCSII Tissue solubiliser (GE Technology, Buckinghamshire) for 12 hours at 20°C**.** A 100µL sample was taken. The Ussing chambers were disassembled, and the mucosal reservoir was rinsed with 1.6M NA. A 100µL sample was taken. All samples were combined with 1.6mL OptiPhase HiSafe 2 scintillation cocktail (PerkinElmer 1200-436, Waltham, MA, USA) and analysed immediately by liquid scintillation counting (Wallac Trilux 1450 Microbeta PerkinElmer, Waltham, Massachusetts, USA).

All samples were corrected for their respective dilution factors. The fraction of radionuclide present within the intestinal section was expressed as a percentage to the radionuclide concentration within the mucosal reservoir after 1 minute circulation to allow values to be compared. All other samples were used to calculate total iron recovery from the Ussing chamber apparatus. Tissues with a change in resistance between time zero and 60 minutes by 30% or a serosal radioactivity over 100cpm were discontinued.

All radionuclide results were standardised per µg of beef, NZGLM, egg albumin or ascorbic acid. Briefly, the protein concentration of each 300mOsm digestate was compared to protein concentration values generated from the proximal analysis of NZGLM and egg albumin (less the digestive enzymes). Iron uptake for both digestates and ascorbic acid were then calculated per gram of treatment. Iron absorption in all treatments was expressed as percent iron absorbed per treatment compared to egg albumin digestate in order to be comparable to previous *in vitro* studies.

Statistical analysis was performed using SAS 9.1 statistical software (SAS Institute Inc., Cary, NC, USA). The iron uptake results were analysed by 1-way and 2-way ANOVA using the General Linear Model procedure. Mouse iron status and growth results were analysed by Student's t-test. Where appropriate, post-hoc analysis was carried out using LSD analysis.

5.4. Results

The body weight, liver weight and liver iron concentration of mice raised on the iron-deplete and iron-replete SS diets are described in Table 5.3. The body weight of mice raised on the iron-deplete diet was significantly lower than mice raised on the iron-replete diet. There was no significant difference in liver weight between mice raised on the iron-replete or iron-deplete diets. The liver iron concentration in mice raised on the iron-deplete diet was significantly lower than mice raised on the iron-replete diet.

Table 5.3. Measurements of mice maintained in an iron-replete diet and an iron-deplete diet

Parameter	*Iron replete mice*	*Iron-deplete mice*	*P*
Body weight (g)	28.02 ± 0.45	23.14 ± 0.95	0.013
Liver weight (g)	1.07 ± 0.04	1.07 ± 0.09	0.986
Liver iron (µg/g)	43.22 ± 1.24	19.55 ± 1.46	<0.0001

Iron absorption was significantly enhanced in the media containing iron and ascorbic acid compared to the media containing iron and cooked egg albumin digestate (4.7 fold; P<0.0001), raw egg albumin digestate (6.9 fold; P<0.0001) or cooked NZGLM digestate (1.4 fold; P=0.034). There was no significant difference in iron absorption when the iron supplement was in the presence of either ascorbic acid or raw NZGLM digestate (P=0.103).

Combining raw NZGLM digestate with an iron supplement significantly enhanced iron absorption compared to the raw egg albumin digestate (5.2 fold; P=0.002) or cooked egg albumin digestate (3.5 fold; P=0.002). There was no significant difference in iron absorption when the iron supplement was combined with either raw or cooked NZGLM digestate (P>0.05). These results are illustrated in Figure 5.2.

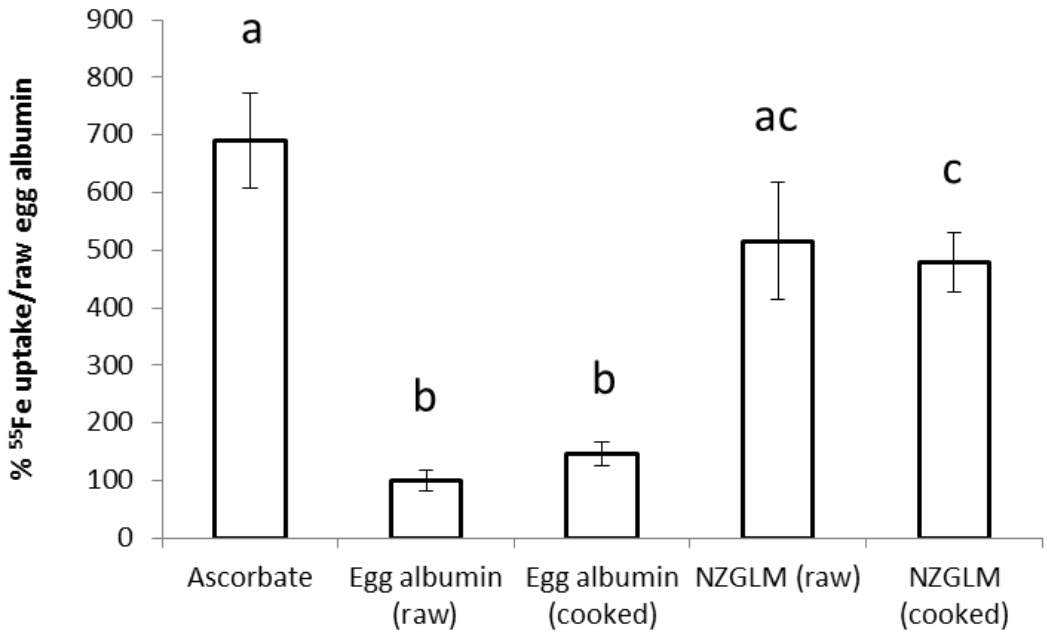

Figure 5.2. ^{55}Fe enrichment of the small intestine.

Sections of proximal small intestine were mounted on Ussing chamber inserts and incubated with a ^{55}Fe-enriiched iron supplement combined with ascorbic acid, raw or cooked egg albumin digestate and raw or cooked NZGLM digestate. Tissue ^{55}Fe uptake was calculated as a percentage compared to the initial ^{55}Fe concentration within the mucosal reservoir. Results are expressed as a fraction of ^{55}Fe uptake in all treatments compared to raw egg albumin. Results represent means ± SEM, (n=14). Bars marked with dissimilar letters are significantly different from one another (P<0.05).

5.5. Discussion

The exclusive provision of a semi-synthetic diet containing ≤5ppm iron for 2-4 weeks with deionised water has been repeatedly reported to reduce total liver iron concentrations by approximately 50% in mice (Muir et al. 1984; Trinder et al. 2002; Gardenghi et al. 2010). Previous results within our laboratory and that of Trinder et al. (2002) suggest that this protocol promotes iron deficiency without anaemia.

Under these conditions the expression of proteins involved in mucosal iron transport in mouse duodenum are enhanced (McKie et al. 2001). This procedure was undertaken in the current study in order to increase the rate of iron absorption by the small intestine.

Iron absorption by the small intestinal sections was significantly higher when combined with ascorbic acid compared to egg albumin digestate. The enhancing effect of ascorbic acid and the inhibitory effect of egg albumin have been both discussed and modelled previously (Hallberg and Hulthen 2000).

The magnitude of iron absorption-enhancement in the presence of ascorbic acid compared to egg albumin in the current study is in agreement with previous studies *in vitro* (Garcia et al. 1996). The enhancing effect of ascorbic acid in this model is higher than that previously observed in our laboratory using Caco-2 cells.

The enhancing effect of raw NZGLM digestate on iron absorption compared to egg albumin is similar to that observed in Caco-2 cells as discussed in Chapter Four This result suggests that the enhancing effect of NZGLM on iron absorption is repeatable *in vitro* using two separate models and therefore further investigation on iron retention *in vivo* is required.

The combination of iron with cooked NZGLM digestate promoted no further increase in iron absorption-enhancement compared to raw NZGLM digestate. Both NZGLM treatments enhanced iron absorption by a similar magnitude compared to egg albumin. The results suggest that heat treatment has no further enhancing or inhibiting effect on MPF factor release or activity from NZGLM.

The NZGLM homogenate contains a high concentration of cysteine-rich myofibrils (Shelud'ko et al. 1999). Myofibrils are released during gastric digestion and interact with iron to promote iron solubility and mucosal iron transport (Storcksdieck and Hurrell 2007). Denaturation of NZGLM proteins prior to digestion by cooking may further promote gastric digestion (Evenepoel et al. 1998). This may explain why the magnitude of iron absorption-enhancement is higher in cooked pork compared to raw pork (Sørensen et al. 2007).

However cooking NZGLM may also promote oxidation of cysteine to cystine therefore reducing the affinity of the myofibrils for iron (Martínez-Torres et al. 1981). The risk of oxidation is significant in NZGLM during cooking because of the high concentration of PUFAs (Tuckey et al. 2013). This may explain why no further enhancing effect of cooking NZGLM on iron absorption was observed in the current study.

The results suggest that cooking egg albumin prior to digestion has no additional effect on iron uptake compared to raw egg albumin digestate. This is not surprising because although the digestion of cooked egg albumin is significantly improved above raw egg albumin (Evenepoel et al. 1998), iron-binding peptides released from the gastric digestion of egg albumin are of a high molecular weight and restrict mucosal transport of iron (Storcksdieck and Hurrell 2007). This may not be further influenced by the cooking. Both raw and cooked egg inhibit iron absorption in humans (Cook and Monsen 1976).

In summary the enhancing effect of raw NZGLM on iron absorption compared to egg albumin as previously reported in Caco-2 cell monolayers is repeatable in isolated sections of mouse intestine *in vitro*. Cooking NZGLM prior to digestion provides no further enhancing effect on iron absorption in this model. The results suggest that either raw or cooked NZGLM may be used to promote iron absorption from a vegetable-based meal or iron supplement *in vitro*.

Chapter Six: Effect of New Zealand green-lipped mussels on iron absorption in iron deficient mice*

The results discussed in Chapter Four and Chapter Five suggest that combining New Zealand green-lipped mussel with an iron supplement enhances iron absorption *in vitro*. The primary objective of this study was to investigate the effect of combining an iron supplement with New Zealand green-lipped mussel, ascorbic acid or egg albumin on iron absorption in iron deficient mice. Prior to consuming these treatments by gastric gavage, mice were raised on either a control iron-deplete diet or a New Zealand green-lipped mussel-enriched iron-deplete diet for two weeks. The following chapter describes the results of the study and discusses the contrasting effects of short and prolonged New Zealand green-lipped mussel supplementation on iron absorption.

*Part of the material presented in this section has been accepted for presentation at the IUNS 20th International Congress of Nutrition: Stewart, R.J.C., Morton, H., Coad, J., Reynolds, G.W., Pedley, K. C. (2013). New Zealand Green-Lipped Mussels (Perna *canaliculus*) both enhance and inhibit non-haem iron absorption in mice. 15th to 20th September. Grenada, Spain.

6.1. Abstract

Non-haem iron bioavailability is affected by the nutritional composition of a meal. Like red meat, NZGLM have been reported to improve non-haem iron absorption from a second dietary iron source *in vitro*. The objective of this study was to investigate whether NZGLM enhance non-haem iron absorption compared to egg albumin or ascorbic acid in iron-deficient mice, and to investigate the effects of repeated exposure to NZGLM extract on iron uptake from a single iron dose. Six week-old male Swiss mice were maintained on a low-iron diet (5ppm) supplemented with or without NZGLM extract (3.3% w/w) *ad libitum* for two weeks. Fasted mice were provided with a single dose of ascorbic acid, NZGLM or egg albumin combined with $^{59}FeCl_3$ radionuclide and $^{56}FeCl_3$ by gavage after a 12 hour fast. Control and NZGLM-supplemented diets were re-introduced *ad libitum* for four days. Iron loading was determined by measuring the incorporation of radiolabelled iron into whole blood and the small intestine. Ascorbic acid and NZGLM significantly enhanced blood ^{59}Fe enrichment and total body ^{59}Fe enrichment compared to egg albumin in mice raised on the NZGLM-free diet. Blood ^{59}Fe enrichment, total body ^{59}Fe enrichment and whole liver iron content was significantly reduced in mice repeatedly exposed to the NZGLM extract compared to mice maintained on the control diet. NZGLM enhance blood and small intestinal iron loading with a similar magnitude to ascorbic acid when provided as a single dose to iron deficient mice. This result is similar to that reported *in vitro*. Repeated exposure to NZGLM extract significantly reduces iron uptake from a single iron dose.

6.2. Introduction

Red meat, pork, poultry and fish all enhance non-haem iron absorption when consumed as part of a vegetable-based meal in human participants (Cook and Monsen 1976). Like MPF, NZGLM contains myofibrils (Shelud'ko et al. 1999), GAGs (Rubin et al. 2006), PUFAs and phospholipids (Murphy et al. 2002) which have all been previously reported to improve mucosal iron transport when isolated and combined with dietary iron (Mulvihill et al. 1998; Huh et al. 2004; Armah et al. 2008).

Additionally NZGLM contains a range of novel n-3 PUFAs (Murphy et al. 2002). Specific fatty acids within this lipid fraction may promote iron solubility or compete with iron absorption-inhibitors when combined with iron within the diet (Simpson et al. 1988; Armah et al. 2008). Furthermore since prolonged MUFA or PUFA supplementation can increase membrane fluidity, promote brush border enzyme activity (Daveloose et al. 1993) and promote DMT1 expression in extra-intestinal tissues (Schonfeld et al. 2007), prolonged NZGLM supplementation may promote mucosal transport and extra-intestinal distribution of iron.

Combining NZGLM digestate with iron has been previously reported to promote iron transport across the brush border membrane of Caco-2 cells and intact mouse intestine as described in Chapter Four and Chapter Five. The effect of NZGLM supplementation on mucosal iron transport and distribution to extra-intestinal tissues requires further investigation *in vivo*. The effect of repeated NZGLM supplementation on brush border iron transport is unknown.

The aim of this study was to use a mouse model to investigate iron absorption and distribution when iron is co-supplemented with ascorbic acid, NZGLM or egg albumin. The effect of prolonged NZGLM supplementation on iron absorption and distribution to extra-intestinal tissues was also investigated.

6.3. Materials and methods

6.3.1. Treatments

Treatments were prepared as described in Chapter 3, Section 3.2.3. Freeze dried NZGLM and freeze dried egg albumin (Zeagold, New Zealand) were reconstituted to 280mg/mL with deionised water in order to ensure that the treatment viscosity was suitable for the gavage procedure. The ascorbate treatment was reconstituted to 17.7mM ascorbate with deionised water.

The total and non-haem iron concentration of each treatment was analysed and calculated as described in Chapter 3, Section 3.2.5 and Section 3.2.6. The iron concentration of all treatments was standardised to 4mM by the addition of $FeCl_3$ in 0.5M HCl and combined with a ^{59}Fe radionuclide tracer (Perkin Elmer NEZ037500UC, Massachusetts, USA) immediately prior to gavage. Treatment preparations are described in Table 6.1.

Table 6.1. Gavage treatment preparation

Treatment	Volume (µL)	Radionuclide	Carrier iron (mM)	Total iron (mM)	Radioactivity (kBq)
Ascorbic acid	180	$^{59}FeCl_3$	3.76	4	50
NZGLM	180	$^{59}FeCl_3$	2.41	4	50
Egg albumin	180	$^{59}FeCl_3$	3.76	4	50
Ascorbic acid	180	-	4	4	-
NZGLM	180	-	2.64	4	-
Egg albumin	180	-	4	4	-

6.3.2. Animals

Three week old weaned male Swiss mice were obtained from Plant and Food Research Small Animal Unit (Palmerston North, New Zealand). Mice were maintained at 22°C ± 1°C on a 12 hour light/dark cycle with 50% ± 10% humidity on a standard commercial iron-replete diet (Purina 5001, St. Louis, MO, USA) *ad libitum* for 21 days. Mice were randomly allocated into 4 treatment groups, each containing 13 mice and housed in perspex cages.

6.3.3. Experimental

Three groups of iron-replete mice were provided with the low-iron control diet and one group was provided with the NZGLM-supplemented diet. The composition of both diets is summarised in Table 6.2.

Table 6.2. Composition of low-iron diets

Ingredient	Diet composition	
	Control diet	NZGLM supplemented diet
	g/Kg diet	
Lactic casein	200	194
Methionine	3	3
Wheaten starch	313.5	313.5
Sucrose	313.5	313.5
Microcrystalline cellulose	50	50
Corn oil	80	78
NZGLM (wet weight)[1]	0	33
Vitamin premix [2]	10	10
Iron-free mineral premix[3]	30	30
Water	300	259

[1] NZGLM diet was formulated to provide approximately 1.5% total dietary energy from NZGLM
[2]Unitech Industries Limited, Auckland, New Zealand (formulation in Appendix 1)
[3]Plant and Food, Palmerston North, New Zealand (formulation in Appendix 1)

Mice were provided *ad libitum* with their respective diet and deionised water for 14 days. Both diets contained a total iron concentration of 5ppm. The exclusive provision of a diet with an iron content ≤5ppm, together with deionised water, has previously been reported to promote iron deficiency without anaemia over 14 days (Gardenghi et al. 2010).

Mouse body weight and food consumption were recorded every 2.5 and 5 days respectively at day 1 and over the 14 day feeding period. All experimental procedures were approved by the Massey University Animal Ethics Committee (11/42). The experimental protocol is illustrated in Figure 6.1.

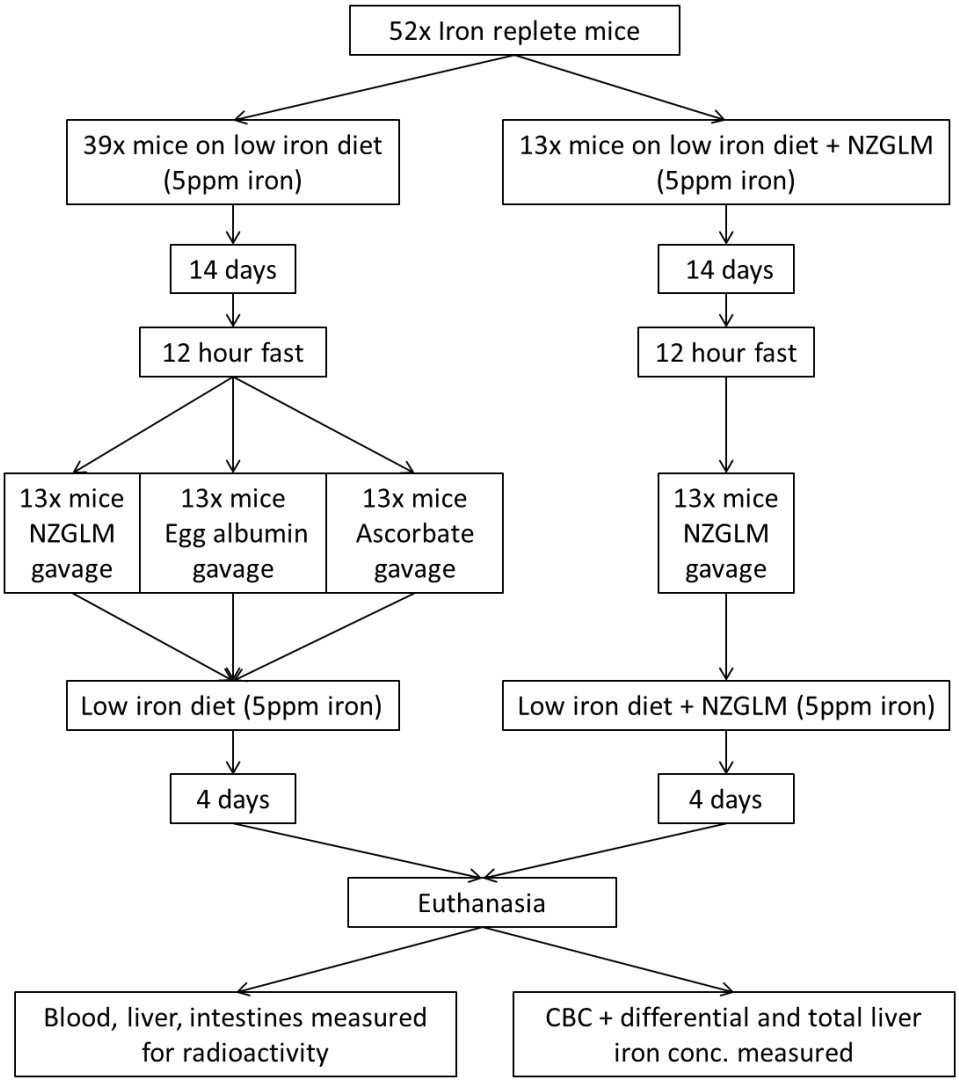

Figure 6.1. Experimental design flow chart.

Both low-iron diets were removed on day 14 of the experimental protocol. Mice were provided exclusively with sugar cubes (Chelsea, Auckland, NZ) and deionised water for 12 hours. A 200µL sample of iron-supplemented treatment was then administered to fasted mice in each treatment group by gastric gavage.

Four out of 13 mice within each treatment group were provided exclusively with 200µL non-radiolabeled treatment by gastric gavage in order to generate biological

samples suitable for haematological analysis. All other mice received radiolabelled treatments in order to measure iron absorption and distribution. All treatments are defined in Table 6.1.

All mice were provided with deionised water *ad libitum* and fasted for 4 hours post-gavage. Both low iron diets were re-introduced to respective groups *ad libitum* for 4 days. Food consumption and body weight measurements were recorded daily until euthanasia. Due to the high rate of erythropoiesis in mice, blood ^{59}Fe enrichment at 5 days post-gavage is expected to reflect haemoglobin enrichment (Brodsky et al. 1966).

6.3.4. Tissue collection

Mice were anesthetised with 3% isoflurane and 500µL of blood was drawn by cardiac puncture. Mice were euthanased by cervical dislocation prior to recovery from the aesthetic and placed immediately on ice.

The small and large intestine of each mouse were removed using the anatomical locations as described by Jin et al (1994). The spleen, liver and kidneys were removed from the carcass and the remaining carcass was homogenised.

Whole blood, organs and carcass homogenate obtained from mice provided with the radionuclide-enriched treatment were analysed for radioactivity (Wallac Wizard 1470-005 γ-counter).

Blood samples from the mice provided with the radionuclide-free treatment were submitted for haematological analysis (New Zealand Veterinary Pathology Limited, Palmerston North, NZ). Liver samples were weighed and the total iron content was measured as described in Chapter 3, Section 3.2.5.

6.3.5. Calculations and Statistical analysis

Changes in mouse body weight and diet consumption at any time point during the experimental phase were compared to the initial body weight and diet consumption weight on days 1 and 2 of the experimental phase respectively. All changes were calculated as a percentage change from weaning (time zero). Time zero was set at 100 percent.

Liver total iron concentrations were calculated per whole liver. Complete blood count results were compared directly. Tissue enrichment of ^{59}Fe was calculated as percent radionuclide retained in individual organs compared to the gavage dose corrected for radioactive decay.

Statistical analysis was performed using SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Growth results were analysed by repeated measures ANOVA. Total liver iron concentrations, CBC and tissue ^{59}Fe enrichment were analysed by 1-way ANOVA using the General Linear Model procedure. Where appropriate, post-hoc analysis was carried out using LSD analysis.

6.4. Results

6.4.1. Diet consumption and mouse growth

Changes in mouse body weight over the 14 day experimental period prior to gavage are illustrated in Figure 6.2. There was no statistical difference in body weight between mouse groups provided with either diet within any time point ($P>0.05$). There was a significant increase in mouse weight over time ($P<0.0001$) with a mean growth rate of 0.58% body weight per day.

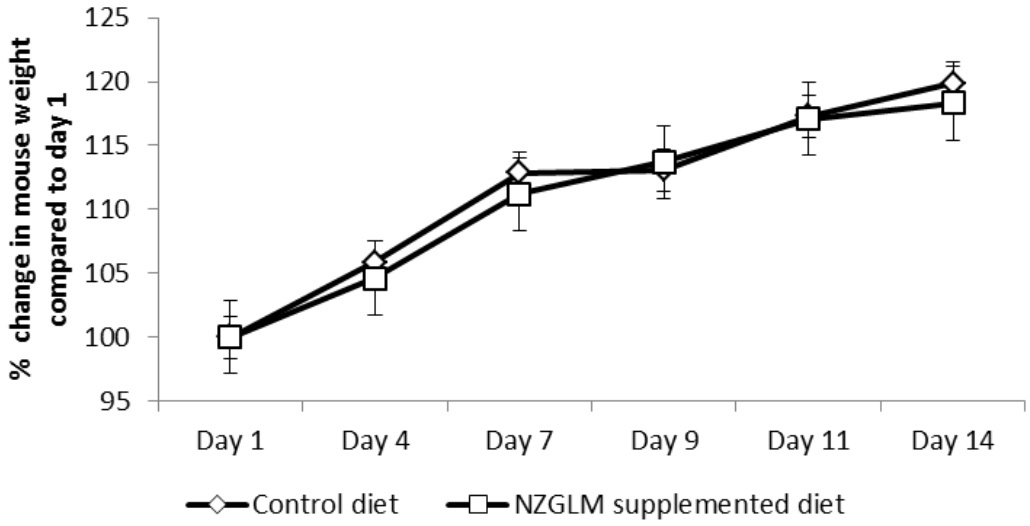

Figure 6.2. *Effect of diet on mouse weight gain.*

Changes in mouse body weight over the 14 day experimental period prior to gavage. Mice fed either the control diet or NZGLM-supplemented diet. Results are expressed as mean % change in body weight compared to initial bodyweight on day 1 of the experimental phase.

6.4.2. Complete blood counts

Mouse RBC concentrations and haemoglobin concentrations were within the normal range for all mice (Hedrich and Bullock 2004) which suggests that mice were not anaemic. The effects of individual gavage treatments and dietary treatments on mouse red blood cell (RBC) count are illustrated in Figure 6.3. There was no statistical effect of gavage treatment or dietary treatment on RBC count ($P>0.05$), however a trend was noted. The mean RBC count was higher in mice raised on the control diet and provided with the NZGLM gavage treatment compared to the egg albumin gavage treatment ($P=0.062$). The mean RBC count was higher in mice raised on the control diet compared to mice raised on the NZGLM-supplemented diet when both were provided with the NZGLM gavage treatment ($P=0.072$).

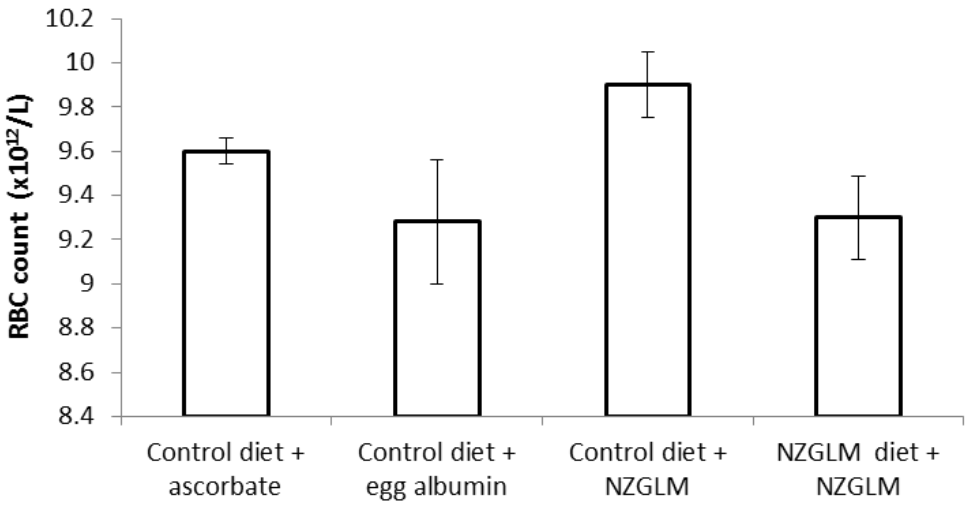

Figure 6.3. Mouse RBC count.

Red blood cell concentrations in mice maintained on a control or NZGLM-supplemented diet for 14 days. At day 15 a single dose of iron combined with ascorbic acid, egg albumin or NZGLM was provided by gavage. Blood was drawn 4 days later. Results are expressed as mean values ± SEM, (n=4).

The effects of treatment gavage or diet on mouse haemoglobin concentrations are illustrated in Figure 6.4. There was no statistical difference in haemoglobin concentration between any gavage treatment or dietary treatment (P>0.05).

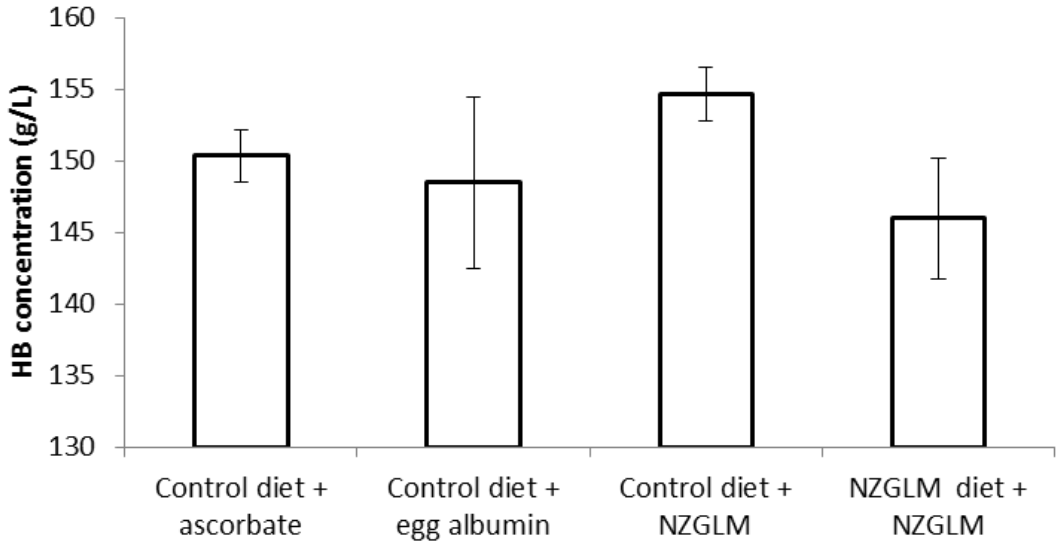

Figure 6.4. Mouse haemoglobin concentrations.

Haemoglobin concentrations in mice maintained on a control or NZGLM-supplemented diet for 14 days. At day 15 a single dose of iron with ascorbic acid, egg albumin or NZGLM was provided by gavage. Blood was drawn 4 days later. Results are expressed as mean values ± SEM, (n=4).

6.4.3. Radionuclide enrichment and liver iron stores

Combining the iron supplement with ascorbic acid significantly enhanced blood iron enrichment compared to combining the iron supplement with egg albumin (1.3 fold; P=0.01). A similar effect was observed when the iron supplement was combined with NZGLM compared to egg albumin (1.4 fold; P=0.0002). These results are illustrated in Figure 6.5.

The enhancing effect of NZGLM gavage was only noted in mice raised on the control diet. Blood iron enrichment was significantly higher in mice which were raised on the control diet prior to receiving the NZGLM gavage treatment compared to mice raised on the NZGLM supplemented diet prior to receiving the NZGLM gavage treatment (1.3 fold; P=0.009).

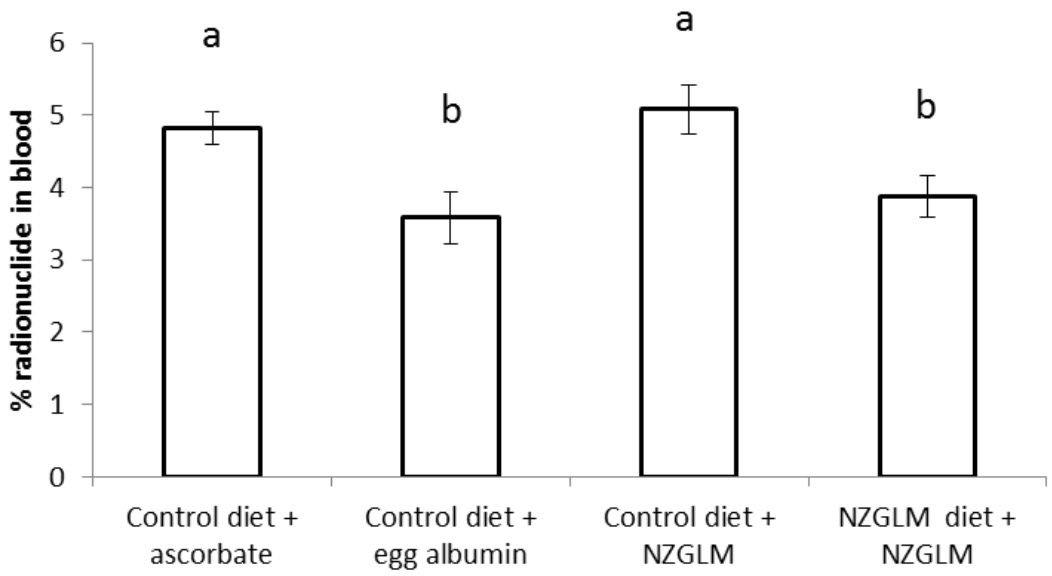

Figure 6.5. *Blood ^{59}Fe enrichment.*

Mice were fed either a control diet or NZGLM-supplemented diet for 14 days. At day 15 a single dose of ^{59}Fe-labelled iron combined with ascorbic acid, egg albumin or NZGLM was provided by gavage. Blood was drawn 4 days later. ^{59}Fe enrichment in 200µL blood was calculated as a percentage from ^{59}Fe within the entire gavage dose. Results are expressed as mean percent ^{59}Fe retained ± SEM, (n=9). Bars marked with dissimilar letters are significantly different from one another (P<0.05).

Combining the iron supplement with ascorbic acid also significantly enhanced liver iron enrichment compared to combining the iron supplement with egg albumin (1.6 fold; P=0.002), NZGLM in mice raised on the control diet (1.3 fold; P=0.019) or NZGLM in mice raised on the NZGLM diet (1.9 fold; P=0.0002).

There was no significant difference in liver iron enrichment between mice provided egg albumin or NZGLM (either dietary treatment) when provided by gavage (P>0.05). These results are illustrated in Figure 6.6.

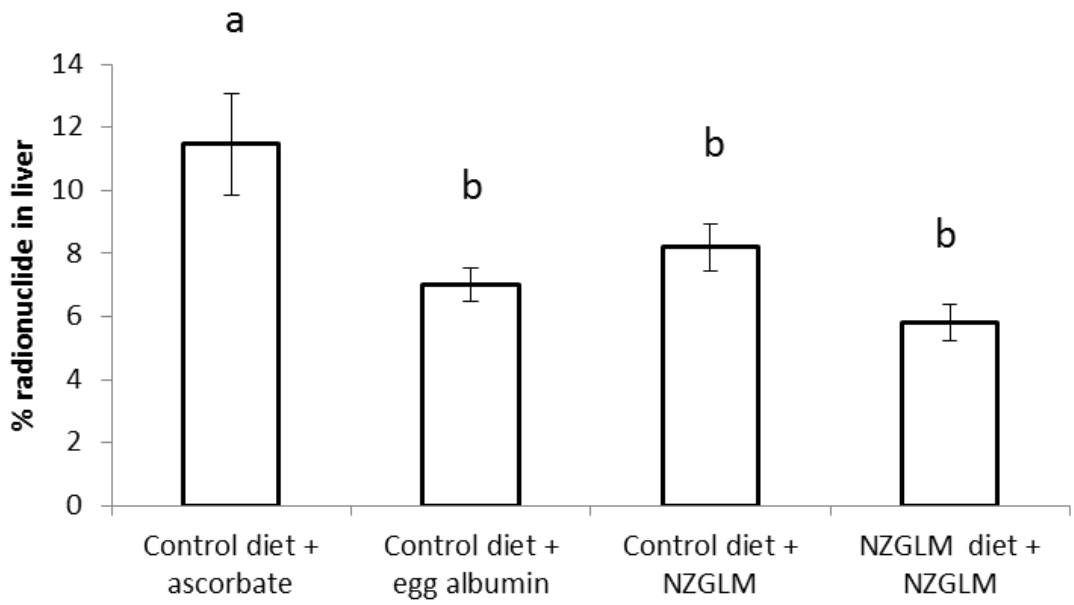

Figure 6.6. Liver ^{59}Fe enrichment.

Mice were fed either a control diet or NZGLM-supplemented diet for 14 days. At day 15 a single dose of ^{59}Fe-labelled iron combined with ascorbic acid, egg albumin or NZGLM was provided by gavage. Mice were euthanased 4 days later. ^{59}Fe enrichment in the whole liver was calculated as a percentage from ^{59}Fe within the entire oral dose. Results are expressed as mean percent ^{59}Fe retained ± SEM, (n=9). Bars marked with dissimilar letters are significantly different from one another (P<0.05).

Combining an iron supplement with either ascorbic acid or NZGLM (mice raised on the control diet) had no significant effect on total liver iron stores compared to the egg albumin (P>0.05). Total liver iron concentrations were significantly higher in mice raised on the control diet compared to mice raised on the NZGLM diet when both dietary groups were provided with the iron supplement combined with NZGLM (1.4 fold; P<0.0001).

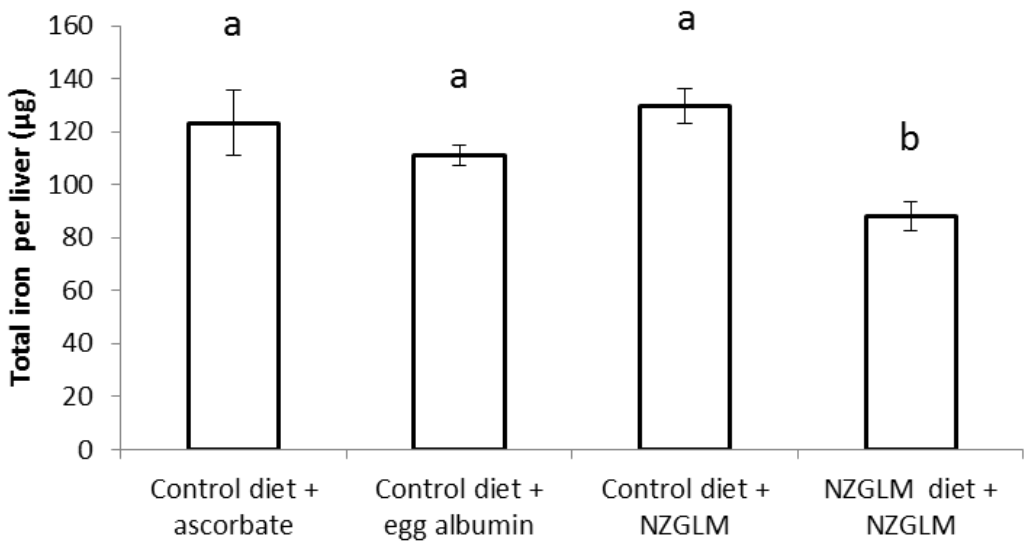

Figure 6.7. Total liver iron stores.

Mice were fed either a control diet or NZGLM-supplemented diet for 14 days. At day 15 a single dose of radionuclide-free iron, combined with ascorbic acid, egg albumin or NZGLM was provided by gavage. The liver was removed 4 days later. The iron concentration of each whole liver was quantified using the wet ashing/ferrozine method. Results are expressed as mean total liver iron concentrations ± SEM, (n=9). Bars marked with dissimilar letters are significantly different from one another (P<0.05).

The measure of total iron retention represents the sum of radionuclide in all organs removed from the mouse and the remaining homogenised carcass. Ascorbic acid significantly improved total iron retention compared to egg albumin (1.5 fold; P<0.0001) or NZGLM (1.3 fold; P=0.026). NZGLM also significantly improved total iron retention compared to egg albumin (1.25 fold; P=0.02) in mice raised on the control diet. There was no significant difference between total iron retention between mice raised on the control diet or the NZGLM diet when both were provided with the iron supplement combined with NZGLM (P=0.167).

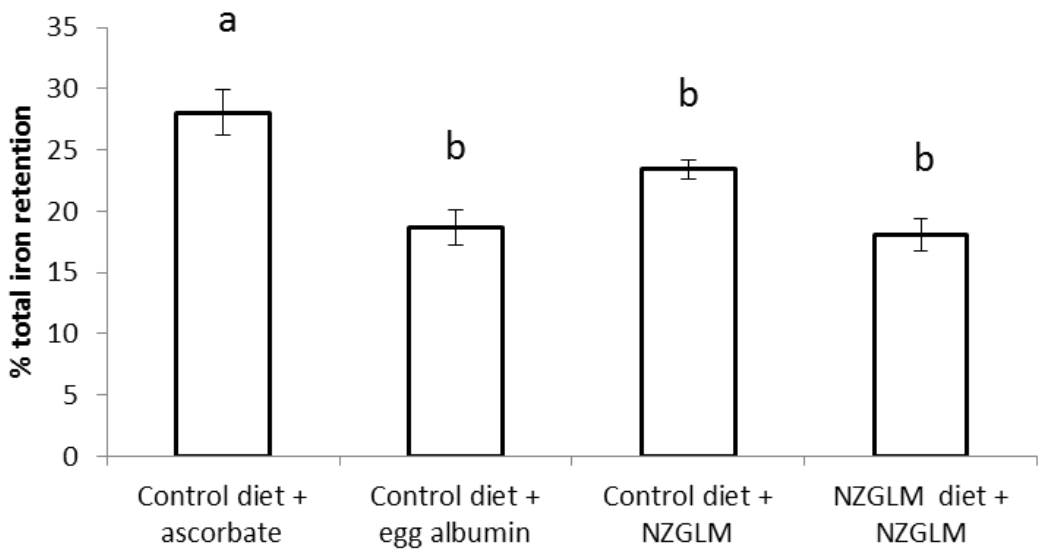

Figure 6.8. Total iron retention.

Mice were fed either a control diet or NZGLM-supplemented diet for 14 days. At day 15 a single dose of ^{59}Fe-labelled iron combined with ascorbic acid, egg albumin or NZGLM was provided by gavage. Mice were euthanased 4 days later. The liver, small and large intestine, spleen, liver and kidneys were removed and the remaining carcass was homogenised. The sum of ^{59}Fe enrichment in all tissues and carcass homogenate was calculated as a percentage from ^{59}Fe within the entire oral dose. Results are expressed as mean percent ^{59}Fe retained ± SEM, (n=9). Bars marked with dissimilar letters are significantly different from one another (P<0.05).

6.5. Discussion

Iron uptake by the absorptive enterocyte can be significantly reduced if the cell has already been recently exposed to dietary iron (Johnson et al. 2005). For this reason the iron concentration of both diets was standardised and differences in weight gain or dietary behaviour between the control and NZGLM-supplemented diets was monitored. The results suggest that there was no aversion to either experimental diet and that growth rates were similar between both experimental groups.

The haematological results suggest that the mice were not suffering from iron-deficiency anaemia or chronic inflammatory disease which may increase WBC concentrations. Although not statistically significant, a treatment-specific trend in RBC count and haemoglobin concentrations was noted. The combination of an iron supplement with either NZGLM or ascorbic acid increased RBC count and haemoglobin concentrations compared to the iron supplement combined with egg albumin. The enhancing effect of NZGLM was only observed in mice maintained on the control diet.

The enhancing effect of ascorbic acid on iron absorption in rodents has been reported to be less than that of humans or cell cultures due to the endogenous production of ascorbic acid in these species (Reddy and Cook 1991). This is in agreement with the current study where blood and liver iron enrichment and total body iron retention were increased by approximately 40% when combined with ascorbic acid treatment compared to combining iron with egg albumin.

The magnitude of iron absorption enhancement by ascorbic acid compared to egg albumin in the current study was significantly lower than that observed *in vitro* (Chapters Four and Five) or in human participants (Cook and Monsen 1977).

Like ascorbic acid, combining NZGLM with an iron supplement increased blood and carcass iron enrichment compared to egg albumin. This enhancing effect was only observed in mice maintained on the control diet. This relationship is also similar to that previously reported in Chapters Four and Five. This suggests that combining NZGLM with an iron supplement may be effective for promoting iron absorption in iron-deficient participants.

Interestingly, the enhancing effect of combining iron with NZGLM on blood iron enrichment was not observed in mice that were raised on the NZGLM diet. Additionally the total liver iron concentration of these mice was significantly reduced compared to mice raised on the control diet. RBC count and haemoglobin concentrations were also reduced, however this result was not statistically significant.

The inhibitory effect of continuous NZGLM supplementation on iron absorption is of considerable interest because both diets were matched for iron load. Although NZGLM also contains manganese, copper and calcium which inhibit iron transport by direct and indirect competitive inhibition (Garrick et al. 2003; Shawki and Mackenzie 2010), the inhibitory effect was only noted during prolonged NZGLM supplementation. This suggests that mechanisms other than direct and indirect competitive inhibition may occur during prolonged NZGLM supplementation.

Unlike manganese or cadmium, copper may reduce DMT1 expression in enterocytes when provided at a concentration of 100μM for 24 hours (Tennant et al. 2002). However the addition of NZGLM to the mouse diet was calculated to only provide an additional 2.4nmoles copper (Vlieg et al. 1991) therefore copper is unlikely to affect DMT1 expression at this concentration.

The addition of NZGLM to the diet within the current study increased the dietary calcium concentration by approximately ~315ppm (Vlieg et al. 1991). Calcium has been repeatedly reported to reduce mucosal iron transport when both divalent cations are consumed together (Barton et al. 1983). Calcium concentrations at or above 500ppm significantly reduces iron uptake in humans (Hallberg et al. 1991), potentially by reducing DMT1 iron transport (Shawki and Mackenzie 2010) or by temporarily removing DMT1 from the brush border membrane (Thompson et al. 2010). If the mechanism of DMT1 removal from the brush border membrane is similar to DMT1 internalisation in response to cytosolic iron loading (Johnson et al. 2005), iron absorption from subsequent meals may be significantly reduced.

The pattern of NZGLM consumption in the current study is unlike that typically seen in humans and therefore limits the applicability of this study to human populations. The AHA recommends consuming 200g oily fish per week as one or two portions. This provides approximately 1.5% total dietary energy. Although this dose was used in the current study (corrected for mouse energy requirements), the feeding regime of one to two portions per week could not be simulated due to possible aversion of mice to meals containing a high NZGLM content.

For the above reasons NZGLM was incorporated evenly within the diet. The effects of weekly or bi-weekly NZGLM supplementation may have a different effect on mucosal iron transport compared to continuous supplementation.

In summary combining NZGLM with an iron supplement can promote enterocyte iron uptake and subsequent liver and blood iron repletion in iron-deficient mice when provided as a single dose. This enhancing effect was not observed when NZGLM was provided at a low dose for multiple days prior to provision of the NZGLM/iron load. The inhibitory effect might be due to the calcium load of NZGLM. Further investigation into this inhibitory mechanism and its effects on iron absorption from subsequent meals or iron supplements is required.

Chapter Seven: Effect of pre-loading Caco-2 cells with calcium on iron absorption from a supplement

The results discussed in Chapter Six suggest that prolonged New Zealand green-lipped mussel supplementation prior to the consumption of an iron supplement may reduce iron absorption. The inhibiting mechanism was proposed to be associated with the high calcium content of New Zealand green-lipped mussel. The primary objective of this study was to investigate the effect of exposing Caco-2 cells to calcium over a range of calcium concentrations and exposure times on brush border iron absorption. The effect of exposing Caco-2 cells to calcium prior to the addition of iron was compared to co-supplementation of calcium and iron. The following chapter describes the results of the study and discusses the inhibitory effect of prolonged calcium supplementation on iron absorption.

7.1. Abstract

Calcium inhibits the mucosal transport of dietary iron. The effect of consuming calcium prior to consuming iron on mucosal iron transport is unknown. The objectives of this study were to investigate whether pre-incubating Caco-2 cells to calcium prior to exposing Caco-2 cells to iron will also inhibit iron absorption and to investigate the conditions required to stimulate this inhibitory effect. Caco-2 cells were pre-treated with either 3mM $CaCl_2$ for 1-24 hours or 0.3-3mM $CaCl_2$ for 3 hours. All extracellular calcium was removed from the mucosal reservoir and the cells were incubated with either $^{55}FeCl_3$ in calcium-free iron uptake media or 3mM calcium-enriched iron uptake media for 60 minutes. Iron uptake from a calcium-free media was attenuated when Caco-2 cells were pre-incubated for 3 hours with 1mM or 3mM $CaCl_2$ compared to 0.3mM $CaCl_2$. Iron uptake from a calcium-free media was also attenuated when Caco-2 cells were pre-incubated with 3mM $CaCl_2$ for 3-6 hours but not 12 hours or 24 hours. Iron uptake was attenuated within 60 minutes when combined directly with 3mM $CaCl_2$ compared to a calcium-free control. Iron solubility was reduced when combined with calcium-enriched media. These observations suggest that when combined directly with iron, calcium reduces iron solubility and the rate of iron uptake across the brush border membrane. Prolonged calcium supplementation prior to the consumption of iron may reduce mucosal iron transport by an independent mechanism. This inhibitory mechanism is slowly reversible and may reduce iron absorption from subsequent meals.

7.2. Introduction

The addition of calcium to a meal significantly reduces iron absorption from that meal by up to 60% (Hallberg et al. 1991). This inhibitory effect has been reported both *in vivo* (Barton et al. 1983; Hallberg et al. 1992) and *in vitro* (Thompson et al. 2010). Iron absorption from subsequent meals may also be affected.

The inhibitory effect of calcium on iron absorption cannot be overcome by co-supplementation with iron-absorption enhancers such as ascorbic acid (Hallberg et al. 1992) or red meat (Monsen and Cook 1976). This indicates that calcium does not interact with iron or directly affect iron solubility or dialisability. Iron absorption in the presence of calcium is reduced further by the addition of phytates and tannins (Hallberg et al. 1991), suggesting that the inhibitory mechanism of calcium is independent of other iron-absorption inhibitors.

Calcium specifically reduces iron transport across the absorptive mucosa (Barton et al. 1983). The inhibitory mechanism does not appear to be competitive inhibition of DMT1 (Garrick et al. 2006). The inhibitory mechanisms may include non-competitive reduction in DMT1 iron transport rate (Shawki and Mackenzie 2010) or a reduction in brush-border membrane DMT1 concentration by stimulating DMT1 internalisation (Thompson et al. 2010). The effect of each of these proposed mechanisms on iron absorption from a meal or iron supplement requires further investigation.

The aims of this study were to investigate the effect of pre-incubating Caco-2 cells with calcium-enriched culture media on iron absorption from a calcium-free iron supplement and to identify the specific calcium concentration range required for this inhibition to occur. These results were compared with iron absorption when combined directly with calcium. The effect of calcium on iron solubility was also assessed.

7.3. Material and methods

7.3.1. Cell cultures

All cell cultures were attained and split as described in Chapter 3, Sections 3.2.10 and 3.2.11.

Pre-incubation treatments were based on the FBS-free cell culture media supplemented with calcium chloride ($CaCl_2$) ranging between 0.3mM-3mM $CaCl_2$ as described in Chapter 3, Section 3.2.8.

Caco-2 cells were cultured on 12-well bicameral Transwell chambers (Corning CLS3460, MA, USA) and collagen-treated 24-well plates (Costar A11428-02, MA USA) as described in Chapter 3, Section 3.2.11.

7.3.2. Iron uptake media

Iron uptake media were prepared as described in Chapter 3, Section 3.2.13.

7.3.3. Calcium pre-incubation time

Caco-2 cells were incubated with 3mM $CaCl_2$-enriched FBS-free culture media for 1 to 24 hours. All cells were rinsed with calcium-free, FBS-free culture media prior to mixing with the iron uptake media supplemented with 200µM ascorbic acid (A0278) and then incubated for 60 minutes at 37°C with 5% CO_2 and 90% humidity.

Non-specifically bound iron on the Caco-2 cell brush border membrane was removed and cell monolayers were prepared for analysis as described in Chapter 3, Section 3.2.15.

7.3.4. Calcium pre-incubation concentration

Caco-2 cells were incubated with 0.3mM $CaCl_2$-enriched FBS-free culture media for 3 hours in order to standardise the baseline calcium load across all cells. This culture medium was replaced with FBS-free culture media enriched with $CaCl_2$ concentrations ranging from 0.3mM $CaCl_2$ to 3mM $CaCl_2$ for a further 3 hours. All Caco-2 cells were rinsed with calcium-free, FBS-free media prior to incubation with iron uptake media supplemented with 200µM ascorbic acid (A0278). Cells were exposed to the iron uptake media for 60 minutes at 37°C with 5% CO_2 and 90% humidity.

Non-specifically bound iron on the Caco-2 cell brush border membrane was removed and cell monolayers were prepared for analysis as described in Chapter 3, Section 3.2.15.

7.3.5. Iron-calcium co-supplementation

Samples of calcium-free, FBS-free culture media were supplemented with a combination of ^{55}Fe and ^{56}Fe to form a series of iron–enriched solutions ranging from 6µM iron to 100µM iron (92kBq/mL). All treatments were combined with ascorbic acid (A0278) ranging from 24µM to 400µM in a four-fold molar excess to iron. Half of each treatment was enriched with 3mM $CaCl_2$.

Caco-2 cells were rinsed with calcium-free FBS-free culture media and combined with iron-enriched treatments for 60 minutes at 37°C with 5% CO_2 and 90% humidity.

Non-specifically bound iron on the Caco-2 cell brush border membrane was removed and cell monolayers were prepared for analysis as described in Chapter 3, Section 3.2.15.

7.3.6. Uptake media iron solubility

Iron solubility within the uptake media was assessed as described by Glahn (1996) using iron uptake media alone, or supplemented with either 200µM ascorbic acid (A0278), $CaCl_2$ over a range of 0.1mM to 5mM or a combination of ascorbic acid and calcium. The fraction of ^{55}Fe remaining in the supernatant was measured after centrifugation of the uptake media at 15,600 X *g* for 5 minutes.

7.3.7. Analysis

All experiments unless otherwise stated assessed the fraction of ^{55}Fe absorbed by the Caco-2 cells from the iron uptake media. Uptake media or cell lysate were combined with OptiPhase HiSafe 2 scintillation cocktail (PerkinElmer 1200-436, Waltham, MA, USA) and the iron concentration was determined by scintillation counting (Wallac Trilux 1450 Microbeta PerkinElmer, Waltham, Massachusetts, USA) to assess the ^{55}Fe content. The cell protein concentration within each monolayer was analysed as described in Chapter 3, Section 3.2.16. Iron absorption by each monolayer was standardised by monolayer protein concentration as described in Chapter 3, Section 3.2.16.

Statistical analysis was performed using SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Treatments was analysed by 1-way and 2-way ANOVA using the General Linear Model procedure. Where appropriate, post-hoc analysis was carried out using LSD analysis.

7.4. Results

Iron absorption from the iron uptake media was significantly higher when cells were pre-incubated with 3mM $CaCl_2$ for 1 hour compared to cells pre-incubated for 3 hours (1.4 fold; P=0.015) or 6 hours (1.3 fold; P=0.008) as illustrated in Figure 7.1. There was no significant effect of time on iron uptake when cells were pre-incubated with 3mM $CaCl_2$ for either 12 or 24 hours compared to 1 hour (P>0.05).

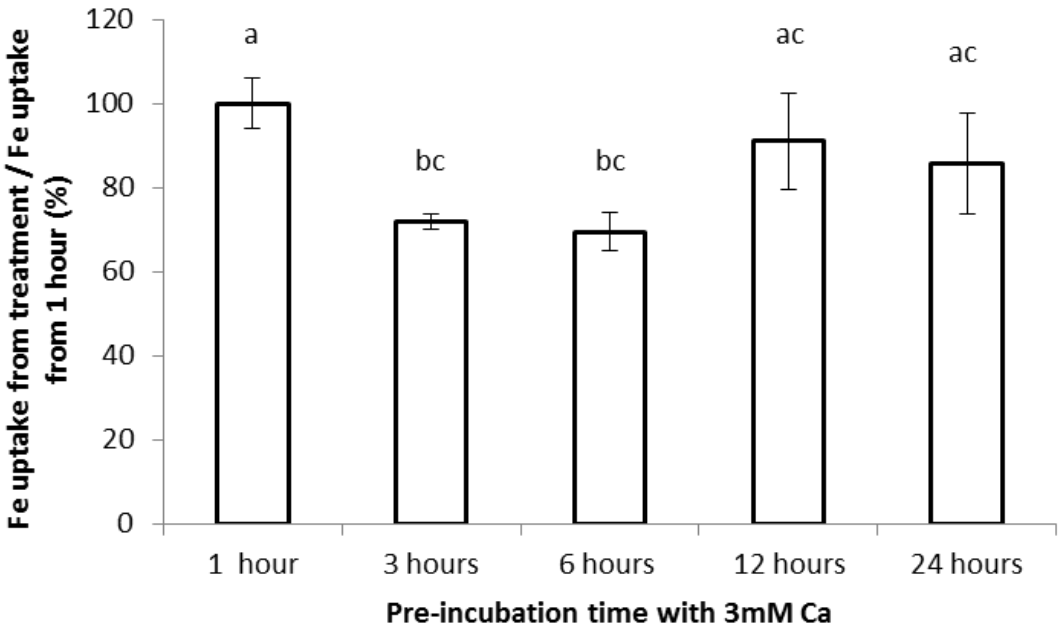

Figure 7.1. Caco-2 cell iron uptake after pre-incubation with 3mM calcium.

Cells were pre-incubated with FBS-free culture media containing 3mM $CaCl_2$ from 1 hour to 24 hours prior to the addition of a standardised iron uptake media. Bars represent means ± SEM, n=6. Bars marked with dissimilar letters are significantly different from each other (P≤ 0.05, LSD analysis).

Caco-2 cell iron uptake was significantly higher in cells pre-incubated for 3 hours with 0.3mM $CaCl_2$ compared to cells pre-incubated for 3 hours with 1mM $CaCl_2$ (1.2 fold; P≤0.0001) or 3mM $CaCl_2$ (1.2 fold; P≤0.0001) as illustrated in Figure 7.2. There was no significant difference in iron uptake between cells pre-incubated with 1mM $CaCl_2$ or 3mM $CaCl_2$.

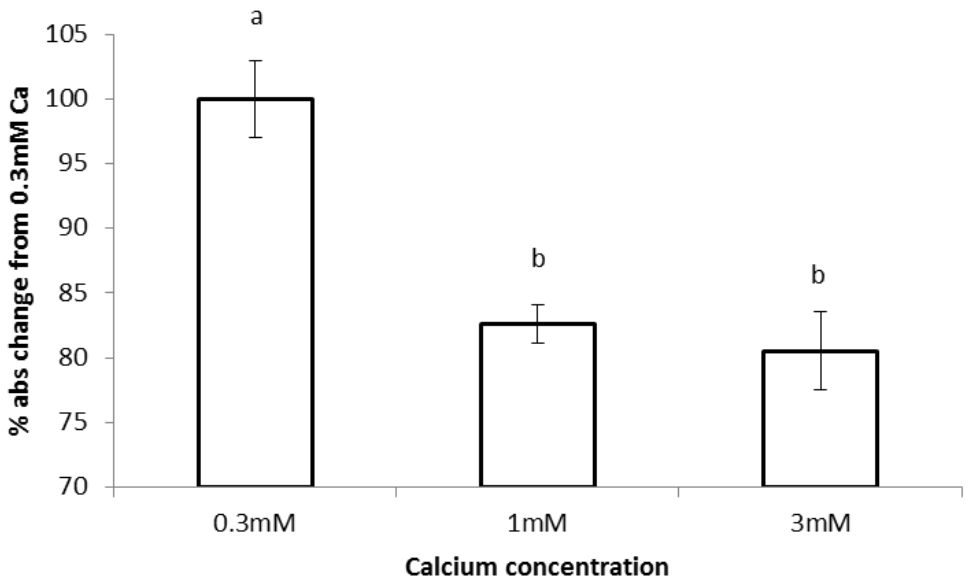

Figure 7.2. Caco-2 cell iron uptake after pre-incubation with calcium-enriched D0 media.

Cells were pre-incubated with FBS-free culture media containing $CaCl_2$ ranging from 0.3mM to 3mM prior to the addition of a standardised iron uptake media. Bars represent means ± SEM, n=14. Bars marked with dissimilar letters are significantly different from each other (P≤0.05, LSD analysis).

Iron solubility within the FBS-free culture media was significantly higher when combined with ascorbic acid compared to iron alone regardless of the $CaCl_2$ concentration (P<0.0001) as illustrated in Figure 7.3. Iron solubility was highest when combined with ascorbic acid without $CaCl_2$. Iron solubility in the presence of ascorbic acid was significantly reduced by the addition of 1mM $CaCl_2$ (1.17 fold; P=0.003), 3mM $CaCl_2$ (1.48 fold; P<0.0001), 4mM $CaCl_2$ (1.55 fold; P<0.0001) or 5mM $CaCl_2$ (1.62 fold; P<0.0001) to the culture media. There was no further decrease in iron solubility when the $CaCl_2$ concentration was increased above 3mM $CaCl_2$ in the presence of ascorbic acid (P>0.05).

Iron solubility in the absence of ascorbic acid was significantly higher in the 0.7mM Calcium-enriched FBS-free culture media compared to the calcium-free culture media (P=0.02). There was no significant effect of increasing the calcium concentration above 0.7mM on iron solubility (P>0.05).

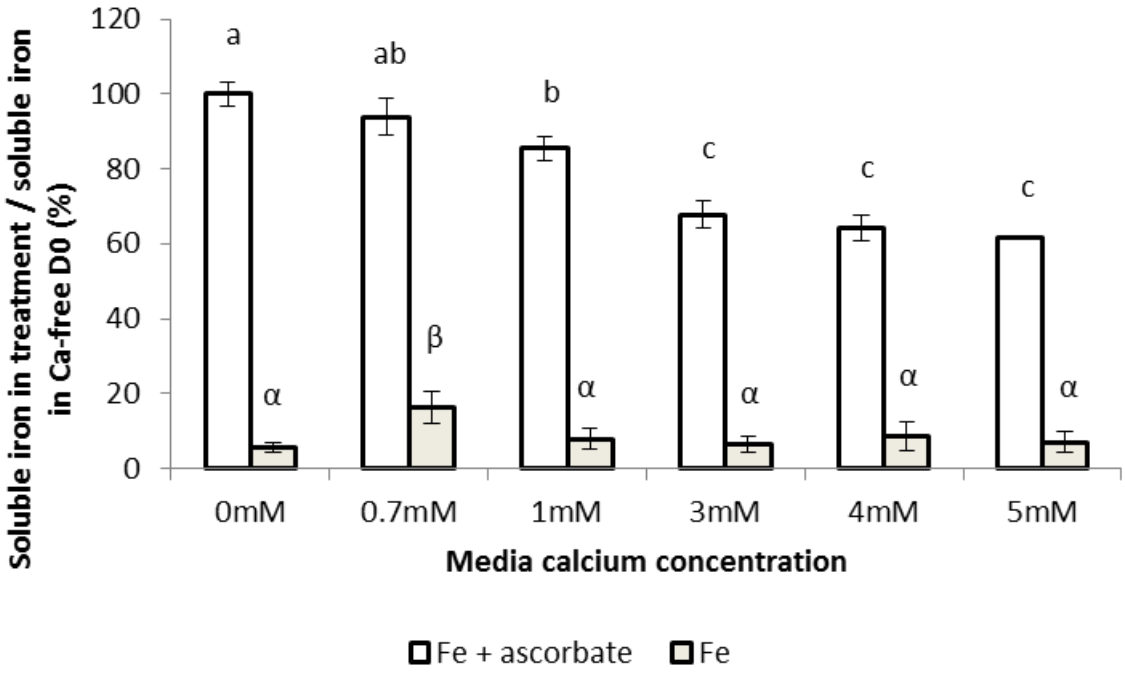

Figure 7.3. Iron solubility in the presence of ascorbic acid and calcium.

Iron solubility was measured alone, or in the presence of 200µM ascorbic acid and/or $CaCl_2$ ranging from 0.7mM to 5mM. Iron solubility was measured as the fraction of iron present within the supernatant after centrifugation at 15,600x g for 5 minutes. Bars represent means ± SEM, n=4. Bars marked with dissimilar letters within the same syntax are significantly different from each other (P≤ 0.05, LSD analysis). Bars marked with a dissimilar syntax are significantly different from each other (P≤0.05, LSD analysis).

Iron uptake was significantly higher in cells provided with the calcium-free iron uptake media compared to the 3mM $CaCl_2$-enriched uptake media ($P<0.0001$). The magnitude of this inhibitory effect of $CaCl_2$/iron co-supplementation was not significantly different between different iron-load treatments ($P>0.05$) as illustrated in Figure 7.4.

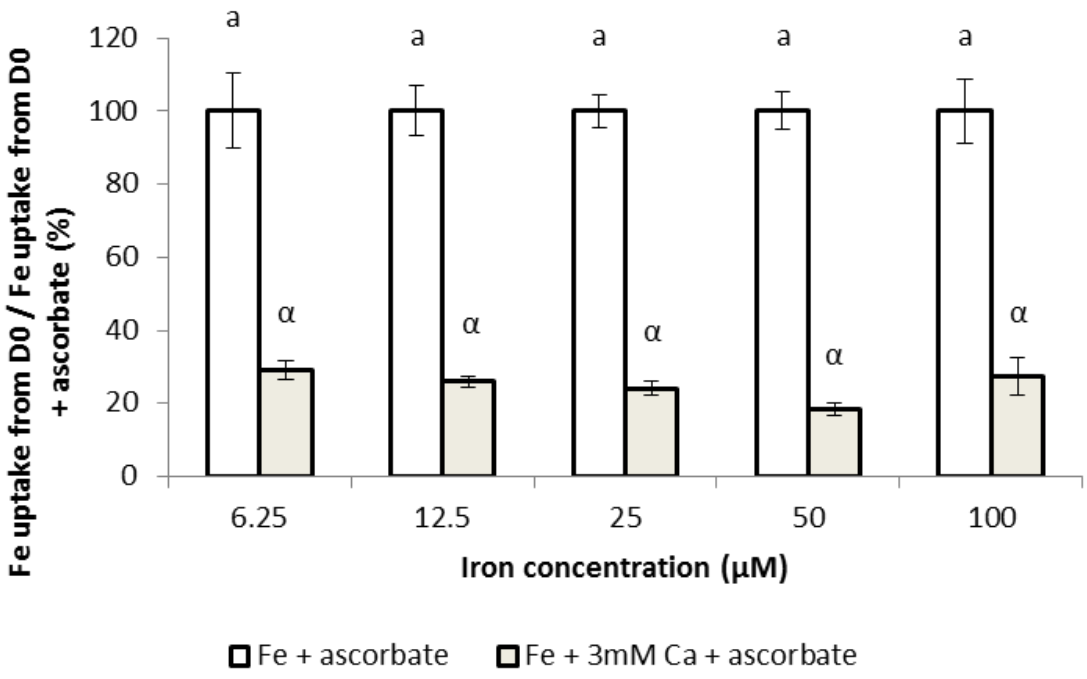

Figure 7.4. Caco-2 cell iron uptake at different concentrations in the presence or absence of 3mM $CaCl_2$. Cells were incubated with iron uptake media, or iron uptake media supplemented with 3mM $CaCl_2$ for 60 minutes. The experiment was repeated with different iron concentrations ranging from 6.25µM to 100 µM with a 4x molar excess of ascorbic acid. Bars represent means ± SEM, n=4. Bars marked with dissimilar letters within the same syntax are significantly different from each other (P≤ 0.05, LSD analysis). Bars marked with a dissimilar syntax are significantly different from each other (P≤0.05, LSD analysis).

7.5. Discussion

Ascorbic acid promotes iron absorption within the small intestine by maintaining iron solubility under neutral and alkaline conditions (Lynch and Cook 1980) and by competing with iron absorption inhibitors (South and Miller 1998). In the current study iron solubility was significantly lower in the un-supplemented culture media compared to the culture media supplemented with a 4-fold molar excess of ascorbic acid. Previous experiments suggest that ascorbic acid supplementation does not counter the inhibitory effects of calcium on iron absorption (Hallberg et al. 1992). For this reason all experiments in this study were undertaken with ascorbic acid-supplemented culture media.

7.5.1. Calcium pre-incubation

A dose-dependent inhibitory effect of calcium on iron absorption was observed when the calcium concentration of the pre-incubation culture media was increased from 0.3mM to 1mM. 0.3mM. There was no significant inhibitory effect when the culture media calcium concentration was further increased from 1mM calcium to 3mM. A similar dose-dependent inverse relationship was reported previously at these calcium concentrations on iron absorption and ferritin production in Caco-2 cells (Thompson et al. 2010). Calcium concentrations below 0.3mM have been previously observed within our laboratory to compromise tight junction integrity under the culture conditions used for these experiments.

The inhibitory effect of calcium on iron absorption when Caco-2 cells were pre-incubated with 3mM calcium was also time-dependent. The strongest effect was noted when Caco-2 cells were pre-incubated with calcium for 3-6 hours. Interestingly the inhibitory effect of calcium pre-incubation on iron absorption was lost when cells were pre-incubated with calcium for 12 hours or 24 hours. This may suggest that the enterocyte can adapt to prolonged calcium exposures in order to ensure that cellular iron requirements are met. This may explain why longitudinal studies show little inhibitory effect of calcium supplementation on iron status (Mølgaard et al. 2005).

Overall, the results of the current study suggest that the inhibitory effect of calcium pre-incubation on iron absorption is slow-acting and dose dependent. Thompson et al. (2010) have previously reported that incubating Caco-2 cells with 2.5mM calcium-enriched DMEM or 30µM iron for 4 hours both stimulate DMT1 internalisation from the plasma membrane into cytosolic vesicles by a similar magnitude. This effect was also dose-dependent.

Brush border DMT1 concentrations can be reduced by incubating enterocytes with iron-enriched culture media (Johnson et al. 2005). Under these conditions brush border iron transport is reduced for up to eight hours. A similar time-dependent inhibitory mechanism may also exist for calcium. These previous reports combined with the observations of the current study suggest that high luminal iron or calcium concentrations may reduce the ability of absorptive enterocytes to take up iron from subsequent meals.

7.5.2. Calcium-iron co-supplementation

Combining iron directly with calcium-enriched media significantly reduced iron absorption compared to combining iron with a calcium-free control medium. The direct inhibitory effect was observed over a range of iron/calcium ratios. The mechanism by which calcium inhibits iron absorption when both are combined within the same meal may be independent to that observed in Section 7.5.1. The inhibitory effect of calcium pre-incubation is only evident after 3 hours which is significantly longer than the duration of these experiments.

Although calcium significantly reduces the solubility of iron within the culture media, this alone does not account for the entire inhibitory effect. Another mechanism of inhibition must also exist. This inhibitory mechanism is rapidly reversible because rinsing Caco-2 cell monolayers, which have been pre-incubated with calcium for less than 60 minutes, with calcium-free media reverses the majority of the inhibitory effect

Calcium has been reported to reduce the iron transport properties of DMT1 by reducing DMT1 proton transport (Shawki and Mackenzie 2010). This inhibitory mechanism is non-competitive and is immediately reversible when calcium is removed from the extracellular fluid. This proposed mechanism is compatible with the findings described here.

Non-competitive inhibition of DMT1 by calcium can also explain why short-term calcium-iron co-supplementation has been repeatedly associated with poor iron absorption (Woodruff 1975; Dawson-Hughes et al. 1986; Benkhedda et al. 2010) and why spacing calcium-rich meals from iron-rich meals can be successfully used to overcome the majority of the calcium-associated inhibition of iron absorption (Gleerup et al. 1995).

In summary our observations suggest that concurrent calcium-iron supplementation significantly reduces iron absorption by the absorptive enterocyte. This inhibitory effect may be associated with a reduced DMT1 transport rate. Furthermore dietary calcium may reduce iron absorption from subsequent meals by promoting time and dose-dependent DMT1 internalisation. The magnitude of this effect in humans and animals is unknown. Dietary guidelines may require adjustment in order to insure that the absorption of iron is maximised not only from a single meal, but also from subsequent meals.

Chapter Eight: Effect of pre-incubating Caco-2 cells with New Zealand green-lipped mussels on iron absorption from a supplement

The results discussed in Chapter Six suggest that prolonged New Zealand green-lipped mussel supplementation reduces iron absorption in mice. The inhibiting mechanism was proposed to be associated with the high calcium content of New Zealand green-lipped mussel. The results discussed in Chapter Seven suggest that pre-exposing Caco-2 cells to calcium reduces iron absorption from an iron supplement. The primary objective of this study was to investigate the effect of pre-exposing Caco-2 cells to New Zealand green lipped mussel or calcium over a range of calcium concentrations on brush border iron absorption. The following chapter describes the results of the study and discusses the inhibitory role of endogenous calcium on iron absorption.

8.1. Abstract

Prolonged supplementation of NZGLM reduces iron uptake in mice. The inhibitory effect of NZGLM supplementation may be due to its high calcium content. Calcium may stimulate DMT1 internalisation from the plasma membrane and reduce iron uptake from subsequent meals. The objective of this study was to investigate the effect of pre-incubating Caco-2 cells with NZGLM digestate, or $CaCl_2$-enriched cell culture media, on iron absorption from a second dietary iron source. Differentiated Caco-2 cells were incubated with NZGLM digestate or cell culture media enriched with $CaCl_2$ for 3 hours. All treatments were standardised for iron concentration. NZGLM and calcium treatments were removed, cells were rinsed with calcium-free media then combined with calcium-free culture media enriched with ^{55}Fe. The fraction of iron transported across the brush border membrane was determined. Iron uptake was attenuated when Caco-2 cells were pre-incubated with NZGLM digestate, 0.65mM $CaCl_2$, 1mM $CaCl_2$ or 3mM $CaCl_2$ treatments compared to the 0.3mM $CaCl_2$ treatment. Iron uptake was significantly reduced in cells pre-incubated with NZGLM digestate compared to the 0.65mM $CaCl_2$ treatment but not the 1mM $CaCl_2$ or 3mM $CaCl_2$ treatments. Our data suggests that pre-incubating Caco-2 cells with NZGLM reduces iron uptake from a second dietary iron source. The preload inhibitory effect includes, but is not limited to, the calcium concentration of NZGLM.

8.2. Introduction

Raising mice on a low-iron diet enriched with NZGLM reduces liver iron stores and decreases mucosal iron transport compared to mice raised on a control low iron diet. As discussed in Chapter Six, these observations suggest that as well as containing iron absorption enhancers, NZGLM also contains iron absorption inhibitors and that frequent NZGLM consumption may not be beneficial for promoting iron absorption.

The inhibitory effect of prolonged NZGLM supplementation on iron absorption may be associated with the high concentration of endogenous calcium which is approximately 40-fold higher than red meat (Vlieg et al. 1991). Calcium has been reported to significantly reduce mucosal iron transfer (Barton et al. 1983). Two independent inhibitory mechanisms have been proposed and include non-competitive inhibition of DMT1 (Shawki and Mackenzie 2010) and DMT1 internalisation from the enterocyte brush border membrane (Thompson et al. 2010).

Once DMT1 internalisation has been stimulated, iron absorption from subsequent meals or supplements may be reduced for up to eight hours (Frazer et al. 2003; Johnson et al. 2005). The inhibitory effects of calcium pre-incubation on iron absorption from subsequent meals or supplements are described in Chapter Seven. These observations suggest that iron absorption from subsequent meals within this eight hour period may be significantly reduced. The effect of pre-loading enterocytes with calcium-rich foods such as NZGLM on brush border iron transport

requires further investigation. The aim of this study was to investigate the effect of pre-incubating Caco-2 cells with NZGLM digestate-enriched culture media, culture media matched for the calcium concentration of NZGLM, or culture media exceeding the calcium concentration of NZGLM on iron absorption from an iron-enriched culture medium.

8.3. Material and methods

8.3.1. Cell cultures

All cell cultures were attained and split as described in Chapter 3, Sections 3.2.9 and 3.2.10.

Pre-incubation treatments were based on the FBS-free cell culture media, with calcium concentrations ranging between 0.3mM-3mM $CaCl_2$, as stipulated within the experimental procedures.

Caco-2 cells were cultured on 12-well bicameral Transwell chambers (Corning CLS3460, MA, USA) and collagen-treated 24-well plates (Costar A11428-02, MA USA) as described in Chapter 3, Section 3.2.11.

8.3.2. Iron uptake media

Iron uptake media was prepared as described in Chapter 3, Section 3.2.13 and supplemented with 200µM ascorbic acid.

8.3.3. *In vitro* digestion

The *in vitro* digestion protocol was modified from Chapter 3, Section 3.2.4 as discussed in Chapter 3, Section 3.4. Lyophilised NZGLM was prepared as described in Chapter 3, Section 3.2.3. For the digestion, 0.5g NZGLM was reconstituted with 9.5mL saline solution (120mmol/L NaCl and 5mmol/L KCl).

Immediately before use, porcine pepsin (P7000, 800-2500 units/mg protein) was activated in 0.1M HCL (25mg/mL) for 5 minutes. For the simulated gastric digestion, treatments were slowly titrated to pH 2.5 with 1M HCl prior to the addition of a 0.5mL activated pepsin aliquot. Samples were incubated at 37°C on a rocking shaker for 60 minutes.

Immediately before use, porcine pancreatin (P1625, activity 3 x USP specifications) and bile salts (B8631) were combined with 0.1M $NaHCO_3$ (S5761) (9.26mg/mL and 55.5mg/mL respectively). For the simulated intestinal digestion, whole gastric digestates were titrated to pH 6.0 with 1M $NaHCO_3$ (S5761) and combined with 0.5mL activated pancreatin/bile solution. All digestates was then adjusted to 300mOsm, titrated to pH 7.0 and made up to a final volume of 15mL with saline solution before incubation at 37°C on a rocking shaker for 120 minutes.

The completed digestate was centrifuged immediately at 172x *g* for 5 minutes and the supernatant was separated by molecular weight using centrifugal ultrafiltration (10kDa MWCO membrane) (Vivaspin 20®, Vivaproducts, MA, USA) according to the manufacturer's directions.

Samples were sterilised through 0.2µm Minisart® SRP syringe filters (Sartorius AG, Goettingen Germany) and analysed for iron concentration as described in Chapter 3, Section 3.2.3 and Section 3.2.4. The calcium concentration of the NZGLM digestate was analysed as described in Chapter 3, Section 3.2.14.

8.3.4. NZGLM pre-incubation

All pre-incubation treatments were based on FBS-free, calcium-free cell culture media, supplemented with $CaCl_2$ as described is Chapter 3, Section 3.2.8. NZGLM digestate was combined 1:1 with FBS-free, calcium-free cell culture media enriched with 0.65mM $CaCl_2$. The iron load in all treatments was standardised to 4.4µM with a 3mM $FeCl_3$ (157740) stock solution in order to match the iron concentration of the diluted NZGLM digestate.

Caco-2 cells were incubated in calcium-free cell culture media supplemented with 0.3mM $CaCl_2$ for 3 hours. Cells were subsequently incubated with NZGLM digestate, 0.3mM $CaCl_2$, 0.65mM $CaCl_2$ 1mM $CaCl_2$ or 3mM $CaCl_2$ for a further 3 hours. All Caco-2 cells were rinsed with calcium-free/FBS-free culture media.

After pre-incubation with NZGLM digestate or calcium-enriched culture media, cells were exposed to the iron uptake media for 60 minutes at 37°C with 5% CO_2 and 90% humidity. Non-specifically bound iron on the Caco-2 cell brush border membrane was removed and cell monolayers were prepared for analysis. These processes are described in Chapter 3, Section 3.2.15.

8.3.5. Analysis

All experiments assessed the fraction of ^{55}Fe absorbed from the iron uptake media. Samples of uptake media and cell lysate were combined with OptiPhase HiSafe 2 scintillation cocktail (PerkinElmer 1200-436, Waltham, MA, USA) and determined by liquid scintillation counting (Wallac Trilux 1450 Microbeta PerkinElmer, Waltham, Massachusetts, USA). The protein concentration within each monolayer was analysed as described in Chapter 3, Section 3.2.6 and iron absorption by each monolayer was standardised by monolayer protein concentration. Statistical analysis was performed using SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Treatments were analysed by 2-way ANOVA using the General Linear Model procedure. Post-hoc analysis was carried out using LSD analysis.

8.4. Results

Caco-2 cell iron uptake was significantly higher in cells pre-incubated for 3 hours with 0.3mM calcium compared to cells pre-incubated for 3 hours with 0.65mM calcium (1.12 fold; P=0.007), 1mM calcium (1.2 fold; P=0.004) 3mM calcium (1.24 fold; P<0.0001) or NZGLM digestate (1.23 fold; P<0.0001). Iron absorption was significantly higher in the 0.65mM calcium treatment compared to the 1mM calcium (1.07 fold; P=0.05), 3mM calcium (1.1 fold; P=0.041) or NZGLM digestate (1.09 fold; P=0.049). There was no significant difference in iron uptake between cells pre-incubated with 1mM $CaCl_2$, 3mM $CaCl_2$ or NZGLM digestate (P>0.05). These results are illustrated in Figure 8.1.

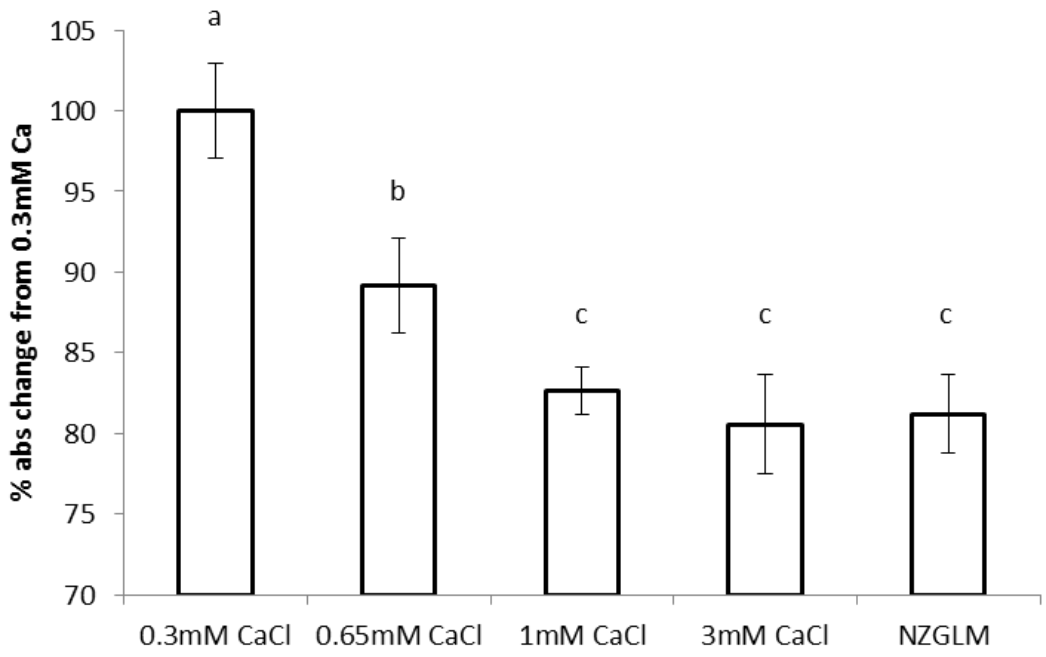

Figure 8.1. Caco-2 cell iron uptake after pre-incubation with calcium-enriched D0 media or NZGLM digestate for 3 hours.

Cells were pre-incubated for three hours in FBS-free culture media supplemented with $CaCl_2$ ranging from 0.3mM to 3mM, or NZGLM digestate prior to the addition of a standardised iron uptake media. Bars represent means ± SEM, n=14. Bars marked with dissimilar letters are significantly different from one another (P≤0.05, LSD analysis).

8.5. Discussion

As discussed in Chapter Seven, iron absorption is significantly reduced when Caco-2 cells are pre-incubated with calcium concentrations ranging from 0.3mM $CaCl_2$ to 3mM $CaCl_2$. This inhibitory mechanism requires a minimum of three hours to manifest. For this reason a three hour pre-incubation period was used for the current study.

The 0.65mM $CaCl_2$ pre-incubation treatment was developed in order to match the concentration of soluble calcium within the NZGLM digestate. All treatments were standardised to 4.4µM iron, which was the iron concentration of NZGLM digestate-enriched cell culture media. This iron concentration was not expected to influence DMT1 internalisation or expression in Caco-2 cells (Sharp et al. 2002).

The inhibitory effect of pre-incubating Caco-2 cells with $CaCl_2$ was similar to that observed in Chapter Seven. The results further suggest that pre-incubating Caco-2 cells with $CaCl_2$ at concentrations ranging between 0.3mM and 3mM inhibits iron absorption. The calcium concentration of NZGLM digestate was within this range and was therefore expected to also inhibit iron absorption.

Calcium-stimulated DMT1 internalisation is dose-dependent (Thompson et al. 2010). In the current study, there was no additional inhibitory effect of calcium when Caco-2 cells were pre-incubated with cell culture media containing $CaCl_2$ concentrations above 1mM. The results suggest that DMT1 internalisation is only partially stimulated by the 0.65mM $CaCl_2$ culture media.

The inhibitory effect of pre-incubating Caco-2 cells with NZGLM digestate on iron absorption was similar to pre-incubating Caco-2 cells with culture media supplemented with 1mM or 3mM $CaCl_2$. This result suggests that that the calcium concentration of NZGLM is only partly responsible for the inhibitory effect of NZGLM digestate. Nutrients other than iron or calcium within the NZGLM digestate may further inhibit iron uptake when provided as a pre-treatment.

DMT1 transports a range of divalent metals including manganese, copper and cadmium (Garrick et al. 2003). The concentration of these divalent metals within NZGLM exceeds that of other MPF factor sources such as beef, lamb or fish by up to 10-fold (Vlieg et al. 1991). Manganese, copper, cadmium and iron directly compete for transport by DMT1 when present together within the intestinal lumen (Garrick et al. 2006). However competitive inhibition can only occur if competing metals are present within the small intestinal lumen at the same time. This was not the case in the current study.

A reduction in DMT1 expression or enhanced DMT1 internalisation from the plasma membrane has not been reported in absorptive enterocytes pre-incubated with cadmium or zinc (Yamaji et al. 2001; Noël et al. 2006) or choroidal epithelia pre-incubated with manganese (Wang et al. 2006). However, pre-incubating absorptive enterocytes with copper for 24 hours significantly reduces iron uptake, reduces DMT1 expression and promotes DMT1 internalisation from the plasma membrane (Tennant et al. 2002).

Copper-stimulated internalisation of DMT1 is significantly less than that reported for iron or calcium (Sharp et al. 2002). Furthermore alterations in DMT1 expression or internalisation has only been reported when copper concentrations were approximately 100-fold higher than that of NZGLM digestate (Tennant et al. 2002). For this reason, the significance of copper within the NZGLM digestate, on the ability of absorptive enterocytes to take up iron, may be limited.

The inhibitory effect of pre-incubating Caco-2 cells with NZGLM digestate is somewhat conflicting to our earlier observations. In Chapters Four, Five and Six we reported that combining iron directly with NZGLM digestate significantly promotes iron uptake *in vitro* and *in vivo*. This suggests that NZGLM contains both enhancers and inhibitors of iron absorption.

Iron absorption-enhancing nutrients within NZGLM may promote mucosal iron transport by improving iron availability within the digesta, similar to that proposed for the MPF factor (Glahn et al. 1996; Mulvihill et al. 1998). However other nutrients within NZGLM may reduce the iron transport ability of the brush border membrane if in close proximity with the absorptive enterocyte for a prolonged period of time.

The effect of prolonged NZGLM consumption on iron absorption and iron status requires further investigation in human participants, especially those who are iron deplete. Shellfish such as mussels and clams are currently recommended for populations at risk of iron deficiency such as blood donors (NZBlood 2013). However, if repeated consumption of shellfish reduces the ability of the enterocyte to absorb iron from a subsequent meal or iron supplement, then these guidelines need to be updated in order to best promote iron repletion.

In summary we have reported that pre-incubating Caco-2 cells with NZGLM digestate for 3 hours reduces the ability of the cells to absorb iron. This inhibitory effect is significantly stronger than pre-incubating Caco-2 cells with a control culture medium standardised for calcium and iron load, and suggests that factors

other than calcium within the digestate may reduce the ability of the enterocyte to take up iron. The impact of prolonged or repeated NZGLM exposure on iron absorption in humans requires further investigation.

Chapter Nine: Proposed human trial: The use of iron supplements combined with absorption-enhancers in blood donors

The human trial described in this chapter was designed to investigate the effect of combining New Zealand green-lipped mussel, beef, ascorbic acid or egg albumin with an iron supplement on iron repletion in blood donors post-phlebotomy. Unfortunately the New Zealand Blood service (NZBS) is currently undertaking a prospective iron-supplementation trial on all consenting blood donors nationwide. For this reason the proposed study could not be undertaken on New Zealand blood donors over the duration of my PhD study.

9.1. Background

Approximately 42,000 patients in New Zealand hospitals are treated with blood or blood products per year. To fulfil this demand, the New Zealand Blood Service (NZBS) must receive 3000 blood donations per week from the New Zealand public, of which approximately 4% are regular donors (NZBlood 2013). If all health criteria are met, blood may be donated up to four times per year whereby approximately 450ml (1 unit) of blood is drawn by venepuncture. Each donation results in a loss of 210-240mg of iron (Alvarez-Ossorio et al. 2000) which must be replaced from body iron stores and dietary sources.

Blood donation studies have repeatedly reported that regular blood donors, especially women of reproductive age, are at considerable risk of becoming iron-deplete (Coad and Conlon 2011). The NZBS recommends consuming iron-rich foods such as beef and NZGLM daily in order to promote iron repletion (NZBlood 2013). This is because these foods contain a high endogenous iron load and they have the ability to further enhance iron absorption from other dietary iron sources.

Although NZGLM enhances iron absorption from a single meal, repeated NZGLM supplementation is associated with reduced iron absorption from subsequent meals in iron-deplete mice and cell cultures as described in Chapters Six and Eight. For this reason the frequent consumption of beef or NZGLM on iron repletion requires further investigation in iron-deplete human participants.

Iron supplementation post-donation has been reported to replete blood donor iron stores (Waldvogel et al. 2012), however due to the low bioavailability of iron supplements, iron supplementation may be required for prolonged periods of time. Unfortunately supplementing blood donors with iron for extended periods of time may exacerbate iron overloading in participants with hemochromatosis. Long-term iron supplementation may also be associated with increased risk of colorectal cancer (Simon 2002). An alternative approach is clearly required.

Supplementing blood donors with 100-150mg elemental iron per day for 8-10 days directly after phlebotomy, can significantly improve iron status within 10 days (Røsvik et al. 2010). Supplementing donors with iron exclusively during this time period has been reported to promote iron repletion within 4 weeks (Maghsudlu et al. 2008). Although the provision of iron for a short period of time (<10 days) lowers the risk of iron loading and its associated pathologies, supplementation of blood donors with 100+ mg/day is associated with gastrointestinal discomfort and poor compliance. Gastrointestinal discomfort is less common when iron doses at or below 40mg per day are used (Radtke et al. 2004) especially if these are consumed with a meal (Newman 2006).

Iron absorption-enhancers can increase iron absorption from a meal (or supplement) by up to four fold in iron-deficient participants (Layrisse and Martinez-Torres 1968). For this reason the provision of a 40mg iron supplement with an iron enhancer may be as effective as providing a 100-150mg iron supplement alone. This may significantly improve iron status and replete iron stores without promoting

iron loading-associated pathologies, gastrointestinal distress or poor compliance. The effect of iron supplementation on iron repletion can be determined using SF measurements 9 days post-donation (Røsvik et al. 2010).

The objective of this proposed study is to investigate the effects of daily supplementation of beef, NZGLM, ascorbic acid (positive control) and egg albumin (negative control), combined with a 40mg iron supplement and a vegetable-based meal, on iron repletion in blood donors. All treatments will be provided for 9 days immediately post donation in order to measure changes in iron status.

9.2. Participant selection

Pre-menopausal women who are regular blood donors (have donated blood at least once within the last 12 months) will be recruited from the NZBS register for Palmerston North. All candidates will be contacted directly by the researchers, and letters informing potential participants of the proposed procedures and contact information will be sent to those who express interest.

9.3. Screening and randomisation

Participant selection will be In accordance with NZBS guidelines. All participants will be healthy based on their reported medical history, over 16 years old, over 50kg weight, and have a haemoglobin concentration ≥ 120g/L as assessed by finger prick.

Additionally participants will be under 45 years old and have a serum ferritin concentration ≤50µg/L and have a c-reactive peptide concentration below 10mg/L in order to improve the sensitivity of this trial (Røsvik et al. 2010; Beck et al. 2011).

Exclusion criteria will include pregnancy within the last 12 months or breastfeeding within the last 3 months, underlying inflammatory condition (e.g. arthritis, gastric ulcers, inflammatory bowel disease, ulcerative colitis, Crohn's disease or morbid obesity (BMI>30), current use of iron supplements, vegetarians, or allergies to shellfish, egg or gluten. Participants will be provided with a sample of all 3 test meals to taste in order to assure compliance.

9.4. Recruitment numbers

We will recruit 84 participants in total on the assumption that approximately 25% of participants may be excluded due to serum ferritin or CRP measurements exceeding the designated cut-off, or by participants dropping out of the trial for personal reasons as previously reported by Leonard et al. (2014). Participants will be stratified by BMI into 4 treatment groups each containing 16 participants with a calculated power of 80%, a significance level of 5% and a clinically significant change in serum ferritin concentration of 7-10ug/L Serum Ferritin. The power calculation used was derived from Snedecor and Cochran (1980). The power calculation parameters used in the current study are based on previous observations for serum ferritin reported by Røsvik et al. (2010).

9.5. Treatments

Fresh NZGLM flesh (including adductor muscle) and lean beef sirloin (Countdown, New Zealand) will be homogenised separately, lyophilised and ground to a fine powder. Lyophilised egg albumin (Zeagold, New Zealand) and ascorbic acid (AA) (Sigma MO, USA) will be used as a negative and positive control respectively.

Three vegetable-based test meals have been designed to disguise the taste, smell and texture of the experimental treatments. These three meals will be used for each treatment in order to promote compliance over the duration of the trial. Each meal was designed to provide a similar volume and energy content. All meals were designed to be produced as one batch and frozen in individual portions. The weight, energy and macronutrient composition is described in Table 9.1.

Table 9.1. Weight, energy and macronutrient profile of test meals

	Weight (g)	**Energy (kJ)**	**Protein (%)**	**CHO (%)**	**Fat (%)**	**Iron (mg)**
Chowder	368	1179	13	70	17	3.32
Pasta	345	1835	19	58	22	2.01
Risotto	387	1369	13	72	15	1.54

All meals were designed to contain a similar concentration of phytates, tannins, chlorogenic acid, ascorbic acid and calcium. The content of ascorbic acid is significantly reduced during the cooking, storage and reheating processes (Gil et al. 1999). The concentration of inhibitors and enhancers is described in Table 9.2.

Table 9.2. Iron absorption enhancer and inhibitor concentration per test meal prior to cooking and storage

	Phytate (mg)	**Tannin (mg)**	**Chlorogenic acid (mg)**	**Total tannins (mg)**	**Calcium (mg)**	**Ascorbic acid (mg)**
Chowder	51.1	9.1	3.6	10.9	127.7	10.6
Pasta	94.2	9	3.6	10.8	370.4	10.32
Risotto	61.3	9.9	3.7	11.73	162.3	8.2

Each meal will be combined with 50g (wet weight) NZGLM (5.45mg iron), beef (1.9mg iron) or egg albumin (0mg iron). This weight has been chosen because the addition of 50g cooked pork to a vegetable-based meal has been previously reported to enhance iron absorption from a single meal in humans (Baech et al. 2003). For the positive control, 500mg AA treatments will be dissolved in 200mL deionised water and consumed with the meal. The iron concentration of all meals will be adjusted to 10mg with a 0.18M $FeCl_3$ iron solution.

The theoretical amount of iron absorbed from the meals with and without the treatments in humans was calculated using a set of algorithms previously published by (Hallberg and Hulthen 2000). This algorithm calculates the percentage of iron obtainable from a meal by multiplying the content of each known iron absorption-enhancer or inhibitor within a meal by a numerical value generated from previously published human trials. The effect of NZGLM on iron uptake from an inhibitory meal was calculated with the same values as beef since no specific value has been generated for NZGLM and both enhance iron absorption by a similar magnitude *in vitro* as discussed in Chapter Four. These values are described in Table 9.3.

Table 9.3. Calculated iron bioavailability from test meals.

	% iron absorbed from meal			
	Meal alone	**Meal with AA**	**Meal with egg**	**Meal with beef or NZGLM**
Chowder	2.48	14.89	1.93	3.26
Pasta	1.72	10.31	1.33	2.26
Risotto	2.43	14.56	1.88	3.19

9.6. Study design

Following normal donation procedures, screened participants will attend the Palmerston North NZBS donation centre in the morning after an overnight fast. Participants will donate 1 unit of whole blood. Two 10mL samples of this blood will be stored for measurement of serum ferritin, C-reactive protein and complete blood count analysis. This protocol is illustrated in Figure 9.1. Blood will be taken by accredited NZBS phlebotomists.

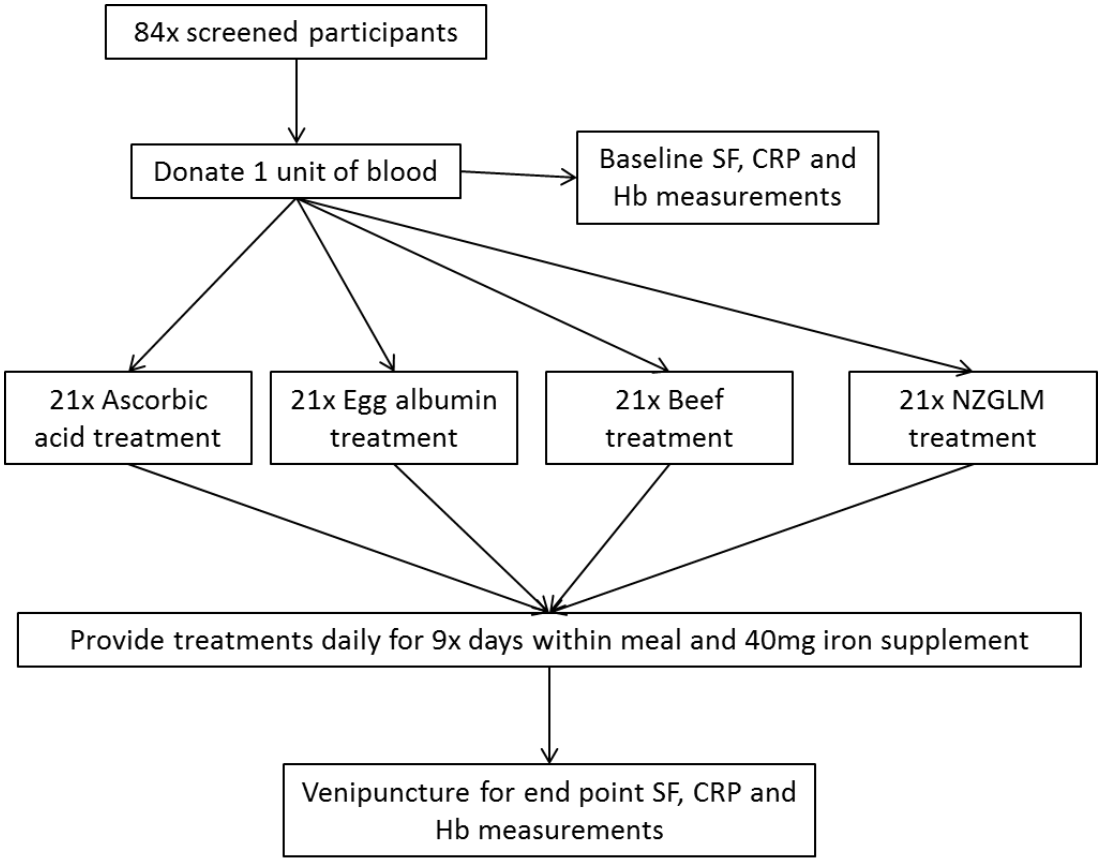

Figure 9.1. Flow chart of experiment protocol.

Participants will be allocated to one of four treatment groups. Meal allocation will be double blinded. The first meal will be consumed with a $FeSO_4$ iron supplement (containing 40mg elemental iron) and 200mL deionised water at the NZBS donation centre immediately after donation. The remaining eight treatment-supplemented meals and iron supplements will be provided daily in a randomised order at noon at the Massey Human Nutrition laboratory. Participants will be required to consume the entire meal. Participants will be asked to not consume any food or beverage apart from deionised water (provided in advance) for four hours prior to the test meal and four hours after the test meal.

Participants will be asked to refrain from consuming meat, pork, chicken, fish and shellfish over the experimental period. Advice on meal preparation for participants who normally eat meat at main meals will be provided. Bread and a selection of spreads will be provided to each participant in order to replace iron-fortified breakfast cereals. Participants will be asked to keep a dietary record diary over this experimental period and to record any gastrointestinal discomfort.

On the morning of day 10 participants will come to the Massey University Human Nutrition laboratory. Two 10mL fasting blood samples will be taken by an accredited phlebotomist for measurement of serum ferritin, C-reactive protein and complete blood count analysis. Weight and height will be measured. Participants will be asked to recall any gastrointestinal discomfort over the trial period.

9.7. Laboratory methods

All serum samples will be stored at -80°C until analysis. Serum samples will be analysed by Canterbury Health Laboratories (Christchurch, NZ). Briefly, serum ferritin will be analysed by Chemiluminescent Microparticle Immunoassay (Abbott i2000 Architect). C-reactive protein will be analysed by and immunoturbidometric method (Abbott c8000).

Whole blood will be submitted immediately to Medlab Central (Palmerston North, NZ) for complete blood counts by Coulter counter (Coulter® Ac·T™ 5diff CP, Beckman Coulter, USA).

9.8. Calculations and statistical analysis

Participants with CRP concentrations exceeding the 10mg/L cut-off will be discontinued in order to identify and eliminate increased serum ferritin concentrations in response to inflammation. Changes in serum ferritin and Hb concentration will be calculated as a percentage within each participant. The change in serum ferritin concentration and haemoglobin will be analysed by 1-way ANOVA after data normalisation if required. LSD analysis will be used to identify differences between treatment groups.

9.9. Dissemination of results

The results gained from this trial will improve our understanding of which dietary factors promote iron absorption from a medium-dose iron supplement combined within a meal, and how we can quickly and safely replete iron stores in blood donors with minimal gastrointestinal distress. Results gained from this trial will be published in an international peer-reviewed journal such as the American Journal of Clinical Nutrition, the British Journal of Nutrition or Transfusion.

Chapter Ten: Discussion, conclusions and future directions

10.1. Introduction:

The experiments described in this thesis sought to characterise the effects of a novel enhancer of iron absorption, New Zealand Green-lipped mussels (NZGLM). These experiments were conducted initially in a well-established experimental model, Caco-2 cells, which have been used extensively for studies on non-haem iron absorption. Caco-2 cells grown as confluent, differentiated monolayers on transwells provide the opportunity to study the mechanisms of iron transport processes at the cellular level and to isolate and manipulate these processes selectively at the apical and basolateral membranes. Thus the effects of dietary components, digested *in vitro*, could be studied directly.

Initial observations in Caco-2 cells were followed by studies in isolated small intestine from mice to investigate the repeatability of the results observed in Caco-2 cells using 'authentic' tissue *in vitro*. These studies also investigated the effects of cooking NZGLM and egg albumin prior to digestion. These *in vitro* experiments were subsequently followed by NZGLM supplementation studies in iron deficient mice to confirm, *in vivo*, the results observed *in vitro*.

Finally, the use of Caco-2 cells in vitro, were used to investigate the role of one of the key components of NZGLM, the high calcium content, on the acute and chronic effects of NZGLM supplementation on iron absorption. This approach was used to differentiate the inhibitory effects of short-term and long-term calcium supplementation, over a range of calcium concentrations, in order to elucidate the mechanisms by which calcium supplementation reduces iron absorption.

10.2. Effect of New Zealand green-lipped mussels on non-haem iron absorption *in vitro*

This series of studies showed that ascorbic acid, NZGLM and beef enhance non-haem iron absorption *in vitro* compared to egg albumin. The enhancing effect was reported in two separate *in vitro* models which simulate nutrient absorption within the small intestine. Both models provided repeatable results.

Ascorbic acid is a potent enhancer of iron dialisability, reduction and absorption compared to egg albumin (Hurrell et al. 1988; Storcksdieck and Hurrell 2007). The enhancing effects of ascorbic acid and the mechanisms involved were discussed in Chapter Two. These enhancing effects of ascorbic acid were observed in both *in vitro* models. The magnitude of iron absorption-enhancement is in agreement with the current literature (Cook and Monsen 1977; Garcia et al. 1996) and demonstrates that both models can be used to isolate enhancers and inhibitors of iron absorption.

Like ascorbic acid, beef has also been previously reported to enhance iron absorption within a meal (Layrisse et al. 1984). Although the enhancing effect is dependent on dose, the enhancing effect of beef is typically less than that of ascorbic acid (Hallberg and Hulthen 2000). This was also observed in the current studies.

NZGLM enhanced iron absorption with a similar magnitude to beef. The enhancing effect was not abated when subjected to heat and demonstrates that NZGLM may be a suitable, heat-stable alternative to ascorbic acid. The potent enhancing effect of NZGLM is of considerable interest because NZGLM also contains a high concentration of iron and PUFAs (Vlieg et al. 1991; Murphy et al. 2002).

NZGLM contains a novel fatty acid profile. The consumption of these fatty acids have been previously associated with therapeutic anti-inflammatory properties (Bui and Bierer 2003) and are proposed to be cardio-protective (Astrup et al. 2011). Repeated PUFA supplementation has been previously reported to enhance the absorption of iron (Seiquer et al. 2002) and calcium (Kruger and Schollum 2005). These observations may suggest that NZGLM supplementation may provide a healthier approach than increased meat consumption for repletion of iron stores.

10.3. Effect of New Zealand green-lipped mussels on non-haem iron absorption iron-deficient mice

The results of the above studies indicate that a single dose of ascorbic acid or NZGLM combined with an iron supplement significantly enhances iron absorption and retention, and improves iron status in iron deficient mice.

The initial results of this study are similar to those reported within Caco-2 cells and isolated sections of mouse small intestine, as discussed in Chapter Four and Chapter Five. These results further suggest that the enhancing effect of NZGLM on

iron absorption is repeatable both *in vitro* and *in vivo* and indicates that consuming an iron supplement with NZGLM may improve iron absorption. This practice may be useful in order to promote iron repletion in human participants.

However the results also indicate that the enhancing effect of NZGLM is abated if the intestinal mucosa is constantly exposed to NZGLM for two weeks prior to the provision of the supplement. The inhibitory effect of prolonged NZGLM supplementation is independent of dietary iron load.

Further investigation into the effect of prolonged NZGLM supplementation in humans is warranted in order to see if daily NZGLM supplementation will reduce mucosal iron absorption. Further investigation into the inhibitory mechanism is also warranted. On the completion of the current study, we hypothesised that the calcium load of NZGLM may be an inhibitory factor.

10.4. Effects of Calcium or NZGLM digestate pre-incubation on enterocyte iron absorption

The aim of this series of studies was to investigate the effect of calcium supplementation on brush border iron transport in Caco-2 cells. The effect of pre-incubating Caco-2 cells with calcium over a range of calcium concentrations and time points was undertaken in order to investigate the minimum concentration and duration of exposure required to influence iron absorption. This was compared to direct co-supplementation of iron and calcium.

The results of this study show that co-supplementation of iron with calcium severely reduces brush border iron transport over a range of iron concentrations. The mechanism is rapidly reversible when calcium is removed from the brush border membrane. The results suggest that calcium inhibits iron absorption by a non-competitive mechanism. These observations have also been previously reported by Shawki and Mackenzie (2010).

The results from these studies also indicate that pre-incubating Caco-2 cells with calcium for three to six hours reduces brush border iron transport. This mechanism is not rapidly reversible when calcium is removed from the brush border membrane which suggests that high luminal calcium concentrations may inhibit iron absorption from subsequent meals.

Like calcium-enriched cell culture media, our results also show that pre-incubating Caco-2 cells with NZGLM digestate-enriched media for three hours significantly reduces iron uptake from a subsequent meal. Furthermore the inhibitory effect of NZGLM on brush border iron transport is of a significantly greater magnitude compared to a calcium-matched control. This suggests that the endogenous calcium content of NZGLM is partly (but not completely) responsible for the inhibitory effect of prolonged NZGLM supplementation on brush border membrane iron transport. Factors other than calcium within NZGLM appear to have an additional inhibitory effect on brush border membrane iron transport.

The results of these studies and those of previous publications (Barton et al. 1983; Thompson et al. 2010) support the observation that calcium inhibits mucosal iron

transfer, possibly by promoting DMT1 internalisation. Since the time required for internalised DMT1 to be re-inserted within the brush border membrane may be a minimum of 8 hours (Johnson et al. 2005), brush border iron transport may be significantly reduced within this time period. This result and interpretation may explain why iron-deficient mice provided with a NZGLM-supplemented diet absorbed less iron than mice maintained on the NZGLM-free diet as observed in Chapter Six.

The current cell culture trials were undertaken under the assumption that dietary calcium is maintained at a relatively consistent concentration within the small intestine for a prolonged period of time. This assumption may be appropriate for laboratory rodents that are fed a calcium-rich meal *ad libitum* and graze continuously but may be less applicable in humans or animals which consume calcium-rich meals periodically. For this reason the effects of repeated NZGLM supplementation on iron repletion in human participants requires further investigation.

From the collection of experiments described in Chapters Four, Five, Six, Seven and Eight the combined results suggest:

- Combining NZGLM with a non-haem iron supplement enhances brush border iron transport when investigated using *in vitro* models or animal trials. The enhancing effect may be associated with the high concentration of myofibrillar proteins and glycosaminoglycans within NZGLM.

- Long-term NZGLM supplementation is associated with a reduction in iron absorption from a single, subsequent iron supplement and a reduction in hepatic iron stores in iron-deficient mice.
- Pre-incubating Caco-2 cells with calcium-enriched media reduces brush border iron transport. This reduction is not rapidly reversible and may compromise iron absorption from subsequent meals or iron supplements.
- The calcium concentration of NZGLM is partly, but not completely responsible for the inhibitory effect of prolonged NZGLM exposure on iron absorption by enterocytes. Other factors such as copper within NZGLM may also contribute to reducing brush border transport.

10.5. Further notes on the proposed human trial

The effects of repeated NZGLM supplementation on iron absorption requires further investigation in human participants in order to recognise the effect of intermittent NZGLM consumption on iron repletion from an iron supplement.

Tagging a meal with radioactive or stable iron isotopes is a common method to investigate iron bioavailability in human participants with a high degree of sensitivity (Roughead et al. 2005; Hurrell et al. 2006). However due to financial limitations during the course of my PhD study a stable isotope study was not feasible, while the use of radioactive isotopes in humans was considered unethical. For this reason an alternative protocol was developed.

Serum ferritin (SF) and haemoglobin (Hb) measurements are currently used to analyse the iron status of human participants in New Zealand. The cost of these measurements is significantly lower than the use of an extrinsic tag; therefore a study protocol was developed in order to investigate the effects of NZGLM on iron absorption by measuring changes in plasma SF. Soluble transferrin receptor concentration was also considered, however again this was not feasible due to financial limitations.

The sensitivity of the SF measurement to changes in iron absorption from dietary sources over a short experimental duration is low. For this reason the use of SF measurements alone to indicate changes in iron bioavailability is not considered appropriate in humans (Hunt and Roughead 1999).

The sensitivity of the SF measurement is significantly enhanced in participants who have undergone phlebotomy and are receiving daily iron supplements (Milne et al. 1990; Røsvik et al. 2010). Under these conditions SF measurements have been used to successfully to investigate the effects of iron supplement bioavailability on iron repletion (Gordeuk et al. 1990).

The human trial protocol described in Chapter Nine was therefore developed to measure the effect of NZGLM when combined with an iron supplement and provided daily to blood donors post-phlebotomy for nine days in order to measure the effect of NZGLM on iron repletion. Iron absorption-enhancers ascorbic acid and beef were also included as positive controls. Egg albumin was included as a negative control.

10.6. Recommended future research

The following recommendations can be suggested for future research:

- In the present series of studies, the effects of NZGLM on iron absorption were undertaken in the absence of phytate and polyphenols. The effect of NZGLM on iron uptake from an inhibitory meal requires further investigation.
- In the present series of studies, the effects of NZGLM on iron absorption were measured using *in vitro* techniques (cell cultures and intestinal sections) and *in vivo* techniques (rodent trials). Further investigation into the effects of single and repeated NZGLM supplementation on iron absorption in human participants is strongly advised.
- Like beef, a single dose of NZGLM enhanced iron absorption compared to egg albumin. However repeated supplementation of NZGLM was associated with reduced brush border iron transport. Although we have reported that the inhibitory effect of repeated NZGLM supplementation is partly associated with its high endogenous calcium concentration compared to other MPF factor sources, the effects of repeated MPF supplementation on brush border iron transport also requires further investigation in human participants.

- The current series of studies suggest that when compared directly by weight, NZGLM enhances iron absorption from a supplement compared to a negative control. NZGLM digestate has a similar enhancing magnitude to beef digestate. Beef has been reported to have a dose-dependent effect on iron absorption (Hallberg and Hulthen 2000). Further investigation into the effect of NZGLM dose on iron absorption is warranted in order to identify the minimum and maximum dose required to enhance iron absorption in human participants.
- The results suggest that fresh, cooked and lyophilised NZGLM enhances iron absorption from a single meal. All treatments were utilised directly after processing in order to minimise the risk of NZGLM oxidation. The high PUFA content of NZGLM significantly increases its susceptibility to oxidative damage (Kosuge and Sugiyama 1989). Oxidative damage of potential MPF factors such as cysteine significantly reduces their ability to enhance iron absorption (Glahn and Van Campen 1997). Further investigation into the effect of NZGLM storage and processing on cysteine oxidation and the ability of stored or processed NZGLM to promote iron absorption is required.

- The studies described in this thesis have shown that prolonged NZGLM supplementation reduces iron transport across the brush border membrane with similar characteristics to calcium-stimulated internalisation of DMT1 (Thompson et al. 2010). Further investigation into the location of DMT1 within the brush border membrane and cytosolic vesicles is required using a combination of confocal microscopy and Western blotting. Further investigation into the DMT1 expression profile after repeated NZGLM supplementation by quantitative PCR would further our understanding on how factors other than calcium and iron within NZGLM influence DMT1 expression.

Appendix 1: Iron free mineral and vitamin mix formulations

Iron-free vitamin mix formulation

Supplier: Unitech Industries, Auckland, New Zealand

Ingredient	**mg/kg diet**
Vitamin A Palmitate 250 Flour	9.6
Vitamin D3 100 GFP	10
Dry vitamin E acetate SD 50	65.67
Vitamin K1 5% SD GFP	20
Vitamin B6 HCL USP	8
Vitamin B12 0.1% SD GFP Pharma	10
D-biotin USP	0.2
Choline Bitatrate	4273
Folic acid USP/PB -UIL/NZDB	0.547
Nicotinamide/Niacinamide USP	15
Calcium D-Pantothenate USP – UIL	17.46
Vitamin B2 BP/USP	7.14
Vitamin B1 HCl USP	5.79
Maltodextran 23-27 DE	5557

Iron-free mineral mix formulation:

Supplier: Crop and Food Research, Palmerston North, New Zealand

Ingredient	**mg/kg diet**
Calcium carbonate	8.4
Potassium (dihydrogen) phosphate	7.5
Calcium hydrogen phosphate	5.7
Magnesium oxide	0.84
Sodium chloride	1.275
Manganous sulphate	0.031
Zinc oxide	0.012
Cupric carbonate	0.012
Sodium selenite	3.3×10^{-4}
Potassium iodate	2.73×10^{-4}
Ammonium molybdate	2.67×10^{-4}
Cellulose	6.23

Appendix 2: Statement of contribution to doctoral thesis containing publications

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Robin John Chrys Stewart

Name/Title of Principal Supervisor: Associate Professor Kevin C Pedley

Name of Published Research Output and full reference:

Stewart, R. J. C., J. Coad, et al. (2012). New Zealand green-lipped mussels (Perna canaliculus) enhance non-haem iron absorption in vitro. British Journal of Nutrition 1(1): 1-4.

In which Chapter is the Published Work: Four

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

Robin John Chrys Stewart — Candidate's Signature

19/11/12 — Date

Kevin Pedley — Principal Supervisor's signature

20/11/12 — Date

GRS Version 3– 16 September 2011

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