CHAPTER 4

RESULTS

4.1 Nutritional composition of date palm and goat milk

Table 6 showed the chemical composition of Ajwa date palm and goat milk. The Ajwa date flesh contains a high level of sugar with every 100g contains 31.6g glucose and 29.5g fructose, respectively. Sucrose, galactose, lactose and maltose were not detected. For mineral trace, in every kilogram of date palm flesh, it contains 536 mg calcium, 418 magnesium, 10.70mg of iron, 5.23mg manganese and 2.39mg zinc. Copper was not determined. 9.80mg ascorbic acid was detected in 100g date. For goat milk, 3.38g of lactose was detected in every 100mℓ milk. No other sugars were detected. Goat milk also contains 0.90mg iron, 0.99mg zinc, 722mg calcium and 1mg magnesium per 1ℓ of milk. Ascorbic acid and manganese, however, were not detected in goat milk.

Table 6: Nutritional composition of date palm and goat milk. Composition of sugars, minerals and vitamin (in fresh weight) of Ajwa date palm and goat milk

Chemical composition	Date Palm (Ajwa)	Goat Milk
Sugars	(g/100g)	(g/100mℓ)
Glucose	31.60	nd
Sucrose	nd	nd
Galactose	nd	nd
Fructose	29.50	nd
Lactose	nd	3.38
Maltose	nd	nd
Mineral	(mg/kg)	(mg/ℓ)
Iron	10.70	0.90
Manganese	5.23	nd
Zinc	2.39	0.99
Copper	nd	nd
Calcium	536	722
Magnesium	418	100
Vitamin	(mg/100g)	(mg/100mℓ)
Ascorbic Acid	9.80	nd

*nd indicate not determined

4.2 Analysis of body weight

Table 7 depicts the variations in body weight of Wistar rats. After 2 weeks of a low iron diet, the mean bodyweight of the rats in the normal control and IDA induced group (Group 2 – Group 6) did not differ significantly (p>0.05). After 4 weeks of intervention, all groups showed a significant increase (p<0.05) in mean body weight. The bodyweight of positive control rat post-intervention showed a significant difference (p<0.05) when compared to the negative control. Higher body weight increment was also detected in all intervention groups as compared to normal and negative control.

Table 7: The effects of date palm and goat milk supplementation on the bodyweight of IDA-induced rats.

Group	Bodyweight (kg) (2 weeks after iron deprivation)	Bodyweight (kg) (4 weeks after iron repletion)	Bodyweight differences (kg)
Normal control	0.21 ± 0.01	0.30 ± 0.01*	0.09 ± 0.00
Negative control	0.21 ± 0.00	0.28 ± 0.01*	0.07 ± 0.01
Positive control	0.21 ± 0.01	0.35 ± 0.01*, [a]	0.14 ± 0.00
Date Palm (DP)	0.21 ± 0.00	0.33 ± 0.02*	0.12 ± 0.01
Goat Milk (GM)	0.20 ± 0.01	0.32 ± 0.01*	0.12 ± 0.01
Date palm and goat milk (DPGM)	0.22 ± 0.01	0.32 ± 0.02*	0.11 ± 0.01

All parameters are presented as mean ± SEM.
* indicates significant differences (p <0.05) pre - and post- treatment.
[a] indicates significant difference (p <0.05) compared to negative control.

4.3 Analysis of RBC parameter and iron profile

IDA is a nutritional condition in which the blood lacks sufficient healthy red blood cells as a consequence of the body's inadequate iron levels. IDA is diagnosed by performing laboratory tests such as full blood count which include the measurement of RBC, Hb, MCV, MHC and platelet count. Additional tests, iron profile, was also done to further evaluate the level of iron in the body. The RBC parameter and iron profile were shown below and the normal reference range for Wistar rat was obtained from Clinical Laboratory Parameter For Crl: WI (Han) (Giknis & Clifford, 2008) and Thakur *et al.*, (2019), respectively.

4.3.1 Red blood cell (RBC) count

Iron deficiency in IDA disturbed the production of red blood cells reflected by the decreased level of RBC count. Based on figure 10, after 4 weeks of intervention, the RBC count returned to normal range with significant increased were seen in group supplemented with date palm (DP), date palm and goat milk (DPGM) and ferrous fumarate tablet (positive control) with final RBC value of 8.25 $x10^{12}$/L, 8.60$x10^{12}$/L and 8.63$x10^{12}$/L respectively. Negative control with continuous low iron diet showed significant deterioration (p<0.05) of RBC with a final count of 3.65$x10^{12}$/L. All groups showed significant differences when compared to the negative control.

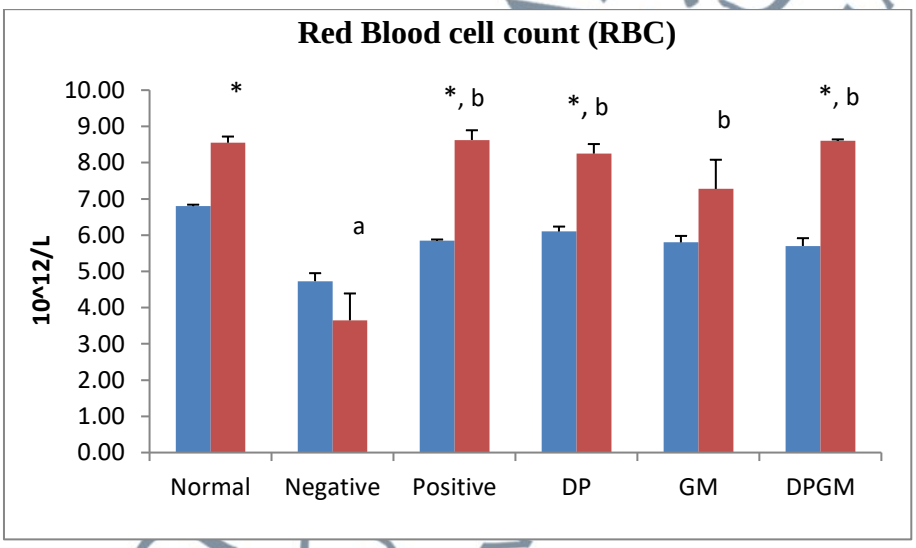


Figure 10: Red blood cell count in IDA induced rats supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre - and post-intervention. a and b indicate significant differences (p<0.05) as compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk. Reference range: 7.27- 9.65 x 10^{12}/L.

4.3.2 Haemoglobin level

A reduced Hb level indicates impaired Hb production. Figure 11 showed that after the consumption of a low iron diet for 2 weeks, the rats were anaemic with a mean blood Hb level of 10g/dL, significantly lower (p<0.05) than the normal control group with a Hb level of 13.50g/dL. After the supplementation with date palm and goat milk, the Hb level significantly increased (p<0.05), with date palm group (DP), showed improvement in Hb level from 10.30g/dL to 15.55g/dL, date palm and goat milk group (DPGM) from 10.35 g/dL to 15.58 g/dL and lastly positive control group from 10.35g/dL to 16.18. There was an increase in Hb concentration in the goat milk group (GM), from 10.03 g/dL to 13.85 g/dL, however, the increase was not significant. The continued supply of low iron diet significantly decreased (p<0.05) Hb level with a final Hb concentration of 6.68g/dL was observed in the negative control group.

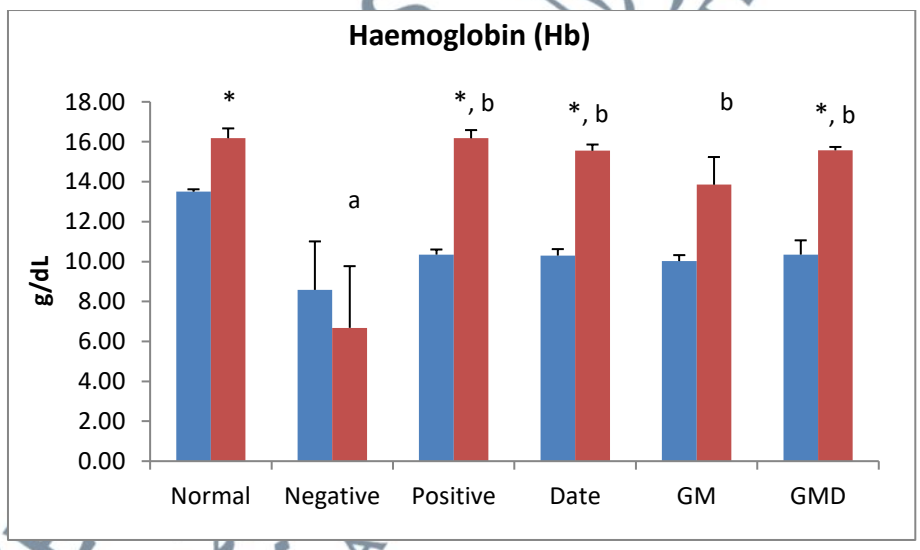


Figure 11: Hemoglobin concentration in IDA-induced rats supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre - and post-intervention. a and b indicate significant differences (p<0.05) as compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk. Reference range: 13.7-17.6 g/dL.

4.3.3 Packed cell volume (PCV)

Packed cell volume (PCV) commonly known as haematocrit, is a measurement of red blood cell volume in circulating blood, determined by cell number and size. Based on figure 12, after 2 weeks of iron depletion, the baseline PCV value is below the normal range of 39.6%. PCV level increased after the IDA induced rats were given intervention supplementation for 4 weeks, in which significant increase (p<0.05) were seen in IDA induced rats supplemented with date palm (DP) with PCV value of 49.25%, in date palm and goat milk (DPGM); 50.25% and positive control; 52.50%. The negative control group displayed a significant decrease (p<0.05) in PCV level with a final value of 16.75% All intervention groups showed a significant difference (p<0.05) when compared to the negative control.

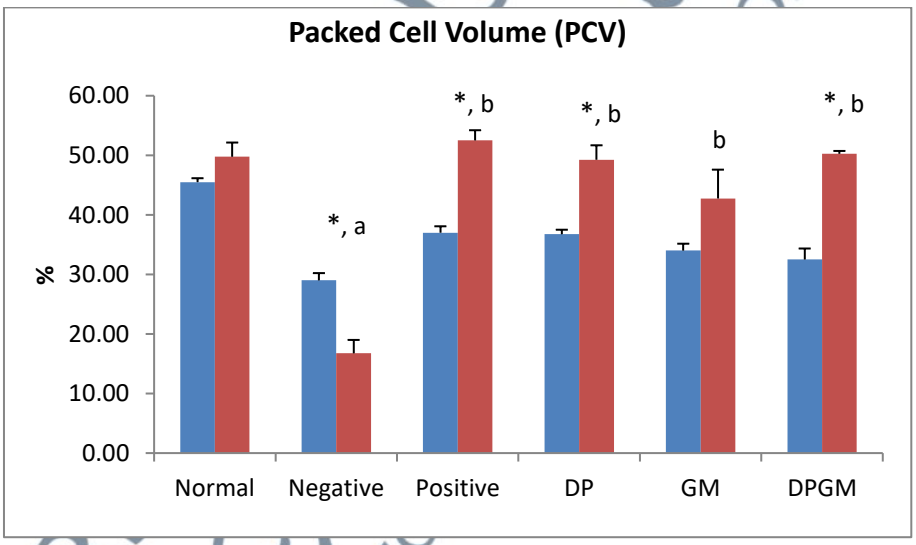


Figure 12: Packed cell volume in IDA induced rats supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre-and post-intervention. a and b indicate significant differences (p<0.05) as compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk. Reference range: 39.6-52.5%

4.3.4 Mean corpuscular volume (MCV)

Mean corpuscular volume (MCV) is the average volume of RBC, that signify the average size of RBC. From figure 13, the negative control group showed a significant decrease (p<0.05) in MCV level, declining from 61.50fl to 44.50 fL. A decrease in MCV value was also observed in the normal control group, decreasing from 67fl to 58fl, however, it is not significant. The level of MCV for the rest of the study groups showed insignificant different pre and post treatment.

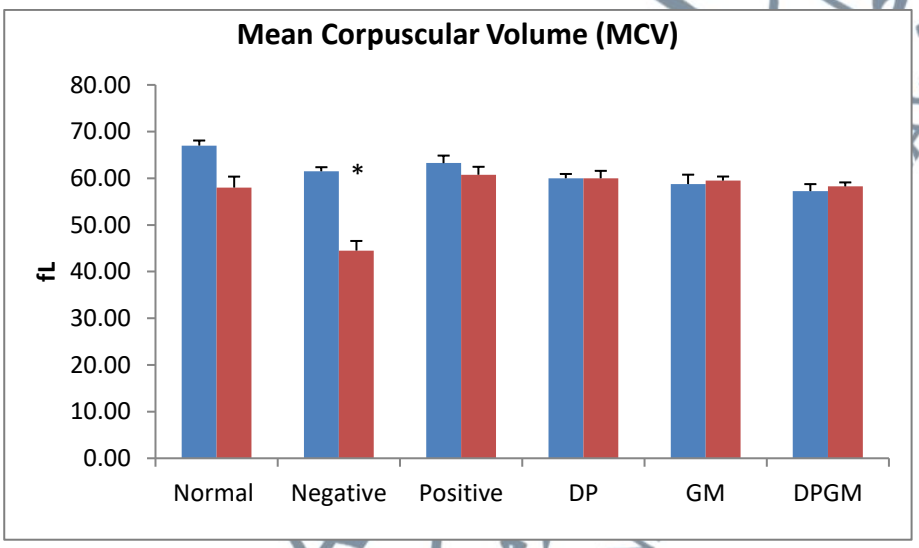


Figure 13: Mean Corpuscular Volume in IDA induced rat supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre-and post-intervention. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk. Reference range: 48.9 -57.9 fL.

4.3.5 Mean corpuscular haemoglobin (MCH)

Mean corpuscular haemoglobin (MCH) measure the average mass of haemoglobin per red blood cell. MCH level was significantly decreased ($p<0.05$) from 18.25 to 15.75pg in negative control group (figure 14). The rest of study groups showed insignificant changes in the MCH value pre and post treatment.

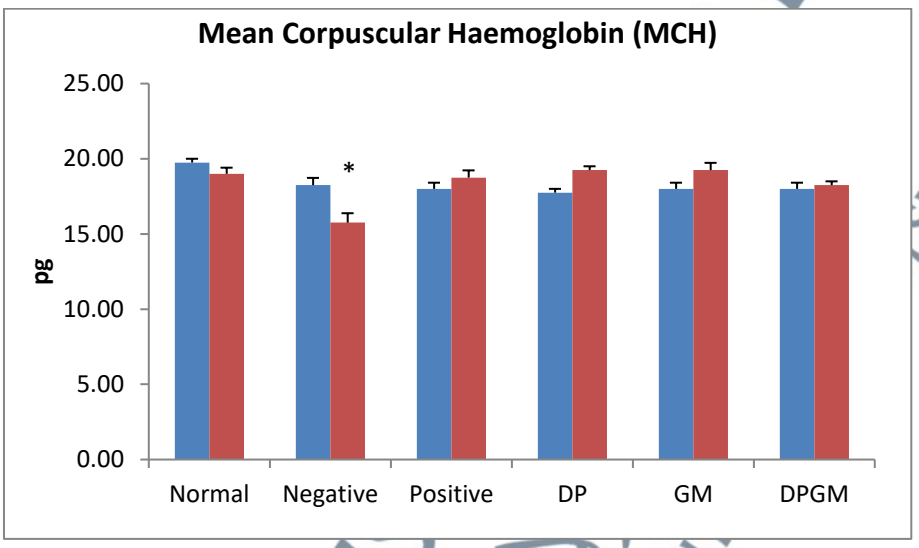


Figure 14: Mean Corpuscular Haemoglobin level in IDA induced rat supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences ($p<0.05$) pre-and post-intervention. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk. Reference range: 17.1 – 20.4pg.

4.3.6 Mean corpuscular hemoglobin concentration (MCHC)

Mean corpuscular hemoglobin concentration (MCHC) measure the haemoglobin concentration in each volume of packed red blood cells. Based on figure 15, after 2 weeks of iron depletion, the baseline value of MCHC in all study groups were below the normal range of 32.9g/dL with value ranging from 28.75g/dL to 31.50g/dL. A significant increase (p<0.05) was seen in negative control where the MCHC level increased from 29.75g/dL to 37.00g/dL. The rest of the intervention groups showed insignificant changes in the MCHC value pre and post treatment.

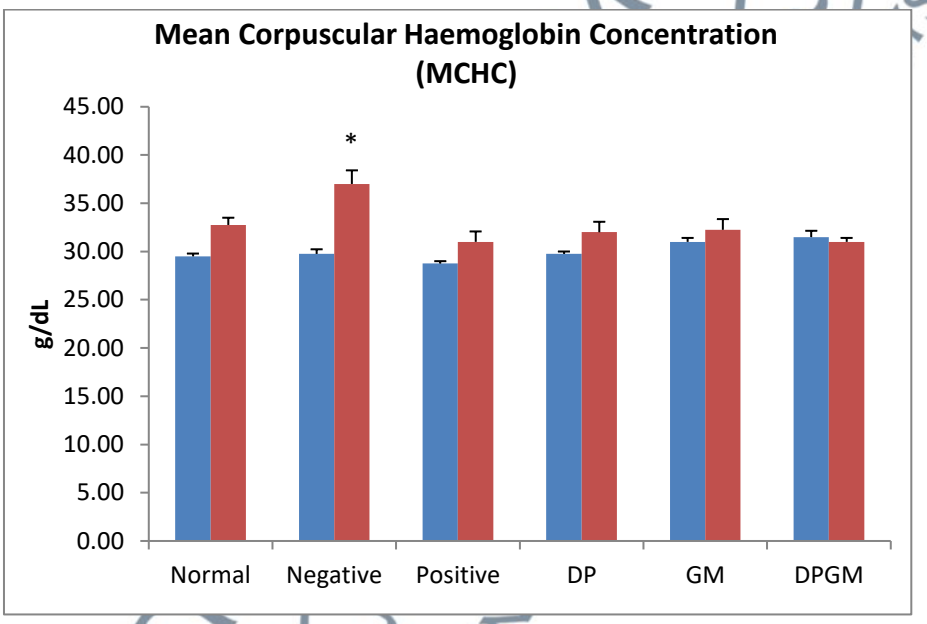


Figure 15: Mean Corpuscular Haemoglobin Concentration (MCHC) in IDA induced rats supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre-and post-intervention. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk. Reference range: 32.9- 37.5 g/dL

4.3.8 Serum Iron

Figure 16 showed that a low iron diet for 2 weeks successfully induced iron deficiency in rats, with serum iron levels ranging from 6.45µmol/L to 11.30µmol/L, as compared to normal control (34.15 µmol/L). Following 4 weeks of intervention, a significant increase (p<0.05) in serum iron level was observed in all intervention groups. The serum iron level was significantly increased from 8.53µmol/L to 31.95µmol/L for date palm group (DP), from 11.30µmol/L to 30.48µmol/L for goat milk group (GM), from 7.23µmol/L to 31.93µmol/L for date palm and goat milk group (DPGM) and from 6.45 µmol/L to 29.35 µmol/L for positive control. A significant decrease (p<0.05) in serum iron level was seen in the negative control group with a final value of 3.13µmol/L from 11.53 µmol/L. All groups showed significant differences (p<0.05) when compared to the normal and negative control.

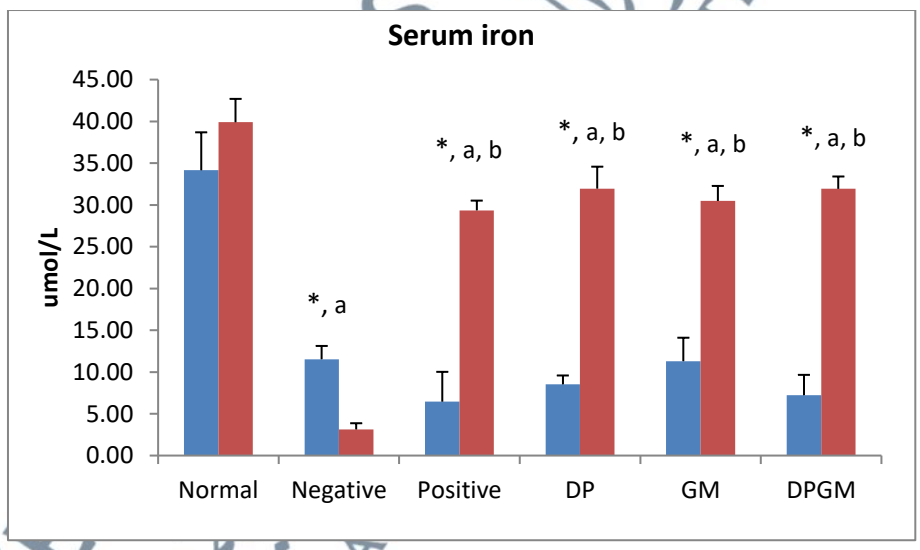


Figure 16: Serum iron level in IDA induced rat supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre-and post-intervention. a and b indicate significant differences (p<0.05) as compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.3.9 Transferrin saturation

Iron circulates throughout the body in transferrin bound form. Transferrin maintains Fe^{3+} in a redox-inert state and deliver it safely to the body's tissue. Figure 17 showed the level of transferrin saturation. After iron depletion, transferrin saturation level was significantly reduced (p<0.05) in all IDA induced groups with transferrin saturation levels ranging from 11.25% to 25%, as compared to normal control with transferrin saturation of 53%. A significant increase (p<0.05) was seen in the positive control, date palm (DP), goat milk (GM) and date palm and goat milk (DPGM) post-intervention. Even though not significant, the final transferrin level in intervention groups was still lower than that of normal control with a final transferrin level of 89.25%. Meanwhile, negative control showed expected declination with transferrin saturation significantly reduced (p<0.05) from 20.50% to 6.75%.

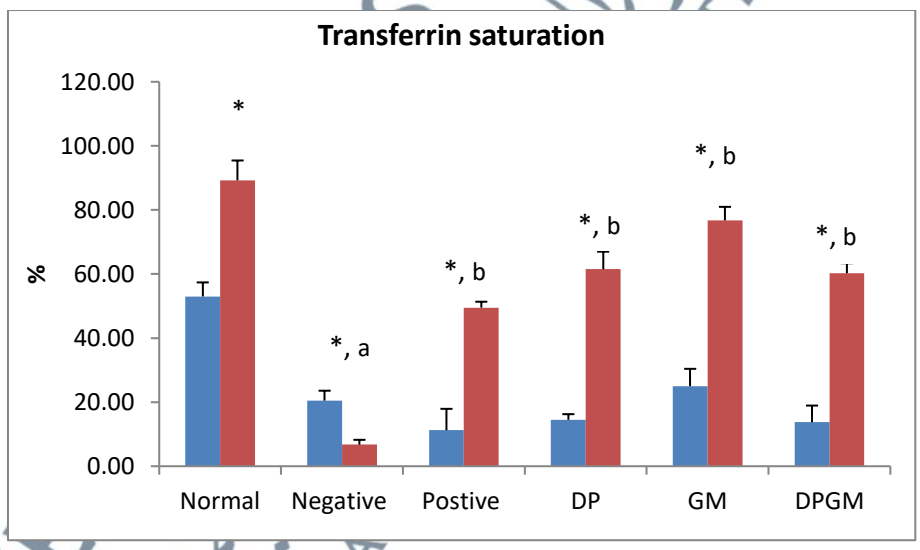


Figure 17: Transferrin saturation level in IDA induced rats supplemented with date palm and goat milk. All parameters are presented as mean ± SEM. * indicates significant differences (p<0.05) pre-and post-intervention. a and b indicate significant differences (p<0.05) as compared to normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.3.10 Ferritin

Ferritin is the intracellular iron storage protein that store and release iron in a tightly regulated manner. The expression of ferritin increases when the cellular iron level high, and vice versa. As hepatocytes have a high capacity for the iron storage, rat's liver was used to determine the level of ferritin. In this study, the level of ferritin was determined using ELISA method. Figure 18 showed that no significant difference was observed between all study groups regardless of diet.

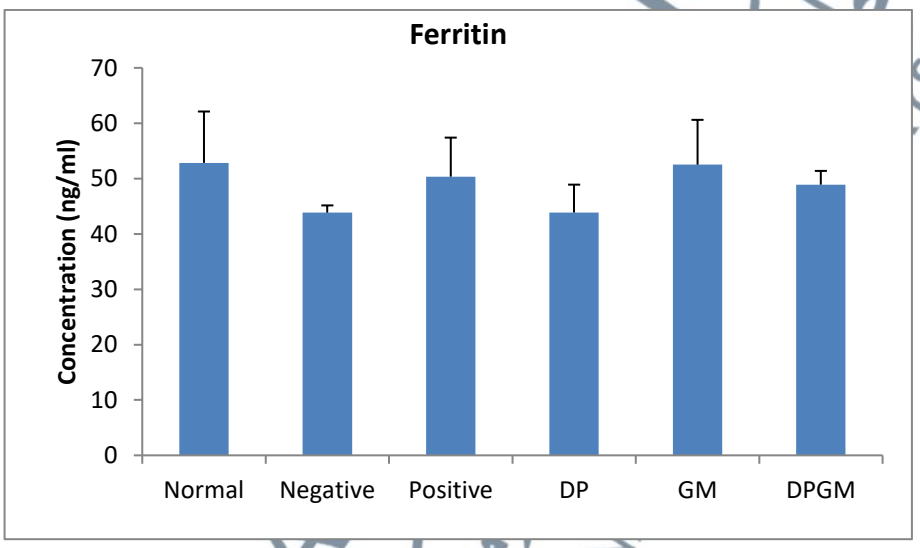


Figure 18: Ferritin level in IDA induced rats supplemented with date palm and goat milk. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk

Table 8: RBC parameters and iron profile of the rat after 2 weeks of anaemia induction

Haematological value	Normal control	Negative control	Positive control	Date Palm (DP)	Goat milk (GM)	Date Palm and goat milk (DPGM)
RBC (109/L)	6.80 ± 0.04	4.73±0.22a	5.85 ± 0.06 [a, b]	6.10 ± 0.27 [b]	5.80 ± 0.36 [a, b]	5.70 ± 0.43 [a, b]
Hb (g/dL)	13.50 ± 0.12 [a, b]	8.58 ± 2.43 [a, b]	10.35 ± 0.51 [a, b]	10.30 ± 0.65 [a, b]	10.03 ± 0.60 [a, b]	10.35 ± 1.43 [a, b]
PCV (%)	45.50 ± 0.65	29.00 ± 1.22 [a]	37.00 ± 2.16 [a, b]	36.75 ± 1.50 [a, b]	34.00 ± 2.31[a]	32.50 ± 3.70 [a]
MCV (fL)	67.00 ± 1.08	61.50 ± 0.87	63.25 ± 3.20	60.00 ± 1.83 [a]	58.75 ± 4.03 [a]	57.25 ± 2.99 [a]
MCH (pg)	19.75 ± 0.25	18.25 ± 0.48	18.00 ± 0.82	17.75 ± 0.50 [a]	18.00 ± 0.82 [a]	18.00 ± 0.82 [a]
MCHC (g/dL)	29.50 ± 0.29	29.75 ± 0.48	28.75 ± 0.50	29.75 ± 0.50	31.00 ± 0.82	31.50 ± 1.29 [a]
Serum Iron (µmol/L)	34.15 ± 4.54	11.53 ± 1.59 [a]	6.45 ± 7.14 a	8.53 ± 2.12 [a]	11.30 ± 5.61 [a]	7.23 ± 4.87 [a]
Transferrin (%)	53.00 ± 4.38	20.50 ± 3.07 [a]	11.25 ± 13.33 [a]	14.50 ± 3.51 [a]	25.00 ± 10.80 [a]	13.75 ± 10.37 [a]

All parameters are presented as mean ± SEM

[a, b] within the same row indicates significant differences ($p<0.05$) as compared to the normal and negative control, respectively.

Table 9: RBC parameters and iron profile of the IDA-induced rat after 4 weeks of intervention with date palm and goat milk

Haematological value	Normal control	Negative control	Positive control	Date palm (DP)	Goat milk (GM)	Date palm and goat milk (DPGM)
RBC (109/L)	8.55 ± 0.17*	3.65 ± 0.74 [a]	8.63 ± 0.54*	8.25 ± 0.53*	7.28 ± 1.61	8.60 ± 0.08*
Hb (g/dL)	16.18 ± 0.49*	6.68 ± 3.09	16.18 ± 0.82*	15.55 ± 0.62*	13.85 ± 2.77	15.58 ± 0.33*
PCV (%)	49.75 ± 2.39	16.75 ± 2.25*	52.50 ± 3.42*	49.25 ± 4.86*	42.75 ± 9.67	50.25 ± 0.96*
MCV (fL)	58.00 ± 2.35	44.50 ± 2.06*	60.75 ± 3.40	60.00 ± 3.16	59.50 ± 1.73	58.25 ± 1.71
MCH (pg)	19.00 ± 0.41	15.75 ± 0.63*	18.75 ± 0.96	19.25 ± 0.50*	19.25 ± 0.96	18.25 ± 0.50
MCHC (g/dL)	32.75 ± 0.75*	37.00 ± 1.41*	31.00 ± 2.16 [a]	32.00 ± 2.16 [a]	32.25 ± 2.22 [a]	31.00 ± 0.82 [a]
Serum Iron (µmol/L)	39.90 ± 2.79*	3.13 ± 0.74*, [a]	29.35 ± 2.3*, [a, b]	31.95 ± 5.26*, [b]	30.48 ± 3.59*, [a, b]	31.93 ± 2.95*, [b]
Transferrin (%)	89.25 ± 6.18*	6.75 ± 1.49*, [a]	49.50 ± 3.70*, [a, b]	61.50 ± 10.97*, [a, b]	76.75 ± 8.42*, [a, b, c]	60.25 ± 6.24*, [a, b]
[∞]Ferritin	52. 84 ± 9.27	43.85 ± 1.29	50.35 ± 7.045	43.87 ± 5.03	52.52 ± 8.09	48.90 ± 2.48

All parameters are presented as mean ± SEM

* indicates significant differences ($p<0.05$) pre and post-intervention.

[a, b, c] within the same row indicates significant differences ($p<0.05$) as compared to the normal, negative and positive control, respectively

[∞]Ferritin was determined using ELISA method

4.4 Analysis of iron bioavailability using HRE.

Haemoglobin regeneration efficiency (HRE) estimates the percentage of ingested iron that is absorbed. To calculate the HRE, the rat's weight and Hb iron content, both in the initial stage and final stage were used (table 10). The negative control group showed a reduced final Hb iron content of 4.06mg, lower compared to normal control, positive control and intervention groups with final Hb iron content ranging from 9.77mg to 12.71mg. HRE percentage in negative control was also significantly higher (p<0.05) with the value of 21.7% compared to normal control with 3.7%, positive control with 5.2%, date palm group with 5.58%, goat milk group with 4.58% and date palm and goat milk group with 5.06%. This result indicates that all available iron is fully used and utilised for the erythropoiesis process. Additionally, all intervention groups showed no significant difference when compared to normal control and they also showed a similar HRE percentage

Table 10: Haemoglobin regeneration efficiency (HRE) of IDA-induced rat supplemented with date palm and goat milk.

Group	Normal control	Negative control	Positive control	Date palm	Goat milk	Date palm and goat milk
Initial body weight (kg)	0.21 ± 0.01	0.21 ± 0.00	0.21 ± 0.01	0.21 ± 0.00	0.20 ± 0.01	0.22 ± 0.01
Initial Hb (g/L)	135.0 ± 1.22	83.0 ± 3.94	103.5 ± 2.53	103.0 ± 3.24	100.3 ± 2.98	103.5 ± 7.14
Initial Hb Fe (mg)	6.36 ± 0.24	3.91 ± 0.21	4.89 ± 0.29	4.80 ± 0.20	4.5 ± 0.29	5.01 ± 0.42
Final Body weight (kg)	0.30 ± 0.01	0.28 ± 0.01	0.35 ± 0.01	0.33 ± 0.02	0.32 ± 0.01	0.32 ± 0.02
Final Hb (g/L)	161.75 ± 4.87	65.3 ± 1.93	161.7 5± 4.11	155.50 ± 3.12	138.50 ± 13.87	155.75 ± 1.65
Final Hb Fe (mg)	10.91 ± 0.42	4.06 ± 0.14	12.71 ± 0.54	11.36 ± 0.68	9.77 ± 0.77	11.29 ± 0.69
HRE (%)	3.7 ± 0.44 *	21.7 ± 4.25	5.2 ± 0.19*	5.58 ± 0.41*	4.58 ± 0.92 *	5.06 ± 0.65*

All parameters are presented as mean ± SEM, (n=24).

* indicates significant differences (p<0.05) as compared to negative control

4.5 Expression of iron metabolism-related genes by qPCR

4.5.1 RNA concentration and purity

The concentration of the RNA for the small intestine and liver were listed below; showing the high quality of RNA obtained from the respective organ in all different intervention groups. The nucleic acid concentrations of the samples were high, ranging from 204ng/mℓ to 2146ng/mℓ for the small intestine (table 11) and 432ng/µl to 3168ng/µl for the liver (table 12).

Table 11: RNA concentration and purity of small intestine in rats

Sample	RNA concentration (ng/µl)	260/280	260/230
Normal control 1	1184	2.063	2.168
Normal control 2	1150	2.061	2.416
Normal control 3	442	2.046	1.826
Normal control 4	410	2.000	1.787
Negative control 1	1042	2.076	2.255
Negative control 2	372	2.090	2.188
Negative control 3	1658	2.057	2.217
Negative control 4	410	2.071	2.135
Positive control 1	850	2.043	1.932
Positive control 2	364	2.068	2.022
Positive control 3	2146	2.056	2.249
Positive control 4	2084	2.063	2.241
Date palm 1	714	2.052	2.151
Date palm 2	976	2.050	1.906
Date palm 3	754	2.083	2.271
Date palm 4	374	2.055	1.731
Goat milk 1	372	2.044	1.603
Goat milk 2	886	2.060	2.161
Goat milk 3	716	2.094	1.642
Goat milk 4	456	1.744	1.968
Date palm & goat milk 1	204	2.040	1.759
Date palm & goat milk 2	246	2.050	1.892
Date palm & goat milk 3	716	2.081	2.325
Date palm & goat milk 4	1230	2.085	2.261

Table 12: RNA concentration and purity of liver in rats

Sample	RNA concentration (ng/µl)	260/280	260/230
Normal control 1	1444	2.045	2.017
Normal control 2	1264	2.052	2.478
Normal control 3	1402	2.068	2.184
Normal control 4	546	2.053	1.468
Negative control 1	1362	2.057	2.225
Negative control 2	540	2.077	1.929
Negative control 3	1658	2.057	2.217
Negative control 4	2814	2.039	1.813
Positive control 1	432	2.057	2.038
Positive control 2	2922	2.075	2.220
Positive control 3	2144	2.086	2.276
Positive control 4	1818	2.066	2.222
Date palm 1	2622	2.023	2.153
Date palm 2	2998	2.015	2.135
Date palm 3	2318	2.048	2.077
Date palm 4	792	2.063	1.021
Goat milk 1	1036	2.056	2.223
Goat milk 2	2346	2.026	2.152
Goat milk 3	2698	2.053	2.141
Goat milk 4	1898	2.059	2.133
Date palm & goat milk 1	852	2.058	1.954
Date palm & goat milk 2	2500	2.073	2.205
Date palm & goat milk 3	3168	2.031	2.161
Date palm & goat milk 4	2966	2.048	2.200

4.5.2 Gel electrophoresis of extracted RNA

The extracted RNA was analysed through agarose gel electrophoresis. A mixture of 2µl of RNA sample, 2µl of Loading Dye and 3µl of RNase free water was loaded into the gel. 28S and 18S rRNA band were observed in all groups for both the small intestine and liver. No other higher molecular weight molecules were detected. Figure 19 to Figure 24 showed the results of the RNA extraction.

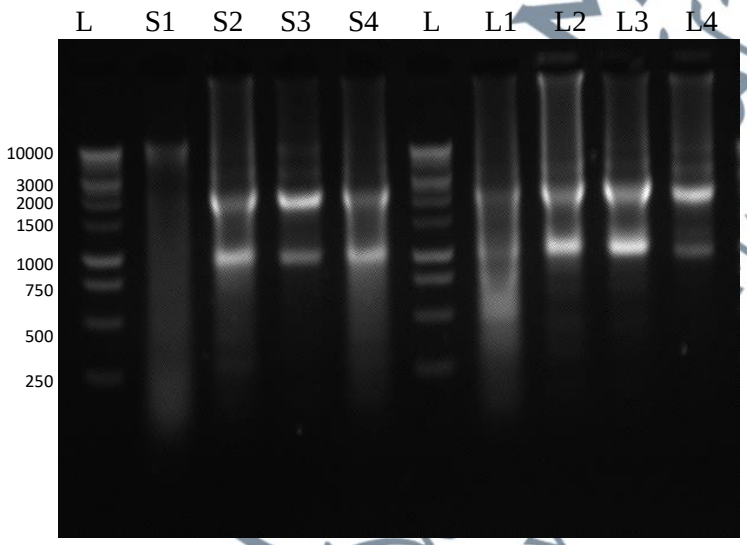


Figure 19: Total RNA extracted from small intestine and liver of normal control group were fractioned on 1% agarose gel, which showed 28S and 18S rRNA band. L: 1kb DNA ladder, Lane S1-S4: Small intestine from rat 1-4, Lane L1 - L4: Liver from rat 1-4.

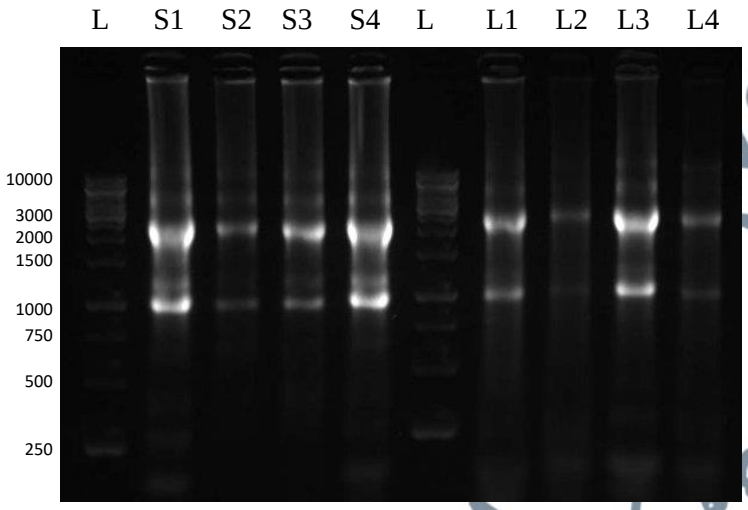


Figure 20: Total RNA extracted from small intestine and liver of negative control group were fractioned on 1% agarose gel, which showed 28S and 18S rRNA band. L: 1kb DNA ladder, Lane S1-S4: Small intestine from rat 1-4, Lane L1-L4: Liver from rat 1-4.

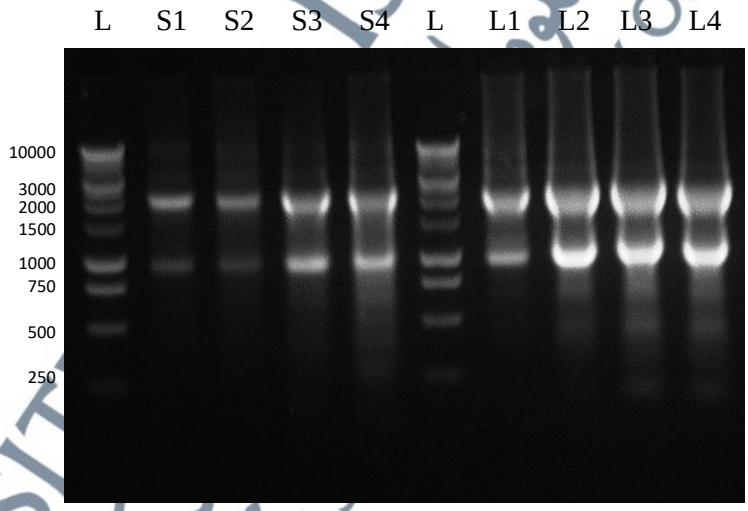


Figure 21: Total RNA extracted from small intestine and liver of positive control group were fractioned on 1% agarose gel, which showed 28S and 18S rRNA band. L: 1kb DNA ladder, Lane S1-S4: small intestine from rat 1-4, Lane L1-L4: liver from rat 1-4.

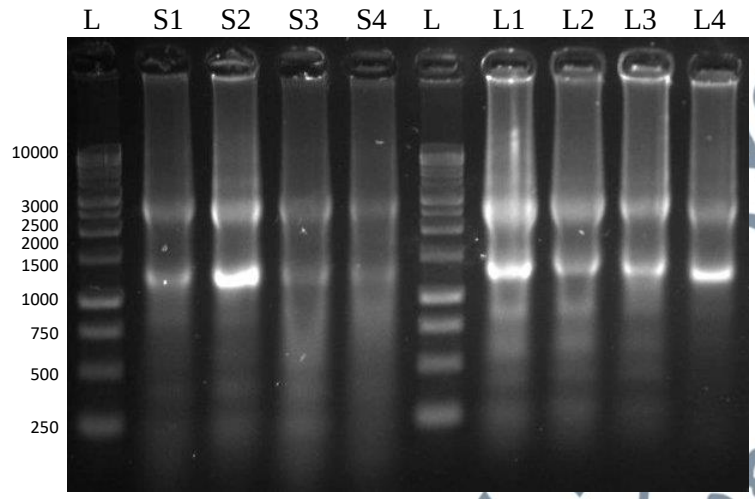


Figure 22: Total RNA extracted from small intestine and liver of date palm treated group were fractioned on 1% agarose gel, which showed 28S and 18S rRNA band. L: 1kb DNA ladder, Lane S1-S4: Small intestine from rat 1-4, Lane L1-L4: Liver from rat 1-4.

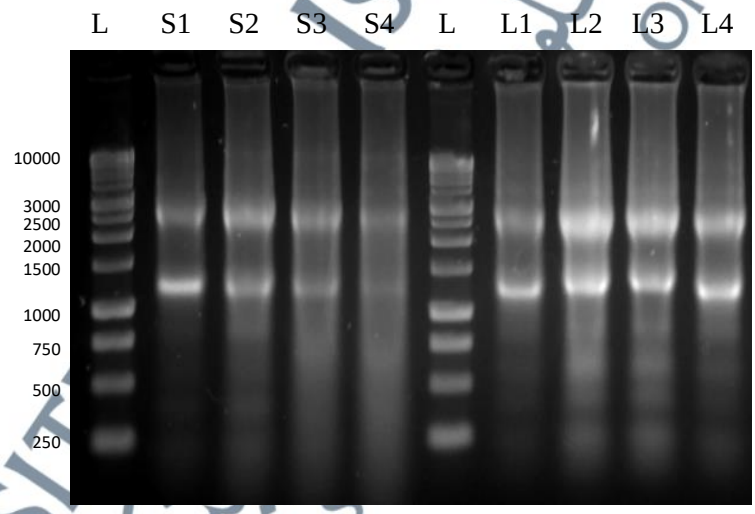


Figure 23: Total RNA extracted from small intestine and liver of goat milk treated group were fractioned on 1% agarose gel, which showed 28S and 18S rRNA band. L: 1kb DNA ladder, Lane S1-S4: Small intestine from rat 1-4, Lane L1-L4: Liver from rat 1-4.

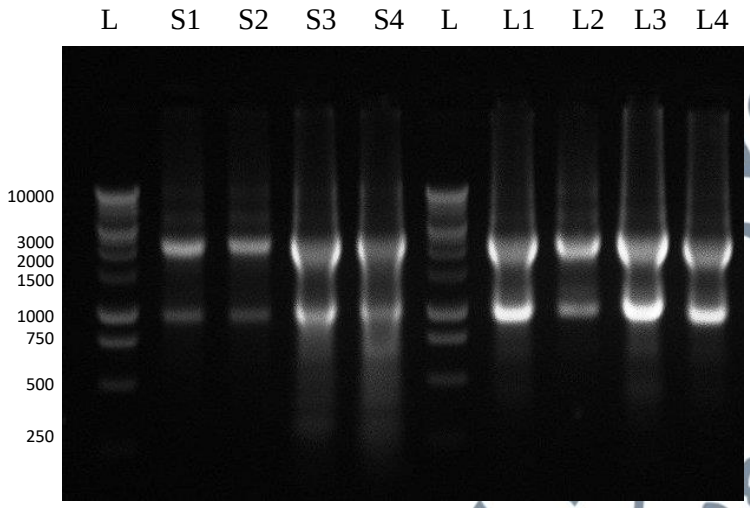
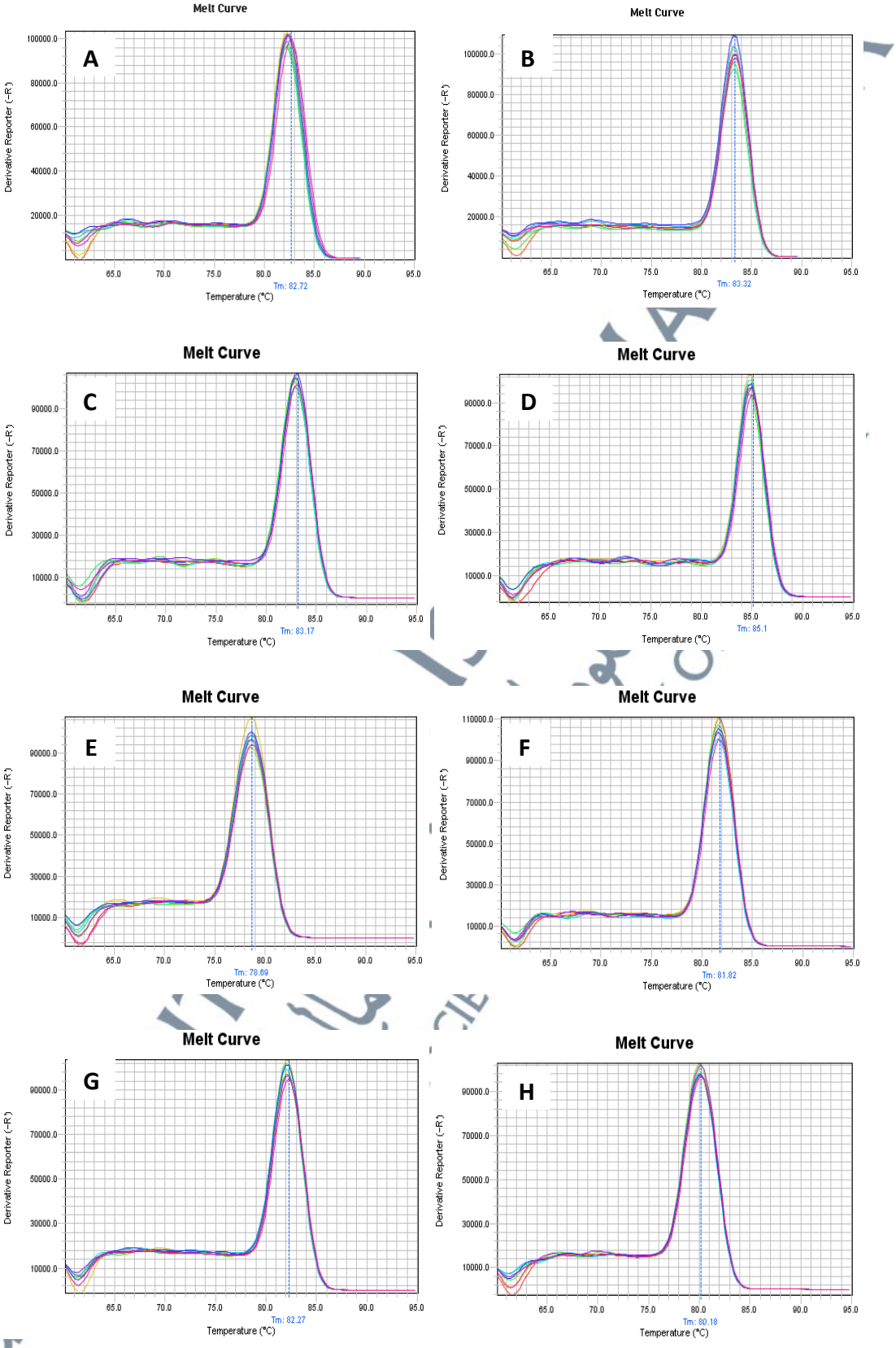


Figure 24: Total RNA extracted from small intestine and liver of date palm and goat milk treated group were fractioned on 1% agarose gel, which showed 28S and 18S rRNA band. L: 1kb DNA ladder, Lane S1-S4: Small intestine from rat 1-4, Lane L1-L4: Liver from rat 1-4.

4.5.3 Validation of reference gene and gene of interest primers

Primer pair specificity was tested in qPCR by examining the melting curve performed at the end of the reaction. The single peak with no shouldering suggested the specificity of primer annealing. Figure 25 (A) and (B) showed sharp melting profiles were observed for housekeeping gene, β-actin, and ribosomal protein S18. A similar pattern was detected for all the iron metabolism-related genes (figure 25 (C) – (I)) in which a single peak of respective temperature was observed. The melt curve of genomic DNA contamination (GDC) (figure 25 (J)) displayed multiple individual peaks indicating that the degree of genomic DNA contamination was too low to influence the gene expression profiling results. Table 13 showed melting point (T_m) for respective iron metabolism-related gene primer.


Melt Curve
A
Derivative Reporter (-R')
Temperature (°C)
Tm: 82.72
Melt Curve
B
Derivative Reporter (-R')
Temperature (°C)
Tm: 83.32
Melt Curve
C
Derivative Reporter (-R')
Temperature (°C)
Tm: 83.17
Melt Curve
D
Derivative Reporter (-R')
Temperature (°C)
Tm: 85.1
Melt Curve
E
Derivative Reporter (-R')
Temperature (°C)
Tm: 78.69
Melt Curve
F
Derivative Reporter (-R')
Temperature (°C)
Tm: 81.82
Melt Curve
G
Derivative Reporter (-R')
Temperature (°C)
Tm: 82.27
Melt Curve
H
Derivative Reporter (-R')
Temperature (°C)
Tm: 80.18

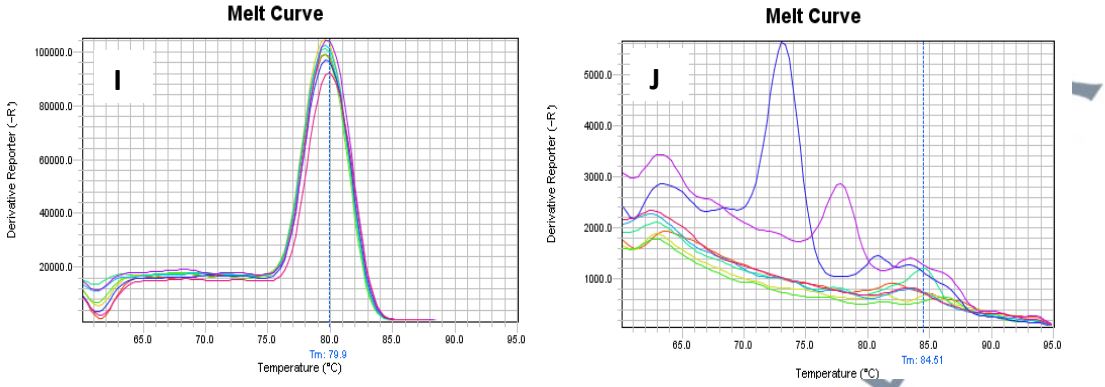


Figure 25: The melting curve of housekeeping genes and iron metabolism-related genes. Clockwise from top left was melt curve for (A) β-actin, (B) ribosomal protein S18 (rps18), (C) divalent metal transporter 1 (DMT1), (D) duodenal cytochrome b reductase (Dcytb), (E) ferroportin, (F) ferritin, (G) transferrin, (H) transferrin receptor, (I) hepcidin and (J) genomic DNA control (GDC).

Table 13: Melting temperature (T_m) for respective iron metabolism-related gene primer

Primer (gene)	Tm (°C)
β-actin	82.72
Ribosomal protein S18	83.32
Divalent metal transporter 1 (DMT1)	83.17
Duodenal cytochrome b reductase (Dcytb)	85.1
Ferroportin	78.69
Ferritin	81.82
Transferrin	82.27
Transferrin receptor (TfR)	80.18
Hepcidin	79.9

4.5.4 mRNA expression of the iron metabolism-related gene

qPCR was employed to investigate the expression of iron metabolism-related genes in IDA induced rats supplemented with date palm and goat milk. The result was analysed using the $\Delta\Delta C_T$ Method, where the mRNA level of iron metabolism-related genes was normalised to the housekeeping gene, expressed as the ratio of Gene of Interest (GOI)/ Housekeeping Gene (HKG). In this study, Rps18 and β-Actin were used as the housekeeping gene for the small intestine and liver, respectively. The data were represented as mean ± S.E.M and data were analysed for significant using Analysis of Variance (ANOVA), followed by Tukey Post-Hoc analysis using SPPS software (Chicago, US) where *p-value* less than 0.05 was statistically significant.

4.5.4.1 Divalent metal transporter (DMT1)

Divalent metal transporter (DMT1) is a membrane-bound solute carrier protein that transports Fe^{2+} across the apical membrane of enterocytes into the cell. Figure 26 showed that after IDA induced rats were supplemented with date palm and goat milk for 4 weeks, no significant changes were seen in the level of duodenal DMT1 expression. A similar expression was also observed in the liver (figure 27). Overall, an insignificant difference was observed across all groups regardless of the body iron status both in the small intestine and liver.

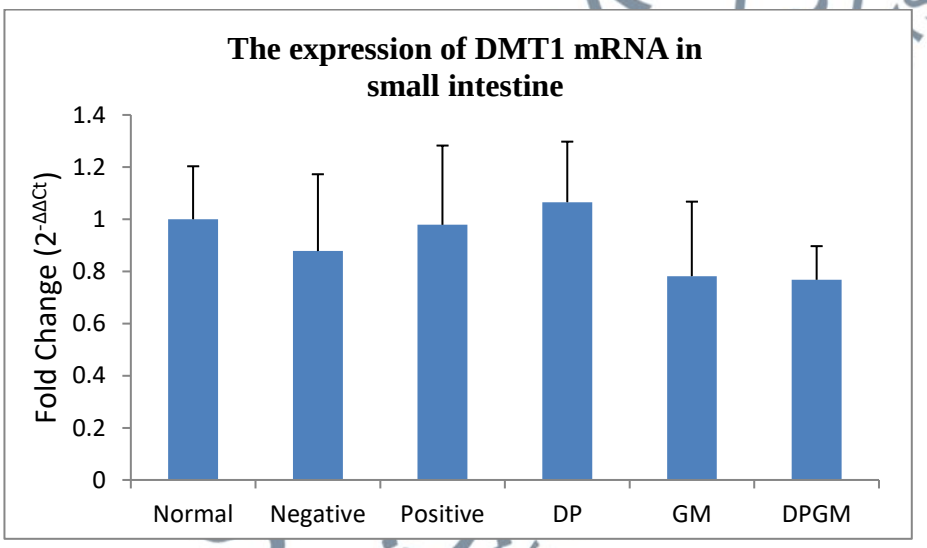


Figure 26: DMT1 mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of DMT1/Rps18. Data represents mean ± SEM. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

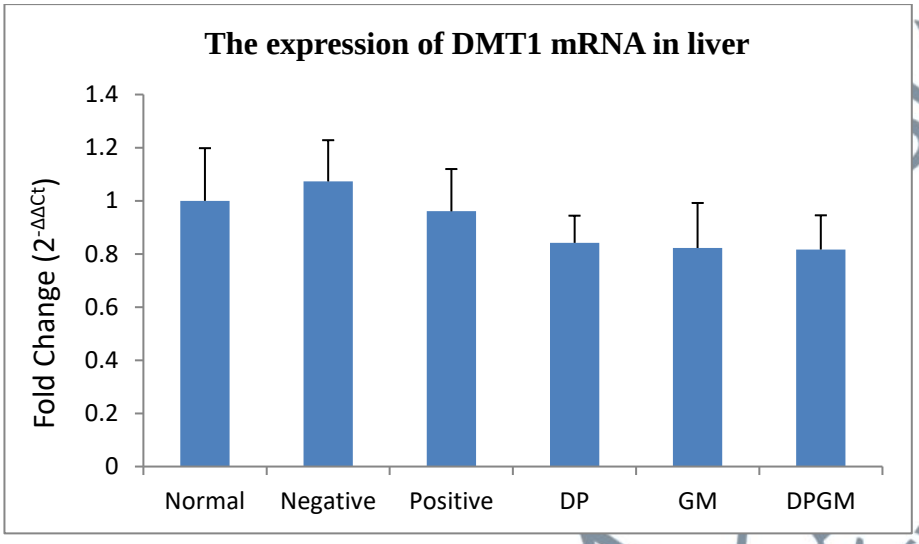


Figure 27: DMT1 mRNA expression in the liver of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of DMT1/β-Actin. Data represents mean ± SEM. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.5.4.2 Cytochrome b reductase (Dcytb)

Duodenal cytochrome b reductase (Dcytb) is an oxidoreductase that reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), before being transported into the small intestine through DMT1. Figure 28 showed that iron deprivation in negative control significantly increased (p<0.05) Dcytb expression by 13-fold as compared to normal control. Date palm and goat milk significantly decreased (p<0.05) duodenal Dcytb mRNA levels as compared to the negative control. A similar trend was also observed in the positive control group in which ferrous fumarate was given. No significant difference was seen when intervention groups were compared to normal control. However, in the liver, the expression of Dcytb mRNA showed an insignificant difference regardless of iron status (figure 29).

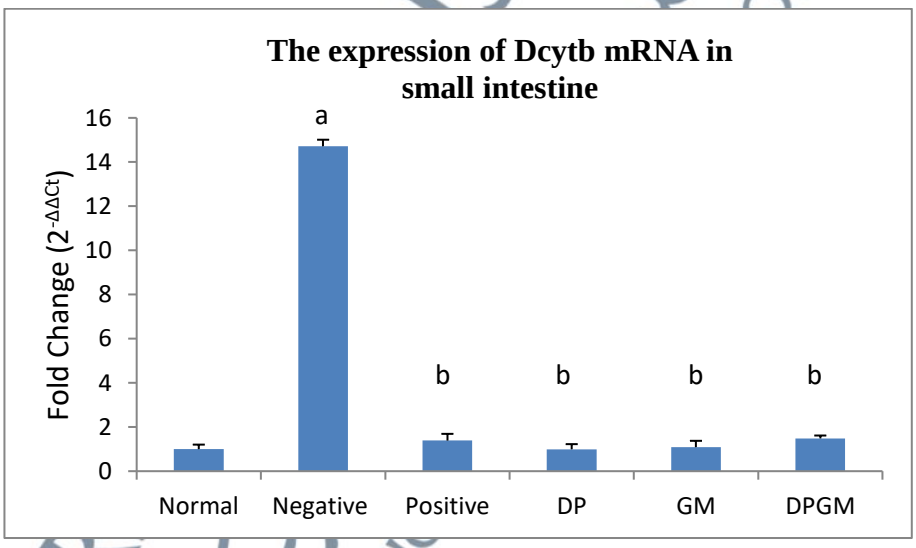


Figure 28: Dcytb DMT1 mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Dcytb/Rps18. Data represents mean ± SEM. a and b indicate significant differences (p<0.05) when compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

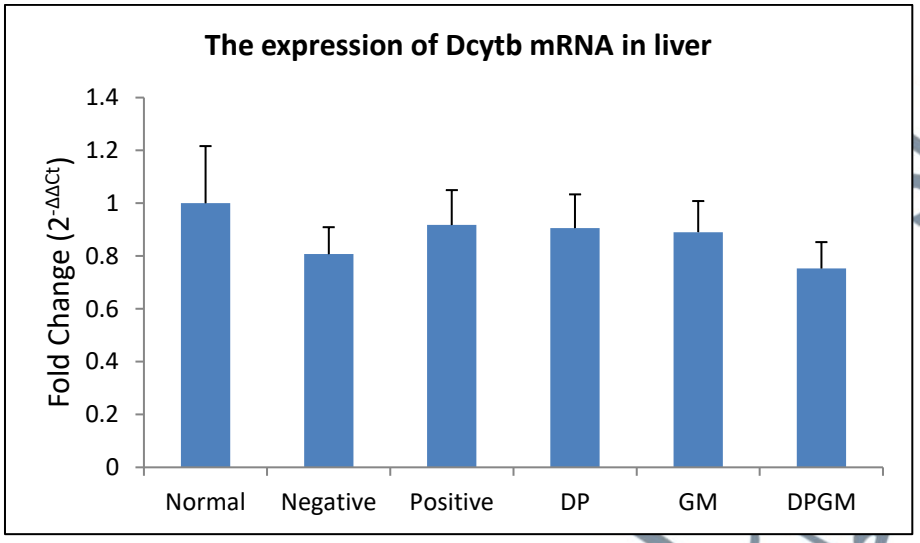


Figure 29: Dcytb mRNA expression in the liver of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of DMT1/β-Actin. Data represents mean ± SEM. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.5.4.3 Ferroportin

Ferroportin is a solute carrier protein, responsible for exporting iron into the bloodstream. Figure 30 showed that in the small intestine, rats kept on an iron-deficit diet showed a significant 1.5-fold increase (p<0.05) in ferroportin mRNA expression when compared to normal control. Supplementation with date palm and goat milk significantly reduced (p<0.05) ferroportin level as compared to the negative control. Significant differences were also observed when all intervention groups were compared to normal control (p<0.0). In the liver, although not significant, ferroportin mRNA level was slightly upregulated (0.5-fold) in negative control as compared to normal control. The level of ferroportin was also indistinguishable in all other intervention groups when compared to the negative control (figure 31).

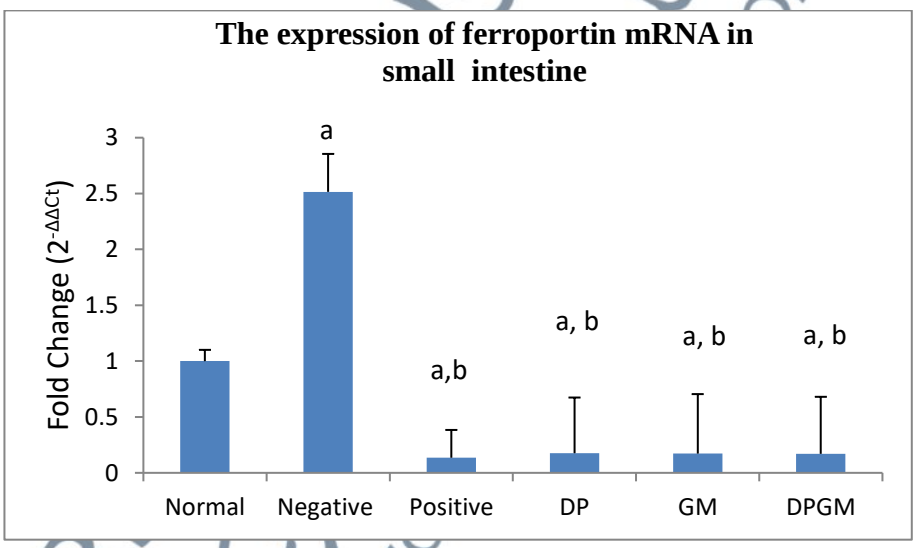


Figure 30: Ferroportin mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Ferroportin/Rps18. Data represents mean ± SEM. a and b indicate significant differences (p<0.05) when compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

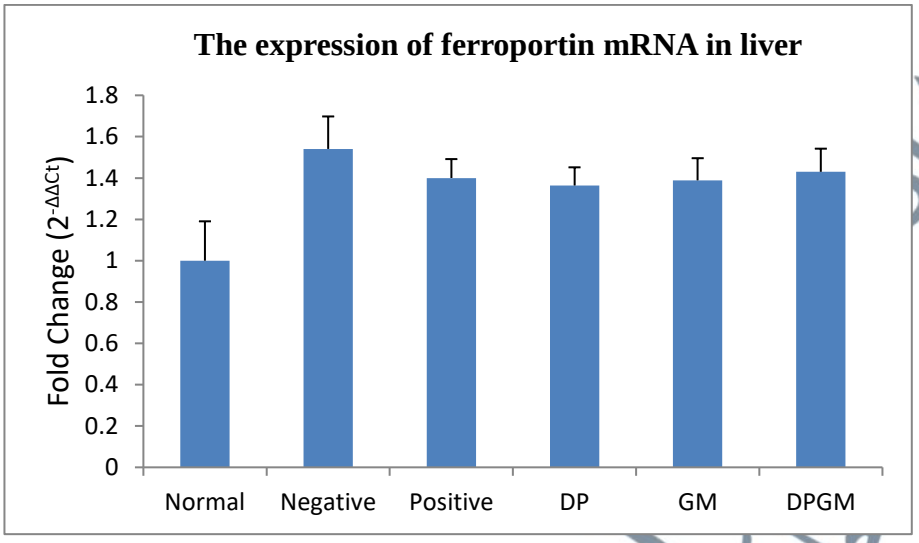


Figure 31: Ferroportin mRNA expression in the liver of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Ferroportin/β-Actin. Data represents mean ± SEM. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.5.4.4 Ferritin

Ferritin is an intracellular iron protein that stores and releases iron in a regulated manner. It is triggered by the presence of iron. When required, iron is released into the bloodstream through ferritin degradation by lysosomal or proteasomal pathways. Based on figure 32, a significant decrease (p<0.05) in ferritin mRNA in the small intestine was observed in all intervention groups as compared to the normal control. A similar pattern was seen in the liver (figure 33), in which all intervention groups showed significant declination (p<0.05) of ferritin mRNA level as compared to normal control. It showed that iron was not retained in both the small intestine and liver.

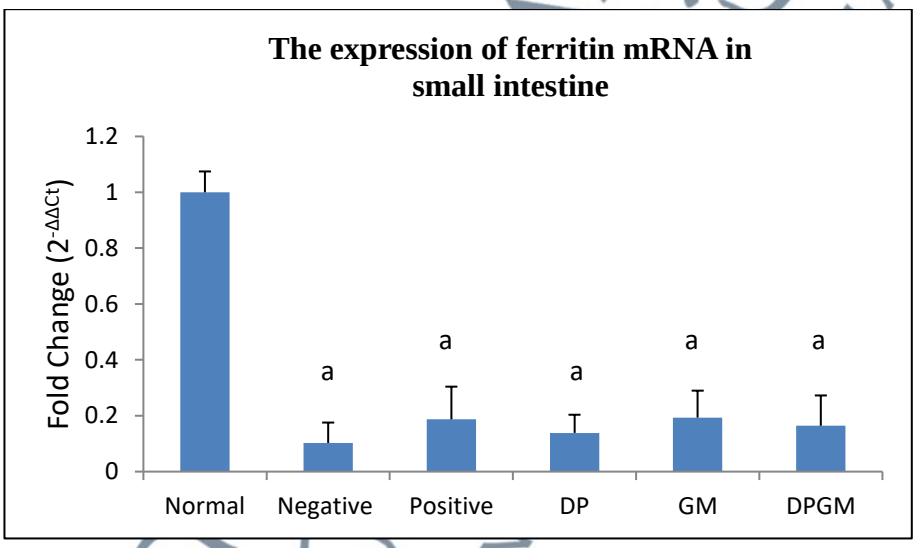


Figure 32: Ferritin mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Ferritin/Rps18. Data represents mean ± SEM. a indicates significant differences (p<0.05) when compared to normal control. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

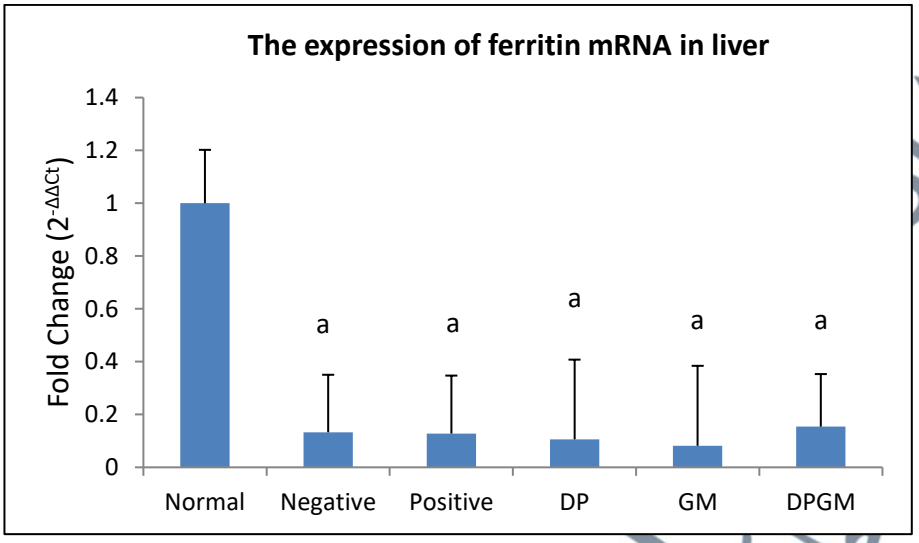


Figure 33: Ferritin mRNA expression in rat's liver after 4 weeks of intervention with date palm and goat milk. All parameters are presented as the ratio of Ferritin/β-Actin. Data represents mean ± SEM. a indicates significant differences ($p<0.05$) when compared to normal control. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.5.4.5 Transferrin

Transferrin is a glycoprotein that binds to the circulating Fe^{3+} preventing it from travelling throughout the body in its toxic form. Referring to figure 34, the group of rats kept on a continuous low iron diet demonstrated a significant 9-fold increase (p<0.05) in duodenal transferrin mRNA level when compared to normal control. All intervention groups also showed a significant decrease (p<0.05) in mRNA transferrin level post-intervention and showed significant as compared to the negative control group. No significant difference was seen between intervention groups and normal control. Meanwhile, in the liver, the expression of transferrin mRNA was significantly increased (p<0.05) in negative control as compared to normal control with an increment of 1.7-fold (figure 35). All intervention groups showed insignificant differences when compared to the negative control.

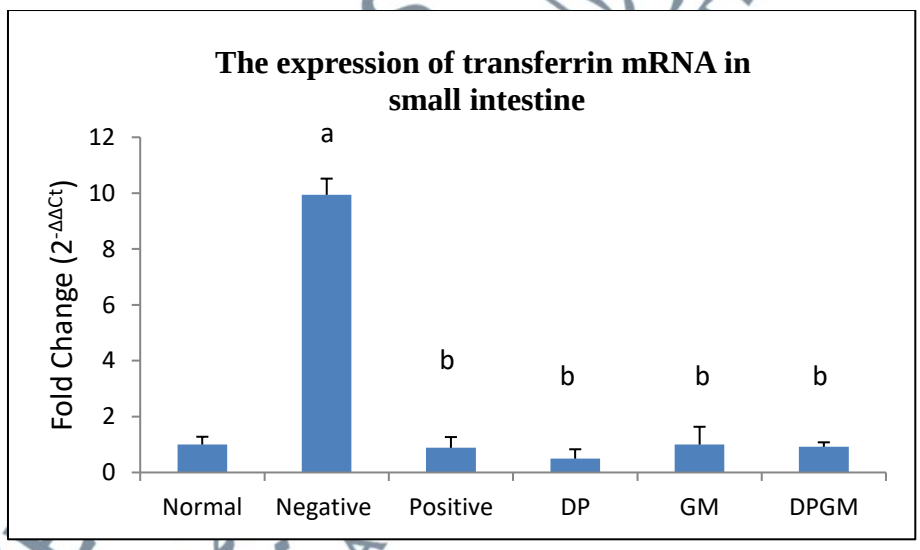


Figure 34: Transferrin mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Transferrin/Rps18. Data represents mean ± SEM a and b indicate significant differences (p<0.05) when compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

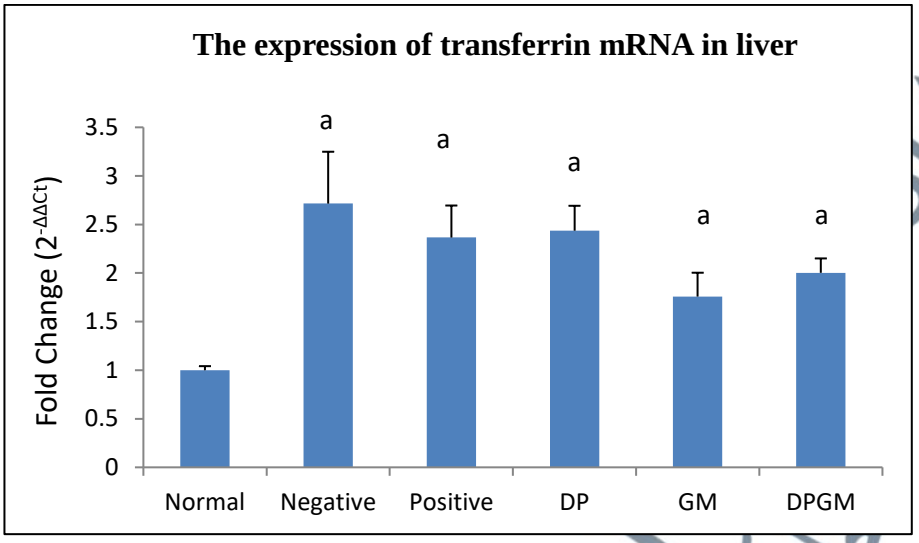


Figure 35: Transferrin mRNA expression in the liver of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Transferrin/β-Actin. Data represents mean ± SEM. a indicates significant differences ($p<0.05$) when compared to normal control. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.5.4.6 Transferrin receptor (TfR)

Transferrin receptor (TfR) is a cell surface receptor, that regulates the uptake of transferrin bound iron into the cell. Continuous iron deprivation significantly increased duodenal TfR mRNA expression in negative control as compared to normal control (figure 36). A significant reduction (p<0.05) in TfR level was noticed in all intervention groups post-intervention when compared to the negative control. All intervention groups also show significant differences (p<0.05) when compared to normal control. A similar trend was observed in TfR mRNA expression in the liver where a significant upregulation of 11.6-fold (p<0.05) was seen in negative control as compared to normal control (figure 37). Date palm and goat milk supplementation significantly downregulate (p<0.05) TfR level by 10-fold change as compared to the negative control. No significant changes were seen in between all intervention groups and when compared to normal control.

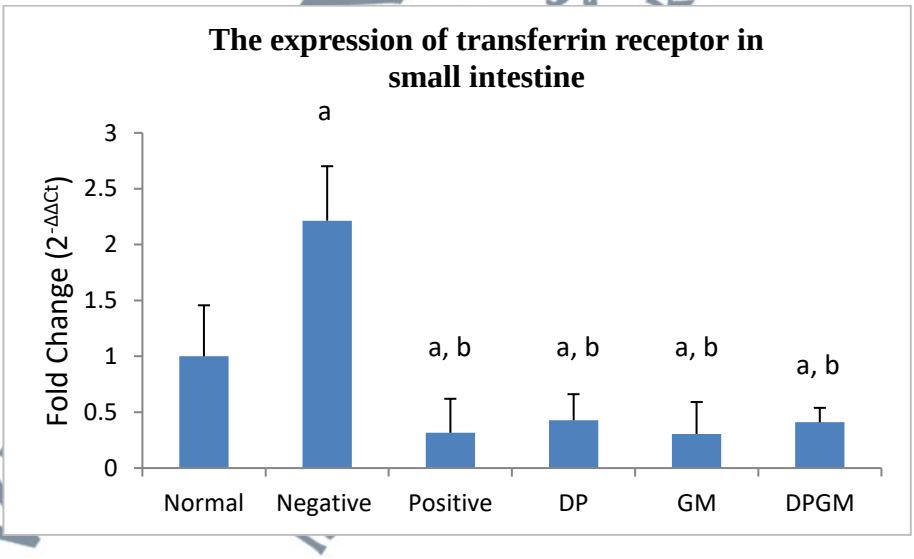


Figure 36: Transferrin receptor (TfR) mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of transferrin receptor /Rps18. Data represents mean ± SEM. a and b indicate significant differences (p<0.05) when compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

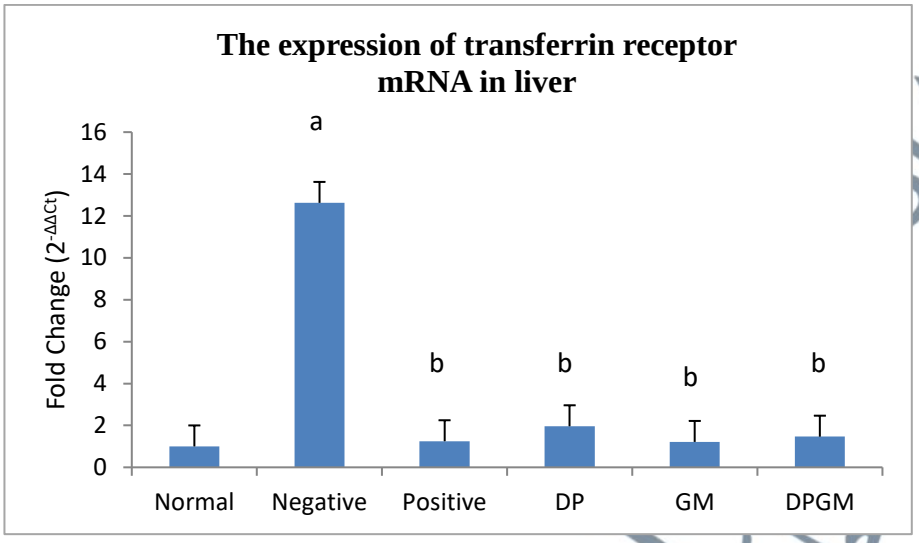


Figure 37: Transferrin receptor mRNA expression in the liver of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of Transferrin receptor/β-Actin. Data represents mean ± SEM. a and b indicate significant differences ($p<0.05$) when compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

4.5.4.7 Hepcidin

Hepcidin is the primary iron regulatory hormone, controlling iron homeostasis. According to figure 38, in the small intestine, hepcidin mRNA expression was significantly reduced (p<0.05) in the negative control as compared to the normal control. A similar expression was seen in all intervention groups. There were also significant differences when goat milk, combination date palm and goat milk and positive control group were compared to both the negative control group and date palm group. Meanwhile, in the liver, the hepcidin mRNA level was significantly reduced (p<0.05) in the negative control as compared to the normal control (figure 39). Supplementation of date palm and goat milk increase the expression of hepcidin, where a significant increase was seen in all intervention groups as compared to the negative control (p<0.05). Positive control showed the highest hepcidin rise with a 3.8-fold difference when compared to normal control.

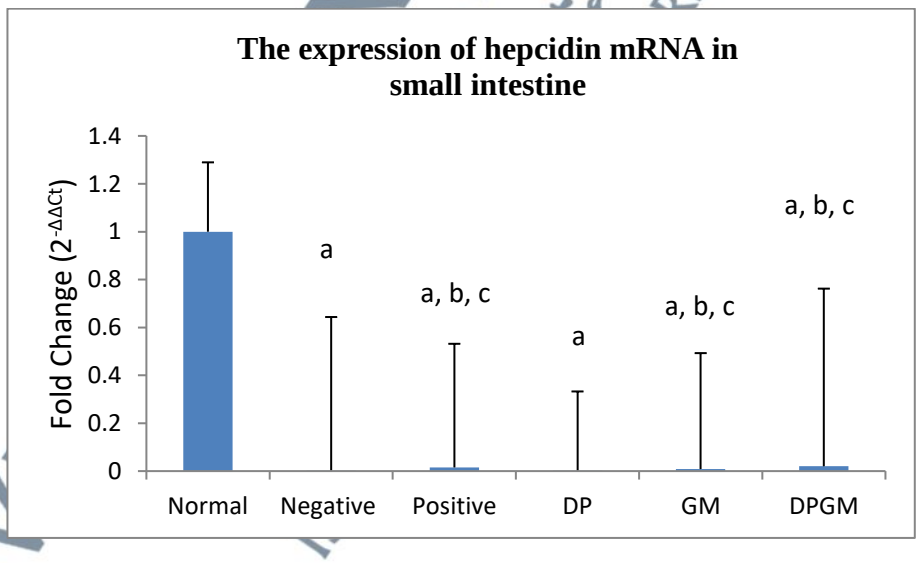


Figure 38: Hepcidin mRNA expression in the small intestine of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of hepcidin/Rps18. Data represents mean ± SEM. a, b, c indicates significant differences (p<0.05) when compared to normal control, negative control, and date palm, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk.

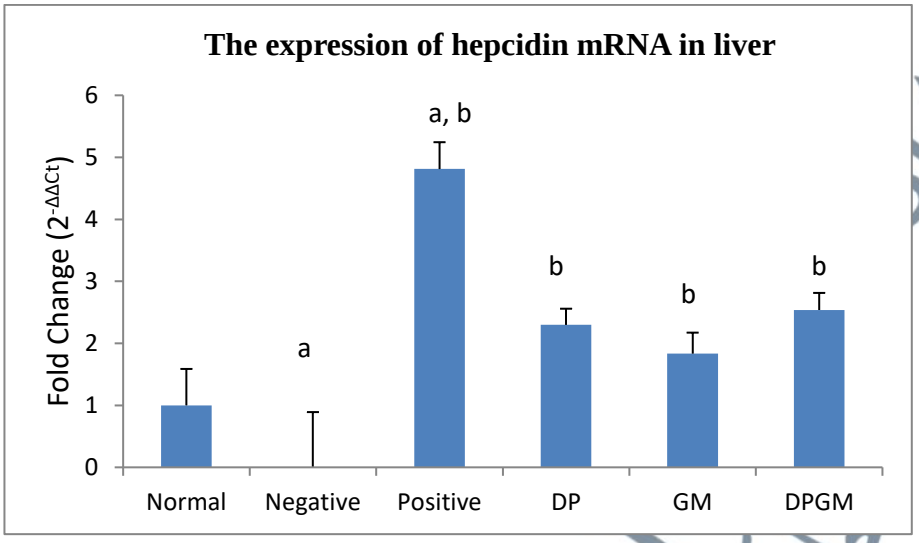


Figure 39: Hepcidin mRNA expression in the liver of IDA induced rat after intervention with date palm and goat milk. All parameters are presented as the ratio of hepcidin/β-Actin. Data represents mean ± SEM. a and b indicate significant differences ($p<0.05$) when compared to the normal and negative control, respectively. DP: Date palm; GM: Goat milk; DPGM: Date palm and goat milk

Table 14: The mRNA expression of the iron metabolism-related gene in small intestine after intervention of date palm and goat milk

Gene	Fold Change ($2^{-\Delta\Delta Ct}$)					
	Normal control	Negative control	Positive control	Date palm (DP)	Goat milk (GM)	Date palm and goat milk (DPGM)
DMT1 (Slc11a2)	1.00 ± 0.20	0.88 ± 0.20	0.98 ± 0.30	1.07 ± 0.23	0.78 ± 0.29	0.77 ± 0.13
Dcytb (Cybrd1)	1.00 ± 0.76	14.72 ± 0.62[a]	1.39 ± 0.29[b]	0.99 ± 0.54[b]	1.09 ± 0.62[b]	1.49 ± 0.32[b]
Ferroportin (Slc40a1)	1.00 ± 0.10	2.52 ± 0.34	0.14 ± 0.25[a, b]	0.18 ± 0.50[a, b]	0.17 ± 0.53[a, b]	0.17 ± 0.51[a, b]
Ferritin (Fth)	1.00 ± 0.07	0.10 ± 0.07[a]	0.19 ± 0.12[a]	0.14 ± 0.07[a]	0.19 ± 0.10[a]	0.16 ± 0.11[a]
Transferrin (Tf)	1.00 ± 0.28	9.94 ± 0.58[a]	0.88 ± 0.39[b]	0.50 ± 0.33[b]	1.00 ± 0.63[b]	0.92 ± 0.16[b]
Transferrin receptor (TfR)	1.00 ± 0.35	2.21 ± 0.47[a]	0.32 ± 0.26[a, b]	0.43 ± 0.37[a, b]	0.31 ± 0.62[a, b]	0.41 ± 0.27[a, b]
Hepcidin (Hamp)	1.00 ± 0.29	0.00 ± 0.64[a]	0.02 ± 0.52[a]	0.00 ± 0.33[a]	0.01 ± 0.48[a]	0.02 ± 0.74[a]

All parameters are presented as mean ± SEM

[a, b] within the same row indicates significant differences (p<0.05) as compared to normal and negative control, respectively

Table 15: The mRNA expression of the iron metabolism-related gene in liver after supplementation of date palm and goat milk.

Gene	Fold Change ($2^{-\Delta\Delta Ct}$)					
	Normal control	Negative control	Positive control	Date palm (DP)	Goat milk (GM)	Date palm and goat milk (DPGM)
DMT1 (Slc11a2)	1.00 ± 0.20	1.07 ± 0.16	0.96 ± 0.16	0.84 ± 0.10	0.82 ± 0.17	0.82 ± 0.13
Dcytb (Cybrd1)	1.00 ± 0.22	0.81 ± 0.10	0.92 ± 0.13	0.91 ± 0.13	0.89 ± 0.12	0.75 ± 0.10[a]
Ferroportin (Slc40a1)	1.00 ± 0.19	1.54 ± 0.16	1.40 ± 0.09	1.36 ± 0.09	1.39 ± 0.11	1.43 ± 0.11
Ferritin (Fth)	1.00 ± 0.20	0.13 ± 0.22[a]	0.13 ± 0.22[a]	0.11 ± 0.30[a]	0.08 ± 0.30[a]	0.15 ± 0.20[a]
Transferrin (Tf)	1.00 ± 0.04	2.72 ± 0.53[a]	2.37 ± 0.33[a]	2.44 ± 0.26[a]	1.76 ± 0.25[a]	2.00 ± 0.15[a]
Transferrin receptor (TfR)	1.00 ± 0.66	12.62 ± 0.52[a]	1.25 ± 0.48[b]	1.96 ± 0.26[b]	1.21 ± 0.30[b]	1.46 ± 0.30[b]
Hepcidin (Hamp)	1.00 ± 0.59	0.00 ± 0.89[a]	4.81 ± 0.43[a, b]	2.30 ± 0.26[b]	1.83 ± 0.34[b]	2.54 ± 0.28[b]

All parameters are presented as mean ± SEM

[a, b] within the same row indicates significant differences (p<0.05) as compared to normal and negative control, respectively.

4.6 Analysis of the expression of iron metabolism-related protein by IHC

Immunohistochemistry (IHC) was performed using primary rabbit polyclonal antibodies with respective dilution. Rabbit specific HRP/DAB Detection IHC Detection Kit Micro-polymer was used as the secondary antibody. Results were analysed using Olympus BX51 microscope and digital images were captured by Olympus C7070 camera using an image analyser, Olympus Analysis LS Research. Cellular localisation and staining intensity were examined and scored independently by two pathologists (blinded) and were graded; accordingly, 0: No expression, 1: Weak expression, 2: Moderate expression, 4: Strong expression. Statistically significant was calculated using the Mann-Whitney test and significant was accepted at $p<0.05$. For no primary antibody control, tissue was incubated with the antibody diluent alone, followed by incubation with secondary antibody and detection reagent. This control was done in each IHC cycle to ensure that staining is specifically produced from the detection of the antigen by primary antibody and not by the non-specific binding of the detection system or the tissue specimen. Figure 40 showed no primary antibody control in the small intestine and liver tissue, showing clear negative staining.

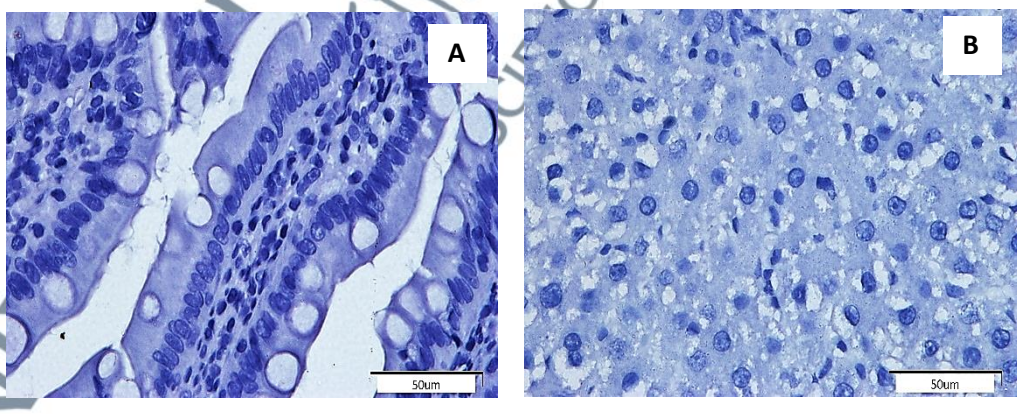


Figure 40: No primary antibody control. Negative reactivity was observed in (A) small intestine and (B) liver tissue. Original magnification x40.

4.6.1 Divalent metal transporter (DMT1)

Analysis of immunoreactivity of iron transport protein, DMT1 in the small intestine of the rat was shown in figure 41. In normal control rats, DMT1 was expressed within the enterocytes, with intense reactivity on the brush border membrane. No staining was observed in the nuclei and adjacent goblet cells (figure 41 A). Similar cellular localisation was observed in all other groups in which DMT1 protein was retained within the brush border membrane of the villi (figure 41 B-F). Only the negative control group showed a significant difference ($p<0.05$) in staining intensity when compared to the positive control.

Analysis of immunoreactivity of iron transport protein, DMT1 in the liver of rats was shown in Figure 41. In normal control liver, DMT1 protein was localised intracellularly with an approximately even distribution throughout the cytoplasm. Kupffer cell and sinusoid were unreactive (figure 42 A). All groups showed similar heterogeneous expression of DMT1 within the hepatocytes (figure 42 B-F). No significant difference was observed between all groups.

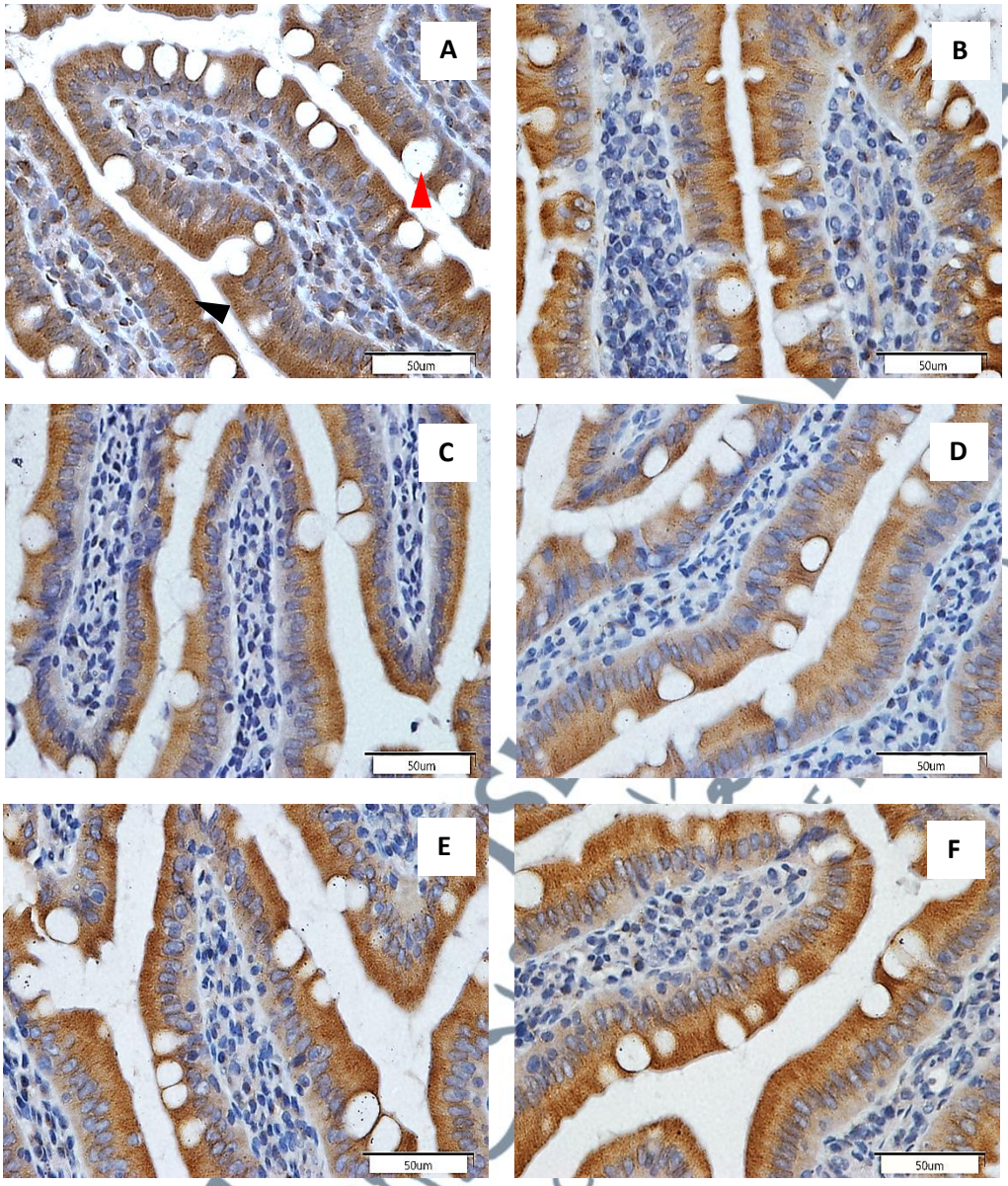


Figure 41: Immunohistochemical localisation of DMT1 protein in the small intestine of the rat. (A) Normal control showed staining localised in epithelium villi with stronger immunoreactivity on the brush border membrane (black arrowhead). No staining was detected in the goblet cell (red arrowhead). (B) Negative control (C) Positive control (D) Date Palm (E) Goat milk (F) Date palm and goat milk. All groups showed similar localisation of DMT1 protein. All images were captured at the magnification of 40x.

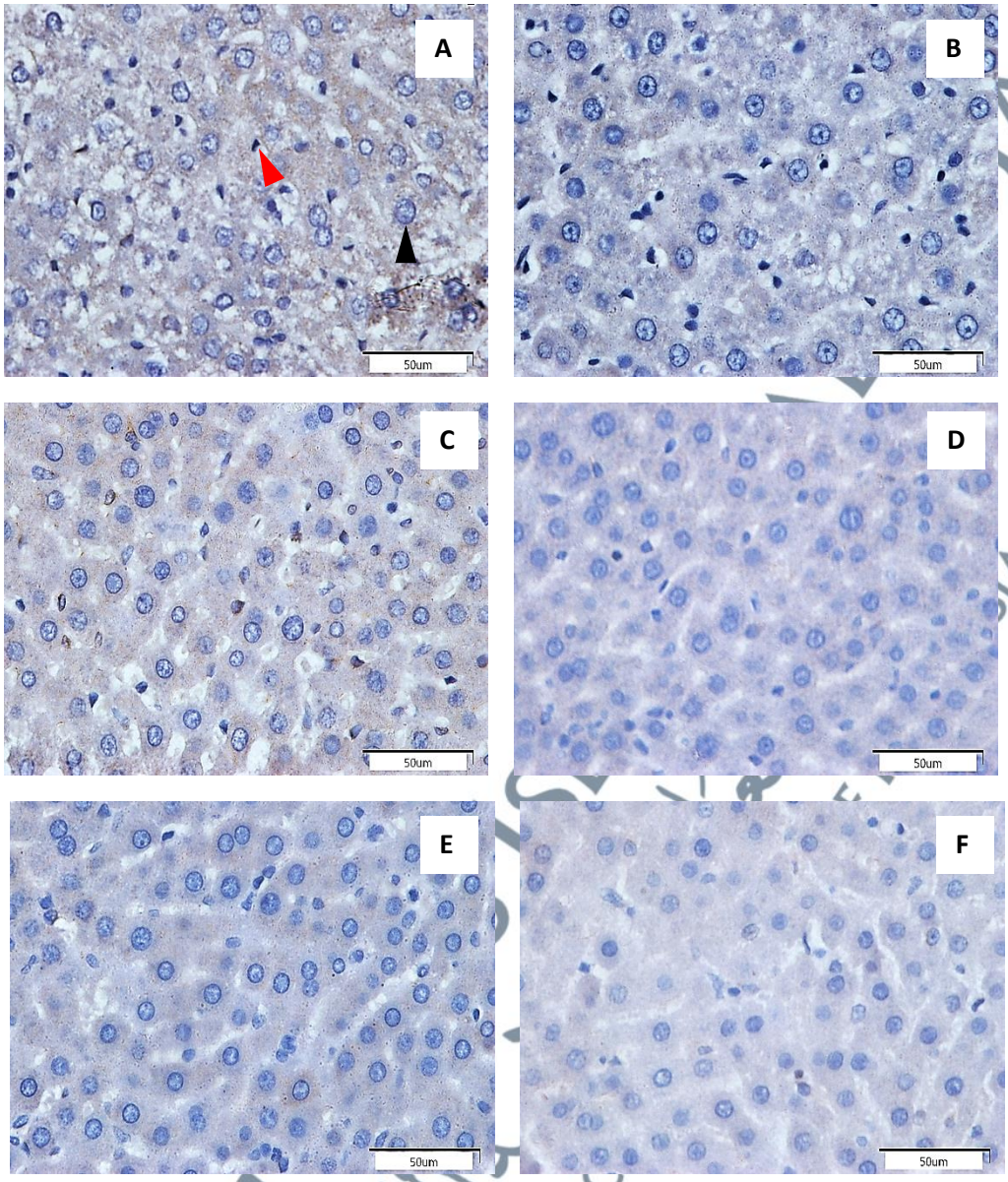


Figure 42: Immunohistochemical localisation of DMT1 protein in the liver of the rat. (A) Hepatic tissue of the normal control rat was heterogeneous concerning DMT1 reactivity. Within the hepatocytes, DMT1 expression was predominantly cytoplasmic, being negative in the nuclei region (black arrowhead). Kupffer cell stained negative (red arrowhead). (B) Negative control (C) Positive control (D) Date Palm (E) Goat milk (F) Date palm and goat milk. All groups showed similar localisation of DMT1 protein. All images were captured at the magnification of 40x.

4.6.2 Cytochrome b reductase (Dcytb)

In normal control small intestine, Dcytb was detected inside the enterocytes, with an even intracellular cytoplasmic distribution. Dcytb expression was found to be concentrated on the brush border of enterocytes along the entire length of villi with goblet cells stained negative (figure 43A). In negative control, staining was localised intracellularly within the enterocytes with an even cytoplasmic distribution. Goblet cell remains negative (figure 43B). Similar Dcytb distribution was observed in all intervention groups, with staining localised within the enterocyte cytoplasm (figure 43).

In a normal healthy liver, Dcytb protein showed cytoplasmic localisation within the hepatocytes with negative staining in the Kupffer cell and nuclei (figure 44A). In the negative control, positive immunoreactivity was detected in both nuclei and Kupffer cells (figure 44B). In positive control liver, the Dcytb protein was detected weakly within the hepatocytes, showing similar cellular localisation with the normal control group, with no reactivity in Kupffer cell and sinusoid region (figure 44C). Date palm showed a similar pattern of localisation with negative control liver, in which Kupffer cell and nuclei showed positive immunoreactivity for Dcytb protein (figure 44D). On the other hand, both goat milk and combination date palm and goat milk group showed a similar pattern of Dcytb localisation with the normal control group, with negative reactivity in Kupffer cell and nuclei (figure 44E-F).

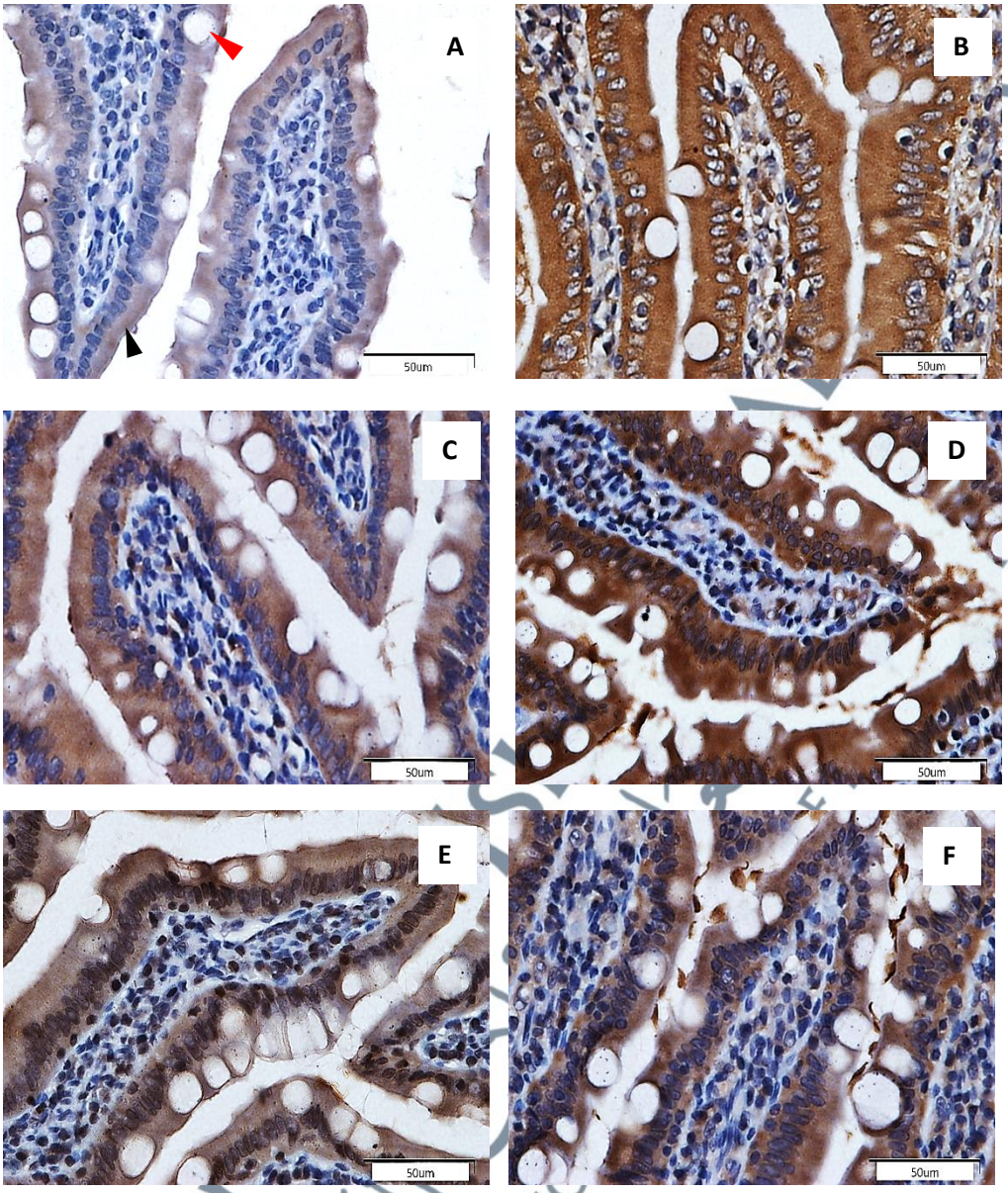


Figure 43: Immunohistochemical localisation of Dcytb protein in the small intestine of the rat. (A) Normal control showed staining localised along with the epithelial cell of the villi (black arrowhead). No staining was detected in the goblet cell (red arrowhead). (B) Negative control (C) Positive control (D) Date Palm (E) Goat milk (F) Date palm and goat milk. All groups showed similar localisation of Dcytb protein. All images were captured at the magnification of x40.

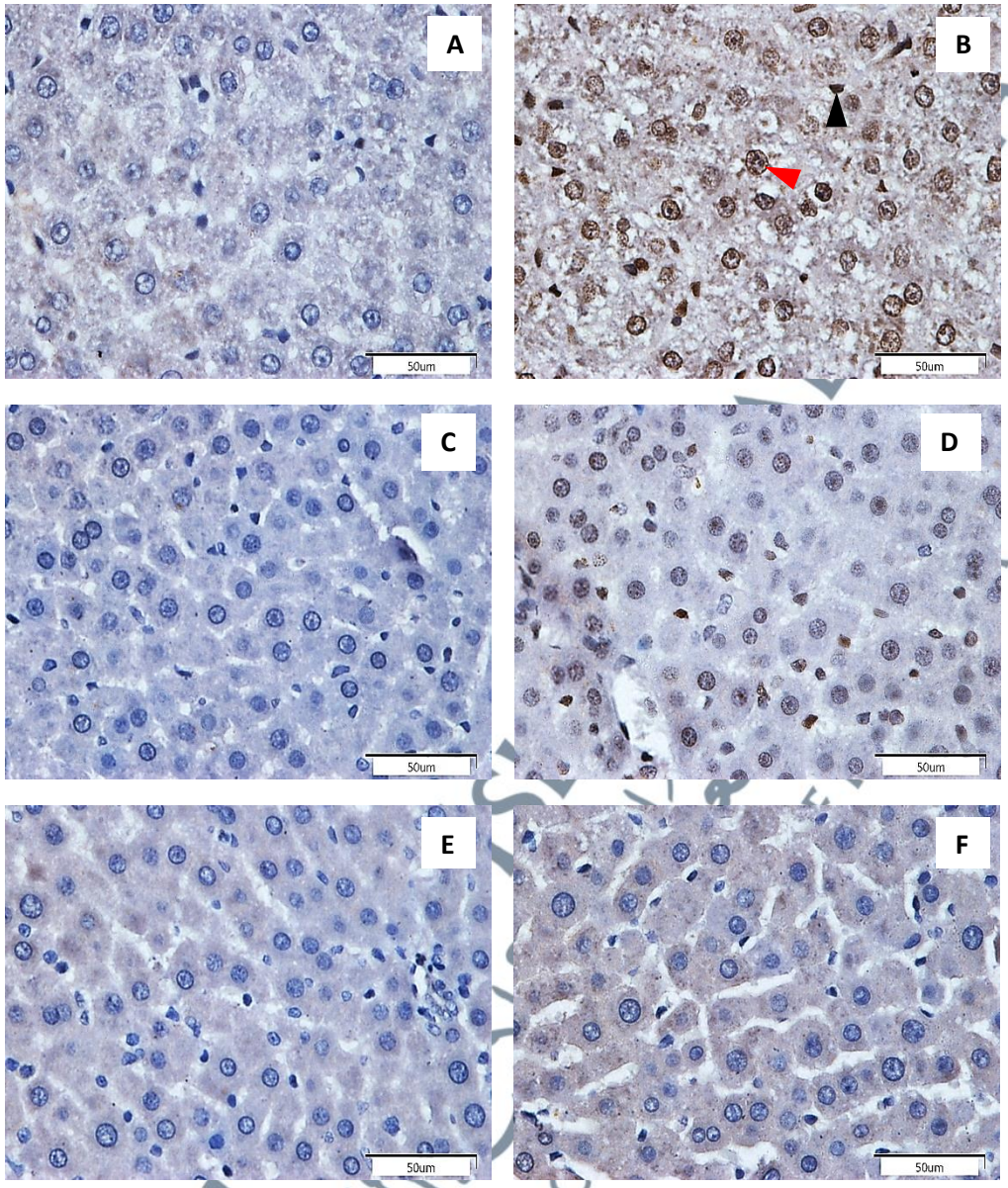


Figure 44: Immunohistochemical localisation of Dcytb protein in the liver of the rat. (A) Normal control showed an even cytoplasmic distribution of Dcytb protein within the hepatocytes. Kupffer cells and nuclei were stained negative. (B) Negative control showed positive reactivity in the cytoplasmic region, Kupffer cells (black arrowhead) and nuclei (red arrowhead). (C) Positive control showed Dcytb was expressed cytoplasmically with negative reactivity in nuclei and Kupffer cells. (D) Date palm expressed a similar expression as a negative control with Kupffer cell and nuclei stained positive. (E) Goat milk (F) Date palm and goat milk. All images were captured at the magnification of x40.

4.6.3 Ferroportin

In normal control small intestine, ferroportin was found throughout the enterocytes along the villus axis. Ferroportin was distributed with an even cytoplasmic distribution but is concentrated at the basal membrane and immediately above the nucleus along the apical membrane (figure 45A). No staining was seen in the nuclei and goblet cells. In the negative control, staining was localised intracellularly within the enterocytes with intensity concentrated on both apical and basal membrane and to a lesser extend along the lateral membrane of enterocytes (figure 45B). For the positive control group and all intervention groups, ferroportin expression was found to be cytoplasmically distributed within the villi epithelium with no stain in nuclei and goblet cells (figure 45).

In normal control liver, ferroportin expression was limited to hepatocytes, where expression was found to be evenly cytoplasmic. No staining was found in Kupffer cells (figure 46A). Similar trends were observed in all groups (Figure 46).

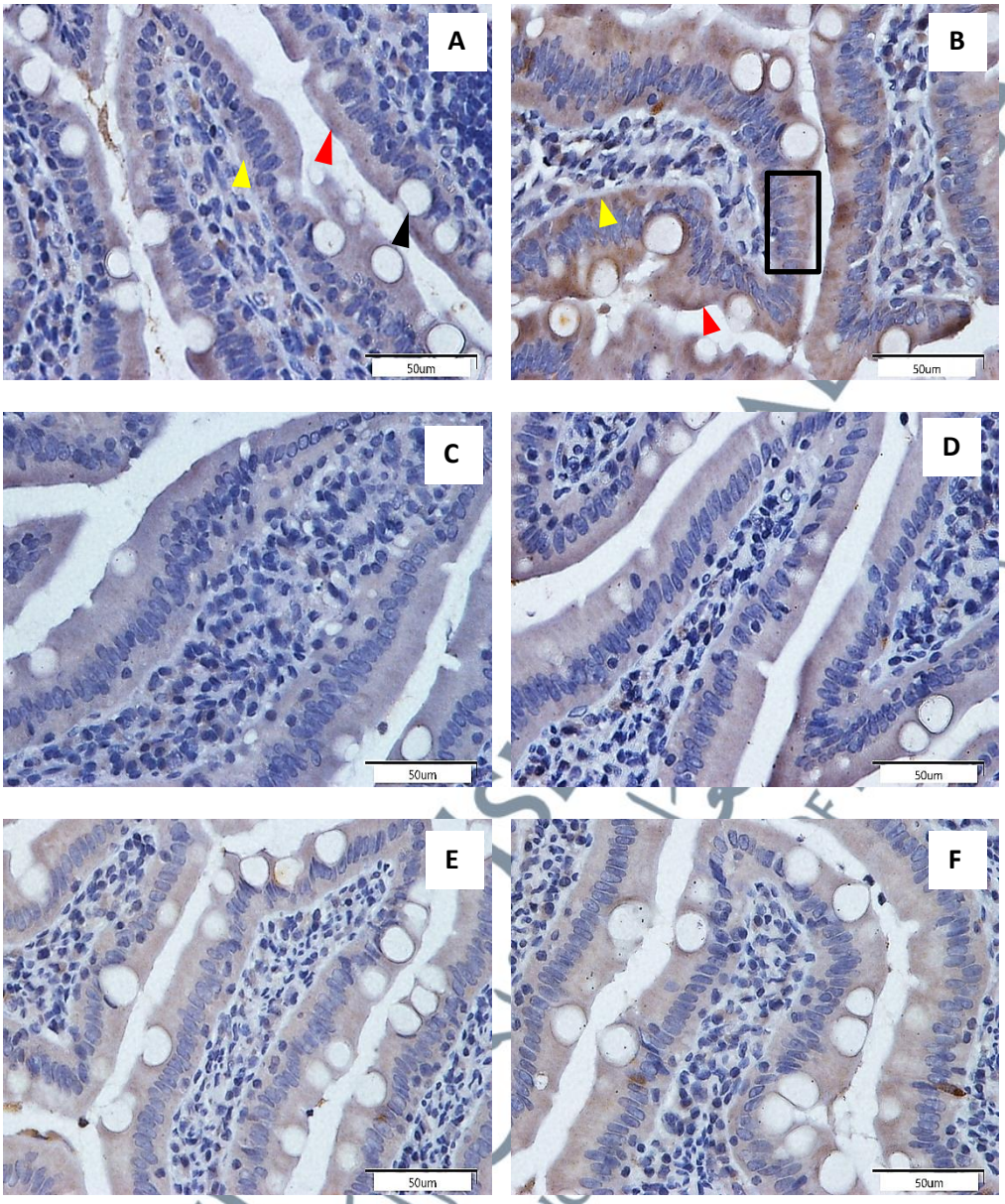


Figure 45: Immunohistochemical localisation of ferroportin protein in the small intestine of the rat. (A) Normal control showed ferroportin throughout the enterocyte with high concentration at the apical (red arrowhead) and basal membrane (yellow arrowhead). No staining was detected in the goblet cell (black arrowhead). (B) Negative control showed immunoreactivity along the epithelium villi on the apical (red arrowhead) and basal membrane (yellow arrowhead) with weak expression on the lateral membrane (black box). (C) Positive control (D) Date Palm (E) Goat milk (F) Date palm and goat milk. All images were captured at the magnification of x40.

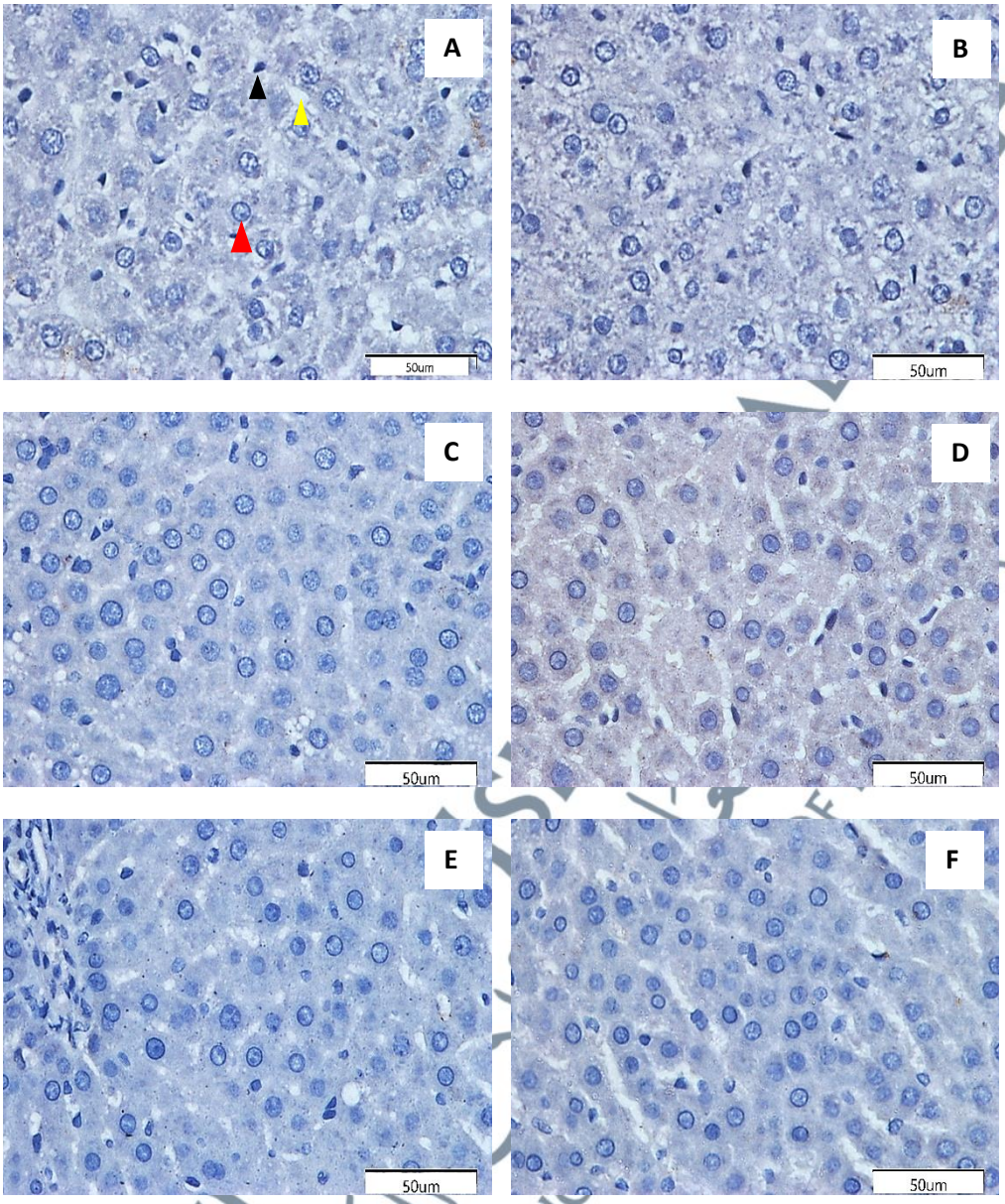
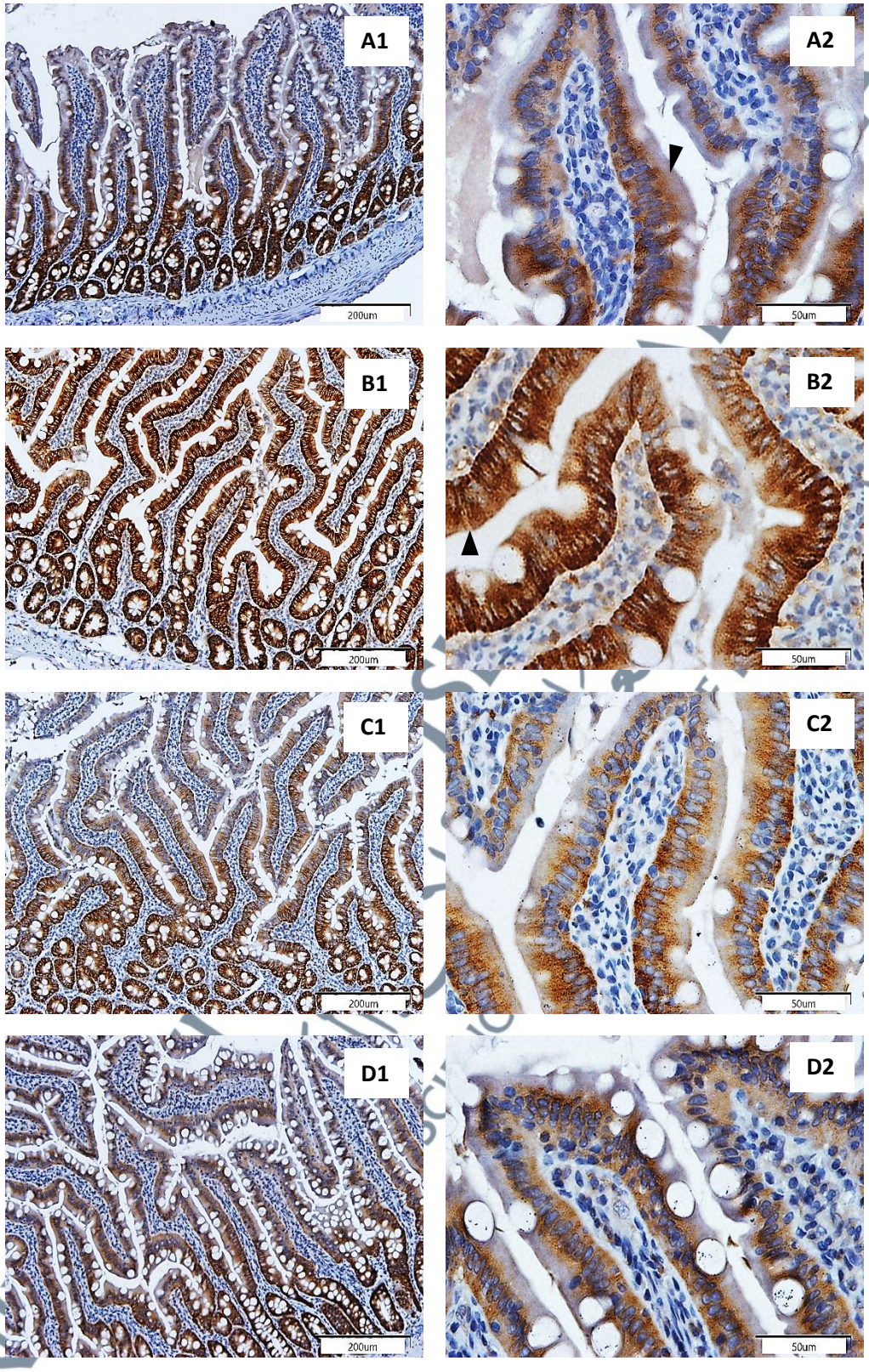


Figure 46: Immunohistochemical localisation of ferroportin protein in the liver of the rat. (A) Normal control expresses an even cytoplasmic distribution of ferroportin protein within the hepatocytes with no immunoreactivity on the Kupffer cell (black arrowhead), sinusoid (yellow arrowhead) and nuclei (red arrowhead). (B) Negative control. (C) Positive control. (D) Date palm (E) Goat milk (F) Date palm and goat milk. All images were captured at the magnification of x40.

4.6.4 Transferrin receptor (TfR)

In normal control small intestine, TfR protein was expressed by the epithelial cells of the crypts and the villi, with decreasing intensity when epithelial cells migrated apically toward the villus tip (figure 47A1). The reaction was predominantly cytoplasmic, with no nuclear staining. No stain was observed in the brush border membrane of the villi (black arrowhead) and goblet cells (figure 47A2). In the negative control, significantly higher ($p<0.05$) staining intensity was observed in the enterocytes as compared to normal control (figure 47B). Staining intensity was also evenly distributed throughout the enterocytes with brush border membrane also stained positive. The goblet cell was stained negative. Positive control small intestine retained similar expression to normal control with staining intensity decreased from crypts region to villus tip (figure 47C). No staining was observed on the brush border region of the villi. All intervention groups expressed a similar trend of TfR protein localisation with normal control (figure 47 D-F).

In normal control liver, TfR localised within hepatocytes, with even cytoplasmic distribution. No staining was observed in Kupffer cells and the sinusoid region (figure 48A). Nuclei also stained negative. A similar profile of expression was observed in all liver tissue regardless of the diet (figure 48 B-F).


A1
200um
A2
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C1
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C2
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D1
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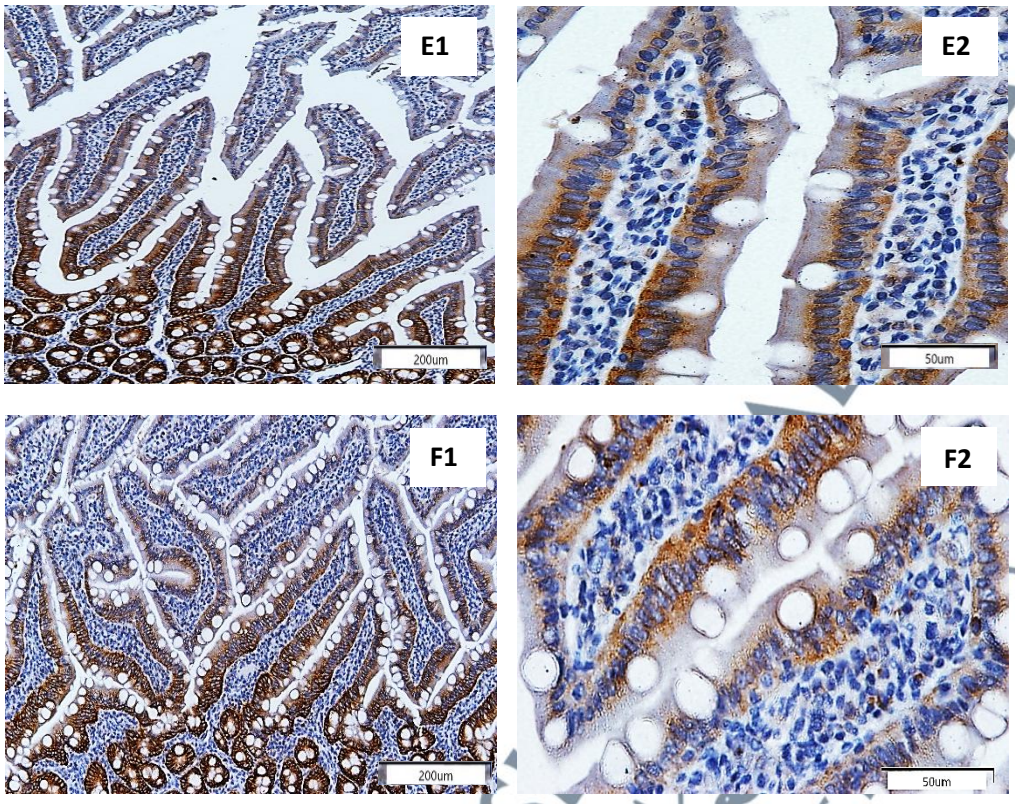

Figure 47: Immunohistochemical localisation of transferrin receptor (TfR) protein in the small intestine of the rat. (A) Normal control showed TfR protein expression within the epithelial cells of the crypts and the enterocytes villi. Staining intensity decreased while epithelial cells migrated toward the villus tip (Figure A1). No staining in the mucus-secreting goblet cells and brush border membrane (black arrowhead in A2). (B) Negative control showed prominent staining on crypts and villi with strong reactivity on both the apical and basolateral sides of the epithelial villi. The brush border membrane was also stained positive (black arrowhead in B2). No staining in nuclei and goblet cells. (C) Positive control (D) Date Palm (E) Goat milk (F) Date palm and goat milk. All images were captured at the magnification of x40.

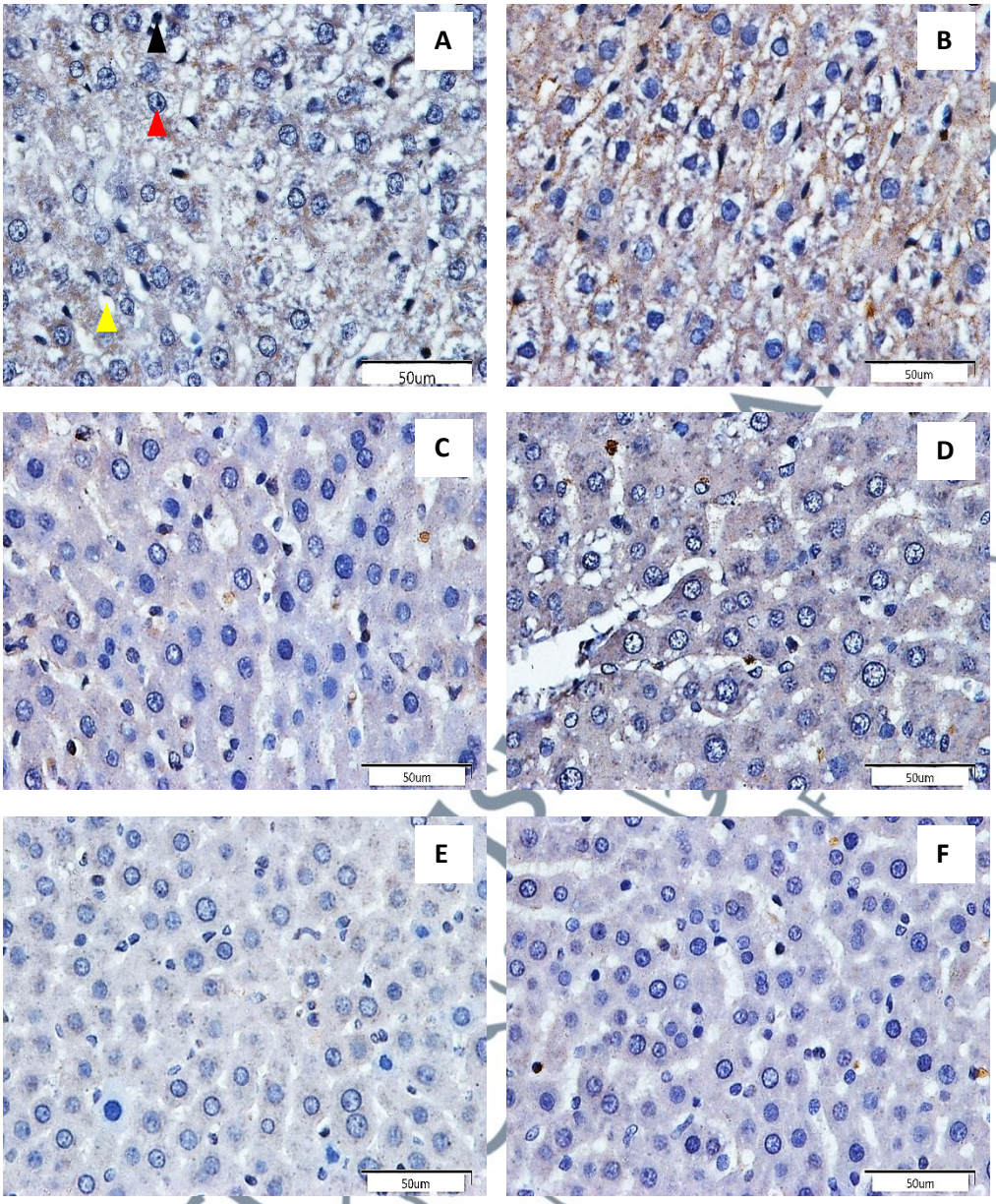

Figure 48: Immunohistochemical localisation of TfR protein in the liver of the rat. (A) Normal control expresses an even cytoplasmic distribution of TfR protein within the hepatocytes with no immunoreactivity on the Kupffer cell (black arrowhead), sinusoid (yellow arrowhead) and nuclei (red arrowhead). (B) Negative control (C) Positive control (D) Date palm (E) Goat milk (F) Date palm and goat milk. All images were captured at the magnification of x40.

Table 16: Semiquantitative analysis of immunoreactivity of iron-related protein in the small intestine of IDA induced rats supplemented with date palm and goat milk

Protein	Normal control	Negative control	Positive control	Date palm	Goat milk	Date palm and goat milk
DMT1	1.88 (2.00)	2.00 (2.00)	1.38 (1.25)	1.75 (1.75)	2.00 (2.0)	1.88 (1.75)
Dcytb	1.63 (1.75)	2.00 (2.25)	1.50 (1.50)	2.50 (2.50) *	1.88 (1.75)	1.63 (1.50)
Ferroportin	2.00 (2.00)	1.50 (1.75)	2.00 (2.00)	1.25 (1.00)	1.25 (1.25) *	1.13 (1.00) *
TfR	1.75 (1.75)	2.75 (3.00) *	2.50 (2.75)	2.25 (2.25)	2.25 (2.25)	1.88 (1.75)

The mean and median of each group is presented, mean (median)
* represent a value that is significantly different when compared to normal control ($p < 0.05$).

Table 17: Semiquantitative analysis of immunoreactivity of iron-related protein in the liver of IDA induced rats supplemented with date palm and goat milk.

Protein	Normal control	Negative control	Positive control	Date palm	Goat milk	Date palm and goat milk
DMT1	1.13 (1.00)	0.63 (0.50)	0.88 (1.00)	0.75 (0.50)	1.00 (1.00)	0.88 (1.00)
Dcytb	0.50 (0.50)	0.88 (0.50)	0.75 (0.75) *	1.38 (1.50) *	1.38 (1.25) *	1.13 (1.00)
Ferroportin	0.50 (0.50)	0.75 (0.50)	0.88 (0.75)	0.88 (1.00) *	0.88 (0.75)	0.88 (1.00) *
TfR	0.88 (1.00)	1.13 (1.00)	1.00 (1.00)	0.75 (0.75)	0.75 (0.75)	1.13 (1.25)

The mean and median of each group is presented, mean (median)
* represent a value that is significantly different when compared to normal control ($p < 0.05$).