THE USE OF THE USSING CHAMBER SYSTEM TO INVESTIGATE IRON ABSORPTION BY THE DUODENUM, JEJUNUM AND ILEUM IN THE MOUSE.

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SHELLEY JANE EDMUNDS
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ABSTRACT

Iron deficiency anaemia is found in approximately 30% of the worlds population and is particularly prevalent in developing countries. The majority of these deficiencies are due to insufficient absorption of iron from the diet. Iron is absorbed primarily by the proximal small intestine, however, there is evidence for a gradient of absorption along the full length of the small intestine. In 1951 Ussing and Zerahn developed a bicameral method for studying iron transport by *in vitro* epithelia. This method has been used previously to investigate iron transport mechanisms in the proximal small intestine.

In the present study Ussing chambers were used to investigate iron absorption by the full length of the mouse small intestine. Consistently high levels of iron were removed from the mucosal compartment by all regions of the small intestine. This iron removal was due to the physiological actions of the tissue and was not caused by iron adhering to the interior of the Ussing chamber apparatus. There was no change in iron uptake when large intestine or caecum was used in place of small intestine.

Ferrous gluconate was chosen as the reference test chemical as it is a readily bioavailable form of iron which has been used previously to investigate iron absorption with the Ussing chamber model. There was a consistently high level of iron uptake when 27.9 mg/L or 9.3 mg/L was added to the mucosal compartment, with no significant differences between results for either concentration.

When 9.15 mg/L manganese sulphate was combined with 9.3 mg/L ferrous gluconate in the mucosal compartment, iron removal was significantly lower in the proximal than the mid small intestine. This was presumably due to competition between the iron and the manganese for transport by the DCT1 protein.

When 200 mg/L calcium chloride and 9.3 mg/L ferrous gluconate were added to the mucosal compartment, there was no significant difference to results compared to ferrous gluconate alone.

The addition of glucose to the intestinal lumen has been shown previously to increase the passive transport of solutes across the intestinal mucosa. However, in the present

experiments when glucose was added to the mucosal Ringer's solution in place of mannitol there was a significant decrease in iron removed from the mucosal compartment by all intestinal regions.

There was evidence that the gluconate portion of ferrous gluconate increased iron absorption in the distal small intestine. This was supported by a significant decrease in iron uptake by the distal small intestine when ferrous sulphate was used in place of ferrous gluconate.

Ferric chloride was unsuitable for use in this system as it precipitated out of the Ringer's solution.

Histological examination of jejunal samples after a typical Ussing chamber experiment found there was no damage to the tissue and the epithelial layer remained intact.

There were significant levels of iron found in both the intestinal tissue and secreted mucus for all intestinal segments. The binding of iron to secreted mucus appears to involve a significant proportion of iron and should be measured in all future Ussing chamber studies.

ACKNOWLEDGMENTS

I am especially grateful to my supervisor Dr. Gordon Reynolds for his continual advice and guidance throughout this project and for always being willing to provide input on all aspects of this thesis.

I would like to thank Dr. David Simcock and Lisa Walker for their time spent in the laboratory, teaching me the Ussing chamber technique and providing ongoing practical assistance. I would also like to thank them for their advice, friendship and for listening to me babble.

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Thank you to Dr. Mervyn Birtles and Guy Hessell for conducting the histological preparation and staining of intestinal tissues used in this thesis and for providing me with such pretty pictures.

I am indebted to Mike Killick and John Sykes for all their advice and assistance with the use of the Flame Atomic Absorption Spectrophotometer. In particular I would like to thank them for stopping their own work on a number of occasions to help me fix the machine when it was being temperamental.

I would like to thank Duncan Hedderley for his advice on the statistical analysis included in this thesis.

Thank you to Boyd Ludlow, as without his love, support and sandwiches this thesis would not have been written.

Finally I would like to thank Wolfgang, Mandy and Rastus Bucket for allowing me to keep my perspective and reminding me of the joys of the whole animal when all I could think of were intestines.

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ABBREVIATIONS

ANOVA	Analysis of Variance
$CaCl_2$	calcium chloride
Caco2	human colon carcinoma epithelial cells
Cd	cadmium
Co	cobalt
Cu	copper
°C	degrees Celsius
DCT1	divalent cation transporter one
DMT1	divalent metal transporter one
FAAS	flame atomic absorption spectrophotometer
Fe	iron
$FeCl_3$	ferric chloride
$FeSO_4$	ferrous sulphate
g	gram
HCl	hydrochloric acid
HNO_3	nitric acid
ICP	inductively coupled plasma emission spectrophotometry
IRE	iron response element
IRP	iron regulatory protein
I_{sc}	short circuit current
HFE	major histocompatibility complex class I-like molecule
K	potassium
KCl	potassium chloride
kg	kilogram
L	Litre
M	molar
mg	milligram
mg/L	micrograms per litre
$MgCl_2.6H_2O$	manganese chloride
mL	millilitre
mM	millimolar
Mn	manganese

$MnSO_4$	manganese sulphate
n	number
Na	sodium
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate monobasic
$NaHCO_3$	sodium bicarbonate
Ni	nickel
nramp2	natural-resistance-associated macrophage protein 2
%	percent
P	probability
p.d.	potential difference
Pb	lead
Rf	fluid resistance
Rt	tissue resistance
se	standard error
SGLT1	Na^+/glucose co-transport protein
Zn	zinc
$\bar{x}$	mean
μAmps	microamperes
μM	micromolar
μg	microgram
Ω	ohm

ERRATA

p ii line 6	"… iron …" should read: "… ion …"
p xii 5th to last line	"… micrograms …" should read: "… milligrams …"
p 3 line 9	"… Ussing Chamber …" should read: "… Ussing chamber …"
p 6 line 7	"… call I-like …" should read: "… class I-like …"
p 13 last line	"… a effective …" should read: "… an effective …"
p 30 Table 3.1	"… Weight (mM) …" should read: "… Concentration (mM) …"
p 30 line 12	"… began …" should read: "… begun …"
p 30 paragraph 3 line 1	"… was …" should read: "… were …"
p 31 paragraph 5 line 4	"… Figure 4.2 …" should read: "… Figure 3.2 …"
p 35 paragraph 3 line 3	"… (Table 4.2) …" should read: "… (Table 3.2) …"
p 36 line 2	"… Table 3.3 …" should read: "… Table 3.2 …"
p 42 paragraph 2 line 4	"… preformed …" should read: "… performed …"
p 43 last line	"… Figure 4.1 …" should read: "… Figure 4.2 …"
p 44 line 1	"… Figure 4.2 …" should read: "… Figure 4.3 …"
p 44 paragraph 2 line 3	"… was no significant variation …" should read: "… were no significant differences …"
p 44 paragraph 2 line 3	"… Figure 4.3 …" should read: "… Figure 4.1 …"
p 46	"… Figure 4.2 …" should read: "… Figure 4.3 …"
p 47 line 6	"… was no significant variation …" should read: "… were no significant differences …"
p 48 last line	"… was no significant variation …" should read: "… were no significant differences …"

p 53 paragraph 3 line 1 "… was no significant variation …" should read: "… were no significant differences …"

p 56 line 1 "… was also significant variation …" should read: "… were also significant differences …"

p 57 line 2 "… was no significant variation …" should read: "… were no significant differences …"

p 58 Figure 4.11 "… x200 .." should read: "… x100 …"

p 61 paragraph 3 line 3 "… proir …" should read: "… prior …"

p 63 3rd to last line "… added to …" should read: "… present in …"

p 63 penultimate line "… thesnon …" should read: "… the …"

p 64 penultimate line "… affects …" should read: "… effects …"

p 64 last line "… in Ussing …" should read: "in an Ussing …"

p 65 line 9 "… solutuion …" should read: "solution …"

p 69 paragraph 2 line 1 "… was no significant variation …" should read: "… were no significant differences …"

p 73 paragraph 4 line 1 "… ere …" should read: "… were …"

p 76 paragraph 2 line 6 "… rom …" should read: "… from …"

p 76 paragraph 5 line 2 "… was no significant variation …" should read: "… were no significant differences …"

p 79 number 5 "… than absorbed …" should read: "… than being absorbed …"

p 79 point 7 "… added the …" should read: "… added to the …"

p 119 reference 6 "… Coning …" should read "… Cloning …"

p 121 line 1 "… 210(), 694-. …"should read: "… 210, 694-700. …"

p 124 reference "… Scricker …" should read: "… Schricker …"

CORRIGENDA

p ii paragraph 2, the final sentence should read:-

"There was no change in iron uptake when the small intestine was replaced with large intestine or caecum."

p 7-8, Section 2.4.1 should read:-

"As iron is not excreted as a waste product, physiological losses are small and iron homeostasis is maintained by regulation of absorption from the diet (McCance and Widdowson, 1938, cited in Hallberg, 2001). The purpose of the regulatory process is to limit iron absorption to the amount needed to cover losses. Regulation of iron uptake is a complex process whereby the iron status of the animal and iron content of the enterocytes affect iron uptake (Conrad, Weintraub and Crosby, 1964; Bothwell et al., 1958). This process is not yet fully understood.

The synthesis of a number of the proteins responsible for iron absorption is controlled by iron regulatory proteins (IRPs). These bind to specific sections of mRNA called iron response elements (IREs) when the body iron content is low (Eisenstein, 2000; Leibold and Guo, 1992) causing the translation of mRNA to be either increased or decreased depending on which protein is being synthesised (Eisenstein, 2000; Leibold and Guo, 1992). IREs are present in the mRNA of many proteins involved in the luminal uptake (e.g. Divalent Cation Transporter 1 (DCT1), see Section 2.4.4.2), intracellular storage (e.g. ferritin, see Section 2.4.5) and serosal release (e.g. transferrin, see Section 2.4.6) of iron (Eisenstein, 2000) indicating that these also play a role in the regulation of iron absorption."

p 18 end of first paragraph, add the following sentence:-

"Small amounts of endogenous iron are also excreted into the urine with values estimated as being up to 0.3 mg/day (Beard et al., 1996)."

p 20 paragraph four, the first sentence should read:-

"Everted intestinal sacs are an *in vitro* method frequently used in the study of iron absorption."

p37 Table 3.2:-

"Final Concentration (mg/L)" should read: "*Initial Test Chemical Concentration in the Mucosal Ringers Solution (mg/L)*"

p 39 third paragraph, the last sentence should read:-

"Before analysis all samples were collected and digested were necessary as described in Sections 3.3.3 and 3.3.4, then diluted as necessary to ensure the iron concentration was within this range."

p 39 penultimate line:-

"... removed from the chambers and immediately fixed ..." should read: "... removed from the chambers, rinsed in 1% HNO_3, and immediately fixed ..."

p 43 Table 3.1, the final sentence of the title should read:-

"All values are mean ± se (number of intestinal segments) and are expressed per square centimetre of tissue."

p47 paragraph one, the last sentence should read:-

"Individual Student's T tests showed that this was caused by the average percentage of iron removed by the first intestinal segment being significantly ($P<0.05$) lower than the average percentage removed by all other segments; there was no significant differences between segments 2 to 8."

p50 paragraph 1, the last sentence should read:

"These control experiments showed a significant ($P=0.01$) change in iron concentration after the 90 minute experimental period which could not be accounted for by measurement error (Table 4.6) ."

p 54 first paragraph, the final sentence should read:-

"Data for each test chemical were then grouped into three intestinal regions; proximal, mid and distal; representing the duodenal, jejunal and ileal sections of the small intestine"

p 55 paragraph 3, the last sentence should read:-

"This was within the range of iron removed from the mucosal solution by small intestinal tissues, and was not significantly different to iron uptake averaged over all regions of the small intestine."

p56 paragraph four, the third sentence should read:-

"Although iron concentration tended to be higher in the proximal region of the small intestine, a two way ANOVA showed no significant difference between the individual intestinal segments ($P=0.4$)."

p 64 paragraph 7 should read:-

"3) There was no significant difference between the percentage iron removed when starting concentrations of either 27.9 mg/L or 9.3 mg/L ferrous gluconate were present in the mucosal Ringer's solution."

p 70 third paragraph, the second sentence should read:-

"This could explain why there was a decrease in iron concentration after the control experiments containing both iron and calcium but no the tissue-mounted experiments showed no significant difference between the percentage of iron absorbed with or without calcium added to the mucosal Ringer's solution."

p 79 number 7:-

"... was removed ..." should read "... may have been removed ..."

p80 number 11, the second sentence should read:-

"However, a qualitative difference in iron staining between tissue which had or had not been exposed to iron in the mucosal Ringer's solution could not be demonstrated with Perl's Prussian blue reaction."

p 82, add the following paragraphs:-

"Where the mucosal Ringer's samples were diluted to obtain iron concentrations within the detection limits of the FAAS, the percentage iron removed from the mucosal Ringer's solution was calculated as follows:

$$\% \text{ removed} = \left[\frac{\text{start} - (\text{end}*\text{dil})}{\text{start}}\right] * 100$$

start = concentration of iron in the mucosal solution at the start of the experimental period

end = concentration of iron measured in the mucosal solution at the end of the experimental period

dil = dilution factor

The percentage iron removed from the mucosal solution for all other samples was calculated as follows:

$$\% \text{ removed} = \left[\frac{\text{start} - \text{end}}{\text{start}} \right] * 100$$

start = concentration of iron in the mucosal solution at the start of the experimental period

end = concentration of iron in the mucosal solution at the end of the experimental period"

p 124 reference 7 should read:-

"Schachter, D., Rosen, S. M. (1959). Active Transport of $_{45}$Ca by the Small Intestine and its Dependence on Vitamin D. American Journal of Physiology, 196, 357- 362."

1. INTRODUCTION

Iron deficiency is found in approximately 30% of the worlds population and is particularly prevalent in developing countries (DeMaeyer and Adiel-Tegman, 1985). The majority of these deficiencies occur when iron absorption from the diet is insufficient to compensate for any physiological iron losses and fulfil the body's metabolic requirements for iron (Hallberg, 2001; Baynes and Bothwell, 1990).

As iron is not actively excreted from the body, iron homeostasis is maintained by the regulation of iron absorption from the diet (McCance and Widdowson, 1937, cited in Hallberg, 2001). However iron absorption is a complex process influenced by a number of factors. For example, the iron status of the individual may affect iron uptake, with anaemia increasing total iron absorption (Bothwell, Pirzio-Biroli and Finch, 1958). An important factor influencing iron uptake is the bioavailability of iron in the diet. One measurement of iron bioavailability is how readily the iron is absorbed by the small intestine. This is influenced by both dietary constituents, which may interact with the iron in the intestinal lumen, and the chemical forms of iron present (Wienk, Marx and Beynen, 1999; Lynch, 1997). In order to prevent the development of iron deficiency it is important to ensure adequate levels of bioavailable iron are present in the diet (DeMaeyer and Adiel-Tegman, 1985). However iron bioavailability and uptake are difficult to predict. Further understanding of the absorption process will aid this prediction.

The majority of iron absorption occurs in the duodenum and many iron absorption studies focus on this region (Rucker, Lonnerdal and Keen, 1994). However there is evidence of iron absorption in the full length of the intestine, with a gradient of absorption from high uptake in the duodenum to low uptake in the terminal ileum (Chowrimootoo, Debnam, Srai and Epstein, 1992). Therefore investigation of absorption in all regions is worthwhile.

In 1951, Ussing and Zerahn developed a method for studying nutrient transport using *in vitro* tissues mounted in a bicameral chamber apparatus. This system has been modified over time and has been used to successfully investigate iron transport in the proximal small intestine (Costa, da Costa and de Sousa, 2000; Vaghefi, Nedjaoum, Guillochon, Bureau, Arhan and Bougle, 2000; Vaghefi, Guillochon, Bureau, Neuvill, Jacob, Arhan

and Bougle, 1998; Helbock and Saltman, 1967). It allows the investigation of specific iron transport processes in different intestinal regions while retaining the physiological processes present in the tissue. Therefore the Ussing chamber apparatus has been used in the following experiments to investigate iron absorption along the full length of the mouse small intestine.