USE OF SEMI-ANAEMIC PIGLETS TO MEASURE IRON BIOAVAILABILITY OF MEAT AND MEAT FRACTIONS

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ABSTRACT

Iron deficiency is a major nutritional problem. According to the World Health Organization (WHO), there are approximately 3.7 billion people in the world with iron deficiency (WHO, 2000). Red meat is known to enhance iron absorption due in part to the presence of a meat factor. Domestic pigs (*Sus domesticus*) have been utilised as models for humans in many medical and nutritional studies. The first experiment reported here used 20 4-week-old piglets to compare the bioavailability of iron in diets containing meat (ME), a water-soluble extract of meat (SA), a water-insoluble fraction of meat (SR), and a meat-free control diet (CO). Bioavailability of iron was assessed on the basis of changes in iron-related blood parameters over a 4-week feeding trial. Iron retention in haemoglobin, red blood cell counts (RBC), haemoglobin levels (HGB), haematocrit (HCT), and mean corpuscular volumes (MCV) for ME, SR, and SA was significantly higher ($p < 0.05$) than for CO indicating that all meat fractions enhanced the bioavailability of iron. For some blood parameters the iron status of group SA was significantly lower ($p<0.05$) than for groups ME or SR. It is concluded that the meat-factor is primarily present in the water-insoluble fraction of beef.

In the second experiment nine four weeks old of age female pigs were allocated into three groups of diet treatments, i.e. E200, which contained 48 g/kg live weight$^{0.75}$ of meat extrinsically labelled with Fe^{57}; I200 and I300 , which contained 48 g/kg liveweight$^{0.75}$ and 69 g/kg liveweight$^{0.75}$ of meat intrinsically labelled with $Fe^{57.}$ The isotope labelled diets were fed only on day 0. Afterwards all pigs received the same weaner diet. The pigs were also injected by ^{58}Fe via *intra venous.* E200, I 200, and I 300 were not significantly different ($p > 0.05$) in growth parameters (i.e. average daily gain and average daily feed intake) and in the blood parameters (i.e. white blood cells and RBC, HGB, HCT, MCV, the mean corpuscular hemoglobin and the total iron body in the blood circulation) and iron absorptions. The findings indicate that the different labeling method and different meat levels gave same results.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Iron deficiency is one of the major nutritional problems. According to the World Health Organization (WHO), there are approximately 3.7 billion people with iron deficiency status (WHO, 2000). Iron deficiency, especially iron deficiency anemia conditions, reduces human productivity and increases public health costs enormously.

Domestic pigs (*Sus domesticus*) have been utilised as a model for human in many medical and nutritional studies. It is a mammal, monogastric animal and its physiological/digestive system has many similarities to human.

Red meat is known as a good source of haem iron, which has a higher bioavailability than non-haem irons. A meat factor is involved in this enhanced effect. To find out more about the meat factor, beef meat was separated into a water-soluble fraction and a water-insoluble fraction. In the first experiment, these fractions were fed to semi-anaemic pigs and their iron status monitored

Stable isotopes are preferred to radioisotopes in nutritional studies for ethical and health reasons. In the second experiment, lamb meat was, intrinsically and extrinsically, labelled with iron stable isotopes to study iron absorptions in semi-anaemic pigs. The aim was to determine whether the level of stable isotope in the lamb meat was high enough to detect clear changes in the level in red blood cells following consumption of the meat, before conducting a human study.

Chapter 1. LITERATURE REVIEW

This review starts by considering iron as an essential nutrient for humans from the point of view of the physiological functions of iron and the effects of iron deficiency. The mechanisms of iron absorption from the gastrointestinal tract are reviewed.

The second part of the review covers the literature showing that pigs are a good model for humans. There are many nutritional and medical studies that support this claim, which is based to some extent on the fact that the pigs' digestive system has many similarities to that of the human.

Part three of the review considers the evidence that meat is a particularly good source of iron, with the bioavailability of iron from meat being higher than that from vegetables or inorganic iron because much of meat iron is as haem iron and there is also a meat factor, which enhances iron absorption.

At the end of the review, there is a section on iron isotopes and on methods to analyse the abundance of stable isotopes of iron. Stable isotopes are preferred, in nutritional studies, for ethical and health reasons.

1.1 IRON AS A NUTRIENT

1.1.1 CHANGES IN LIFESTYLE

Our ancestors have consumed meat for several million years. The changes in food sources and lifestyle, which can be determined from fossil evidence, have

led to a decrease in tooth size that suggests diet changes from being based on flesh (large tearing canine tooth) to one based on fruits, vegetables, and cereal grains (grinding teeth) (Underwood, 2001). Lifestyle has changed from a nomadic, travelling, and hunting one to a sedentary one with the cultivation of grains and vegetables.

About 10,000 years ago, cultivated cereal, grains, and vegetables started to become a major part of the human diet (Newman, 1975). As a consequence, intakes of iron absorption inhibitors such as phytates and polyphenols were boosted, while the intake of iron absorption enhancers such as vitamin C and preformed vitamin A shrank (Underwood, 2001).

This inadequate iron uptake is worse in developing countries, because the major iron source is from non-haem iron (MacPail, 2001) compared to developed countries where the level of meat consumption is higher. Hunt (2002) suggested that there should be a national programme to overcome iron intake problems in developing countries.

Some people, due to their beliefs or religion, are vegetarian. Some studies have revealed that vegetarians are more likely to suffer iron and vitamin B12 deficiencies (Iamaroon *et al.,* 2002; Plevrakis, 2002). In a study with Australian women vegetarians, although total daily iron intakes were not significantly different between vegetarians and omnivores, the mean serum ferritin concentrations were significantly lower (P = 0.025) in vegetarians (25.0 +/- 16.2 microg/L) than in omnivores women (45.5 +/- 42.5 microg/L) (Ball and Bartlett, 1999).

1.1.2 FUNCTIONS OF IRON

Iron is the fourth most abundant metal in the earth's crust and the most abundant element in earth as a whole including the inner core, outer core, mantle, and crust (Ehrlich, 2002). The atomic number of iron is 26, and it is mostly found in nature with an atomic weight of 55.85 daltons. Iron is present freely in two forms, ferrous (Fe^{2+}) and ferric (Fe^{3+}).

Over 500 millions years ago when the earth was covered by a low-oxygen environment, iron mostly existed in ferrous form, but at present much iron is in the less soluble oxidized ferric form (Fairweather-Tait, 1995a). This change, along with changing food sources, might explain why so many people (3.7 billion people, WHO, 2000) in the world suffer from iron deficiency, with at least 2 billion of them having iron deficiency anemia (IAEA, 1996).

The chemistry of iron is complex. The dual valency and reactivity with oxygen are the major problems (Fairweather-Tait, 1995b). Ionic iron is an active promoter of free radical reactions. In the tolerance range the body has self-protection mechanisms to prevent body iron reaching toxic levels and to regulate the amount that is safely absorbed (Fairweather-Tait, 1995b).

1.1.2.1 Functional Form of iron

There are at least six major functional forms of iron in the body (Fairweather-Tait, 1995b) :

a) Haemoglobin (Hb)

The molecular weight of Hb, which is made up of four polypeptide chains, is 65,400 and contains approximately 0.34 % iron. Iron is

stabilized in the ferrous state and is ready to be reversibly bound to oxygen (Fairweather-Tait, 1995b).

b) Myoglobin

Myoglobin is known as the red pigment of muscle. The structure is a single peptide homologue of haemoglobin. It provides oxygen storage for muscle contraction processes (Fairweather-Tait, 1995b).

c) Cytochromes

Cytochromes a, b and c are mainly in cells within the cristae of mitochondria. They are involved in electron transport and are essential for the oxidative processes in cellular energy production (Fairweather-Tait, 1995b).

Cytochrome c has a molecular weight of 13,000 and is made up of a single peptide chain and one haem group containing one atom of iron.

Cytochrome P-450, the extra-mitochondrial cytochrome, is located in the liver and is involved in oxidative degradation of drugs and steroids (Fairweather-Tait, 1995b).

d) Iron-sulfur proteins

The iron-sulfur proteins are involved in electron transportation by the reversible changing of ferrous to ferric iron (Beinerd *et al.*, 1997). Examples are NADH dehydrogenase, succinic dehydrogenase, and aconitase (Fairweather-Tait, 1995b).

e) Iron enzymes

The iron-containing enzymes are widely distributed in the body, but are predominantly found in red blood cells and liver. Their major function is to reduce hydrogen peroxide in the body (Fairweather-Tait, 1995b). Some iron-containing enzyme or metalloenzymes are involved in reactions that use O_2 as a substrate such as aromatic amino acid hydrolases and 5-lypogenase (Baynes and Stipanuk, 2000).

f) Lactofferrin

Lactoferrin has a molecular weight of 80,000 and is present in human milk at a concentration of 1 mg iron per 1 ml milk. It binds two atoms of ferric iron per molecule, and is located in neutrophillic granulocytes and on mucosal surfaces (Fairweather-Tait, 1995b).

1.1.2.2 Storage Forms of iron

The storage forms of iron are:

a. **Ferritin**

Ferritin, which is a storage form of iron in cells, is water soluble (Vymazal *et al.*, 2000), and has a spherical protein covering the iron core. High concentrations are found in the liver, spleen, and bone marrow (Worwood, 1995). Ferritin is present at a low concentration in plasma and urine (Worwood, 1986).

Ferritin is an oligomeric protein of 24 identical subunits, each with a

molecular weight of around 20 kD, to form a hollow protein shell with an external diameter of 12 – 13 nm and an inside diameter of 7 – 8 nm. With a molecular weight about 500 kD, it has an ability to store up to 4500 iron atoms in a water-soluble, non-toxic, bioavailable form as ferric hydroxyphosphate micelles (Chricton, 2001).

Two isomers make up human ferritin, the 'L' subunit dominates in liver and spleen ferritin, whilst the 'H' subunits are in the more acidic environments, such as the heart and red cells. The gene for the 'L' subunit is located on chromosome 19q13.3-q13.4 and the gene for the 'H' subunit is on chromosome 11q13 (Worwood, 1990).

Andrews *et al.* (1987) found that electrophoretically fast ferritin had a lower iron content (2000 Fe atoms/molecule) than electrophoretically slow ferritin (3000 Fe atoms/molecule).

b. **Haemosiderin**

Haemosiderin is a degraded and water-insoluble form of ferritin (Vymazal *et al.*, 2000). It is usually found in lysosomes and the amount might be an indicator of increasing iron accumulations (Worwood, 1995; Baynes and Stipanuk, 2000).

1.1.2.3 Transport Form of Iron

Transferrin is the main protein that acts as an iron transporter in the blood and has a half-life of 8 to 12 days in the blood (Wick *et al.*, 1995). Milman *et al.* (2000) found the following correlations between marrow haemosiderin iron and ferritin ($r = 0.64$, $p = 0.0001$), transferrin saturation ($r = 0.56$, $p = 0.001$), and

plasma transferrin ($r = 0.53$, $p = 0.001$).

Haem iron is a known cofactor for nitric oxide synthesis. The neurotransmitters nitric oxide and acetylcholine play a key role in ileal smooth muscle relaxation and contraction. In a study with prairie dogs, Goldblatt *et al.* (2001) found that iron deficiency decreased ileal smooth muscle relaxation.

1.1.3 IRON DEFICIENCY

There is a positive association between iron deficiency and ischaemic stroke or venous thrombosis onset in children between 6 to 18 months of age (Harfield *et al.*, 1997). The iron deficiency status altered motor activities in a study with six-month old infants. Angulo-Kinzler (2002) reported that iron deficient patients were more active and it was assumed that the condition was related to the restless leg syndrome.

Iron deficiency conditions in pregnant women contribute to higher rates of premature delivery and perinatal mortality, weaker immune responses, gastrointestinal abnormalities (Bini *et al.*, 1998; Lee *et al.*, 1998), epidermal appendage changes, impaired thermogenesis, changes in thyroid metabolism and altered catecholamine turnover (Baynes, 1994). An oesophagogastroduodenoscopy study in elderly iron-deficient patients showed that some of the patients had erosive and ulcerative lesions in the stomach, in hiatal hernia, or in the esophagus (van Mook *et al.*, 2001).

Thyroid peroxidase is a haem-containing enzyme that plays an important role in thyroid hormone synthesis. Iron deficiency in male weanling Sprague-Dawley rats reduced thyroid peroxidase activity (Hess *et al.*, 2002).

Iron deficiency might cause neurodegenerative diseases. In a study with rat spinal cords, Cairo *et al.* (2002) found that iron was required for various essential functions including myelinogenesis. The haem synthesis in mitochondria is a process of joining ferrous iron and protoporphyrin IX. Haem deficiency in brain cells decreased mitochondrial complex IV, activated nitric oxide synthase, altered amyloid precursor protein, and corrupted iron and zinc homeostasis (Atamna *et al.*, 2002). Thus, the metabolic consequences were similar to dysfunctional neurons as in Alzheimer disease (Atamna *et al.*, 2002).

In a study with twenty young women, Brutsaert *et al.* (2003) found that the lower-serum-iron concentration group had lower tissue levels of iron, and greater fatigue of the quadriceps muscle during dynamic knee-extension exercises.

1.1.4 ANAEMIA

Iron-deficiency anaemia (IDA) is a global public health problem influencing an estimated 51% of children below 4 years of age in developing countries and 12% in developed countries (Domeloff and Hernell, 2002).

Among some nutrition deficiency cases, iron deficiency anaemia is one of the four most important forms of malnutrition worldwide; the others are protein-energy malnutrition, vitamin A deficiency, and iodine deficiency disorders (Stephenson *et al.*, 2000).

The iron concentration in the human body is normally 40 – 50 mg Fe / kg body weight. The value is influenced by sex, physical activity, health status, and other factors. In adults, the lower limit for blood haemoglobin concentration is about 130 g/l for males and 120 g/L for females (Bothwell *et al.*, 1979; Worwood,

1995). Below those thresholds, the patients are classed as iron deficient. Severe iron deficiency is classed as iron deficiency anaemia.

1.1.4.1 Chlorosis

Anaemia was firstly named as chlorosis, a Greek word meaning green. Ancient Greeks found that muscular weakness in injured war veterans might be cured when they drank water in which a sword had rusted (Hughes, 1977).

Thomas Sydenham (1624-1689), a graduate of Oxford University, and considered as the father of English medicine, showed the value of iron filings or chalybate for the treatment of chlorosis (Valli and Jacobs, 2000). Later, in the 18th century, it was known that iron was one of the elements of blood. From 1832 to 1843 the association between chlorosis and iron was further observed.

1.1.4.2 Regenerative and Non-regenerative Anaemia

Anaemia might be caused by

a) Non-regenerative factors including malnutrition and disease. In this case the production of red blood cells by bone marrow is not adequate to fulfil normal body needs. The forms of red blood cells that are present in the blood circulation often appear normal (Reagan *et al.*, 1998).

b) Regenerative factors. The bone marrow still produces red blood cells and releases them into the blood circulation, but the red blood cells are immature and are known as polychromatophils. These cells are bluish to reddish-blue in colour (Reagan *et al.*, 1998).

Low numbers (up to 1 %) of polychromatophilic erythrocytes are normally found in pigs even when the haematocrit is normal (Jain, 1993a).

Some examples of regenerative and non-regenerative factors that induce anaemia are as follows:

1. Malnutrition, e.g. iron deficiency, folate deficiency, Vitamin A deficiency, Vitamin B_{12} deficiency. Folate and vitamin B_{12} deficiency may generate a megaloblastic anaemia.

2. Parasites, e.g. malaria

3. Blood loss, e.g. enteritis haemmoraghica.

4. Abnormalities in genes, e.g. α-globin gene cluster on chromosome 16 and β-globine gene cluster on chromosome 11 (Pippard, 1995).

5. The defects in ABC7 (ATP-binding cassette) protein affect the iron homeostasis in mitochondria. The condition is related to the inherited disease X-linked sideroblastic anaemia/ataxia (Taketani *et al.*, 2002).

6. Human transferrin G277S mutation (Lee *et al.*, 2001).

7. Abnormalities at any stages of red cell and haemoglobin production or in red cell destruction, i.e. the haemolysis (red cell destruction) rate exceeds the red cell production rate,. For example:

    a. Haemoglobinopathies (e.g. sickle cell disease with most cases being among Africans; HbH disease,; unstable haemoglobine

variants)

b. Red cell membrane defects (e.g. hereditary spherocytosis)

c. Red cell metabolism defects (e.g. glucose-6-phosphate dehydrogenase deficiency)

d. Red cell enzymopathies (inherited abnormalities of red cell enzymes) also known as chronic haemolytic anemia (Luzzatto and Karadimitris, 2000).

e. Infections, such as in *Helicobacter pylori* infection (Sugiyama *et al.*, 2002)

f. or in drug cases.

1.1.5 INDICATORS OF IRON DEFICIENCY

In the 19th century Hoppe-Seylers discovered haemoglobin and demonstrated that the blood pigment contained haematin (iron and protein). In 1880, Growers described a simple method to measure the haemoglobin concentration by comparing the patient's blood colour to a standard haemoglobin colour.

Iron deficiency anaemia patients have low levels of iron and red cells in the blood (Beard *et al.*, 1996; Thorn, 2000; Wallace, 1995). Furthermore, Ioannou *et al.* (2002) suggested a measurement method for serum ferritin levels for anaemic patients with a MCV (mean corpuscular volume) </= 95 fL. If the serum ferritin is under 45 ng/ml, a further endoscopic evaluation is needed.

In the past few years, a soluble transferrin receptor (sTfR) has been used as an

indicator of iron deficiency in humans (Gaillard *et al.*, 2001; R'zik and Beguin, 2001) although in some cases sTfR cannot precisely predict the iron status of patients (Diaz de Domingo *et al.*, 2001). The ratio of sTfR to serum transferrin was also suggested as a means of checking for iron deficiency in doubtful cases (Ruivard *et al.*, 2000; Dimitriou *et al.*, 2000; Raya *et al.*, 2001).

1.1.6 IRON ABSORPTION

Iron absorption is a complicated process with some parts not yet clearly understood. At least three factors influence both the level of haem and non-haem iron absorption. These are the iron content in the diet, the bioavailibility of the iron, and the availability of the iron-transport-related vehicles such as iron transporters and receptors (Baynes and Stipanuk, 2000).

It is more important to know the quality rather than the quantity of iron in diet, as this could be used by governmental institutions to design national food-fortification programmes or to develop bioavailability logarithm equations (Hunt, 1996) for the food industry to help produce iron fortified products.

Iron is mainly absorbed as the Fe^{2+} (ferrous form) in the duodenum and in the upper jejunum (Cavanna *et al.*, 1983, Srai *et al.*, 2002). The iron absorption rate in the duodenum is, however, relatively lower than in the jejunum in pig (Furugouri and Kawabata, 1979). In an *in vitro* rats-intestine experiment with an Ussing chamber, Bougle *et al.* (2002) found that a significant amount of iron was absorbed in the proximal colon as well, although less than in the duodenum.

1.1.6.1 NON-HAEM versus HAEM IRON ABSORPTION

Haem and non-haem iron have different pathways of absorption (Figure 1.1).

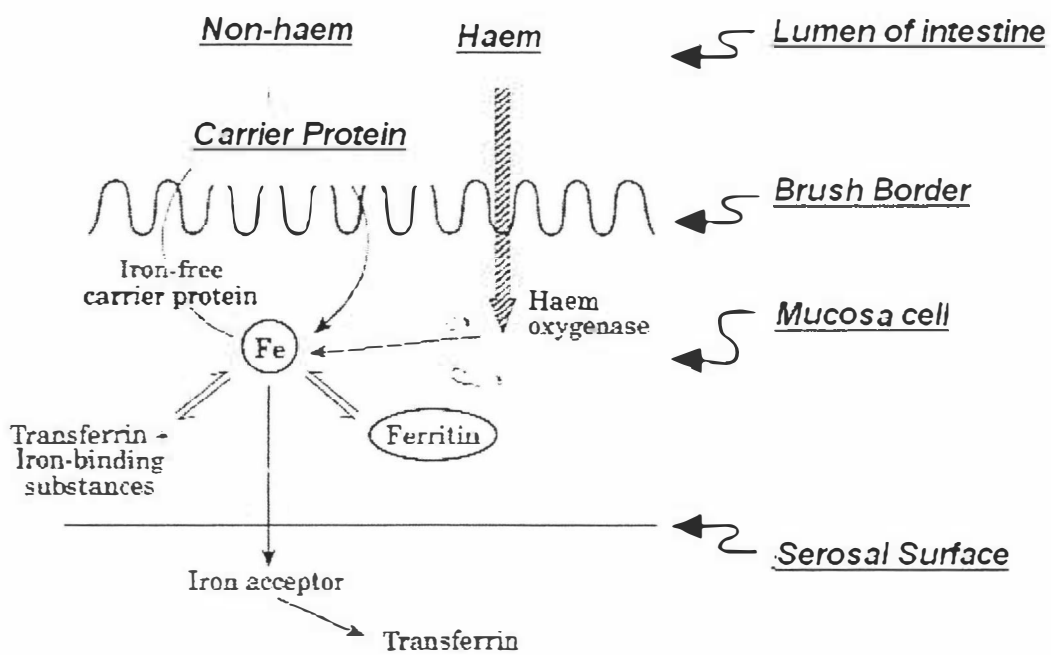

Figure 1.1 The mechanism of iron absorption (adapted from Fairweather-Tait, 1995a).

1.1.6.1.1 Non-haem Iron Absorption

Some factors that influence non-haem-iron absorption are:

- The iron status of subjects. Levels of iron stores have a negative correlation with iron absorption capacity (Baynes *et al.*, 1987). This is a

homeostatic process to help maintain body iron levels.

- The bioavailability and solubility of the iron sources. For instance, Fe-citric acid and Fe-methionine complexes are more available than $FeSO_4.H_2O$ (Zhang *et al.*, 2000).

- The presence of enhancers, such as ascorbic acid (Fairweather-Tait, 1995a; Hallberg *et al.*, 2003; Hashimoto *et al.*, 1992; Davidsson *et al.*, 1998) and certain amino acids, and inhibitors such as phytates, iron-binding polyphenols, calcium, zinc (Herman *et al.,* 2002; Ruz *et al.*, 2002; Chung *et al.*, 2002) or other di-valent ions.

- The presence of meat (Baech, 2002; Baech *et al.*, 2003; South *et al.*, 2000) or animal protein in the diet (Cook and Monsen, 1976).

There are two types of non-haem-iron resources: organic and inorganic. Examples of inorganic iron are ferrous sulphate, ferrous gluconate, ferrous lactate, ferric ammonium citrate, ferric saccharate, ferrous fumarate, ferrous succinate, ferric pyrophosphate, and ferric orthophosphate (Bothwell and MacPhail, 1992).

Organic non-haem iron comes from both plant and animal sources. Dietary iron coming from organic non-haem iron is normally in the trivalent form (Fe^{3+}), which should be converted into the divalent form (Fe^{2+}) before absorption. Vitamin C is an example of a compound that can change Fe^{3+} to Fe^{2+} (Wick *et al.*, 1995; Fairweather-Tait, 1995a). Examples of organic iron are ferritin and haemosiderin (in meat), iron bound to phosphoprotein, phospovitin (in egg yolk), and iron bound to lactoferrin (in milk) (Baynes and Stipanuk, 2000).

1.1.6.1.2 Divalent Ion Minerals and the Transport Agents

For non-haem iron absorption, the process involves several stages. In order to enter cells of the mucosa, Fe^{2+} ions are bound to the di-valent metal transporters 1 (Ma *et al.*, 2002). This explains why other di-valent minerals including Ca^{2+}, Mg^{2+}, and Mn^{2+} become competitors in the iron-transporting process and might reduce iron bioavailibility. Solomon and Jacob (1981) found that inorganic zinc competes with non-haem iron in the absorption process.

The transport agent is also known as MIBP (membrane iron binding protein). The process is followed by the change from appoferritin into ferritin (Wick *et al.*, 1995). Then iron is carried by an intracellular transport agent that passes to the basolateral enterocyte membrane where iron is transferred to transferrin in the portal blood. The transport of iron from intestinal enterocytes into the circulation is carried out by hephaestin (Sakakibara and Aoyama, 2002).

After passing the brush border membrane but before entering into the blood stream, Fe^{2+} is oxidised into Fe^{3+} by endooxidase I (ceruplasmin) (Wick *et al.*, 1995). The change from apotransferin into transferrin follows this process, with the specific binding transport agent, transferrin, Fe^{3+}, passing into the blood plasma (Wick *et al.*, 1995). Non-transferrin-bound iron (NTBI) is also present in the circulation especially in overloaded iron cases (Liu *et al.*, 2003). A study of the absorption of non-organic iron chelated with amino acids has shown that the absorption pathway of iron bis-glycine chelate follows the non-haem iron absorption pathway (Pizzaro *et al.*, 2002).

Hephaestin is an iron-binding protein that, it has been suggested, transports iron from within intestinal enterocytes into the circulation (Sakakibara and Aoyama, 2002).

1.1.6.1.3 Physiological Factors

Some physiological factors that might influence non-haem iron absorption include the following:

-A short-term regulator effect by iron levels in the intestinal mucosal cells (Fairweather-Tait, 1986).

-Long-term regulator effects by serum ferritin levels (Cook *et al.*, 1974; Murray-Kolb *et al.*, 2003) and the rate of erythropoiesis (Bothwell *et al.*, 1979).

-Gastric juice has an effect on the release of non-haem iron from food, but not of haem iron (Fairweather-Tait, 1995b). Slower rates of gastric emptying (longer in the acidic environment) resulted in a higher proportion of iron being solubilized. Patients with partial gastrectomy had an impaired iron absorption. There was no evidence that the emptying rate of the small intestine had any effect on iron absorption (Fairweather-Tait and Wright, 1991).

-Bicarbonate produced in the pancreas promoted the formation of unavailable iron hydroxide polymers. However, pancreatic secretions also play a role in releasing amino acids and polypeptides from food, and these may act as absorption-promoting ligands (Fairweather-Tait,

1995b).

-Bile salts have cholanic ring 7 alpha-OH and/or 12 alpha-OH groups, which can bind Fe^{2+} with high affinity at pre-micellar concentrations and thereby enhance iron absorption (Sanyal *et al.*, 1994).

-Studies in rats have shown that fasting increased the quantity and iron - binding properties of mucus layers, which led to increased iron transport (Quaterman, 1987).

-Mikami *et al.* (2000) found that low non-haem iron concentrations in intestinal mucosa might improve the intestinal iron absorption in the early stage of iron deficiency for rats.

-A study with humans suggested that women in late pregnancy had a higher iron absorption compared to women in early pregnancy or non-pregnant women (Whittaker *et al.*, 2001).

1.1.6.1.4 Haem iron Absorption

Haem iron, which is mainly found in haemoglobin and myoglobin derived from animal tissues, is known to be more bioavailable than non-haem iron (Baynes and Stipanuk, 2000; Ekman and Reizeisntein, 1993).

Some factors that might influence haem-iron absorption include its solubility, the calcium content in diet (Hallberg *et al.*, 1992), and the balance between the

strength of haem-peptides and the polymerization rate of haem (Vaghefi, 2002). Haem-iron is absorbed into the mucosal cell as a porphyrin complex. The iron status of the body might influence haem iron absorption but not as strongly as non-haem-iron absorption.

Approximately 70 % of a specific dose of haemoglobin is converted to haem within 30 minutes of ingestion in a dog model (Baynes and Stipanuk, 2000). Haem fed in a free form is poorly absorbed, probably due to the haem polymerisation (Baynes and Stipanuk, 2000). However, free haem-iron is not present in the normal diet.

Haem enters the intestinal absorptive cells as an intact metalloporphyrin. In the absorptive cells, the porphyrin is separated from iron by haem oxygenase within the cells (Uzel and Conrad, 1998).

Haem binds to a specific protein receptor on the luminal intestinal surface before passing through into the enterocyte via endocytosis (Baynes and Stipanuk, 2000). After haem degradation, the iron enters the iron pool, which is the same pool as for the non-haem iron. The haem iron absorbed increases linearly with increasing iron intake in the physiological range of 0.25 mg to 6 mg (Baynes and Stipanuk, 2000).

1.1.6.2 IRON OVERLOAD

An excessive level of iron in the body might be lethal, but unless iron intakes are excessive the body can coordinate iron absorption and excretion to maintain iron homeostasis (Linder *et al.*, 2003).

In some cases gene-abnormalities influence iron absorption and body iron homoeostasis as well so that iron overload results. For example, HFE, a

haemochromatosis gene named by the nomenclature committee (Baynes and Stipanuk, 2000) and TFR2, a transferrin receptor isoform (Camaschella *et al.*, 2000; Melis *et al.*, 2002), lead to haemochromatosis or overloaded iron.

Studies have shown no correlations between high intakes of haem-iron from meat and risk of mycocardial infarction (Malaviarachchi *et al.*, 2002), colon cancer (Hill, 2002), or breast cancer (Holmes, *et al.*, 2003).

However, a high overdose of iron by injection might be lethal. Tollerz (1965) observed that a dose of 375 mg iron dextrin per kg live weight killed a piglet within 12 hours after *intra muscular* injection. The symptoms of iron poisoning were:

-Initially an increased drowsiness and insipient dyspnoea. The pig was restless and continuously rose to its feet, took a few steps and then lay down again.

-The piglet did not respond immediately when the sow called for a suckling time, whereas the healthy ones ran to find their places at the teats.

-The pig lay in the box with noticeable dyspnoea. If touched, it moved at first, but later it did not respond at all.

1.2 THE PIGLET AS A MODEL FOR HUMANS

The piglet may be a good model for the human being, and especially for infants, as physiologically their digestive system and metabolic processes are similar (Pond and Houpt, 1978).

Piglets are born in an iron-deficient condition. Without an iron supplement the piglet develops iron deficiency within 14 days postpartum (Zimmermann, 1995).

1.2.1 NUTRITIONAL STUDIES

Bertolo *et al.* (1999) used neonatal piglets to study metabolic and physiological changes caused by the route of feeding and the adequacy of amino acid patterns for a paediatric elemental diet. Darragh and Moughan (1998) examined the true (corrected for endogenous amino acid excretions) ileal amino acid digestibility of human milk in 3-week old piglets. Darragh and Moughan (1995) looked at the digestibility of high-quality protein in 3-week old piglets compared to 3-months old male infants. The results showed that the faecal digesitibilities were not different and it was concluded that piglets were a suitable model to study protein digestibility in infants. Borum (1993) studied the use of colostrum in piglets to evaluate parenteral feeding formulas.

Rowan *et al.* (1994) studied the true ileal amino acid digestibility in growing pigs compared to adult humans. The findings showed that there were no differences for true ileal dietary amino acid digestibility except for threonine, phenylalanine, cystine and methionine, and there were no differences between adults and pigs for the ileal digestibility of DM and the faecal digestibility of gross energy.

Innish (1993) utilised piglets to determine omega-6 and omega-3 fatty acid requirements for infants. Darragh and Moughan (1992) determined the amino acid requirements for infants by using piglets. Wykes *et al.* (1991) observed the amino acid metabolism in neonatal piglets as a model for low-birth-weight infants (LBWI) fed parenterally (TPN). Moughan *et al.* (1991) determined the effect of the protein source of a milk formula on the stomach-emptying rate, and the post-prandial changes in gastric pH and milk-clotting enzyme activity. Moughan *et al.* (1992) studied the digestion and absorption of milk-fed

human infants. Baltzell *et al.* (1987) studied the metabolism of carnitine in infants. Harada *et al.* (1999) studied the transport of lactoferrin from the intestinal lumen into the bile by using piglets as a model for infants. Schaafsma and Beelen (1999) compared egg shell powder and purified calcium carbonate as calcium sources for piglets and concluded that chicken eggshell powder was a promising source of calcium for human nutrition.

Some nutritional studies in which the effect of iron on piglet performance has been assessed include the following:

a) In a study with 1144 piglets, Markowska-Daniel *et al.* (2002a) reported that groups with iron treatment had the highest body weight gain and the lowest percentage of death from birth to the fattening stage.

b) In another study with 88 piglets (from 9 litters), groups treated with iron before weaning demonstrated increased leukocyte numbers (Markowska-Daniel *et al.*, 2002b).

c) Piglets treated with an iron injection 3 days after birth had a 21% improvement in both average daily gain and average daily feed intake ($p<0.05$), while a double dose of iron at 3 and 10 days after birth was no better than the single dose (Acda *et al.*, 2002).

d) Yu *et al.* (2000) compared organic and inorganic iron resources for 72 30-day-old piglets and found that the organic iron resource had a better bioavailability. The blood parameters were haematocrit or packed cell volume, haemoglobin concentration, plasma iron and total iron binding capacity in the blood, and haemosiderin and ferritin iron in the liver and spleen.

e) Intoccia *et al.* (1977) assessed the iron bioavailibility of an inorganic iron source (ferrous sulphate) in anaemic piglets before applying the results to human subjects. The piglets were made anaemic by being given just a commercial milk and haemoglobin, and haematocrit were used as the indicators of the iron bioavailibility. It was concluded that haemoglobin and haematocrit were sensitive and reliable indicators to measure iron bioavailability in piglets.

1.2.2 MEDICAL STUDIES

Stepanek *et al.* (1998) used 4 10-week-old piglets to study hypobaric hypoxia and reported that the piglet is an appropriate animal model for the study of high altitude-related diseases in humans. Foster *et al.* (2001) utilised piglets as a model of hypoxia/ischaemia in neuroprotection studies. Schlosser *et al.* (2002) studied meconium aspiration processes by using the piglet as a model in a lung injury study. Berul *et al.* (2001) used piglets as a model for infants to study an invasive cardioverter defibrillator (ICD) implantation for infants with smaller venous capacity.

von der Hardt *et al.* (2002) examined a new ventilation strategy (i.e. an aerosol therapy with perfluorocarbon) in a surfactant-depleted piglet model in proposing to reduce the initial pulmonary inflammatory reaction. Jeng *et al.* (2002) studied effects of partial liquid ventilation with FC-77 in acute lung injury incidents.

Poutahidis *et al.* (2001) utilised piglets to study human *Helicobacter pylori* – associated gastritis. Hoshino *et al.* (1995), and Torres and Ji-Huang (1987) investigated human rotavirus strains by using the piglet as a model. Shu *et al.* (2001) used piglets to study diarrhoea associated with rotavirus and *Escherichia*

coli, one of the major gastrointestinal problems in infants.

Sakamoto *et al.* (2001) examined advantages and disadvantages of near-infrared spectroscopy during cardiopulmonary bypass in a piglet model. Kusuda *et al.* (2000) studied respiratory distress syndrome in a piglet model. Barrington *et al.* (2000) investigated the haemodynamic effects of magnesium sulfate (MgSO4) in a neonatal piglet model of the meconium aspiration syndrome.

In brain studies, because of developmental and morphological similarities between the piglet and the human brain, researchers have utilised piglets as a model. Examples include:

a) Duhaime *et al.* (2000), who studied the mechanical trauma effect of a focal brain injury.

b) Feng *et al.* (2000), who reported that a high-dose of desmethyl tirilazad improved the neurogical function after a hypoxic ischemic brain injury.

c) Undar *et al.* (1999), who examined effects of the perfusion mode on regional and global cerebral, renal, and myocardial blood flow using a neonatal piglet model.

d) Erikson *et al.* (1998), who studied the effects of iron deficiency on regional brain iron, ferritin, transferrin concentrations in 28 day-old piglets.

1.2.3 THE DIGESTIVE SYSTEM

The digestive system of a pig includes the mouth, pharynx, alimentary canal, and several glands. The alimentary canal is made up of the oesophagus, stomach, small intestine, and large intestine. As a monogastric animal, the pig has many similarities to humans, except that the caecum in pig is relatively highly developed.

Digestion might be described as a degradation process, which includes mechanical, chemical, and microbial activities. With respect to mechanical digestion, the pig has the most complete dentition among the domestic animals (Dyce *et al.*, 1996).

The similarities of digestion in the pig and the human include:

- As in the human, saliva in the pig contains a mixture of α-amylase enzymes that are not present in the dog, cat, and horse (Maskell and Johnson, 1993). However, the contribution of saliva amylase is relatively very small compared to pancreatic amylase. In a 5-hour monitoring after food ingestion, Corring (1980) found that the ratio of the total saliva amylase to the total pancreatic amylase was 1:250,000.

- Protein digestion begins in the stomach through gastric proteases and HCl. Pepsins are secreted as inactive pepsinogens and under acidic condition pepsinogens are converted to pepsins by autocatalytic catalysis. Chymosin secreted as prochymosin is unique for suckling pigs (Low, 1990). Chymosin is the most important protease in the immediate post-natal period. Chymosin acts mainly on the casein of milk protein and clots milk without further proteolytic breakdown of the peptide bonds, which allows peptides, such as growth factors and

immunoglobulins in the colostrum, to proceed into the small intestine (Cranwell, 1995). The clotting of milk plays an important role in gastric emptying and stomach development (Yen, 2001).

- Gastric emptying time is affected considerably by various protein sources and methods of processing (Low, 1990). The proteolysis process of bovine milk proteins was slower than for fish proteins, isolated soybean protein, and whey-supplemented milk. Heat-damaged milk protein was less readily digested than undamaged milk protein (Low and Zebrowska, 1989; Low, 1990). The ability of the duodenum and other parts of the small intestine to process chyme or digesta (the stomach contents) controls gastric emptying for humans and pigs (Kutchai, 1998; Bastianelli and Sauvant, 1999).

- The pig, like the human has three major parts of the small intestine (the duodenum, jejunum, and ileum). The small intestine of newborn piglets is 2 to 4 m long. The proportion of duodenum is about 5 percent, which is similar for humans (Kutchai, 1998). In growing pigs the jejunum makes up about 90 percent of the total small intestine length (Schummer *et al.*, 1979). In grown pigs the ileum can be distinguished from the jejunum by its slightly thicker muscular coats and its junction with the large intestine.

  Once the chyme enters the duodenum the pH is raised to outside the pepsin-active pH and into the pH range suitable for the pancreatic enzymes. Digestion in the small intestine includes a luminal phase and a mucosal phase (Herd, 1992).

1.2.4 BLOOD VOLUME

The blood volume of the pig foetus is around 11.7% of body weight (MacDonald *et al.*, 1987). The volume of blood (litres per kilogram live weight) in the first hours *postnatal* is about 9-10% of body weight (Ramirez *et al.*, 1963). After the first few weeks of age, the blood volume becomes approximately 6-8% of body weight (Talbot and Swenson, 1970; Hannon *et al.*, 1990).

1.3 MEAT COMPOSITION

1.3.1 MEAT FRACTIONS

Protein represents about 20 % of muscle or meat weight, with approximately 60 % associated with the myofibrils, 30 % as soluble sarcoplasmic proteins (Greaser, 1986), and about 10 % as connective tissue (Lawrie, 1998).

1.3.1.1 Connective Tissue

The connective tissue surrounding the whole muscle is the epimysium, while muscle-fibre bundles are separated by the perimysium, which contains larger blood vessels and nerves. Inside the perimysium each individual muscle fibre is covered by endomysium (Figure 1.2).

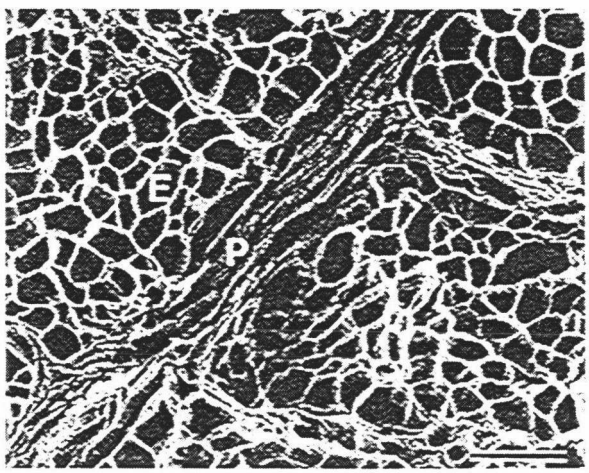

Figure 1.2 A scanning electron micrograph of bovine *semi- tendonosus* muscle immediately post-mortem. The network of endomysial connective tissue (E) and perimysial connective tissue (P) are clearly presented after the muscle fibres have been removed. The bar is 200 μm (Adapted from Nishimura *et al.*, 1996).

Endomysium has the functions of (1) connecting adjacent muscle-fibres, (2) providing a muscle-fibre-capillary connection, and (3) providing networks of collagen intimately associated with the basal laminae of the muscle fibres (Borg and Caulfield, 1980).

The basement membrane that links the collagenous fibres of the endomysium to the muscle cell membrane contains only approximately 40 % collagen (dry weight base), whilst the other components are complex polysaccharides (proteoglycans and glycoprotein) (Bailey,1989). Some functions of the plasma membrane are (1) to form a selective barrier that controls transport of ions in and out of the fibre, (2) to transmit action potentials generated by nerves, and (3) to take a part in the transmission of energy produced by the contractile apparatus within muscle cells (McCormick, 1995).

The solubility of collagen decreases with the increasing age of the animal because collagen becomes more resistant to breakdown with increasing age.

It is not caused by an increase in the number of intermolecular cross-links but by the formation of non-reducible links involving three or more chains.

Long and bar-like tropocollagen molecules build up the collagen fibres. Each tropocollagen molecule is composed of three polypeptide chains (alpha chains) twisted together into triple helix. About 1000 amino acids are in each polypeptide chain (Warriss, 2000).

Collagen is a protein with many hydroxyproline residues, which is relatively uncommon for other proteins (Bowes *et al.*, 1957; Warriss, 2000). The common amino acid sequences of the primary structure are usually, but not always (Warriss, 2000), the repeatition of glycine- proline- hydroxyproline-glycine. The amount of proline and hydroxyproline contributes nearly a quarter of amino acid residues (Warriss, 2000) and determines the thermal stability of the triple helix (Lawrie, 1998). Hydroxyproline might influence the structural stability of the collagen triple helix (Burjanadze and Veis, 1997). By using ion exchange chromatography, the collagen content in food might be estimated by the hydroxyproline concentration (Cardillo *et al.*, 1993).

On heating to 65^0 C the collagen shrinks and above 85^0 C, collagen is converted into soluble gelatin (Lawrie, 1998).

1.3.1.2 The Muscle Fibre

The basic structure of all muscles is the fibre. The size of the fibre (i.e. the diameter and the length) is affected by age, type of muscle, training, species, breed, and sex.

The parallel units (myofibrils), which are the principal components of muscle fibres, have the same diameter irrespective of the size of the fibres (Davies,

1989). Surrounding the fibre is the sarcolemma and endomysium. Within the fibre are myofibrils surrounded by a fluid phase, the sarcoplasm. Within the sarcoplasm are mitochondria (involved in cell respiration and synthesis of ATP), lysosomes (containing catabolic enzymes) (De Duve, 1959), peroxixomes (containing fatty acyl oxidase and catalase) (De Duve and Baudhuin, 1966), and sarcoplasmic lipid bodies (Lawrie, 1998).

Two major contractile proteins in myofibrils, which make up 70 % of myofibril weight, are actin and myosin (Quinn *et al.*, 1980). The myosin molecule consists of a head region joined via a neck to a tail part. The myosin has a length of 520 nm and molecular weight of 520,000 daltons, whilst G-actin has a molecular weight of 42,000 daltons. The thin actin filaments consist of actin together with troponin and tropomyosin (Warriss, 2000). Myosin makes up 50 – 60 % of the total myofibrillar protein or about one third of total muscle fibre protein (Pellet and Young, 1990).

1.3.1.3 Muscle Water

Water makes up approximately 75 % of fresh muscle weight, and is located within the filaments, in the interfilamental spaces, and in extracellular spaces (Huang and Nip, 2001).

The water-holding-capacity (WHC) of muscle tissue is affected by the proportion of water that is either *bound water* (the water that is linked to myofibrillar protein through hydrogen bonding), or *immobilized water* (the capillary water entrapped in myofibrils (Lawrie, 1998). About 80 % of total water in muscle is in the myofibriallar space and about 20 % is in the sarcoplasm (Offer and Knight, 1988).

1.3.1.4 Sarcoplasm

Sarcoplasm is a solution containing salts and proteins, which contributes 5.5 % of total muscle weight and about 30 % of total muscle protein weight (Greaser *et al.*, 1981). The main sources of iron, haemoglobin and myoglobin, are in the water-soluble fraction of muscle. Myoglobin represents 0.2 % of total muscle weight or about 3.6 % of total sarcoplasmic protein weight, whilst haemoglobin and other unspecified extracellular proteins are about 0.6 % of total muscle weight or about 10 % of total sarcoplasmic protein weight (Lawrie, 1975 and Greaser *et al.*, 1981).

South *et al.* (2000) conducted a study to compare the iron bioavailibility of diets containing non-haem iron (ferric citrate), non-haem iron with meat and haem iron (dried animal red blood cells) in iron-deficient weanling pigs. The results showed that haemoglobin repletion efficiencies were 21.8 %, 11.2 %, and 9.1 % for non-haem iron with meat, non-haem iron, and haem iron, respectively. The results showed that the bioavailability of the haem iron itself was very poor, and lower than non-haem iron. These results also showed that there is something inside the meat, often referred to as the "meat factor", which improves iron absorption.

In an *in vitro* study with rat intestinal tissue in an Ussing chamber, Vaghefi *et al.* (2000) showed that purified haem-iron enhanced iron bioavailability provided it still had peptides or amino acids present to maintain its soluble form. It is not clear which meat fractions are involved in enhancing iron absorption.

1.3.2 HAEMOGLOBIN

Approximately 65 % of the 3-5 grammes of adult body iron is in haemoglobin, 10 % in myoglobin, 3 % is in iron containing enzymes, and the remaining iron is stored in ferritin (Baynes and Stipanuk, 2000).

L.J. Henderson, an American biochemist, described haemoglobin as "the second most interesting substance in the world" (Lehmann and Huntsman, 1974). Haemoglobin was the first protein to be crystallized by K.B. Reichert in 1849. The capacity of haemoglobin to bind oxygen is linked to concentrations of carbon dioxide (especially to the protons derived from CO_2). This connection is known as the Bohr effect, (Dickerson and Geis, 1983).

Haemoglobin is made up of globin (protein) and four ferroprotoporphyrin or haem moieties (Figure 1.3). In 1864 George Srokes, an English mathematician and physicist, showed that haemoglobin reversibly binds and releases oxygen (Dickerson and Geis, 1983). Later, it was demonstrated that the ferrous state of iron allows it to carry out this reversible function (Baynes and Stipanuk, 2000). The biosynthesis of haem and its joining with globin takes place in the later stages of red cell development in the bone marrow (Worthington-Roberts and Monsen, 1990).

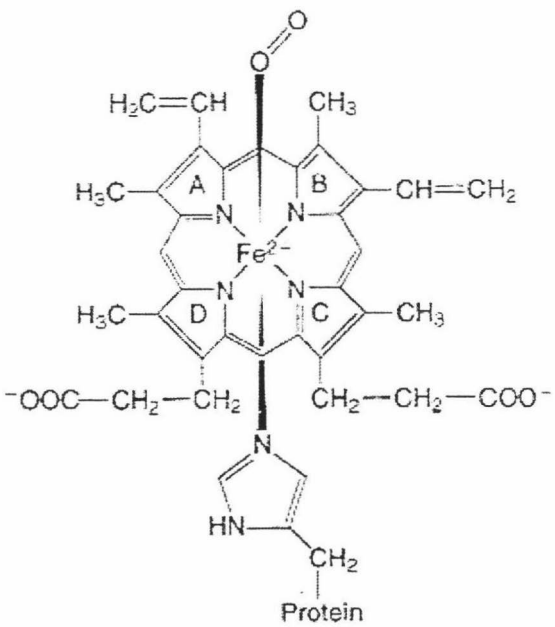

Figure 1.3 The structure of haem (adapted from Baynes and Stipanuk, 2000).

Haem has a diameter of about 12 Å. In comparison, the diameters of water and benzene are about 4 Å, and 6 Å, respectively, and lysine has a length of about 12 Å (Dickerson and Geis, 1983). Haemoglobin is about 64 Å long and myoglobin is about. 44 by 25 Å[1] (Dickerson and Geis, 1983).

[1] One centimetre equals 100 million angstrom units (Å).

Iron comes to the bone marrow in the ferric form with transferrin, and is then reduced to the ferrous form and is incorporated into the proporphyirin structure (Rimington, 1959).

The haem part of haemoglobin consists of iron (Fe^{2+}) at the centre of the structure. Ferroprotopophyrin IX, as an example of haem iron, has six ligands including four ligands connected to nitrogen, one proximal ligand connected to a histidyl residue on a protein and the sixth ligand or distal ligand bound to oxygen (O_2) (Baynes and Stipanuk, 2000).

In anaemic conditions, the amount of haemoglobin decreases with the consequence that the number of red blood cells is lower and the size of red blood cells is smaller. Iron plays an important role in haemoglobin synthesis as the haem part apparently governs ribosomal translation for globin chain synthesis (Thraugh, 1989).

The haemoglobin levels of pregnant women who received iron supplementation were higher than the control group in the study of Milman *et al.*, (2000). In a study with schoolchildren in Mali, the haemoglobin concentrations of the subjects who received iron supplementation were higher than controls (Hall *et al.*, 2002).

Globin chains in haemoglobin differ between animal species (Dickerson and Geis, 1983). At the embryonic stage, most animals only synthesize embryonic ε chains. At the foetal stage, γ chains are synthesized. Two γ chains combined with two ε chains build up to form foetal HgbF (haemoglobin foetal). Pigs and other animals, such as cats, dogs and horses don't have HgbF; the position is replaced by adult Hgb during the foetal period (Kitchen and Brett, 1974).

1.3.3 MYOGLOBIN

Myoglobin is found in muscle, where its main role is as an oxygen storage protein. Oxygen is required for metabolic oxidation processes in muscle and oxymyoglobin can temporarily provide oxygen under anaerobic conditions (Dickerson and Geis, 1983; Andrews and Smith, 2000).

The red colour in meat comes from myoglobin, which has one haem group and one protein chain. At low oxygen concentrations as in muscle, myoglobin has a higher affinity oxygen than haemoglobin (Dickerson and Geis, 1983).

Myoglobin has one polypeptide chain of 153 amino acids with a molecular weight of 17,199 and the haem group has a molecular weight of 616, so the total weight of myoglobin is 17,815 (Dickerson and Geis, 1983).

1.4 BLOOD CHARACTERISTICS

1.4.1 HAEMOPOIESIS

Blood is the smallest tissue in the body, but is the life-sustaining medium of all the approximately 30 to 40 trillion cells in a mammal's body (Gasper, 2000). In the first few weeks of gestation, the main site of the blood cell formation or haemopoiesis is the yolk sac. The liver and spleen are the main organs involved in blood cell formation or haemopoiesis from 6 weeks to 7 months of foetal life, while the bone marrow is the most important site of haemopoiesis from 7 months of foetal life to infancy (Hoffbrand *et al.*, 2001).

Erythropoiesis, or the formation of erythrocytes, is regulated by the hormone erythropoietin. The hormone has a molecular weight of 30,400 and is a heavily glycosylated polypeptide of 165 amino acids. It is normally produced in the

peritubular interstitial cells of the kidney (90 %) and in the liver and other organs (10%) (Hoffbrand *et al.*, 2001). Low oxygen (O_2) tension in tissues of the kidney stimulates the production of this hormone. In anaemia, where the amount of haemoglobin is low, and hence the oxygen supply to the kidney is not sufficient, the production of erythropoietin is stimulated (Hoffbrand *et al.*, 2001).

1.4.2 ERYTHROCYTE

The erythrocyte or red blood cell (RBC) number per unit volume is well known as an indicator of body iron status.The first complete account of red blood cells was made by Antoni van Leeuwenhoek (1632-1723) of Delft, whose hobby was grinding lenses (Haden, 1939). The erythrocyte is a sac of haemoglobin. In humans, one red blood cell contains 250 million haemoglobin molecules, and one haemoglobin molecule can bind four oxygen molecules (Marieb, 2003).

The physiology of erythrocytes varies between species. For instance, erythrocytes from cats and most dogs do not have Na^+/K^+ pump activity (Harvey, 2000a). The membrane permeability to glucose for human erythrocytes is high, but is poor for pig erythrocytes (Harvey, 1997). The membrane permeability for other domestic animals lies between these extremes.

Erythrocytes from adult pigs do not have functional glucose transporters and so have a limited ability to use glucose as an energy source (Harvey, 2002a). Inosine produced in the liver plays a role as an energy alternative for pig erythrocytes (Young *et al.*, 1985).

In a study with 83 human subjects, the ability of erythrocytes to deform was reduced and the fragility was increased in iron deficient subjects (Anderson *et*

al., 2000). The number/volume of erythrocytes was lower in iron deficient human subjects than ini a control group (Reinhard, 1992).

Abnormalities of red blood cells can be analysed by a series of quantitative parameters, including MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), and RDW (red cell distribution width); and qualitative parameters including the size, the shape, and the colour of the cells.

RBC size can be classified as macrocytic, microcytic, normocytic or anisocytic. Normocytosis is the normal size of red blood cells.

Macrocytosis is occurs when RBCs are larger than normal, and may be caused either by a defect in either nuclear maturation or by stimulated erythropoiesis (Turgeon, 1999; Kociba 2000). Defects in nuclear maturation are associated with vitamin B_{12} or folate deficiency (Turgeon, 1999). Macrocitosis is also known as megaloblastic anaemia (Roper *et al.*, 1995).

Microcytosis results from a decrease in haemoglobin synthesis for which iron deficiency is one of several causes; the other causes are impaired globulin synthesis, or a mitochondrial abnormality affecting the synthesis of the haem unit (Turgeon, 1999).

Anisocytosis indicates a high variation in the size of RBCs. It is prominent in severe anaemia.

1.5 IRON ISOTOPES

Isotopes of an element are forms that have the same atomic number but different atomic weights and mass numbers. Isotopes of the same element have

the same number of protons, but different numbers of neutrons (Bernat, 1983).

The atomic number of iron is 26. Its nucleus contains 26 protons and 26 to 35 neutrons, thus the atomic weight of iron is in the range of 52 to 61. Physically, four rings or "shells" surround the nucleus, containing 2, 8, 14, and 2 electrons, respectively (Bernat, 1983).

Six of the ten isotopes of iron are radioactive, i.e.: ^{52}Fe, ^{53}Fe, ^{55}Fe, ^{59}Fe, ^{60}Fe, and ^{61}Fe, with half-lives of 8.4 hours, 9 minutes, 2.6 years, 45.1 days, $3x10^5$ years , and 6.1 minutes, respectively (Bernat, 1983).

Radio-labelled isotopes have been used for many experiments in nutritional research and have helped to solve a lot of nutritional and metabolic mysteries. Their utilisation is, however, becoming restricted for ethical and safety reasons due to the radioactive effects (Fairweather-Tait and Dainty, 2002).

For the last two decades many scientists have gradually switched from radioactive isotopes to stable isotopes. As noted by Hans Blix, the Director General of the International Atomic Energy Agency (IAEA) at that time, isotope techniques provide the best methods for measuring the bioavailability of many important vitamins and minerals (IAEA, 1996).

As a biological tracer, stable isotopes are a useful tool to use in the study of nutrient absorption or bioavailability for animals or humans. Methods have been developed to analyse the level of enrichment of stable isotope in samples of faeces, blood, liver, spleen, and other organs.

Several principal methods are used to analyse the enrichment of stable isotopes in samples. These include neutron activation analyses (Janghorbani and Young, 1980), isotope dilution analyses (Sariego-Muniz *et al.*, 2001),

liquid chromatography (Harrington *et al.*, 2001), and mass spectrometry (Zlotkin *et al.*, 1995).

Mass spectrometry methods include thermal ionization mass spectrometry (Turnlund, 1983), inductively-coupled plasma mass spectrometry (Zlotkin *et al.*, 1995; Kmetov *et al.*, 2003), accelerator mass spectrometry, resonance ionization mass spectrometry (Walczik, 2001), gas chromatography, fast atom bombardment mass spectrometry techniques (Christie *et al.*, 1984; Lehmann *et al.*, 1984; Flory *et al.*, 1993), electron-impact ionization mass spectrometry, and negative thermal ionization techniques (Walczyk and von Blanckenburg, 2002)

1.5.1 NUTRITIONAL STUDIES WITH IRON ISOTOPES

Some studies that have utilised iron isotopes for medical and nutritional investigations are listed and discussed below.

- Turnlund (1983) used an atomic absorption spectrophotometry and thermal ionization, magnetic sector mass spectrometry to analyse the amount of ^{58}Fe tracers in faeces for 15 days following feeding and found that in elderly men the level of iron absorption was 8.7 %.

- Qian *et al.* (2002) conducted an experiment on eight juvenile athletes by utilising ^{58}Fe as the tracer. Iron absorption during the high intensity long-term training was lower than in to the non-training period, i.e. 9.1% +/- 2.8 vs. 11.9% +/- 4.7, respectively.

-The iron isotope ^{56}Fe is relatively more abundant in nature compared to other iron isotopes. The natural quantity and ratio of ^{56}Fe and ^{54}Fe reflect variations between genotypes and

individuals. The average $^{56}Fe/\ ^{54}Fe$ isotope ratio in the blood of males was 0.03 percent lower than that of females (Walczyk and von Blanckenburg, 2002). Probably in the future, these characteristics of iron isotopes could be used as an individual "finger print" in humans. The ratio of $^{56}Fe/\ ^{54}Fe$ is also applied as biosignatures in micro organisms to identify the contribution to sedimentary rocks in modern and ancient earth (Beard *et al.*, 1996).

- In a study utilising double stable-iron-isotope labelling in infants, Kastenmayer *et al.* (1994) found that the absorption of ^{57}Fe and ^{58}Fe were not significantly different. The isotopic enrichment of the iron isotopes in erythrocytes was measured by thermal ionization mass spectrometry.

- Ehrenkranz *et al.* (1992) studied iron absorption in premature infants by using a single dose of about 228 micrograms of ^{58}Fe/kg of body weight (as $FeSO_4$, with 10 mg/kg of vitamin C) administrated by nasogastric tube. The Gastrointestinal absorption of the ^{58}Fe dose as measured by faecal isotope balance was 41.6 +/- 17.6% (mean +/- SD), whilst only 12.0 +/- 9.6% of the ^{58}Fe dose (28.7 +/- 22.3% of the absorbed ^{58}Fe dose) was incorporated into red blood cells (RBCs) on day 15.

- In a study with very low birth weight infants, the percentage of intravenously-infused iron incorporated into haemoglobin on day 15 was 17.8 % (Zlotkin *et al.*, 1995). The stable isotopes were ^{57}Fe and ^{58}Fe.

2 THE MEAT FRACTION EXPERIMENT

2.1 INTRODUCTION

Iron deficiency is one of the major nutritional problems in humans. According to the World Health Organization (WHO), there are approximately 3.7 billion people with iron deficiency status (WHO, 2000). Iron deficiency, especially iron deficiency anemia conditions, reduces human productivity and increase enormously public health costs. Eventually these would impact on the national economic growth.

Many scholars have studied iron and its availability. The domestic piglet (*Sus domesticus*) has been utilised as a model for humans in many medical and nutritional studies. It is a monogastric mammal and its physiological/digestive system has many similarities to that of human beings.

It is well known that haem iron is more available than non-haem iron and the presence of red meat in a diet helps non-haem iron absorption. The objective of this study was to investigate the "meat factor" that assists the iron absorption, by using pigs as a model for humans.

2.2 MATERIALS AND METHODS

2.2.1 MEAT EXTRACTION

A meat extraction process was conducted to separate different meat fractions. Ninety nine % VL (visually lean) bull meat was used in this study. The meat was from an inside cut and primarily semi membranosus muscle. For homogenising purpose, the meat was minced under commercial conditions.

Further steps in the meat extraction process were conducted at the Food Technology Pilot Plant at Massey University. The flow steps are described in Figure 2.1.

Five kg lots of mincemeat were chopped in a bowl cutter machine[1] (Figure A.1 Appendix) for two minutes.

Five kg of chilled water were added and chopping continued for one minute (Figure A.2 Appendix).

Another five kg of chilled water was added and chopping continued for another minute.

The mixture was moved out from the bowl cutter machine into a plastic (non-iron) bucket and stirred with an overhead stirrer[2] with speed no.7 for ten minutes.

Every minute, the mixture was checked and some fibre-like stuff or the collagen attached to the spinner was taken out.

The mixture was left overnight in a pillowcase. The pillowcase had 220 threads per ten cm^2 and was made of 50 percent cotton and 50 percent polyester.

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[1] Talsa Bowl Cutter , type C 35 STP *43. Talsa, Xirivella, Spain.

[2] Heidolph Overhead Stirrer. Heidolph Instruments, GmbH & Co. Germany.

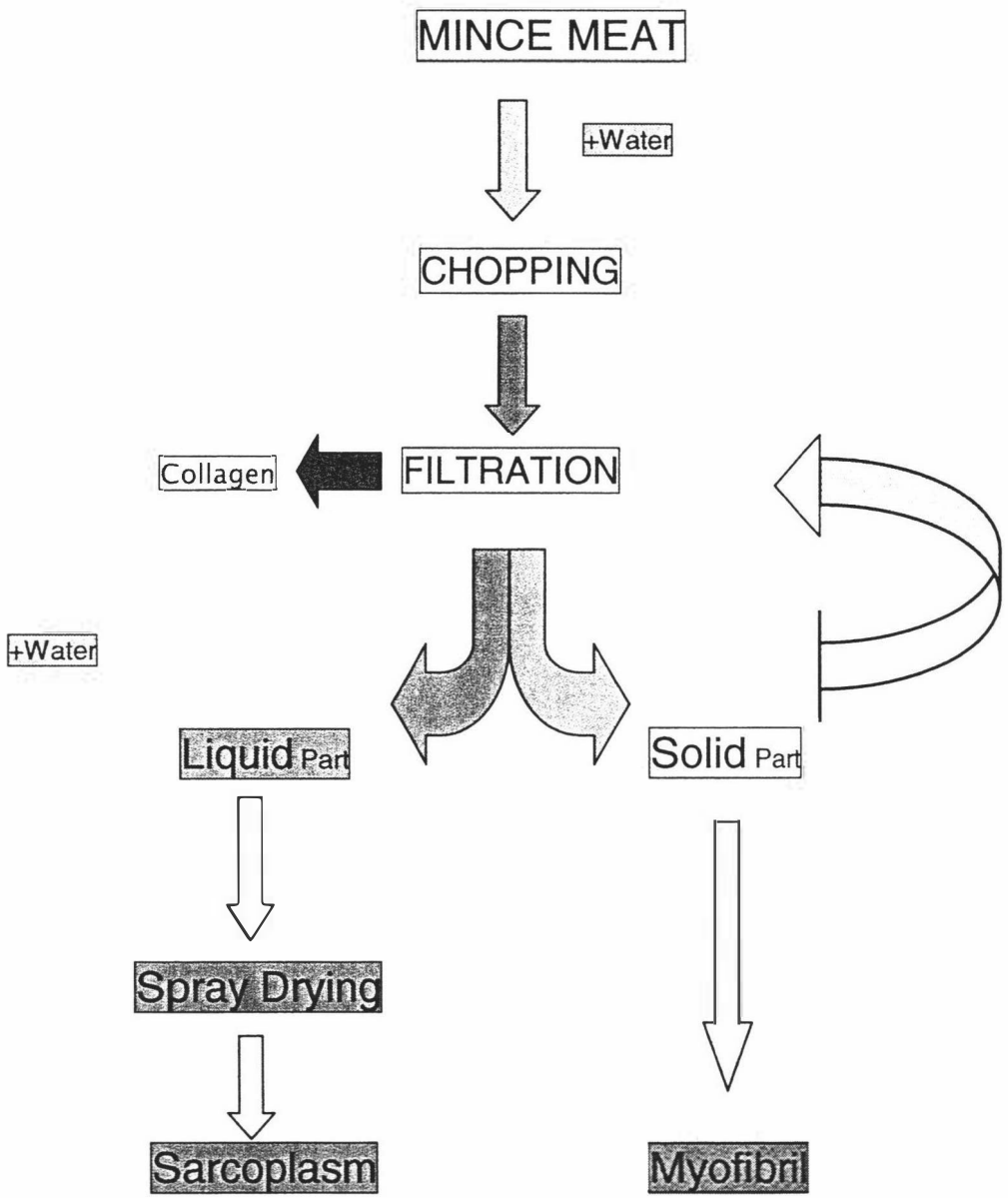

Figure 2.1. The meat extraction steps.

The next morning the liquid part was collected and spray-dried to get the sarcoplasmic fraction (water-soluble), which was used as the iron source for the Sarcoplasmic treatment group.

The solid part left inside the pillowcase was resuspended with chilled water and left another night in the pillowcase. The solid part represented the water-insoluble fraction (myofibril/surimi), which was used as the iron source for the surimi raw group.

2.2.2 EXPERIMENTAL ANIMALS

2.2.2.1 THE ANIMALS

Twenty pigs four weeks of age from 5 litters, comprising 12 females and 8 males, were used in this study. The pigs were all of the same genotype (Large-White x Landrace cross breed) and were obtained randomly from a commercial farm. All procedures involving animals were conducted with the approval of, and under the guidelines established by, the Massey University Animal Ethics Committee.

The pigs received only 60 mg iron intramuscularly injected after birth to create a semi-anaemic condition. Although it was only 30 percent of the normal dose, the iron found in experiment-diets gradually fulfilled the iron requirements. At the end of the trial the pigs that remained in an iron deficient condition received iron injection intramuscularly.

Pigs were reared on the sow with no access to creep feed, weaned at 3 weeks of age, and moved to the metabolic crates.

The experiment was conducted at the Massey University Animal Physiology Unit. The experimental design is random block design. Four pigs were

selected from five litters and randomly placed into one of four groups of treatments. The pigs were individually penned in metabolic crates.

2.2.2.2 METABOLIC CRATES

The size of the metabolic crate was about 60 cm x 160 cm with punched metal floors. Recycled plastics were placed over 40 percent of the punched metal floor area providing a warm and dry lying area. The height of the punched metal floor from the bottom cement floor was about 80 cm. The 250-watt heat lamps were placed approximately 90 cm height from the punched metal floor all the time in each metabolism crate providing additional heating to maintain pigs in their thermo-comfort zones. The room temperatures were recorded twice daily.

Water was provided ad libitum. A push water nipple in each crate was placed on the left side of the crate, which is not too close to the feeder in order to avoid any miscalculation of feed consumption.

2.2.3 EXPERIMENTAL DIETS

The study was conducted over five weeks. The first week was a period of adaptation to the environment, the pigs were only fed commercial milk. The pigs received the experimental diets from the second week to the fifth week of experiment.

The four experimental diet groups were:

1) Control diet (CO) group
2) Meat diet (ME) group
3) Sarcoplamic diet (SA) group
4) Surimi raw diet (SR) group

The ingredients and nutrient contents of all diets are listed in Table 2.1 and Table 2.2. All diets met the nutrient requirement recommended by NRC

(1998) and were balanced in energy, amino acids, mineral, and vitamins.

Table 2.1 The material ingredients for each diet (as fed).

Ingredients	CO[1]	ME	SR	SA
Soybean oil	5	5	5.02	5.01
Cellulose	1.5	1.5	1.5	1.5
Wheat Starch	13.03	13.02	13.08	13.04
Min + Vit. + AA	2.15	2.03	2.15	2.03
Casein	10.012	3.445	5.543	8.514
Meat	0	25	0	0
Water-soluble fraction	0	0	21.9	0
Water-insoluble fraction	0	0	0	1.5
Water	68.3	50	50.8	68.4
Fe Heptasulfate	0.008	0.005	0.007	0.006
Total (%)	100	100	100	100

Where:
- [1] CO= Control Group; ME=Meat Group; SR=Surimi Raw Group; SA=Sarc Group

Table 2.2 The nutrients contents for each diet, meat and meat fractions.

	CO[1]	ME	SR	SA
Dry Matter (g/kg)[2]	276	273	272	276
Total Fe (ppm)[2]	50.5	50.6	50.4	49.1
Digestible Energy (DE) (MJ/kg)[3]	5.86	5.85	5.78	5.78
Protein (g/kg)[3]	88.4	88.3	86.2	85.4
Lysine/DE[3]	1.43	1.32	1.37	1.37

	Meat	WI MF[4]	WS MF[4]
Dry Matter (g/kg)[2]	231	161	923
Total Fe (ppm)[2]	25	8.8	248

Where:
- [1] CO= Control Group; ME=Meat Group; SR=Surimi Raw Group; SA=Sarc Group.
- [2] Analysed.
- [3] Calculated. The other nutrients contents are listed at Table A.3.
- [4] WIMF is water-insoluble meat fraction; WS MF is water-soluble meat fraction.

The pigs of all groups received an equal amount of feed and were fed twice daily (9 a.m. and 4 p.m.). The pigs were initially fed 400 gram per piglet per day.

Due to the increasing nutrient requirements of pigs, the amounts of feed were increased by 50 gram every four days. The diet was given in a liquid form by adding an equal volume of water to the dry diet. The feed consumption was the difference between feed offered and the feed left in the feeder.

2.2.4 BLOOD SAMPLING

Blood samples were taken on days 0, 7, 14, 21, and 28 of the trial. Two 5 ml blood samples (about 2 % of the total blood volume) were taken from each piglet via the vena jugularis by using the vacutainer collection system.

The piglet was inhalationly anaesthetised during the blood sampling with a mixture of Halothane / Fluothane (4 %) and oxygen. After the piglet was unconscious, it was briefly laid in the full recumbent position (Figure A.3 Appendix). Two vacuum blood tubes were used to collect 2 x 5 ml blood samples; one of them was an anticoagulant-containing tube (heparin).

Immediately after the blood collection from each piglet, the anticoagulant-content tube was briefly shaken to ensure the anticoagulant completely mixed with the blood. The blood samples were taken to the Institute of Veterinary, Animal and Biomedical Science laboratory to get the blood parameter data, such as the number of red blood cells (RBC), the number of white blood cell (WBC), the hematocrite concentration (HCT) the hemoglobin concentration (HGB), the mean cell hemoglobin (MCH), the mean cell hemoglobin volume (MCHC), and other information. The instrument was an "Advia 120" electronic cell counting apparatus manufactured by Bayer Corporation Tarrytown, New York.

To prevent the double handling and to reduce stress the pigs were weighed immediately after the blood sampling.

2.2.5 STATISTICAL ANALYSES

The repeated measurement analyses of variance was used to compare the effect of the dietary treatment, the interaction between treatment and time, and time itself. The analyses were performed using the SAS System for Windows, version 8.2 (SAS Institute Inc, Cary, NC, USA).

The statistical model is:

$$Y_{ijk} = \mu + D_i + P_j(D_i) + W_k + D_iW_k + e_{ijk}$$

Where ;

- Y_{ijk} is an observation in the k^{th} week of the j^{th} piglet with the i^{th} diet treatment.
- μ is the general mean.
- D_i is the fixed effect of the i^{th} diet treatment.
- $P_j(D_i)$ is the random effect of the j^{th} piglet within the i^{th} diet treatment.
- W_k is the fixed effect of the k^{th} day time.
- D_iW_k is the interaction effect between the i^{th} diet treatment and the k^{th} day.
- e_{ijk} is a random (residual) error unique to Y_{ijk} assumed to be normally and independently distributed with mean θ and variance $\delta^2 r$.

The data were unbalanced because one piglet from the control group was culled out in the fourth week because of its low feed intake. The least significant different (LSD) test was applied to examine the difference between classes. A P-value of < 0.05 was considered statistically significant for all analyses.

Microsoft Excel 2000 (Microsoft Corporation, USA) was used to draw the graphs.

2.2.5.1 Iron Intake-Hemoglobin Incorporation (IIIHI)

The iron intake-hemoglobin incorporation or the whole body hemoglobin retention efficiency could be calculated if the data of some parameters were provided including the feed intake, the iron content in feed, and the hemoglobin concentration.

The feed iron intake was calculated by an equation as follows:

$$\textbf{FEI = (Feed intake x iron content in feed) x } 10^{-6}$$

(g) (g) (ppm or μg/g)

Where: - FEI is the total iron intake (g)

The body hemoglobin iron was determined by an equation as follows:

HGBFe = Live weight x 0.07 x hemoglobin concentration x 0.00346625

(g) (Kg) (L/Kg) (g/L)

Where:

- HGBFe is the body hemoglobin iron (g)
- 0.07 is the blood volume (7 % of body live weight).
- 0.00346625 is the iron contribution in hemoglobin (0.346625 % by weight). How to get this value will be explained below.

The hemoglobin has a total of four chains, two identical α-chains with 141 amino acids in each chain and a molecular weight of 15,126 each and another two identical groups of amino acids with β-chains of 146 amino acids in each chain with 15,867 molecular weight each. Hemoglobin also contains four haem groups, of which one has an atomic iron at the centre.

The molecular weight of haem is 616. Thus the total molecular weight of hemoglobin is

((2 x 15,126) + (2 x 15,867) + (4 x 616)) = 64,450 daltons

(Dickerson and Geis, 1983).

An atomic iron has an atomic weight of 55.85 daltons. One hemoglobin has four atomic irons, so the total percentage iron in one hemoglobin is

(4 x 55.85)/64,450 x 100 % = 0.346625 %

After FEI and HGBFe are available, the regression analyses for each pig was calculated by a model:

$$\mathbf{HGBFe_i = \alpha + \beta\ FEI_i + e_i}$$

Where:

- $HGBFe_i$ is the body hemoglobin iron (g) for i^{th} pig.
- α is the intercept or the expected value of dependant variable when the FEI-variable is zero;
- β is the slope or the expected changing in the HGBFe-variable given a unit change in the FEI-variable;
- FEI_i is the total iron intake (g) for the i^{th} pig; and
- e_i is a random (residual) error unique to $HGBFe_i$, which is assumed to be normally and independently distributed with mean θ and variance $\delta^2 r$.

A simple anova was applied to analyse the effect of diets to the β or IIHI. The statistical model was:

$$\beta_{ij} = \mu + D_i + e_{ij}$$

Where:

- β_{ij} is the j^{th} slope of the i^{th} Diet treatment,
- μ is the general mean,
- D_i is the fixed effect of the i^{th} diet treatment, and
- e_{ij} is a random (residual) error unique to β_{ij} assumed to be normally and independently distributed with mean θ and variance $\delta^2 r$.

2.3 RESULTS AND DISCUSSIONS

In the last week of the experiment, one pig from the control group was removed from the experiment because of its low feed intake. The pig was given an iron injection and moved to a piggery. The averages room temperatures over the experiment were 20.5^0 (minimum) and 27.6^0 (maximum) Celsius.

2.3.1. Average Daily Gain (ADG)

The least-squares means of ADG (g/day) on day 0 and from day 1 to day 28 for each diet are presented in Table 2.3.

Table 2.3 Least-squares means from days 1 to 28 for the average daily gain (ADG) (g/day), the average daily feed intake (ADFI) (g/day), the feed conversion ratio (FCR) (g/g), and live weight on day 0 (g) for each diet, with residual standard deviations (RSD).

	Parameters			
Diet[1]	LW Day 0 (g)	ADFI (g/day)	ADG (g/day)	FCR
CO	7003[a]	694.6[a]	165.6[a]	5.55
ME	7110[a]	847.1[b]	251.7[b]	4.88
SR	6477[a]	876.7[b]	224.9[b]	6.86
SA	7352[a]	880.9[b]	259.4[b]	6.18
RSD	1157	93.1	62.2	2.77

Where:
- [1] CO= Control Group; ME=Meat Group; SR=Surimi Raw Group; SA=Sarc Group
- [a,b] Values in the same column with a common superscript letter or without a superscript letter are not significantly different ($p>0.05$).

As expected the least-squares means for live weights on day 0 were not significantly different between diets ($p = 0.6819$).

Table 2.3 shows the ADG over the experiment for the ME group was 52.0 percent higher ($p < 0.05$) than the ADG for the CO group. SR was 35.8 percent higher ($p < 0.05$) than CO and SA was 56.6 percent higher ($p < 0.05$) than CO.

The ADG for all treatment groups (ME, SR, and SA) were higher ($p < 0.05$) than the control group. The ADG between the meat and meat-fractions groups were not significantly different ($p > 0.05$) from each other.

In the haemoglobin section (2.3.7), the data shows that the meat and meat fractions groups had a higher haemoglobin level than to the control group, which might indicate that the higher iron absorbed improved the average daily gain. This finding was in close agreement with some other studies with piglets that found iron treatments resulted in a higher ADG (Sarma *et al.*, 2000; Acda *et al.*, 2002).

2.3.2 Average Daily Feed Intake (ADFI)

The average daily feed intake (g/day) is the total feed consumption in gram (as fed basis) divided by the number of days. The least-squares means of ADFI from are listed at Table 2.3.

Average daily feed intake (ADFI) from day 1 to 28 was different between diets ($p=0.018$). The least-squares means of ADFI for ME, SR, and SA were 21.95 %, 26.21 %, and 26.82 % higher ($p < 0.05$) than CO.

The ADFI of non-haem iron group was lower ($p < 0.05$) than in the haem

groups. This might be a consequence of the gastrointestinal effect. The explanation is as follows: the haem iron is, as a cofactor, involved in nitric oxide synthesis, whilst the neurotransmitters nitric oxide and acetylcholine play a key role in ileal smooth muscle relaxation and contraction (Goldblatt *et al.,* 2001). The abnormality in the ileal peristaltic movement might affect the digesta transit time and eventually influence the feed intake (probably through neurotransmitters to the hypothalamus) and appetite.

2.3.3 Feed Conversion Ratio (FCR)

The feed conversion ratio (FCR) is the amount of feed intake (g) divided by the amount of weight gain (g) in the same specific period. The least-squares means of FCR from day 1 to 28 for each diet are listed in Table 2.3. The effects of diets were not significantly different between diets ($p = 0.0824$)

2.3.4 Blood parameters

The statistical significance of group differences in levels for blood parameters (white blood cells, red blood cells, haemoglobin level, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, red distribution width, microcytic, and platelet counts) is shown at Table 2.4.

2.3.5 Red Blood Cells (RBC)

2.3.5.1 Comparisons Between Groups

The red blood cell counts (RBC) or erythrocyte counts were different between diets ($p = 0.0288$). The meat and meat fractions groups had higher counts than the control group. LSMeans for CO, ME, SR, and SA were 4.71, 5.79, 6.08, and 5.62 x 10^{12} cells/L consecutively. ME and SR were 22.93 %

and 29.02 % higher ($p < 0.05$) than CO, respectively. SA was 19.18 % higher than CO, however it was not significantly different ($p > 0.05$) (Figure 2.2).

There was also an increased in RBC over time ($p < 0.0001$) for all diets. The least-squares means across all diets on days 0, 7, 14, 21, and 28 were 3.53, 4.41, 5.81, 6.66, and 7.34 x 10^{12} cells/L, respectively. The residual standard deviation (RSD) was 0.69.

Table 2.4. The statistical significance of the effects of diet, animal, time effect, and their interactions on blood parameters.

Parameter	Diet	Piglet(Diet)	Time	Diet*Time
RBC	*	***	***	NS
HGB	**	***	***	***
HCT	**	***	***	***
MCV	**	***	***	***
MCH	NS	***	***	***
RDW	NS	***	***	NS
Microcytic	NS	***	***	***
PLT	NS	***	***	**
WBC	NS	*	*	NS

Where:

- NS = not significant ($p > 0.05$).
- * = $p < 0.05$.
- ** = $p < 0.01$.
- *** = $p < 0.001$
- WBC is white blood cells; RBC is red blood cells; HGB is haemoglobin; HCT is haematocrit; MCV is mean corpuscular volume; MCH is mean corpuscular haemoglobin; RDW is red distribution width; PLT is platelet. Microcytic is an abnormality in size decreasing of the red blood cells volume

Figure 2.2 shows that from day 7 the RBC value for the meat and meat fractions groups (ME, SR, and SA) was above the control group. This finding indicated that there was a higher iron absorption in the meat and meat

fractions groups, which resulted in an increased erythrocyte production. This result was similar to studies in humans (Engelmann *et al.,* 1998; Baech, 2002; Hallberg *et al.,* 2003) where the addition of meat has increased RBC counts.

This finding supports the presence of a "meat factor" in meat that assists iron absorption, and suggests that there was more "meat factor" in the water-insoluble fraction (SR) than in the water-soluble fraction (SA).

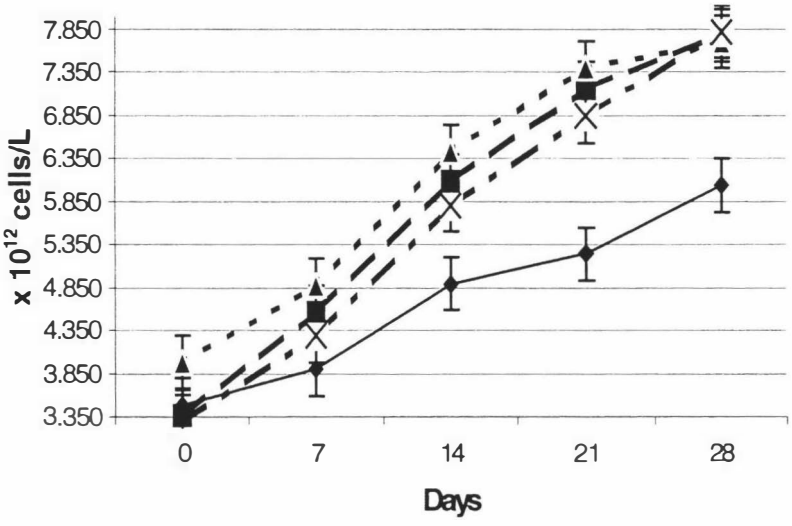

Figure 2.2 Least-squares means (±SE) for red blood cell counts (x 10^{12} cells/L) for each diet on days 0, 7, 14, 21 and 28.

Where :

-♦- = Control Group (CO) ; -■- = Meat Group (ME);

-▲-= Surimi Raw Group (SR) ; -x- = Sarc Group (SA).

2.3.5.2 Relation between RBC and HGB

The red blood cell (RBC) is a sac of haemoglobin; hence the size and number of RBC have a significant influence on blood haemoglobin (HGB) concentration.

A linear regression analysis across all samples showed a positive correlation between RBC and HGB. The linear regression equation for RBC and HGB, where RBC is the independent variable and HGB (g/L) is the dependent variable:

$$\mathbf{HGB = 14.3748 + 12.2060\ RBC}$$

Where: - RBC is the red blood cells count (X 10^{12} cells/L)
- HGB is the haemoglobin concentration (g/L)

The coefficient of determination (R^2) was 0.91 and the slope (β) was significantly different from zero ($p < 0.0001$). The standard error of the slope was 0.39.

2.3.6 Haemoglobin (HGB)

The haemoglobin concentration (g/L)(HGB) was significantly different between diets ($p = 0.0016$). There were significant differences over time ($p < 0.0001$) and a significant interaction between diets and time ($p = 0.0008$). The least-squares means for HGB for each diet are presented at Table 2.5.

On days 0 and 7, the haemoglobin of all groups was not significantly different ($p>0.05$). In the early stages of iron deficiency, where iron intake is inadequate, the iron stores are used to fulfil the iron requirement (Kaneko,

2000). But if it occurs continuously, the iron store could not meet the requirements and caused a decreasing in the haemoglobin production.

At Table 2.5 on day 14, ME and SR were 28.2 and 29.4 % higher ($P<0.05$) than CO. SA was 15.0 % higher than CO, but it was not significantly different ($p > 0.05$). ME and SR were 11.5 and 12.5 % higher ($p < 0.05$) than SA (Figure 2.3).

On day 21, ME, SR, and SA were 49.0, 49.0, and 25.4 % higher ($p < 0.05$) than CO. ME and SR were 18.9 and 18.9 % higher ($p < 0.05$) than SA.

On days 14 and 21, SA was lower ($p < 0.05$) than ME and SR, which were similar to RBC although they were not significantly different ($p > 0.05$).

On day 28, ME, SR, and SA were 47.4, 45.9, and 27.2 % higher ($p < 0.05$) than CO. ME and SR were 15.9 and 14.7 % higher ($p < 0.05$) than SA. This result was not replicated in RBC data where RBC of SA was higher than ME and SR (although they were not significantly different ($p >0.05$). RBC is a sac of haemoglobin; normally a higher HGB is followed by a higher RBC or they should have a positive correlation. These contrary results encourage further study.

Table 2.5 Least-squares means for blood haemoglobin levels (HGB) (g/L), haematocrit (HCT) (L/L), mean corpuscular volume (MCV) (fL), and mean corpuscular haemoglobin (MCH) (pg) for each diet on days 0, 7, 14, 21 and 28, with residual standard deviations (RSD).

		Parameters			
Day	**Diet**[1]	**HGB**	**HCT**	**MCV**	**MCH**
0	CO	55.2^{a}	0.146^{a}	41.26^{ab}	16.14^{a}
	ME	56.0^{a}	0.138^{a}	40.92^{a}	16.80^{a}
	SR	63.6^{a}	0.170^{a}	42.72^{b}	16.16^{a}
	SA	56.8^{a}	0.132^{a}	40.56^{a}	17.26^{a}
7	CO	64.8^{a}	0.148^{a}	38.34^{a}	17.12^{a}
	ME	73.4^{a}	0.184^{ab}	39.78^{a}	16.42^{a}
	SR	72.2^{a}	0.204^{b}	41.52^{b}	14.90^{b}
	SA	68.2^{a}	0.166^{ab}	38.28^{a}	16.08^{ab}
14	CO	70.8^{a}	0.178^{b}	35.80^{a}	14.62^{a}
	ME	90.8^{b}	0.258^{ac}	41.62^{b}	15.26^{a}
	SR	91.6^{b}	0.276^{c}	43.18^{b}	14.28^{a}
	SA	81.4^{a}	0.222^{ab}	38.06^{c}	14.16^{a}
21	CO	71.0^{a}	0.178^{a}	33.72^{a}	13.74^{ab}
	ME	105.8^{b}	0.314^{b}	43.70^{b}	14.84^{a}
	SR	105.8^{b}	0.324^{b}	43.70^{b}	14.28^{ab}
	SA	89.0^{c}	0.256^{c}	37.18^{c}	13.02^{b}
28	CO	81.3^{a}	0.216^{a}	35.17^{a}	13.36^{a}
	ME	119.8^{b}	0.364^{b}	46.78^{b}	15.34^{b}
	SR	118.6^{b}	0.358^{bd}	46.50^{b}	15.40^{b}
	SA	103.4^{c}	0.314^{cd}	39.96^{c}	13.24^{a}
RSD		8.68	0.037	1.47	1.01

Where:
- [1] CO= Control Group; ME=Meat Group; SR=Surimi Raw Group; SA=Sarc Group
- [a,b] Values in the same column and the same day with a common superscript letter are not significantly different ($p>0.05$).

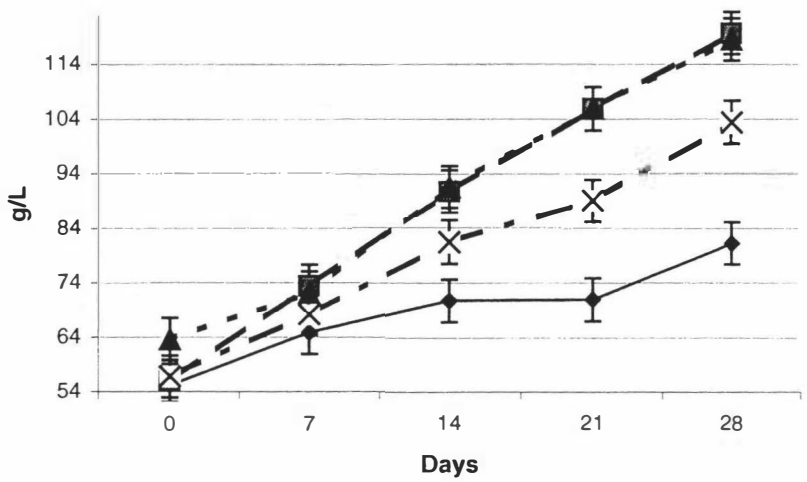

Figure 2.3 Least-squares means (±SE) haemoglobin levels (g/L) for each diet on days 0, 7, 14, 21 and 28.

Where :

-♦- = Control Group (CO) ; -■- = Meat Group (ME);
-▲-= Surimi Raw Group (SR) ; -x- = Sarc Group (SA).

- The RSD (residual standard deviation) was 8.68.

From day 14 to 28, the haemoglobin level for the meat group was higher ($p < 0.05$) than the control group (CO). This finding is in close agreement with some studies in humans (Hallberg *et al.*, 2003; Hazell *et al.*, 1982) and in pigs (South *et al.,* 2000) that found meat additions increased haemoglobin level compared to control.

2.3.7 Haematocrit (HCT)

Haematocrit (HCT) or packed red blood cell volume or packed cell volume

(PCV) is the percentage of red blood cells in whole blood. Hematocrit is a late stage indicator of iron deficiency. Patients with a tendency to iron deficiency anemia have a lower hematocrit due to a decrease in red blood cell production and haemoglobin concentration (MMWR, 1998).

The least-squares means of PCV for each diet on day 0, 7, 14, 21, and 28 are presented in Table 2.5 and the graphs is illustrated at Fig. 2.4.

On day 0, there were no significant differences ($p>0.05$) among the diet groups. On day 7, ME was not significantly different ($p > 0.05$) from the other groups. SR was 37.8 % higher ($p <0.05$) than CO.

From day 0 to day 7, the PCV of all groups was increased, but ME had the highest increasing by 33.3 % compared to 1.4 % (CO), 20.0 % (SR) and 25.8 % (SA).

On day 14, CO was 31.0 and 35.5 % lower ($p < 0.05$) than ME and SR, respectively. SA was 19.6 % lower ($p < 0.05$) than SR.

On day 21, HCT for the meat and meat fractions groups (ME, SR, and SA) were 76.4, 82.0, 43.8 %, respectively higher ($p < 0.05$) than the control group.

HCT of CO, from day 14 to 21 did not change, which it might be an indicator that the iron uptake was enough only to maintain RBC level without any expansion of production. The inferiority of the control group was obvious on days 21 and 28, where CO was lower ($p < 0.05$) than ME , SR , and SA. On day 28, ME was 15.9 % higher ($p < 0.05$) than SA.

Fig 2.4 demonstrates that initially ME was at the lowest position but after 28

days of receiving the meat diet, eventually ME achieved the highest position. The overall changing of HCT for all groups from day 0 to 28 was 0.070, 0.226, 0.188, and 0.182 (L/L) for CO, ME, SR, and SA, respectively. ME achieved the highest increase ($p<0.05$) among the groups.

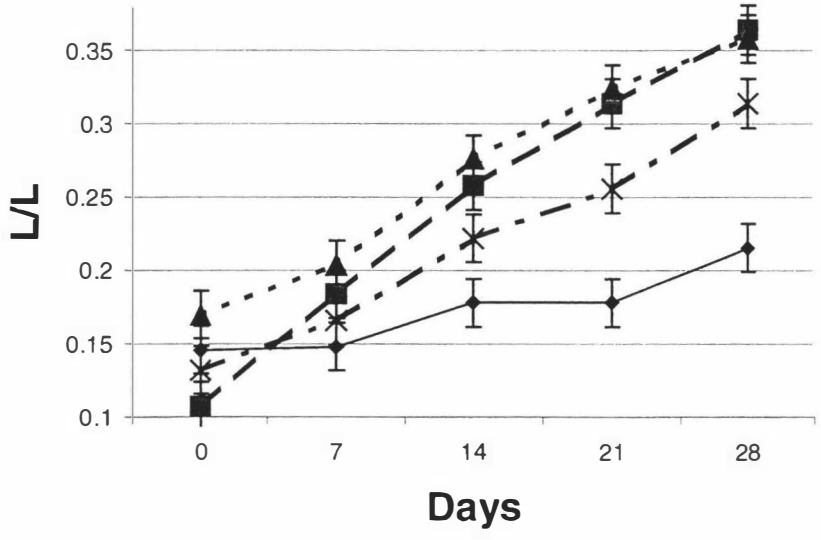

Figure 2.4 Least-squares means (± SE) hematocrit levels (L/L) for each diet on days 0, 7, 14, 21 and 28.

Where :

-♦- = Control Group (CO) ; -■- = Meat Group (ME);

-▲-= Surimi Raw Group (SR) ; -x- = Sarc Group (SA).

This result shows that the least-squares means of HCT from day 0 to day 28, which represent the percentage of red blood cells volume of the meat and meat fractions groups (ME, SR, and SA), were 45.3 %, 53.8 %, and 26.0 %, respectively, higher than the control group. It could be postulated that meat and meat fractions enhanced iron absorptions. The findings are close to other comparable values reported by South *et al.* (2000), who found the addition of 100 g/kg-diet of dried beef meat increased HCT by 19.4 % in pigs.

2.3.7.1 Relationship between HCT and HGB

HCT is the percentage of blood volume occupied by RBC, whilst RBC is a sac of haemoglobin, so the more haemoglobin content in RBC, the bigger they are and the higher the percentage of HCT should be. There was a positive correlation between the haemoglobin level and the hematocrit. R^2 = 0.93, the standard error for the slope was 0.0001 and the slope (β) was different from zero ($p < 0.0001$), the regression equation was:

$$\mathbf{HCT = - 0.0869 + 0.0038\ HGB}$$

Where: - HCT is the hematocrit (%)
- HGB is the haemoglobin level (g/L)

2.3.8 Iron Intake-Haemoglobin Incorporation (IIIHI)

The iron intake-haemoglobin incorporation (IIHI) is probably the only true method for determining the iron bioavailability, because it is a direct assessment of the iron utilisation (Fairweather-Tait, 1995a).

The iron incorporation into haemoglobin or iron intake-haemoglobin incorporation (IIHI) is simply defined as a percentage of iron intake, incorporated into haemoglobin. The total iron intake and the body haemoglobin iron (HgbFe) for each individual pig are listed in Table A.1 (Appendix), whilst the individual IIHI are showed in Table A.2 (Appendix).

The IIHI values for each dietary group are presented in Table 2.6.

Table 2.6 Least-squares means for iron intake-haemoglobin incorporation (IIHI) for each diet from days 0 to 28, with a residual standard deviations (RSD).

Diet[1]	IIHI (%)
CO	14.15[a]
ME	25.31[b]
SR	21.98[b]
SA	21.58[b]
RSD	3.45

Where:
- [1] CO= Control Group; ME=Meat Group; SR=Surimi Raw Group; SA=Sarc Group
- [a,b] Values in the same column with a common superscript letter are not significantly different ($p>0.05$).

The values in Table 2.6 were calculated by multiplying the slope of the regression equation (β) by 100 %.

IIHI of all meat and meat fractions groups, i.e. ME, SR, and SA were higher ($p < 0.05$) than the control group. IIHI for ME, SR, and SA were **78.9 %, 55.3 %,** and **52.5 %** respectively higher than CO. This finding provides more evidence that a meat factor in meat or meat fraction is involved in the iron absorption process.

2.3.9 Mean Corpuscular Volume (MCV)

MCV is an average volume of a single cell of the red blood cells measured in femtoliters[1] (Harvey, 2000b). MCV are normally used as a morphology indicator of anemia, because the value describes the normality size of the red blood cell.

MCV were different between diets ($p = 0.0049$) and there was a significant interaction ($p < 0.0001$) between diet and time. The least-squares means of MCV for each diet are listed in Table 2.5 and the graph is illustrated in Figure 2.5.

On day 0, CO was not significantly different ($p > 0.05$) from all other diets. SR was higher ($p < 0.05$) than SA and ME. On day 7, CO was not significantly different ($p > 0.05$) to ME and SA, but lower ($p < 0.05$) than SR. SR was higher ($p < 0.05$) to SA and ME.

MCV of all groups dropped in the first week of experiment (from day 0 to day 7). This is comparable to MCV in human, which gradually decreased during the first sixth months of life then increased during childhood to adult stage of life (Osky, 1993).

[1] one femtoliter (fL) equals to 10^{-15} liter.

HGB of all diets groups were increased. RBCs of all groups were increased as well, i.e. from 3.482 to 3.896 x 10^{12} cells/L (CO); 3.362 to 4.558 x 10^{12} cells/L (ME); 3.978 to 4.876 x 10^{12} cells/L (SR); 3.296 to 4.292 x 10^{12} cells/L (SA). From these facts, it might be concluded that this period was the time of maturing processes for red blood cells (RBC count increased but the size decreased).

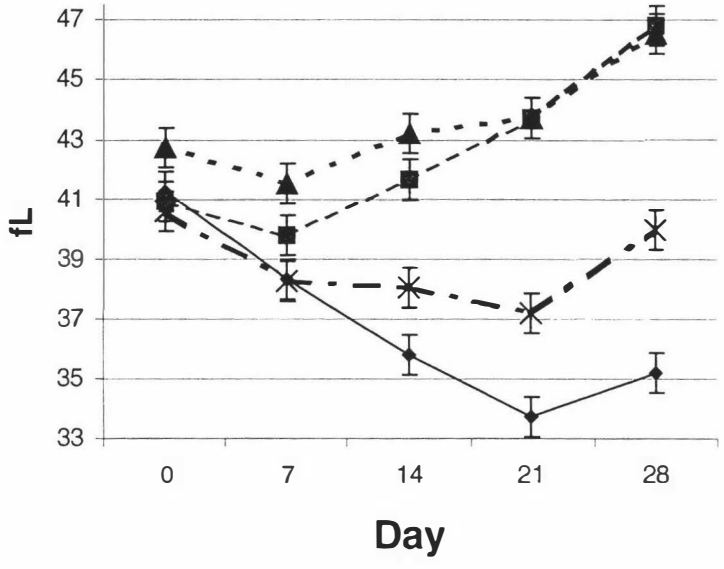

Figure 2.5 Least-squares means (± SE) of mean corpuscular volume (fL) for each diet on day 0, 7, 14, 21 and 28.

Where:
-♦- = Control Group (CO) ; -■- = Meat Group (ME);
-▲-= Surimi Raw Group (SR) ; -x- = Sarc Group (SA).

Because the development process of red blood cells (from haemocytoblast (stem cells) to mature RBC) in humans takes 3 to 5 days (Marieb, 2003), the

result showed that the process in pigs might take longer than in human.

On day 14, MCV of ME and SR were raised, but MCV of CO continued to fall, whilst MCV of SA decreased a little. MCV of the non-haem group (CO) was lower than the haem groups (ME, SR, and SA). The inadequate iron uptake in the non-haem group (CO) obviously caused insufficient iron precursor to synthesize haemoglobin. The red blood cells, in turn, had a smaller size and number. SA was lower ($p < 0.05$) than ME and SR.

On day 21, the non-haem group (CO) and the sarc group continued to decline, whilst the meat and surimi raw group was increased. CO was lower ($p < 0.05$) than ME, SR, and SA. SA was lower ($p < 0.05$) than ME and SR.

On day 28, all groups increased and the results were similar to day 21, i.e. ME, SR, and SA were 33.0, 33.2, and 13.6 %, respectively higher ($p <0.05$) than CO. ME and SR were 17.6 and 16.4 %, respectively higher ($p <0.05$) than CO.

The lowest points of MCV for CO and SA were at day 21, then they bounced back on the next days (Fig. 2.5), but on the final (day 28) CO and SA were still lower ($p < 0.05$) than ME and SR.

Overall from day 14 to day 28, the mean corpuscular volume for the haem iron diet groups (ME, SR, and SA) were higher ($p < 0.05$) than the non-haem iron group. This gave further evidence that the "meat factor" assists iron absorption.

2.3.10 Mean Corpuscular Haemoglobin (MCH)

MCH is derived by dividing HGB (g/L) by RBC ($x10^{12}$ cells/L), which means

the mean haemoglobin weight in a red blood cell . The effect of diets on MCH was not significant ($p = 0.3692$). However, there was an interaction between diet and time ($p = 0.0019$). The detail of MCH on day 0, 7, 14, 21, and 28 are listed in Table 2.5, which shows that the effects of diets were not consistent over time.

On day 0, there were no significant differences between diets ($p > 0.05$). On day 7, ME were lower ($p < 0.05$) than CO and SR, but on day 14 they were all not significantly different ($p > 0.05$). On day 21, only ME was higher ($p < 0.05$) than SA, whilst the other comparisons were not significantly different ($p > 0.05$). On day 28, ME and SR were higher ($p <0.05$) than CO and SA.

2.3.11 Red Distribution Width (RDW)

RDW is the expression of the coefficient variation (CV) of the RBC volume distribution calculated directly from the histogram. The calculation only takes the middle area and excludes both extreme ends (left and right sides). In humans, iron deficiency anemia patients have indicators of higher RDW but lower MCV, whilst megaloblastic anemia, folate and vitamin B_{12} deficiency pattern have higher RDW and higher MCV (Turgeon, 1999).

The least-squares means of red distribution width for all diets are presented in Table 2.7.

Table 2.7 Least-squares means of RDW for all diets on days 0, 7, 14, 21 and 28, with residual standard deviation (RSD).

Time	Least-Squares Means
Day 0	35.17[a]
Day 7	34.01[b]
Day 14	31.22[c]
Day 21	28.55[d]
Day 28	27.72[d]
RSD[1]	1.61

Where :

- [1]RSD is residual standard deviation.
- [a,b] Values in the same column with a common superscript letter are not significantly different (p>0.05).

Table 2.7 shows that RDW were declining over time and that they were different ($p < 0.0001$). Day 0 was higher ($p < 0.05$) than day 7, 14, 21, and 28.

Day 7 was higher ($p < 0.05$) than days 14, 21, and 28. Day 14 was higher

($p < 0.05$) than days 21 and 28, whilst day 21 was not significantly different ($p > 0.05$) from day 28.

RDW on day 28 was 21.2 % lower ($p < 0.05$) than on day 0. There were differences effects between piglets within diet ($p < 0.0001$), which described the high variation of RDW between individual pigs. This shows that RDW is not appropriate as an indicator of iron deficiency anemia in piglets. The findings were similar to those of Piedras *et al.* (1993) who reported that RDW is not appropriate for identifying iron deficiency in non-anaemic human female.

2.3.12 Microcytic

Microcytosis is an abnormality in size, a decrease in red blood cell volume, i.e. smaller than 30 fL. The microcytosis might be happening only in the small groups of red blood cells or in general. If the microcytosis occurs in general it will reduce MCV (Bain, 1995).

The value of microcytic could be used as an indicator of iron deficiency anemia (Watson and Canfield, 2000). In the early stages of iron deficiency anemia, the number of microcytic cells is often too small to affect the value of all RBC, such as MCV and MCHC (Tvedtan and Weiss, 2000), because they appeared as normocytic and normochromic until reticulocytes appear in blood circulation. Reticulocytosis is initially monitored two to four days after the insufficient intake and reaches peaks at four to seven days (Fernandez and Grindem, 2000). Reticulocytes are immature red blood cells. Two types of reticulocytes are known: aggregate and punctate. Aggregate reticulocytes are larger cells with coarsely clumped reticulum, whilst punctate reticulocytes are the more mature RBC, which contains dots and granules of residual RNA (Jain, 1993b).

There was no difference between diets ($p = 0.0684$), but the interaction between diet and time was different ($p < 0.0001$). The least-squares means of microcytic percentage for each diet are listed at Table 2.8.

On day 0, CO was not significantly different ($p > 0.05$) from ME, SR, and SA. However, SR was lower ($p < 0.05$) than ME and SA.

Table 2.8 Least-square means of microcytic (%) and platelet counts (PLT) (x 10^9 cells/L) for each diet on day 0, 7, 14, 21 and 28, with residual standard deviations (RSD).

		Parameters	
Day	**Diet**[1]	**Microcytic**	**PLT**
0	CO	22.84^{ab}	714.8^{a}
	ME	25.48^{b}	623.0^{a}
	SR	18.90^{a}	964.0^{b}
	SA	24.88^{b}	971.8^{b}
7	CO	29.02^{b}	1043.4^{a}
	ME	26.24^{b}	846.2^{b}
	SR	20.74^{a}	854.4^{ab}
	SA	30.28^{b}	874.8^{ab}
14	CO	33.90^{c}	1042.4^{a}
	ME	22.62^{b}	763.0^{b}
	SR	16.12^{a}	740.2^{b}
	SA	27.24^{b}	830.6^{b}
21	CO	38.34^{a}	872.6^{a}
	ME	16.30^{b}	565.0^{b}
	SR	13.22^{b}	715.8^{ab}
	SA	25.88^{c}	871.4^{a}
28	CO	32.83^{a}	941.4^{a}
	ME	11.58^{b}	422.4^{b}
	SR	10.36^{b}	530.2^{b}
	SA	19.76^{c}	750.4^{a}
RSD		4.12	152.0

Where:
- [1]CO = Control Group; ME = Meat Group; SR = Surimi Raw Group; SA = Sarc Group
- [a,b] Values in the same column and the same day with a common superscript letter are not significantly different ($p>0.05$).

On day 7, SR was lower ($p < 0.05$) than CO, ME, and SA. On day 14, CO was higher ($p < 0.05$) to ME, SR, and SA. As in days 0 and 7, SR was lower ($p < 0.05$) than ME and SA.

The percentage of microcytic on day 14 showed that within 14 days, the insufficient iron intake in the CO group manifested in reduced the RBC size. This was also shown in the mean corpuscular volume (MCV) data at Table 2.5 that MCV for CO was lower ($p < 0.05$) than all other diets. At this stage the microcytosis happened generally.

On day 21, the microcytic percentage for CO was higher ($p < 0.05$) than ME, SR, and SA. The microcytic percentage for CO was more than double than microcytosis percentage for ME and SR. On the other hand the microcytic percentage for SA was higher ($p < 0.05$) than ME and SR.

On day 28, CO was higher to ME, SR, and SA. Again, SA was higher to ME and SR.

The best progress in reducing the microcytic percentage from day 0 to day 28 had been achieved by ME, i.e. 25.48 % - 11.58 % = 13. 90 % followed by SR (8.84 %), SA (5.12 %). In contrast the microcytic percentage for CO was increased by 9.99 %.

The inadequacy of iron absorption affected the synthesis of haemoglobin and red blood cells in the body. This was shown in the microcytic percentage for the control group.

2.3.13 Platelet

The morphology of pig platelets is similar to those of other species. They are variable in shape and are generally small (1 to 3 μm in diameter) (Evans, 1994).

The effect of diets on PLT was not significant ($p = 0.2509$). However, there was a significant interaction between diet and time ($p = 0.0016$). The least-squares means of PLT for each diet are listed at Table 2.8.

On day 0, PLT from SR and SA were higher ($p < 0.05$) than CO and ME. After seven days of receiving the experimental diets, SR and SA were not significantly different ($p > 0.05$) to CO and ME. But CO was higher ($p < 0.05$) than ME. The inadequate iron absorption to meet iron in the control group had shown the effect on PLT on day 7.

On day 14, CO was higher than ME, SR, and SA. From day 7 to day 14, all groups decreased but CO had a smaller decrease among the others (only 1.0×10^9 cells/L).

On day 21, CO and ME decreased a massive amount, i.e. 169.8 and 198 ($\times 10^9$ cells/L). SR only decreased by 24.4 ($\times 10^9$ cells/L), whilst SA increased by 40.8 ($\times 10^9$ cells/L). CO and SA were higher ($p<0.05$) than ME.

On day 28, PLT for CO increased by 68.8 ($\times 10^9$ cells/L), whilst the other groups decreased to 142.6, 185.6, and 121.0 ($\times 10^9$ cells/L) for SR, SA, and ME, respectively. PLT of CO and SA were higher ($p < 0.05$) than ME and SR.

Overall, the change in PLT from day 0 to day 28 for each group as follows:

an increasing of 226.6 (x 10^9 cells/L) for CO, a decrease of 200.6 for ME, a decreasing of 433.8 for SR, and a decrease of 221.4 for SA.

Because SA was already higher ($p<0.05$) than the rest of the groups on day 0, the decreasing from 971.8 to 750.4 = 221.4 could be acceptable. The biggest decrease was in SR, i.e. 433.8, which probably might be an indicator that the meat extraction process had separated some factors involved in megakaryocytopoiesis[2].

Least-squares means of PLT for ME (422.4[3]) and SR (530.2) were in the reference range from Thorn (2000), i.e. 520 +/- 195. SA (750.4) was a bit higher, whilst CO (941.4) was absolutely out of the range.

This finding is opposite to human studies as the following examples:

Loo and Beguin (1999) reported that the PLT was lower in the iron deficiency group than in the normal group.

Some studies that applied the platelet aggregation as an indicator for PLT also showed the contrary results, i.e. the platelet aggregation was higher in the iron-therapy group compared to the control group (Kurekci *et al.,* 2000; Kabakus *et al.*, 2000; Caliskan *et al.*, 1999;).

On the other hand similar results were also observed in human studies:

A study in children found that a group with iron deficiency anemia had **higher platelet aggregation** than the control group. It was suggested that the antioxidant level, which was lower in the iron deficiency anemia group, was correlated with platelet aggregation(Tekin *et al.*, 2001) .

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[2] Megakaryocytopoiesis is the synthesis of megakaryocytes or the latest stage in the synthesis of thrombocytes.

[3] X 10^9 cells/L

A study in children with iron deficiency anemia showed that **their platelet count** was higher than that of the normal group (Hicsonmez *et al.*, 1978). After oral and/or parenteral iron therapy the platelet count decreased, whilst reticulocytes were seen to increase.

A study with infants 6 to 18 months of age found that there was an association between iron deficiencies with ischemic stroke and venous thrombosis onset (Harfield *et al.*, 1997).

This finding suggests the involvement of some iron-content enzyme in thrombocytopoiesis/megakaryocytopoiesis. The other possibility is that the haemorrhagic condition could increase the releasing of PLT into the blood circulation, as it appears that the megakaryocytes remain in the bone marrow to shed PLT and do not normally enter the blood circulation (Thorn, 2000).

This result is also in opposition to some studies that related the meat consumption to coronary hearth disease, where one of the indicators that could make clogging is the increasing of PLT. This study revealed the PLT of the haem iron group (ME, SR, and SA) was lower ($p < 0.05$) than the non-haem iron group (CO).

2.3.14 White Blood Cells (WBC)

There were significant changes in WBC over time ($p=0.0169$) (Table 2.4). The least-squares means for all diets on days 0, 7, 14, 21, and 28 are listed at Table 2.9.

There were no significant differences in the number of white blood cells between diets ($p = 0.0636$) and no interaction between diet and time

(p=0.5530).

The WBC on day 7 was 29.7 % increased ($p < 0.05$) over day 0 (Table 2.9). This indicates that for all diet groups the iron intake in the first week induced white blood cell synthesis. The finding in the first week is similar with that one of Kleinbeck and McGlone (1999) who also found that supplemental iron increased WBC in pigs.

Table 2.9 Least-squares means for white blood cell counts (WBC) on days 0, 7, 14, 21 and 28 (x 10^{12} cells/L), with residual standard deviation (RSD).

Days	WBC
0	14.80^{a}
7	19.19^{b}
14	18.28^{ab}
21	18.67^{ab}
28	16.48^{ab}
RSD[1]	4.42

Where :
- [1]The RSD is residual standard deviation.
- [a,b] Values in the same column with a common superscript letter are not significantly different ($p>0.05$).

From days 14 up to 28, WBC declined and became not significantly different ($p > 0.05$) from WBC on day 0. WBC in the experiment were in the reference range given by Thorn (2000), i.e. 11 to 20 x 10^{12} cells/L.

2.4 GENERAL DISCUSSIONS

The summary of the least square means over the four weeks for blood parameters related to iron are listed at Table 2.10.

Table 2.10 Least-squares means for CO, ME, SR and SA on RBC, HGB, HCT, IIHI, MCV and MCH.

	CO[1]	ME	SR	SA
RBC[2]	4.7113[a]	5.7916[bd]	6.0788[cd]	5.6152[b]
HGB	68.6[a]	89.2[c]	90.4[c]	79.8[b]
HCT	0.173[a]	0.245[c]	0.266[c]	0.218[b]
IIHI	14.15[a]	25.31[b]	21.98[b]	21.58[b]
MCV	36.86[a]	42.56[c]	43.52[c]	38.81[b]
MCH	15.00[a]	15.73[b]	15.00[a]	14.75[a]

Where:

- 1CO = Control Group; ME = Meat Group; SR = Surimi Raw Group; SA = Sarc Group.
- [2]RBC = red blood cells (x 10^{12} cells/L); HGB = haemoglobin level (g/L); HCT = haematocrit (L/L); IIHI = iron-intake-haemoglobin incorporated (%); MCV = mean corpuscular volume fL; MCH = mean corpuscular haemoglobin (pg).
- [a,b] Values in the same column with a common superscript letter are not significantly different ($p>0.05$).

The liquid part of the meat extraction contains water-soluble proteins, whereas the solid part contains water-insoluble protein. This is to investigate the possibility that the meat factor has a relation with the solubility characters of meat proteins as reported by Baech (2002).

The meat extraction method in this experiment is similar to the one used in a study in humans by Baech (2002) who separated meat fractions into water-soluble, salt-soluble and insoluble meat proteins. The salt soluble and insoluble meat proteins were products resulting from further extractions of the water-insoluble fractions. It might be said that our water-insoluble fraction

(surimi) is similar with the salt-soluble plus insoluble proteins in Baech's experiment.

The blood parameters presented at Table 2.10 show that the meat, surimi raw and sarc groups achieved better performances than the control group. Between the two meat fraction groups (water-insoluble and water-soluble) there was a trend for the surimi raw group to have better result than the sarc group. RBC, HGB, HCT and MCV of water-insoluble fractions group were 19.3, 13.3, 22.0, and 12.1 % respectively higher ($p < 0.05$) than the water-soluble fractions group. In other words the meat factor that assists iron absorption is present more in water-insoluble meat fractions than in water soluble fractions.

2.4.1 IRON ABSORPTION

IIHI or erythrocyte incorporation was used as an indicator of iron absorption. The meat group had 78.9 % higher IIHI than the control group ($p < 0.05$). The finding is in close agreement with the following studies:

South *et al.* (2000) who found that the addition of 100 g/kg-diet dried beef in pigs improved iron absorption by almost 2-fold.

Hallberg *et al.* (2003) found that the addition of 20 g/kg diet powdered red meat increased iron absorption by 84.6 % in human. Engelmann *et al.*, (1998) reported that the addition of 25 g/kg-diet lean beef improved the iron absorption by 15 % in infants.

In a study in humans, the addition of 50 g of pork meat improved iron absorption by 44 % (Baech *et al.*, 2003).

These findings support that piglets may be utilised as a model for human.

2.4.2 IIHI of The Control Group

IIHI of the control group (14.15 +/- 1.73)[1] in this study was higher than the one reported for women with a normal iron condition (4.59 +/- 3.4). (Ekman and Reizaeinstein, 1993). Both of the studies used the same non-haem iron source, i.e. FeSO4.

Some conditions discussed below might explain why IIHI of the non-haem iron group was higher than those reported in other studies in human or in pigs.

The pigs were in an anemic condition. Since the body-iron level influences non-haem iron absorption (Cook *et al.*, 1974; Murray-Kolb *et al.*, 2003), the lower the body-iron level is, the higher the non-haem iron absorption is.

It is known that iron absorption in infants or in younger pigs is better than in older ages. The subjects in this study are pigs 28 days of age that could be comparable with infants in human and, hence, have a better iron absorption compared to women.

The individual variation of the subjects in Ekman and Reizaeinstein's (1993) study was another possible explanation. The CV[2] of the women subjects was very high, i.e. 74.1 % and 82.5 % for the normal and the iron deficiency group, respectively, whilst in this study the CV was 16.38 % in the control group.

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[1] Least square mean +/- standard error

[2] CV is the coefficient of variation (SD/mean x 100 %).

2.4.3 The Sarc Diet

IIHI for the Sarc group was 52.5 % higher ($p < 0.05$) than for the control group. The finding was similar to a study in human that found the addition of 12 g water-soluble protein increased iron absorption by 40 % (Baech *et al.*, 2003). However, this result is opposite to that of South *et al.* (2000) who found the haemoglobin repletion efficiency[3] of haem iron (9.1 %) in pigs was lower than the non-haem iron (11.2 %).

In South *et al.* (2000), the iron source in the haem iron diet was dried animal red blood cells, whilst in this study the iron source for the sarc diet group came from the liquid part of the meat extraction process and $FeSO_4$. The liquid part contained sarcoplasm[4] and thus some haemoglobin, myoglobin, and other proteins. This finding might reveal that haem iron itself is poorly absorbed, but when it is given together with non-haem iron, it might assist the non-haem iron absorption. Another possibility is that the purification of haem-iron should be in the presence of peptides or amino acids to maintain its availability (Vaghefi *et al.*, 2000).

2.4.4 The Surimi diet

Data at Table 2.10 shows that IIHI for the surimi raw group is 55.3 % higher ($p < 0.05$) than for the control group (Table 2.10). IIHI for the surimi raw group was higher than for the sarc group, but it was not significantly different. The surimi or the solid part or water-insoluble fractions in this experiment was

[3] The hemoglobin repletion efficiency is another term of IIHI.

[4] Hemoglobin and myoglobin are relatively presented in sarcoplasm (Lawrie, 1975 and Greaser *et al.*, 1981).

similar to the water-insoluble fractions, salt soluble proteins plus insoluble proteins, in Bæch's study (2003). And the results in Baech's experiment was similar to this experiment where the addition of 12 g salt soluble proteins improved iron absorptions by 79 %.

2.4.5 Comparisons Between Meat and Meat Fractions Groups

IIHI between meat and meat fractions groups (ME, SR, and SA) were not significantly different ($p > 0.05$); however ME (25.31 +/- 1.55) was **15.2 %** and **17.3** % higher than SR (21.98 +/- 1.55) and SA (21.58 +/- 1.55), respectively.

The superiority of these groups (ME, SR, and SA) indicated that the meat factor was definitely present in meat. The separation into water-soluble and water-insoluble fractions decreased the activity of the "meat factor" and hence its role in assisting iron absorption.

Probably, some of the "meat factor" was attached into the "fibre-like" substance attached to the spinner machine during the meat extraction process. The 'fibre-like' material was similar to collagen and was pulled out from the mixture and excluded from the experiment.

In a study with an intestinal rat Ussing chamber model, Vaghefi *et al.* (2002) identified a protein with a loosely bound low-molecular weight peptide, that assisted the iron absorption by maintaining the haem iron solubility. The meat factor might be a low-molecular weight peptide (Vaghefi *et al.* 2002) and because it is light, this low-molecular weight peptide could be dragged into the centre of the bucket during mixing become attached to the "collagen" and be excluded from the experiment. This prediction needs to be clarified in further experiments.

However, in another study in humans, Bæch (2003) found that a heavier

molecular weight peptide (just over 200 kDa), i.e. myosin was a potential candidate for the protein containing the meat factor.

2.4.6 Possible Mechanisms

IIHI of all meat and meat fraction groups (ME, SR, and SA) were significantly higher ($p < 0.05$) than in the control group. This result provided further evidence that there is a "meat factor" playing an important role in the iron absorption process.

1. There is a possible mechanism that protein and amino acids in food stimulated gastrin secretion (Korman *et al.*, 1971). The production of gastric acid and gastrin increased 30-40 % and 65-75 %, respectively when beef meat was consumed compared to soy protein in human subjects (McArthur *et al.*, 1988). Anaemic patients with chlorhydria (lack of gastric acid production) have a decreased rate of non-haem iron absorption and an addition of hydrochloric acid or gastric juice improved the absorption (Cook *et al.*, 1964).

2. Probably the meat factor pulls the "agent" that binds non-haem iron and together passes it through intestinal lumen. A study in man, Bjorn-Rasmussen and Hallberg (1979) found that beef meat enhanced the iron absorption only when the iron salt was trivalent or when sodium phytate was added to the solution. They concluded that the meat factor counteracted the activity of inhibitor factors and making formations of a luminal carrier, which transports the iron to the mucosal cell membrane

3. Proteins are candidates for the meat factor. Some amino acids were observed to bind iron (Albert, 1950; Opatz and Liskamp, 2002). A study in rats by Kroe *et al.* (1962) found that histidine, glutamine, glutamic acid, and asparagine enhanced iron absorption. Cysteine-containing peptides, rather

than the free cysteine, enhanced iron absorption in human (Taylor *et al.*, 1986). Cysteine enhanced passive uptakes of iron and the passive processes involved in the enterocyte transfer of the common pool made of both sources (heme and nonheme) of iron (Vaghefi *et al.*, 2000).

4. In an *in vitro* study, the product of globin hydrolysis during digestion process, which maintains the solubility of the haem (Vaghefi *et al.*, 2000), also played a role in enhancing the availability of haem iron.

2.5 CONCLUSIONS

The addition of meat and meat fractions (ME, SR, and SA) enhanced iron absorptions and improved values for some blood parameters (RBC, HGB, HCT, MCV, and MCH). The mechanism is not clear, but there was definitely a meat factor that played an important role in improving iron absorptions.

The meat extraction process separated meat into the water-insoluble or solid part (surimi) and the water-soluble or liquid part (sarc). In the last two weeks of the experiment, water-insoluble fractions showed better results ($p < 0.05$) in improving some blood parameters than water-soluble fractions. The parameters were the haemoglobin concentration, the mean corpuscular volume or the size of red blood cell, the microcytosis percentage, and the platelet count. Also on day 28, water-insoluble fractions had a higher ($p < 0.05$) MCH value than water-soluble fractions.

Meat and meat fractions improved iron absorption. This finding had many similarities with a study in humans, which used a similar meat extraction method (Baech, 2002).

Comparisons between meat and meat fractions groups indicated the meat factor is more present in the water-insoluble fractions rather than in the water-soluble fractions.

The red-cell distribution width (RDW) is not an appropriate iron deficiency anemia indicator in pigs.

2.6 FUTURE PERSPECTIVES

Beef meat and meat fractions may be utilised to improve iron bioavailability to fight the iron deficiency endemic in some countries.

The findings show that the 28 to 56 day of age pig is a reliable model animal for human iron absorption studies. The utilisation of pigs as a model for human studies has some advantages; one of them is relatively easier to get the homogeny subjects. This is shown by the coefficient of variation (CV), which is smaller in pigs than in human.

It is suggested that the following studies to be conducted:

To clarify the two opposite findings of Vaghefi *et al.* (2002) with a low-weight molecular peptide theory and Bæch (2003) with the heavy-molecular weight theory.

To analyse molecular weights of peptides in each meat fraction group. Meat fractions are separated into water soluble, salt soluble, and salt-insoluble proteins.

To measure effects of meat fractions on production of gastric acid secretion and gastrin release to learn a possible relation between the solubility characteristics of meat protein and those gastric juice productions.

Chapter 3.

USE OF STABLE ISOTOPES TO MEASURE IRON BIOAVAILABILITY IN PIGLETS

3.1 INTRODUCTION

Using radioisotopes as tracers in nutritional studies is not popular at the present for ethical and health reasons (Fairweather-Tait and Dainty, 2002).

Isotope labelling has been utilised in many nutritional studies to learn about the activity, the movement, and the fate of nutrients in the body, and to determine levels of absorption.

The best way to measure iron bioavailability is to use human subjects, but it is an expensive approach and subjects should be carefully selected as the rate of absorption of iron is influenced by several factors such as age and body iron level. Therefore pigs have been used, as a model for humans, in many nutritional studies.

In the experiment described herein lamb meat, as a good source of iron, was intrinsically and extrinsically labelled with a stable isotope. The aim of the study was to validate the analytical laboratory procedures for measuring ^{57}Fe and ^{58}Fe in blood and to learn about iron absorption and metabolism by using pigs as a model before the isotope-labelled lamb meat is used with humans. In particular, the aim was to determine whether the level of stable isotope in the lamb meat was high enough to detect clear changes in its level in red blood cells following consumption of the meat.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Animals

Eleven pigs were initially included in the experiment. All pigs were female to reduce the variation in iron isotope ratio between sexes. A study in humans showed that the ratio of $^{56}Fe/^{54}Fe$ in males was 0.03 percent lower than in females (Walczyk and von Blanckenburg, 2002).

The pigs came from three litters of the same genotype (Duroc x (Large-White x Landrace cross breed)) and were obtained from a commercial farm. To avoid genetic effects the litter of origin was taken account when pigs were allocated into treatments. All procedures involving animals were conducted with the approval of, and under the guidelines established by, the Massey University Animal Ethics Committee.

The pigs received only 50 mg iron intramuscularly injected immediately after birth to create a semi-anaemic condition. Although it was only about 25 percent of the normal dose, the iron in the diets gradually fulfilled the iron requirements. Pigs were reared on the sow, weaned at 3 weeks of age, and moved to individual metabolism crates at that time.

Nine pigs were chosen from the eleven, based on their high feed intake during the first week of adaptation. Two pigs were intramuscularly injected with iron and excluded from the experiment.

The experiment was conducted at the Massey University Animal Physiology Unit. The experiment design was a random block design. Three groups of three pigs were blocked according to weight and litter of origin and randomly

allocated ton one of the three treatment groups.

3.2.2 Individual Metabolic Crates

The metabolic crates used are described in section 2.2.2.2.

3.2.3 Experimental Diets

The experiment was conducted over three weeks. The first seven days from day -7 to day -1 were adaptation days. The pigs were fed a diet similar to the experimental diet, except that the meat was normal lamb meat (Table 3.1).

The three experimental groups were:

1) I200: received 48 g/kg$^{0.75}$ of meat intrinsically labelled with Fe^{57}.
2) I300: received 69 g/kg$^{0.75}$ of meat intrinsically labelled with Fe^{57}.
3) E200: received 48 g/kg$^{0.75}$ of meat extrinsically labelled with Fe^{57}.

On day 0, the pigs were fed the experimental diets, which were labelled with ^{57}Fe. The pigs were fed individually at 10 minute intervals between pigs. The amount of diet was based on the metabolic live weight (kg $^{0.75}$).

One and a half hours after feeding, the feeders were taken out and weighed, to get the amount of diet consumed. All feeders were kept away from the pigs until the blood sampling was finished, but were returned after this.

On day 1, in the morning all the diet left in each feeder was weighed. Then from day 1 to the end of the experiment (day 14), the pigs were fed the weaner diet without any stable labelled iron isotope.

3.2.4 Meat Preparation

The meat for the I200 and I300 diets was intrinsically labelled with

intra venous infusions during lamb growth. The meat meal contained 20 ppm Fe with 35 % enrichment with ^{57}Fe.

The meat for E200 was extrinsically labelled with ^{57}Fe. The meat contained 33 ppm Fe at 53 % enrichment of ^{57}Fe, which was made up of 15 ppm of Fe at 2 % ^{57}Fe-enrichment (regular lamb meat) plus 18 ppm of extrinsically added Fe as $FeSO_4$ at 95 % ^{57}Fe-enrichment.

The ingredients of the diets are listed in Table 3.1. All diets met the nutrient requirements recommended by NRC and were balanced in energy, amino acids, minerals, and vitamins.

Table 3.1 The diet composition of the experimental diets and the weaner diet (% as-fed basis).

Ingredient	Experimental Diet	Weaner Diet
Fish meal	0	1.72
Maize	0	11.49
Soybean oil	4.5	3.45
Wheat	14.4	22.41
Methionine	0.2	0.17
Threonine	0.2	0.17
Vitamin + Mineral	0.1	0.11
DiCP	1.5	1.72
NaCl	0.1	0.11
Lamb Meat	25	0.00
Water	50	50.00
Casein	4	8.62

Where : - DiCP is dicalcium phosphate
- NaCl is sodium chloride

The meats were prepared by Dr Scott Knowles at Ag-Research, Palmerston North. The labelled iron for I200 and I300 was primarily organic iron, whilst for E200 it was inorganic iron. The analysed iron content of experimental and weaner diets were 39 and 112 ppm iron consecutively.

3.2.5 Blood Sampling

Blood samples on days -1, 0, 7, and 14 were collected via the *vena jugularis* using the vacutainer collection system. The piglets were anaesthetised during blood sampling with a mixture of Halothane / Fluothane (4 %) and oxygen.

On day -1, there were two tubes of blood samples, one 5 ml of blood was for a complete blood counting and the other was brought to AgResearch for an initial record. To reduce double handling, the pigs were weighed immediately after the blood sampling.

On day 0, 4.5 hours after the experimental iron isotope diet had been offered at morning feeding, the pigs were anaesthetized for another blood sampling. Immediately after that the pigs were also injected by ^{58}Fe via *intra venous* to compare the erythrocyte incorporation efficiency between per-oral (^{57}Fe) and per-*intra venous* (^{58}Fe).

On days 7 and 14, the pigs were anaesthetized for blood samplings and to avoid double handling the pigs were immediately weighed. About 7 ml of blood were taken to count the isotope and other blood parameters.

The ICP-MS (inductive couple plasma-mass spectrometry) method, which was used to count the enrichment of the isotope tracers, was carried out under supervision of Dr Scott Knowles from Ag-Research, Palmerston North.

3.2.6 Calculation of Erythrocyte Incorporation

3.2.6.1 Erythrocyte incorporation

The quantity of the tracers (i.e. ^{57}Fe and ^{58}Fe) at a specified time 't' was calculated as:

$$^{57}Fe_{inc} = \frac{(R_t\ ^{57}Fe/^{56}Fe - R_0\ ^{57}Fe/^{56}Fe)}{R_0\ ^{57}Fe/^{56}Fe} \times Fe_{circ} \times 0.0219$$

$$^{58}Fe_{inc} = \frac{(R_t\ ^{58}Fe/^{56}Fe - R_0\ ^{58}Fe/^{56}Fe)}{R_0\ ^{58}Fe/^{56}Fe} \times Fe_{circ} \times 0.0030$$

Where:

- ^{57}Fe and ^{58}Fe are expressed in mg.
- $R_t\ ^{57}Fe/^{56}Fe$ Rt $^{58}Fe/^{56}Fe$ are the isotopes ratio at time 't'.
- $R_0\ ^{57}Fe/^{56}Fe$ and $R_0\ ^{58}Fe/^{56}Fe$ are the isotopes ratio at time '0'. In this calculation, the value is 0.02339 for $R_0\ ^{57}Fe/^{56}Fe$ and 0.003155 for $R_0\ ^{58}Fe/^{56}Fe$ (Fomon *et al*, 2000).
- Fe_{circ} is the total circulating iron in blood at time 't'.
- 0.0219 = (2.15 x 56.935)/(100 x 55.845) an intermediate calculation from Fomon *et al*., 2000 (2.15 is the natural abundance (%) of ^{57}Fe; 56.935 is the atomic weight of ^{57}Fe; 55.845 is the atomic weight of Fe).
- 0.0030 = (0.29 x 57.933)/ (100 x 55.845) an intermediate calculation from Fomon *et al*., 2000 (0.29 is the natural abundance (%) of ^{58}Fe; 57.933 is the atomic weight of ^{58}Fe; 55.845 is the atomic weight of Fe).

The formula for Fe_{circ}, expressed in mg, was estimated as

$$\mathbf{Fe_{circ} = Live\ weight \times 0.07 \times Hb \times 3.46625}$$

Where:

- Live weight is expressed in kg.
- 0.07 is the blood volume (L/kg).
- Hb is the haemoglobin concentration (g/L).
- 3.46625 is the iron contribution in haemoglobin. The number comes from the explanation in section 2.2.5.1.

Iron absorption is defined as the amount of iron in the blood circulation divided by the amount of iron intake (dose or administered).

$$\%\ ^{57}Fe_{inc} = (^{57}Fe_{inc} / ^{57}Fe_{dose}) \times 100\ \%$$

$$\%\ ^{58}Fe_{inc} = (^{58}Fe_{inc} / ^{58}Fe_{dose}) \times 100\ \%$$

The isotope ^{58}Fe was infused/administered intra-venously, which means that all iron was assumed to be100 % absorbed into the blood circulation. The quantity of ^{57}Fe absorption corrected for ^{58}Fe absorption is:

$$\%^{57}Fe_{abs} = (\%\ ^{57}Fe_{inc} / \%\ ^{58}Fe_{inc}) \times 100\ \%.$$

3.2.7 Statistical Analyses

Simple analyses of variance were used to compare the effect of diets on live weights, average daily gains (ADG) from day 1 to 14, average daily feed intake (ADFI) from day 1 to 14, and ^{57}Fe intakes and ^{58}Fe doses on day 0.

The statistical model was:

$$Y_{ij} = \mu + D_i + e_{ij}$$

Where ;

- Y_{ij} is an observation of the j^{th} piglet with the i^{th} diet treatment.
- μ is the general mean.
- D_i is the fixed effect of the i^{th} diet treatment.
- e_{ij} is a random (residual) error unique to Y_{ij} assumed to be normally and independently distributed with mean θ and variance $\delta^2 r$.

Repeated measures analyses of variance were used to compare the effects of the different dietary treatments, time, and the interaction between treatment and time on all parameters. The analyses were performed using the SAS® System for Windows®, version 8.2 (SAS Institute Inc, Cary, NC, USA).

The statistical model was:

$$Y_{ijk} = \mu + D_i + P_j(D_i) + W_k + D_iW_k + e_{ijk}$$

Where ;

- Y_{ijk} is an observation in the k^{th} week of the j^{th} piglet with the i^{th} diet treatment.
- μ is the general mean.
- D_i is the fixed effect of the i^{th} diet treatment.
- $P_j(D_i)$ is the random effect of the j^{th} piglet within the i^{th} diet treatment.
- W_k is the fixed effect of the k^{th} day.

- D_iW_k is the interaction effect between the i^{th} diet treatment and the k^{th} day.
- e_{ijk} is a random (residual) error unique to Y_{ijk} assumed to be normally and independently distributed with mean θ and variance $\delta^2 r$.

The least significant different (LSD) test was applied, to examine differences between classes. A p-value of < 0.05 was considered statistically significant for all analyses.

3.3 RESULTS AND DISCUSSIONS

3.3.1 Double Labeled Stable Iron Isotopes

The analyses required to measure the enrichment of iron isotopes were carried out by Dr Scott Knowles from AgResearch Palmerston North.

The values for Fe_{inc}, Fe_{circ}, Fe dose and the percentage of Fe_{abs} are presented in Table A.4 (Appendix). The formulae used to calculate these parameters are given in the materials and methods section of this chapter.

Least-squares means for the percentage enrichment for ^{57}Fe and ^{58}Fe in red blood cells on days -1, 7 and 14 are listed in Table 3.2. Only on days 7 and 14, the percentage enrichment for the pigs fed meat lamb extrinsically labeled with ^{57}Fe was statistically different from zero ($p < 0.0001$).

Table 3.2 Least-squares means for the percentage enrichment for ^{57}Fe and ^{58}Fe in red blood cells on days -1, 7 and 14, with residual standard deviations (RSD).

Day	Diet	^{57}Fe (%)	^{58}Fe (%)
-1	E200	-0.04	-0.8
	I200	0.4	0.7
	I300	0.1	0.1
7	E200	22.8[b]	23.9
	I200	4.3[a]	20.4
	I300	7.7[a]	24.1
14	E200	16.7[b]	18.5
	I200	2.2[a]	15.9
	I300	3.4[a]	16.2
RSD		3.95	4.77

[a, b] Values in the same column and within sampling days without superscript letters or with common superscript letters do not differ significantly ($P < 0.05$).

As planned, the ^{57}Fe intakes were significantly different ($p < 0.0001$), but ^{58}Fe doses were not (Table 3.3).

Table 3.3 Means for ^{57}Fe intakes and ^{58}Fe doses on day 0 (mg), with residual standard deviations (RSD).

Diet	57Fe	58Fe
E200	3.32^{a}	0.0961
I200	1.31^{b}	0.0961
I300	1.79^{c}	0.0961
RSD	0.09	0.0189

Where:

- ^{a,b} Values in the same column with a different superscript are significantly different ($p>0.05$).

The statistical analysis showed that the effect of diets on $^{57}Fe_{abs}$ was not significant ($p = 0.3707$). The least-squares means of $^{57}Fe_{abs}$ are shown in Table 3.4.

Table 3.4 Least-squares means for $^{57}Fe_{abs}$ (%) for each diet on days 7 and 14, with residual standard deviations (RSD).

Diet	Day 7	Day 14
E200	19.99	18.67
I200	12.18	8.25
I300	13.99	10.31
RSD	1.58	1.58

There was a significant change over the time ($p = 0.0072$). The mean for $^{57}Fe_{abs}$ for day 7 at 15.38 % was significantly higher ($p < 0.05$) than for day 14 (12.41 %).

The $^{57}Fe_{abs}$ data were highly variable (Table A.4 Appendix). This may have been caused by the high individual variation between piglets and/or by the fact that the mass spectrometry method used to count the iron isotope did not give reliable results (possibly due to low levels of enrichment).

The E200 group had the highest $^{57}Fe_{abs}$ with values ranging from 9.60 to 29.12 % on day 7 and 8.29 to 27.18 % on day 14. The I200 group was the lowest with ranges of 7.97 to 19.05 % on day 7 and 5.95 to 12.1 % on day 14. I 300 was intermediate with the ranges of 4.26 to 20.51 % on day 7 and 1.42 to 16.38 % on day 14. However, there were no significant differences between groups ($p = 0.3707$).

This finding is similar to a study of zinc absorption in young women, where Egan *et al.* (1991) found that the zinc absorption from a milk-based formulated diet extrinsically labeled was higher than an intrinsically labeled one, although they were not significantly different.

This finding is in contrast to a study of zinc absorption in men, where Janghorbani *et al.* (1982) reported that the zinc absorption in chicken meat intrinsically labeled was significantly higher than the extrinsically labeled product.

The ^{57}Fe enrichment level for intrinsic and extrinsic labeling for the meat used in the current experiment is too low to be applied to human subjects. Based on the pig data for this trial a 70 kg human subject would need to eat 1.2 to 1.7 kg of labeled meat to get similar enrichment values, which is much higher than the normal serving size of 100 to 200 grams (Athar *et al.*, 2001) used in human studies.

3.3.2 Growth Parameters

The mean live weights for the diet groups on day 0 (Table 3.5) were not significantly different ($p = 0.9707$). This is an advantage of using piglets as

subjects rather than humans, as having a similar initial live weight eliminates the possible effect of live weight.

Table 3.5 Means for live weight on day 0, average daily gain (ADG), and average daily feed intake (ADFI) for each dietary group with residual standard deviations (RSD).

Parameter	E 200	I 200	I 300	RSD
LW Day 0 (kg)	6.73	6.58	6.93	1.72
ADG (gram/day)	254.9	261	211.6	53.2
ADFI (gram/day)	637.8	656.9	627.5	106.9

Where :

- LW is live weight, ADG is average daily gain for 14 days; ADFI is average daily feed intake for 14 days.

The effect of diets on averaged daily gain was not significant ($p = 0.5034$). The average daily feed intake (gram per day) is the total feed consumption in grams divided by the number of days (day). There were no significant differences between diets ($p = 0.9941$) (Table 3.5).

3.3.3 Blood Parameters

A summary of the significance levels for blood parameters is shown in Table 3.6.

The effect of diets on WBC was not significant ($p = 0.5854$). There were no significant differences over time ($p = 0.0751$) as well. The least-squares means of white blood cells for E 200, I 200, and I 300 were 16.21, 13.82, and 14.79 $\times 10^9$ cells/L with RSD = 2.67.

Table 3.6 Statistical significance levels for the diet, animal and time effects, and their interactions on blood parameters.

Parameter	Diet	Piglet(Diet)	Time	Diet*Time
WBC	NS	***	NS	NS
RBC	NS	***	***	NS
HGB	NS	***	***	NS
HCT	NS	***	***	NS
MCV	NS	***	NS	NS
MCH	NS	**	NS	NS
HGBFe	NS	***	***	NS

Where:
- NS = not significant ($p > 0.05$).
- * = $p < 0.05$.
- ** = $p < 0.01$.
- *** = $p < 0.001$
- WBC is white blood cells; RBC is red blood cells; HGB is haemoglobin; HCT is haematocrit; MCV is mean corpuscular volume; MCH is mean corpuscular haemoglobin; HGBFe is iron content in haemoglobin.

There was a significant increase of RBC over time ($p < 0.0001$). The least-squares means of RBC for days 0, 7 and 14 are showed in Table 3.7. RBC on days 7 and 14 significantly increased ($p< 0.05$) by 28.3 and 45.1 %, respectively.

The effect of diets on RBC was not significant ($p = 0.6387$). The least-squares means for E 200, I 200, and I 300 were 5.161, 4.643, and 5.440 x 10^{12} cells/L, respectively.

There were no significant differences between diets for haemoglobin (HGB) ($p = 0.9579$). The least-squares means (g/L) for E200, I200, and I300 were 80.0, 77.2 and 77.7, respectively.

Table 3.7 Least-squares means for red blood cells (RBC x 10^{12} cells/L), haemoglobin (HGB g/L), haematocrit (HCT L/L), and haemoglobin iron (HGBFe mg) on days 0, 7, and 14, with residual standard deviations (RSD).

Day	RBC	HGB	HCT	HGBFe
0	4.083^{a}	65.6^{a}	0.188^{a}	105.08^{a}
7	5.238^{b}	79.9^{b}	0.229^{b}	156.27^{b}
14	5.923^{c}	89.4^{b}	0.270^{c}	220.39^{c}
RSD	0.59	11.4	0.037	32.82

Where :

- ^{a,b} Values in the same column with a common superscript are not significantly different ($p>0.05$).

There was a significant change in HGB over time ($p = 0.0027$). The least-squares means for days 0, 7, and 14 are listed in Table 3.7. The HGB level on days 7 and 14 were significantly 21.8 and 36.3 % higher ($p < 0.05$) than day 0.

Haematocrit (HCT) or packed red blood cells volume or packed cell volume (PCV) is the percent of whole blood that is comprised of red blood cells. There were no significant differences between diets for haematocrit ($p = 0.9918$). The least-squares means for hematocrit for E 200, I 200, and I 300 were 0.231, 0.224, and 0.231 (L/L), respectively.

There was a significant difference over the time ($p = 0.0019$). The least-squares means of hematocrit on days 0, 7, and 14 are presented at Table 3.6. HCT on day 7 and 14 were significantly increased by 21.8 and 43.66 %, respectively compared to day 0.

MCV is the average volume of a single red blood cell measured in femtolitres[1] (Harvey, 2000b). There were no significant differences between diets and over time for MCV ($p = 0.7399$ and $p = 0.2396$). The least-squares means for the mean corpuscular volume (fL) for E 200, I 200, and I 300 were 44.19, 46.64, and 42.27, respectively.

MCH is derived by dividing HGB (g/L) with RBC ($x10^{12}$ cells/L), which means the mean hemoglobin weight in a red blood cell. No significant differences between diets were observed ($p = 0.1860$) for mean corpuscular hemoglobin (MCH). There was no significant difference over the time ($p = 0.1327$). The least square means of MCH for E 200, I 200, and I 300 were 15.69, 17.01, and 14.40 pg, respectively.

HGBFe or haemoglobin Fe in the circulation is the total iron in blood circulation derived from a formula as shown in the methods section with some assumptions including that blood volume is 7 % (L/kg) of live weight and that the weight of iron in hemoglobin is 0.346625 % (for the calculation see section 2.2.5.1).

The effects of diets on HGBFe were not significant ($p = 0.9926$). The least-squares means of HGBFe for E 200, I 200, and I 300 were 161.75, 158.35, and 161.65, respectively.

HGBFe on days 7 and 14 were significantly 48.7 and 109.7 % higher than on day 0 ($p < 0.05$). The least-squares means of HGBFe for day 0, 7, and 14 are listed in Table 3.7.

The effects of diets were not significant on all blood parameters. This is reasonable because the diets were different only on day 0. ^{57}Fe intakes between diet groups were significantly different on day 0, but from day 1 to day 14 all animals received the same weaner diet.

[1] one femtolitre (fL) equals to 10^{-15} litre.

Iron intakes from experimental diets and weaner diets obviously improved the performance of some blood parameters, i.e. RBC, HGB, HCT, and HGBFe in all diet groups.

3.4 CONCLUSION

The different labeling method (intrinsically and extrinsically) and the different dosage of meat had no significant effects on either the growth parameters (i.e. the average daily gain and average daily feed intake) or the blood parameters (i.e. the white blood cells and red blood cells count, the haemoglobin level, the haematocrit, the mean corpuscular volume, the mean corpuscular haemoglobin, and the total iron body in the blood circulation).

The ^{57}Fe enrichment level of the intrinsically labeled lamb is too low to be applied in human studies.

3.5 FUTURE PERSPECTIVES

The use of stable isotopes of iron, both extrinsic and intrinsic, is a promising method to use in the study of iron absorption and bioavailability.

Twenty eight-day-old piglets can be used as laboratory animals to test some methods before they are applied to humans.

The following study are suggested for the future:

To conduct further experiments with double isotope labeling methods, with higher levels of iron isotope enrichment and with different analytical ways to count the isotope tracers.

Appendices

APPENDICES

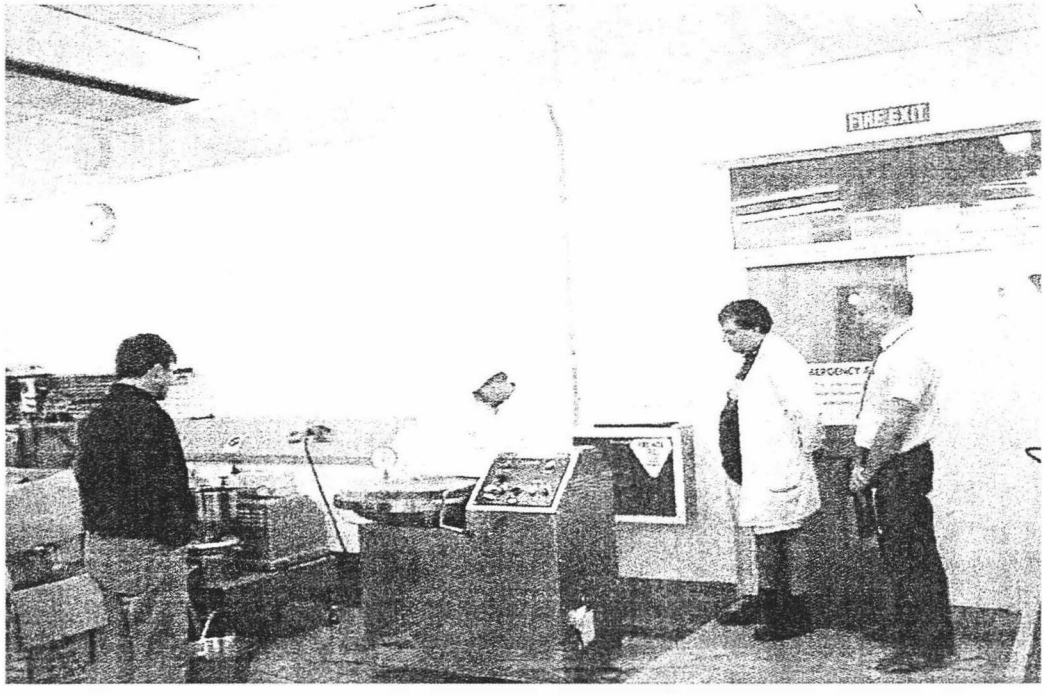

Figure A.1. The bowl cutter machine.

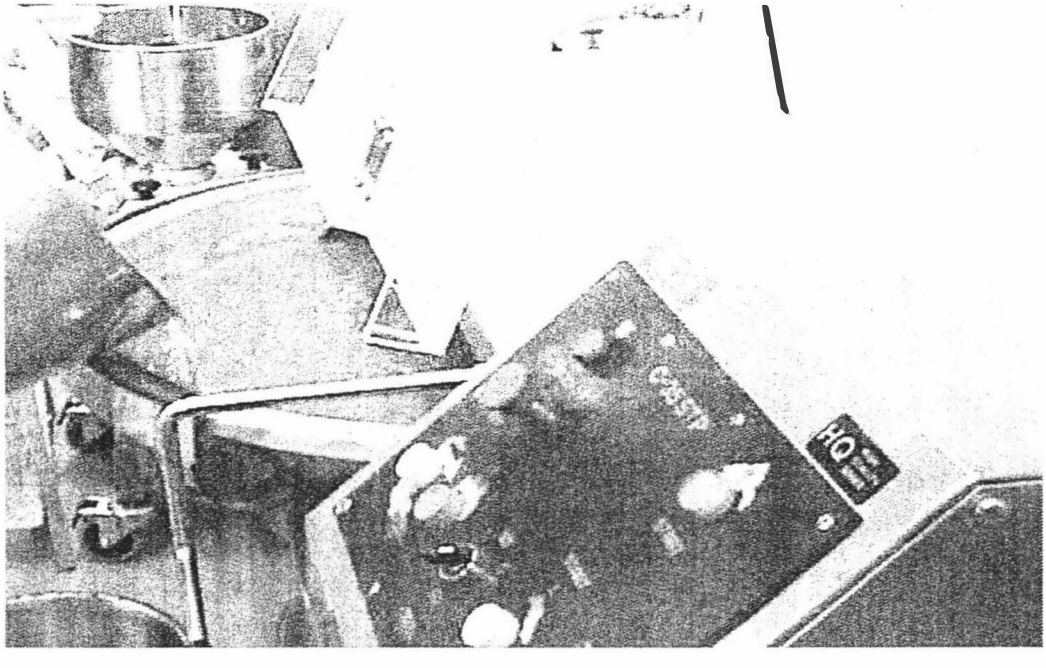

Figure A.2. Adding some chilled water into the bowl cutter machine.

Table A.1. The individual data of iron intake and body iron level, which were used in the calculation of the iron intake-hemoglobin incorporation (IIHI).

Piglet	Diet**	Day	Total feed Intake(g)	Iron content in diet (ppm)	Total Iron Intake (g)	Hemoglobin (g/L)	Liveweight(g) (g)	Body Iron (g)*
2	CO	0-28	.	50.52	.	.	.	.
3	SR	0	0	50.36	0	84	7152	0.145769
3	SR	7	4751	50.36	0.23926036	91	8052	0.177788
3	SR	14	11038	50.36	0.55587368	102	10107	0.250139
3	SR	21	17401	50.36	0.87631436	105	12092	0.308067
3	SR	28	24458	50.36	1.23170488	112	13950	0.379097
4	SA	0	0	49.14	0	59	6140	0.087898
4	SA	7	2344	49.14	0.11518416	62	6269	0.094308
4	SA	14	8563	49.14	0.42078582	76	8175	0.150751
4	SA	21	15787	49.14	0.77577318	83	10852	0.218548
4	SA	28	23216	49.14	1.14083424	101	13179	0.32297
5	ME	0	0	50.61	0	57	7968	0.1102
5	ME	7	545	50.61	0.02758245	64	6712	0.104229
5	ME	14	3062	50.61	0.15496782	57	8191	0.113284
5	ME	21	9717	50.61	0.49177737	72	10070	0.175922
5	ME	28	17635	50.61	0.89250735	91	12413	0.274079
6	CO	0	0	50.52	0	62	5842	0.087884
6	CO	7	3011	50.52	0.15211572	69	6110	0.102294
6	CO	14	6306	50.52	0.31857912	72	7151	0.124927
6	CO	21	10402	50.52	0.52550904	70	8153	0.138476
6	CO	28	17007	50.52	0.85919364	73	9993	0.177002
7	SR	0	0	50.36	0	73	5255	0.093079
7	SR	7	3216	50.36	0.16195776	71	6477	0.111581
7	SR	14	9560	50.36	0.4814416	103	8183	0.204507
7	SR	21	16522	50.36	0.83204792	121	9765	0.286692
7	SR	28	24234	50.36	1.22042424	129	12451	0.38972
8	SA	0	0	49.14	0	61	8740	0.12936
8	SA	7	4713	49.14	0.23159682	70	10232	0.173787
8	SA	14	10958	49.14	0.53847612	73	12670	0.224418
8	SA	21	18232	49.14	0.89592048	82	14605	0.290585
8	SA	28	26150	49.14	1.285011	102	16619	0.411304
10	ME	0	0	50.61	0	57	5803	0.080258
10	ME	7	4693	50.61	0.23751273	87	6984	0.147429
10	ME	14	10977	50.61	0.55554597	107	9279	0.240904
10	ME	21	18394	50.61	0.93092034	109	11839	0.313112
10	ME	28	26257	50.61	1.32886677	118	14410	0.412576
11	SR	0	0	50.36	0	51	7345	0.090891
11	SR	7	4818	50.36	0.24263448	69	8408	0.140767
11	SR	14	11187	50.36	0.56337732	88	10318	0.220311
11	SR	21	18207	50.36	0.91690452	100	12268	0.297668
11	SR	28	25919	50.36	1.30528084	113	13053	0.357888
12	ME	0	0	50.61	0	56	6881	0.093497
12	ME	7	3200	50.61	0.161952	70	7556	0.128336
12	ME	14	9444	50.61	0.47796084	100	9449	0.229268
12	ME	21	16274	50.61	0.82362714	118	11181	0.320126
12	ME	28	24086	50.61	1.21899246	144	13756	0.480632

Piglet	Diet**	Day	Total feed Intake(g)	Iron content in diet (ppm)	Total Iron Intake (g)	Hemoglobin (g/L)	Liveweight(g) (g)	Body Iron (g)*
13	CO	0	0	50.52	0	63	6486	0.099146
13	CO	7	4130	50.52	0.2086476	62	7537	0.113383
13	CO	14	9500	50.52	0.47994	69	8981	0.15036
13	CO	21	14635	50.52	0.7393602	70	9878	0.167774
13	CO	28	22158	50.52	1.11942216	79	12267	0.235138
14	SR	0	0	50.36	0	58	6705	0.094359
14	SR	7	2406	50.36	0.12116616	63	6751	0.103197
14	SR	14	8026	50.36	0.40418936	80	8789	0.170603
14	SR	21	14704	50.36	0.74049344	100	10019	0.243099
14	SR	28	22624	50.36	1.13934464	117	12603	0.357782
16	SA	0	0	49.14	0	49	7875	0.093628
16	SA	7	4664	49.14	0.22918896	67	8992	0.14618
16	SA	14	10928	49.14	0.53700192	91	10811	0.238707
16	SA	21	17649	49.14	0.86727186	101	12715	0.311599
16	SA	28	25409	49.14	1.24859826	104	15126	0.381694
17	ME	0	0	50.61	0	49	8227	0.097813
17	ME	7	253	50.61	0.01280433	61	6882	0.10186
17	ME	14	6118	50.61	0.30963198	88	8790	0.187685
17	ME	21	13190	50.61	0.6675459	115	10928	0.304928
17	ME	28	20995	50.61	1.06255695	121	13279	0.38986
18	CO	0	0	50.52	0	35	8759	0.074384
18	CO	7	4615	50.52	0.2331498	57	9624	0.133103
18	CO	14	10843	50.52	0.54778836	78	11338	0.21458
18	CO	21	18007	50.52	0.90971364	80	13801	0.267891
18	CO	28	25316	50.52	1.27896432	92	15408	0.343948
19	SR	0	0	50.36	0	52	5927	0.074782
19	SR	7	4409	50.36	0.22203724	67	6511	0.105848
19	SR	14	10563	50.36	0.53195268	85	8320	0.171593
19	SR	21	17809	50.36	0.89686124	103	9765	0.244044
19	SR	28	25496	50.36	1.28397856	122	11811	0.349627

Piglet	Diet**	Day	Total feed Intake(g)	Iron content in diet (ppm)	Total Iron Intake (g)	Hemoglobin (g/L)	Liveweight(g) (g)	Body Iron (g)*
20	ME	0	0	50.61	0	61	6671	0.098737
20	ME	7	3546	50.61	0.17946306	85	7283	0.150206
20	ME	14	9811	50.61	0.49653471	102	9023	0.223311
20	ME	21	16170	50.61	0.8183637	115	10383	0.28972
20	ME	28	24203	50.61	1.22491383	125	12930	0.392163
21	SA	0	0	49.14	0	55	5575	0.074399
21	SA	7	3633	49.14	0.17852562	68	6348	0.104738
21	SA	14	9042	49.14	0.44432388	81	8157	0.160315
21	SA	21	15442	49.14	0.75881988	89	9913	0.214069
21	SA	28	23211	49.14	1.14058854	105	12266	0.3125
23	CO	0	0	50.52	0	47	6030	0.068766
23	CO	7	2745	50.52	0.1386774	57	6360	0.087961
23	CO	14	7383	50.52	0.37298916	66	7722	0.123661
23	CO	21	12242	50.52	0.61846584	70	8971	0.152369
23	CO	28	19255	50.52	0.9727626	76	10980	0.202476
25	SA	0	0	49.14	0	60	8428	0.122697
25	SA	7	3475	49.14	0.1707615	74	9274	0.166516
25	SA	14	9818	49.14	0.48245652	86	11542	0.240845
25	SA	21	17326	49.14	0.85139964	90	13678	0.298692
25	SA	28	25338	49.14	1.24510932	105	15890	0.404829

Where : * Blood Volume = Live weight (g) x 7 %
Percentage iron in Hb = 3.46629 %
Body Hb Iron = Live weight (g) x 0.07 x Hb (g/L) x 0.00346629
**CO is the control group, ME is the meat group;
SR is the surimi raw group; SA is the sarcoplasm group.

Table A.2 The individual iron intake-hemoglobin incorporation (IIHI)

Piglet	Diet*	Slope (b)**	Piglet	Diet*	Slope (b)**
2	CO	. ***	13	CO	0.1198
3	SR	0.1930	14	SR	0.2340
4	SA	0.2059	16	SA	0.2352
5	ME	0.1885	17	ME	0.2814
6	CO	0.1026	18	CO	0.2069
7	SR	0.2488	19	SR	0.2140
8	SA	0.2125	20	ME	0.2347
10	ME	0.2465	21	SA	0.2062
11	SR	0.2094	23	CO	0.1366
12	ME	0.3147	25	SA	0.2191

Where:

* CO= Control Group; ME=Meat Group; SR=Surimi Raw Group; SA=Sarcoplasma Group

** Slope (b) reflects the iron repletion efficiency or IIHI.

*** The piglet was excluded.

Table A.3 The nutrients details for each diet (as calculated).

Nutrients	CO	ME	SR	SA
DM (g/kg)	276.40	272.59	272.51	276.20
GE (MJ/kg)	6.15	6.11	6.06	6.07
CP (g/kg)	88.38	88.30	86.19	85.40
DE (MJ/kg)	5.86	5.85	5.80	5.79
AIDP (g/kg)	79.46	81.01	78.36	76.99
App.Ileal.D.AA	g/kg	g/kg	g/kg	g/kg
ASPARTIC ACID	7.80	7.80	7.70	7.55
THREONINE	5.67	5.46	5.56	5.45
SERINE	5.03	3.61	4.03	4.60
GLUTAMIC ACID	10.53	11.74	11.46	10.13
PROLINE	8.51	5.21	6.28	7.66
GLYCINE	1.45	2.88	2.09	1.74
ALANINE	2.86	4.13	3.54	3.03
VALINE	6.11	4.56	5.04	5.79
ISOLEUCINE	5.16	4.33	4.64	4.78
LEUCINE	9.32	7.62	7.95	8.73
TYROSINE	2.86	2.88	2.88	2.71
PHENYLALANINE	5.09	3.91	4.18	4.71
TRY+Phen	7.95	6.79	7.06	7.42
HISTIDINE	2.56	3.01	2.32	3.05
LYSINE	8.40	7.74	7.90	7.93
ARGININE	3.82	4.81	4.53	3.69
Cystine	0.31	0.30	0.29	0.30
METHIONINE	4.27	3.89	4.06	4.03
Met+Cyst	4.58	4.18	4.35	4.33
FAT(g/kg)	51.18	54.08	50.80	51.09
NDF (g/kg)	15	15	15.1	15
ADF (g/kg)	15	15	15.1	15
g/kg				
CA	4.850	4.469	4.599	4.774
P	3.677	3.507	3.382	3.886
Na	0.582	0.626	0.574	0.710
Cl	0.525	0.499	0.507	0.519
K	1.027	1.041	1.024	1.001
FE	0.018	0.017	0.017	0.017

Table A.4 The iron isotopes ratio, $Fe_{circulation}$, $^{57}Fe_{incorporated\text{-}corrected}$ for each individual pig.

Piglet	Treat-ment	days	57/56	58/56	57Feinc	%57Feinc	58Fe*inc	%58Feinc	Fecirc	57Fedose	58Fedose	57Fe IIHI-correction
G3	I200	-1	0.0237	0.0033	0.0242	0.0179	0.0073	0.1010	74.3094	1.3469	0.0723	
G4	I300	-1	0.0237	0.0032	0.0341	0.0198	0.0083	0.0842	111.2388	1.7248	0.0987	
B5	E200	-1	0.0234	0.0032	0.0058	0.0017	0.0021	0.0244	114.3174	3.3747	0.0857	
B6	I200	-1	0.0236	0.0032	0.0221	0.0170	0.0022	0.0196	110.6031	1.3011	0.1108	
B8	I300	-1	0.0234	0.0031	0.0013	0.0007	-0.0009	-0.0078	109.4550	1.8042	0.1116	
R9	I200	-1	0.0231	0.0031	-0.0278	-0.0219	-0.0055	-0.0523	103.3153	1.2712	0.1052	
R10	E200	-1	0.0231	0.0031	-0.0250	-0.0079	-0.0029	-0.0338	95.4389	3.1645	0.0849	
R11	E200	-1	0.0233	0.0031	-0.0107	-0.0031	-0.0068	-0.0579	116.1010	3.4125	0.1176	
R12	I300	-1	0.0231	0.0031	-0.0186	-0.0102	-0.0045	-0.0582	82.3480	1.8275	0.0780	
B5	E200	7	0.0288	0.0038	0.8248	0.2444	0.0960	1.1199	162.1187	3.3747	0.0857	22.1665
R10	E200	7	0.0256	0.0038	0.2669	0.0843	0.0757	0.8910	131.3348	3.1645	0.0849	9.6007
R11	E200	7	0.0315	0.0041	1.3527	0.3964	0.1626	1.3827	178.7462	3.4125	0.1176	29.1154
G3	I200	7	0.0241	0.0037	0.1032	0.0766	0.0701	0.9701	145.7943	1.3469	0.0723	7.9738
B6	I200	7	0.0247	0.0037	0.2377	0.1827	0.1076	0.9711	195.4457	1.3011	0.1108	19.0490
R9	I200	7	0.0243	0.0039	0.1093	0.0860	0.0921	0.8758	123.3004	1.2712	0.1052	9.9234
G4	I300	7	0.0255	0.0038	0.3811	0.2210	0.1078	1.0924	188.8283	1.7248	0.0987	20.5139
B8	I300	7	0.0257	0.0040	0.4017	0.2227	0.1418	1.2703	185.0451	1.8042	0.1116	17.7755
R12	I300	7	0.0242	0.0040	0.0718	0.0393	0.0725	0.9301	95.4439	1.8275	0.0780	4.2640
B5	E200	14	0.028	0.0037	0.8480	0.2513	0.1023	1.1930	203.4261	3.3747	0.0857	21.3934
R10	E200	14	0.025	0.0036	0.2710	0.0856	0.0888	1.0459	209.2571	3.1645	0.0849	8.2962
R11	E200	14	0.029	0.0039	1.2582	0.3687	0.1621	1.3777	236.5898	3.4125	0.1176	27.1787
G3	I200	14	0.0237	0.0034	0.0700	0.0520	0.0632	0.8750	239.2418	1.3469	0.0723	5.9480
B6	I200	14	0.0239	0.0035	0.1490	0.1145	0.1056	0.9533	286.7267	1.3011	0.1108	12.1055
R9	I200	14	0.0241	0.0039	0.0863	0.0679	0.1053	1.0008	139.4959	1.2712	0.1052	6.8381
G4	I300	14	0.024	0.0034	0.1933	0.1121	0.0829	0.8400	306.5657	1.7248	0.0987	13.4762
B8	I300	14	0.025	0.0037	0.3281	0.1818	0.1255	1.1244	239.1008	1.8042	0.1116	16.3843
R12	I300	14	0.024	0.0038	0.0256	0.0140	0.0764	0.9799	123.1121	1.8275	0.0780	1.4156

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