CHAPTER 3

MATERIALS AND METHODS

This study was a laboratory experimental study using animals (Wistar rat) that aims to investigate the effects of date palm and goat milk supplementation on iron metabolism in IDA induced rat. This study was carried out in Faculty of Medicine and Health Science University Sains Islam Malaysia. The handling and use of these laboratory animals received the approval from Animal Ethical Committee University Sains Islam Malaysia, Kuala Lumpur (USIM/AEC/AUP/2016).

3.1 Materials

3.1.1 Raw materials

Phoenix dactylifera L. (Ajwa variety) was bought from Saudagar Kurma, Malaysia. Commercial fresh goat milk (*Capra aegagrus hircus* Saanen breed), was bought from the goat farm from Sepang, Selangor. Date palm and goat milk were kept in a 4°C and -20°C freezers, respectively.

3.1.2 Chemicals

Tribromoethanol (Nacalai Tesque, Japan), undenatured 99.5% ethanol (Nacalai Tesque, Japan), tert-amyl alcohol (Nacalai Tesque, Japan), 70%, 80%, 90%, 95% and 100% alcohol all from HmbG ® Chemicals (Malaysia), RNAlater RNA Stabilization Reagent solution (Qiagen, Germany), Halt™ Protease Inhibitor Cocktail EDTA-Free (Thermofisher Scientific, USA), agarose powder (Vivantis, USA), 1 x LB buffer (Faster Better Media, Baltimore), bovine serum albumin (Sigma Aldrich, USA) Diamond™ Nucleic Acid Dye (Promega, USA), DNA ladder (Promega, USA), 10% neutral formalin solution (Sigma Aldrich, USA), phosphate buffered saline (Sigma Aldrich, USA), Target Retrieval Solution pH 6.0 (DAKO, USA), Richard – Allan Scientific ™

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Haematoxylin (Thermo Scientific, USA), Richard-Allan Scientific bluing reagent (Thermo Scientific, USA), DPX Mountant (Sigma Aldrich, USA), xylene substitute (Sigma-Aldrich, USA), Tween-20 (Vivantis, USA), RNase-OFF™ (Takara Bio, Japan).

3.1.3 Commercial kits

Rat FE (Ferritin) ELISA kit from Elabscience Biotechnology (China), T-PER® Protein Extraction Kit (Thermo Fisher Scientific, USA), RNAesy Mini Kit (Qiagen, Germany), RT^2 First Strand Kit (Qiagen, Germany), RT^2 Profiler PCR Array (Qiagen, Germany), Rabbit specific HRP/DAB Detection IHC Detection Kit -Micro-polymer (ab236469) (Abcam, USA).

3.1.4 Equipment

Animal cage OptiRAT ® RACK (Animal Care System Inc, USA), domestic hand blender (Philip HR1600/01), DiaSpect Hemoglobin TM Analyzer (DiaSpect, Germany), Sysmex XE – 2100 Automated Hematology Analyser (Sysmex, Japan), Applied Biosystem StepOnePlus™ RealTime PCR System (ThermoFisher Scientific, USA), Advia 2400 Chemistry System (Siemens, Germany), Implen Nanophotometer (Implen GmbH, Germany), IEC MicroCL 17 Centrifuge (Thermo Electron Corporation, USA), DragonLab Mini Centrifuge (DLab Scientific Co, Beijing), -80°C Freezer (Sastec Laboratory Equipment, Malaysia), microwave (Sharp, Japan), electrophoresis chamber, vortex mixture (Jeio Tech, Korea), Tecan Infinite m200pro Microplate Reader (Tecan, Switzerland), water bath (Memmert, Germany), water stills (Hamilton Laboratory Glass Limited, UK), Esco Ductless Fume Cabinet (Ascent™ Max, USA), electronic weighing balance (A&D, Japan), Leica TP1020 Automated Tissue Processor (Leica Biosystem, Germany), Leica Paraffin Embedding Station, Histoembedder EG 1160 (Leica Biosystem, Germany), Leica RM2235 Manual Rotary Microtome (Leica

Biosystem, Germany), DYY-8C Electrophoresis Power Supply (Liuyi, Beijing), Olympus Microscope BX51 (Olympus, Japan), micropipettes (Eppendorf, Germany), multichannel pipette (Eppendorf, Germany).

3.1.5 Apparatus

Corn cob bedding, rodent standard diet 1324 P – 10mm pellets (Altromin, Germany), iron deficient diet, TD80396 (Teklad, USA), 3cc/mℓ syringe, 5cc/mℓ syringe and 10cc/mℓ syringe all from Terumo, Japan, 4mm curved animal oral gavage (Popper and Sons, USA), needle 25G X 11' (Terumo, Japan), surgical kit (Albion, UK) consist of scissors, scalpel handle, forceps and razor, alcohol swab (), 2.8 stainless steel beads (Benchmark Scientific, USA), 1.5 mℓ microcentrifuge tube (Watson Biolab, Japan), 2.0 mℓ screw cap microcentrifuge tube round bottom (Watson Biolab, Japan), plastic reagent reservoir, 10µl, 200µl and 1mℓ pipette tips (Axygen, USA), aluminium foil, paraffin film (Benis, USA), Platinum Pro Adhesive Glass Slide (Matsunami, Japan), paraffin wax (Thermo Scientific, USA), 50mℓ Conical Tube (SPL Life Sciences, Korea), Slide Staining Coplin Jar (DWK, Germany), 200mℓ and 500 mℓ reusable glass media bottle (Fisher Scientific, USA).

3.2 Experimental animal

Twenty-four (24) male Wistar rats of 4 weeks of age were purchased from Animal House, Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur. The rats were housed in groups of 4 rats per cage in an ambient condition with a temperature of 25°C, and a humidity of 60–70%, in a 12 light/12 dark cycle. The rats were given commercial rodents' pellets and distilled water *ad libitum*. After acclimatization for 7 days, the rats were then assigned randomly into six (6) groups of four (4) animals; (1) normal control group; (2) negative control group, (3) positive control group, (4) date palm group, (5)

goat milk group, (6) date palm and goat milk group. The normal control group were given normal commercial pellets whereas the rest groups (Group 2 -6) were given low iron diets for two weeks to generate an anaemic condition. After IDA detection, negative control (Group 2) was continuously supplied with a low iron diet and acted as an IDA model group while the positive control group (Group 3) were given iron tablet that act as a standard treatment for IDA (Susanti *et al*., 2018). The date palm group (Group 4) and goat milk group (Group 5) were given date palm solution and goat milk, respectively while the date palm and goat milk group (Group 6) were supplemented with both date palm and goat milk. The supplements were given once every morning for 28 consecutive days in accordance with animal equivalent dosage (AED) calculation in conjunction with distilled water and commercial rat pellet. A simplified experimental procedure was shown in table 1.

Table 1: Animal grouping according to intervention

Group	Acclimatization (7 days)	Anaemia induction (14 days)	Intervention (28 days)
Normal control (Group 1)	Commercial pellet	Commercial pellet	Commercial pellet
Negative control (Group 2)	Commercial pellet	Low iron pellet	Low iron pellet
Positive control (Group 3)	Commercial pellet	Low iron pellet	Commercial pellet + iron tablet
Date palm (DP) (Group 4)	Commercial pellet	Low iron pellet	Commercial pellet + date palm
Goat milk (GM) (Group 5)	Commercial pellet	Low iron pellet	Commercial pellet + goat milk
Date palm and goat milk (DPGM) (Group 6)	Commercial pellet	Low iron pellet	Commercial pellet + date palm + goat milk

Body weights and food intake were recorded daily. Rats were sacrificed after 28days and were kept fasted for 12 hours before dissection. The rats were anaesthetized with avertin, administered intraperitoneally. Blood was collected via cardiac puncture and rats were sacrificed by excessive bleeding before cervical dislocation was done to confirm the death of animals. Blood was analysed for RBC parameter (RBC, Hb, PCV, MCV, MCH, MCHC) while serum was analysed for iron profile (serum iron and

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transferrin saturation) and iron bioavailability. For serum collection, 2mℓ of collected blood was left standing at room temperature for 2 hours before centrifugation. The blood was spun at 3000 rpm for 15minutes, before stored at -80 °C for further use. Liver was harvested for ELISA to determine the liver ferritin content while both small intestine and liver were harvested for qPCR to study the expression of iron metabolism related gene and for immunohistochemistry (IHC) to study the localisation and distribution of iron metabolism related protein. For qPCR, harvested organs were kept in RNAlater RNA Stabilization Reagent solution at -80 °C while for IHC, organs were stored in 10% formalin buffered solution.

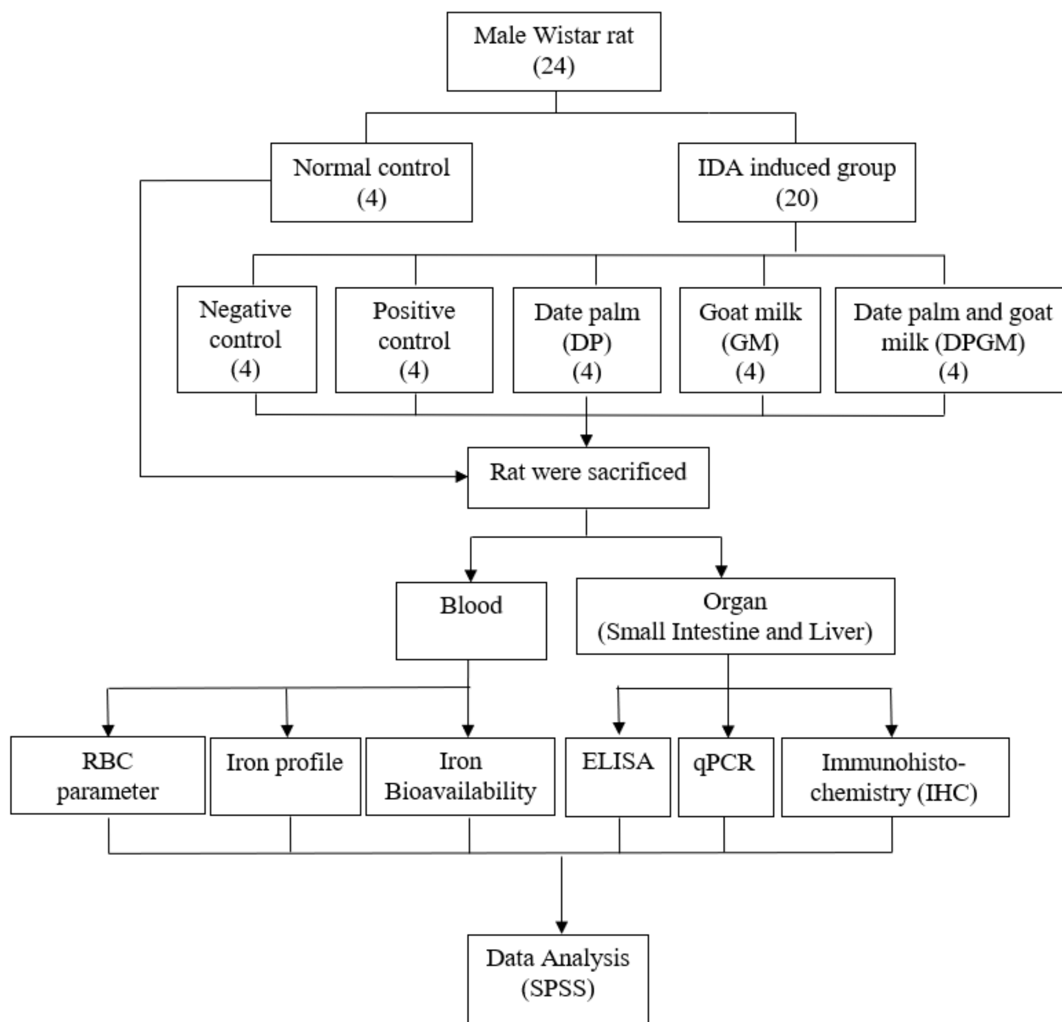

Figure 7: Experimental flowchart of the study design.

3.3 Sample size calculation

Snedecor and Cochran's formula was employed to determine the number of animals allocated for each group. The calculation was performed based on the change of serum iron level between the normal control group and iron-deficient anaemia rats determined by a prior study by Wang *et al*., (2014). The difference in serum iron level between normal control and IDA rats was 6.61mg/L. The standard deviation for the normal and IDA control group was 1.19 and 1.43 respectively. The sample size was calculated based on a 90% power (1-β = 0.9) and a 99% significance level (α = 0.01), thus by referring to figure 8, the constant (C) value was 14.88. The optimal group size was determined to be three. Based on this, it was decided that the optimal group size would be four rats each group. Rats were randomised into control and treatment groups using a simple random sampling method (draw lot).

A

$$n = 1 + 2C\left(\frac{s}{d}\right)^2$$

B

	α	0.05	0.01
1-β	0.8	7.85	11.68
	0.9	10.51	14.88

Figure 8: Sample size calculation. A. Equation by Snedecor and Cochran (1984) was used to compute sample size, where s is the standard deviation, d is the difference to be detected based on previous findings and C is a constant value derived from the power and significance level. B. Table for determining the value of C for the sample size calculation in which α represents a significant level (95% or 99%) while 1- β = power (80% or 90%).

3.4 Preparation of chemical

3.4.1 Tribromoethanol (Avertin)

A mixture of tribromoethanol (Avertin) and *tert*-amyl alcohol was used as anaesthesia to rats, being administered intraperitoneally. A total of 2.5g of tribromoethanol was added into 10mℓ undenatured 99.5% ethanol and 1.5mℓ *tert*-amyl

alcohol. Then, 108.5mℓ normal saline was added to make up 120mℓ mixture solution. The solution was stirred overnight at room temperature until the avertin was completely dissolved. The solution was kept in a tightly sealed dark bottle at 4°C for further use.

$$\text{Amount of Avertin (m}\ell\text{)} = \frac{\text{Recommended dose (mg/kg) x Rat's weight (kg)}}{\text{Avertin concentration (mg/m}\ell\text{)}}$$

$$= \frac{\text{250mg/kg x 0.2kg}}{\text{20.83mg/m}\ell}$$

$$= \underline{\text{2.4m}\ell}$$

3.4.2 Phosphate Buffer Saline / Tween-20

A tablet of phosphate buffer saline (PBS) was dissolved in 200 mℓ of distilled water, yielded 0.01M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride. 0.2% of Tween-20 was added into PBS solution, stirring until completely dissolved. The mixture was kept at 4°C for further use.

3.4.3 Antibody diluent

Tween-20 of 0.1% was added into 200mℓ of 0.01 M PBS, mix thoroughly followed by 1% of Bovine Serum Albumin (BSA) powder. The mixture was stored at 4°C for further use.

3.5 Treatment preparation

3.5.1 Preparation of date palm

The method of Al-Qarawi *et al*., 2005 with some modifications was adopted for the preparation of aqueous date palm extracts. Briefly, dried date palm fruit (*Phoenix dactylifera*) were weighed, and seeds were removed manually. The flesh was ground into smaller particles and mixed with distilled water (1/3, w/v) using a domestic hand blender. The mixtures were stored at -4°C for further analysis.

3.5.2 Preparation of goat milk

Fresh goat milk was directly given to the rat using 10mℓ syringe. The milk was stored at -4°C.

3.5.3 Preparation of ferrous fumarate

Ferrous fumarate 33mg/100mg Tablet was prepared by mixing two iron tablets into 5mℓ of distilled water.

3.6 Animal Equivalent Dosage (AED)

The daily dose of date palm and goat milk per rat was calculated according to Animal Equivalent Dose (AED) formula (Nair & Jacob, 2016) below;

$$\text{AED (mg/kg)} = \text{Human dose (mg/kg)} \times [\text{Human } K_m / \text{Animal } K_m]$$

Where the correction factor (K_m) for humans and rats is 37 and 6 respectively. The K_m is derived by dividing the species' the average body weight in kg to its body surface area in m^2. Thus, the average human body weight of 60kg with a body surface area of $1.62m^2$ results in K_m of 37; while for a rat, the average body weight of 150g with a body surface of $0.025m^2$ results in K_m of 6. The human dose is obtained using the formula;

$$\text{Human dose (mg/kg)} = \frac{\text{Food concentration (mg/m}\ell\text{) x Volume of food (m}\ell\text{)}}{\text{Weight (kg)}}$$

Food concentration was obtained according to usual human intake: for date palm; 5 date palm flesh was weighed and mixed with distilled water (1/3, w/v) using a domestic hand blender, while for goat milk; 250mℓ goat milk is considered as a usual intake in a meal (Abreu *et al.,* 2014). Once the human dose was obtained, it is converted to animal equivalent dose (AED) stated above using constant K_m value, and the final volume of food that will be given to each individual rat was calculated using the formula;

$$\text{Volume of food (m}\ell\text{)} = \frac{\text{Animal weight (kg) x Animal dose (mg/kg)}}{\text{Food concentration (mg/m}\ell\text{)}}$$

Animal weight was the weight for each individual rat while animal dose was the AED value. Calculation of date palm, goat milk and iron tablet dosage were as follows;

A) Date palm;

Human dose $= \frac{333.33\text{mg/m}\ell \times 126\text{m}\ell}{60\text{kg}}$

$= \underline{700\text{mg/kg}}$

AED (mg/kg) $= 700\text{mg/kg} \times \frac{37}{6}$

$= \underline{431.6\text{mg/kg}}$

Volume of food (mℓ) $= \frac{0.3\text{kg} \times 4316.6\text{mg/kg}}{333.33\ \text{mg/m}\ell}$

$= \underline{\underline{3.88\text{m}\ell}}$

B) Goat milk;

Human Dose $= 250\text{m}\ell/60\text{kg}$

$= \underline{4.17\text{m}\ell/\text{kg}}$

AED (mg/kg) $= 4.17\text{m}\ell/\text{kg} \times \frac{37}{6}$

$= \underline{25.70\text{m}\ell/\text{kg}}$

Volume of food (mℓ) $= 0.3\text{kg} \times 25.70\text{m}\ell/\text{kg}$

$= \underline{\underline{7.71\text{m}\ell}}$

C) Ferrous fumerate;

Human dose $= \frac{13.2\text{mg/m}\ell \times 5\text{m}\ell}{60\text{kg}}$

$= \underline{1.1\text{mg/m}\ell}$

AED (mg/kg) $= 1.1\text{mg/kg} \times \frac{37}{6}$

$= \underline{6.78\text{mg/kg}}$

Volume of food (mℓ) $= \frac{0.3\text{kg} \times 6.78\text{mg/kg}}{13.2\ \text{mg/m}\ell}$

$= \underline{\underline{0.15\text{m}\ell}}$

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3.7 Nutritional analysis of date palm (*Phoenix dactylifera*) and goat milk

The nutritional content of date palm and goat milk was determined by sending the sample to Permulab Sdn Bhd in Petaling Jaya, Selangor. The sugar profile (glucose, galactose, fructose, lactose, maltose, and sucrose) and Vitamin C (ascorbic acid) were analysed using High-Performance Liquid Chromatography (HPLC), while Inductively Coupled Plasma Emission Spectrometry (ICP-OES) was used to investigate the mineral elements (iron, calcium, magnesium, zinc, copper, and manganese).

3.8 RBC parameters and iron profile

The collected blood was sent to the Pathlab Sdn. Bhd. Petaling Jaya, for haematological analysis. Complete Blood Count (CBC) was analysed using Sysmex XE – 2100 Automated Haematology Analyser, which employ flow cytometry concept while the iron profile was determined using Advia 2400 Chemistry System (Siemens, Germany). The reference range for Wistar rat RBC parameters and iron profile were obtained from Clinical Laboratory Parameter For Crl: WI (Han) (Giknis & Clifford, 2008), and Thakur *et al*., (2019), respectively and showed below;

Table 2: RBC parameter for Wistar rat

RBC Parameter	Reference range
Red blood cell (10^{12}/L)	7.27 - 9.65
Haemoglobin (g/dL)	13.7 - 17.6
Packed cell volume (PCV) (%)	39.6 - 52.5
Mean Corpuscular Volume (MCV) (fL)	48.9 -57.9
Mean Corpuscular Haemoglobin (MCH) (pg)	17.1 – 20.4
Mean Corpuscular Haemoglobin Concentration (MCHC) (g/dL)	32.9 - 37.5

Table 3: Iron profile for Wistar rat

Iron profile	Reference range
Serum iron (µg/dL)	114.8 ± 5.4
Transferrin saturation (%)	41.2 ± 1.4

3.9 Enzyme-Linked Immunosorbent Assay (ELISA)

Liver ferritin was determined using Rat FE (Ferritin) ELISA kit. A total of 30mg of rat's liver was homogenised in 600µl T-PER® Protein Extraction Kit and 6µl Halt™ Protease Inhibitor Cocktail EDTA-Free solution in 2.8mm acid stainless bead prefilled in 2mℓ tube for 45 seconds at 450 rpm in using The BeadBug™ Microtube Homogeniser. The homogenates were placed in a new 2mℓ microcentrifuge tube and centrifuged for 5 minutes at 10000rpm to get the supernatant. Then, 100µl of Reference Standard, Blank and liver supernatant was added per well and the plate was incubated for 90 minutes at 37°C. The liquid was removed from each wall and 100µl of Biotinylated Detection Antibody was added into each well and incubated for 1 hour at 37°C. Each well was aspirated and washed using 350µl wash buffer three times, and after the last wash, the plate was inverted and pat against thick, clean absorbent paper. After that, 100µl HRP Conjugate was added and incubated for 30 minutes at 37°C, followed by a washing step five times. Then, 90µl of Substrate Solution was added to each well and incubated for 15 minutes at 37°C, protected from light. Finally, 50µl of Stop Solution was added into the well and the optical density (OD) of each well was determined using a microplate reader set at 450 nm. The standard curve was determined using CurveExpert Basic software (Hyams Development, USA).

3.10 Calculation of iron bioavailability

The iron bioavailability was calculated as haemoglobin regeneration efficiency (HRE) (Lobo *et al.,* 2011; Mahoney *et al.*, 1974). HRE estimates of the percentage of ingested iron that is absorbed and was calculated according to total body haemoglobin iron of rats using the formula below;

$$\text{Hb Fe} = \text{BW} \times 0.067 \times \text{Hb} \times 3.35$$

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Where Hb Fe is the total body haemoglobin iron (mg); Hb is the concentration of haemoglobin (g/dL); BW is the body weight in kg; 0.067 is the volume of blood in litre per kilogram body weight (L/kg), and 3.35 is the weight of iron in mg per gram haemoglobin (mg/g). From the formula, the HRE was calculated as follow;

$$(HRE\ \%) = \frac{[Hb\ Fe\ Final - Hb\ Fe\ initial \times 100]}{Total\ Fe\ intake}$$

3.11 Gene expression of the iron metabolism-related gene

3.11.1 RNA extraction

Liver and small intestine were harvested from rats and kept in RNAlater RNA Stabilization Reagent solution to prevent RNA degradation. Tissues were cut and subjected to the RNA extraction procedure using RNAesy Mini Kit. A total of 30mg tissues were disrupted in Buffer RLT and homogenised for 45 seconds at 450 rpm using The BeadBug^{TM} Microtube Homogeniser. The solution was transferred into a new 1.5mℓ microcentrifuge tube and was centrifuged for 3 min at 13,000 rpm. The supernatant (lysate) was transferred into a new microcentrifuge and 1 volume of 50% (for small intestine) or 70% (liver) ethanol was added, and the solution was mixed immediately by pipetting up and down. The solution then was transferred into the RNeasy spin column placed in a 2mℓ collection tube and was centrifuged for 15 seconds at 13,000rpm. The flow-through in 2ml collection tube was discarded. 700µl Buffer RW1 was added into the spin column, followed by centrifugation for 15 seconds at 13,000rpm. The resulting flow through in the 2ml collection tube was discarded. Then, 500µl of Buffer RPE was added into the spin column was centrifuged for 15 seconds at 13,000 rpm. The resulting flow through in the 2ml collection tube was discarded. Then, 500µl RPE Buffer was added into the spin column and centrifuged at 10,000 rpm for 2 minutes. The resulting flow through in the 2ml collection tube was discarded. The spin

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column was placed into a new 2ml collection tube and centrifuged at full speed for 1 minute. The spin column was placed into a new 1.5ml collection tube and 60µ RNase-free water was added into the spin column and centrifuged for 1 min at 10,000rpm to elute the RNA. The RNA concentration and purity were examined using Implen Nanophotometer. The RNA was kept at -80°C for further use.

3.11.2 Gel electrophoresis

A total of 1g of agarose powder was dissolved into 100mℓ 1xLB buffer and boiled in a microwave oven for 2 minutes. The mixture was cooled down under a running water. 6µl of Diamond™ Nucleic Acid Dye was added and the mixture was stirred to disperse the dye. Then, the mixture was poured into the gel rack and a comb was inserted into the gel. The gel was left to cool down at room temperature for 1 hour. After the gel was completely solid, the comb was removed, and the gel was transferred into the chamber filled with 1xLB buffer until the gel was completely immersed. Then, 5µl of 1kb DNA ladder was loaded into the well followed by the mixture of 3µl of RNA sample, 2µl of loading dye and 2µl of distilled water. The electrophoresis was run at 120V for 25 minutes.

3.11.3 cDNA synthesis

cDNA synthesis was done using the RT^2 First Strand Kit according to the manufacturer's instructions. A separate 10µl genomic DNA elimination mix was first prepared for each RNA sample, containing 1µl of RNA (25ng-5µg), 2µl Buffer GE (gDNA elimination buffer) and 7µl RNase free water. The solution was incubated for 5 minutes at 42°C and immediately placed on ice for 1 minute. Then, 10µl reverse transcription mix containing 4µl 5x Buffer BC3, 1µl Control P2, 2µl RE3 Reverse Transcriptase Mix and 3µl RNase free water, was added into each 10µl genomic DNA

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elimination mixture for a final volume of 20µl. The solution was mixed gently by pipetting up and down before being incubated at 42°C for exactly 15 minutes. Then the mixture was incubated at 95°C for 5 minutes. 92µl of RNase free water was then added into the solution. The reaction was put on ice and proceeds with real-time PCR or kept at -20°C until further use.

3.11.4 Real-time polymerase chain reaction (qPCR)

The expression of mRNA levels in the liver and small intestine subjected to different diet regimes was investigated by qPCR using Qiagen RT^2 Profiler PCR Array.

3.11.4.1 Primer selection

Table 4 showed the Entrez gene ID and catalogue number of individual primers used in this study together with the patented controls provided with the Qiagen RT^2 Profiler PCR Array. The genomic DNA control (GDC) detects the presence of genomic DNA, the reverse transcription control (RTC) detects an artificial RNA template while positive PCR control (PPC) monitor for PCR inhibitor.

Table 4: Primer lists. The list of primers used in detecting the expression level of iron metabolism-related genes in rats. (–) denote not provided.

Gene name	Gene symbol	Catalogue number	Entrez gene ID
Divalent metal transporter 1 (DMT1)	SLC11A2	PPR45424A	25715
Duodenal cytochrome b reductase (Dcytb)	CYBRD1	PPR59406B	295669
Ferroportin (Fpn)	SLC40A1	PPR46085A	170840
Ferritin (Fth)	FTH1	PPR50795A	25319
Transferrin (Tf)	TF	PPR52888A	24825
Transferrin receptor (TfR)	TFRC	PPR067324	64678
Hepcidin (Hamp)	HAMP	PPR43953A	84604
Beta (β) actin (housekeeping gene)	ACTB	PPR06570C	-
Rps18 (housekeeping gene)	RPS18	PPR62140A	-
Genomic DNA Control (GDC)	GDC	PPR63338A	-
Reverse transcription control (RTC)	RTC	PPX63340	-
Positive PCR control (PPC)	PPC	PPX63339	-

3.11.4.2 Preparation of master mixture

The PCR components consisting of 1350µl 2x RT^2 SYBR Green Mastermix, 102µl cDNA synthesis reaction and 1248µl RNase free water totalling to 2700µl master

mix were prepared in a 5mℓ tube at room temperature. All samples were run in duplicate in 25 µL reaction mixtures, dispensed into PCR array plate and sealed with optical adhesive film.

3.11.4.3 qPCR condition

qPCR was run on Applied Biosystem StepOnePlus™ RealTime PCR System. The primer was readily coated on the bottom of 96 well plates and the layout as shown in figure 9. The PCR profile was performed with an initial denaturation at 95ºC for 10 minutes, followed by 40 cycles of denaturation step at 95˚C for 15 seconds and annealing at 60°C for 1 minute. The threshold values were defined at the value of 0.015 and ΔC_T values were determined using the following formula;

$\Delta C_T = C_T^{GOI} - C_T^{HKG}$

$\Delta\Delta C_T$ = Average C_T (Treatment Group) – Average C_T (Control Group)

Fold change expression = $2^{(-\Delta\Delta CT)}$

Where GOI: gene of interest, HKG: housekeeping gene

A1 DMT1	A2 DCYTB	A3 FPN	A4 FTH	A5 TF	A6 TFR	A7 HAMP	A8 ACTB	A9 RPS18	A10 GDC	A11 RTC	A12 PPC
B1 DMT1	B2 DCYTB	B3 FPN	B4 FTH	B5 TF	B6 TFR	B7 HAMP	B8 ACTB	B9 RPS18	B10 GDC	B11 RTC	B12 PPC
C1 DMT1	C2 DCYTB	C3 FPN	C4 FTH	C5 TF	C6 TFR	C7 HAMP	C8 ACTB	C9 RPS18	C10 GDC	C11 RTC	C12 PPC
D1 DMT1	D2 DCYTB	D3 FPN	D4 FTH	D5 TF	D6 TFR	D7 HAMP	D8 ACTB	D9 RPS18	D10 GDC	D11 RTC	D12 PPC
E1 DMT1	E2 DCYTB	E3 FPN	E4 FTH	E5 TF	E6 TFR	E7 HAMP	E8 ACTB	E9 RPS18	E10 GDC	E11 RTC	E12 PPC
F1 DMT1	F2 DCYTB	F3 FPN	F4 FTH	F5 TF	F6 TFR	F7 HAMP	F8 ACTB	F9 RPS18	F10 GDC	F11 RTC	F12 PPC
G1 DMT1	G2 DCYTB	G3 FPN	G4 FTH	G5 TF	G6 TFR	G7 HAMP	G8 ACTB	G9 RPS18	G10 GDC	G11 RTC	G12 PPC
H1 DMT1	H2 DCYTB	H3 FPN	H4 FTH	H5 TF	H6 TFR	H7 HAMP	H8 ACTB	H9 RPS18	H10 GDC	H11 RTC	H12 PPC

Figure 9: 96 well plate layout for qPCR. DMT1: divalent metal transporter 1; DCYTB: duodenal cytochrome b reductase; FPN: ferroportin; FTH: ferritin; TF: transferrin; TFR: transferrin receptor; HAMP: hepcidin; ACTB: beta (β) actin; RPS18: ribosomal protein S18; GDC: genomic DNA control; RTC: reverse transcription control; PPC: positive PCR control.

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3.12 Immunohistochemistry

Small intestine and liver were cut into approximately 5cm pieces before putting into histological cassettes and stored in 10% neutral formalin solution for 12 hours. The tissues were fixed in Leica TP1020 Automated Tissue Processor consisting of a series of toluene and graded alcohol solution, started with 70% alcohol for 1 hour, 90% alcohol for 1 hour, and series of absolute alcohol for 4hours with 1 hour in each chamber followed by three series of toluene for 1 hour per chamber. The fixed tissue was soaked in heated paraffin wax before being embedded into paraffin block using Histo Embedder (EG 1160). The paraffin block was sectioned by Manual Rotary Microtome (RM2235) producing a 3µm section ribbon. The section was floated on 40°C water and transferred to an adhesive platinum glass slide. The slide was dried for 1 hour at 37°C in drying oven before the tissue was deparaffinised and rehydrated through a series of xylene and graded alcohol; xylene 1 for 5minutes, xylene 2 for 5minutes, absolutes alcohol 1 for 3minutes, absolute alcohol 2 for 3minutes, 90% alcohol for 1minute and 80% alcohol for 1minute. The tissue then was rinsed in distilled water for 3minutes and immersed in 10% neutral buffered formalin for 30minutes at room temperature. Then, the slide was washed with distilled water and any remaining liquid was carefully wiped off using Kim wipe tissue. After deparaffinization and rehydration, the slide was incubated with hydrogen peroxidase block for 10minutes. Then, the slide was rinsed with PBS/Tween-20 (0.02% Tween-20) twice, 3minutes each. Then, the tissue section was subjected to heat-induced epitope retrieval (HIER) treatment where the slide was immersed in a Coplin jar containing Target Retrieval Solution pH 6. The slide was incubated at 95°C for 1 hour and was left cooled at room temperature for 20minutes before rinsing with PBS/Tween 20 three times, 3minutes each. Protein block was applied on the slide for 10minutes at room temperature, before rinsing once in

PBS/Tween-20 for 3minutes. Then, the slide was incubated with a primary antibody with appropriate dilution overnight at 4°C. The slide was then rinsed with PBS/Tween-20 three times; 3minutes each. The slide then was incubated in labelled polymer-HRP anti-Rabbit (Rabbit specific HRP/DAB Detection Kit-Micro-polymer), for 15 minutes at room temperature. Table 5 showed the details of each antibody with respective optimised dilution;

Table 5: The antibodies used in IHC. The antibody catalogue number and optimised dilution used in the IHC

Protein	Antibody	Catalogue Number	Dilution
DMT1	Anti-DMT1 antibody	ab140977 (Abcam)	1:4000
TfR	Anti-Transferrin Receptor antibody	ab84036 (Abcam)	1:6000
Ferroportin	Slc40A1 Polyclonal Antibody	bs-4906R (Bioss Antibodies)	1:4000
Dcytb	CYBR1 Polyclonal Antibody	bs-8297R (Bioss Antibodies)	1:1000

After that, all the tissue was rinsed with PBS/Tween20 four times, 3minutes each before visualizing using diluted DAB chromogen for 5minutes. The reaction was stopped with PBS/Tween-20 rinsing, 4 times, 3minutes each before being counterstained with haematoxylin for 1minute. The slide was rinsed with distilled water and dipped in bluing reagent for 1minute followed by distilled water rinsing for 2minutes. The slide was then dehydrated in a series of graded alcohol started with 95% alcohol for 2minutes, absolute alcohol twice for 3minutes each and lastly soaked twice in xylene solution for 5minutes each, before mounting coverslip onto the slide. The slide was left dried for 2 days before being examined under a microscope. Immunostaining was analysed using an Olympus BX51 microscope, equipped with x10, x20, and x40 dry objectives and an x100 oil immersion objective. Images were collected digitally with an Olympus C7070 camera and analysed with LS Research software. Cellular localisation (nuclear, cytoplasmic, cell surface) and staining intensity were determined and scored independently by two pathologists. The intensity was graded as 0 (no expression), 1 (weak), 2 (moderate) and 3 (strong) (Brookes *et al*., 2006).

3.13 Statistical analysis

The results were presented as mean ± SEM. All statistical data were analysed using SPSS 23.0 software (SPSS Inc, Chicago, USA). Paired T-Test was used to evaluate significant differences pre- and post- treatment, whereas one-way ANOVA was used to analyse the significant differences between groups, followed by Tukey Post Hoc test. Significant differences were defined as p values less than 0.05. For IHC, statistically significant was analysed using Mann-Whitney test and significance was accepted at $p<0.05$.

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