

Erythrocyte morphology and filterability in rats fed on diets containing different fats and oils

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The present study aimed to determine if the type of dietary fat or oil affects erythrocyte morphology and/or filterability in normal healthy rats. A feeding trial was carried out, in which nine groups of nine rats were fed on diets containing one of the following treatments (test fats or oils): anhydrous milk fat, anhydrous milk fat after passage through a column of active carbon, palm oil, MaxEPA fish oil, hydrogenated coconut oil, anhydrous tallow shortening, margarine hardstock, olive oil and soyabean oil. The test fats or oils supplemented with 10 g safflower-seed oil/kg were incorporated into otherwise nutritionally adequate diets so that the test fat or oil plus safflower-seed oil contributed 35% of the gross energy of the diet. The rats were fed for 10 weeks. Diet had a significant effect on five of the six classes of erythrocytes identified, and the proportion of cells in each class was shown to be dependent on diet. However, the attribute causing the dependence was not clear. There was no significant effect of diet on erythrocyte filterability index. There was no statistical correlation between erythrocyte filterability index and morphology. Although it has been observed that diet, particularly fish oil, can improve the filterability of erythrocytes once filterability is impaired, the effect of diet on erythrocyte filterability in normal healthy animals including humans is unclear. The importance of the differences in erythrocyte morphology due to diet is also unclear. Both areas deserve further investigation.

Erythrocytes: Fatty acids: Dietary fat

Most concepts of the physics of haemodynamics are based on erythrocytes as biconcave discs. Samples of blood from normal healthy human donors and from non-human mammals contain substantial proportions of non-discocytic erythrocytes (Simpson, 1989). It has been observed that non-discocytes occur in chronic disorders and thus may contribute to the pathology of the disease perhaps by impairing blood flow in the microcirculation (Simpson *et al.* 1987). Simpson (1989) classified erythrocytes into six morphological classes: biconcave discs, flat cells, cells with surface changes, early cup forms, late cup forms and cells with altered margins.

The ability of erythrocytes to change their shape in order to travel through capillary beds can be estimated by measuring erythrocyte filterability. Erythrocyte filterability has been shown to be reduced in a variety of disease states including peripheral vascular disease (Reid *et al.* 1976*b*; Drummond *et al.* 1980), hypercholesterolaemia (Levenson *et al.* 1992) and acute myocardial infarction (Drummond *et al.* 1980). However, it is not known whether the reduced filterability is a consequence of the disease state or a contributing factor.

Diet is known to contribute to the risk of cardiovascular disease. Although non-discocytic cells have been observed in healthy animals, the influence of diet, particularly dietary fat, on morphology has not been reported. Likewise, the influence of dietary fat on

erythrocyte filterability in normal healthy animals, as opposed to those with already impaired filterability, has not been studied.

If it is assumed that the biconcave-disc-shaped erythrocyte is the most easily filterable, then it might be expected that the diet group with the highest proportion of disc-shaped cells would have the highest erythrocyte filterability. The present study aimed to determine if the type of dietary fat or oil, particularly milk fat, affects erythrocyte morphology and/or filterability in normal healthy rats.

METHODS

Feeding trial

The experimental design was nine treatments (test fats or oils) with nine rats/treatment. The nine test treatments (fats or oils) were: anhydrous milk fat (AMF1), anhydrous milk fat after passage through a column of active carbon to remove the minor components (AMF2), palm oil (PALM), MaxEPA fish oil (MaxE), hydrogenated coconut oil (COCO), anhydrous tallow shortening (TALL), margarine hardstock (MARG), olive oil (OLIVE) and soyabean oil (SOY). The ingredient composition of the diets is given in Table 1. The test fats were supplemented with 15 g safflower-seed oil/kg to ensure that all diets contained sufficient linoleic acid to meet the essential fatty acid requirement of the rat. The test fat plus safflower-seed oil contributed 35% of the gross energy of the diet.

Weaned male Sprague-Dawley rats (21–23 d, 45–55 g) were housed individually in raised stainless steel cages with mesh floors (New Zealand Institute for Crop and Food Research Food Evaluation Unit, Palmerston North, New Zealand) and randomly allocated to the experimental treatments. The environmental conditions were: temperature $22 \pm 1^\circ$, humidity $60 \pm 5\%$, air exchange 12 times/h and a 12 h light-dark cycle. The experimental diets were fed *ad libitum* for 70 d and water was continuously available. Fresh diet was provided daily to reduce the risk of rancidity and its consequent effects. The rats were weighed initially and then at 7 d intervals. Feed intake was recorded daily and calculated for each 7 d period.

Blood collection

On day 70 the rats were weighed, anaesthetized with diethyl ether, and blood was collected from the abdominal aorta into sodium EDTA as anticoagulant (1.5 mg/ml blood). Immediately after collection, three drops of whole blood were mixed with 1 ml of 10 ml/l glutaraldehyde in 0.1 M-phosphate buffer (pH 7.2) to fix the erythrocytes for the morphology determination. Whole blood (2 ml) was used for the packed cell volume and filtration tests.

Erythrocyte morphology

After fixation for at least 30 min at room temperature, the whole-blood sample was centrifuged (BTL bench centrifuge) at 450 g for 30 s. The supernatant fraction was discarded and the blood cells were washed twice with phosphate buffer (0.1 M), dehydrated in ascending concentrations of acetone (25, 50, 75, 90, 100%) and suspended in 1 ml of 100% acetone.

Two drops of the final suspension were placed on a clean coverslip and allowed to air dry in a desiccator. A piece of coverslip was mounted on an Al scanning electron microscopy stub with conductive silver paint and sputter coated with gold (Polarion E5100 cold stage sputter coater, Polarion Equipment, Watford, Herts.). Specimens were examined using a scanning electron microscope (Cambridge 250 MK111, Cambridge Instruments Ltd, Cambridge, Cambs.) with an accelerating voltage of 10 kV. Suitably spread areas of cells were photographed and printed at a final magnification of $\times 1500$. All identifiable cells on one micrograph from each rat were classified and counted according to the procedure of

Table 1. *Ingredient composition of diets (g/kg diet, as fed)*

Ingredient	Diet								
	AMF1	AMF2	PALM	MaxE	COCO	TALL	MARG	OLIVE	SOY
Egg albumin*	238	238	238	238	238	238	238	238	238
Vitamin mix†	50	50	50	50	50	50	50	50	50
Mineral mix‡	50	50	50	50	50	50	50	50	50
AMF1	196.3								
AMF2		197.3							
PALM			191.7						
MaxE				189.2					
COCO					199.6				
TALL						191.1			
MARG							189.4		
OLIVE								190.5	
SOY									188.3
Safflower-seed oil§	15	15	15	15	15	15	15	15	15
Cellulose	20	20	20	20	20	20	20	20	20
Starch¶	430.7	429.7	435.6	437.8	427.4	435.9	437.6	436.5	438.7

AMF1, anhydrous milk fat (New Zealand Dairy Research Institute (NZDRI), Palmerston North, New Zealand); AMF2, anhydrous milk fat, after passage through a column of active C (NZDRI); PALM, palm oil (Abels Ltd, Auckland, New Zealand); MaxE, MaxEPA (British Cod Liver Oils Ltd, Hull, Yorks); COCO, hydrogenated coconut oil (92 confectionery fat, Abels Ltd, Auckland, New Zealand); TALL, anhydrous tallow shortening (PB special shortening, Abels Ltd); MARG, margarine hardstock (Abels Ltd); OLIVE, olive oil (Industias Pont, S A, Tarrega, Lerida, Spain); SOY, soyabean oil (Abels Ltd).

* Spray-dried egg albumin, Harvey Farms, Tauranga, New Zealand.

† Composition of vitamin mix (g/kg): retinyl acetate (500 000 IU/g) 0.1, ergocalciferol (1:100 in cellulose) 0.2, DL- α -tocopheryl acetate (250 IU/g) 8.0, menadione 0.06, riboflavin 0.1, thiamin hydrochloride 0.1, pyridoxine hydrochloride 0.1, nicotinic acid 0.1, calcium pantothenate 0.4, pteroylmonoglutamic acid 0.04, biotin 0.02, cyanocobalamin (1:100 in sucrose) 0.1, myoinositol 4.0, choline chloride 30.0, sucrose to make 1 kg.

‡ Composition of mineral mix (g/kg): CaHPO₄ 427, MgO 35, KCl 200, iodized NaCl 100, Fe₂(SO₄)₃ 28, ZnO 1.2, CuCO₃.Cu(OH)₂.H₂O 0.4, MnSO₄.H₂O 4.8, Na₂SeO₃ 0.00452, CoCl₂.6H₂O 0.00232, CrK(SO₄)₂.12H₂O 0.38, cellulose to make 1 kg.

§ Faggs Products Ltd, Wellington, New Zealand.

|| Avicell PH 101, Commercial Minerals Ltd, Auckland, New Zealand.

¶ Wheaten cornflour, Goodman Fielder Industries Ltd, Summerhill, New South Wales, Australia.

Simpson (1989) (Fig. 1). Whole blood was used as there is evidence that washing erythrocytes before fixation affects erythrocyte shape, leading to an increase in the proportion of discocytes present (Simpson, 1993).

Packed cell volume

Packed cell volume measurements were made on anticoagulated whole blood using a microhaematocrit centrifuge and microcapillary reader (Model MB and Model CR respectively, International Equipment Co., Needham Hts, MA, USA).

Erythrocyte filterability

Within 1 h of blood collection, samples of whole blood (1 ml) were filtered using a filtration apparatus modified from that of Reid *et al.* (1976*a*). The vacuum and filtrate reservoirs were combined and a tap was fitted between the filter holder and the reservoir. Polycarbonate membrane filters (Hemafil, SN110424, 13 mm; Nucleopore Corporation, Pleasanton, CA, USA) mounted in 'pop top' holders (SN42100; Nucleopore Corporation) were used. The filters were washed with 5 ml saline (9 g NaCl/l) and then with 5 ml

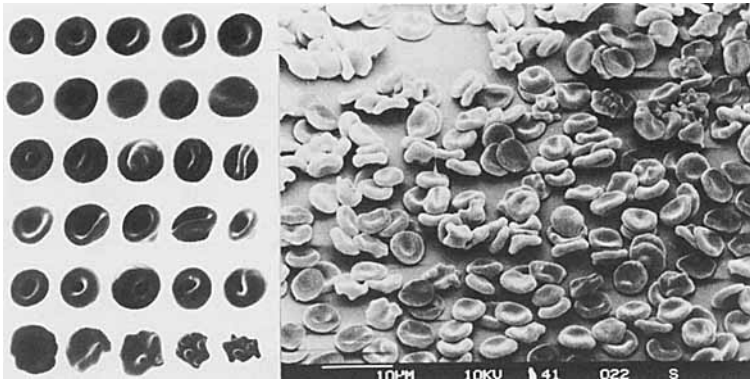


Fig. 1. Photomicrographs of erythrocytes from rats. On the left, erythrocytes from a 21-d-old female rat bled by heart puncture and selected as being representative of the shape classes, magnified $\times 1370$. Top row, normal discocytes as biconcave discs; second row, flat cells; third row, cells with surface changes; fourth row, early cup forms; fifth row, late cup forms; bottom row, cells with altered margins (from Simpson (1989); used with permission). On the right, a typical photomicrograph of erythrocytes from a male rat fed on one of the test diets for 70 d from weaning, magnified $\times 1500$. Blood was collected from the abdominal aorta.

deionized water and were placed on blotting paper to air-dry overnight before use. The 0.5 ml and 1.0 ml flow times were recorded and the erythrocyte filterability index (based on that of Reid *et al.* 1976a) was calculated as follows:

$$\text{filterability index} = (60 / (2 \times 0.5 \text{ ml flow time})) \times (\text{packed cell volume} / 100).$$

Chien (1977) suggested that suspensions of erythrocytes or washed erythrocytes should be used, rather than whole blood, to prevent blocking of the filter pores. However, it was considered that whole blood gave a better reflection of the real physiological state. Therefore, to overcome this potential problem, the filterability index was calculated using twice the flow time for the first 0.5 ml rather than the flow time for 1 ml. The CV was calculated using the overall standard deviation divided by the overall mean of the trial data set.

Statistical analysis

ANOVA was carried out, using the generalized linear model in SAS (Statistical Analysis Systems, 1994), on the live weight, feed intake, packed cell volume and filterability index data to test the effect of diet. Where a significant effect of diet was found, it was further examined by calculating the least significant differences between pairs of treatments.

The effect of diet on erythrocyte morphology (%) was assessed using the logit link function $\{\log_e(p/1-p)\}$ from S-Plus (StatSci, 1994). The values predicted by the model for each diet are reported. A χ^2 analysis using the actual count data was performed to test for independence between erythrocyte class and diet (Minitab, 1994).

RESULTS

Feeding trial

The mean live weights of the rats at the start of the trial and after 10 weeks of feeding are given in Table 2. There was no significant difference in the mean live weight of rats in the dietary groups at the start of the trial. There was a statistically significant difference ($P < 0.05$) in the mean live weight between treatments, with the lowest live weight for the MARG treatment (378 g) and the highest live weight for the AMF2 treatment (422 g). The live weights of the rats in the two AMF treatments were not significantly different when tested at the 5% level.

The mean feed intakes of the rats over the 10-week treatment period are given in Table

Table 2. Live weight (initial and after 10 weeks) and feed intake (after 10 weeks) of rats fed on diets containing different fats and oils
(Mean values for nine rats per dietary group except where noted)

	Dietary fat or oil									Pooled SD	Statistical significance of effect of diet (ANOVA)
	AMF1	AMF2	PALM	MaxE	COCO	TALL	MARG	OLIVE	SOY†		
Live weight (g) initial	48.0	48.3	47.5	46.8	47.3	47.1	47.6	46.9	47.6	1.99	NS
Live weight (g) after 10 weeks	404	422	389	405	395	393	378	385	416	24.5	**
Feed intake (Σg DM/7 d per kg ^{0.75})‡	3005	3076	3001	2936	3027	3021	3121	2873	2956	77.5	***

AMF1, anhydrous milk fat; AMF2, anhydrous milk fat, after passage through a column of active C; PALM, palm oil; MaxE, MaxEPA; COCO, hydrogenated coconut oil; TALL, anhydrous tallow shortening; MARG, margarine hardstock; OLIVE, olive oil; SOY, soyabean oil.

** $P < 0.01$, *** $P < 0.001$.

† $n = 7$.

‡ $\text{kg}^{0.75}$ is metabolic body weight and for each 7 d period it was calculated as the mean live weight (kg) over the 7 d period raised to the power of 0.75. $\Sigma \text{g DM}/7 \text{ d per kg}^{0.75} = \text{g DM}/7 \text{ d per kg}^{0.75}$ for week 1 + $\text{g DM}/7 \text{ d per kg}^{0.75}$ for week 2 + ... $\text{g DM}/7 \text{ d per kg}^{0.75}$ for week 10.

Table 3. *Morphological classification of erythrocytes from rats fed on diets containing different fats and oils*

(Values are the back-transformed means of logit transformed data; it is not appropriate to quote back-transformed pooled standard deviations)

	Dietary fat or oil									Statistical significance of effect of diet
	AMF1	AMF2	PALM	MaxE	COCO	TALL	MARG	OLIVE	SOY	
<i>n</i>	9	9	9	9	9	9	9	8	7	
Cell shape										
Normal	0.481	0.500	0.475	0.555	0.559	0.634	0.592	0.617	0.488	**
Flat	0.140	0.125	0.066	0.061	0.102	0.076	0.116	0.107	0.075	**
Surface changes	0.079	0.052	0.037	0.039	0.028	0.051	0.048	0.033	0.031	NS
Early cup	0.074	0.036	0.049	0.041	0.064	0.080	0.112	0.046	0.041	**
Late cup	0.050	0.045	0.053	0.068	0.050	0.063	0.120	0.095	0.054	**
Altered margins	0.174	0.243	0.321	0.236	0.197	0.096	0.012	0.103	0.310	**

AMF1, anhydrous milk fat; AMF2, anhydrous milk fat, after passage through a column of active C; PALM, palm oil; MaxE, MaxEPA; COCO, hydrogenated coconut oil; TALL, anhydrous tallow shortening; MARG, margarine hardstock; OLIVE, olive oil; SOY, soyabean oil.

** $P < 0.01$.

2. There was a statistically significant effect of treatment ($P < 0.05$), with the MARG-treated rats having the highest feed intake (3121 Σ g DM/7 d per kg^{0.75}) and the OLIVE-treated group having the lowest intake (2873 Σ g DM/7 d per kg^{0.75}).

Erythrocyte morphology

Table 3 indicates that all six classes of erythrocytes identified by Simpson (1989) were present in the blood of normal healthy rats regardless of the experimental diet fed. Across the diet groups, normal discocytic cells represented 47–63% of the total cells present, with rats fed on the TALL diet having the highest (63.4%) and those fed on the PALM diet having the lowest (47.5%) proportion of discocytic cells. The proportion of cells with altered margins was the most variable, with a range from 1.2% for the MARG group to 32.1% for the PALM group. Each of the other classes represented less than 14% of the total cells. The proportion of cup cells was about equally divided between early and late forms. Changes in the proportion of normal-shaped cells seemed to be mirrored by changes in the altered margins class.

There was a statistically significant ($P < 0.05$) effect of diet on the proportion of erythrocytes in each class, except for the cells with surface changes where diet did not significantly affect the proportion of cells (Table 3). The χ^2 test indicated that the distribution across the classes was dependent on the diet treatment. The proportion of cells in each class did not differ significantly between the two AMF treatments and the proportions were in the middle of the range for each class.

Erythrocyte filterability and packed cell volume

The range of values for the filterability index was from 1.17 for rats fed on the AMF1 diet to 1.65 for rats fed on the SOY diet. There was no statistically significant effect ($P > 0.05$) of diet on the erythrocyte filterability index (Table 4). The technique has a high degree of variability as indicated by a CV of 38.3% which reflects the inherent variability of the technique as also observed by Reid *et al.* (1976b).

Table 4. Packed cell volume and filterability index in whole blood from rats fed on diets containing different fats and oils

(Mean values and pooled standard deviation)

	Dietary fat or oil									Pooled SD	Statistical significance of effect of diet (ANOVA)†
	AMF1	AMF2	PALM	MaxE	COCO	TALL	MARG	OLIVE	SOY		
<i>n</i>	6	8	8	7	8	9	7	7	6		
Packed cell volume (%)	40.8	40.3	40.1	41.5	40.2	41.3	43.1	40.5	40.2	1.32	**
Filterability index‡	1.17	1.58	1.56	1.22	1.20	1.39	1.59	1.36	1.65	0.50	NS

AMF1, anhydrous milk fat; AMF2, anhydrous milk fat after passage through a column of active C; PALM, palm oil; MaxE, MaxEPA; COCO, hydrogenated coconut oil; TALL, anhydrous tallow shortening; MARG, margarine hardstock; OLIVE, olive oil; SOY, soyabean oil.

** $P < 0.01$.

† Significance of difference was calculated for each pair of means.

‡ Filterability index = $(60/(2 \times 0.5 \text{ ml flow time})) \times (\text{packed cell volume} \times 100)$.

Rats fed on the MARG diet had a significantly higher ($P < 0.05$) packed cell volume (43.1%) than rats fed on the other dietary treatments (40.1–41.5%).

DISCUSSION

Although it has been shown that non-discocytes are present in the blood of animals and humans with chronic disorders (Simpson *et al.* 1987), the influence of diet on the presence of non-discocytes in healthy animals has not been reported. In the present study the χ^2 test indicated that the distribution in proportions of the cell classes depended on diet but it was not clear what attribute of the diet (fat or oil) caused the dependence. There were no clear trends in changes of proportions of cell shapes with change in degree of saturation or unsaturation of fatty acids. Blood samples from rats fed on the AMF1 and AMF2 diets were not significantly different from each other, which suggests that the minor components in milk fat do not influence erythrocyte morphology.

The results suggest that, in normal healthy animals, diet does not have a significant effect on the filterability of erythrocytes. When fish-oil supplements (1.2–3.6 g eicosapentaenoic acid (EPA)/d) were given to healthy human male volunteers, an increase in deformability index was observed after 4 weeks of supplementation (Terano *et al.* 1983; Ernst, 1989). In patients with hyperlipoproteinaemia, supplementation of the normal diet of the volunteers with salmon-oil-concentrate capsules increased erythrocyte deformability after 8 weeks of supplementation. A much lower concentration of EPA was used (Ernst, 1989).

Supplementation, particularly with fish oil, may offer pharmacological benefits in the prevention and treatment of disorders that affect erythrocyte filterability. Dietary manipulation may be useful in the treatment of conditions where filterability has already been impaired. However, it appears from our present study in rats that the presence of different dietary fats or oils may not have a significant effect on filterability in normal healthy animals. If it is assumed that the biconcave disc cell is the most easily filterable cell shape, then it may be expected that the diet group with the highest proportion of discocytic cells would have the highest filterability. However, in this study, no correlation was observed between filterability index and morphology.

Although it has been observed that diet can improve the filterability of erythrocytes once filterability is impaired, the significance of erythrocyte filterability in normal healthy animals including humans is unclear. The importance of the differences in erythrocyte morphology due to diet is also unclear. Both areas deserve further investigation.

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