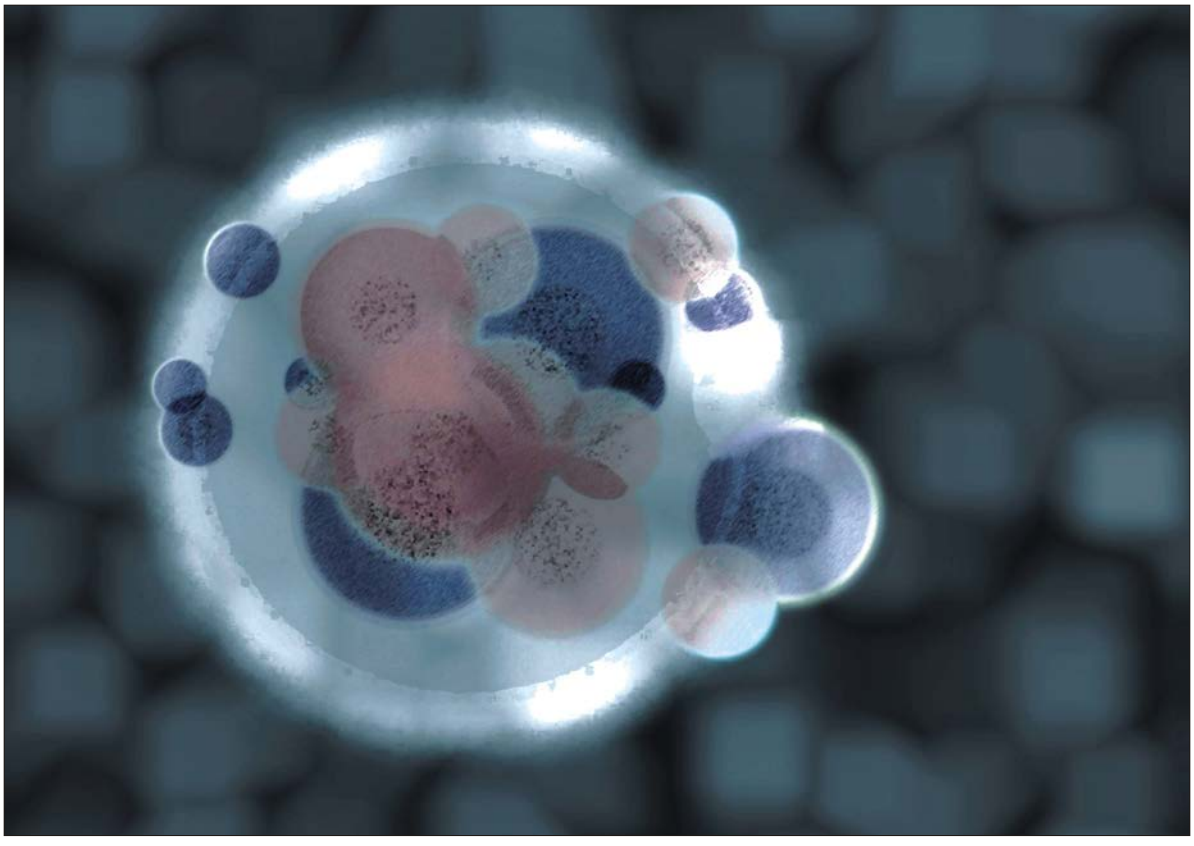

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Research Article
Quercetin Does Not Alter Erythrocyte Osmotic Fragility in Female, Sprague Dawley Rats Fed a High-fructose, High-cholesterol Diet

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Abstract

Background and Objectives: Increased dietary cholesterol and fructose intake is associated with increased erythrocyte membrane rigidity and damage as a result of lipid peroxides, leading to increased erythrocyte osmotic fragility. Flavonoids possess anti-oxidant properties associated with their ability to stabilize cell membranes. The current study investigated the effects of a high-fructose, high-cholesterol diet, together with quercetin (a flavonoid), on erythrocyte osmotic fragility and erythrocyte indices of female Sprague Dawley rats. **Methods and Materials:** Rats received their respective diets and drinking solutions *ad libitum* for 8 weeks, following which blood samples were collected and analysed. **Results:** No significant differences ($p>0.05$) in terminal body mass was observed between groups. Serum cholesterol concentrations were increased ($p<0.05$) in all groups receiving the high-fructose, high-cholesterol diet compared to those receiving standard rat chow and tap water to drink. Serum triglyceride concentrations and blood haemoglobin concentrations, haematocrit and mean corpuscular haemoglobin concentrations were not significantly different between groups. Fragiligrams of erythrocytes from all rats were similar, with no differences in any of the osmotic fragility indices examined. **Conclusion:** Feeding rats a high-fructose, high-cholesterol diet for 8 weeks resulted in hypercholesterolemia but had no effect on erythrocyte osmotic fragility or serum triglyceride concentrations. The administration of quercetin had no effect on the parameters assessed.

Key words: Quercetin, erythrocyte osmotic fragility, rat, high-fructose, high-cholesterol diet

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Erythrocyte osmotic fragility is a measure of the erythrocyte's ability to resist haemolysis, which is dependent on the rigidity of the erythrocyte membrane (EM). The rigidity of the EM is influenced by the fatty acid and cholesterol content of the membrane[1], which in turn can be altered by diet[2]. The lipid composition of the EM has been shown to reflect long term dietary lipid intake[3]. Previous studies have shown that increased cholesterol intake in rats, resulting in hypercholesterolemia, causes significant cholesterol accumulation within the EM[4]. Increased EM cholesterol content, increases the membrane cholesterol:phospholipid ratio, which in turn can increase the rigidity of the membrane, increasing the osmotic fragility of the erythrocytes[5].

Increased erythrocyte osmotic fragility also occurs following alterations in membrane structure and function due to lipid peroxidation by-products[6]. Excessive fructose consumption, which has been linked to development of the metabolic syndrome[7], increases lipogenesis and the formation of lipid peroxides[8] and advanced glycation end products (AGEs)[9], which are linked to EM damage and thus increased erythrocyte osmotic fragility and altered erythrocyte indices[8,10]. By virtue of their exposure to high levels of oxygen, as well as the rich polyunsaturated fatty acid content within their cell membranes, the erythrocytes are susceptible to both intracellular and extracellular sources of reactive oxygen species which induce lipid peroxidation[11,12]. Plant flavonoids, such as quercetin (3,3',4',5,7-pentahydroxyflavone), have been reported to possess antioxidant activity and are able to inhibit lipid peroxidation induced by free radicals[13].

The ability of flavonoids to interact with and stabilize cell membranes is associated with their antioxidant activity[14]. The degree of incorporation of flavonoids within the membrane, their orientation within the membrane and the uniformity of their distribution, contribute to their effectiveness as antioxidants[15]. Since cholesterol has been shown to regulate membrane structure and function, alterations in EM cholesterol content may alter incorporation of the antioxidants (i.e., flavonoids) into the EM, thus modifying their antioxidant properties and ability to stabilize the EM[11,16]. In addition to cholesterol diet-induced plasma lipid profile modifications, fructose consumption is associated with the development of hypertriglyceridemia[2]. Thus, the current study made use of a high-fructose, high-cholesterol fed rat model to assess the effects of quercetin administration (a polyphenol flavonoid-antioxidant) on erythrocyte osmotic fragility and other erythrocyte indices. There is increasing use of phytochemicals to manage diseases, however these need to be scientifically validated under controlled conditions.

Therefore, this study investigated the effects of a high-fructose, high-cholesterol diet on erythrocyte osmotic fragility, erythrocyte indices and the possible beneficial or detrimental effects of quercetin administration. Research on male rats has also been over represented, despite the fact that there are widely reported sexually dimorphic differences in susceptibility to metabolic disorders[17] and hence this study focused on the lesser studied female rats.

MATERIALS AND METHODS

The study was carried out in the School of Physiology of the University of the Witwatersrand, South Africa, between April and August of 2017.

Ethical approval: The study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (Ethics clearance number: 2017/02/07B).

Animals, housing and diets: Thirty six, 21-day old, female, Sprague Dawley rats (*Rattus Norvegicus*) (38.1±5.8 g) were used in the study. The rats were randomly divided into 6 dietary groups (n = 6 rats in each group). Group 1 (C) received standard rat chow (SRC) (LabChef, North West University/RB2005) and plain gelatine cubes, group 2 (Q) received SRC and gelatine cubes containing quercetin (75 mg kg^{-1} body mass) (Sigma-Aldrich Co., St Louis, MO USA), group 3 (Fe) received SRC and gelatine cubes containing fenofibrate (100 mg kg^{-1} body mass) (Sigma-Aldrich Co., St Louis, MO USA), group 4 (HFHC) received SRC enriched with 2% cholesterol (Life Science, SIGMA®), 20% fructose (Nature's Choice, Germany) drinking solution and plain gelatine cubes, group 5 (HFHC-Q) received SRC enriched with 2% cholesterol, 20% fructose drinking solution and gelatine cubes containing quercetin (75 mg kg^{-1} body mass) and group 6 (HFHC-Fe) received SRC enriched with 2% cholesterol, 20% fructose drinking solution and gelatine cubes containing fenofibrate (100 mg kg^{-1} body mass). The rats received their respective diets *ad libitum* for the 8 week experimental period and had free access to their water or fructose drinking solution. Quercetin or fenofibrate was administered in flavoured gelatine cubes, made to a volume of 2 mL. The gelatine cubes were prepared using methods modified from Kamerman *et al.*[18]. Briefly, 8 g gelatine powder, 8 g brown sugar and 3 mL of savoury bread spread, Bovril (Unilever, South Africa) were mixed into 100 mL hot water. The quercetin or fenofibrate (or neither for the plain gelatine cubes) was added to the gelatine solution, vortexed and poured into 2 mL moulds and allowed to set.

Rats were individually housed in perspex cages in the Central Animal Service's animal unit, at the Faculty of Health Sciences, University of the Witwatersrand. Ambient temperature was maintained at 26±2°C and lighting restricted to 12 h, with lights on from 06:00.

General experimental procedure: Before the experimental period, the rats were allowed a three-day adaptation period, to become accustomed to the housing, handling and feeding procedures. During the adaptation period, all rats were fed SRC and received plain gelatine cubes. During the 8 week experimental period the rats were fed their respective diets/treatments (as previously described). The rats were weighed twice a week using a pre-weighed cage on a scale (Precisa 310 M, Laser, Johannesburg, South Africa). Following the experimental period the rats were fasted for approximately 15 h overnight. A drop of blood was collected from the tail vein of the rats via pin prick with a sterile needle. Blood haemoglobin concentrations and haematocrit (Hct) were measured. The rats were then anaesthetized using an anaesthetic overdose of sodium pentobarbital (Eutha-naze, Centaur Labs, South Africa) (200 mg kg^{-1}), administered intraperitoneally. Blood samples were collected by cardiac puncture (using 20 G hypodermic needles) into vacutainer tubes (Vacuette, Greiner Bio-One, Amphur Phantong, Chonburi, Thailand) containing either lithium heparin (for osmotic fragility determinations) or into serum separator, clot activator vacutainer tubes (Vacuette) (for serum determinations).

Blood parameters

Haematocrit, haemoglobin concentration and mean corpuscular haemoglobin concentration: Blood haematocrit and haemoglobin concentration was measured using a veterinary Hct meter (InSight HCT meter, Woodley Equipment Company, Bolton, UK), according to manufacturer's instructions. Mean corpuscular haemoglobin concentration was then calculated as follows:

$$\text{Mean cell haemoglobin concentration} = \frac{\text{Hb (g/100 mL)}}{\text{Hct (\%)}} \times 100$$

Erythrocyte osmotic fragility: Erythrocyte osmotic fragility was determined using methods previously described by Donaldson *et al.*[19]. Briefly, 50 µL of whole blood was added to tubes containing 5 mL of varying concentrations (between 0 and 0.85% saline) of phosphate-buffered saline (PBS) (pH 7.4). The solutions were allowed to stand at room temperature (24°C) for 30 min and then centrifuged (Sorvall RT 6000 B, Du Pont, Hertfordshire, UK) at 370 g and 22°C for 5 min. Absorbance of the supernatant was determined using a spectrophotometer (Ultrospec II, LKB Biochrom, Cambridge, England) at 540 nm, using distilled water as the blank. The highest absorbance reading was considered 'maximal/complete haemolysis' and subsequently used to calculate the percentage haemoglobin released in each PBS solution, relative to the solution in which maximal haemolysis occurred, for each rat. Fragiligrams were constructed and then indices of fragility determined.

Serum cholesterol and triglyceride measurements: Blood samples collected at euthanasia were centrifuged (Sorvall RT 6000 B, Du Pont) at 370 g and 22°C for 15 min. The serum was then used to determine serum cholesterol and triglyceride concentrations calorimetrically using an IDEXX Vetlab Analysis Machine (IDEXX Laboratories, Westbrook, ME, USA), according to manufacturer's instructions.

Statistical analysis: All data are expressed as Mean±SD, unless otherwise stated. The data were analysed and plotted using Graphpad 6 Prism software (Graph-pad Software Inc, San Diego, USA). A one-way ANOVA was used to compare final body mass, blood haemoglobin concentration, haematocrit, mean corpuscular haemoglobin and serum cholesterol and triglyceride concentrations of the rats between dietary groups. Differences between groups were identified using a Tukey's multiple comparisons *post hoc* test (following the one-way ANOVA). The $p \leq 0.05$ was considered significant.

Mean±SD for the data concerning the percentage haemoglobin released from erythrocytes upon haemolysis, for each PBS solution, was calculated and used to construct the fragiligram for each dietary group. The range of PBS solutions at which initial (4%) haemolysis (IH), 50% haemolysis (MCF) and maximal haemolysis (MH) of the erythrocytes occurred was then read off the graphs.

RESULTS

Body mass: Figure 1 shows the initial and final body masses of the rats in each dietary group. There were no significant differences in initial or final body masses between groups ($p>0.05$). All rats in all dietary groups grew significantly during the feeding period ($p<0.0001$).

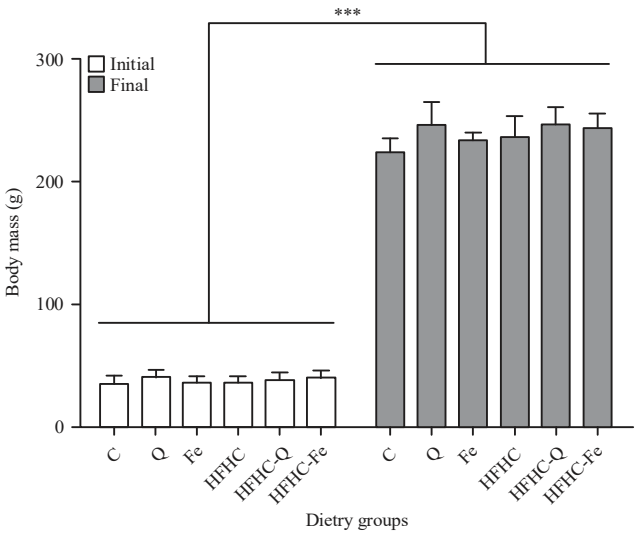

Fig. 1: Initial and final body masses/dietary group of Sprague Dawley rats following the 8 week feeding period

C: Control, Q: Quercetin, Fe: Fenofibrate, HFHC: High-fructose, high-cholesterol, HFHC-Q: High-fructose, high-cholesterol with quercetin, HFHC-Fe: High-fructose, high-cholesterol with fenofibrate, data represented as Mean±SD, n = 6 in each dietary group, ***p<0.0001 when comparing initial to final body mass within a single dietary group

Table 1: Blood haemoglobin concentration, haematocrit and mean corpuscular haemoglobin concentration for female, Sprague Dawley rats following the 8 week feeding period

Dietary groups	Haemoglobin (g dL^{-1})	Haematocrit (%)	MCHC (g dL^{-1})
C	17.82±0.66	53.33±1.86	33.40±0.12
Q	17.05±0.72	51.17±1.94	33.32±0.24
Fe	17.75±1.17	53.33±3.45	33.28±0.19
HFHC	17.20±0.85	51.67±2.73	33.30±0.25
HFHC-Q	17.12±0.55	51.33±1.51	33.34±0.24
HFHC-Fe	17.88±1.21	53.67±3.50	33.32±0.19

Data represented as Mean±SD, MCHC: Mean corpuscular haemoglobin concentration, C: Control, Q: Quercetin, Fe: Fenofibrate, HFHC: High-fructose, high-cholesterol, HFHC-Q: High-fructose, high cholesterol with quercetin, HFHC-Fe: High-fructose, high-cholesterol with fenofibrate

Haemoglobin concentration, haematocrit and mean corpuscular haemoglobin concentration: Table 1 shows the blood haemoglobin concentration (g dL^{-1}), haematocrit (%) and mean corpuscular haemoglobin concentration (g dL^{-1}) for the rats following the 8 week feeding period. No significant differences in haemoglobin concentration, haematocrit or mean corpuscular haemoglobin concentration were observed between groups (p>0.05).

Erythrocyte osmotic fragility: Figure 2 shows the fragiligrams of the erythrocytes from the rats following the 8 week feeding period. The fragiligrams of the erythrocytes from the rats in the various dietary groups were not different from one another. Table 2 indicates the range of PBS solutions (%) at which initial haemolysis (IH), 50% haemolysis (MCF) and maximal haemolysis (MH) occurred for the erythrocytes from the rats in the various dietary groups. There were no differences in any of the osmotic fragility indices examined.

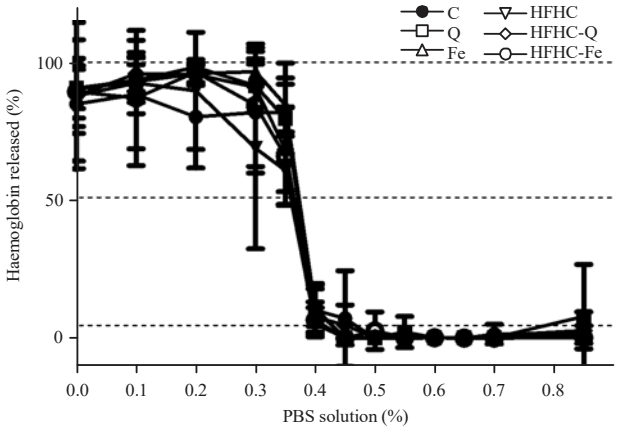

Fig. 2: Fragiligrams obtained from the erythrocytes of Sprague Dawley rats following the 8 week feeding period

C: Control, Q: Quercetin, Fe: Fenofibrate, HFHC: High-fructose, high-cholesterol, HFHC-Q: High-fructose, high-cholesterol with quercetin, HFHC-Fe: High-fructose, high-cholesterol with fenofibrate, data represented as Mean±SD, n = 6 in each dietary group

Table 2: Range of phosphate-buffered saline solutions at which initial haemolysis, 50% haemolysis and maximal haemolysis occurred for the erythrocytes from female, Sprague Dawley rats following the 8 week feeding period

	PBS solution (%)		
Dietary groups	Initial haemolysis	Mean corpuscular fragility	Maximal haemolysis
C	0.45-0.65	0.35-0.40	0.00-0.30
Q	0.45-0.65	0.35-0.40	0.00-0.30
Fe	0.45-0.65	0.35-0.40	0.00-0.30
HFHC	0.45-0.65	0.35-0.40	0.00-0.30
HFHC-Q	0.45-0.65	0.35-0.40	0.00-0.30
HFHC-Fe	0.45-0.65	0.35-0.40	0.00-0.30

PBS: Phosphate-buffered saline solution, C: Control, Q: Quercetin, Fe: Fenofibrate, HFHC: High-fructose, high-cholesterol, HFHC-Q: High-fructose, high cholesterol with quercetin, HFHC-Fe: High-fructose, high-cholesterol with fenofibrate, Mean corpuscular fragility: 50% haemolysis

Serum cholesterol and triglyceride concentration: Figure 3 and 4 show the serum cholesterol and serum triglyceride concentrations (mg dL^{-1}) for rats following the 8 week feeding period, respectively. Serum cholesterol concentrations were significantly increased (p<0.05) in all groups receiving the high-fructose, high-cholesterol diet (HFHC, HFHC-Q and HFHC-Fe) compared to those receiving the normal rat chow and tap water (C, Q and Fe). Serum triglyceride concentrations were not significantly different (p>0.05) between dietary groups following the 8 week feeding period; however there

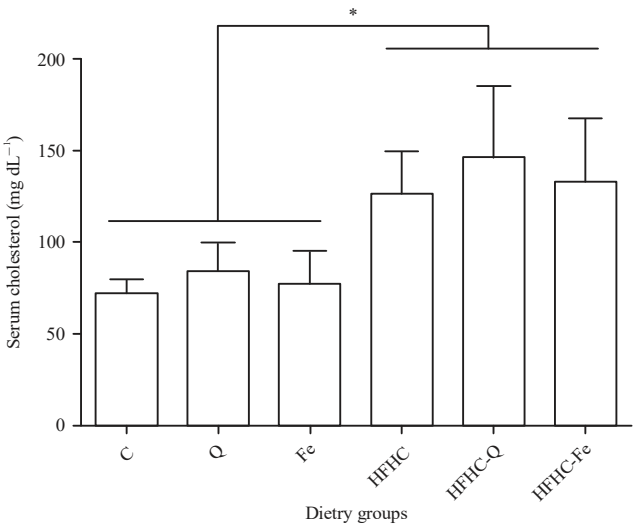

Fig. 3: Serum cholesterol concentrations of Sprague Dawley rats following the 8 week feeding period

C: Control, Q: Quercetin, Fe: Fenofibrate, HFHC: High-fructose, high-cholesterol, HFHC-Q: High-fructose, high-cholesterol with quercetin, HFHC-Fe: High-fructose, high-cholesterol with fenofibrate, data represented as Mean±SD, n = 6 in each dietary group, *$p \leq 0.05$ when comparing groups receiving the high-fructose, high-cholesterol diet (HFHC, HFHC-Q and HFHC-Fe) to those receiving normal rat chow and tap water (C, Q and Fe)

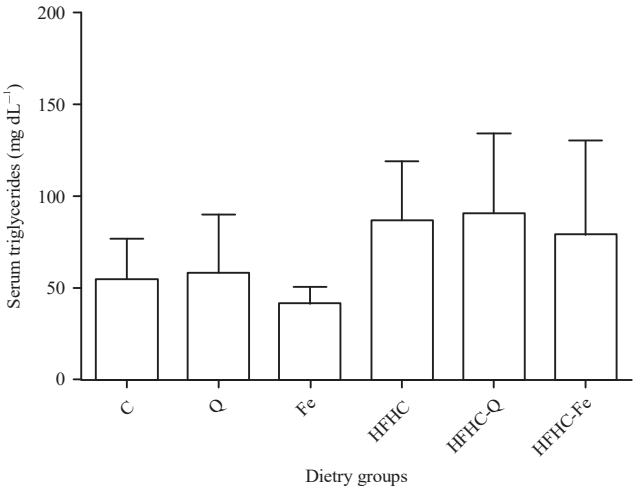

Fig. 4: Serum triglycerides concentrations of Sprague Dawley rats following the 8 week feeding period

C: Control, Q: Quercetin, Fe: Fenofibrate, HFHC: High-fructose, high-cholesterol, HFHC-Q: High-fructose, high-cholesterol with quercetin, HFHC-Fe: High-fructose, high-cholesterol with fenofibrate, data represented as Mean±SD, n = 6 in each dietary group

was a tendency towards increased serum triglyceride concentrations in the groups receiving the high-fructose, high-cholesterol diet (HFHC, HFHC-Q and HFHC-Fe) compared to that observed in the control diet groups (C, Q and Fe).

DISCUSSION

The high-fructose, high-cholesterol diet resulted in hypercholesterolemia in the rats. However, feeding the rats the high-fructose, high-cholesterol diet, with or without quercetin administration post weaning, did not affect erythrocyte osmotic fragility, body mass, blood haemoglobin concentration, haematocrit, mean corpuscular haemoglobin concentration or serum triglyceride concentrations.

High-fructose feeding causes alterations in lipid metabolism resulting in dyslipidemia and the development of lipid peroxides which damage erythrocyte membranes (EMs)[12]. Advanced glycation end products (AGEs) can also result from such diets[9], also negatively impacting the erythrocyte[10]. Additionally, high-cholesterol diets have been shown to modify EM cholesterol content, affecting the stability of the membrane[4,5]. Therefore following the development of hypercholesterolemia in the rats, a change in the lipid composition of the EM was anticipated, which in turn would influence the stability of the membrane and consequently the erythrocyte osmotic fragility. However this was not the case. EM lipid composition was not directly assessed in the current study; however since no observable changes in erythrocyte osmotic fragility were noted it can be assumed that changes (if any) in EM composition induced by the high-fructose, high-cholesterol diet, did not significantly impact the EM fluidity. This finding is similar to previous studies where fructose consumption and administration of *Hibiscus sabdariffa* extracts to neonatal rats was previously shown not to alter erythrocytes osmotic fragility[20,21].

Despite the significantly increased serum total cholesterol concentrations observed in the rats receiving the high-fructose (20%), high-cholesterol diet (2%), the diet, with or without quercetin, had no influence on serum triglyceride concentrations or on the body mass gain across the dietary groups. These results are in agreement with those of Axelsen *et al.*[22] who also observed significant hypercholesterolemia in sprague-dawley rats fed a high-fructose (10%), high-cholesterol (4%) diet for a period of 15 weeks, but no significant differences in serum triglyceride concentrations or body mass compared to the control group. Pahua-Ramos *et al.*[23] observed significantly increased serum total cholesterol and triglyceride concentrations, as well as body mass gain in male Wistar rats fed a hypercholesterolemic diet and a 60% fructose solution for 7 weeks, compared to control rats. Tillman *et al.*[24] observed no differences in body mass gain between mice that were weaned directly on to a high-fructose diet (60% calories from fructose) and those that did not receive

fructose[24]. The authors attributed the lack of diet-induced differences in body mass gain to the age of the mice. The increased metabolic rate of the mice during their growth period could possibly have resulted in the fructose being fully oxidised, thus negating some of the expected diet-induced effects[24]. This could be the case in the current study, since the rats were also weaned directly onto the high-fructose, high-cholesterol diet.

'Weanling' rats (21 days old) were used in the current study in order to mimic children consuming diets high in fats and fructose. Additionally, de Moura *et al.*[25] observed that high-fructose administration was more effective at producing signs of the MetS in adult versus young rats. The discrepancies in previous results with regards to fructose-induced body mass gain and changes in serum/plasma triglyceride concentrations could be due to the method of fructose administration (in feed or liquid form), the amount of fructose, the duration of feeding, the type of animal model used and the age of the rats upon commencing the fructose administration, all of which may have influenced the results presented here.

CONCLUSION

In conclusion, the high-fructose, high-cholesterol diet resulted in the development of hypercholesterolemia without impacting on the erythrocyte fragility or haemoglobin content in the growing female rats. The quercetin was well tolerated by the rats and did not negatively impact the erythrocytes, confirming the perceptions about the safety of quercetin use. Further studies are required to elucidate interactions between dietary, environmental and genetic factors involved in development of diet-induced metabolic abnormalities and their effects on erythrocyte osmotic fragility and other erythrocyte indices.

SIGNIFICANCE STATEMENT

There is increasing consumption of fructose and cholesterol worldwide which causes metabolic problems and therefore places a heavy burden on health care delivery systems worldwide. Phytochemicals can be used as prophylactic agents. However, these substances can have toxic effects. This study advances knowledge by showing that quercetin does not negatively impact erythrocyte osmotic fragility and erythrocyte indices, whether given with or without fructose and cholesterol.

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