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Chronic vitamin A-enriched diet feeding regulates hypercholesterolaemia through transcriptional regulation of reverse cholesterol transport pathway genes in obese rat model of WNIN/GR-Ob strain

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***Background & objectives*: Hepatic scavenger receptor class B1 (SR-B1), a high-density lipoprotein (HDL) receptor, is involved in the selective uptake of HDL-associated esterified cholesterol (EC), thereby regulates cholesterol homoeostasis and improves reverse cholesterol transport. Previously, we reported in euglycaemic obese rats (WNIN/Ob strain) that feeding of vitamin A-enriched diet normalized hypercholesterolaemia, possibly through hepatic SR-B1-mediated pathway. This study was aimed to test whether it would be possible to normalize hypercholesterolaemia in glucose-intolerant obese rat model (WNIN/GR/Ob) through similar mechanism by feeding identical vitamin A-enriched diet.**

***Methods*: In this study, 30 wk old male lean and obese rats of WNIN/GR-Ob strain were divided into two groups and received either stock diet or vitamin A-enriched diet (2.6 mg or 129 mg vitamin A/kg diet) for 14 wk. Blood and other tissues were collected for various biochemical analyses.**

***Results*: Chronic vitamin A-enriched diet feeding decreased hypercholesterolaemia and normalized abnormally elevated plasma HDL-cholesterol (HDL-C) levels in obese rats as compared to stock diet-fed obese groups. Further, decreased free cholesterol (FC) and increased esterified cholesterol (EC) contents of plasma cholesterol were observed, which were reflected in higher EC to FC ratio of vitamin A-enriched diet-fed obese rats. However, neither lecithin-cholesterol acyltransferase (LCAT) activity of plasma nor its expression (both gene and protein) in the liver were altered. On the contrary, hepatic cholesterol levels significantly increased in vitamin A-enriched diet fed obese rats. Hepatic SR-B1 expression (both mRNA and protein) remained unaltered among groups. Vitamin A-enriched diet fed obese rats showed a significant increase in hepatic low-density lipoprotein receptor mRNA levels, while the expression of genes involved in HDL synthesis, namely, ATP-binding cassette protein 1 (*ABCA1*) and apolipoprotein A-I, were downregulated. No such response was seen in vitamin A-supplemented lean rats as compared with their stock diet-fed lean counterparts.**

***Interpretation & conclusions*: Chronic vitamin A-enriched diet feeding decreased hypercholesterolaemia and normalized HDL-C levels, possibly by regulating pathways involved in HDL synthesis and degradation, independent of hepatic SR-B1 in this glucose-intolerant obese rat model.**

Key words Gene expression - lipoprotein - metabolism - obesity - retinoids- vitamin A- enriched diet

Reverse cholesterol transport (RCT) is one of the major pathways by which excess cholesterol from extrahepatic tissues is removed through high-density lipoprotein (HDL)-mediated uptake in the liver[1]. Studies have shown a strong, independent inverse association between plasma HDL-cholesterol (HDL-C) levels and cardiovascular diseases[2,3]. Understanding of RCT has shed light on the role of various factors, enzymes and proteins involved in HDL remodelling and RCT process including apolipoprotein A-I (ApoA-I) and E (ApoE), lecithin-cholesterol acyltransferase (LCAT), scavenger receptor class BI (SR-BI), ATP-binding cassette transporter proteins A1 (ABCA1) and hepatic lipase (HL)[4,5]. In mammals, RCT is one of the key mechanisms in regulating cholesterol homoeostasis through complex process; initially, peripheral tissues transfer the free cholesterol (FC) to nascent HDL, called lipid-poor ApoA-I, and after its transfer, LCAT converts FC into esterified cholesterol (EC) and thereby helps in maintaining the FC gradient between the peripheral cells and the HDL particle, resulting in efflux of more FC from extrahepatic tissues to HDL[4,5].

We have earlier reported in obese rats of WNIN/Ob strain that the observed hypercholesterolaemia with abnormally high plasma HDL-C levels was due to under-expression of hepatic SR-B1 at both protein and gene levels and these abnormalities were normalized by vitamin A-enriched diet feeding through hepatic SR-BI-mediated RCT pathway[6]. However, some of the components of RCT and their transcriptional regulation by vitamin A were not studied. Similar to WNIN/Ob strain, obese rats of WNIN/GR-Ob strain display hypercholesterolaemia and elevated HDL-C levels as compared to their age- and sex-matched lean counterparts, but they are glucose-intolerant. As this trait was unique in this model, this study was undertaken to test whether similar vitamin A-enriched diet (129 mg of vitamin A per kg diet) would be able to improve the hypercholesterolaemia through SR-B1 and also to address the transcriptional regulation of various other components of RCT pathway by vitamin A.

Material & Methods

Cholesterol and HDL-C assay kits were procured from BioSystems S.A. (Barcelona, Spain). Kits for total cholesterol (TC) and esterified cholesterol (EC) assays were purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). LCAT activity assay kit was from Roar Biomedical Inc., USA. SR-B1 primary and secondary antibodies were purchased from Abcam (Cambridge, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Amersham ECL-nitrocellulose membrane and ECL advance western blotting detection kits were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). Total RNA isolation kit was purchased from Qiagen GmbH, Germany. For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, cDNA synthesis kit, pre-validated probes for rat (Universal Probe Library for rat) were purchased from Roche (Roche Diagnostics GmbH, Germany). All other chemicals used were of analytical grade. Gene-specific primers were obtained from Integrated DNA Technologies, BVBA (IDT), Leuven, Belgium.

Animals and experimental design: Adult (30 wk), male lean and obese rats of WNIN/GR-Ob strain were obtained from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India, and randomly divided into two groups A and B, each consisting of 12 lean and 12 obese rats (with impaired glucose tolerance trait), respectively, and further divided into two subgroups (A-I, A-II and B-I, B-II) consisting of six rats each. Subgroups A-I and B-I received the stock diet having 2.6 mg of vitamin A/kg diet, while subgroups A-II and B-II received vitamin A-enriched diet (129 mg of vitamin A/kg diet as retinyl palmitate) for 14 wk. The stock diet and vitamin A-enriched diets were identical with regard to all other ingredients, except the vitamin A content (Table I). The study was approved by the Institutional Animal Ethical Committee. At the end, blood was collected after 12 h fasting and rats were sacrificed. Various tissues were excised, weighed, rapidly frozen in liquid nitrogen and stored at −80°C for the further analysis.

Analysis of plasma parameters: Plasma TC and FC levels were measured and EC levels were calculated as per the manufacturer's instruction. LCAT activity was measured as the ratio of change in fluorescence intensity (at 470/390 nm) as instructed in the manufacturer's protocol. A higher ratio indicates lesser activity. Liver total lipids were extracted by chloroform:methanol mixture (2:1v/v) and assayed for cholesterol as described earlier[7].

Hepatic immunoblot analysis: Liver samples were homogenized in a Tris buffer containing 250 mM sucrose, 10 mM Tris (*p*H 7.4), 1 mM EDTA, 1 mM dithiothreitol (DTT) supplemented with protease and phosphatase inhibitor cocktails and various cellular fractions were collected after differential centrifugation.

Table I. Diet composition

Diet constituents	Quantity (g/kg)
Wheat flour	225
Roasted Bengal gram powder	600
Skim milk powder	50
Casein	40
Refined groundnut oil	45
Mineral mixture[a]	40
Vitamin mixture[b]	5

Prepared and supplied by National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad; [a]Composition of mineral mixture (g/kg mineral mixture): Dicalcium phosphate, 312.5 g; $CaCO_3$, 138.7 g; NaCl, 75 g; $MgSO_47H_2O$, 57.3 g; $FeSO_47H_2O$, 12.5; $MnSO_4H_2O$, 4.01; KI, 0.25 g; $ZnSO_47H_2O$, 0.55 g; $CuSO_45H_2O$, 0.48 g and $CoCl_26H_2O$, 0.003 g; [b]Composition of vitamin mixture (g/kg vitamin mixture): (dl)-α tocopherol acetate, 24 g; menadione, 0.3g; thiamine, 2.4 g; riboflavin, 1.0 g; pyridoxine, 1.2 g; niacin, 2.0 g; pantothenic acid, 2.4 g; cyanocobalamine, 1.0 µg; folic acid, 0.2 g; para amino benzoic acid, 20 g; biotin, 0.08 g; inositol, 20 g; and choline chloride, 200 g. vitamin A of 2.6 mg and vitamin D of 10 µg in the form of vanitin were added per kg diet. In addition, for vitamin A-enriched diet, 126.4 mg of vitamin A was added per kg diet as retinyl palmitate

Microsomal fraction was used to detect the SR-BI protein as described earlier[7]. β-actin was used as loading control. Images were analysed using Image J 1.46r software (National Institute of Health, USA).

RNA isolation and gene expression analysis by quantitative reverse transcriptase (RT) PCR: Total RNA from the liver was isolated using RNeasy mini kit according to the manufacturer's instructions. Reverse transcription reaction was performed using 5.0 µg of total RNA, according to the manufacturer's protocol for Transcriptor First Strand cDNA Synthesis kit (Roche, Germany). Quantitative PCR (real-time) was performed in 20 µl reaction, using 2.0 µl of cDNA, pre-validated probes (UPL probes for rat; Roche) and gene-specific primers, using LightCycler 480 real-time PCR system (Roche). The reaction was performed in duplicate. Primer pairs used for the amplification reactions of various genes from cDNAs are listed in Table II. Endogenous expression of acidic ribosomal phosphoprotein was used to normalize the expression data, and relative expression levels were calculated as described earlier[7].

Statistical analysis: Comparisons were made between stock diet versus vitamin A-enriched diet groups of each phenotype by one-way ANOVA (with *post hoc* test). Data were analysed using SPSS software package version 11.0 (IBM Corp. Armonk, NY, USA).

Results

Impact of vitamin A on cholesterol levels: Plasma TC and HDL-C levels of obese rats were nearly 3.5- and 2.5-fold higher than their age- and sex-matched lean counterparts fed with stock diet, respectively. Further, compositional analysis of TC revealed that obese rats had higher FC (49 %) and lower EC (51 %) compared to their stock diet-fed lean rats, whose FC and EC levels were 13 and 87 per cent of TC, respectively. Feeding of vitamin A-enriched diet to obese rats significantly reduced the abnormally high plasma TC and HDL-C levels by 56 and 54 per cent, respectively. However, TC compositional analysis showed that there was a significant increase in EC content to 63 per cent, while FC decreased to 37 per cent in obese rats upon vitamin A-enriched diet consumption as compared to their stock diet-fed obese rats, which had 49 per cent of EC and 51 per cent of FC levels. This was reflected in higher ratio of EC to FC. Although there were changes in all these parameters in lean phenotype by high vitamin A diet feeding, those were not significantly different in comparison with the values of stock diet-fed lean controls. In contrast to the circulatory levels, cholesterol levels were elevated in the livers of vitamin A-enriched diet-fed obese rats by 0.8-fold, while in lean rats, no such increase was observed (Table III).

Impact of vitamin A on plasma LCAT activity, hepatic LCAT and SR-B1 expression: Plasma LCAT activity was found comparable between stock diet fed lean and obese rats. Although vitamin A-enriched diet consumption increased its activity in both the phenotypes, it was not significantly different as compared with their respective controls consuming stock diet. Gene expression data also showed no significant change in LCAT mRNA levels in both lean and obese phenotype receiving either stock or high vitamin A diet (Fig. 1A and B). Western blot analysis revealed that hepatic SR-B1 protein and gene expression levels were not significantly different between phenotypes; further, chronic feeding of vitamin A-enriched diet had no effect on these parameters (Fig. 1C & D).

Vitamin A on other component of RCT pathway genes expression: Although not directly linked to RCT, a key transcriptional regulator of cholesterol biogenesis is sterol regulatory element binding protein 2 (SREBP2),

Table II. Gene-specific primers used for quantitative reverse transcriptase polymerase chain reaction

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ABCA1	CAGACGATATCTCGATTCATGG	GAGCGTGACTTCGGTTGG
APOA-I	TTCTGGCAGCAAGATGAGC	ACACAGTGGCGAAATCCTTC
APOA-II	GCTGGTCACCATCTGTAGCC	GGGTCTGCACATCCGTCT
APOE	GTTGGTCCCATTGCTGACA	CGCAGGTAATCCCAGAAGC
ARPP	GATGCCCAGGGAAGACAG	CACAATGAAGCATTTTGGGTAG
HL	GAGGTGGCTGCTCTTCTCC	TTAAGTGAACTTTGCTCCGAGA
LCAT	CACACGGCCTGTCATCCT	GGTTTATCCAGCTTGGCTTCT
LDL-R	TGCTACTGGCCAAGGACAT	CTGGGTGGTCGGTACAGTG
SR-B1	GGTGCCCATCATTTACCAAC	GCGAGCCCTTTTTACTACCA
SREBP2	GTGCAGACAGTCGCTACACC	AATCTGAGGCTGAACCAGGA

ABCA1, ATP-binding cassette transporter proteins A1; *APOA-I*, apolipoprotein A-I; *APOA-II*, apolipoprotein A-II; *ARPP*, acidic ribosomal phosphoprotein; *HL*, hepatic lipase; *LCAT*, lecithin cholesterol acyltransferase; *LDL-R*, low density lipoprotein receptor; *SR-B1*, scavenger receptor class B1; *SREBP2*, sterol regulatory element binding protein 2; *APOE*, apolipoprotein E

Table III. Impact of vitamin A on biochemical parameters of lean and obese rats

Plasma parameters (mg/dl)	Lean rats		Obese rats	
	A-I	A-II	B-I	B-II
TC	147.7±12.0	95.7±5.1	494.9±120.8$^{\#}$	220.4±21.6*
FC	19.0±5.7	7.09±3.0	242.1±97.6$^{\#}$	81.9±22.5*
EC	128.7±7.1	88.6±5.9	252.8±41.3$^{\#}$	138.5±33.8*
Ratio of EC to FC	8.7±1.9	27.0±10.9	1.3±0.41$^{\#}$	2.3±0.82
HDL-C	51.8±7.7	40.9±1.7	134.2±21.0$^{\#}$	60.5±12.1*
Liver cholesterol (mg/g)	8.6±1.6	12.1±1.8	9.5±0.84	16.9±0.98*

Data represent the means±SEM of 6, except for liver cholesterol; 4 rats from each group. $^{\#}P<0.05$ compared to stock diet-fed lean rats; $^{*}P<0.05$ compared to stock diet-fed obese rats. A-I, B-I-stock diet and A-II, B-II-vitamin A-enriched diet fed groups. SEM, standard error of mean; TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol; HDL-C, high density lipoprotein cholesterol

whose mRNA levels were similar among all the groups. Of the various hepatic RCT-associated gene expression profiles, ABCA1, HL and low-density lipoprotein receptor (LDL-R) did not differ between age- and sex-matched, stock diet-fed lean and obese rats. However, hepatic LDL-R expression was upregulated to nearly 3-fold in obese rats, while hepatic ABCA1 levels were downregulated in both the phenotypes (*i.e.,* 77 % in lean and 64 % in obese) by feeding of high vitamin A-containing diet as compared with their respective stock diet receiving control groups. *HL* mRNA levels remained unaltered among all the groups (Fig. 2A). Gene expression data on various apoproteins showed that ApoA-I and ApoA-II levels were significantly higher (2.5- and 4.5-fold, respectively) while ApoE levels were not different between stock diet-fed lean and obese rats. *APOA-I* mRNA was downregulated in obese rats fed on vitamin A-enriched diet, while ApoA-II expressions levels were not altered. Conversely, no such effects were seen in lean rats fed on an identical diet (Fig. 2B).

Discussion

It was observed that chronic vitamin A supplementation reduced abnormally-elevated circulatory cholesterol levels in obese rats, which corroborated with decreased HDL-C levels. Further, vitamin A-enriched diet feeding brought down the FC and EC levels. Although not significant, an increase in the EC to FC ratio was in line with elevated LCAT activity. Importantly, most of these changes were not seen in lean phenotype fed on the identical dietary regimen.

Although HDL synthesis is a complex process, biogenesis of nascent HDL is an important determinant of circulatory HDL levels[8,9]. In Tangier disease, which is characterized by low plasma HDL-C levels, role of

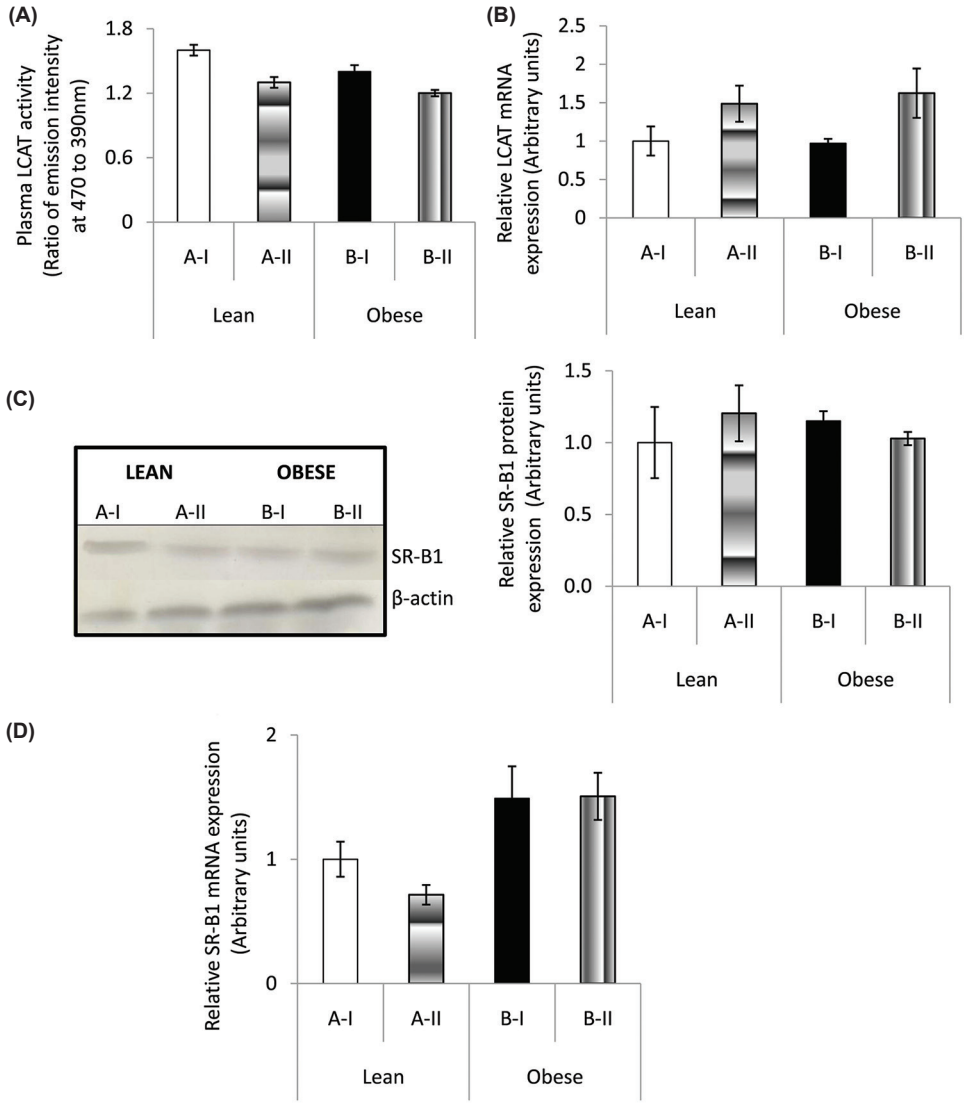

Fig. 1. Impact of vitamin A on plasma lecithin-cholesterol acyltransferase (LCAT) activity, hepatic LCAT and scavenger receptor class B1 (SR-B1) expression. **(A)** Plasma LCAT activity, represented as ratio of change in emission intensity at 490-370 nm. Higher ratio indicates lesser activity and *vice versa*, **(B)** Hepatic *LCAT* mRNA expression levels, **(C)** Representative Western blot showing hepatic SR-B1 protein levels and histogram represents the densitometric (arbitrary units) values of blot relative to stock diet-fed lean rats, **(D)** Hepatic *SR-B1* mRNA expression levels. Data represent the means ± standard error of mean of 4-6 rats from each group. A-I, B-I-stock diet and A-II, B-II-vitamin A-enriched diet fed groups.

functional ABCA1 in HDL-biogenesis, particularly at initial stage, has been reported[10,11]. In general, ABCA1 facilitates the transfer of phospholipids and FC from cells to ApoA-I or lipid-poor HDL particle and thereby initiates its synthesis as nascent HDL[12,13]. In the current study, the observed reduction in the expression of *ABCA1* and *APOA-I* suggests that vitamin A supplementation negatively regulates hepatic HDL biogenesis *per se* at least in obese phenotype.

Unlike in the euglycaemic obese rats (WNIN/Ob strain), hepatic SR-B1 expression both at protein and gene levels showed no significant differences between lean and obese phenotypes fed either stock or vitamin A-enriched diet, suggesting that SR-B1 might not be the key player of HDL-C regulation, at least in this strain rats, and vitamin A had no impact on hepatic SR-B1 either at transcription or translational levels. It is well known that hepatic SR-BI is involved in the selective uptake of CE from HDL, and in rats, it contributes to nearly 65 per cent plasma HDL-CE clearance and uptake by the liver[14]. Therefore, the present findings suggested that

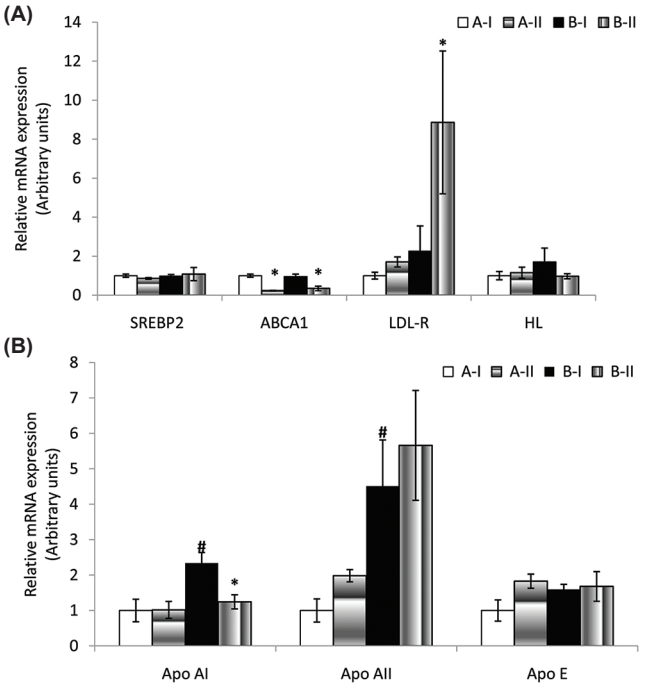

Fig. 2. Impact of vitamin A on transcriptional regulation of reverse cholesterol transport (RCT)-associated genes. **(A)** Hepatic RCT-associated gene expression levels determined by quantitative reverse transcriptase polymerase chain reaction analysis, **(B)** Hepatic apolipoproteins mRNA expression. Data represent the means ± standard error of mean of four rats from each group. $^{\#}P<0.05$ compared to A-I group; $^{*}P<0.05$ compared to B-I group. A-I, B-I-stock diet and A-II, B-II-vitamin A-enriched diet fed groups. *SREBP2*, sterol regulatory element binding protein 2; *ABCA1*, ATP-binding cassette transporter proteins A1, *LDL-R*; Low density lipoprotein receptor, HL, hepatic lipase.

there was no defective hepatic SR-B1 expression in these obese rats and vitamin A-mediated regulation of hypercholesterolaemia and HDL-C levels was independent of hepatic SR-B1.

To explain vitamin A-mediated hypocholesterolaemic effect in obese rats, we studied other components of RCT. Besides SR-B1, hepatic LDL-R is known to play a major role in regulating circulatory cholesterol levels. Wide difference exists in lipid profile and its metabolism between human and rodents; in the former, nearly 75 per cent of plasma cholesterol is transported by LDL, but in latter, through HDL. Previously, several studies have shown the involvement of LDL-R in cholesterol homoeostasis in rodent models and LDL-R knockout mice displayed defective HDL metabolism, resulting in elevated circulatory cholesterol levels[15-19]. In the current study, vitamin A supplementation resulted in elevated hepatic LDL-R expression, which corroborated with normalization of abnormally high plasma HDL-C levels and thereby decreased hypercholesterolaemia in obese phenotype. Further, increased hepatic cholesterol accumulation supports our hypothesis that vitamin A improves circulatory cholesterol levels, possibly through LDL-R-mediated HDL-C regulation. However, the present study did not address the cholesterol catabolic pathway, the final stage of RCT.

It has been demonstrated that SREBP2 is the key transcriptional regulator of LDL-R[20]. In the present study, *LDL-R* mRNA levels were significantly elevated after vitamin A supplementation without affecting SREBP2 mRNA levels. Lu *et al*[21] have also reported hypocholesterolaemic effect of gossypin through transcriptional regulation of *LDL-R* gene, which is independent of SREBP2, but through activation of extracellular signal-regulated kinase (ERK) pathway. Although it is not reasonable to conclude in the absence of protein data, gene expression results suggest that vitamin A-mediated transcriptional regulation of LDL-R may be independent of SREBP2, at least in this glucose-intolerant obese rat model, and warrants further study to prove this hypothesis. Overall, the data presented here demonstrated that vitamin A supplementation regulated cholesterol homoeostasis by modulating transcript levels of its metabolic pathway genes, particularly in obese phenotype. Identical dietary regimen failed to elicit such a response in normocholesterolaemic lean rats and thus underscores the role of genetic factors in bringing about a nutrient-specific physiological response.

In conclusion, the present results showed that chronic feeding of vitamin A-enriched diet to glucose-intolerant obese rats (WNIN/GR-Ob) regulated hypercholesterolaemia by normalizing abnormally-elevated HDL-C levels, possibly by regulating pathways involved in HDL biogenesis and degradation, independent of hepatic SR-B1. These results not only emphasize the role of genetic factors in determining the disease and health conditions but also their modulation by nutrients in eliciting physiological response, which forms the basis for nutritional supplementation based therapeutic strategies.

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Conflicts of Interest: None.

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