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Curcuma oil ameliorates insulin resistance & associated thrombotic complications in hamster & rat

Vishal Singh[*,†], Manish Jain[*,†], Ankita Misra[*], Vivek Khanna[*], Prem Prakash[*], Richa Malasoni[**], Anil Kumar Dwivedi[**], Madhu Dikshit[*] & Manoj Kumar Barthwal[*]

[*]*Pharmacology Division & [**]Division of Pharmaceutics, CSIR-Central Drug Research Institute, Lucknow, India*

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***Background & objectives*: *Curcuma* oil (C. oil) isolated from turmeric (*Curcuma longa* L.) has been shown to have neuro-protective, anti-cancer, antioxidant and anti-hyperlipidaemic effects in experimental animal models. However, its effect in insulin resistant animals remains unclear. The present study was carried out to investigate the disease modifying potential and underlying mechanisms of the C. oil in animal models of diet induced insulin resistance and associated thrombotic complications.**

***Methods*: Male Golden Syrian hamsters on high fructose diet (HFr) for 12 wk were treated orally with vehicle, fenofibrate (30 mg/kg) or C. oil (300 mg/kg) in the last four weeks. Wistar rats fed HFr for 12 wk were treated orally with C. oil (300 mg/kg) in the last two weeks. To examine the protective effect of C. oil, blood glucose, serum insulin, platelet aggregation, thrombosis and inflammatory markers were assessed in these animals.**

***Results*: Animals fed with HFr diet for 12 wk demonstrated hyperlipidaemia, hyperglycaemia, hyperinsulinaemia, alteration in insulin sensitivity indices, increased lipid peroxidation, inflammation, endothelial dysfunction, platelet free radical generation, tyrosine phosphorylation, aggregation, adhesion and intravascular thrombosis. *Curcuma* oil treatment for the last four weeks in hamsters ameliorated HFr-induced hyperlipidaemia, hyperglycaemia, insulin resistance, oxidative stress, inflammation, endothelial dysfunction, platelet activation, and thrombosis. In HFr fed hamsters, the effect of C. oil at 300 mg/kg was comparable with the standard drug fenofibrate. *Curcuma* oil treatment in the last two weeks in rats ameliorated HFr-induced hyperglycaemia and hyperinsulinaemia by modulating hepatic expression of sterol regulatory element binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor-gamma co-activator 1 (PGC-1)α and PGC-1β genes known to be involved in lipid and glucose metabolism.**

***Interpretation & conclusions*: High fructose feeding to rats and hamsters led to the development of insulin resistance, hyperglycaemia, endothelial dysfunction and oxidative stress. C. oil prevented development of thrombotic complications associated with insulin resistance perhaps by modulating genes involved in lipid and glucose metabolism. Further studies are required to confirm these findings.**

Key words Curcuma oil - hamster - hyperinsulinaemia - platelets - *SREBP-1c*

[†]The first two authors have contributed equally.

Turmeric (*Curcuma longa* L.) widely used in the herbal medicine, possesses anti-oxidant and anti-inflammatory activities[1]. The chemical constituents and detailed gas chromatography-mass spectrometry (GC-MS) profile of *Curcuma* oil (C. oil) has been reported earlier[2]. Anti-hyperlipidaemic activity of C. oil through modulation of hepatic peroxisome proliferator-activated receptor alpha (PPARα), liver X receptor-α (LXRα) and associated genes involved in lipid metabolism and transport has also been reported[3]. Enhanced thrombotic events observed during insulin resistance and diabetic dyslipidaemia can be attributed to endothelial activation, hyperactivity of platelets, inflammation, hypercoagulability, and hypofibrinolysis[4,5]. Diets high in saturated fats and cholesterol have been demonstrated to induce weight gain, insulin resistance, hyperlipidaemia, and impaired oxidative defense in animals[6].

Feeding Golden Syrian hamsters with diet rich in fructose and fat leads to the development of diabetic dyslipidaemia[7]. Therefore, these are considered as a good model for the evaluation of anti-hyperlipidaemic and anti-diabetic test agents[7]. On the contrary, rats have high density lipoprotein (HDL) as the major circulating lipoprotein and develop insulin resistance after high fructose diet (HFr) feeding[8]. It has been shown that HFr feeding in Wistar rats for 12 wk leads to the development of impaired glucose tolerance, insulin resistance, endothelial dysfunction, oxidative stress and inflammation[5]. The present study was undertaken to evaluate the effect of C. oil on diabetic dyslipidaemia, insulin sensitivity and associated deleterious effects in the hamster and rat models of diet induced insulin resistance.

Material & Methods

All fine chemicals including apyrase, acetylcholine (ACh), phenylephrine (PE), bovine serum albumin, adenosine 5'-diphosphate (ADP), collagen and thrombin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dichlorohydrofluorescein diacetate (DCF-DA) was purchased from Invitrogen (CA, USA). Anti-phosphotyrosine clone PY20, 4G10 and Insulin kit were obtained from Santa Cruz biotechnology (CA, USA) and Millipore (MA, USA), respectively. Fructose was procured from Sisco Research Laboratories (India). Chrono-par Collagen was procured from Chrono-Log Corp. (Havertown, PA, USA). BCA™ Protein Assay Kit (Pierce, Rockford, USA) and Coagulation kits (Diagnostica Stago, Vilvoorde, Belgium) were also procured. The modified high fat and fructose diet (Research diet D.99122211) for hamsters was procured from Research Diets Inc, Burnswick USA. High fructose diet for rats was prepared at the in-house diet production facility of Central Drug Research Institute (CDRI), Lucknow, India.

Animal diet and treatment: The study was conducted in the Pharmacology Division of CDRI, Lucknow. Preparation and quality assessment of C. oil was performed as described elsewhere[2]. Male Golden Syrian hamsters (100-120g) and Wistar rats (180-200g) were received from Animal house of CDRI. The study protocol was approved by the Institutional Animal Ethics Committee. After acclimatization, hamsters were randomly divided into two groups, chow and HFr fed (normal diet supplemented with 9% fructose, 0.5% cholesterol and 30% saturated fat). After eight weeks of HFr feeding, these animals were again grouped into those receiving HFr along with vehicle (0.25% carboxymethylcellulose) or fenofibrate (30 mg/kg/day; per oral, po)[9] or C. oil (300 mg/kg/day; po) for an additional four weeks. Similarly, rats were randomly distributed into two groups, chow and HFr fed [35% fructose, 5% saturated fat (coconut oil) along with 25% fructose in drinking water][5]. After 10 wk of HFr feeding, these animals were again grouped into those receiving HFr along with vehicle (0.25% carboxymethylcellulose) or C. oil (300 mg/kg/day; po) for an additional two weeks. At the end of 12 wk, overnight fasted animals were anaesthetized and blood was collected by cardiac puncture in tubes containing 2.5 per cent trisodium citrate. Platelet rich plasma (PRP) was isolated from three ml of whole blood by centrifugation at 180×*g* for 10 min. Platelets were isolated by centrifugation of PRP at 600×*g* in the presence of 10 per cent acid citrate dextrose (ACD).

Blood samples from the overnight fasted animals were collected by cardiac puncture and plasma was prepared. Plasma total cholesterol (TC), low density lipoprotein (LDL) cholesterol (LDL-C), high density lipoprotein (HDL) cholesterol (HDL-C), triglycerides (TGs) and glucose were estimated using commercial kits from Pointe Scientific, USA and RFCL Limited, India[4]. The plasma insulin level was measured by using ELISA kit (Merck. Millipore, USA) as per manufacturer's protocol[5].

Assessment of insulin resistance: To evaluate if the animals were insulin resistant, the quantitative insulin sensitivity check index *i.e.* QUICKI = 1/ log (fasting insulin, μU/ml) + log (fasting glucose, mg/dl)[10] and homeostasis model assessment for insulin resistance

i.e. HOMA-IR = [fasting insulin (µU/ml) × fasting glucose (mmol/l)]/22.5[11] were calculated.

Measurement of malondialdehyde: Malondialdehyde (MDA) was measured spectrophotometrically using 1,1,3,3-tetraethoxypropane as standard. To 200µl of plasma, 300µl of 30 per cent trichloroacetic acid, 150 µl of 5N hydrochloric acid and 300 µl of 2 per cent w/v 2-thiobarbituric acid were added and the volume was made upto one ml by phosphate buffer (*p*H 7.4). The mixture was then heated for 15 min at 90°C. The mixture was centrifuged at 11000×*g* rpm for ten min. Intensity of pink colour in the supernatant was measured spectrophotometrically at 532 nm[5].

Aortic cholesterol assessment: After collecting blood, animals were perfused with cold PBS containing 5 mM EDTA. Whole aorta was removed, cleaned, weighed and lipid was extracted with hexane: isopropanol (3:2)[3]. The extracted lipids were dried, resuspended in reagent grade ethanol containing NP40 (9:1). Tissue TC was measured using cholesterol assay kit according to manufacturer's protocol. In brief, 50µl of samples were incubated with 50µl of working reagent from cholesterol assay kit for 30 min in dark. After incubation, plate was read by fluorescence plate reader (BMG LABTECH, Offenburg, Germany) at the excitation wavelength of 540 nm and emission wavelength of 590 nm.

Semi-quantitative and real-time quantitative reverse transcription-PCR for inflammatory and lipogenic gene expression: Spleens from hamsters and liver from rats were aseptically removed and placed in RPMI (Roswell Park Memorial Institute) medium. Single cell suspensions were prepared and RBC lysed as described previously[4]. RNA was isolated using Trizol (Life Technologies, USA) as per manufacturer's protocol. cDNA was synthesized using RevertAidTM H Minus First strand cDNA Synthesis Kit (Fermentas, USA). Gene amplification of tumour necrosis factor (TNF)α and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) of hamsters was achieved using specific primers (Table I)[4] and amplification conditions as described previously[4]. The PCR products were analyzed on agarose gel and quantified using calibrated densitometer (BioRad Inc. USA). Hepatic mRNA expression of sterol regulatory element binding protein 1c (*SREBP-1c*), peroxisome proliferator-activated receptor-gamma co-activator (*PGC*)-1α and 1β in rats were monitored by using gene specific primers (Table I)[12-14]. Real-time RT-PCR was carried out in LightCycler®480II Real-Time PCR system (Roche, Indianapolis, USA) with the help of SYBR green maxima reagents (Fermentas, Maryland, USA) and amplification conditions as described previously[3,15]. Melting curve analysis was performed immediately after amplification using manufacturer's protocol.

Estimation of inflammatory cytokines by ELISA: Circulating level of C-reactive protein (CRP), interleukin-6 (IL-6), interferon-gamma (IFN-γ),

Table I. Sequence of primers used in this study

Species	Name of the gene	Primer sequence 5' to 3'
Rat	*PGC-1α*[12]	F: ACCCACAGGATCAGAACAAACC R: GACAAATGCTCTTTGCTTTATTGC
Rat	*PGC-1β*[12]	F: CAAGAAGCGGCGGGAAA R: GCTCATGTCACCGGAGAGATTT
Rat	*SREBP-1C*[13]	F: CATCGACTACATCCGCTTCTTACA R: GTCTTTCAGTGATTTGCTTTTGTGA
Rat	*GAPDH*[14]	F: TGCCACTCAGAAGACTGTGG R: TTCAGCTCTGGGATGACCTT
Hamster	*TNFα*[4]	F: GACCACAGAAAGCATGATCC R: TGACTCCAAAGTAGACCTGC
Hamster	*HGPRT*[4]	F: ATCACATTATGGCCCTCTGTG R: CTGATAAAATCTACAGTYATGG

F, forward; R, reverse primer. Superscript numerals denote reference numbers.
PGC-1α , peroxisome proliferator-activated receptor-gamma co-activator-1α ; *PGC-1β,* peroxisome proliferator-activated receptor-gamma co-activator-1β; *SREBP-1C,* sterol regulatory element binding protein 1c; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *TNFα,* tumour necrosis factor α; *HGPRT,* hypoxanthine guanine phosphoribosyltransferase

and tumour necrosis factor (TNF) were measured in plasma samples from rats using ELISA kits from BD Biosciences (California, USA) as per manufacturer's protocol. Plates were read at 450 and 570 nm (wavelength correction) on ELISA plate reader (Bio-Tek Instruments, Winooski, VT). Standards provided in the kit were used for drawing the standard curves.

Assessment of vasoreactivity: Transverse four mm wide rings of thoracic aorta were cut and mounted in ten ml organ baths containing Kreb's solution. After equilibration, the aortic rings were exposed to KCl Krebs buffer (80 mM) to assess the maximum tissue contractility. The presence of a functional endothelium was verified by the occurrence of significant relaxation to acetyl choline (Ach, 3 to 30 mM) in phenylephrine (PE, 1 μM) pre-contracted aortic rings. Cumulative concentration dependent contraction responses to PE were also assessed. Finally, tissue contractility and viability were assessed by exposing the rings to KCl Krebs buffer (80 mM) in all the groups[16].

Platelet reactive oxygen species (ROS) generation: Platelets from hamsters were resuspended in Tyrode's HEPES [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] buffer (Sigma-Aldrich, San Diego, USA) with 5 mM glucose, 1 mM EDTA, and allowed to rest at 37°C for 30 min before being labelled as previously described[17]. The platelet samples were incubated with 10 μM dihydrodichlorofluorescein diacetate (DCF-DA) for 30 min at 37°C. Labelled cells were stimulated with collagen (5 μg/ml) or thrombin (0.5U/ml) for 10 min at 37°C. Fluorescence was analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Immunoblotting, platelet adhesion and aggregation: Phosphotyrosine blotting was performed to examine platelet activation in Hfr fed hamsters as described previously[5]. Platelet adhesion to collagen and fibrinogen was measured in polystyrene 96-well microtiter plates as described earlier[4]. Whole blood and PRP were used to study platelet aggregation. Aggregation response was induced by ADP (10 μM), collagen (2.5 μg/ml) and thrombin (1 U/ml) and was monitored on a dual channel aggregometer (Chrono log Corp., USA). The percentage of aggregation was calculated by conventional method as described earlier[4].

Coagulation parameters: Coagulation parameters namely thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen time (FT) were assayed in plasma samples of all the groups within two hours of sample collection. All assays were performed using commercial kits as per manufacturer's instructions and measured by using a coagulometer (Start4 Semi automated, Young Instruments, Stago, France).

Arterio-venous (AV) shunt in hamsters: The AV shunt method modified by Kim *et al*[18] was employed. Animal was anaesthetized with urethane (1.25 g/kg). The shunt assembly, filled with saline was cannulated between the jugular vein and contralateral carotid artery and blood was allowed to circulate through the shunt. Blood flow through the shunt was maintained for 10 min, and thrombus adhered or deposited on thread was calculated by subtracting the wet weight of the silk thread.

Ferric chloride induced arterial thrombosis in rats: Carotid artery from anaesthetized animals was carefully dissected and a pulsed Doppler Probe (DBF-120A-CPx, Crystal Biottech, USA) was placed around it to record the blood flow by using data acquisition system software (MP150 Data Acquisition System, BIOPAC Systems, USA). Thrombosis was monitored as the time taken for the cessation of carotid artery blood flow, time to occlusion (TTO) after the application of 20 per cent ferric chloride soaked filter paper as described earlier[19].

Statistical analysis: The significance of difference between the means of three or more groups was determined by one way ANOVA followed by Tukey-Kramer post-hoc multiple comparison test using GraphPad Prism 6.0 software (GraphPad Software, Inc., CA, USA).

Results

Effect of C. oil in hamsters: Twelve week feeding of HFr diet increased the body weight of hamsters (168 ± 12g) as compared to chow fed groups (148 ± 6g). HFr feeding led to enhanced plasma glucose level (1.6 fold) and hyperlipidaemia with significant increase in the circulating triglyceride (3.6 fold), total cholesterol (4.1 fold), LDL-C (5 fold), HDL-C (2 fold), as compared to chow fed hamsters (Table II). A significant increase in fasting insulin was observed (2 fold). Insulin sensitivity indices, QUICKI and HOMA-IR were significantly altered with reduced QUICKI index (1.2 fold), and elevated HOMA-IR (3 fold, Table II) in HFr fed group compared with chow fed group, thus displaying reduced insulin sensitivity. To assess the effect of HFr on oxidative stress, plasma MDA levels were estimated. Consumption of HFr resulted in enhanced plasma MDA (about 2 fold) indicating general oxidative stress under hyperinsulinaemia.

Table II. Effect of *Curcuma* oil hyperlipidaemia, insulin sensitivity and oxidative stress in hamsters

Parameters	Chow	High fructose (HFr) diet	HFr + Fenofibrate	HFr + *Curcuma* oil
Glucose (mmol/l)	3.63 ± 0.43***	5.64 ± 0.27	3.82 ± 0.92**	4.51 ± 0.98*
TGs (mmol/l)	1.017 ± 0.14***	3.64 ± 0.95	1.36 ± 0.62***	2.24 ± 0.4**
TC (mmol/l)	2.2 ± 0.23***	9.1 ± 1.2	5.6 ± 0.9***	5.8 ± 1.8***
LDL (mmol/l)	1.06 ± 0.31***	5.3 ± 0.5	4.3 ± 0.67**	4.05 ± 0.7***
HDL (mmol/l)	0.8 ± 0.13***	1.7 ± 0.3	2.5 ± 0.07***	2.18 ± 0.24***
Plasma-insulin (ng/ml)	1.6 ± 0.8**	3 ± 0.7	1.6 ± 0.38**	1.9 ± 0.25*
QUICKI	0.3 ± 0.02***	0.25 ± 0.01	0.29 ± 0.01*	0.3 ± 0.02***
HOMA-IR	6 ± 2.8***	17.9 ± 4.3	6.2 ± 3.6**	9.5 ± 3.4**
MDA (μM)	0.32 ± 0.02***	0.6 ± 0.12	0.45 ± 0.08*	0.4 ± 0.07**
Aortic cholesterol (μg/mg tissue)	0.34 ± 0.32***	2.4 ± 0.3	1.9 ± 0.23*	1.8 ± 0.25**

Values are mean ± SD (n=6)
TG, triglycerides; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; MDA, malondialdehyde; QUICKI, quantitative insulin sensitivity check index
P^{*}<0.5, **<0.01, ***<0.001 compared with HFr

No change in body weight was observed following fenofibrate (164 ± 2 g) and C. oil (166 ± 5.3 g) treatment. *Curcuma* oil and fenofibrate treated and HFr fed animals showed significant reduction in plasma glucose, TGs, TC and LDL. HDL levels were elevated in C. oil or fenofibrate fed groups as compared to only HFr fed groups. Augmented plasma insulin level was significantly reduced after C. oil or fenofibrate treatment. C. oil or fenofibrate treatment also restored altered insulin sensitivity indices *i.e.* QUICKI and HOMA-IR. Both C. oil and fenofibrate treatment significantly reduced plasma MDA levels as compared to HFr fed alone (Table II).

Feeding hamster with HFr diet for 12 wk led to an increase in aortic cholesterol accumulation (7 fold) as compared with chow fed hamsters (Table II). To assess the effect of HFr on systemic inflammation, TNFα mRNA transcript was measured in the spleen lymphocytes. Significant induction of TNFα (1.6 fold) was observed in HFr fed groups. PE (1 nM to 100 μM) produced concentration dependent contraction in endothelium intact aortic rings and this was similar among all the groups (Fig. 1a). ACh (3 nM to 30 mM) produced concentration dependent relaxations in these PE pre-contracted rings. Maximal vasodilator response to ACh in aortic rings from HFr fed hamsters was impaired (56.0 ± 2.8%) as compared to chow fed control (86.0 ± 4.8%, Fig. 1b). Both C. oil (1.3 fold) and fenofibrate (1.3 fold) prevented aortic lipid accumulation (Table II). TNFα transcript in spleen lymphocyte of HFr fed hamsters was significantly reduced after C. oil (1.5 fold) and fenofibrate treatment (1.3 fold). No change in PE induced contraction was observed by C. oil or fenofibrate (Fig. 2a). Impaired vasorelaxation response to ACh in HFr fed hamsters was restored after C. oil (87.9 ± 4%) and fenofibrate (71.2 ± 2.6%) treatment (Fig. 1b).

Phosphotyrosine blotting results revealed increased tyrosine phosphorylation of several signaling proteins in the platelets obtained from HFr fed hamsters when stimulated with collagen or thrombin. Platelets isolated from HFr fed groups showed elevated free radical generation when stimulated with collagen (1.7 fold, Table III & Fig. 2a) and thrombin (1.6 fold, Table III & Fig. 2b). HFr fed animals showed significantly enhanced platelet adhesion on collagen (1.5 fold) and fibrinogen (2 fold) coated surface when compared with their respective chow fed control group (Table III). As determined by whole blood aggregometry, platelets isolated from HFr fed hamsters showed enhanced platelet sensitivity to ADP (1.8 fold), collagen (2.2 fold) and thrombin (1.5 fold) in comparison to chow fed control indicating hyper-aggregability of platelets (Table III).

Both C. oil and fenofibrate treatment in hamsters reduced collagen and thrombin induced platelet tyrosine phosphorylation. Likewise, C. oil prevented collagen (1.5 fold, Table III & Fig. 1a) or thrombin (P<0.001) induced platelet free radical generation, however, no significant change was observed with fenofibrate. C. oil and fenofibrate reduced platelet adhesion on both

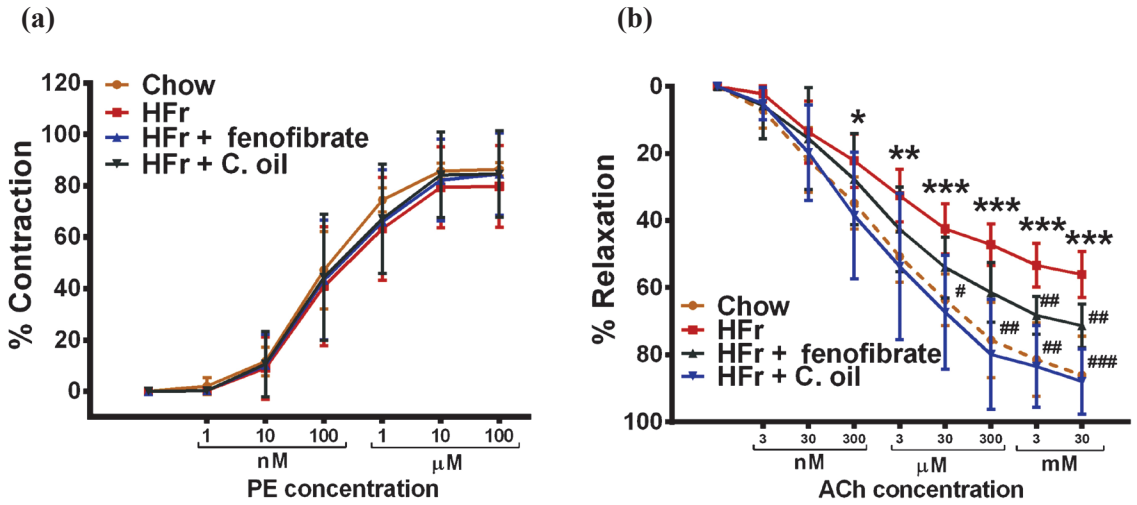

Fig. 1. Curcuma oil reduces endothelial dysfunction in hamster. **(a)** Line graph represents concentration dependent response of phenylephrine (PE, 1 nM to 100 µM) induced vascular contraction (n=6) and **(b)** acetylcholine (Ach, 3 nM to 30 mM) induced vascular relaxation (n=6) in the aortic rings of hamsters from different groups. Results are expressed as mean ± standard deviation. P *<0.05, **<0.01, ***<0.001 vs chow; $^{\#}$<0.05, $^{\#\#}$<0.01, $^{\#\#\#}$<0.001 vs HFr.

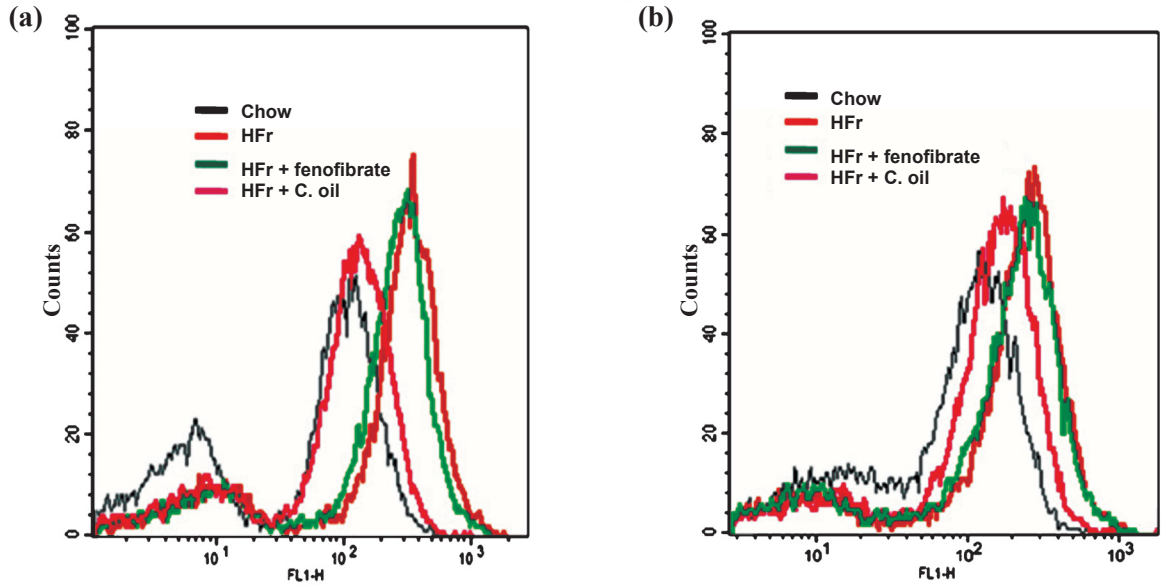

Fig. 2. Curcuma oil reduces platelet free radical generation in hamster. Histogram representing mean fluorescence index by dichlorohydrofluorescein diacetate (DCF-DA) in platelets stimulated with **(a)** collagen (5 µg/ml, n=4) or (**b)** thrombin (0.5 U/ml, n=4). Histogram represents one of three to four similar experiments.

collagen (1.5 & 1.3 fold, respectively) and fibrinogen (1.4 & 1.3 fold, respectively) coated surface when compared with HFr fed groups (Table III). *Curcuma* oil and fenofibrate reduced HFr induced elevated whole blood aggregation after ADP (2.6 & 2 fold, respectively), collagen (2.3 & 2 fold, respectively), and thrombin (~2.5 & 2 fold, respectively) stimulation (Table III).

A significant decrease in the TT (1.2 fold) was observed in HFr fed animals (Table III) in comparison to the chow fed control, indicating hypercoagulability

Table III. *Curcuma* oil reduces platelet activation and thrombosis in hamsters

Parameters	Chow	High fructose (HFr) diet	HFr + Fenofibrate	HFr+ *Curcoma* oil
Platelet free radical generation (n=4)				
MFI (collagen stimulated)	149 ± 5.6***	256.5 ± 6.35	244.7 ± 11.15	169.3 ± 1.5***
MFI (thrombin stimulated)	138 ± 2.6***	225 ± 8.2	225.3 ± 12.2	146.3 ± 13***
Platelet adhesion (n=6)				
Collagen coated surface (× 10^6 cells)	0.98 ± 0.09***	1.5 ± 0.2	1.2 ± 0.04**	1 ± 0.19***
Fibrinogen coated surface (× 10^6 cells)	0.53 ± 0.08***	1 ± 0.14	0.74 ± 0.19*	0.68 ± 0.17**
Platelet aggregation (n=6)				
ADP (% aggregation)	21.5 ± 6.1***	40.5 ± 9.8	20.6 ± 9***	15 ± 3.2***
Collagen (% aggregation)	25.5 ± 4.2***	55.5 ± 5.4	28 ± 3.2***	24 ± 3.2***
Thrombin (% aggregation)	27 ± 3.2***	42 ± 3	20.5 ± 4.2***	16.5 ± 4.9***
Coagulation parameters (n=6)				
TT (sec)	28.5 ± 4.1**	23.9 ± 2.6	27.9 ± 4.4	30.7 ± 1.5**
PT (sec)	8.6 ± 1.4	9.5 ± 1.3	10 ± 1.1	10 ± 0.76
aPTT (sec)	15.4 ± 1.3	16 ± 0.9	14 ± 2.3	14.7 ± 2
FT (sec)	18.2 ± 0.4	17.4 ± 3.6	16.6 ± 3	18.3 ± 1.8
Thrombosis (n=6)				
Thrombus weight (mg/kg)	44.8 ± 2.5***	59.8 ± 2.4	45 ± 4	36 ± 6.8***

MFI, mean fluorescence index; TT, thrombin time; PT, prothrombin time; aPTT, activated partial thromboplastin time; FT, fibrinogen time
*P**<0.05, **<0.01, ***<0.001 compared with HFr

state in HFr fed hamsters. However, no differences were observed in aPTT, FT and PT in HFr fed group when compared to their respective control (Table III). To assess the prothrombotic state induced by HFr, thrombus formation was monitored in AV shunt model in all the groups. A significant increase in thrombus weight was observed after HFr treatment (1.3 fold) as compared to the controls (Table III). C. oil significantly ameliorated the HFr induced coagulation dysfunction (1.2 fold), however, fenofibrate was found to be ineffective (Table III). A significant and similar reduction in thrombus weight was observed in both C. oil (1.7 fold) and fenofibrate (1.3 fold) fed groups (Table III).

Effect of Curcuma oil in rats: HFr diet feeding for 12 wk in rats significantly augmented plasma glucose (1.2 fold), triglyceride (1.7 fold), and insulin levels (4.3 fold) as compared to chow fed animals. Insulin sensitivity indices were significantly altered since reduced QUICKI index (1.7 fold) and elevated HOMA-IR (4 fold, Table IV) were observed in HFr group when compared to control. HFr treatment increased plasma MDA (3 fold) as compared to chow fed alone, indicating general oxidative stress under hyperinsulinaemic conditions. *Curcuma* oil treatment in HFr fed animals significantly improved plasma glucose (1.2 fold) and triglycerides (1.2 fold). Altered insulin level (1.9 fold, Table IV) as well as insulin sensitivity indices, *i.e.* QUICK I (1.2 fold) and HOMA-IR (1.7 fold) were also restored by C. oil as compared to HFr fed alone (Table IV). MDA levels were also found to be reduced following C. oil treatment (1.4 fold). HFr feeding in rats resulted in enhanced hepatic expression of sterol regulatory element binding protein 1c (SREBP-1c) (39 fold), peroxisome proliferator-activated receptor-gamma co-activator-1α (PGC-1α) (1.9 fold), and peroxisome proliferator-activated receptor-gamma co-activator-1β (PGC-1β) (3 fold) as compared to chow fed group. C. oil treatment in HFr fed rats repressed hepatic expression of SREBP-1c (3.5 fold), PGC-1α (1.5 fold) and PGC-1β (1.6 fold) as compared to HFr fed alone (Table IV), suggesting protective effect of C. oil is by modulating expression of lipogenic genes in liver.

HFr feeding for 12 wk resulted into enhanced plasma TNF (1.9 fold), IFN-γ (3.1 fold), IL-6 (9.5 fold) and CRP (1.4 fold) level as compared to chow fed alone (Table IV). PE (1 nM to 100 μM) induced

Table IV. *Curcuma* oil reduces insulin resistance, lipogenic gene expression, inflammation and thrombosis in rats

Parameters	Chow	High fructose (HFr) diet	HFr+ *Curcuma* oil
Plasma biochemistry (n=6)			
Glucose (mmol/l)	4.2 ± 0.42**	5 ± 0.3	4.3 ± 0.4**
TGs (mmol/l)	1 ± 0.14***	1.7 ± 0.4	1.2 ± 0.2*
Plasma insulin (mmol/l)	2 ± 0.33***	8.7 ± 3.1	4.6 ± 1.6**
QUICKI	0.57 ± 0.02***	0.41 ± 0.03	0.48 ± 0.023**
HOMA-IR	0.14 ± 0.03***	0.57 ± 0.2	0.33 ± 0.08*
MDA (nM)	27 ± 7.3***	84 ± 14.6	59 ± 2.7***
Hepatic expression of lipogenic genes (fold change, n=6)			
SREBP1c	1 ± 0.2***	39 ± 7.1	11 ± 2***
PGC1α	1 ± 0.08***	1.9 ± 0.5	1.3 ± 0.27*
PGC1β	1 ± 0.3***	3.1 ± 0.8	1.8 ± 0.9*
Inflammatory parameters (pg/ml, n=6)			
TNF	69 ± 7.8***	129.3 ± 17.4	107 ± 10*
IFNγ	37.2 ± 6.3***	116 ± 3.4	91 ± 13.2***
IL-6	8 ± 0.27***	76.2 ± 7	48.2 ± 5.6***
CRP	68.2 ± 6.6***	97 ± 9.3	64 ± 5.3***
Thrombotic parameters (n=6)			
Platelet aggregation (%)	52 ± 12.2**	67 ± 8.6	53 ± 3.9*
Platelet adhesion (×10^6 cells)	0.97 ± 0.03*	1.46 ± 0.4	0.95 ± 0.27**
Time to occlusion (fold change)	1 ± 0.04***	0.72 ± 0.15	0.88 ± 0.03*

Values are mean ± SD (n=6)
QUICKI, quantitative insulin senstivity check index; HOMA-IR, homeostatic model assessment of insulin resistance; MDA, malondialdehyde; TNF, tumour necrosis factor; IFNγ, interferon gamma; CRP, c-reactive protein; TG, triglycerides
*P**<0.01, **<0.01, ***<0.001 compared with HFr

concentration dependent contraction in endothelium intact aortic rings was similar among all the groups (data not shown). Vasodilator response to ACh from HFr fed rats was found to be reduced (52 ± 5.3%) as compared to chow fed control (92 ± 2.3%). High fructose diet feeding in rats for 12 wk resulted in significant increase in ADP induced platelet aggregation (1.3 fold) and platelet adhesion on collagen coated surface (1.5 fold). HFr feeding resulted into reduced TTO (1.4 fold), measured after topical application of ferric chloride on the carotid artery of rats (Table IV).

Curcuma oil treatment in HFr fed animals resulted in significant reduction of TNF (1.2 fold), IFN-γ (1.3 fold), IL-6 (1.6 fold), and CRP (1.5 fold) in comparison to HFr fed alone. Impaired relaxant response to ACh was preserved in C. oil fed groups (73 ± 1.9%). *Curcuma* oil treatment significantly reduced ADP induced platelet aggregation (1.2 fold) and platelet adhesion (1.5 fold) on collagen coated surface in HFr fed rats and significantly increased TTO (1.2 fold) thus signifying its antithrombotic potential (Table IV).

Discussion

High fructose diet used in the current study has been previously shown to induce diabetic dyslipidaemia in hamsters[7,9]. An earlier study demonstrated that HFr feeding in rat leads to impaired glucose tolerance and insulin resistance[5]. In the present study HFr feeding to hamsters and rats led to enhanced plasma glucose and insulin levels and alteration in insulin level sensitivity indices QUICKI and HOMA-IR, indicating development of an experimental condition that closely mimics insulin resistance[20].

Enhanced expression of TNFα in spleen lymphocytes of hamsters as well as in plasma of rats, following HFr feeding, indicates role of systemic inflammation in these animals. Reversion in TNF level

in plasma of rat or spleen lymphocytes of hamster by C. oil and fenofibrate was comparable. Previous reports have suggested a role of TNFα in inducing endothelial dysfunction[21]. Therefore, to assess the effect of HFr on parameters pertaining to the maintenance of haemostasis, endothelial dysfunction, platelet activation and coagulation cascade were assessed in these animals. HFr feeding demonstrated significant alteration in ACh induced endothelium dependent vasorelaxation, indicating endothelial dysfunction in these animals. Correction of vascular dysfunction in C. oil treated hamsters or rat might be due to the reduction in insulin resistance and TNFα levels.

Platelets from HFr fed hamsters showed significant increase in collagen and thrombin induced free radical generation and protein tyrosine phosphorylation thus indicating the mechanisms involved in its activation. *Curcuma* oil treatment reduced HFr induced platelet activation as evident by reduction in platelet free radical generation and tyrosine phosphorylation. At the same time a significant increase in ADP, collagen and thrombin induced platelet aggregation was also observed that indicated hyperaggregability of platelets in HFr fed groups. Both C. oil and fenofibrate reduced platelet adhesion and aggregation. Similar to a previous report in which fenofibrate has been shown to have anti-platelet activity[22], a significant reversion in platelet adhesion and aggregation response was observed by fenofibrate in our study. From these studies, it can be concluded that platelets from HFr fed animals adhere and aggregate more than their respective controls and fenofibrate and C. oil show anti-platelet activity under such conditions. We found significant decrease in the TT indicating enhanced thrombin activity and activation of coagulation cascade. No significant change was seen in other coagulation parameters, as reported earlier[23]. In arteriovenous shunt (AV shunt) model of thrombosis, a significant increase was observed in the thrombus weight in HFr fed groups indicating an elevated tendency of thrombosis in these animals. This might be due to changes in platelet activity, coagulation cascade and endothelium functionality, suggesting that hamster model used in the present study exhibited enhanced pro-thrombotic state associated with metabolic syndrome. Both fenofibrate and C. oil significantly reduced thrombus weight in the AV shunt model. The reduction in thrombus weight by fenofibrate seems to be dependent on its anti-platelet activity, rather than its effect on coagulation factors analyzed in this study.

The translation of accurate dose from animal studies to human use is difficult, and this has to be done with extreme caution. In a previous human study, turmeric oil (600 mg and 1 g/day) was found to be safe on haematological, renal and hepatotoxicity parameters[24]. However, detailed toxicity and safety evaluations of C. oil need to be carried out in animals before its translation for human use. To delineate the probable mechanism of C. oil induced reversion in insulin resistance, we did mRNA expression analysis of genes involved in glucose metabolism in the rat model. Changes in hepatic expression of *SREBP1c*, *PGC1α*, and *PGC1β* are often associated with hyperglycaemia and insulin resistance in rodents[25,26], which is quite similar to humans[27]. Therefore, rat model was considered more relevant for deciphering the protective mechanism of C. oil.

Increased *PGC-1α* expression in liver elevates hepatic glucose output leading to type II diabetes in rodent, possibly due to alterations of the insulin-glucagon axis and hepatic insulin resistance, respectively[28]. Dietary fructose upregulates PGC-1β mediated transcriptional activation of *SREBP* to stimulate *de novo* hepatic lipogenesis[29]. Therefore, increased liver *PGC-1α* mRNA expression in fructose fed animals might alter the systemic glucose homeostasis, further leading to hyperglycaemia and insulin resistance.

In conclusion, our results demonstrated that HFr feeding to hamsters and rats led to the development of insulin resistance, hyperglycaemia, oxidative stress, endothelium dysfunction, inflammation, platelet activation, and prothrombotic state. *Curcuma* oil treatment significantly attenuated diabetic dyslipidaemia, insulin resistance and associated deleterious effects in HFr fed hamsters and rats. Studies in the rat model suggest that perhaps C. oil mediates its protective effect on diabetic dyslipidaemia and insulin resistance by suppressing hepatic *PGC-1α*, *PGC-1β*, and *SREBP1c* mRNA transcripts but further studies need to be done to confirm these findings.

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Reprint requests: Dr Manoj Kumar Barthwal, Pharmacology Division, CSIR-Central Drug Research Institute
B.S. 10/1, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow 226 031, Uttar Pradesh, India
e-mail: manojbarthwal@cdri.res.in